

**GENETIC AND METABOLIC MARKERS
OF AGEING IN SHEEP OOCYTES**

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Abstract

Declining fertility in reproductive ageing is recognizable in both natural conception and assisted reproduction. Although diminished ovarian reserve and reduced receptivity of uterus may play a role, oocyte quality seems to be a major cause of deterioration of reproductive function since donor oocytes improving the success rate of IVF program. This thesis aims to evaluate the impact of age on the oocyte by using a monovular species sheep as a representative of the human model. The study has been conducted in germinal vesicle (GV) oocytes derived from abattoir tissues in order to understand the biological dysfunction of aged oocytes in terms of chromatin rearrangement, noninvasive functional analyses (i.e., amino acid and carbohydrate metabolism), mitochondrial DNA copy number, and genetic and epigenetic regulation.

Initially, due to germinal vesicle oocytes used in a series of experiments, the method needs to simulate the natural condition in order to minimize confounding results. Thus, the *in vitro* culture employed a PDE inhibitor, cilostamide, to prevent the spontaneous meiotic resumption. The studies depicted the different potential of chromatin rearrangement of prepubertal oocytes compared to adult oocytes as well as the functional analyses such as amino acid turnover, glucose and pyruvate consumption. In addition, the later experiments discovered that age altered a surrogate of mitochondrial number, mitochondrial DNA copy number, and found inversed relation to the mitochondrial unit function. Finally, genetic studies demonstrate changes in genes governing metabolisms and genes involved in epigenetic marks.

Collectively these results indicate that age profoundly influences oocyte cellular marker, metabolic function, and genetic and epigenetic regulations, is responsible to the impairment of oocyte development in monovular species. Also the findings may be used to determine oocyte quality *in vitro*, which is an initial step to study in depth in human and other species.

Table of Contents

Acknowledgements	ii
Abstract	iii
Table of Contents	iv
List of Tables	ix
List of Figures	xi
List of Abbreviations	xiv
List of Presentations and Publications	xx
CHAPTER 1. GENERAL INFORMATION	1
1.1 INTRODUCTION	1
1.2 OVARIAN RESERVE	2
1.3 FOLLICULOGENESIS	4
1.4 OOCYTE MATURATION	7
1.4.1 Nuclear Maturation, Meiotic Arrest And Resumption	7
1.4.2 Cytoplasmic Maturation	12
1.4.3 Molecular Maturation	15
1.4.4 Cumulus-Oocyte Interactions	16
1.5 OOCYTE AND ENERGY METABOLISM	18
1.6 METABOLIC PATHWAYS FOR ENERGY GENERATION	19
1.6.1 Glycolysis	19
1.6.2 The Tricarboxylic Cycle	21
1.6.3 Oxidative Phosphorylation	23
1.6.4 Pentose Phosphate Pathway	24
1.6.5 Beta-Oxidation Of Fatty Acid	26
1.7 ENERGY SUBSTRATES IN OOCYTES AND EMBRYO METABOLISM	28
1.7.1 Carbohydrates	28
1.7.2 Amino Acids	32
1.7.3 Fatty Acids	33
1.8 MITOCHONDRIA	36
1.8.1 Mitochondrial Genetic System	37
1.8.2 Mitochondria During Oocyte Development	38
1.8.3 ATP Requirement During Oocyte Maturation	39
1.9 PREIMPLANTATION EMBRYO DEVELOPMENT AND EMBRYONIC GENOME ACTIVATION	40
1.10 AGEING AND OOCYTE QUALITY	41
1.10.1 Oocyte Ageing And Developmental Potential	43
1.10.2 Prepubertal Oocytes And Developmental Potentials	46
1.10.3 Molecular Mechanisms Of Age Oocyte Competence	48
1.10.4 Study Model Of Ageing	48
1.11 HYPOTHESIS	49
CHAPTER 2. GENERAL MATERIALS AND METHODS	52
2.1 PREPERATION OF GLASSWARE	52
2.2 PREPARATION, CALIBRATION AND MAINTENANCE OF SILICONISED NANOLITRE PIPETTES	52
2.2.1 Preparation Siliconised Glass Nanolitre Pipettes	52
2.2.2 Nanolitre Pipette Calibration	54
2.2.3 Nanolitre Pipette Maintenance	54

2.3	PREPARATION OF SILICONISED OIL WELLS	54
2.4	PREPARATION OF MINERAL OIL FOR CULTURE	55
2.5	MEDIA PREPARATION	55
2.5.1	Ovary Washing Medium	55
2.5.2	Ovary Holding Medium (Follicle Isolation Medium)	55
2.5.3	Oocyte Holding Medium (H199+)	56
2.5.4	Oocyte Maturation Medium	57
2.5.5	Modified Oocyte Maturation Medium For Amino Acid Profiling	57
2.6	OVINE REPRODUCTIVE TISSUE COLLECTION	59
2.7	HARVESTING OF SHEEP CUMULUS OOCYTE COMPLEX	60
2.8	OOCYTE DENUDATION AND <i>IN VITRO</i> MATURATION	61
2.9	<i>IN VITRO</i> MATURATION OF OVINE CUMULUS OOCYTE COMPLEX	65
2.10	OOCYTE CULTURE FOR MEASUREMENT OF AMINO ACID TURNOVER PROFILING	65
2.11	OOCYTE CULTURE FOR MEASUREMENT OF GPL METABOLISM	67
2.12	STORAGE OF SAMPLES	68
2.13	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY	69
2.13.1	HPLC Buffers, Solutions And Amino Acid Standards	69
2.13.2	HPLC Sample Preparation And Analysis	72
2.13.3	Amino Acid Calculation	75
2.14	DATA MANAGEMENT AND STATISTICAL ANALYSIS	78
CHAPTER 3: EVALUATION OF CHROMATIN CONFIGURATION IN GERMINAL VESICLE OOCYTES IN RELATION TO AGE AND QUALITY		81
3.1	INTRODUCTION	81
3.2	MATERIAL AND METHODS	84
3.2.1	Harvesting And Denudation of Cumulus Oocyte Complexes	84
3.2.2	Chromatin Staining	85
3.2.3	System Used to Classify Ovine Germinal Vesicle Oocyte	86
3.2.4	Composition, Preparation And Culture Of Modified Amino Acid Profiling Medium	88
3.2.5	Experimental Design	91
	Experiment 1: Characterization Of The Pattern Of Chromatin Configuration In Ovine GV-Staged Oocytes Using DAPI Staining	91
	Experiment 2: Assessment Of Ovine GV Chromatin Configuration After A 6 Hours Incubation In AAP Medium	91
	Experiment 3: Evaluation Of The Effect Of Denudation On The Dynamics Of Oocyte Chromatin Configuration Over 6 Hours	92
	Experiment 4: Effect Of The PDE Inhibitor Cilostamide On Chromatin Configuration	93
	Experiment 5: Effect of Cilostamide on Amino Acid Metabolism	94
	Experiment 6: Age And Chromatin Configuration	94
3.2.6	Statistical Analysis	95
3.3	RESULTS	95
3.3.1	Experiment 1: Characterization Of The Pattern Of Chromatin Configuration In Ovine GV-Staged Oocytes Using DAPI Staining	95
3.3.2	Experiment 2: Ovine Follicular Size And GV Oocyte Chromatin Configuration	96
3.3.3	Experiment 3: Effect Of Denudation On The Dynamics Of Oocyte Chromatin Configuration Over 6 Hours	98

3.3.4	Experiment 4: Effect Of Cilostamide On Oocyte Chromatin Configuration	99
3.3.5	Experiment 5: Effect Of The Phosphodiesterase Inhibitor On Amino Acid Metabolism	101
3.3.6	Experiment 6: Impact Of Age On Chromatin Configuration	106
3.4	DISCUSSION	108
3.4.1	Classification Of Ovine Chromatin Configuration	108
3.4.2	Effect Of Ovine Follicular Size On Chromatin Configuration	109
3.4.3	Effect Of Denudation On The Dynamics Of Oocyte Chromatin Configuration	112
3.4.4	Delayed Spontaneous Premature Oocyte Maturation By Cilostamide, A Phosphodiesterase-Inhibitor	114
3.4.5	Effect Of Cilostamide On GV-Staged Oocyte Amino Acid Metabolism	116
3.4.6	Pilot Study On Impact Of Age On Chromatin Configuration	118
3.4.7	Conclusion	121
CHAPTER 4. IMPACT OF MATERNAL AGE ON METABOLIC INDICES OF OOCYTE COMPETENCE		125
4.1	INTRODUCTION	125
4.2	MATERIALS AND METHODS	128
4.2.1	Experimental Design	128
	Experiment 1: Effect Of Reproductive Age On Oocyte Amino Acid Turnover	128
	Experiment 2: Effect Of Reproductive Age On Oocyte Energy Metabolism	128
4.2.2	Harvesting Cumulus Oocyte Complexes And Denudation	129
4.2.3	Carbohydrate Quantification	129
4.2.4	Quantification Of Oocyte Carbohydrate Metabolism Using An Ultramicrofluorometric Assay	133
4.2.5	Statistical Analysis	140
4.3	RESULTS	140
4.3.1	Effect Of Ageing On Oocyte Amino Acid Profiling	140
4.3.2	Effect Of Ageing On Oocyte Energy Metabolism	143
4.3.3	Relationship Between GPL Utilization And Oocyte Chromatin Configuration	145
4.3.4	Relationship Between Amino Acid Metabolism And Oocyte Chromatin Configuration	150
4.4	DISCUSSION	156
4.4.1	Effect Of Reproductive Age On GV-Staged Oocyte Amino Acid Metabolism	156
4.4.2	Effect Of Reproductive Ageing On Oocyte Energy Metabolism	161
4.4.3	Relationship Between Metabolism, Age And Chromatin Configuration	163
4.4.4	Conclusion	165
CHAPTER 5. EFFECT OF AGE ON THE EXPRESSION OF OOCYTE GENETIC MARKERS		167
5.1	INTRODUCTION	167
5.2	MATERIALS AND METHODS	170
5.2.1	Oocyte Sample Preparation And RNA Extraction	170

5.2.2	DNA Decontamination	171
5.2.3	cDNA Library Construction	171
5.2.4	Verification Of cDNA Library Product (cDNA Smear)	176
5.2.5	Bovine Primer Testing	181
5.2.6	Customized Array Using Bovine Primers	186
5.2.7	Gene Expression Analysis by Real-Time PCR	187
5.2.8	Statistical Analysis Of qPCR Data	191
5.3	RESULTS	191
5.3.1	Customized Candidate Genes Into The Study	191
5.3.2	Differential Gene Expression Analysis Across Ovine GV- And MII-Staged Oocytes From Prepubertal And Adult Animals	194
5.4	DISCUSSION	201
5.4.1	Validation of cDNA Libraries Construction	201
5.4.2	Selection Of The Internal Reference Genes	202
5.4.3	Differential Gene Expression In Oocytes Between Prepubertal And Adult Sheep	204
5.4.4	Conclusion	226
CHAPTER 6: AGEING AND CELLULAR MARKERS: EVALUATION OF OOCYTE MITOCHONDRIAL DNA COPY NUMBER AND ENERGY		229
6.1	INTRODUCTION	229
6.2	MATERIALS AND METHODS	232
6.2.1	Experimental Design	232
	Experiment 1: Quantitation Of Mitochondrial DNA Copy Number Of Immature And Mature Ovine Oocytes Harvested From Prepubertal And Adult Sheep	233
	Experiment 2: Measurement Of Energy Metabolism And Mitochondrial DNA Copy Number In Prepubertal And Adult Ovine Oocytes	234
	Experiment 3: The Impact Of Oocyte Culture Media Composition On Mitochondrial DNA Copy Number	235
6.2.2	Oocyte DNA Extraction And Analysis	235
6.2.3	Quantification Of Mitochondrial DNA Copy Number Using Real-Time PCR	238
6.2.4	Statistical Analysis	240
6.3	RESULTS	241
6.3.1	Experiment 1: Quantitation Of mtDNA Copy Number Of Immature And Mature Oocytes Harvested From Prepubertal And Adult Sheep	241
6.3.2	Experiment 2: Measurement Of Energy Metabolism And Mitochondrial DNA Copy Number In Prepubertal And Adult Sheep Oocytes	242
6.3.3	Experiment 3: The Impact Of Oocyte Culture Media Composition On Mitochondrial DNA Copy Number	245
6.4	DISCUSSION	248
6.4.1	High Variation Of Mitochondrial DNA Copy Number Within Oocytes	248
6.4.2	Mitochondrial DNA Copy Number During Oocyte Maturation	250
6.4.3	Impact of Age On Mitochondrial DNA Copy In Immature And Mature Oocytes	251

6.4.4	The Impact Of Age On Carbohydrate Metabolism Especially Pyruvate Consumption	254
6.4.5	Mitochondrial DNA Copy Number And Energy Metabolism	255
6.4.6	The Relationship Between Mitochondrial DNA Copy Number And Mitochondrial Function	256
6.4.7	Effect Of Culture Media On Mitochondrial DNA Copy Number	257
6.4.8	Conclusion	259
CHAPTER 7. GENERAL DISCUSSION		263
7.1	INTRODUCTION	263
7.2	THE STUDY MODEL	264
7.3	AGE ASSOCIATED CHANGES IN CHROMATIN REMODELING	265
7.4	AGE ASSOCIATED WITH ALTERATION OF ENERGY METABOLISM LINKED TO COMPENSATORY MITOCHONDRIAL DNA REPLICATION	267
7.5	THE EFFECT OF OOCYTE AGE ON GENETIC AND EPIGENETIC MARKERS	269
7.6	FUTURE DIRECTIONS AND PERSPECTIVES	270
7.7	CONCLUSION	272
REFERENCES		273
Appendix I	Suppliers Addresses	327
Appendix II	Preparation Stocks For Culture	329
Appendix III	Preparation Stocks For GPL Assays	332
Appendix IV	Preparation Stocks For Molecular Analysis	336

List of Tables

Table 1.1	Maximal oocyte formation and remaining at birth among species	3
Table 1.2	Genes and signaling pathways involved in the formation of germ cells, follicle assembly and follicle activation	5
Table 1.3	Characteristics and dynamics of cytoplasmic organelles during oocyte maturation	14
Table 2.1	Composition of follicle isolation medium (FIM)	56
Table 2.2	Composition of oocyte holding medium 199+ (H199+)	56
Table 2.3	Composition of ovine, serum-free oocyte maturation medium composition	58
Table 2.4	Composition of ovine amino acid profiling-AAP medium	59
Table 2.5	Preparation of buffers A and B for HPLC	70
Table 2.6	Amino acid additives for 2.5mM stock	71
Table 2.7	Preparation of 10 μ M amino acids standard solution	71
Table 3.1	Classification of chromatin configuration of ovine GV-staged oocytes	86
Table 3.2	Composition of ovine modified serum-free AAP medium	89
Table 3.3	Distribution of chromatin configuration patterns of ovine GV-staged oocytes	96
Table 3.4	Distribution of chromatin configuration patterns of oocytes derived from medium (MAF) and large antral follicles (LAF) of prepubertal and adult sheep.	97
Table 3.5	Distribution of chromatin configuration of denuded oocytes derived from prepubertal and adult sheep at 0, 3 and 6 hrs	98
Table 3.6	Comparison of HEPES effect on oocyte chromatin configuration at 0, 3 and 6 hours incubation.	99
Table 3.7	Amino acid profiles from GV-staged ovine oocytes from 4 types of AAP media with /without cilostamide	104
Table 4.1	Composition of modified KSOM for oocyte metabolism incubations	130
Table 4.2	Glucose, pyruvate and lactate reaction mixtures	132
Table 4.3	Preparation of standards and quality controls for ovine oocyte glucose, pyruvate and lactate assays	133
Table 4.4	Amino acid profiles by GV-staged oocytes between prepubertal lambs and adult ewes	142
Table 4.5	Carbohydrate metabolism by GV-staged oocytes between prepubertal lamb and adult ewe oocytes	144
Table 4.6	Carbohydrate metabolism by sheep GV-staged oocytes between prepubertal lambs and adult ewes: subgroup analysis	146
Table 4.7	Amino acid profiles in subgroup analysis by sheep GV-staged oocytes between prepubertal lamb and adult ewes	153
Table 5.1	Composition of RNAGEM Extraction Reagent Mastermix	170
Table 5.2	Composition of the first strand cDNA construction reaction	174
Table 5.3	Preparation for Master Mix Reaction of Long Distance PCR for cDNA Amplification	175
Table 5.4	Primer sequences of housekeeping genes and oocyte specific genes	179

Table 5.5	A customized real-time PCR array primer sequences originally designed to work with bovine oocytes and embryos	182-185
Table 5.6	Function of candidate housekeeping genes for normalisation	187
Table 5.7	Customized array for ovine cDNA libraries testing (validated primers)	188
Table 5.8	Composition of real-time PCR reaction	190
Table 5.9	Thermal cycler program for real-time PCR	190
Table 5.10	Differentially-expressed genes in prepubertal from adult GV-staged oocytes	195
Table 5.11	Age-related differences in gene expression of MII-staged oocytes between prepubertal and adult animals	195
Table 5.12	Summarized potentially stage-specific genes	200
Table 6.1	Composition of lytic cocktail	237
Table 6.2	Quantification of mtDNA copy number in denuded GV and MII oocytes derived from prepubertal and adult sheep	242
Table 6.3	Glucose and pyruvate and lactate consumption/production (pmol/oocyte/H) by prepubertal and adult sheep oocytes	243

List of Figures

Figure 1.1	Model demonstrates how an oocyte maintains the meiotic arrest in the follicle prior to nuclear maturation	8
Figure 1.2	Model demonstrates the bi-directional communication in regulating meiosis arrest and activating meiotic resumption after LH-triggered oocyte maturation.	9
Figure 1.3	Changes in oocyte cAMP levels, MPF and MAPK activity during oocyte maturation.	11
Figure 1.4	Glucose metabolism via glycolysis	20
Figure 1.5	The tricarboxylic acid cycle	22
Figure 1.6	Oxidative phosphorylation (OXPHOS)	24
Figure 1.7	The pentose phosphate pathway	25
Figure 1.8	The carnitine shuttle	27
Figure 2.1	Diagram of a siliconized nanolitre pipette set (top) and schematics of a siliconised glass oil well (bottom)	53
Figure 2.2	Representative reproductive tracts and corpus luteum from sheep tissues	61
Figure 2.3	Cumulus Oocyte Complexes before (A-B) and after (C-D) 24 hours of IVM under brightfield microscope	63
Figure 2.4	Representative denuded MII oocytes from adult sheep animals after 24 hours IVM	63
Figure 2.5	Illustration of the layout equilibration (EQ) and AAP dishes	66
Figure 2.6	Illustration of the layout equilibration (EQ) and GPL dishes	68
Figure 2.7	Amino acid incubation dish and dilution of samples for HPLC analysis into an HPLC vial	72
Figure 2.8	Representative example of HPLC analysis of spent AAP medium by oocytes and control samples	74
Figure 3.1	Schematics of the prepared chromatin staining slide	86
Figure 3.2	Demonstration of classification of sheep GV chromatin configuration	87
Figure 3.3	Examples of dish-layouts used for the AAP media validation experiment	90
Figure 3.4	Preliminary result of age effect on chromatin morphology distribution by using pooled follicle size 2-5mm	97
Figure 3.5	Distribution of chromatin configuration in modified AAP media; negative control (DMN), DMSO vehicle control (DMO), 10 μ M (DM10) and 50 μ M (DM50) cilostamide supplementation	100
Figure 3.6	Average amino acid profiles of sheep GV oocytes following 6 hours incubation in 4AAP media	103
Figure 3.7	Average total net balance, depletion, appearance and turnover of 18 amino acids by sheep GV oocytes following 6 hours incubation in 4 different AAP media.	105
Figure 3.8	Average total net balance, depletion, appearance and turnover of 18 amino acids by sheep GV oocytes following 6 hours incubation in pooled control and pooled cilostamide supplemented AAP media	106
Figure 3.9	Comparison of the chromatin distribution between prepubertal lamb and adult sheep oocytes in relation to types of media	107

Figure 3.10	Comparison of the chromatin distribution between AAP and GPL media supplementation with 50µM cilostamide, in prepubertal lamb (A) and adult sheep (B) oocytes	107
Figure 4.1	Quantification of oocyte carbohydrate metabolism using an ultramicrofluorometric assay	131
Figure 4.2	Demonstration of standard curves used for the calculation of glucose, lactate and pyruvate concentration in spent-KSOM media	134
Figure 4.3	Demonstrate a siliconised glass oil well containing nanolitre volume drops for the quantification of NAD(P)H fluorescence	135
Figure 4.4	Amino acid profiles of sheep GV-staged oocytes obtained from 2-5mm sized follicles from prepubertal and adult ewe oocytes incubated for 6 hours in 50µM cilostamide supplemented AAP media	141
Figure 4.5	Amino acid turnover of sheep GV-staged oocytes obtained from 2-5 mm sized follicles between prepubertal lamb and adult ewe oocytes incubated for 6 hours in 50µM cilostamide supplemented AAP media	143
Figure 4.6	Carbohydrate metabolism of sheep GV-staged oocytes obtained from 2-5mm sized follicles between prepubertal lamb and adult ewe oocytes incubated 6 hours in 50µM cilostamide supplemented modified KSOM medium	144
Figure 4.7	Distribution of chromatin configuration between prepubertal lamb and adult ewe GV-staged oocytes incubated for 6 hours in modified KSOM medium containing 50µM cilostamide supplementation	146
Figure 4.8	Subgroup analysis of GV-staged oocytes incubated in 50µM cilostamide supplemented KSOM medium for 6 hours between prepubertal and adult animals corresponding to carbohydrate metabolism and chromatin configuration.	147
Figure 4.9	Glucose/Pyruvate/Lactate metabolism of GV oocytes from two age groups compared with regard to net-like or clump chromatin patterns	148
Figure 4.10	Glucose/Pyruvate/Lactate metabolism between 2 chromatin patterns (net-like and clump), in GV-staged oocytes incubated for 6 hours in modified KSOM medium with 50µM cilostamide supplementation	149
Figure 4.11	Chromatin configuration of sheep GV-staged oocytes incubated for 6 hours in 50µM cilostamide supplemented AAP medium between prepubertal lambs and adult ewes	151
Figure 4.12	Comparison of amino acid profiles in subgroup analysis for the relationship of amino acid metabolism and chromatin configuration between the 2 animal ages in sheep GV-staged oocytes incubated for 6 hours in AAP medium with 50µM cilostamide supplementation.	152
Figure 4.13	Amino acid turnover of sheep GV-staged oocytes in subgroup analysis between prepubertal lamb and adult ewes incubated for 6 hours in modified AAP media with 50µM cilostamide supplementation	154

Figure 4.14	Amino acid turnover between three chromatin patterns (net-like, condensed and clump), in GV-staged sheep oocytes incubated for 6 hours in modified AAP medium with 50 μ M cilostamide supplementation	155
Figure 5.1	A schematic of RNA extraction and DNase treatment workflow	171
Figure 5.2	Diagrammatic representation of Real Time reaction.	172
Figure 5.3	Representative examples of cDNA libraries quality check for individual oocyte cDNAs using 2% (w/v) agarose gel electrophoresis.	177
Figure 5.4	Agarose gel image of product size verification following real-time qPCR analysis.	178
Figure 5.5	Demonstrate the consistent of levels expression of the 3 housekeeping genes (A) and the 2 oocyte specific genes (B) used to confirm cDNA quality across 24 selected cDNA samples	180
Figure 5.6	Schematic a newly customized array plate using for 2 samples testing simultaneously.	186
Figure 5.7	Representative dissociation curves following real-time PCR	192
Figure 5.8	Representative of real-time PCR products of bovine array primers testing with ovine cDNA libraries (1:50 total RNA) on 2% (w/v) agarose gel.	193
Figure 5.9	Comparative gene expression between prepubertal and adult GV-staged sheep oocytes	196
Figure 5.10	Comparative gene expression between prepubertal and adult MII-staged sheep oocytes	197
Figure 5.11	Comparative gene expression between prepubertal GV- and MII-staged sheep oocytes	198
Figure 5.12	Comparative gene expression between adult GV- and MII-staged sheep oocytes	199
Figure 5.13	Effect of age on relative expression of key genes related to metabolic and growth	200
Figure 6.1	Correlation between mtDNA copy number and each parameter of energy metabolism per oocyte	244
Figure 6.2	Comparison mtDNA copy number postincubation in different culture media in lamb and adult sheep oocytes	246
Figure 6.3	Comparison ranks of mtDNA copy number postincubation in different culture media in lamb and adult sheep oocytes	247

LIST OF ABBREVIATIONS

α -MEM	Minimum Essential Medium Eagle Alpha Modification
AA	Amino acid
AAP	Amino acids profiling
ACAT1	Acetyl-Coenzyme A acetyltransferase 1
ACE	Adenylation control element
ACTB	Beta actin
ADP	Adenosine diphosphate
AHCY	Adenosylhomocysteinase
AKT	Protein Kinase B
AMH	Anti-Mullerian hormone
AMPK	5'AMP activated protein kinase
APC	anaphase-promoting complex
AREG	Amphiregulin
ART	Assisted Reproduction Therapy
ASCL2	Achaete-scute family bHLH transcription factor 2
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
Bax	Bcl-2-like protein 4
Bcl2	B-cell lymphoma 2
BHMT	Betaine-homocysteine S-methyltransferase
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
Bub	Budding Uninhibited By Benzimidazoles
CaMKII	Calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CAT	Choline acetyl transferase
CC	Cumulus cell
Ccnb1	Cyclin B1
CCNG1	Cyclin G1
CD9	Oolemma tetraspanin protein
CDC	cell division cycle
CDH1	CDC20 homologue 1
CDK1	Cyclin Dependent Kinase 1
cDNA	Complementary deoxyribonucleic acid
CDX2	Caudal type homeobox 2
CEEFs	Cumulus-expansion enabling factors
CFL1	Cofilin 1
CG	Cortical granules
cGMP	Cyclic guanosine monophosphate
c-kit	Cognitive Kit ligand receptor
CNP	C-type natriuretic peptide
CO ₂	Carbon dioxide
COCs	Cumulus oocyte complexes
COI	Cytochrome C oxidase I
COX2	Cyclo-oxygenase 2
CPEB	Cytoplasmic polyadenylation element binding protein

CPT	Carnitine palmitoyltransferase
Cx	Connexin
DABA	2,4-diaminobutyric acid
DAPI	4',6-diamidino-2-phenylindole
DAZL	Deleted in azoospermia-like
DHFR	Dihydrofolate reductase
DMN	Medium without DMSO
DMO	Medium with DMSO
DMSO	Dimethyl sulfoxide
DM10	Dimethyl sulfoxide plus cilostamide 10uM
DM50	Dimethyl sulfoxide plus cilostamide 50uM
DNA	Deoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase
DPBS	Dulbecco's phosphate buffered saline
DTT	Threo-1,4-Dimercapto-2,3-butanediol
dsDNA	double stand Deoxyribonucleic acid
EBSS	Earle's Balance Salt Solution
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHMT2	Euchromatic histone-lysine N-methyltransferase 2
ELP3	Elongator acetyltransferase complex subunit 3
EMI1	Early mitotic inhibitor 1
EPPS	N-2-hydroxyethyl piperazine-N'-3-propanesulfonic acid
EQ	Equilibration dish
ER	Estrogen receptor
EREG	Epiregulin
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FAD	flavin adenine dinucleotide
FADH2	flavin adenine dinucleotide
FASN	fatty acid synthase
FF	Follicular fluid
FGF8B	Fibroblast growth factor 8B
FGFR1	Fibroblast Growth Factor Receptor 1
FIGLA	Factor in germline alpha
FILIA	Maternal effect gene
FIM	Follicle isolation medium
FOLR	Folate Receptor
FOXO3	Forkhead box O3
FSH	Follicle Stimulation Hormone
FSHR	Follicle-stimulating hormone receptor
FSK	Forskolin
GAPDH	Glyceraldehyde phosphate dehydrogenase
GC	Granulosa cell
GDF9	Growth-differentiation factor 9
GDHB	NAD-specific glutamate dehydrogenase
GI	Glycolytic Index

GLUT	Glucose transporter
GLYT	Glycine transporter
GMP	Guanosine monophosphate
Gn	Gonadotropin
GPI	Glycosylphosphatidyl-inositol anchored proteins
GPL	Glucose-pyruvate-lactate
GPR	G-protein receptor
GPT2	Glutamic pyruvate transaminase
GREM	Gremlin
GSH	Glutathione peroxidase
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
GTSF	Gametocyte Specific Factor
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
G6PD	Glucose 6 phosphate dehydrogenase
H19	Maternally expressed transcript
H2A	Histone A2
HAS2	Hyaluronan synthase 2
HAT1	Histone acetyltransferase 1
HCL	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEPES-MEM	Minimum Essential Medium Eagle HEPES Modification
HK	Hexokinase
HKs	Housekeeping genes
HPLC	High-performance liquid chromatography
IBMX	3-isobutyl-1-methylxanthine
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3
IGF2	Insulin-like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding protein
IGFR	Insulin Like Growth Factor Receptor
IL	Interleukin
IMM	Inner mitochondrial membrane
INSR	Insulin receptor
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
JAK	Janus Kinase
KAT5	Lysine acetyltransferase 5
KDM1B	Lysine demethylase 1B

KHDC3L	KH Domain Containing 3 Like, Subcortical Maternal Complex Member
KL	Kit ligand
KSOM	Potassium simplex optimised medium
LAF	Large antral follicle
LD PCR	Long distance PCR
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
L-LDH	L-lactate dehydrogenase
MAD2	Mitotic Arrest Deficient 2
Mad2	Mitotic Arrest Deficient 2
MAF	Medium antral follicle
MAPK	Mitogen-activated protein kinase
MAT	Methionine adenosyltransferase
MCT	Monocarboxylate carrier family
MEA	Mouse Embryo Assay
MEG	Maternally expressed
MEM	Minimum Essential Media
MEST	Mesoderm specific transcript
MGC	Mural granulosa cell
MI	Metaphase stage of meiosis I
MII	Metaphase stage of meiosis II
Mili-Q	Deionised water
MIMT1	MER1 repeat containing imprinted transcript 1
MOS	Proto-oncogene serine/threonine-protein kinase mos
MPF	Maturation promoting factor
mRNA	Messenger ribonucleic acid
MSK2[RPS6KA4]	Ribosomal protein S6 kinase, 90kDa, polypeptide 4
mtDNA	Mitochondrial DNA
mtRNAPol	mitochondrial DNA polymerase γ
mtSSBP	mitochondrial single-stranded DNA-binding protein
MTHFR	Methylenetetrahydrofolate reductase
mTOR	Mechanistic target of rapamycin
MYT1	Myelin transcription factor 1
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
nl	Nanolitre
NLRP	NLR Family Pyrin Domain Containing
NO	Nitric oxide
NPPC	Natriuretic peptide protein C
Npr2	Natriuretic peptide receptor 2
Nrf	Nuclear respiratory factor
NSN	Non-surrounded nucleolus
ODS	Octadecylsilyl

OMM	outer mitochondrial membrane
OOEP	Oocyte Expressed Protein
OPA	O-phthaldialdehyde
OXPPOS	Oxidative phosphorylation system
P450	Cytochrome p450
P450SCC	Cytochrome p450 side chain-cleavage enzyme
p53	Tumor protein p53
PADI6	Peptidyl arginine deiminase, type VI
PB	Polar body
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PDE	Phosphodiesterase
PDK	Pyruvate dehydrogenase kinase
PEG10	Paternally expressed 10
PEG3	Paternally expressed 3
PFA	Paraformaldehyde
PGCs	Primordial germ cells
PGE2	Prostaglandin E2
PGK1	Phosphoglycerate kinase 1
PGS-2	Prostaglandin endoperoxide synthase-2
pH	Expression of the acidity or alkalinity of a solution
PHLDA2	Pleckstrin homology-like domain, family A, member 2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PLAGL1	Pleiomorphic adenoma gene-like 1
PLCz	Phospholipase C zeta
PN	Pronuclear
poly-A	polyadenylation
POLG	mitochondrial polymerase gamma
POU5F1	POU Class 5 Homeobox 1
PPP	Pentose phosphate pathway
PRDM	PR Domain Zinc Finger Protein
PRDX	Peroxiredoxin
PRMT5	Protein arginine methyltransferase 5
PRPP	phosphoribosyl pyrophosphate
PTEN	phosphatase and tensin homolog
Ptgs2	Cyclooxygenase 2
PTX	Pentraxin
PVS	Peri-vitteline space
R3 IGF1	Recombinant analogue of human IGF1
RBBP8	RB Binding Protein 8
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPLP0	Ribosomal protein large P0
RT	reverse transcription
SAC	spindle assembly checkpoint

SAM	S-adenosyl-methionine
SCMC	Subcortical maternal complex
sERC	Smooth endoplasmic reticulum cluster
SETD7	SET domain containing lysine methyltransferase 7
SETDB1	SET domain bifurcated 1
SGLT	sodium-glucose linked transporters
SHMT	Serine hydroxymethyltransferase
SLC	Solute carrier family
SMCb1	Structural maintenance of chromosomes protein b1
SN	Surrounded nucleolus
SNRPN	Small nuclear ribonucleoprotein polypeptide N
Sogl2	Shugoshin-like 2
SPOM	Simulated physiological oocyte maturation
SR-BI / SCARB1	Scavenger receptor class B member 1
STK	Serine/Threonine Kinase
SYBR Green	DNA binding stain
TAp73	Transcriptionally active p73
TBE	Tris-borate ethylenediaminetetraacetic acid
TCA	Tricarboxylic acid
TCM199	Tissue culture medium 199
TET	Tet methylcytosine dioxygenase
Tex19.1	Testis-expressed protein 19.1
TFAM	Mitochondrial Transcription Factor
TGFB	Transforming growth factor beta
TLE6	Transducin Like Enhancer Of Split 6
TNFAIP	Tumor necrosis factor alpha-induced protein
Tpx2	Targeting protein for Xklp2
TRDMT1	tRNA aspartic acid methyltransferase 1
TRIM28	Tripartite motif containing 28
Trp	Triphthophan
TSC1	Tumor suppressor tuberous sclerosis complex 1
TSSC4	Tumor suppressing subtransferable candidate 4
TUBB	Tubulin beta class
TZP	Transzonal cytoplasmic projections
UHRF1	Ubiquitin like with PHD and ring finger domains 1
USP29	Ubiquitin specific peptidase 29
WEE1	Wee1-like protein kinase
XIST	X (inactive)-specific transcript
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase
ZNF	Zinc finger-containing
ZP	zona Pellucida

List of Presentations

- Lu, J., **Topipat, C.**, Iles, D., McKeegan, P., Warburton, P., Huntriss, J.D., and Picton, H.M. (2018) Characterization of ovine GV and MII oocyte RNA polyadenylation length modifications using single cell RNA-sequencing. Oral presentation (Short Paper Session B3.2) at the Fertility Meeting, Liverpool, UK.
- **Topipat, C.**, McKeegan, P., Lu, J., Collado Fernandez, E., Huntriss, J.D., and Picton, H.M. (2017) Energy metabolism, chromatin configuration, and gene expression between prepubertal and adult germinal-stage oocytes as indices to determine oocyte quality. Poster presentation (P1-33) at the Fourth World Congress of Reproductive Biology, Okinawa, Japan.
- **Topipat, C.**, Lu, J., McKeegan, P., Collado Fernandez, E., Huntriss, J.D., and Picton, H.M. (2017) The impact of reproductive age on metabolism and gene expression indices of oocyte quality in germinal staged oocytes. E-poster presentation (P-404) at the 33rd Annual Meeting of European Society of Human Reproduction and Embryology, Geneva, Switzerland.
- Gnanaprabha, K., **Topipat, C.**, Lu, J., McKeegan, P., and Picton, H.M. (2017) Mitochondrial indices of ovine oocyte maturation *in vitro*. Oral presentation (Short Paper Session B1.4) at the Fertility Meeting, Edinburgh, UK.
- **Topipat, C.**, Lu, J., Collado Fernandez, E., McKeegan, P., Huntriss, J.D., and Picton, H.M. (2017) Mitochondrial markers associated with maternal age during ovine oocyte maturation *in vitro*. Oral presentation (Short Paper Session B1.5) at the Fertility Meeting, Edinburgh, UK.
- **Topipat, C.**, Lu, J., Collado Fernandez, E., Huntriss, J.D., and Picton, H.M. (2015) The effect of reproductive ageing on chromatin configuration and amino acid metabolism in germinal vesicle staged sheep oocytes *in vitro*. Poster presentation (P-052) at the Society of Reproduction and Fertility Annual Conference, Oxford, UK.
- **Topipat, C.**, Lu, J., Collado Fernandez, E., Huntriss, J.D., and Picton, H.M. (2015) The dynamics of chromatin configuration during ovine oocyte maturation *in vitro*. Oral Presentation (Short Paper Session A1.1) at the Fertility Meeting, Birmingham, UK.

CHAPTER 1: GENERAL INFORMATION

1.1. INTRODUCTION

Reproduction is one of the most important biological processes that enable the existence of species. In the female, the fundamental unit of mammalian reproduction is the oocyte. It cooperates with sperm from the male to form an embryo that will ultimately develop into the next generation. Numbers of studies have established the link between oocyte competence and embryo development prior to embryonic genome activation (Heikinheimo and Gibbons, 1998, Smith, 2001, Swain and Pool, 2008). Impaired oocyte growth leads to incomplete meiotic maturation, failed fertilisation, and arrested embryo development (Swain and Pool, 2008). Even though this relationship is well accepted, many aspects of the interrelation between oocyte quality and embryo development remain to be established.

The human ovary is generally believed to contain a limited number of primordial follicles in the ovarian pool. It is established during fetal life around mid-gestation before the number of resting follicles decline in later stages of life from late gestation and after birth through puberty until menopause, see review (Hartshorne *et al.*, 2009). The decrease in fertility potential in advanced age women is evident by diminished ovarian reserve, which reflects a decrease in both follicle number and oocyte quality (Faddy *et al.*, 1992).

The exhaustion of the ovarian reserve concerning primordial follicle depletion results in the subsequent decrease in the number of growing follicles as well as a decline in their oocyte quality. This depletion may occur as a result of ageing, chemotherapy or radiation treatment, or other factors including genetic causes such as pre-mutation of fragile X (Tejada *et al.*, 2008), Turner syndrome (Lunding *et al.*, 2015), and premature ovarian insufficiency all of which lead to fertility problems. The effects of age on fertility are significantly more pronounced in women after the age of 35 (Karamahmutoglu *et al.*, 2014). These phenomena are evident not only in natural conception but also in assisted reproduction cycles (Heffner, 2004).

A striking trend in developed societies is that women are likely to postpone parenthood and expect to have their children towards the latter stages of their

fertile lifespan; in other words, conception will be delayed until the late reproductive years. Unsurprisingly, these individuals are confronted with an age-related decline in their fecundity which is accompanied by an increased risk of adverse pregnancy outcome (Dunson *et al.*, 2004, Steuerwald *et al.*, 2007, Tatone *et al.*, 2008, Wilding *et al.*, 2005). In the face of this societal change further research is needed to define and explore how oocyte biology and fertility changes with age. Besides this, research helps us to gain knowledge and to improve the culture condition *in vitro* as well as to select potential oocytes for further development. This review will provide an overview of the present knowledge concerning folliculogenesis and oogenesis and the impact of ageing on ovarian function and oocyte competency. It will also provide an understanding of the genetic and metabolic markers used to determine the developmental potential of oocytes. Data have been reviewed from mammalian models and human studies.

1.2. OVARIAN RESERVE

During ovarian follicle development and selection, the fate of the vast majority of follicles is for them to become atretic and degenerate. In contrast, few follicles will complete development and progress to the final stages of follicular maturation with the ovulation of a mature oocyte into the oviduct. The mammalian ovary contains a limited supply of oocytes. In humans, primordial germ cells (PGCs) are in the active stage of germline expansion during PGCs migration to the ovary, and they endure extensive proliferation by mitosis to form diploid oogonia from the 7th week of gestation (Kurilo, 1981). Oogonia entering meiosis are now termed 'oocytes' and they progress to meiosis-I, through the leptotene, zygotene and pachytene stages before meiotic arrest at the diplotene stage of prophase I. When the maximum quantity of approximately 6-7 million germ cells is reached during mid-gestation, oocytes become encapsulated into pre-granulosa cells (GCs) to make up the primordial follicles (Hartshorne *et al.*, 2009). Oogonial mitosis synchronously deteriorates and stops by the end of the second trimester, in parallel, the rate of atresia gradually rises. By the term, only 1-2 million primordial follicles remain in human ovary, and the figure significantly reduces to 200,000-300,000 germ cells at puberty (Oktem and Oktay, 2008, Oktem and Urman, 2010, Pepling and Spradling, 2001) (Table 1.1).

Table 1.1. Maximal oocyte formation and remaining at birth among species
(van den Hurk and Zhao, 2005)

Species	Max number of oocytes	Number of oocytes at birth	Atretic rate (%)
Human	7,000,000	700,000	90
Cow	2,700,000	135,000	95
Sheep	900,000	82,000	91
Pig	1,200,000	500,000	58
Mouse	50,000-75,000	10,000-15,000	80

In other species including bovine and ovine, PGCs differentiate into oogonia between 3-6 weeks after conception with the key cellular events taking place as mentioned in human germ cells before the PGCs mitotically proliferate to reach a peak number in mid-gestation (Gondos *et al.*, 1971, Gosden, 2002, McNatty *et al.*, 2000, van den Hurk and Zhao, 2005). The maximum number of non-follicular germ cells is estimated from approximately 1 million in sheep and sows to 3-7 million in the ovary of cows and women (van den Hurk and Zhao, 2005). After that, when follicle formation begins, apoptosis accounts for approximately 90% of the oocyte loss in late fetal life within ovarian follicles (Juengel *et al.*, 2002, Reynaud and Driancourt, 2000, Smith *et al.*, 1993). Nonetheless, hundreds of thousands of oocytes are left over within the calf, gilt, sheep and human ovaries at birth (Palma *et al.*, 2012, van den Hurk and Zhao, 2005). In mice, ovarian programming occurs over a different time frame. It has been estimated that several thousand PGCs reach the gonads and carry on mitosis until the maximum number is present at the transition from mitosis to meiosis (Gondos *et al.*, 1971). The population of oocytes then dramatically decline such that approximately two-thirds are lost in the first week of postnatal life (Kerr *et al.*, 2006, Pepling and Spradling, 1998, Pepling and Spradling, 2001). Apoptosis seems to be the primary mechanism controlling the continuous loss of oocytes both in the pre- and post-natal periods in all vertebrate species (Oktem and Urman, 2010, Vaskivuo *et al.*, 2002).

1.3. FOLLICULOGENESIS

Both male and female germline formation begin with the traverse of PGCs along with the germ cell proliferation into gonadal ridges to form a cluster called the 'germ cell nest', which is encapsulated PGCs by the pre-GCs cells (de Cuevas *et al.*, 1997, Gondos *et al.*, 1971, Pepling *et al.*, 1999, Pepling and Spradling, 1998, Pepling and Spradling, 2001). The incomplete cytokinesis allows intercellular communication via cytoplasmic bridges within the individuals' nest. It is believed to promote some genotypes over others (see reviewed in (Pepling *et al.*, 1999) and to reduce defective germ cells (Braun *et al.*, 1989). When the developing fetus grows to the later stage of gestation, the germ cell nests breakdown, the cytoplasmic bridges disappear, and pre-GCs invade the nests and encompass oocytes to form individual primordial follicles (Pepling and Spradling, 2001). This is accompanied by a substantial loss of oocytes as a quality control mechanism to promote the preferential destruction of deficient oocytes via apoptosis (Albamonte *et al.*, 2008, De Felici *et al.*, 2008, Ghafari *et al.*, 2007, Jefferson *et al.*, 2006, Lobascio *et al.*, 2007, Pepling and Spradling, 2001). Several molecular mechanisms control the intricate processes from PGC formation, germ cell nest breakdown, and follicle assembly until primordial follicle formation, which require the coordination of transcription factors, multiple signalling pathways, and transposon repression as summarized in Table 1.2 (see reviews in (Grive and Freiman, 2015, Oktem and Urman, 2010, Sanchez and Smitz, 2012).

The process of folliculogenesis begins with primordial follicle formation and establishes the stockpile of the finite numbers of germ cells available for the entire female reproductive lifespan. The morphological hallmark of a primordial follicle is characterised by the presence of a single layer of flattened pre-GCs enclosing the meiotic arrested oocyte (primary oocyte). When animals reach puberty, the recruited follicles will grow through the primary, secondary, and preantral stage before the antral cavity acquires. At this stage, most of the antral follicles undergo atresia; however, under the influence of gonadotrophin, only few antral follicles can reach the preovulatory phase. In response to a gonadotropin surge, a dominant preovulatory-stage follicle will ovulate and release the mature, fertilizable oocyte into the oviduct awaiting sperm penetration for fertilisation (Gosden *et al.*, 1989, Gougeon, 1996, McGee and Hsueh, 2000, Picton *et al.*, 2003).

Table 1.2. Genes and signaling pathways involved in the formation of germ cells, follicle assembly and follicle activation. (Adapted from Collado-Fernandez, 2013, Grive and Freiman, 2015, Sanchez and Smitz, 2012)

Gene	Factor	Role
PGC formation		
BMP2	extracellular growth factor TGF B from endoderm	PGC induction
BMP4	extracellular growth factor TGF B from ectoderm origin	PGC formation; targeting mutation leading to the severe defect of PGC formation
BMP8b	extracellular growth factor TGF B from ectoderm origin	PGC formation; targeting mutation leading to the severe defect of PGC formation
BMP7	extracellular growth factor TGF B from ectoderm origin	PGF formation
Smad-1	intracellular signaling molecule of TGF B	lacking found fewer PMF formation
Smad-4	intracellular signaling molecule of TGF B	no PMF formation
Prdm1 (Blimp1)	transcriptional repressor	transcriptional repressor
Prdm14	transcriptional regulator	transcriptional regulator
PGC proliferation		
HOXA1	Homeobox protein Hox-A1	PGC specification
HOXB1	Homeobox protein Hox-B1	PGC specification
c-kit/KL	Tyrosine kinase receptor and its ligand	PGC migration and proliferation
PGC pluripotency		
DPPA3/Stella/PGC7	Developmental pluripotency associated 3, germ cell marker gene	Retention of germ cell fate and pluripotency
SOX2	SRY-related HMG box 2	maintain pluripotency
POU5F1 (OCT4)	POU class 5 homeobox transcription factor 1	maintain pluripotency
NANOG		maintain pluripotency
NANOS3	Nanos homolog3, anti-apoptotic activity	prevent apoptosis of PGC during migration to gonad
Fragilis	An interferon inducible gene	Germ cell competence
Oogonia to oocyte transition: Entry to MPI		
BAX	pro-apoptotic member of the Bcl2 family	require for the death of ectopic PGC during their migration
RSPO1/ β -catenin	proliferation and meiotic initiation	signaling promotes XX germ cell proliferation and entry into meiosis
BCL2	pro-apoptotic member of the Bcl2 family	oocyte program cell death
BCLX	anti-apoptotic member of the Bcl2	premature PGC cell death
Primordial follicle formation		
FIGLA	factor in germline alpha	essential for primordial follicle development
NOTCH	Notch signalling pathway	essential for primordial follicle development
Daz la	Deleted in azoospermia	cytoplasmic protein expressed in PGCs and pre-meiotic and meiotic germ cells
NGF	Nerve growth factor	reduced number of PMF in Ngf deficient mice; in vitro culture mouse ovaries, Ngf decreased apoptotic genes
AMH	Anti-mullerian hormone, TGF-B family	AMH-null mice increased recruitment of PMF into growing pool suggesting negative regulator during PMF to primary follicle transition
Activin A	TGF-B family	increase the number of PGCs
Nobox	homeobox-containing transcription factor newborn ovary homeobox	essential for oocyte survival; germ cell cyst breakdown and PMF development
Primordial follicle activation		
Foxo3	Forkhead transcription factor	PMF activation; Foxo3-null mice exhibit global follicular activation leading oocyte death and early depletion
AMH	Anti-mullerian hormone, TGF-B family	AMH-null mice increased recruitment of PMF into growing pool suggesting negative regulator during PMF to primary follicle transition
Tsc/mTORC1	Tuberous sclerosis complex, tumor suppressor gene, a negative regulator of mammalian target of rapamycin	keep PMF quiescent, lacking Tsc1 gene in mouse oocytes caused the entire PMF activated prematurely leading to POF
PTEN/PI3K	Phosphatase and tensin homolog, tumor suppressor, a major negative regulator of phosphatidylinositol 3-kinase	lacking PTEN the entire PMF pool become activate
p27	Cyclin-dependent kinase inhibitor 1B	Premature activation of PMF pool; proliferation and differentiation of pre-GC; early follicular depletion and POF
Foxl2	Winged-helix transcription factor	Premature growth of oocyte; arrested proliferation and differentiation of pre-GC; lack of primary follicles
TAF4B	TBP-associated Factor 4b	Impaired cyst breakdown; loss of PMF

Local autocrine and paracrine mediators initially drive dynamics of follicle development until growing follicles reach the preantral stage, they become responsive to gonadotropin exposure as evidence of follicle stimulating hormone (FSH) and luteinizing hormone (LH) receptors on the follicular granulosa and theca cells. However, developing follicles at this stage are dependent on the influence of a multitude of autocrine and paracrine signals and local intra-ovarian factors, rather than endocrine signals (McNatty *et al.*, 2007, Picton and Gosden, 2000). Progression to the preantral/early antral stage involves oocyte enlargement, zona pellucida formation, extensive proliferation to form a multilayered GCs, formation of a basal lamina, and recruitment of stromal cells to form theca layers enclosing the follicle basement membrane, followed by developing fluid-filled spaces between granulosa layers to gradually form a single antral cavity (Orisaka *et al.*, 2009, van den Hurk and Zhao, 2005, Young and McNeilly, 2010). Since follicles at this stage start to have a vascular supply, they can also respond to other endocrine factors transported through blood circulation apart from local intraovarian mediators signalling follicle development. Evidence shows the androgen receptor expression predominates in preantral follicles suggesting a physiological role of ovarian androgens in supporting follicle growth beyond that of a substrate precursor for estrogen production. Studies confirmed the androgen role in promoting the development of preantral to small antral stage follicles in several species such as rat (Tetsuka *et al.*, 1995), sheep (Juengel *et al.*, 2006), rhesus (Vendola *et al.*, 1998) and human (Otala *et al.*, 2004, Rice *et al.*, 2008).

At the final stage of follicular development, growing antral follicles to the preovulatory stage become critically dependent on FSH support. However, roles of autocrine and paracrine still influence the antral development in promoting granulosa cell proliferation and subsequently modulating FSH-dependent follicles. Growth-differentiation factor 9 (GDF9), Bone morphogenetic protein 15 (BMP15), and Bone morphogenetic protein 6 (BMP6), for example, are secreted factors derived from the oocytes while activin, inhibin, and BMP6 originate from the granulosa cells as reviewed (Knight and Glister, 2006). Incorporate with the regulatory control, changes of the follicular structures characterise by the enlargement of oocyte size and volume accompanied by a rapid increase in the volume of the fluid-filled antral cavity, combined with the continued proliferation of

granulosa and theca cells and increased thecal vascularisation. The histotypic complexity of the follicle structure imposes a limitation on the efficacy of secreted signalling molecules derived from theca, mural granulosa (MGC), cumulus granulosa cells (CC) and oocytes to transfer via diffusion dependent processes. Therefore, concentration gradients of secretory signalling molecules within follicles are generated in the different compartments to facilitate further growth and cellular differentiation (Knight and Glister, 2006).

1.4. OOCYTE MATURATION

1.4.1. Nuclear Maturation, Meiotic Arrest And Resumption

Meiotic progression initiates in the early stages of oocyte and follicle development, but following the first meiotic checkpoint the germinal vesicle (GV) oocyte is maintained at the diplotene stage of prophase of meiosis I until the oocyte becomes fully grown and meiosis is resumed following the preovulatory LH surge. It is well established that an elevation of intraoocyte cyclic adenosine monophosphate (cAMP) plays a crucial role in the maintenance of meiotic arrest within the oocyte in rodents (Mehlmann, 2005b) and humans (Mehlmann, 2005b, Nogueira *et al.*, 2003a). It is believed that cAMP is predominantly derived from exogenous influx from the adjacent CCs via gap junctions (Chaube and Misro, 2002, Dekel *et al.*, 1981, Sela-Abramovich *et al.*, 2006, Webb *et al.*, 2002) whereas endogenous generation of cAMP within the oocyte occurs via G-protein coupled receptor and subsequently adenylate cyclase activation is limited (DiLuigi *et al.*, 2008, Masciarelli *et al.*, 2004, Mehlmann, 2005a, Vaccari *et al.*, 2008). Like cAMP, later studies reported another factor (i.e., cyclic guanosine monophosphate (cGMP)) from the CCs may also act as an indirect regulator for the inhibition of meiotic resumption, as cGMP influx prevents the activation of the cAMP-PDE3A (cAMP-phosphodiesterase 3A) enzyme, which is responsible for the degradation of cAMP within the oocyte (Norris *et al.*, 2009) (Figure 1.1). Therefore the assumption could be drawn that high levels of intraoocyte cAMP are possibly the key determinant to maintain oocyte arrest before undergoing meiotic resumption.

The well-orchestrated interaction between the oocytes and the surrounding GCs is essential to the regulation of oocyte meiotic arrest. Oocyte-secreted factors promote the natriuretic peptide receptor 2 (Npr2) expression on CCs. In turn, increased

activation of Npr2 by its ligand, C-type natriuretic peptide (CNP) produced by the mural GCs, induces the conversion of guanosine triphosphate (GTP) to produce cGMP and this inhibits the activity of phosphodiesterase-3A (PDE3A) (Norris *et al.*, 2009, Vaccari *et al.*, 2008, Zhang *et al.*, 2010)(Figure 1.1). A recent study has identified oocyte-secreted factors, GDF9, BMP15 and Fibroblast growth factor 8B (FGF8B), as well as, oestradiol play a direct role in the regulation of the Nrp2 expression in CCs, and consequently in controlling oocyte meiotic arrest before ovulation (Zhang *et al.*, 2011a).

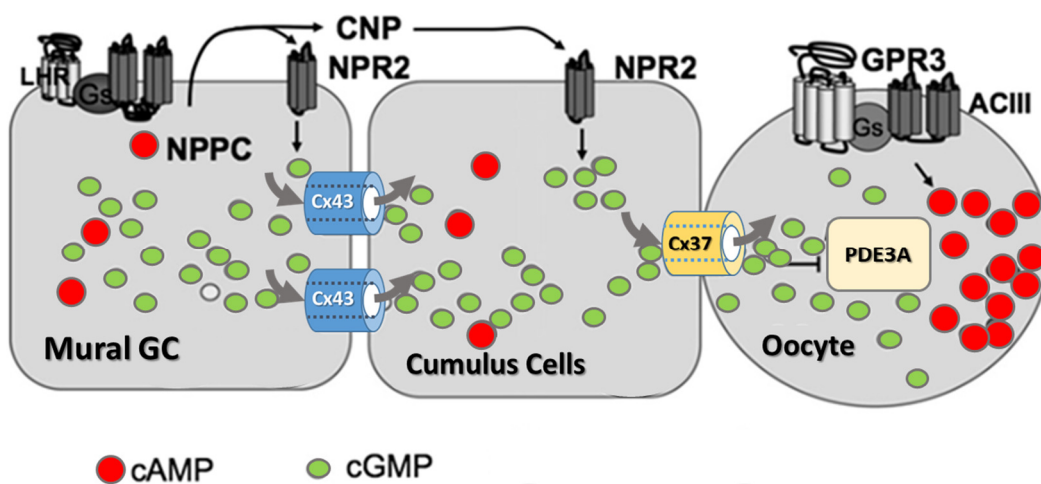


Figure 1.1. Model demonstrates how an oocyte maintains the meiotic arrest in the follicle prior to nuclear maturation. The natriuretic peptide protein C (NPPC) expressed in the mural granulosa cells (GC), produces C-type natriuretic peptide (CNP) ligands and probably secretes to extracellular space. Activated natriuretic peptide receptor 2 (NPR2) by CNP increases cGMP accumulation in both the mural and cumulus cells compartment. Then it diffuses via gap junctions (connexin-43 and -37) to inhibit phosphodiesterase-3A (PDE3A) enzyme hydrolyzing cAMP in the oocyte compartment. (Adapt from Conti *et al.*, 2012.)

The preovulatory LH surge initiates a cascade of meiotic maturation events that transform the arrested GV oocytes in the stage of prophase I through metaphase I (MI) of the first meiotic division and ending up with extrusion of the first polar body (PB). At this stage, the oocyte enters the second meiotic checkpoint and arrests at the metaphase II (MII) stage of meiosis II. Following the LH surge, the meiotic arrest of the oocyte at the GV stage is again overridden by lowering the total follicular cGMP content, initiating a drastic decline in cAMP levels within the oocyte as a result

of PDE3A activation, which actively hydrolysis intraoocyte cAMP (Zhang *et al.*, 2011a, Zhang *et al.*, 2010). Since the inhibitory factor(s) released from theca (e.g., hypoxanthine) and granulosa cells (e.g., cGMP, cAMP) pass to the oocyte via gap junctions, and the closure of these channels in response to the LH-surge induces the extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signalling pathway, this contributes to meiotic resumption as well (De Loos *et al.*, 1994, Sirard and Bilodeau, 1990, Sirard and First, 1988) (Figure 1.2). This evidence is supported by a decline of *Nppc* mRNA levels (encoding CNP) in mural GCs, leading to the limited production of cGMP in CCs to transport into the oocyte. Moreover, closure of the gap junctions may also contribute to this process, as a result of Epidermal growth factor receptor (EGFR) activation (Norris *et al.*, 2008, Sela-Abramovich *et al.*, 2005) (Figure 1.2).

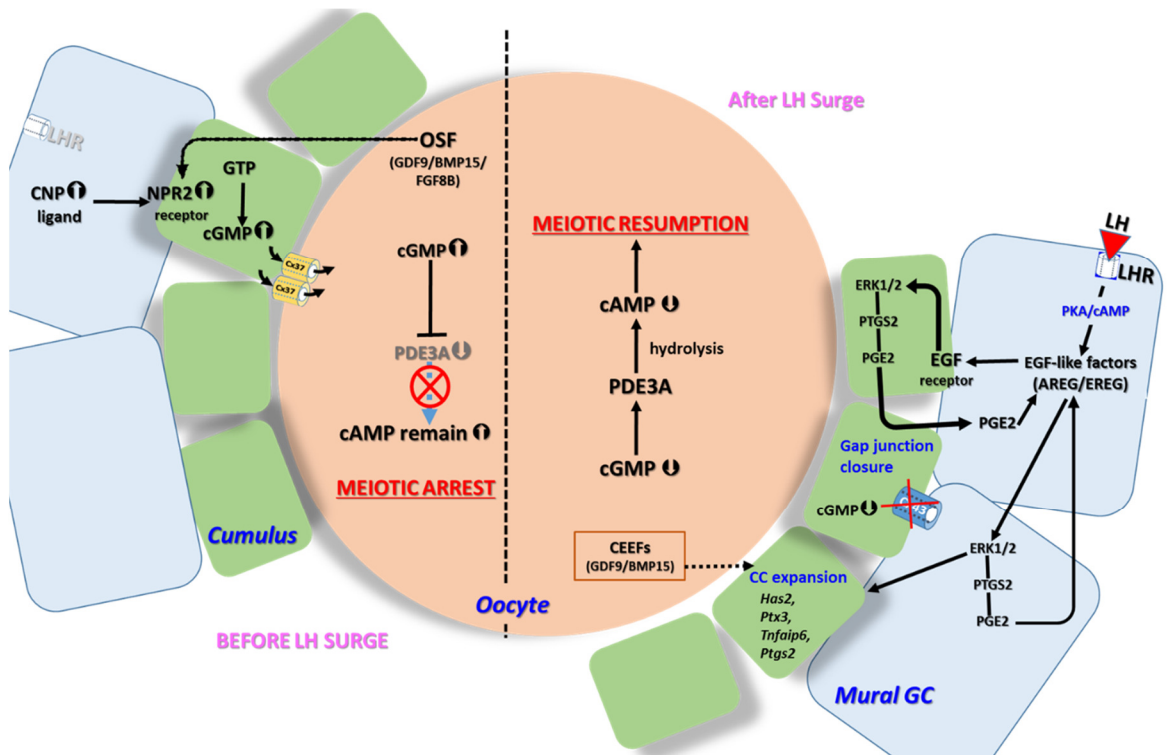


Figure 1.2. Model demonstrates the bi-directional communication in regulating meiosis arrest and activating meiotic resumption after LH-triggered oocyte maturation. Oocyte secreted factors (i.e., GDF9, BMP15, and FGF8B) upregulate NPR2 expressed in cumulus cells to induce GTP conversion to cGMP, which the high level then diffuses to the oocyte via gap junctions in order to maintain meiotic arrest (left panel). After LH-surge (right panel), the PKA signaling pathway is activated and induces the production of the EGF-like factors (i.e., AREG, EREG, etc.). These factors via EGFR activate a ERK1/2 cascade resulting in; 1) upregulating transcripts involved in cumulus expansion, 2)

enhancing the LH stimuli response through the production of PGE2 and subsequent EGF-like cascade, and 3) closing gap junction to inhibit cGMP transport into the oocyte and subsequently release an immature oocyte to progress to the meiotic resumption. GDF9: Growth-differentiation factor-9; BMP15: Bone morphogenetic protein-15; FGF8B: Fibroblast growth factor-8B; NPR2: Natriuretic peptide receptor 2; LH: Luteinising hormone; PKA: Protein kinase A; EGF: Endothelial growth factor; EGFR: Endothelial growth factor receptor; ERK: Extracellular signal-regulated kinase; AREG: Amphiregulin; EREG: Epiregulin; PGE2: Prostaglandin E2; PDE3A: Phosphodiesterase enzyme 3A; and cGMP: Cyclic guanosine monophosphate (Adapt from Sanchez, 2012).

Together with meiotic resumption, the nuclear maturation is driven by the activation of the M-phase promoting factor or maturation-promoting factor (CDK). This is a heterodimer that consists of the catalytic subunit (cyclin dependent kinase 1, CDK1) and the regulatory subunit (cyclin B). The MPF complex phosphorylates special gene targets participating in GVBD, chromosome condensation, cytoskeletal rearrangements and arrest in transcriptional activity (Dunphy *et al.*, 1988, Moreno and Nurse, 1990, Oh *et al.*, 2010, Vogt *et al.*, 2008). The MPF inactivation has been observed in the GV arrest and during the transition from meiosis I to meiosis II with a peak at metaphase I. It then maintains at a high level during the MII arrest until fertilization (Fulka *et al.*, 1992, Josefsberg *et al.*, 2003, Mehlmann, 2005b, Wu *et al.*, 1997) (Figure 1.3.A).

The protein kinases WEE1/MYT1 regulate the CDK1 activity by inhibitory action while the cell division cycle (CDC) phosphatase 25 (CDC25) paradoxically plays an activating role. Besides, MPF activity is dependent on the cyclin B ubiquitination and degradation which is mediated by the anaphase-promoting complex (APC) and APC co-activator proteins CDC20 (APC_{CDC20}) and CDC20 homologue 1 (CDH1). The GV arrest is also characterised by high levels of cAMP, as well as low levels of CDK1 and cyclin B1. High cAMP levels sustain meiotic arrest of the immature oocyte at the GV stage by activating PKA leading to WEE1/MYT1 activation and CDC25 inhibition, and ultimately resulting in CDK1 inactivation (Bornslaeger *et al.*, 1986, Han and Conti, 2006, Kovo *et al.*, 2006, Pirino *et al.*, 2009). On the other hand, APC activity marks cyclin B1 for degradation observed in murine GV oocytes (Reis *et al.*, 2006) (Figure 1.3.B).

A reduction in the intraocyte cAMP and GC-derived cGMP concentration after LH triggering causes the PKA inactivation and, in turn, the MPF activation to drive nuclear progression through meiosis I (Norris *et al.*, 2009, Schultz *et al.*, 1983, Vaccari *et al.*, 2008)). In addition, immediately after GVBD, inhibition of APCCDH1 activity by the early mitotic inhibitor 1 (EMI1) facilitates cyclin B1 accumulation and spindle formation (Marangos *et al.*, 2007) (Figure 1.3.C). Consequently, during prometaphase I, degradation of APC_{CDC20} allows chromosome congregation at metaphase I (Reis *et al.*, 2007). Later, releasing degradation marks of cyclin B1 and securin result in anaphase progression, meiosis I completion and extrusion of the first PB (Herbert *et al.*, 2003, Reis *et al.*, 2007) (Figure 1.3.C).

After that, the oocyte driven by MPF with participation of the MOS/MEK/MAPK pathway immediately progresses to meiosis II without chromatin decondensation while the sister chromatids become rapidly realigned and form the MII plate and maintain MII arrest (Fan and Sun, 2004, Madgwick and Jones, 2007, Tripathi *et al.*, 2010). As soon as fertilization resulting in APC activation and cyclin B1 and securin degradation, the MII arrest is released (Hansen *et al.*, 2007, Madgwick and Jones, 2007, Nixon *et al.*, 2002).

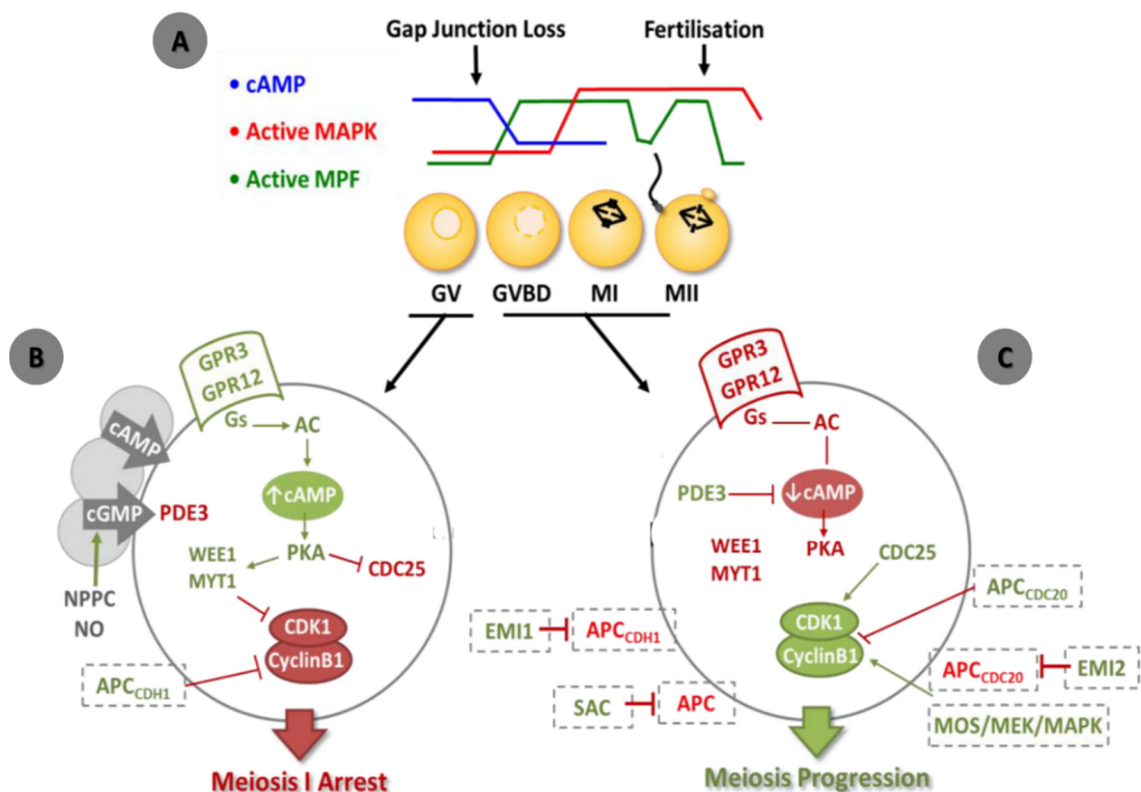


Figure 1.3. Changes in oocyte cAMP levels, MPF and MAPK activity during oocyte maturation. (A) Dynamics of cAMP, active MAPK and active MPF levels during nuclear maturation. (B) Signaling pathway regulating meiosis arrest in the GV oocyte. (C) Meiosis progression during transition from GVBD to MII oocyte, the downstream pathway after Gn triggering lost gap junction. Stimulation of GPR and CC-derived cAMP/cGMP maintain high cAMP levels, PKA activity and inactivation of MPF (CDK1+CyclinB1). Gn stimulation and/or gap junction loss triggers a drop in cAMP followed by MPF activation, GVBD and progression of MI. The spindle assembly checkpoint (SAC) ensuring the correct segregation of chromosomes can delay progression to anaphase through inhibition of APCCDC20 if aberrations in the spindle or the alignment of chromosomes detected. (Brunet *et al.*, 2003; Homer *et al.*, 2009; McGuinness *et al.*, 2009). MPF inactivation is required during MI to MII transition. Two APC coactivators (CDC20 and CDH1) and an inhibitor (early mitotic inhibitor, EMI) are necessary in mouse oocyte meiotic progression (Reproduced and modified from Collado-Fernandez 2013 with permission).

1.4.2. Cytoplasmic Maturation

Regulation of oocyte maturation commences during oocyte development and entails coordination of both nuclear and numerous cytoplasmic processes. Acquisition of ooplasmic signalling cascades is a fundamental regulatory control mechanism that is needed to ensure oocytes accumulate sufficient carbohydrate and protein synthesis and storage, successful organelle replication, and redistribution and regulation of metabolic/molecular mechanisms required for a mature MII oocyte to finalise subsequent fertilisation occurrences. These events include sperm/oocyte binding and fusion, oocyte activation, sperm processing and pronuclear (PN) formation, as well as preimplantation embryo development.

Since the zona pellucida (ZP), a conglomerate of glycoproteins synthesized and secreted by the developing oocyte (Calafell *et al.*, 1992, Nogues *et al.*, 1988), is the outermost part of the oocyte, abnormal ZP protein production causes abnormal sperm recognition/binding and leads to subsequent fertilization failure (Oehninger *et al.*, 1996, Rankin *et al.*, 1999, Rankin *et al.*, 2001). Also insufficiency or disorientation of proteins in oolemma including oocyte protein products (i.e., the oolemma tetraspanin protein (CD9) (Chen *et al.*, 1999, Kaji *et al.*, 2000, Miller *et al.*, 2000, Miyado *et al.*, 2000), glycosylphosphatidyl-inositol anchored proteins (GPI) and oolemma microvilli (Runge *et al.*, 2007) may impede this fusion process (Komorowski *et al.*, 2006).

There are two critical events taking place during oocyte activation; (i) a cortical granule (CG) reaction that modifies the ZP to prevent polyspermy, and (ii) the release from MII arrest to complete meiosis. During fertilization, PLC ζ , a sperm specific protein, is released into ooplasm to induce the endoplasmic reticulum to release Ca²⁺ and initiate the prolonged Ca²⁺ oscillation (Carroll, 2001, Kashir *et al.*, 2010, Nomikos *et al.*, 2013). This process begins soon after sperm/oocyte fusion and stops when PN formation arises (Larman *et al.*, 2004, Marangos *et al.*, 2003). Alteration of a Ca²⁺ rise and oscillations (i.e., frequency, amplitude and duration) have drastic impacts on the following events in both fertilisation and the early embryonic developmental stage (Ducibella *et al.*, 2002, Gordo *et al.*, 2000, Ozil, 1998, Rogers *et al.*, 2006). The prerequisite for correct Ca²⁺ signalling in oocytes is that the presumptive gametes must not only reach full size, but they also contain absolute oscillation capacity following cytoplasmic maturation (Carroll *et al.*, 1994).

Failure to complete meiosis II theoretically can occur as a result of perturbation of any of the steps in the meiotic maturation process and can involve failure of MII oocyte meiotic resumption, impaired segregation of sister chromatids, and/or failure of extrusion of the second polar body (PB) extrusion. Clinical manifestations of these problems can result in retention of the extra copy of maternal genetic material leading to a 3PN zygote (Flaherty *et al.*, 1995), incorrect maternal chromosome segregation leading to multiple small female PN, karyomeres, or complete absence of the female pronucleus (Asch *et al.*, 1995). Completion of the second meiotic division in oocytes is also dependent on Ca²⁺ oscillations as Ca²⁺ transients signal various downstream pathways involving several phosphatases/kinases including- calmodulin-dependent kinase II (CaMKII) and MAPK (Gordo *et al.*, 2001, Lorca *et al.*, 1993). In summary, incomplete cytoplasmic maturation may compromise fertilisation due to inadequate protein synthesis or aberration in associated signalling cascades involved in oocyte activation that subsequently result in cortical reaction failure, as well as polyspermic penetration. Alternatively, oocytes may demonstrate premature CG exocytosis and zona hardening, resulting in an impedance of sperm penetration into the oocyte.

Such events as described above occur during the transition from the GV stage to MII stage of oocyte development, in which gamete quality is defined by the processes of

cytoplasmic maturation, and is divided into three different elements: (1) cytoplasmic organelles redistribution, (2) cytoskeletal dynamics, and (3) molecular mechanism controlled maturation. The latter will be discussed in more detail in the next section.

During oocyte growth, the spatial rearrangement of organelles is accompanied by the reorganisation of the cytoplasmic microtubular network in which they are encased in order to occupy defined locations within the cell (Albertini, 1992). Dramatic changes in actin microfilaments and microtubules are required to

Table 1.3. Characteristics and dynamics of cytoplasmic organelles during oocyte maturation

Organelle	Characteristics and dynamics	References
Perivitelline space	PVS enlargement and retraction of TZP of corona cells	Hyttel et al., 1997; Mihm et al., 1999
Nucleus	migrates to the periphery and nuclear envelop breaks down	Kruip et al., 1983; Hyttel et al., 1997
Mitochondria	location preference varies in species, and during stage of oocyte development; close to nucleus during GVBD; close to spindle at MI and MII	Hyttel et al., 1997; Van Blerkom, 1984; 1991; Calarco, 1995; Nishi, 2003
	hood shape predominate, aggregate around lipid droplets, vesicles and smooth endoplasmic reticulum	Kruip et al., 1983; Hyttel et al., 1997; Sturmey et al, 2006; Motta et al., 2000
Endoplasmic Reticulum	reduced, more cortical and fragmented during final maturation	Kruip et al., 1983; Fair 2003
Golgi complexes	decrease size from GVBD until almost disappear at MII	Hyttel et al., 1997; Van Blerkom, 1984; 1991; Calarco, 1995; Nishi, 2003; Fair 2003
Cortical granules	localize to the periphery underneath the plasma membrane	Hyttel et al., 1998; Fair 2003
Lipid droplets	increase and more central localization following maturation	Hyttel et al., 1999
	more peripheral localization	Sturmey et al., 2006

facilitate GV positioning and oocyte polarity, for meiotic spindle formation and migration, for asymmetrical meiotic divisions and PB extrusion of the oocyte and for trafficking of cellular organelles (Schuh, 2011, Schuh and Ellenberg, 2008, Sun and Schatten, 2006, Yu *et al.*, 2010). Impaired cytoskeleton dynamics are linked to a failure of the second PB extrusion in mouse oocytes (FitzHarris *et al.*, 2007a), and to incomplete meiosis I division in the cow (Roth and Hansen, 2005), and are related to oocyte developmental competence in the pig (Brevini *et al.*, 2007). Details of morphological and ultrastructural dynamics are describes in Table 1.3.

1.4.3. Molecular Maturation

Apart from meiotic and cytoplasmic maturation, oocyte competence requires sophisticated molecular machinery to drive the processes needed to finalise saturation and support subsequent cellular events such as fertilisation, pronuclear formation, and preimplantation embryogenesis. Since numbers of transcripts and proteins will be consumed during early embryo development before embryonic genome activation, accumulation of these factors are necessary and required for resumption of meiosis, control of the mitotic cell cycle and activation of the embryonic genome (Sirard, 2001).

The first detectable mRNA synthesis takes place in the bovine oocyte of the preantral stage follicle (Fair *et al.*, 1997) and carries on until the oocyte is fully grown, approximately 110 μ m diameter, which corresponds to a 3mm follicle when transcription becomes less active (Fair *et al.*, 1995, Fair *et al.*, 1996). Low levels of transcriptional activity continue up to GVBD before a drastic decline at the MII stage oocyte (Memili *et al.*, 1998). Therefore mRNA transcripts that regulate development through fertilisation and up to the time of embryonic genome activation must be synthesised prior to ovulation. During maturation, oocytes contain huge amounts of RNA stored in the cytoplasm. The total RNA oocyte content estimates 0.6ng in mouse (Sternlicht and Schultz, 1981) and 2.0ng in human (Neilson *et al.*, 2000). Large amounts of the mRNA are degraded during the maturation process of oocyte development. Less mRNA is detected in an MII oocyte compared to a GV oocyte, approximately 19% in mouse (Bachvarova *et al.*, 1985) and 40% in human (Dobson *et al.*, 2004). The rate of RNA accumulation varies throughout the oocyte maturation and a sophisticated system involves in control of synthesis and expression of the

mRNA. One important mechanism of control is the polyadenylation (poly-A) of which the process is adding poly-A tails to transcripts to render them activated (Shim *et al.*, 1997) that leads to protein synthesis (Couttet *et al.*, 1997, Tomek *et al.*, 2002). Messenger RNAs with a long poly-A tail around 150 A-residues are rapidly transcribed while mRNAs with a shorter poly-A tail around 90 A-residues are masked and stored for later use (Curtis *et al.*, 1995). The masked mRNAs control the stored mRNA expression via an adenylation control element (ACE) as provoking elongation of the poly-A tail directs to the subsequent translation (Bachvarova, 1992, Fox *et al.*, 1989, Verrotti *et al.*, 1996). Incompetent oocytes tend to have shorter poly-A tails on the stored mRNA in comparison to their developmentally competent counterparts (Brevini-Gandolfi *et al.*, 1999). Therefore, impedance during transcription, or integrity of mRNA stability affects the oocytes developmental potential for fertilisation and embryogenesis (Brevini *et al.*, 2002).

1.4.4. Cumulus-Oocyte Interactions

When the follicle commences the growing pool, the oocyte within it starts growing and communicating with its surrounding somatic cells, CC and MGC, through gap junctions that mainly composed of connexins (Cx) (Anderson and Albertini, 1976, Eppig, 1982). Cx43 and Cx37 predominantly expressed in the ovary. Disruption of the Cx43 gene causes the arrest of follicular growth at early stages and due to the GC developmental defect, the oocyte fails to undergo meiotic maturation (Ackert *et al.*, 2001, Juneja *et al.*, 1999). While absence of Cx37 compromises female fertility as blocking formation of a mature preovulatory follicle and occurrence of premature luteinisation, oocyte growth is thus detrimental and meiotic competence is not achieved (Simon *et al.*, 1997).

A bi-directional communication via flow of metabolites, nutrients and paracrine factors, between the oocyte and the GC, drives the maturation process towards a fertilizable oocyte. Recruiting the follicle into the growing pool involves kit-ligand (KL) production from the GC to bind its receptor, Cognitive Kit ligand receptor (c-kit), on the oocyte (Packer *et al.*, 1994). Another is the retinoblastoma protein that is involved in initial cell differentiation. The peak expression demonstrates in preantral human oocytes (Bukovsky *et al.*, 1995). In the following, a developing follicle is moderately controlled by oocyte secreted factors from the transforming

growth factor β (TGF β) family such as GDF-9, GDF-9B also known as BMP15, anti mullerian hormone (AMH), activin and inhibin. Other important growth factors support follicular development and oocyte maturation include growth hormone (GH), insulin growth factor-I (IGF-I) and members of the epidermal growth factor (EGF) family. The gonadotrophins and hormones are, of course, also major players in this intrinsic cross-talk between follicular cells.

Together with meiotic resumption, cumulus expansion is also induced by oocyte derived cumulus-expansion enabling factors (CEEFs) (i.e., GDF9, BMP15, and others) (Dragovic *et al.*, 2005, Dragovic *et al.*, 2007)(Figure 1.2). In response to LH surge, the PKA signalling pathway is activated in the MGC and the CC enhancing signals to produce the EGF-like factors (i.e. AREG/EREG /betacellulin). Subsequently, they promote the downstream ERK1/2 pathway mediated by the EGFR mediated signaling stimulates the expression of cumulus expansion-related genes such as *COX2*, *gremlin1 (GREM1)*, *HAS2*, and *PTX3*, *Ptgs2* and *Tnfaip6* as downstream GDF-9 target genes (Ashkenazi *et al.*, 2005, Fulop *et al.*, 2003, Ochsner *et al.*, 2003, Varani *et al.*, 2002).

The regulation of oocyte transcripts that actively participate in meiotic maturation is central to the acquisition of oocyte developmental competence. A proportion of oocyte transcripts, however, end up degraded during the process of maturation (Su *et al.*, 2007). Essential factors that retain their function include transcripts involved in the signalling pathways for the regulation of oocyte meiosis and the maintenance of meiotic arrest at MII, such as ERK/MAPK and PI3/AKT (Cui *et al.*, 2007, Su *et al.*, 2007). The aberrant degradation or maintenance of these key transcripts during oocyte maturation and fertilisation may compromise oocyte quality and developmental competence (Alizadeh *et al.*, 2005).

Recent genome-wide profiling studies of murine maternal mRNAs have revealed approximately 7600 transcripts are actively translated during oocyte maturation, whereas other transcripts are repressed (Chen *et al.*, 2011). Two different mechanisms have been reported regarding translational repression during oocyte maturation; by degradation process as previously report (Cui *et al.*, 2007, Su *et al.*, 2007), and by the translocation of stable transcripts from the polysome to the

subpolysome/RNP fraction (Chen *et al.*, 2011). For instance, cell cycle regulators (e.g., *Ccnb1* and *Mos*) are shown to have active translation during the GV–MII transition. Similarly, components of the anaphase-promoting complex and components of the spindle assembly checkpoint (e.g., *Mad2*, *Bub1b*, and *Sog12*), as well as transcripts encoding transcriptional regulators and chromatin remodelers also maintain dynamic translation. Recently, cytoplasmic polyadenylation element binding protein (CPEB) and azoospermia-like (DAZL) have been identified as two essential modulators during oocyte maturation. CPEB protein promotes translation of *Dazl* mRNA, which is important during the transition to the MI stage. DAZL protein, on the other hand, subsequently enhances its mRNA translation during oocyte maturation (Cauffman *et al.*, 2005, Chen *et al.*, 2011, Collier *et al.*, 2005). Down-regulation of DAZL protein by injection of specific antisense morpholino oligonucleotides has resulted in delayed meiotic resumption and inhibition of the extrusion of the first PB in mice. Also, DAZL was found to be necessary for the translation of spindle assembly transcripts (i.e., *Tex19.1*, *Tpx2* and *Dazl* itself) (Chen *et al.*, 2011). This observation explains the link between insufficient DAZL and an increase in spindle defects in oocytes.

1.5. OOCYTE AND ENERGY METABOLISM

Oocyte during development required energy support to maintain oocyte viability as well as to support growth and maturation to acquire the developmental competence. Improper energy support leads to incomplete progression of nuclear maturation and insufficient oocyte cytoplasmic machineries such as mitochondrial activity and distribution, and oxidative and ionic stresses. These may result in unsuccessfully driven fertilisation and early embryo development to blastocyst formation as before embryonic genome activation they require maternal originating metabolic supply.

Mammalian cells including oocytes produce energy in the form of adenosine triphosphate (ATP) and intermediate molecules by utilizing potential substrates from exogenous supply as well as metabolizing endogenous storage, including pyruvate, glucose, oxygen, amino acids, fatty acids, purines and pyrimidines for energy production, nuclear maturation, production of extracellular matrices, signalling molecules and maintenance of cellular homeostasis/REDOX potential as

reviewed (Collado-Fernandez *et al.*, 2012). Varying ATP expenses at the different stage of development have been observed. Fluctuation of ATP stored within oocytes has been demonstrated during nuclear maturation (Yu *et al.*, 2010). Developing embryos into blastocyst stage require higher ATP supply and this linked to higher protein production (Thompson, 1997) and increased Na^+/K^+ ATPase activity to drive the blastocoel formation (Watson, 1992). Accumulation of intra-oocyte ATP seems to be crucial for fertilisation events and embryo cleavage (see review in (Krisher *et al.*, 2007).

Like other eukaryotic cells, mammalian oocytes synthesise ATP production by two major mechanisms; aerobic respiration via oxidative phosphorylation and the anaerobic pathway by utilising substrates via glycolysis (Thompson, 1997). Under the oxygen availability circumstance, the breakdown product pyruvate from the glycolytic pathway is entering the tricarboxylic acid (TCA) cycle to produce intermediate energy substrates such as NADH and FADH_2 , through the electron transport chain (ETC) to generate more ATP supply. Apart from glycolysis, although the pentose phosphate pathway (PPP) cannot directly metabolise glucose to ATP, it can produce other intermediates that are vital for cellular function; NADPH and phosphoribosyl pyrophosphate (PRPP). Moreover, the beta-oxidation pathway is involved in breaking down the long chain fatty acid molecules to produce small fatty acid molecules, acetyl co A, for the TCA cycle along with intermediate energy substrates NADH and FADH_2 for ETC as well.

1.6. METABOLIC PATHWAYS FOR ENERGY GENERATION

1.6.1. Glycolysis

Glycolysis is a universal catabolic pathway of a living organism. It is the first step of glucose metabolism to extract energy by splitting the six-carbon molecule glucose into two three-carbon pyruvate molecules. The process begins when glucose is transported into the cytoplasm either by facilitated diffusion via glucose transporter proteins (i.e., GLUT1-4 family) or active transport via Sodium-Dependent Glucose Transporter (i.e., SGLT1-2) and involves two major phases; the energy-requiring phase and the energy-releasing phase. In the first half of glycolysis, two ATP molecules are invested, and the phosphorylated glucose (six-carbon) is cleaved to form two glyceraldehyde-3-phosphate (three-carbon). During the second half, or

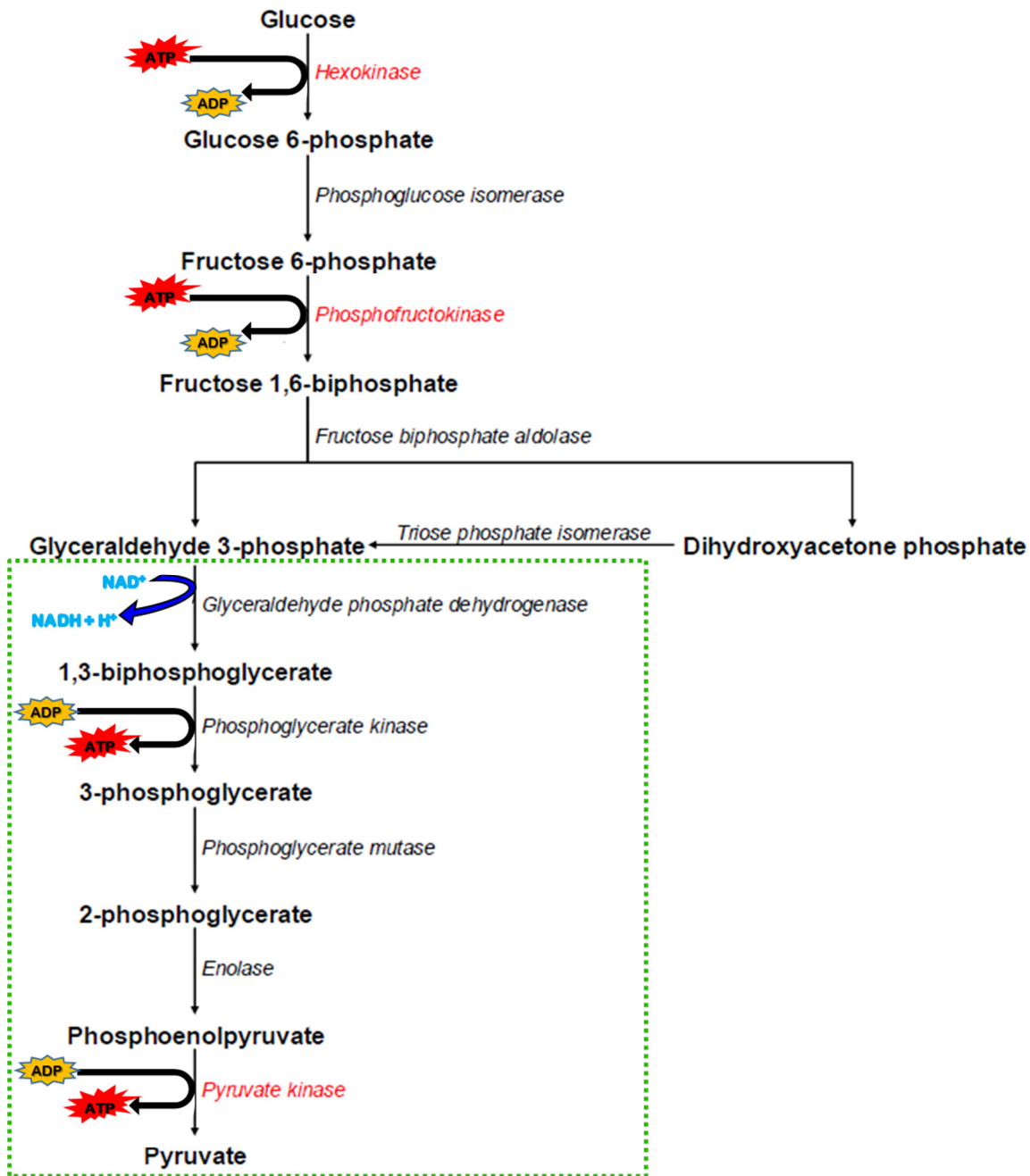


Figure 1.4. Glucose metabolism via glycolysis. Key regulator enzymes of the glycolytic pathway are highlighted in red. The first half of glycolysis (Investment phase) uses two ATP molecules in the phosphorylation of glucose, which is then split into two three-carbon molecules. The second half of glycolysis (pay off phase in green box) involves phosphorylation without ATP investment, produces two NADH and four ATP molecules per glucose (Modified from Lowe, 2014).

energy-releasing phase, each three-carbon sugar, glyceraldehyde-3-phosphate, is converted into pyruvate (three-carbon) through a series of reactions that produce two ATPs and one NADH molecules. As one glucose breakdown forms two three-carbon molecules, the process takes place twice so overall four ATPs and two NADHs are finally released from this stage. The net products are finally provided by

glycolysis including two ATPs, two NADHs and two pyruvate molecules which are precursors maintaining the TCA cycle or the PPP. As glycolysis is a multi-step pathway, it is thus regulated by inhibition or activation of enzymes in certain levels. Among several enzymes involved the three key regulatory enzymes; hexokinase, phosphofructokinase (PFK), and pyruvate kinase are rate-limiting due to their involvement in irreversible reactions, which serve as points of control for glycolytic flux. While an ADP:ATP ratio will serve as the sensing switch for the adjustment of glycolytic activity (Figure 1.4).

1.6.2. The Tricarboxylic Cycle

The tricarboxylic acid (TCA) cycle is also known as the citric acid cycle or the Krebs cycle, an aerobic pathway taking place in the mitochondrial matrix to metabolise pyruvate and produce acetyl-CoA, NADH and FADH₂. As ATP is the final target, intermediate metabolites, NADH and FADH₂, transfer their electrons to the next step of oxidative phosphorylation, the electron transport chain, which is consuming oxygen for energy synthesis. Furthermore, many intermediaries from the pathway are also used for the biosynthetic process of amino acids and purine nucleotides, the crucial elements for growth and DNA replication.

The TCA cycle is a series of eight steps, a closed-loop pathway, which consists of redox, dehydration, hydration and decarboxylation reactions. It begins with the entry of acetyl coenzyme A (Acetyl CoA), a two-carbon metabolite from energy substrate breakdown, condensed with oxaloacetate by the aid of citrate synthase enzyme to form citrate along with releasing CoA. The following enzymatic reactions include the conversion citrate to isocitrate, oxidised to α -ketoglutarate and subsequent succinyl coenzyme A before it is phosphorylated to produce GTP/ATP and then transforming to succinate, fumarate and malate, respectively. At the final step of the TCA cycle, oxaloacetate is regenerated by oxidising malate (Figure 1.5). In summary, each round of the TCA cycle yields two carbon dioxide molecules, three NADHs, three protons (H⁺), one FADH₂ and one GTP/ATP molecule.

The TCA cycle is regulated through the availability of substrate precursors (i.e., pyruvate, acetyl CoA, propionyl CoA and glucogenic amino acids) that are metabolites from several different metabolic pathways including glycolysis, β -

oxidation cycle, and protein catabolism. Apart from that, abundant products, as well as feedback inhibition, also play essential roles in the TCA cycle control.

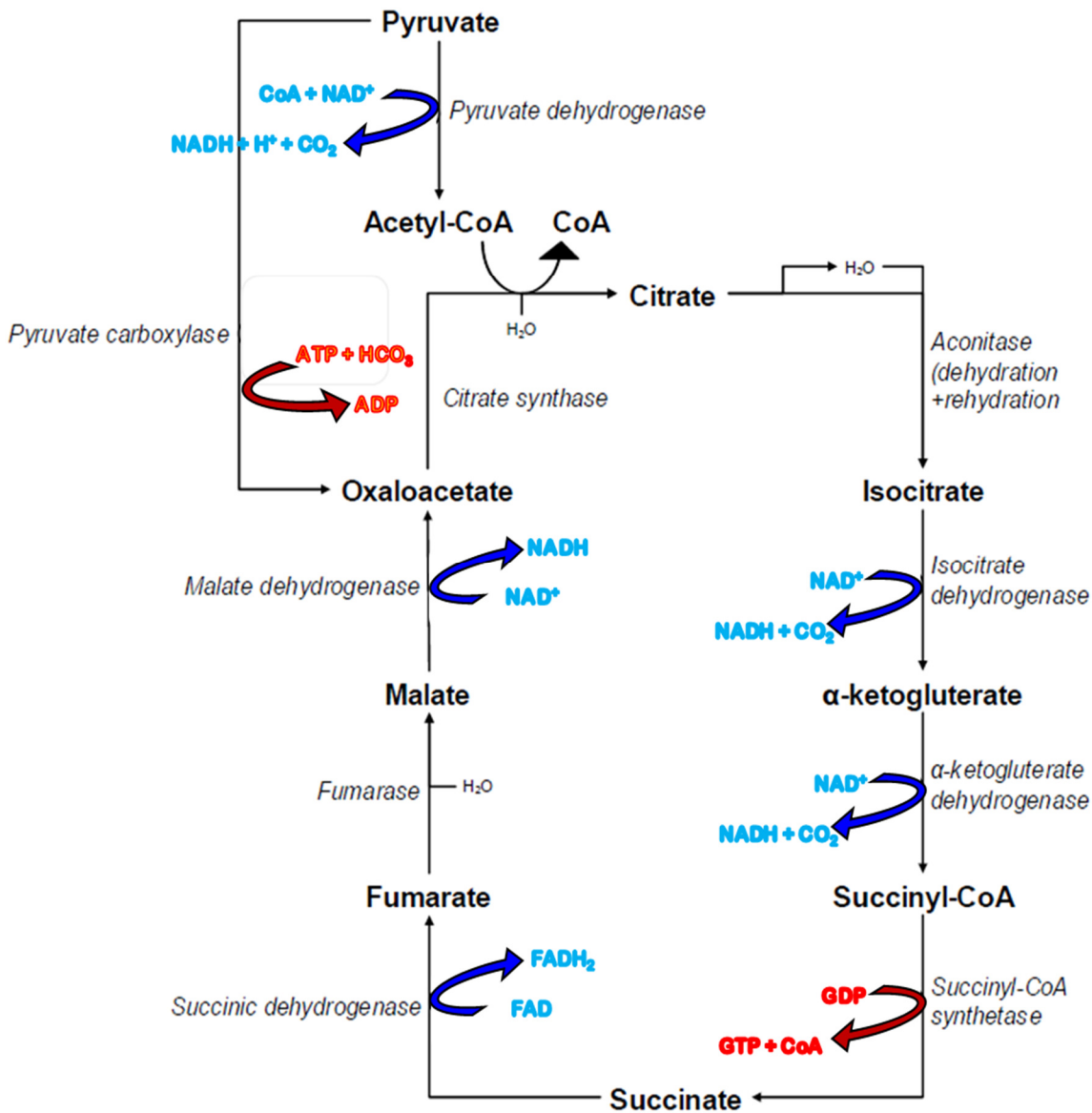


Figure 1.5. The tricarboxylic acid cycle. In each cycle, the acetyl group from acetyl CoA is attached to an oxaloacetate molecule (four-carbon) to form a citrate molecule (six-carbon). Through a series of steps, citrate is oxidized to release two carbon dioxide molecules for each acetyl group. Final products consist of three NADH, one FADH₂, and one ATP or GTP (depending on the cell type) in each cycle. The cycle runs continuously in the presence of sufficient reactants (i.e., Acetyl CoA) (Modified from Lowe, 2014).

1.6.3. Oxidative Phosphorylation

In almost all aerobic organisms, oxidative phosphorylation as the final step of the metabolic pathway in which nutrients are catabolized to generate ATP takes place within mitochondria. This process consists of two connected elements; ETC and chemiosmosis. During this process, the reduced oxygen as the electron acceptor picks up two protons (H^+) to form water molecules (Figure 1.6).

Most of the ATP synthesised from the glucose catabolism when oxygen is available, is derived from the ETC process. The process takes place when NADH, a strong, reduced electron carrier, donates its electron entering the ETC and passes them through a series of electron transporter protein complexes; I, III and IV, and mobile carriers such as ubiquinone Q and cytochrome C, that are embedded in the inner mitochondrial membrane. Ultimately, oxygen as a terminal electron acceptor is reduced and takes up two protons to form a molecule of water. The process of proton removal from the matrix creates the proton gradient across the inner mitochondrial membrane, and this will be utilised during the process of chemiosmosis. Another electron donor, $FADH_2$, bypasses enzyme protein complex I and passes its electron through the electron transporter protein II, which cannot contribute the proton pump like the electron transporter protein complex I, before electrons are passed through the enzyme protein complex III and IV. Therefore, $FADH_2$ becomes a weak, reduced electron donor compared to NADH (Berg *et al.*, 2002).

The uneven distribution of hydrogen ions across the membrane leads to different concentrations and establishes the electrical gradient (proton gradient) in which chemiosmosis is functioning to pump hydrogen ions (protons) that highly aggregate the inside to the outside of the inner mitochondrial membrane. Like other membrane, hydrogen ions cannot diffuse through the nonpolar regions of phospholipid membranes, therefore, can only pass through an integral membrane protein called ATP synthase. This molecular machinery facilitates the addition of a phosphate to ADP forming potential energy in the form of ATP.

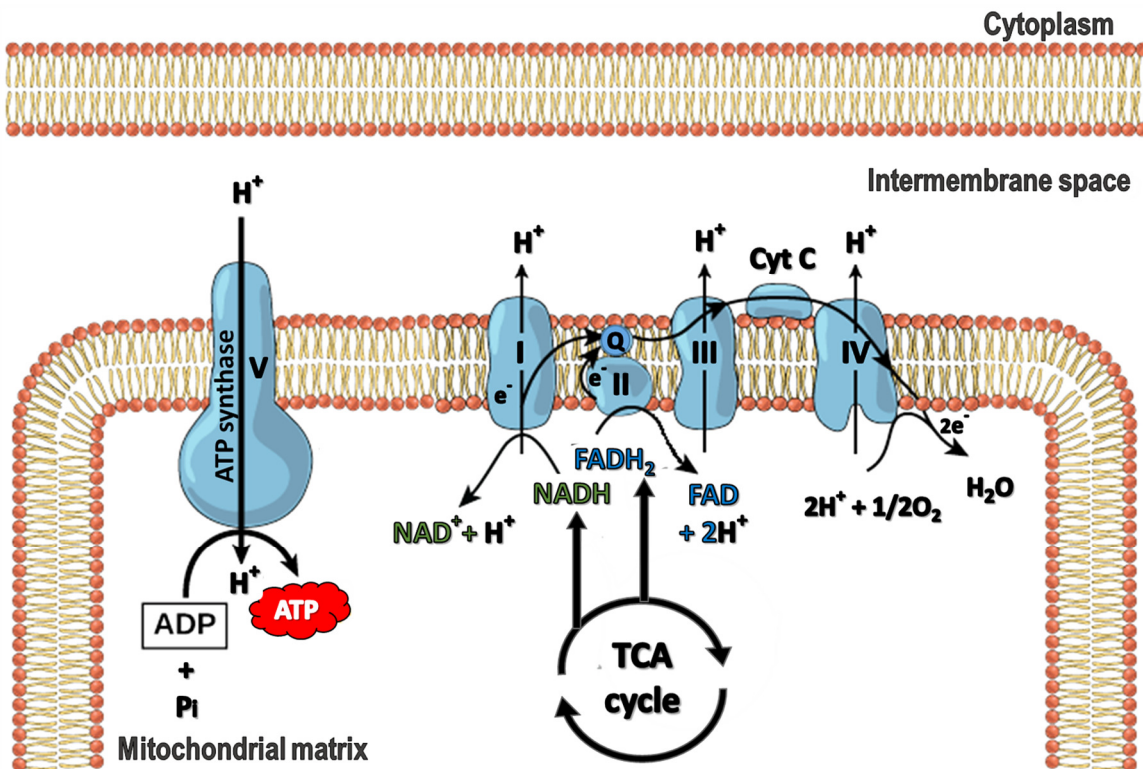


Figure 1.6. Oxidative phosphorylation (OXPHOS). The first four enzyme complexes (I, II, III and IV) in the electron transfer chain (ETC) pumping proton (H^+) from the mitochondrial matrix generate the pH gradient between mitochondrial matrix and intermembrane space. By the electrochemical gradient, the fifth enzyme complex facilitate proton pump to generate ATP via ATP synthase. Q: Coenzyme Q; Cyt C: Cytochrome C; ADP: Adenosine diphosphate; ATP: Adenosine Triphosphate; Pi: Inorganic Phosphate; e^- : Electron; NAD^+ : the oxidized form of nicotinamide adenine dinucleotide; $NADH$: the reduced form of nicotinamide adenine dinucleotide; FAD : flavin adenine dinucleotide; $FADH_2$: the reduced form of flavin adenine dinucleotide, and TCA: the tricarboxylic acid. *Image modified from "Oxidative phosphorylation: Figure 3," by Openstax College, Biology (CC BY 4.0).*

1.6.4. Pentose Phosphate Pathway

The pentose phosphate pathway is an alternative pathway of glycolysis, which is fundamental in an anabolic role rather than energy production. At the end of reactions, it generates $NADPH$ that transfers an electron to generate ATP via the ETC, pentoses (five-carbon sugar) and ribose-5-phosphate, which are essential for DNA and RNA synthesis. $NADPH$ produced from this pathway is used in reductive biosynthetic reactions within cells, for example, fatty acid synthesis, as well as serving to reduced glutathione production. On the other hand, the ribose-5-phosphate compounds from the PPP pathway are required for the biosynthesis of nucleotides and nucleic acids and synthesis of aromatic amino acids, respectively;

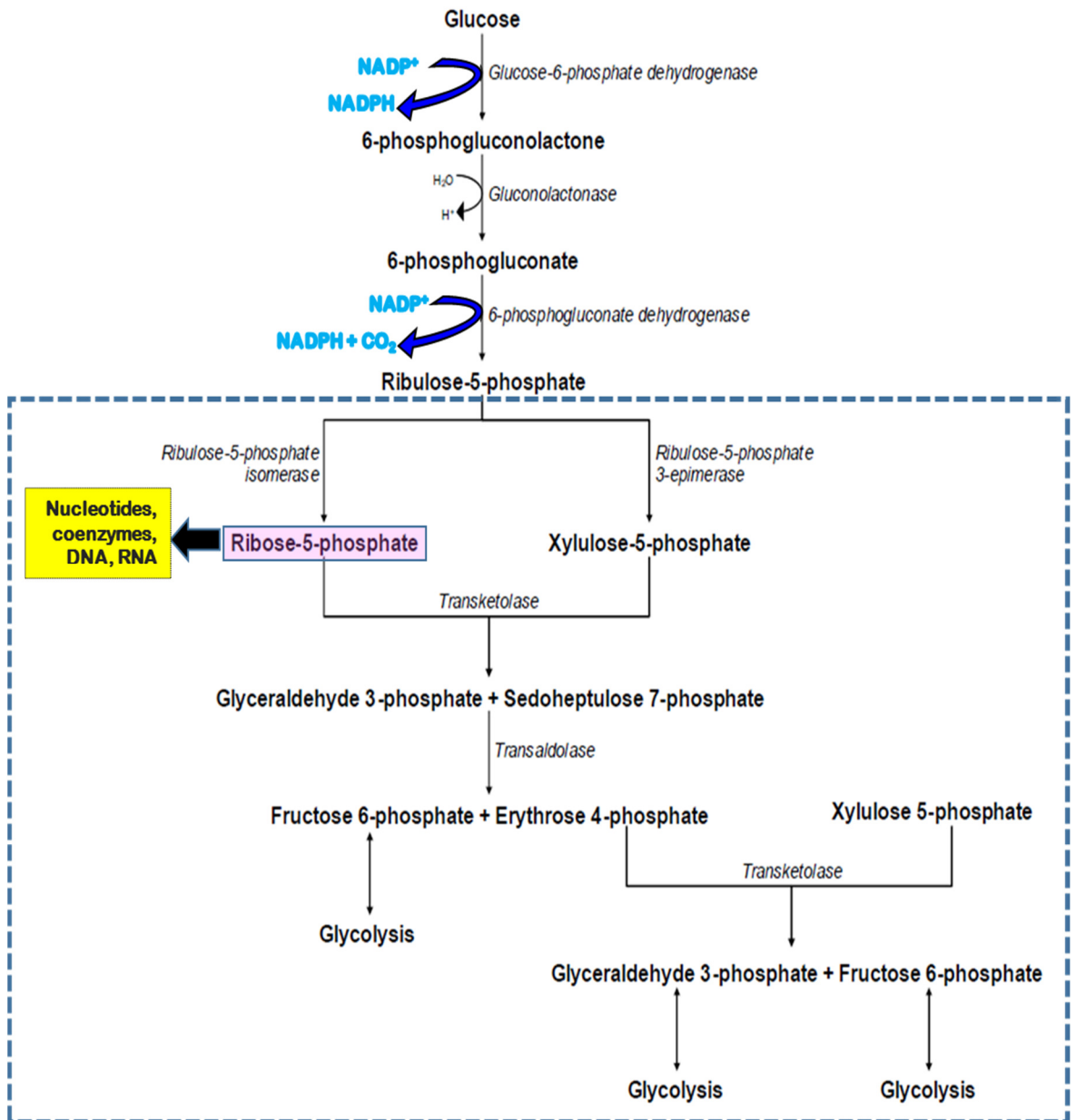


Figure 1.7. The pentose phosphate pathway. An alternative pathway to glycolysis and TCA cycle for oxidation of glucose. This process occurs in cytoplasm and is divided into two phases; the first part is oxidative phase, which is irreversible, and the second half is non-oxidative phase, which is a series reversible sugar phosphate inter-conversion reaction (in blue box) (Modified from Lowe, 2014).

these are all precursors for cell growth and DNA synthesis (Kruger and von Schaewen, 2003).

Similar to glycolysis, the PPP takes place in the cytoplasm. The pathway separates two distinct phases; oxidative and non-oxidative [figure 1.7]. The kick-off phase begins by glucose being metabolised to glucose-6-phosphate, the precursor for the PPP pathway, and during the subsequent oxidative reaction, it is converted into

ribose-5-phosphate. At the end of the oxidative phase, it contributes one ribulose-5-phosphate sugar, two NADPHs, two proton and one carbon dioxide molecules. The oxidative reaction is an irreversible process regulated by the rate-limiting step enzyme, glucose-6-phosphate dehydrogenase (G6PD) whose activity is dependent on the availability of NADP⁺/NADPH. The pathway is stimulated by increasing concentrations of NADP⁺ by the utilisation of NADPH in reductive biosynthetic pathways and strongly inhibited by NADPH (Voet and Voet, 2011). Several reversible reactions in the non-oxidative phase start when the precursor molecule ribulose-5-phosphate is converted to ribose-5-phosphate for DNA and RNA synthesis. While the subsequent reactions depend on the different cell demands; three-carbon and six-carbon molecules for glycolysis and four-carbon molecules for amino acid synthesis, for example (Keller *et al.*, 2014).

1.6.5. Beta-Oxidation Of Fatty Acid

Fatty acid metabolism plays important roles in both catabolic and anabolic processes. In animal metabolism, catabolism of fatty acids yields the highest ATP production per gram compared to carbohydrates and protein, when they complete oxidation via beta-oxidation and the TCA cycle. In the anabolic process, its metabolism contributes essential biomolecules such as triglycerides, phospholipids, local hormones and ketone bodies. In a sufficient nutritional supply milieu, the excess acetyl-CoA is synthesised to store in the form of energy substrates (i.e., fatty acids) instead of fatty acid oxidation for ATP production (Stryer, 1995).

Fatty acids primarily enter a cell via fatty acid protein transporters on the cell surface. Then long-chain free fatty acid molecules are activated by acyl-CoA synthase to form fatty acyl CoA on the outer mitochondrial membrane before being transported into mitochondria by the carnitine shuttle for energy production via beta-oxidation (Lopaschuk and Jaswal, 2010). Inside the mitochondrial matrix, the fatty acyl-CoA enters the beta-oxidation pathway, which is a recurring, four-step degradation process to break down saturated fatty acyl-CoA molecules through fatty acyl-CoA oxidation with a FAD, transalkene hydration, L- β -hydroxyacyl CoA oxidation with NAD⁺ and acetyl-CoA cleavage by thiolysis. Finally, each beta-oxidation cycle yields one FADH₂, one NADH, one acetyl CoA and a fatty acid

molecule with two-carbon units removed from the carboxyl end to produce acetyl-CoA (Figure 1.8).

In order to produce final ATP, an acetyl CoA is further oxidised in the mitochondrial TCA cycle and then the NADH and FADH₂ produced by both fatty acid β -oxidation and the TCA cycle pass their electron in the ETC for the energy production. An overview of fatty acid oxidation is provided in Figure 1.8. Also, the other acyl fatty acid with two carbons less will return into the beta-oxidation until no more fatty acid molecule can break down to gain an acetyl CoA molecule.

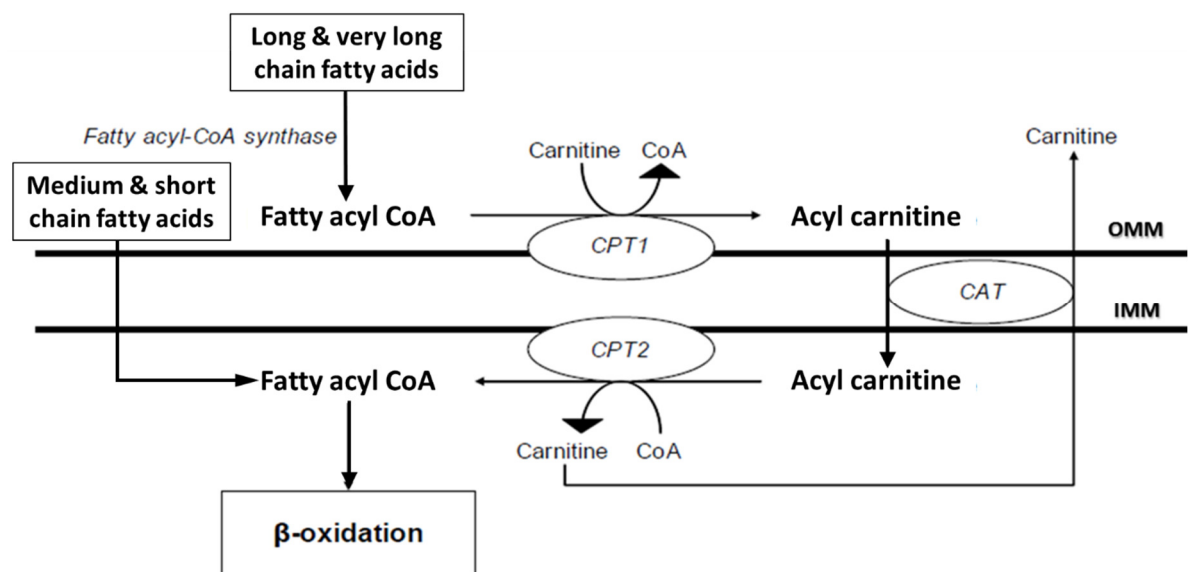


Figure 1.8. The carnitine shuttle. Mitochondrial carnitine palmitoyl transferase system uptake and transport of plasma fatty acids to the mitochondrial matrix. The shuttle is necessary for long and very long chain fatty acids transport through non-polarized mitochondrial membranes. Esterification of these fatty acids to form acyl-CoA is performed at the plasma membrane or mitochondrial outer membrane by a family of acyl-CoA synthases. Transport of long-chain fatty acids across the inner mitochondrial membrane acquires (i) CPT1 to generate acylcarnitines, (ii) the CAT to move the acylcarnitine across the inner membrane, and (iii) CPT2, on the matrix side of the inner membrane to recreate the long-chain acyl-CoA ester, the precursor for beta-oxidation. Medium- and short-chain fatty acids possibly traverse from plasma into the mitochondrial matrix by passive diffusion. CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; CAT, carnitine:acylcarnitine translocase; OMM and IMM, outer and inner mitochondrial membrane (Modified from Lowe, 2014).

1.7. ENERGY SUBSTRATES IN OOCYTES AND EMBRYO METABOLISM

1.7.1. Carbohydrates

The ability of oocytes to directly take up glucose for metabolism is still debated (Clark *et al.*, 2011, Downs *et al.*, 1998) as numbers of evidence showed that exogenous glucose is utilized by cumulus cells via glycolysis to provide pyruvate metabolite through the gap junctions (Biggers *et al.*, 1967, Clark *et al.*, 2011, Gilchrist *et al.*, 2008, Sugiura and Eppig, 2005, Sutton-McDowall *et al.*, 2010, Wang *et al.*, 2012, Xie *et al.*, 2016). Using live fluorescent staining demonstrated that murine COCs take up glucose by cumulus cells via facilitative glucose transporters (GLUTs) before it is transferred to the oocyte via gap junctions (Wang *et al.*, 2012). The oocyte *per se* seems to have a limited capacity for glucose utilisation (Biggers *et al.*, 1967, Xie *et al.*, 2016). Denuded oocytes also showed reduced glucose uptake compared to COCs in mice (Downs, 1999, Saito *et al.*, 1994, Wang *et al.*, 2012) and cattle (Zuelke and Brackett, 1993). Indirect evidence from bovine observed lower levels of phosphofructokinase activity in oocytes in comparison to the levels detected in the cumulus cells supports that oocytes have a poor capacity to utilise glucose (Cetica *et al.*, 2002). A recent report demonstrated mouse denuded oocytes matured *in vitro* in maturation media containing only glucose as an energy substrate were unable to complete nuclear maturation (Xie *et al.*, 2016). Inhibition of GLUTs reduced glucose uptake in CCs and oocytes of COCs, but the same effect cannot be demonstrated in denuded oocytes (Wang *et al.*, 2012). It suggests that in mouse CCs and oocytes might use different glucose transport system. In contrast, 2.78mM glucose supplement in maturation medium resulted in greater nuclear maturation rates of denuded porcine oocytes when compared to the counterpart cultured in maturation medium without glucose (Tsuzuki *et al.*, 2008), but this may be species-specific.

Both glycolysis and the PPP seem to have crucial roles in the meiotic resumption (Cetica *et al.*, 2002, Downs, 1999, Wang *et al.*, 2012, Xie *et al.*, 2016). This is supported by an increase in enzymatic activities in both the glycolytic (i.e., phosphofructokinase) and the PPP (i.e., G6PDH) pathways during bovine oocytes maturation, indicating that oocytes predominantly utilise glucose through the PPP pathway (Cetica *et al.*, 2002). However, it is hard to define as this can be either a

cause or a consequence of oocyte maturation. Similar to bovine, ATP content in porcine oocytes was not different when maturation *in vitro* occurred either in the presence or absence of glucose (Tsuzuki *et al.*, 2008). Inhibition of the PPP reduced porcine oocyte development to the MII stage and indicated insufficient energy production by glycolysis alone to maintain nuclear maturation (Funahashi *et al.*, 2008, Herrick *et al.*, 2006a, Sato *et al.*, 2007). However, very high glucose levels, in turn, may have a detrimental effect to oocyte maturation in murine (Downs and Mastropolo, 1994), porcine (Tsuzuki *et al.*, 2008) and bovine (Kumar *et al.*, 2012). High concentrations of glucose during bovine oocyte maturation *in vitro* increases intracellular ROS formation and decreases intracellular glutathione levels resulting in increased oxidative stress and impaired nuclear maturation (Hashimoto *et al.*, 2000)

Recently an effect of glucose metabolism on cytoplasmic maturation has been proposed. A mouse experiment found that glucose in culture media can promote cytoplasmic maturation of oocyte culture *in vitro* through the glycolytic pathway whereas, to a greater extent through the PPP, it facilitates ooplasmic maturation by reducing oxidative stress and providing energy substrate, fructose-6-phosphate for glycolysis (Xie *et al.*, 2016). Apart from glycolysis and the PPP pathway, glucose is also utilised via hexosamine biosynthesis pathway during oocyte maturation, where CCs use glucose and glucosamine to synthesise hyaluronic acid, a primary substrate of extracellular matrix to support cumulus expansion (Gutnisky *et al.*, 2007, Sutton-McDowall *et al.*, 2004).

Pyruvate is considered the main energy substrate utilised by oocytes for the meiotic resumption and further embryonic development (Gonzales-Figueroa and Gonzales-Molfini, 2005). Without pyruvate, most oocytes are unable to undergo GV breakdown (Funahashi *et al.*, 2008, Xie *et al.*, 2016). Complete nuclear maturation was observed in mouse (Biggers *et al.*, 1967, Xie *et al.*, 2016) and cattle (Geshi *et al.*, 2000) oocytes when cultured in maturation media containing only pyruvate as the energy substrate. Studies in porcine showed pyruvate supplementation in a glucose-free medium improved maturation rates in a dose-dependent manner, but maturation rate is still significantly lower when compared to media containing both glucose and pyruvate (Funahashi *et al.*, 2008, Sato *et al.*, 2007). The result suggested

that pyruvate supplies energy production via the TCA cycle whereas glucose primarily is metabolised via the PPP at this stage.

Energy production dramatically changes throughout the mammalian preimplantation stages regarding both the amount of ATP required and the energy substrate that is preferred. Although glucose, pyruvate, lactate and glutamine are the predominate energy substrates utilised during the embryo culture period, the preference of each varies according to the stage of embryo development and animal species (Dumollard *et al.*, 2009). At cleavage stages, developing embryos rely predominantly on pyruvate, lactate and glutamine utilisation for ATP production (Gardner, 1998, Leese, 1995). As both conversion of lactate to pyruvate and exogenous pyruvate supply can enter the TCA cycle and be metabolised to produce ATP. In addition, early embryos are capable of oxidising glutamine to generate ATP in the mitochondria until the morula stage is reached (Dumollard *et al.*, 2009). Embryos prior to compaction have limited capacity to metabolise glucose despite the presence of glycolytic and PPP enzymes (Biggers *et al.*, 1967, Cetica *et al.*, 2002, Flood and Wiebold, 1988). High glucose utilisation detected at the early embryo stage demonstrated abnormal glucose metabolism during *in vitro* embryo production (Gardner and Davies, 2000, Krisher and Bavister, 1998) resulting in poor embryo development outcomes (Gardner and Lane, 1998, Lane and Gardner, 1996). This abnormal glucose metabolism might be explained in part as a consequence of increasing glycolytic pathway sensitivity (Bavister, 1995, Khurana and Niemann, 2000). The proposed mechanism was supported by improved blastocyst development and quality in bovine and porcine when temporary, inhibiting oxidative phosphorylation in compact embryos (Machaty *et al.*, 2000, Thompson *et al.*, 2000) with coincidentally decreasing oxygen tension found in utero (Fischer and Bavister, 1993).

Like it was shown in oocytes, high glucose concentrations in culture media compromised embryonic development at early cleavage stages in pigs (Flood and Wiebold, 1988, Leese, 2003). Using sequential media that contains glucose support only from day three onwards, higher numbers of embryos reached blastocyst stage, as well as, more cell numbers in blastocyst than embryos cultured in media containing glucose for the entire period (Karja *et al.*, 2006, Kikuchi *et al.*, 2002). The

finding was correlated with higher H₂O₂ levels observed in embryos cultured with glucose for the entirely 7 days culture period (Karja *et al.*, 2006). This may suggest that an increase in metabolic rate leading to a subsequent rise in ROS production may cause compromised developmental embryos.

Developing embryo to the blastocyst stage require high ATP demands to drive fluid-filled cavity through the higher activity of Na⁺/K⁺ pump (Donnay *et al.*, 1999, Leese *et al.*, 1991) and to produce proteins (Thompson *et al.*, 1998). A decline in ATP: ADP ratio is sensing phosphofructokinase enzyme activity driving the glycolytic pathway and subsequently increase of ATP production (Dumollard *et al.*, 2009, Flood and Wiebold, 1988).

Due to the PPP metabolism contributing to the lowest ratio of total glucose utilisation at the blastocyst stage (Flood and Wiebold, 1988), the majority of glucose is being shunted through the glycolytic pathway for energy production. Coincide with an increase in oxygen consumption (Leese, 1995, Sturme and Leese, 2003), and it is indicative of an increase in overall metabolic rate and energy production. Following blastocyst expansion, oxygen consumption decreases as it needs only to maintain homeostasis (Sturme and Leese, 2003).

The difference in quality between *in vitro* and *in vivo* cultured embryos is well documented. *In vitro* produced embryos typically show delayed development, decreased cell number, altered morphology and metabolism, and produce poorer outcomes following transfer (reviewed in (Thompson, 1997)). *In vitro* culture caused an increase in the glycolytic rate in freshly flushed *in vivo* derived cattle (Khurana and Niemann, 2000) and mouse (Gardner and Leese, 1990) embryos with lactate production (indicative of glycolytic activity) significantly lower in *in vivo* bovine embryos than those cultured *in vitro* (Khurana and Niemann, 2000). Conflicting data found in a porcine report, instead of using lactate production for measuring glycolytic activity, embryos were assessed by glucose uptake, which may not have accounted for any glucose utilised via the PPP, found higher in glucose utilisation (Swain *et al.*, 2002). Alternatively, this may be due to species differences in embryonic metabolism. One possible explanation for increased glycolysis in *in vitro* produced embryos is stress-induced inactivation of ATP-consuming pathways. As

the culture *in vitro* activates stress 5'AMP activated protein kinase (AMPK), inactivates ATP-consuming pathways, and then activates ATP-regenerating pathways, including glucose transport and glycolysis (Leese, 2002).

1.7.2. Amino acids

Several roles of amino acids within biological systems have been observed including energy substrates, precursors for proteins and nucleotides synthesis, chelating agents preventing heavy metal toxicity, antioxidants against oxidative stress, osmolytes regulatory control intracellular osmotic pressure, pH buffering acidic environment, and signalling molecules. A wide range of amino acids is present in both oviductal and uterine fluids at a range of concentrations (Iritani *et al.*, 1974). The role of amino acids as an energy source is emphasised as the supplementation of non-essential and essential amino acids into chemically defined media demonstrated an increase of cell numbers in the blastocyst, as well as, oocyte maternal mRNA levels (Watson *et al.*, 2000).

Intact COCs are capable of utilising amino acids as an energy source during maturation *in vitro*. Supplementation of non-essential and essential amino acids when cattle oocytes matured *in vitro* increased subsequent cleavage, blastocyst development rates and cell number, and subsequently resulted in higher oocyte maternal mRNA levels compared with media without amino acid or serum supplementation (Watson *et al.*, 2000). In porcine, oocytes matured *in vitro* by using amino acid mix as the sole energy substrate increased meiosis and glycolytic rates compared to those equivalents cultured in media containing only pyruvate and lactate as the energy substrates; however, it could not support development to blastocyst stage to the extent of complete media (Tubman *et al.*, 2005). A mouse study showed a prominent role of glutamine in oocyte maturation. When it was used as an energy substrate, the initiation of meiotic resumption was demonstrated by the presence of GVBD, but oocytes were unable to complete meiotic maturation (Downs, 2000). In contrast to oocyte maturation *in vivo*, LH surge stimulates cumulus cells to convert glutamine to α -ketoglutarate, which is subsequently oxidised via the TCA cycle for ATP production (Rose-Hellekant *et al.*, 1998). This indicated that glutamine alone could not supply sufficient ATP to overcome the meiotic block due to inadequate glycolysis and the PPP activity.

Apart from the role as energy substrates, cysteine, glycine and glutamic acid are precursors of the antioxidant glutathione (GSH), reducing agent and scavenger against ROS. Cysteine is highly desirable for GSH synthesis during oocyte maturation since GSH pools during this period are critical for sperm decondensation and male pronuclear formation (Ali *et al.*, 2001, Sutovsky and Schatten, 1997). The addition of the low molecular-weight thiol compounds (i.e., cysteamine and β -mercaptoethanol) contributing cysteine or cystine during IVM enhances the developmental potential of oocytes (de Matos *et al.*, 1995, de Matos *et al.*, 1996). Supplementation with cysteine has demonstrated an increase in glutathione production, thus protecting the embryo from oxidative damage and ROS (Guerin *et al.*, 2001, Harvey *et al.*, 2002). Furthermore, supplementation of cysteamine to IVC media increased development of cleavage stage bovine embryos to blastocysts, compared with the equivalents cultured in TCM199 alone (Takahashi *et al.*, 2013). Therefore, an essential component of a comprehensive IVM system is the provision of adequate levels of GSH precursors such as glutamine and cysteamine.

1.7.3. Fatty acids

Even though triglycerides are the most abundant lipids constituting over 50% of all lipids within mammalian oocytes, few studies have been conducted to assess functions of both intracellular and extracellular lipids during oocyte maturation. Although they may have a potential role as reserve fuels, it seems to be unclear how fatty acids participate in maturational processes (Leese, 2002). Indirect evidence such as the availability of lipid reserves and levels of lipase activity suggested that oocytes perhaps are capable of oxidising lipids as substrates after disconnection from the CCs during maturation (Cetica *et al.*, 2002). However, wide varying reports both against and in support of this idea result in an inconclusive answer.

Endogenous lipids play an as yet undetermined role during oocyte maturation. Decreased triglyceride levels within the pig ooplasm throughout the maturation (Romek *et al.*, 2011, Sturmeay and Leese, 2003) has been reported with a concomitant increase in oxygen consumption being correlated to levels of triglyceride depletion (Sturmeay and Leese, 2003). This finding supports an earlier report in cattle oocytes (Ferguson and Leese, 1999, Kim *et al.*, 2001). However, the

upregulation of β -oxidation in porcine during oocytes maturation demonstrated varied results. Few studies reported improved rates of nuclear maturation and blastocyst development when β -oxidation was manipulated (Somfai *et al.*, 2011, Wu *et al.*, 2011), whereas others found no effect (You *et al.*, 2012). An explanation for these different results may lie in the different media compositions used.

During porcine oocyte maturation *in vitro*, inhibition of β -oxidation impairs meiotic maturation and hampers cleavage embryo developed to the blastocyst (Sturmey *et al.*, 2006). The intermediary changes in the fatty acid oxidation pathway may imply an important role of lipids during oocyte maturation in a range of species, including mice (Dunning *et al.*, 2010) and cattle (Ferguson and Leese, 2006, Yamada *et al.*, 2007). Changes in lipid droplet size, density and arrangement during IVM are evidence of the metabolic role of endogenous fatty acids. Using an inhibitor of nuclear maturation, olomoucine, oocytes showed no reduction in lipid droplet size, whereas, without the inhibitor, oocytes displayed a reduced droplet size, which was correlated with meiotic progression (Niimura *et al.*, 2004).

Similar to triglyceride, as observed by fluorescent staining, phospholipid and cholesterol content of lipid droplets decrease throughout *in vitro* maturation (Romek *et al.*, 2011). In the absence of exogenous energy substrates, bovine oocytes consumed oxygen not different from the equivalents in media containing energy substrates (Ferguson and Leese, 2006). Furthermore, bovine oocytes showed higher lipase enzyme activities than either glucose-6-phosphate dehydrogenase or phosphofructokinase activity (Cetica *et al.*, 2002), determined by a greater rate of lipolysis than either PPP or glycolysis. Whereas murine oocytes contain very low triglyceride component compared to cattle oocytes (Loewenstein and Cohen, 1964, McEvoy *et al.*, 2000), they significantly decreased maturation rates when cultured in media in the absence of exogenous substrates (Downs and Hudson, 2000). As observed in cattle, porcine oocytes contain a large number of triglyceride reserves (McEvoy *et al.*, 2000), suggesting that the capability abundance of endogenous reserves is used for energy production. Without glucose or pyruvate, porcine oocytes in maturation media supplemented with β -oxidation stimulant significantly increased nuclear maturation (Wu *et al.*, 2011). Finally, it has been suggested that exogenous fatty acids do not play a role in energy production.

Alterations of metabolic processes during *in vitro* maturation has been observed. Porcine COCs matured *in vitro* demonstrated dysregulation of gene transcripts associated with lipid metabolism (Yuan *et al.*, 2011) and mouse COCs decreased fatty acid oxidation compared to the equivalents matured *in vivo* (Dunning *et al.*, 2014). In porcine, inhibition of lipid metabolism during oocyte maturation decreased the abundance of gene transcripts involved in fatty acid oxidation, glycolysis and oxidative stress (Paczkowski *et al.*, 2013). This is consistent with findings seen in cattle oocytes matured *in vitro*, genes related to lipid metabolisms such as Chol acyl-transferase (ACAT1) and fatty acid synthase (FASN); were downregulated compared to oocytes matured *in vivo* (Gonzalez-Serrano *et al.*, 2013). Surprisingly, these genes become overexpressed in *in vitro* produced cattle blastocysts compared with the *in vivo* counterpart. A large body of evidence supports that endogenous lipids play a role in energy production throughout preimplantation embryo development *in vitro*. Developing embryos are capable of growth in the absence of exogenous energy substrates, suggesting that endogenous fatty acids are reserved for ATP production.

Inhibition of β -oxidation during embryo culture resulted in developmental arrest in various species (Dunning *et al.*, 2010, Ferguson and Leese, 2006, Sturmey *et al.*, 2008). Porcine zygotes cultured with a β -oxidation inhibitor increased glucose consumption, and lactate production (Sturmey *et al.*, 2008) indicating an increase in glycolytic rate to compensate for the absence of lipids as an energy substrate.

Almost half of IVP cattle zygotes cultured in the absence of exogenous energy substrates, can pass at least one cleavage division (Ferguson and Leese, 1999) while oxygen consumption by 5-to 8-cell cattle embryos remains stable whether cultured in the presence or absence of exogenous nutrients (Ferguson and Leese, 2006) implying oxidation and metabolism of energy substrates likely from endogenous sources. Moreover, adding a β -oxidation inhibitor during maturation in bovine oocytes reduced embryo capacity to form the blastocyst, the similar pattern though to a lesser extent was also observed when zygotes were exposed to the inhibitor during embryo development (Ferguson and Leese, 2006). In contrast, mouse zygotes cultured in deprivation of exogenous energy substrates were found to arrest and degenerate early (Manser and Leese unpublished, cited in (Ferguson and Leese,

2006)). The difference may be due to cattle embryos containing a far more significant amount of endogenous lipid than mouse embryos. A study in porcine support the findings in cattle that endogenous lipids alone may be insufficient to support viable embryo development (Park *et al.*, 2005b). Zygotes cultured in defined medium excluding exogenous energy substrates showed reduced cleavage and blastocyst rates. Incomplete oxidation of metabolites from β -oxidation is believed a defect in preventing the final ATP production as embryos cultured in a defined media without carbohydrates supplemented with oxaloacetate showed no difference of blastocyst rates compared to the equivalents cultured in media containing glucose (Park *et al.*, 2005a, Sturmey *et al.*, 2008).

Analysis of lipid content via the fluorescent probe Nile Red showed that while fatty acid content remained constant from the zygote to morula stage, it decreased at the blastocyst stage and again at hatching (Romek *et al.*, 2011, Romek *et al.*, 2009). Formation of the blastocoel and blastocyst hatching are two stages of development which are energetically expensive; thus decreased fatty acid content at these stages is consistent with a metabolic role.

1.8. MITOCHONDRIA

Mitochondria are maternally inherited organelles within the cytoplasm that have a major role in energy production. These organelles separate the mitochondrial matrix from other cytoplasmic structures by a double-membrane system, consisting of the outer and inner mitochondrial membranes. Unlike the outer membrane functioning as the membrane permeabilisation, the inner mitochondrial membrane where the ETC takes place folds to make up cristae to increase the area surface for more ATP production (Sherratt, 1991). Within the mitochondrial matrix, this site contains the enzymes responsible for metabolic functions including the TCA cycle and oxidative phosphorylation, as well as the mitochondrial genetic system (Cooper, 2000).

For their role of generating ATP the mitochondrial membranes comprise numerous transport systems that import metabolites and high energy intermediates, export ATP molecules to the cytosol, as well as, return inorganic phosphate through a phosphate-proton pump. The mitochondrial machineries are driven by the

chemiosmotic gradient which is not only essential for ATP production, but it is also used for other purposes.

Not only do mitochondria play a key role in ATP synthesis, but they are also important for several cellular processes, including fatty acid synthesis, Ca²⁺ homeostasis and the biogenesis of haem and iron-sulphur proteins. Their own independent machinery contributes to protein synthesis involved by DNA, messenger and transfer RNAs and ribosomes. Mitochondria, on the other hand, are also an essential calcium storage. Calcium signalling is an important second messenger in intracellular compartments to maintain accurate cell functions. Therefore, they may enact buffering calcium overload against calcium mediating toxins.

1.8.1. Mitochondrial Genetic System

Mitochondrial genomes are packaged as circular DNA molecules, like their bacterial origin (Andersson *et al.*, 2003). The genomes of mammalian mitochondria are approximately 16 kb in size but substantially vary in different species (Leblanc *et al.*, 1997). In addition, the well-conserved mitochondrial genomes encode only 37 genes; 13 proteins coding sequence involved in the electron transport chain and oxidative phosphorylation (Schon *et al.*, 2012) 22 transfer RNAs and two ribosomal RNAs (Bibb *et al.*, 1981, Jansen, 2000). Since more than eighty proteins are required for oxidative phosphorylation, most of the proteins encoded by the nuclear genome are imported into the mitochondria to control the cellular function in a tissue-specific fashion (Benkhalifa *et al.*, 2014, Cummins, 2001, Johnson *et al.*, 2007, Van Blerkom, 2011). Considering the genome structure, the mitochondrial DNA is vulnerable to mutation due to the lack of a protective mechanism to maintain DNA integrity (i.e., histones and non-coding regions, introns) as well as close proximity to the electron transport, which is recognizable the source of reactive oxygen species (ROS) production (Bentov and Casper, 2013, Parsons, 1997).

Mitochondria are not self-supporting entities in the cell. The mitochondrial DNA is replicated independently of the cell cycle DNA replication for review see (Holt, 2009). However, it is subjected to the cellular requirement for mtDNA synthesis (Bogenghagen and Clayton, 1976) such as replenishment of damaged molecules or

an increase of ATP requirement within a particular cell or in the specific condition (Moyes *et al.*, 1998). Replication and transcription depends upon trans-acting nuclear-encoded regulatory proteins; mitochondrial DNA polymerase γ (POLG) (Hubscher *et al.* 1979), mitochondrial single-stranded DNA-binding protein (mtSSBP) (Farr *et al.*, 1999), mitochondrial RNA polymerase (mtRNAPol) (Tiranti *et al.*, 1997), the mitochondrial transcription factor TFAM (Fisher & Clayton, 1985; 1988), and the nuclear respiratory factors NRF-1 and NRF-2 (Gleyzer *et al.* 2005; Virbasius & Scarpulla 1994), for example.

1.8.2. Mitochondria During Oocyte Development

In order to acquire adequate ATP supply within the cell, mitochondria necessitate apposite transcription and translation of the proteins that comprise the ETC (Moghaddas *et al.*, 2003). As the mitochondrial genome encodes protein subunits of the respiratory chain complexes, therefore an adequate mtDNA copy number is necessary for maintaining an electron transport chain function. Like other somatic cells, embryonic development requires ATP to support the chromosomal segregation and cell division, in which the ATP levels observed in blastomeres is correlated with mtDNA abundance (Van Blerkom *et al.*, 2000). On the other hand, the fully-grown oocytes at fertilisation acquire threshold levels of the mtDNA/mitochondrial pool in order to successfully reach implantation and carry on future preimplantation embryonic development (Wai *et al.*, 2010). Though the rapid growth of the embryo at the cleavage stage is characterised by the speed of dividing cells, their replication still pauses until blastocyst implantation (Larsson *et al.*, 1998, Piko and Taylor, 1987, Thundathil *et al.*, 2005). Processes of cellular division and chromosomal segregation are energy expenses, so if the mature oocyte lacks the proper mtDNA copy numbers it will lead to insufficient energy and will create functional deficiencies and likely compromise the embryo capability to progress through the preimplantation stages, and perhaps postimplantation embryogenesis as well (Wai *et al.*, 2010). Greater amounts of mtDNA are observed in competent oocytes than those found in incompetent oocytes, and impairment of mtDNA replication during oocyte maturation arrests mouse embryo development beyond the 6-cell stage (Spikings *et al.*, 2007). Besides, the poor fertility outcomes of incompetent oocytes can be rescued by augmenting with mitochondria from competent oocytes (El Shourbagy *et al.*, 2006).

Morphological changes of mitochondria within mammalian oocytes and embryos have been observed (Au *et al.*, 2005, Motta *et al.*, 2000). This is accompanied by stage-specific dynamic distribution with other related organelles during maturation and early development (Dumollard *et al.*, 2004, Motta *et al.*, 2000). Growing oocytes as well as fully-grown GV- and MII-staged oocytes attain a roundish or oval shape mitochondrial structure containing fewer cristae (peripheral arch, vacuole) and high-density matrix (Motta *et al.*, 2000). Nevertheless, the undifferentiated morphology of mitochondria in mature oocytes does not link to their ability in ATP synthesis (Campbell and Swann, 2006, Dumollard *et al.*, 2004). In contrast to mitochondria in other differentiated cells, fission and fusion infrequently occur in growing oocytes (Sathananthan and Trounson, 2000). After fertilisation, already at the 4-cell stage in the human, more elongated forms (with a length up to 2.5 μm) with numerous transverse cristae appear and become more and more abundant. Concomitantly, the density of the matrix decreases (Motta *et al.*, 2000).

1.8.3. ATP Requirement During Oocyte Maturation

The distribution and reorganisation of mitochondria during oocyte maturation are dynamic. The movement purposes to provide energy supply to areas of high energy consumption (Ferreira *et al.*, 2009, Krisher and Bavister, 1998, Mao *et al.*, 2014, Stojkovic *et al.*, 2001). Mitochondria are accumulated surrounding the GV membrane at the GV-stage oocyte and then moved apart from the perinuclear region when GVs breakdown. Ultimately, they are distributed in ooplasm in a mature MII-stage oocyte (Dumollard *et al.*, 2006). Concomitant with redistribution, the size of mitochondrial clusters also changes from the high proportion of more massive mitochondrial clusters upon GVBD to the large population of smaller mitochondrial clusters throughout the ooplasm (Yu *et al.*, 2010).

Due to the crucial role in energy supply during oocyte maturation, the reorganisation of mitochondria is possibly linked to the ATP concentration. This idea is supported by a bovine study (Stojkovic *et al.*, 2001) and was later confirmed by a study in mouse (Yu *et al.*, 2010). The latter study observed three phases of higher ATP production taking place around the time of GVBD, the phase of spindle migration, and during MI to MII stage transition. Numerous events occur during

nuclear and cytoplasmic maturation driven by ATP; spindle formation and chromosome movements require the motor proteins activities, for example. Previous studies in bovine and human demonstrated that an increase in ATP concentration is necessary for oocyte maturation, fertilisation and blastocyst formation (Duran *et al.*, 2011, Nagano *et al.*, 2006a, Stojkovic *et al.*, 2001). Insufficient ATP supply during oocyte maturation leads to a decrease of positive spindle formation rates (Eichenlaub-Ritter *et al.*, 2004, Zeng *et al.*, 2007) whereas inhibition of oxidative phosphorylation reducing ATP content results in a reduction of nuclear maturation, normal spindle formation, chromosome alignment, evenly mitochondrial redistribution, and blastocyst formation rates (Ge *et al.*, 2012b).

ATP content reflects oocyte developmental capacity in various species. A high rate of successful pregnancies are observed in human oocytes undergoing IVF that reported average ATP above 2 pmol/oocyte (Van Blerkom *et al.*, 1995). In bovine, increased ATP levels were observed in oocytes matured *in vitro* from 1.8 pmol in GV-stage compared to 2.5 pmol in MII-stage oocytes (Stojkovic *et al.*, 2001) and another study found the levels changed from 1.2 pmol in GV-stage to 2.0 pmol in MII-stage oocytes (Iwata *et al.*, 2011). Unlike the maturation *in vitro*, ATP content in bovine oocytes matured *in vivo* showed either no or little change during the oocyte maturation (Tamassia *et al.*, 2004).

1.9. PREIMPLANTATION EMBRYO DEVELOPMENT AND EMBRYONIC GENOME ACTIVATION

Precise oocyte maturation is critical for fertilization and early embryo development. Maternal factors stored in the egg control the first cell divisions. The transition from maternal to embryonic control, referred to as the embryonic or zygotic gene activation, is a gradual process and varies in different species. In the mouse the major embryonic genome activation happens at the 2-cell stage, whereas in pig and cow, it occurs at the 8-cell stage (Telford *et al.*, 1990). In order to time the onset of embryonic transcription in human pre-implantation embryos, different approaches have been employed in various stages of embryos such as transcription inhibitors (Braude *et al.*, 1988) or newly synthesized RNAs, proteins labelling (Braude *et al.*, 1988, Tesarik *et al.*, 1986), specific mRNA molecules quantification (Heikinheimo

and Gibbons, 1998) as well as parentally imprinted genes analysis (Lighten *et al.*, 1997b). These studies all indicate that the embryonic genome activation in the human occurs between the 4- and 8-cell stages.

Several mechanisms may underline impaired or delayed genome activation in preimplantation embryos. High concentrations of histones binding the DNA molecules may interfere the accessibility for the transcription (Prioleau *et al.*, 1994). Alteration of chromatin remodeling processes may impact the transcription activation or repression (Wolffe and Hayes, 1999). Also, structural differences between maternal and paternal genomes in terms of protamine-histone exchange and histone modification can be factors influencing the transcriptional activity during embryonic genome activation (Aoki *et al.*, 1997) (Adenot *et al.*, 1997, Ura *et al.*, 1997, Wade *et al.*, 1997). Also, the length of the cell cycle may be an influencing factor that affect the embryonic genome activation (Memili and First, 1998). At the onset of EGA the maternally derived mRNAs gradually disappear, this process is initiated by shortening of the poly-A tail (Decker and Parker, 1994). This is a gradual process and in human embryos the maternally inherited c-mos mRNA decreased following fertilization and cannot be detected after the 6-cell stage (Heikinheimo *et al.*, 1995).

1.10. AGEING AND OOCYTE QUALITY

Female ovarian function declines when women are approaching the later stage of their reproductive lifespan. In humans, age-decline fecundity shows not only in natural conception, but it is also reported in ART cycles (Broekmans, 2009, Heffner, 2004). The similar phenomenon has been observed across mammalian species (Alviggi *et al.*, 2009, Broekmans *et al.*, 2009, Fissore *et al.*, 2002). It is believed that decline in age-associated female reproduction is largely attributable to a diminished primordial follicle pool within the ovary, as well as a decline in quality of oocyte (Baird *et al.*, 2005, Hansen *et al.*, 2008, te Velde and Pearson, 2002). Mechanism underlying age-declined oocyte quality remains vaguely determined (Tatone *et al.*, 2008). Evidence suggested that increased rates of aneuploidy with advancing maternal age is possibly a key determinant of oocyte quality (Hassold *et al.*, 2007, Hassold and Hunt, 2001, Pellestor *et al.*, 2003). However, little is known about how aneuploidy is conducted by an increase of maternal age (Hussein, 2005, Matikainen

et al., 2001, Pru and Tilly, 2001, Tilly, 2001). Similarly, the mechanism of ovarian follicle loss is still investigated although it is widely accepted that age-dependent ovarian follicle atresia is possibly driven by the activation of the apoptotic pathway since this leads to ovarian follicle atresia (Hussein, 2005, Matikainen *et al.*, 2001, Pru and Tilly, 2001, Tilly, 2001).

Recent studies have reported the existence of mitotically active oogonial stem cells in the ovaries of mice and humans (Johnson *et al.*, 2004, Woods and Tilly, 2013), though in general it is well accepted that women are born with a limited ovarian reserve. The fixed number of primordial follicles is established in the human ovaries since early fetal life and serves as the source of developing follicles and oocytes for reproduction for the whole reproductive lifespan (Johnson *et al.*, 2004, White *et al.*, 2012). Oocytes enter meiosis I since the second trimester of fetal development before they have suspended in diplotene stage of prophase I until ovulation. The range of individual oocyte pause may last up to 50 years (Fragouli *et al.*, 2011b). Oocyte numbers progressively decrease following human development as the evidence observed 1,000,000 million follicles at birth before the decline to 450,000 follicles at the puberty and approximately 1000 oocytes at menopause (Broekmans *et al.*, 2007, Eijkemans *et al.*, 2014). The follicle cohort near the end of the reproductive lifespan has a reduced quality, as they originated from oogonial stem cells that had undergone more cell divisions, and thus may contain more cellular and DNA damage (Eichenlaub-Ritter, 1998).

Previous studies observed an association of maternal age with a decline in oocyte quality (Gaulden, 1992, Hassold and Chiu, 1985, Rowsey *et al.*, 2013, Schwarzer *et al.*, 2014). This result in decline in female fertility, increased miscarriage rates and developmental abnormalities in fetuses in advanced age women (Fragouli *et al.*, 2011b, Hassold *et al.*, 2007, Hassold and Hunt, 2001). Indeed, 10-30% of all oocytes and 50% of the blastocyst stage embryos over all maternal ages may carry a numerical chromosomal aberration (Fragouli *et al.*, 2011a, Hassold and Hunt, 2001). Nevertheless, it is undetermined as to what exactly causes oocyte quality impairment (Hulten *et al.*, 2010, Rowsey *et al.*, 2013). Evidence suggests that maternal age is the leading cause of embryonic aneuploidy (Hassold and Hunt, 2001, Hassold *et al.*, 2007, Pellestor *et al.*, 2003). More than 90% of the chromosome

imbalance results from chromosomal missegregation, of which the majority, more than 70% of nondisjunction, occurs in meiosis I (Nagaoka *et al.*, 2012).

Although many cases of age-associated decline in female fertility are attributable to aneuploidy (Mai *et al.*, 2013, Sandalinas *et al.*, 2002, Treff *et al.*, 2016), other contributing factors may underlie diminished oocyte and embryo developmental potential leading to poor pregnancy outcomes. Due to the crucial roles in ATP supply, apoptosis and calcium homeostasis, compromised mitochondrial function, a characteristic of aged oocytes, stems from ranged aberrations including but not limited to mtDNA damage and changes in mitochondrial gene expression and dynamics, as well as decreases in mitochondrial membrane potential reviewed by (Schatten *et al.*, 2014).

The differences in structural features of the oocyte mitochondria from advanced age women have been commonly described such as mitochondrial swelling, vacuolization, and cristae alteration (Muller-Hocker *et al.*, 1996, Van Blerkom, 2011). Apart from mitochondrial morphology, a progressive decline in mitochondrial function as evidenced by low mitochondrial membrane activity, reduced ATP production, and decreased mtDNA copy number or mitochondrial number in aged oocyte has also been observed (Chan *et al.*, 2005, de Bruin *et al.*, 2004, Eichenlaub-Ritter, 2012). From these data it may be speculated that alterations in mitochondrial quality and quantity may be contributing factors in abnormal meiosis, as mitochondrial dysfunction disturbs meiotic spindle integrity leading to defects in meiotic spindle assembly, cell cycle regulation, chromosome segregation, embryo development, and finally (Eichenlaub-Ritter, 2012, Van Blerkom, 2011).

1.10.1. Oocyte Ageing And Developmental Potential

The terminology of oocyte ageing can be confusing. According to the stage of developing oocytes, it can easily be classified into two main categories: ovarian ageing and postovulatory oocyte ageing. The first category, ovarian ageing by means of the prolonged arrest of immature oocytes within ovaries until the late reproductive life (Broekmans *et al.*, 2009, Takahashi *et al.*, 2011, Tarin, 1996). The second category, postovulatory oocyte ageing is a time-dependent process

whenever fertilisation does not occur during an optimal period; postovulation or post oocyte collection during ART. This category counts on both the mature oocyte-derived *in vivo* and the mature oocyte generated *in vitro* (Tarin, 1996). Ovarian ageing thus largely contributes to age-decline female fertility caused by a decrease in oocyte quality (Alviggi *et al.*, 2009). These occurrences in later reproductive life are consistent in most long-lived mammalian species such as cow, sheep, pigs and human (Alviggi *et al.*, 2009, Broekmans *et al.*, 2009, Fissore *et al.*, 2002). Albeit the concept of postovulatory oocyte ageing is distinct from maternal ovarian ageing, both categories of aged oocytes demonstrate similar phenotypic characteristics in terms of reproductive failure (Miao *et al.*, 2009b). However, the mechanisms that underpin the decline in oocyte quality are not necessarily equivalent (Takahashi *et al.*, 2011).

In postovulatory oocyte ageing, numerous morphological and cellular changes have been identified such as irregularities in the oolemma, a 'cobblestone' appearance or hardening of zona pellucida, disruption of meiotic spindle assembly, swelling of mitochondrial matrix, displacement of cortical granules, increased number of lysosomes, as well as aggregation of tubular smooth endoplasmic reticulum and of small mitochondria-vesicle complexes (see review in (Miao *et al.*, 2009a, Tarin *et al.*, 2000). However, morphological changes in oocytes derived from maternal ovarian ageing have been rarely described. Cellular fragmentation, milky or dark cytoplasm, and the presence of cellular remains enclosed by the zona pellucida have been characterised in oocytes of aged mice (Fujino *et al.*, 1996, Tarin *et al.*, 2001). On the contrary, using microscopic observation, Takahashi (2011) cannot distinguish between the oocytes harvested from aged and young mice despite the lower ovulation number found in aged animals (Takahashi *et al.*, 2011).

A large body of evidence supports aneuploidy observed in ovarian ageing caused by a defect of cell cycle control in meiosis I, especially metaphase I to anaphase I (Tatone *et al.*, 2008). Several reports found a reduction of transcript levels for 'cohesion proteins' (i.e., SMCb1) and for 'spindle checkpoint proteins' (i.e., MAD2), which are key molecules involved in cell cycle control in ovarian ageing (Cukurcam *et al.*, 2007, Hodges *et al.*, 2005, Steuerwald, 2005). Unlike aneuploidy observed in maternal ageing, both *in vivo* and *in vitro* experimental studies and reports from

domestic animals observed postovulatory oocyte ageing impairs female reproduction in other aspects; lower fertilisation, polyspermy, chromosomal anomalies, abnormal embryo development and increased fetal mortality (Huhtinen *et al.*, 1996, Tarin *et al.*, 2000, Wilcox *et al.*, 1998).

There are many reports that disruption and loss of the meiotic spindle assembly have been demonstrated in postovulatory ageing of oocytes in experimental animals and humans (Eichenlaub-Ritter *et al.*, 1988, Mailhes *et al.*, 1998, Szollosi, 1971, Van Wissen *et al.*, 1991). In mouse, barrel-shaped and microtubules of the meiotic spindle are clearly detected in fresh oocytes; however, microtubules are gradually lost from the spindle in postovulatory aged oocytes (Miao *et al.*, 2009b). As has been reported in experimental animals, *in vitro*-aged human oocytes aberration of c-tubulin expression was observed, which indicates disruption of centrosome structure at the meiotic poles (Schatten, 2008, Sun *et al.*, 2007). These changes in aged oocytes lead to premature chromosomal separation, which is strongly associated with aneuploidy (Mailhes *et al.*, 1998, Steuerwald, 2005).

Mitochondria are the most important organelles supplying ATP via oxidative phosphorylation to maintain oocyte quality. Ovarian ageing alters not only mitochondrial morphology but it also mitochondrial function in oocytes. In mouse, aged oocytes have aberrant mitochondrial arrangement as well (Tarin *et al.*, 1999). A report found abnormal characteristics of mitochondrial ultra-structures in aged-human oocytes including a high density of the matrix, vacuolization, and swelling (de Bruin *et al.*, 2004). In comparison to young women, decreased mitochondrial membrane potential indicating the change in oocyte mitochondrial function from reproductive-aged women has been observed (Wilding *et al.*, 2001). Moreover, the accumulation of the mitochondrial DNA point mutations and levels of mitochondrial DNA deletions in oocytes derived from reproductive-aged women were higher compared to oocytes from young women (Barritt *et al.*, 2000, Keefe *et al.*, 1995). Therefore, the data suggest age-dependent impairment of mitochondrial function may be one of the leading causes of a decline of oocyte quality.

As it provides the primary energy source for the oocyte, it can be anticipated that impaired mitochondrial function causes detrimental oocyte quality (Bentov *et al.*,

2011, Ford, 2013, Takeo *et al.*, 2014, Van Blerkom *et al.*, 1995). When insufficient ATP levels interfere with the cellular functions of the oocyte, problems arise in oogenesis and embryogenesis once fertilised (May-Panloup *et al.*, 2007, Van Blerkom *et al.*, 1995). This typically manifests as either pre- or postimplantation developmental arrest, implantation failure, miscarriage, or developmental defects in the offspring resulting from aneuploidy (Allen *et al.*, 2000, Gauden, 1992, Hassold and Chiu, 1985, Keefe *et al.*, 1995, Kushnir *et al.*, 2012).

1.10.2. Prepubertal Oocytes And Developmental Potentials

Although prepubertal oocytes exhibit comparable meiotic maturation rates with adult oocytes (Ledda *et al.*, 2001, O'Brien *et al.*, 1997b, O'Brien *et al.*, 1996), a higher prevalence of abnormal fertilization (Kochhar *et al.*, 2002, Ledda *et al.*, 2001, O'Brien *et al.*, 1997b, O'Brien *et al.*, 1996) and lower blastocyst formation during *in vitro* culture (Kochhar *et al.*, 2002, Leoni *et al.*, 2007b, O'Brien *et al.*, 1997b, O'Brien *et al.*, 1996, Ptak *et al.*, 1999) have been observed. The features are likely attributed to the disturbance of cytoplasmic maturation, which commonly presents as failed fertilisation, polyspermy, cleavage arrest, failure to reach blastocyst stage, and postimplantation embryonic losses (Armstrong, 2001). Morphological studies found blastocysts produced *in vitro* and derived from prepubertal oocytes, were undistinguishable from those derived from adult oocytes (O'Brien *et al.*, 1997b), in terms of cell number (Ledda *et al.*, 1997, Leoni *et al.*, 2007b, O'Brien *et al.*, 1996) and inner cell mass to total cell number ratio (Kochhar *et al.*, 2002, Leoni *et al.*, 2007b). An altered speed of development has been observed in oocytes derived from prepubertal animals. Previous studies found a two-hour delay in developing lamb oocytes matured *in vitro* (Kochhar *et al.*, 2002), as well as, delays in the two-cell (Leoni *et al.*, 2007b), four-cell (Ptak *et al.*, 1999) and blastocyst development (Leoni *et al.*, 2007b, O'Brien *et al.*, 1997a, Ptak *et al.*, 1999). Also other reports demonstrated such a difference (Ledda *et al.*, 1997, O'Brien *et al.*, 1996), though a later study (Morton *et al.*, 2005) could not detect such speed differences in embryonic development. This may be due to interference of higher FSH administration during ovarian stimulation, or differences in culture media (KM Morton and SL Catt, unpublished data cited in (Morton, 2008) or oxygen tension (Leoni *et al.*, 2007a). As the kinetics of both oocyte maturation (Dominko and First, 1997) and embryo development (van Soom *et al.*, 1997) may impair the

developmental potential of embryos, the reduction in developmental speed of prepubertal derived embryos may explain the diminish survival *in vivo* (Kelly *et al.*, 2005, O'Brien *et al.*, 1997a, O'Brien *et al.*, 1997b, Ptak *et al.*, 1999, Ptak *et al.*, 2006) and the high foetal loss rates (O'Brien *et al.*, 1997a, Ptak *et al.*, 1999).

According to the morphology, the only difference observed between prepubertal and adult oocyte animals is the size of the oocyte. Prepubertal oocytes have a smaller size when compared to those derived from adult animals. In GV stage, the morphology of cytoplasmic organelles (i.e., endoplasmic reticula, Golgi complexes, mitochondria and lipid droplets) does not differ between oocytes derived from prepubertal and adult groups (Ledda *et al.*, 1997). However, prepubertal oocytes demonstrated a lower number of transzonal projections (Ledda *et al.*, 2001) and a delay in the migration of the cortical granules observed during *in vitro* maturation (O'Brien *et al.*, 1996) when compared with adult oocytes.

Also, metabolic differences between adult and prepubertal oocytes have been reported. Lower glutamine metabolism during IVM was observed in prepubertal than adult oocytes while they were similar in glucose and pyruvate metabolism (O'Brien *et al.*, 1996). During oocyte maturation, protein synthesis is essential for normal meiotic progression and embryo development (Moor and Crosby, 1986). Lower amino acid uptake (Kochhar *et al.*, 2002, Ledda *et al.*, 1996, Ledda *et al.*, 2001) and protein (Kochhar *et al.*, 2002) were observed during prepubertal oocytes maturation *in vitro*, as well as, different time period of peak protein synthesis of prepubertal oocytes observed when compared with adult oocytes (Kochhar *et al.*, 2002). Although the level and pattern of MPF activity was not different in adult and prepubertal oocytes, the MPF activity after maturation was detectably lower in oocytes derived from prepubertal than those derived from adult animals (Ledda *et al.*, 2001). These results point to the effect of oocyte competence concerning morphologic, metabolic and ultrastructural differences between oocytes from adult and prepubertal animals. However, data may be confounded by the different age ranging between studies as well as hormonal administration before oocyte harvesting.

1.10.3. Molecular mechanisms of Age and Oocyte Competence

Transcriptome studies have confirmed different levels of transcript abundance in MII oocytes linked to maternal age (Grondahl *et al.*, 2010). During the oocyte growth phase, growing oocytes produce and accumulate necessary transcripts and proteins stored for oocyte maturation and further embryonic development, which upon induction undergo coordinated translation and degradation (Su *et al.*, 2007). Despite transcriptional quiescence, maturing fully grown GV oocytes and MII oocytes enable synthesis of proteins which are essential for biological processes by regulating transcript poly(A) tail length (Reyes *et al.*, 2017).

1.10.4. Study model of ageing

Since ageing is a complexity of biological process in which both genetics and environment interact with each other, studies in living organisms especially in humans are not so easy to distinguish the environmental effects from the underlying genetic cause. It is impossible to control the environment for humans. Further, a group of people carrying the same genetic identity to stay in the same environment never happens for the experimental study. Moreover, mechanisms contributing to the ageing process are so complex and diverse, it is nearly impossible to test separate hypotheses in humans.

Several age-related biological pathways have been evolutionarily conserved among species, data concluded from model organisms to some extent, are also applicable to humans. No universal model system proposes to be suitable for testing all the different hypotheses in either basic research or applied medicine. Different models varied in cell lines, unicellular organisms or multicellular organisms are distinct in biological characteristics; therefore, the model system used is necessary depending on the scientific questions and the hypothesis.

The decline in female fertility in the late reproductive age is largely attributable to the poor oocyte quality driven by ovarian ageing. (Heffner, 2004, Practice Committee of the American Society for Reproductive, 2006). Although the precise mechanism of oocyte ageing remains unknown, evidence suggests this might be chronic damage to the oocytes and/or the surrounding, follicular cells. To conduct an experimental study of oocyte quality in animal models concerning ovarian ageing

seems to have some challenges. Limitations of oocyte availability in aged-animal models could be a major problem. Apart from that, the study time-interval requires more than one year to attain the animals getting old enough to compare the biological ageing differences (Takahashi *et al.*, 2011). Moreover, there is no appropriate animal model analogous to the one that is used in ovarian ageing studies. Therefore, the postovulatory ageing oocytes, which is easily accessible and given the same phenotype of reproductive failure, have been employed to study the mechanism underpinning oocyte ageing instead of reproductive-aged oocytes (Takahashi *et al.*, 2011).

Another common model used to overcome the limitations of the ovarian ageing is the prepubertal oocyte as it can be an excellent negative model for fertility (Khatir *et al.*, 1996). Therefore, knowledge about the reproductive system at the prepubertal age could further expand our scientific understanding of human infertility. Sheep resembles human as it is monovular species and similar preimplantation embryonic development period. Also, the availability of tissues is easily accessible from abattoirs, which is a major UK industry. In this thesis, a series of experiment used the prepubertal sheep oocyte to a representative of the poor quality oocyte model in comparison to the adult sheep oocyte to represent the better oocyte quality model to study the impact of age on molecular and metabolic characteristics. Moreover, a better understanding of the reproductive capacity of prepubertal animals may lay down the groundwork for discoveries in future research in many fields such as advanced agricultural production, conservation of endangered species, and improved fertility treatment.

1.11. HYPOTHESIS

The evidence presented here indicates that the developmental potential and hence quality of GV and MII oocytes is gradually acquired during oogenesis and folliculogenesis. The capacity of the GV oocyte to complete meiotic maturation and to support fertilisation and the early development of the embryo before embryonic genome activation. It is dependent on multiple and interrelated parameters of oocyte biology including cumulus-oocyte communication, oocyte metabolic capacity, DNA and RNA packaging, and regulation of transcription during maturation as well as maternal age, chromosomal health and the impact of the

follicular environment *in vivo* or culture environment *in vitro*. On this basis, we hypothesise that the interaction between key molecular and metabolic markers will ultimately define oocyte quality and that these markers will be altered in oocytes of different reproductive ages.

Aims And Objectives

This thesis used the sheep as a physiologically relevant model for human reproduction to study the interaction between different *in vivo* and *in vitro* parameters and their relationship to markers of oocyte quality. The aims and objectives of the experimental studies conducted during the course of this project were as follows:

- (i) To evaluate the impact of age on sheep GV oocyte competence
- (ii) To validate the system for measuring the oocyte metabolism and chromatin remodelling
- (iii) To study chromatin configuration pattern in sheep GV oocytes
- (iv) To compare metabolic process between pre- and adult derived GV oocytes
- (v) To quantify the mtDNA surrogate of mitochondrial number between pre- and adult derived GV oocytes
- (vii) To study genetic and epigenetic differences between pre- and adult derived GV oocytes

CHAPTER 2: GENERAL MATERIAL AND METHODS

All reagents used in this series of the experiment were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, UK) unless otherwise specified. Laboratory consumables such as sterile pipette tips, Eppendorf tubes and universals were purchased from Starlab Ltd. (Milton Keynes, Blakelands, UK) while other plastic wares including all MEA Nunc® culture dishes were from Thermo Fisher Scientific UK Ltd. (Loughborough, UK). All supplier details are provided in Appendix I.

2.1. PREPERATION OF GLASSWARE

All new glass wares were treated as detailed in the following steps before first use. The glassware was soaked in a 2% (v/v) Hydrochloric acid (HCl) in deionized water solution overnight to eliminate impurities, rinsed thoroughly three times in tap water, and, finally cleansed 3 more times with Milli-Q® water (Millipore UK Ltd.). The new glass ware was left in an oven at 30°C (Philip Harris Ltd, Hyde, UK) for the drying process prior to sterilisation by heating in an autoclave at 180°C before use in sterile culture work. Instead of using 2% (v/v) HCl in the first step, other used glass wares were soaking in weak Trigene® solution (Medichem International, Kent, UK) and then cleaned as in steps described above.

2.2. PREPARATION, CALIBRATION AND MAINTENANCE OF SILICONISED NANOLITRE PIPETTES

2.2.1. Preparation Siliconised Glass Nanolitre Pipettes

Nanolitre (nl) pipettes were used in two aspects of the metabolism measurement in the experiments. First, they were used to set-up drops in the culture dish for carbohydrate metabolism assays of glucose (G), pyruvate (P) and lactate (L). And the other purpose was for analytical assays to quantify amounts of carbohydrates (GPL) in spent potassium simplex optimised medium (KSOM) based medium. The pipettes were made of borosilicate glass capillary tubes (Drummond Scientific Co., preantral, US) size 0.80mm (outer) x 0.60mm (inner) x 70mm (length). A pipette puller (Research Instruments Limited, Cornwall, UK) was used to assist capillary tube pulling. The pipette puller was adjusted to facilitate narrowing of the pipettes to the diameter desired and then the pulled capillary tubes were snapped at the narrowest point. To further refine the pipette orifice was clamped and held the

micropipette in a horizontal position in a microforge holder (Research Instruments Limited). The pipette tip was moved towards the heated platinum-iridium filament. To create a constriction inside the micropipette (Figure 2.1.), the microforge holder was rotated to a vertical position and the filament moved towards the desired position. The micropipette was heated up until it started bending, the holder was then set to apply heat to the opposite side of the micropipette. After a 45° rotation of the micropipette tip clamp holder was performed, heating steps were applied again to both sides of the pipette circumference. This created an internal constriction on all 4 sides within the pipette tip lumen from the melted glass.

To siliconise the nanolitre micropipette, tips were used for repeat aspiration of 5% (v/v) dimethyl-dichlorosilane in heptane solution (silanization solution I) (Sigma, 85126) several times in a fume extraction hood. They were then left at room temperature to dry overnight. Each finished pipette was attached to a stainless steel rod of 10cm length and <1mm inner diameter (New England Small Tube Corporation, Litchfield, US) with sealing wax (Figure 2.1.). In order to operate the nanolitre pipette, the set-up had to be connected to a 50 cm rubber tube which attached to a 10 mL syringe (Becton Dickinson, Oxford, UK) at the other end. Finally, the nanolitre pipettes were cleansed with acetone by repeating aspiration before and after use.

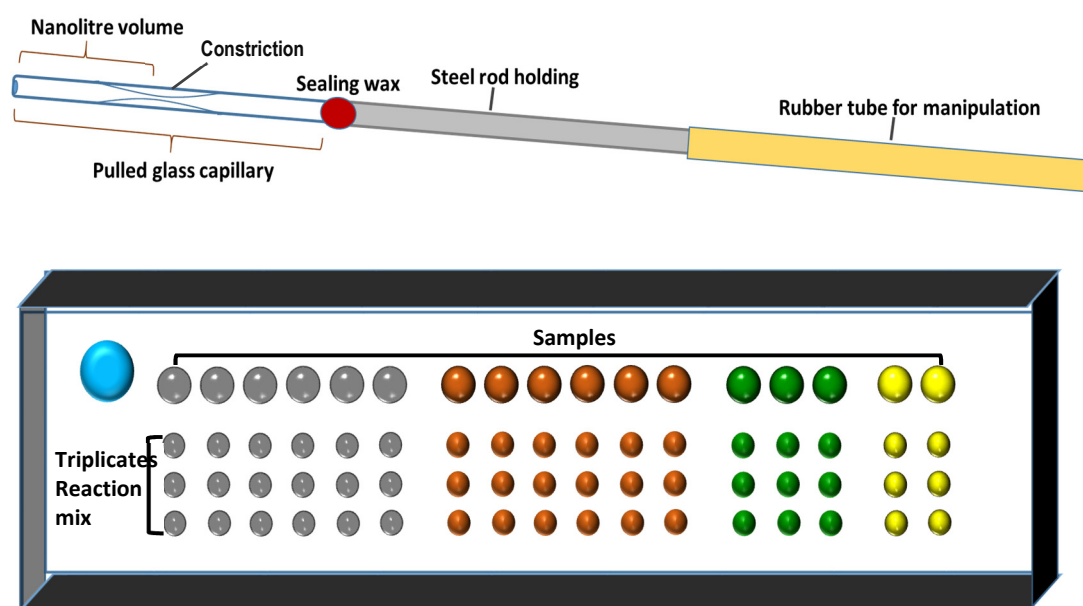


Figure 2.1. Diagram of a siliconized nanolitre pipette set (top) and a siliconised glass oil well (bottom).

2.2.2. Nanolitre Pipette Calibration

To calibrate nanolitre pipettes, a microdrop of H₂O of known volume (between 1-10 μ L) was set on a siliconised oil well (Figure 2.1.) and overlaid with mineral oil. Using the nanolitre pipette as many drops of water as possible were set up, until the known volume water was finished. This procedure was performed in triplicate, with at least 2 different initial volumes. The pipette volume was calculated by division of the initial known H₂O volume by the actual number of nanodrops produced and averaged across the set of triplicate measures. Only pipettes for which each triplicate value differed from at least one of the others by less than 10% were used for nanolitre metabolism assays.

2.2.3. Nanolitre Pipette Maintenance

Cleansing nanolitre pipettes was performed before and after each use by several repeated aspiration episodes of acetone to avoid culture medium build up inside the pipette lumen. As a result of the extremely fine diameter of the pipettes, blockage in pipette lumen was not an uncommon occurrence, therefore the resiliconization process was needed for maintenance. Reversal of micropipette blockage was achieved by repeated passing of 37% (v/v) HCl solution in and out until the block disappeared.

2.3. PREPARATION OF SILICONISED OIL WELLS

Homemade glass wells, size 5cm (length) x 2cm (width) x 1cm (height), were prepared for the quantification of 3 replicate measures of carbohydrate concentrations within the spent culture media used for GPL assays. Glass bars were cut into four pieces of the desired length and each piece was mounted onto a microscope slide with Araldite[®] resin (Bostik Ltd, Leicester, UK) to produce 4 rims of a glass well. Wells were left overnight to dry. The constructed wells were then cleansed with 70% (v/v) ethanol followed by soaking and rinsing several times in Milli-Q[®] water. Wells were siliconised by pipetting 3 ml of silanization solution I into each well before the wells were laid in a fume extraction hood for 3-5 minutes before being left to dry overnight. The spent reusable oil wells were cleansed with Trigene[®] solution (Medichem International, Kent, UK) using a soft sponge to remove the oil followed by rinsing with 50 mL Milli-Q[®] water and hanging to dry overnight. Like the siliconised nanolitre pipettes, the maintenance of oil wells required regular

resiliconising with silanization solution I to avoid culture medium being mounted on the surface of the well.

2.4. PREPARATION OF MINERAL OIL FOR CULTURE

All mineral oils used in the experimental series were embryo tested (Sigma, M5310) and purchased from Sigma-Aldrich Co. Ltd. Before the oil was used to overlay culture media, 1 ml of the corresponding culture medium was added and mixed into 50 ml of sterile mineral oil to wash the oil. The washed oil was then equilibrated in a humidified CO₂ incubator (Galaxy 170S; New Brunswick-Eppendorf UK Ltd, Cambridge, UK) at 38.5°C and 5% CO₂ in air at least 2 days prior to use.

2.5. MEDIA PREPARATION

2.5.1. Ovary Washing Medium

The ovary medium for washing abattoir-derived ovaries was made up by adding one tablet of 5 grams Phosphate-buffered saline (PBS) (Gibco®, 18912014; Life Technologies Ltd., Paisley, UK) into 500 ml Milli-Q® water (resistance 18.2 MΩ·cm at 25°C, 0.22 µm membrane filter). No adjustment was required in the dissolved PBS solution as the pH will be 7.45. A 5 ml aliquot of 100X Antibiotic-Antimycotic Solution (ABAM) (Sigma, A5955) containing 10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B per ml was added to the PBS solution. This medium was freshly prepared prior to each culture and incubated overnight at 38.5°C in a non-gassed incubator (SI30H hybridization oven/shaker, Scientific Laboratory Supplies Ltd) before use.

2.5.2. Ovary Holding Medium (Follicle Isolation Medium)

The follicle isolation medium (FIM) was used for holding pre-washed ovaries in the following experiments in the whole thesis. The FIM was made up according to the protocol developed by our group (Collado Fernandez, 2013, Hemmings, 2007). The composition of FIM was demonstrated in Table 2.1. All culture media and additives were made up in a 500ml volumetric flask. Precise volumes of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered, Minimal Essential Medium (HEPES-MEM) and other stock solutions were aliquoted by sterile tips or pipettes. The final solution was then filter sterilised using a 500ml of TPP® vacuum

Filtermax rapid bottle filter unit containing a 0.22µm premium PES filter membrane (Techno Plastic Products, TPP, Trasadingen, Switzerland). All spare sterile medium was kept at 4°C in refrigerator up to 2 weeks. Prior to use, the medium was warmed up overnight at 38.5°C in a non-gassed incubator.

Table 2.1. Composition of follicle isolation medium (FIM). The use was for 500ml preparation. HEPES-MEM; BSA: bovine serum albumin, IU: international unit. *See Appendix II for stock preparation.

Component	Stock	Volume (ml)	Final working concentration
MEM Eagle HEPES (Sigma,M7278)	-	470	-
Penicillin-streptomycin (100X) (Sigma,P4333)	10,000 IU/ml, 10,000 µg/ml	5	1,000 IU/ml, 1,000 µg/ml
Sodium pyruvate stock*	47 mM	0.5	47 µM
≥96% lyophilized powder, cell culture, BSA stock* (20X)	20 mg/ml	25	1 mg/ml

Table 2.2. Composition of oocyte holding medium 199+ (H199+). The use was for 250ml preparation. *See Appendix II for stock preparation.

Components	Stock	Volume (ml)	Final working concentration
M199 10X (Gibco,21180)	10X	25	1X
Sodium bicarbonate stock*	250 mM	4	4 mM
HEPES stock*	250 mM	21	21mM
Penicillin-streptomycin (100X) (Sigma,P4333)	10,000 IU/ml 10,000 µg/ml	5	1,000 IU/ml 1,000 µg/ml
Heparin (Mucous) injection BP (Leo. Labs,009876-04)	5,000 IU/ml	0.180 (900 IU)	3.6 IU/ml
≥96% lyophilized powder, cell culture, BSA (Sigma, A9418)	-	1 g	4 mg/ml
Tissue culture water (Sigma, W3500)	-	Up to 250	-

2.5.3. Oocyte Holding Medium (H199+)

Instead of FIM, H199+ medium was prepared for holding COCs while they were harvested during oocyte recovery before they were passed into defined amino acid profiling (AAP) or GPL media. This solution was prepared using the protocol detailed in Table 2.2 (Cotterill, 2008). After filter sterilisation using a 250 ml TPP®

vacuum Filtermax rapid bottle filter system (Techno Plastic Products, TPP, Trasadingen, Switzerland), the spare medium was kept at 4°C in refrigerator up to 2 weeks. Before use, the medium was equilibrated overnight at 38.5°C in a non-gassed incubator.

2.5.4. Oocyte Maturation Medium

Because of the undefined nature of culture medium with serum supplement, heterogeneity between serum batches may lead to inconsistent results. Therefore, physiological serum-free culture medium has been employed throughout the series of experiment in this thesis to more accurately define and control culture conditions and promote consistency between experiments (Bavister, 1995, Freshney, 2010). Previous studies have confirmed that serum-free oocyte maturation medium can support oocyte development *in vitro* in most species including humans (Cobo *et al.*, 1999, Wynn *et al.*, 1998) and other mammalian species (Hemmings *et al.*, 2012, Serta *et al.*, 1995). The IVM medium formulation used here was based on the medium previously described and validated by (Cotterill *et al.*, 2012, Wynn *et al.*, 1998, Danfour, 2001). The composition of serum-free IVM medium is shown in Table 2.3. The final solution was sterilised with a 0.2µm cellulose acetate syringe filter (Acrodisc, Pall Corporation, Portsmouth, UK) and was then stored at 4°C in refrigerator up to 1 week. Before culture, IVM media dishes were prepared and allowed to equilibrate overnight at 38.5°C in a humidified 5%CO₂ incubator.

2.5.5. Modified Oocyte Maturation Medium For Amino Acid

Profiling

AAP medium is a derivative of the defined, serum-free, oocyte maturation medium that is needed to facilitate measurement of very tiny amounts of amino acids production and utilization by oocytes *in vitro*. According to the published protocol (Danfour, 2001, Hemmings, 2007), the defined serum-free IVM medium has been modified in 6-fold reduction of 18 amino acids while other components were retained at the same concentration by replacement with the appropriate amount of Earle's balanced salt solution (Sigma, E2888) instead of MEM Eagle, α -modification (Sigma, M4526). This media adjustment maintained oocyte development but enabled the detection of amino acids turnover (Danfour, 2001, Hemmings, 2007). In

addition to those reagents required in maturation medium, DL-2,4-diaminobutyric acid dihydrochloride (DABA) was also added to the AAP medium as an internal control to enhance the accuracy of the AAP assay technique. DABA is non-toxic and inert and so not able to be metabolized by an oocyte or embryo. The pH of the medium was adjusted to approximately 7.9 by the addition of 5M HCl or 1M NaOH as appropriate. The correct medium pH was visualized by display in pale orange rather than pink. The composition of AAP medium is shown in Table 2.4. Like the serum-free IVM medium, the sterilised AAP medium was stored at 4°C in a refrigerator for up to 1 week. To set up the AAP assays the dishes were prepared on the day before use as they required overnight incubation in a humidified 5% CO₂ incubator at 38.5°C.

Table 2.3. Composition of ovine, serum-free oocyte maturation medium composition. The use is for 10 ml preparation. MEM; BSA; R3 IGF-1: Recombinant analog of human insulin like growth factor-1. *See Appendix II for stock preparation.

Component	Stock	Volume (µl)	Final working concentration
MEM Eagle, α-modification (Sigma, M4526)	-	9,577	-
≥96% lyophilized powder, essentially fatty acid free BSA (Sigma, A6003)	-	0.01 g	1 mg/ml
Penicillin-streptomycin (100X) (Sigma,P4333)	10,000 IU/ml, 10,000 µg/ml	96	960 IU/ml, 960 µg/ml
Sodium pyruvate stock*	47 mM	100	0.47 mM
L-Glutamine stock*	100 mM	200	2 mM
Bovine holo-transferrin stock*	5 mg/ml	10	5 µg/ml
Sodium selenite stock*	50 µg/ml	1	5 ng/ml
Bovine insulin stock* (10mg/ml) (Diluted 1:1000)	10 µg/ml	10	10 ng/ml
R3 IGF-1 stock*	100 µg/ml	1	10 ng/ml
Ovine FSH stock*	2 IU/ml	3	0.6 mIU/ml
Ovine LH stock*	2 IU/ml	1.5	0.3 mIU/ml

Table 2.4. Composition of ovine amino acid profiling-AAP medium. The use is for 10ml preparation. EBSS: Earle's balanced salt solution; and, DABA: DL-2,4-Diaminobutyric acid dihydrochloride. *See Appendix II for stock preparation.

Component	Stock	Volume (µl)	Final working concentration
MEM Eagle, α-modification (Sigma, M4526)	-	1,210	-
≥96% lyophilized powder, essentially fatty acid free BSA (Sigma, A6003)	-	0.01 g	1 mg/ml
Penicillin-streptomycin (100X) (Sigma,P4333)	10,000 IU/ml, 10,000 µg/ml	50	500 IU/ml, 500 µg/ml
Sodium pyruvate stock*	47 mM	100	0.47 mM
L-Glutamine stock*	100 mM	6.25	62.5 µM
Bovine holo-transferrin stock*	5 mg/ml	10	5 µg/ml
Sodium selenite stock*	50 µg/ml	1	5 ng/ml
Bovine insulin stock* (10mg/ml) (Diluted 1:1000)	10 µg/ml	10	10 ng/ml
R3 IGF-1 stock*	100 µg/ml	1	10 ng/ml
Ovine FSH stock*	2 IU/ml	3	0.6 mIU/ml
Ovine LH stock*	2 IU/ml	1.5	0.3 mIU/ml
EBSS (Sigma, E2888)	-	8,450	-
DABA stock*	50 mM	12.5	62.5 µl

2.6. OVINE REPRODUCTIVE TISSUE COLLECTION

Sheep reproductive tissues were obtained from the local abattoir (J Penny and sons, Rawdon, Leeds, UK) and transported in an insulated carrier at ambient temperature. On arrival, tissues were assessed prior to ovarian isolation according to two criteria. Adult tissues were identified by size of ovaries and uterine horns and by the morphological features of uneven ovarian surface incorporate with the presence of one fresh ovulation point and the old corpora lutea or corpus albicans presenting by yellowish points, and/or hemorrhagic or clear cystic appearance containing blood vessels on the membrane surface of each pair of ovaries (Figure 2.2). Prepubertal tracts were characterized morphologically by small uterine horns without rugae appearance on the tracts, smooth ovarian surface and complete lack of corpora lutea

or previous ovulation scars. The lamb tissues were collected during the summer nonbreeding season (April-August) while the ewe tracts were characterized as yielding good quality oocytes were prepared during autumn-winter breeding season (October-February). Ovaries were collected in a pre-warmed beaker filled up with pre-warmed ovary wash medium (see section 2.5.1) while processing and then these were rinsed at least 3 times before undergoing for follicle aspiration.

2.7. HARVESTING OF SHEEP CUMULUS OOCYTE COMPLEX

Ovaries were prepared and placed in FIM at 38.5°C in a non-gassed incubator until processing. Antral follicle diameter between 2-5 mm was chosen and aspirated by using a 19-gauge short bevelled needle (Terumo UK Ltd, Surrey, UK) connected to a 10ml syringe (BD Plastipak®, Drogheda, Ireland) prefilled with 1ml pre-warmed oocyte holding medium (H199+) (See section 2.5.3). According to validation trials that size of follicle was taken into account, medium (2-3mm) vs large (4-5mm) antral follicles were discriminated by using the largest bevel diameter of a 19-gauge short bevelled needle as a measurement reference (4mm). Follicles with diameter between $\frac{1}{2}$ and almost full length of reference diameter were classified into medium size antral follicles group (2-3mm) whereas aspirates from antral follicle diameter that was equal to or just above the reference diameter were classified into large size antral follicles group (4-5mm). And the whole processes of harvesting were accommodated with the two equipment settings including two sets of harvesting plastic wares, syringes and needles for separation follicular aspirates of medium sized (2-3mm) from the aspirates derived from large sized (4-5mm) antral follicles.

Each aspirate contained follicular fluid with cumulus oocyte complexes (COC) was dispensed into a 90mm sterile tissue culture dishes containing pre-warmed H199+ medium, which were placed on a 38.5°C heated stage (MTG Medical Technology Vertriebs GmbH, Bruckberg, DE) throughout the process. Then COC harvesting was performed and examined to separate COC from follicular fluid under a stereomicroscope (Olympus UK Ltd., Southall, UK) fitted with a heated stage (Tokai HitCo., Ltd, Shizuoka-ken, JP) at 38.5°C. COCs were pooled together in a 35mm nunc® petri dish filled up with pre-warmed H199+ and kept at 38.5°C in a non-gassed incubator until the recovery process finished. Time spent from follicle aspiration to finished COCs harvesting was no longer than 1 hour.

Only oocytes enclosed by 3 or more intact layers of unexpanded cumulus cells that were medium-brown in colour and composed of a finely granulated homogenous ooplasm were selected and pooled in a 4-well dish containing fresh pre-warmed H199+ (800µl/well) at 38.5°C. The holding plate was kept in a non-gassed incubator until further analysis. In order to assess the chromatin organization, GV staged oocytes were freed of CCs by being soaked in 800µl of H199+ containing 80IU/ml of hyaluronidase enzyme in the first well of a 4-well dish for 30 seconds to 1 minute prior to being transferred and soaked in 800µl of new pre-warmed H199+, this was repeated twice more in the other 2 wells of the same 4-well dish with gently repeated pipetting in and out through narrow bore glass pipettes (see section 2.8). Denuded oocytes underwent a final examination under the stereomicroscope and those without the first polar body were recruited for the following chromatin studies. Each experiment was performed in 3-6 replicates depending on the quantity of tissue availability. All processes were conducted under sterile conditions in a laminar flow hood class II on the heated stage to maintain the temperature at 38.5°C.



Figure 2.2. Representative reproductive tracts and corpus luteum from sheep tissues. Size and characteristics of lamb and ewe reproductive tracts compared (left picture) while ewe ovaries (right picture) demonstrate follicles (red arrow head) and

2.8. OOCYTE DENUDATION AND *IN VITRO* MATURATION

In many of the experiments, conducted denudation of the CC surrounding each oocyte was a prerequisite for maturational assessment and for the measurement of single-oocyte metabolism in GV stage oocytes, as well as for the preparation of each single cell for molecular analyses. Due to the fact that determination of the actual

number of GCs and CC that enclose an oocyte can be problematic, to study oocyte amino acid turnover and carbohydrate metabolism it is necessary to completely remove the surrounding cumulus and follicular cells from oocytes (Hemmings, 2007, Harris, 2002). Moreover, cumulus and GCs are metabolically active so the presence of these cells may interfere with the oocyte and the AAP measurements. Oocytes enclosed within 3 or more intact layers of CCs were chosen under the stereo microscope (Olympus UK Ltd, Southall, Middlesex) for all oocyte experiments (Figure 2.3.A and B). Before denudation, the COCs were washed twice through 800µl of H199+ medium and groups of 10 to 20 COCs were then placed into 500µl of pre-warmed 80 IU/ml hyaluronidase (Hyase®) solution (see appendix II) in H199+ medium up to 60 seconds in 4-well nunc® dish. Denudation was done by using 2-step pipetting techniques. The first step involved narrowing the pipette size to 156-190µm diameter (Swemed®) to eliminate large pieces of CCs followed by the second step where the pipette size was reduced to 130-133µm diameter (Swemed®). The COCs were repeatedly passed through the narrow pipettes until all CCs had been totally removed. Denuded GV oocytes were then passed through a few washes in new 800µl of H199+ medium in a 4-well nunc® dish and were ready for inclusion into the experiments. Some of the denuded GV oocytes were used as the control group at the time of harvest, time 0 hour (Figure 2.4). Single-GV oocyte time 0 control samples were rinsed twice in 800µl 1X Dulbecco's phosphate buffered saline (DPBS; Gibco™,14190). Prior to being stored for future molecular analysis they were loaded into 0.5ml Eppendorf tubes containing either 10µl 1X PBS buffer (Sigma, P5493) for mitochondrial (mt)DNA analysis, 10µl Dynabead® buffer supplied for complementary deoxyribonucleic acid (cDNA) construction, or 2µl RNA-GEM buffer supplied for next generation sequencing according to the experimental design. The individually stored oocytes were stored at -80°C until analysis.

To facilitate the resumption of meiotic maturation and oocyte IVM, groups of 30-35 COCs were incubated in serum-free oocyte IVM medium, for 24 hours at 38.5°C (Figure 2.3.C and D) in a humidified atmosphere 5%CO₂ before undergoing denudation as detailed previously. Assessment of nuclear maturation was checked under a stereomicroscope with a magnification range from 4X to 125X (Olympus UK Ltd, Southall, Middlesex) after denudation by gently pipetting and rolling oocytes to

determine the presence or absence of the 1st PB (Figure 2.4). Both denudation and assessment of nuclear maturation were carried out in H199+ dishes placed on heated stage at 38.5°C under a stereomicroscope.

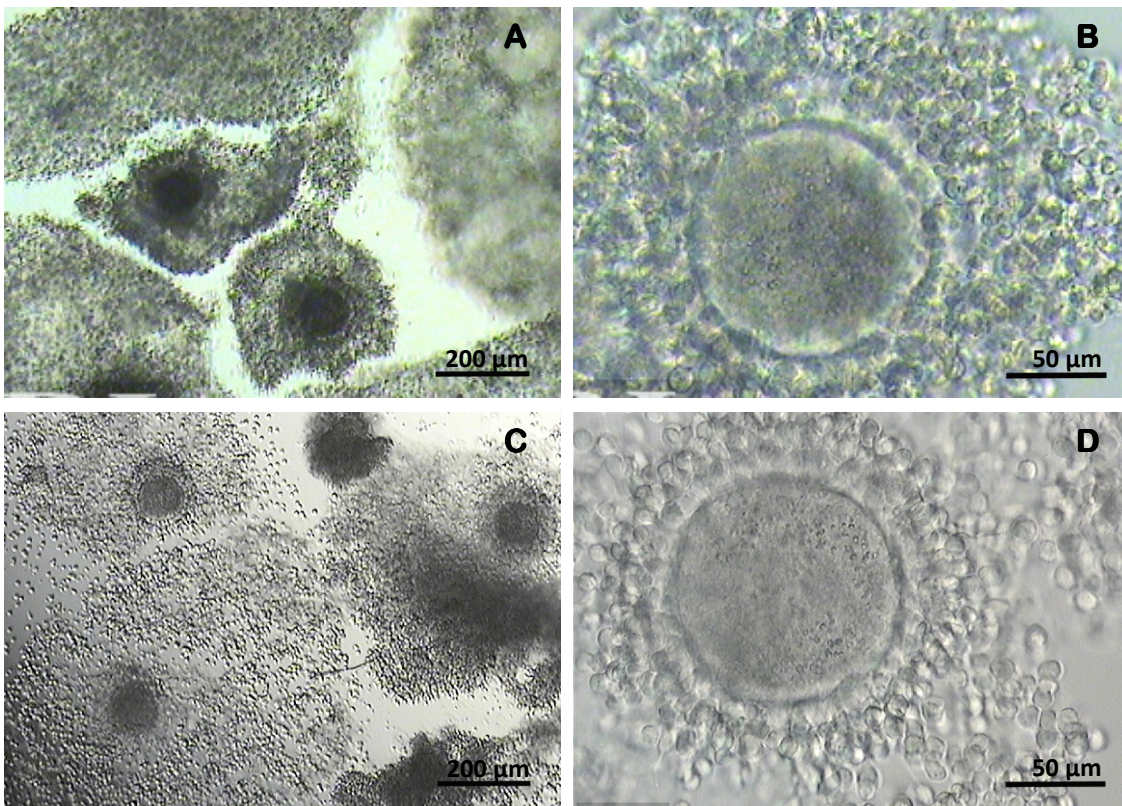


Figure 2.3. Cumulus Oocyte Complexes before (A-B) and after (C-D) 24 hours of IVM under brightfield microscope. Compaction of CCs demonstrates in A and B. Dispersed and organized cumulus layers demonstrate in C and D.

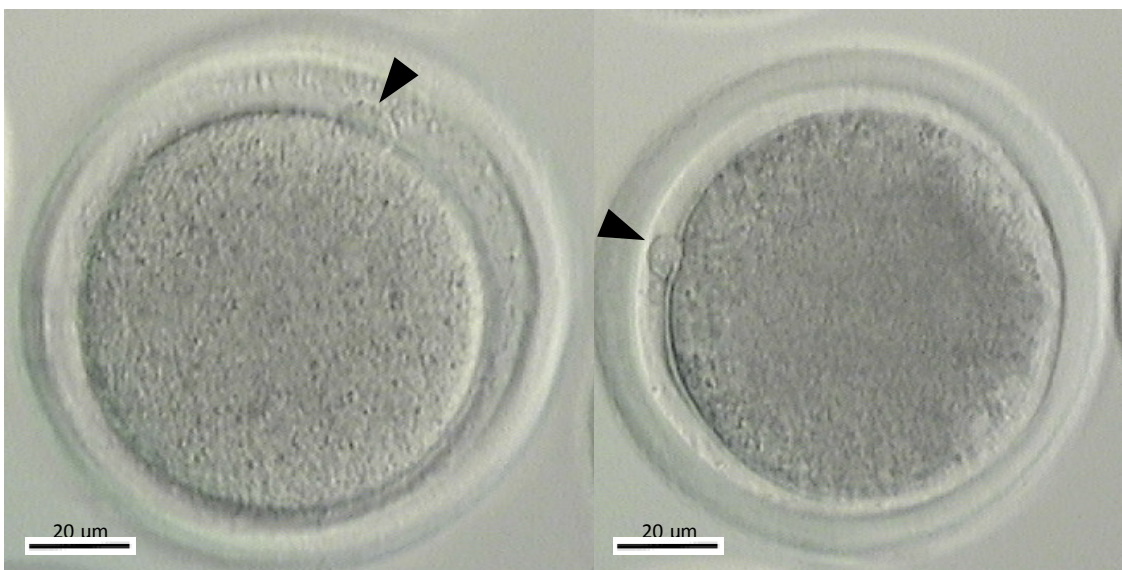


Figure 2.4. Representative denuded MII oocytes from adult sheep animals after 24 hours IVM. Each of them demonstrate the first PB underneath ZP (arrow head).

2.9. *IN VITRO* MATURATION OF OVINE CUMULUS OOCYTE COMPLEX

The methodology for the serum-free ovine *in vitro* maturation (IVM) has been extensively validated and reported by our group (Cotterill *et al.*, 2012, Danfour, 2001). The majority of experiments conducted here prepared oocytes for maturation in groups of 30-35 COCs. IVM media was prepared in 4-well nunc® dishes on the day before tissue collection. The top row two wells on each plate contained 800µL of IVM medium for washing whereas the bottom two wells contained 500µL of IVM medium overlaid with 300µL of pre-equilibrated and washed mineral oil. Dishes were then incubated overnight at 38.5°C and 5%CO₂ in air in a humidified incubator.

On the day of tissue collection, harvested COCs presenting a homogeneous compact cumulus of at least 3 layers were first rinsed in 1 well from the top prior to placing for culture in a well on the bottom of IVM dish at 38.5°C and 5% CO₂ in air in a humidified incubator for 24 hours (Figure 2.3.C and D). Cumulus morphology was evaluated at the end of the IVM culture before the denudation process was initiated. After denudation, single-MII oocyte samples in the MII control group at time 24 hours, were rinsed twice in 1X DPBS prior to being stored for molecular analysis by loading into 0.5ml Eppendorf tubes containing either 10µl 1XPBS buffer for mtDNA analysis, 10µl Dynabead® buffer or 2µl RNA-GEM buffer for cDNA construction according to the experimental design.

2.10. OOCYTE CULTURE FOR MEASUREMENT OF AMINO ACID TURNOVER PROFILING

Equilibration (EQ) and AAP dishes were prepared on the day prior to tissue handling from the abattoir. Dishes were prepared according to the number of oocytes to be tracked. A maximum 4 sets of EQ and AAP plates were prepared for each culture replicate. Each set of plates could be used for tracking up to 9 oocytes (Figure 2.5). In each EQ dish, 2 wash drops of 10µl AAP medium were dispensed at the top row of a 35mm MEA tested, CE marked nunc® dish for IVF supplies, and the other 9 drops of 5µl AAP medium were setup for individual oocytes equilibration and overlaid with pre-warmed mineral oil (Figure 2.5).

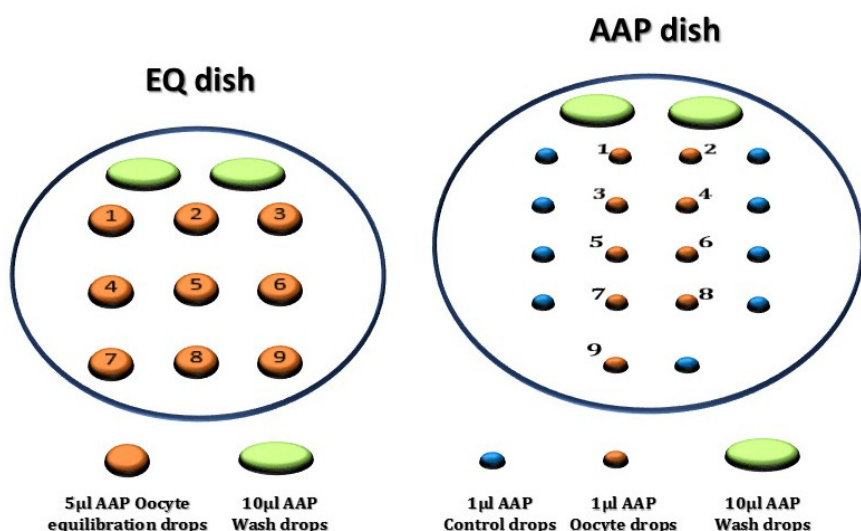


Figure 2.5. Illustration of the layout equilibration (EQ) and AAP dishes. Both dishes were prepared by using modified AAP medium with 50µM cilostamide supplementation.

The AAP tracking dish contained 2 wash drops of 10µl AAP medium on the top row of a 60mm MEA tested, CE marked nunc® dish for IVF, and the other 18 drops of the 1µl AAP medium were setup for a maximum of 9 oocytes trackings in corresponding 9 drops of control AAP medium (without oocyte incubation) (Figure 2.5). All dishes were prepared in a sterile flow hood at room temperature. Due to the very tiny volume of a 1µl drop of AAP, it is not manageable to use classic Gilson pipettes (Gilson Scientific Ltd, Bedfordshire, UK). To achieve the 1µl volume precision the use of a Microgram M10 positive displacement pipette (Gilson Scientific Ltd, Bedfordshire, UK) was required. Same as in the EQ dish, pre-incubated mineral oil was placed on top. Both EQ and AAP dishes were allowed to equilibrate to 38.5°C and 5%CO₂ in a humidified incubator overnight.

On the day of the experiment, after the denudation process, individual ovine oocytes underwent washing twice in the 2-wash drops prepared in each EQ dish before passing on to 1 of the holding drops for an hour equilibration. After that, the oocytes were transferred to incubate for 6 hours in AAP assay dishes. As well as oocytes loading to EQ dish, the individual oocytes were washed twice in wash-drops before gently being placed into the corresponding drops at the equivalent position to the EQ dish with volume of medium carry over as less as possible. Both equilibration and AAP incubation were performed in a humidified CO₂ incubator at 38.5°C in 5%

CO₂ conditions. After 6 hours of culture incubated oocytes were removed individually from 1µl AAP drops taking extreme care not to change the volume of spent culture media. Finally, spent AAP dishes were sealed with tape before storage at -80°C until analysis. Each individual oocyte was rinsed twice in 1X DPBS prior to being stored for future molecular analysis by loading into 0.5ml Eppendorf tubes containing either 10µl PBS buffer for mtDNA analysis or 2µl RNA-GEM buffer for next generation sequencing according to experimental design. A small number of individual oocyte single samples were fixed in 4% paraformaldehyde (PFA) for chromatin staining with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) in the next step (see details in chapter 3).

2.11. OOCYTE CULTURE FOR MEASUREMENT OF GPL METABOLISM

EQ and GPL assay dishes were prepared on the day prior to tissue handling from the abattoir. Dishes were set-up according to a number of oocytes to be tracked; however, a maximum of 4 sets of EQ and GPL dishes were prepared for each culture replicate. Each set of dishes could be used for tracking up to 9 oocytes (Figure 2.6). Carbohydrate metabolism analysis was performed in modified KSOM medium. An EQ dish contained two 10µl wash drops at the top row and another 9 drops of 5µl modified KSOM medium for oocyte equilibration overlaid with pre-warmed mineral oil in a 35mm MEA tested, CE marked nunc® dish for IVF. A GPL tracking dish contained two 10µl wash drops on the top row and a further 12 drops of 130-160nl modified KSOM medium set-up for 9 individual oocytes incubation and the final 3 drops were left blank as a control at the bottom row of a 60mm MEA tested, CE marked nunc® dish for IVF. Like an EQ dish, GPL tracking dishes were overlaid with pre-warmed mineral oil. Apart from the 2-wash drops, nanolitre-drop size required a set of nanolitre pipettes for manipulation. As stated elsewhere, they needed to be cleaned before and after use as well as between each drop set-up to wash out residuals of media mounted inside pipettes' wall. Both EQ and GPL dishes were allowed to equilibrate to 38.5°C and 5% CO₂ in a humidified incubator overnight.

On the day of the experiment, after the process of denudation, individual oocytes were handled as described above (see in section 2.10). Briefly, oocytes were rinsed twice before being incubated individually for 1 hour in the EQ dish and again rinsed

twice before a 6 hour incubation in GPL tracking dish in a humidified CO₂ incubator at 38.5°C in 5% CO₂ conditions. Previously the optimal incubation was validated for metabolism assays of murine (Harris, 2002)] and human oocytes (Harris *et al.*, 2010). Quantification of carbohydrate metabolism for an oocyte in large antral follicle needs only a few hours incubation time; however, this thesis has designed the use of a 6 hours incubation period in parallel with amino acid metabolism. After 6 hours incubated oocytes had to be removed from nanodrops with minimal change in volume of spent culture media. Finally, spent GPL dishes were sealed with tape before storage at -80°C until analysis. Single-oocyte samples were rinsed twice in 1X DPBS prior to being stored for future molecular analysis by loading into 0.5ml Eppendorf tubes containing either 10µl PBS buffer for mtDNA analysis or 2µl RNA-GEM buffer for next generation sequencing according to experimental design. A part of single samples were fixed in 4% PFA for chromatin staining with DAPI (see details in chapter 3).

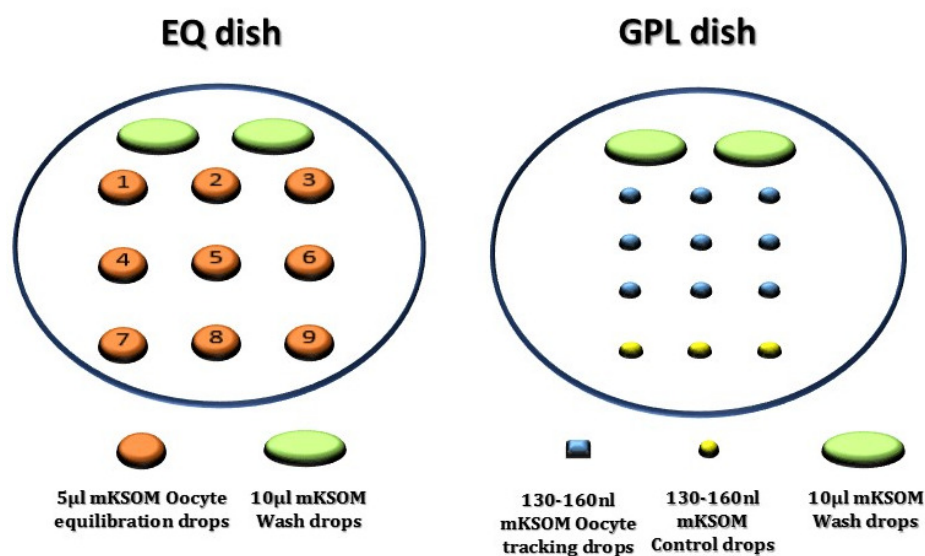


Figure 2.6. Illustration of the layout equilibration (EQ) and GPL dishes. Both dishes were prepared by using modified GPL medium with 50µM cilostamide supplementation.

2.12. STORAGE OF SAMPLES

Spent culture media were left in the original culture dish, sealed and labelled with insulating tape and then kept frozen at -80°C until further analysis. In the validation trial, all oocytes were employed for chromatin configuration evaluation while in the experimental study the cultured oocytes were used for different purposes; including chromatin configuration, mitochondrial copy number, and for genetic

studies. Details of each method used in collecting samples was described in relation to the experiment.

2.13. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

To isolate different molecules dissolved in aqueous samples, the technique of High Performance Liquid Chromatography (HPLC) was employed. The components in individual samples are separated from each other by the liquid phase forcing the aqueous solution with high pressure pump through the stationary phase within the HPLC column. The non-volatile components with different chemical and/or biological properties such as polarities and hydrophobicities, therefore, interact with the packing particles within the stationary phase with different affinities. This enables components within samples to be separated and identified in a reasonable timescale according to differing polarities of the solvents passing through the HPLC column. Analytes from a flow-through device are then displayed as peaks eluted in specific timescales on a chromatogram and the amount of these separated components may be quantified by measuring the relative area beneath each peak. Here reverse phase HPLC was employed to separate 18 amino acids in the spent culture media. This method of analysis has previously been validated for use in single embryo cultures of various species by the Leese group (Booth *et al.*, 2005, Houghton *et al.*, 2002, Houghton *et al.*, 2003, Humpherson *et al.*, 2005, Orsi and Leese, 2004b, Orsi and Leese, 2004a, Stokes *et al.*, 2007), and for bovine IVM-derived oocytes (Collado Fernandez, 2013, Hemmings, 2007, Hemmings *et al.*, 2012) and human oocytes (Hemmings *et al.*, 2013).

2.13.1. HPLC Buffers, Solutions And Amino Acid Standards

a) Sodium Acetate Buffer

Sodium acetate buffer was prepared by dissolving 56.5g sodium acetate trihydrate (Fisher Scientific UK Ltd, Loughborough, UK) in 5 litres of HPLC grade water (ELGA LabWater, High Wycombe, UK). The solution was stored in two 2.5 litre bottles. The pH was adjusted to 5.9 by slowly adding approximately 600µl glacial acetic acid (Fisher Scientific UK Ltd) while the buffer was mixing by use of a magnetic stirrer (Bibby Sterilin Ltd, Stone, UK).

b) Buffers A and B

Buffers A and B were used as the mobile phase for amino acids separation in the HPLC system. The buffers' composition as shown in Table 2.5 were prepared in an extraction hood by mixing sodium acetate buffer, methanol (Thermo Fisher Scientific UK Ltd.) with or without tetrahydrofuran (Thermo Fisher Scientific UK Ltd.) according to the protocol in a 1 litre volumetric flask before filtering through a 0.45µm Durapore® membrane (Millipore) mounted in a glass vacuum filtration set up (Millipore). The volumes of the reagents used are specified in Table 2.5).

Table 2.5. Preparation of buffers A and B for HPLC. *see Appendix III for stock preparation

	Sodium acetate buffer* (ml)	Methanol (ml)	Tetrahydrofuran (ml)	Working Concentration (v/v)
Buffer A (aqueous)	800	200	5	80% Sodium acetate 20% Methanol
Buffer B (organic)	200	800	-	20% Sodium acetate 80% Methanol

c) O-Phthaldialdehyde Solution (OPA)

An OPA solution was used as the derivatizing agent for the HPLC system. This was prepared by adding 100µl of β-mercaptoethanol (Thermo Fisher Scientific UK Ltd.) into a 50ml bottle of OPA (Sigma, P0532). The solution was aliquoted into brown glass HPLC vials (Chromacol Ltd., Herts, UK) and stored at -20°C until required.

d) HPLC Standards

HPLC standards were prepared to standardize the peak areas of the unknown samples. 500µl of 18aa standard (Sigma, AAS18) was mixed with 50ml HPLC grade water (ELGA) in a volumetric flask to make a 25µM stock concentration of the 18 amino acids, then it was aliquoted into sterile white microfuge tubes and stored in freezer at -20°C.

e) D-Aminobutyric Acid, Tryptophan And Asparagine Standard

The 18aa standard mixture lacks tryptophan, asparagine or D-aminobutyric acid. Therefore, a 2.5mM stock solution of these amino acids was prepared by dissolving the amino acids listed in Table 2.6 in 50ml HPLC grade water (ELGA). The use of a sonicator bath (Grant Instruments plc, Chelmsford, UK) is needed to completely dissolve this mixture in solution. A 500µl of solution was aliquoted from the 2.5mM

stock and diluted in a 50ml volumetric flask with HPLC grade water (ELGA) to produce a 25 μ M stock. This final 25 μ M stock was aliquoted into sterile blue microfuge tubes and stored at -20°C.

Table 2.6. Amino acid additives for 2.5mM stock

Reagent	Weight	Stock Concentration
L-Asparagine monohydrate	0.01876g	2.5 mM
L-glutamine	0.01751g	2.5 mM
L-Tryptophan	0.02553g	2.5 mM
DABA	0.01289g	2.5 mM
HPLC grade water	50ml	

f) 10 μ M Amino Acids Standard Solution

Amino acid standards of known concentration (10 μ M) were used for quantification and a monitor of the working condition to ensure accuracy of the HPLC system. The 10 μ M amino acids standard solution was prepared just prior to use by mixing the reagents listed in Table 2.7 in a 1ml sterile microfuge tube following the thawing of the 25 μ M amino acid stocks (see Appendix III for details). In order to ensure accurate measurements, 25 μ l of the 10 μ M amino acids standard solution was dispensed into glass HPLC vials and analysed after every 6 samples.

Table 2.7. Preparation of 10 μ M amino acids standard solution

Reagent	Volume (μ l)	Working conc (μ M)	pmoles in 25 μ M
25 μ M stock of 18 AAs	100	10	250
25 μ M stock of DABA, Gln, Trp, Asn	100	10	250
HPLC grade H2O (ELGA process water, Marlow, UK)	50	-	-

g) Wash Buffer D

Buffer D was used for rinsing the column before and after a complete set of samples was run through. It was prepared in an extraction hood by mixing 500ml of methanol and 500ml of HPLC grade water into a 1 litre volumetric flask. The solution was filter-sterilised through a 0.45 μ m Durapore® membrane mounted in a glass vacuum filtration set up (Millipore UK Ltd.).

2.13.2. HPLC Sample Preparation And Analysis

Dishes containing spent AAP culture medium droplets and controls were thawed at room temperature. Regardless of whether measuring oocyte or control drops, 9 μl of HPLC grade water was added to each 1 μl drop of spent culture media then a total 10 μl volume was passed into a glass HPLC vial. To make up 25 μl in each vial, 15 μl of HPLC grade water was added. This gave a final dilution of 1:25 for each medium drop (Figure 2.7).

The samples were analysed by reverse-phase HPLC, using an Agilent 1100 HPLC system (Agilent Technologies UK Ltd., UK, Stockport, UK) to separate 18 amino acids in spent media and using OPA as the derivatizing agent. Each 25 μl of sample reacted with 25 μl of OPA prior to 25 μl of the mixture being injected onto the column. Samples were separated by a 250 \times 4.6 mm HyperClone 5 μm octadecylsilyl (ODS) (C18) column (Phenomenex, Macclesfield, UK) with 2 buffers: Buffer A and Buffer B, which contained a sodium acetate (pH 5.9) and methanol volume ratio of 80:20 and 20:80, respectively. Tetrahydrofluran was added in buffer A to decrease the buffer's polarity, making up a 0.5% concentration in final solution. The flow rate was set at 1.3ml/min throughout. During the separation process, mixtures of buffer A and buffer B as the mobile phase were passed through the column, and the compositions were changed gradually from 100:0 (A:B) at the beginning to 0:100 (A:B) by the end of the analysis. Each separation cycle lasted 45 minutes of duration (Hemmings *et al.*, 2012, Hemmings *et al.*, 2013).

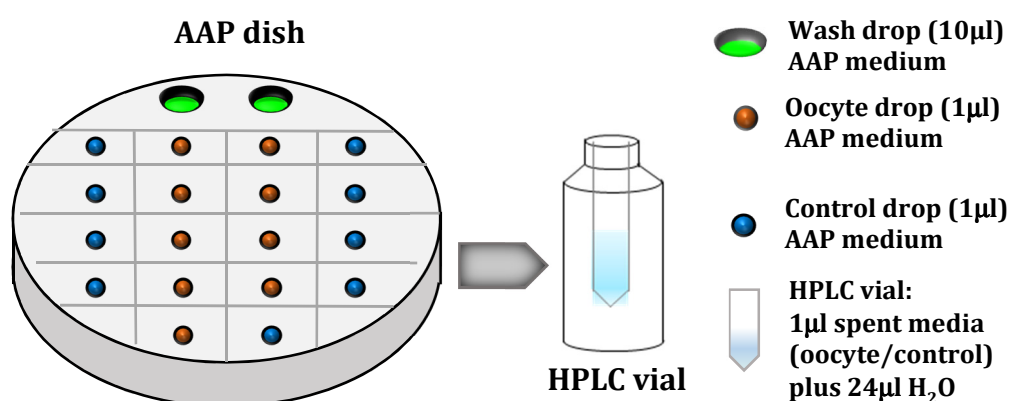


Figure 2.7. Amino acid incubation dish and dilution of samples for HPLC analysis into an HPLC vial.

Amino acids were eluted sequentially based on their different binding affinities to the column. Fluorescence intensity was monitored at an excitation wavelength of 340nm and an emission wavelength of 450nm. The baseline concentration of amino acids within the control droplets was calculated from the medium formulation available from the manufacturer. A chromatogram was produced for each sample and the area beneath each peak was given in arbitrary units which were converted to units of concentration (Figure 2.8). Any dilution errors were corrected by the inclusion of DABA at a known concentration in all oocyte incubation media as the internal standard. To monitor the consistency of the HPLC separation, amino acid standards were analysed every 6 samples. The area beneath each peak was given in arbitrary units which were converted to units of concentration (Booth *et al.*, 2005, Houghton *et al.*, 2002, Stokes *et al.*, 2007). An internal standard to correct for dilution errors was provided by the inclusion of DABA at a concentration of 62.5 μ M in all incubation medium. The net appearance or depletion of each amino acid per sample was calculated with reference to the mean of the control droplets cultured alongside.

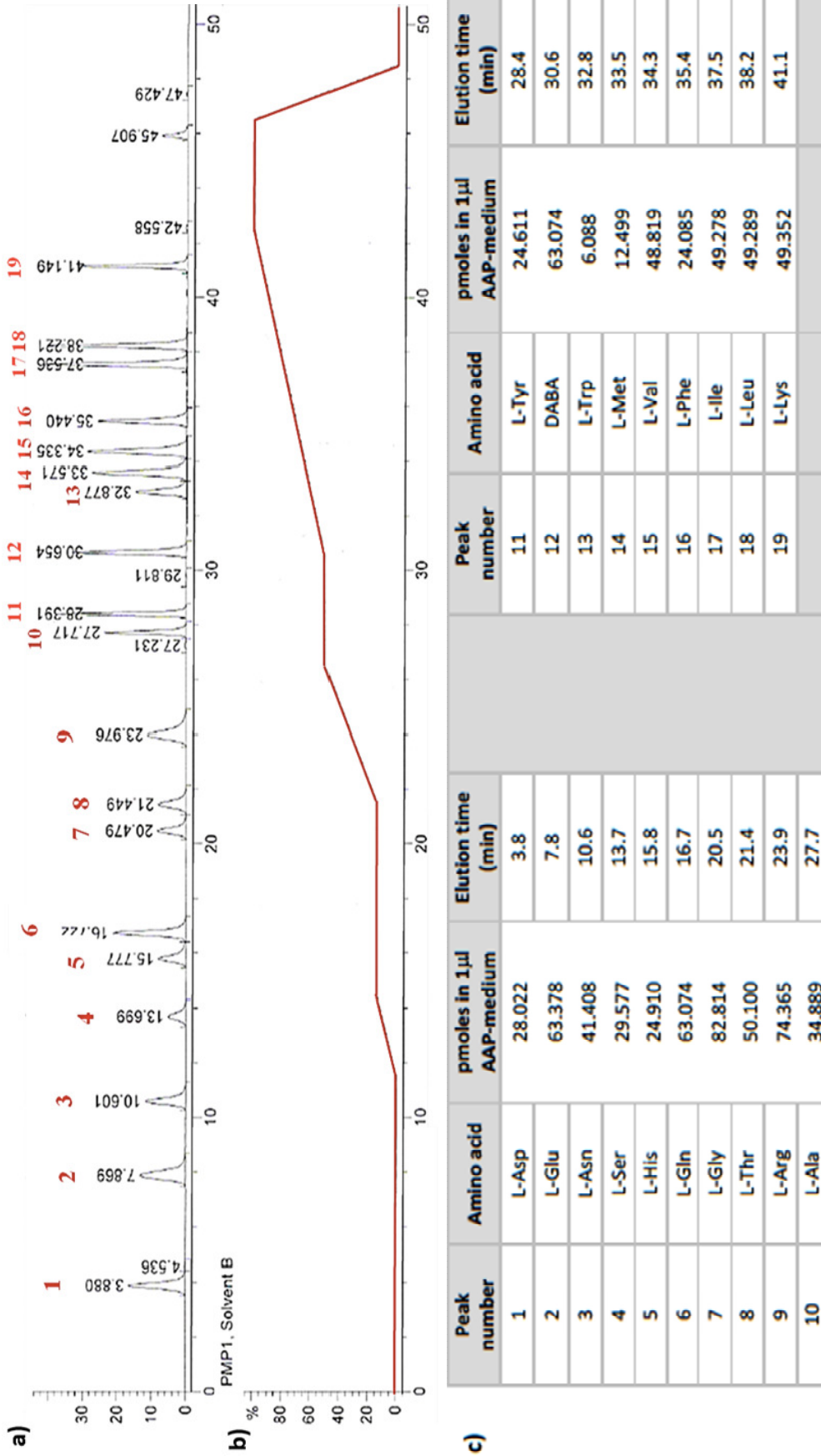


Figure 2.8. Representative example of HPLC analysis of spent AAP medium by oocytes and control samples. a) Representative chromatogram. b) Gradual change in the composition of the mobile phase over time, from 0% Buffer B (100% Buffer A) to 100% Buffer B (0% Buffer A). c) Table for the identification of peaks according to each amino acid elution time. The amount in pmoles of each amino acid in the AAP medium is also shown.

2.13.3. Amino Acid Calculation

The concentration of the amino acids in each drop of medium was calculated from the area beneath each peak corresponding to each amino acid. Identification was performed based on the individual AA standards as the amino acids are always eluted in the same order and at the same elution time. Initially, to correct for non-specific dilution errors each amino acid peak area value was divided by the peak area of the internal standard DABA, and multiplied by 100. This value was the corrected amino acid area. Corrected amino acid areas corresponding to each amino acid for both control and experimental drops were therefore obtained (see example below):

- Volume of α MEM in modified AAP medium = 1.232ml (plus vol in stocks)
- Total volume of modified AAP medium prepared = 9.909ml
- Concentration of Asp in α MEM = $225.378 * 10^{-6}M$

Thus, the equation used for each amino acid (AA) calculation in modified AAP medium demonstrated as the following;

$$\text{The initial amount of AA} = \frac{(\text{concentration in } \alpha\text{MEM}) \times (\alpha\text{MEM volume})}{\text{modified AAP volume}}$$

$$\begin{aligned} \text{Initial amount of Asp} &= \frac{(225.38 * 10^{-6}M) \times (1.232\text{ml})}{9.91 \text{ ml}} \\ &= 28.02 * 10^{-6}M \\ &= 28.02 * 10^{-6} * 10^{12}\text{pmoles in } 10^6 \mu\text{l} \\ &= 28.02 \text{ pmoles in } 1\mu\text{l} \end{aligned}$$

- And the initial amount of Asp in a $1\mu\text{l}$ ($1 * 10^{-6}l$) drop of modified AAP medium contained **28.02 pmoles**.
- Therefore, the initial amount of Asp in a control drop as well as in an oocyte drop was equal to 28.02 pmoles (Figure 2.8.C).

Analytes from the HPLC system were detected in arbitrary units by calculating the area under the peak. Each spent culture medium sample analysed was expected to provide 18 amino acid peaks plus 1 internal control DABA peak, which was used to correct for any non-specific dilution, degradation or appearance, occurring in oocyte and control drops for correcting amino acid peak area calculation. Asp is taken as an example for demonstration:

- Asp Peak Area in control drop 1 = 76,887 arbitrary units
- DABA Peak Area in control drop 1 = 160,019 arbitrary units

Thus, the equation used for Corrected Peak Area demonstrated as the following;

$$\begin{aligned}
 \text{Asp Corrected Peak Area in control drop 1} &= \frac{\text{Asp peak area}}{\text{DABA peak area}} \times 100 \\
 \text{(arbitrary units)} &= \frac{76,887}{160,019} \times 100 \\
 &= 48.05 \text{ arbitrary units}
 \end{aligned}$$

The Asp Mean Corrected Control area was specific for each AAP dish; therefore, all 9 control drops from the same dish were calculated the same way and an average of 9 Asp measurements was corrected for the control peak area used for the further calculation or correction in oocyte drops.

$$\begin{aligned}
 &\text{Asp mean corrected control area (arbitrary units)} \\
 &= \text{Average of the Asp corrected peak area in all 9 control drops} \\
 &= \frac{(41.12 + 42.35 + 43.64 + 43.39 + 44.91 + 44.29 + 44.51 + 44.56 + 44.95)}{9} \\
 &= 43.75 \text{ arbitrary units}
 \end{aligned}$$

Therefore, the Asp Mean Corrected Control area = **43.75 arbitrary units**

To calculate the amount of amino acid in pmoles contained in each oocyte drop after 6 hours' incubation is to use the Rate of Peak Area Variation (increase or decrease in peak area for a given amino acid before and after 6h oocyte culture) per oocyte. Values presented >1 indicate production while values <1 indicate depletion. The Rate of Peak Area Variation of aspartate in oocyte drop 1 is demonstrated by the following;

- Asp Peak area in Oocyte drop 1 = 14.90 arbitrary units
- DABA Peak area in Oocyte drop 1 = 31.49 arbitrary units
- Asp Mean Corrected Control Area considered as the concentration before incubation = 43.75 arbitrary units

$$\begin{aligned}
 \text{Asp Corrected Peak area in Oocyte drop 1} &= \frac{\text{Asp Peak Area}}{\text{DABA Peak Area}} \times 100 \\
 &= \frac{14.90}{31.49} \times 100 \\
 &= 47.31 \text{ arbitrary units}
 \end{aligned}$$

$$\begin{aligned}
 \text{Rate of Peak Area Variation} &= \frac{\text{Asp Corrected Peak area in Oocyte drop 1}}{\text{Asp Mean Corrected Control area}} \\
 &= 47.31 / 43.75 \\
 &= 1.08 \text{ arbitrary units}
 \end{aligned}$$

The amount of each amino acid in pmoles presented in the oocyte drops after the 6 hours incubation was then calculated as the following;

➤ Asp amount in a Control drop = 28.02 pmoles

$$\begin{aligned}
 \text{AA amount in oocyte drop post-incubation} &= (\text{Rate of Peak Area Variation}) \times (\text{AA amount in a control drop}) \\
 &= 1.082 \times 28.02 \\
 &= 30.32 \text{ pmoles of Asp}
 \end{aligned}$$

Finally, the amount in pmoles of amino acid production or consumption by each oocyte during the 6 hours incubation period was calculated by the following equation;

$$\begin{aligned}
 \text{AA amount produced during incubation (amount difference)} &= \text{AA after 6h incubation} - \text{AA amount in control drop} \\
 &= 30.32 \text{ pmoles} - 28.02 \text{ pmoles} \\
 &= 2.30 \text{ pmoles of Asp}
 \end{aligned}$$

Therefore, **2.30 pmoles** of Asp were produced by oocyte 1 during 6 hours incubation.

The Production or Consumption Rate for AA

$$\begin{aligned}
 &= \frac{\text{the amount different in pmoles during incubation}}{\text{incubation period}} \\
 \text{Asp Production Rate} &= \frac{2.30 \text{ pmoles}}{6 \text{ hours}} \\
 &= 0.85 \text{ pmoles/oocyte per hour}
 \end{aligned}$$

In the HPLC system, 25µl of OPA were added to 25µl of sample within each HPLC vial, and the fact that only 40µl of each HPLC vial was injected into the column; therefore, analytes from the column represented 80% of the original amount present in the sample. However, this factor is not taken into account in the calculation due to the fact that it is constant for all samples.

According to previous studies, four parameters were used to determine the amino acid metabolism of oocytes/embryos; overall amino acid depletion, overall amino acid appearance, net amino acid balance, and amino acid turnover (Hemmings *et al.*, 2012, Houghton *et al.*, 2002). Each parameter was calculated as the following;

- The overall amino acid depletion was calculated by sum of all amino acids depletion.
- The overall amino acid appearance was calculated by sum of all amino acids appearance.
- The net nitrogen balance was calculated by subtracting the overall amino acid depletion from the overall amino acid appearance.
- The amino acid turnover was calculated by sum of the amount of overall amino acid depletion and the amount of overall amino acid appearance.

2.14. DATA MANAGEMENT AND STATISTICAL ANALYSIS

All experimental data were analysed using either the SPSS statistics program (SPSS V.21, SPSS Inc., Chicago, IL, USA) or GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). The Pearson's chi-square analysis was used to determine the differences in nominal data; the proportion of oocytes in the various classifications. The normality of distribution was tested by Kolmogorov-Smirnov or Shapiro-Wilk in SPSS or D'Agostino & Pearson or Shapiro-Wilk in GraphPad, programs applied when appropriate. Normally distributed data are presented as mean values \pm SEM, whilst not normally distributed data are presented as boxplots. In boxplots, the boxes represent the 50% of data points (25th to 75th percentile) and the whiskers represent the maximum and minimum data points within 1.5 times of the interquartile range. The horizontal line within each box represents the median. Levene's test was used for testing variance of data. Normally distributed data were analysed by student's t-test if two groups were compared while ANOVA was used in comparison of more than 2 groups, followed by Tukey's post hoc test to specify the contrast between two means. For data not normally distributed non-parametric

tests were applied. Those included Mann Whitney U-tests for 2 group comparisons or Kruskal-Wallis tests if more than 2 groups were considered. For all analyses, significant differences were regarded if the P-value was < 0.05 unless stated otherwise.

CHAPTER 3: EVALUATION OF CHROMATIN CONFIGURATION IN GV OOCYTES IN RELATION TO AGE AND QUALITY

3.1. INTRODUCTION

Acquisition of nuclear and cytoplasmic maturation of an oocyte is required to undergo successful fertilization and further embryonic development. The morphological characteristics of meiotic competence can be defined by the disappearance of the GV (GV) membrane, so called GV breakdown, and extrusion of the first polar body (PB); however, changes comprising cytoplasmic maturity are poorly described (Lasiene *et al.*, 2009, Swain and Pool, 2008, Ferrell, 1999). Various mechanisms have been proposed as the means of maintaining meiotic cell cycle arrest in GV oocytes. These mechanisms are not necessarily mutually exclusive. First, transferring cAMP and the maintenance of high intracytoplasmic cAMP from CCs into the oocyte via gap junctions has long been established as a means of maintaining meiotic arrest (Chaube and Misro, 2002, Dekel *et al.*, 1981, Webb *et al.*, 2002). Besides cAMP, CCs also pass cGMP via gap junction to the oocyte to inhibit hydrolysis of cAMP by the PDE3A enzyme (Norris *et al.*, 2009, Sun *et al.*, 2009a). In addition to external cAMP transport, the meiotic arrest is also maintained by intraoocyte cAMP production via GPR and adenylate cyclase in regulatory control maturation promoting factors synthesis (Han and Conti, 2006, Ledan *et al.*, 2001). Although these mechanisms harmonize in maintaining meiotic resumption, the machinery driving spontaneous nuclear maturation resulting in the transformation of an immature oocyte to MII stage is less than clear (Mehlmann, 2005b, Mehlmann *et al.*, 2002, Vaccari *et al.*, 2008, Zhang *et al.*, 2010).

Previous studies have been widely addressing the lower success rate of clinical IVM when compared to IVF in terms of embryo yields and survival of offspring across mammalian species (Albuz *et al.*, 2010, Child *et al.*, 2002, Eppig *et al.*, 2009, Thompson *et al.*, 1995). Incomplete cytoplasmic maturation has been blamed as a well-accepted downside of *in vitro* maturation (IVM) leading to the low potential of IVM derived oocytes despite the high rate of nuclear maturation. Among numbers of strategies proposed to overcome the limitation of oocytes culture *in vitro*, the

promising ones included delayed spontaneous meiotic maturation as well as maintaining gap junction communication between oocyte and surrounding CCs to attain the acquisition of cytoplasmic maturation (Jamnongjit and Hammes, 2005). Due to manipulation of cAMP or cGMP, this method sustains high level of cAMP within CCs and the oocyte either to the regulatory synthesis of cAMP or inhibiting degradation of cAMP via cGMP blocking PDE3A associating with prolonged bidirectional communication of CC-oocyte via gap junction (Albuz *et al.*, 2010, Franciosi *et al.*, 2012, Luciano *et al.*, 2004, Shu *et al.*, 2008, Thomas *et al.*, 2004a, Thomas *et al.*, 2004b). Numerous establishing mechanisms contribute to enhance the oocyte developmental competence by simulating physiological oocyte maturation (SPOM) in a delayed GV breakdown (GVBD) by increasing intra-oocyte (GSH), lowering intra-oocyte hydrogen peroxide (H₂O₂) (Li *et al.*, 2016), enhancing COC oxygen consumption and oocyte oxidative metabolism and accentuating epidermal growth factor signalling in CCs (Richani *et al.*, 2014, Zeng *et al.*, 2013, Zeng *et al.*, 2014). Recent studies have also reported the improvement of IVM outcome by adding adenylate cyclase activators and/or PDE3A inhibitors (Conti *et al.*, 2002), as well as applying a 2-step culture system with adding PDE3A (Albuz *et al.*, 2010, Gharibi *et al.*, 2013, Li *et al.*, 2016, Rose *et al.*, 2013), C-Type Natriuretic Peptide (CNP) (Sanchez *et al.*, 2017), or BMP-15 (Santiquet *et al.*, 2017) into the prematuration step.

During the meiotic maturation, one of the less well characterized but nonetheless remarkable modifications associated with oocyte developmental competence is the dynamic change of GV chromatin configuration that occurs following the reinitiation of meiosis. This process is essential to support the structural remodelling of the oocyte in that it is necessary for the completion of meiotic maturation. This process involves several different oocyte functions (for review see (De La Fuente, 2006, Luciano and Lodde, 2013). At this stage of development, individual oocyte chromosomes loosen their structure to disperse the chromatin mass. And then, within the GV, the chromatin undergoes profound rearrangement prior to the meiotic resumption. This phenomenon may determine the oocyte/embryo potential, as previously reported in various species observed it links to subsequent embryonic development (Lodde *et al.*, 2007, Luciano *et al.*, 2011, Zuccotti *et al.*, 1998, Zuccotti *et al.*, 2002), oocyte morphology (Assey *et al.*,

1994, Hyttel *et al.*, 1997, Lodde *et al.*, 2008), transcriptional activity (Abe *et al.*, 2010, Bouniol-Baly *et al.*, 1999, Christians *et al.*, 1999, De La Fuente and Eppig, 2001, Liu and Aoki, 2002, Lodde *et al.*, 2008, Luciano *et al.*, 2011, Miyara *et al.*, 2003) and the epigenetic signature of the oocyte (Akiyama *et al.*, 2004, Andreu-Vieyra *et al.*, 2010, Ivanovska *et al.*, 2005, Ivanovska and Orr-Weaver, 2006, Lodde *et al.*, 2009). Acquisition of oocyte competence to facilitate further development requires nuclear chromatin transforming from a diffused/fibrillar pattern to a more condensed stage. Nonetheless, physiological factors such as gamete size and follicle development, may influence chromatin morphology observed by (Liu *et al.*, 2006, Russo *et al.*, 2007a, Schramm *et al.*, 1993, Sui *et al.*, 2005). According to the main objective of the thesis, it aims to investigate the impact of animal age (prepubertal vs. adult) on the key functional indices of oocyte developmental competence such as chromatin configuration, metabolism and molecular genetics. Sheep oocytes have been used as a physiologically relevant model for human oocyte development. In order to establish what is the impact of oocyte age and the culture environment of assisted reproduction techniques on the dynamics, to investigate the effect of animal age on oocyte quality, to establish whether a direct relationship exists between chromatin configuration and other oocyte functional markers, it was necessary to classify the chromatin configuration of sheep oocytes *in vitro* and to explore if/how this classification is confounded by follicular size, the denudation process, or other aspects of handling and culture such as the culture media environment. Oocyte denudation is essential for the precise analysis of oocyte metabolism as the presence of CCs enclosed oocytes impedes metabolic measurement of oocytes due to CCs having more metabolic activity (Biggers *et al.*, 1967, Preis *et al.*, 2005, Sugiura *et al.*, 2005). However, the process of denudation may facilitate spontaneous premature meiotic maturation. Therefore, it can be hypothesized that the oocyte chromatin configuration can be indicative of its fundamental developmental capacity after it is released from the ovulatory follicles which in itself is considered to be one of the markers of oocyte competence or quality.

Aims And Objectives

The aim of this chapter was to examine the impact of ageing on oocyte chromatin configuration and to evaluate how this chromatin pattern and non-invasive

metabolism assays may be used as a tool for oocyte quality assessment. The specific objectives were;

- (i) to develop a classification system for chromatin configuration in the sheep GV oocytes model based on information from other species
- (ii) to research and standardize the confounding factors that may influence changes in chromatin configuration such as the follicular size, time released from the follicle, HEPES buffered and the process of oocyte denudation.
- (iii) to examine the effect of age on the pattern of chromatin configuration in GV-staged sheep oocyte.
- (iv) to validate the measurement of energy metabolism and AAP of individual GV oocytes as a non-invasive tool for quality assessment to differentiate high potential oocyte for further embryonic development.

3.2. MATERIALS AND METHODS

3.2.1. Harvesting And Denudation of Cumulus Oocyte Complexes

Prepubertal and adult sheep ovaries were obtained from a local abattoir during the non-breeding (July to September) and breeding (October to December) seasons, respectively, and then transported to the laboratory, within 2 hours of slaughter. Immediately on arrival, reproductive tissues were separated according to the characteristics described previously (see section 2.6). After that ovaries were dissected and washed 3 times with pre-warmed phosphate buffer saline containing 100units/ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml amphoterin B (see section 2.6 and 2.5.1.) and maintained at 38.5°C in ovary holding medium (see section 2.5.2.) during the process of follicle aspiration. All subsequent procedures, unless differently specified, were performed at 38.5°C.

COCs were harvested as described in section 2.7. Briefly, two sets of harvesting plastic wares, syringes and needles were prepared to separate follicular aspirates of medium sized (2-3mm) from large sized (4-5mm) antral follicles, using a 19-gauge short bevelled needle connected to a 10ml syringe prefilled with 1ml pre-warmed oocyte holding medium (H199+)(see section 2.7). Approximated follicle size was determined by the facet of bevel length as a cut point (4mm). Follicles equal to or larger than this cut off were allocated into the large antral follicle group, while follicles between half and the full length of bevel were categorized as medium

sized follicles. From this step onwards to the end of the experiment, oocytes derived from different sized follicles were conducted separately. The aspirated contents from each group were pooled together in the new pre-warmed H199+ medium in 35mm dishes until examination under a stereomicroscope to recover and remove COCs for the following experiment. Only oocytes enclosed by 3 or more intact layers of unexpanded CCs that were medium-brown in colour and composed of a finely granulated homogenous ooplasm were selected for the study. In order to assess the chromatin organization or metabolism, oocytes were freed of CCs, this denudation process was previously described (see section 2.8). Denuded oocytes from each group were separately pooled in a 4-well dish containing fresh pre-warmed H199+ at 38.5°C until a final examination under the stereomicroscope and those without a PB were recruited for the following chromatin studies. Each experiment was replicated 3-6 times depending on the quantity of tissue availability.

3.2.2. Chromatin Staining

Denuded GV oocytes were immediately fixed in freshly made 4% PFA (v/v) and kept overnight at 4°C for chromatin staining the next morning. The fixative solution was then removed by washing and the oocytes underwent permeabilisation for 5 minutes, twice in 500µl phosphate buffer saline (PBS) with 0.1% Triton X-100. DAPI staining was performed by loading individual oocytes into a 5µl drop of 10µg/ml DAPI on a clean slide (Superfrost® plus; Thermo Scientific (fisher scientific), Loughborough, UK) containing five drops of 5µl DAPI solution that was prepared immediately before use. Each oocyte drop remained separated from the others by a round 10mm coverslip (Scientific Laboratory Supplies, Nottingham, UK) (Figure 3.1). Oocyte containing slides were stored in darkening slide folders until chromatin analysis was performed within 3 hours. Chromatin configuration was evaluated under a 400X of fluorescent microscopy (Zeiss Axioplan2 imaging®; Zeiss, Cambridge, UK). Oocytes were classified and grouped according to the degree of chromatin condensation by our own classification that was modified from a previously reported classification in bovine (Liu *et al.*, 2006, Lodde *et al.*, 2007).

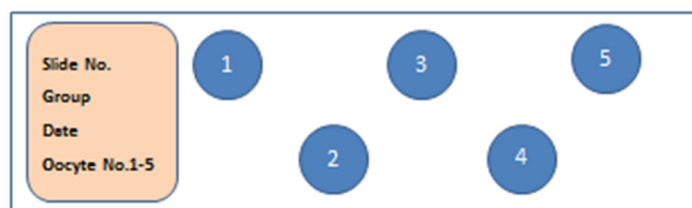


Figure 3.1. Schematics of the prepared chromatin staining slide. Each slide contains maximum 5 drops of DAPI (blue circle). The numbers on top of the droplets are representative for the experimental oocyte number. Every slide has the same layout.

3.2.3. System Used to Classify Ovine GV Oocyte

Ovine GV oocyte chromatin classification was conducted following fixation in 4% (w/v) PFA and 10 μ g/ml DAPI labelling. GV staged chromatin of ovine oocytes was characterized into 4 configurations, based on the degree of chromatin condensation as demonstrated in Table 3.1 and Figure 3.2.

Table 3.1. Classification of chromatin configuration of ovine GV-staged oocytes

	Classification	Characteristics
GV configuration	I: Diffused	Diffused, filamentous pattern with rarely defined empty space entire the nucleus (Figure 3.2.A)
	II: Net-like	Net-like filamentous structure with empty spaces formation throughout the nucleoplasm (Figure 3.2.B)
	III: Condensed (like goat)	Condensed thread-like structure without (Figure 3.2.C) or with enhancement foci (Figure 3.2.D)
	IV: Clumped	Single clumping or clumped chromatin (Figure 3.2.E)
Resumed meiosis	V: MI	Metaphase plate identification (Figure 3.2.F)
	VI: MII	Metaphase plate differentiate from minute single clump outside oolemma (Figure 3.2.G)

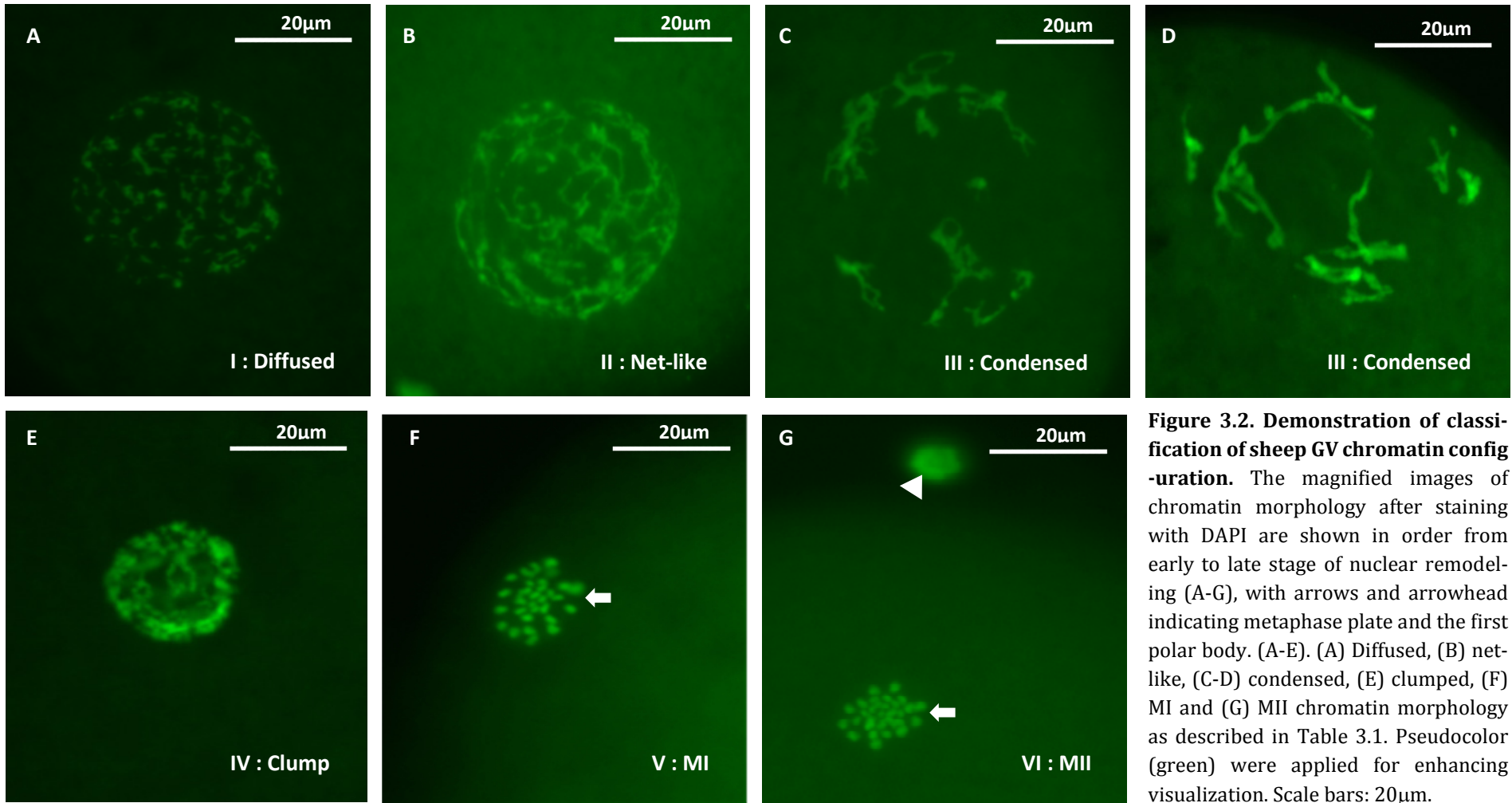


Figure 3.2. Demonstration of classification of sheep GV chromatin configuration. The magnified images of chromatin morphology after staining with DAPI are shown in order from early to late stage of nuclear remodeling (A-G), with arrows and arrowhead indicating metaphase plate and the first polar body. (A-E). (A) Diffused, (B) net-like, (C-D) condensed, (E) clumped, (F) MI and (G) MII chromatin morphology as described in Table 3.1. Pseudocolor (green) were applied for enhancing visualization. Scale bars: 20µm.

3.2.4. Composition, Preparation And Culture Of Modified Amino Acid Profiling Medium

To assess the amino acid metabolism of the GV-staged oocyte requires that the modified AAP medium is complemented with cilostamide, a PDE3A inhibitor, to block acceleration of premature oocyte maturation facilitated by denudation. As its action prevents the inactivation of the intracellular second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), high levels of intraocyte cAMP thus delays or prevents meiotic resumption. Cilostamide needs to be dissolved in DMSO; therefore, apart from cilostamide, DMSO *per se* may have some effect on cell metabolism. To test the impact of cilostamide requires two control media; AAP control medium-no DMSO as a negative control (DMN), and AAP medium containing DMSO vehicle at the equivalent volume used for the cilostamide treatment 8.56 μ l DMSO as a DMSO vehicle control (DMO). Following the previous work done by our group (Cotterill, 2008), 10 and 50 μ M cilostamide (DM10 and DM50) supplementation was tested. The composition of the modified AAP medium was modified from a previously described protocol in bovine (Hemmings, 2007, Hemmings *et al.*, 2012), by incorporating DMSO with or without cilostamide as shown in Table 3.2. Modified AAP medium was prepared fresh on the day before incubation, all reagents listed (Table 3.2) were mixed in a 15ml sterile universal and the pH reduced to approximately 7.9 by adding 5M HCl whenever needed. Sterilisation was done by filtering the solution through a 0.2 μ m Acrodisc[®] syringe filter and the sterile media was stored at 4°C until use. For the measurement, the modified AAP medium was prepared in 2 steps; equilibration (EQ) and incubation (AAP). Dish preparation was done under a sterile condition at room temperature and drops of medium were immediately overlaid with prewashed and equilibrated mineral oil and left overnight at 38.5°C in a 5% CO₂ humidified incubator. The next day, pooled denuded-oocytes were randomly allocated in the 4 different types of AAP media (Figure 3.3). Individual oocytes were washed twice and equilibrated in an EQ dish for 1 hour before being transferred to incubate for 6 hours in the AAP dish. Transfers were conducted using 130-133 μ m stripping pipettes (Swemed[®]) to ensure the volume was maintained during careful oocyte displacement into the AAP culture drops. Previous work from our own group (Hemmings, 2007, Hemmings *et al.*, 2012), has shown that incubating 6 hours was optimal to detect significant

Table 3.2. Composition of ovine modified serum-free AAP medium. Stock preparations are shown in Appendix II.

<i>Reagent</i>	<i>Stock concentration</i>	<i>Working concentration</i>	<i>Control-no cilostamide</i>		<i>Cilostamide</i>	
			<i>DMN (no DMSO)</i>	<i>DMO (with DMSO)</i>	<i>DM10 (10 µM in DMSO)</i>	<i>DM50 (50 µM in DMSO)</i>
<i>α MEM eagle (Sigma,M4526)</i>	-	-	1.210 ml	1.210 ml	1.210 ml	1.210 ml
<i>BSA Cell Culture Grade (Sigma, A6003) (≥96% essentially fatty acid free).</i>	-	-	0.01 g	0.01 g	0.01 g	0.01 g
<i>Pen/Strep</i>	10,000 IU/ml, 10,000 µg/ml	50 IU/ml, 50 µg/ml	50 µl	50 µl	50 µl	50 µl
<i>Na pyruvate stock</i>	47 mM	0.47 mM	100 µl	100 µl	100 µl	100 µl
<i>L-Glutamine stock</i>	100 mM	62.5 µM	6.25 µl	6.25 µl	6.25 µl	6.25 µl
<i>Bovine holo-transferrin</i>	5 mg/ml	5 µg/ml	10 µl	10 µl	10 µl	10 µl
<i>Na selenite stock</i>	50 µg/ml	5 ng/ml	1 µl	1 µl	1 µl	1 µl
<i>Bovine insulin 10mg/ml (Diluted 1:1000 immediately before use)</i>	10 µg/ml	10 ng/ml	10 µl	10 µl	10 µl	10 µl
<i>Long R3 IGF-1</i>	100 µg/ml	10 ng/ml	1 µl	1 µl	1 µl	1 µl
<i>Ovine FSH</i>	2 IU/ml	0.0006 IU/ml	0	0	0	0
<i>Ovine LH</i>	2 IU/ml	0.0003 IU/ml	0	0	0	0
<i>EBSS</i>	-	-	8.5 ml (plus 8.56µl)	8.5 ml	8.5 ml	8.5 ml
<i>cilostamide</i>	20 mg/ml	-	0	0	8.56 µl of 5X dilution (8.56 µl stock: 34.24µlDMSO)	8.56 µl
<i>DMSO</i>	-	-	0	8.56 µl	0	0
<i>DABA stock</i>	50 mM	62.5 µM	12.5 µl	12.5 µl	12.5 µl	12.5 µl
<i>Total volume</i>			9.909 ml	9.909 ml	9.909 ml	9.909 ml

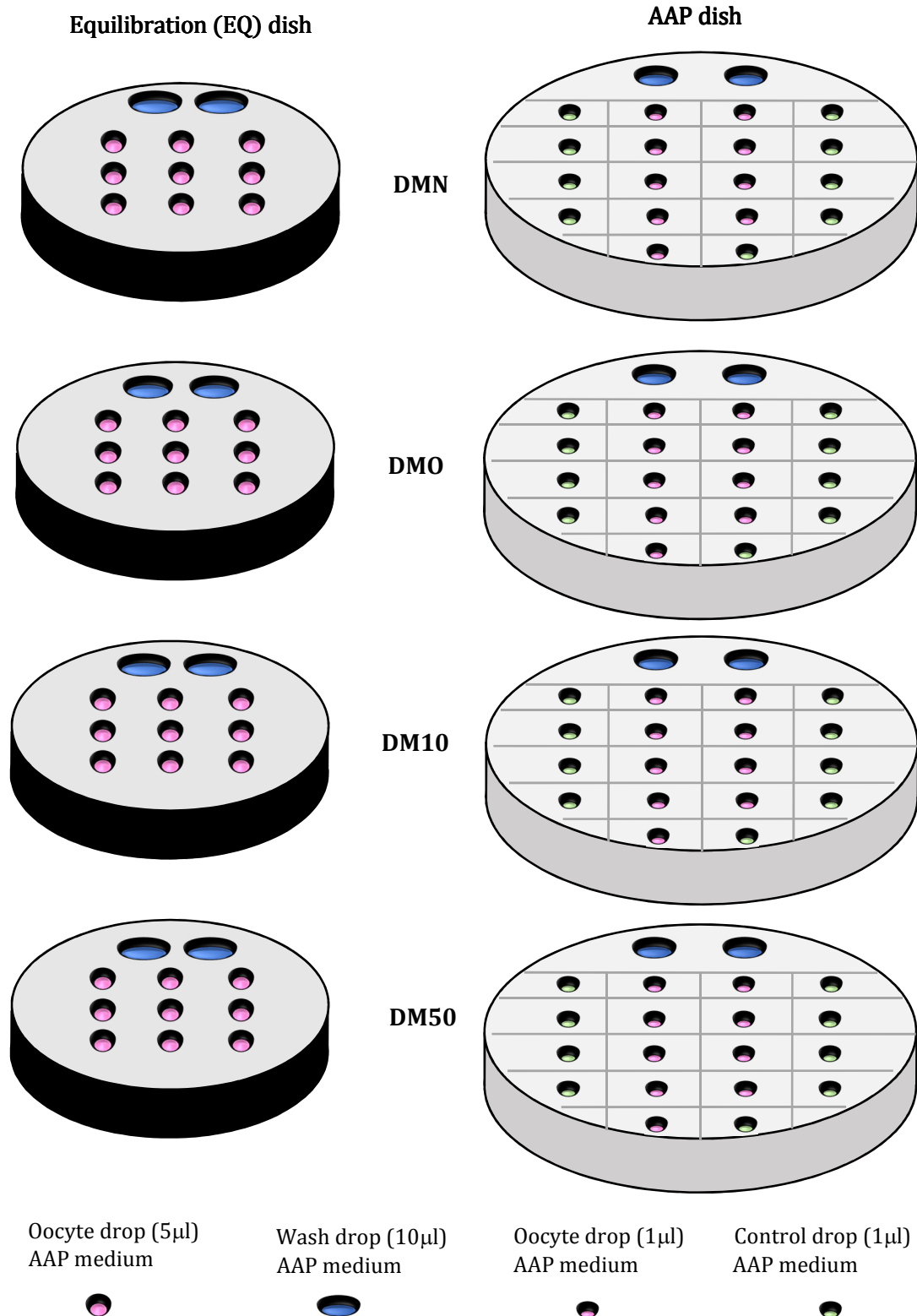


Figure 3.3. Examples of dish-layouts used for the AAP media validation experiment. Each EQ dish contains 2 wash drops of 10 μ l AAP medium (blue) and 9 drops of 5 μ l AAP medium (large pink) for individual oocyte incubation, while each AAP dish contains 2 wash drops of 10 μ l AAP medium (blue), 9 control drops of 1 μ l AAP medium (green), and 9 drops of 1 μ l AAP medium (small pink) for individual oocyte incubation.

changes in AA turnover by individual bovine oocytes without compromising the subsequent developmental potential of the gamete. Therefore, in the following experiments, denuded oocytes were prepared for a for 6 hour AAP assay after a 1-hour equilibration period.

3.2.5. Experimental Design

a) Experiment 1: Characterization Of The Pattern Of Chromatin Configuration In Ovine GV-Staged Oocytes Using DAPI Staining

According to several published reports in various species, classification of chromatin morphology is still inconclusive since a variety of classifications established. This may result from species specific chromatin patterns, as well as, the different methods used for evaluation; staining or types of microscopes, for example (Luciano and Lodde, 2013). Among several reports, only one study has been conducted in sheep using SYBR-Green staining of chromatin (Russo *et al.*, 2007a). This approach was quite distinct from other studies in terms of the appearance of chromatin that integrated the patterns found in both mammals and ruminants. The current experiment, therefore, was conducted to examine the chromatin appearance and classification of sheep GV-staged oocyte. The simple nuclear staining DAPI was used for evaluation. In this experiment, all GV oocytes from prepubertal and adult arms were harvested in breeding season. Tissues were immediately processed followed by the COCs harvesting and denudation before immediate PFA fixation and chromatin staining. Hence, chromatin configuration in this first experiment is representative of GV oocyte chromatin patterns at time 0 point.

b) Experiment 2: Assessment Of Ovine GV Chromatin Configuration After A 6 Hours Incubation In AAP Medium

During mammalian oocyte development, the transition of chromatin pattern from a less condensed to a more condensed state represents oocyte growth and differentiation. Oocytes enclosed in large antral follicles have a greater potential to mature *in vitro* and demonstrate a higher proportion of oocytes with advanced stages of chromatin condensation in comparison to those oocytes derived from small antral follicles where uncondensed chromatin patterns predominate (Fuhrer *et al.*, 1989, Hinrichs and Schmidt, 2000, Hirao *et al.*, 1995, Schramm *et al.*, 1993, Sui

et al., 2005, Wang *et al.*, 2009a, Wickramasinghe *et al.*, 1991, Yousaf and Chohan, 2003). To evaluate whether this marker chromatin configuration pattern can be used to discriminate between oocytes of different developmental potential on oocyte chromatin patterns from animals of different age, a validation trial is conducted to exclude the impact of follicular size. Moreover, this study also evaluated the maturation potential of oocytes recruited from follicles of 2-3mm and of 4-5mm diameter. This experiment was performed in triplicate. Ovarian tissues were classified according to prepubertal or adult ovarian morphology (see section 2.6). The COCs derived from both prepubertal and adult animals were harvested following follicle aspiration and assigned into 2 groups according to the follicular size of 2-3mm or 4-5mm diameter. Thereafter, denudation was immediately performed as detailed above.

c) Experiment 3: Evaluation Of The Effect Of Denudation On The Dynamics Of Oocyte Chromatin Configuration Over 6 Hours

Following the release of fully grown oocytes from the follicle, premature meiotic maturation spontaneously occurs (Edwards, 1965a, Edwards, 1965b). It has been suggested that the follicular mechanisms play roles in controlling meiotic arrest by the regulation of cGMP, purine levels and natriuretic peptide protein C (NPPC) to inactivate PDE enzyme which is essential for cAMP degradation (Norris *et al.*, 2009, Sun *et al.*, 2009b). It thereby was necessary to test whether the dynamics of chromatin can be driven by denudation. Moreover, this experiment was able to confirm the changes in chromatin patterns in sequence from the early to the later stage as compared to the established classification of sheep GV oocyte chromatin configuration. Therefore, the experiment was designed to explore the dynamics of chromatin reorganization at 3 consecutive time points; 0 hrs (T0), 3 hrs (T3), and 6 hrs (T6).

Apart of denudation, this experiment also tested the effect of H199+ oocyte holding medium containing HEPES buffer on alteration of the dynamics of chromatin remodelling. According to the safety reports, using HEPES buffering system with sperm is widely supported (Will *et al.*, 2011). Debates remain about the use with oocytes and embryos despite HEPES buffer being widely incorporated in media used in animal and human ART. Previous studies in varied species actually indicated

that HEPES efficiently supports oocyte maturation (Byrd *et al.*, 1997, Downs and Mastropolo, 1997), fertilization (Bhattacharyya and Yanagimachi, 1988, Hagen *et al.*, 1991) and embryo development (Ali *et al.*, 1993, Hagen *et al.*, 1991, Mahadevan *et al.*, 1986, Ozawa *et al.*, 2006) at room atmosphere. However, few reports observed the presence of HEPES compromising glucose uptake (Butler *et al.*, 1988) and lowering fertilisation rates (Lee and Storey, 1986) in mice, as well as compromising blastocyst formation in sheep (Walker *et al.*, 1989a). To explore the HEPES effect, the experiment setting using H199+ oocyte medium without HEPES was conducted in the same three consecutive time points (T0, T3 and T6). Regarding the oocyte handling process, this experiment will help us to clarify if denudation *per se* effects the chromatin configuration and whether HEPES in media may be needed to take into account in the following experiments.

d) Experiment 4: Effect Of The PDE Inhibitor Cilostamide On Chromatin Configuration

Several reports ensure that sustained functional communication between oocyte and CCs via gap junctions during culture *in vitro* is crucial for proper differentiation and meiotic maturation of oocytes in bovine, mouse and human (Luciano *et al.*, 2011, Shu *et al.*, 2008, Vanhoutte *et al.*, 2009). In addition, a recent bovine report demonstrated that a proper functional coupling between oocyte and CCs supports an order of large-scale chromatin remodelling (Luciano *et al.*, 2011). To assess the accurate oocyte metabolism in the series of experiments, CCs removal is an inevitable process that facilitates premature chromatin remodelling and/or metabolic changes of oocytes. Therefore, cilostamide supplementation was applied to simulate the physiological milieu during the culture *in vitro* media since the transient blocking agent inhibits PDE enzymes hydrolysing cAMP to prolong meiotic arrest (Lodde *et al.*, 2013, Mayes and Sirard, 2002, Nogueira *et al.*, 2006, Vanhoutte *et al.*, 2009, Vanhoutte *et al.*, 2008). This may result in harmonizing the nuclear and cytoplasmic maturation and so enhance the oocyte competence (Gilchrist and Thompson, 2007, Luciano *et al.*, 2004, Ponderato *et al.*, 2002). This experiment was to evaluate the inhibitory effect of cilostamide on delaying the progression of chromatin dynamics. Therefore, it was designed to examine chromatin morphology at 6 hrs incubation in four different modified AAP media; AAP negative control (DMN) without DMSO, AAP control with DMSO (DMO), AAP supplemented with

10 μ M (DM10) and 50 μ M (DM50) cilostamide. Two selective concentrations of cilostamide used in this trial (10 μ M and 50 μ M) were tested in a previous study from our own group (Cotterill, 2008).

e) Experiment 5: Effect of Cilostamide on Amino Acid Metabolism

In an assay similar to one that determines chromatin configuration, cilostamide was used to evaluate its effect on oocyte amino acid metabolism since it has yet to be defined. The experiment was conducted during the non-breeding season, using only prepubertal tissues. Cilostamide is prepared by dissolving 5mg of cilostamide (Sigma, C7971) in 250 μ l DMSO to make the 20mg/ml concentration as detailed in Appendix II. The pilot study was designed to compare the amino acid turnover/profiling among four different modified AAP media including two control media (DMN and DMO) and two media with different cilostamide concentrations (DM10 and DM50) as described in previous experiments. Details regarding media preparation, incubation process and culture condition are shown in section 3.2.4. After 6 hrs incubation, individual oocytes and dishes contained spent AAP culture media were stored frozen as detailed in section 2.10. Each arm of this experiment was performed in quadruplo.

f) Experiment 6: Age And Chromatin Configuration

The final experiment was designed to examine whether chromatin configuration can be used as a marker to determine oocyte development potential by using the ageing model between prepubertal and adult oocytes. Prepubertal oocytes were processed during outbreeding season from June to August 2014 while adult oocytes were collected in late October to December 2014. COCs were harvested from reproductive tissues which had no fibrotic scars on either the ovarian surface or both sides, small uterine horns with no rugae appearance, as well as absence of corpus luteum and/or albicans. After denudation, oocytes were individually incubated in modified AAP medium containing 50 μ M cilostamide in 5% CO₂ humidified incubator at 38.5°C for 6 hours prior to overnight fixation in 4%PFA (v/v). Chromatin staining was conducted with 10 μ g/ml DAPI which was performed the next morning as described in section 3.2.2. Apart from modified AAP medium, a parallel experiment was conducted by 6 hours incubation of denuded oocytes in

GPL medium supplement with 50 μ M cilostamide at the same settings as described in modified AAP medium.

3.2.6. Statistical Analysis

All experiments were conducted at least in triplicate. Statistical differences in pattern of chromatin distributions in comparison of animal ages, incubation time point and media composition were analysed by the Chi-square test. A significant difference was considered if $P < 0.05$. When data were not normally distributed, individual amino acid measurements and variables of amino acid turnover, statistical analysis performed by Mann-Whitney U-test and Kruskal-Wallis if two groups and more than two groups compared, respectively. All data obtained were analysed using GraphPad (GraphPad Prism 7 for Mac OS X, GraphPad Software, Inc., La Jolla, CA, USA).

3.3. RESULTS

3.3.1. Experiment 1: Characterization Of The Pattern Of Chromatin Configuration In Ovine GV-Staged Oocytes Using DAPI Staining

GV oocytes were recovered, denuded, fixed and DAPI stained for immediate analysis according to the established classification in Figure 3.2 and Table 3.1. The observation of chromatin configuration from the earliest until the late stage showed 5.3% in diffused (class I), 54.7% in netlike (class II), 15.1% in condensed (class III), and 21.5% in clumped (class IV) pattern. A few oocytes, less than 4% (9 of 265 oocytes) had meiotic resumption as metaphase plate identified with or without a clump outside the nuclear envelope (class VI and V). Whilst 145 of 265 oocytes (54.7%) demonstrated the net-like chromatin pattern (class II), which was the highest chromatin pattern depicted in GV oocytes (Table 3.3) regardless of animal age.

Table 3.3. Distribution of chromatin configuration patterns of ovine GV-staged oocytes

	Classification	No. of oocytes	Percent (%)
GV configuration	I: Diffused	14	5.3
	II: Net-like	145	54.7
	III: Condensed	40	15.1
	IV: Clumped	57	21.5
Meiotic resumption	V: MI	5	1.9
	VI: MII	4	1.5
	Total	265	100.0

3.3.2. Experiment 2: Ovine Follicular Size And GV Oocyte

Chromatin Configuration

This validation trial was conducted between February and March 2014. Both oocytes from prepubertal and adult animals were analysed in triplicate. A total of 30 oocytes (3 replicates) were harvested for study in each group. Therefore, 26 and 23 prepubertal GV oocytes, as well as, 25 and 24 adult GV oocytes harvested from medium (2-3mm) and large (4-5mm) antral follicles, respectively, remained for chromatin analysis. Concerning follicle sizes, the distribution of chromatin pattern displayed in percentages of total oocytes was shown in Figure 3.4.A while the chromatin pattern displayed in regard to age group was shown in Figure 3.4.B and C. After 6 hours incubation in AAP medium, all oocytes transformed to more advanced stages of chromatin condensation; condensed, clumped and a small number even progressed to MI. More than 70% of oocytes in each groups reached at least the clumped stage while above 30% of each group progress to GVBD reached GV breakdown stage. However, none of the oocytes has completed meiotic maturation to MII within 6 hours of incubation. Regardless animal age, there was no significant difference ($P>0.05$) detected in distribution of chromatin pattern between oocytes derived from 2-3mm and 4-5mm diameter follicles (Table 3.4.). Therefore, oocytes derived from both medium and large antral follicles of each age group were pooled together and reanalysed using the Chi-test to see if there was a significant effect of animal age on the pattern of chromatin configuration. The preliminary results found a significant difference of chromatin distribution ($P<0.05$) as shown in Figure 3.4.

Table 3.4. Distribution of chromatin configuration patterns of oocytes derived from medium (MAF) and large antral follicles (LAF) of prepubertal and adult sheep.

Classification		Diffused	Netlike	Condensed	Clumped	MI	MII	Total	P
Adult (%)	MAF	0	0	4 (16)	13 (52)	8(32)	0	25	NS
	LAF	0	0	7 (29.2)	9 (37.5)	8(33.3)	0	24	
Lamb (%)	MAF	0	0	0	11 (42.3)	15(57.7)	0	26	NS
	LAF	0	0	1 (4.3)	15 (65.2)	7(30.4)	0	23	
Pooled (%)	MAF	0	0	4 (7.8)	24 (47.1)	23(45.1)	0	51	NS
	LAF	0	0	8 (17)	24 (51.1)	15(31.9)	0	47	

No significant (NS) differences found between sizes of antral follicles. A statistical significance were determined if Chi-tests, $p < 0.05$.

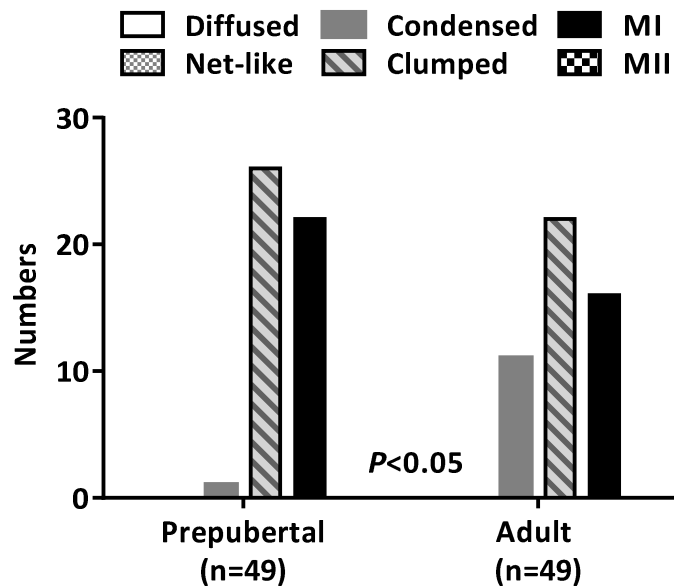


Figure 3.4. Preliminary result of age effect on chromatin morphology distribution by using pooled follicle size 2-5 mm. Data were recalculated from Table 3.4 and a statistically significant difference between prepubertal and adult oocytes was indicated by Chi-tests, $P < 0.05$.

3.3.3. Experiment 3: Effect Of Denudation On The Dynamics Of Oocyte Chromatin Configuration Over 6 Hours

The chromatin configuration at 0 hours incubation were higher distributed in lesser condensed chromatin morphology compared to the appearances observed at 3 and 6 hours post-incubation. The proportion of net-like chromatin pattern declined from 70.4% at the time 0 control to 25.8% and 11.8% at 3 and 6 hours incubation, respectively, in the prepubertal group (Table 3.5). By contrast, it was reduced from 58.8% at time 0 to 15.4% and 0% at 3 and 6 hours, respectively, in the adult group. Approximately, 50% of oocytes in both age groups reached MI stage after 6 hours incubation. Transformation to MI stage chromatin was detected as early as 3 hours post-incubation regardless of animal age (Table 3.5). Data demonstrated significant differences ($p < 0.001$; one-way ANOVA) of means of the pattern of chromatin distribution among 3 different time points in both prepubertal and adult oocytes (Table 3.5). Evidence showed significant differences may be contributed by two patterns: netlike and MI, which seemed to demonstrate the negative correlation of the netlike pattern, as well as the positive correlation of clumped chromatin and duration of incubation time. Unfortunately, the study was unable to identify differences in each pattern of chromatin.

Table 3.5. Distribution of chromatin configuration of denuded oocytes derived from prepubertal and adult sheep at 0, 3 and 6 hrs (T0, T3 and T6).

Classification		Diffused	Netlike	Condensed	Clumped	MI	MII	Sum	P
Prepubertal	T0	1 (3.7)	19 (70.4)	4 (14.8)	3 (11.1)	0	0	27	<0.001 [±]
	T3*	0	8 (25.8)	15 (48.4)	3 (9.7)	5 (16.1)	0	31	
	T6*	0	4 (11.8)	13 (38.2)	1 (2.9)	16 (47.1)	0	34	
Adult	T0	3 (17.6)	10 (58.8)	3 (17.6)	1 (5.9)	0	0	17	<0.001 [±]
	T3*	1 (7.7)	2 (15.4)	6 (46.2)	1 (7.7)	3 (23.1)	0	13	
	T6*	0	0	5 (20.8)	5 (20.8)	13 (54.2)	1 (4.2)	24	
Pooled	T0	4 (9.1)	29 (65.9)	7 (15.9)	4 (9.1)	0	0	44	<0.001 [±]
	T3*	1 (2.3)	10 (22.7)	21 (47.7)	4 (9.1)	8 (18.2)	0	44	
	T6*	0	4 (6.9)	18 (31.0)	6 (10.3)	29 (50)	1 (1.7)	58	

([±]) indicated statistical significances of means of chromatin distribution among 3 different time points; one-way ANOVA, $p < 0.05$, while (*) indicated significant differences if compared to T0 control; unpaired student *t*-test, $p < 0.05$. No significant differences detected in each pattern of chromatin within the animal ages when T0 control compared to T3 or T6; Kruskal-Wallis test, $p > 0.05$.

This experiment has extended to evaluate a confounding effect of HEPES on the dynamics of chromatin patterns in comparison of using oocyte holding medium with and without HEPES supplementation. As shown in Table 3.6, it demonstrated that oocytes incubated in H199+ holding medium with and without HEPES buffer for 3 (T3) or 6 (T6) hours were significantly different ($p < 0.05$) when compared to time 0 control (T0). However, no significant differences ($p > 0.05$) of means in chromatin morphology was detected between oocytes incubated in H199+ holding medium with or without HEPES after both 3 and 6 hours incubation.

Table 3.6. Comparison of HEPES effect on oocyte chromatin configuration at 0, 3 and 6 hours incubation.

Classification		Diffused	Netlike	Condensed	Clumped	MI	MII	Sum	P
T0	Control	2 (3.8)	33 (62.3)	5 (9.4)	13 (24.5)	0	0	53	-
T3	HEPES*	0	3 (10.7)	1 (3.6)	24 (85.7)	0	0	28	0.16
	No HEPES*	0	1 (2.4)	1 (2.4)	40 (95.2)	0	0	42	
T6	HEPES*	0	9 (14.1)	5 (7.8)	45 (70.3)	3 (4.7)	2 (3.1)	64	0.23
	No HEPES*	0	7 (14.9)	6 (12.8)	34 (72.3)	0	0	47	

A significant difference of means of chromatin distribution; One-way ANOVA, $p < 0.0001$, among 5 groups: T0 control, T3 with and without HEPES media, and T6 with and without HEPES media, while (*) indicated significant differences by Dunn's multiple comparisons t -test ($p < 0.05$) if compared to T0 control. No significant differences detected regarding oocyte chromatin morphology when incubation in H199+ holding medium with and without HEPES were compared using Mann-Whitney test, $p < 0.05$.

3.3.4. Experiment 4: Effect Of Cilostamide On Oocyte Chromatin Configuration

This experiment was conducted to estimate the effective dosage of cilostamide supplementation into AAP medium to prevent oocyte meiotic resumption. The two concentrations, 10 μ M and 50 μ M, were selected to be compared with the conventional AAP medium as a control. In addition, the experiment also tested the confounding effect of the solvent vehicle, dimethylsulfoxide (DMSO), on the dynamics of chromatin configuration. Therefore, GV-staged oocytes incubated in DMSO vehicle control (DMO) medium for 6 hours were also compared to those incubated in the negative control AAP medium.

As shown in Figure 3.5.A, no differences were detected ($p>0.05$) in term of distribution of chromatin configuration between DMN and DMO, as well as between the 2 dosages of cilostamide supplementation in AAP media. Therefore, data from 2 control groups, as well as data from 2 AAP media supplemented with cilostamide were pooled to increase sample size in order to emphasize the effect of cilostamide in inhibiting meiotic resumption. The pooled data showed significant differences ($p<0.05$) in chromatin distribution of the cilostamide group when compared to the control group (Figure 3.5.B).

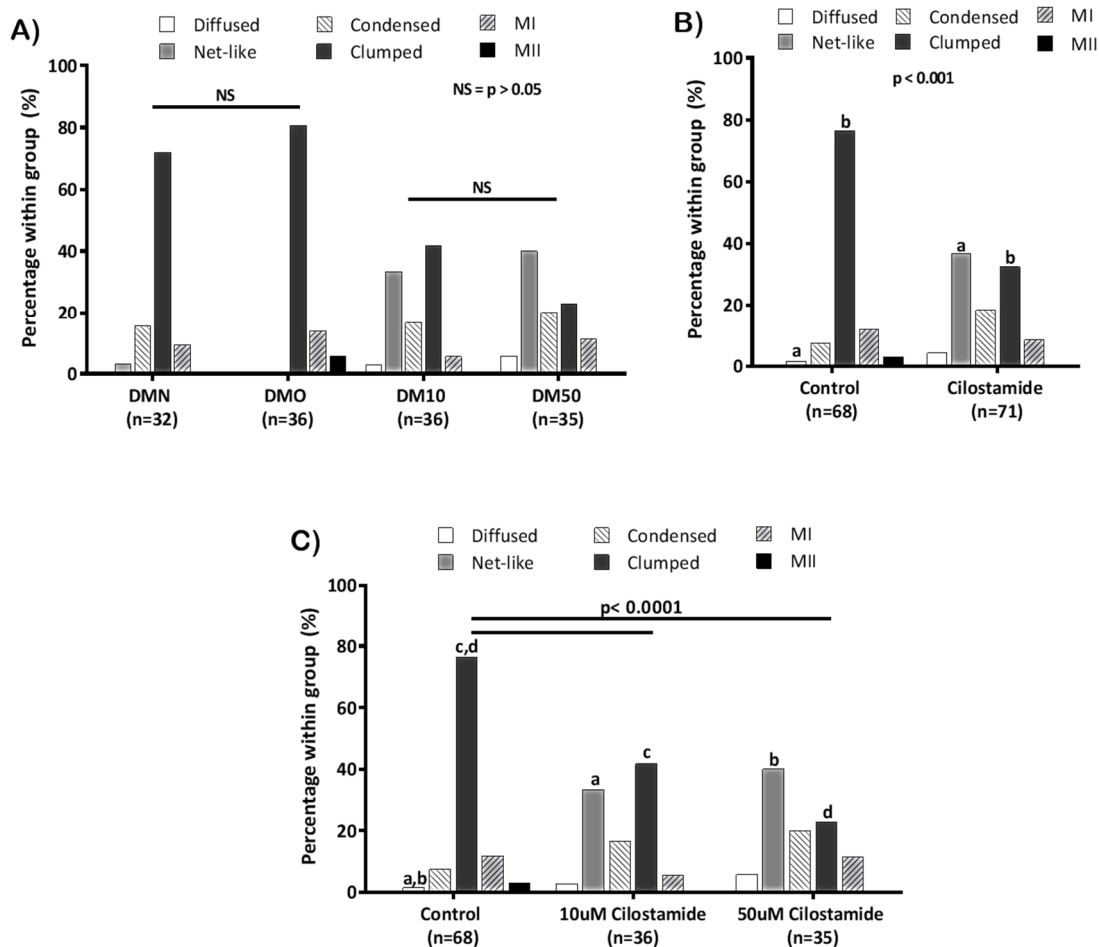


Figure 3.5. Distribution of chromatin configuration in modified AAP media; negative control (DMN), DMSO vehicle control (DMO), 10 μ M (DM10) and 50 μ M (DM50) cilostamide supplementation. Significant differences of chromatin distribution between 2 groups comparison indicated by (—) if Chi-test, $p<0.05$. (Panel A) Comparison among 4 AAP media. No significant differences of chromatin distribution were detected between the 2 controls and between the 2 concentrations of cilostamide. NS: not significant. (Panel B) Comparison between pooled control and pooled cilostamide media found a significant difference of chromatin distribution ($p<0.001$) and comparison between 2 groups regarding net-like (a) and clumped (b) chromatin showed significant differences; Mann-Whitney test, $p<0.05$. (Panel C) Comparison of pooled control and either 10 μ M or 50 μ M cilostamide. Significant differences ($p<0.0001$) of chromatin distribution found in both 10 μ M and 50 μ M cilostamide if pooled control compared. The same letter indicated significant differences of both net-like (a,b) and clumped (c,d) if the 2 groups compared; Mann-Whitney test, $p<0.05$.

control (Figure 3.5.C). Although the dosage of 10 μ M (DM10) cilostamide demonstrated no different distribution in chromatin pattern compared to the 50 μ M (DM50) cilostamide, the higher concentration of cilostamide supplemented AAP media was better able to inhibit progression to advanced stage of chromatin condensation than the lower concentration (Figure 3.5.C).

3.3.5. Experiment 5: Effect Of The Phosphodiesterase Inhibitor On Amino Acid Metabolism

Although the previous section validated the appropriate dosage of cilostamide to be used in the series of experiments, it was still necessary to examine whether the cilostamide may interfere with oocyte metabolic function. According to the chromatin configuration, 50 μ M cilostamide seemed to be the most effective dose to prevent the spontaneously premature oocyte meiotic maturation. This experiment demonstrated that AAP may be used as a marker to determine the oocyte amino acid metabolism in the GV-staged ovine oocytes (Figure 3.6) as it has been reported in the mature MII stages of oocyte development for bovine and human oocytes (Collado Fernandez, 2013, Hemmings, 2007) and across various stages of follicle development (Hemmings, 2007).

Results from 36 individual oocytes in total incubated in 4 different AAP media regarding changes in peak area for each amino acid are summarised in Figure 3.6 and Table 3.7 revealing the extent to which amino acids were depleted from, or appeared in, the medium. Besides this, the amino acid turnover in 4 different modified AAP media was demonstrated in Figure 3.7.

From 18 amino acids measured overall, glycine, alanine and lysine were the 3 main amino acids appearing while threonine, arginine, leucine and glutamine were the 4 highly depleted amino acids from the AAP medium (Figure 3.6). No significant differences ($p>0.05$) were detected in terms of individual amino acids when compared to the 2 control media (DMN vs DMO). Similarly, there were no significant differences ($p>0.05$) between 2 concentrations of cilostamide supplemented AAP media (DM10 (10 μ M) vs DM50 (50 μ M)) in term of individual amino acids measured.

Although the peak areas of the most individual AAs were not significantly different between the 4 different media, tryptophan ($P < 0.001$) was found to be significantly different in appearance between the DMN and either DM10 or DM50 group. Moreover, tryptophan ($P < 0.005$), histidine ($P < 0.005$) and glutamate ($P < 0.001$) were significantly appearing/depleted when DMO and DM10 were compared. On the other hand, appearance of methionine ($P < 0.001$) was found to be significantly different only when DMN and DM10 were compared (Figure 3.6).

Accumulative amino acid measurements found a significant difference in overall net balance ($p < 0.05$) among 4 different AAP media while no significance was found in overall depletion/appearance and turnover. Subgroup analysis was then performed and it confirmed that the significant difference in overall net balance was caused by differences of net balance between DM50 and either DMO ($P = 0.05$) or DM10 ($P < 0.05$) (Figure 3.7.).

When cumulative two control compared to cumulative two concentration of cilostamide, no differences were detected from all 4 parameters of amino acid measurement (Figure 3.8.).

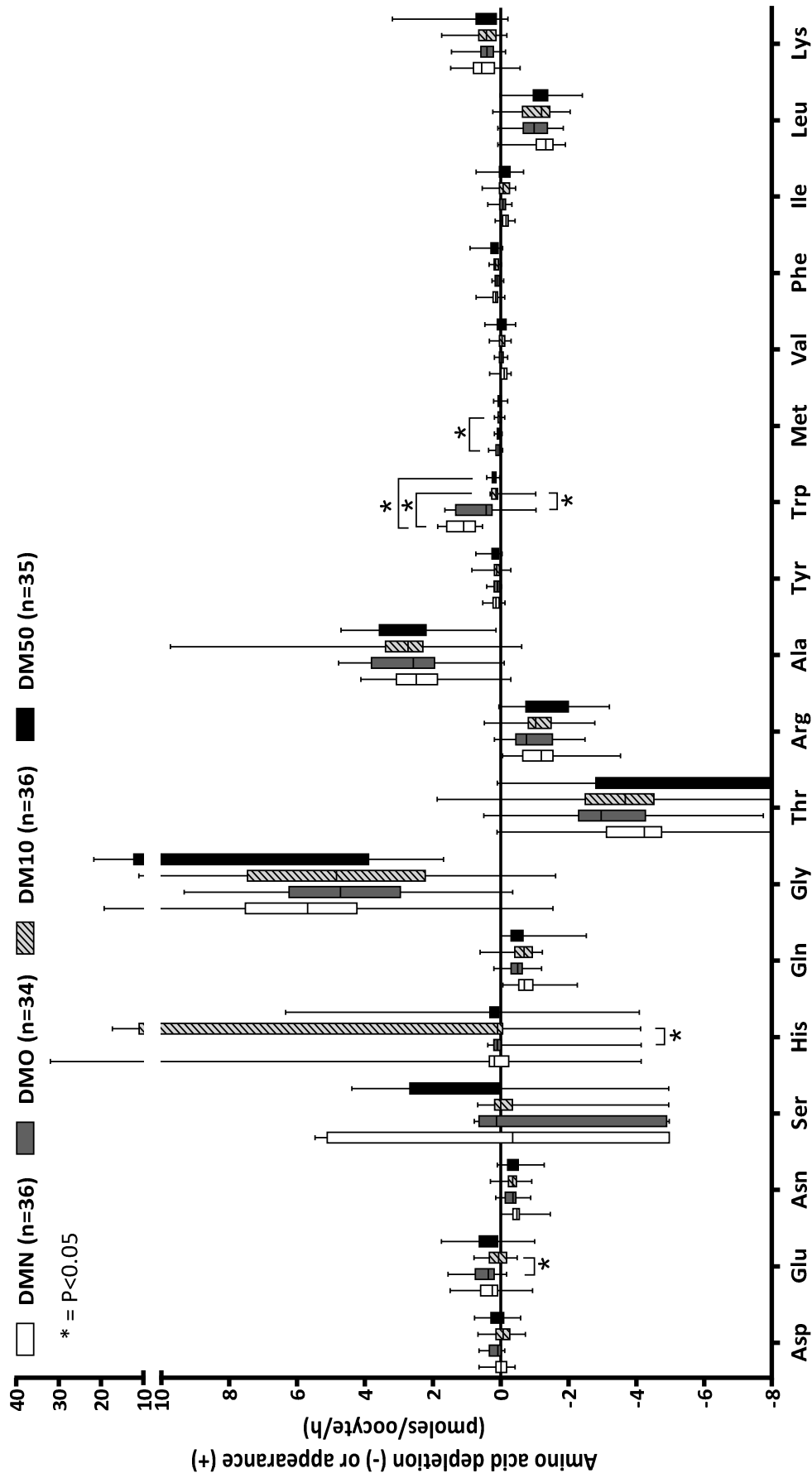


Figure 3.6. Average amino acid profiles of sheep GV oocytes following 6 hours incubation in 4 AAP media. Positive values represent a means appearance; negative values a means depletion. Boxes represent the 25th to 75th percentile, the median is represented by the line within the box, and the whiskers represent the maximum and minimum data point. Changes in amino acid concentration either depletion or appearance were assessed by Kruskal–Wallis. Asterisks (*) indicate significant differences in amino acid profiles between control media, and between 10µM and 50µM cilostamide supplemented AAP media were compared by Mann–Whitney U-test; $p < 0.05$ was assumed to be significant difference. Negative control: DMO (open box); 10µM: DM10 (shading gray) and 50µM: DM50 (black box) cilostamide supplemented AAP media.

Table 3.7 Amino acid profiles from GV-staged ovine oocytes from 4 types of AAP media with/without cilostamide

(pmoles/oocyte/h)	DMN (n=36)			DMO (n=34)			DM10 (n=36)			DM50 (n=35)		
	Median	Mean	SEM	Median	Mean	SEM	Median	Mean	SEM	Median	Mean	SEM
Asp	-0.014	-0.005	0.042	0.082	0.150	0.038	-0.073	-0.037	0.052	-0.009	0.072	0.051
Glu	0.251	0.311	0.080	0.362	0.461	0.066	0.061	0.070	0.058	0.421	0.356	0.076
Asn	-0.477	-0.489	0.046	-0.362	-0.309	0.041	-0.356	-0.361	0.041	-0.295	-0.399	0.063
Ser	0.000	0.030	0.602	0.000	-0.990	0.382	0.000	-0.266	0.196	0.000	0.305	0.306
His	0.181	0.120	0.960	0.100	-0.020	0.127	0.093	3.729	1.147	0.194	0.747	0.442
Glu	-0.701	-0.781	0.071	-0.501	-0.513	0.054	-0.686	-0.653	0.062	-0.420	-0.621	0.101
Gly	5.691	6.046	0.555	4.722	4.660	0.405	4.842	4.782	0.597	5.176	7.812	0.946
Thr	-4.240	-4.340	0.352	-2.967	-3.407	0.389	-3.670	-3.811	0.401	-3.769	-4.496	0.409
Arg	-1.198	-1.208	0.124	-0.762	-0.956	0.124	-1.030	-1.135	0.106	-1.098	-1.301	0.138
Ala	2.487	2.374	0.164	2.573	2.615	0.238	2.728	2.827	0.274	2.816	2.882	0.182
Tyr	0.142	0.143	0.024	0.094	0.120	0.019	0.117	0.119	0.029	0.126	0.186	0.033
Trp	0.268	0.584	0.112	0.309	0.419	0.113	0.000	0.022	0.047	0.142	0.140	0.020
Met	0.080	0.086	0.013	0.066	0.066	0.008	0.023	0.025	0.011	0.035	0.030	0.013
Val	-0.111	-0.072	0.024	-0.023	-0.016	0.016	-0.044	-0.044	0.024	-0.051	-0.045	0.033
Phe	0.145	0.164	0.024	0.108	0.107	0.014	0.156	0.140	0.016	0.127	0.182	0.031
Ile	-0.154	-0.149	0.023	-0.069	-0.054	0.023	-0.073	-0.082	0.037	-0.121	-0.110	0.046
Leu	-1.333	-1.223	0.076	-0.990	-0.950	0.090	-1.206	-1.054	0.096	-1.173	-1.194	0.088
Lys	0.556	0.480	0.077	0.403	0.470	0.064	0.419	0.477	0.074	0.535	0.634	0.118
Net balance	1.520	2.070	1.460	1.830	1.850	0.608	1.090	4.750	1.560	3.820	5.180	0.863
Depletion	-8.600	-10.800	1.080	-7.530	-7.760	0.650	-7.250	-8.330	0.689	-7.670	-9.090	0.870
Appearance	11.200	12.800	1.120	9.910	9.610	0.597	9.500	13.100	1.660	11.300	14.300	1.340
Turnover	22.500	23.600	1.640	18.700	17.400	1.090	17.700	21.400	2.010	19.900	23.400	2.080

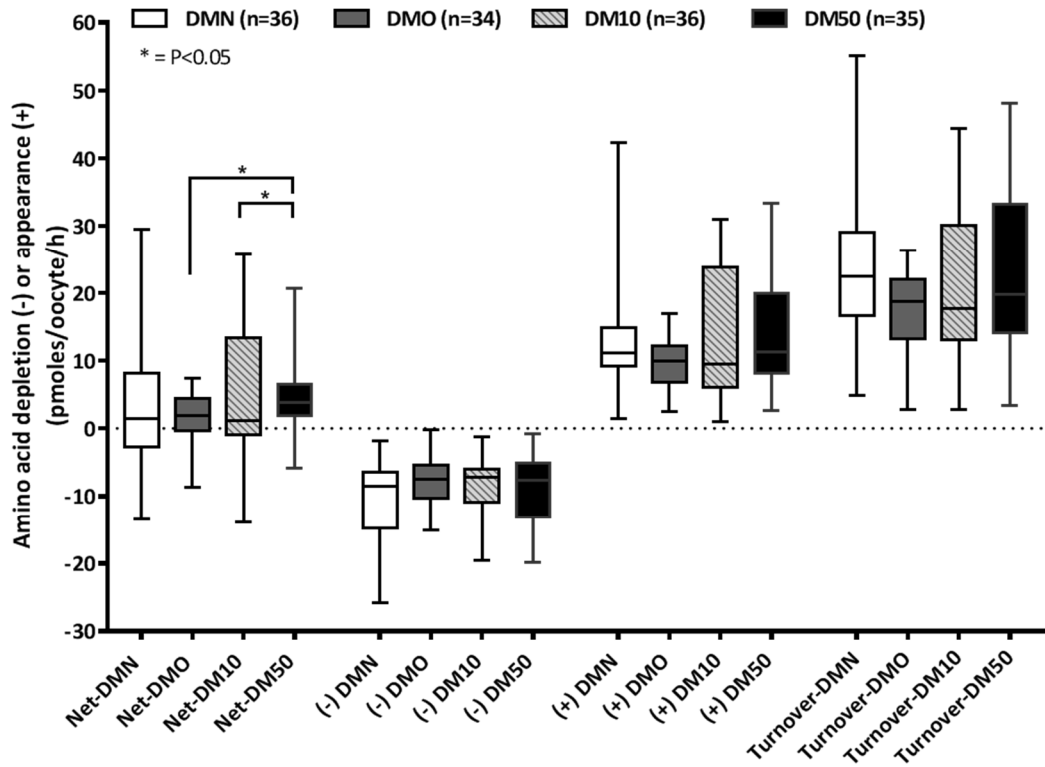


Figure 3.7. Average total net balance, depletion, appearance and turnover of 18 amino acids by sheep GV oocytes following 6 hours incubation in 4 different AAP media. Negative control: DMN (open box); DMSO control: DMO (gray box); 10 μ M: DM10 (shading gray box) and 50 μ M: DM50 (black box) cilostamide supplemented AAP media. Boxes represent the 25th to 75th percentile, the median is represented by the line within the box, and the whiskers represent the maximum and minimum data point. No significant differences ($p > 0.05$) were apparent by Kruskal–Wallis in the summed net appearance, net depletion or “turnover” among 4 different media except net balance. Asterisks (*) indicate significant differences between each group; post hoc Mann–Whitney U-test, $p < 0.05$.

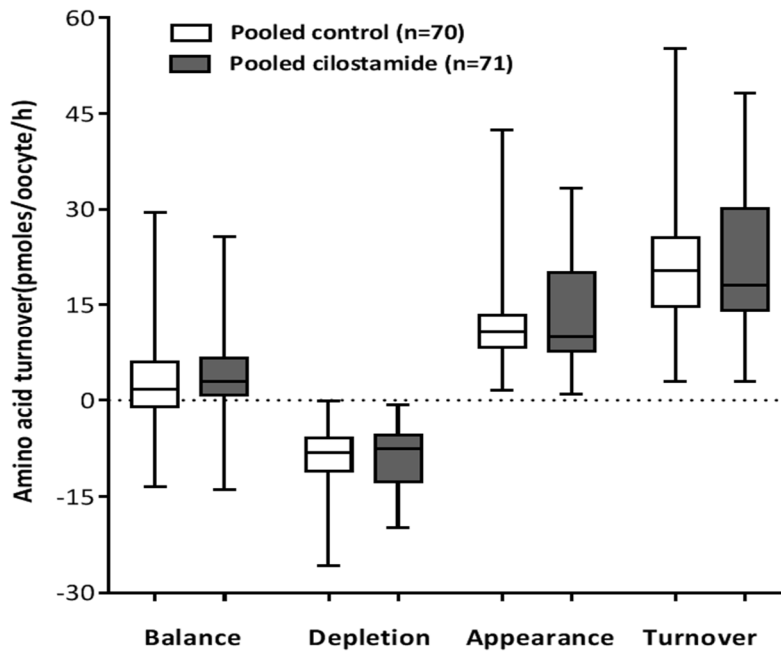


Figure 3.8. Average total net balance, depletion, appearance and turnover of 18 amino acids by sheep GV oocytes following 6 hours incubation between pooled control and pooled cilostamide supplemented AAP media. Pooled control: DMN and DMSO (open box) and pooled cilostamide: DM10 and DM50 (shade box). No significant differences were apparent by Mann–Whitney U-test; $p>0.05$.

3.3.6. Experiment 6: Impact Of Age On Chromatin Configuration

The preliminary data were analysed to investigate the impact of animal age on the dynamics of chromatin configuration by using oocytes derived from prepubertal and adult sheep. Significant differences ($p<0.05$) in oocyte chromatin patterns after 6 hours incubation were found between the two age groups regardless of media used for incubation, AAP and GPL media supplemented with $50\mu\text{M}$ cilostamide. (Figure 3.9.A and B). In contrast, no differences were observed between the two age groups at the time 0, no incubation control (Figure 3.9.C).

When the types of culture media was taken into account, results showed a significant difference in term of patterns of chromatin distribution between oocytes incubated in AAP medium supplemented with $50\mu\text{M}$ cilostamide compared to those cells incubated in GPL medium supplemented with $50\mu\text{M}$ cilostamide observed in prepubertal age animal (Figure 3.10.A). However, such a difference was not detected ($p>0.05$) in adult sheep oocytes incubated in AAP and GPL media supplemented with $50\mu\text{M}$ cilostamide (Figure 3.10.B).

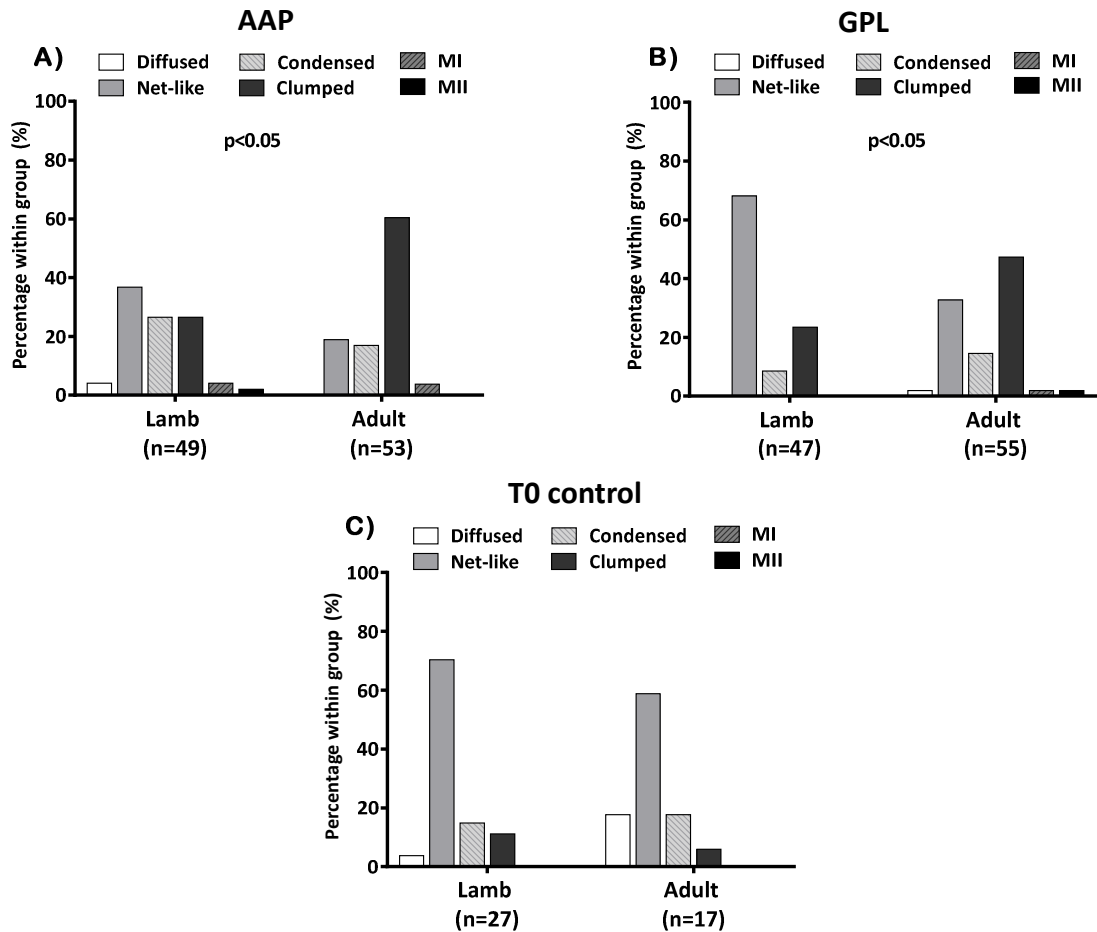


Figure 3.9. Comparison of the chromatin distribution between prepubertal lamb and adult sheep oocytes in relation to types of media. Bar charts represents data derived from oocytes incubated in (A) AAP and (B) GPL media containing 50 μ M cilostamide supplementation. No incubation time 0 (T0) as a control data are shown in (C). Chi-tests determined a significant difference between age groups if $p < 0.05$.

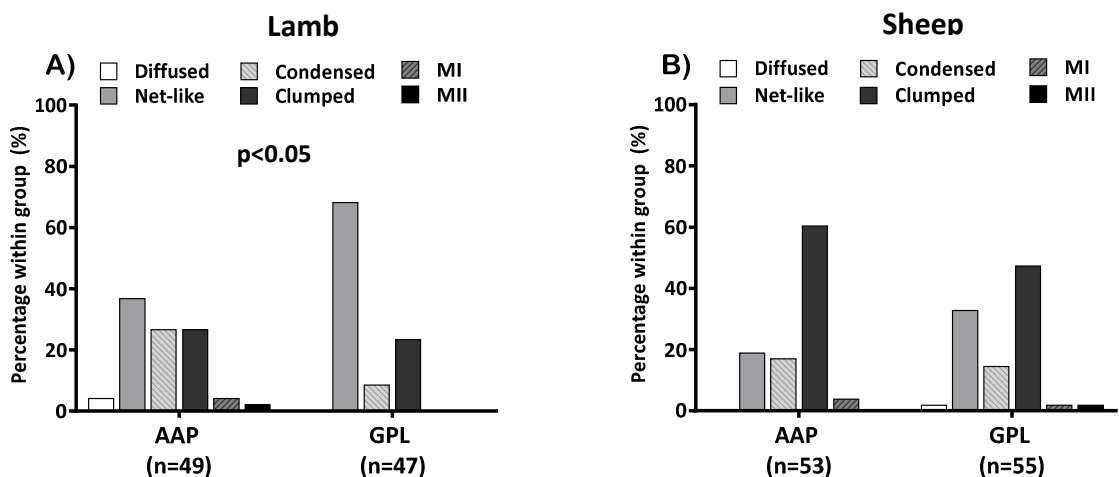


Figure 3.10. Comparison of the chromatin distribution between AAP and GPL media supplementation with 50 μ M cilostamide, in prepubertal lamb (A) and adult sheep (B) oocytes. Chi-square test determined a significant difference between two culture media compared if $p < 0.05$.

3.4. DISCUSSION

3.4.1. Classification Of Ovine Chromatin Configuration

The chromatin configuration found in this study closely resembled the patterns displayed in large monovular domestic species such as buffalo (Yousaf and Chohan, 2003), bovine (Liu *et al.*, 2006, Lodde *et al.*, 2007) and goat (Sui *et al.*, 2005). This is in contrast to the previous report described in ovine oocytes using SYBR-Green staining (Russo *et al.*, 2007a, Russo *et al.*, 2007b), which divided the classification into 3 major categories: the NSN, condensed chromatin SN, and condensed chromatin near the nucleolus and the nuclear envelope. Their pattern of GV chromatin was intriguingly like described in human (Combelles *et al.*, 2003), mouse (Debey *et al.*, 1993, Zuccotti *et al.*, 1995), rabbit (Wang *et al.*, 2009a) and bovine after culture (Liu *et al.*, 2006). Instead of forming condensed chromatin SN as demonstrated in mouse (Bouniol-Baly *et al.*, 1999, Mattson and Albertini, 1990, Zuccotti *et al.*, 1995), monkey (Schramm *et al.*, 1993), pig (Sun and Machaca, 2004) and human (Combelles *et al.*, 2003, Miyara *et al.*, 2003), this experiment has revealed at least 4 patterns of chromatin configuration in oocytes from medium and large antral follicles, which corresponds to the previous reports in goats (Sui *et al.*, 2005), horse (Franciosi *et al.*, 2012, Hinrichs *et al.*, 1993) and cows (Chohan and Hunter, 2003, Liu *et al.*, 2006).

During the oocyte growth and differentiation, no matter what classification is used, the advanced level of chromatin condensation typically acquired the appearance of either a single compact clump or a SN morphology before meiotic resumption (Chohan and Hunter, 2003, Comizzoli *et al.*, 2011, Hinrichs *et al.*, 2005, Hinrichs and Schmidt, 2000, Lodde *et al.*, 2007, Schramm *et al.*, 1993, Sui *et al.*, 2005, Wang *et al.*, 2009c, Wickramasinghe *et al.*, 1991, Yousaf and Chohan, 2003, Zuccotti *et al.*, 1998). In addition, the higher level of oocyte chromatin condensation was associated with a greater embryo developmental potential compared to those with a less condensed chromatin pattern (Lodde *et al.*, 2007, Luciano *et al.*, 2011, Zuccotti *et al.*, 1998, Zuccotti *et al.*, 2002).

The established classification of chromatin configuration in this study was different from a previous report in the same species (Russo *et al.*, 2007a). The chromatin

labelling technique is possibly to explain the different pattern of chromatin morphology. As compared to SYBR Green, used in the Russo's study, DAPI, chosen in this experiment, has different binding properties and sensitivity as it selectively binds to the minor groove of the double strand deoxyribonucleic acid (dsDNA) at A-T rich regions (Banerjee and Pal, 2008a). Some studies found SYBR Green to be far more sensitive in dsDNA staining than other common fluorescent dye due to multiple modes of interaction not only as a DNA minor groove binder but also as a result of intercalation between base pairs and stabilisation of the electrostatic SYBR Green-DNA complex (Dragan *et al.*, 2012, Zipper *et al.*, 2004). Therefore, the SYBR Green could take up more details of chromatin structure, which might influence the appearance of chromatin morphology. As a common staining is used in most laboratories, for the chromatin classification system here we have chosen DAPI for chromatin labelling to study dynamic changes of chromatin configuration of sheep GV oocytes. This method will be used in all of the subsequent experiments in this thesis.

3.4.2. Effect Of Ovine Follicular Size On Chromatin Configuration

It is well established that oocytes derived from prepubertal and adult animals have different developmental potentials (Armstrong, 2001, Kochhar *et al.*, 2002, Leoni *et al.*, 2007a, O'Brien *et al.*, 1997b). It has been suggested that incomplete oocyte growth of prepubertal oocytes may result from insufficiency in protein accumulation and RNA synthesis, which is indicated by the size of the oocytes (Lazzari *et al.*, 1994). Moreover, some reports in the bovine found an influence of follicle size on oocyte performance during maturation and culture *in vitro* (Lonergan *et al.*, 1994, Pavlok *et al.*, 1992), as well as a link between follicle diameter and oocyte size (Arlotto *et al.*, 1996, Hyttel *et al.*, 1997, Lechniak *et al.*, 2002). In several species the capability of oocytes to resume and complete meiosis *in vitro* has been attributed to oocyte diameter (Durinzi *et al.*, 1995, Fair *et al.*, 1995, Ledda *et al.*, 1999, Motlik *et al.*, 1986, Otoi *et al.*, 2000, Raghu *et al.*, 2002), with the smaller sized oocytes tending to undergo abnormal meiotic development (Lechniak *et al.*, 2002).

The current validation experiment was carried out to determine if there was an effect of follicle size on oocyte chromatin morphology in sheep GV oocytes recovered from medium- and large-sized antral follicles. The selective follicle size may link to the clinical application as a representative marker for developmental

potential of follicles and oocytes in *in vitro* production whereas the large antral follicles were hardly to exclude the effect of follicular atresia. Although a number of previous studies have reported the relationship between follicle diameter, oocyte size, and pattern of chromatin distribution (Liu *et al.*, 2006, Russo *et al.*, 2007a, Schramm *et al.*, 1993, Sui *et al.*, 2005, Sun and Machaca, 2004, Wang *et al.*, 2009c), the result of the pilot experiment did not support such a finding. Data from this experiment demonstrated a similar distribution of chromatin patterns between follicles of 2-3mm diameter and those of 4-5mm diameter in both age groups after incubated in AAP medium for 6 hours. This observation is in agreement with the finding of a previous report (Shirazi and Sadeghi, 2007). These authors found that sheep oocytes obtained from antral follicles ranging from 2-6mm were fully grown and able to support nuclear maturation despite the heterogeneity observed in oocyte diameter. Russo *et al.* (2007) have classified sheep oocytes according to the degree of chromatin condensation and distribution; NSN, SN, and surrounded nucleolus and nuclear envelope. They found the link between the pattern of chromatin configuration displayed and the stage of folliculogenesis observed including preantral, early antral, and medium antral follicles (Russo *et al.*, 2007a). There are two reasons that can help to explain the discrepancy between the current work and the published study. First, our experiment compared only two follicle sizes, medium (2-3mm) and large antral follicle (4-5mm), which is equivalent to the size of medium antral follicles in the published study. As mentioned before, ovine GV oocytes reached the full-grown state in medium-size antral follicles and several previous studies have demonstrated the ovulatory potential of these gametes (Barboni *et al.*, 2011, Bartlewski *et al.*, 1999, Ptak *et al.*, 2006, Russo *et al.*, 2007a).

Apart from sheep, other species also demonstrated an increase in chromatin condensation in correlation with the follicle development (Chohan and Hunter, 2003, Comizzoli *et al.*, 2011, Hinrichs and Schmidt, 2000, Liu *et al.*, 2006, Lodde *et al.*, 2007, Schramm *et al.*, 1993, Sui *et al.*, 2005, Wang *et al.*, 2009c, Yousaf and Chohan, 2003). Due to varying systems used for grouping the size of follicle, head to head comparison between studies become difficult because most previous reports assigned follicle size 2-5mm in the same category. The particular studies that have demonstrated a relationship between follicle size and chromatin configuration, were conducted to compare early stage (i.e., preantral follicle) with the later stage

of folliculogenesis (i.e., medium, large or preovulatory follicles). Only one report in bovine categorized equivalent follicle size to this current study and demonstrated some difference between the two follicle sizes (Liu *et al.*, 2006). Liu's study (2006), demonstrated significantly more heterochromatin SN pattern in oocytes recruited from large antral follicles (3-5.9mm) compared to medium follicles (1.5-2.9mm diameter). However, this may be subjected to species difference since sheep achieve the fully grown oocytes from smaller antral follicles (2mm) compared to bovine(4mm) (Picton *et al.*, 2008, van den Hurk and Zhao, 2005, Ginther *et al.*, 1997). Another study conducted in goats supported the relationship between chromatin morphology and follicle size from preantral stage until late antral follicle (Sui *et al.*, 2005). However, medium- and large-size follicles (>3mm diameter) were pooled (Sui *et al.*, 2005) same as in a monkey study (Schramm *et al.*, 1993). In contrast, Comizzoli *et al.* (2011) could not find any differences in chromatin pattern between early antral to late antral stages in a cat model (Comizzoli *et al.*, 2011). Therefore, these are no consistent differences between two particular stages of follicle data, which is consistent to our finding. In other words, this suggests that growth of oocytes rather than follicle size should be taken into consideration as follicle growth during the late stages of preovulatory follicular development may not reflect oocyte competence. In the sheep, antral follicles ranging from 2-6mm diameter contain fully grown oocytes with good competence for IVM despite their variability in diameter (Shirazi and Moalemian, 2007).

The current experiment has revealed at least 4 chromatin patterns are present in sheep GV oocytes. Therefore, it is difficult to compare the two studies. Instead of forming condensed chromatin SN as demonstrated in mouse (Bouniol-Baly *et al.*, 1999, Mattson and Albertini, 1990, Zuccotti *et al.*, 1995), monkey (Schramm *et al.*, 1993), pig (Sun and Machaca, 2004) and human (Combelles *et al.*, 2003, Miyara *et al.*, 2003), our findings demonstrate the majority of GV oocytes are characterized by a single compact clump of chromatin, which closely corresponded to previous reports in goats (Sui *et al.*, 2005), horse (Franciosi *et al.*, 2012, Hinrichs *et al.*, 1993) and bovine (Chohan and Hunter, 2003, Liu *et al.*, 2006). However, the higher level of chromatin condensation whether single compact clump or SN is typically required during the oocyte growth and differentiation before meiotic resumption (Chohan and Hunter, 2003, Comizzoli *et al.*, 2011, Hinrichs *et al.*, 2005, Hinrichs and Schmidt,

2000, Lodde *et al.*, 2007, Schramm *et al.*, 1993, Sui *et al.*, 2005, Wang *et al.*, 2009c, Wickramasinghe *et al.*, 1991, Yousaf and Chohan, 2003, Zuccotti *et al.*, 1998). In addition, this chromatin configuration exhibited a greater potential during embryo development compared to oocytes with less condensed chromatin (Lodde *et al.*, 2007, Luciano *et al.*, 2011, Zuccotti *et al.*, 1998, Zuccotti *et al.*, 2002). In conclusion, this experiment has shown no impact of follicle size on chromatin configuration between GV oocytes from medium- and large-sized sheep follicles. On this basis oocytes from both follicle sizes can be pooled in the future investigations of this thesis.

3.4.3. Effect Of Denudation On The Dynamics Of Oocyte Chromatin Configuration

The results of this pilot experiment indicate that denudation of COCs influences chromatin morphology and distribution in gametes from both adult and prepubertal sheep. Our data have shown that in simple holding medium, around half of the denuded oocytes could reach MI after only 6 hours of incubation post-denudation (47.1-54.2%). In other words, approximately 50% of oocytes resume in meiosis 6 hours post-denudation. As Yadav *et al.* (1997) reported that 22.9% of sheep COCs resumed in meiosis after 6 hours as evidenced by MI oocytes (Yadav *et al.*, 1997), a higher speed of meiotic resumption was found in the current work (50% vs 22.9%). This data indicates that denudation triggers the resumption of meiosis and has the potential to accelerate nuclear maturation.

Recently, a study supported the effect of cilostamide in inhibiting meiotic resumption. They found cilostamide reduced meiotic resumption as progression of oocytes reached MI stage drop from approximately 50% after 6 hours incubation during maturation *in vitro* to almost 30% in the group of oocytes using pre-IVM media protocol (Gharibi *et al.*, 2013). Acceleration of meiotic resumption in their study was comparable to our finding in denuded oocytes after 6 hours incubation most likely from different types of IVM media used for culture. High dosage of gonadotrophin (10µg/ml of FSH and LH) used in combination with oestradiol and other growth factors such as EGF and IGF1 might speed up the maturational process. In contrast to our finding, as these additives were absent from the H199+ oocyte holding medium; therefore, apart from the follicle released from ovary expediting

nuclear maturation in the current work, it is possibly influenced by the denudation process as well.

The cAMP modulators, such as PDE inhibitors (i.e., cilostamide and milrinone), have been proposed to improve synchronization of nuclear and cytoplasmic function during oocyte IVM (Anderiesz *et al.*, 2000, Nogueira *et al.*, 2003b, Wu *et al.*, 2006, Cotterill, 2008). The addition of cAMP modulators into oocyte maturation medium SPOM has been demonstrated to improve embryo quality in various species such as bovine, porcine and cattle (Aktas *et al.*, 1995a, Aktas *et al.*, 1995b, Grupen *et al.*, 2006, Luciano *et al.*, 1999). As the current experiment aimed to validate chromatin morphology as a marker of oocyte quality, it was essential to eliminate cumulus cells removal as a confounding factor in the oocyte handling process and to establish the simulated physiological condition for all subsequent experiments in this thesis that utilized denuded oocytes incubations. As mentioned earlier, in order to measure the metabolism of GV staged oocyte, the denudation process is unavoidable. On the basis of the current observation, conventional AAP medium, combined with cumulus removal, accelerates spontaneous nuclear maturation in denuded oocytes. In contrast, the addition of the PDE inhibitor, cilostamide, prevented this effect and created a media environment that was suitable for metabolism testing in GV oocytes.

The H199+ medium used in this experiment contained HEPES as the buffer system in order to prevent the periodic fluctuations of *in vitro* culture conditions. Intracellular perturbations following pH alteration may be pronounced in denuded oocytes because they loss cumulus cells which is the robust machinery to regulate pH (Fitzharris and Baltz, 2006, FitzHarris *et al.*, 2007b). It is possible that the effect of HEPES *per se* may facilitates atresia induction and so leads to acceleration of chromatin reorganization in oocytes. The results shown here demonstrate no difference of chromatin distribution between holding medium supplemented with and without HEPES at 3 and 6 hours of culture. Although HEPES-containing media has been extensively used in ART and the efficacy of HEPES use is widely accepted with sperm preparation methods (Crabo *et al.*, 1972, Molinia *et al.*, 1994, Molinia *et al.*, 1996), the safety of this buffer is still considered, when applied to oocytes and embryos (Will *et al.*, 2011). Reports observed the use of ICSI medium containing

HEPES compromised human embryo quality (Morgia *et al.*, 2006) as well as reduced blastocyst formation in sheep zygote culture using HEPES buffer (Walker *et al.*, 1989b). Importantly, a previous study found using 10% HEPES during mouse oocyte maturation triggered cell degeneration (Bagger *et al.*, 1987). In contrast, numerous other studies have indicated the improved efficiency when HEPES was used during oocyte maturation (Byrd *et al.*, 1997, Downs and Mastropolo, 1997), fertilisation (Behr *et al.*, 1990, Bhattacharyya and Yanagimachi, 1988, Hagen *et al.*, 1991), and embryo development (Ali *et al.*, 1993, Hagen *et al.*, 1991, Mahadevan *et al.*, 1986, Ozawa *et al.*, 2006) at room temperature. Various confounding factors such as pH, concentration and ionic composition, may contribute to the inconsistent results between studies. The H199+medium used in this experiment contained only 21mM HEPES which have previously been confirmed yield in maturation (Geshi *et al.*, 1999), fertilisation (Geshi *et al.*, 1999, Lee and Storey, 1986), and embryo cleavage rates (Geshi *et al.*, 1999, Iwasaki *et al.*, 1999, Liu *et al.*, 1996) that are similar to media without HEPES. Moreover, in this study, H199+ medium also incorporated bicarbonate as an additional buffering system. This combination was previously shown to support embryo development (Mahadevan *et al.*, 1986). Collectively, these reasons, therefore, support our results that the presence of HEPES in oocyte holding medium has not contributed to accelerated changes in GV oocyte chromatin configuration dynamics.

3.4.4. Delayed Spontaneous Premature Oocyte Maturation By Cilostamide, A Phosphodiesterase-Inhibitor

The low developmental potential of oocytes matured *in vitro* is thought to be caused by premature nuclear maturation with inadequate cytoplasmic competence (Gilchrist and Thompson, 2007). The moderation of cAMP turnover by PDE inhibitors is one strategy proposed to synchronize the discrepancy between nuclear of cytoplasmic maturation by maintaining high intra-oocyte cAMP (Bornslaeger and Schultz, 1985, Gilchrist *et al.*, 2011, Nogueira *et al.*, 2003a, Nogueira *et al.*, 2003b, Nogueira *et al.*, 2006). This SPOM rationale has been shown to improve the developmental capacity of embryos and consequently increase live birth rate after IVM (Nogueira *et al.*, 2006, Thomas *et al.*, 2004a). The SPOM strategy was used here to overcome the effect of denudation by modification of AA profiling medium by supplementing it with cilostamide. It was therefore necessary to ensure that the

vehicle used to deliver the cilostamide (i.e., DMSO) had no impact on oocyte behaviour, such as chromatin changes and metabolism. No difference between DMN and vehicle DMO control groups were detected with regard to chromatin dynamics, indicating that DMSO vehicle has no confounding effect on chromatin morphology. Furthermore, this experiment confirmed that cilostamide supplementation significantly attenuated the spontaneous resumption of meiosis seen following denudation of sheep GV oocytes. The result agrees with previous ovine studies (Gharibi *et al.*, 2013, Rose *et al.*, 2013). Although data between two different cilostamide concentrations used, 10 μ M and 50 μ M, showed a trend toward a dose-dependent effect, this did not reach statistical significance. Rose *et al.* (2013) demonstrated that the use of cAMP modulators in a SPOM system was dose dependent in term of delayed ovine nuclear maturation. Differences between this experiment and what Rose and colleagues reported may have at least 3 explanations. First, our chromatin assessment was done within the first 6 hours after AAP incubation instead of after 21 to 30 hours of IVM culture. This time period used in our study may therefore be too short to see any difference. Second, the different observations are likely to be due in part to the different protocols, which includes the use of different culture conditions and media preparation, and also different type of cAMP modulators used. In Rose's study 2 types of cAMP modulators, IBMX (3-isobutyl-1-methyxanthine) and forskolin (FSK) were used in pre-IVM culture, followed by supplementation with cilostamide in the IVM culture. Our study used only a 1-step protocol with cilostamide added throughout the short incubation. The third possible cause of the different findings is related to animal age-differences, in our study only oocytes from prepubertal sheep were used whereas oocytes from peripubertal sheep were investigated in the published study. Gharibi *et al.* (2013) have also reported the use of a 2-step IVM protocol in ovine oocytes by incorporating a low dose cilostamide concentration (1 to 20 μ M) in only the 22 hour pre-IVM culture. Their results did not demonstrate a dose-dependent effect of cilostamide treatment (Gharibi *et al.*, 2013). Higher dosages of 10 and 50 μ M cilostamide were compared in the current study but no significant differences were found after 6 hours of incubation.

On the basis of these results, supplementation of oocyte incubation media with a dose of 50 μ M cilostamide was selected as the most effective way of preventing

accelerated chromatin configuration changes following the denudation of oocytes that was necessary to quantify GV oocyte metabolism.

3.4.5. Effect Of Cilostamide On GV-Staged Oocyte Amino Acid

Metabolism

No differences were detected between individual AA nor overall AA depletion/appearance, net-balance, and turnover changed between control media; DMN and DMO. This data can be interpreted as showing that DMSO vehicle *per se* has no impact on oocyte AA metabolism during the 6 hours incubation period. This is in agreement with previous finding by our own group (Cotterill, 2008). Although the toxicity of the solvent in culture has been reported in several studies, all data (Ezz Eldin and Sarhan, 2014, Syed *et al.*, 2013) agreed that at very low concentration (<1% v/v) no toxicity effect was detected.

Likewise, in comparison of AAP medium supplemented with 10 μ M (DM10) and 50 μ M cilostamide (DM50), no significant differences were detected ($p>0.05$) in terms of individual AA changes, overall AA depletion/ appearance and turnover except for the nitrogen balance ($p<0.05$) (see Figure 3.10). Therefore, data within control and cilostamide groups can be pooled together to empower the statistical rigour of testing the effect, if any, of cilostamide supplementation. When data were reanalysed between pooled control and pooled cilostamide, the difference of net balance disappeared, which might reflect the heterogeneity of the study population in immature GV-staged oocytes. Also, it is important that the appropriate concentration of solvents for use in toxicity testing is determined for the system employed and that the biological effects of bioactive compounds should be evaluated *a priori* in order to take into accounts the negative effects of the solvent itself (Forman *et al.*, 1999), GV-staged oocytes *per se* may present in various stages of chromatin configuration. A previous study found differences between MII oocytes derived *in vitro* and *in vivo* by using amino acid metabolism assays (Collado Fernandez, 2013). However, no difference were detected when immature oocytes were included. This may evidence that immature oocytes vary in stages of development *in vitro* in terms of cytoplasmic maturation (Collado Fernandez, 2013).

Focusing on profiles of AA metabolism, five of them including serine, histidine, glycine, threonine and alanine showed diversity in range of individual AA detection. However, they were consistent among 4 AAP media tested. The finding is consistent with what has previously been observed in a human study of immature denuded oocytes (Hemmings *et al.*, 2013). They also found profiles of some AAs (serine, glycine, threonine, alanine and leucine) having varying detection range. Despite species differences, the previous report and the current work, observed four amino acids in common. Specifically, AAP from GV-staged sheep oocytes were more variable when compared to a study of MII-staged human oocytes (Hemmings *et al.*, 2013).

Some differences were detected for individual AAs between control media and culture medium supplemented with cilostamide. Two observations can be drawn here from individual amino acid profiling of the validation trial. First, a consistent difference between the DMO and DM10 groups was found in 3 out of 4 differing AAs, DM10 resulted in significantly less tryptophan and glutamate but more histidine appearance in spent culture media compared to the DMO group. The other striking observation among 4 different AAP media has shown that the appearance of tryptophan was significantly different between control (DMN) and both cilostamide supplemented media (DM10 and DM50), and also between DMO and 10 μ M cilostamide supplemented medium (DM10) groups. These results may suggest an effect of the addition of cilostamide to the AAP medium. However, since no significant differences were found within either of the 2-control or 2-cilostamide supplemented media as mentioned above, the data were reanalysed for all individual AAs to compare between pooled control and pooled cilostamide supplemented media. There were 2 (tryptophan and methionine) out of the 4 (histidine, glutamate, tryptophan and methionine) differing AA profiles remained persistently different ($p < 0.05$). Interpretation of these findings suggest there is considerable variability in the data and also heterogeneity in the GV-staged oocytes used in the present study. There may also have been a breeding seasonal effect as the tissues used in the validation trial were recruited from prepubertal animals during nonbreeding season. Another limitation is that in approximately one third of the validation samples it was not possible to detect tryptophan. This may influence the representation of tryptophan metabolism for each oocyte population analysed

although it remains possible that tryptophan and methionine metabolism maybe affected by the cilostamide supplement. Looking in more detail at the function of these individual AAs, both tryptophan and methionine are categorized as essential AAs, which cannot be produce from other precursors therefore intake is required. They both have a principle role in protein synthesis and can be catabolized to yield glucogenic precursors (Richard *et al.*, 2009). In addition, tryptophan is also involved in the NAD(P) biosynthetic pathway (Salway, 2004) while methionine participates in the nucleotide synthesis, also it is involved in both DNA and histone methylation processes (Ikeda *et al.*, 2010).

Although it cannot be ruled out at this stage whether there is /or is not a direct effect of DMSO and cilostamide on tryptophan and methionine metabolism by GV oocytes, further studies are clearly required to examine the reagents impacted on the function of tryptophan and methionine on oocyte maturation potential and in embryo development. However, it is less likely that these two amino acids have any effect on overall AA depletion/appearance and AA turnover since they contributed minimal quantities to the overall picture of amino acid profiling.

3.4.6. Pilot Study On Impact Of Age On Chromatin Configuration

Investigations of the effect of oocyte age on chromatin configuration has mostly concentrated on the mature MII stages rather than the immature or GV staged oocytes (Ottolenghi *et al.*, 2004, Tarin *et al.*, 2000). The effect of age has been linked to alterations of chromatin modifications either at the DNA or chromatin levels such as DNA methylation or histone acetylation. In recent years, the remodelling of nuclear chromatin configuration at the end of oocyte differentiation has gained more attention although as detailed earlier there have been few reports in sheep. Furthermore, most studies investigated chromatin configuration in relation to maturation and developmental potential and they have rarely taken ageing into account. This pilot experiment demonstrated the impact of ageing on dynamics of chromatin configuration.

It is generally accepted that the uncondensed chromatin of quiescent GV oocytes is reorganized into a more condensed state prior to the initiation of oocyte maturation (De La Fuente, 2006). Following our own classification system as reported here,

oocytes were classified with 4 types of GV staged chromatin configuration: diffused, net-like, condensed and clumped pattern. The morphology resembles the previous reports in cattle (Chohan and Hunter, 2003, Lodde *et al.*, 2007, Luciano *et al.*, 2012), buffalo (Yousaf and Chohan, 2003), horse (Hinrichs *et al.*, 2005, Lodde *et al.*, 2007, Luciano *et al.*, 2012) and goat (Sui *et al.*, 2005). As reported in these species, GV oocyte chromatin progressed from a less condensed state-diffused filamentous pattern to remodel and form more condensed pattern-distinct clumps of chromatin condensation without the presence of perinucleolar rim formation (Hinrichs *et al.*, 2005, Lodde *et al.*, 2007, Luciano *et al.*, 2012, Yousaf and Chohan, 2003). The chromatin appearance in other species demonstrated initially decondensed chromatin in a configuration termed NSN and subsequently changes to a more condensed, so-called SN form (Bouniol-Baly *et al.*, 1999, Bui *et al.*, 2017, Debey *et al.*, 1993, Mattson and Albertini, 1990, Zuccotti *et al.*, 1995).

This preliminary analysis regarding animal age shows the similar pattern of chromatin distribution of oocytes recruited from medium and large antral follicles when prepubertal and adult sheep were compared. In addition, the higher proportion of oocytes with less condensed chromatin (diffused and net-like stages: 75%) in comparison to those with advanced chromatin condensation (condensed and clumped stages: 25%) was detected in both age groups. This proportion is rather equivalent to what was observed in a buffalo report, in which less condensed chromatin predominated in oocytes derived from middle sized antral follicles (Yousaf and Chohan, 2003), but greater than an observation in cattle (Liu *et al.*, 2006, Lodde *et al.*, 2007). At the time of study, only Russo *et al.* (2007) reported in the same species and found conflicting results, showing medium follicles demonstrated a higher proportion of advanced oocyte chromatin condensation. However, different patterns of chromatin classification used makes it hard to compare as it was previously mentioned. Moreover, differences may be influenced by animal selection criteria, in that this work identified corpus luteum and/or corpus albican for adult animals while in their study animals were controlled by animal weight (>50kg).

After a 6 hours incubation, the chromatin pattern between prepubertal and adult GV oocytes was significantly different ($p < 0.05$) regardless of the types of media

used. This preliminary evidence is suggestive of different developmental potential in oocytes of the two animal ages that is associated with delayed chromatin reorganization in prepubertal oocytes despite no differences in chromatin morphology being evident prior to incubation. Numerous reports in different species including bovine (Khatir *et al.*, 1998), ovine (Ledda *et al.*, 1997), and caprine (Romaguera *et al.*, 2011) have shown that prepubertal oocytes have a reduced developmental competence, it is assumed that the insufficient cytoplasmic maturation of oocyte is responsible for this defect (Sirard *et al.*, 2006). It is possible that this difference could be explained by differences in metabolic capacity between prepubertal and adult oocytes that may reflect that different nutritional requirements are linked to developmental capacity (Downs and Verhoeven, 2003, Nogueira *et al.*, 2006, Vanhoutte *et al.*, 2009, Vanhoutte *et al.*, 2008). Moreover, these different nutrient requirements may be critically linked to the transcriptional activity of the oocyte during the maintenance of meiotic arrest as oocyte transcriptional silencing seems to have been completed by the time chromatin condensation is initiated (Bouniol-Baly *et al.*, 1999, Lodde *et al.*, 2008, Luciano *et al.*, 2011). The relationship between chromatin status and oocyte metabolism will be evaluated in more detail in the following chapters.

It was interesting to note that different incubation media affected chromatin changes in oocytes of different ages differently. Prepubertal oocytes, but not adult oocytes, incubated in AAP medium supplementation with cilostamide demonstrated chromatin remodelling to the more advanced stages of chromatin configuration than similar aged oocytes incubated in GPL medium. This preliminary evidence raises some important questions. As previously reported in mice, cytoplasmic factors are critically important to determine meiotic competence of oocytes, whereas, nuclear factors are essential to support embryonic developmental competence (Inoue *et al.*, 2008, Lowther and Mehlmann, 2015). Recently, few studies found differences of prepubertal oocytes in various aspects of oocyte biology in prepubertal oocytes from different species, such as the dynamics of the first meiosis, active mitochondrial distribution, transcript abundance, and glutathione and apoptosis levels (Jiao *et al.*, 2013, Kohata *et al.*, 2013, Pawlak *et al.*, 2015). The preliminary findings were that culture media containing different nutritional supplements such as AAs and energy sources have greater or less capacity to

promote nuclear chromatin remodelling in oocytes of different ages reinforces the idea of biological differences in the competence of adult vs prepubertal oocytes. Since the components of GPL medium contains mostly balanced salt solution, it represents a simple culture environment media supplemented with not only energy sources such as pyruvate and glucose, but also with several AAs. Apart of nutrition support, AA functions as key organic osmolytes, antioxidants as well (Wu, 2009). This is in agreement with several reports that found improvement of maturation and culture *in vitro* by adding glutamine and/or PDE inhibitors (Downs and Verhoeven, 2003, Geshi *et al.*, 2000, Zuelke and Brackett, 1993).

The pilot studies presented have indicated that discrete differences exist between the nuclear chromatin pattern and the response to the culture environment between juvenile and adult GV sheep oocytes. These results merit further investigation in later chapters of this thesis.

3.4.7. Conclusion

This chapter has established the classification system for sheep oocyte chromatin configuration in the context of chromatin markers to differentiate the potential of oocytes for further development. Similar to other ruminants, evidence suggest that the dynamics of sheep chromatin morphology changed from diffused state to reach the state of chromatin condensation when meiotic resumption initiates. The progression of chromatin morphology is time-dependent and can be facilitated by the denudation process regardless of animal age, HEPES buffer or culture media. By using this chromatin marker, oocytes derived from medium- to large-size follicles seem to be equivalent in context of the oocyte potential.

As the main objective of this thesis is to link the metabolic, chromatin and genetic markers; the simulation culture *in vitro* resembles physiological condition required. Cilostamide was selected to prevent acceleration of nuclear maturation due to the fact that lacking of cumulus cells is prerequisite for oocyte metabolism assessment. The validation trial suggested that supplementing maturation media with 50 μ M cilostamide was superior to 10 μ M cilostamide in term of the efficacy to delay chromatin dynamics and was less likely to interfere the oocyte metabolic assays. On the basis of the results presented here, the classification of chromatin configuration

and the 50 μ M cilostamide supplemented incubation media were set for evaluation of alteration of chromatin dynamics and for quantifying the changes of oocyte metabolism. The strategy will be used in all subsequent oocyte incubation experiments reported in this thesis. Finally, the preliminary analysis indicated that there were discrete differences existing with regard to the nuclear chromatin pattern and the response to the culture environment of oocytes derived from prepubertal compared to adult sheep. These results merit further investigation in later chapters of this thesis.

CHAPTER 4: IMPACT OF MATERNAL AGE IN METABOLIC INDICES OF OOCYTE COMPETENCE

4.1. INTRODUCTION

Ageing is a complex biological process that is associated with the deterioration of the biological function of the organism at different levels; organs, tissues, cells and molecular levels. Several mechanisms have been proposed in contribution to ageing such as nuclear and/or mitochondrial genome instability, epigenetic regulation, protein homeostasis, ROS, metabolic deregulation and telomerase attrition (Oh *et al.*, 2014). Ovarian senescence is a continuous process of physiological ageing, clinically characterized by gradual depletion of ovarian oocyte reserve and in parallel decline in oocyte quality (Eichenlaub-Ritter *et al.*, 2011, Takahashi *et al.*, 2013). As one of the major causes of female infertility (CENTERS FOR DISEASE CONTROL AND PREVENTION, Heffner, 2004), understanding of mechanisms underpinning ovarian ageing would contribute better knowledge in female reproduction and improve the fertility management.

Depletion of ovarian follicles during ovarian ageing may be simply explained by the exhaustion of the follicular pool, which is dependent on two components; initial size and rate of follicular atresia. This is possibly linked to a functioning organelle, mitochondrion, as it serves as central energy supply and utilization by generating most of the ATP in response to the cell demands (May-Panloup *et al.*, 2016, Otten and Smeets, 2015, Tilly and Sinclair, 2013). Mitochondrial biogenesis during foetal life may determine the initial size of the follicular pool (Aiken *et al.*, 2016). As its role in regulation of ROS production and control of apoptosis (Tait and Green, 2010), mitochondria also response in follicular atresia (Ene *et al.*, 2013, Hsueh *et al.*, 1996, Ratts *et al.*, 1995), as well as providing mediators regulating cellular and organismal ageing (Balaban *et al.*, 2005, Bratic and Larsson, 2013, Dumollard *et al.*, 2007a). This is in accordant to two ageing theories proposed, the free radical theory and mitochondrial theory, which are linked to mitochondria (da Costa *et al.*, 2016, May-Panloup *et al.*, 2016, Oh *et al.*, 2014). Therefore, impaired mitochondrial function leading to metabolic alterations may possibly cause oocyte ageing via several mechanisms mentioned above (da Costa *et al.*, 2016, Oh *et al.*, 2014, Mercado-Saenz *et al.*, 2010).

In parallel to quantitative aspects, impairment of oocyte quality also contributes to the gradual decline in age-related fertility rather than uterine receptivity (Bancsi *et al.*, 2002) as the outcomes from ART cycle using donor oocytes in aged women are comparable to the success rate of young age group (CENTERS FOR DISEASE CONTROL AND PREVENTION 2017). Oocyte development, a highly demanding process, requires the effective mandate of energy metabolism. Perturbations of the metabolic milieu either organism or cellular level upon the extent of impairment can interfere with the normal physiological function of the oocyte. Since each oocyte is a unique cell hosted within each ovarian follicle without a direct vascular supply, the metabolism of each oocyte relies upon the co-operation with its surrounding somatic follicular cells to support nutrients, energy substrates as well as signaling molecules to pass throughout its life cycle. Unlike any other cell types, oocyte development progress slowing through their growth and maturation phase during which they progressively acquire the catalogue of proteins, RNA, cellular organelles needed to support fertility and early embryo development. The metabolic needs of the oocyte are met by the follicle cells and are tightly controlled. Thus, the tight metabolic control persists after fertilization in order to prevent abnormal embryonic development or demise (Seli *et al.*, 2014).

Metabolic assessment to predict embryo and oocyte development potential has been studied for more than 40 years (Biggers and Stern, 1973, Brinster, 1973). Most of the reports related to metabolism have focused on the measurement of major substrates added to/or utilized from culture media, which contribute to energy supply such as pyruvate for oocytes or glucose for embryos (Downs *et al.*, 2002, Dumollard *et al.*, 2007b, Harris *et al.*, 2007, Harris *et al.*, 2009, Khurana and Niemann, 2000, Thompson *et al.*, 1996), and lactate production (Harris *et al.*, 2007, Harris *et al.*, 2009, Khurana and Niemann, 2000, Thompson *et al.*, 1996). Prior to embryonic genome activation, the oocyte and early mammalian embryo rely on their own endogenous stores and energetic substrates milieu for ATP generation (Ferguson and Leese, 1999, Gardner *et al.*, 2001). Measuring metabolic demands thus determine the oocyte quality and the embryo's ability for further development. Similar to energy metabolism, the oocyte and embryo are also dependent on their own amino acid pool to overcome any limitations in fixed nitrogen resources (Caro and Trounson, 1984) until post compaction (Barnett and Bavister, 1996). Alteration

of the presence of amino acids in culture milieu whether decrease by consumption or increase by protein degradation (Orsi and Leese, 2004b, Sturmey *et al.*, 2010, Thompson *et al.*, 1998), can be used to determine developmental potential of oocyte and developing mammalian embryo. The measurement of amino acid turnover has also been linked to oocyte (Hemmings *et al.*, 2012, Hemmings *et al.*, 2013) and embryo health and developmental competence (Houghton *et al.*, 2002, Stokes *et al.*, 2007). The preliminary evidence on the human egg from Hemmings and colleagues found age effect leading to differences in amino acid profiles (Hemmings *et al.*, 2013). Prior to oocyte maturation, glycolysis conserves energy and oxygen resources for mouse follicular growth and synthesis of steroid hormones (Boland *et al.*, 1994b). Aged oocytes accumulate oxidized proteins, DNA, and lipids via oxidation processes subsequently increasing free radical or ROS production leading to mitochondrial dysfunction by mtDNA damage, activated caspases, impaired calcium signaling metabolic enzyme in electron transport chain and altered activity of key enzymes involved in energy metabolism; therefore, metabolic changes may, in turn, be markers to determine the quality of oocytes. However, it is still unclear whether there are metabolic manifestations of age in oocytes.

Aims and Objectives

The aim of this chapter was to examine the impact of maternal reproductive age on oocyte metabolism in terms of both energy metabolism and AA turnover by individual oocytes, in relation to the other markers of oocyte health such as chromatin configuration. The specific objectives were;

- (i) to evaluate the effect of maternal age on the immature oocyte metabolism in terms of AA turnover and carbohydrate metabolism using the sheep as a model for human oocytes
- (ii) to investigate the link between metabolic markers and oocyte chromatin configuration

4.2. MATERIALS AND METHODS

4.2.1. Experimental Design

Denudation of cumulus oocyte complexes is a prerequisite for the accurate determination of the GV-staged oocyte metabolism, and so modification of oocyte culture media was necessary in order to prevent the spontaneous premature oocyte maturation. Cilostamide was chosen to block or delay oocyte meiotic resumption as described previously in chapter section 3.2.4. Two discrete experiments were conducted.

a) Experiment 1: Effect Of Reproductive Age On Oocyte Amino Acid Turnover

In this experiment, 50µM cilostamide was used to supplement the AAP incubation medium in order to quantify how GV-staged oocyte amino acid metabolism was affected by maternal age. This experiment was conducted in two different sessions using prepubertal tissues from the non-breeding season (July-August) and tissues from animals with established reproductive cycles in the breeding season (November-December). According to previous publications (Kochhar *et al.*, 2002, Ledda *et al.*, 1997, Leoni *et al.*, 2007a, O'Brien *et al.*, 1997b, Reader *et al.*, 2015), pregnancy and live birth outcomes from IVM in prepubertal lamb oocytes are significantly reduced compared to similar procedures conducted in adult oocytes suggesting that prepubertal lamb oocytes represent poor quality when compared to adult adult oocytes.

b) Experiment 2: Effect Of Reproductive Age On Oocyte Energy Metabolism

The experiment was conducted to examine the effect of oocyte reproductive age on energy metabolism using the sheep model. As detailed in the previous chapter, 50µM cilostamide was added into the potassium simplex optimised medium (KSOM) to inhibit spontaneous premature oocyte maturation by the oocyte released from follicle and CCs removal during a 6 hour energy metabolism assessment for individual oocytes.

4.2.2. Harvesting Cumulus Oocyte Complexes And Denudation

Cumulus enclosed oocytes were harvested from abattoir derived tissues as described previously (see Section 2.7). The CCs were immediately removed after being incubated in H199+ containing 80IU/ml hyaluronidase for 30 seconds to 1 minute by gently, repeated pipetting in and out through narrow bore glass pipettes (see Section 2.8). Denuded oocytes underwent a final examination under a stereomicroscope and immature oocytes without evidence PB were pooled to be used for metabolic assessment. Each experiment was performed in quadruplo, with approximately equal numbers of oocytes in each group in order to prevent the bias from different batches of tissues. All procedures were conducted on microscopes fitted with heated stages at 37°C.

4.2.3. Carbohydrate Quantification

a) Preparation Of Modified Potassium Simplex Optimised Medium

KSOM was originally formulated in the embryo culture system to overcome the 2-cell block in mouse embryos (Erbach *et al.*, 1994). The complexity of KSOM can be modified by omitting some components (i.e., lactate, glutamine) for culture and carbohydrate metabolism quantification of early oocytes (oocyte-KSOM) and follicles (follicle-KSOM) as previously described (Collado Fernandez, 2013, Harris *et al.*, 2007). Briefly, the reagents listed in Table 4.1 were mixed together and a calibrated electronic osmometer was used to measure osmolarity. Stock A solution was used to increase the osmolarity if necessary. The medium was filter-sterilised through a 0.2µm syringe filter into a 30ml universal and stored at 4°C for a maximum of 1 week.

Table 4.1. Composition of modified KSOM for oocyte metabolism incubations.

Note that BSA was only added after the osmolarity had been measured and adjusted to 256 ± 2 mOsm/kg. (*) indicated the amounts and concentrations above refer to those used for amino acid metabolism (modified from Collado Fernandez, 2013).

Stock (strength)		Amount for oocyte KSOM (ml)	Final concentration of oocyte KSOM (mM)
A (10X)*	NaCl	1	94.97
	KCl		2.50
	KH ₂ PO ₄		0.35
	MgSO ₄ ·7H ₂ O		0.20
	EDTA		0.01
B (10X)*	NaHCO ₃	1	25.01
	Phenol red		
C (100X)*	Sodium pyruvate	0.2	0.40
D (100X)*	CaCl ₂ ·2H ₂ O	0.1	1.71
Glucose 0.2 (100X)*		0.1	0.20
H ₂ O		7.5	-
Penicillin/Streptomycin		0.1	100IU/ml and 0.1mg/ml
Cilostamide (20mg/ml)		8.56 µl	50
Osmolarity: 256 ± 2 mOsm/kg			
BSA tissue culture grade (>96% fatty acid free)		10 mg	1mg/ml

b) Preparation Of Glucose, Pyruvate And Lactate Reaction Mixtures

Reaction mixtures containing the enzymes and co-factors required for the redox reactions shown in Figure 4.1.a were prepared by mixing the reagents listed in Table 4.2. into a sterile universal and vortexing thoroughly. Solutions were filter-sterilised, aliquoted into 1.5ml sterile microfuge tubes and stored at -20°C for up to 6 weeks. Just prior to use, 30µl of an L-Lactate dehydrogenase (L-LDH) suspension (10107085001, Roche Diagnostics Ltd., Burgess Hill, UK) was added to each 1ml of pyruvate reaction mix, or 26µl of L-LDH in the case of lactate reaction mix. The reaction times for the glucose, pyruvate and lactate were 10min, 3min and 30min respectively, upon addition of substrate.

a) Enzyme-linked ultramicrofluorimetric REDOX reactions

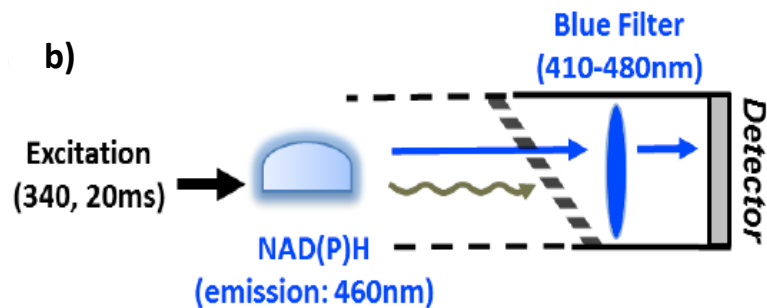
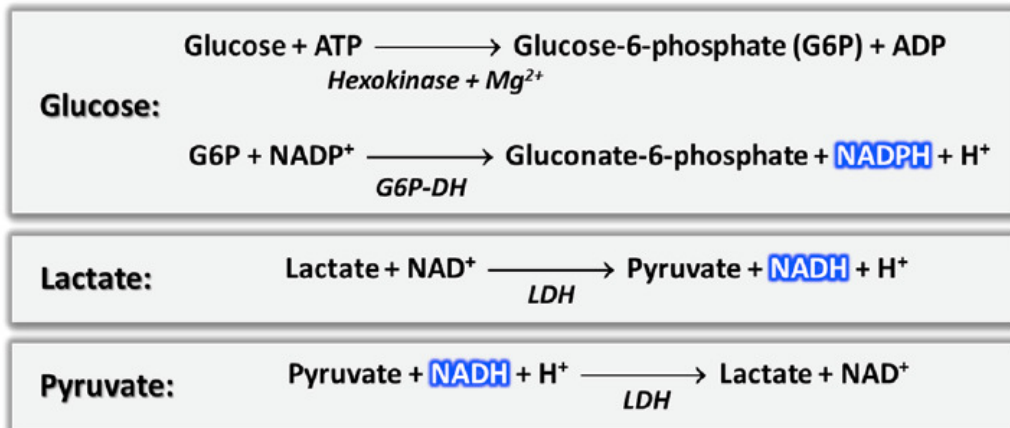


Figure 4.1. Quantification of oocyte carbohydrate metabolism using an ultramicrofluorometric assay. a) Fluorimetric redox reactions used for the measurement of glucose, lactate and pyruvate in spent culture medium. Note that NADH (in blue) is the fluorescent product or substrate. Enzymes are indicated in italics: G6PDH: glucose 6 phosphate dehydrogenase; LDH: lactate dehydrogenase. b) Representation of the photometry system used for quantification of NAD(P)H fluorescence in spent culture media (From Collado-Fernandez, 2013 with permission).

Table 4.2. Glucose, pyruvate and lactate reaction mixtures. (*) indicated enzyme added upon thawing. HK: hexokinase; GPDH: glucose 6-phosphate dehydrogenase; L-LDH is from hog muscle.

	Stock Concentration	Volume	Working Concentration
Glucose Reaction Mix			
EPPS buffer		15 ml	
DTT stock	5 mM	2 ml	0.42 mM
MgSO4 stock	37 mM	2 ml	3.08 mM
ATP stock	10 mM	1 ml	0.42 mM
NADP stock	10 mM	3 ml	1.25 mM
HK / G6PDH	340 IU HK/ml; 170 IU/ml G6PDH	1 ml	24.29 IU/ml; 12.14 IU/ml
Pyruvate Reaction Mix			
EPPS Buffer		28 ml	
NADH stock	5 mM	600 µl	0.11 mM
L-LDH*	5500 IU/ml	30 µl	165 IU/ml
Lactate Reaction Mix			
Glycine/Hydrazine Buffer		18 ml	
MilliQ® H2O		16 ml	
NAD stock	60 mM	3 ml	4.86 mM
L-LDH *	5500 IU/ml	26 µl	143 IU/ml

c) Preparation Of Glucose, Pyruvate And Lactate Standards And Quality

Controls

A series of glucose, pyruvate and lactate standards of known concentration were required for the standard curves construction. These standards were prepared by diluting a volume of the required glucose, pyruvate or L-lactate stock (detailed in Appendix III) with sterile tissue culture grade water as demonstrated in Table 4.3. Quality controls were prepared as well as the standards and consisted of 2 solutions of a concentration as demonstrated in Table 4.3. Quality controls were analysed in parallel to samples of oocyte spent culture media and control samples.

Table 4.3. Preparation of standards and quality controls for ovine oocyte glucose, pyruvate and lactate assays.

Oocyte Glucose Standard (mM)	1 mM Glucose Stock (ml)	H2O (ml)
0	0	1
0.1(QC low)	0.1(QC low)	0.9
0.2	0.2	0.8
0.4	0.4	0.6
0.6	0.6	0.4
0.8	0.8	0.2
0.9 (QC high)	0.9 (QC high)	0.1
1.0	1.0	0
Oocyte Pyruvate Standard (mM)	1 mM Pyruvate Stock (ml)	H2O (ml)
0	0	1
0.1(QC low)	0.1(QC low)	0.9
0.2	0.2	0.8
0.3	0.3	0.7
0.4	0.4	0.6
0.5 (QC high)	0.5 (QC high)	0.5
0.6	0.6	0.4
Oocyte Lactate Standard (mM)	1 mM Lactate Stock (ml)	H2O (ml)
0	0	1
0.1(QC low)	0.1(QC low)	0.9
0.2	0.2	0.8
0.4	0.4	0.6
0.6	0.6	0.4
0.8	0.8	0.2
0.9 (QC high)	0.9 (QC high)	0.1
1.0	1.0	0

4.2.4. Quantification Of Oocyte Carbohydrate Metabolism Using An Ultramicrofluorometric Assay

Nanolitre assays were carried out for the measurement of glucose, pyruvate and lactate concentrations in spent-KSOM media from sheep GV-staged oocytes, following an established non-invasive ultramicrofluorometric protocol (Gardner and Leese, 1986, Harris *et al.*, 2007, Harris *et al.*, 2009, Leese and Barton, 1984). The technique is a modified version of conventional coupled enzymatic redox reactions into a small scale, which bring out the reduction of NAD(P)⁺ from glucose and lactate metabolism or the oxidation of NADH from pyruvate metabolism (Figure 4.1.a) as the nucleotide NAD(P)H in its reduced form is given different excitation (340nm) and emission (460nm) wavelength of fluorescence (blue). Therefore, the proportional difference of fluorescence in a nanodrop of culture medium (before

and after the redox reaction) can be used to calculate the amount of glucose and pyruvate consumed as well as lactate produced from the reaction (Figure 4.1.a and b) by using standard curves as demonstrated below (see Figure 4.2).

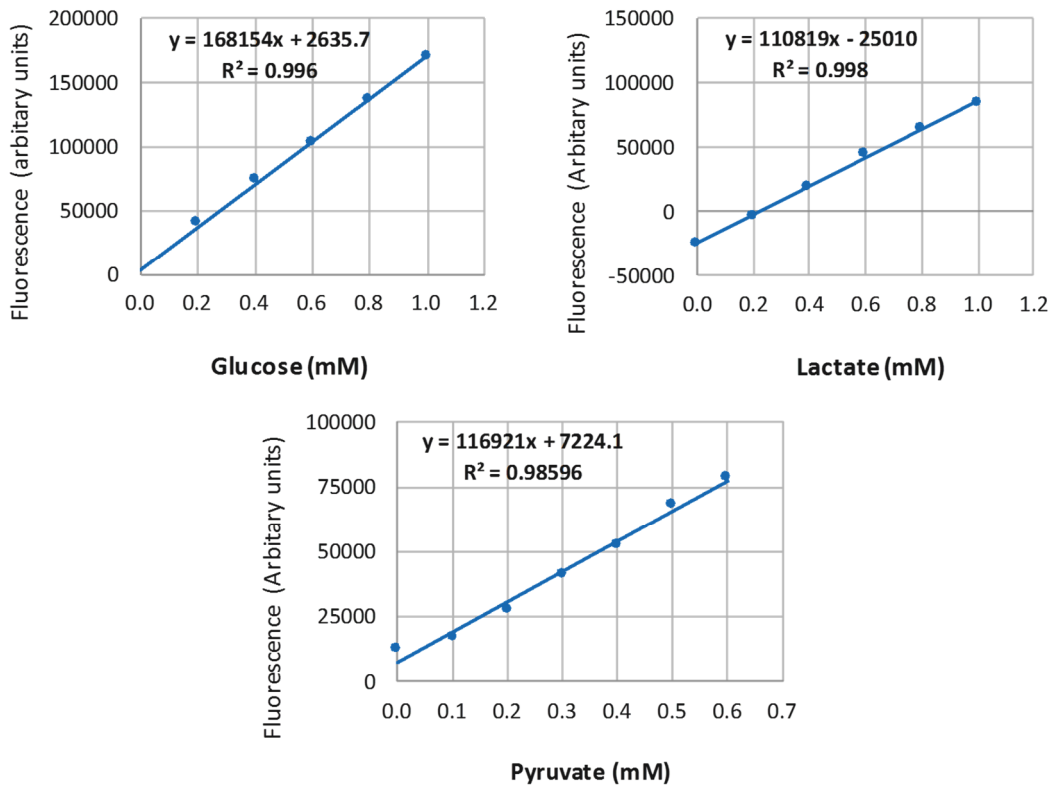


Figure 4.2. Demonstration of standard curves used for the calculation of glucose, lactate and pyruvate concentration in spent-KSOM media. Data are presented as means \pm SEM of triplicates. Blue lines show the lines of best fit for the standard curves.

a) Oocyte Incubations

The quantification of glucose, pyruvate and lactate consumption or production, by individual oocytes, required the measurement of nanolitre volume of spent culture medium. The measurement of control media enables detection of tiny changes of concentration of metabolites within the culture medium prior to any utilization by oocytes (i.e. viability, changes due to metabolism) (Harris, 2002). The time and volume of the metabolism incubations have been optimized previously by our group (Collado Fernandez, 2013, Harris, 2002, Harris *et al.*, 2010). Harris *et al.* (2002) demonstrated that 2 hours' incubation for glucose/pyruvate /lactate assays was sufficient to give rise to acceptable changes of between 25-35% concentration before and after the assay. In addition, incubation volumes were kept as small as

possible to enable changes to be detectable. As mentioned above, the 6 hour period of incubation in the current study was longer than previous reports, and designed in order to evaluate the link of measuring markers. Oocyte metabolism medium was incubated in 35x10mm Easy Grip™ falcon dishes (Becton Dickinson, Oxford, UK), prepared on the day before the experiment. Briefly, two wash drops of 10µl KSOM were laid and covered with 3ml of sterile mineral oil. Nanolitre incubation drops were laid onto the bottom of the dishes and allowed to equilibrate to 37°C and 5%CO₂ in air overnight.

Denuded oocytes were washed twice through 2, 10µl wash drops of KSOM in the GPL dishes before being transferred individually to nanolitre drops. In order to displace the tiniest of volume, calibrated, siliconised, thin pulled glass pipettes were used. Oocyte incubation and control (oocyte-free media) assays were incubated under oil at 37°C and 5%CO₂ in a humidified incubator. The incubation volumes and times were 144nl and 1 hour equilibration before 6 hours assay incubation. After incubation, oocytes were removed from nanodrops and stored for different purposes including measurement of chromatin, mitochondrial copy number, and for genetic studies. Methods have been described in section 2.12. The culture dishes containing the spent culture medium were stored at -80°C as described for the AAP dishes for up to 3 months.

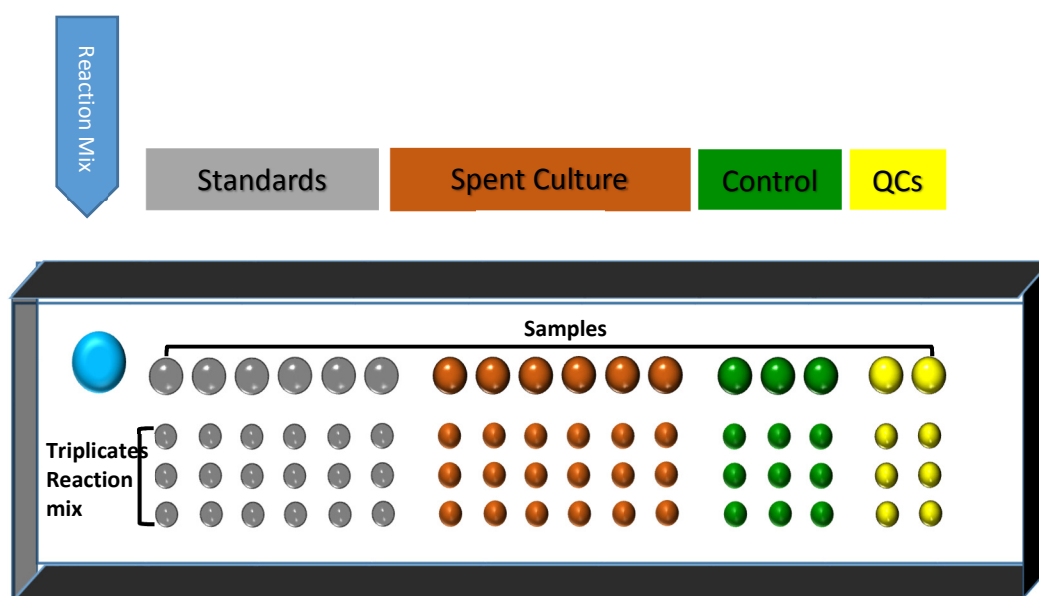


Figure 4.3. Demonstrate a siliconised glass oil well containing nanolitre volume drops for the quantification of NAD(P)H fluorescence

b) Ultramicrofluorometric Analysis Of Samples

Dishes containing spent KSOM medium were thawed and analysed using the non-invasive ultramicrofluorometric assay (Harris *et al.*, 2007, Harris *et al.*, 2005, Harris *et al.*, 2009, Leese and Barton, 1984). Appearance or disappearance of the NAD(P)H fluorescence was measured in nanolitre volumes of spent KSOM medium following its reaction with each of the reaction mixtures detailed in Table 4.2. A photometric imaging system consisting of a Zeiss Axiovert 200 epifluorescence microscope (Carl Zeiss Ltd., Cambridge, UK) fitted with a dichroic mirror, emitter filter, photon multiplier tube and photometer (Photon Technology International, West Sussex, UK) was used as previously described (Harris *et al.*, 2007, Harris *et al.*, 2009) (Figure 4.1.b). The 20X objective was selected. The intensity of the NAD(P)H blue fluorescence was monitored with a 372nm excitation wavelength and a 460nm emission wavelength, using the Felix 32 software. Spent culture medium samples, control drops and quality control samples were measured and conducted in triplicate. Data were analysed against standard curves.

Initially, standard curves for each metabolite were constructed by quantifying a fluorescence detected from a series of known concentration of standards. Under stereomicroscopy, a 5µl drop of reaction mix was placed on the top left of the oil well by using a nanolitre pipette, grids of triplicate reaction mix droplets (14-32nl) were laid on an in house manufactured oil well containing ~3ml of mineral oil (Figure 4.3) see section 2.3 for preparation of the oil wells. The oil well was then placed on the microscope stage, the target drop was centred using a calibrated ocular graticule and the aperture adjusted. The “blank” fluorescence was measured for each set of triplicate drops. Next, a row of 21.3-37.2nl (depending on the assay) droplets of the glucose/pyruvate/lactate standard solutions were laid along the top of the grid of triplicate reaction mix droplets (Figure 4.3). A 2.8nl volume from each of the standard droplets was added to the 3 reaction mix droplets below. The reaction was allowed to take place for 3min, 10min or 30min for pyruvate, glucose or lactate, respectively. The fluorescence was then measured and recorded (“post-reaction” reading). A standard curve was produced by plotting the data obtained. Demonstration of standard curves is shown in Figure 4.2.

In addition, dishes containing spent culture medium and control droplets were laid down on the bench top to thaw for a few minutes. Nanodrops of spent KSOM, control drops and quality controls were then assayed using the same procedure as that described for the standards, but now using the droplets of spent culture medium. First “blank” readings were measured before a 2.8nl volume of spent culture medium was added to the reaction mix droplets. After reactions, the “post-reaction” reading was measured per nanodrop. In order to monitor the working conditions of the system, quality controls were repeated every 9 samples of spent culture medium.

c) Calculations

The glucose and pyruvate consumption rate as well as the lactate production rate were calculated per oocyte per hour. In order to correct for non-specific fluorescence, a corrected fluorescence value was obtained for each standard, sample and control droplet by subtracting the fluorescence measured from the blank readings from those of the “post-reaction” readings (for glucose and lactate assays, due to the appearance of NAD(P)H), or vice versa (for pyruvate assay, due to the disappearance of NADH). In this case, glucose is taken as an example of the calculations for each metabolite:

AFU : Arbitrary Fluorescent Unit

Average triplicate of post-reaction of oocyte sample 1 = 344191 AFU

Blank reading = 306419 AFU

$$\begin{aligned} \text{Corrected fluorescence} &= | \text{post-reaction AFU} - \text{blank reading AFU} | \\ &= | 344191 - 306419 | \\ &= 37772 \text{ AFU} \end{aligned}$$

Therefore, oocyte sample 1 (triplicate1) Corrected Fluor is **37772** AFU.

The corrected fluorescence values of the standards (averages per triplicate) were plotted against their known concentration, producing a standard curve whose equation was used to calculate the average concentration of glucose/pyruvate/lactate for each of the triplicate sample droplets (representative examples of standard curves are shown in Figure 4.2):

Glucose Standard curve in Figure 4.2. demonstrated in linear correlation as the following;

$$Y = 168154X + 2635.7$$

X = standard concentration (mM)

Y = standard fluorescence (AFUs)

In order to measure the glucose concentration in samples, known post-reaction fluorescence was used for calculation in the modified equation;

$$X = \frac{(Y - 2635.7)}{168154}$$

Therefore, glucose concentration in each sample was calculated by the following equation;

Sample-1 Glucose Concentration (triplicate-1)

$$\begin{aligned} &= \frac{(\text{Sample-1 Corrected Fluorescence} - 2635.7)}{168154} \\ &= \frac{(37772 - 2635.7)}{168154} \\ &= 0.209\text{mM} \end{aligned}$$

Finally, Sample-1 Glucose Concentration was calculated from an **average of the triplicates' value** of Sample-1 Glucose concentration.

The method of the calculation above was applied to all of the triplicates of each control drop within the same dish. The amount of glucose in individual droplets, either samples or controls, was calculated separately as shown in the equation below;

- Average Glucose Conc. in Sample-1 = 0.217 mM (from triplicates)
- Average Glucose Conc. in Control-1 = 0.305 mM
- Nanodrop volume in the assay = 21.3 nl

$$\text{Glucose amount (pmoles)} = \text{Glucose concentration (M)} \times \text{Volume (L)} \times 10^{12}$$

$$\begin{aligned} \text{Glucose amount in Sample 1} &= (0.217 \times 10^{-3}) \times (21.3 \times 10^{-9}) \times 10^{12} \\ &= 4.622 \text{ pmoles} \end{aligned}$$

$$\begin{aligned} \text{Glucose amount in Control 1} &= (0.305 \times 10^{-3}) \times (21.3 \times 10^{-9}) \times 10^{12} \\ &= 6.497 \text{ pmoles} \end{aligned}$$

To correct the baseline glucose amount, an average of glucose amount from 3 control droplets in each dish was calculated prior to calculating the glucose consumption rate of individual oocytes.

Average Control Glucose amount in each dish

$$\begin{aligned}
 &= \frac{(\text{Glucose amount in Control 1} + \text{in Control 2} + \text{in Control 3})}{3} \\
 &= \frac{6.497 + 6.386 + 6.509}{3} \\
 &= 6.464 \text{ pmoles}
 \end{aligned}$$

The consumption/production rate of glucose, lactate and pyruvate was obtained by subtracting the amount of glucose/lactate/pyruvate in pmoles present in an individual sample from the average control amount presented in each dish. The rate was finally divided by the duration of the oocyte incubation (in hours):

- Incubation time = 6 hours

Glucose Consumption rate in Sample1

$$\begin{aligned}
 &= \frac{(\text{Average Control Glucose amount} - \text{Glucose amount in Sample1})}{\text{Duration of incubation (h)}} \\
 &= \frac{6.464 - 4.622}{6} \\
 &= 0.307 \text{ pmoles/oocyte/h}
 \end{aligned}$$

Regarding Glycolytic Index (GI), this has been reported as a marker to estimate the efficiency of anaerobic glycolysis (Boland *et al.*, 1994a, Lane and Gardner, 1996) as well as ATP production from glycolytic pathway (Harris *et al.*, 2007). Accordingly, 1 mole of glucose is metabolised to produce 2 moles of lactate, it is therefore possible to determine the percentage of glycolysis by calculating the ratio between glucose consumption and lactate production. GI was calculated using the following equation;

$$\text{Glycolytic Index (GI)} = \frac{\text{lactate production rate}}{2 \times \text{glucose consumption rate}}$$

Therefore, when GI equals 1 it was assumed that all glucose consumed had contributed to lactate production by the anaerobic pathway of glycolysis and this represented a GI value of 100% (Boland *et al.*, 1994a, Harris *et al.*, 2007, Lane and Gardner, 1996). In other words, a GI of less than 1 indicated that anaerobic

glycolysis was less than 100% efficient. While a GI higher than 1 indicated that more lactate was production than glucose was consumed (GI >100%).

4.2.5. Statistical Analysis

All statistical tests were performed using either SPSS statistics V.21 or GraphPad Prism 7 software. Normality was tested for all groups using the Kolmogorov-Smirnov or Shapiro-Wilk test when using the SPSS statistics program while D'Agostino & Pearson or Shapiro-Wilk tests were applied if the GraphPad Prism programme was used as appropriate. Differences between the animal age groups were analysed using the unpaired Student t-test if the data were normally distributed or by Mann-Whitney U tests if data did not fit a normal distribution curve. Statistical significance was assumed for p-values of ≤ 0.05 . Data presented as boxplots are the data that did not fit a normal distribution. Data that fitted a normal distribution are presented as mean \pm SEM, with N values indicating the number of samples analysed.

4.3. RESULTS

4.3.1. Effect Of Ageing On Oocyte Amino Acid Profiling

The experiment was performed in quadruplo in both age groups. A total of 144 oocytes from four replicates in each group were cultured in AAP medium. After exclusion of some samples that experienced technical problems during sample preparation, 135 and 133 AAP-incubated GV oocyte samples from prepubertal and adult animals were finally analysed. The AAP of the GV-staged oocytes was analysed according to animal age; prepubertal vs adult sheep. As shown previously in section 3.3.5, glycine, alanine and lysine were the major AAs appearing in the spent culture media from both age groups while asparagine, glutamine, threonine, arginine, and leucine are representative AAs depletion from the culture media.

Aspartic acid, isoleucine and leucine depletion were significantly greater in the AAP media samples incubated with adult GV oocytes compared to those incubated with prepubertal GV oocytes ($p < 0.05$) (Figure 4.4). Significant differences were also detected with less glutamic acid and tyrosine but more alanine appearance in AAP

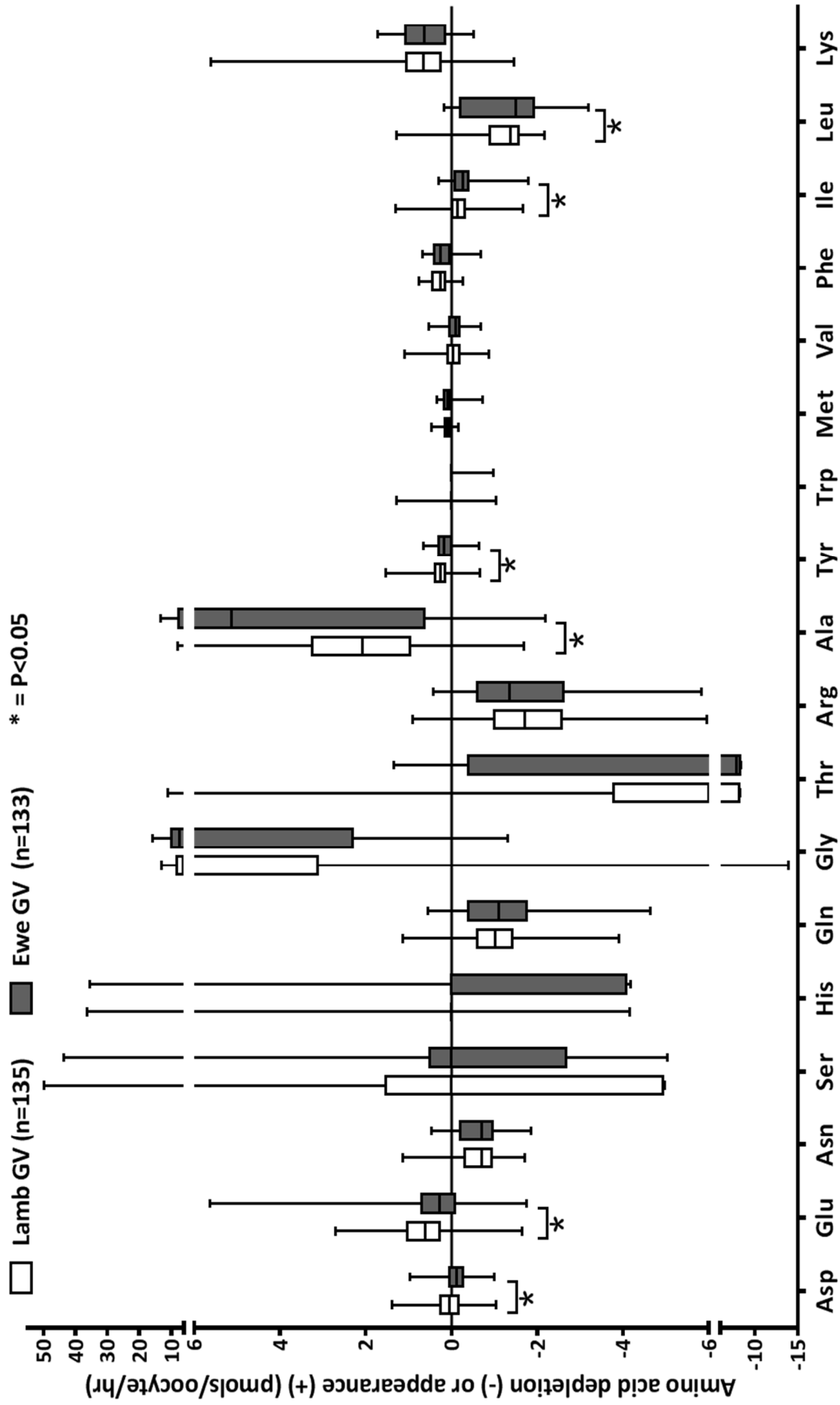


Figure 4.4. Amino acid profiles of sheep GV-staged oocytes obtained from 2-5mm sized follicles from prepubertal (open box) and adult ewe oocytes (shaded box) incubated for 6 hours in 50µM cilostamide supplemented AAP media. (*) indicates significant differences; Mann-Whitney test, p<0.05.

Table 4.4. Amino acid profiles by GV-staged oocytes between prepubertal lambs and adult ewes. Significant differences, $p < 0.05$, between the 2 groups are indicated by a, b, c, d and (*). a demonstrates the AAs that have differentially expressed between two groups; b, c, d demonstrates the amino acids having less production, more production and more consumption in adult oocytes incubated in AAP medium supplemented with $50\mu\text{M}$ cilostamide. (*) indicated the significant parameter of amino acid turnover.

(pmoles/OC/h)	Lamb (n=135)			Ewe (n=133)		
	Median	Mean	SEM	Median	Mean	SEM
Asp^a	0.044	0.073	0.034	-0.119	-0.107	0.028
Glu^b	0.610	0.622	0.069	0.283	0.392	0.075
Asn	-0.701	-0.616	0.040	-0.698	-0.625	0.041
Ser	0.000	2.121	0.991	0.000	1.176	0.814
His	0.000	0.322	0.468	0.000	0.179	0.621
Glu	-1.013	-1.016	0.063	-1.092	-1.149	0.088
Gly	6.197	5.580	0.318	7.305	6.515	0.379
Thr	-5.986	-5.142	0.320	-7.853	-5.113	0.313
Arg	-1.697	-1.772	0.100	-1.346	-1.609	0.107
Ala^c	2.069	2.166	0.143	5.136	4.800	0.311
Tyr^b	0.265	0.265	0.021	0.178	0.132	0.022
Trp	0.000	-0.098	0.030	0.000	-0.066	0.021
Met	0.092	0.095	0.008	0.087	0.055	0.019
Val	-0.025	-0.041	0.022	-0.105	-0.074	0.019
Phe	0.261	0.284	0.017	0.263	0.219	0.024
Ile^a	-0.131	-0.170	0.030	-0.264	-0.257	0.022
Leu^a	-1.375	-1.138	0.061	-1.504	-1.277	0.076
Lys	0.647	0.730	0.080	0.642	0.634	0.047
Net balance*	-2.284	2.265	1.227	1.147	3.824	1.126
Depletion	-14.223	-13.574	0.536	-14.555	-13.480	0.632
Appearance	14.089	15.839	1.128	17.025	17.304	1.240
Turnover*	28.953	29.412	1.271	33.486	30.784	1.615

media incubated with adult GV oocytes compared to those incubated with prepubertal GV oocytes ($p < 0.05$) (Figure 4.4 and Table 4.4).

Data from Figure 4.5 show that AA turnover was significantly higher ($p < 0.05$) in spent culture media incubated with adult oocytes than those incubated with prepubertal oocytes. Whilst overall AA net-balance was switched from a positive

balance in the adult group to a negative balance in the prepubertal group ($p < 0.05$) (Figure 4.5). However, no significant difference was found between overall AA depletion and appearance ($p > 0.05$).

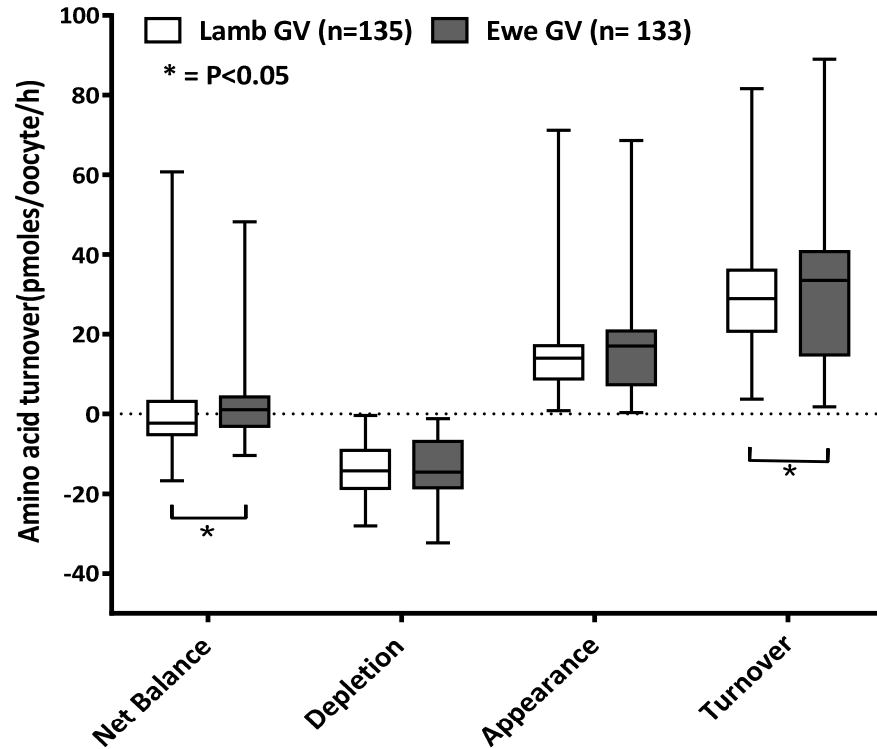


Figure 4.5. Amino acid turnover of sheep GV-staged oocytes obtained from 2-5 mm sized follicles between prepubertal lamb (open bar) and adult ewe (shade bar) oocytes incubated for 6 hours in 50 μ M cilostamide supplemented AAP media. (*) indicates significant differences; Mann-Whitney test, $p < 0.05$.

4.3.2. Effect Of Ageing On Oocyte Energy Metabolism

Four culture replicates were carried out from both prepubertal and adult tissues. A total of 288 GV-staged oocytes retrieval from 2-5mm sized follicles (144 from both prepubertal and adult tissues) were obtained for carbohydrate metabolism quantification in the spent culture medium. At the count, a total of 270 oocyte incubation drops, 126 from prepubertal and 144 from adult GV oocytes, were analysed. Due to the lack of normal distribution, data were presented in median and range using Mann-Whitney test for statistical analyses.

Median pyruvate consumption rate in spent culture media of GV oocytes derived from adult ewes was significantly greater than those derived from prepubertal lambs (8.46 (0.00 - 7.90) vs 5.95 (0.00 - 12.76) pmoles/oocyte/h; $p < 0.001$).

However, no significant difference of lactate production ($p>0.05$) was found in spent culture media between the two age groups, the production rates was 1.01 (0.00-18.90) pmoles/oocyte/h for adult and 1.02 (0.00-6.49) pmoles/oocyte/h for prepubertal oocytes, respectively. On the other hand, more glucose was significantly consumed by prepubertal GV oocytes than adult oocytes ($p<0.05$), rates were 0.45 (0.32 – 2.27) compared to 0.39 (0.00 – 3.10) pmoles/oocyte/h, respectively (Figure 4.6).

Table 4.5. Carbohydrate metabolism by GV-staged oocytes between prepubertal lambs and adult ewes. (*) and (**) indicate significant differences $P<0.05$ and $P<0.001$, respectively.

(pmoles/oocyte/h)	Lamb (n=126)			Ewe (n=144)		
	Median	Mean	SEM	Median	Mean	SEM
Glucose consumption*	-0.450	-0.724	0.062	-0.385	-0.475	0.041
Pyruvate consumption**	-5.950	-5.291	0.175	-8.460	-7.549	0.261
Lactate production	1.015	1.129	0.082	1.010	1.501	0.170

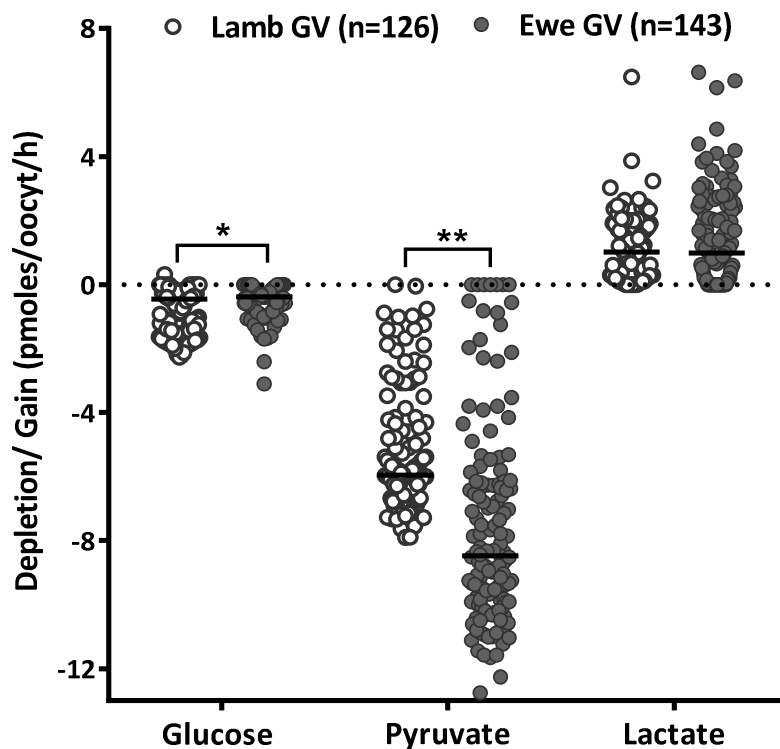


Figure 4.6. Carbohydrate metabolism of sheep GV-staged oocytes obtained from 2-5mm sized follicles between prepubertal lamb (open circle) and adult ewe (shade circle) oocytes incubated 6 hours in 50 μ M cilostamide supplemented modified KSOM medium. (*) and (**) indicate significant differences; Mann-Whitney test, $p<0.05$ and $p<0.001$, respectively. An outlier excluded from the adult group did not interfere overall outcomes.

4.3.3. Relationship Between GPL Utilization And Oocyte Chromatin Configuration

To examine the relationship between carbohydrate metabolism and oocyte chromatin configuration, a subset of spent culture media corresponding to carbohydrate metabolism and chromatin morphology was reanalyzed. A total of 113 oocytes (55 from prepubertal and 58 from adult animals) were incubated in KSOM medium and subsequently allocated for chromatin study. Of these, 8 out of 55 oocytes were excluded from the prepubertal group due to 4 samples lost during transfer and DAPI staining and 4 samples uninterpretable. 3 out of 58 oocytes from the adult group were excluded due to 2 dead oocytes (no metabolism detected) and 1 sample lost during transfer for staining. Thus reanalyzed data were generated from 47 prepubertal and 55 adult GV oocytes according to the chromatin pattern and metabolism by the same cell.

Subgroup analysis showed GV chromatin patterns were significantly different between prepubertal and adult GV oocytes (Chi-square test; $p < 0.05$) (Figure 4.7), as it was previously shown in the results of chapter 3. Net-like and clumped chromatin patterns seemed to dominate the results in both age groups with more than 80% of each age group (68% and 19% in prepubertal and 33% and 50% in the adult group, respectively). Collectively, this data confirms a strong impact of maternal age on chromatin configuration in GV oocytes.

Carbohydrate metabolism in this subgroup analysed also demonstrated the same differences as shown in the previous section for the total number of oocytes incubated in KSOM (see section 4.3.2). In this regard, there was significantly less glucose (0.42 (0.00-3.10) vs 0.85 (0.00-2.27) pmoles/oocyte/h; $p = 0.002$) and higher pyruvate (8.48 (1.97-11.58) vs 5.68 (0.05-7.33) pmoles/oocyte/h; $p < 0.001$) consumption rates in spent culture media derived from adult ewe oocytes when compared to those from prepubertal lamb oocytes while no differences in lactate production rate was detected (0.85 (0.00-6.37) vs 0.81 (0.00-2.40) pmoles/oocyte/h; $p < 0.05$) (Figure 4.8). With regard to the same set of data, none of prepubertal but 2 of the adult GV oocytes incubated 6 hours in modified KSOM medium with 50 μ M cilostamide supplementation resumed meiosis as they reached MI and MII stages.

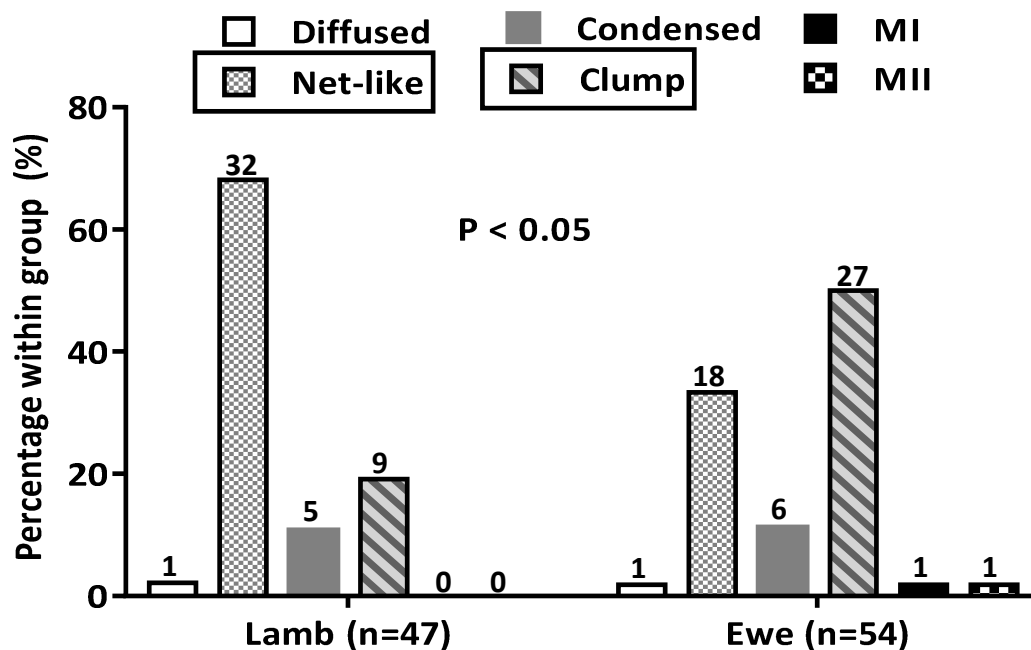


Figure 4.7. Distribution of chromatin configuration between prepubertal lamb (n=47) and adult ewe (n=54) GV-staged oocytes incubated for 6 hours in modified KSOM medium containing 50 μ M cilostamide supplementation. A significant difference between age groups was determined by Chi-square test, $p < 0.05$. Figures on top of each bar represent sample size (N) in each chromatin pattern.

Table 4.6. Carbohydrate metabolism by sheep GV-staged oocytes between prepubertal lambs and adult ewes: subgroup analysis. (*) and (**) indicate significant differences $p < 0.05$ and $p < 0.001$, respectively.

(pmoles/oocyte/h)	Lamb (n=47)			Ewe (n=54)		
	Median	Mean	SEM	Median	Mean	SEM
Glucose consumption*	0.850	0.894	0.099	0.417	0.504	0.075
Pyruvate consumption**	5.680	5.027	0.251	8.403	7.804	0.380
Lactate production	0.810	0.849	0.098	0.848	1.269	0.207

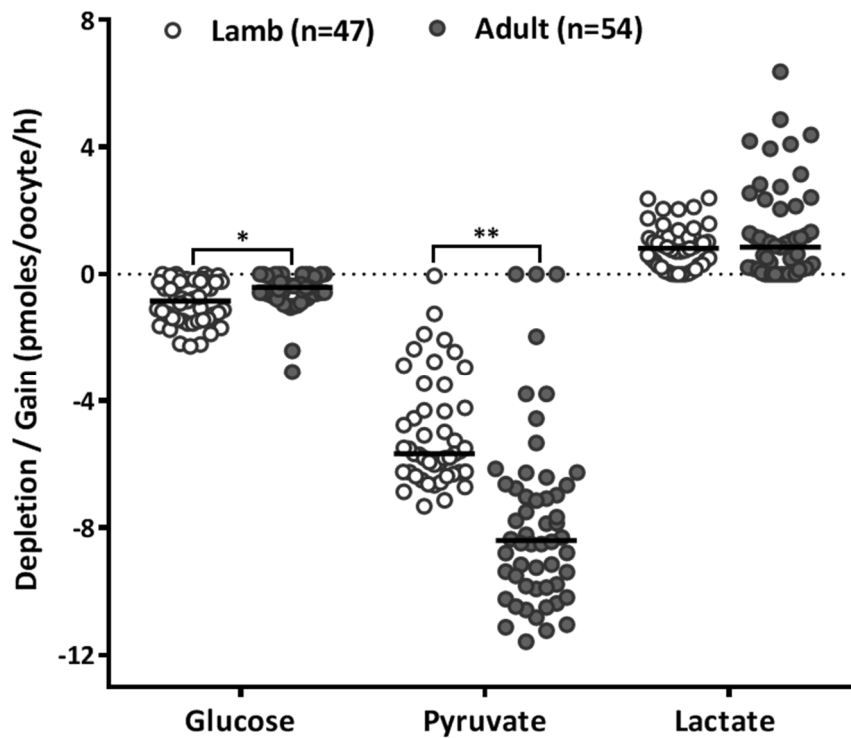


Figure 4.8. Subgroup analysis of GV-staged oocytes incubated in 50 μ M cilostamide supplemented KSOM medium for 6 hours between prepubertal and adult animals corresponding to carbohydrate metabolism and chromatin configuration. Open (○) and shade (●) circles represent data taken from spent culture media incubated the prepubertal lamb and the adult ewe oocytes, respectively. (*) and (**) indicate significant differences using Mann-Whitney test; $p < 0.05$ and $p < 0.001$, in individual assays.

As mentioned above, the predominate net-like and clump chromatin patterns were taken into account in order to examine whether the age effect still persisted in each pattern of chromatin configuration in terms of carbohydrate metabolism. When the metabolic data of the subgroup of oocytes presenting net-like patterns from adult ewe oocytes were analysed they still showed significantly ($p < 0.05$) less glucose and greater pyruvate consumption than equivalent chromatin status GV oocytes from lambs. However, when subgroup analysis was conducted on oocytes presenting a clumped pattern, there was only a significant difference between age groups in the rate of pyruvate consumption ($p < 0.05$) but not in either glucose consumption or lactate production ($p > 0.05$) (Figure 4.9). With regard to the pattern of chromatin configuration, further analyses were conducted to test whether the pattern of chromatin configuration itself could have an impact on carbohydrate metabolism. Although it was shown that oocytes presenting net-like chromatin patterns tended to have higher pyruvate consumption than GV oocytes presenting clumped

chromatin patterns in both age groups, the data from only prepubertal lamb GV oocytes reached statistical significance ($p < 0.05$; Figure 4.10).

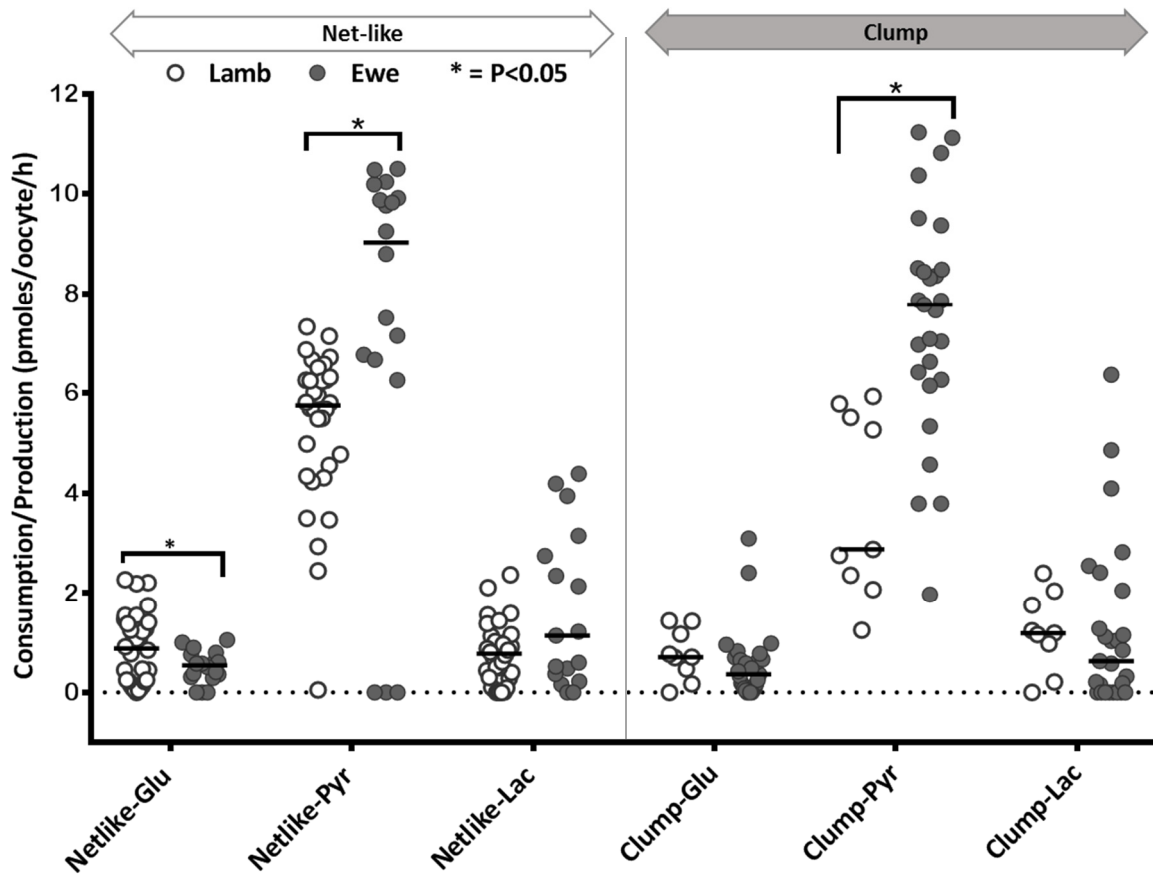


Figure 4.9. Glucose/Pyruvate/Lactate metabolism of GV oocytes from two age groups compared with regard to net-like (n=32 in lamb vs. 18 in ewes) or clump chromatin patterns (n=9 in lamb vs. 27 in ewes). The first three columns on the left of chart represent data of net-like chromatin while three column right column represent data of clumped chromatin patterns. (*) indicates a significant difference by using t-test or Mann-Whitney test when appropriate; $p < 0.05$, between the 2 age groups in individual oocyte assays.

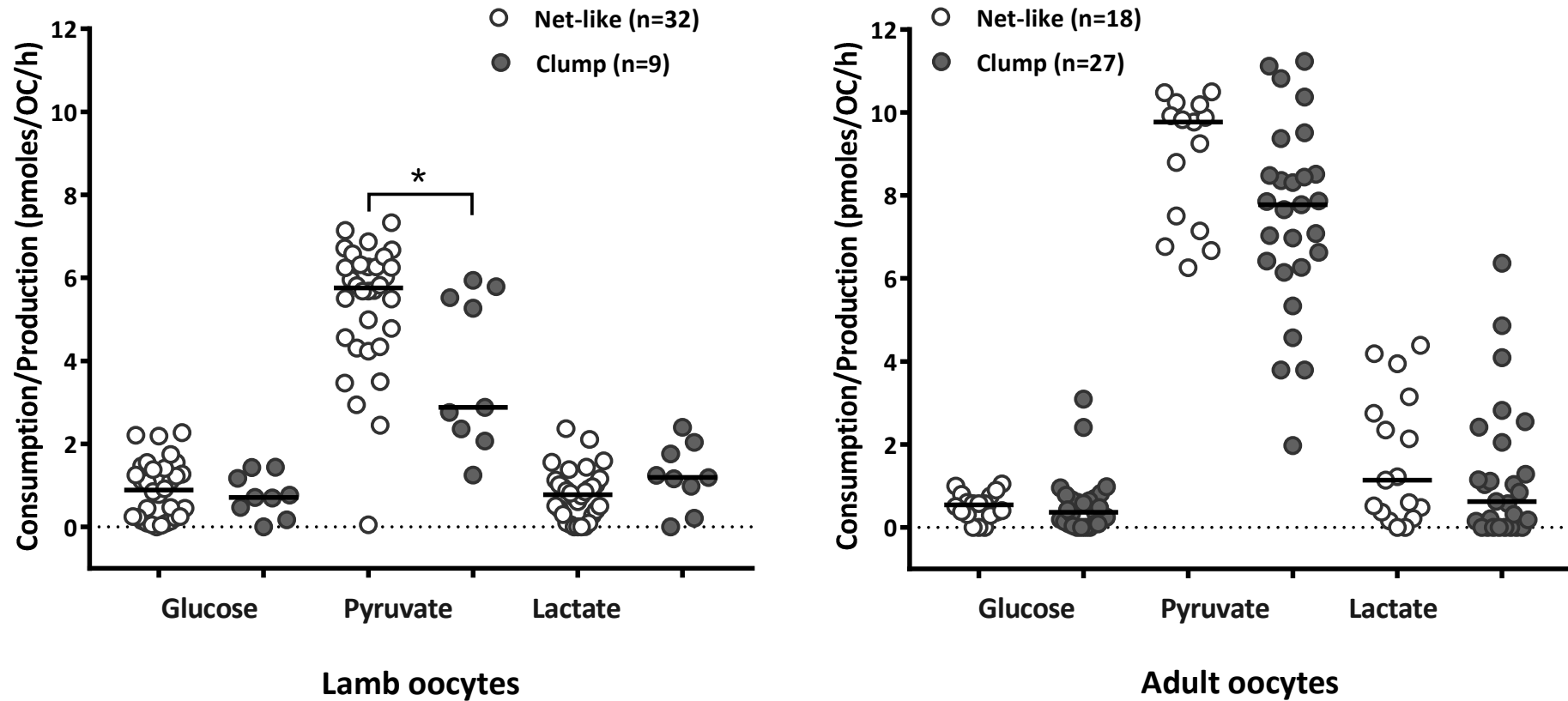


Figure 4.10. Glucose/Pyruvate/Lactate metabolism between 2 chromatin patterns (net-like and clump), in GV-staged oocytes incubated for 6 hours in modified KSOM medium with 50 μ M cilostamide supplementation. Data on the left chart represent metabolic rate derived from prepubertal lamb oocytes (n=32 net-like vs. 9 clumped chromatin) while on the right chart represent data from adult oocytes (n=18 net-like vs. 27 clumped chromatin). (*) indicates a significant difference using unpaired t-test or Mann-Whitney test when appropriate; $p < 0.05$, in individual oocyte assays.

4.3.4. Relationship Between Amino Acid Metabolism And Oocyte Chromatin Configuration

The relationship between oocyte chromatin configuration and AA metabolism was also inspected in relation to chromatin configuration of the source oocyte. Subgroup analysis was performed on the spent culture medium droplets that were incubated with oocytes used for chromatin analysis. A total of 111 oocytes (55 from prepubertal and 56 from adult oocytes) were incubated in AAP medium and subsequently allocated for evaluation of chromatin configuration. Of these, 5 out of 55 oocytes were excluded from the prepubertal group because 2 samples were lost during transfer and DAPI staining and 3 samples were uninterpretable; while 4 out of 56 oocytes from the adult group were excluded - 3 were lost during transfer and 1 sample was contaminated. Thus, 50 oocytes from prepubertal and 52 oocytes from adult sheep were retained for chromatin pattern analysis in relation to amino acid turnover by the same cell.

Even in this subset of data the chromatin configuration between prepubertal and adult GV oocytes was found to be significantly different ($p < 0.05$; Chi-square test) (Table 4.8 and Figure 4.11). This confirmed that ageing affected the chromatin configuration of GV oocytes during *in vitro* incubation. However, in this data set not only did the net-like and clump chromatin pattern dominate the chromatin results but also the condensed chromatin pattern became more prominent in both age groups when compared to the chromatin findings derived from oocytes used for studies of carbohydrate metabolism. Here, the prevalence of net-like, condensed and clumped chromatin in prepubertal GV oocytes were 38.8%, 24.5% and 26.5%, respectively, while they were 19.2%, 17.3% and 59.6% respectively in adult GV oocytes. These 3 chromatin patterns were evaluated in relation to their amino acid metabolism.

The chromatin morphology showed that 3 denuded oocytes from prepubertal and 2 denuded oocytes from adult sheep resumed meiosis within 6 hours of incubation with modified AAP medium with 50 μ M cilostamide supplementation (Figure 4.11). Amino acid metabolism in this subgroup analysis also demonstrated significant changes of glutamic acid, alanine and tyrosine between

the 2 age groups ($p < 0.05$). In this regard, there was significantly less glutamic acid (0.438 ± 0.100 vs. 0.638 ± 0.137 pmoles/oocyte/h; $p < 0.05$) and tyrosine consumed (0.143 ± 0.024 vs. 0.271 ± 0.046 pmoles/oocyte/h; $p < 0.05$) and greater alanine production (4.028 ± 0.454 vs. 1.963 ± 0.227 pmoles/oocyte/h; $p < 0.001$) in spent culture media derived from adult oocytes when compared to the prepubertal lamb oocytes (Figure 4.12 and Table 4.9). In respect of the total of AAs, greater AA net-balance (1.802 ± 1.260 vs. 1.320 ± 2.366 pmoles/oocyte/h; $p < 0.05$) but lower overall AAs depletion (11.482 ± 0.992 vs. 15.796 ± 0.864 pmoles/oocyte/h; $p < 0.05$) and turnover (24.765 ± 2.000 vs. 32.913 ± 2.112 pmoles/oocyte/h; $p < 0.05$) was demonstrated in spent culture media from adult oocyte samples compared to prepubertal oocyte samples (Table 4.7. and Figure 4.13).

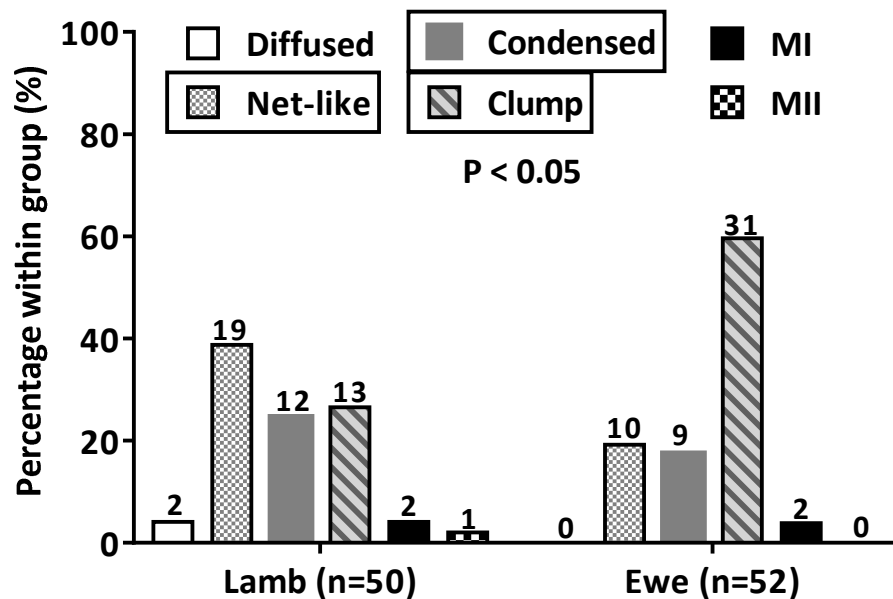


Figure 4.11. Chromatin configuration of sheep GV-staged oocytes incubated for 6 hours in 50 μ M cilostamide supplemented AAP medium between prepubertal lambs and adult ewes. A significant difference demonstrates when pattern of chromatin distribution between two-age groups compared; Chi-test, $p < 0.05$. Figures on top of each bar represent sample (N) size in each chromatin pattern.

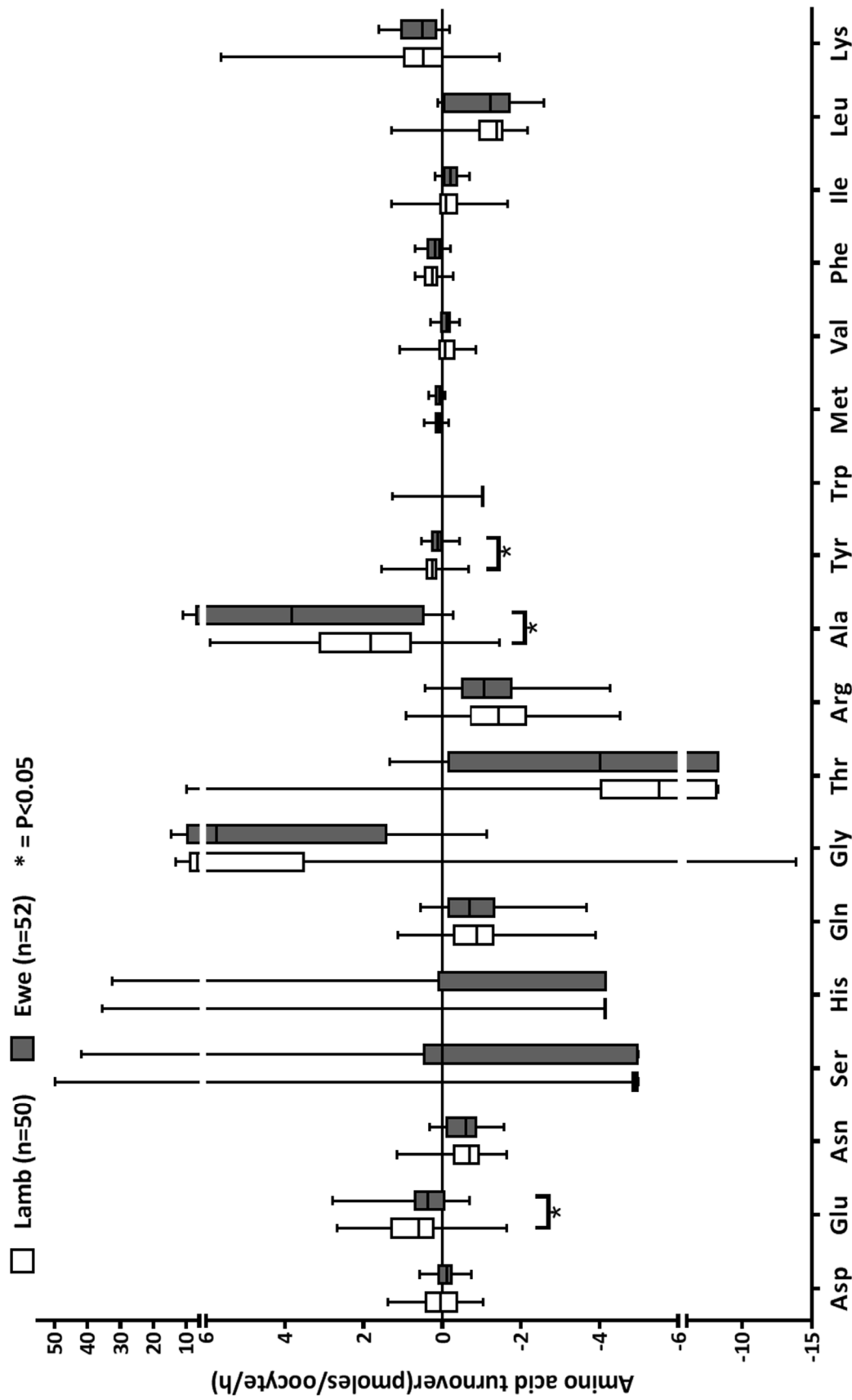


Figure 4.12. Comparison of amino acid profiles in subgroup analysis for the relationship of amino acid metabolism and chromatin configuration between the 2 animal ages in sheep GV-staged oocytes incubated for 6 hours in AAP medium with 50µM cilostamide supplementation. (*) indicates significant differences; Mann-Whitney test, p < 0.05.

Table 4.7. Amino acid profiles in subgroup analysis by sheep GV-staged oocytes between prepubertal lamb and adult ewes. ^a, ^b and (*) indicate significant differences, $p < 0.05$, between two-age groups. ^a represents amino acids with less produced while ^b represents amino acids with more produced by adult ewe oocytes.

(pmoles/OC/h)	Lamb (n=50)			Ewe (n=52)		
	Median	Mean	SEM	Median	Mean	SEM
Asp	0.043	0.077	0.074	-0.106	-0.078	0.035
Glu^a	0.603	0.698	0.137	0.357	0.438	0.100
Asn	-0.681	-0.583	0.072	-0.588	-0.527	0.066
Ser	-4.892	0.520	2.022	-0.004	-0.768	1.231
His	-4.144	1.383	3.754	-4.144	-0.747	1.642
Gln	-0.884	-0.864	0.112	-0.687	-0.897	0.131
Gly	6.530	6.102	0.624	5.732	5.691	0.611
Thr	-5.510	-5.220	0.524	-4.013	-4.337	0.506
Arg	-1.424	-1.469	0.150	-1.071	-1.337	0.151
Ala^b	1.817	1.963	0.227	3.819	4.028	0.454
Tyr^a	0.255	0.271	0.046	0.122	0.143	0.024
Trp	-1.008	-0.733	0.161	-	-	-
Met	0.096	0.101	0.016	0.070	0.090	0.014
Val	-0.061	-0.076	0.050	-0.111	-0.078	0.023
Phe	0.258	0.272	0.030	0.195	0.222	0.028
Ile	-0.096	-0.182	0.071	-0.203	-0.208	0.028
Leu	-1.392	-1.160	0.099	-1.221	-1.066	0.114
Lys	0.490	0.809	0.194	0.496	0.593	0.068
Net balance*	-3.961	1.320	2.366	1.801	1.802	1.260
Depletion*	-16.814	-15.796	0.864	-10.988	-11.482	0.992
Appearance	14.475	17.117	2.070	13.122	13.283	1.345
Turnover*	32.047	32.913	2.112	25.407	24.765	2.000

In order to examine whether chromatin configuration can influence AA metabolism, 3 main chromatin patterns were taken for subcategory analysis. Figure 4.14 illustrates the overall AA metabolism in terms of net-balance, depletion, appearance and turnover of both prepubertal and adult oocytes. After 6 hours of incubation in modified AAP medium, oocytes that reached the later stages of chromatin configuration demonstrated a lower overall AA depletion, lower overall AA appearance and lower overall AA turnover in both age groups. Concerning differences of AA metabolism between each pattern of chromatin

configuration, there was a significant difference of overall AA depletion ($p < 0.05$) detected between condensed and clump chromatin in both age groups. Whilst overall AA appearance was found to be significantly different between clumped and net-like chromatin patterns ($p < 0.005$), as well as, between clump and condensed chromatin patterns ($p < 0.005$) only in spent culture media derived from prepubertal oocytes and not in those samples derived from adult oocytes. Moreover, overall AA turnover also showed to be significantly different ($p < 0.05$) between clumped and condensed chromatin in samples obtained from prepubertal oocytes but not those from adult oocytes (Figure 4.14).

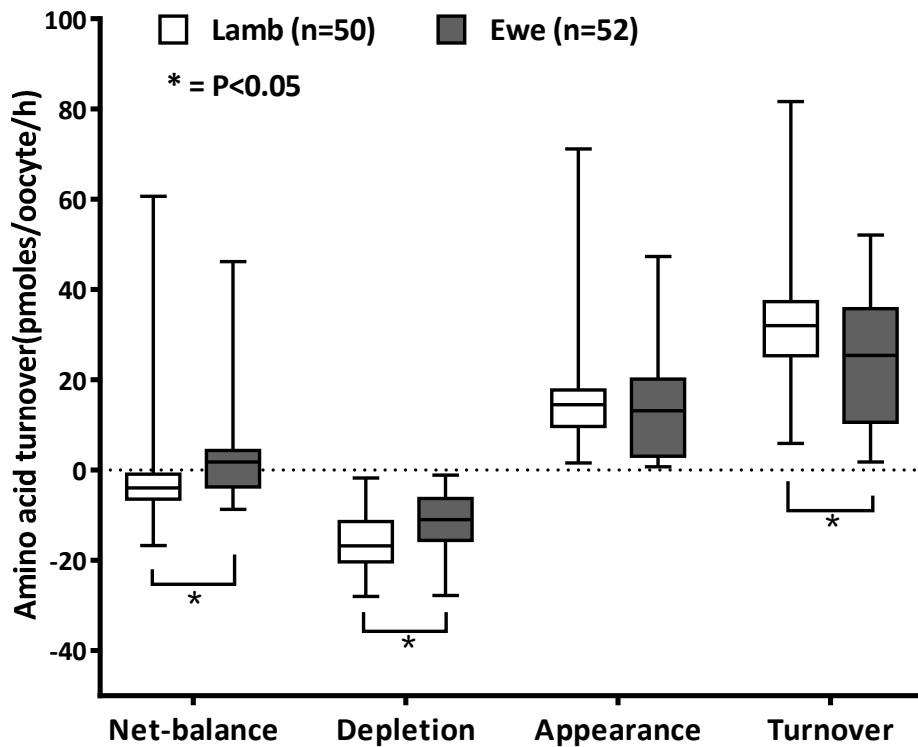


Figure 4.13. Amino acid turnover of sheep GV-staged oocytes in subgroup analysis between prepubertal lambs (open box) and adult ewes (shade box) incubated for 6 hours in modified AAP media with 50 μ M cilostamide supplementation. (*) indicates significant differences; $p < 0.05$.

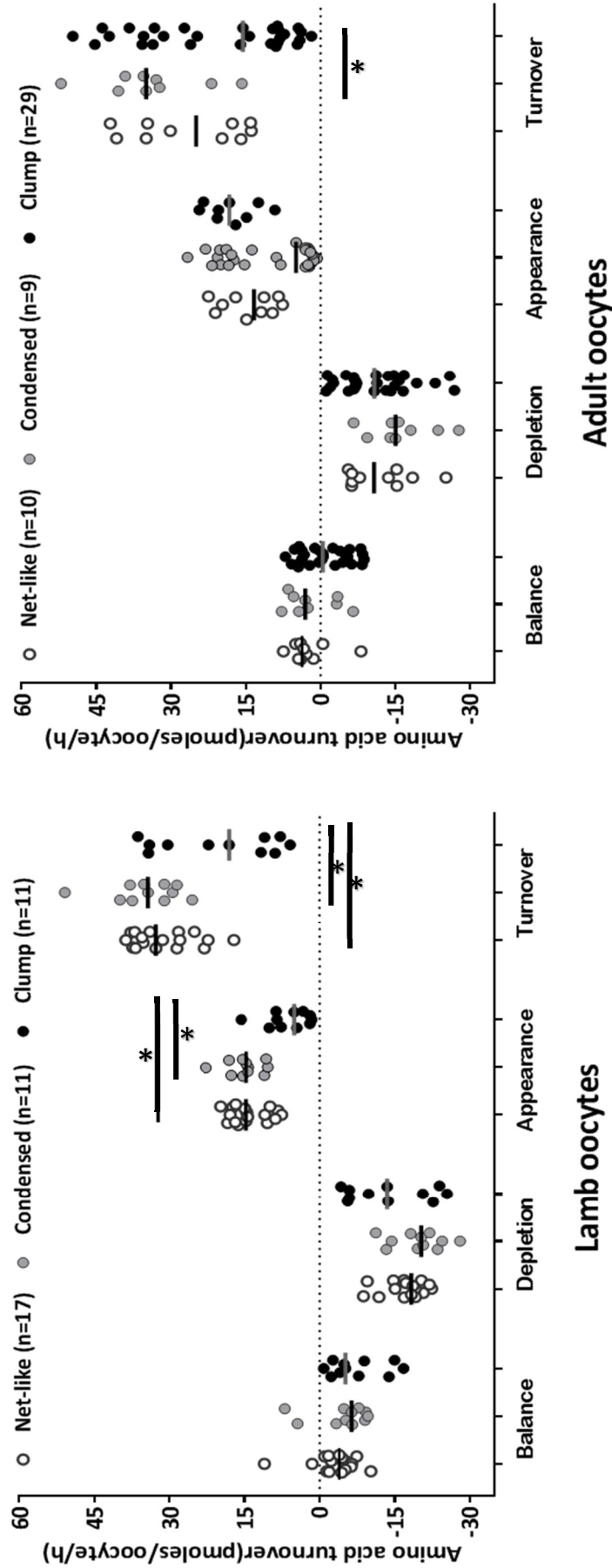


Figure 4.14. Amino acid turnover between three chromatin patterns (net-like, condensed and clump), in GV-staged sheep oocytes incubated for 6 hours in modified AAP medium with 50 μ M cilostamide supplementation. Data on the left chart represent metabolic rate derived from prepubertal lamb oocytes (n=17 net-like, 11 condensed, and 11 clumped chromatin) while on the right chart represent data from adult oocytes (n=10 net-like, 9 condensed and 29 clumped patterns). (*) indicates a significant difference using unpaired t-test or Mann-Whitney test when appropriate; p<0.05, in individual oocyte assays. Data excluded 5 outliers from lamb (2, 1, and 2 samples) and 2 outliers from adult (0, 0, and 2 samples) did not compromise the overall outcomes.

4.4. DISCUSSION

4.4.1. Effect Of Reproductive Age On GV-Staged Oocyte Amino Acid Metabolism

When maternal (i.e., reproductive) age was taken into account, this study demonstrated significant differences in amino acid profiles between prepubertal and adult GV-staged oocytes. The GV-staged oocytes from adult sheep represented the competent oocyte model that depicted significantly higher amino acid turnover than those oocytes from prepubertal lambs. On the other hand, overall oocyte amino acid net-balance switched from negative in young animals to positive in adult sheep. The observation in this study reflects a previous report in human, which found significantly higher amino acid turnover and overall amino acid depletion in donated oocytes matured *in vitro* from patients under the age of 35 years that underwent ICSI with normal ovarian morphology (better quality) compared to the counterpart oocytes recruited from patients aged 35 years and over (poor quality) (Hemmings *et al.*, 2013). Indeed, prepubertal lamb oocytes were utilized here as a model of poor oocyte quality as indicated from ART outcomes using juvenile lamb oocytes (Kochhar *et al.*, 2002, Ledda *et al.*, 1997, Leoni *et al.*, 2007a, O'Brien *et al.*, 1997b, Ptak *et al.*, 1999). However, a recent report from our group using an *in vivo* ageing model with sheep MII-staged oocytes could detect a significant difference in only the overall amino acid depletion (Collado Fernandez *et al.*, 2015). This may indicate that there is a temporal variability in amino acid metabolism during stages of maturation as GV-staged oocyte amino acid metabolism assays were immediately incubated after denudation instead of after 18hrs of oocyte *in vitro* maturation as performed in MII staged oocytes. However, it still cannot rule out the effect of the ageing model used in this study, of which the main defect of prepubertal oocytes during ageing *in vitro* was believed to be due to incomplete cytoplasmic maturation while the reproductive ageing process may increase free radical oxygen species residues of oxidative phosphorylation (da Costa *et al.*, 2016).

According to the individual amino acid profiles, the current study demonstrated significantly more aspartic acid, isoleucine and leucine depletion as well as less

glutamic acid and tyrosine but more alanine appearance in adult GV oocytes in comparison to prepubertal GV oocytes ($p < 0.05$) (Figure 4.4 and Table 4.4). These may reflect aged impairment on sheep oocyte development. In contrast to Hemmings, *et al.* (2013), the patient's age altered different amino acid profiles of donated GV oocytes from ICSI patients matured *in vitro*, of which oocytes from women under 35 years exhibited more glutamine, arginine, methionine and phenylalanine depletion in comparison to oocytes from women over 35 (Hemmings *et al.*, 2013). Picton and colleagues (2010) reported age influenced changes in several amino acid metabolisms of embryos on day 1 or day 2 post insemination. More aspartate, glutamine, threonine, tyrosine, valine and isoleucine as well as less lysine consumption were demonstrated in day 1 or day 2 embryos from women under 37 years compared to those from women over 37 (Picton *et al.*, 2010). It seems that embryos require an increased amount of amino acids presumably for protein synthesis and energy conversion during embryonic growth than oocytes do. Although maternal age is likely to have an impact on amino acid turnover, the variation was observed in individual amino acids. This could be explained by developmental stage differences between oocytes and embryos, as well as species differences between sheep and human. It is notable that age significantly affected differences of glutamine consumption in the two human studies mentioned (Hemmings *et al.*, 2012, Picton *et al.*, 2010); however, in this study, this did not reach statistical significance. As its crucial roles in generating metabolites; glutamate and then α -ketoglutarate for ATP synthesis via TCA cycle, the consistent glutamine consumption among studies may support other roles beyond energy fuel and protein precursors such as an organic osmotic regulator (Lawitts and Biggers, 1993), antioxidant (Suzuki *et al.*, 2007), carbon and nitrogen provider for purine and pyrimidine synthesis (Leese *et al.*, 1993) as well as a glucose metabolism regulator (Zielke *et al.*, 1976).

Collado-Fernandez, *et al.* reported lower glutamine and leucine depletion as well as lower tyrosine appearance but higher glutamic acid appearance found in sheep oocytes derived from early (9-12 months) compared to late reproductive age (6-8 years) in an *in vivo* ageing model (Collado Fernandez *et al.*, 2015). Age altered 3 amino acid metabolisms in that report which is consistent with the finding in

this work (i.e., glutamic acid, tyrosine and leucine). Changes in tyrosine and leucine metabolism reported in Collado-Fernandez, *et al.* (2015) and the current work may imply three possibilities. The two amino acids are fundamental elements for oocyte rather than embryo development determining stage-specific. According to the model used instead of bovine or human as previous reports, it may influence by species effect as sheep oocytes employed in both studies. Finally, the impact of maternal age interferes with the metabolism of both tyrosine and leucine.

Apart from protein synthesis, tyrosine is broken down to provide two key elements; fumarate and acetyl-CoA for generating ATP via TCA cycle, which the metabolic enzyme activities related to oocyte maturation have been determined in several species; mouse (Johnson *et al.*, 2007), rat (Tsutsumi *et al.*, 1992), and bovine (Cetica *et al.*, 2003). Moreover, it is also part of signal transduction processes via protein kinases phosphorylation and may be part of a signalling cascade via SH2 domain binding (Pawson, 2004). Like other amino acids, leucine metabolites participate in the TCA cycle as energy precursors for ATP production. In particular, robust evidence exert that leucine as a nutrient regulator promotes growth via the upregulation of the IGF-1/AKT/mTOR [mammalian target of rapamycin complex] signalling system, the evolutionary conserved master of protein synthesis (Gangloff *et al.*, 2004, Gonzalez *et al.*, 2012, Martin and Sutherland, 2001, Dreyer *et al.*, 2008, Richards, 2002), as well as inhibiting proteolysis by repression of proteasomal degradation (Drummond and Wilke, 2009), and/or the PI3K and protein kinase C signalling pathways (Doi *et al.*, 2003). Further studies are needed to substantiate the current findings in different species and other ageing models, and also to verify the linkage of molecular pathways with these changes in amino acids.

The results from the current work support and expand the capability of using amino acid turnover of 18-amino acid mixtures to determine biological characteristics of embryo health as reported previously in human (Hemmings *et al.*, 2013, Houghton *et al.*, 2002, Picton *et al.*, 2010, Brison *et al.*, 2004, Drabkova *et al.*, 2016) and of MII oocytes in bovine (Hemmings *et al.*, 2012, Collado

Fernandez, 2013). The current work highlights a quantitative amino acid turnover of GV-staged oocytes prior to maturation *in vitro*. The “Quiet Embryo Hypothesis” was originally used for embryo selection to determine the potential to reach blastocyst stage (Leese, 2002), and the finding was later confirmed when using cryopreserved-thawed day 2 human embryos with lost blastomeres as a model (Picton *et al.*, 2010), as well as, in bovine oocytes (Hemmings *et al.*, 2012). In contrast, in this study adult ewe-GV oocytes with better developmental competence have been linked to higher overall amino acid turnover, and is compatible with the results from another sheep *in vivo* ageing model (Collado Fernandez *et al.*, 2015). Differences in the observed results from the different studies may occur for a number of reasons. First, oocyte dynamics during maturation may have biological differences leading to the different manifestation of amino acid patterns. Some reports have for example shown that differences exist in transport systems during meiotic maturation as previously described in mouse (Pelland *et al.*, 2009, Tartia *et al.*, 2009) and bovine (Hemmings, 2007). Second, the strong impact of ageing may alter amino acid metabolism via several mechanisms. Regarding numbers of ageing theories proposed such as DNA damage, mitochondrial dysfunction, ROS, these might involve in multi-organelles as well as molecular signalling pathways resulting in the different amino acid metabolism among studies. And finally, the species difference is possibly a major contributing factor for metabolic changes.

With regards to individual amino acids, the current study is consistent with the findings of other published works and suggest that during development key amino acids play important roles that are over and above that of protein synthesis. For example, glycine is one of the key organic osmotic regulators to balance the embryo cell volume when culturing in high osmolarity medium (Baltz, 2001, Dawson and Baltz, 1997, Hammer *et al.*, 2000, Steeves and Baltz, 2005, Steeves *et al.*, 2003, Tartia *et al.*, 2009, Zander-Fox *et al.*, 2013). In addition, glycine acts as a heavy metal chelator (Sturmey *et al.*, 2008) and is involved in the synthesis of purine nucleotides (Salway, 2004) and GSH with glutamate and cysteine corporation (Dumollard *et al.*, 2007a, Sturmey *et al.*, 2008). Many studies demonstrated the similar pattern of glycine appearance in oocyte/embryo amino

acid metabolism assays. A previous report from our group demonstrated differences in glycine turnover linked to genetic health in human embryos (Picton *et al.*, 2010). Recently, it has been evidenced that glycine supplementation during vitrification and/or maturation of mouse GV oocytes increased cleavage and blastocyst rates as well as an improved MII-spindle assembly (Cao *et al.*, 2016). This could be the protective effect of glycine against alteration of oocyte physiological responses to oxidative stress in relation to spindle assembly, chromosome configuration, and mitochondrial homeostasis, as well as metabolic gene expression (Zander-Fox *et al.*, 2013).

Arginine is another important amino acid utilized in the cell signalling process that is consistently consumed by oocytes and embryos during *in vitro* culture. With regard to its function, arginine is metabolized to nitric oxide (NO) by NO synthase by mammalian cells (Cendan *et al.*, 1996) as an absolute requirement for normal embryonic growth (Kim *et al.*, 2004, Manser *et al.*, 2004). Moreover, it may play another role to establish pregnancy (Sengupta *et al.*, 2005). Similar to this study, the greater consumption of arginine has been shown to be consistently linked to the poor embryo/oocyte competence in several reports in varied species (Brison *et al.*, 2004, Collado Fernandez *et al.*, 2015, Hemmings *et al.*, 2012, Hemmings *et al.*, 2013).

Alanine is a key amino acid, which plays an essential role in the disposal of ammonium ions (Donnay *et al.*, 1999). The observation consistently found across published studies links the appearance of alanine in AAP assay to a significant depletion of arginine in the culture medium (Collado Fernandez, 2013, Collado Fernandez *et al.*, 2015, Hemmings *et al.*, 2012, Hemmings *et al.*, 2013, Houghton *et al.*, 2002, Gopichandran and Leese, 2003). Previous reports found that differences of alanine production can be used to discriminate quality of oocyte/embryo (Hemmings *et al.*, 2012, Hemmings *et al.*, 2013, Houghton *et al.*, 2002) as was also shown in this work. However, the most interesting point of the current study still needs to be explained namely why immature prepubertal oocytes with poor developmental competence produced less alanine compared to those oocytes derived from the animals in reproductive age. Lower alanine

production of immature oocytes may possibly be a consequence of lower pyruvate and glutamate utilization via the TCA cycle (Sellick *et al.*, 2011).

Unlike many reports that use retrograde data to analyse amino acid profiling in relation to the effect of maternal age, this work has designed a cohort study to compare the impact of age on the GV-staged oocyte between prepubertal and reproductive age animal model. Age impact on bovine oocyte has given a similar pattern of amino acid turnover (Hemmings, 2007, Hemmings *et al.*, 2013) to those observed in this work, unlike human data which used only the oocytes/embryos discarded from the clinic which perhaps led to biased results. A heterogeneity of individual amino acids demonstrated in various reports suggests a functional variation of different metabolism to support oocyte or embryo viability to subsequent growth and development. As previously reported, embryos derived *in vitro* have greater variation of amino acid turnover than embryos derived *in vivo* (Sturmeay *et al.*, 2010). Therefore, this functional biomarker, in turn, may reflect oocyte competency and predict embryo quality. Further studies are clearly needed to substantiate the current finding using different ageing models and species.

4.4.2. Effect Of Reproductive Ageing On Oocyte Energy Metabolism

Prior to the embryonic genome activation, developing embryos survive on their own supports to maintain the basal metabolic activities. This depends on DNA, RNA and proteins catalogues accumulated during oocyte development. Therefore, developing oocytes require abundant ATP to support transcription and translation as well as to prepare for cytoplasmic and final nuclear maturation for further development. Numbers of substrates (i.e., glucose, pyruvate, lipids and amino acids) required to be metabolized for ATP generation either via glycolysis or oxidative phosphorylation. During maturation, pyruvate seems to be the major substrate that oocytes use for energy production (Collado Fernandez, 2013, Downs *et al.*, 2002, Harris *et al.*, 2007, Leese, 2002, Xie *et al.*, 2016). During the later stage of embryonic development, the substrate preference is switched to utilize glucose via glycolysis. Therefore, carbohydrate metabolized to ATP may

be used as a potential marker to evaluate oocyte quality or competency in the current ovine ageing model, where oocytes from pre- and adult animals were used to represent poor and good quality oocytes. In support of the validity of this concept, the results showed significant differences in glucose and pyruvate consumption between pre- and adult oocytes. Greater pyruvate than glucose consumption by oocytes regardless of animal age in the current work confirms the observation from many previous reports that oocytes mainly utilize pyruvate for ATP synthesis (Harris *et al.*, 2007, Harris *et al.*, 2009, Xie *et al.*, 2016). Like others previously reported using the prepubertal and adult oocytes model, decrease in pyruvate consumption demonstrated in prepubertal oocytes represents a poor developmental competence (Ledda *et al.*, 2001, O'Brien *et al.*, 1996) compared to adult oocytes.

It is perhaps not surprising that the GV oocyte does not support the “Quiet Embryo” hypothesis or indeed to reflect the metabolism of mature MII oocytes. First, most of the previous reports measured carbohydrate metabolism of the preimplantation embryo to determine embryo potential (i.e., implantation and pregnancy) (Leese, 2002) while the present study focused on an earlier stage of oocyte development (i.e., GV). An explanation of the current findings may therefore relate to biological differences between oocyte developmental stages. Oocyte developmental competence requires both nuclear and cytoplasmic maturation to support progression through oocyte and embryo development. Completion of the first meiotic division is manifest by extrusion of the first polar body. Cytoplasmic maturation is critically important to preimplantation embryo development post-fertilization before embryonic genome activation. Inadequate cytoplasmic maturation has been blamed for the lower productivity of oocytes derived *in vitro* compared to those derived *in vivo*.

Energy metabolism is critical for oocyte maturation since all processes are dynamic and require ATP synthesis from various substrates including carbohydrate, amino acids and lipids (Collado-Fernandez *et al.*, 2012, Songsasen *et al.*, 2012). Apart from requiring extrinsic substrates/nutrients from the environment, intrinsic enzyme activities, intracellular mediators and plasma

membrane transporters are also necessary for oocytes to complete such functions (Gu *et al.*, 2015). Pyruvate uptake into cells occurs via the proton-linked monocarboxylate carrier family (MCT) (Halestrap and Price, 1999). It has been reported that mouse oocytes are rich in the SLC16A members of MCT (Herubel *et al.*, 2002). In addition, the importance of pyruvate is emphasized by pyruvate dehydrogenase E1 α (Pdhx1) specific deletion, which results in severe meiotic defects (Johnson *et al.*, 2007). A recent report has clarified different roles of pyruvate dehydrogenase kinases (PDKs) in mouse oocyte maturation related to meiotic maturation in terms of meiotic spindle morphology and chromosome alignment, and ATP generation (Hou *et al.*, 2015). Therefore, it can be speculated that prepubertal GV-stages oocytes may be deficient in one of the crucial transporters and/or enzymes related to glycolytic and/or oxidative phosphorylation pathways that are evident in later stages of MII development. Further studies are needed to elucidate the essential components of immature oocytes that contribute to developmental competence and support altered molecular mechanisms linked to oxidative phosphorylation, and organelle replication and redistribution.

Due to having a reduced capacity for glucose uptake despite the presence of facilitative glucose transporters (i.e., SLC2A1, SLC2A3 SLC2A8) (Augustin *et al.*, 2001, Dan-Goor *et al.*, 1997, Pisani *et al.*, 2008, Zheng *et al.*, 2007), oocytes depend upon CCs to convert glucose to utilizable substrates such as pyruvate or lactate (Biggers *et al.*, 1967, Sugiura *et al.*, 2005). A study demonstrated that bovine oocytes have low phosphofructokinase activity, a rate-limiting enzyme in the glycolytic pathway (Cetica *et al.*, 2002), leading to the restriction in glucose utilization (Harris *et al.*, 2007, Saito *et al.*, 1994).

4.4.3. Relationship Between Metabolism, Age And Chromatin Configuration

The investigation of the links between AAs and energy metabolism in relation to age and chromatin configuration in GV oocytes is novel. To investigate whether the nuclear chromatin pattern was linked to oocyte metabolism, subgroup analysis was performed only in those samples that provided information on both

carbohydrate metabolism and chromatin configuration of the same cell in each age group of animals or conversely on AAP and chromatin pattern. The robust impact of age on oocytes is emphasized by the consistent relationship demonstrated between oocyte chromatin configuration and pyruvate consumption or amino acid metabolism in the subgroup analyses (section 4.3.3 and 4.3.4). Moreover, pyruvate consumption also differed between age groups in both subgroups of net-like and clumped chromatin patterns (Figure 4.9.). In comparisons of prepubertal oocytes, the chromatin pattern change between net-like and clumped, was associated with a significant difference in pyruvate consumption but no differences in glucose consumption and lactate production (Figure 4.10). However, such a relationship disappeared when pyruvate consumption was reanalysed by using adult oocytes. This brought up a possible hypothesis. Since chromatin configuration occurs during the final stage of oocyte development prior to meiotic maturation, metabolism of adult oocytes may reflect improved inherent cytoplasmic maturation compared to juvenile oocytes such that these oocytes are more likely to have sufficient energy storage capacity to carry them through meiotic resumption. The data from the current study also suggest that a relationship exists between oocyte pyruvate consumption and the patterns of chromatin configuration, of which the net-like pattern exhibited higher pyruvate utilization than the clumped chromatin. In addition, the relationship may be a reflection of oocyte maturation potential; however, this is not yet established in lamb GV oocytes. Further studies are required to confirm how insufficient cytoplasmic development in prepubertal oocytes relate to adult oocytes in terms of transporter system and enzymatic activities.

An impact of age was also demonstrated in the subgroup analysis of chromatin configuration in relation to AA turnover. Despite the fact that age had a significant effect on AA profiles, the subgroup analysis revealed differences of the overall AA depletion, appearance and turnover between chromatin patterns (net-like, condensed and clump) that were more pronounced when prepubertal oocyte data were reanalysed. In contrast, the data from adult oocytes demonstrated only a significant difference in overall AA depletion between condensed and clumped chromatin ($p < 0.05$) (Figure 4.14). This may possibly be indicative of cytoplasmic

maturation since immature small prepubertal oocytes depicted characteristics of active growth phase by presenting high level of transcription and translation, as well as demanding more amino acids as building blocks for establishing cytoplasmic organelles (i.e., mitochondria) and for synthesizing proteins, enzymes and signalling molecules for further development (Fair *et al.*, 1997). As it was previously reported in porcine and bovine, an increase in total mitochondrial number occurs in developing prepubertal oocytes in correlation with the oocyte volume until the final stage of the growth phase approaches (Fair *et al.*, 1996, Pedersen *et al.*, 2016).

4.4.4. Conclusion

This chapter demonstrated the strong evidence of maternal age impact on differences in amino acid and energy metabolism by using prepubertal lamb and adult ewe GV-staged oocytes as a model. AAP and energy metabolism assays as the noninvasive prospective biomarkers can be used to predict the cytoplasmic competency of oocytes and to reflect biological differences in terms of ultrastructural organelles and functions. Further studies are required to elucidate the relevance of maternal age-altered metabolism and the clinical outcomes.

CHAPTER 5: EFFECT OF MATERNAL AGE ON THE EXPRESSION OF OOCYTE GENETIC MARKERS

5.1. INTRODUCTION

The relationship between maternal age and declining reproductive success has been associated with the impairment of oocyte maturation and embryonic development (Rambags *et al.*, 2014, Simsek-Duran *et al.*, 2013). The mechanisms underpinning this phenomenon are poorly described. Although diminished ovarian reserve and reduced uterine receptivity, in part, can explain this phenomenon, it is hypothesised that decreasing oocyte quality may be the cause leading to impaired developmental potential.

After completion of primordial germ cell (PGC) migration in early foetal life, non-growing oocytes enter the first meiosis and remain arrested at the diplotene stage of prophase I. At this stage, oocytes displaying no changes in cell-cycle phase and cell volume may be considered quiescent, although some crucial activities are necessary to maintain the viability. The potential gametes are encapsulated by flattened pre-granulosa cells within primordial follicles from the perinatal period throughout the female's reproductive lifespan, and these oocytes become sustained for prolonged periods-several decades later. When primordial follicle activation occurs, the follicle and the primary oocyte contained within it grow to attain preovulatory status within a matter of weeks or months depending on the species. During development, they will increase in cell volume and will accumulate cytoplasmic organelles before redistribution at the specific stage to acquire full oocyte competence. Meanwhile, ooplasm, in turn, become the site of RNA and protein storages. After that, the secondary oocyte becomes quiescent again just before ovulation when gene transcription is silenced (Bouniol-Baly *et al.*, 1999).

Robust evidence of competence markers of oocyte quality is based on the follicle size since oocytes from large follicles tend to show better quality. Several studies have demonstrated that gene transcripts vary in oocytes of differing quality. As such, these potential markers of competence are categorized by their functions

such as cell cycle control (i.e., *TAp73*, *p53*, *CCNG1*, *CDK7*, *BUB3* and *RBBP8*), transcription factor (i.e., *HOXA10*, *HOXB7*, *HOXA7*, *POU5F1*, *FIGLA*, *PRDM4* and *SOX15*), metabolism (i.e., *GDHB*, *G6PDH*, *SMARCA5*, *ADH5*, and *STK3*) and molecules involved in gene or protein processing (i.e., *TFAM*, *MAPK1*, *MAPK13*, *FGFR1*, *GSK3A*, *IGF1R*, *JAK1*) (Orozco-Lucero *et al.*, 2014, Labrecque and Sirard, 2014, Labrecque *et al.*, 2013, Romar *et al.*, 2010, Torner *et al.*, 2008, Bermudez *et al.*, 2004, Hamatani *et al.*, 2004, Steuerwald *et al.*, 2007, Virant-Klun *et al.*, 2013). Recent studies indicate that some essential maternal effect genes specifically expressed in oocytes are required for zygote progression beyond the first embryonic cell divisions such as the subcortical maternal complex, a multiprotein complex, (i.e., *MATER (NLRP5)*, *FILIA (OOEP)*, *FLOPED*, *TLE6*, *NLRP7* and *KHDC3L*), has been identified in few mammalian species (i.e., mouse and human) after fertilization during early embryogenesis (Bebbere *et al.*, 2016, Zhu *et al.*, 2015).

The biological age of an individual progress some cellular and molecular regulatory mechanisms within the aged oocytes may deteriorate in terms of cellular homeostasis, altered energy and amino acid metabolism as shown in Chapters 4, accumulation of mitochondrial mutations and alteration of the scavenging reactive oxygen species resulting from aberrant mitochondrial function (Miao *et al.*, 2009a, Wilding *et al.*, 2005) and disruption of chromosomal segregation (Jessberger, 2012, Lister *et al.*, 2010) and the meiotic apparatus (Petronczki *et al.*, 2003, Revenkova *et al.*, 2004, Tsutsumi *et al.*, 2014). As mentioned before, oocytes accumulate regulatory transcripts mainly during the later stages of folliculogenesis and immediately before ovulation it can thus be hypothesised from previous reports that multiple aspects of oocyte transcription are fundamental for oocyte quality maybe, in part, even govern age-diminished oocyte quality. Therefore, these could be candidate molecular markers of oocyte quality.

A growing number of reports revealed changes in the epigenomes accompanying ageing in various cell types in both dividing and non-dividing cells. These epigenetic alterations are manifest at multiple levels such as a global histone

protein reduction, a global decrease in DNA methylation, replacement of canonical histones with histone variants, alteration of histone posttranslational modifications and DNA methylation, and altered noncoding RNA expression (Pal and Tyler, 2016). Likewise, mechanisms of epigenetic changes in ageing oocytes lead to declining oocyte quality are similar to other tissues. Alteration of the DNA methylation pattern was found in mouse oocytes (Hamatani *et al.*, 2004, Mertineit *et al.*, 1998, Pan *et al.*, 2005, Yue *et al.*, 2012), as well as, histone modification (Akiyama *et al.*, 2006, Hamatani *et al.*, 2004, Manosalva and Gonzalez, 2009, Shao *et al.*, 2014, Suo *et al.*, 2010). Finally, post-transcriptional regulation of gene expression by non-coding RNA may result in suppression of miRNA function. However, results remain inconsistent across species, and further research is needed to clarify the impact of ageing on molecular markers of oocyte quality. (da Silveira *et al.*, 2012, Chen *et al.*, 2012, Cui *et al.*, 2013, Diez-Fraile *et al.*, 2014, Suh *et al.*, 2010).

Aims and Objectives

To elucidate the impact of ageing on oocyte gene expression profiles, molecular analysis of ovine oocytes from animals of different reproductive ages was conducted using a focused, customised real-time PCR arrays developed in house. The repertoire of genes included on this bespoke array included i) oocyte-specific genes, ii) members of the subcortical maternal complex (SCMC), iii) imprinted genes, iv) epigenetic regulators, v) genes involved in one-carbon metabolism, and vi) genes involved in growth and metabolism, and vii) housekeeping genes. The expression of these transcripts was surveyed in individual oocytes acquired from both prepubertal and adult sheep. This analysis may generate essential information that can be used to create a better understanding of oogenesis and to elucidate how the developmental potential of oocytes can be affected by this process. Moreover, this study may identify new molecular markers that could be used for the selection of high-quality oocytes that will support improved embryo developmental potential.

5.2. MATERIALS AND METHODS

5.2.1. Oocyte Sample Preparation And RNA Extraction

Oocytes were harvested from prepubertal and adult sheep ovaries obtained from a local abattoir during the non-breeding (July to September) and breeding (October to December) seasons for processing as described previously (see section 2.6). Only GV oocytes enclosed by 3 or more intact layers of unexpanded CCs, medium-brown in colour and exhibiting a finely granulated homogenous ooplasm underwent the immediate denudation after collection (see section 2.7). Oocytes were cultured in groups of 30-35 for 24 hours in IVM medium, followed by denuding, checking for nuclear maturation to MII and storage in RNAGEM® lysis buffer for processing for molecular analysis as detailed in the next section. All instruments and plastic wares used in the following experiments were DNA/RNA-free (Starlab, Ltd.) unless otherwise indicated. Between 20-25 denuded oocytes were collected in each experimental group over 3-4 replicate oocyte harvests. Each individual, denuded oocyte was rinsed/soaked twice in DPBS before it was carefully dispensed into 0.5ml RNase-free PCR tubes containing 2µl of RNAGEM-extraction reagent mastermix (for preparation, see Table 5.1) (RNAGEM Tissue Plus®, RTP0500, Southampton Science Park, Southampton, UK) and immediately snap frozen in liquid nitrogen before being transferred for storage in a -80°C freezer until further analysis (see section 2.12). To minimise the volume transferred, individual oocytes were passed into the PCR tube under a stereomicroscope at room temperature by using a 133µm Swemed stripping pipette. Post-transfer, the pipette was washed in a large volume of DPBS by pipetting up and down several times before the next oocyte was transferred. Fresh RNAGEM-extraction reagent mastermix was prepared before use.

Table 5.1. Composition of RNAGEM-Extraction Reagent Mastermix.
As supplied in RNAGEM™ Tissue PLUS kit according to the manufacturers recommendations

Reagent	Volume
10X Buffer Silver	5µl
RNAGEM enzyme	1µl
RNase-free H ₂ O	44µl
<i>Total</i>	<i>50 µl</i>

5.2.2. DNA Decontamination

For each experimental age group, three equivalent staged oocyte samples were processed simultaneously for cDNA library construction. The samples were kept on ice during preparation for the DNase treatment. The DNase cocktail was prepared by mixing 0.3µl/reaction of 10X DNase buffer with 0.2µl/reaction of 1U/µl DNase I. For the next step sample tubes were incubated in a Veriti™ thermal cycler (Applied Biosystems) at 75°C for 10 minutes to achieve cell lysis. After cooling down to 37°C, the 0.5µl of prepared DNase cocktail was added to each sample tube, and the tube was incubated for 5 minutes to allow completion of the DNA decontamination reaction. Next, reactions were heated up to 75°C for 5 minutes for degradation of DNase activity before cooling down to store at 4°C for the subsequent process (Figure 5.1).

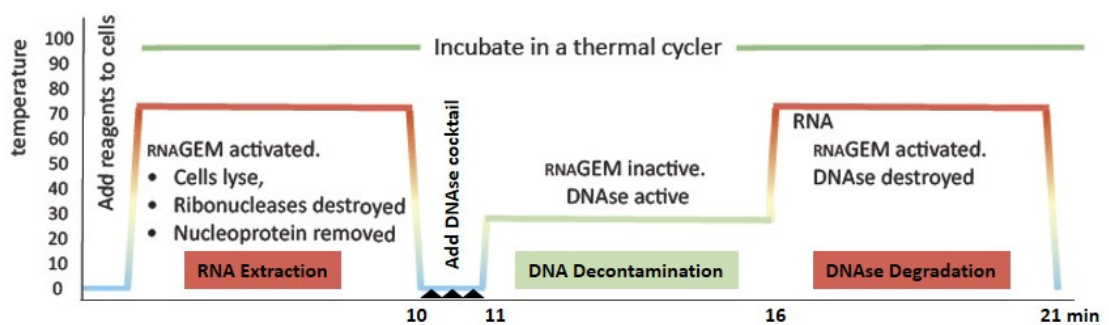
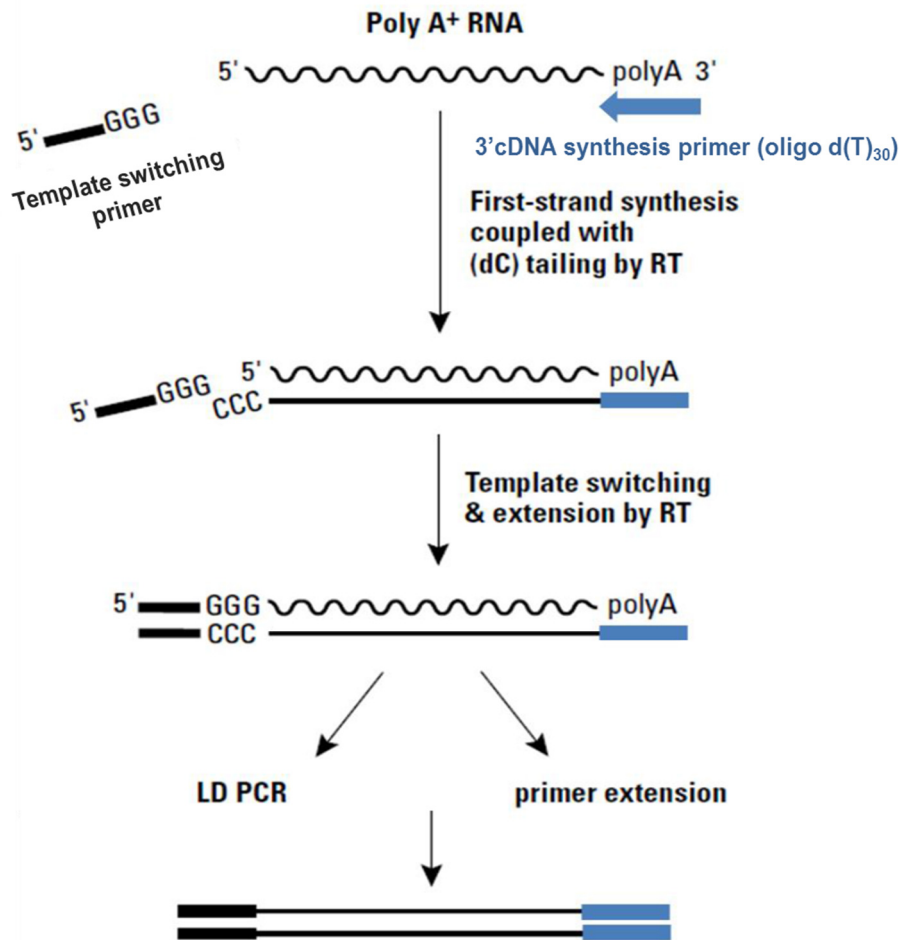


Figure 5.1. A schematic of RNA extraction and DNase treatment workflow (modified from www.Zygem.com, RNAGEM tissue plus® product detail).

5.2.3. cDNA Library Construction

a) mRNA Purification

It has been estimated that an individual sheep oocyte contains 760 pg RNA regardless of the stage of maturity (Olszanska and Borgul, 1993). Another report found that the RNA content may vary according to stages of development and age of oocytes (Bebbere *et al.*, 2014). A greater amount of RNA was experimentally identified within GV staged oocyte as compared to MII-staged oocytes. Moreover, the study also demonstrated that adult oocytes contain a higher concentration of RNA than prepubertal oocytes, 942 vs 538 pg/oocyte respectively in GV, and 285 vs 238 pg/oocyte respectively in MII-staged oocytes (Bebbere *et al.*, 2014).



Primer	Sequence
3'-CDS (Oligo d(T) ₃₀) (cDNA Synthesis)	5'- AAGCAGTGGTATCAACGCAGAGTAC T(30)VN-3'
5'-TSO (Template Switching Oligonucleotides)	5'- AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3' (adapted from SMART™ RACE cDNA Amplification)
Nested Universal Primer (TUP)	5'- AAGCAGTGGTATCAACGCAGAGT-3'

Figure 5.2. Diagrammatic representation of Real Time reaction. An oligo d(T)₃₀-T7 primer which binds to the poly(A) tails of the isolated mRNA and mediates the 1st strand cDNA synthesis reaction by SuperScript II reverse transcriptase, whose terminal transferase activity adds extra C residues at the 3' end of the cDNA. A second primer containing an oligo (G) at its 3' end template switching oligonucleotides binds to the extra C residues on the cDNA, creating an extended template. SuperScript II reverse transcriptase switches to this extended template and cDNA sequences contain the full 5' end of the mRNA plus an additional sequence complementary to the template switching primer that can be used for subsequent LD PCR amplification. The sequences of both primers are provided. Modified from SMART® cDNA Library Construction Kit User Manual (Takara Bio Europe/Clontech).

Due to the SMART™ protocol, which works on very low levels of purified RNA, as low as a few picograms (pg)(Picelli *et al.*, 2014), no RNA purification of single cells from the method described in the previous section was required. Therefore, this method was employed in the following series of experiments.

b) First Stranded DNA Construction (Reverse Transcription)

The procedure for reverse transcription (RT) in brief is summarized in Figure 5.2. The protocol has been modified in-house by Dr J. Lu, from the previously optimised protocol used by our own group (Hemmings, 2007), developed from the published protocol used for the analysis of single neurons (Eberwine *et al.*, 1992, Wang *et al.*, 2000). The primers used for PCR were designed and manufactured by Clontech Laboratories, Inc. and provided in the SMART™ cDNA Library Construction Kit (Switching Mechanism at 5' End of RNA Template) purchased from Clontech (Takara Bio Europe - Clontech, Saint-Germain-en Laye, France).

This protocol allows the enrichment of high-quality, full length cDNA transcripts with complete 5' ends whereas truncated cDNAs or premature termination of reverse transcription reaction, genomic DNA, RNA without poly (A) cannot be converted to dsDNA and will, therefore, be eliminated. The 3 SMART™ primers were employed for cDNA synthesis in this study, primer sequences are provided in Figure 5.2, designed according to Wang *et al.*, (2000) and were produced by Life Technologies Ltd.

The master mix of the RT reaction for the first stranded cDNA was prepared by adding the reagents listed in Table 5.2 and mixed in a nuclease free microcentrifuge tube. A 13µl aliquot of this completed reaction mix was added to each DNA-decontaminated oocyte sample tube containing approximately 3µl of total oocyte RNA (sample volume ~2.5µl plus 0.5µl DNase buffer) and kept on ice. Samples were loaded in a Veriti™ thermal cycler for incubation at 42°C temperature for 90 min. Subsequently, samples underwent two additional incubations for 2 min at 50°C and 2 min at 42°C for ten cycles. At the last step of the reaction, 16µl of the 1st strand cDNA synthesised reactions were incubated at

70°C for 15 min before cooling down, and non-amplified cDNA was kept standing at 4°C for further processing with the cDNA amplification protocol.

Table 5.2. Composition of the first strand cDNA construction reaction

Reagent	Concentration	Vol per reaction (µl)
5X First strand buffer	5X	3.2
Betaine	5 M	3.0
MgCl ₂	50 mM	1.8
Superscript II Reverse Transcriptase (Invitrogen, CA / Life Sciences)	200 U/µL	0.75
RNase-out* (<i>RNase inhibitor</i>)	40 U/µL	0.4
DTT	0.1 M	0.75
TSO (5'primer)*	10 mM	1.0
3CDS (3'primer)*	10 mM	1.0
dNTP*	10 mM	1.0
DNA-decontaminated RNA template	-	3.0
Total		15.9

c) Long Distance PCR For cDNA Amplification

The long distance PCR for cDNA amplification has been adapted from the SMART™ RACE cDNA Amplification kit and the previously published protocol (Picelli *et al.*, 2014) by our group for use in single oocytes. Because the limited quantity of source material is approximately 0.76ng in an unidentified stage oocyte (Olszanska and Borgul, 1993), the first strand DNA product synthesised from total RNA in single oocyte lysates subsequently needed to be amplified to construct the full length cDNA by using a Long Distance PCR (LD PCR)-based method. The reagents as shown in Table 5.3 were prepared to make an LD PCR master mix. Then 34µl of the reaction mix was added to each sample tube (the same first strand cDNA tubes from the previous step), producing a final volume of 50µl. The LD PCR was performed in a Veriti™ thermal cycler and comprised of 2 stages;

- (i) a first stage of denaturing step: one cycle at 95°C for 1 min
- (ii) a second stage consisting of 25 cycles of 3 steps;
 - 95°C for 15 sec melting step
 - 60°C for 1 min annealing step
 - 72 °C for 5 min elongation step

The LD PCR product was held at 4°C after cDNA amplification completed. And the cDNA libraries were kept at -20°C until later use.

Table 5.3. Preparation for Master Mix Reaction of Long Distance PCR for cDNA Amplification

Reagents	Concentration	Vol per reaction (µl)
DNA/RNA-free H ₂ O	-	25.5
10X Advantage 2 PCR Buffer (Takara Bio Europe/Clontech)	10X	5.0
50X Advantage 2 Polymerase Mix (Takara Bio Europe/Clontech)	50X	1.0
TaKara LA Taq® Hot-Start (Takara Bio Europe/Clontech)	5U/µL	0.5
dNTPs	10 mM	1.0
Nested universal primer (TUP)	10 mM	1.0
1 st strand DNA template	-	16.0
Total		50

d) Agarose Gel Electrophoresis For Visualisation Of PCR Products

A 2% (w/v) agarose gel was prepared by mixing 2 grams of molecular grade agarose powder (Bioline Reagents Ltd., London, UK) with 100ml of 1X tris-borate ethylenediaminetetraacetic acid (1X TBE) buffer (Appendix II) in a glass beaker then heated up in a 750 V microwave oven up to 2 min until the agarose dissolved and formed a homogeneous turbid, clear liquid. 10µl of the Gel Red 10,000X in H₂O stock was immediately diluted and mixed into the agarose gel solution; the gel was then poured into a gel clamp (Bio-Rad laboratories Ltd, Hertfordshire, UK) containing a 10, 15 or 20 well comb to allow regular spacing. The gel was left to cool down and set at room temperature for 40 min before placed in a Sub Cell GT Tank with Powerpac 300 (Bio-Rad) and covered with 1X TBE. The comb was removed to make reveal the wells. Test samples were loaded into each well by

mixing 5µl of each sample with 1µl of 6X Gel loading dye blue (New England Biolabs, Inc., Fisher scientific, UK). 10µl of a Quick-Load® 100bp DNA ladder (New England Bio Labs Ltd., Fisher scientific, UK) was loaded into 1-2 wells of each gel as a marker. The subsequent samples were loaded separately into consecutive wells followed by the positive and negative controls. Agarose gel electrophoresis was performed immediately after the verification PCRs. The TBE electrophoresis was performed at 100 V for 60 min at room temperature. The gel was visualised in a Molecular Imager® Gel Doc™ XR+ System (Bio-Rad Laboratories Ltd.), and images were acquired using the Image Lab™ software (Bio-Rad Laboratories Ltd.).

5.2.4. Verification Of cDNA Library Product (cDNA Smear)

The successful construction of each cDNA library was verified in 2 steps. First, the cDNA libraries were visualized when smeared on 2% weight per volume (w/v) agarose gel electrophoresis as described in the previous published protocol (Kang *et al.*, 2015, Yue *et al.*, 2009) (Figure 5.3.). The next step was performed by using specific housekeeping primers, for which the protocol has previously been validated by Huntriss and colleagues (Huntriss *et al.*, 2002) for work on oocyte specific genes. The housekeeping primers and oocytes-specific target genes used in this study have been designed and optimised by our own group (Cotterill, 2008, Liperis, 2013) (Figure 5.4.).

Three housekeeping genes including *GAPDH*, *H2A* and *YWHAZ*, were used to verify the cDNA libraries in this study. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, the most common used reference housekeeper gene, was included as the first housekeeper because it is relatively stable and highly expressed in most tissues and cells (Barber *et al.*, 2005). Moreover, it is involved in almost fundamental cellular processes with both a glyceraldehyde-3-phosphate dehydrogenase activity associated with glycolysis and a nitrosylate activity involved with nuclear functions (Barber *et al.*, 2005, Ercolani *et al.*, 1988, Jeong *et al.*, 2006). Another housekeeper was histone 2A (*H2A*). This gene is also commonly used as a reference gene for several tissues. It encodes a conserved nuclear protein that together with other histones forms the eukaryotic

nucleosome core and has high expression in oocytes regulating chromatin formation (Kleinschmidt *et al.*, 1985, Thatcher and Gorovsky, 1994). Finally, the third housekeeping gene was the *YWHAZ* gene, (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide), which is highly conserved across species. And it is implicated in the protection of cells from apoptosis through binding to the pro-apoptotic protein (Mack and Munger, 2012, Mack *et al.*, 2012) as well as encoding an adaptor protein involved in various signal transduction pathways such as metabolism, transcription, apoptosis, protein transport, and cell cycle regulation. This gene has been shown to have a stable expression across folliculogenesis and early embryo development (Mamo *et al.*, 2008, Tommerup and Leffers, 1996).

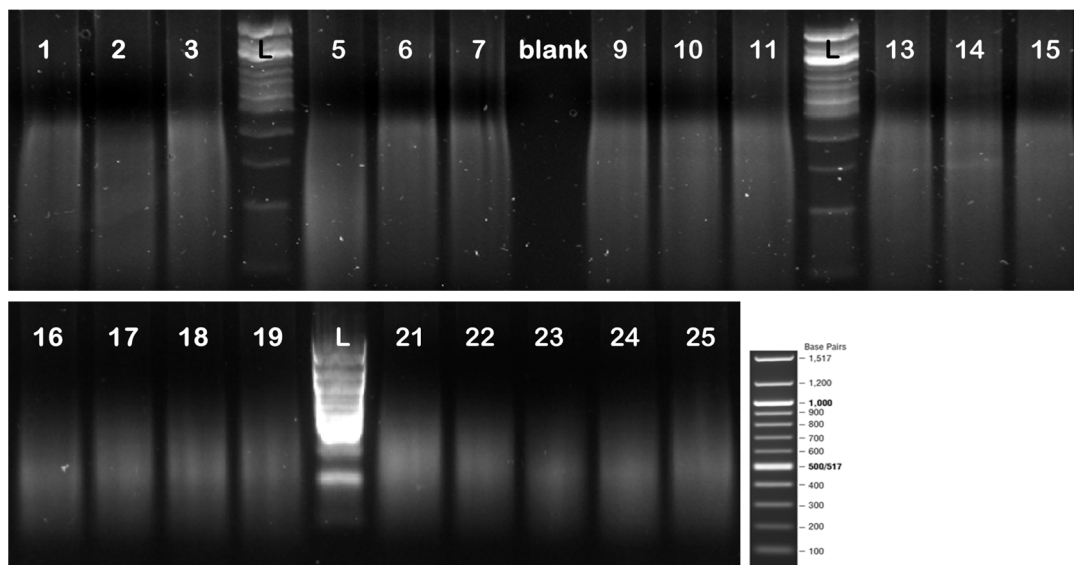


Figure 5.3. Representative examples of cDNA libraries quality check for individual oocyte cDNAs using 2% (w/v) agarose gel electrophoresis. The uniform smear of cDNA samples indicated the good quality of the cDNA libraries. (*Top*) The gel was run at 110 volts for 60 min; lanes 1-3, lanes 5-7, lanes 9-11 and lanes 13-15 (*from left to right*) indicate cDNA libraries from an individual oocyte of: Lanes 1-3: prepubertal GV; Lanes 5-7: adult GV; Lanes 9-11 and Lanes 13-15: adult MII oocytes, while lane 4 and 12 contain 1 kb molecular weight markers (New England Biolabs Ltd., Herts, UK). (*Bottom*) The gel was run at 100 volts for 60 min; lane 16-19 and lane 21-25 (*from left to right*) indicate cDNA libraries from an individual oocyte of: Lanes 16-17: prepubertal GV; Lanes 18-19: prepubertal MII; Lanes 21-22: adult GV; Lanes 23-25:

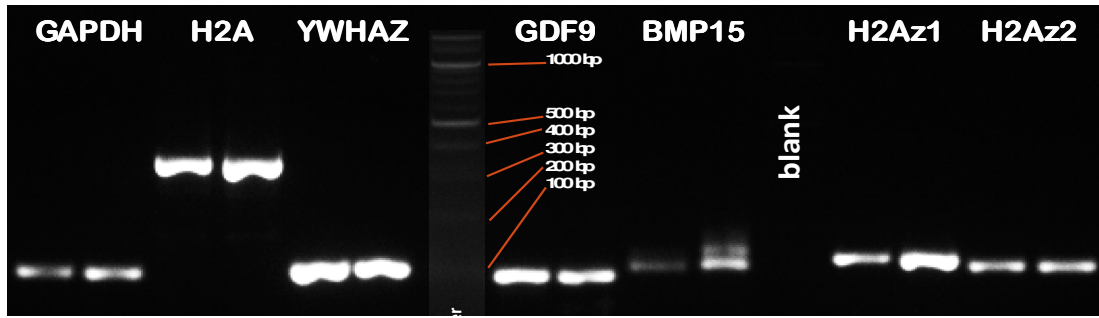


Figure 5.4. Agarose gel image of product size verification following real-time qPCR analysis. The amplification product of an individual oocyte sample was performed by 2% (w/v) agarose gel electrophoresis. Housekeeping genes include *GAPDH*, *YWHAZ*, and *H2A* (derived from 3 primers; *H2A*, *H2Az1* and *H2Az2*). The specific genes used to confirm oocyte cDNA sample specificity were *BMP15* and *GDF9*.

In addition to the housekeeping genes, the newly generated cDNA libraries were further tested using primers for the *GDF9* and *BMP15* genes that are specifically expressed in oocytes, as another quality control. *GDF9* is essential for all stages of follicular development and maturation (Otsuka *et al.*, 2011) as well as in supporting metabolic cascades such as glycolysis and sterol biosynthesis (Sugiura and Eppig, 2005). In addition, it regulates CC metabolism via augmentation of intrinsic prostaglandin-E2/EP2 receptor signalling pathway leading to the suppression of FSH-induced steroidogenesis while promoting CC progesterone production (Elvin *et al.*, 1999, Sugiura and Eppig, 2005). Moreover, *GDF9* enhances cumulus expansion by promoting hyaluronan synthase 2 (*HAS2*) and cyclooxygenase 2 (*PTGS2*) gene expression when in the presence of FSH (Elvin *et al.*, 1999).

BMP15 is another oocyte-specific gene that is important from the early gonadotrophin-independent phases and prevention of GC apoptosis (Persani and Bonomi, 2014). All primers used in the study have been confirmed previously to detect expression of their respective target in ovine oocytes (Cotterill, 2008, Liperis, 2013). Preparation of primers was performed by dissolving them in nuclease-free water to create a 100mM solution and then vortexing them. Dilutions of stock primers were performed by dilution with nuclease free water to a concentration of 10mM before use. The primer sequences of the housekeepers and oocyte specific genes used for library verification are shown in

Table 5.4. Primer sequences of housekeeping genes and oocyte specific genes

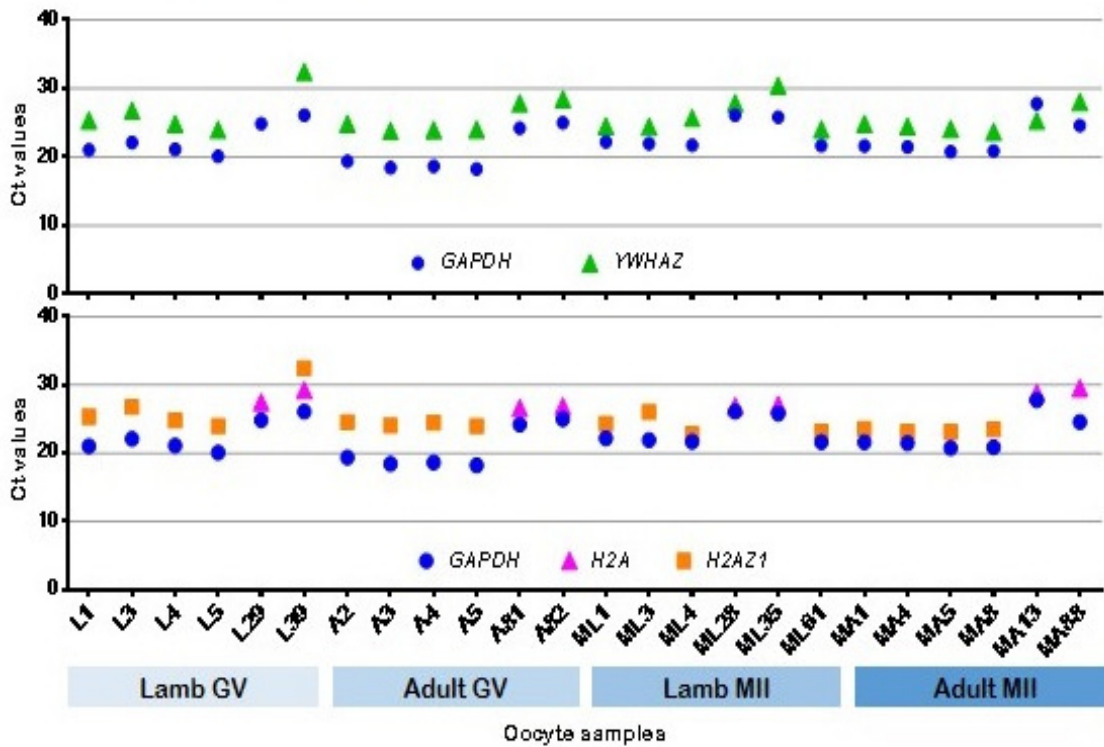
Gene		Primer Sequence	Size (bp)	Temp (°C)	Reference
Housekeeping genes	<i>GAPDH</i>	F: 5' TGATTCCACCCATGGCAAGT 3' R: 5' CGCTCCTGGAAGATGGTGAT 3'	93	60	AF030943 (ovine)
	<i>H2A</i>	F: 5' AGGTATGTCAGGCCGTGGTA 3' R: 5' AGCACAGCCTGGATGTTAGG 3'	300	60	AY074805
	<i>YWHAZ</i>	F: 5' TGTAGGAGCCCGTAGGTCATCT 3' R: 5' TTCTCTCTGTATTCTCGAGCCATCT 3'	102	60	AY970970
	<i>oH2AZ1</i>	F: 5' ACAGCTGTCCAGTGTGGTG 3' R: 5' GCAGAAATTTGGTTGGTTGG 3'	124	60	AY074805 (ovine)
	<i>oH2AZ2</i>	F: 5' AAGCGTATTACCCCTCGTCA 3' R: 5' TGGATGTGTGGAATGACACC 3'	106	60	AY074805 (ovine)
Oocyte specific	<i>GDF9</i>	F: 5' AGTAAGCTGGAACCGGAATCG 3' R: 5' GGTGGCCGACAAGAGAAGTCT 3'	95	60	AF078545 (ovine)
	<i>BMP15</i>	F: 5' GGCAAAGCTCTGGAATCACA 3' R: 5' TGCCATGCCACCAGAACTC 3'	102	60	AF236079 (ovine)
	<i>GTSF1</i>	F: 5' CTCTGGACCCTGAAAAGCTG 3' R: 5' GTTTGTTCGACATCAGGA 3'	120	60	Ensembl:ENSOAR- G00000016162

Table 5.4. The specificity of the primers' validation was confirmed by the gel electrophoresis and dissociation curve as shown in Figure 5.4.

A total of 20 to 25 individual oocyte samples collected from either GV- or MII-staged oocytes from both prepubertal and adult animals were finally processed and constructed into 45 cDNA libraries of individual oocytes. Following this quality testing, 4 out of the 45 libraries generated were excluded from further analysis due to the absence of a cDNA smear on the agarose gel. Hence, only ten cDNA libraries from each group including prepubertal GV-, prepubertal MII-, and adult GV-derived oocytes, as well as 11 cDNA libraries from adult MII-derived oocytes have been recruited for further steps. However, 4 out of 41 libraries including 2 and 1 GV-derived samples from prepubertal and adult groups, respectively, and 1 MII-derived cDNA library from the adult group that failed to demonstrate the expression of at least two housekeeping genes and at least one oocyte specific genes were discarded. Ultimately, 37 out of 41 newly established cDNA libraries passed the minimal requirement tests for gene expression

analysis. Of these, six individual cDNA samples originated the full-length clones by using the total RNA sequences from 4 different groups were chosen for transcriptional studies (Figure 5.5. A and B).

A: Housekeepers



B: Oocyte specific genes

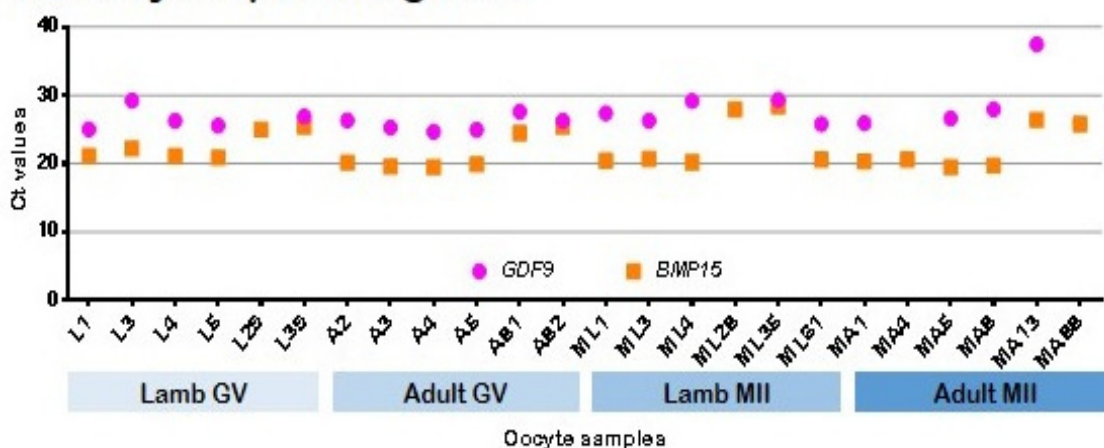


Figure 5.5. Demonstrate the consistent of levels expression of the 3 housekeeping genes (A) and the 2 oocyte specific genes (B) used to confirm cDNA quality across 24 selected cDNA samples. Data are presented as mean Ct values \pm SEM of the triplicate analysis per sample. Note that due to the very small SEM, error bars cannot be visualized on the graph.

5.2.5. Bovine Primer Testing

Given the relatively highly conserved DNA sequences in bovine and ovine cells, as well as the lack of annotation of the ovine genome, this study used the primers originally designed to work with bovine oocytes and embryos to test the cDNA libraries from ovine oocytes. The customized primers (Order reference: SIPR 662036; Real Time Primers, LLC, Elkins Park, PA, USA) have been previously validated in those tissues in-house (unpublished data). Primer sequences are demonstrated in Table 5.5. Initially, to test whether the bovine PCR primer sequences on the custom array were capable of amplifying the respective gene transcripts in ovine oocytes, primer testing was performed in 3 steps as detailed below. The method for PCR testing is clarified in section 5.2.6. Overall, many genes were successfully amplified despite the species difference between primers and cDNA. Experiments were considered successful for a given gene if the cycle threshold (Ct) value generated from PCR amplification of the ovine oocyte cDNA was between 15 and 35, and if a clean band was generated on subsequent analysis by agarose gel electrophoresis. Successful gene amplification validated primers, which were selected to facilitate experiments comparing gene expression across different age groups and maturational stages of ovine oocytes in the subsequent analyses.

a) Testing Primers By Using cDNA Derived From Individual Oocytes

Two cDNA libraries were selected to be representative samples including 1 sample derived from prepubertal GV oocytes (L9) and another sample from prepubertal MII oocytes (ML39).

b) Testing Primers By Using cDNA Derived From 10-Oocytes Pools

Ten oocytes were used to construct each pooled cDNA library. One pool was derived from 10 GV-staged (AG104) adult animals whereas another was formed using 10 MII-staged oocytes (AM104) from adult animals.

Table 5.5. A customized real-time PCR array primer sequences originally designed to work with bovine oocytes and embryos. (Order reference: SIPR 662036; Real Time Primers, LLC, Elkins Park, PA, USA).

Gene	Description	Forward sequences	Reverse sequences
<i>ACTB</i>	Actin, beta	CTCTTCCAGCCTTCCTTCCCT	GGGCAGTGATCTCTTTCTGCG
<i>AHCY</i>	adenosylhomocysteinease	TGACAGGGGGAATTGAGAAG	GCCTGGATGGTAAAGTGCAT
<i>APEG3</i>	antisense transcript gene of PEG3	not available	not available
<i>ASCL2</i>	achaete-scute family bHLH transcription factor 2	ACCCAAGGCTAGTGTGCAAG	CATAAAGCCCTCTCCCCCTTC
<i>ATF2</i>	activating transcription factor 2	TCCTCAACCAGTGCAGTCAG	TGGAGTTGTGTGAGCTGGAG
<i>AURKB</i>	AURKB	GGAGGGAGGTCCTAGTCTG	TCTCAGTGCTAAGGGGTGCT
<i>BHMT</i>	betaine--homocysteine S-methyltransferase	GCTCATGAAAAGAAGGCTTGG	AGGGTTCAAAATCCACAGCAC
<i>CDX2</i>	caudal type homeobox 2	AAGACAAATACCGGGTCTGTG	CTGCGGTTCTGAAACCAAAAT
<i>CFL1</i>	cofilin 1	TGGGGTCATCAAAGTGTTC	TTGTCTGGCAGCATCTTGAC
<i>DHFR</i>	dihydrofolate reductase	AGAACATGGGTATCGGCAAG	ATGAGCTCCCTTTGGAGGTT
<i>DNMT1</i>	DNA (cytosine-5-)-methyltransferase 1	AGTGGGGACTGTGTTTCTG	TGCTGTGGATGTACGAGAGC
<i>DNMT3A</i>	DNA (cytosine-5-)-methyltransferase 3 alpha	AGCACAAACGGAGAAGCCTAA	CAGCAGATGGTGCAGTAGGA
<i>DNMT3B</i>	DNA (cytosine-5-)-methyltransferase 3 beta	TCAGGATGGGAAGGAGTTTG	CTGCTGGAATCTCGGAGAAC
<i>DNMT3L</i>	DNA (cytosine-5-)-methyltransferase 3-like	ATGAGCAACTGGGTCTGCTT	GGCTCTCTCTTCCACACAGG
<i>EHMT2</i>	euchromatic histone-lysine N-methyltransferase 2	ACCTCAGATGTGGCCAAAAG	GTTCAGCCAGAGCTTCAACC
<i>ELP3</i>	elongator acetyltransferase complex subunit 3	AGGGCTCTATGAGCTGTGGA	TGAGCTGACTAACGGCATTG
<i>GAPDH</i>	Glyceraldehyde phosphate dehydrogenase	GGGTCATCATCTTGCACCT	GGTCATAAGTCCCCTCCACGA
<i>GNAS</i>	GNAS complex locus	GGGTGCTGGAGAATCTGGTA	TGGTTTCAATGGCCTCTTTTC
<i>GPT2</i>	glutamic pyruvate transaminase	ACTGTAACCCCAAGGTGCTG	CTCAGGCCCATCTCATAAAA
<i>GPX1</i>	glutathione peroxidase	TTCTTGAGCATTCACTGTGC	GGAAGGTGCCATCATTCTGT
<i>GSK3A</i>	glycogen synthase kinase 3 alpha	GAGCGACTGGACTTCGTTTC	AAGAGTCCGGGGCAATAAGT
<i>GSK3B</i>	glycogen synthase kinase 3 beta	CAGTGGTGTGGATCAGTTGG	CAGACGGCTACAAAAGTGCAA
<i>H19</i>	H19, imprinted maternally expressed transcript	GACACCCAGAACCCCTCAAGA	CCTTCCAGAGCTGATTCCTG

Table 5.5. A customized real-time PCR array primer sequences originally designed to work with bovine oocytes and embryos. (Order reference: SIPR 662036; Real Time Primers, LLC, Elkins Park, PA, USA).

Gene	Description	Forward sequences	Reverse sequences
<i>H2A</i>	Histone H2A	ATGTTGGACGTGGAAAAGG	ATCACAGCGGCCAAAATAAAC
<i>HAT1</i>	histone acetyltransferase 1	GCAACATGCTAGACGGGTTT	GCTGTTTCATGTTGCATGCTT
<i>IGF1R</i>	insulin-like growth factor 1 receptor	CCAAAACCGAAGCTGAGAAG	TCCGGGTCTGTGATGTTGTA
<i>IGF2</i>	insulin-like growth factor 2	GCCCTGCTGGAGACTTACTG	GGTGACTCTTGGCCTCTCTG
<i>IGF2BP1</i>	insulin-like growth factor 2 mRNA binding protein 1	AAGTGGAGCTGCAAGGAAAA	CGTCTCACTCTCCGTGTTCA
<i>IGF2BP2</i>	insulin-like growth factor 2 mRNA binding protein 2	CGCAAGATCAGGGAAATTGT	TTCCTCAGGATGGTCTTTGG
<i>IGF2BP3</i>	insulin-like growth factor 2 mRNA binding protein 3	CAGTTCAAAGGCTCAGGGAAG	ACGGGGAACAACAACACTTCTG
<i>IGF2R</i>	insulin-like growth factor 2	GTCTGCAGATCAGTCTCTCA	TCGTTCTGGAGCTGAAAAGGT
<i>IGF2R</i>	insulin-like growth factor 2 receptor	GTCTGCAGATCAGTCTCTCA	TCGTTCTGGAGCTGAAAAGGT
<i>INSR</i>	insulin receptor	TTCTGGGGGAAGAATGACTG	AACTGGGATGCGTGTCTACC
<i>KAT5</i>	lysine acetyltransferase 5	TCGACTCCAAGTGTCTGCAC	CTTCTGGAGTGGTCTCAGC
<i>KDM1B</i>	lysine demethylase 1B	AGTGTCAAGAGTGCCTCAACC	TCGTTGGGTTGGTAGAAAAGG
<i>KHDC3L</i>	KH domain containing 3-like, subcortical maternal complex member	GACTACAGCATGGCCTCTCC	CCTCCAGATGAACTGCCTTC
<i>MAT1A</i>	methionine adenosyltransferase I, alpha	AGACGAGCCTGTTGGCTTTA	GCCCCTCTCCTACAGATTCC
<i>MAT2B</i>	methionine adenosyltransferase II, beta	TGCAGTTCAGCAACAAGTCC	ATTGCACAGGCCATTTCATA
<i>MEG3</i>	maternally expressed 3	ACCTGTCTCACGCTTCTCGT	TCCTGAGAGCTGGTGGAGTT
<i>MEG9</i>	maternally expressed 9	GCCTGCCACACTTTATGGTT	CAGAGACAGCTTTTGCCAACA
<i>MEST</i>	mesoderm specific transcript	AAGGGACTGGGCATCTTCTA	TGAAGCCAAAAGCCTAGGAAA
<i>MIMT1</i>	MER1 repeat containing imprinted transcript 1	GCTCTTAAAAGGGCATGTG	CCATCATCTCTTCTGGAGAA
<i>MSK2[<i>RPS6K44</i>]</i>	ribosomal protein S6 kinase, 90kDa, polypeptide 4	CGAAAATGTTACCCACCCTCT	GACAATGTGACCCTCGGAGT
<i>MTHFR</i>	methylenetetrahydrofolate reductase	GAGACCTCGTCCATGGTGAT	TCCTTCCCTCTTCCCATT
<i>MTR</i>	5-methyltetrahydrofolate-homocysteine methyltransferase	CTTCCCAATACCTTCGGTGA	AAAAACAGTGGCAGGTGGAAC
<i>NAP1L5</i>	nucleosome assembly protein 1-like 5	TTCCAGGCTCTGGAGAAAAA	CTCTGCTGCAGGCTCTTCTT

Table 5.5. A customized real-time PCR array primer sequences originally designed to work with bovine oocytes and embryos. (Order reference: SIPR 662036; Real Time Primers, LLC, Elkins Park, PA, USA).

Gene	Description	Forward sequences	Reverse sequences
<i>NLRP2</i>	NLR family, pyrin domain containing 2	GTGCGAGGCTTTGAAGAAAC	TTACTCCACTGGACCCCAAG
<i>NLRP5</i>	NLR family, pyrin domain containing 5	CGGAGGCTCCTACTGTTCTG	CCTGGTCTCTGAAGGTGAGC
<i>NNAT</i>	neuronatin	CGACAACCTCTGTGCCTGTGT	AGATGGGATTCGTTTTCCGTG
<i>NR3C1</i>	nuclear receptor subfamily 3, group C, member 1	CCATTTCTGTTACACGGTGTG	CAAAATCCCAAGGAAGTCA
<i>OOEP1</i>	oocyte expressed protein 1	TTGACGCTGGAAACCTAGTC	TCTCACGCTCCTGACAAAC
<i>PADI6</i>	peptidyl arginine deiminase, type VI	TCGGAGACTTCTGCTCCTGT	CTGGAGACGCATAGGGAGAG
<i>PEG10</i>	paternally expressed 10	ATTGTTTCATTGGCTGGAAGG	GCTTTGGGTTGCTTTCTGAG
<i>PEG3</i>	paternally expressed 3	CTGTACGTGGATTGGCCTTT	TAGGCACGGGTGATCTAGTG
<i>PGK1</i>	Phosphoglycerate kinase 1	CTGCTGTTCCAAGCATCAAA	GCACAAGCCTTCTCCACTTC
<i>PHLDA2</i>	pleckstrin homology-like domain, family A, member 2	CCAGGTGTGGAAGAAGAAGC	GACGGTTCACAGTAGCTCTC
<i>PLAGL1</i>	pleiomorphic adenoma gene-like 1	GGACCCCAAGCTTAGAAAAGG	TTTGGAGGTGGTTCTTCAGG
<i>POU5F1</i>	POU class 5 homeobox 1	GTTTTGAGGCTTTGCAGCTC	CTCCAGGTTGCCCTCTCACTC
<i>PRDX2</i>	peroxiredoxin 2	GGTCCAGGCTTTCCAGTACA	TGGAGTCTGAAGGAGCAGGT
<i>PRMT5</i>	protein arginine methyltransferase 5	AAGCAGGGGTTTGATTTCTCT	TATGCCCCAAAAATTCAGCTC
<i>RPLP0</i>	ribosomal protein, large, P0	CAACCCCTGAAGTGTCTTGACA	GCCTTGACCTTTTTCAGCAAG
<i>SETD7</i>	SET domain containing lysine methyltransferase 7	GCCCCGTGATGTTCTACACT	TGGTGGTAACGGAAAAAGGAG
<i>SETDB1</i>	SET domain bifurcated 1	GCCCCGTGATGTTCTACACT	TGGTGGTAACGGAAAAAGGAG
<i>SHMT1</i>	serine hydroxymethyltransferase 1	ACTGGGACCTTTACCCCATC	TTGGGATTTCTGGTTGAAAAGC
<i>SHMT2</i>	serine hydroxymethyltransferase 2 (mitochondrial)	ATTGTCACCACGACCCTCA	GGATGGAAACACAGCAAAGT
<i>SLC16A1</i>	solute carrier family 16, member 1	TTAATGCCACCACCAGTGAA	GAAGGAAGCAGCAATCAAGC
<i>SLC16A3</i>	solute carrier family 16, member 3	GGTCAGGGTCTTCTTCAAGG	AAGCCAGTAAGGACCCCTGT
<i>SLC16A4</i>	solute carrier family 16, member 4	GAAGGCACCTCAGAGCAAAC	CAAGAAAAGCAGAGCCCAAAC
<i>SLC16A7</i>	solute carrier family 16, member 7	AATTAATCCGACCCCGAATC	GTCCACAGCACTGGAAAAAT

Table 5.5. A customized real-time PCR array primer sequences originally designed to work with bovine oocytes and embryos. (Order reference: SIPR 662036; Real Time Primers, LLC, Elkins Park, PA, USA).

Gene	Description	Forward sequences	Reverse sequences
<i>SLC2A1</i>	solute carrier family 2, member 1	CCCCAGAAGGTGATTGAAGA	GCCGAAAACGGTTAACAAAAA
<i>SLC2A12</i>	solute carrier family 2, member 12	TTGGCCTTGTATTCCCTTG	CGGGTCCTCATGTGTCTTT
<i>SLC2A2</i>	solute carrier family 2, member 2	TTGGTGTGATCAATGCACCT	AGACAGGACCAGAACATGG
<i>SLC2A3</i>	solute carrier family 2, member 3	ACTTTGGAAGAGCGGTCAGA	TGGCCAACAGGTTGACAATA
<i>SLC2A4</i>	solute carrier family 2, member 4	ACCTTATGGCCACTCCCTCT	CTCAGCCAACACCCTCAGACA
<i>SLC2A6</i>	solute carrier family 2, member 6	CTGGGGCTATACGTGCATTT	CCATGATGAAGAGCATGGTG
<i>SLC2A8</i>	solute carrier family 2, member 8	CCTGTGATTTGGGAGAAGGA	ATGACAGGAGAACCCACAGG
<i>SLC2A9</i>	solute carrier family 2, member 9	TGCTGTCTGCAGGTCCTTG	GTTACCACGGGAGAGGTTGT
<i>SLC46A1</i>	solute carrier family 46 (folate transporter), member 1	GGCAAAACCAAGGTCAGTTGT	GCTGAGTACCCCTTCACAG
<i>SNRPN</i>	small nuclear ribonucleoprotein polypeptide N	GTTCCAGCTGGTGTTCCTCAAT	TGGAGGAGCCATAAATTCCTG
<i>TET1</i>	tet methylcytosine dioxygenase 1	CCTCTCCAACCAACCAGTGT	GAATTTGTGTGGGTCTGGT
<i>TET2</i>	tet methylcytosine dioxygenase 2	GTAAGGCCGGTGACAGTGAT	TTTCTCGCCAGAGGTTCTGT
<i>TET3</i>	tet methylcytosine dioxygenase 3	GAAAGGCCAGAAGCACTCAC	TGAAGGGAAGGTTGTCTGTC
<i>TLE6</i>	transducin like enhancer of split 6	TGACCTCTTGGGGTCATCTC	GGAAGTTTTCTGCCTGCTTG
<i>TRDMT1</i>	tRNA aspartic acid methyltransferase 1	GGTGGCTGCTGTTGATGTAA	GACAGGGTGGGCTCATTAAA
<i>TRIM28</i>	tripartite motif containing 28	ACTCCACCTTCTCCCCAGAT	TCCGTCAGCTTGTGAACTG
<i>TSSC4</i>	tumor suppressing subtransferable candidate 4	CGACAGGAAGAGGGTATCCA	AAACCCACTGTCTCCACCAG
<i>TUBB</i>	Tubulin beta class	ACCTACAACCTGGACCCGATC	ACATCCAGGACCGAGTCAAC
<i>UHRF1</i>	ubiquitin like with PHD and ring finger domains 1	ACACACTCCTGGTGACTCC	TGACCTTCAGCGATCTTCT
<i>USP29</i>	ubiquitin specific peptidase 29	ACAGAGCTGGGCCTCTACAA	CATCTGGACCACCTCCGTTCT
<i>XIST</i>	X (inactive)-specific transcript	TTGAATGGGATTTGGGGTAA	AGTAGTGTGGCCTTGGGATG
<i>YWHAZ</i>	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	AGACGGAAAGGTGCTGAGAAA	CCTCAGCCAAAGTAGCGGTAG

c) Testing Primers By Using Libraries Constructed By Pooled Aliquots From 10 cDNA Libraries

A new pooled-library, a 10 μ l aliquot of the equivalent stage and age groups of cDNA libraries, was pooled together. As a result, four constructed pools representative of prepubertal GV (10LGV) and MII (10LMII), and also adult GV (10AGV) and MII groups (10AMII) were tested.

5.2.6. Customized Array Using Bovine Primers

The validated primers from the previous section were used to establish a new customised array PCR to compare candidate gene targets between different age groups and maturational stages of ovine oocytes. The bovine primers that were identified as being able to amplify the respective gene transcript from ovine cDNA libraries as deduced in the experiments using cDNA from 10 pooled sheep were selected for further analysis. Each new double array testing was prepared in 96-well PCR plates (Figure 5.6) for simultaneous analysis of 2 established cDNA samples, one from each age group. Results are shown in the following section 5.3.2.

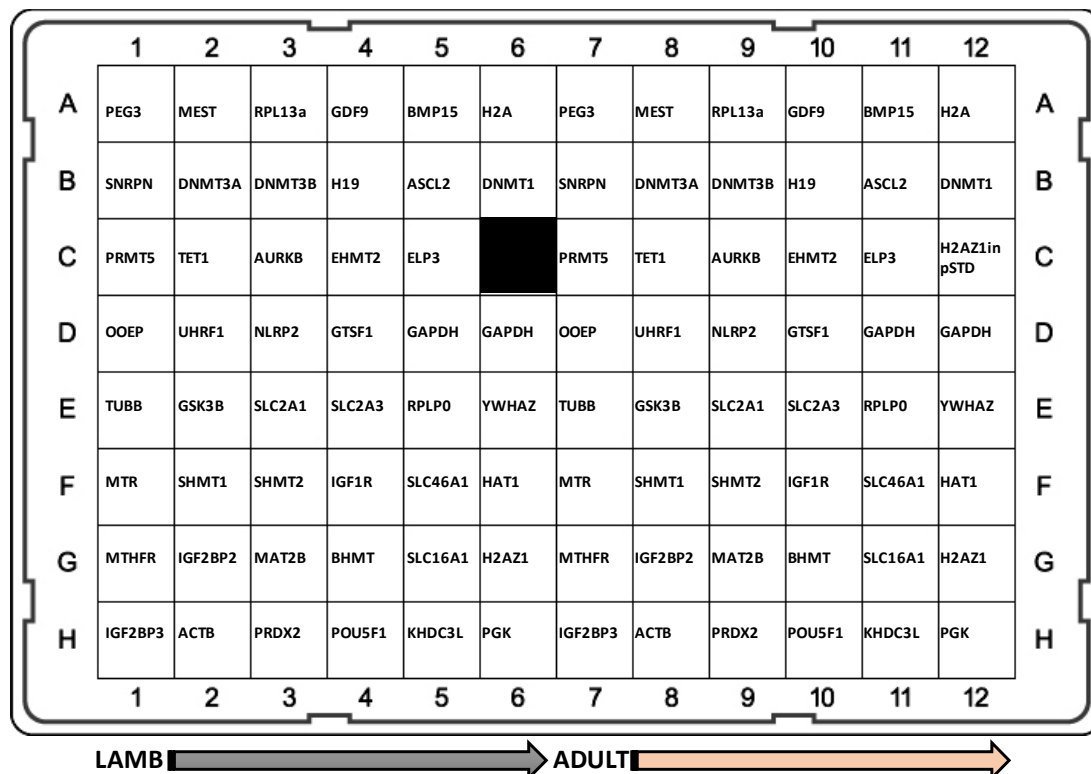


Figure 5.6. Schematic a newly customized array plate using for 2 samples testing simultaneously. Target genes in column 1-6 for a lamb cDNA sample (white background) are equivalent to those in column 7-12 for an adult cDNA samples (pink background) Well C6 (black background) is blank while C12-well is used for loading the control sample to see interassay variation control between plate. Regarding the experimental design, each plate run 2 cDNA samples derived from oocytes at the same maturational stage.

5.2.7. Gene Expression Analysis by Real-Time PCR

Finally, the analysis of gene expression in individual oocytes was conducted on the amplified cDNA libraries from 6 selected high quality oocyte samples in each group, using qPCR relative quantification. The customised array analysis has been prepared following the validated primers from section 5.2.6. Each array consisted of 45 genes, which includes seven housekeeping (*TUBB*, *ACTB*, *RPL13a*, *GAPDH*, *RPLP0*, *H2A*, and *YWHAZ*) and 38 functional genes (see Table 5.6 and 5.7.).

Table 5.6. Function of candidate housekeeping (HK) genes for normalization

	Gene	Function	Size (bp)	Temp (°C)	Reference
Selective HKs	<i>GAPDH</i>	Carbohydrate metabolism	93	60	AF030943 (ovine)
	<i>H2A</i>	Nucleosome structure (H2A) histone family member 2	300	60	AY074805
	<i>RPLP0</i>	<ul style="list-style-type: none"> Protein metabolism and modification Structural component of the 60S large ribosomal subunit 	227	60	NM_001012682
	<i>RPL13a</i>	Structural component of the 60S large ribosomal subunit	179	60	NM_012423
Excluded HKs	<i>YWHAZ</i>	<ul style="list-style-type: none"> Protein domain specific binding Signal transduction 	102	60	AY970970
	<i>ACTB</i>	Cytoskeletal structural actin	178	60	AY141970
	<i>TUBB</i>	Microtubule globular protein component	228	60	NC_037350

a) Set-Up And Optimisation Of Real Time Quantitative PCR

In order to measure gene expression in test samples, it was necessary to normalise the transcription level of each gene of interest against the expression level of relevant housekeeping genes. Therefore, it is critically important to select

Table 5.7. Customized array for ovine cDNA libraries testing (validated primers).

Categories	Transcripts	
Housekeepers	<i>ACTB</i>	<i>RPLP0</i>
	<i>GAPDH</i>	<i>RPL13a</i>
	<i>H2A</i>	<i>YWHAZ</i>
	<i>TUBB</i>	
Oocyte specific genes	<i>BMP15</i>	<i>GTSF1</i>
	<i>GDF9</i>	<i>POU5F1 (OCT4)</i>
Subcortical maternal complex (SCMC)	<i>NLRP2</i>	<i>OOEP(KHDC2)</i>
		<i>KHDC3L</i>
Imprinted genes	<i>ASCL2</i>	<i>PEG3</i>
	<i>H19</i>	<i>SNRPN</i>
	<i>MEST</i>	
One carbon metabolism	<i>BHMT</i>	<i>MTR</i>
	<i>MAT2B</i>	<i>PRMT5</i>
	<i>MTHFR</i>	<i>SHMT1</i>
Folate transporters		<i>SHMT2</i>
	<i>SLC46A1</i>	
Epigenetic regulators	<i>DNMT1</i>	<i>ELP3</i>
	<i>DNMT3A</i>	<i>HAT1</i>
	<i>DNMT3B</i>	<i>TET1</i>
	<i>EHMT2</i>	<i>UHRF1</i>
Meiosis division	<i>AURKB</i>	
Metabolism and Growth	<i>GSK3B</i>	<i>PRDX2</i>
	<i>IGF1R</i>	<i>SLC2A1</i>
	<i>IGF2BP2</i>	<i>SLC2A3</i>
	<i>IGF2BP3</i>	<i>SLC16A1</i>
	<i>PGK</i>	

the appropriate reference genes chosen for normalisation to facilitate accurate interpretation of real-time PCR results in order to both interpret the real-time PCR data and to understand the biological dynamics of events of oocyte and embryo development (Ginzinger, 2002, Kubista *et al.*, 2006, Livak and Schmittgen, 2001, Mamo *et al.*, 2007, Papin *et al.*, 2004). Because no universal housekeepers exist for all cell types, it is essential to test a wide range of the most common housekeepers to identify the correct reference genes for normalisation in quantitative real-time PCR of candidate genes in immature and mature oocytes

(Gal *et al.*, 2006, Jeong *et al.*, 2006, Mamo *et al.*, 2007). According to the minimum information required for publication of qRT-PCR experiments (MIQE) guidelines, the chosen housekeepers for normalisation must be experimentally validated for particular tissues or cells, or have been fully validated elsewhere. In addition, normalisation should be performed against multiple reference genes chosen from various candidate housekeepers to reduce the variation and analytical errors in the expression level of targeting genes (Bustin, 2010, Vandesompele *et al.*, 2002). In the experimental series of this chapter, normalisation of gene expression was performed against four housekeepers including *GAPDH*, *H2A*, *RPL13a* and *RPLP0*. *GAPDH* and *H2A* have been validated in the oocyte (Cotterill *et al.*, 2012, Jeong *et al.*, 2005, Kuijk *et al.*, 2007, Macabelli *et al.*, 2014, Mamo *et al.*, 2007, Ross *et al.*, 2010) whereas *RPLP0* and *RPL13a* were also stable enough to be used for normalisation of gene expression in ovine and rat tissues (Svingen *et al.*, 2015, Zaros *et al.*, 2010). Moreover, *RPLP0* and *RPL13a* have been previously used as an internal reference in human blastocysts by our own group (Huntriss *et al.*, 2017).

b) Analysis Of Oocyte Gene Expression By qPCR

Quantitative real-time PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Software Version SDS 2.2, Life Technologies Ltd.) using a 2X SYBR® Green PCR Master Mix (Life Technologies Ltd.). Each reaction was prepared in a 25µl final volume for either verification of cDNA libraries or measuring oocyte gene expression (Table 5.8). The thermal cycler was set-up with a standard mode to run 40 cycles, then the melting curve was run once the relative quantification (RQ) file was complete to verify the presence of gene-specific peaks and to confirm the absence of primer dimer formation. The relative quantification protocol was programmed in 4 stages as shown in Table 5.9.

The Ct value was used to determine the abundance of transcript level within individual oocytes due to its inverse correlation with the amount of target cDNA and is defined as the number of cycles required for the fluorescence signal or amplification curve to reach a particular threshold level in a real-time PCR cycle (Kubista *et al.*, 2006). The mean Ct value of the housekeeping and oocyte specific

genes of all cDNA libraries as described in section 5.2.3 was calculated from the three replicates of the PCR reaction. And this mean Ct value as a determining sample quality was used to decide sample selection for further comparison concerning the effect of age and maturational stage of oocytes.

To analyse transcripts level, individual cDNA samples were run on the customised array. The verification process had identified that, out of a total of 96 bovine PCR primers, a total of 44 PCR primer sets (44 genes) correctly amplified PCR products

Table 5.8. Composition of real-time PCR reaction

Reagent	Concentration	Volume (μ l)
2X SYBR® Green PCR Master Mix	2X	12.5
DNase/RNase free H ₂ O	-	10.5
cDNA sample	1/50	1
Mixed forward and reverse primers	10* or 0.4 μ M**	1
Total		25

* 10 μ M used for verification of cDNA libraries; *GDF9*, *BMP15*, *GAPDH*, *H2A*, *YWHAZ*

** 0.4 μ M used for measuring the level of gene expression

Table 5.9. Thermal cycler program for real-time PCR

Relative quantification			Dissociation curve
Stage 1	Stage 2	Stage 3	Stage 4
50°C for 2 min	95°C for 10 min	95°C for 15 sec	95°C for 15 sec
		60°C for 1 min	60°C for 1 min
			95°C for 15 sec

from ovine oocyte cDNA. To maximise our throughput of samples on the real-time machine, a customised 96-well PCR plate has been prepared for 2 times 44 verified primers, so that two cDNA samples (1 from each group; prepubertal and adult oocytes) could be analysed in a single plate run. Also, assessment of all transcript levels of individual samples within the customised array can be

compared. The reproducibility of this method has been reported by the recent work of our own group (Huntriss *et al.*, 2017). Since the Ct value is inversely correlated to the amount of target gene in cDNA samples, in comparison to the abundance of the transcript levels, the relative quantity (Rq) of the target gene expression for each sample was calculated by using the formula $2^{(-\Delta Ct)}$ (Livak and Schmittgen, 2001). To obtain normalised Ct values (ΔCt), raw Ct of individual samples were subtracted from the geometric mean of the Ct value of the four selected internal reference genes (Goossens *et al.*, 2005, Radonic *et al.*, 2004, Vandesompele *et al.*, 2002). Finally, the Rq of target genes between age groups and between stages of maturity were compared.

5.2.8. Statistical Analysis Of qPCR Data

All statistical analyses were performed using GraphPad Prism software (GraphPad Prism 7 for Mac OS X, GraphPad Software, Inc., La Jolla, CA, USA). All data from the qPCR analysis (Ct and $2^{(-\Delta Ct)}$ values) were tested for normality using the D'Agostino-Pearson and Shapiro-Wilk normality test. Statistical differences in expression levels ($2^{(-\Delta Ct)}$ values) between GV and MII stages of oocyte or between prepubertal and adult animal ages were determined if $p < 0.05$ by using multiple t-test comparisons, (unpaired and without assuming a consistent SD) or by ANOVA or using the non-parametric Kruskal Wallis test according to whether data were normally distributed or not, (Anderson *et al.*, 2009, Assidi *et al.*, 2008) as appropriate. Ct values were all normally distributed and are therefore presented as means \pm SEM, while Rq values did not follow a normal distribution for all genes. Results are therefore presented as boxplots.

5.3. RESULTS

5.3.1. Customized Candidate Genes Into The Study

The customised array for the genetic study was generated following the validated primers from section 5.2.6. Each array consisted of 45 genes which include seven housekeeping (*TUBB*, *ACTB*, *RPL13a*, *GAPDH*, *RPLP0*, *H2A*, and *YWHAZ*) (Table 5.6. and Figure 5.7.) and 38 target genes. The remaining genes were eliminated due to 1 of following exclusion criteria namely a Ct value < 15 or > 35 , the appearance

of primer dimers in dissociation curves, or presence of non-specific band or >1 specific bands by gel electrophoresis using real-time PCR products (Figure 5.8). Finally, a total of 24 amplified cDNA libraries comprising six samples selected from each group, were finally studied by using qPCR relative quantification.

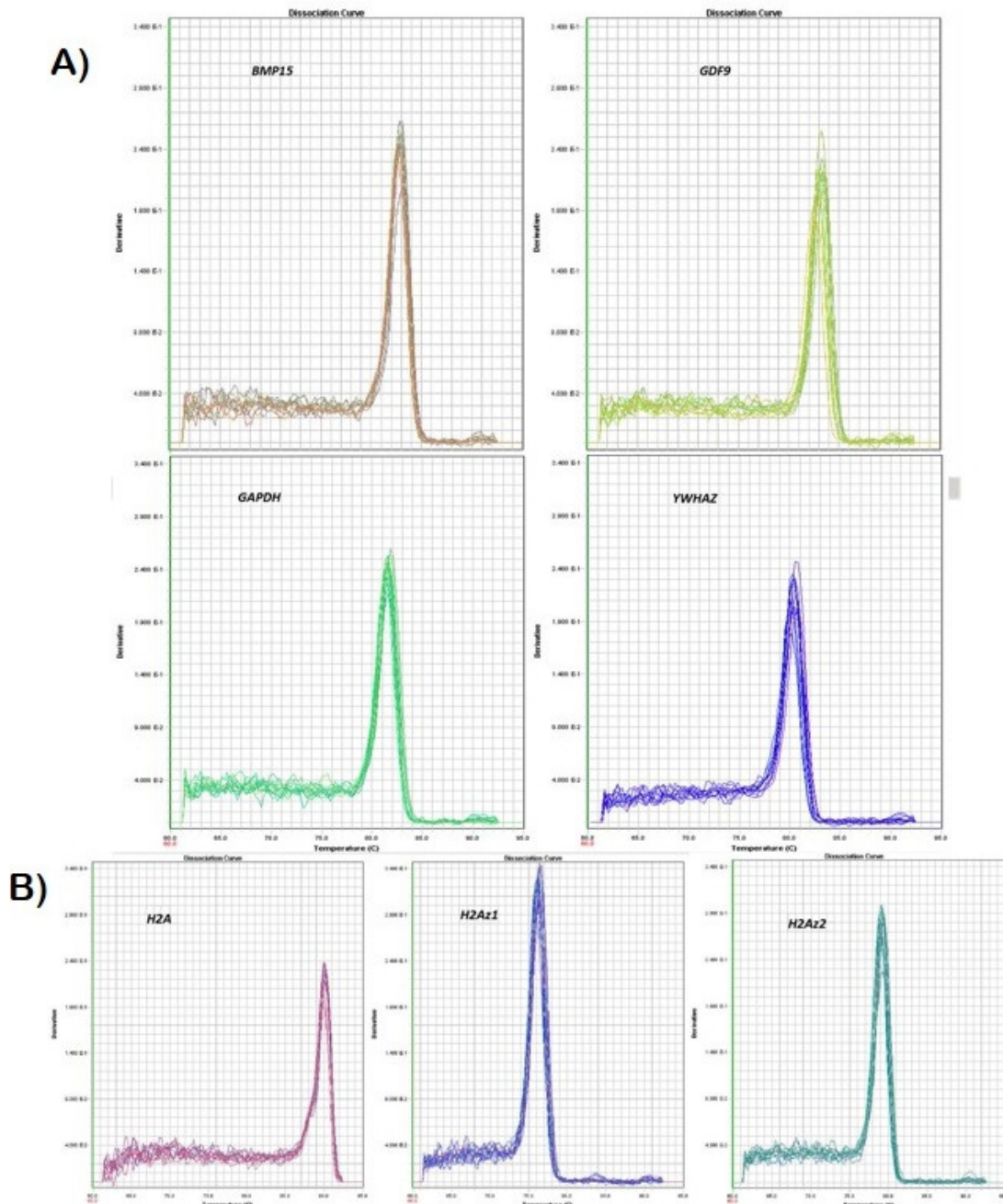


Figure 5.7. Representative dissociation curves following real-time PCR. The dissociation curve was generated in triplicates using cDNA libraries of 4 individual oocyte samples. Both housekeepers and oocyte specific genes indicated high specificity for the target genes. Each of the lines represents the absorbance value at a given temperature. (A) shows dissociation curve of *BMP15*, *GDF9*, *GAPDH* and *YWHAZ* while (B) compares dissociation curve of 3 sets of designed *H2A* primer.

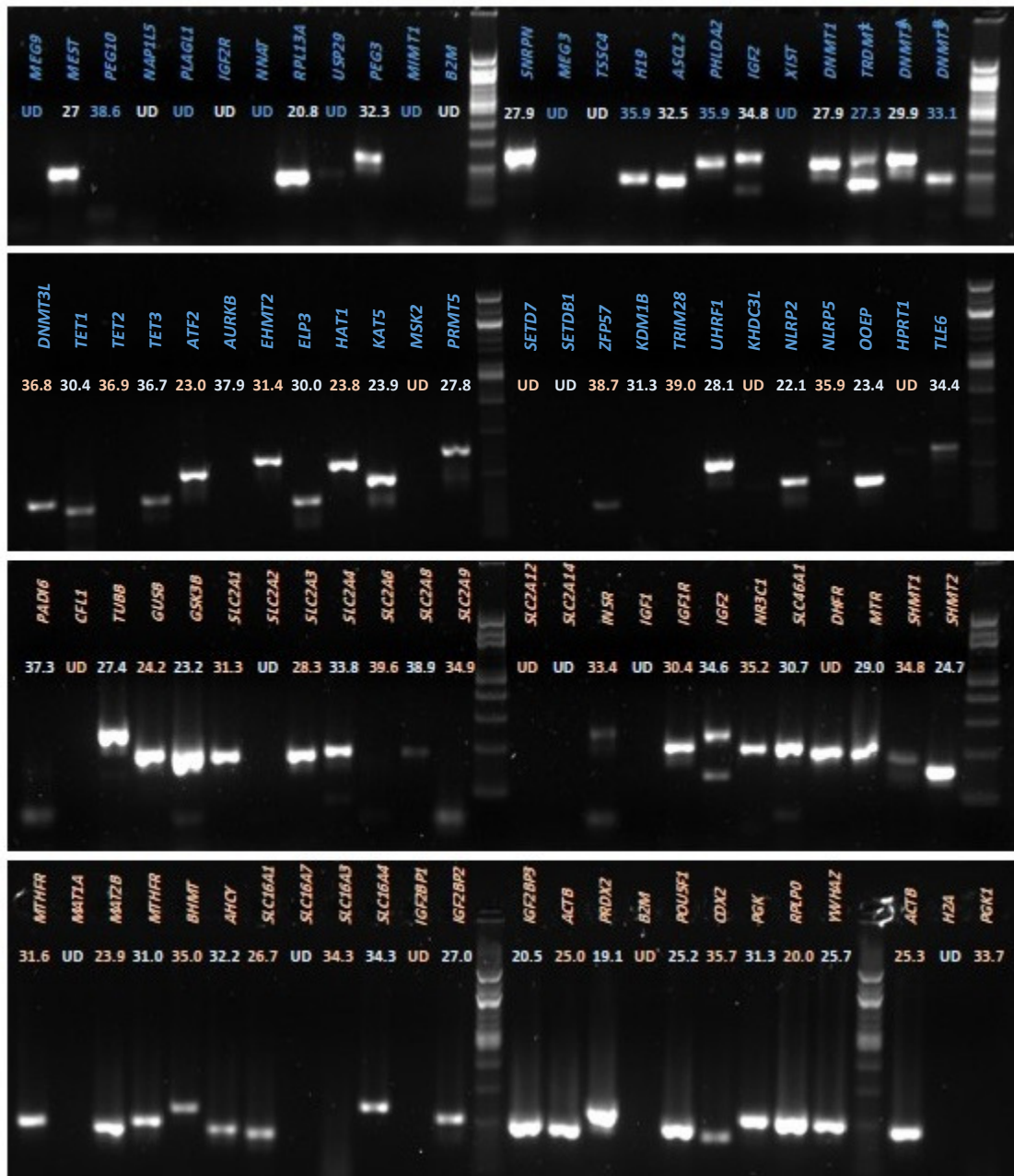


Figure 5.8. Representative of real-time PCR products of bovine array primers testing with ovine cDNA libraries (1:50 total RNA) on 2% (w/v) agarose gel. Specific bands were indicated that ovine DNA samples were successfully identifiable using bovine array primers. The gene symbols are labeled in vertical and the number indicating Ct values are shown on top of each lane. UD represents an undetermined Ct value. Gels were run with 100-110 volts for 60 min. Band sizes in the test samples were compared against a 1 kb DNA ladder.

5.3.2. Differential Gene Expression Analysis Across Ovine GV- And MII-Staged Oocytes From Prepubertal And Adult Animals

The 38 selective target genes analysed in this study were classified into eight categories according to their function and included oocyte specific genes, SCMC genes, imprinted genes, 1-carbon metabolism, folate transporters, epigenetic regulators, chromosome segregation markers and genes associated with metabolism and growth. We observed 17 genes that differentially increased the level of expression in prepubertal-derived GV oocytes (Table 5.10.), and two genes that differentially increased level of expression in prepubertal-derived MII oocytes (Table 5.11.), when compared to the equivalent maturational-stage of oocytes derived from adults (Figure 5.9 and Figure 5.10). Moreover, comparisons between immature and mature oocytes demonstrated 5 (Figure 5.11), and 3 (Figure 5.12) differentially expressed genes in oocytes derived from prepubertal and adult animals, respectively (Table 5.12).

Regardless of animal age, three genes including *GTSF1*, *NLRP2*, and *PRDX2* were found differentially expressed between GV and MII staged oocytes (Table 5.12). Significant differences ($p < 0.05$) were consistently maintained in samples derived from both prepubertal and adult tissues. While the other two genes- *HAT1* and *SLC16A1*, expressed significantly different ($p < 0.05$) between the 2 –maturational stages only in samples originating from prepubertal (Figure 5.11) but no such differences were detected in samples from adult animals (Figure 5.12).

With respects to GV-staged oocytes, the significant 17 genes, which were significantly expressed ($p < 0.05$) between prepubertal and adult samples (Figure 5.9 and Table 5.10), distributed across functional gene categories in the study. They seemed to be predominantly involved in metabolism and growth (i.e., *SLC2A1*, *SLC2A3*, *SLC16A1*, *IGF1R*, *IGF2BP2*, *IGF2BP3*, *GSK3B* and *PRDX2*) and 1-carbon metabolism (i.e., *MAT2B*, *PRMT5* and *SHMT2*) (Figure 5.9 and 5.13). Although the rest of the significant genes have been classified in different categories, they seemed to have a link to epigenetic programming in common. For example, the imprinted gene, *SNRPN*, as well as epigenetic regulators, *DNMT3A* and *DNMT3B*, also differed significantly ($p < 0.05$) in the level of expression

between 2 age groups when GV-stage oocytes were compared. Also, *NLRP2* and *OOEP*, which belong to the SCMC group, showed significant differences ($p < 0.05$) between 2 age groups. Moreover, *SLC2A1* may potentially be another gene differentially expressed since 5 out of the 6 GV samples derived from adult, but none from the prepubertal counterpart.

Table 5.10. Differentially-expressed genes in prepubertal from adult GV-staged oocytes. Data calculated against four housekeeping genes; *GAPDH*, *H2A*, *RPL13a* and *RPLP0*. Significant differences if $p < 0.05$, unpaired *t*-test.

Categories	Transcripts	<i>p</i> -value
Oocyte specific genes	🔊 <i>BMP15</i>	0.032
	🔊 <i>GTSF1</i>	0.041
Subcortical maternal complex (SCMC)	🔊 <i>NLRP2</i>	0.048
	🔊 <i>OOEP(KHDC2)</i>	0.022
Imprinted genes	🔊 <i>SNRPN</i>	0.005
One carbon metabolism	🔊 <i>MAT2B</i> ±	0.027
	🔊 <i>PRMT5</i>	0.047
	🔊 <i>SHMT2</i>	0.049
Epigenetic regulators	🔊 <i>DNMT3A</i>	0.009
	🔊 <i>DNMT3B</i>	0.011
Metabolism and growth	🔊 <i>GSK3B</i>	0.033
	🔊 <i>IGF1R</i>	0.022
	🔊 <i>IGF2BP2</i>	0.006
	🔊 <i>IGF2BP3</i>	0.002
	🔊 <i>PRDX2</i>	0.024
	🔊 <i>SLC16A1</i>	0.042
	🔊 <i>SLC2A1</i> *	-

± Mann-Whitney test due to data not normally distributed

* *SLC2A1* level of gene expressed detected only in adult GV samples but none from prepubertal samples; therefore, comparative statistical analysis between two groups was limited.

Table 5.11. Age-related differences in gene expression of MII-staged oocytes between prepubertal and adult animals.

Categories	Transcripts	<i>p</i> -value
Subcortical maternal complex (SCMC)	🔊 <i>OOEP(KHDC2)</i>	0.012
Metabolism and growth	🔊 <i>IGF2BP3</i>	0.007

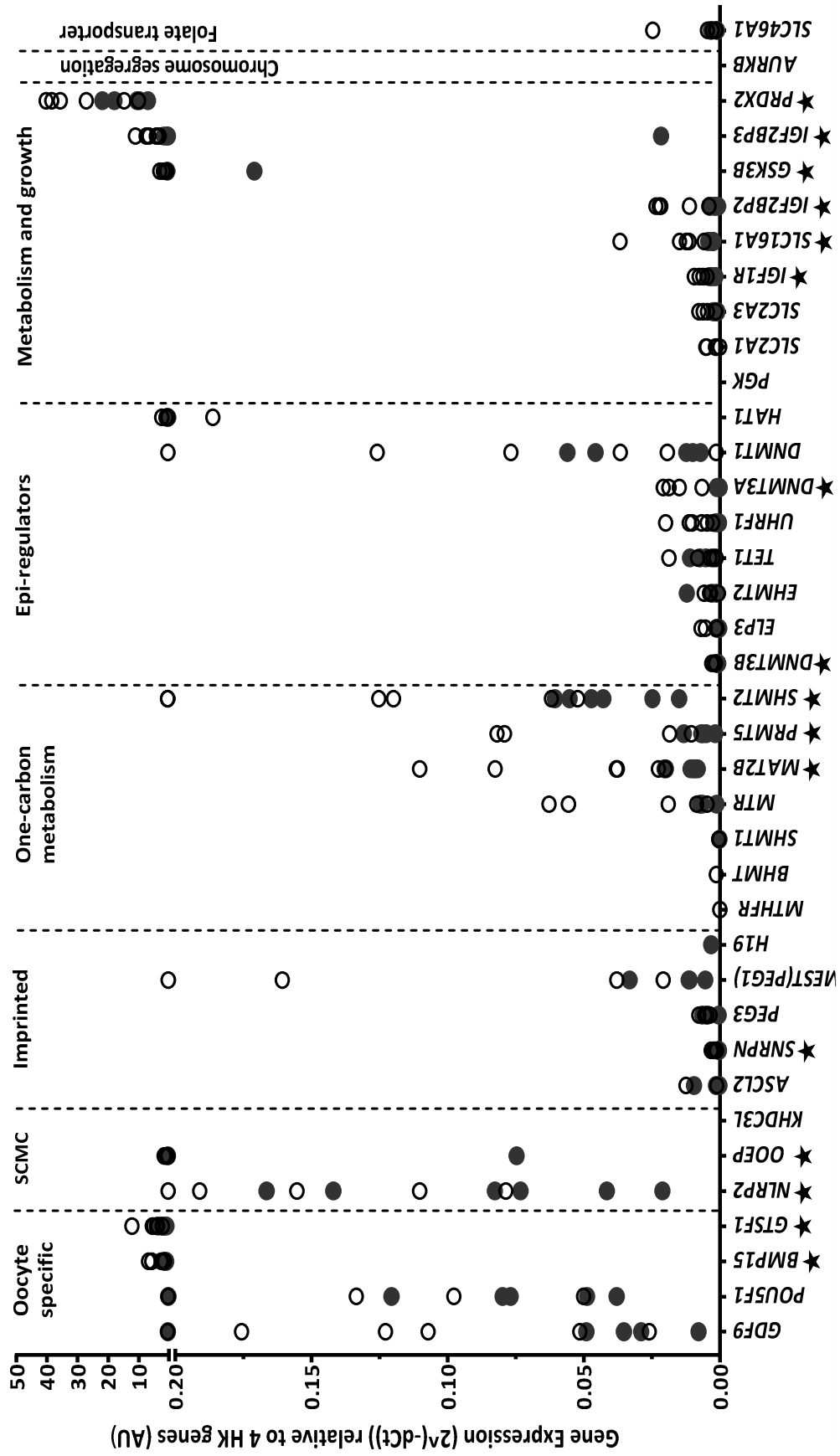


Figure 5.9. Comparative gene expression between prepupal and adult GV-staged sheep oocytes. Data shown in superimposed scatter plot of all genes expressed included in the customized array using bovine primers and designed housekeeping genes. ● Prepupal GV- and ○ adult GV-derived samples. Significant differences (*) between age groups if unpaired t-test, p<0.05.

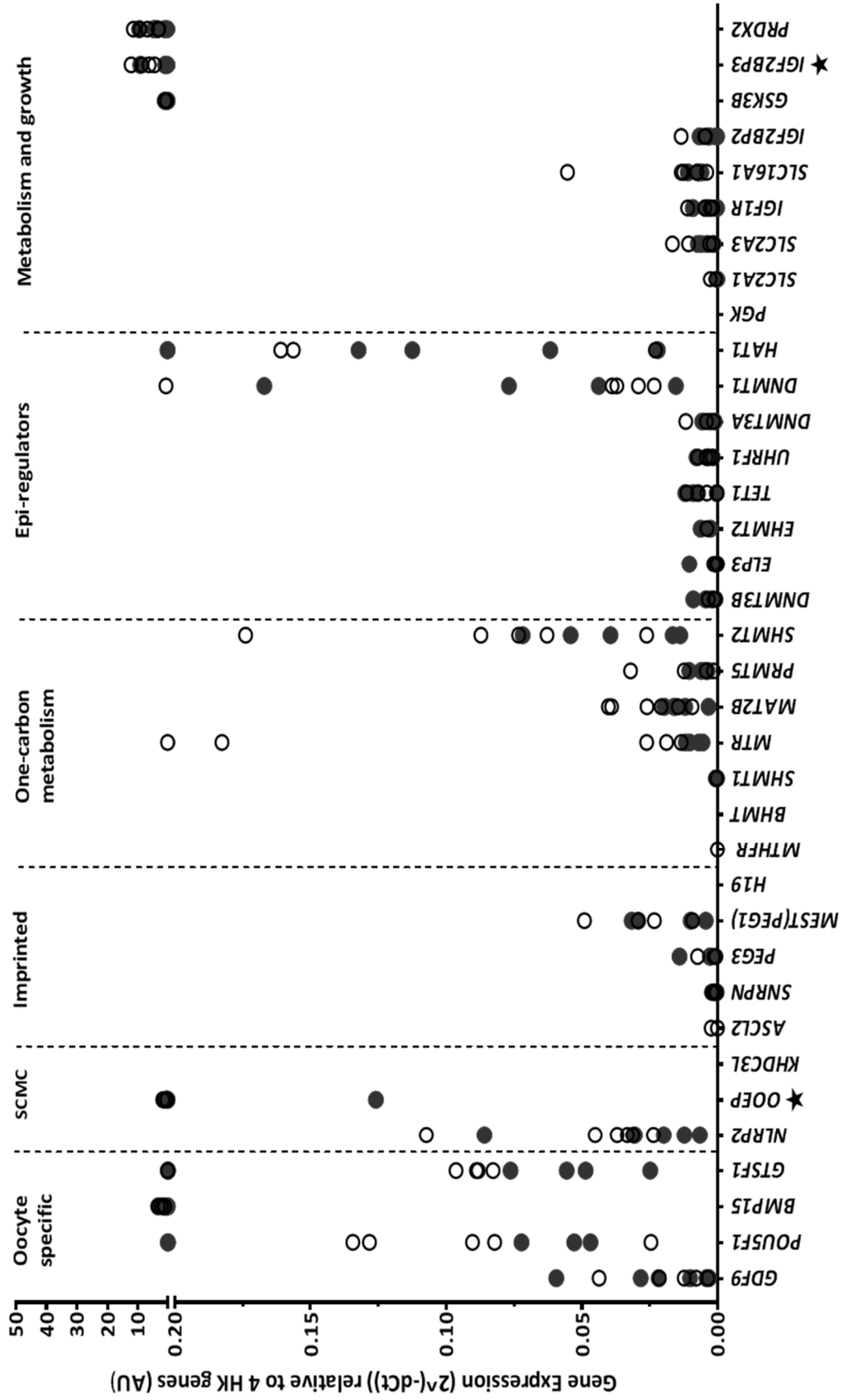


Figure 5.10. Comparative gene expression between prepubertal and adult MII-staged sheep oocytes. Data shown in superimposed scatter plot of all genes expressed included in the customized array using bovine primers and designed housekeeping genes. ● Prepubertal MII- and adult MII-derived samples. Significant differences (*) between age groups if unpaired *t*-test, *p* < 0.05.

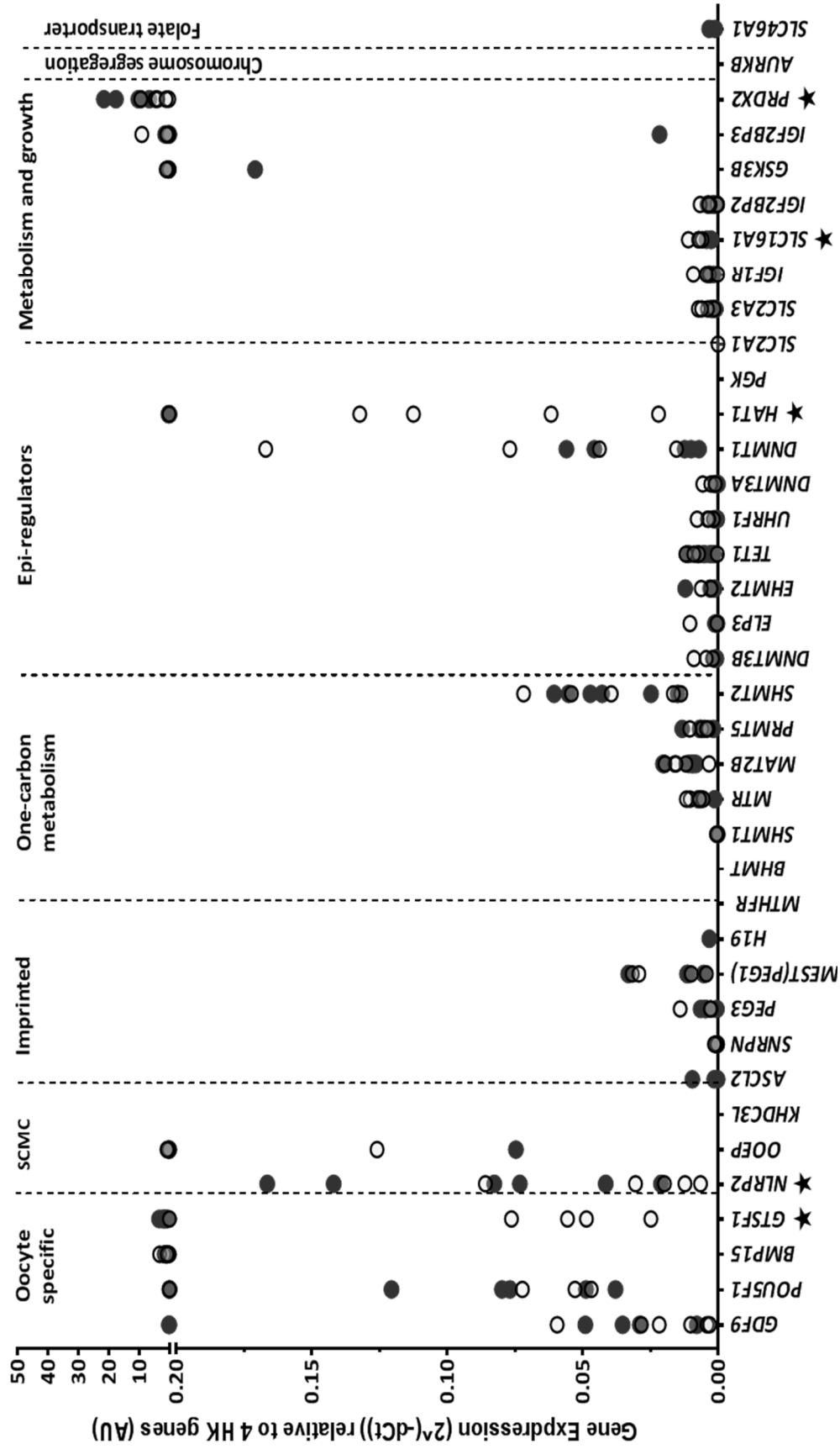


Figure 5.1.1. Comparative gene expression between prepubertal GV- and MII-staged sheep oocytes. Data shown in superimposed scatter plot of all genes expressed included in the customized array using bovine primers and designed housekeeping genes. ● GV- and ○ MII-derived samples. Significant differences (*) between different stages if unpaired *t*-test, *p*<0.05.

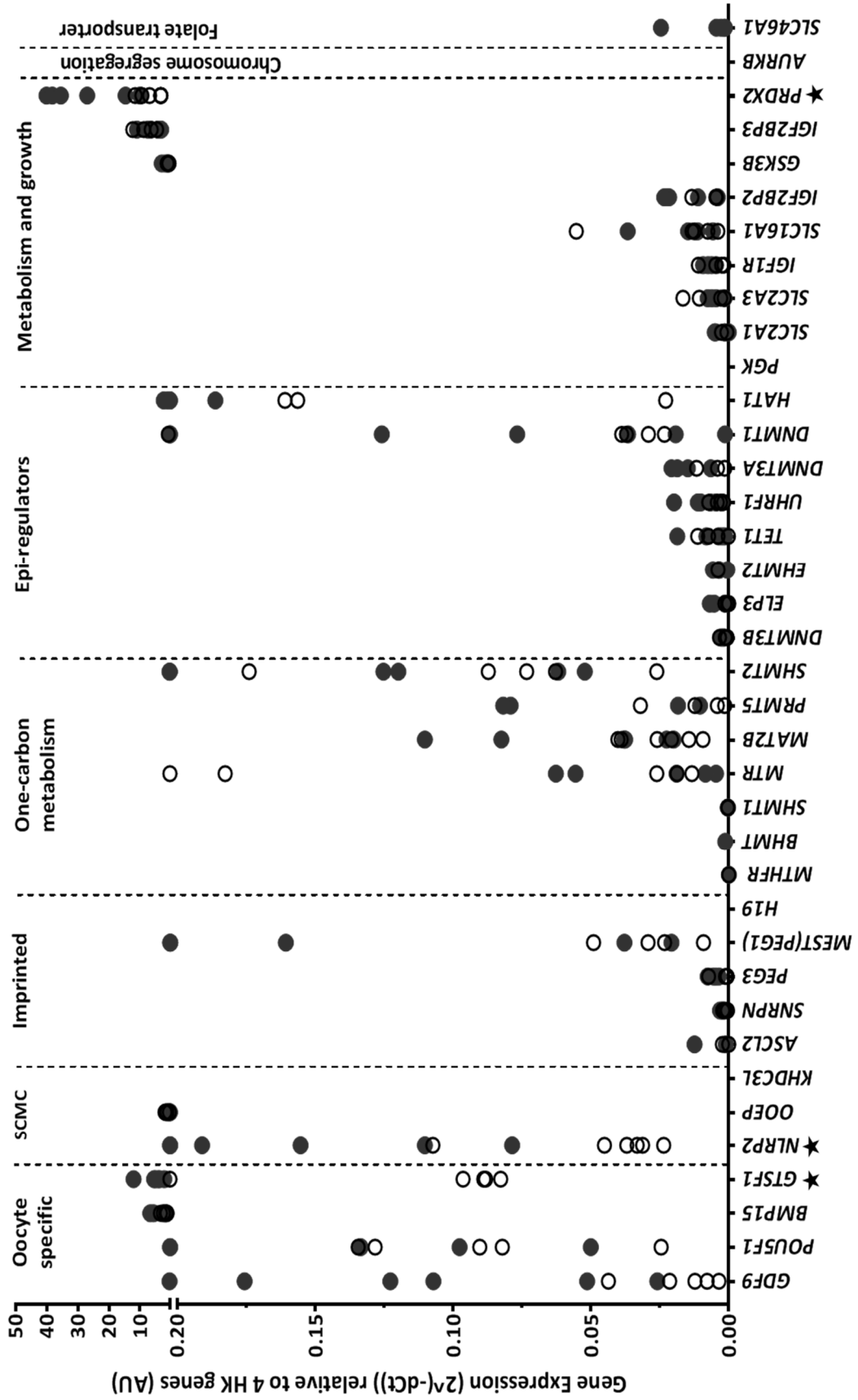


Figure 5.12. Comparative gene expression between adult GV- and MII-staged sheep oocytes. Data shown in superimposed scatter plot of all genes expressed included in the customized array using bovine primers and designed housekeeping genes. ● GV- and ○ MII-derived samples. Significant differences (★) between different stages if unpaired *t*-test, *p*<0.05.

Table 5.12. Summarized potentially stage-specific genes. Differentially increased level of expression in GV- compared to MII-stage oocytes within prepubertal and adult groups.

Categories	Differential Gene Expression (<i>p</i> -value)	
	Lamb	Adult
Oocyte specific genes		
● <i>GTSF1</i>	0.002	0.004
Subcortical maternal complex (SCMC)		
● <i>NLRP2</i>	0.047	0.006
Metabolism and growth		
● <i>PRDX2</i>	0.017	0.003
● <i>SLC16A1</i>	0.004	-
Epigenetic regulators		
● <i>HAT1</i>	0.028	-

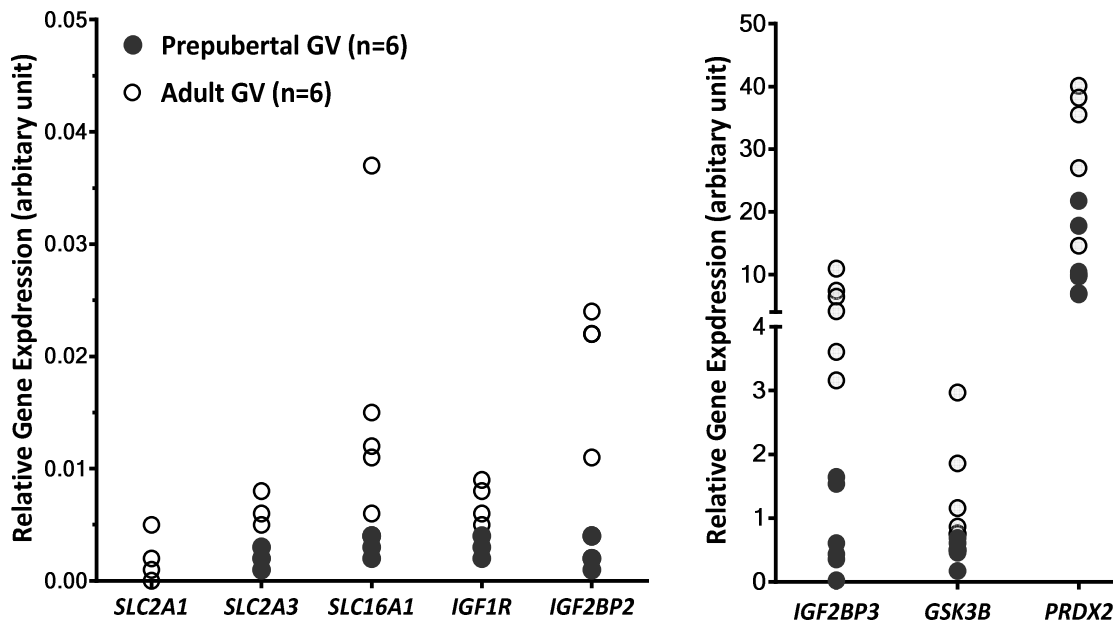


Figure 5.13. Effect of age on relative expression of key genes related to metabolic and growth. Data shown in superimposed scatter plot relative to 4 housekeeping genes; *GAPDH*, *H2A*, *RPLP0* and *RPL13A*. ● Prepubertal GV-, ○ adult GV oocytes, and NS: not significant. *SLC2A1* was undetectable in all prepubertal GV samples. Significant differences found ($p < 0.05$, unpaired *t*-test) between age groups.

5.4. DISCUSSION

The experiments presented in this chapter demonstrate that a panel of candidate markers of oocyte development and metabolism alter with meiotic progression irrespective of animal age. Furthermore, the data demonstrate that the animal age has influenced expression levels of known markers of oocyte quality as well as a range of maternal effect genes and epigenetic regulators. Of particular importance in the context of this thesis is the observation that alteration of oocyte age to the expression patterns in part of genes associated with metabolism and growth as well as genes involved in epigenetic programming can be pilot data for further intensive studies focusing on the relationship of age and the impairment of metabolic function and epigenetic control in various ageing study models. The bovine primer sets that were successfully validated in groups of 10 oocytes but were subsequently not detected by qPCR analysis conducted on a single oocyte analysis were recorded as "absence of gene expression". New genetic study technology (i.e., transcriptome analysis by RNA sequencing) would substantially enhance the findings of the present work by extensively recruiting a number of genes into the study. Also, specific primer sequences for ovine species are required in future studies.

5.4.1. Validation of cDNA Libraries Construction

Apart from avoiding gDNA contamination and correct amplification, cDNA library construction was critically important to the successful outcome of the molecular investigations conducted in this experimental series. In this study, we used a highly efficient method for cDNA synthesis from total RNA. Due to the 2-step criteria used for cDNA verification, 91% (41/45) of cDNA libraries showed smears on an agarose gel; however, 90% (37/41) of those samples verified at least one oocyte specific gene and two housekeepers were recruited for the following genetic study. Sample drop-out was not significant concerning the stage and age of oocytes (Chi-square test; $p > 0.05$). Almost all samples evaluated in this study demonstrated the same pattern of gene expression according to the three housekeepers, *GAPDH*, *YWHAZ*, and *H2A*. Also, the single amplicon generated in melting curve analysis by the end of the real time PCR reaction confirmed the

specificity of both oocyte-specific and housekeeping genes. Therefore, these results were indicative of the quality of established cDNA libraries and provided proof of the success of the new cDNA construction protocol used.

5.4.2. Selection Of The Internal Reference Genes

The reliability of real time-PCR analysis depends on the customisation of appropriate housekeepers as internal references. Theoretically, the stability of the reference gene expression is required across all samples used for analysis regardless of their different physiological states. Furthermore, the reference genes must also behave consistently during the experimental process (Radonic *et al.*, 2004, Wan *et al.*, 2010). To ensure the independence of gene expression from the investigation, the geometric mean of multiple selective-reference genes was recommended for data normalisation (Goossens *et al.*, 2005, Vandesompele *et al.*, 2002).

Herein, 7 housekeeping genes were investigated, but only 4 of them were selected to use as internal references including *GAPDH*, *H2A*, *RPL13a*, and *RPLP0* due to the similar pattern expressed in all samples. Previous studies have reported the successful use of the selective genes, *GAPDH*, *YWHAZ* and *H2A*, as the internal reference of gene expression analysis for oocytes, embryos and blastocysts in various species: human (Robert *et al.*, 2002), bovine (Goossens *et al.*, 2005), cattle and buffaloes (Jeong *et al.*, 2005, Kumar *et al.*, 2012, Macabelli *et al.*, 2014), mouse (Mamo *et al.*, 2007), rabbit (Mamo *et al.*, 2008), sheep (O'Connor *et al.*, 2013, Taylor *et al.*, 2009), pigs (Kuijk *et al.*, 2007, Wang *et al.*, 2017), and horse (Smits *et al.*, 2009). Although *GAPDH*, one of the most common genes tested in oocytes, has previously been reported to stably express in bovine (Goossens *et al.*, 2005), buffaloes (Kumar *et al.*, 2012), pigs (Kuijk *et al.*, 2007) and mouse (Mamo *et al.*, 2007), its use as an internal control in ovine oocytes is less promising owing to a prior report showing it to have the least stability relative to other housekeeping control genes (O'Connor *et al.*, 2013). However, the expression profile for *GAPDH* within single ovine oocyte samples in the current study seemed to be stable, and this was in accordance with another report by our group (Liperis, 2013).

Like *GAPDH*, *H2A* was frequently used as housekeeping gene in oocytes and embryos in mouse (Mamo *et al.*, 2007), rabbit (Mamo *et al.*, 2008), bovine (Goossens *et al.*, 2005), and horse (Smits *et al.*, 2009). Although *H2A* was confirmed as the most stable relative to other reference genes in rabbit (Mamo *et al.*, 2008) and mouse (Mamo *et al.*, 2007) oocytes, inconsistent data was demonstrated in other species (Goossens *et al.*, 2005, Smits *et al.*, 2009) especially ovine (O'Connor *et al.*, 2013). Our data found the pattern of *H2A* expression was rather stable and consistent with the trend seen in *GAPDH*. In this regard, 3 explanations may shed light on different outcomes observed between studies. First, the culture condition differed between studies. Our experiment performed IVM in serum-free defined medium instead of using bicarbonate buffered-TCM as used in the O'Connor study (O'Connor *et al.*, 2013). Second, the samples were processed using different methods. The previous study collected groups of 10 denuded oocytes before holding the cell pools overnight at 4°C in 10µl of RNA later before passing on to store at -80°C. In contrast, in the current study, each sample contained only one single denuded oocyte that was immediately stored in 2µl of RNAGEM buffer and snapped frozen in liquid nitrogen following harvest. As the earlier reports, many factors may influence the stability of reference genes such as the state of development (Luchsinger *et al.*, 2014), experimental conditions (Beekman *et al.*, 2011, De Boever *et al.*, 2008, Maccoux *et al.*, 2007, Penning *et al.*, 2007), tissues (Maroufi *et al.*, 2010, Nygard *et al.*, 2007, Pierzchala *et al.*, 2012) and/or breeds (Pierzchala *et al.*, 2012). The final explanation is possibly related to the primer specificity since differences in primer design can lead to alterations in the PCR products generated.

The current study found ribosomal protein L13 (*RPL13*) and ribosomal protein P0 (*RPLP0*) can be the other two potential reference genes of sheep oocytes since they have been reported in human ovarian tissues (Cai *et al.*, 2014, Fu *et al.*, 2010, Lv *et al.*, 2017) especially in GCs (Lv *et al.*, 2017). As these two genes expressed in the same fashion as *GAPDH* and *H2A* in almost all samples.

5.4.3. Differential Gene Expression In Oocytes Between Prepubertal And Adult Sheep

Testing of the custom, in-house, bovine real time PCR array against sheep oocytes used in the present study revealed that 47 out of 94 ovine genes were amplified. The remaining genes were excluded due to poor primer efficiencies such as an undetectable amplicon, primer-dimer, or more than one specific band depicted. The number of bovine primers amplifying genes from ovine oocytes indicates that there is remarkable cross species homology in gene expression. For further qPCR analysis, the customised array used here gave a comprehensive coverage to 44 genes in 8 categories of gene functions including oocyte specific, subcortical maternal complex, imprinted genes, one-carbon metabolism, folate transporter, epigenetic regulators, chromosomal segregation, and metabolism and growth as well as 7 housekeeping genes.

It is well-established that oocyte ageing causes poor fertilisation and impairs embryo development (Liu and Case, 2011) (see review in (Miao *et al.*, 2009a, Qiao *et al.*, 2014)). Cellular and molecular hallmarks of ageing have been revealed as consequences from various biological systems such as genomic instability, telomere attrition, loss of proteostasis, and epigenetic alterations (Lopez-Otin *et al.*, 2013). Here, the genetic studies of gene transcription using prepubertal and adult sheep oocytes exhibit differential gene expression between two age groups and in so doing provide insights into the molecular mechanism that underpin functional indices of ageing in oocytes. Candidate genes studies provide insights into:

a) Genes Involved In Oocyte And Early Embryonic Development

This study identifies 2 oocyte-specific genes, *BMP15* and *GTSF1*, involved in development, that are affected by animal age. Although these genes demonstrated a low level of expression in GV-staged oocytes from both prepubertal and adult sheep, the study observed the lower level of expression found in GV oocytes derived from prepubertal compared to the level seen in adult animals. It has been suggested that insufficient *BMP15* and *GTSF1* expressed in

prepubertal oocytes may contribute to the clinically low developmental potential reported previously (Reader *et al.*, 2015).

The oocyte secreted factor, *BMP15* plays a crucial role throughout the process of folliculogenesis from primordial follicle formation until ovulation especially in sheep but (not in mice) (Juengel and McNatty, 2005, Kedem *et al.*, 2011, Paulini and Melo, 2011, Trombly *et al.*, 2009). During final stages of folliculogenesis, its involvement can be demonstrated in events associated with coordination of oocyte maturation and ovulation including regulation of follicular cell proliferation and viability, increased AA transport, reduced numbers of LHR, decreased luteinisation and promoting glycolysis in CCs (Gilchrist *et al.*, 2008, Reader *et al.*, 2015, Su *et al.*, 2008, Yan *et al.*, 2001, Banerjee and Pal, 2008b).

Evidence from previous studies have shown the relevance of *BMP15* to oocyte quality and have led to improved preimplantation embryo development via oocyte regulating paracrine signaling pathway (Hussein *et al.*, 2006, Lin *et al.*, 2014, Yeo *et al.*, 2008). Some reports have verified that *BMP15* action is intrinsically linked to *GDF9*, the other oocyte-specific protein, to provide signals promoting the growth and differentiation of ovarian follicles (Gilchrist *et al.*, 2008). A further study has recently demonstrated that *BMP15* and *GDF9* form a heterodimeric protein that is a potent activator of GCs signaling and function, which improves oocyte quality (Mottershead *et al.*, 2015). This finding is consistent with the other reports in equine (Campos-Chillon *et al.*, 2015) and human (Li *et al.*, 2014) observed the higher *GDF9* and *BMP15* mRNA levels correlated with better outcomes regarding oocyte maturation, fertilisation, and embryo quality. Moreover, the aberration of *GDF9* and *BMP15* mRNA levels to gonadotropin stimulation in oocytes and CCs derived from PCOS patients may lead to poor oocyte quality (Wei *et al.*, 2013).

Conflicting results found in cattle suggest that *GDF9*, but not *BMP15*, was upregulated in competent oocytes even though *BMP15* mRNA levels were 20-fold higher than a level expression of *GDF9* (Donnison and Pfeffer, 2004). Just as in this cattle study, the present work demonstrates more than 20-fold higher *BMP15*

expression than *GDF9*; however, the conflict finding depicts the upregulation of *BMP15* in competent adult GV oocytes while no correlation was detected with *GDF9* expression. This indicates that *BMP15* function is species-specific and that an abundance of *BMP15* expression may be indicative of oocyte quality. Moreover, lower *BMP15* mRNA expression observed in prepubertal oocytes here may reflect reduced quality in prepubertal oocytes.

GTSF1 is another gene, gamete-specific, found differentially expressed in this work and showed highly conserved during oogenesis and spermatogenesis (Krotz *et al.*, 2009). Although the *GTSF1* function was clearly revealed in mouse spermatogenesis for transposon suppression to prevent germ cell apoptosis and support meiotic progression (Yoshimura *et al.*, 2009), its role in female is less defined since fertility of *GTSF1*-null female mice was not impaired (Krotz *et al.*, 2009, Yoshimura *et al.*, 2009). The recent study by our group demonstrated that *GTSF1* gene expressed in human ovarian follicles, GV- and MII-staged oocytes throughout blastocyst since oogonia entering meiosis and following primordial follicle formation. This work suggested that the existence of *GTSF1* transcripts was indicative of some roles in oogenesis and early embryogenesis (Huntriss *et al.*, 2017).

Consistent with data in human, a previous study conducted by our group found ovine oocyte-specific *GTSF1* expressed throughout stages of oogenesis and early embryogenesis in contrast to other ovarian somatic cells (Liperis, 2013). This observation agrees with the finding in the current work, where abundance of *GTSF1* mRNA strongly represented in both GV and MII staged oocytes. A higher level of *GTSF1* expression in adult GV oocytes may be indicative for the greater developmental potential of adult oocytes compared to prepubertal oocytes. In mice, reduced *GTSF1* abundance was observed in postovulatory-ageing MII oocytes (Trapphoff *et al.*, 2016). Similar to *BMP15*, insufficient expression of the *GTSF1* gene may reflect an impairment of oocyte quality due to the ageing process.

Apart from oocyte-specific genes, maternal effect genes also play essential roles in mammalian zygote development beyond the first cleavage stage. At least four proteins encoded by *NLRP5*, *OOEP*, *TLE6* and *KHDC3L* forming the multi-protein SCMC, are distinctively expressed in mammalian oocytes and early embryos (Bebbere *et al.*, 2014, Dean, 2002, Tong *et al.*, 2000, Zheng and Dean, 2009). In mice, functional studies suggested that the unique transcripts have fundamental roles in epigenetic reprogramming, embryonic genome activation, and cell specification during oocyte to zygote transition, as well as zygote progression through late stages of embryogenesis (Dean, 2002, Tong *et al.*, 2000, Wu *et al.*, 2003, Zhang *et al.*, 2015, Zheng *et al.*, 2007). Likewise, in sheep, the existence of SCMC components was expressed at the highest level in GV oocytes prior to tailing off during IVM and through early embryogenesis until reaching an undetectable level at the blastocyst stage (Bebbere *et al.*, 2014).

The current study identified two SCMC genes, *NLRP2* and *OOEP*, as being differentially expressed between the two animal ages studied. Similar to the pattern found in oocyte-specific genes, an overall higher expression of both *NLRP2* and *OOEP* were detected in adult GV compared to prepubertal GV oocytes. Furthermore, *NLRP2* but not *OOEP* was expressed significantly different between maturational stages when GV and MII oocytes were compared regardless of animal age. It is unclear why *OOEP* expression patterns in the current study are different from the findings presented in the previous report (Bebbere *et al.*, 2014). This discrepancy may be caused by methodology differences in sample collection influencing study outcome since the current work analysed individual oocytes instead of the 10 pooled oocytes as in the previous report. Alternatively, there could be more sample variation with single oocytes analyses *per se* leading to different results. And the final possible explanation could be a seasonal effect which has not mentioned in the previous studies like it was clarified in this work.

The second member of the SCMC studies- *NLRP2*, has been demonstrated in mouse ovaries but not in other tissues. In mice, the transcript is accumulated during oogenesis before its remarkably rapid degradation during the first cleavage when mouse embryonic genome activation occurs (Mahadevan *et al.*,

2017, Peng *et al.*, 2012). Another study found *NLRP2* to be an essential regulator of oocyte quality (Kuchmiy *et al.*, 2016). In contrast to the finding in mice, the expression level of *NLRP2* in rhesus monkeys is persistent throughout oocyte maturation and preimplantation embryogenesis, suggesting the transcription of *NLRP2* occur from both maternal and embryo sides in this species (McDaniel and Wu, 2009). In contrast, *OOEP* is an essential maternal-effect gene required for oocyte cytoplasmic lattice formation and embryonic development at the maternal-zygotic stage transition (Tashiro *et al.*, 2010). The disruption of this gene leads to female infertility with neither impairment during oogenesis, ovulation nor fertilisation but absence of the SCMC genes does affect embryonic arrest at the cleavage stage (Li *et al.*, 2008, Tashiro *et al.*, 2010, Tong *et al.*, 2004, Tong *et al.*, 2000, Zhang *et al.*, 2015).

The higher levels of *NLRP2* and *OOEP* expression in GV oocytes of adult sheep shown here may be an explanation as to why adult oocytes have greater developmental potential than their prepubertal counterparts. Characteristics previously observed in the maternal age-effect on murine oocytes, which *Nlrp2* significantly decreased in MII oocytes (Dankert *et al.*, 2014). The functional role of *Nlrp2* in this species yet to be elucidated, data from knock-out oocytes result in arrest development of the 2-cell embryo (Peng *et al.*, 2012, Tian *et al.*, 2009). These results have led us to speculate that *Nlrp2* deficiency linked to imprinting defects, as a consequence of *NLRP2* mutations observed in humans (Meyer *et al.*, 2009). However, this is incompatible with proteomic analyses in mice which demonstrate that the abundance of both *Nlrp2* and *Ooep* seemed to be stable during maternal ageing (Schwarzer *et al.*, 2014). Also, when GV and MII stages are compared, reduction of *NLRP2* levels in mature oocytes of both animal ages imply an essential role of this gene during oocyte maturation. Further studies are required to confirm whether an abundance of SCMC mRNA and/or proteins such as *NLRP2* and *OOEP* represent a promising indicator of age compromised oocyte competence.

b) Imprinted Genes And Regulation Of Epigenetic Remodelling

Genomic imprinting is an epigenetic mechanism of transcriptional regulation that is imposed during gametogenesis and embryonic development to distinguish the maternal and paternal gene specific origin (Verona *et al.*, 2003). Evidence suggests a link exists between events associated with ART such as ovarian stimulation and *in vitro* embryo culture and aberration of genetic imprinting in various species (Denomme and Mann, 2012). Notably, an incidence of rare imprinting disorders increased in children conceived after the use of ARTs such as Beckwith-Wiedemann syndrome (Bowdin *et al.*, 2007, Chang *et al.*, 2005, DeBaun *et al.*, 2003, Gicquel *et al.*, 2003, Gomes *et al.*, 2007, Halliday *et al.*, 2004, Maher *et al.*, 2003, Rossignol *et al.*, 2006, Sutcliffe *et al.*, 2006). According to the process of genomic imprinting established during oocyte development, disruption of the imprinting process leads to compromised embryo development.

In the current study, five parental imprinted genes including *ASCL2*, *H19*, *MEST(PEG1)*, *PEG3*, and *SNRPN*, were investigated for oocyte maturation and age. These genes are of particular interest because of their critical biological functions and phenotypic characteristics related to the appearance of their gene products. *ASCL2* (Arnold *et al.*, 2006, Guillemot *et al.*, 1995, Jiang *et al.*, 2015, Wrenzycki *et al.*, 2001) and *H19* (Cai and Cullen, 2007, Hansmann *et al.*, 2011, Nordin *et al.*, 2014, Walsh *et al.*, 1994) are paternally imprinted and non-methylated in oocytes whereas *MEST(PEG1)* (Kaneko-Ishino *et al.*, 1995), *PEG3* (Kuroiwa *et al.*, 1996), and *SNRPN* (Barr *et al.*, 1995) are maternally imprinted and thus fully methylated in oocytes.

The current study confirmed *MEST/PEG1* to be expressed at a high level compared to the remaining 4 genes that were detected at a very low level in sheep oocytes regardless of maturity. *MEST/PEG1* is a classic imprinted gene primarily expressed from the paternal allele in the mesoderm and its derivatives (Kaneko-Ishino *et al.*, 1995). In human studies, it has been shown that *MEST/PEG1* is already unmethylated in spermatogonia (Kerjean *et al.*, 2000) while in the maternal germ line *MEST/PEG1* is entirely methylated in ovulated MII stage oocytes (Lucifero *et al.*, 2002). The current study of sheep oocytes is concomitant

with observations from previous reports that found the highest abundance of *MEST/PEG1* gene in the oocyte and early embryos across species studied including bovine, human, mouse, and pig (Jiang *et al.*, 2015). In addition, dynamic patterns of *MEST/PEG1* expression are evident and show a continuous decline from oocytes until the blastocyst stage of embryonic development (Jiang *et al.*, 2015). No differential *MEST/PEG1* expression was detected in the current study either between GV- and MII-staged oocytes or between animal ages.

It is not surprising that *H19* and *ASCL2*, which represented paternally imprinted genes, when investigated in the current study are almost undetectable from all oocytes regardless of age and/or maturity stage. Earlier studies have confirmed *ASCL2* imprinting status in mice (Guillemot *et al.*, 1995), but later work in cattle also reported sequence homology and gene expression patterns in blastocyst, oocyte, embryo, fetal and placenta tissues (Arnold *et al.*, 2006, Jiang *et al.*, 2015, Tunster *et al.*, 2016, Wrenzycki *et al.*, 2001). In mammals, the biological function of *ASCL2* has been acknowledged in chromosomal segregation and nervous system development (Rebhan *et al.*, 1997) as well as normal placenta development and successful pregnancies (Arnold *et al.*, 2006). Also, *ASCL2* expression dynamics was not established until later preimplantation embryo development (Jiang *et al.*, 2015). In the bovine, *ASCL2* expression was detected at the blastocyst stage of embryo development, whereas in mouse and human temporal expression has been reported at the 4-cell and 8-cell stages, respectively. As mentioned above, *ASCL2* expression in this study was barely detectable in both GV and MII stage of sheep oocytes. This result is consistent with previous reports in bovine (Jiang *et al.*, 2015), porcine (Park *et al.*, 2011) and human (Salpekar *et al.*, 2001).

The paternally imprinted *H19* gene investigated in the current work was detectable neither in GV nor MII of sheep oocytes in 23 of the 24 samples analyzed. The absence of the transcript is in accordance with previous reports in bovine (Jiang *et al.*, 2015) and human (Salpekar *et al.*, 2001). Although in the male germline, *H19* methylation imprints are initiated in fetal germ cells and completed in pachytene phase of meiosis I spermatocytes, an abundance of the

H19 transcript is not expressed until later, at the blastocyst stage (Tremblay *et al.*, 1995) or postimplantation stage (Szabo and Mann, 1995). Evidence has led to the assumption that both *ASCL2* and *H19* genes may be essential in the later stage of development around the period of zygotic genome activation and may influence placental development, but unlikely involved neither in oocyte maturation nor in maintaining pluripotent stem cell properties.

In contrast, *SNRPN* expression is controlled by a differentially methylated region that is fully methylated during oogenesis. Methylation abnormalities within this region are associated with Prader–Willi and Angelman syndromes (Leff *et al.*, 1992, Lucifero *et al.*, 2002). *PEG3* (paternally expressed gene 3) is an imprinted gene encoded zinc finger–containing (ZNF) protein containing putative transcription factor activities (el-Baradi *et al.*, 1991, Pieler and Bellefroid, 1994). Loss of *PEG3* imprinted methylation has been observed in mouse blastocysts derived from superovulated oocytes (Market-Velker *et al.*, 2010). In accordance with an earlier report, the current study demonstrated both *SNRPN* and *PEG3*, maternally imprinted genes, expressed at a low level in oocytes derived from both animal ages. Although in human oocyte and embryo development *SNRPN* was expressed throughout developmental stages, the pattern of expression continuously increased from the lower level expressed in oocytes to higher levels at the blastocyst stage (Salpekar *et al.*, 2001). This is in contrast to a *PEG3* expression which appears to be species specific. As shown in the current study, a low level of *PEG3* expression has been demonstrated in bovine and human oocytes (Jiang *et al.*, 2015). Conflicting results have however been shown in mice, where higher *PEG3* is expressed in oocytes before sharply declining to low levels at the 2-cell stage which is maintained until the later stage of preimplantation embryo development (Jiang *et al.*, 2015). Among five imprinted genes investigated in this study, only *SNRPN* has shown differential expression between prepubertal and adult GV oocytes. This may suggest that *SNRPN* can potentially be a sensitive determinant for oocyte competence; otherwise, most imprinted genes may tolerate external stimuli with regard to explicit time points, dosage of expression level, as well as species specificity *vice versa* (Guenatri *et al.*, 2013,

Hansmann *et al.*, 2011, Kameyama *et al.*, 2007, Lucifero *et al.*, 2002, O'Doherty *et al.*, 2011, Reik and Dean, 2001).

DNA methylation is one of the most crucial epigenetic mechanism regulating imprinted gene expression. As it has been demonstrated that expression of certain genes is required to establish oocyte-specific methylation during oocyte growth during oogenesis and embryogenesis, as well as oocyte maturation (Kageyama *et al.*, 2007, Kaneda *et al.*, 2004, Okano *et al.*, 1999, Reik and Dean, 2001, Yan *et al.*, 2014, Bourc'his *et al.*, 2001). In mammals, genomic imprinting is governed by DNA methyltransferase family including *DNMT3A* and *DNMT3B*, as well as its cofactor *DNMT3L*, and is critical for functioning gametes. Patterns of DNA methylation presenting at maternal and paternal DNA regions ensures expression of one allele, while on the other hand, inactivation of the different parental allele is necessary for imprinted genes.

A total of 8 epigenetic regulatory genes, *DNMT1*, *DNMT3A*, *DNMT3B*, *EHMT2*, *ELP3*, *HAT1*, *TET1* and *UHRF1*, were investigated in this chapter. These include three genes from DNA methyltransferase family: *DNMT1*, *DNMT3A*, and *DNMT3B* enzymes. Significantly lower levels of *DNMT3A* and *DNMT3B* were expressed in GV oocytes derived from prepubertal sheep compared to adult GV oocyte counterparts, while no difference was observed in *DNMT1* expression between age groups. A recent study in the same species found the similar trend in which lamb oocytes showed a decrease of global DNA methylation linked to less abundance of *DNMT1*, *DNMT3A*, and *TET3B* in the oocyte but not *DNMT3B* (Fang *et al.*, 2016). As previously reported, both *DNMT3A* and *DNMT3B* were essential for *de novo* methylation in mouse development (Okano *et al.*, 1999). A recent study in human links alterations of *DNMT1*, *DNMT3A* and *DNMT3B* protein expression in the GV and MII oocytes from superovulation cycle to aberration of DNA methylation processes, resulting in oocyte and embryo developmental defects (Uysal *et al.*, 2017). Moreover, premature acceleration of methylation induced by *DNMT3A* and *DNMT3L* leading to the insufficient acquisition of methylation imprints was also demonstrated in non-growing mouse oocytes versus fully grown oocytes derived from wild-type (Hara *et al.*, 2014). In human

oocytes, cytoplasmic DNMT3B as the major DNA methyltransferase, but not DNMT3A and DNMT3L seemed to be responsible to remethylation of all oocyte developmental stages (i.e., GV, MI and MII) (Petruzza *et al.*, 2014) and consistent to the complete acquisition of methylation in fully grown GV oocytes (Anckaert *et al.*, 2013).

The results of the experimental work in this chapter lead to the assumption that the acknowledged poor developmental potential of prepubertal oocytes, to some extent may be caused by an aberration in epigenetic regulation due to insufficient DNA methyltransferase enzymes for establishing *de novo* methylation. In contrast, other genes which are not members of the DNA methyltransferase family showed no differences in expression patterns between two age groups studied. This suggests that the DNMTs family is perhaps the principle regulatory control mechanism of epigenetic regulation within oocytes. Further studies are required a wide range of genes involved in epigenetic regulatory mechanism to confirm this observation. Such defective genes and patterns of DNA methylation, especially in imprinting control regions, in correlation with the developmental potential may be able to prove the hypothesis above.

Besides the epigenetic regulation of imprinted genes, the maternal and culture environments may cause epigenetic interference. Nutrition is one of many external factors influencing epigenetic aberration so called nutritional epigenomics. Studies of the dynamics of epigenetic reprogramming rely on the extensive amount of DNA synthesis at particular periods (Messerschmidt *et al.*, 2014, Smith *et al.*, 2014). Therefore, events that occur during oogenesis and preimplantation embryo development are vulnerable to environmentally induced epigenetic modification.

This study investigated seven genes involved in one-carbon metabolism, the complex metabolic network, to regulate the methylation pathway. The studied genes including *BHMT*, *MAT2B*, *MTHFR*, *MTR*, *PRMT5*, *SHMT1* and *SHMT2*, encoded essential enzymes participating in an integrated metabolic cascade of the folate and methionine cycle, and *trans*-sulfuration pathway. Their principle

roles are to generate a methyl donor, S-adenosylmethionine, which is the universal donor of methyl groups required for DNA methylation, and then transfer to synthesise *de novo* purines and to remethylate methionine (Laanpere *et al.*, 2010, Locasale, 2013). Therefore, in participating in DNA methylation, these enzymes are critically for the epigenetic controls. Alteration of genes expressed may determine the environmental effect on epigenetic regulation and also the maintenance of meiotic arrest vs the resumption of meiosis in the oocyte.

The level of *BHMT* and *MTHFR* gene expression in this chapter are barely detectable in all samples regardless of age and maturity of oocytes whereas the *SHMT1* transcript was detected at very low levels. This may lead to the assumption that the folate-mediated 1C metabolism decline function at the later stage oocyte development, especially in GV-stage oocyte cytoplasm due to almost undetectable *SHMT1*, a key converting enzyme, to metabolite tetrahydrofolate into 5,10-methelene-tetrahydrofolate in folate cycle of oocyte cytoplasm. The remaining four genes- *MTR*, *MAT2B*, *PRMT5* and *SHMT2*, were found to be expressed at higher levels in both GV and MII samples of both age groups. This may indicate that the methionine cycle maintains functions in GV- and MII-stage sheep oocytes regarding methylation mechanism and epigenetic programming. These findings are in agreement with previous studies. Expression of several methylation pathway enzymes related to folate and methionine cycles (i.e., *AHCY*, *MAT2A*, *MAT2B*, *MTHFR*, *MTR*, *SHMT1* and *SHMT2*) have been demonstrated in bovine GV, MII oocytes and throughout all developmental stages of preimplantation embryos whereas *MAT1A* expression disappeared after the first cleavage (Ikeda *et al.*, 2010). This finding is supported by another group who have identified expression of similar key enzymes involved in methylation (i.e., *CBS*, *DHFR*, *GNMT*, *MAT1A*, *MAT2A*, *MAT2B*, *MTHFR*, *MTR*, *SAHH*, and *mSHMT*) as well as folate receptors (i.e., *FOLR1Pdhx1* and *FOLR2*) and the reduced folate carrier (*SLC19A1*) in bovine oocytes and pre-elongation embryos (Kwong *et al.*, 2010). Altogether this data confirms the importance of 1C metabolism within oocytes. Regarding the abundance of transcripts, this is the first report exhibiting genes encoding key enzymes representative of folate and methionine cycles expressed in sheep oocytes.

Of the seven genes related to 1C metabolism, three genes including *MAT2B*, *SHMT2* and *PRMT5* showed differential expression between GV samples derived from prepubertal oocyte and those from adult counterparts. *MAT2B* encodes a regulatory protein associated with MAT2A enzyme forming a hetero-tetramer that catalyses the synthesis of S-adenosyl-methionine (SAM), the primary methyl donor, via an ATP-driven process in the methionine cycle for biological methylation (Legro, 2000, Legro, 2001, Martinez-Chantar *et al.*, 2003, Nordgren *et al.*, 2011). *SHMT2* is essential for the transformation of glycine to serine within mitochondria. Its abundance can affect the proliferation rate of cancer cells (Amelio *et al.*, 2014, Jain *et al.*, 2012, Tomita and Kami, 2012) and porcine embryonic cells (Redel *et al.*, 2016). A previous study using human embryonic stem cells as an epigenetic model investigated a possible mechanism regulating epigenetic modification (Steele *et al.*, 2005). These authors demonstrated that metrotexate induced epigenetic defects by depletion of folate resulted in elevated intracellular homocysteine concentration after seven days in culture and a concomitant increase in cysteine and glutathione, indicating clearance of homocysteine. Disruption of this methylation pathway by introduced exogenous homocysteine or via exposure to a folate antagonist during the earliest stages in bovine oocytes and preimplantation embryos affects hypermethylation of genomic DNA and causes developmental retardation in bovine embryos (Ikeda *et al.*, 2010, Kwong *et al.*, 2010).

PRMT5, a type II arginine methyltransferase, has been linked to gene silencing through the establishment of repressive histone marks (Bedford, 2007, Fabbrizio *et al.*, 2002, Pal *et al.*, 2004, Pollack *et al.*, 1999). Expression of *PRMT5* participates in both early and late stages of developmental processes. In mice, abundance of *Prmt5* expression is evident in the oocyte cytoplasm until the first cellular differentiation (Tee *et al.*, 2010), as well as in undifferentiated embryonic stem cells (Ancelin *et al.*, 2006), suggesting that it is essential for germ cell development and for maintaining pluripotency of mouse ES cells. The current study is the first report to show that oocytes expressed a high level of *PRMT5* in both GV and MII stages in a large ruminant species. This confirms a putative role for *PRMT5* during oogenesis, as stated previously. Moreover, higher abundance

of transcripts found in greater potential adult GV oocytes suggests that apart of DNA methylation as an active regulating epigenetic mechanism during global DNA methylation, another way may control through histone modification.

The presence of several key genes regulating the methylation pathway enzymes supports an essential role of DNA and histone methylation in imprinting acquisition during oocyte development. Apart from insufficient DNA methyltransferases, dysregulation of the imprinting process by interfering methyl donor metabolism can be another ageing mechanism inducing epigenetic aberration leading to decreased oocyte developmental potentials. Therefore, *MAT2B*, *SHMT2* and *PRMT5* genes may be used as markers of oocyte developmental potential.

c) Genes Involved In Oocyte Metabolism And Growth

Glucose is a fundamental metabolic subunit used to satisfy the energy demands of oocytes and embryos, as well as, to be preserved in the form of glycogen storage (Houghton *et al.*, 1996). Apart from its role as an energy source, it also contributes to CC expansion as a precursor of extracellular matrix macromolecules (Heilig *et al.*, 1995, Sutton-McDowall *et al.*, 2010) and can be used for protein glycosylation (Lis and Sharon, 1993). Also, metabolites from the pentose phosphate pathway, ribose-5-phosphate, can be used to synthesise nucleotides.

Several reports observed increase glucose consumption throughout mammalian embryo development (Harris *et al.*, 2005, Leese *et al.*, 1993, Wirtu *et al.*, 2004) (see review (de Souza *et al.*, 2015)); however, the more significant majority of the evidence presented, including that shown in the earlier chapters (Chapter4) of this thesis clearly indicate that glucose may participate in functions other than energy supply during oocyte maturation (Downs *et al.* 1998; Martin and Leese 1999; Zheng *et al.* 2001; Herrick *et al.* 2006), and that pyruvate remains the primary energy source in this cell type. It is well established that the oocyte *per se* has a poor capacity for glucose utilization and relies on CCs conversion of glucose to oocyte utilizable metabolites such as pyruvate or lactate (Biggers *et al.*,

1967, Harris *et al.*, 2007, Rieger and Loskutoff, 1994, Xie *et al.*, 2016). In the cow, oocytes lack hexokinase and phosphofructokinase activities, which are essential rate-limiting enzymes in the glycolytic pathway (Brinster, 1965, Cetica *et al.*, 2002). In contrast, some studies in other species found that oocytes have capacity to take up glucose (Sutton-McDowall *et al.*, 2010, Wang *et al.*, 2012) as well as the machinery such as glucose transport carriers as characterized by the presence of solute carriers *SLC2A1*, *SLC2A3*, *SLC2A8* (Biggers *et al.*, 1967, Saito *et al.*, 1994, Urner and Sakkas, 1999a, Brinster, 1968, Brinster, 1971).

In order to interpret the observation from the previous chapters regarding alterations of energy metabolism in prepubertal GV-stage oocytes, the same cell model was used here to analyse the expression level of mRNA transcripts involved in the metabolism and growth. Nine genes of them were successfully validated by using pooled sheep-oocyte samples (Table 5.7). Except for *PGK*, the rest eight genes related to oocyte metabolism and development were able to be amplified in cDNA samples derived from individual oocytes. Significantly higher levels of studied transcripts were expressed in adult than prepubertal GV oocytes except for *SLC2A1* and *SLC2A3*. These results may imply that the greater potential of adult GV oocytes may link to glucose metabolism, glucose metabolism regulators and apoptotic regulation. This is consistent with earlier reports in which oocytes exhibited the existence of hexokinase (Tsutsumi *et al.*, 1992) glucose-6-phosphate dehydrogenase (Mangia and Epstein, 1975), and the pentose phosphate pathway (Comizzoli *et al.*, 2003, Conti, 2010, Herrick *et al.*, 2006b, Kaniak *et al.*, 2004, Urner and Sakkas, 1999b), as well as the presence of glucose carriers (Augustin *et al.*, 2001, Dan-Goor *et al.*, 1997, Pisani *et al.*, 2008, Zheng *et al.*, 2007). Moreover, the assumption that can be drawn from this work is that differing oocyte competency between prepubertal and adult GV-staged oocytes may be contributed by several molecular mechanisms; energy metabolism related enzymes, glucose and pyruvate transporters, glucose metabolism regulators and apoptosis.

In support of this idea, *SLC2A1* and *SLC2A3* expression regulate two of the significant types of glucose transporters, GLUT1 and GLUT3, in various species at

different stages of development. Previous studies have demonstrated the expression of the *Glut1* isoform in mouse oocytes and preimplantation embryos whereas *Glut3* was expressed only at the blastocyst stage (Chi *et al.*, 1993, Morita *et al.*, 1992, Pantaleon *et al.*, 1997). Likewise, in human, the appearance of GLUT1 rather than other GLUT (i.e., GLUT2, -3 or -4) transporters was detected in oocytes and preimplantation development (Dan-Goor *et al.*, 1997). In contrast, GLUT1, GLUT3 and GLUT8 demonstrated in bovine oocytes throughout preimplantation embryo stages (Augustin *et al.*, 2001). They found strong GLUT1 expression in bovine from early to late stages whereas increasing levels of GLUT3 expression were depicted from weak levels in oocytes and cleavage stages to high levels of expression from morula stages onwards. Also, GLUT1 and GLUT3 were also detected in sheep oocytes, and GCs derived from medium and large follicles (Pisani *et al.*, 2008). Here, the results of the current study exhibit expression of both *SLC2A1* and *SLC2A3* in sheep oocytes, are consistent with previous reports in the ovine and bovine species. The result reflects the capability of sheep oocytes via glucose transporters to take up glucose into cells as the matter of fact that glucose is not the primary substrate for oocyte metabolism as the oocyte *per se* having a low glycolytic rate (Harris *et al.*, 2007, Saito *et al.*, 1994, Xie *et al.*, 2016). This might be explained, on the one hand, that oocyte lacks phosphofructokinase activity (Cetica *et al.*, 2002), on the other hand, glucose transporters exhibiting by the oocyte (i.e., GLUT1 and GLUT3) have lower affinity to glucose compared to GLUT4 that is predominantly on CCs leading to the less efficient process of glucose metabolism within the oocyte (Charron *et al.*, 1989, Nishimoto *et al.*, 2006, Roberts *et al.*, 2004, Williams *et al.*, 2001). Otherwise, the presence of glucose transporters suggests that it is as part of the oocytes cytoplasmic legacy preparing to support glucose metabolism at the later stage of embryonic development as evidence has previously shown that pyruvate is the primary energy supply in eggs (Harris *et al.*, 2007, Harris *et al.*, 2009, Leese, 2015). Also, the expression of glucose transporters differs among species at least between ruminant and non-ruminant animals. The different glucose metabolism of oocytes is dependent on species-specific regulation.

Unfortunately, none of the samples derived from the prepubertal oocytes in this study has detected the *SLC2A1* transcript while almost all of the samples, 5 out of 6, from the adult counterparts showed expression at a very low level. This observation in sheep GV oocytes is consistent with the findings reported in cattle in which a lower level of *SLC2A1* expression was displayed in calves compared to cow oocytes (Diederich *et al.*, 2012, Oropeza *et al.*, 2004). Hence, differential expression of *SLC2A1* between two-age groups may be used to anticipate poor oocyte potential to glucose uptake, as the cytoplasmic legacy passing on to the developing embryo, leading to compromised embryonic development. Previous studies in mice and sheep support the idea that embryos with reduced glucose uptake are linked to down regulation of GLUT1 expression (Heilig *et al.*, 2003, Leoni *et al.*, 2007a). The outcome of this being similar to the result observed in heterozygous *Glut1*-knock out models in mice (Jensen *et al.*, 2006, Wang *et al.*, 2006). Moreover, recent mouse studies have shown that inhibition of GLUT1 function blocked oocyte meiotic resumption (Han *et al.*, 2012, Yan *et al.*, 2014). This may indicate, to some extent, the essential role of *SLC2A1* expression in oocyte maturation and it is perhaps a useful determining marker of oocyte quality.

Although the current work did not demonstrate differential expression of *SLC2A3* gene between age groups, the abundance of *SLC2A3* observed tended to be lower in prepubertal compared to adult GV oocytes. This is in agreement with previous reports in sheep (Pisani *et al.*, 2008), and rhesus monkeys (Zheng *et al.*, 2007), suggesting poor oocyte developmental potential, to some extent, is linked to reduced expression of *GLUT3* glucose transporters. It is also noteworthy that the current study demonstrates expression of *SLC2A3* in GV oocytes which is similar to other previous reports in cattle (Augustin *et al.*, 2001) and sheep (Pisani *et al.*, 2008). The appearance of the *SLC2A3* regulated *GLUT3* glucose transporter may imply that glucose metabolism in ruminant species has a crucial role not only during trophoblastic development (Ganguly *et al.*, 2007, Steeves and Gardner, 1999) but also during oocyte maturation.

Apart from glucose transporters, this work also evaluated the expression of *SLC16A1* that encodes the monocarboxylate transporter-1 (MCT1) protein. This protein function facilitates the reversible diffusion of the monocarboxylate metabolites (i.e., L-lactate, pyruvate and ketone bodies) together with a proton companion through the plasma membrane (Halestrap and Price, 1999). This protein was also characterised as a pH regulator in *Xenopus* oocytes in response to intracellular pH changes (Broer *et al.*, 1998). The presence of MCT1 has been detected in various species. MCT1 was detected in human and mice from GV and MII stage oocytes throughout preimplantation embryo development (Harding *et al.*, 1999, Herubel *et al.*, 2002). Likewise, expression of MCT1 was detected in denuded bovine oocyte and CCs of both immature and mature COC (Lopes *et al.*, 2015).

The current work represents the first report to identify *SLC16A1* expression in sheep oocytes at the GV and MII stages. This observation is consistent with the presence of MCT carriers previously verified in mice, human and bovine. Therefore, appearance of MCT carrier suggests a permissive role of the MCT1 lactate transporter being identical to other species regarding pH regulation and energy production as exposure to the different glucose concentration of mouse embryos cultured *in vitro* altered MCT1 transport activity (Jensen *et al.*, 2006). Moreover, differential expression of *SLC16A1* between GV and MII stages shown in this report may imply the possibility of involvement of this gene in energy production due to high energy consumption during oocyte maturation. This finding suggests the possibility of *SLC16A1* as a marker for determination of oocyte developmental potential.

Expression of some genes involved in signalling pathways related to glucose metabolism was investigated in the current study including *GSK3B*, *IGF1R*, *IGF2BP2*, and *IGF2BP3*. *GSK3* is a highly conserved serine-threonine kinase that was initially described as a key enzyme with multifunctional properties involved in glucose metabolism. Later evidence found *GSK3* to contribute to protein synthesis, cell proliferation and differentiation, program cell death, oocyte and embryo polarity, gene expression and other functions (see reviews in (Cook *et al.*,

1996, Lau *et al.*, 1999, Yao *et al.*, 2002). Being a part of the insulin signaling pathway, *GSK3B* and *AKT* polymorphisms have been shown in PCOS women and are associated with impaired reproductive function and aberration of metabolism as manifest by as insulin resistance and hyperandrogenemia (Goodarzi *et al.*, 2007, Goodarzi *et al.*, 2008). Modification of GSK3B activity by using specific GSK3 inhibitors in bovine pointed to this kinase participating in follicle and oocyte development during folliculogenesis, regulating chromosome segregation during the first meiosis, as well as signalling cumulus expansion and MI/MII transition of the oocyte (Uzbekova *et al.*, 2009). This chapter, however, represents the first report of *GSK3B* expression in both GV- and MII-staged oocytes in ovine species. The significantly lower abundance of the transcript was shown in prepubertal oocytes compared to level expressed in their adult GV counterparts. *GSK3B* function in sheep oocytes may have a similar role to that observed for bovine oocytes. This study also found *GSK3B* transcripts to be more highly expressed in GV than in MII oocytes. This result is consistent with an observation in *Xenopus*, where fully active *GSK3B* was shown to be responsible for maturation arrest in oocytes (Fisher *et al.*, 1999, Guger and Gumbiner, 1995, Sarkissian *et al.*, 2004). In the bovine, a higher level of *GSK3B* protein phosphorylation was found in MII staged oocytes when compared to GV stage oocytes, as well as, delayed meiotic progression and diminished cumulus expansion when *GSK3B* phosphorylation was inhibited (Uzbekova *et al.*, 2009). Thus it can be implied that *GSK3B* may have at least two regulatory roles during oocyte maturation namely protein synthesis and control of meiotic arrest/progression. *GSK3B* expression, therefore, may be a potential indicator to identify oocyte competence.

The insulin/IGF signalling pathway regulates numbers of cell activities including cell proliferation and differentiation, survival, metabolism and reproductive functions (Boura-Halfon and Zick, 2009, Efstratiadis, 1998, Kanzaki and Pessin, 2001, Monget *et al.*, 2002, Nakae *et al.*, 2001). Its modulation orchestrates via members of the IGF family (i.e., insulin, IGF1, and IGF2), IGF receptors (i.e., INSR, IGF1R, and IGF2R), and insulin-like growth factor binding proteins (IGFBPs). The essential role of insulin/IGF action on oocyte and follicular development has been

revealed by several studies. In human, abnormal expression of IGF-like families: *IGF1R*, *IGF2R*, *IGF2BP2* and *IGFBP2*, in CCs from mature COC in PCOS patients correlated with abnormal folliculogenesis and compromised oocyte competence (Haouzi *et al.*, 2012). In addition, female *IGF1*-null mice can survive to adulthood but follicular development arrests at late preantral stage confirm the role of IGF1 during folliculogenesis in this species (Liu *et al.*, 1993, Zhou *et al.*, 1997). Selective expression of *IGF1* in GCs together with the presence of *IGF1R* expressed in both GCs and oocytes of developing follicles in mice and human, suggests a potential paracrine mechanism of action of IGF1 on oocytes (Zhou and Bondy, 1993, Zhou *et al.*, 1991).

The appearance of *IGF1R* expressed in this study emphasises essential IGF1 signalling in response to activation of insulin or IGF1 in sheep oocyte. This is consistent with several previous reports. In the bovine, *IGF1R* expression has been demonstrated by the oocyte throughout embryo developmental stages with the highest level of expression shown at the hatched blastocyst stage (Yaseen *et al.*, 2001). In mice, the *Igf1r* expression has also been demonstrated in oocytes and at all stages of embryo development with decreasing fashion during embryo development (Inzunza *et al.*, 2007). In addition, blocking the IGF1R was shown to be detrimental to embryo development both *in vivo* and *in vitro* leading to reduced cell numbers in mouse embryo (Inzunza *et al.*, 2007, Markham and Kaye, 2003), as well as delayed early and late embryo development (Inzunza *et al.*, 2007). Some conflicting results have been observed. In humans, for example, *IGF1R* mRNA was found to be more abundant in oocytes and early stage embryos but decreased dramatically after 8-cell stages until the formation of early blastocysts (Lighten *et al.*, 1997a). Another study in mice found the pattern of *Igf1r* reversed what report in human, which was expressed from 8-cell until blastocyst stage onwards (Zheng *et al.*, 2007). The temporal expression of *IGF1R* during development indicates that the differential role of IGF1 and IGF1R is both stage and species specific.

Since there is an absence of *IGF1* expression in oocytes and preimplantation embryos in some species (e.g.: buffalo: (Chandra *et al.*, 2011, Daliri *et al.*, 1999);

cattle: (Wang *et al.*, 2009b, Yaseen *et al.*, 2001); human: (Lighten *et al.*, 1997a); rat: (Chandra *et al.*, 2011, Lonergan *et al.*, 2000, Pandey *et al.*, 2009, Yoshida *et al.*, 1998), the biological function of the IGF system in these species is hypothesized to occur via the binding of locally produced IGF2 to the IGF1R protein exhibiting important paracrine-autocrine effects on bovine ovarian physiology (Lucy, 2000, Spicer and Aad, 2007, Wang *et al.*, 2009b). Therefore, the expression of *IGF1R* mRNA in reproductive function suggests critical local roles of the IGF signalling pathway via paracrine regulator rather than autocrine action. Clinical studies have supported the importance of IGF1-IGF1R signalling and have shown that supplementation of IGF1 into IVM and embryo culture medium is beneficial for further development (Chandra *et al.*, 2011, Herrler *et al.*, 1998, Lighten *et al.*, 1997a, Matsui *et al.*, 1995, Palma *et al.*, 1997, Pawshe *et al.*, 1998). A correlation between *IGF1* mRNA expression and subsequent blastocyst formation indicated the potential of *IGF1* mRNA as a marker of embryo development (Kowalik *et al.*, 1999). Thus expression of IGFs and their receptors can be used as potential markers of oocyte and embryo quality (Liu *et al.*, 1997).

Modifying IGF activity not only subjects to the circulation of its ligand, but it also requires the receptor availability (Poretsky *et al.*, 1999). The presence of the IGF2BPs can be another factor modifying the IGF signalling due to a higher affinity to bind IGFs than its receptors (Bunn and Fowlkes, 2003, Brogan *et al.*, 2010). Therefore, increased inactive IGFs-IGF2BPs complexes lead to declining circulating IGFs. Moreover, IGF2BPs play a role in the suppression of steroid production by human GC (Chang *et al.*, 2002, Devoto *et al.*, 1999, Mason *et al.*, 1998, Spicer and Aad, 2007, Wright *et al.*, 2002). Data indicate that the IGF2BPs may play a biological role in insulin/IGF signalling pathway (Bach *et al.*, 2005, Bunn and Fowlkes, 2003, Hwa *et al.*, 1999, Nakae *et al.*, 2001) and in germ-cell development and maturation. This is in agreement with the essential roles of RNA trafficking and translational control of transcripts of some germ-cell-specific genes (Chennathukuzhi *et al.*, 2003, Ruggiu *et al.*, 1997, Schumacher *et al.*, 1995). Herein, this study demonstrates that *IGF2BP2* and *IGF2BP3* are expressed in sheep GV oocytes and that the expression of the *IGF2BP3* transcript is relatively more abundant than of *IGF2BP2* within oocytes. This suggests an important role

of two IGF2BP proteins during oocyte development and may indicate the different potency/affinity of the binding proteins to growth factors. Moreover, this work shows expression of *IGF2BP2* and *IGF2BP3* in GV-staged oocytes, are significantly more abundant in adult than in the prepubertal group, which is consistent with a few published reports (Nielsen *et al.*, 2001, Yaniv and Yisraeli, 2002).

PRDX2, a member of the *PRDX* family, has a crucial function in the modulation of intracellular apoptosis through the clearance of H₂O₂ produced during cell metabolism (Kim *et al.*, 2000). It also orchestrates other cellular functions including protecting protein and lipid against oxidative injury, cell proliferation and differentiation. *PRDXs* were detected in oocytes both before and after IVM. Differential patterns of expression from a continuous profile (*PRDX1* and *PRDX5*) to a delayed (*PRDX4*) or an interrupted expression around the time of embryonic genome activation (*PRDX2*, *PRDX3*, and *PRDX6*) (Leyens *et al.*, 2004a). The role of *PRDX2* as an antioxidant has been reported in older women where lower *PRDX2* expression in CCs is correlated with reduced oocyte development into poor-quality embryos (Lee *et al.*, 2010). The influence of oxidative stress on oocyte maturation and embryonic development (Ferreira *et al.*, 2009, Guerin *et al.*, 2001) has led to investigations of the expression of the peroxiredoxin genes in gametes or embryos. In bovine, the transcripts are detected in oocytes (Dalbies-Tran and Mermillod, 2003, Leyens *et al.*, 2004a), and *PRDX1* and *PRDX2* transcripts were identified as markers of oocyte maturation, as their polyadenylated form exhibited a major decrease during the process (Dalbies-Tran and Mermillod, 2003, Thelie *et al.*, 2009). However, this data was incompatible with another report in porcine, which found up-regulated *PRDX2* in MII stage oocytes (Kim *et al.*, 2011). This perhaps determines the decrease of *PRDX2* expression leading to insufficient antioxidant processes, thereby promoting apoptosis. The inconsistent finding may evaluate the species-specific response. Therefore, *PRDX2* may serve as biomarkers or therapeutic targets for the developmental potential of oocytes.

PRDX2 and *PRDX6* proteins have been reported to be degraded and synthesised, respectively, during IVM (Bhojwani *et al.*, 2006, Leyens *et al.*, 2004b). This supports the hypothesis that a suboptimal molecular maturation of oocytes from prepubertal animals underlying poor developmental competence. Finally, it reinforces the idea that peroxiredoxin genes as candidate markers of developmental potential (Romar *et al.*, 2010). *In situ* hybridization analysis demonstrated that *PRDX2* mRNA was detected in oocytes and theca cells as well as GCs of some antral and preovulatory follicles (Park *et al.*, 2012). The recent observation of *PRDX2* expression in GCs of preovulatory follicles and oocytes agreed with that reported by others (Wang *et al.*, 2010, Yang *et al.*, 2011). The role of *PRDX2* in the ovulatory process is probably to modulate cell cycle progression. *PRDX2* suppression by small interfering RNA in GCs of the mouse ovary inhibits cell proliferation by augmenting H₂O₂ production (Yang *et al.*, 2011). Here, the study demonstrates that lower *PRDX2* is expressed in prepubertal GV oocytes might be an indicator to predict the low potential of these oocytes during oocyte maturation and embryo development. The reasons may be explained by insufficient antioxidants to deal with oxidative stress. Otherwise, increased *PRDX2* in adult GV oocytes can be a compensation mechanism to neutralise the oxidative process after oocyte development.

The differential gene expression in this study relies on the fact that we have compared only six replicates from each group to analyse the impact of either maturational stages or age. The findings may need to be confirmed by further studies with more replicates as well as studies that link molecular analysis to functional performance on the same cells. According to the customised array used, the current work has confirmed the findings that have been reported in other species about the effect of age on various aspects of oocyte quality, and it has introduced the analysis of some novel transcripts relevant to reproductive ageing and cellular senescence in the ovine species. The results generated suggest that more extensive transcriptome analysis such as provided by, for example, next generation sequencing techniques would be invaluable as a means to confirm and extend the current data set on the effect of age on old vs young GV and MII oocyte genes in sheep and other species including humans. Also, in future,

it would be advantageous to analyse tissue from animals of known age and parity rather than tissue derived from the abattoir as used in the current study. Finally, *in vitro* maturation and culture may contribute some confounders in IVM derived MII compared to *in vivo* MII oocytes regarding gene expression and protein production.

5.4.4. Conclusion

In conclusion, this chapter has investigated the effect of maternal age on the molecular regulation in prepubertal and adult oocytes. Among a wide range of genes studied, the evidence from this work clearly demonstrates that alteration of age contributes to changes of several candidate genes, which may determine oocyte competence (*BMP15*, *GTSF1*, *NLRP2*, *OOEP*, *SNRPN*, *MAT2B*, *PRMT5*, *SHMT2*, *DNMT3A*, *DNMT3B*, *GSK3B*, *IGF1R*, *IGF2BP2*, *IGF2BP3*, *PRDX2*, *SLC16A1* and *SLC2A1*), as well as, some key genes which possibly moderate oocyte maturity (*GTSF1*, *NLRP2* and *PRDX2*). Results also show that maternal age-related gene targets have distributed in various functions but the highlight is metabolism and growth pathway and also the 1C metabolism. Furthermore, alteration were also found in genes related to epigenetic modification such as epigenetic regulatory control of imprinted genes and global gene methylation. This study also detected changes in the expression of oocyte specific genes, maternal genetic factors such as SCMC, and some genes governing oocyte oxidative stress. As the prepubertal and adult oocytes model used, in this work, all transcripts expressed in similar trends, with lower levels detected in prepubertal oocytes compared to adult counterparts, indicating that gene transcription defects may cause insufficient molecular functions driving incomplete cytoplasmic maturation instead of DNA damage or oxidative stress in ovarian ageing. Further studies are necessary to conclude a causal association of altered genes related to maternal age linked to biological functions in term of protein analysis and clinical experiment. Also, the new generation of transcriptomic analysis may enhance our knowledge of the molecular basis underpinning age differing oocyte competence.

CHAPTER 6: AGEING AND CELLULAR MARKERS: EVALUATION OF OOCYTE MITOCHONDRIAL DNA COPY NUMBER AND ENERGY METABOLISM

6.1. INTRODUCTION

The most important role of mitochondria within oocytes is to provide cellular energy in the form of ATP to drive energy-requiring cellular activities during oocyte maturation and fertilization until blastocyst implantation. During maturation, oocytes need to acquire sufficient amounts of ATP to complete meiotic and cytoplasmic maturation and subsequent fertilization. In addition, mitochondria also play essential roles in metabolisms, calcium homeostasis, fatty acid oxidation and regulation of programmed cell death, apoptosis (Balaban *et al.*, 2005, Dumollard *et al.*, 2007b, Naviaux and McGowan, 2000). Because mitochondrial replication ceases when oocytes matured at ovulation (Piko and Taylor, 1987), fertilized oocytes and preimplantation embryos rely on the accumulation of oocyte mitochondrial pool to generate ATP via pathways of oxidative phosphorylation to meet the cellular energy demands for supportive embryo viability and further development.

Oxygen consumption has been regarded as the best determinant of global metabolic activity (Leese, 2003) and this parameter can be used for oocyte and embryo quality assessment (Overstrom *et al.*, 1989, Barnett and Bavister, 1996, Houghton *et al.*, 1996, Lopes *et al.*, 2007, Scott *et al.*, 2008). Mitochondria consume oxygen for the ATP generation via oxidative phosphorylation. The principle of cellular energy required for oocyte maturation during follicular development relies on the number and activity of mitochondria present in the ooplasm (Jansen and de Boer, 1998, Motta *et al.*, 2000). Reports suggest mitochondria link to oocyte developmental competence (El Shourbagy *et al.*, 2006, Reader *et al.*, 2015, Tarazona *et al.*, 2006, Van Blerkom, 2009). Both mitochondrial quality and quantity are considered as promising markers of oocyte quality (Reynier *et al.*, 2001, Santos *et al.*, 2006, Stojkovic *et al.*, 2001). The degree of mitochondrial activity and levels of ATP production in bovine oocytes

have been shown to increase during IVM (Brevini *et al.*, 2007, Gutnisky *et al.*, 2013, Stojkovic *et al.*, 2001, Tarazona *et al.*, 2006). In addition, the quality of mitochondrial ATP generation is essential for sustaining sperm-triggered calcium oscillations to initiate the quiescent mouse oocyte development (Dumollard *et al.*, 2004). Current evidence supports the idea that highly competent oocytes have a greater mtDNA copy number than less competent oocytes. In human and pig, lower mtDNA copy numbers were observed in failed fertilization oocytes compared to normal fertilization cohorts (El Shourbagy *et al.*, 2006, Reynier *et al.*, 2001, Santos *et al.*, 2006, Wai *et al.*, 2010). Moreover, data from other species showed greater mtDNA copy numbers in oocytes with better developmental potential, such as rat oocytes from larger follicles (Zeng *et al.*, 2009), mouse oocytes from a natural cycle as opposed to cohorts from maturation *in vitro* or ovarian hyperstimulation cycles (Ge *et al.*, 2012a), as well as pigs' oocytes with BCB+ (El Shourbagy *et al.*, 2006). On the other hand, mouse oocytes with reducing mtDNA copy number decrease blastocyst rates (Ge *et al.*, 2012b).

In humans, maternal age and oocyte mtDNA copy number have been shown to be negatively correlated (Chan *et al.*, 2005), and oocyte samples from women with ovarian insufficiency were found to have fewer copies of mtDNA (May-Panloup *et al.*, 2005a). In fact, a critical threshold of mtDNA is thought to be required for successful fertilization and early embryonic development, and this threshold is estimated at about 100,000 copies of mtDNA in a mature oocyte for the mouse (Piko and Taylor, 1987), pig (El Shourbagy *et al.*, 2006), and human (Reynier *et al.*, 2001, Santos *et al.*, 2006).

Energy metabolism is vital for oocyte maturation because the dynamic process requires a high energy supply. A wide range of substrates can be metabolised by oocytes for ATP generation such as pyruvate, glucose, oxygen, fatty acids, amino acids, purines and pyrimidines (Collado-Fernandez *et al.*, 2012, Songsasen *et al.*, 2012). Only exogenous energy substrates, i.e. glucose, pyruvate and lactate, are commonly thought the main source of energy metabolism (Berger and Wilde, 2013, Harris *et al.*, 2009, Van Blerkom, 2000, Wilding *et al.*, 2001, Wilding *et al.*, 2002) despite the fact that the ATP synthesis through glycolysis is far less

efficient than through mitochondrial OXPHOS. Moreover, other endogenous stores including glycogen, proteins and lipids also can be potential sources of ATP production. It is therefore essential that abundant mtDNA molecules are present at fertilization to maintain embryo survival until mtDNA replication (Margineantu *et al.*, 2002).

Various tools have been used to evaluate mitochondrial metabolism both directly and indirectly as a means to predict oocyte competence. Measurement of ATP production is a direct measurement of the by-products of the energy supply chain, which links mitochondrial function to oocyte developmental potential (Dalton and Carroll, 2013, Nagano *et al.*, 2006b, Thouas *et al.*, 2004, Van Blerkom and Davis, 2006). Mammalian oocytes and preimplantation embryos prior to embryonic genome activation utilize pyruvate as energy source but not glucose and lactate (Bavister and Squirrell, 2000, Butcher *et al.*, 1998). In the absence of CCs, glucose and lactate utilization are not possible whereas pyruvate utilized through mitochondrial electron transport to sustain ooplasmic maturation by oocyte *per se* may not supply sufficient energy to support embryo development until the blastocyst stage (Dumollard *et al.*, 2007b, Wilding *et al.*, 2009). This suggested that both aerobic and anaerobic respiration contribute to the production of energy in mammalian oocytes/embryos (Dumollard *et al.*, 2007b, Wilding *et al.*, 2009). However, a recent study suggested beneficial effects of glucose metabolism on ooplasmic maturation mediated through glycolytic pathways for energy supply, and facilitating the pentose-phosphate pathway to reduce oxidative stress and to provide fructose-6-phosphate for glycolysis (Xie *et al.*, 2016). Therefore, metabolism of energetic substrates may reflect numbers of mitochondria and mtDNA with regard to ATP production. Collectively, the evidence based on these studies suggests that both mtDNA replication and/or mitochondrial metabolic activity may be used as markers of oocyte quality (May-Panloup *et al.*, 2005a, Santos *et al.*, 2006, Shoubridge and Wai, 2007).

The mechanism of oocyte ageing still remains largely unknown. Both the prolonged process of deterioration through time combined with progressive damage due to environmental exposure may lead to impairment of the

physiological function of the oocytes and/or surrounding somatic cells (Chan *et al.*, 2005, de Bruin *et al.*, 2004, Tarin, 1996, Zhang *et al.*, 2006). Consequently, alterations of morphological or molecular characteristics can be demonstrated in aged oocyte (Miao *et al.*, 2009a). Several theories proposed to explain the mechanisms underpinned oocyte ageing including shortening of telomeres, free radical-induced damage, defective DNA-repair mechanisms, accumulation of mutations in the nuclear genome, and mitochondrial dysfunction (Prasad *et al.*, 2015, Takahashi *et al.*, 2013). As a key role in energy supply, alteration of mitochondrial function seems to be one of the hallmarks of ageing. The underlying mechanisms of ageing, which contribute to mitochondrial dysfunction and decreased fertility, however, have yet to be elucidated.

Aims and Objectives

The aim of this chapter was to review whether mtDNA copy number can be used as a cellular marker to determine the quality of sheep oocytes derived from prepubertal and reproductive age.

The specific objectives were:

- a) To determine the range of mtDNA copy number, a surrogate of mitochondrial number of immature and mature sheep oocytes in *in vitro*.
- b) To examine the impact of reproductive age and maturational stage on the oocyte mtDNA copy number.
- c) To identify the link between the oocyte mtDNA copy number and energy substrate metabolism.
- d) To determine the effect of culture media on the mtDNA copy number.

6.2. MATERIALS AND METHODS

6.2.1. Experimental Design

The methodology used to differentiate between oocytes from prepubertal and adult animals was as detailed in section 2.7. Oocytes were recovered from both age group and cultured using the IVM protocol for group culture in section 2.10. Serum-free IVM medium was used for all incubations detailed. Oocyte AAP and GPL incubations were conducted to assess glucose and pyruvate utilization, as

well as lactate production as shown in sections 2.11 and 2.12, respectively. A series of 2 experiments was conducted.

Experiment 1: Quantitation Of Mitochondrial DNA Copy Number Of Immature And Mature Ovine Oocytes Harvested From Prepubertal And Adult Sheep

Samples used in the first experiment were collected from abattoir-derived sheep ovaries during the breeding season (October-November 2014-2015) and non-breeding season (May-June 2014-2015), respectively. In brief, tissues were selected according to the reproductive tract size and the presence of 2 or more CL to determine adult ovarian status. After tissues were separated, COCs were aspirated, oocytes enclosed within at least 3 cumulus layers were obtained and randomly divided into 2 groups; Time 0 (GV) and Time 24 (MII) hours.

Single oocytes at GV or MII stages of maturation used to quantify mtDNA copy number were prepared as detailed in section 2.7. Once COCs had been harvested, all oocytes were disaggregated from their CCs by placing in holding medium (H199+) containing Hyase® for 30-60 seconds before repeat pipetting up and down through narrow glass striper between 130-133µm and 156-190µm diameter (Swemed®). After denudation oocytes were confirmed by inverted microscopy before samples were stored for later molecular analysis. Each denuded oocyte was washed twice in DPBS (Dulbecco's Phosphate Buffer Saline; Gibco®, 14190136) prior to transfer into a thin walled DNase/RNase 0.5mL PCR tube (Starlab®, I1405) containing 10µL of 1XPBS solution (see section 2.86 for preparation) by a glass pipette diameter 130-133µm (Swemed®) in order to minimize volume transfer in the storage tube. Oocyte samples were immediately snap frozen into prepared containers filled with liquid nitrogen before storing in an -80°C freezer for future analysis.

A second group of COCs were cultured in IVM medium and incubated under 5% CO₂ in air condition for 24 hours (see details in chapter 2). After IVM, oocytes were denuded to confirm the stage of maturation according to the presence of the first polar body. Individual MII oocytes were collected in individual PCR tubes as

described above. This study was conducted to (i) estimate the actual number (amount) of mtDNA in each sheep oocyte and to assess the normal variation of mtDNA copy in both immature and mature stages of oocyte in this species; (ii) to assess the effect of age on mtDNA copy number in both immature and mature stages of oocytes; and (iii) to determine the difference of replication capacity between prepubertal and adult oocytes.

Experiment 2: Measurement Of Energy Metabolism And Mitochondrial DNA Copy Number In Prepubertal And Adult Ovine Oocytes

In the second experiment, carbohydrate metabolism including glucose, pyruvate and lactate, and mtDNA copy number were directly linked by quantification of all 4 parameters in the same individual oocyte. The purposes of study were (i) to study the impact of age on oocyte quality in terms of energy production; (ii) to determine whether energy metabolism could be used as an index of oocyte quality; and (iii) to address whether alterations in mtDNA copy number were responsible for alterations in carbohydrate metabolism. Samples collected from abattoir-derived sheep reproductive tissues were used for this experiment as described above. Oocytes then were immediately freed from their cumulus shells by gentle repeat pipetting up and down using narrow borosilicate glass stripers (Swemed®) of size range from 130-133µm to 156 -190µm to ensure that oocyte carbohydrate utilization and production were entirely attributed to changes of spent-culture media components in comparison to controlled incubated GPL media from the same dish as described in detail in chapter 2. Each individual denuded oocyte was transferred into an individual drop of pre-warmed GPL media which was incubated at 38.5°C in 5% CO₂ in air. After 6 hours of culture, individual oocytes were collected and stored in individual PCR tubes containing 10µl of PBS and snap frozen in liquid nitrogen before storage at -80°C for further mtDNA copy number analysis. Spent culture dishes were sealed and kept frozen at -80°C for later GPL assay.

Experiment 3: The Impact Of Oocyte Culture Media Composition On Mitochondrial DNA Copy Number

A number of studies have reported an exponential replication of mitochondria in various species during oogenesis (Duran *et al.*, 2011, Iwata *et al.*, 2011, Mao *et al.*, 2012, Zeng *et al.*, 2007). Mitochondrial DNA copy number has been linked to oocyte quality (Otten and Smeets, 2015, Takahashi *et al.*, 2013), and it may be influenced by the culture environment as well. This evidence suggests that mtDNA copy number may be useful as a parameter to improve the quality of culture medium for oocyte maturation *in vitro*. The third experiment therefore aimed to examine the effect of different culture media; AAP medium vs balanced salt solution based (GPL) medium vs IVM on the rate of mitochondrial replication in ovine oocytes.

6.2.2. Oocyte DNA Extraction And Analysis

a) Plasmid Spike Construction

Before molecular analysis, oocyte samples were lysed to release genetic materials. During the process, a plasmid DNA sample was used as an internal reference to control the efficiency of the oocyte DNA extraction that may compromise the total amount of mtDNA copy number which might result in reduced amounts of mtDNA to be quantified and subsequently give an incorrect estimation of the actual amount present. The plasmid-spike sample has thus been developed to use as an internal control to determine the efficiency of DNA extraction.

The pGEM[®]-T Easy Vector System I cloning kit (Promega[®], A1360) was used to ligate 500bp of bovine Pfa112b cDNA into a 3kb pGEM[®]-T Easy Vector. The vector was then inserted into *E. coli* for amplification. The ligation reaction mix was composed of: 1) 5 µl of 2X Rapid Ligation Buffer, T4 DNA Ligase, 2) 1 µl of pGEM[®]-T Easy Vector (50ng), 3) 1 µl of T4 DNA Ligase (3 Weiss units/ µL), 4) 120ng bovine Pfa112b cDNA. The volume was made up to 10µL with sterile water. 1µl of pGEM[®]-T Easy Vector (50ng) was added before incubation at room temperature for 1 hour to allow target DNA ligation into the vector. The tubes containing the pGEM[®]-T Easy Vector ligation reactions were centrifuged to

collect the pellet and then 2 μ L of ligation reaction was added to a sterile polypropylene tube (or a 1.5ml microcentrifuge tube) on ice. 50 μ L of the pGEM[®]-T Easy Vector ligation cells was carefully transferred into each setup tube and the tubes were gently flicked to mix before being placed on ice for 20 minutes. The cells were then heat shocked for 45–50 seconds in a water bath at exactly 42°C and the tubes were promptly returned to ice for a further 2 minutes before 950 μ L room-temperature SOC medium was added to the tubes containing transformed cells and the tubes were held for 1.5 hours at 37°C with horizontal shaking. The *E. coli* containing plasmid vector was transferred to 50ml polypropylene tubes (Falcon[®] Cat.No. 2098). Tube lids were screwed on loosely and the tubes were gently shaken horizontally at 37°C overnight.

b) Plasmid Spike Extraction From *E. Coli*

The 50ml Falcon tubes containing *E. coli* were centrifuged at 2000 rpm for 10 mins to spin down the cells. Plasmid spike DNA was extracted using the QIA prep miniprep kit (Qiagen[®], 27104). The protocol was used according to manufacturer instruction. Pellets were resuspended in 250 μ l buffer P1 to lyse the cells. 250 μ l buffer P2 was added and the tubes were inverted 4 to 6 times to mix the solutions. 350 μ l buffer N3 was added to neutralize the acidic lysis solutions and the mixture was centrifuged for 10 mins at 13000 rpm. The resulting supernatant was placed inside a Qiagen spin column and centrifuged to bind the plasmid DNA to the filter. Buffer PB was washed through the filter to remove nucleases followed by buffer PE supplied in the Qiaprep miniprep kit to remove other impurities. The column was then centrifuged again to remove traces of buffer PE. The DNA was eluted into a clean tube using 30 μ l elution buffer. DNA concentrations in 1 μ l were measured by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. USA).

DNA extracted from the transformed *E. coli* was used as a template for PCR using forward (GGCGCTTTCTCATAGCTCAC) and reverse (AGTCGTGTCTTACCGGGTTG) M13 primers, which amplify a region of the pGEM[®]-T Easy cloning vector containing the inserted sequence. Agarose gel electrophoresis was used to confirm the presence of an insert of the correct size. Conventional PCR with

primers specific for the insert sequence was also carried out to confirm insertion of the correct 151bp product. To aid quantification of mtDNA copy number in sheep oocytes, the plasmid vector spike was added into each oocyte sample before the cell lysis process and PCR reaction in order to amplify as endogenous control alongside the oocyte mtDNA.

c) Oocyte Lysis Protocol

Efficient sample lysis was needed to release the DNA out of the oocyte membrane. A lysis cocktail containing 2X PCR buffer with 1% (v/v) Triton X-100 and 200µg/ml proteinase K was freshly made before each use. The reagents used are detailed in the following Table 6.1.

Table 6.1. Composition of lytic cocktail. This is used for 0.5mL preparation.

Reagent/supplier/ catalogue No.	Stock Concentration	Amount for 0.5mL final volume	Final working concentration
DNase/RNase H ₂ O	-	390µL	-
10XPCR buffer (Sigma, P2317)	10X	100µL	2X
Triton X-100	100	5µL	1%
Proteinase K (Invitrogen, 25530049)	20mg/mL	5µL	200µg/mL

Prior to DNA extraction, and immediately after each oocyte sample had thawed at room temperature, 1µL of plasmid-spike was added to each tube at a concentration of 8pg/µl. This obligatory step was employed to estimate the degree of DNA damage and to represent as an exogenous reference for all real-time PCR reactions. After that 10µL of a prepared lysis cocktail was added into each sample before processing by a programmed thermocycler (Applied Biosystems, Veriti® 96-Well Thermal Cycler, MA, USA) using a 2-step protocol, in which samples were heating up and maintaining at 55°C for 30 minutes, followed by warming to 95°C for 5 minutes. The first step activated the proteinase K enzyme to ensure lysis of the oocyte cell membrane and mitochondrial envelope, whilst the latter was to denature the proteinase K enzyme.

6.2.3. Quantification Of Mitochondrial DNA Copy Number Using Real-Time PCR

According to literature oocyte mitochondria contain only 1-2 mitochondrial genomes, thus mtDNA copy number can represent the amount of actual mitochondria (Chiaratti and Meirelles, 2010, Jiao *et al.*, 2007, Piko and Matsumoto, 1976, Piko and Taylor, 1987, Santos *et al.*, 2006). Several quantitative methods have been used to determine mtDNA including dot blot hybridization and densitometric measurements of autoradiographs (Piko and Taylor, 1987), competitive PCR methods have permitted the examination of mtDNA molecules in individual human oocytes (Chen *et al.*, 1995). Since the establishment of real-time PCR this assay to quantify the number of mtDNA, has become well-accepted due to the high sensitivity and minimizing the need for nested amplifications (Wittwer and Garling, 1991, Wittwer *et al.*, 1997). The real-time PCR protocol used in this thesis has been developed by our own group at the University of Leeds (Cotterill *et al.*, 2012, Cotterill *et al.*, 2013).

a) Generation Of Exogenous Mitochondrial DNA And Plasmid-Spike DNA Standards For Real-Time PCR And Standard Curve Preparation

The external mtDNA standard was prepared from a 165 bp fragment of the ovine mitochondrion-encoded cytochrome *C* oxidase I (COI) region in the mitochondrial genome by using whole-genomic DNA isolated from ovine oocytes. The forward primer 5' acgtcgatacacgggcttac 3' and the reverse primer 5' agcctccgactgtgaaaaga 3' (accession number: AF010406.1) were used to generate the COI template by PCR in a 100µL reaction containing 1µL DNA, 10µL of 10X PCR buffer, 3µL MgCl₂ (50mM), 0.5 µL Taq Polymerase (5U/µL) (All supplied by BIOTAQ Polymerase Kit, Bioline Ltd, London, UK), 1µL of each primer (10µM/µL), 2µL dNTP (10mM) and 81.5µL H₂O. The PCR conditions included 95°C for 5 min for 1 cycle, 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, 72°C for 5 min and maintained at 4°C. The PCR product was confirmed by DNA fragment size demonstrated on a 2% (w/v) agarose gel while DNA concentration and quality were measured using a nanodrop ND 1000 spectrophotometer (Thermo-Scientific, Wilmington, USA). The standard curve was constructed using 10-fold

serial dilutions (0.2–0.000002 ng/ μ L) of the 165 bp product of mtDNA to quantify each experimental sample.

A standard curve of the plasmid-spike was generated to determine the extraction efficiency and to ensure the PCR condition attained as an exogenous control. A construct of plasmid-spike template containing a bovine gametocyte-specific factor 1 (GTSF1 or Protein FAM112B) cDNA (167bp) insertion within pGEM®-T-easy vector 3.015kb from section 6.2.2 was prepared in 10-fold serial dilutions (0.4-0.000004ng/ μ L) of a total size plasmid-spike DNA about 3.5kb. However, only part of GTSF1 gene was amplified during quantitative PCR assays due to the obligatory primer sequences; 5' ggcgctttctcatagctcac 3' (forward) and 5' agtcgtgtccttaccgggttg 3' (reverse). The given fragment size of PCR product is 151bp.

b) Real-Time PCR for Quantification Of Mitochondrial DNA Copy Number In Individual Sheep Oocytes

Real-time PCR analysis was performed using 96-well plates (Starlab®, UK) by an ABI7900HT PCR analyser (Applied Biosystems, CA, USA) using SYBR green technology. Each 25 μ L RT-PCR reaction volume comprised of 12.5 μ L of SYBR green master mix (Applied Biosystems), 10.5 μ L H₂O, 1 μ L specific mixed primers (forward and reverse, 10nM each), and 1 μ L of lysed sample. The PCR conditions were setup including initial denaturation at 95°C for 10 minutes followed by 40 cycles of a denaturation step of 15 seconds at 95°C, annealing and extension for 60 seconds at 60°C. A melting curve was routinely included to assure the quality of each amplicon and that no mispriming was detected. All samples from both age groups across all experiments as well as negative controls were run in triplicate, and then average values per sample were used for statistical analysis.

c) Extraction Efficiency

Extraction efficiency of individual PCR reaction was evaluated by comparing actual plasmid-spike Ct detected in each reaction with the Ct reference from the standard curve. The Ct difference of plasmid-spike (Δ Ct) was calculated by subtraction of the actual reading Ct value from the Ct reference and the Δ Ct value

was later used to estimate the extent of DNA reduction during sample processing since cell lysis. Finally, each actual reading of COI Ct for each individual reaction was corrected according to the Δ Ct value of the plasmid-spike in order to precisely estimate mtDNA copy number for each sample. After correction, the normalized COI Ct value was used to calculate mtDNA copy number in the next step.

d) Calculation Of Oocyte Mitochondrial DNA Copy Number

The normalized COI Ct value of each sample was converted into the concentration amount of COI DNA using the given formula from the COI standard curve prepared in section 6.2.3.a. According to an average molecular weight, 1 base pair of DNA in sodium salt is equivalent to 650 daltons (g/mol), therefore the molecular weight of double stranded DNA of the COI gene is equal to 107,250 (165X by 650) g/mol. Regarding the Avogadro's number, 1 mole of substance has 6.022×10^{23} molecules; thus it can be implied that 1 molecule of 165bp-COI PCR product is equivalent to 1.78×10^{-4} fg, which represents a single mitochondrial copy. As a result, mtDNA copy number was calculated by dividing the absolute quantity of sample PCR product derived from the COI standard curve equation in fg by 1.78×10^{-4} .

6.2.4. Statistical Analysis

The number of mitochondria per oocyte was calculated for both prepubertal and adult ewe oocytes at each different time point. All statistical analyses were performed using GraphPad Prism6 Software. Normal distribution was tested for the entire data set using both Shapiro-Wilk and D'Agostino & Pearson normality tests. Data that were not normally distributed were log transformed if possible prior to analysis using unpaired Student's t-test or ANOVA as appropriate; otherwise, nonparametric tests such as Mann-Whitney *U* or Kruskal-Wallis tests were applied. The values presented are means \pm SEM or median \pm range for the number of samples shown. In all analyses, a *P* value of <0.05 was considered to be statistically significant.

6.3. RESULTS

6.3.1. Experiment 1: Quantitation Of Mitochondrial DNA Copy Number Of Immature And Mature Oocytes Harvested From Prepubertal And Adult Sheep

In this experiment, samples were divided into four different groups according to animal age and the stage of oocyte maturity: immature prepubertal (lamb GV), mature prepubertal (lamb MII), immature adult (adult GV) and mature adult (adult MII) oocytes. A total of 180 oocytes samples were processed for mtDNA copy number quantification, results from 177 sample were included in the analysis. Three samples excluded from the analysis were eliminated due to an erroneous Ct reading at the same location, this included 1 sample each from the lamb GV, adult GV and adult MII groups. As a result of the observed high variability of mtDNA copy number in these studies, the data was not normally distributed, therefore, the results are presented as median and range.

Study of the effect of the stage of oocyte maturation revealed that MII oocytes in both age groups had greater mtDNA copy number than immature GV oocytes. The numbers of mtDNA in MII oocytes from lambs and adults were 674,779 (2,245-9,852,000) (median and range) and 290,871 (4,085-3,754,000), respectively. In contrast, in immature oocytes, mtDNA copy per oocyte in lamb GV oocytes was estimated to be 88,619 (2,393-4,358,000) compared to 76,835 (454-5,658,000) in adult GV oocytes. Differences in mtDNA copy number between GV and MII oocytes reached statistical significance regardless of animal age; lamb ($P < 0.0001$) and adult ($P = 0.0031$) as shown in Table 6.2.

The effect of animal age on mtDNA copy number per oocyte from lamb vs adult was analysed. Only a significant difference in mtDNA copy number between the 2 animal-ages was detected in mature MII-staged oocytes 674,779 (2,245-9,852,000) in lamb vs. 290,871 (4,085-3,754,000) in adult; $P = 0.0006$). No significant difference was found in GV-staged oocytes when the two age groups were compared 88,619 (2,393-4,358,000) in lamb vs. 76,835 (454-5,658,000) in adult; $P > 0.05$) (Table 6.2). In adult age, mature MII oocytes showed 3.78 times higher mtDNA copy numbers

than in immature GV oocytes, while 7.61 times higher mtDNA copy numbers were detected in the prepubertal counterpart.

Table 6.2. Quantification of mtDNA copy number in denuded GV and MII oocytes derived from prepubertal and adult sheep

Group	Oocyte maturity	No. Samples	Mean \pm SEM	Median	Range	P-value
Lamb	GV	44	588,917 \pm 156,646	88,619	2,393-4,358,000	<0.0001
	MI	45	1,964,000 \pm 390,928	674,779	2,245-9,852,000	
Adult	GV	44	369,399 \pm 135,791	76,835	454-5,658,000	0.0031
	MI	44	439,605 \pm 90,755	290,871	4,085-3,754,000	

6.3.2. Experiment 2: Measurement Of Energy Metabolism And Mitochondrial DNA Copy Number In Prepubertal And Adult Sheep Oocytes

All samples used for mtDNA quantification in this experiment were derived from abattoir tissues according to the seasonal breeding and animal-age as detailed earlier and underwent 6 hours incubation in GPL media. The GPL assay data linked to the same samples in this molecular analysis have been presented in Chapter 4 section 4.3.2. Quantitative PCR for mtDNA copy number of the stored cells was conducted here on a total of 90 samples (45 from each age group); however, only 88 samples generated results that were included in this analysis; 2 samples were excluded from the lamb dataset due to the erroneous Ct reading by the analyser or a pipetting error.

In addition to the control T0 and T24 time points analysed in the previous experiment, the effect of animal age was also considered in samples used following carbohydrate metabolism assay. In this experiment, prepubertal oocytes after 6 hours in GPL incubation had significantly higher mtDNA copy number than adult oocytes at the equivalent time point in the same media, which were 1,210,000 (1,455-5,390,000) and 479,970 (724-2,420,000), respectively (P=0.0061). Results from the GPL assays are shown in Table 6.3. Prepubertal oocytes had significantly less pyruvate consumption and less lactate production in comparison to those from adult animals; P<0.001 and P=0.014, respectively,

whereas no difference was detected in glucose consumption between the 2 groups (Table 6.3). In comparison to all GPL-assay samples, data were consistent in term of less pyruvate consumption while they were inconsistent if glucose or lactate was considered. For 1/3 of lamb and adult samples, glucose consumption was below the limit of detection of the assay. Glycolytic index was calculated to assess efficiency of glucose metabolism, though in this case this may not be appropriate since 1 third of the samples in both lamb and adult sheep showed zero glucose consumption (14 out of 42; 33.3% and 14 out of 44; 31.8%, respectively). Figure 6.1 shows that no correlation was found between mtDNA copy number and glucose or pyruvate consumption for either groups, lamb ($r^2= 0.003$ and 0.156) and adult sheep ($r^2= 0.048$ and 0.139), the same applied to lactate production in both animal ages ($r^2= 0.001$ and 0.019).

Table 6.3. Glucose and pyruvate and lactate consumption/production (pmol/oocyte/H) by prepubertal and adult sheep oocytes.

	Lamb (n=42)			Adult (n=44)			P-value
	Mean \pm SEM	Median	Range	Mean \pm SEM	Median	Range	
Glucose	0.45 \pm 0.10	0.25	0 - 2.13	0.43 \pm 0.07	0.32	0 - 1.67	0.677
Pyruvate	5.64 \pm 0.32	6.61	0.98-7.89	7.59 \pm 0.37	8.43	0.83-10.30	<0.001
Lactate	1.61 \pm 0.17	1.52	0.10-6.49	2.20 \pm 0.21	2.07	0 - 6.63	0.014

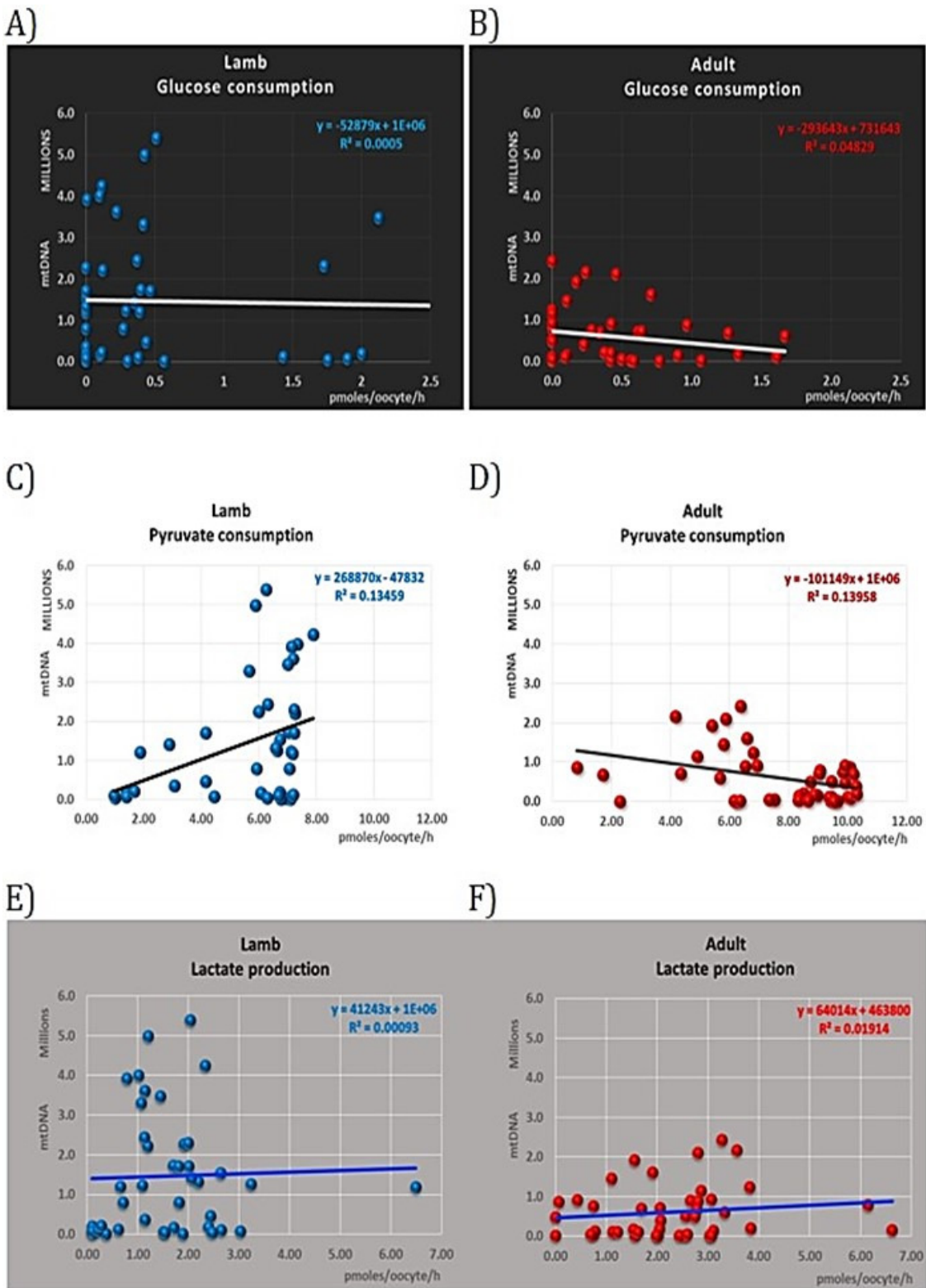


Figure 6.1. Correlation between mtDNA copy number and each parameter of energy metabolism per oocyte (pmol/oocyte/hr). Glucose (A and B), pyruvate (C and D) consumption and lactate production (E and F) compared in both age groups.

6.3.3. Experiment 3: The Impact Of Culture Media On Oocyte Mitochondrial DNA Copy Number

During oocyte maturation, mtDNA copy number was analysed with regard to culture media and duration of incubation by using GV samples without incubation as the time 0 (T0) control. In AAP and GPL groups, the samples were incubated for only 6 hours (T6-AAP and T6-GPL) while MII oocytes recruited from samples incubated 24 hours in serum-free IVM media (T24). Data shown on Figure 6.2 demonstrate significant differences in mtDNA copy number when oocytes incubated in different media were compared with T0 controls regardless of animal age ($p < 0.05$). Moreover, Figure 6.2 also shows differences in mtDNA copy number between two age groups in samples cultured in GPL medium for 6 hours and the MII samples derived from 24 hours incubation in serum free IVM medium. Oocytes cultured in AAP medium depicted the highest mtDNA copy numbers followed by oocytes cultured in GPL medium and serum-free IVM medium. Significant differences ($p < 0.05$) were detected when mtDNA copy number of samples cultured in AAP medium were compared to samples cultured in other culture media (Figure 6.3). No significant differences were detected in mtDNA copy number between samples incubated 6 hours in GPL medium (T6-GPL) and their MII sample (T24) counterparts (Figure 6.3).

Proportions of mtDNA copy number in different culture media were calculated by using the median mtDNA copy number of GV oocytes at the T0 control as reference. AAP medium demonstrated the highest ratio followed by GPL and serum-free IVM media, which were 99.3, 13.7 and 7.6 in lamb and 94.0, 6.2 and 3.8 in adult sheep respectively. Despite the fact that the culture period in AAP and GPL media was 18 hours shorter, these media supported the replication of mtDNA copy number better than the usual oocyte maturation *in vitro* using serum-free IVM medium. These data suggest that mtDNA replication occurs rapidly after the onset of culture during final oocyte maturation.

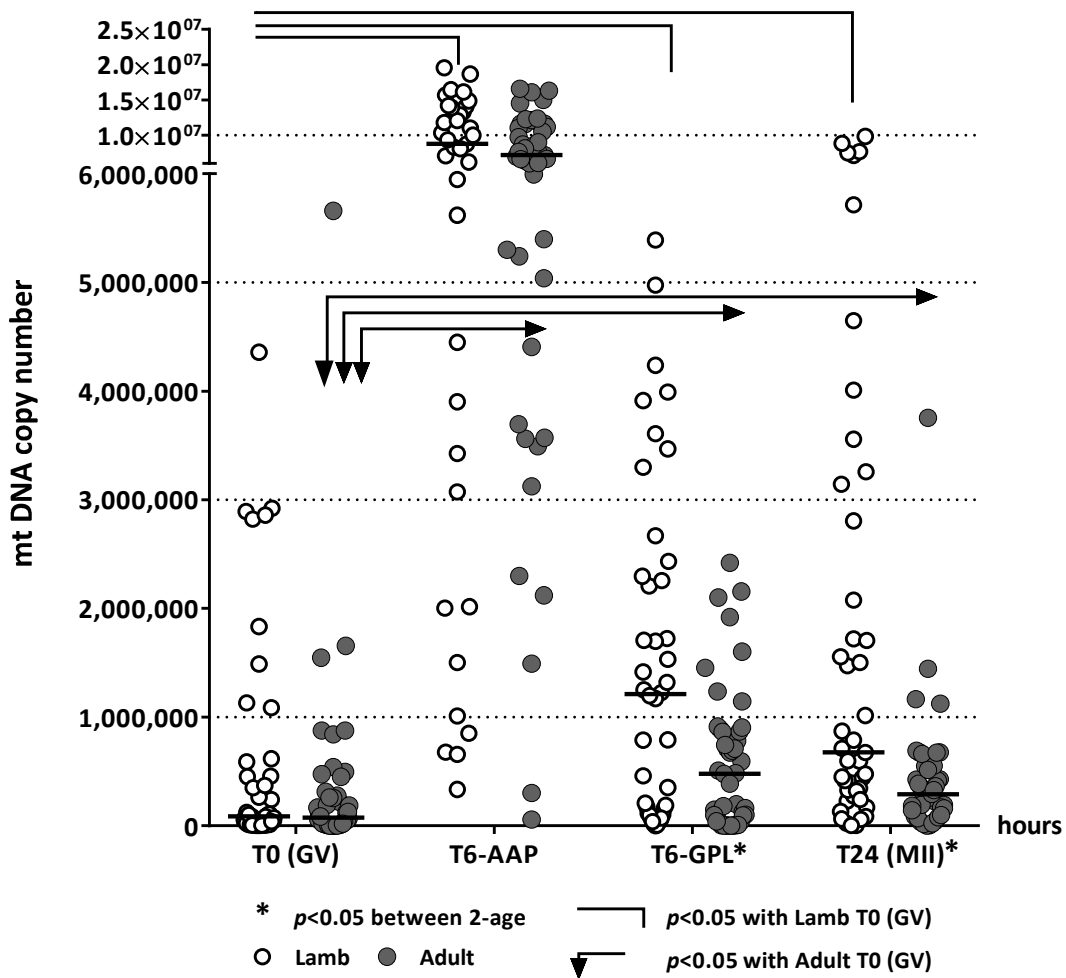


Figure 6.2. Comparison mtDNA copy number postincubation in different culture media in lamb (○) and adult (●) sheep oocytes. Data shown are the actual mtDNA copy number of individual oocytes with the median of each data group represented by black bar (—) on top of each column. T0 and T24 denote GV- and MII- stage of oocytes while T6-AAP and T6-GPL represented oocytes incubated for 6 hours in AAP and GPL media. Asterisks (*) indicate significant differences (Mann-Whitney test; $p < 0.05$) between age groups. Straight lines and double arrow lines indicate significant differences (Mann-Whitney test; $p < 0.05$) between each medium and T0 control within lamb or adult group, respectively.

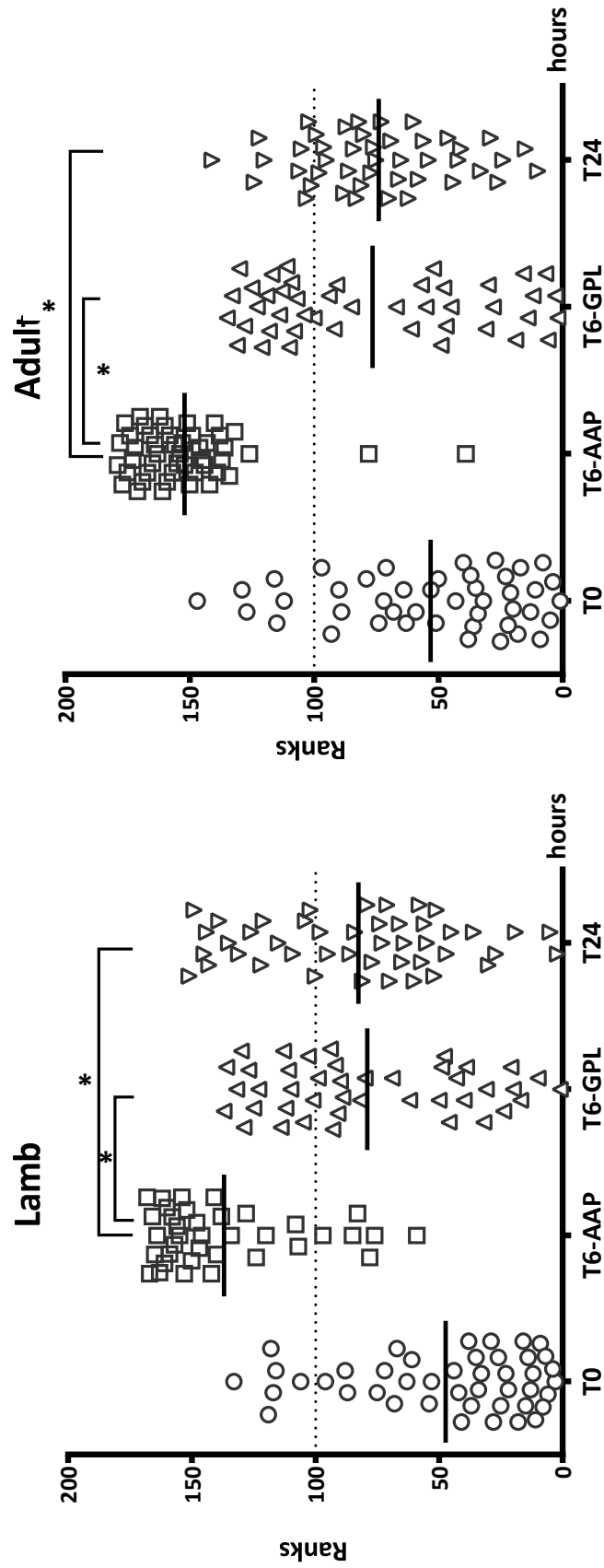


Figure 6.3. Comparison ranks of mtDNA copy number postincubation in different culture media in lamb (left) and adult (right) sheep oocytes. Data shown are the rank mtDNA copy number of individual oocytes with the mean rank of each data represented by () on top of each column. T0 and T24 denote GV- and MII- stage of oocytes while T6-AAP and T6-GPL represented oocytes incubated for 6 hours in AAP and GPL media. Asterisks (*) indicate significant differences ($p < 0.05$) by Kruskal-Wallis test if > 2 groups and multiple comparisons if 2 groups compared. Post-hoc significant differences ($p < 0.05$) confirmed by Mann-Whitney test if data was not normally distributed.

6.4. DISCUSSION

Results from the here presented series of experiments indicate that biological age has an impact on the replication of mitochondrial DNA during the final stages of oocyte maturation in sheep oocytes. Although no significant differences were detected in mtDNA copy number between age groups in GV oocytes, the data showed higher rates of mtDNA amplification in prepubertal oocytes regardless of whether they were incubated in standard serum-free IVM medium or in GPL balanced-salt solution based medium when compared to adult oocyte counterparts. Moreover, the data presented indicate that alteration in mtDNA copy number is linked to age related changes in the observed changes in carbohydrate metabolism, especially pyruvate consumption, as previously reported in chapter 4 section 4.3.2. Finally, this study also demonstrated that the composition of oocyte culture media *per se* could have a critical impact on mtDNA replication *in vitro*.

6.4.1. High Variation Of Mitochondrial DNA Copy Number Within Oocytes

There was a high variation of mtDNA copy number in individual ovine oocytes, which ranged from 454 to 5,658,000 corresponding to a 12462 fold difference in GV stage and from 2,245 to 9,852,000 corresponding to a 4388 fold difference in MII oocytes. The variability in the number of mtDNA copies in this study is consistent with several reports previously published in other species (Otten and Smeets, 2015). Besides individual oocyte differences in copy number, interspecies differences in mtDNA copy number have also been observed and this is related to an invasive pattern and the length of the preimplantation phase (Otten and Smeets, 2015). For example, human and mice blastocysts have an immediate, fast implantation rate that is characterized by rapid invasion of the endometrial stroma and embedding into the uterine wall in order to access high levels of nutritional support from the female reproductive tract (Leese, 2002, Wimsatt, 1975). In contrast, in cows, pigs and sheep, the blastocyst transforms through an elongated stage and has a significantly delayed implantation (diapause) before fusion with the luminal epithelium occurs (Lee and DeMayo, 2004). It is possible that in order to distribute mtDNA molecules to the daughter blastomeres in the embryo

with the absence of mtDNA replication during early embryogenesis (May-Panloup *et al.*, 2005b, Thundathil *et al.*, 2005), oocyte and embryo ruminants may require higher mtDNA copy number than other species. In support of this idea, the present study shows sheep oocytes have a higher level of mtDNA content when compared to other reports for example in humans (Chen *et al.*, 1995, Reynier *et al.*, 2001, Steuerwald *et al.*, 2000), and mice (Cao *et al.*, 2007, Cummins, 1998, Ge *et al.*, 2012b, Thundathil *et al.*, 2005, Zhang *et al.*, 2011b).

Using median estimation, the approximate mtDNA copy number in GV stage lamb and adult oocytes were 88,619 (2,393-4,358,000) and 76,835 (454-5,658,000) copies, whereas that in MII oocytes was 674,779 (2,245-9,852,000) and 290,871 (4,085-3,754,000), respectively. The amounts of mtDNA described here in mature oocytes are compatible with the earlier reports in the same species, which were approximately ~400,000 and ~700,000 in adult animals (Cotterill *et al.*, 2013, Reader *et al.*, 2015). In contrast to mature oocytes, the unexpected estimation of mtDNA copy number of GV oocytes in this study was shown 6 to 10 times lower than the others. Cotterill and colleagues using a real-time PCR based assay estimated ~700,000 mitochondrial DNA copies (range 143,210-1,358,988) in 16 GV oocytes derived from 3-5 mm follicles (Cotterill *et al.*, 2013). Whilst Reader and group reported between ~500,000 and ~700,000 mtDNA copies (range 140,850-1,468,000) from 12 oocytes each in lamb and adult animals by real-time PCR (Reader *et al.*, 2015). Although this study has shown a similar estimation of mitochondrial DNA both prepubertal and adult animals, there is no simple explanation why such a big discrepancy of mtDNA content was observed in immature oocytes here and previous reports. Explanations might be that a larger sample size (n=88) was included in our study, that different breeds of sheep were used or that a seasonal effect occurred; also the quantification method in the current studies using different internal reference control at the DNA extraction process (Steuerwald *et al.*, 2000). However, the variability of mtDNA copies reported here may reflect the different patterns of nuclear chromatin configuration exhibited in immature oocytes as described in chapter 3. As a result, we suggest that an estimation of normal variability of mtDNA copy number be approximated by the interquartile range between 25th and 75th percentile.

6.4.2. Mitochondrial DNA Copy Number During Oocyte Maturation

The onset of the preovulatory LH surge leads to meiotic resumption in oocytes that are accompanied by several changes within cytoplasm and nucleus that are essential to ensure the oocyte has the capacity for fertilization and for subsequent support of early embryogenesis. A substantial amount of energy production is required to support structural changes such as chromosome condensation and alignment, and the pulling apart of spindle fibres in order to separate chromosomes or chromatin during the extrusion of the first and the second polar bodies. Several previous reports in frogs (el Meziane *et al.*, 1989), mice (Ebert *et al.*, 1988, Piko and Taylor, 1987), rats (Kameyama *et al.*, 2007) and pigs (El Shourbagy *et al.*, 2006) for example have shown no mtDNA amplification takes place between early stages of embryogenesis until postimplantation. During the terminal stage of maturation, a substantial amount of mitochondria is required to ensure enough energy support to complete oocyte maturation and subsequent preimplantation embryo development (Chiaratti and Meirelles, 2010, Cummins, 1998, May-Panloup *et al.*, 2005b, Smith and Alcivar, 1993). Here, the data strongly supported roles of the essential organelles in oocyte maturation in terms of ATP synthesis via oxidative phosphorylation, regulation of apoptosis and calcium exchange.

Unlike other sheep studies, the current study demonstrates a significant increase in mtDNA contents in MII oocytes matured *in vitro* compared to GV oocytes during GV-MII transition in both age groups (median 88,619 vs. 674,779, $P < 0.0001$ in lamb; and, 76,835 vs. 290,871, $P = 0.0031$ in adult sheep). This finding is consistent with previous reports in other experimental models including cows (Iwata *et al.*, 2011), mice (Piko and Taylor, 1987), pigs (Mao *et al.*, 2012), and humans (Duran *et al.*, 2011, Zeng *et al.*, 2007), all of which have revealed the occurrence of significant mtDNA replication during the final stage of oocyte maturation. These data could collectively harmonize with the stereology observed by the electron microscope, which determined an increase in total mitochondrial number and volume (de Paz *et al.*, 2001, Reader *et al.*, 2015), as well as reports of increases in ATP content during oocyte maturation in bovine and porcine (Brevini *et al.*, 2005, Stojkovic *et al.*, 2001).

However, some other studies in pigs (Pawlak *et al.*, 2016), human (May-Panloup *et al.*, 2005a), also in sheep (Cotterill *et al.*, 2013, Reader *et al.*, 2015) did not detect differences in mtDNA copy number during oocyte maturation. The difficulty in identifying differences during the GV and MII transitions period in Cotterill *et al.* (2013) and Reader *et al.* (2015) may consequently result from higher detection of mtDNA copies in GV oocytes. Whilst in human studies, all samples represented defective oocytes since they were discarded from IVF and ICSI cycle due to arrest underdevelopment. This could be another possibility why difference of mtDNA copy number cannot be identified (Barritt *et al.*, 2002, May-Panloup *et al.*, 2005a, Reynier *et al.*, 2001). As a result, various factors might explain the discrepancy outcome including inter-oocyte variability, small sample-size, culture medium, environmental factors, such as seasonal effect, different quantitative PCR techniques, as well as reliability of mtDNA *per se* as a representative to mitochondrial number.

6.4.3. Impact of Age On Mitochondrial DNA Copy In Immature And Mature Oocytes

A significant difference in mtDNA copy number was only observed in MII-stage (but not in GV-stage oocytes) between prepubertal and adult animals (674,779 vs. 290,871; $P < 0.001$). In contrast, Reader and colleagues using a similar ageing model could not detect a difference in mtDNA contents between immature and mature oocytes (Reader *et al.*, 2015). However, Reader's results demonstrated a conflict between two mitochondrial estimation methods; stereology (Transmission EM) and mtDNA copy number quantified using real-time PCR. Only the stereological approximation of mitochondria in MII oocytes reached a significant difference with age while the molecular method could not demonstrate such an impact. A possible interpretation of this data is that ageing process deteriorate mtDNA replication during the latest stage of ovine oocyte development. Although the ageing model, prepubertal vs. adult oocytes, used in the current study is similar to Reader's report, there are several differences between the experimental approaches used that could account for the conflicting outcome. Instead of harvesting oocytes during the same period (early breeding season), our prepubertal oocytes were harvested during non-breeding season while adult oocytes were collected during the breeding season. Thus the results may be influenced

by the seasonal effect. Next, the criteria used to differentiate reproductive age in this study included reproductive characteristics such as uterine size and internal rugae and the requirement of at least 2 corpora lutea presented on the ovaries in order to exclude the first oestrous cycle in contrast to single corpus luteum in Reader's study. In addition, the maturation medium used here was serum-free rather than the serum-based medium used by Reader's study. Finally, differences in the qPCR technique used for mtDNA quantification were also evident. Thus it is possible that the 2 groups of animals in our model clearly present differences in oocyte quality and developmental competence that were not observed in the published study.

Another difference between our results and Reader's is a significant higher level of mtDNA copy number detected in our prepubertal MII oocytes when compared to those from adult animals rather than lower mitochondrial number in immature oocytes as reported (Reader *et al.*, 2015). Like our results, Steuerwald *et al.* (2000) using MII oocytes matured *in vitro* demonstrated an increased mtDNA copy number in poor quality oocytes derived from elderly aged women. This suggests that different source of samples whether oocyte matured *in vivo* or *in vitro* could impact on the study findings. For example, studies in human analysed unfertilized MII oocyte samples discarded from the clinical practice may be interfered by postovulatory ageing as the phenomenon may cause mtDNA degradation. Numerous factors therefore contribute to mtDNA replication and copy number measurement including mechanism of oocyte maturation, sample selection and hormonal stimulation. An unexpected increase in mtDNA contents in poor quality oocyte models leads to the intriguing question of whether this replication occurs as part of the mechanism to compensate to mitochondrial dysfunction.

An impact of age on mtDNA copy number has been observed in other species (Chan *et al.*, 2005, Murakoshi *et al.*, 2013, Reynier *et al.*, 2001, Steuerwald *et al.*, 2000). Most human studies have found a negative correlation between age and mtDNA copy number (Chan *et al.*, 2005, Murakoshi *et al.*, 2013, Steuerwald *et al.*, 2000). A conflicting result found by Reynier and colleagues using unfertilized human MII-oocyte samples did not support such a correlation (Reynier *et al.*, 2001). In fact, the studies in human oocytes

may be difficult to interpret, although samples discarded from IVF or ICSI demonstrated rather homogeneity of the samples analysed. However, that the effect of *in vitro* ageing while oocytes undergo incubation may interfere or superimpose the results, cannot be excluded.

In porcine studies, two reports that compared prepubertal and adult porcine oocytes could not reveal a remarkable effect of age on mtDNA copies (Pawlak *et al.*, 2011, Pedersen *et al.*, 2014). Similarly in the bovine, no strong correlation between age and mitochondrial replication has been reported, although Iwata and colleagues found an increasing trend towards correlation when MII oocytes were derived from cows above 50 months old (Iwata *et al.*, 2011).

The present study demonstrates that reproductive age may impact on oocyte cytoplasmic competence at the final stage of oocyte maturation through alteration of mtDNA replication. Regarding the critical threshold of mtDNA copy number required to support oocyte developmental competence (Wai *et al.*, 2010), the optimal range also needs to be defined since high levels of mtDNA copy number may designate the prognosis of diminished quality oocytes (Wang and Moley, 2010). It has been reported here that the greater ratios of mature to immature mtDNA copy number in oocytes derived from prepubertal sheep compared to the ratios found in oocytes derived from adult sheep were observed as a more rapid speed of mtDNA replication during GV-MII transition observed in prepubertal oocytes, this was 2-3 times faster than it was seen in the other group.

Although the relationship between mtDNA copy and oocyte quality are still inconclusive, the data presented here using oocytes from prepubertal animals to represent low quality oocytes turned up overtly increased number of mtDNA copies compared to those shown in adult oocytes. This intriguing observation raises questions whether an increase in mtDNA copy number is the compensatory mechanism required to meet energy metabolism for oocyte maturation and fertilization. It also suggested that the numbers of mtDNA copies and mitochondrial replication can be influenced by numerous factors including age, culture media and culture period. Also uncountable

effects of mitochondrial fission and fusion still need to be evaluated. Determination of oocyte competence in terms of cytoplasmic maturation must therefore include measurements of mtDNA content and estimates of mtDNA function.

6.4.4. The Impact Of Age On Carbohydrate Metabolism Especially Pyruvate Consumption

It is widely accepted that the age-related decline in fertility may result in defective oocyte competence. However, the mechanisms involved in such impairments are still unclear. Mitochondria are highly abundant organelles that play key roles in calcium oscillations and ATP generation through OXPHOS. Numerous studies have noted that mitochondrial dysfunction leads to an impairment of oocyte competence (Fernandez-Silva *et al.*, 2003, Hamatani *et al.*, 2004, Iwata *et al.*, 2011, Jeng *et al.*, 2008, Nagano *et al.*, 2006a). It is critically important to quantify mitochondrial function needs by evaluation of mitochondrial OXPHOS activity such as mtDNA copy number, mitochondrial membrane potential and ROS and/or ATP production, carbohydrate metabolism, substrates required for respiration.

According to the metabolism measured in immature oocytes in Chapter 4 (section 4.2.3), the current study has shown a significant effect of age on 2 parameters of energy metabolism; glucose and pyruvate consumption. In order to establish if a link existed between mtDNA copy number and energy metabolism, the current experiment was designed to measure mtDNA copy number and metabolic assay in the same cells. The data of GPL assay in this experiment were a subset of those detailed in Chapter 4. As the significance of metabolic difference between prepubertal and adult oocytes still persisted in this subgroup analysis, which adult oocytes have significantly consumed higher pyruvate ($P=0.0002$) and have produced higher lactate ($P=0.0144$) than the prepubertal samples counterpart. Though some minor differences in glucose consumption and lactate production found between the subgroup and total cohort could be explained by excluding some outliers in the subgroup, this is not recommended. Since there was no prior, valid reason for sample exclusion we must assume, for now, that the data obtained are true.

According to carbohydrate metabolism results, approximately a third of the samples assigned for GPL turnover did not record any glucose utilization, while the rest detected very low levels; therefore, lactate production of oocytes was unlikely from glucose breakdown via the glycolysis pathway. It is notable that pyruvate, not glucose, was the major source of energy production in this study (Harris *et al.*, 2009, Leese, 2002). Pyruvate consumption is, therefore, implicated as a possible use of marker of oocyte quality in sheep oocytes.

6.4.5. Mitochondrial DNA Copy Number And Energy Metabolism

The final stage of oocyte maturation to acquire the developmental competence requires both nuclear and cytoplasmic maturation, and all these processes need energy supply (Ferreira *et al.*, 2009, Sun and Nagai, 2003). The nuclear process includes GV breakdown, condensation and segregation of chromosomes, and extrusion of the first PB whereas the cytoplasmic maturation series involves organelles relocation, increases Ca^{2+} stores, and mRNAs and proteins synthesis (Ferreira *et al.*, 2009). An increase in ATP content at this stage was observed in cattle (Stojkovic *et al.*, 2001), pig (Brevini *et al.*, 2005), and mouse (Yu *et al.*, 2010) oocytes. Therefore, energy substrate utilization for ATP production was expected as during the here presented experiments. Contrary to our expectation, using either the energy metabolism or mtDNA copy number alone was not sufficient to detect differences between oocytes from prepubertal and adult animals, and we failed to find any direct correlation between these 2 markers. It is possible that both aerobic and anaerobic respiration are involved in oocyte respiration (Wilding *et al.*, 2009). Moreover, different mitochondrial structures such as round or ellipsoid types of mitochondria may have different energy productivity (Eichenlaub-Ritter *et al.*, 2011, Van Blerkom, 2009, Van Blerkom, 2011).

Debate continues as to whether decreased (May-Panloup *et al.*, 2005a, Murakoshi *et al.*, 2013) or increased (Steuerwald *et al.*, 2000) mtDNA copy number or mitochondrial number relate to declining oocyte quality owing to the limited number of studies directly comparing mitochondrial function and mitochondrial DNA content. To address this controversial issue, mitochondrial activity per unit function has been considered in the current study. We found that the individual unit function of mitochondrion declined

exponentially in correlation to an increase in mtDNA copy number. These patterns were observed for all 3 elements; glucose, pyruvate and lactate. Also there was a high degree of similarity in the patterns recorded for oocytes derived from both prepubertal and adult animals. This observation is consistent with previous reports. Wang and Moley found diabetic mice oocytes had low ATP content but higher mitochondrial number than healthy oocytes (Wang and Moley, 2010). Furthermore, Harris and coworkers demonstrated oocytes derived from PCOS patients had an increased uptake of glucose compared to controls (Harris *et al.*, 2010, Maruthini *et al.*, 2014). As a result, it can be interpreted that a large amount of mtDNA copy number in this study are likely a compensatory mechanism in response to oocyte mitochondrial dysfunction.

6.4.6. The Relationship Between Mitochondrial DNA Copy Number And Mitochondrial Function

Data from the prepubertal model used in the current study is representative of incompetent oocytes which have shown an upsurge in mtDNA copy number with 2-3 fold more copy numbers present than mtDNA in oocytes derived from reproductive age animals. As mentioned in section 6.4.3, our data suggests a compensation mechanism of poor quality oocytes by increase the amounts of mitochondria to meet their energy requirement. The question still exists as to why some groups reported low levels of mtDNA instead of having high level of mtDNA detection like in our results. The plausible explanation of these conflicting result needs to consider several aspects. Firstly, the model of aged oocytes is one of the important factors effecting the outcome since it is the complex process linked to numbers of ageing theories. Unfertilized MII samples discarded from human IVF or ICSI cycles may not be a perfect model for ageing study because mechanisms underpinning failed fertilization or cleavage arrest may involve many functional defects associated with degenerative changes due to chronological age. Also failed fertilization of MII oocytes may result from maturation defects. Moreover, the effect of postovulatory ageing *in vitro* may also superimpose the outcomes. For example, degenerative change in mitochondrial turnover, which degradation is beyond the mitochondrial replication process, and as such may not be able to detect if it thus occurs during the high phase of replication. Some studies suggested that the mitochondrial degradation rate is greater than the replication rate (Spikings *et al.*,

2007). Next, regarding age and increased mtDNA mutation, it is possible that defects in clearance of mutated mitochondria may contribute. Moreover, as mentioned previously, there are several confounding factors involved in oocyte mitochondrial replication such as stimulation protocol, hormones, culture media (serum-based or serum-free), samples recruited. These might lead to developing a potential marker of oocyte quality (Pawlak *et al.*, 2016).

6.4.7. Effect Of Culture Media On Mitochondrial DNA Copy Number

A recent study reported the effect of hormonal stimulation on mtDNA copy number (Ge *et al.*, 2012a). Ge and coworkers found that mouse oocytes derived from ovarian hormonal stimulation had less ATP and higher ROS contents within mitochondria and lower membrane potential than control groups. Whilst immature oocytes in IVM medium showed higher ROS production but had no differences in ATP production or membrane potential when compared to controls (Ge *et al.*, 2012a). This evidence agrees with our finding. A significant difference in mitochondrial genome replication during IVM was observed in the AAP and GPL media compared to standard oocyte maturation medium. From this it can be implied that mtDNA replication may be sensitive to changes of environmental milieu such as culture media, temperature and oxygen condition. On the other hand, regarding mtDNA, if this marker has been validated, it can be used as a test for culture media optimization.

The present study confirms mitochondrial replication during the final stage of oocyte maturation. However, this observation can partially be explained by two main factors. First, the different composition within each culture medium may influence mtDNA replication. Here we prepared AAP medium by 8 times dilution of 18 amino acids concentration used in serum-free derivative IVM medium on purpose to examine oocyte metabolism. The low amino acid concentration medium allowed minute changes in amino acid utilization and production to be able to detect by high performance liquid chromatography (Harris *et al.*, 2009, Collado Fernandez, 2013, Hemmings, 2007) while GPL medium is a balanced salt solution contained tiny amount of glucose for carbohydrate metabolism. Amino acids play many roles in culture medium including precursors of protein production, supportive osmotic pressure, pH buffering, as well as

chelating agent role. Changes to deprive amino acid concentration medium may induce a stressful milieu surrounding oocytes leading to an increase in mtDNA replication to meet the energy required for oocyte survival.

The denudation process to free cumulus cell of oocytes could be another main issue concerned with regard to increase in mtDNA replication. In the normal circumstance, oocyte and cumulus cells support each other in terms of growth and development via bidirectional communication in a follicular milieu as a fundamental unit development (Eppig, 2001, Knight and Glister, 2006). Disaggregation of cumulus cells from individual oocytes, therefore, may alter metabolic function of the small unit and may induce stressful and detrimental effects to oocyte metabolism during oocyte maturation *in vitro*. This might explain differences of mtDNA copy number between oocytes incubated in AAP/GPL media with the control in standard serum-free IVM medium. As a previous report, more quiescent oocytes/embryos have shown better quality (Leese, 2002). Under the stressful condition, denuded oocytes may have to compensate by increase mtDNA replication to produce sufficient ATP for development. Further studies are needed to determine whether this may be a useful marker for culture media testing.

During the early stages of meiotic resumption, the cytoskeletal network of microtubules mediates dynamics of ultrastructure within nucleus and cytoplasm in forming the meiotic spindle and organelles mobilization (Van Blerkom, 1991). Mitochondrial relocalization and changes in mitochondrial activity are needed to ensure the capacity of oocyte to achieve fertilizing competence and further embryonic development. Brevini *et al.* (2005) found mitochondrial reorganization and increased ATP content during IVM related to the development capacity after IVF (Brevini *et al.*, 2005). Duran and colleagues revealed a significantly increasing ATP content in parallel with the maturational stage of human oocytes (Duran *et al.*, 2011) as well as Zeng *et al.*, who demonstrated higher ATP levels and higher total mtDNA copy numbers in oocytes that reached maturation compared to those who failed maturation (Zeng *et al.*, 2007). Like humans, a study in pigs found mtDNA copy has a strong correlation to fertilization outcome (Pedersen *et al.*, 2014). This strongly indicated that mitochondrial number,

mtDNA copy number, mitochondrial structure, distribution and functions are all essential for the final stage of oocyte development.

6.4.8. Conclusion

The chapter provides some evidence to support the impact of reproductive age on mtDNA copy number although the data clearly demonstrated only in mature (MII) oocytes. Differences between two stages of oocytes (GV vs MII) confirmed the mitochondrial DNA replication during oocyte maturation *in vitro*. A quantitative assessment of mitochondrial number by using a surrogate mtDNA copy number estimation observed a wide range of variation in mitochondrial number among individual oocytes. Further investigation is needed to identify the cut-off level in different circumstances prior to conclude on the normal range of oocyte mitochondrial number.

Although numbers of evidences in the literature as well as this current work suggest age-altered mitochondrial number and energy metabolism, the study could not identify the link between the two factors. It is possible that both parameters are easily interfered with by the environmental milieu such as culture media or cumulus free environment. Moreover, amounts of mitochondria by the estimation of mtDNA copy number did not take the mitochondrial structure into account. Therefore, data concerning mtDNA copy number as a representative of mitochondrial number to determine oocyte quality should be carefully interpreted since they lack functional evaluation.

The findings in this chapter may demonstrate an impact of culture media *per se* on oocyte mitochondrial number; this is possibly related to the low IVM outcomes of oocytes matured *in vitro* in comparison to those matured *in vivo*. A contradiction observed between this work and previous reports is that an increase in mtDNA copy number seems to determine compromised oocytes rather than the better quality oocytes. The speculation has focused on the different model used in this experiment, is compatible to test competency of oocyte for further development instead of the model for reproductive ageing.

Additional studies are required to clarify the effect of maternal ageing not only on mitochondrial number in different methods of estimation in different species but also on other aspects of mitochondrial function like regulation of Ca^{2+} signalling, apoptosis induction and fertilization. This work has challenged the appliance of mitochondrial replacement therapy to improve oocyte quality in terms of the correct quantity of mitochondrial substitution, the selective methods to identify which oocytes required mitochondrial replacement, as well as the functional properties of mitochondrial supplement. Oversupply of mitochondria into oocytes may lead to compromising oocytes from excessive energy burn out and overt ROS production and subsequently facilitating apoptosis. Moreover, the natural history of mitochondrial adaptation after replacement still needs to be elucidated.

CHAPTER 7: GENERAL DISCUSSION

7.1. INTRODUCTION

Identifying the mechanisms that underpin age impaired oocyte growth, maturation and subsequent embryo development would have a great impact on women's reproductive life in this era in terms of improvement of female conception, infertility diagnosis and treatment, and family planning (Picton *et al.*, 2003; Telfer and McLaughlin, 2007). It would also expand current knowledge of the treatment of the age related diseases and potentially lead to increased productivity of farm species. The intrinsic quality of the oocyte is widely accepted as being critically important to the success of female reproduction *in vivo* and *in vitro* in both animals and humans (Gandolfi and Gandolfi, 2001; Jurisicova and Acton, 2004; Krisher, 2004; Sirard *et al.*, 2006). Cytoplasmic and molecular maturation insufficiency disrupts oocyte growth and developmental potential. As the cytoplasmic machinery of the zygote includes mRNA, proteins, mitochondria, that are maternally inherited, it is therefore essential that all of the proper biological processes of oocyte development must be completed and in a coordinated manner to ensure the full capacity of oocyte to undergo fertilization and to support development of preimplantation embryo before EGA.

The success rate of infertility treatment in aged women undergoing ART is significantly lower than that of women of earlier reproductive age, no matter what ovarian stimulation protocols have been offered or the quality of embryo selected for transfer. Moreover, pregnancy in aged women is reported to result in increased chances of miscarriage and obstetric complications (Grotegut *et al.*, 2014, McDonnell *et al.*, 2014). For example, the increased prevalence of congenital malformations, preterm delivery, newborn with small for gestational age, age related diseases, childlessness and short lifespan (Doyle *et al.*, 1992; Farin *et al.*, 2001; Shiota and Yamada, 2005; Eppig *et al.*, 2009; Ge, 2015). Thus, a better understanding how age changes oocyte biology and metabolism may lead to the development of optimised culture conditions as well as improved, individualized ovarian stimulation protocols that match the infertile pathology and thereby improve the quality of the oocytes and embryos in the ART program.

7.2. THE STUDY MODEL

Ageing is a complicated process associated with stochastic changes in cell structure and functions. Models used for the study of ageing are important and need to be aware of the diversity of the outcomes. This thesis used oocytes from the prepubertal and adult sheep as a model to study the impact of age on human oocyte quality. One of the models that commonly used to study oocyte ageing is prepubertal oocytes as it is accessible resource, and have a lower potential to grow and develop into an offspring than adult oocytes. Therefore, this thesis has taken the prepubertal oocyte during off-breeding season representative the model of poor quality oocyte and employed adult oocytes during breeding season to represent the oocyte with better developmental potential. It should be pointed out that the findings in all of the enclosed series of studies should be interpreted with caution as our chosen *in vitro* model may not be equivalent to ovarian ageing *in vivo*. More specifically our *in vitro* model of oocyte ageing may be subtly or grossly different to oocyte ageing *in vivo*. Indeed, it is for this reason we elected to focus the majority of the research in this thesis on the biology of GV oocytes as these more closely resemble oocytes *in vivo* than cells that have undergone IVM following denudation. Nevertheless, the results generated here can be used to investigate defects in the oocyte cytoplasmic machinery as the molecular and metabolic links that causes poor reproductive outcomes. Although further focused studies are however needed to confirm that the ageing process is conserved in our chosen model, the advantages of our *in vitro* model have enable us to conduct well controlled studies in oocyte biology is not affected by *in vivo* nutrition, genetic background and health status, environmental factors and, as is relevant to the current sheep studies, seasonal effects of the breeding season.

Studies of oocyte metabolism, are hardly to do using COCs since no methods are available to control or subtract the metabolism of CCs which contribute to the nutrients utilized or metabolites produced by the intact COCs. The current work therefore aimed to focus on the use of serum-free culture conditions that simulated the normal physiological conditions that were appropriate for the culture of denuded oocytes. Supplementation of the defined oocyte culture media with 50µM cilostamide was necessary to ensure inhibition of meiotic progression in GV oocytes. The extensive

validation studies of the culture system conducted in chapter 3 aimed to demonstrate that the culture strategy used did not impact contribute the outcomes measured outcomes such as metabolism and chromatin configuration. However, since this thesis did not evaluate the impact of the culture environment on subsequent oocyte developmental competence, and without cumulus enclosed the oocytes, detrimental effect may be anticipated. Further studies are required to evaluate the oocyte's capacity to undergo fertilization to support embryo development to the blastocyst stage, as well as the optimizing *in vitro* culture condition needed to be established.

7.3. AGE ASSOCIATED CHANGES IN CHROMATIN REMODELING

As we know, human oocytes enter meiosis I division and then maintain meiotic arrest since foetus *in utero* before becoming cell cycle arrested at diplotene stage of prophase I during mid-gestation is similar to sheep and cow. At this stage, oocyte chromosomes present less distinct and forming faint network and resting inside a primordial follicle (Mandl and Zuckerman, 1952) until it has been activated into a preovulatory pool. Concomitant to follicle development, the oocytes grow and substantially increase in size which it is considerable as part of cytoplasmic development. The oocyte chromosome turns into decompensation phase accompanying with chromatin becomes transcriptionally active. As the dynamics occurs towards the end of oocyte differentiation prior to the meiotic resumption of oocyte. This may be considered as an indicative marker of oocyte competence as the meiotic competence acquisition is timely dependent on the appearance of the early signs of chromatin condensation whereas subsequently embryonic developmental competence is acquired almost the highest level of chromatin condensation (Albertini *et al.*, 2003, De La Fuente, 2006).

A number of studies in various species such as mouse (Wickramasinghe *et al.*, 1991) (Zuccotti *et al.*, 1995), cow (Fuhrer *et al.*, 1989, Lodde *et al.*, 2007), goat (Sui *et al.*, 2005), horse (Hinrichs and Schmidt, 2000) and human (Combelles *et al.*, 2003, Miyara *et al.*, 2003), have reported that chromatin remodeling occurs during the developmental period before oocytes undergoing competence. Although the pattern of chromatin

configuration in different species showed differences to some extent, they were consistent in term of moving from diffused stage of chromatin towards the condensed chromatin pattern. Several studies found pattern of chromatin configuration in mouse closed to human oocytes as the staining showed NSN-SN pattern in which condensed chromatin were formed at the perinucleolar region (Wickramasinghe *et al.*, 1991, Zuccotti *et al.*, 1995, Miyara *et al.*, 2003). On the other hand, other ruminant species such as cow and goats, could not identify such characteristics but seem to have spreading patterns from either diffuse filamentous pattern, few foci condensed, distinct clump condensed and single clump condensation(Lodde *et al.*, 2008).

Only one published study has been reported with regard to chromatin configuration pattern of sheep oocytes (Russo *et al.*, 2007b). Characteristics of chromatin configuration in sheep seemed to be the combination of both NSN-SN pattern like mouse and human, and discrete pattern as found in ruminants. Therefore, we questioned whether this could be the effect of the SYBR staining used instead of conventional staining such as Hoechst or DAPI. The current work therefore conducted to confirm and redo the classification of sheep chromatin configuration by DAPI staining as the results seen in the chapter 3. The evidence clearly demonstrated that the staining method *per se* influences chromatin pattern manifestation due to the different properties of dye labeling. Although our established chromatin classification is closed to what have been presented in other ruminant species (i.e., cow and goat), it was not identical. The characteristics was far from NSN-SN pattern as shown in mouse and human. Dynamics of chromatin configuration have progressed through diffused, net-like, condensed, and clump stages prior to oocyte nuclear membrane breakdown as can determine by meiotic spindle plate with presence or absence of the first PB. Therefore, the established classification was applied for the following series of experiments in this thesis. However, it has been aware of that reliability of chromatin configuration may depend on the correct staining methods, quality of sample tissues batch to batch variation, and especially the interpretation skills of scientists. The experiments were strictly followed the protocol in all replicates and conducted by only 1 interpretator (CT) to avoid the interpretator effect in the study.

Data also showed that immature GV sheep oocytes (chapter 3) clearly demonstrated that the dynamics of chromatin remodeling has been delayed in GV samples derived from prepubertal compared to adult tissues. Due to ATP required during meiotic maturation, a low pyruvate consumed by prepubertal oocytes (chapter 4) may explain that insufficient energy production to drive the meiotic maturation causes delayed development. Besides, GV-staged oocytes derived from prepubertal animals seem to have transcriptionally active, higher level of several genes expressed, whereas the equivalent from adult animals showed less abundance of the transcripts. The data suggests that prepubertal GV oocytes have less sufficient molecular machineries to drive meiotic maturation process. Such an effect was pronounced when the condition *in vitro* culture has less support as using GPL medium (KSOM based medium). Further studies require to confirm this finding not only in other models both *in vitro* and *in vivo*, but also in different species. Moreover, it is worth to study both genetics and proteins underlying meiotic maturation.

7.4. AGE ASSOCIATED WITH ALTERATION OF ENERGY METABOLISM LINKED TO COMPENSATORY MITOCHONDRIAL DNA REPLICATION

The results presented in chapter 4 show that the overall pattern of AA depletion and appearance was maintained in immature GV sheep oocytes. Glycine, alanine and lysine production and asparagine, glutamine, threonine, arginine and leucine consumption were the major AAs that contributed to GV oocytes AAP regardless of animal age. With regard to AAP, adult GV oocytes showed a higher consumption of aspartic acid, isoleucine and leucine. On the other hand, they turn to consume less glutamic acid and tyrosine but produced more alanine when incubated in AAP media. The current work demonstrated that adult GV oocytes showed a higher AA turnover and presented a positive AA net-balance when compared to prepubertal GV oocytes.

This study identified a few AAs, such as aspartate and isoleucine that were linked to oocyte age. These candidate AAs had previously been shown to be associated with embryo quality on day 1 or day 2 of embryo culture *in vitro*. Specifically, the effect of

age was significantly associated with altered glutamine consumption in most previous studies in both human oocytes (Hemmings *et al.*, 2012) and embryos (Picton *et al.*, 2010) and ovine oocytes *in vivo* (Collado Fernandez *et al.*, 2015). The same trend has found in the study but differences in glutamine consumption did not reach statistical significance. This suggests that glutamine should be one of the affected AA selected for further study since it plays several roles in oocytes as it is a protein precursors for purine and pyrimidine synthesis and glutathione production as well as an antioxidant, and an organic osmotic regulator. Moreover, it can also be converted and used as an energy supply. This may link its metabolism to many ageing theories regardless cell type studied. Another AA which was consistently affected by age was tyrosine (Collado Fernandez *et al.*, 2015, Hemmings *et al.*, 2012, Picton *et al.*, 2010). As it involves in number of signaling pathways. Further studies are required to focus on these AAs and age related alterations in their metabolism. Future studies of the associated signaling pathways may enhance knowledge and so help elucidate the mechanism of ageing. Although glycine, arginine and alanine made major contribution to AAP measurement in the current study their biology overall is already known to be linked to oocyte competence and/or embryo quality, and the current work could not demonstrate any changes in these AAs in relation to age.

The work reported in this thesis confirmed that pyruvate rather than glucose is the major substrate for energy metabolism of oocytes due to lack of rate limiting enzyme in the glycolytic pathway, phosphofructokinase, as has been reported previously. Oocyte development and maturation demand that sufficient ATP synthesis is available to drive the process. They therefore require the mandate of energy metabolism. This study also demonstrated that age-related changes in glucose and pyruvate consumption occurred due to the fact that these nutrients are precursors needed for energy production. Adult oocytes with better quality have more capacity to take up more pyruvate than prepubertal oocytes whereas the poor quality oocytes utilized more glucose. The data suggests that prepubertal oocytes may have defects in the cytoplasmic and/or molecular machinery needed to uptake pyruvate and synthesize enough ATP to support further development. Therefore, in the last thesis chapter- chapter 6, mtDNA copy number was studied to see whether such an impairment was

reflected by an insufficient mitochondrial number to produce energy using as mtDNA copy number as a surrogate for quantitation of mitochondrial number. Surprisingly, we found an increased mtDNA copy number in prepubertal oocytes with lower developmental potential compared to adult oocytes. The conclusion from this finding was that mitochondrial number alone may not be accurate enough to evaluate oocyte quality since the organelles may replicate to compensate for compromised cell function and/or not all mitochondria present in the oocyte may be equally active.

7.5. THE EFFECT OF OOCYTE AGE ON GENETIC AND EPIGENETIC MARKERS

In recent years, evidence has grown regarding the molecular mechanisms that govern the ageing process. That effect of ageing has been shown to involve several mechanisms and interactions (Kirkwood *et al.*, 2005, Mihalas *et al.*, 2017) for example deterioration and ageing in oocytes may involve increased ROS production, DNA damage, impaired DNA repair mechanisms, mitochondrial defects. Moreover, epigenetic modification may contribute to the effects of ageing.

This work investigated the potential impact of age on the molecular regulation of a range of genes involved in sheep oocyte maturation in prepubertal and adult animals. The effect of age was studied on a range of potential genetic markers including oocyte-specific genes involved in metabolism and growth, the subcortical maternal protein complex, imprinted genes, genes involved in one-carbon metabolism, and epigenetic regulators. The evidence presented clearly identified candidate genes that contributed to the establishment of oocyte competence and demonstrated that the expression of the key genes was moderated by oocyte stage of maturity (*GTSF1*, *NLRP2* and *PRDX2*) and animal age (*BMP15*, *GTSF1*, *NLRP2*, *OOEP*, *SNRPN*, *MAT2B*, *PRMT5*, *SHMT2*, *DNMT3A*, *DNMT3B*, *GSK3B*, *IGF1R*, *IGF2BP2*, *IGF2BP3*, *PRDX2*, *SLC16A1* and *SLC2A1*).

Studies of genes related metabolism were highlighted as gene targets that were moderated by age either through the involvement of the glycolytic pathway or 1C metabolism. While the SCMC drives the development of the later stages of the

preimplantation embryo it also is relevant to oocyte-specific genes. Furthermore, alteration in epigenetic modification of aged oocytes may involve the regulatory control of imprinted gene expression and global gene methylation. This study also detected changes in oocyte genes governing oxidative stress. In general, from the data generated using the oocyte ageing model studied here, it can be speculated that the impairment of gene transcription with ovarian ageing is more closely associated with alteration of oocyte cytoplasmic maturation rather than with DNA damage related to ovarian ageing. Since the similar trends were shown in all transcripts, which were found to be expressed at lower levels in prepubertal compared to adult oocyte counterparts.

On the basis of the collective results here, the ageing process has shown that it is involved in several functions of oocyte and these support many theories of ageing mechanism in general. As we demonstrated the alteration of a limited number of genes related to metabolic pathways, some missing genetic and epigenetic control from the current study may be required for further inspection by using modern technologies such as RNA sequencing and proteomics to fill the missing gap of knowledge of oocyte ageing. Moreover, it would have more beneficial to support the findings of this study if future studies provide tracking process of culture in vitro from oocyte to embryo development by linkage to the noninvasive metabolism assays both energy and AAs metabolisms at each stage. Finally, key markers found may be worth to be studied in other species including human in both GV and MII stages to elucidate the age effect in women both fertile and infertile to assemble the more large pictures of age to oocyte defects.

7.6. FUTURE DIRECTIONS AND PERSPECTIVES

Although the pattern of chromatin configuration is closed to other ruminant species, the study to link the morphological finding and oocyte transcriptional status or clinically developmental potential are still required. Likewise, extensive works on transcriptomics and proteomics will provide further information in term of molecular machinery and cytoplasmic maturation; RNA, proteins or organelles, for example. The current work contradicts what has been observed in previous reports by demonstrating the correlation between poor quality oocyte and the greater replication

of mitochondrial DNA copy number during oocyte maturation regardless types of media incubated in vitro. This finding suggests the compensatory mitochondrial replication to stress-induced response in order to supply energy enough for driven the maturational processes including GVBD, meiotic spindle formation and relocalisation, and the first PB extrusion. As the oocyte per se has limited capacity to utilise carbohydrate; it, therefore, synthesises energy through alternative pathways using amino acids and endogenous fatty acids as precursors. Further studies are needed to be validated the observation and assumption from this study. Moreover, the direct measurement of oocyte energy production (i.e., ATP production or oxygen consumption) may be appropriate options to confirm the hypothesis.

The substantial evidence from this work provokes the revision of mitochondrial DNA copy number as a surrogate of mitochondrial number since the interference of organelles adaptation by fission and fusion may involve leading to incorrect estimation of the mitochondrial number. Furthermore, alteration of mitochondrial structures also influences the mitochondrial function in term of ATP production. An increase of mitochondrial DNA copy number in this work may be caused by the failure to dispose of the defective mitochondrial DNA leading to the accumulation of mitochondrial DNA mutation within oocytes. Several questions in this regard are still opened for discussion and looking for scientific answers in the future.

The simulated in vitro culture condition used in this thesis primarily purposes to address the accurate evaluation of oocyte metabolism per se without the interference of other surrounding granulosa cell metabolism. Instead, it may induce the stress response, which compromises to the further embryonic development. Therefore, for clinical application, the culture work is needed to be optimised.

As the sheep oocyte is the model chosen in this research may have some biological differences from human oocytes regarding seasonal breeding species, lipid droplet composition in ooplasm despite a monovular species. Application of knowledge transferred to human oocyte needs to be cautious; however, it may be attributable to the development of sheep in vitro production and improved knowledge gap about what

made prepubertal sheep oocytes differ from the oocyte-derived from adult sheep. Considerable research data may use to develop efficiency in vitro production to reduce the generation gap in order to increase more agricultural and business production.

7.7. CONCLUSION

This thesis provides further evidence in support of the hypothesis that is central to this thesis namely that age leads to the impairment of the biological functions of immature GV oocytes in many aspects. The parameters altered by age include the dynamics of oocyte meiotic maturation as well as the impairment of energy metabolism and AA metabolism. Importantly, these defects associated with age are linked through the important organelle, the mitochondrion, whose altered function may itself contribute the age-related impairment. Moreover, some of the genetic determinants found in the study may explain this relationship further. The studies presented confirmed that the classification of chromatin configuration in sheep GV oocytes closely resembles that of other ruminant species. Moreover, this is the first report that has estimated mtDNA copy number and carbohydrate metabolism in the same individual cells, and it represents the 1st reports of the expression of a range of novel candidate genes in sheep oocytes. The work undertaken will significantly advance our understanding of the biology of ageing in ruminant oocytes and is highly relevant to the reduction of oocyte quality seen with advancing maternal age in oocytes from women. The findings of this work have therapeutic impacts and may help to improve ovarian stimulation protocols in older patients. Furthermore, the results suggest that estimation of mitochondrial number alone is insufficient to support the therapeutic application of mitochondrial replacement as a means of improving the quality of oocytes with poor developmental competence.

REFERENCES

- ABE, K., INOUE, A., SUZUKI, M. G. & AOKI, F. 2010. Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes. *J Reprod Dev*, 56, 502-7.
- ACKERT, C. L., GITTENS, J. E., O'BRIEN, M. J., EPPIG, J. J. & KIDDER, G. M. 2001. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol*, 233, 258-70.
- ADENOT, P. G., MERCIER, Y., RENARD, J. P. & THOMPSON, E. M. 1997. Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development*, 124, 4615-25.
- AIKEN, C. E., TARRY-ADKINS, J. L., PENFOLD, N. C., DEARDEN, L. & OZANNE, S. E. 2016. Decreased ovarian reserve, dysregulation of mitochondrial biogenesis, and increased lipid peroxidation in female mouse offspring exposed to an obesogenic maternal diet. *FASEB J*, 30, 1548-56.
- AKIYAMA, T., KIM, J. M., NAGATA, M. & AOKI, F. 2004. Regulation of histone acetylation during meiotic maturation in mouse oocytes. *Mol Reprod Dev*, 69, 222-7.
- AKIYAMA, T., NAGATA, M. & AOKI, F. 2006. Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice. *Proc Natl Acad Sci U S A*, 103, 7339-44.
- AKTAS, H., WHEELER, M. B., FIRST, N. L. & LEIBFRIED-RUTLEDGE, M. L. 1995a. Maintenance of meiotic arrest by increasing [cAMP]_i may have physiological relevance in bovine oocytes. *J Reprod Fertil*, 105, 237-45.
- AKTAS, H., WHEELER, M. B., ROSENKRANS, C. F., JR., FIRST, N. L. & LEIBFRIED-RUTLEDGE, M. L. 1995b. Maintenance of bovine oocytes in prophase of meiosis I by high [cAMP]_i. *J Reprod Fertil*, 105, 227-35.
- ALBAMONTE, M. S., WILLIS, M. A., ALBAMONTE, M. I., JENSEN, F., ESPINOSA, M. B. & VITULLO, A. D. 2008. The developing human ovary: immunohistochemical analysis of germ-cell-specific VASA protein, BCL-2/BAX expression balance and apoptosis. *Hum Reprod*, 23, 1895-901.
- ALBERTINI, D. F. 1992. Cytoplasmic microtubular dynamics and chromatin organization during mammalian oogenesis and oocyte maturation. *Mutat Res*, 296, 57-68.
- ALBERTINI, D. F., SANFINS, A. & COMBELLES, C. M. 2003. Origins and manifestations of oocyte maturation competencies. *Reprod Biomed Online*, 6, 410-5.
- ALBUZ, F. K., SASSEVILLE, M., LANE, M., ARMSTRONG, D. T., THOMPSON, J. G. & GILCHRIST, R. B. 2010. Simulated physiological oocyte maturation (SPOM): a novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod*, 25, 2999-3011.
- ALI, A., HOEFLICH, K. P. & WOODGETT, J. R. 2001. Glycogen synthase kinase-3: properties, functions, and regulation. *Chem Rev*, 101, 2527-40.
- ALI, J., WHITTEN, W. K. & SHELTON, J. N. 1993. Effect of culture systems on mouse early embryo development. *Hum Reprod*, 8, 1110-4.
- ALIZADEH, Z., KAGEYAMA, S. & AOKI, F. 2005. Degradation of maternal mRNA in mouse embryos: selective degradation of specific mRNAs after fertilization. *Mol Reprod Dev*, 72, 281-90.
- ALLEN, D. D., MARTIN, J., ARRIAGADA, C., CARDENAS, A. M., RAPOPORT, S. I., CAVIEDES, R. & CAVIEDES, P. 2000. Impaired cholinergic function in cell lines derived from the cerebral cortex of normal and trisomy 16 mice. *Eur J Neurosci*, 12, 3259-64.
- ALVIGGI, C., HUMAIDAN, P., HOWLES, C. M., TREDWAY, D. & HILLIER, S. G. 2009. Biological versus chronological ovarian age: implications for assisted reproductive technology. *Reprod Biol Endocrinol*, 7, 101.

- AMELIO, I., CUTRUZZOLA, F., ANTONOV, A., AGOSTINI, M. & MELINO, G. 2014. Serine and glycine metabolism in cancer. *Trends Biochem Sci*, 39, 191-8.
- ANCELIN, K., LANGE, U. C., HAJKOVA, P., SCHNEIDER, R., BANNISTER, A. J., KOUZARIDES, T. & SURANI, M. A. 2006. Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat Cell Biol*, 8, 623-30.
- ANCKAERT, E., SANCHEZ, F., BILLOOYE, K. & SMITZ, J. 2013. Dynamics of imprinted DNA methylation and gene transcription for imprinting establishment in mouse oocytes in relation to culture duration variability. *Biol Reprod*, 89, 130.
- ANDERIESZ, C., FONG, C. Y., BONGSO, A. & TROUNSON, A. O. 2000. Regulation of human and mouse oocyte maturation in vitro with 6-dimethylaminopurine. *Hum Reprod*, 15, 379-88.
- ANDERSON, E. & ALBERTINI, D. F. 1976. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. *J Cell Biol*, 71, 680-6.
- ANDERSON, R. A., SCIORIO, R., KINNELL, H., BAYNE, R. A., THONG, K. J., DE SOUSA, P. A. & PICKERING, S. 2009. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction*, 138, 629-37.
- ANDERSSON, S. G., KARLBERG, O., CANBACK, B. & KURLAND, C. G. 2003. On the origin of mitochondria: a genomics perspective. *Philos Trans R Soc Lond B Biol Sci*, 358, 165-77; discussion 177-9.
- ANDREU-VIEYRA, C. V., CHEN, R., AGNO, J. E., GLASER, S., ANASTASSIADIS, K., STEWART, A. F. & MATZUK, M. M. 2010. MLL2 is required in oocytes for bulk histone 3 lysine 4 trimethylation and transcriptional silencing. *PLoS Biol*, 8.
- AOKI, F., WORRAD, D. M. & SCHULTZ, R. M. 1997. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev Biol*, 181, 296-307.
- ARLOTTO, T., SCHWARTZ, J. L., FIRST, N. L. & LEIBFRIED-RUTLEDGE, M. L. 1996. Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes. *Theriogenology*, 45, 943-56.
- ARMSTRONG, D. T. 2001. Effects of maternal age on oocyte developmental competence. *Theriogenology*, 55, 1303-22.
- ARNOLD, D. R., LEFEBVRE, R. & SMITH, L. C. 2006. Characterization of the placenta specific bovine mammalian achaete scute-like homologue 2 (Mash2) gene. *Placenta*, 27, 1124-31.
- ASCH, R., SIMERLY, C., ORD, T., ORD, V. A. & SCHATTEN, G. 1995. The stages at which human fertilization arrests: microtubule and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in humans. *Hum Reprod*, 10, 1897-906.
- ASHKENAZI, H., CAO, X., MOTOLA, S., POPLIKER, M., CONTI, M. & TSAFRIRI, A. 2005. Epidermal growth factor family members: endogenous mediators of the ovulatory response. *Endocrinology*, 146, 77-84.
- ASSEY, R. J., HYTTTEL, P., GREVE, T. & PURWANTARA, B. 1994. Oocyte morphology in dominant and subordinate follicles. *Mol Reprod Dev*, 37, 335-44.
- ASSIDI, M., DUFORT, I., ALI, A., HAMEL, M., ALGRIANY, O., DIELEMANN, S. & SIRARD, M. A. 2008. Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate in vitro. *Biol Reprod*, 79, 209-22.
- AU, H. K., YEH, T. S., KAO, S. H., TZENG, C. R. & HSIEH, R. H. 2005. Abnormal mitochondrial structure in human unfertilized oocytes and arrested embryos. *Ann N Y Acad Sci*, 1042, 177-85.

- AUGUSTIN, R., POCAR, P., NAVARRETE-SANTOS, A., WRENZYCKI, C., GANDOLFI, F., NIEMANN, H. & FISCHER, B. 2001. Glucose transporter expression is developmentally regulated in in vitro derived bovine preimplantation embryos. *Mol Reprod Dev*, 60, 370-6.
- BACH, L. A., HEADEY, S. J. & NORTON, R. S. 2005. IGF-binding proteins--the pieces are falling into place. *Trends Endocrinol Metab*, 16, 228-34.
- BACHVAROVA, R., DE LEON, V., JOHNSON, A., KAPLAN, G. & PAYNTON, B. V. 1985. Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. *Dev Biol*, 108, 325-31.
- BACHVAROVA, R. F. 1992. A maternal tail of poly(A): the long and the short of it. *Cell*, 69, 895-7.
- BAGGER, P. V., BYSKOV, A. G. & CHRISTIANSEN, M. D. 1987. Maturation of mouse oocytes in vitro is influenced by alkalization during their isolation. *J Reprod Fertil*, 80, 251-5.
- BAIRD, D. T., COLLINS, J., EGOZCUE, J., EVERS, L. H., GIANAROLI, L., LERIDON, H., SUNDE, A., TEMPLETON, A., VAN STEIRTEGHEM, A., COHEN, J., CROSIGNANI, P. G., DEVROEY, P., DIEDRICH, K., FAUSER, B. C., FRASER, L., GLASIER, A., LIEBAERS, I., MAUTONE, G., PENNEY, G., TARLATZIS, B. & GROUP, E. C. W. 2005. Fertility and ageing. *Hum Reprod Update*, 11, 261-76.
- BALABAN, R. S., NEMOTO, S. & FINKEL, T. 2005. Mitochondria, oxidants, and aging. *Cell*, 120, 483-95.
- BALTZ, J. M. 2001. Osmoregulation and cell volume regulation in the preimplantation embryo. *Curr Top Dev Biol*, 52, 55-106.
- BANCSI, L. F., BROEKMANS, F. J., EIJKEMANS, M. J., DE JONG, F. H., HABBEMA, J. D. & TE VELDE, E. R. 2002. Predictors of poor ovarian response in in vitro fertilization: a prospective study comparing basal markers of ovarian reserve. *Fertil Steril*, 77, 328-36.
- BANERJEE, D. & PAL, S. K. 2008a. Dynamics in the DNA recognition by DAPI: exploration of the various binding modes. *J Phys Chem B*, 112, 1016-21.
- BANERJEE, D. & PAL, S. K. 2008b. Excited-state solvation and proton transfer dynamics of DAPI in biomimetics and genomic DNA. *J Phys Chem A*, 112, 7314-20.
- BARBER, R. D., HARMER, D. W., COLEMAN, R. A. & CLARK, B. J. 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics*, 21, 389-95.
- BARBONI, B., RUSSO, V., CECCONI, S., CURINI, V., COLOSIMO, A., GAROFALO, M. L., CAPACCHIETTI, G., DI GIACINTO, O. & MATTIOLI, M. 2011. In vitro grown sheep preantral follicles yield oocytes with normal nuclear-epigenetic maturation. *PLoS One*, 6, e27550.
- BARNETT, D. K. & BAVISTER, B. D. 1996. What is the relationship between the metabolism of preimplantation embryos and their developmental competence? *Mol Reprod Dev*, 43, 105-33.
- BARR, J. A., JONES, J., GLENISTER, P. H. & CATTANACH, B. M. 1995. Ubiquitous expression and imprinting of Snrpn in the mouse. *Mamm Genome*, 6, 405-7.
- BARRITT, J. A., COHEN, J. & BRENNER, C. A. 2000. Mitochondrial DNA point mutation in human oocytes is associated with maternal age. *Reprod Biomed Online*, 1, 96-100.
- BARRITT, J. A., KOKOT, M., COHEN, J., STEUERWALD, N. & BRENNER, C. A. 2002. Quantification of human ooplasmic mitochondria. *Reprod Biomed Online*, 4, 243-7.
- BARTLEWSKI, P. M., BEARD, A. P., COOK, S. J., CHANDOLIA, R. K., HONARAMOOZ, A. & RAWLINGS, N. C. 1999. Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. *J Reprod Fertil*, 115, 111-24.
- BAVISTER, B. D. 1995. Culture of preimplantation embryos: facts and artifacts. *Hum Reprod Update*, 1, 91-148.
- BAVISTER, B. D. & SQUIRRELL, J. M. 2000. Mitochondrial distribution and function in oocytes and early embryos. *Hum Reprod*, 15 Suppl 2, 189-98.

- BEBBERE, D., ARIU, F., BOGLIOLO, L., MASALA, L., MURRONE, O., FATTORINI, M., FALCHI, L. & LEDDA, S. 2014. Expression of maternally derived KHDC3, NLRP5, OOE1 and TLE6 is associated with oocyte developmental competence in the ovine species. *BMC Dev Biol*, 14, 40.
- BEBBERE, D., MASALA, L., ALBERTINI, D. F. & LEDDA, S. 2016. The subcortical maternal complex: multiple functions for one biological structure? *J Assist Reprod Genet*, 33, 1431-1438.
- BEDFORD, M. T. 2007. Arginine methylation at a glance. *J Cell Sci*, 120, 4243-6.
- BEEKMAN, L., TOHVER, T., DARDARI, R. & LEGUILLETTE, R. 2011. Evaluation of suitable reference genes for gene expression studies in bronchoalveolar lavage cells from horses with inflammatory airway disease. *BMC Mol Biol*, 12, 5.
- BEHR, B. R., STRATTON, C. J., FOOTE, W. D., KNUTZEN, V. & SHER, G. 1990. In vitro fertilization (IVF) of mouse ova in HEPES-buffered culture media. *J In Vitro Fert Embryo Transf*, 7, 9-15.
- BENKHALIFA, M., FERREIRA, Y. J., CHAHINE, H., LOUANJLI, N., MIRON, P., MERVIEL, P. & COPIN, H. 2014. Mitochondria: participation to infertility as source of energy and cause of senescence. *Int J Biochem Cell Biol*, 55, 60-4.
- BENTOV, Y. & CASPER, R. F. 2013. The aging oocyte--can mitochondrial function be improved? *Fertil Steril*, 99, 18-22.
- BENTOV, Y., YAVORSKA, T., ESFANDIARI, N., JURISICOVA, A. & CASPER, R. F. 2011. The contribution of mitochondrial function to reproductive aging. *J Assist Reprod Genet*, 28, 773-83.
- BERG, J. M., TYMOCZKO, J. L. & STRYER, L. 2002. *Biochemistry. 5th edition.*, New York, W. H. Freeman.
- BERGER, L. & WILDE, A. 2013. Glycolytic metabolites are critical modulators of oocyte maturation and viability. *PLoS One*, 8, e77612.
- BERMUDEZ, M. G., WELLS, D., MALTER, H., MUNNE, S., COHEN, J. & STEUERWALD, N. M. 2004. Expression profiles of individual human oocytes using microarray technology. *Reprod Biomed Online*, 8, 325-37.
- BHATTACHARYYA, A. & YANAGIMACHI, R. 1988. Synthetic organic pH buffers can support fertilization of guinea pig eggs, but not as efficiently as bicarbonate buffer. *Gamete Res*, 19, 123-9.
- BHOJWANI, M., RUDOLPH, E., KANITZ, W., ZUEHLKE, H., SCHNEIDER, F. & TOMEK, W. 2006. Molecular analysis of maturation processes by protein and phosphoprotein profiling during in vitro maturation of bovine oocytes: a proteomic approach. *Cloning Stem Cells*, 8, 259-74.
- BIBB, M. J., VAN ETTEN, R. A., WRIGHT, C. T., WALBERG, M. W. & CLAYTON, D. A. 1981. Sequence and gene organization of mouse mitochondrial DNA. *Cell*, 26, 167-80.
- BIGGERS, J. D. & STERN, S. 1973. Metabolism of the preimplantation mammalian embryo. *Adv Reprod Physiol*, 6, 1-59.
- BIGGERS, J. D., WHITTINGHAM, D. G. & DONAHUE, R. P. 1967. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A*, 58, 560-7.
- BOGENHAGEN, D. & CLAYTON, D. A. 1976. Thymidylate nucleotide supply for mitochondrial DNA synthesis in mouse L-cells. Effect of 5-fluorodeoxyuridine and methotrexate in thymidine kinase plus and thymidine kinase minus cells. *J Biol Chem*, 251, 2938-44.
- BOLAND, N. I., HUMPHERSON, P. G., LEESE, H. J. & GOSDEN, R. G. 1994a. Characterization of follicular energy metabolism. *Hum Reprod*, 9, 604-9.
- BOLAND, N. I., HUMPHERSON, P. G., LEESE, H. J. & GOSDEN, R. G. 1994b. The effect of glucose metabolism on murine follicle development and steroidogenesis in vitro. *Hum Reprod*, 9, 617-23.

- BOOTH, P. J., HUMPHERSON, P. G., WATSON, T. J. & LEESE, H. J. 2005. Amino acid depletion and appearance during porcine preimplantation embryo development in vitro. *Reproduction*, 130, 655-68.
- BORNSLAEGER, E. A., POUYMIROU, W. T., MATTEI, P. & SCHULTZ, R. M. 1986. Effects of protein kinase C activators on germinal vesicle breakdown and polar body emission of mouse oocytes. *Exp Cell Res*, 165, 507-17.
- BORNSLAEGER, E. A. & SCHULTZ, R. M. 1985. Regulation of mouse oocyte maturation: effect of elevating cumulus cell cAMP on oocyte cAMP levels. *Biol Reprod*, 33, 698-704.
- BOUNIOL-BALY, C., HAMRAOUI, L., GUIBERT, J., BEAUJEAN, N., SZOLLOSI, M. S. & DEBEY, P. 1999. Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes. *Biol Reprod*, 60, 580-7.
- BOURA-HALFON, S. & ZICK, Y. 2009. Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab*, 296, E581-91.
- BOURC'HIS, D., XU, G. L., LIN, C. S., BOLLMAN, B. & BESTOR, T. H. 2001. Dnmt3L and the establishment of maternal genomic imprints. *Science*, 294, 2536-9.
- BOWDIN, S., ALLEN, C., KIRBY, G., BRUETON, L., AFNAN, M., BARRATT, C., KIRKMAN-BROWN, J., HARRISON, R., MAHER, E. R. & REARDON, W. 2007. A survey of assisted reproductive technology births and imprinting disorders. *Hum Reprod*, 22, 3237-40.
- BRATIC, A. & LARSSON, N. G. 2013. The role of mitochondria in aging. *J Clin Invest*, 123, 951-7.
- BRAUDE, P., BOLTON, V. & MOORE, S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*, 332, 459-61.
- BRAUN, R. E., BEHRINGER, R. R., PESCHON, J. J., BRINSTER, R. L. & PALMITER, R. D. 1989. Genetically haploid spermatids are phenotypically diploid. *Nature*, 337, 373-6.
- BREVINI-GANDOLFI, T. A., FAVETTA, L. A., MAURI, L., LUCIANO, A. M., CILLO, F. & GANDOLFI, F. 1999. Changes in poly(A) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence. *Mol Reprod Dev*, 52, 427-33.
- BREVINI, T. A., CILLO, F., ANTONINI, S. & GANDOLFI, F. 2007. Cytoplasmic remodelling and the acquisition of developmental competence in pig oocytes. *Anim Reprod Sci*, 98, 23-38.
- BREVINI, T. A., LONERGAN, P., CILLO, F., FRANCISCI, C., FAVETTA, L. A., FAIR, T. & GANDOLFI, F. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol Reprod Dev*, 63, 510-7.
- BREVINI, T. A., VASSENA, R., FRANCISCI, C. & GANDOLFI, F. 2005. Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. *Biol Reprod*, 72, 1218-23.
- BRINSTER, R. L. 1965. Lactate dehydrogenase activity in the preimplanted mouse embryo. *Biochim Biophys Acta*, 110, 439-41.
- BRINSTER, R. L. 1968. Lactate dehydrogenase activity in the oocytes of mammals. *J Reprod Fertil*, 17, 139-46.
- BRINSTER, R. L. 1971. Oxidation of pyruvate and glucose by oocytes of the mouse and rhesus monkey. *J Reprod Fertil*, 24, 187-91.
- BRINSTER, R. L. 1973. Parental glucose phosphate isomerase activity in three-day mouse embryos. *Biochem Genet*, 9, 187-91.
- BRISON, D. R., HOUGHTON, F. D., FALCONER, D., ROBERTS, S. A., HAWKHEAD, J., HUMPHERSON, P. G., LIEBERMAN, B. A. & LEESE, H. J. 2004. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod*, 19, 2319-24.
- BROEKMANS, F. J. 2009. Testing for Ovarian Reserve in Assisted Reproduction programs: the current point of view. *Facts Views Vis Obgyn*, 1, 79-87.

- BROEKMANS, F. J., KNAUFF, E. A., TE VELDE, E. R., MACKLON, N. S. & FAUSER, B. C. 2007. Female reproductive ageing: current knowledge and future trends. *Trends Endocrinol Metab*, 18, 58-65.
- BROEKMANS, F. J., SOULES, M. R. & FAUSER, B. C. 2009. Ovarian aging: mechanisms and clinical consequences. *Endocr Rev*, 30, 465-93.
- BROER, S., SCHNEIDER, H. P., BROER, A., RAHMAN, B., HAMPRECHT, B. & DEITMER, J. W. 1998. Characterization of the monocarboxylate transporter 1 expressed in *Xenopus laevis* oocytes by changes in cytosolic pH. *Biochem J*, 333 (Pt 1), 167-74.
- BROGAN, R. S., MIX, S., PUTTABYATAPPA, M., VANDEVOORT, C. A. & CHAFFIN, C. L. 2010. Expression of the insulin-like growth factor and insulin systems in the luteinizing macaque ovarian follicle. *Fertil Steril*, 93, 1421-9.
- BUI, T. T. H., BELLI, M., FASSINA, L., VIGONE, G., MERICO, V., GARAGNA, S. & ZUCCOTTI, M. 2017. Cytoplasmic movement profiles of mouse surrounding nucleolus and not-surrounding nucleolus antral oocytes during meiotic resumption. *Mol Reprod Dev*, 84, 356-362.
- BUKOVSKY, A., CAUDLE, M. R., KEENAN, J. A., WIMALASENA, J., FOSTER, J. S., UPADHYAYA, N. B. & VAN METER, S. E. 1995. Expression of cell cycle regulatory proteins (p53, pRb) in the human female genital tract. *J Assist Reprod Genet*, 12, 123-31.
- BUNN, R. C. & FOWLKES, J. L. 2003. Insulin-like growth factor binding protein proteolysis. *Trends Endocrinol Metab*, 14, 176-81.
- BUSTIN, S. A. 2010. Developments in real-time PCR research and molecular diagnostics. *Expert Rev Mol Diagn*, 10, 713-5.
- BUTCHER, L., COATES, A., MARTIN, K. L., RUTHERFORD, A. J. & LEESE, H. J. 1998. Metabolism of pyruvate by the early human embryo. *Biol Reprod*, 58, 1054-6.
- BUTLER, J. E., LECHENE, C. & BIGGERS, J. D. 1988. Noninvasive measurement of glucose uptake by two populations of murine embryos. *Biol Reprod*, 39, 779-86.
- BYRD, S. R., FLORES-FOXWORTH, G., APPLEWHITE, A. A. & WESTHUSIN, M. E. 1997. In vitro maturation of ovine oocytes in a portable incubator. *Theriogenology*, 47, 857-64.
- CAI, J., LI, T., HUANG, B., CHENG, H., DING, H., DONG, W., XIAO, M., LIU, L. & WANG, Z. 2014. The use of laser microdissection in the identification of suitable reference genes for normalization of quantitative real-time PCR in human FFPE epithelial ovarian tissue samples. *PLoS One*, 9, e95974.
- CAI, X. & CULLEN, B. R. 2007. The imprinted H19 noncoding RNA is a primary microRNA precursor. *RNA*, 13, 313-6.
- CALAFELL, J. M., NOGUES, C., PONSÁ, M., SANTALO, J. & EGOZCUE, J. 1992. Zona pellucida surface of immature and in vitro matured mouse oocytes: analysis by scanning electron microscopy. *J Assist Reprod Genet*, 9, 365-72.
- CAMPBELL, K. & SWANN, K. 2006. Ca²⁺ oscillations stimulate an ATP increase during fertilization of mouse eggs. *Dev Biol*, 298, 225-33.
- CAMPOS-CHILLON, F., FARMERIE, T. A., BOUMA, G. J., CLAY, C. M. & CARNEVALE, E. M. 2015. Effects of aging on gene expression and mitochondrial DNA in the equine oocyte and follicle cells. *Reprod Fertil Dev*, 27, 925-33.
- CAO, L., SHITARA, H., HORII, T., NAGAO, Y., IMAI, H., ABE, K., HARA, T., HAYASHI, J. & YONEKAWA, H. 2007. The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat Genet*, 39, 386-90.
- CAO, X. Y., ROSE, J., WANG, S. Y., LIU, Y., ZHAO, M., XING, M. J., CHANG, T. & XU, B. 2016. Glycine increases preimplantation development of mouse oocytes following vitrification at the germinal vesicle stage. *Sci Rep*, 6, 37262.
- CARO, C. M. & TROUNSON, A. 1984. The effect of protein on preimplantation mouse embryo development in vitro. *J In Vitro Fert Embryo Transf*, 1, 183-7.
- CARROLL, J. 2001. The initiation and regulation of Ca²⁺ signalling at fertilization in mammals. *Semin Cell Dev Biol*, 12, 37-43.

- CARROLL, J., SWANN, K., WHITTINGHAM, D. & WHITAKER, M. 1994. Spatiotemporal dynamics of intracellular $[Ca^{2+}]_i$ oscillations during the growth and meiotic maturation of mouse oocytes. *Development*, 120, 3507-17.
- CAUFFMAN, G., VAN DE VELDE, H., LIEBAERS, I. & VAN STEIRTEGHEM, A. 2005. DAZL expression in human oocytes, preimplantation embryos and embryonic stem cells. *Mol Hum Reprod*, 11, 405-11.
- CENDAN, J. C., TOPPING, D. L., PRUITT, J., SNOWDY, S., COPELAND, E. M., 3RD & LIND, D. S. 1996. Inflammatory mediators stimulate arginine transport and arginine-derived nitric oxide production in a murine breast cancer cell line. *J Surg Res*, 60, 284-8.
- CENTERS FOR DISEASE CONTROL AND PREVENTION 2017. AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE, SOCIETY FOR ASSISTED REPRODUCTIVE TECHNOLOGY. *2015 Assisted Reproductive Technology National Summary Report*. Atlanta (GA): US Dept of Health and Human Services.
- CETICA, P., PINTOS, L., DALVIT, G. & BECONI, M. 2002. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. *Reproduction*, 124, 675-81.
- CETICA, P., PINTOS, L., DALVIT, G. & BECONI, M. 2003. Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. *Reproduction*, 126, 753-63.
- CHAN, C. C., LIU, V. W., LAU, E. Y., YEUNG, W. S., NG, E. H. & HO, P. C. 2005. Mitochondrial DNA content and 4977 bp deletion in unfertilized oocytes. *Mol Hum Reprod*, 11, 843-6.
- CHANDRA, V., KUMAR, G. S. & SHARMA, G. T. 2011. Temporal expression pattern of insulin-like growth factors (IGF-1 and IGF-2) ligands and their receptors (IGF-1R and IGF-2R) in buffalo (*Bubalus bubalis*) embryos produced in vitro. *Livestock Science*, 135, 225-230.
- CHANG, A. S., MOLEY, K. H., WANGLER, M., FEINBERG, A. P. & DEBAUN, M. R. 2005. Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. *Fertil Steril*, 83, 349-54.
- CHANG, S. Y., TSAI, M. Y., HUANG, F. J. & KUNG, F. T. 2002. Expression of insulin-like growth factor (IGF), IGF receptor, and IGF-binding protein messenger ribonucleic acids in luteinized granulosa cells from different size follicles after controlled ovarian hyperstimulation. *J Assist Reprod Genet*, 19, 121-6.
- CHARRON, M. J., BROSIUS, F. C., 3RD, ALPER, S. L. & LODISH, H. F. 1989. A glucose transport protein expressed predominately in insulin-responsive tissues. *Proc Natl Acad Sci U S A*, 86, 2535-9.
- CHAUBE, S. K. & MISRO, M. M. 2002. Effect of styrene maleic anhydride (SMA) on viability and integrity of isolated rat oocytes in vitro. *Contraception*, 66, 469-72.
- CHEN, J., MELTON, C., SUH, N., OH, J. S., HORNER, K., XIE, F., SETTE, C., BLELLOCH, R. & CONTI, M. 2011. Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (*Dazl*) at the oocyte-to-zygote transition. *Genes Dev*, 25, 755-66.
- CHEN, L., HU, X., DAI, Y., LI, Q., WANG, X., LI, Q., XUE, K., LI, Y., LIANG, J., WANG, Y., LIU, X. & LI, N. 2012. MicroRNA-27a activity is not suppressed in porcine oocytes. *Front Biosci (Elite Ed)*, 4, 2679-85.
- CHEN, M. S., TUNG, K. S., COONROD, S. A., TAKAHASHI, Y., BIGLER, D., CHANG, A., YAMASHITA, Y., KINCADE, P. W., HERR, J. C. & WHITE, J. M. 1999. Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization. *Proc Natl Acad Sci U S A*, 96, 11830-5.
- CHEN, X., PROSSER, R., SIMONETTI, S., SADLOCK, J., JAGIELLO, G. & SCHON, E. A. 1995. Rearranged mitochondrial genomes are present in human oocytes. *Am J Hum Genet*, 57, 239-47.
- CHENNATHUKUZZHI, V., MORALES, C. R., EL-ALFY, M. & HECHT, N. B. 2003. The kinesin KIF17b and RNA-binding protein TB-RBP transport specific cAMP-responsive element

- modulator-regulated mRNAs in male germ cells. *Proc Natl Acad Sci U S A*, 100, 15566-71.
- CHI, M. M., MANCHESTER, J. K., BASURAY, R., MAHENDRA, S., STRICKLER, R. C., MCDUGAL, D. B., JR. & LOWRY, O. H. 1993. An unusual active hexose transport system in human and mouse preimplantation embryos. *Proc Natl Acad Sci U S A*, 90, 10023-5.
- CHIARATTI, M. R. & MEIRELLES, F. V. 2010. Mitochondrial DNA copy number, a marker of viability for oocytes. *Biol Reprod*, 83, 1-2.
- CHILD, T. J., PHILLIPS, S. J., ABDUL-JALIL, A. K., GULEKLI, B. & TAN, S. L. 2002. A comparison of in vitro maturation and in vitro fertilization for women with polycystic ovaries. *Obstet Gynecol*, 100, 665-70.
- CHOHAN, K. R. & HUNTER, A. G. 2003. Meiotic competence of bovine fetal oocytes following in vitro maturation. *Anim Reprod Sci*, 76, 43-51.
- CHRISTIANS, E., BOIANI, M., GARAGNA, S., DESSY, C., REDI, C. A., RENARD, J. P. & ZUCCOTTI, M. 1999. Gene expression and chromatin organization during mouse oocyte growth. *Dev Biol*, 207, 76-85.
- CLARK, A. R., STOKES, Y. M. & THOMPSON, J. G. 2011. Estimation of glucose uptake by ovarian follicular cells. *Ann Biomed Eng*, 39, 2654-67.
- COBO, A. C., REQUENA, A., NEUSPILLER, F., ARAGON S, M., MERCADER, A., NAVARRO, J., SIMON, C., REMOHI, J. & PELLICER, A. 1999. Maturation in vitro of human oocytes from unstimulated cycles: selection of the optimal day for ovum retrieval based on follicular size. *Hum Reprod*, 14, 1864-8.
- COLLADO-FERNANDEZ, E., PICTON, H. M. & DUMOLLARD, R. 2012. Metabolism throughout follicle and oocyte development in mammals. *Int J Dev Biol*, 56, 799-808.
- COLLADO FERNANDEZ, E. 2013. *Molecular and metabolic measures of oocyte developmental competence in vivo and in vitro*. PhD, University of Leeds.
- COLLADO FERNANDEZ, E., CAMPBELL, B. K., LU, J. & PICTON, H. M. Impact of Maternal Age on Oocyte Amino Acid Turnover and Mitochondria DNA Copy Number in Sheep. SRF Annual Meeting, 2015 Oxford.
- COLLIER, B., GORGONI, B., LOVERIDGE, C., COOKE, H. J. & GRAY, N. K. 2005. The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. *EMBO J*, 24, 2656-66.
- COMBELLES, C. M., ALBERTINI, D. F. & RACOWSKY, C. 2003. Distinct microtubule and chromatin characteristics of human oocytes after failed in-vivo and in-vitro meiotic maturation. *Hum Reprod*, 18, 2124-30.
- COMIZZOLI, P., PUKAZHENTHI, B. S. & WILDT, D. E. 2011. The competence of germinal vesicle oocytes is unrelated to nuclear chromatin configuration and strictly depends on cytoplasmic quantity and quality in the cat model. *Hum Reprod*, 26, 2165-77.
- COMIZZOLI, P., URNER, F., SAKKAS, D. & RENARD, J. P. 2003. Up-regulation of glucose metabolism during male pronucleus formation determines the early onset of the s phase in bovine zygotes. *Biol Reprod*, 68, 1934-40.
- CONTI, M. 2010. Signaling networks in somatic cells and oocytes activated during ovulation. *Ann Endocrinol (Paris)*, 71, 189-90.
- CONTI, M., ANDERSEN, C. B., RICHARD, F., MEHATS, C., CHUN, S. Y., HORNER, K., JIN, C. & TSAFRIRI, A. 2002. Role of cyclic nucleotide signaling in oocyte maturation. *Mol Cell Endocrinol*, 187, 153-9.
- COOK, D., FRY, M. J., HUGHES, K., SUMATHIPALA, R., WOODGETT, J. R. & DALE, T. C. 1996. Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. *EMBO J*, 15, 4526-36.
- COOPER, G. M. 2000. *The Cell: A Molecular Approach*, Sunderland, MA, Sinauer Associates.
- COTTERILL, M. 2008. *Gene function during the development of ovine oocytes and ovarian follicles*. PhD thesis, University of Leeds.

- COTTERILL, M., CATT, S. L. & PICTON, H. M. 2012. Characterisation of the cellular and molecular responses of ovine oocytes and their supporting somatic cells to pre-ovulatory levels of LH and FSH during in vitro maturation. *Reproduction*, 144, 195-207.
- COTTERILL, M., HARRIS, S. E., COLLADO FERNANDEZ, E., LU, J., HUNTRISS, J. D., CAMPBELL, B. K. & PICTON, H. M. 2013. The activity and copy number of mitochondrial DNA in ovine oocytes throughout oogenesis in vivo and during oocyte maturation in vitro. *Mol Hum Reprod*, 19, 444-50.
- COUTTET, P., FROMONT-RACINE, M., STEEL, D., PICTET, R. & GRANGE, T. 1997. Messenger RNA deadenylylation precedes decapping in mammalian cells. *Proc Natl Acad Sci U S A*, 94, 5628-33.
- CRABO, B. G., BROWN, K. I. & GRAHAM, E. F. 1972. Effect of some buffers on storage and freezing of boar spermatozoa. *J Anim Sci*, 35, 377-82.
- CUI, X. S., LI, X. Y., YIN, X. J., KONG, I. K., KANG, J. J. & KIM, N. H. 2007. Maternal gene transcription in mouse oocytes: genes implicated in oocyte maturation and fertilization. *J Reprod Dev*, 53, 405-18.
- CUI, X. S., SUN, S. C., KANG, Y. K. & KIM, N. H. 2013. Involvement of microRNA-335-5p in cytoskeleton dynamics in mouse oocytes. *Reprod Fertil Dev*, 25, 691-9.
- CUKURCAM, S., BETZENDAHL, I., MICHEL, G., VOGT, E., HEGELE-HARTUNG, C., LINDENTHAL, B. & EICHENLAUB-RITTER, U. 2007. Influence of follicular fluid meiosis-activating sterol on aneuploidy rate and precocious chromatid segregation in aged mouse oocytes. *Hum Reprod*, 22, 815-28.
- CUMMINS, J. 1998. Mitochondrial DNA in mammalian reproduction. *Rev Reprod*, 3, 172-82.
- CUMMINS, J. M. 2001. Mitochondria: potential roles in embryogenesis and nucleocytoplasmic transfer. *Hum Reprod Update*, 7, 217-28.
- CURTIS, D., LEHMANN, R. & ZAMORE, P. D. 1995. Translational regulation in development. *Cell*, 81, 171-8.
- DA COSTA, J. P., VITORINO, R., SILVA, G. M., VOGEL, C., DUARTE, A. C. & ROCHA-SANTOS, T. 2016. A synopsis on aging-Theories, mechanisms and future prospects. *Ageing Res Rev*, 29, 90-112.
- DA SILVEIRA, J. C., VEERAMACHANENI, D. N., WINGER, Q. A., CARNEVALE, E. M. & BOUMA, G. J. 2012. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. *Biol Reprod*, 86, 71.
- DALBIES-TRAN, R. & MERMILLOD, P. 2003. Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. *Biol Reprod*, 68, 252-61.
- DALIRI, M., RAO, K. B., KAUR, G., GARG, S., PATIL, S. & TOTTEY, S. M. 1999. Expression of growth factor ligand and receptor genes in preimplantation stage water buffalo (*Bubalus bubalis*) embryos and oviduct epithelial cells. *J Reprod Fertil*, 117, 61-70.
- DALTON, C. M. & CARROLL, J. 2013. Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte. *J Cell Sci*, 126, 2955-64.
- DAN-GOOR, M., SASSON, S., DAVARASHVILI, A. & ALMAGOR, M. 1997. Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Hum Reprod*, 12, 2508-10.
- DANFOUR, M. A. 2001. *Influence of the environment on mammalian oocyte development*. PhD thesis, University of Leeds.
- DANKERT, D., DEMOND, H., TRAPPHOFF, T., HEILIGENTAG, M., RADEMACHER, K., EICHENLAUB-RITTER, U., HORSTHEMKE, B. & GRUMMER, R. 2014. Pre- and postovulatory aging of murine oocytes affect the transcript level and poly(A) tail length of maternal effect genes. *PLoS One*, 9, e108907.

- DAWSON, K. M. & BALTZ, J. M. 1997. Organic osmolytes and embryos: substrates of the Gly and beta transport systems protect mouse zygotes against the effects of raised osmolarity. *Biol Reprod*, 56, 1550-8.
- DE BOEVER, S., VANGESTEL, C., DE BACKER, P., CROUBELS, S. & SYS, S. U. 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. *Vet Immunol Immunopathol*, 122, 312-7.
- DE BRUIN, J. P., DORLAND, M., SPEK, E. R., POSTHUMA, G., VAN HAAFTEN, M., LOOMAN, C. W. & TE VELDE, E. R. 2004. Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. *Biol Reprod*, 70, 419-24.
- DE CUEVAS, M., LILLY, M. A. & SPRADLING, A. C. 1997. Germline cyst formation in *Drosophila*. *Annu Rev Genet*, 31, 405-28.
- DE FELICI, M., LOBASCIO, A. M. & KLINGER, F. G. 2008. Cell death in fetal oocytes: many players for multiple pathways. *Autophagy*, 4, 240-2.
- DE LA FUENTE, R. 2006. Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. *Dev Biol*, 292, 1-12.
- DE LA FUENTE, R. & EPPIG, J. J. 2001. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. *Dev Biol*, 229, 224-36.
- DE LOOS, F. A., ZEINSTRA, E. & BEVERS, M. M. 1994. Follicular wall maintains meiotic arrest in bovine oocytes cultured in vitro. *Mol Reprod Dev*, 39, 162-5.
- DE MATOS, D. G., FURNUS, C. C., MOSES, D. F. & BALDASSARRE, H. 1995. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. *Mol Reprod Dev*, 42, 432-6.
- DE MATOS, D. G., FURNUS, C. C., MOSES, D. F., MARTINEZ, A. G. & MATKOVIC, M. 1996. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. *Mol Reprod Dev*, 45, 451-7.
- DE PAZ, P., SANCHEZ, A. J., DE LA FUENTE, J., CHAMORRO, C. A., ALVAREZ, M., ANEL, E. & ANEL, L. 2001. Ultrastructural and cytochemical comparison between calf and cow oocytes. *Theriogenology*, 55, 1107-16.
- DE SOUZA, D. K., SALLES, L. P. & ROSA E SILVA, A. A. 2015. Aspects of energetic substrate metabolism of in vitro and in vivo bovine embryos. *Braz J Med Biol Res*, 48, 191-7.
- DEAN, J. 2002. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. *J Reprod Immunol*, 53, 171-80.
- DEBAUN, M. R., NIEMITZ, E. L. & FEINBERG, A. P. 2003. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet*, 72, 156-60.
- DEBEY, P., SZOLLOSI, M. S., SZOLLOSI, D., VAUTIER, D., GIROUSSE, A. & BESOMBES, D. 1993. Competent mouse oocytes isolated from antral follicles exhibit different chromatin organization and follow different maturation dynamics. *Mol Reprod Dev*, 36, 59-74.
- DECKER, C. J. & PARKER, R. 1994. Mechanisms of mRNA degradation in eukaryotes. *Trends Biochem Sci*, 19, 336-40.
- DEKEL, N., LAWRENCE, T. S., GILULA, N. B. & BEERS, W. H. 1981. Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. *Dev Biol*, 86, 356-62.
- DENOMME, M. M. & MANN, M. R. 2012. Genomic imprints as a model for the analysis of epigenetic stability during assisted reproductive technologies. *Reproduction*, 144, 393-409.
- DEVOTO, L., CHRISTENSON, L. K., MCALLISTER, J. M., MAKRIGIANNAKIS, A. & STRAUSS, J. F., 3RD 1999. Insulin and insulin-like growth factor-I and -II modulate human granulosa-lutein cell steroidogenesis: enhancement of steroidogenic acute regulatory protein (StAR) expression. *Mol Hum Reprod*, 5, 1003-10.

- DIEDERICH, M., HANSMANN, T., HEINZMANN, J., BARG-KUES, B., HERRMANN, D., ALDAG, P., BAULAIN, U., REINHARD, R., KUES, W., WEISSGERBER, C., HAAF, T. & NIEMANN, H. 2012. DNA methylation and mRNA expression profiles in bovine oocytes derived from prepubertal and adult donors. *Reproduction*, 144, 319-30.
- DIEZ-FRAILE, A., LAMMENS, T., TILLEMANN, K., WITKOWSKI, W., VERHASSELT, B., DE SUTTER, P., BENOIT, Y., ESPEEL, M. & D'HERDE, K. 2014. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization. *Hum Fertil (Camb)*, 17, 90-8.
- DILUIGI, A., WEITZMAN, V. N., PACE, M. C., SIANO, L. J., MAIER, D. & MEHLMANN, L. M. 2008. Meiotic arrest in human oocytes is maintained by a Gs signaling pathway. *Biol Reprod*, 78, 667-72.
- DOBSON, A. T., RAJA, R., ABEYTA, M. J., TAYLOR, T., SHEN, S., HAQQ, C. & PERA, R. A. 2004. The unique transcriptome through day 3 of human preimplantation development. *Hum Mol Genet*, 13, 1461-70.
- DOI, M., YAMAOKA, I., FUKUNAGA, T. & NAKAYAMA, M. 2003. Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. *Biochem Biophys Res Commun*, 312, 1111-7.
- DOMINKO, T. & FIRST, N. L. 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Mol Reprod Dev*, 47, 456-67.
- DONNAY, I., PARTRIDGE, R. J. & LEESE, H. J. 1999. Can embryo metabolism be used for selecting bovine embryos before transfer? *Reprod Nutr Dev*, 39, 523-33.
- DONNISON, M. & PFEFFER, P. L. 2004. Isolation of genes associated with developmentally competent bovine oocytes and quantitation of their levels during development. *Biol Reprod*, 71, 1813-21.
- DOWNS, S. M. 1999. Uptake and metabolism of adenosine mediate a meiosis-arresting action on mouse oocytes. *Mol Reprod Dev*, 53, 208-21.
- DOWNS, S. M. 2000. Adenosine blocks hormone-induced meiotic maturation by suppressing purine de novo synthesis. *Mol Reprod Dev*, 56, 172-9.
- DOWNS, S. M. & HUDSON, E. D. 2000. Energy substrates and the completion of spontaneous meiotic maturation. *Zygote*, 8, 339-51.
- DOWNS, S. M., HUMPHERSON, P. G. & LEESE, H. J. 1998. Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. *Biol Reprod*, 58, 1084-94.
- DOWNS, S. M., HUMPHERSON, P. G. & LEESE, H. J. 2002. Pyruvate utilization by mouse oocytes is influenced by meiotic status and the cumulus oophorus. *Mol Reprod Dev*, 62, 113-23.
- DOWNS, S. M. & MASTROPOLO, A. M. 1994. The participation of energy substrates in the control of meiotic maturation in murine oocytes. *Dev Biol*, 162, 154-68.
- DOWNS, S. M. & MASTROPOLO, A. M. 1997. Culture conditions affect meiotic regulation in cumulus cell-enclosed mouse oocytes. *Mol Reprod Dev*, 46, 551-66.
- DOWNS, S. M. & VERHOEVEN, A. 2003. Glutamine and the maintenance of meiotic arrest in mouse oocytes: influence of culture medium, glucose, and cumulus cells. *Mol Reprod Dev*, 66, 90-7.
- DRABKOVA, P., ANDRLOVA, L., HAMPL, R. & KANDAR, R. 2016. Amino acid metabolism in human embryos. *Physiol Res*, 65, 823-832.
- DRAGAN, A. I., PAVLOVIC, R., MCGIVNEY, J. B., CASAS-FINET, J. R., BISHOP, E. S., STROUSE, R. J., SCHENERMAN, M. A. & GEDDES, C. D. 2012. SYBR Green I: fluorescence properties and interaction with DNA. *J Fluoresc*, 22, 1189-99.
- DRAGOVIC, R. A., RITTER, L. J., SCHULZ, S. J., AMATO, F., ARMSTRONG, D. T. & GILCHRIST, R. B. 2005. Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology*, 146, 2798-806.

- DRAGOVIC, R. A., RITTER, L. J., SCHULZ, S. J., AMATO, F., THOMPSON, J. G., ARMSTRONG, D. T. & GILCHRIST, R. B. 2007. Oocyte-secreted factor activation of SMAD 2/3 signaling enables initiation of mouse cumulus cell expansion. *Biol Reprod*, 76, 848-57.
- DREYER, H. C., DRUMMOND, M. J., PENNING, B., FUJITA, S., GLYNN, E. L., CHINKES, D. L., DHANANI, S., VOLPI, E. & RASMUSSEN, B. B. 2008. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *Am J Physiol Endocrinol Metab*, 294, E392-400.
- DRUMMOND, D. A. & WILKE, C. O. 2009. The evolutionary consequences of erroneous protein synthesis. *Nat Rev Genet*, 10, 715-24.
- DUCIBELLA, T., HUNEAU, D., ANGELICHIO, E., XU, Z., SCHULTZ, R. M., KOPF, G. S., FISSORE, R., MADOUX, S. & OZIL, J. P. 2002. Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev Biol*, 250, 280-91.
- DUMOLLARD, R., CARROLL, J., DUCHEN, M. R., CAMPBELL, K. & SWANN, K. 2009. Mitochondrial function and redox state in mammalian embryos. *Semin Cell Dev Biol*, 20, 346-53.
- DUMOLLARD, R., DUCHEN, M. & CARROLL, J. 2007a. The role of mitochondrial function in the oocyte and embryo. *Curr Top Dev Biol*, 77, 21-49.
- DUMOLLARD, R., DUCHEN, M. & SARDET, C. 2006. Calcium signals and mitochondria at fertilisation. *Semin Cell Dev Biol*, 17, 314-23.
- DUMOLLARD, R., MARANGOS, P., FITZHARRIS, G., SWANN, K., DUCHEN, M. & CARROLL, J. 2004. Sperm-triggered [Ca²⁺] oscillations and Ca²⁺ homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development*, 131, 3057-67.
- DUMOLLARD, R., WARD, Z., CARROLL, J. & DUCHEN, M. R. 2007b. Regulation of redox metabolism in the mouse oocyte and embryo. *Development*, 134, 455-65.
- DUNNING, K. R., CASHMAN, K., RUSSELL, D. L., THOMPSON, J. G., NORMAN, R. J. & ROBKER, R. L. 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol Reprod*, 83, 909-18.
- DUNNING, K. R., RUSSELL, D. L. & ROBKER, R. L. 2014. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. *Reproduction*, 148, R15-27.
- DUNPHY, W. G., BRIZUELA, L., BEACH, D. & NEWPORT, J. 1988. The *Xenopus cdc2* protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell*, 54, 423-31.
- DUNSON, D. B., BAIRD, D. D. & COLOMBO, B. 2004. Increased infertility with age in men and women. *Obstet Gynecol*, 103, 51-6.
- DURAN, H. E., SIMSEK-DURAN, F., OEHNINGER, S. C., JONES, H. W., JR. & CASTORA, F. J. 2011. The association of reproductive senescence with mitochondrial quantity, function, and DNA integrity in human oocytes at different stages of maturation. *Fertil Steril*, 96, 384-8.
- DURINZI, K. L., SANIGA, E. M. & LANZENDORF, S. E. 1995. The relationship between size and maturation in vitro in the unstimulated human oocyte. *Fertil Steril*, 63, 404-6.
- EBERT, K. M., LIEM, H. & HECHT, N. B. 1988. Mitochondrial DNA in the mouse preimplantation embryo. *J Reprod Fertil*, 82, 145-9.
- EBERWINE, J., YEH, H., MIYASHIRO, K., CAO, Y., NAIR, S., FINNELL, R., ZETTEL, M. & COLEMAN, P. 1992. Analysis of gene expression in single live neurons. *Proc Natl Acad Sci U S A*, 89, 3010-4.
- EDWARDS, R. G. 1965a. Maturation in vitro of human ovarian oocytes. *Lancet*, 2, 926-9.
- EDWARDS, R. G. 1965b. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*, 208, 349-51.
- EFSTRATIADIS, A. 1998. Genetics of mouse growth. *Int J Dev Biol*, 42, 955-76.
- EICHENLAUB-RITTER, U. 1998. Genetics of oocyte ageing. *Maturitas*, 30, 143-69.
- EICHENLAUB-RITTER, U. 2012. Oocyte ageing and its cellular basis. *Int J Dev Biol*, 56, 841-52.

- EICHENLAUB-RITTER, U., CHANDLEY, A. C. & GOSDEN, R. G. 1988. The CBA mouse as a model for age-related aneuploidy in man: studies of oocyte maturation, spindle formation and chromosome alignment during meiosis. *Chromosoma*, 96, 220-6.
- EICHENLAUB-RITTER, U., VOGT, E., YIN, H. & GOSDEN, R. 2004. Spindles, mitochondria and redox potential in ageing oocytes. *Reprod Biomed Online*, 8, 45-58.
- EICHENLAUB-RITTER, U., WIECZOREK, M., LUKE, S. & SEIDEL, T. 2011. Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion*, 11, 783-96.
- EIJKEMANS, M. J., VAN POPPEL, F., HABBEMA, D. F., SMITH, K. R., LERIDON, H. & TE VELDE, E. R. 2014. Too old to have children? Lessons from natural fertility populations. *Hum Reprod*, 29, 1304-12.
- EL-BARADI, T., BOUWMEESTER, T., GILTAY, R. & PIELER, T. 1991. The maternal store of zinc finger protein encoding mRNAs in fully grown *Xenopus* oocytes is not required for early embryogenesis. *EMBO J*, 10, 1407-13.
- EL MEZIANE, A., CALLEN, J. C. & MOUNOLOU, J. C. 1989. Mitochondrial gene expression during *Xenopus laevis* development: a molecular study. *EMBO J*, 8, 1649-55.
- EL SHOURBAGY, S. H., SPIKINGS, E. C., FREITAS, M. & ST JOHN, J. C. 2006. Mitochondria directly influence fertilisation outcome in the pig. *Reproduction*, 131, 233-45.
- ELVIN, J. A., YAN, C., WANG, P., NISHIMORI, K. & MATZUK, M. M. 1999. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. *Mol Endocrinol*, 13, 1018-34.
- ENE, A. C., PARK, S., EDELMANN, W. & TAKETO, T. 2013. Caspase 9 is constitutively activated in mouse oocytes and plays a key role in oocyte elimination during meiotic prophase progression. *Dev Biol*, 377, 213-23.
- EPPIG, J. J. 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev Biol*, 89, 268-72.
- EPPIG, J. J. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, 122, 829-38.
- EPPIG, J. J., O'BRIEN, M. J., WIGGLESWORTH, K., NICHOLSON, A., ZHANG, W. & KING, B. A. 2009. Effect of in vitro maturation of mouse oocytes on the health and lifespan of adult offspring. *Hum Reprod*, 24, 922-8.
- ERBACH, G. T., LAWITTS, J. A., PAPAIOANNOU, V. E. & BIGGERS, J. D. 1994. Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol Reprod*, 50, 1027-33.
- ERCOLANI, L., FLORENCE, B., DENARO, M. & ALEXANDER, M. 1988. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. *J Biol Chem*, 263, 15335-41.
- EZZ ELDIN, H. M. & SARHAN, R. M. 2014. Cytotoxic effect of organic solvents and surfactant agents on *Acanthamoeba castellanii* cysts. *Parasitol Res*, 113, 1949-53.
- FABBRIZIO, E., EL MESSAOUDI, S., POLANOWSKA, J., PAUL, C., COOK, J. R., LEE, J. H., NEGRE, V., ROUSSET, M., PESTKA, S., LE CAM, A. & SARDET, C. 2002. Negative regulation of transcription by the type II arginine methyltransferase PRMT5. *EMBO Rep*, 3, 641-5.
- FADDY, M. J., GOSDEN, R. G., GOUGEON, A., RICHARDSON, S. J. & NELSON, J. F. 1992. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Hum Reprod*, 7, 1342-6.
- FAIR, T., HULSHOF, S. C., HYTTEL, P., GREVE, T. & BOLAND, M. 1997. Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. *Mol Reprod Dev*, 46, 208-15.
- FAIR, T., HYTTEL, P. & GREVE, T. 1995. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Mol Reprod Dev*, 42, 437-42.
- FAIR, T., HYTTEL, P., GREVE, T. & BOLAND, M. 1996. Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle. *Mol Reprod Dev*, 43, 503-12.

- FAN, H. Y. & SUN, Q. Y. 2004. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod*, 70, 535-47.
- FANG, Y., ZHANG, X., ZHANG, J., ZHONG, R. & ZHOU, D. 2016. Global DNA methylation and related mRNA profiles in sheep oocytes and early embryos derived from pre-pubertal and adult donors. *Anim Reprod Sci*, 164, 144-51.
- FERGUSON, E. M. & LEESE, H. J. 1999. Triglyceride content of bovine oocytes and early embryos. *J Reprod Fertil*, 116, 373-8.
- FERGUSON, E. M. & LEESE, H. J. 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. *Mol Reprod Dev*, 73, 1195-201.
- FERNANDEZ-SILVA, P., ENRIQUEZ, J. A. & MONTOYA, J. 2003. Replication and transcription of mammalian mitochondrial DNA. *Exp Physiol*, 88, 41-56.
- FERREIRA, E. M., VIREQUE, A. A., ADONA, P. R., MEIRELLES, F. V., FERRIANI, R. A. & NAVARRO, P. A. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*, 71, 836-48.
- FERRELL, J. E., JR. 1999. Xenopus oocyte maturation: new lessons from a good egg. *Bioessays*, 21, 833-42.
- FISCHER, B. & BAVISTER, B. D. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil*, 99, 673-9.
- FISHER, D. L., MORIN, N. & DOREE, M. 1999. A novel role for glycogen synthase kinase-3 in Xenopus development: maintenance of oocyte cell cycle arrest by a beta-catenin-independent mechanism. *Development*, 126, 567-76.
- FISSORE, R. A., KUROKAWA, M., KNOTT, J., ZHANG, M. & SMYTH, J. 2002. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction*, 124, 745-54.
- FITZHARRIS, G. & BALTZ, J. M. 2006. Granulosa cells regulate intracellular pH of the murine growing oocyte via gap junctions: development of independent homeostasis during oocyte growth. *Development*, 133, 591-9.
- FITZHARRIS, G., MARANGOS, P. & CARROLL, J. 2007a. Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein. *Dev Biol*, 305, 133-44.
- FITZHARRIS, G., SIYANOV, V. & BALTZ, J. M. 2007b. Granulosa cells regulate oocyte intracellular pH against acidosis in preantral follicles by multiple mechanisms. *Development*, 134, 4283-95.
- FLAHERTY, S. P., PAYNE, D., SWANN, N. J. & MATTEWS, C. D. 1995. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod*, 10, 2623-9.
- FLOOD, M. R. & WIEBOLD, J. L. 1988. Glucose metabolism by preimplantation pig embryos. *J Reprod Fertil*, 84, 7-12.
- FORD, J. H. 2013. Reduced quality and accelerated follicle loss with female reproductive aging - does decline in theca dehydroepiandrosterone (DHEA) underlie the problem? *J Biomed Sci*, 20, 93.
- FORMAN, S., KAS, J., FINI, F., STEINBERG, M. & RUMMLER, T. 1999. The effect of different solvents on the ATP/ADP content and growth properties of HeLa cells. *J Biochem Mol Toxicol*, 13, 11-5.
- FOX, C. A., SHEETS, M. D. & WICKENS, M. P. 1989. Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUUU. *Genes Dev*, 3, 2151-62.
- FRAGOULI, E., ALFARAWATI, S., DAPHNIS, D. D., GOODALL, N. N., MANIA, A., GRIFFITHS, T., GORDON, A. & WELLS, D. 2011a. Cytogenetic analysis of human blastocysts with the use of FISH, CGH and aCGH: scientific data and technical evaluation. *Hum Reprod*, 26, 480-90.

- FRAGOULI, E., ALFARAWATI, S., GOODALL, N. N., SANCHEZ-GARCIA, J. F., COLLS, P. & WELLS, D. 2011b. The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy. *Mol Hum Reprod*, 17, 286-95.
- FRANCIOSI, F., LODDE, V., GOUDET, G., DUCHAMP, G., DELEUZE, S., DOUET, C., TESSARO, I. & LUCIANO, A. M. 2012. Changes in histone H4 acetylation during in vivo versus in vitro maturation of equine oocytes. *Mol Hum Reprod*, 18, 243-52.
- FRESHNEY, R. I. 2010. Database of misidentified cell lines. *Int J Cancer*, 126, 302.
- FU, J., BIAN, L., ZHAO, L., DONG, Z., GAO, X., LUAN, H., SUN, Y. & SONG, H. 2010. Identification of genes for normalization of quantitative real-time PCR data in ovarian tissues. *Acta Biochim Biophys Sin (Shanghai)*, 42, 568-74.
- FUHRER, F., MAYR, B., SCHELLANDER, K., KALAT, M. & SCHLEGER, W. 1989. Maturation competence and chromatin behaviour in growing and fully grown cattle oocytes. *Zentralbl Veterinarmed A*, 36, 285-91.
- FUJINO, Y., OZAKI, K., YAMAMASU, S., ITO, F., MATSUOKA, I., HAYASHI, E., NAKAMURA, H., OGITA, S., SATO, E. & INOUE, M. 1996. DNA fragmentation of oocytes in aged mice. *Hum Reprod*, 11, 1480-3.
- FULKA, J., JR., JUNG, T. & MOOR, R. M. 1992. The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. *Mol Reprod Dev*, 32, 378-82.
- FULOP, C., SZANTO, S., MUKHOPADHYAY, D., BARDOS, T., KAMATH, R. V., RUGG, M. S., DAY, A. J., SALUSTRI, A., HASCALL, V. C., GLANT, T. T. & MIKECZ, K. 2003. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development*, 130, 2253-61.
- FUNAHASHI, H., KOIKE, T. & SAKAI, R. 2008. Effect of glucose and pyruvate on nuclear and cytoplasmic maturation of porcine oocytes in a chemically defined medium. *Theriogenology*, 70, 1041-7.
- GAL, A. B., CARNWATH, J. W., DINNYES, A., HERRMANN, D., NIEMANN, H. & WRENZYCKI, C. 2006. Comparison of real-time polymerase chain reaction and end-point polymerase chain reaction for the analysis of gene expression in preimplantation embryos. *Reprod Fertil Dev*, 18, 365-71.
- GANGLOFF, Y. G., MUELLER, M., DANN, S. G., SVOBODA, P., STICKER, M., SPETZ, J. F., UM, S. H., BROWN, E. J., CEREGHINI, S., THOMAS, G. & KOZMA, S. C. 2004. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol*, 24, 9508-16.
- GANGULY, A., MCKNIGHT, R. A., RAYCHAUDHURI, S., SHIN, B. C., MA, Z., MOLEY, K. & DEVASKAR, S. U. 2007. Glucose transporter isoform-3 mutations cause early pregnancy loss and fetal growth restriction. *Am J Physiol Endocrinol Metab*, 292, E1241-55.
- GARDNER, D. K. 1998. Development of serum-free media for the culture and transfer of human blastocysts. *Hum Reprod*, 13 Suppl 4, 218-25.
- GARDNER, D. K. & LANE, M. 1998. Culture of viable human blastocysts in defined sequential serum-free media. *Hum Reprod*, 13 Suppl 3, 148-59; discussion 160.
- GARDNER, D. K., LANE, M., STEVENS, J. & SCHOOLCRAFT, W. B. 2001. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril*, 76, 1175-80.
- GARDNER, D. K. & LEESE, H. J. 1986. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. *Hum Reprod*, 1, 25-7.
- GARDNER, D. K. & LEESE, H. J. 1990. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil*, 88, 361-8.
- GARDNER, R. L. & DAVIES, T. J. 2000. Mouse chimeras and the analysis of development. *Methods Mol Biol*, 135, 397-424.

- GAULDEN, M. E. 1992. Maternal age effect: the enigma of Down syndrome and other trisomic conditions. *Mutat Res*, 296, 69-88.
- GE, H., TOLLNER, T. L., HU, Z., DA, M., LI, X., GUAN, H., SHAN, D., LU, J., HUANG, C. & DONG, Q. 2012a. Impaired mitochondrial function in murine oocytes is associated with controlled ovarian hyperstimulation and in vitro maturation. *Reprod Fertil Dev*, 24, 945-52.
- GE, H., TOLLNER, T. L., HU, Z., DAI, M., LI, X., GUAN, H., SHAN, D., ZHANG, X., LV, J., HUANG, C. & DONG, Q. 2012b. The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. *Mol Reprod Dev*, 79, 392-401.
- GESHI, M., TAKENOUCI, N., YAMAUCHI, N. & NAGAI, T. 2000. Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. *Biol Reprod*, 63, 1730-4.
- GESHI, M., YONAI, M., SAKAGUCHI, M. & NAGAI, T. 1999. Improvement of in vitro co-culture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with beta-mercaptoethanol. *Theriogenology*, 51, 551-8.
- GHAFFARI, F., GUTIERREZ, C. G. & HARTSHORNE, G. M. 2007. Apoptosis in mouse fetal and neonatal oocytes during meiotic prophase one. *BMC Dev Biol*, 7, 87.
- GHARIBI, S., HAJIAN, M., OSTADHOSSEINI, S., HOSSEINI, S. M., FOROUZANFAR, M. & NASR-ESFAHANI, M. H. 2013. Effect of phosphodiesterase type 3 inhibitor on nuclear maturation and in vitro development of ovine oocytes. *Theriogenology*, 80, 302-12.
- GICQUEL, C., GASTON, V., MANDELBAUM, J., SIFFROI, J. P., FLAHAULT, A. & LE BOUC, Y. 2003. In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN10T gene. *Am J Hum Genet*, 72, 1338-41.
- GILCHRIST, R. B., DE VOS, M., SMITZ, J. & THOMPSON, J. G. 2011. IVM media are designed specifically to support immature cumulus-oocyte complexes not denuded oocytes that have failed to respond to hyperstimulation. *Fertil Steril*, 96, e141; author reply e142.
- GILCHRIST, R. B., LANE, M. & THOMPSON, J. G. 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update*, 14, 159-77.
- GILCHRIST, R. B. & THOMPSON, J. G. 2007. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology*, 67, 6-15.
- GINTHER, O. J., KOT, K., KULICK, L. J. & WILTBANK, M. C. 1997. Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology*, 48, 75-87.
- GINZINGER, D. G. 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol*, 30, 503-12.
- GOMES, M. V., GOMES, C. C., PINTO, W., JR. & RAMOS, E. S. 2007. Methylation pattern at the KvDMR in a child with Beckwith-Wiedemann syndrome conceived by ICSI. *Am J Med Genet A*, 143A, 625-9.
- GONDOS, B., BHIRALEUS, P. & HOBEL, C. J. 1971. Ultrastructural observations on germ cells in human fetal ovaries. *Am J Obstet Gynecol*, 110, 644-52.
- GONZALES-FIGUEROA, H. & GONZALES-MOLFINO, H. M. 2005. Maturation of pig oocytes in vitro in a medium with pyruvate. *Braz J Med Biol Res*, 38, 869-72.
- GONZALEZ-SERRANO, A. F., PIRRO, V., FERREIRA, C. R., OLIVERI, P., EBERLIN, L. S., HEINZMANN, J., LUCAS-HAHN, A., NIEMANN, H. & COOKS, R. G. 2013. Desorption electrospray ionization mass spectrometry reveals lipid metabolism of individual oocytes and embryos. *PLoS One*, 8, e74981.
- GONZALEZ, I. M., MARTIN, P. M., BURDSAL, C., SLOAN, J. L., MAGER, S., HARRIS, T. & SUTHERLAND, A. E. 2012. Leucine and arginine regulate trophoblast motility through mTOR-dependent and independent pathways in the preimplantation mouse embryo. *Dev Biol*, 361, 286-300.
- GOODARZI, M. O., ANTOINE, H. J., PALL, M., CUI, J., GUO, X. & AZZIZ, R. 2007. Preliminary evidence of glycogen synthase kinase 3 beta as a genetic determinant of polycystic ovary syndrome. *Fertil Steril*, 87, 1473-6.

- GOODARZI, M. O., JONES, M. R., CHEN, Y. D. & AZZIZ, R. 2008. First evidence of genetic association between AKT2 and polycystic ovary syndrome. *Diabetes Care*, 31, 2284-7.
- GOOSSENS, K., VAN POUCKE, M., VAN SOOM, A., VANDESOMPELE, J., VAN ZEVEVEREN, A. & PEELMAN, L. J. 2005. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. *BMC Dev Biol*, 5, 27.
- GOPICHANDRAN, N. & LEESE, H. J. 2003. Metabolic characterization of the bovine blastocyst, inner cell mass, trophectoderm and blastocoel fluid. *Reproduction*, 126, 299-308.
- GORDO, A. C., HE, C. L., SMITH, S. & FISSORE, R. A. 2001. Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. *Mol Reprod Dev*, 59, 106-14.
- GORDO, A. C., WU, H., HE, C. L. & FISSORE, R. A. 2000. Injection of sperm cytosolic factor into mouse metaphase II oocytes induces different developmental fates according to the frequency of $[Ca^{2+}]_i$ oscillations and oocyte age. *Biol Reprod*, 62, 1370-9.
- GOSDEN, R. G. 2002. Oogenesis as a foundation for embryogenesis. *Mol Cell Endocrinol*, 186, 149-53.
- GOSDEN, R. G., TELFER, E., FADDY, M. J. & BROOK, D. J. 1989. Ovarian cyclicity and follicular recruitment in unilaterally ovariectomized mice. *J Reprod Fertil*, 87, 257-64.
- GOUGEON, A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev*, 17, 121-55.
- GRIVE, K. J. & FREIMAN, R. N. 2015. The developmental origins of the mammalian ovarian reserve. *Development*, 142, 2554-63.
- GRONDAHL, M. L., YDING ANDERSEN, C., BOGSTAD, J., NIELSEN, F. C., MEINERTZ, H. & BORUP, R. 2010. Gene expression profiles of single human mature oocytes in relation to age. *Hum Reprod*, 25, 957-68.
- GROTEGUT, C. A., CHISHOLM, C. A., JOHNSON, L. N., BROWN, H. L., HEINE, R. P. & JAMES, A. H. 2014. Medical and obstetric complications among pregnant women aged 45 and older. *PLoS One*, 9, e96237.
- GRUPEN, C. G., FUNG, M. & ARMSTRONG, D. T. 2006. Effects of milrinone and butyrolactone-I on porcine oocyte meiotic progression and developmental competence. *Reprod Fertil Dev*, 18, 309-17.
- GU, L., LIU, H., GU, X., BOOTS, C., MOLEY, K. H. & WANG, Q. 2015. Metabolic control of oocyte development: linking maternal nutrition and reproductive outcomes. *Cell Mol Life Sci*, 72, 251-71.
- GUENATRI, M., DUFFIE, R., IRANZO, J., FAUQUE, P. & BOURC'HIS, D. 2013. Plasticity in Dnmt3L-dependent and -independent modes of de novo methylation in the developing mouse embryo. *Development*, 140, 562-72.
- GUERIN, P., EL MOUATASSIM, S. & MENEZO, Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update*, 7, 175-89.
- GUGER, K. A. & GUMBINER, B. M. 1995. beta-Catenin has Wnt-like activity and mimics the Nieuwkoop signaling center in *Xenopus* dorsal-ventral patterning. *Dev Biol*, 172, 115-25.
- GUILLEMOT, F., CASPARY, T., TILGHMAN, S. M., COPELAND, N. G., GILBERT, D. J., JENKINS, N. A., ANDERSON, D. J., JOYNER, A. L., ROSSANT, J. & NAGY, A. 1995. Genomic imprinting of *Mash2*, a mouse gene required for trophoblast development. *Nat Genet*, 9, 235-42.
- GUTNISKY, C., DALVIT, G. C., PINTOS, L. N., THOMPSON, J. G., BECONI, M. T. & CETICA, P. D. 2007. Influence of hyaluronic acid synthesis and cumulus mucification on bovine oocyte in vitro maturation, fertilisation and embryo development. *Reprod Fertil Dev*, 19, 488-97.
- GUTNISKY, C., MORADO, S., DALVIT, G. C., THOMPSON, J. G. & CETICA, P. D. 2013. Glycolytic pathway activity: effect on IVM and oxidative metabolism of bovine oocytes. *Reprod Fertil Dev*, 25, 1026-35.

- HAGEN, D. R., PRATHER, R. S., SIMS, M. M. & FIRST, N. L. 1991. Development of one-cell porcine embryos to the blastocyst stage in simple media. *J Anim Sci*, 69, 1147-50.
- HALESTRAP, A. P. & PRICE, N. T. 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J*, 343 Pt 2, 281-99.
- HALLIDAY, J., OKE, K., BREHENY, S., ALGAR, E. & D, J. A. 2004. Beckwith-Wiedemann syndrome and IVF: a case-control study. *Am J Hum Genet*, 75, 526-8.
- HAMATANI, T., FALCO, G., CARTER, M. G., AKUTSU, H., STAGG, C. A., SHAROV, A. A., DUDEKULA, D. B., VANBUREN, V. & KO, M. S. 2004. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet*, 13, 2263-78.
- HAMMER, M. A., KOLAJOVA, M., LEVEILLE, M., CLAMAN, P. & BALTZ, J. M. 2000. Glycine transport by single human and mouse embryos. *Hum Reprod*, 15, 419-26.
- HAN, S. J. & CONTI, M. 2006. New pathways from PKA to the Cdc2/cyclin B complex in oocytes: Wee1B as a potential PKA substrate. *Cell Cycle*, 5, 227-31.
- HAN, Y., YAN, J., ZHOU, J., TENG, Z., BIAN, F., GUO, M., MAO, G., LI, J., WANG, J., ZHANG, M. & XIA, G. 2012. Acute fasting decreases the expression of GLUT1 and glucose utilisation involved in mouse oocyte maturation and cumulus cell expansion. *Reprod Fertil Dev*, 24, 733-42.
- HANSEN, D. V., POMERENING, J. R., SUMMERS, M. K., MILLER, J. J., FERRELL, J. E., JR. & JACKSON, P. K. 2007. Emi2 at the crossroads: where CSF meets MPF. *Cell Cycle*, 6, 732-8.
- HANSEN, K. R., KNOWLTON, N. S., THYER, A. C., CHARLESTON, J. S., SOULES, M. R. & KLEIN, N. A. 2008. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Hum Reprod*, 23, 699-708.
- HANSMANN, T., HEINZMANN, J., WRENZYCKI, C., ZECHNER, U., NIEMANN, H. & HAAF, T. 2011. Characterization of differentially methylated regions in 3 bovine imprinted genes: a model for studying human germ-cell and embryo development. *Cytogenet Genome Res*, 132, 239-47.
- HAOUZI, D., ASSOU, S., MONZO, C., VINCENS, C., DECHAUD, H. & HAMAMAH, S. 2012. Altered gene expression profile in cumulus cells of mature MII oocytes from patients with polycystic ovary syndrome. *Hum Reprod*, 27, 3523-30.
- HARA, S., TAKANO, T., FUJIKAWA, T., YAMADA, M., WAKAI, T., KONO, T. & OBATA, Y. 2014. Forced expression of DNA methyltransferases during oocyte growth accelerates the establishment of methylation imprints but not functional genomic imprinting. *Hum Mol Genet*, 23, 3853-64.
- HARDING, E. A., DAY, M. L., GIBB, C. A., JOHNSON, M. H. & COOK, D. I. 1999. The activity of the H⁺-monocarboxylate cotransporter during pre-implantation development in the mouse. *Pflugers Arch*, 438, 397-404.
- HARRIS, S. E. 2002. *Experimental and clinical investigation into mammalian oocyte metabolism, nutrition and fertility*. PhD thesis, University of Leeds.
- HARRIS, S. E., ADRIAENS, I., LEESE, H. J., GOSDEN, R. G. & PICTON, H. M. 2007. Carbohydrate metabolism by murine ovarian follicles and oocytes grown in vitro. *Reproduction*, 134, 415-24.
- HARRIS, S. E., GOPICHANDRAN, N., PICTON, H. M., LEESE, H. J. & ORSI, N. M. 2005. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology*, 64, 992-1006.
- HARRIS, S. E., LEESE, H. J., GOSDEN, R. G. & PICTON, H. M. 2009. Pyruvate and oxygen consumption throughout the growth and development of murine oocytes. *Mol Reprod Dev*, 76, 231-8.
- HARRIS, S. E., MARUTHINI, D., TANG, T., BALEN, A. H. & PICTON, H. M. 2010. Metabolism and karyotype analysis of oocytes from patients with polycystic ovary syndrome. *Hum Reprod*, 25, 2305-15.

- HARTSHORNE, G. M., LYRAKOU, S., HAMODA, H., OLOTO, E. & GHAFARI, F. 2009. Oogenesis and cell death in human prenatal ovaries: what are the criteria for oocyte selection? *Mol Hum Reprod*, 15, 805-19.
- HARVEY, A. J., KIND, K. L. & THOMPSON, J. G. 2002. REDOX regulation of early embryo development. *Reproduction*, 123, 479-86.
- HASHIMOTO, S., MINAMI, N., YAMADA, M. & IMAI, H. 2000. Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: relevance to intracellular reactive oxygen species and glutathione contents. *Mol Reprod Dev*, 56, 520-6.
- HASSOLD, T. & CHIU, D. 1985. Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. *Hum Genet*, 70, 11-7.
- HASSOLD, T., HALL, H. & HUNT, P. 2007. The origin of human aneuploidy: where we have been, where we are going. *Hum Mol Genet*, 16 Spec No. 2, R203-8.
- HASSOLD, T. & HUNT, P. 2001. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet*, 2, 280-91.
- HEFFNER, L. J. 2004. Advanced maternal age--how old is too old? *N Engl J Med*, 351, 1927-9.
- HEIKINHEIMO, O. & GIBBONS, W. E. 1998. The molecular mechanisms of oocyte maturation and early embryonic development are unveiling new insights into reproductive medicine. *Mol Hum Reprod*, 4, 745-56.
- HEIKINHEIMO, O., LANZENDORF, S. E., BAKA, S. G. & GIBBONS, W. E. 1995. Cell cycle genes c-mos and cyclin-B1 are expressed in a specific pattern in human oocytes and preimplantation embryos. *Hum Reprod*, 10, 699-707.
- HEILIG, C., ZALOGA, C., LEE, M., ZHAO, X., RISER, B., BROSIUS, F. & CORTES, P. 1995. Immunogold localization of high-affinity glucose transporter isoforms in normal rat kidney. *Lab Invest*, 73, 674-84.
- HEILIG, C. W., SAUNDERS, T., BROSIUS, F. C., 3RD, MOLEY, K., HEILIG, K., BAGGS, R., GUO, L. & CONNER, D. 2003. Glucose transporter-1-deficient mice exhibit impaired development and deformities that are similar to diabetic embryopathy. *Proc Natl Acad Sci U S A*, 100, 15613-8.
- HEMMINGS, K. E. 2007. *Cellular and molecular markers of oocyte quality*. PhD, University of Leeds.
- HEMMINGS, K. E., LEESE, H. J. & PICTON, H. M. 2012. Amino acid turnover by bovine oocytes provides an index of oocyte developmental competence in vitro. *Biol Reprod*, 86, 165, 1-12.
- HEMMINGS, K. E., MARUTHINI, D., VYJAYANTHI, S., HOGG, J. E., BALEN, A. H., CAMPBELL, B. K., LEESE, H. J. & PICTON, H. M. 2013. Amino acid turnover by human oocytes is influenced by gamete developmental competence, patient characteristics and gonadotrophin treatment. *Hum Reprod*, 28, 1031-44.
- HERBERT, M., LEVASSEUR, M., HOMER, H., YALLOP, K., MURDOCH, A. & MCDUGALL, A. 2003. Homologue disjunction in mouse oocytes requires proteolysis of securin and cyclin B1. *Nat Cell Biol*, 5, 1023-5.
- HERRICK, J. R., BRAD, A. M. & KRISHER, R. L. 2006a. Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation in vitro. *Reproduction*, 131, 289-98.
- HERRICK, J. R., LANE, M., GARDNER, D. K., BEHBOODI, E., MEMILI, E., BLASH, S., ECHELARD, Y. & KRISHER, R. L. 2006b. Metabolism, protein content, and in vitro embryonic development of goat cumulus-oocyte complexes matured with physiological concentrations of glucose and L-lactate. *Mol Reprod Dev*, 73, 256-66.
- HERRLER, A., KRUSCHE, C. A. & BEIER, H. M. 1998. Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis. *Biol Reprod*, 59, 1302-10.

- HERUBEL, F., EL MOUATASSIM, S., GUERIN, P., FRYDMAN, R. & MENEZO, Y. 2002. Genetic expression of monocarboxylate transporters during human and murine oocyte maturation and early embryonic development. *Zygote*, 10, 175-81.
- HINRICHS, K., CHOI, Y. H., LOVE, L. B., VARNER, D. D., LOVE, C. C. & WALCKENAER, B. E. 2005. Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. *Biol Reprod*, 72, 1142-50.
- HINRICHS, K. & SCHMIDT, A. L. 2000. Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. *Biol Reprod*, 62, 1402-8.
- HINRICHS, K., SCHMIDT, A. L., FRIEDMAN, P. P., SELGRATH, J. P. & MARTIN, M. G. 1993. In vitro maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. *Biol Reprod*, 48, 363-70.
- HIRAO, Y., TSUJI, Y., MIYANO, T., OKANO, A., MIYAKE, M., KATO, S. & MOOR, R. M. 1995. Association between p34cdc2 levels and meiotic arrest in pig oocytes during early growth. *Zygote*, 3, 325-32.
- HODGES, C. A., REVENKOVA, E., JESSBERGER, R., HASSOLD, T. J. & HUNT, P. A. 2005. SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction. *Nat Genet*, 37, 1351-5.
- HOLT, I. J. 2009. Mitochondrial DNA replication and repair: all a flap. *Trends Biochem Sci*, 34, 358-65.
- HOU, X., ZHANG, L., HAN, L., GE, J., MA, R., ZHANG, X., MOLEY, K., SCHEDL, T. & WANG, Q. 2015. Differing roles of pyruvate dehydrogenase kinases during mouse oocyte maturation. *J Cell Sci*, 128, 2319-29.
- HOUGHTON, F. D., HAWKHEAD, J. A., HUMPHERSON, P. G., HOGG, J. E., BALEN, A. H., RUTHERFORD, A. J. & LEESE, H. J. 2002. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod*, 17, 999-1005.
- HOUGHTON, F. D., HUMPHERSON, P. G., HAWKHEAD, J. A., HALL, C. J. & LEESE, H. J. 2003. Na⁺, K⁺, ATPase activity in the human and bovine preimplantation embryo. *Dev Biol*, 263, 360-6.
- HOUGHTON, F. D., THOMPSON, J. G., KENNEDY, C. J. & LEESE, H. J. 1996. Oxygen consumption and energy metabolism of the early mouse embryo. *Mol Reprod Dev*, 44, 476-85.
- HSUEH, A. J., EISENHAEUER, K., CHUN, S. Y., HSU, S. Y. & BILLIG, H. 1996. Gonadal cell apoptosis. *Recent Prog Horm Res*, 51, 433-55; discussion 455-6.
- HUHTINEN, M., KOSKINEN, E., SKIDMORE, J. A. & ALLEN, W. R. 1996. Recovery rate and quality of embryos from mares inseminated after ovulation. *Theriogenology*, 45, 719-26.
- HULTEN, M. A., PATEL, S., JONASSON, J. & IWARSSON, E. 2010. On the origin of the maternal age effect in trisomy 21 Down syndrome: the Oocyte Mosaicism Selection model. *Reproduction*, 139, 1-9.
- HUMPHERSON, P. G., LEESE, H. J. & STURMEY, R. G. 2005. Amino acid metabolism of the porcine blastocyst. *Theriogenology*, 64, 1852-66.
- HUNTRISS, J., ERIKA BERENYI, MATT HINKINS, KIRSTEN RICHES, ANTHONY RUTHERFORD, ADAM BALEN, KAY ELDER & PICTON, H. M. A Consistent Set of Imprinted Gene Transcripts are Expressed in Human Blastocysts: Preliminary Evidence for an Imprinted Gene Network Operating in Human Preimplantation Development. *Fertility* 2017, 5-7 Jan 2017 2017 Edinburgh, UK.
- HUNTRISS, J., GOSDEN, R., HINKINS, M., OLIVER, B., MILLER, D., RUTHERFORD, A. J. & PICTON, H. M. 2002. Isolation, characterization and expression of the human Factor In the Germline alpha (FIGLA) gene in ovarian follicles and oocytes. *Mol Hum Reprod*, 8, 1087-95.
- HUSSEIN, M. R. 2005. Apoptosis in the ovary: molecular mechanisms. *Hum Reprod Update*, 11, 162-77.

- HUSSEIN, T. S., THOMPSON, J. G. & GILCHRIST, R. B. 2006. Oocyte-secreted factors enhance oocyte developmental competence. *Dev Biol*, 296, 514-21.
- HWA, V., OH, Y. & ROSENFELD, R. G. 1999. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev*, 20, 761-87.
- HYTTEL, P., FAIR, T., CALLESEN, H. & GREVE, T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, 47, 23-32.
- IKEDA, S., NAMEKAWA, T., SUGIMOTO, M. & KUME, S. 2010. Expression of methylation pathway enzymes in bovine oocytes and preimplantation embryos. *J Exp Zool A Ecol Genet Physiol*, 313, 129-36.
- INOUE, A., NAKAJIMA, R., NAGATA, M. & AOKI, F. 2008. Contribution of the oocyte nucleus and cytoplasm to the determination of meiotic and developmental competence in mice. *Hum Reprod*, 23, 1377-84.
- INZUNZA, J., MORANI, A., CHENG, G., WARNER, M., HREINSSON, J., GUSTAFSSON, J. A. & HOVATTA, O. 2007. Ovarian wedge resection restores fertility in estrogen receptor beta knockout (ERbeta^{-/-}) mice. *Proc Natl Acad Sci U S A*, 104, 600-5.
- IRITANI, A., SATO, E. & NISHIKAWA, Y. 1974. Secretion rates and chemical composition of oviduct and uterine fluids in sows. *J Anim Sci*, 39, 582-8.
- IVANOVSKA, I., KHANDAN, T., ITO, T. & ORR-WEAVER, T. L. 2005. A histone code in meiosis: the histone kinase, NHK-1, is required for proper chromosomal architecture in *Drosophila* oocytes. *Genes Dev*, 19, 2571-82.
- IVANOVSKA, I. & ORR-WEAVER, T. L. 2006. Histone modifications and the chromatin scaffold for meiotic chromosome architecture. *Cell Cycle*, 5, 2064-71.
- IWASAKI, T., KIMURA, E. & TOTSUKAWA, K. 1999. Studies on a chemically defined medium for in vitro culture of in vitro matured and fertilized porcine oocytes. *Theriogenology*, 51, 709-20.
- IWATA, H., GOTO, H., TANAKA, H., SAKAGUCHI, Y., KIMURA, K., KUWAYAMA, T. & MONJI, Y. 2011. Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes. *Reprod Fertil Dev*, 23, 424-32.
- JAIN, R., DEY, B. & TYAGI, A. K. 2012. Development of the first oligonucleotide microarray for global gene expression profiling in guinea pigs: defining the transcription signature of infectious diseases. *BMC Genomics*, 13, 520.
- JAMNONGJIT, M. & HAMMES, S. R. 2005. Oocyte maturation: the coming of age of a germ cell. *Semin Reprod Med*, 23, 234-41.
- JANSEN, R. P. 2000. Origin and persistence of the mitochondrial genome. *Hum Reprod*, 15 Suppl 2, 1-10.
- JANSEN, R. P. & DE BOER, K. 1998. The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. *Mol Cell Endocrinol*, 145, 81-8.
- JEFFERSON, W., NEWBOLD, R., PADILLA-BANKS, E. & PEPLING, M. 2006. Neonatal genistein treatment alters ovarian differentiation in the mouse: inhibition of oocyte nest breakdown and increased oocyte survival. *Biol Reprod*, 74, 161-8.
- JENG, J. Y., YEH, T. S., LEE, J. W., LIN, S. H., FONG, T. H. & HSIEH, R. H. 2008. Maintenance of mitochondrial DNA copy number and expression are essential for preservation of mitochondrial function and cell growth. *J Cell Biochem*, 103, 347-57.
- JENSEN, P. J., GITLIN, J. D. & CARAYANNOPOULOS, M. O. 2006. GLUT1 deficiency links nutrient availability and apoptosis during embryonic development. *J Biol Chem*, 281, 13382-7.
- JEONG, H. J., KIM, H. J., LEE, S. H., KWACK, K., AHN, S. Y., CHOI, Y. J., KIM, H. G., LEE, K. W., LEE, C. N. & CHA, K. Y. 2006. Gene expression profiling of the pre-implantation mouse embryo by microarray analysis: comparison of the two-cell stage and two-cell block. *Theriogenology*, 66, 785-96.
- JEONG, Y. J., CHOI, H. W., SHIN, H. S., CUI, X. S., KIM, N. H., GERTON, G. L. & JUN, J. H. 2005. Optimization of real time RT-PCR methods for the analysis of gene expression in mouse eggs and preimplantation embryos. *Mol Reprod Dev*, 71, 284-9.

- JESSBERGER, R. 2012. Age-related aneuploidy through cohesion exhaustion. *EMBO Rep*, 13, 539-46.
- JIANG, Z., DONG, H., ZHENG, X., MARJANI, S. L., DONOVAN, D. M., CHEN, J. & TIAN, X. C. 2015. mRNA Levels of Imprinted Genes in Bovine In Vivo Oocytes, Embryos and Cross Species Comparisons with Humans, Mice and Pigs. *Sci Rep*, 5, 17898.
- JIAO, F., YAN, J. B., YANG, X. Y., LI, H., WANG, Q., HUANG, S. Z., ZENG, F. & ZENG, Y. T. 2007. Effect of oocyte mitochondrial DNA haplotype on bovine somatic cell nuclear transfer efficiency. *Mol Reprod Dev*, 74, 1278-86.
- JIAO, G. Z., CAO, X. Y., CUI, W., LIAN, H. Y., MIAO, Y. L., WU, X. F., HAN, D. & TAN, J. H. 2013. Developmental potential of prepubertal mouse oocytes is compromised due mainly to their impaired synthesis of glutathione. *PLoS One*, 8, e58018.
- JOHNSON, J., CANNING, J., KANEKO, T., PRU, J. K. & TILLY, J. L. 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature*, 428, 145-50.
- JOHNSON, M. T., FREEMAN, E. A., GARDNER, D. K. & HUNT, P. A. 2007. Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo. *Biol Reprod*, 77, 2-8.
- JOSEFSBERG, L. B., GALIANI, D., LAZAR, S., KAUFMAN, O., SEGER, R. & DEKEL, N. 2003. Maturation-promoting factor governs mitogen-activated protein kinase activation and interphase suppression during meiosis of rat oocytes. *Biol Reprod*, 68, 1282-90.
- JUENGEL, J. L. & MCNATTY, K. P. 2005. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum Reprod Update*, 11, 143-60.
- JUENGEL, J. L., READER, K. L., BIBBY, A. H., LUN, S., ROSS, I., HAYDON, L. J. & MCNATTY, K. P. 2006. The role of bone morphogenetic proteins 2, 4, 6 and 7 during ovarian follicular development in sheep: contrast to rat. *Reproduction*, 131, 501-13.
- JUENGEL, J. L., SAWYER, H. R., SMITH, P. R., QUIRKE, L. D., HEATH, D. A., LUN, S., WAKEFIELD, S. J. & MCNATTY, K. P. 2002. Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. *Mol Cell Endocrinol*, 191, 1-10.
- JUNEJA, S. C., BARR, K. J., ENDERS, G. C. & KIDDER, G. M. 1999. Defects in the germ line and gonads of mice lacking connexin43. *Biol Reprod*, 60, 1263-70.
- KAGEYAMA, S., LIU, H., KANEKO, N., OOGA, M., NAGATA, M. & AOKI, F. 2007. Alterations in epigenetic modifications during oocyte growth in mice. *Reproduction*, 133, 85-94.
- KAJI, K., ODA, S., SHIKANO, T., OHNUKI, T., UEMATSU, Y., SAKAGAMI, J., TADA, N., MIYAZAKI, S. & KUDO, A. 2000. The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat Genet*, 24, 279-82.
- KAMEYAMA, Y., FILION, F., YOO, J. G. & SMITH, L. C. 2007. Characterization of mitochondrial replication and transcription control during rat early development in vivo and in vitro. *Reproduction*, 133, 423-32.
- KANEDA, M., OKANO, M., HATA, K., SADO, T., TSUJIMOTO, N., LI, E. & SASAKI, H. 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, 429, 900-3.
- KANEKO-ISHINO, T., KUROIWA, Y., MIYOSHI, N., KOHDA, T., SUZUKI, R., YOKOYAMA, M., VIVILLE, S., BARTON, S. C., ISHINO, F. & SURANI, M. A. 1995. Peg1/Mest imprinted gene on chromosome 6 identified by cDNA subtraction hybridization. *Nat Genet*, 11, 52-9.
- KANG, Y., MCMILLAN, I., NORRIS, M. H. & HOANG, T. T. 2015. Single prokaryotic cell isolation and total transcript amplification protocol for transcriptomic analysis. *Nat Protoc*, 10, 974-84.
- KANIAK, A., XUE, Z., MACCOOL, D., KIM, J. H. & JOHNSTON, M. 2004. Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. *Eukaryot Cell*, 3, 221-31.
- KANZAKI, M. & PESSIN, J. E. 2001. Signal integration and the specificity of insulin action. *Cell Biochem Biophys*, 35, 191-209.

- KARAMAHMUTOGLU, H., ERDEM, A., ERDEM, M., MUTLU, M. F., BOZKURT, N., OKTEM, M., ERCAN, D. D. & GUMUSLU, S. 2014. The gradient technique improves success rates in intrauterine insemination cycles of unexplained subfertile couples when compared to swim up technique; a prospective randomized study. *J Assist Reprod Genet*, 31, 1139-45.
- KARJA, N. W., KIKUCHI, K., FAHRUDIN, M., OZAWA, M., SOMFAI, T., OHNUMA, K., NOGUCHI, J., KANEKO, H. & NAGAI, T. 2006. Development to the blastocyst stage, the oxidative state, and the quality of early developmental stage of porcine embryos cultured in alteration of glucose concentrations in vitro under different oxygen tensions. *Reprod Biol Endocrinol*, 4, 54.
- KASHIR, J., HEINDRYCKX, B., JONES, C., DE SUTTER, P., PARRINGTON, J. & COWARD, K. 2010. Oocyte activation, phospholipase C zeta and human infertility. *Hum Reprod Update*, 16, 690-703.
- KEDEM, A., FISCH, B., GAROR, R., BEN-ZAKEN, A., GIZUNTERMAN, T., FELZ, C., BEN-HAROSH, A., KRAVARUSIC, D. & ABIR, R. 2011. Growth differentiating factor 9 (GDF9) and bone morphogenetic protein 15 both activate development of human primordial follicles in vitro, with seemingly more beneficial effects of GDF9. *J Clin Endocrinol Metab*, 96, E1246-54.
- KEEFE, D. L., NIVEN-FAIRCHILD, T., POWELL, S. & BURADAGUNTA, S. 1995. Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women. *Fertil Steril*, 64, 577-83.
- KELLER, M. A., TURCHYN, A. V. & RALSER, M. 2014. Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean. *Mol Syst Biol*, 10, 725.
- KELLY, J. M., KLEEMANN, D. O. & WALKER, S. K. 2005. Enhanced efficiency in the production of offspring from 4- to 8-week-old lambs. *Theriogenology*, 63, 1876-90.
- KERJEAN, A., DUPONT, J. M., VASSEUR, C., LE TESSIER, D., CUISSET, L., PALDI, A., JOUANNET, P. & JEANPIERRE, M. 2000. Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. *Hum Mol Genet*, 9, 2183-7.
- KERR, J. B., DUCKETT, R., MYERS, M., BRITT, K. L., MLADENOVSKA, T. & FINDLAY, J. K. 2006. Quantification of healthy follicles in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply. *Reproduction*, 132, 95-109.
- KHATIR, H., LONERGAN, P. & MERMILLOD, P. 1998. Kinetics of nuclear maturation and protein profiles of oocytes from prepubertal and adult cattle during in vitro maturation. *Theriogenology*, 50, 917-29.
- KHURANA, N. K. & NIEMANN, H. 2000. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod*, 62, 847-56.
- KIKUCHI, K., EKWALL, H., TIENHAI, P., KAWAI, Y., NOGUCHI, J., KANEKO, H. & RODRIGUEZ-MARTINEZ, H. 2002. Morphological features of lipid droplet transition during porcine oocyte fertilisation and early embryonic development to blastocyst in vivo and in vitro. *Zygote*, 10, 355-66.
- KIM, B. H., KIM, C. H., JUNG, K. Y., JEON, B. H., JU, E. J. & CHOO, Y. K. 2004. Involvement of nitric oxide during in vitro fertilization and early embryonic development in mice. *Arch Pharm Res*, 27, 86-93.
- KIM, H., LEE, T. H., PARK, E. S., SUH, J. M., PARK, S. J., CHUNG, H. K., KWON, O. Y., KIM, Y. K., RO, H. K. & SHONG, M. 2000. Role of peroxiredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide-induced apoptosis in thyroid cells. *J Biol Chem*, 275, 18266-70.
- KIM, J., KIM, J. S., JEON, Y. J., KIM, D. W., YANG, T. H., SOH, Y. M., LEE, H. K., CHOI, N. J., PARK, S. B., SEO, K. S., CHUNG, H. M., LEE, D. S. & CHAE, J. I. 2011. Identification of maturation and protein synthesis related proteins from porcine oocytes during in vitro maturation. *Proteome Sci*, 9, 1477-5956.

- KIM, J. Y., KINOSHITA, M., OHNISHI, M. & FUKUI, Y. 2001. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. *Reproduction*, 122, 131-8.
- KIRKWOOD, T. B., FEDER, M., FINCH, C. E., FRANCESCHI, C., GLOBERSON, A., KLINGENBERG, C. P., LAMARCO, K., OMHOLT, S. & WESTENDORP, R. G. 2005. What accounts for the wide variation in life span of genetically identical organisms reared in a constant environment? *Mech Ageing Dev*, 126, 439-43.
- KLEINSCHMIDT, J. A., FORTKAMP, E., KROHNE, G., ZENTGRAF, H. & FRANKE, W. W. 1985. Co-existence of two different types of soluble histone complexes in nuclei of *Xenopus laevis* oocytes. *J Biol Chem*, 260, 1166-76.
- KNIGHT, P. G. & GLISTER, C. 2006. TGF-beta superfamily members and ovarian follicle development. *Reproduction*, 132, 191-206.
- KOCHHAR, H. P., WU, B., MORRIS, L. H., BUCKRELL, B. C., POLLARD, J. W., BASRUR, P. K. & KING, W. A. 2002. Maturation status, protein synthesis and developmental competence of oocytes derived from lambs and ewes. *Reprod Domest Anim*, 37, 19-25.
- KOHATA, C., IZQUIERDO-RICO, M. J., ROMAR, R. & FUNAHASHI, H. 2013. Development competence and relative transcript abundance of oocytes derived from small and medium follicles of prepubertal gilts. *Theriogenology*, 80, 970-8.
- KOMOROWSKI, S., BARANOWSKA, B. & MALESZEWSKI, M. 2006. CD9 protein appears on growing mouse oocytes at the time when they develop the ability to fuse with spermatozoa. *Zygote*, 14, 119-23.
- KOVO, M., KANDLI-COHEN, M., BEN-HAIM, M., GALIANI, D., CARR, D. W. & DEKEL, N. 2006. An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes. *Reproduction*, 132, 33-43.
- KOWALIK, A., LIU, H. C., HE, Z. Y., MELE, C., BARMAT, L. & ROSENWAKS, Z. 1999. Expression of the insulin-like growth factor-1 gene and its receptor in preimplantation mouse embryos; is it a marker of embryo viability? *Mol Hum Reprod*, 5, 861-5.
- KRISHER, R. L. & BAVISTER, B. D. 1998. Responses of oocytes and embryos to the culture environment. *Theriogenology*, 49, 103-14.
- KRISHER, R. L., BRAD, A. M., HERRICK, J. R., SPARMAN, M. L. & SWAIN, J. E. 2007. A comparative analysis of metabolism and viability in porcine oocytes during in vitro maturation. *Anim Reprod Sci*, 98, 72-96.
- KROTZ, S. P., BALLOU, D. J., CHOI, Y. & RAJKOVIC, A. 2009. Expression and localization of the novel and highly conserved gametocyte-specific factor 1 during oogenesis and spermatogenesis. *Fertil Steril*, 91, 2020-4.
- KRUGER, N. J. & VON SCHAEWEN, A. 2003. The oxidative pentose phosphate pathway: structure and organisation. *Curr Opin Plant Biol*, 6, 236-46.
- KUBISTA, M., ANDRADE, J. M., BENGTTSSON, M., FOROOTAN, A., JONAK, J., LIND, K., SINDELKA, R., SJOBACK, R., SJOGREEN, B., STROMBOM, L., STAHLBERG, A. & ZORIC, N. 2006. The real-time polymerase chain reaction. *Mol Aspects Med*, 27, 95-125.
- KUCHMIY, A. A., D'HONT, J., HOCHEPIED, T. & LAMKANFI, M. 2016. NLRP2 controls age-associated maternal fertility. *J Exp Med*, 213, 2851-2860.
- KUIJK, E. W., DU PUY, L., VAN TOL, H. T., HAAGSMAN, H. P., COLENBRANDER, B. & ROELEN, B. A. 2007. Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. *BMC Dev Biol*, 7, 58.
- KUMAR, P., YADAV, P., VERMA, A., SINGH, D., DE, S. & DATTA, T. K. 2012. Identification of stable reference genes for gene expression studies using quantitative real time PCR in buffalo oocytes and embryos. *Reprod Domest Anim*, 47, e88-91.
- KURILO, L. F. 1981. Oogenesis in antenatal development in man. *Hum Genet*, 57, 86-92.
- KUROIWA, Y., KANEKO-ISHINO, T., KAGITANI, F., KOHDA, T., LI, L. L., TADA, M., SUZUKI, R., YOKOYAMA, M., SHIROISHI, T., WAKANA, S., BARTON, S. C., ISHINO, F. & SURANI, M. A.

1996. Peg3 imprinted gene on proximal chromosome 7 encodes for a zinc finger protein. *Nat Genet*, 12, 186-90.
- KUSHNIR, M. M., NAESSEN, T., WANGGREN, K., ROCKWOOD, A. L., CROCKETT, D. K. & BERGQUIST, J. 2012. Protein and steroid profiles in follicular fluid after ovarian hyperstimulation as potential biomarkers of IVF outcome. *J Proteome Res*, 11, 5090-100.
- KWONG, W. Y., ADAMIAK, S. J., GWYNN, A., SINGH, R. & SINCLAIR, K. D. 2010. Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo. *Reproduction*, 139, 705-15.
- LAANPERE, M., ALTMAE, S., STAVREUS-EVERS, A., NILSSON, T. K., A, Y. & SALUMETS, A. 2010. Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. *Nutr Rev*, 68, 99-113.
- LABRECQUE, R. & SIRARD, M. A. 2014. The study of mammalian oocyte competence by transcriptome analysis: progress and challenges. *Mol Hum Reprod*, 20, 103-16.
- LABRECQUE, R., VIGNEAULT, C., BLONDIN, P. & SIRARD, M. A. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev*, 80, 428-40.
- LANE, M. & GARDNER, D. K. 1996. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod*, 11, 1975-8.
- LARMAN, M. G., SAUNDERS, C. M., CARROLL, J., LAI, F. A. & SWANN, K. 2004. Cell cycle-dependent Ca²⁺ oscillations in mouse embryos are regulated by nuclear targeting of PLC ζ . *J Cell Sci*, 117, 2513-21.
- LARSSON, N. G., WANG, J., WILHELMSSON, H., OLDFORS, A., RUSTIN, P., LEWANDOSKI, M., BARSH, G. S. & CLAYTON, D. A. 1998. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet*, 18, 231-6.
- LASIENE, K., VITKUS, A., VALANCIUTE, A. & LASYS, V. 2009. Morphological criteria of oocyte quality. *Medicina (Kaunas)*, 45, 509-15.
- LAU, K. F., MILLER, C. C., ANDERTON, B. H. & SHAW, P. C. 1999. Molecular cloning and characterization of the human glycogen synthase kinase-3 β promoter. *Genomics*, 60, 121-8.
- LAWITTS, J. A. & BIGGERS, J. D. 1993. Culture of preimplantation embryos. *Methods Enzymol*, 225, 153-64.
- LAZZARI, G., GALLI, C. & MOOR, R. M. 1994. Functional changes in the somatic and germinal compartments during follicle growth in pigs. *Anim Reprod Sci*, 35, 119-130.
- LEBLANC, C., RICHARD, O., KLOAREG, B., VIEHMANN, S., ZETSCHKE, K. & BOYEN, C. 1997. Origin and evolution of mitochondria: what have we learnt from red algae? *Curr Genet*, 31, 193-207.
- LECHNIAK, D., KACZMAREK, D., STANISLAWSKI, D. & ADAMOWICZ, T. 2002. The ploidy of in vitro matured bovine oocytes is related to the diameter. *Theriogenology*, 57, 1303-8.
- LEDAN, E., POLANSKI, Z., TERRET, M. E. & MARO, B. 2001. Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation. *Dev Biol*, 232, 400-13.
- LEDDA, S., BOGLIOLO, L., CALVIA, P., LEONI, G. & NAITANA, S. 1997. Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. *J Reprod Fertil*, 109, 73-8.
- LEDDA, S., BOGLIOLO, L., LEONI, G. & NAITANA, S. 1999. Follicular size affects the meiotic competence of in vitro matured prepubertal and adult oocytes in sheep. *Reprod Nutr Dev*, 39, 503-8.
- LEDDA, S., BOGLIOLO, L., LEONI, G. & NAITANA, S. 2001. Cell coupling and maturation-promoting factor activity in in vitro-matured prepubertal and adult sheep oocytes. *Biol Reprod*, 65, 247-52.

- LEDDA, S., LOI, P., BOGLIOLO, L., MOOR, R. M. & FULKA, J., JR. 1996. The effect of 6-dimethylaminopurine (6-DMAP) on DNA synthesis in activated mammalian oocytes. *Zygote*, 4, 7-9.
- LEE, K. Y. & DEMAYO, F. J. 2004. Animal models of implantation. *Reproduction*, 128, 679-95.
- LEE, M. A. & STOREY, B. T. 1986. Bicarbonate is essential for fertilization of mouse eggs: mouse sperm require it to undergo the acrosome reaction. *Biol Reprod*, 34, 349-56.
- LEE, M. S., LIU, C. H., LEE, T. H., WU, H. M., HUANG, C. C., HUANG, L. S., CHEN, C. M. & CHENG, E. H. 2010. Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. *J Assist Reprod Genet*, 27, 629-39.
- LEESE, H. J. 1995. Metabolic control during preimplantation mammalian development. *Hum Reprod Update*, 1, 63-72.
- LEESE, H. J. 2002. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays*, 24, 845-9.
- LEESE, H. J. 2003. What does an embryo need? *Hum Fertil (Camb)*, 6, 180-5.
- LEESE, H. J. 2015. History of oocyte and embryo metabolism. *Reprod Fertil Dev*, 27, 567-71.
- LEESE, H. J. & BARTON, A. M. 1984. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil*, 72, 9-13.
- LEESE, H. J., CONAGHAN, J., MARTIN, K. L. & HARDY, K. 1993. Early human embryo metabolism. *Bioessays*, 15, 259-64.
- LEESE, H. J., HUMPHERSON, P. G., HARDY, K., HOOPER, M. A., WINSTON, R. M. & HANDYSIDE, A. H. 1991. Profiles of hypoxanthine guanine phosphoribosyl transferase and adenine phosphoribosyl transferase activities measured in single preimplantation human embryos by high-performance liquid chromatography. *J Reprod Fertil*, 91, 197-202.
- LEFF, S. E., BRANNAN, C. I., REED, M. L., OZCELIK, T., FRANCKE, U., COPELAND, N. G. & JENKINS, N. A. 1992. Maternal imprinting of the mouse *Snrpn* gene and conserved linkage homology with the human Prader-Willi syndrome region. *Nat Genet*, 2, 259-64.
- LEGRO, R. S. 2000. The genetics of obesity. Lessons for polycystic ovary syndrome. *Ann NY Acad Sci*, 900, 193-202.
- LEGRO, R. S. 2001. Polycystic ovary syndrome: the new millenium. *Mol Cell Endocrinol*, 184, 87-93.
- LEONI, G. G., BEBBERE, D., SUCCU, S., BERLINGUER, F., MOSSA, F., GALIOTO, M., BOGLIOLO, L., LEDDA, S. & NAITANA, S. 2007a. Relations between relative mRNA abundance and developmental competence of ovine oocytes. *Mol Reprod Dev*, 74, 249-57.
- LEONI, G. G., ROSATI, I., SUCCU, S., BOGLIOLO, L., BEBBERE, D., BERLINGUER, F., LEDDA, S. & NAITANA, S. 2007b. A low oxygen atmosphere during IVF accelerates the kinetic of formation of in vitro produced ovine blastocysts. *Reprod Domest Anim*, 42, 299-304.
- LEYENS, G., KNOOPS, B. & DONNAY, I. 2004a. Expression of peroxiredoxins in bovine oocytes and embryos produced in vitro. *Mol Reprod Dev*, 69, 243-51.
- LEYENS, G., VERHAEGHE, B., LANDTMETERS, M., MARCHANDISE, J., KNOOPS, B. & DONNAY, I. 2004b. Peroxiredoxin 6 is upregulated in bovine oocytes and cumulus cells during in vitro maturation: role of intercellular communication. *Biol Reprod*, 71, 1646-51.
- LI, H. J., SUTTON-MCDOWALL, M. L., WANG, X., SUGIMURA, S., THOMPSON, J. G. & GILCHRIST, R. B. 2016. - Extending prematuration with cAMP modulators enhances the cumulus contribution to oocyte antioxidant defence and oocyte quality via gap junctions. *Hum Reprod*, 31, 810-21.
- LI, L., BAIBAKOV, B. & DEAN, J. 2008. A subcortical maternal complex essential for preimplantation mouse embryogenesis. *Dev Cell*, 15, 416-25.
- LI, Y., LI, R. Q., OU, S. B., ZHANG, N. F., REN, L., WEI, L. N., ZHANG, Q. X. & YANG, D. Z. 2014. Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. *Reprod Biol Endocrinol*, 12, 81.

- LIGHTEN, A. D., HARDY, K., WINSTON, R. M. & MOORE, G. E. 1997a. Expression of mRNA for the insulin-like growth factors and their receptors in human preimplantation embryos. *Mol Reprod Dev*, 47, 134-9.
- LIGHTEN, A. D., HARDY, K., WINSTON, R. M. & MOORE, G. E. 1997b. IGF2 is parentally imprinted in human preimplantation embryos. *Nat Genet*, 15, 122-3.
- LIN, Z. L., LI, Y. H., XU, Y. N., WANG, Q. L., NAMGOONG, S., CUI, X. S. & KIM, N. H. 2014. Effects of growth differentiation factor 9 and bone morphogenetic protein 15 on the in vitro maturation of porcine oocytes. *Reprod Domest Anim*, 49, 219-27.
- LIPERIS, G. 2013. *The function of gametocyte specific factor 1 (GTSF1) in mammalian oocyte and ovarian follicle development*. PhD, University of Leeds.
- LIS, H. & SHARON, N. 1993. Protein glycosylation. Structural and functional aspects. *Eur J Biochem*, 218, 1-27.
- LISTER, L. M., KOUZNETSOVA, A., HYSLOP, L. A., KALLEAS, D., PACE, S. L., BAREL, J. C., NATHAN, A., FLOROS, V., ADELFAK, C., WATANABE, Y., JESSBERGER, R., KIRKWOOD, T. B., HOOG, C. & HERBERT, M. 2010. Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2. *Curr Biol*, 20, 1511-21.
- LIU, H. & AOKI, F. 2002. Transcriptional activity associated with meiotic competence in fully grown mouse GV oocytes. *Zygote*, 10, 327-32.
- LIU, H. C., HE, Z. Y., MELE, C. A., VEECK, L. L., DAVIS, O. K. & ROSENWAKS, Z. 1997. Expression of IGFs and their receptors is a potential marker for embryo quality. *Am J Reprod Immunol*, 38, 237-45.
- LIU, J. P., BAKER, J., PERKINS, A. S., ROBERTSON, E. J. & EFSTRATIADIS, A. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell*, 75, 59-72.
- LIU, K. & CASE, A. 2011. Advanced reproductive age and fertility. *J Obstet Gynaecol Can*, 33, 1165-1175.
- LIU, Y., SUI, H. S., WANG, H. L., YUAN, J. H., LUO, M. J., XIA, P. & TAN, J. H. 2006. Germinal vesicle chromatin configurations of bovine oocytes. *Microsc Res Tech*, 69, 799-807.
- LIU, Z., FOOTE, R. H. & SIMKIN, M. E. 1996. Effect of amino acids and alpha-amanitin on the development of rabbit embryos in modified protein-free KSOM with HEPES. *Mol Reprod Dev*, 45, 157-62.
- LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(-Delta Delta C(T)) Method. *Methods*, 25, 402-8.
- LOBASCIO, A. M., KLINGER, F. G., SCALDAFERRI, M. L., FARINI, D. & DE FELICI, M. 2007. Analysis of programmed cell death in mouse fetal oocytes. *Reproduction*, 134, 241-52.
- LOCASALE, J. W. 2013. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat Rev Cancer*, 13, 572-83.
- LODDE, V., FRANCIOSI, F., TESSARO, I., MODINA, S. C. & LUCIANO, A. M. 2013. Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte. *J Assist Reprod Genet*, 30, 1219-26.
- LODDE, V., MODINA, S., GALBUSERA, C., FRANCIOSI, F. & LUCIANO, A. M. 2007. Large-scale chromatin remodeling in germinal vesicle bovine oocytes: interplay with gap junction functionality and developmental competence. *Mol Reprod Dev*, 74, 740-9.
- LODDE, V., MODINA, S., MADDOX-HYTTEL, P., FRANCIOSI, F., LAURIA, A. & LUCIANO, A. M. 2008. Oocyte morphology and transcriptional silencing in relation to chromatin remodeling during the final phases of bovine oocyte growth. *Mol Reprod Dev*, 75, 915-24.
- LODDE, V., MODINA, S. C., FRANCIOSI, F., ZUCCARI, E., TESSARO, I. & LUCIANO, A. M. 2009. Localization of DNA methyltransferase-1 during oocyte differentiation, in vitro maturation and early embryonic development in cow. *Eur J Histochem*, 53, 199-207.
- LOEWENSTEIN, J. E. & COHEN, A. I. 1964. Dry Mass, Lipid Content and Protein Content of the Intact and Zona-Free Mouse Ovum. *J Embryol Exp Morphol*, 12, 113-21.

- LONERGAN, P., GUTIERREZ-ADAN, A., PINTADO, B., FAIR, T., WARD, F., FUENTE, J. D. & BOLAND, M. 2000. Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro. *Mol Reprod Dev*, 57, 146-52.
- LONERGAN, P., MONAGHAN, P., RIZOS, D., BOLAND, M. P. & GORDON, I. 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Mol Reprod Dev*, 37, 48-53.
- LOPASCHUK, G. D. & JASWAL, J. S. 2010. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. *J Cardiovasc Pharmacol*, 56, 130-40.
- LOPES, A. S., MADSEN, S. E., RAMSING, N. B., LOVENDAHL, P., GREVE, T. & CALLESEN, H. 2007. Investigation of respiration of individual bovine embryos produced in vivo and in vitro and correlation with viability following transfer. *Hum Reprod*, 22, 558-66.
- LOPES, E. F., MARQUES, L. S., DURANTI, R. G., DE OLIVEIRA, A. T., LOPES, R. F. & RODRIGUES, J. L. 2015. Gene Expression of Monocarboxylate Transporters and Oocyte-secreted Factors in Bovine Cumulus-oocyte Complexes Selected by Brilliant Cresyl Blue. *Reprod Domest Anim*, 50, 763-70.
- LOPEZ-OTIN, C., BLASCO, M. A., PARTRIDGE, L., SERRANO, M. & KROEMER, G. 2013. The hallmarks of aging. *Cell*, 153, 1194-217.
- LORCA, T., CRUZALEGUI, F. H., FESQUET, D., CAVADORE, J. C., MERY, J., MEANS, A. & DOREE, M. 1993. Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature*, 366, 270-3.
- LOWE, J. L. 2014. *Lipid metabolism during the in vitro production of porcine embryos*. PhD, University of Sydney.
- LOWTHER, K. M. & MEHLMANN, L. M. 2015. Embryonic Poly(A)-Binding Protein Is Required During Early Stages of Mouse Oocyte Development for Chromatin Organization, Transcriptional Silencing, and Meiotic Competence. *Biol Reprod*, 93, 43.
- LUCHSINGER, C., ARIAS, M. E., VARGAS, T., PAREDES, M., SANCHEZ, R. & FELMER, R. 2014. Stability of reference genes for normalization of reverse transcription quantitative real-time PCR (RT-qPCR) data in bovine blastocysts produced by IVF, ICSI and SCNT. *Zygote*, 22, 505-12.
- LUCIANO, A. M., FRANCIOSI, F., MODINA, S. C. & LODDE, V. 2011. Gap junction-mediated communications regulate chromatin remodeling during bovine oocyte growth and differentiation through cAMP-dependent mechanism(s). *Biol Reprod*, 85, 1252-9.
- LUCIANO, A. M. & LODDE, V. 2013. Changes of large-scale chromatin configuration during mammalian oocyte differentiation. In: COTICCHIO, G., ALBERTINI, D. F. & DE SANTIS, L. (eds.) *Oogenesis*. Springer, London, pp. 93-108.
- LUCIANO, A. M., LODDE, V., FRANCIOSI, F., TESSARO, I., CORBANI, D. & MODINA, S. 2012. Large-scale chromatin morpho-functional changes during mammalian oocyte growth and differentiation. *Eur J Histochem*, 56, e37.
- LUCIANO, A. M., MODINA, S., VASSENA, R., MILANESI, E., LAURIA, A. & GANDOLFI, F. 2004. Role of intracellular cyclic adenosine 3',5'-monophosphate concentration and oocyte-cumulus cells communications on the acquisition of the developmental competence during in vitro maturation of bovine oocyte. *Biol Reprod*, 70, 465-72.
- LUCIANO, A. M., POCAR, P., MILANESI, E., MODINA, S., RIEGER, D., LAURIA, A. & GANDOLFI, F. 1999. Effect of different levels of intracellular cAMP on the in vitro maturation of cattle oocytes and their subsequent development following in vitro fertilization. *Mol Reprod Dev*, 54, 86-91.
- LUCIFERO, D., MERTINEIT, C., CLARKE, H. J., BESTOR, T. H. & TRASLER, J. M. 2002. Methylation dynamics of imprinted genes in mouse germ cells. *Genomics*, 79, 530-8.
- LUCY, M. C. 2000. Regulation of ovarian follicular growth by somatotropin and insulin-like growth factors in cattle. *J Dairy Sci*, 83, 1635-47.

- LUNDING, S. A., AKSGLAEDE, L., ANDERSON, R. A., MAIN, K. M., JUUL, A., HAGEN, C. P. & PEDERSEN, A. T. 2015. AMH as Predictor of Premature Ovarian Insufficiency: A Longitudinal Study of 120 Turner Syndrome Patients. *J Clin Endocrinol Metab*, 100, E1030-8.
- LV, Y., ZHAO, S. G., LU, G., LEUNG, C. K., XIONG, Z. Q., SU, X. W., MA, J. L., CHAN, W. Y. & LIU, H. B. 2017. Identification of reference genes for qRT-PCR in granulosa cells of healthy women and polycystic ovarian syndrome patients. *Sci Rep*, 7, 6961.
- MACABELLI, C. H., FERREIRA, R. M., GIMENES, L. U., DE CARVALHO, N. A., SOARES, J. G., AYRES, H., FERRAZ, M. L., WATANABE, Y. F., WATANABE, O. Y., SANGALLI, J. R., SMITH, L. C., BARUSELLI, P. S., MEIRELLES, F. V. & CHIARATTI, M. R. 2014. Reference gene selection for gene expression analysis of oocytes collected from dairy cattle and buffaloes during winter and summer. *PLoS One*, 9, e93287.
- MACCOUX, L. J., CLEMENTS, D. N., SALWAY, F. & DAY, P. J. 2007. Identification of new reference genes for the normalisation of canine osteoarthritic joint tissue transcripts from microarray data. *BMC Mol Biol*, 8, 62.
- MACHATY, Z., BONK, A. J., KUHHOLZER, B. & PRATHER, R. S. 2000. Porcine oocyte activation induced by a cytosolic sperm factor. *Mol Reprod Dev*, 57, 290-5.
- MACK, H. I. & MUNGER, K. 2012. Modulation of autophagy-like processes by tumor viruses. *Cells*, 1, 204-47.
- MACK, H. I., ZHENG, B., ASARA, J. M. & THOMAS, S. M. 2012. AMPK-dependent phosphorylation of ULK1 regulates ATG9 localization. *Autophagy*, 8, 1197-214.
- MADGWICK, S. & JONES, K. T. 2007. How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor. *Cell Div*, 2, 4.
- MAHADEVAN, M. M., FLEETHAM, J., CHURCH, R. B. & TAYLOR, P. J. 1986. Growth of mouse embryos in bicarbonate media buffered by carbon dioxide, hepes, or phosphate. *J In Vitro Fert Embryo Transf*, 3, 304-8.
- MAHADEVAN, S., SATHAPPAN, V., UTAMA, B., LORENZO, I., KASKAR, K. & VAN DEN VEYVER, I. B. 2017. Maternally expressed NLRP2 links the subcortical maternal complex (SCMC) to fertility, embryogenesis and epigenetic reprogramming. *Sci Rep*, 7, 44667.
- MAHER, E. R., BRUETON, L. A., BOWDIN, S. C., LUHARIA, A., COOPER, W., COLE, T. R., MACDONALD, F., SAMPSON, J. R., BARRATT, C. L., REIK, W. & HAWKINS, M. M. 2003. Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet*, 40, 62-4.
- MAI, C. T., KUCIK, J. E., ISENBURG, J., FELDKAMP, M. L., MARENGO, L. K., BUGENSKA, E. M., THORPE, P. G., JACKSON, J. M., CORREA, A., RICKARD, R., ALVERSON, C. J., KIRBY, R. S. & NATIONAL BIRTH DEFECTS PREVENTION, N. 2013. Selected birth defects data from population-based birth defects surveillance programs in the United States, 2006 to 2010: featuring trisomy conditions. *Birth Defects Res A Clin Mol Teratol*, 97, 709-25.
- MAILHES, J. B., YOUNG, D. & LONDON, S. N. 1998. Postovulatory ageing of mouse oocytes in vivo and premature centromere separation and aneuploidy. *Biol Reprod*, 58, 1206-10.
- MAMO, S., GAL, A. B., BODO, S. & DINNYES, A. 2007. Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured in vivo and in vitro. *BMC Dev Biol*, 7, 14.
- MAMO, S., GAL, A. B., POLGAR, Z. & DINNYES, A. 2008. Expression profiles of the pluripotency marker gene POU5F1 and validation of reference genes in rabbit oocytes and preimplantation stage embryos. *BMC Mol Biol*, 9, 67.
- MANDL, A. M. & ZUCKERMAN, S. 1952. The growth of the oocyte and follicle in the adult rat. *J Endocrinol*, 8, 126-32.
- MANGIA, F. & EPSTEIN, C. J. 1975. Biochemical studies of growing mouse oocytes: preparation of oocytes and analysis of glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. *Dev Biol*, 45, 211-20.

- MANOSALVA, I. & GONZALEZ, A. 2009. Aging alters histone H4 acetylation and CDC2A in mouse germinal vesicle stage oocytes. *Biol Reprod*, 81, 1164-71.
- MANSER, R. C., LEESE, H. J. & HOUGHTON, F. D. 2004. Effect of inhibiting nitric oxide production on mouse preimplantation embryo development and metabolism. *Biol Reprod*, 71, 528-33.
- MAO, J., WHITWORTH, K. M., SPATE, L. D., WALTERS, E. M., ZHAO, J. & PRATHER, R. S. 2012. Regulation of oocyte mitochondrial DNA copy number by follicular fluid, EGF, and neuregulin 1 during in vitro maturation affects embryo development in pigs. *Theriogenology*, 78, 887-97.
- MAO, L., LOU, H., LOU, Y., WANG, N. & JIN, F. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reprod Biomed Online*, 28, 284-99.
- MARANGOS, P., FITZHARRIS, G. & CARROLL, J. 2003. Ca²⁺ oscillations at fertilization in mammals are regulated by the formation of pronuclei. *Development*, 130, 1461-72.
- MARANGOS, P., VERSCHUREN, E. W., CHEN, R., JACKSON, P. K. & CARROLL, J. 2007. Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC(Cdh1). *J Cell Biol*, 176, 65-75.
- MARGINEANTU, D. H., BROWN, R. M., BROWN, G. K., MARCUS, A. H. & CAPALDI, R. A. 2002. Heterogeneous distribution of pyruvate dehydrogenase in the matrix of mitochondria. *Mitochondrion*, 1, 327-38.
- MARKET-VELKER, B. A., ZHANG, L., MAGRI, L. S., BONVISSUTO, A. C. & MANN, M. R. 2010. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet*, 19, 36-51.
- MARKHAM, K. E. & KAYE, P. L. 2003. Growth hormone, insulin-like growth factor I and cell proliferation in the mouse blastocyst. *Reproduction*, 125, 327-36.
- MAROUFI, A., VAN BOCKSTAELE, E. & DE LOOSE, M. 2010. Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. *BMC Mol Biol*, 11, 15.
- MARTIN, P. M. & SUTHERLAND, A. E. 2001. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol*, 240, 182-93.
- MARTINEZ-CHANTAR, M. L., LATASA, M. U., VARELA-REY, M., LU, S. C., GARCIA-TREVIJANO, E. R., MATO, J. M. & AVILA, M. A. 2003. L-methionine availability regulates expression of the methionine adenosyltransferase 2A gene in human hepatocarcinoma cells: role of S-adenosylmethionine. *J Biol Chem*, 278, 19885-90.
- MARUTHINI, D., HARRIS, S. E., BARTH, J. H., BALEN, A. H., CAMPBELL, B. K. & PICTON, H. M. 2014. The effect of metformin treatment in vivo on acute and long-term energy metabolism and progesterone production in vitro by granulosa cells from women with polycystic ovary syndrome. *Hum Reprod*, 29, 2302-16.
- MASCIARELLI, S., HORNER, K., LIU, C., PARK, S. H., HINCKLEY, M., HOCKMAN, S., NEDACHI, T., JIN, C., CONTI, M. & MANGANIELLO, V. 2004. Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. *J Clin Invest*, 114, 196-205.
- MASON, H. D., CWYFAN-HUGHES, S., HOLLY, J. M. & FRANKS, S. 1998. Potent inhibition of human ovarian steroidogenesis by insulin-like growth factor binding protein-4 (IGFBP-4). *J Clin Endocrinol Metab*, 83, 284-7.
- MATIKAINEN, T., PEREZ, G. I., ZHENG, T. S., KLUZAK, T. R., RUEDA, B. R., FLAVELL, R. A. & TILLY, J. L. 2001. Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. *Endocrinology*, 142, 2468-80.
- MATSUI, M., TAKAHASHI, Y., HISHINUMA, M. & KANAGAWA, H. 1995. Insulin and insulin-like growth factor-I (IGF-I) stimulate the development of bovine embryos fertilized in vitro. *J Vet Med Sci*, 57, 1109-11.
- MATTSON, B. A. & ALBERTINI, D. F. 1990. Oogenesis: chromatin and microtubule dynamics during meiotic prophase. *Mol Reprod Dev*, 25, 374-83.

- MAY-PANLOUP, P., BOUCRET, L., CHAO DE LA BARCA, J. M., DESQUIRET-DUMAS, V., FERRE-L'HOTELLIER, V., MORINIERE, C., DESCAMPS, P., PROCACCIO, V. & REYNIER, P. 2016. Ovarian ageing: the role of mitochondria in oocytes and follicles. *Hum Reprod Update*, 22, 725-743.
- MAY-PANLOUP, P., CHRETIEN, M. F., JACQUES, C., VASSEUR, C., MALTHIERY, Y. & REYNIER, P. 2005a. Low oocyte mitochondrial DNA content in ovarian insufficiency. *Hum Reprod*, 20, 593-7.
- MAY-PANLOUP, P., CHRETIEN, M. F., MALTHIERY, Y. & REYNIER, P. 2007. Mitochondrial DNA in the oocyte and the developing embryo. *Curr Top Dev Biol*, 77, 51-83.
- MAY-PANLOUP, P., VIGNON, X., CHRETIEN, M. F., HEYMAN, Y., TAMASSIA, M., MALTHIERY, Y. & REYNIER, P. 2005b. Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. *Reprod Biol Endocrinol*, 3, 65.
- MAYES, M. A. & SIRARD, M. A. 2002. Effect of type 3 and type 4 phosphodiesterase inhibitors on the maintenance of bovine oocytes in meiotic arrest. *Biol Reprod*, 66, 180-4.
- MCDANIEL, P. & WU, X. 2009. Identification of oocyte-selective NLRP genes in rhesus macaque monkeys (*Macaca mulatta*). *Mol Reprod Dev*, 76, 151-9.
- MCDONNELL, N. A., WALSH, J. M., CARRUTHERS, S. C. & MORRISON, J. J. 2014. Obstetric and neonatal outcomes for women \geq 45 years of age: a cohort study. *Am J Perinatol*, 31, 823-8.
- MCEVOY, T. G., SINCLAIR, K. D., YOUNG, L. E., WILMUT, I. & ROBINSON, J. J. 2000. Large offspring syndrome and other consequences of ruminant embryo culture in vitro: relevance to blastocyst culture in human ART. *Hum Fertil (Camb)*, 3, 238-246.
- MCGEE, E. A. & HSUEH, A. J. 2000. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev*, 21, 200-14.
- MCNATTY, K. P., FIDLER, A. E., JUENGEL, J. L., QUIRKE, L. D., SMITH, P. R., HEATH, D. A., LUNDY, T., O'CONNELL, A. & TISDALL, D. J. 2000. Growth and paracrine factors regulating follicular formation and cellular function. *Mol Cell Endocrinol*, 163, 11-20.
- MCNATTY, K. P., READER, K., SMITH, P., HEATH, D. A. & JUENGEL, J. L. 2007. Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. *Soc Reprod Fertil Suppl*, 64, 55-68.
- MEHLMANN, L. M. 2005a. Oocyte-specific expression of Gpr3 is required for the maintenance of meiotic arrest in mouse oocytes. *Dev Biol*, 288, 397-404.
- MEHLMANN, L. M. 2005b. Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction*, 130, 791-9.
- MEHLMANN, L. M., JONES, T. L. & JAFFE, L. A. 2002. Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science*, 297, 1343-5.
- MEMILI, E., DOMINKO, T. & FIRST, N. L. 1998. Onset of transcription in bovine oocytes and preimplantation embryos. *Mol Reprod Dev*, 51, 36-41.
- MEMILI, E. & FIRST, N. L. 1998. Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of alpha-amanitin on embryo development. *Mol Reprod Dev*, 51, 381-9.
- MERCADO-SAENZ, S., RUIZ-GOMEZ, M. J., MORALES-MORENO, F. & MATINEZ-MORILLO, M. 2010. Cellular Aging: Theories and Technological Influence. *Braz. Arch. Biol. Technol.*, 53, 1319-1332.
- MERTINEIT, C., YODER, J. A., TAKETO, T., LAIRD, D. W., TRASLER, J. M. & BESTOR, T. H. 1998. Sex-specific exons control DNA methyltransferase in mammalian germ cells. *Development*, 125, 889-97.
- MESSERSCHMIDT, D. M., KNOWLES, B. B. & SOLTER, D. 2014. DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes Dev*, 28, 812-28.

- MEYER, E., LIM, D., PASHA, S., TEE, L. J., RAHMAN, F., YATES, J. R., WOODS, C. G., REIK, W. & MAHER, E. R. 2009. Germline mutation in NLRP2 (NALP2) in a familial imprinting disorder (Beckwith-Wiedemann Syndrome). *PLoS Genet*, 5, e1000423.
- MIAO, Y. L., KIKUCHI, K., SUN, Q. Y. & SCHATTEN, H. 2009a. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum Reprod Update*, 15, 573-85.
- MIAO, Y. L., SUN, Q. Y., ZHANG, X., ZHAO, J. G., ZHAO, M. T., SPATE, L., PRATHER, R. S. & SCHATTEN, H. 2009b. Centrosome abnormalities during porcine oocyte aging. *Environ Mol Mutagen*, 50, 666-71.
- MIHALAS, B. P., REDGROVE, K. A., MCLAUGHLIN, E. A. & NIXON, B. 2017. Molecular Mechanisms Responsible for Increased Vulnerability of the Ageing Oocyte to Oxidative Damage. *Oxid Med Cell Longev*, 2017, 4015874.
- MILLER, B. J., GEORGES-LABOUESSE, E., PRIMAKOFF, P. & MYLES, D. G. 2000. Normal fertilization occurs with eggs lacking the integrin alpha6beta1 and is CD9-dependent. *J Cell Biol*, 149, 1289-96.
- MIYADO, K., YAMADA, G., YAMADA, S., HASUWA, H., NAKAMURA, Y., RYU, F., SUZUKI, K., KOSAI, K., INOUE, K., OGURA, A., OKABE, M. & MEKADA, E. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. *Science*, 287, 321-4.
- MIYARA, F., MIGNE, C., DUMONT-HASSAN, M., LE MEUR, A., COHEN-BACRIE, P., AUBRIOT, F. X., GLISSANT, A., NATHAN, C., DOUARD, S., STANOVICI, A. & DEBEY, P. 2003. Chromatin configuration and transcriptional control in human and mouse oocytes. *Mol Reprod Dev*, 64, 458-70.
- MOGHADDAS, S., HOPPEL, C. L. & LESNEFSKY, E. J. 2003. Aging defect at the QO site of complex III augments oxyradical production in rat heart interfibrillar mitochondria. *Arch Biochem Biophys*, 414, 59-66.
- MOLINIA, F. C., EVANS, G. & MAXWELL, W. M. 1994. In vitro evaluation of zwitterion buffers in diluents for freezing ram spermatozoa. *Reprod Nutr Dev*, 34, 491-500.
- MOLINIA, F. C., EVANS, G. & MAXWELL, W. M. 1996. Fertility of ram spermatozoa pellet-frozen in zwitterion-buffered diluents. *Reprod Nutr Dev*, 36, 21-9.
- MONGET, P., FABRE, S., MULSANT, P., LECERF, F., ELSEN, J. M., MAZERBOURG, S., PISSELET, C. & MONNIAUX, D. 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domest Anim Endocrinol*, 23, 139-54.
- MOOR, R. M. & CROSBY, I. M. 1986. Protein requirements for germinal vesicle breakdown in ovine oocytes. *J Embryol Exp Morphol*, 94, 207-20.
- MORENO, S. & NURSE, P. 1990. Substrates for p34cdc2: in vivo veritas? *Cell*, 61, 549-51.
- MORGIA, F., TORTI, M., MONTIGIANI, M., PISCITELLI, C., GIALLONARDO, A., SCHIMBERNI, M., GIANNINI, P. & SBRACIA, M. 2006. Use of a medium buffered with N-hydroxyethylpiperazine-N-ethanesulfonate (HEPES) in intracytoplasmic sperm injection procedures is detrimental to the outcome of in vitro fertilization. *Fertil Steril*, 85, 1415-9.
- MORITA, Y., TSUTSUMI, O., HOSOYA, I., TAKETANI, Y., OKA, Y. & KATO, T. 1992. Expression and possible function of glucose transporter protein GLUT1 during preimplantation mouse development from oocytes to blastocysts. *Biochem Biophys Res Commun*, 188, 8-15.
- MORTON, K. M. 2008. Developmental capabilities of embryos produced in vitro from prepubertal lamb oocytes. *Reprod Domest Anim*, 43 Suppl 2, 137-43.
- MORTON, K. M., CATT, S. L., MAXWELL, W. M. & EVANS, G. 2005. In vitro and in vivo developmental capabilities and kinetics of in vitro development of in vitro matured oocytes from adult, unstimulated and hormone-stimulated prepubertal ewes. *Theriogenology*, 64, 1320-32.
- MOTLIK, J., FULKA, J. & FLECHON, J. E. 1986. Changes in intercellular coupling between pig oocytes and cumulus cells during maturation in vivo and in vitro. *J Reprod Fertil*, 76, 31-7.

- MOTTA, P. M., NOTTOLA, S. A., MAKABE, S. & HEYN, R. 2000. Mitochondrial morphology in human fetal and adult female germ cells. *Hum Reprod*, 15 Suppl 2, 129-47.
- MOTTERSHEAD, D. G., SUGIMURA, S., AL-MUSAWI, S. L., LI, J. J., RICHANI, D., WHITE, M. A., MARTIN, G. A., TROTTA, A. P., RITTER, L. J., SHI, J., MUELLER, T. D., HARRISON, C. A. & GILCHRIST, R. B. 2015. Cumulin, an Oocyte-secreted Heterodimer of the Transforming Growth Factor-beta Family, Is a Potent Activator of Granulosa Cells and Improves Oocyte Quality. *J Biol Chem*, 290, 24007-20.
- MOYES, C. D., BATTERSBY, B. J. & LEARY, S. C. 1998. Regulation of muscle mitochondrial design. *J Exp Biol*, 201, 299-307.
- MULLER-HOCKER, J., SCHAFER, S., WEIS, S., MUNSCHER, C. & STROWITZKI, T. 1996. Morphological-cytochemical and molecular genetic analyses of mitochondria in isolated human oocytes in the reproductive age. *Mol Hum Reprod*, 2, 951-8.
- MURAKOSHI, Y., SUEOKA, K., TAKAHASHI, K., SATO, S., SAKURAI, T., TAJIMA, H. & YOSHIMURA, Y. 2013. Embryo developmental capability and pregnancy outcome are related to the mitochondrial DNA copy number and ooplasmic volume. *J Assist Reprod Genet*, 30, 1367-75.
- NAGANO, M., KATAGIRI, S. & TAKAHASHI, Y. 2006a. ATP content and maturational/developmental ability of bovine oocytes with various cytoplasmic morphologies. *Zygote*, 14, 299-304.
- NAGANO, M., KATAGIRI, S. & TAKAHASHI, Y. 2006b. Relationship between bovine oocyte morphology and in vitro developmental potential. *Zygote*, 14, 53-61.
- NAGAOKA, S. I., HASSOLD, T. J. & HUNT, P. A. 2012. Human aneuploidy: mechanisms and new insights into an age-old problem. *Nat Rev Genet*, 13, 493-504.
- NAKAE, J., KIDO, Y. & ACCILI, D. 2001. Distinct and overlapping functions of insulin and IGF-I receptors. *Endocr Rev*, 22, 818-35.
- NAVIAUX, R. K. & MCGOWAN, K. A. 2000. Organismal effects of mitochondrial dysfunction. *Hum Reprod*, 15 Suppl 2, 44-56.
- NEILSON, L., ANDALIBI, A., KANG, D., COUTIFARIS, C., STRAUSS, J. F., 3RD, STANTON, J. A. & GREEN, D. P. 2000. Molecular phenotype of the human oocyte by PCR-SAGE. *Genomics*, 63, 13-24.
- NIELSEN, F. C., NIELSEN, J. & CHRISTIANSEN, J. 2001. A family of IGF-II mRNA binding proteins (IMP) involved in RNA trafficking. *Scand J Clin Lab Invest Suppl*, 234, 93-9.
- NIIMURA, S., KAWAKAMI, S. Y. & TAKANO, H. 2004. Changes in the amount of cytoplasmic inclusions in mouse oocytes during meiotic maturation in vivo and in vitro. *Reprod Med Biol*, 3, 231-236.
- NISHIMOTO, H., MATSUTANI, R., YAMAMOTO, S., TAKAHASHI, T., HAYASHI, K. G., MIYAMOTO, A., HAMANO, S. & TETSUKA, M. 2006. Gene expression of glucose transporter (GLUT) 1, 3 and 4 in bovine follicle and corpus luteum. *J Endocrinol*, 188, 111-9.
- NIXON, V. L., LEVASSEUR, M., MCDUGALL, A. & JONES, K. T. 2002. Ca(2+) oscillations promote APC/C-dependent cyclin B1 degradation during metaphase arrest and completion of meiosis in fertilizing mouse eggs. *Curr Biol*, 12, 746-50.
- NOGUEIRA, D., ALBANO, C., ADRIAENSSENS, T., CORTVRINDT, R., BOURGAIN, C., DEVROEY, P. & SMITZ, J. 2003a. Human oocytes reversibly arrested in prophase I by phosphodiesterase type 3 inhibitor in vitro. *Biol Reprod*, 69, 1042-52.
- NOGUEIRA, D., CORTVRINDT, R., DE MATOS, D. G., VANHOUTTE, L. & SMITZ, J. 2003b. Effect of phosphodiesterase type 3 inhibitor on developmental competence of immature mouse oocytes in vitro. *Biol Reprod*, 69, 2045-52.
- NOGUEIRA, D., RON-EL, R., FRIEDLER, S., SCHACHTER, M., RAZIEL, A., CORTVRINDT, R. & SMITZ, J. 2006. Meiotic arrest in vitro by phosphodiesterase 3-inhibitor enhances maturation capacity of human oocytes and allows subsequent embryonic development. *Biol Reprod*, 74, 177-84.

- NOGUES, C., PONSA, M., VIDAL, F., BOADA, M. & EGOZCUE, J. 1988. Effects of aging on the zona pellucida surface of mouse oocytes. *J In Vitro Fert Embryo Transf*, 5, 225-9.
- NOMIKOS, M., KASHIR, J., SWANN, K. & LAI, F. A. 2013. Sperm PLCzeta: from structure to Ca²⁺ oscillations, egg activation and therapeutic potential. *FEBS Lett*, 587, 3609-16.
- NORDGREN, K. K., PENG, Y., PELLEYMOUNTER, L. L., MOON, I., ABO, R., FENG, Q., ECKLOFF, B., YEE, V. C., WIEBEN, E. & WEINSHILBOUM, R. M. 2011. Methionine adenosyltransferase 2A/2B and methylation: gene sequence variation and functional genomics. *Drug Metab Dispos*, 39, 2135-47.
- NORDIN, M., BERGMAN, D., HALJE, M., ENGSTROM, W. & WARD, A. 2014. Epigenetic regulation of the *Igf2/H19* gene cluster. *Cell Prolif*, 47, 189-99.
- NORRIS, R. P., FREUDZON, M., MEHLMANN, L. M., COWAN, A. E., SIMON, A. M., PAUL, D. L., LAMPE, P. D. & JAFFE, L. A. 2008. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development*, 135, 3229-38.
- NORRIS, R. P., RATZAN, W. J., FREUDZON, M., MEHLMANN, L. M., KRALL, J., MOVSESIAN, M. A., WANG, H., KE, H., NIKOLAEV, V. O. & JAFFE, L. A. 2009. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development*, 136, 1869-78.
- NYGARD, A. B., JORGENSEN, C. B., CIRERA, S. & FREDHOLM, M. 2007. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *BMC Mol Biol*, 8, 67.
- O'BRIEN, J. K., BECK, N. F., MAXWELL, W. M. & EVANS, G. 1997a. Effect of hormone pre-treatment of prepubertal sheep on the production and developmental capacity of oocytes in vitro and in vivo. *Reprod Fertil Dev*, 9, 625-31.
- O'BRIEN, J. K., CATT, S. L., IRELAND, K. A., MAXWELL, W. M. & EVANS, G. 1997b. In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. *Theriogenology*, 47, 1433-43.
- O'BRIEN, J. K., DWARTE, D., RYAN, J. P., MAXWELL, W. M. & EVANS, G. 1996. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. *Reprod Fertil Dev*, 8, 1029-37.
- O'CONNOR, T., WILMUT, I. & TAYLOR, J. 2013. Quantitative evaluation of reference genes for real-time PCR during in vitro maturation of ovine oocytes. *Reprod Domest Anim*, 48, 477-83.
- O'DOHERTY, A. M., RUTLEDGE, C. E., SATO, S., THAKUR, A., LEES-MURDOCK, D. J., HATA, K. & WALSH, C. P. 2011. DNA methylation plays an important role in promoter choice and protein production at the mouse *Dnmt3L* locus. *Dev Biol*, 356, 411-20.
- OCHSNER, S. A., RUSSELL, D. L., DAY, A. J., BREYER, R. M. & RICHARDS, J. S. 2003. Decreased expression of tumor necrosis factor- α -stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice. *Endocrinology*, 144, 1008-19.
- OEHNINGER, S., HINSCH, E., PFISTERER, S., VEECK, L. L., KOLM, P., SCHILL, W. B., HODGEN, G. D. & HINSCH, K. D. 1996. Use of a specific zona pellucida (ZP) protein 3 antiserum as a clinical marker for human ZP integrity and function. *Fertil Steril*, 65, 139-45.
- OH, J., LEE, Y. D. & WAGERS, A. J. 2014. Stem cell aging: mechanisms, regulators and therapeutic opportunities. *Nat Med*, 20, 870-80.
- OH, J. S., HAN, S. J. & CONTI, M. 2010. Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption. *J Cell Biol*, 188, 199-207.
- OKANO, M., BELL, D. W., HABER, D. A. & LI, E. 1999. DNA methyltransferases *Dnmt3a* and *Dnmt3b* are essential for de novo methylation and mammalian development. *Cell*, 99, 247-57.
- OKTEM, O. & OKTAY, K. 2008. The ovary: anatomy and function throughout human life. *Ann N Y Acad Sci*, 1127, 1-9.
- OKTEM, O. & URMAN, B. 2010. Understanding follicle growth in vivo. *Hum Reprod*, 25, 2944-54.

- OLSZANSKA, B. & BORGUL, A. 1993. Maternal RNA content in oocytes of several mammalian and avian species. *J Exp Zool*, 265, 317-20.
- ORISAKA, M., TAJIMA, K., TSANG, B. K. & KOTSUJI, F. 2009. Oocyte-granulosa-theca cell interactions during preantral follicular development. *J Ovarian Res*, 2, 9.
- OROPEZA, A., WRENZYCKI, C., HERRMANN, D., HADELER, K. G. & NIEMANN, H. 2004. Improvement of the developmental capacity of oocytes from prepubertal cattle by intraovarian insulin-like growth factor-I application. *Biol Reprod*, 70, 1634-43.
- OROZCO-LUCERO, E., DUFORT, I., ROBERT, C. & SIRARD, M. A. 2014. Rapidly cleaving bovine two-cell embryos have better developmental potential and a distinctive mRNA pattern. *Mol Reprod Dev*, 81, 31-41.
- ORSI, N. M. & LEESE, H. J. 2004a. Amino acid metabolism of preimplantation bovine embryos cultured with bovine serum albumin or polyvinyl alcohol. *Theriogenology*, 61, 561-72.
- ORSI, N. M. & LEESE, H. J. 2004b. Ammonium exposure and pyruvate affect the amino acid metabolism of bovine blastocysts in vitro. *Reproduction*, 127, 131-40.
- OTALA, M., MAKINEN, S., TUURI, T., SJOBERG, J., PENTIKAINEN, V., MATIKAINEN, T. & DUNKEL, L. 2004. Effects of testosterone, dihydrotestosterone, and 17beta-estradiol on human ovarian tissue survival in culture. *Fertil Steril*, 82 Suppl 3, 1077-85.
- OTOI, T., FUJII, M., TANAKA, M., OOKA, A. & SUZUKI, T. 2000. Oocyte diameter in relation to meiotic competence and sperm penetration. *Theriogenology*, 54, 535-42.
- OTSUKA, F., MCTAVISH, K. J. & SHIMASAKI, S. 2011. Integral role of GDF-9 and BMP-15 in ovarian function. *Mol Reprod Dev*, 78, 9-21.
- OTTEN, A. B. & SMEETS, H. J. 2015. Evolutionary defined role of the mitochondrial DNA in fertility, disease and ageing. *Hum Reprod Update*, 21, 671-89.
- OTTOLENGHI, C., UDA, M., HAMATANI, T., CRISPONI, L., GARCIA, J. E., KO, M., PILIA, G., SFORZA, C., SCHLESSINGER, D. & FORABOSCO, A. 2004. Aging of oocyte, ovary, and human reproduction. *Ann N Y Acad Sci*, 1034, 117-31.
- OVERSTROM, E. W., BENOS, D. J. & BIGGERS, J. D. 1989. Synthesis of Na⁺/K⁺ ATPase by the preimplantation rabbit blastocyst. *J Reprod Fertil*, 85, 283-95.
- OZAWA, M., NAGAI, T., KANEKO, H., NOGUCHI, J., OHNUMA, K. & KIKUCHI, K. 2006. Successful pig embryonic development in vitro outside a CO₂ gas-regulated incubator: effects of pH and osmolality. *Theriogenology*, 65, 860-9.
- OZIL, J. P. 1998. Role of calcium oscillations in mammalian egg activation: experimental approach. *Biophys Chem*, 72, 141-52.
- PACKER, A. I., HSU, Y. C., BESMER, P. & BACHVAROVA, R. F. 1994. The ligand of the c-kit receptor promotes oocyte growth. *Dev Biol*, 161, 194-205.
- PACZKOWSKI, M., SILVA, E., SCHOOLCRAFT, W. B. & KRISHER, R. L. 2013. Comparative importance of fatty acid beta-oxidation to nuclear maturation, gene expression, and glucose metabolism in mouse, bovine, and porcine cumulus oocyte complexes. *Biol Reprod*, 88, 111.
- PAL, S. & TYLER, J. K. 2016. Epigenetics and aging. *Sci Adv*, 2, e1600584.
- PAL, S., VISHWANATH, S. N., ERDJUMENT-BROMAGE, H., TEMPST, P. & SIF, S. 2004. Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. *Mol Cell Biol*, 24, 9630-45.
- PALMA, G. A., ARGANARAZ, M. E., BARRERA, A. D., RODLER, D., MUTTO, A. A. & SINOWATZ, F. 2012. Biology and biotechnology of follicle development. *ScientificWorldJournal*, 2012, 938138.
- PALMA, G. A., MULLER, M. & BREM, G. 1997. Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced in vitro. *J Reprod Fertil*, 110, 347-53.
- PAN, H., O'BRIEN M, J., WIGGLESWORTH, K., EPPIG, J. J. & SCHULTZ, R. M. 2005. Transcript profiling during mouse oocyte development and the effect of gonadotropin priming and development in vitro. *Dev Biol*, 286, 493-506.

- PANDEY, A., SINGH, N., GUPTA, S. C., RANA, J. S. & GUPTA, N. 2009. Relative expression of cell growth regulatory genes insulin-like growth factors (IGF-1 and IGF-2) and their receptors (IGF-1R and IGF-2R) in somatic cell nuclear transferred (SCNT) and in vitro fertilized (IVF) pre-implantation buffalo embryos. *Cell Biol Int*, 33, 555-64.
- PANTALEON, M., HARVEY, M. B., PASCOE, W. S., JAMES, D. E. & KAYE, P. L. 1997. Glucose transporter GLUT3: ontogeny, targeting, and role in the mouse blastocyst. *Proc Natl Acad Sci U S A*, 94, 3795-800.
- PAPIN, J. F., VAHRSON, W. & DITTMER, D. P. 2004. SYBR green-based real-time quantitative PCR assay for detection of West Nile Virus circumvents false-negative results due to strain variability. *J Clin Microbiol*, 42, 1511-8.
- PARK, C. H., UH, K. J., MULLIGAN, B. P., JEUNG, E. B., HYUN, S. H., SHIN, T., KA, H. & LEE, C. K. 2011. Analysis of imprinted gene expression in normal fertilized and uniparental preimplantation porcine embryos. *PLoS One*, 6, e22216.
- PARK, J. I., JEON, H. J., JUNG, N. K., JANG, Y. J., KIM, J. S., SEO, Y. W., JEONG, M., CHAE, H. Z. & CHUN, S. Y. 2012. Periovarian expression of hydrogen peroxide-induced sulfiredoxin and peroxiredoxin 2 in the rat ovary: gonadotropin regulation and potential modification. *Endocrinology*, 153, 5512-21.
- PARK, Y., HONG, J., YONG, H., LIM, J. & LEE, E. 2005a. Effect of exogenous carbohydrates in a serum-free culture medium on the development of in vitro matured and fertilized porcine embryos. *Zygote*, 13, 269-75.
- PARK, Y. S., KIM, S. S., KIM, J. M., PARK, H. D. & BYUN, M. D. 2005b. The effects of duration of in vitro maturation of bovine oocytes on subsequent development, quality and transfer of embryos. *Theriogenology*, 64, 123-34.
- PARSONS, P. G. 1997. Can skin cancer in humans be prevented by alleviation of oxidative stress? *Redox Rep*, 3, 77-83.
- PAULINI, F. & MELO, E. O. 2011. The role of oocyte-secreted factors GDF9 and BMP15 in follicular development and oogenesis. *Reprod Domest Anim*, 46, 354-61.
- PAVLOK, A., LUCAS-HAHN, A. & NIEMANN, H. 1992. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol Reprod Dev*, 31, 63-7.
- PAWLAK, P., CHABOWSKA, A., MALYSZKA, N. & LECHNIAK, D. 2016. Mitochondria and mitochondrial DNA in porcine oocytes and cumulus cells--A search for developmental competence marker. *Mitochondrion*, 27, 48-55.
- PAWLAK, P., PERS-KAMCZYC, E., RENSKA, N., KUBICKOVA, S. & LECHNIAK, D. 2011. Disturbances of nuclear maturation in BCB positive oocytes collected from peri-pubertal gilts. *Theriogenology*, 75, 832-40.
- PAWLAK, P., WARZYCH, E., HRYCIUK, M. & LECHNIAK, D. 2015. Transcript abundance, glutathione and apoptosis levels differ between porcine oocytes collected from prepubertal and cyclic gilts. *Theriogenology*, 84, 86-93.
- PAWSHE, C. H., RAO, K. B. & TOTTEY, S. M. 1998. Effect of insulin-like growth factor I and its interaction with gonadotropins on in vitro maturation and embryonic development, cell proliferation, and biosynthetic activity of cumulus-oocyte complexes and granulosa cells in buffalo. *Mol Reprod Dev*, 49, 277-85.
- PAWSON, T. 2004. Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems. *Cell*, 116, 191-203.
- PEDERSEN, H. S., CALLESEN, H., LOVENDAHL, P., CHEN, F., NYENGAARD, J. R., NIKOLAISEN, N. K., HOLM, P. & HYTTEL, P. 2014. Ultrastructure and mitochondrial numbers in pre- and postpubertal pig oocytes. *Reprod Fertil Dev*.
- PEDERSEN, H. S., CALLESEN, H., LOVENDAHL, P., CHEN, F., NYENGAARD, J. R., NIKOLAISEN, N. K., HOLM, P. & HYTTEL, P. 2016. Ultrastructure and mitochondrial numbers in pre- and postpubertal pig oocytes. *Reprod Fertil Dev*, 28, 586-98.

- PELLAND, A. M., CORBETT, H. E. & BALTZ, J. M. 2009. Amino Acid transport mechanisms in mouse oocytes during growth and meiotic maturation. *Biol Reprod*, 81, 1041-54.
- PELLESTOR, F., ANDREO, B., ARNAL, F., HUMEAU, C. & DEMAILLE, J. 2003. Maternal aging and chromosomal abnormalities: new data drawn from in vitro unfertilized human oocytes. *Hum Genet*, 112, 195-203.
- PENG, H., CHANG, B., LU, C., SU, J., WU, Y., LV, P., WANG, Y., LIU, J., ZHANG, B., QUAN, F., GUO, Z. & ZHANG, Y. 2012. Nlrp2, a maternal effect gene required for early embryonic development in the mouse. *PLoS One*, 7, e30344.
- PENNING, L. C., VRIELING, H. E., BRINKHOF, B., RIEMERS, F. M., ROTHUIZEN, J., RUTTEMAN, G. R. & HAZEWINKEL, H. A. 2007. A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues. *Vet Immunol Immunopathol*, 120, 212-22.
- PEPLING, M. E., DE CUEVAS, M. & SPRADLING, A. C. 1999. Germline cysts: a conserved phase of germ cell development? *Trends Cell Biol*, 9, 257-62.
- PEPLING, M. E. & SPRADLING, A. C. 1998. Female mouse germ cells form synchronously dividing cysts. *Development*, 125, 3323-8.
- PEPLING, M. E. & SPRADLING, A. C. 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol*, 234, 339-51.
- PERSANI, L. & BONOMI, M. 2014. Uncertainties in endocrine substitution therapy for central endocrine insufficiencies: hypothyroidism. *Handb Clin Neurol*, 124, 397-405.
- PETRONCZKI, M., SIOMOS, M. F. & NASMYTH, K. 2003. Un menage a quatre: the molecular biology of chromosome segregation in meiosis. *Cell*, 112, 423-40.
- PETRUSSA, L., VAN DE VELDE, H. & DE RYCKE, M. 2014. Dynamic regulation of DNA methyltransferases in human oocytes and preimplantation embryos after assisted reproductive technologies. *Mol Hum Reprod*, 20, 861-74.
- PICELLI, S., FARIDANI, O. R., BJORKLUND, A. K., WINBERG, G., SAGASSER, S. & SANDBERG, R. 2014. Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc*, 9, 171-81.
- PICTON, H. M., DANFOUR, M. A., HARRIS, S. E., CHAMBERS, E. L. & HUNTRISS, J. 2003. Growth and maturation of oocytes in vitro. *Reprod Suppl*, 61, 445-62.
- PICTON, H. M., ELDER, K., HOUGHTON, F. D., HAWKHEAD, J. A., RUTHERFORD, A. J., HOGG, J. E., LEESE, H. J. & HARRIS, S. E. 2010. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod*, 16, 557-69.
- PICTON, H. M. & GOSDEN, R. G. 2000. In vitro growth of human primordial follicles from frozen-banked ovarian tissue. *Mol Cell Endocrinol*, 166, 27-35.
- PICTON, H. M., HARRIS, S. E., MURUVI, W. & CHAMBERS, E. L. 2008. The in vitro growth and maturation of follicles. *Reproduction*, 136, 703-15.
- PIELER, T. & BELLEFROID, E. 1994. Perspectives on zinc finger protein function and evolution—an update. *Mol Biol Rep*, 20, 1-8.
- PIERZCHALA, M., PAREEK, C. S., URBANSKI, P., GOLUCH, D., KAMYCZEK, M., ROZYCKI, M., SMOZYNSKI, R., HORBANCZUK, J. O. & KURYL, J. 2012. Study of the differential transcription in liver of growth hormone receptor (GHR), insulin-like growth factors (IGF1, IGF2) and insulin-like growth factor receptor (IGF1R) genes at different postnatal developmental ages in pig breeds. *Mol Biol Rep*, 39, 3055-66.
- PIKO, L. & MATSUMOTO, L. 1976. Number of mitochondria and some properties of mitochondrial DNA in the mouse egg. *Dev Biol*, 49, 1-10.
- PIKO, L. & TAYLOR, K. D. 1987. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev Biol*, 123, 364-74.
- PIRINO, G., WESCOTT, M. P. & DONOVAN, P. J. 2009. Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes. *Cell Cycle*, 8, 665-70.

- PISANI, L. F., ANTONINI, S., POCAR, P., FERRARI, S., BREVINI, T. A., RHIND, S. M. & GANDOLFI, F. 2008. Effects of pre-mating nutrition on mRNA levels of developmentally relevant genes in sheep oocytes and granulosa cells. *Reproduction*, 136, 303-12.
- POLLACK, B. P., KOTENKO, S. V., HE, W., IZOTOVA, L. S., BARNOSKI, B. L. & PESTKA, S. 1999. The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. *J Biol Chem*, 274, 31531-42.
- PONDERATO, N., CROTTI, G., TURINI, P., DUCHI, R., GALLI, C. & LAZZARI, G. 2002. Embryonic and foetal development of bovine oocytes treated with a combination of butyrolactone I and roscovitine in an enriched medium prior to IVM and IVF. *Mol Reprod Dev*, 62, 513-8.
- PORETSKY, L., CATALDO, N. A., ROSENWAKS, Z. & GIUDICE, L. C. 1999. The insulin-related ovarian regulatory system in health and disease. *Endocr Rev*, 20, 535-82.
- PRASAD, S., TIWARI, M., KOCH, B. & CHAUBE, S. K. 2015. Morphological, cellular and molecular changes during postovulatory egg aging in mammals. *J Biomed Sci*, 22, 36.
- PREIS, K. A., SEIDEL, G., JR. & GARDNER, D. K. 2005. Metabolic markers of developmental competence for in vitro-matured mouse oocytes. *Reproduction*, 130, 475-83.
- PRIOLEAU, M. N., HUET, J., SENTENAC, A. & MECHALI, M. 1994. Competition between chromatin and transcription complex assembly regulates gene expression during early development. *Cell*, 77, 439-49.
- PRU, J. K. & TILLY, J. L. 2001. Programmed cell death in the ovary: insights and future prospects using genetic technologies. *Mol Endocrinol*, 15, 845-53.
- PTAK, G., LOI, P., DATTENA, M., TISCHNER, M. & CAPPAL, P. 1999. Offspring from one-month-old lambs: studies on the developmental capability of prepubertal oocytes. *Biol Reprod*, 61, 1568-74.
- PTAK, G., MATSUKAWA, K., PALMIERI, C., DELLA SALDA, L., SCAPOLO, P. A. & LOI, P. 2006. Developmental and functional evidence of nuclear immaturity in prepubertal oocytes. *Hum Reprod*, 21, 2228-37.
- QIAO, J., WANG, Z. B., FENG, H. L., MIAO, Y. L., WANG, Q., YU, Y., WEI, Y. C., YAN, J., WANG, W. H., SHEN, W., SUN, S. C., SCHATTE, H. & SUN, Q. Y. 2014. The root of reduced fertility in aged women and possible therapeutic options: current status and future prospects. *Mol Aspects Med*, 38, 54-85.
- RADONIC, A., THULKE, S., MACKAY, I. M., LANDT, O., SIEGERT, W. & NITSCHKE, A. 2004. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun*, 313, 856-62.
- RAGHU, H. M., NANDI, S. & REDDY, S. M. 2002. Follicle size and oocyte diameter in relation to developmental competence of buffalo oocytes in vitro. *Reprod Fertil Dev*, 14, 55-61.
- RAMBAGS, B. P., VAN BOXTEL, D. C., THARASANIT, T., LENSTRA, J. A., COLENBRANDER, B. & STOUT, T. A. 2014. Advancing maternal age predisposes to mitochondrial damage and loss during maturation of equine oocytes in vitro. *Theriogenology*, 81, 959-65.
- RANKIN, T., TALBOT, P., LEE, E. & DEAN, J. 1999. Abnormal zona pellucida in mice lacking ZP1 result in early embryonic loss. *Development*, 126, 3847-55.
- RANKIN, T. L., O'BRIEN, M., LEE, E., WIGGLESWORTH, K., EPPIG, J. & DEAN, J. 2001. Defective zona pellucida in Zp2-null mice disrupt folliculogenesis, fertility and development. *Development*, 128, 1119-26.
- RATTS, V. S., FLAWS, J. A., KOLP, R., SORENSON, C. M. & TILLY, J. L. 1995. Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology*, 136, 3665-8.
- READER, K. L., COX, N. R., STANTON, J. A. & JUENGEL, J. L. 2015. Mitochondria and vesicles differ between adult and prepubertal sheep oocytes during IVM. *Reprod Fertil Dev*, 27, 513-22.
- REBHAN, M., CHALIFA-CASPI, V., PRILUSKY, J. & LANCET, D. 1997. GeneCards: integrating information about genes, proteins and diseases. *Trends Genet*, 13, 163.

- REDEL, B. K., SPATE, L. D., LEE, K., MAO, J., WHITWORTH, K. M. & PRATHER, R. S. 2016. Glycine supplementation in vitro enhances porcine preimplantation embryo cell number and decreases apoptosis but does not lead to live births. *Mol Reprod Dev*, 83, 246-58.
- REIK, W. & DEAN, W. 2001. DNA methylation and mammalian epigenetics. *Electrophoresis*, 22, 2838-43.
- REIS, A., CHANG, H. Y., LEVASSEUR, M. & JONES, K. T. 2006. APCcdh1 activity in mouse oocytes prevents entry into the first meiotic division. *Nat Cell Biol*, 8, 539-40.
- REIS, A., MADGWICK, S., CHANG, H. Y., NABTI, I., LEVASSEUR, M. & JONES, K. T. 2007. Prometaphase APCcdh1 activity prevents non-disjunction in mammalian oocytes. *Nat Cell Biol*, 9, 1192-8.
- REVENKOVA, E., EIJPE, M., HEYTING, C., HODGES, C. A., HUNT, P. A., LIEBE, B., SCHERTHAN, H. & JESSBERGER, R. 2004. Cohesin SMC1 beta is required for meiotic chromosome dynamics, sister chromatid cohesion and DNA recombination. *Nat Cell Biol*, 6, 555-62.
- REYES, J. M., SILVA, E., CHITWOOD, J. L., SCHOOLCRAFT, W. B., KRISHER, R. L. & ROSS, P. J. 2017. Differing molecular response of young and advanced maternal age human oocytes to IVM. *Hum Reprod*, 32, 2199-2208.
- REYNAUD, K. & DRIANCOURT, M. A. 2000. Oocyte attrition. *Mol Cell Endocrinol*, 163, 101-8.
- REYNIER, P., MAY-PANLOUP, P., CHRETIEN, M. F., MORGAN, C. J., JEAN, M., SAVAGNER, F., BARRIERE, P. & MALTHIERY, Y. 2001. Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol Hum Reprod*, 7, 425-9.
- RICE, S., OJHA, K. & MASON, H. 2008. Human ovarian biopsies as a viable source of pre-antral follicles. *Hum Reprod*, 23, 600-5.
- RICHANI, D., WANG, X., ZENG, H. T., SMITZ, J., THOMPSON, J. G. & GILCHRIST, R. B. 2014. Prematuration with cAMP modulators in conjunction with EGF-like peptides during in vitro maturation enhances mouse oocyte developmental competence. *Mol Reprod Dev*, 81, 422-35.
- RICHARD, D. M., DAWES, M. A., MATHIAS, C. W., ACHESON, A., HILL-KAPTURCZAK, N. & DOUGHERTY, D. M. 2009. L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. *Int J Tryptophan Res*, 2, 45-60.
- RICHARDS, J. S. 2002. Delivery of the oocyte from the follicle to the oviduct: a time of vulnerability. *Ernst Schering Res Found Workshop*, 43-62.
- RIEGER, D. & LOSKUTOFF, N. M. 1994. Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. *J Reprod Fertil*, 100, 257-62.
- ROBERT, C., MCGRAW, S., MASSICOTTE, L., PRAVETONI, M., GANDOLFI, F. & SIRARD, M. A. 2002. Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol Reprod*, 67, 1465-72.
- ROBERTS, R., STARK, J., IATROPOULOU, A., BECKER, D. L., FRANKS, S. & HARDY, K. 2004. Energy substrate metabolism of mouse cumulus-oocyte complexes: response to follicle-stimulating hormone is mediated by the phosphatidylinositol 3-kinase pathway and is associated with oocyte maturation. *Biol Reprod*, 71, 199-209.
- ROGERS, N. T., HALET, G., PIAO, Y., CARROLL, J., KO, M. S. & SWANN, K. 2006. The absence of a Ca(2+) signal during mouse egg activation can affect parthenogenetic preimplantation development, gene expression patterns, and blastocyst quality. *Reproduction*, 132, 45-57.
- ROMAGUERA, R., MOLL, X., MORATO, R., ROURA, M., PALOMO, M. J., CATALA, M. G., JIMENEZ-MACEDO, A. R., HAMMAMI, S., IZQUIERDO, D., MOGAS, T. & PARAMIO, M. T. 2011. Prepubertal goat oocytes from large follicles result in similar blastocyst production and embryo ploidy than those from adult goats. *Theriogenology*, 76, 1-11.
- ROMAR, R., DE SANTIS, T., PAPILLIER, P., PERREAU, C., THELIE, A., DELL' AQUILA, M. E., MERMILLOD, P. & DALBIES-TRAN, R. 2010. Expression of Maternal Transcripts During Bovine Oocyte In Vitro Maturation is Affected by Donor Age. *Reprod Dom Anim*.

- ROMEK, M., GAJDA, B., KRZYSZTOFOWICZ, E., KEP CZYNSKI, M. & SMORAG, Z. 2011. Lipid content in pig blastocysts cultured in the presence or absence of protein and vitamin E or phenazine ethosulfate. *Folia Biol (Krakow)*, 59, 45-52.
- ROMEK, M., GAJDA, B., KRZYSZTOFOWICZ, E. & SMORAG, Z. 2009. Lipid content of non-cultured and cultured pig embryo. *Reprod Domest Anim*, 44, 24-32.
- ROSE-HELLEKANT, T. A., LIBERSKY-WILLIAMSON, E. A. & BAVISTER, B. D. 1998. Energy substrates and amino acids provided during in vitro maturation of bovine oocytes alter acquisition of developmental competence. *Zygote*, 6, 285-94.
- ROSE, R. D., GILCHRIST, R. B., KELLY, J. M., THOMPSON, J. G. & SUTTON-MCDOWALL, M. L. 2013. Regulation of sheep oocyte maturation using cAMP modulators. *Theriogenology*, 79, 142-8.
- ROSS, P. J., WANG, K., KOCABAS, A. & CIBELLI, J. B. 2010. Housekeeping gene transcript abundance in bovine fertilized and cloned embryos. *Cell Reprogram*, 12, 709-17.
- ROSSIGNOL, S., STEUNOU, V., CHALAS, C., KERJEAN, A., RIGOLET, M., VIEGAS-PEQUIGNOT, E., JOUANNET, P., LE BOUC, Y. & GICQUEL, C. 2006. The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *J Med Genet*, 43, 902-7.
- ROTH, Z. & HANSEN, P. J. 2005. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction*, 129, 235-44.
- ROWSEY, R., KASHEVAROVA, A., MURDOCH, B., DICKENSON, C., WOODRUFF, T., CHENG, E., HUNT, P. & HASSOLD, T. 2013. Germline mosaicism does not explain the maternal age effect on trisomy. *Am J Med Genet A*, 161A, 2495-503.
- RUGGIU, M., SPEED, R., TAGGART, M., MCKAY, S. J., KILANOWSKI, F., SAUNDERS, P., DORIN, J. & COOKE, H. J. 1997. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature*, 389, 73-7.
- RUNGE, K. E., EVANS, J. E., HE, Z. Y., GUPTA, S., MCDONALD, K. L., STAHLBERG, H., PRIMAKOFF, P. & MYLES, D. G. 2007. Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution. *Dev Biol*, 304, 317-25.
- RUSSO, V., MARTELLI, A., BERARDINELLI, P., DI GIACINTO, O., BERNABO, N., FANTASIA, D., MATTIOLI, M. & BARBONI, B. 2007a. Modifications in chromatin morphology and organization during sheep oogenesis. *Microsc Res Tech*, 70, 733-44.
- RUSSO, V., MARTELLI, M., MAURO, A., DI GIACINTO, O., NARDINOCCHI, D. & BERARDINELLI, P. 2007b. Nuclear remodelling in growing oocytes of sheep. *Vet Res Commun*, 31 Suppl 1, 201-4.
- SAITO, T., HIROI, M. & KATO, T. 1994. Development of glucose utilization studied in single oocytes and preimplantation embryos from mice. *Biol Reprod*, 50, 266-70.
- SALPEKAR, A., HUNTRISS, J., BOLTON, V. & MONK, M. 2001. The use of amplified cDNA to investigate the expression of seven imprinted genes in human oocytes and preimplantation embryos. *Mol Hum Reprod*, 7, 839-44.
- SALWAY, J. G. 2004. *Metabolism at a glance*, Oxford, Blackwell Publishing Ltd.
- SANCHEZ, F., LOLICATO, F., ROMERO, S., DE VOS, M., VAN RANST, H., VERHEYEN, G., ANCKAERT, E. & SMITZ, J. E. J. 2017. An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield. *Hum Reprod*, 32, 2056-2068.
- SANCHEZ, F. & SMITZ, J. 2012. Molecular control of oogenesis. *Biochim Biophys Acta*, 1822, 1896-912.
- SANDALINAS, M., MARQUEZ, C. & MUNNE, S. 2002. Spectral karyotyping of fresh, non-inseminated oocytes. *Mol Hum Reprod*, 8, 580-5.
- SANTIQUET, N. W., GREENE, A. F., BECKER, J., BARFIELD, J. P., SCHOOLCRAFT, W. B. & KRISHER, R. L. 2017. A pre-in vitro maturation medium containing cumulus oocyte complex ligand-receptor signaling molecules maintains meiotic arrest, supports the cumulus

- oocyte complex and improves oocyte developmental competence. *Mol Hum Reprod*, 23, 594-606.
- SANTOS, T. A., EL SHOURBAGY, S. & ST JOHN, J. C. 2006. Mitochondrial content reflects oocyte variability and fertilization outcome. *Fertil Steril*, 85, 584-91.
- SARKISSIAN, M., MENDEZ, R. & RICHTER, J. D. 2004. Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and glycogen synthase kinase-3. *Genes Dev*, 18, 48-61.
- SATHANANTHAN, A. H. & TROUNSON, A. O. 2000. Mitochondrial morphology during preimplantational human embryogenesis. *Hum Reprod*, 15 Suppl 2, 148-59.
- SATO, H., IWATA, H., HAYASHI, T., KIMURA, K., KUWAYAMA, T. & MONJI, Y. 2007. The effect of glucose on the progression of the nuclear maturation of pig oocytes. *Anim Reprod Sci*, 99, 299-305.
- SCHATTEN, H. 2008. The mammalian centrosome and its functional significance. *Histochem Cell Biol*, 129, 667-86.
- SCHATTEN, H., SUN, Q. Y. & PRATHER, R. 2014. The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility. *Reprod Biol Endocrinol*, 12, 111.
- SCHON, E. A., DIMAURO, S. & HIRANO, M. 2012. Human mitochondrial DNA: roles of inherited and somatic mutations. *Nat Rev Genet*, 13, 878-90.
- SCHRAMM, R. D., TENNIER, M. T., BOATMAN, D. E. & BAVISTER, B. D. 1993. Chromatin configurations and meiotic competence of oocytes are related to follicular diameter in nonstimulated rhesus monkeys. *Biol Reprod*, 48, 349-56.
- SCHUH, M. 2011. An actin-dependent mechanism for long-range vesicle transport. *Nat Cell Biol*, 13, 1431-6.
- SCHUH, M. & ELLENBERG, J. 2008. A new model for asymmetric spindle positioning in mouse oocytes. *Curr Biol*, 18, 1986-92.
- SCHULTZ, R. M., MONTGOMERY, R. R. & BELANOFF, J. R. 1983. Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Dev Biol*, 97, 264-73.
- SCHUMACHER, J. M., LEE, K., EDELHOFF, S. & BRAUN, R. E. 1995. Distribution of Tenr, an RNA-binding protein, in a lattice-like network within the spermatid nucleus in the mouse. *Biol Reprod*, 52, 1274-83.
- SCHWARZER, C., SIATKOWSKI, M., PFEIFFER, M. J., BAEUMER, N., DREXLER, H. C., WANG, B., FUELLEN, G. & BOIANI, M. 2014. Maternal age effect on mouse oocytes: new biological insight from proteomic analysis. *Reproduction*, 148, 55-72.
- SCOTT, L., BERNTSEN, J., DAVIES, D., GUNDERSEN, J., HILL, J. & RAMSING, N. 2008. Symposium: innovative techniques in human embryo viability assessment. Human oocyte respiration-rate measurement--potential to improve oocyte and embryo selection? *Reprod Biomed Online*, 17, 461-9.
- SELA-ABRAMOVICH, S., CHOREV, E., GALIANI, D. & DEKEL, N. 2005. Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology*, 146, 1236-44.
- SELA-ABRAMOVICH, S., EDRY, I., GALIANI, D., NEVO, N. & DEKEL, N. 2006. Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology*, 147, 2280-6.
- SELI, E., BABAYEV, E., COLLINS, S. C., NEMETH, G. & HORVATH, T. L. 2014. Minireview: Metabolism of female reproduction: regulatory mechanisms and clinical implications. *Mol Endocrinol*, 28, 790-804.
- SELLICK, C. A., HANSEN, R., STEPHENS, G. M., GOODACRE, R. & DICKSON, A. J. 2011. Metabolite extraction from suspension-cultured mammalian cells for global metabolite profiling. *Nat Protoc*, 6, 1241-9.

- SENGUPTA, J., DHAWAN, L., LALITKUMAR, P. G. & GHOSH, D. 2005. Nitric oxide in blastocyst implantation in the rhesus monkey. *Reproduction*, 130, 321-32.
- SERTA, R. T., MICHALOPOULOS, J., SEIBEL, M. M. & KIESSLING, A. A. 1995. The developmental potential of mouse oocytes matured in serum-free culture conditions. *Hum Reprod*, 10, 1810-5.
- SHAO, G. B., CHEN, J. C., ZHANG, L. P., HUANG, P., LU, H. Y., JIN, J., GONG, A. H. & SANG, J. R. 2014. Dynamic patterns of histone H3 lysine 4 methyltransferases and demethylases during mouse preimplantation development. *In Vitro Cell Dev Biol Anim*, 50, 603-13.
- SHERRATT, H. S. 1991. Mitochondria: structure and function. *Rev Neurol (Paris)*, 147, 417-30.
- SHIM, C., LEE, S. G., SONG, W. K., LEE, C. S., LEE, K. K. & KIM, K. 1997. Laminin chain-specific gene expression during mouse oocyte maturation. *Mol Reprod Dev*, 48, 185-93.
- SHIRAZI, A. & MOALEMIAN, Z. 2007. Ovine cumulus cells estradiol-17Beta production in the presence or absence of oocyte. *Anim Reprod Sci*, 101, 125-33.
- SHIRAZI, A. & SADEGHI, N. 2007. The effect of ovine oocyte diameter on nuclear maturation. *Small Ruminant Research*, 69, 103-107.
- SHOUBRIDGE, E. A. & WAI, T. 2007. Mitochondrial DNA and the mammalian oocyte. *Curr Top Dev Biol*, 77, 87-111.
- SHU, Y. M., ZENG, H. T., REN, Z., ZHUANG, G. L., LIANG, X. Y., SHEN, H. W., YAO, S. Z., KE, P. Q. & WANG, N. N. 2008. Effects of cilostamide and forskolin on the meiotic resumption and embryonic development of immature human oocytes. *Hum Reprod*, 23, 504-13.
- SIMON, A. M., GOODENOUGH, D. A., LI, E. & PAUL, D. L. 1997. Female infertility in mice lacking connexin 37. *Nature*, 385, 525-9.
- SIMSEK-DURAN, F., LI, F., FORD, W., SWANSON, R. J., JONES, H. W., JR. & CASTORA, F. J. 2013. Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PLoS One*, 8, e64955.
- SIRARD, M. A. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology*, 55, 1241-54.
- SIRARD, M. A. & BILODEAU, S. 1990. Granulosa cells inhibit the resumption of meiosis in bovine oocytes in vitro. *Biol Reprod*, 43, 777-83.
- SIRARD, M. A. & FIRST, N. L. 1988. In vitro inhibition of oocyte nuclear maturation in the bovine. *Biol Reprod*, 39, 229-34.
- SIRARD, M. A., RICHARD, F., BLONDIN, P. & ROBERT, C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*, 65, 126-36.
- SMITH, G. D. 2001. In vitro maturation of oocytes. *Curr Womens Health Rep*, 1, 143-51.
- SMITH, L. C. & ALCIVAR, A. A. 1993. Cytoplasmic inheritance and its effects on development and performance. *J Reprod Fertil Suppl*, 48, 31-43.
- SMITH, P., O, W. S., HUDSON, N. L., SHAW, L., HEATH, D. A., CONDELL, L., PHILLIPS, D. J. & MCNATTY, K. P. 1993. Effects of the Booroola gene (FecB) on body weight, ovarian development and hormone concentrations during fetal life. *J Reprod Fertil*, 98, 41-54.
- SMITH, Z. D., CHAN, M. M., HUMM, K. C., KARNIK, R., MEKHOUBAD, S., REGEV, A., EGGAN, K. & MEISSNER, A. 2014. DNA methylation dynamics of the human preimplantation embryo. *Nature*, 511, 611-5.
- SMITS, K., GOOSSENS, K., VAN SOOM, A., GOVAERE, J., HOOGEWIJS, M., VANHAESEBROUCK, E., GALLI, C., COLLEONI, S., VANDESOMPELE, J. & PEELMAN, L. 2009. Selection of reference genes for quantitative real-time PCR in equine in vivo and fresh and frozen-thawed in vitro blastocysts. *BMC Res Notes*, 2, 246.
- SOMFAI, T., KANEDA, M., AKAGI, S., WATANABE, S., HARAGUCHI, S., MIZUTANI, E., DANG-NGUYEN, T. Q., GESHI, M., KIKUCHI, K. & NAGAI, T. 2011. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod Fertil Dev*, 23, 912-20.

- SONGSASEN, N., WESSELOWSKI, S., CARPENTER, J. W. & WILDT, D. E. 2012. The ability to achieve meiotic maturation in the dog oocyte is linked to glycolysis and glutamine oxidation. *Mol Reprod Dev*, 79, 186-96.
- SPICER, L. J. & AAD, P. Y. 2007. Insulin-like growth factor (IGF) 2 stimulates steroidogenesis and mitosis of bovine granulosa cells through the IGF1 receptor: role of follicle-stimulating hormone and IGF2 receptor. *Biol Reprod*, 77, 18-27.
- SPIKINGS, E. C., ALDERSON, J. & ST JOHN, J. C. 2007. Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development. *Biol Reprod*, 76, 327-35.
- STEELE, W., ALLEGRUCCI, C., SINGH, R., LUCAS, E., PRIDDLE, H., DENNING, C., SINCLAIR, K. & YOUNG, L. 2005. Human embryonic stem cell methyl cycle enzyme expression: modelling epigenetic programming in assisted reproduction? *Reprod Biomed Online*, 10, 755-66.
- STEEVES, C. L. & BALTZ, J. M. 2005. Regulation of intracellular glycine as an organic osmolyte in early preimplantation mouse embryos. *J Cell Physiol*, 204, 273-9.
- STEEVES, C. L., HAMMER, M. A., WALKER, G. B., RAE, D., STEWART, N. A. & BALTZ, J. M. 2003. The glycine neurotransmitter transporter GLYT1 is an organic osmolyte transporter regulating cell volume in cleavage-stage embryos. *Proc Natl Acad Sci U S A*, 100, 13982-7.
- STEEVES, T. E. & GARDNER, D. K. 1999. Metabolism of glucose, pyruvate, and glutamine during the maturation of oocytes derived from pre-pubertal and adult cows. *Mol Reprod Dev*, 54, 92-101.
- STERNLICHT, A. L. & SCHULTZ, R. M. 1981. Biochemical studies of mammalian oogenesis: kinetics of accumulation of total and poly(A)-containing RNA during growth of the mouse oocyte. *J Exp Zool*, 215, 191-200.
- STEUERWALD, N. 2005. Meiotic spindle checkpoints for assessment of aneuploid oocytes. *Cytogenet Genome Res*, 111, 256-9.
- STEUERWALD, N., BARRITT, J. A., ADLER, R., MALTER, H., SCHIMMEL, T., COHEN, J. & BRENNER, C. A. 2000. Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. *Zygote*, 8, 209-15.
- STEUERWALD, N. M., BERMUDEZ, M. G., WELLS, D., MUNNE, S. & COHEN, J. 2007. Maternal age-related differential global expression profiles observed in human oocytes. *Reprod Biomed Online*, 14, 700-8.
- STOJKOVIC, M., MACHADO, S. A., STOJKOVIC, P., ZAKHARTCHENKO, V., HUTZLER, P., GONCALVES, P. B. & WOLF, E. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod*, 64, 904-9.
- STOKES, P. J., HAWKHEAD, J. A., FAWTHROP, R. K., PICTON, H. M., SHARMA, V., LEESE, H. J. & HOUGHTON, F. D. 2007. Metabolism of human embryos following cryopreservation: implications for the safety and selection of embryos for transfer in clinical IVF. *Hum Reprod*, 22, 829-35.
- STRYER, L. 1995. *Biochemistry (Fourth ed.)*. New York, W.H. Freeman and Company.
- STURMEY, R. G., BERMEJO-ALVAREZ, P., GUTIERREZ-ADAN, A., RIZOS, D., LEESE, H. J. & LONERGAN, P. 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Mol Reprod Dev*, 77, 285-96.
- STURMEY, R. G., BRISON, D. R. & LEESE, H. J. 2008. Symposium: innovative techniques in human embryo viability assessment. Assessing embryo viability by measurement of amino acid turnover. *Reprod Biomed Online*, 17, 486-96.
- STURMEY, R. G. & LEESE, H. J. 2003. Energy metabolism in pig oocytes and early embryos. *Reproduction*, 126, 197-204.

- STURMEY, R. G., O'TOOLE, P. J. & LEESE, H. J. 2006. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction*, 132, 829-37.
- SU, Y. Q., SUGIURA, K., WIGGLESWORTH, K., O'BRIEN, M. J., AFFOURTIT, J. P., PANGAS, S. A., MATZUK, M. M. & EPPIG, J. J. 2008. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development*, 135, 111-21.
- SU, Y. Q., SUGIURA, K., WOO, Y., WIGGLESWORTH, K., KAMDAR, S., AFFOURTIT, J. & EPPIG, J. J. 2007. Selective degradation of transcripts during meiotic maturation of mouse oocytes. *Dev Biol*, 302, 104-17.
- SUGIURA, K. & EPPIG, J. J. 2005. Society for Reproductive Biology Founders' Lecture 2005. Control of metabolic cooperativity between oocytes and their companion granulosa cells by mouse oocytes. *Reprod Fertil Dev*, 17, 667-74.
- SUGIURA, K., PENDOLA, F. L. & EPPIG, J. J. 2005. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev Biol*, 279, 20-30.
- SUH, N., BAEHNER, L., MOLTZAHN, F., MELTON, C., SHENOY, A., CHEN, J. & BLELLOCH, R. 2010. MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr Biol*, 20, 271-7.
- SUI, H. S., LIU, Y., MIAO, D. Q., YUAN, J. H., QIAO, T. W., LUO, M. J. & TAN, J. H. 2005. Configurations of germinal vesicle (GV) chromatin in the goat differ from those of other species. *Mol Reprod Dev*, 71, 227-36.
- SUN, L., CHAI, Y., HANNIGAN, R., BHOGARAJU, V. K. & MACHACA, K. 2007. Zinc regulates the ability of Cdc25C to activate MPF/cdk1. *J Cell Physiol*, 213, 98-104.
- SUN, L. & MACHACA, K. 2004. Ca²⁺(cyt) negatively regulates the initiation of oocyte maturation. *J Cell Biol*, 165, 63-75.
- SUN, Q. Y., MIAO, Y. L. & SCHATTEN, H. 2009a. Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. *Cell Cycle*, 8, 2741-7.
- SUN, Q. Y. & NAGAI, T. 2003. Molecular mechanisms underlying pig oocyte maturation and fertilization. *J Reprod Dev*, 49, 347-59.
- SUN, Q. Y. & SCHATTEN, H. 2006. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction*, 131, 193-205.
- SUN, X., LI, Z., YI, Y., DING, W., CHEN, J., ENGELHARDT, J. F. & LENO, G. H. 2009b. Chromatin configurations in the ferret germinal vesicle that reflect developmental competence for in vitro maturation. *Reprod Domest Anim*, 44, 320-5.
- SUO, L., MENG, Q. G., PEI, Y., YAN, C. L., FU, X. W., BUNCH, T. D. & ZHU, S. E. 2010. Changes in acetylation on lysine 12 of histone H4 (acH4K12) of murine oocytes during maternal aging may affect fertilization and subsequent embryo development. *Fertil Steril*, 93, 945-51.
- SUTCLIFFE, A. G., PETERS, C. J., BOWDIN, S., TEMPLE, K., REARDON, W., WILSON, L., CLAYTON-SMITH, J., BRUETON, L. A., BANNISTER, W. & MAHER, E. R. 2006. Assisted reproductive therapies and imprinting disorders--a preliminary British survey. *Hum Reprod*, 21, 1009-11.
- SUTOVSKY, P. & SCHATTEN, G. 1997. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol Reprod*, 56, 1503-12.
- SUTTON-MCDOWALL, M. L., GILCHRIST, R. B. & THOMPSON, J. G. 2004. Cumulus expansion and glucose utilisation by bovine cumulus-oocyte complexes during in vitro maturation: the influence of glucosamine and follicle-stimulating hormone. *Reproduction*, 128, 313-9.
- SUTTON-MCDOWALL, M. L., GILCHRIST, R. B. & THOMPSON, J. G. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction*, 139, 685-95.

- SUZUKI, C., YOSHIOKA, K., SAKATANI, M. & TAKAHASHI, M. 2007. Glutamine and hypotaurine improves intracellular oxidative status and in vitro development of porcine preimplantation embryos. *Zygote*, 15, 317-24.
- SVINGEN, T., LETTING, H., HADRUP, N., HASS, U. & VINGGAARD, A. M. 2015. Selection of reference genes for quantitative RT-PCR (RT-qPCR) analysis of rat tissues under physiological and toxicological conditions. *PeerJ*, 3, e855.
- SWAIN, J. E., BORMANN, C. L., CLARK, S. G., WALTERS, E. M., WHEELER, M. B. & KRISHER, R. L. 2002. Use of energy substrates by various stage preimplantation pig embryos produced in vivo and in vitro. *Reproduction*, 123, 253-60.
- SWAIN, J. E. & POOL, T. B. 2008. ART failure: oocyte contributions to unsuccessful fertilization. *Hum Reprod Update*, 14, 431-46.
- SYED, M., SKONBERG, C. & HANSEN, S. H. 2013. Effect of some organic solvents on oxidative phosphorylation in rat liver mitochondria: Choice of organic solvents. *Toxicol In Vitro*, 27, 2135-41.
- SZABO, P. E. & MANN, J. R. 1995. Allele-specific expression and total expression levels of imprinted genes during early mouse development: implications for imprinting mechanisms. *Genes Dev*, 9, 3097-108.
- SZOLLOSI, D. 1971. Morphological changes in mouse eggs due to aging in the fallopian tube. *Am J Anat*, 130, 209-25.
- TAIT, S. W. & GREEN, D. R. 2010. Cell survival in tough times: The mitochondrial recovery plan. *Cell Cycle*, 9, 4254-5.
- TAKAHASHI, T., IGARASHI, H., AMITA, M., HARA, S. & KURACHI, H. 2011. Cellular and molecular mechanisms of various types of oocyte aging. *Reprod Med Biol*, 10, 239-249.
- TAKAHASHI, T., IGARASHI, H., AMITA, M., HARA, S., MATSUO, K. & KURACHI, H. 2013. Molecular mechanism of poor embryo development in postovulatory aged oocytes: mini review. *J Obstet Gynaecol Res*, 39, 1431-9.
- TAKEO, S., SATO, D., KIMURA, K., MONJI, Y., KUWAYAMA, T., KAWAHARA-MIKI, R. & IWATA, H. 2014. Resveratrol improves the mitochondrial function and fertilization outcome of bovine oocytes. *J Reprod Dev*, 60, 92-9.
- TAMASSIA, M., NUTTINCK, F., MAY-PANLOUP, P., REYNIER, P., HEYMAN, Y., CHARPIGNY, G., STOJKOVIC, M., HIENDLEDER, S., RENARD, J. P. & CHASTANT-MAILLARD, S. 2004. In vitro embryo production efficiency in cattle and its association with oocyte adenosine triphosphate content, quantity of mitochondrial DNA, and mitochondrial DNA haplogroup. *Biol Reprod*, 71, 697-704.
- TARAZONA, A. M., RODRIGUEZ, J. I., RESTREPO, L. F. & OLIVERA-ANGEL, M. 2006. Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. *Reprod Domest Anim*, 41, 5-11.
- TARIN, J. J. 1996. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol Hum Reprod*, 2, 717-24.
- TARIN, J. J., PEREZ-ALBALA, S., AGUILAR, A., MINARRO, J., HERMENEGILDO, C. & CANO, A. 1999. Long-term effects of postovulatory aging of mouse oocytes on offspring: a two-generational study. *Biol Reprod*, 61, 1347-55.
- TARIN, J. J., PEREZ-ALBALA, S. & CANO, A. 2000. Consequences on offspring of abnormal function in ageing gametes. *Hum Reprod Update*, 6, 532-49.
- TARIN, J. J., PEREZ-ALBALA, S. & CANO, A. 2001. Cellular and morphological traits of oocytes retrieved from aging mice after exogenous ovarian stimulation. *Biol Reprod*, 65, 141-50.
- TARTIA, A. P., RUDRARAJU, N., RICHARDS, T., HAMMER, M. A., TALBOT, P. & BALTZ, J. M. 2009. Cell volume regulation is initiated in mouse oocytes after ovulation. *Development*, 136, 2247-54.
- TASHIRO, F., KANAI-AZUMA, M., MIYAZAKI, S., KATO, M., TANAKA, T., TOYODA, S., YAMATO, E., KAWAKAMI, H., MIYAZAKI, T. & MIYAZAKI, J. 2010. Maternal-effect gene *Ces5/Ooep/Moep19/Floped* is essential for oocyte cytoplasmic lattice formation and

- embryonic development at the maternal-zygotic stage transition. *Genes Cells*, 15, 813-28.
- TATONE, C., AMICARELLI, F., CARBONE, M. C., MONTELEONE, P., CASERTA, D., MARCI, R., ARTINI, P. G., PIOMBONI, P. & FOCARELLI, R. 2008. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update*, 14, 131-42.
- TAYLOR, J., MOORE, H., BEAUJEAN, N., GARDNER, J., WILMUT, I., MEEHAN, R. & YOUNG, L. 2009. Cloning and expression of sheep DNA methyltransferase 1 and its development-specific isoform. *Mol Reprod Dev*, 76, 501-13.
- TE VELDE, E. R. & PEARSON, P. L. 2002. The variability of female reproductive ageing. *Hum Reprod Update*, 8, 141-54.
- TEE, W. W., PARDO, M., THEUNISSEN, T. W., YU, L., CHOUDHARY, J. S., HAJKOVA, P. & SURANI, M. A. 2010. Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. *Genes Dev*, 24, 2772-7.
- TEJADA, M. I., GARCIA-ALEGRIA, E., BILBAO, A., MARTINEZ-BOUZAS, C., BERISTAIN, E., POCH, M., RAMOS-ARROYO, M. A., LOPEZ, B., FERNANDEZ CARVAJAL, I., RIBATE, M. P. & RAMOS, F. 2008. Analysis of the molecular parameters that could predict the risk of manifesting premature ovarian failure in female premutation carriers of fragile X syndrome. *Menopause*, 15, 945-9.
- TELFORD, N. A., WATSON, A. J. & SCHULTZ, G. A. 1990. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev*, 26, 90-100.
- TESARIK, J., KOPECNY, V., PLACHOT, M. & MANDELBAUM, J. 1986. Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing in vitro. *J Reprod Fertil*, 78, 463-70.
- TETSUKA, M., WHITELAW, P. F., BREMNER, W. J., MILLAR, M. R., SMYTH, C. D. & HILLIER, S. G. 1995. Developmental regulation of androgen receptor in rat ovary. *J Endocrinol*, 145, 535-43.
- THATCHER, T. H. & GOROVSKY, M. A. 1994. Phylogenetic analysis of the core histones H2A, H2B, H3, and H4. *Nucleic Acids Res*, 22, 174-9.
- THELIE, A., PAPIILLIER, P., PERREAU, C., UZBEKOVA, S., HENNEQUET-ANTIER, C. & DALBIESTRAN, R. 2009. Regulation of bovine oocyte-specific transcripts during in vitro oocyte maturation and after maternal-embryonic transition analyzed using a transcriptomic approach. *Mol Reprod Dev*, 76, 773-82.
- THOMAS, R. E., ARMSTRONG, D. T. & GILCHRIST, R. B. 2004a. Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3',5'-monophosphate levels. *Biol Reprod*, 70, 548-56.
- THOMAS, R. E., THOMPSON, J. G., ARMSTRONG, D. T. & GILCHRIST, R. B. 2004b. Effect of specific phosphodiesterase isoenzyme inhibitors during in vitro maturation of bovine oocytes on meiotic and developmental capacity. *Biol Reprod*, 71, 1142-9.
- THOMPSON, J. G. 1997. Comparison between in vivo-derived and in vitro-produced pre-elongation embryos from domestic ruminants. *Reprod Fertil Dev*, 9, 341-54.
- THOMPSON, J. G., MCNAUGHTON, C., GASPARRINI, B., MCGOWAN, L. T. & TERVIT, H. R. 2000. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. *J Reprod Fertil*, 118, 47-55.
- THOMPSON, J. G., PARTRIDGE, R. J., HOUGHTON, F. D., COX, C. I. & LEESE, H. J. 1996. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil*, 106, 299-306.
- THOMPSON, J. G., SHERMAN, A. N., ALLEN, N. W., MCGOWAN, L. T. & TERVIT, H. R. 1998. Total protein content and protein synthesis within pre-elongation stage bovine embryos. *Mol Reprod Dev*, 50, 139-45.
- THOMPSON, K. A., LAPOLT, P. S., RIVER, J., HENDERSON, G., DAHL, K. D. & MELDRUM, D. R. 1995. Gonadotropin requirements of the developing follicle. *Fertil Steril*, 63, 273-6.

- THOUAS, G. A., TROUNSON, A. O., WOLVETANG, E. J. & JONES, G. M. 2004. Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest in vitro. *Biol Reprod*, 71, 1936-42.
- THUNDATHIL, J., FILION, F. & SMITH, L. C. 2005. Molecular control of mitochondrial function in preimplantation mouse embryos. *Mol Reprod Dev*, 71, 405-13.
- TIAN, X., PASCAL, G. & MONGET, P. 2009. Evolution and functional divergence of NLRP genes in mammalian reproductive systems. *BMC Evol Biol*, 9, 202.
- TILLY, J. L. 2001. Commuting the death sentence: how oocytes strive to survive. *Nat Rev Mol Cell Biol*, 2, 838-48.
- TILLY, J. L. & SINCLAIR, D. A. 2013. Germline energetics, aging, and female infertility. *Cell Metab*, 17, 838-50.
- TOMEK, W., TORNER, H. & KANITZ, W. 2002. Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. *Reprod Domest Anim*, 37, 86-91.
- TOMITA, M. & KAMI, K. 2012. Cancer. Systems biology, metabolomics, and cancer metabolism. *Science*, 336, 990-1.
- TOMMERUP, N. & LEFFERS, H. 1996. Assignment of the human genes encoding 14,3-3 Eta (YWHAH) to 22q12, 14-3-3 zeta (YWHAZ) to 2p25.1-p25.2, and 14-3-3 beta (YWHA B) to 20q13.1 by in situ hybridization. *Genomics*, 33, 149-50.
- TONG, Z. B., GOLD, L., DE POL, A., VANEVSKI, K., DORWARD, H., SENA, P., PALUMBO, C., BONDY, C. A. & NELSON, L. M. 2004. Developmental expression and subcellular localization of mouse MATER, an oocyte-specific protein essential for early development. *Endocrinology*, 145, 1427-34.
- TONG, Z. B., GOLD, L., PFEIFER, K. E., DORWARD, H., LEE, E., BONDY, C. A., DEAN, J. & NELSON, L. M. 2000. Mater, a maternal effect gene required for early embryonic development in mice. *Nat Genet*, 26, 267-8.
- TORNER, H., GHANEM, N., AMBROS, C., HOLKER, M., TOMEK, W., PHATSARA, C., ALM, H., SIRARD, M. A., KANITZ, W., SCHELLANDER, K. & TESFAYE, D. 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction*, 135, 197-212.
- TRAPPHOFF, T., HEILIGENTAG, M., DANKERT, D., DEMOND, H., DEUTSCH, D., FROHLICH, T., ARNOLD, G. J., GRUMMER, R., HORSTHEMKE, B. & EICHENLAUB-RITTER, U. 2016. Postovulatory aging affects dynamics of mRNA, expression and localization of maternal effect proteins, spindle integrity and pericentromeric proteins in mouse oocytes. *Hum Reprod*, 31, 133-49.
- TREFF, N. R., KRISHER, R. L., TAO, X., GARNSEY, H., BOHRER, C., SILVA, E., LANDIS, J., TAYLOR, D., SCOTT, R. T., WOODRUFF, T. K. & DUNCAN, F. E. 2016. Next Generation Sequencing-Based Comprehensive Chromosome Screening in Mouse Polar Bodies, Oocytes, and Embryos. *Biol Reprod*, 94, 76.
- TREMBLAY, K. D., SAAM, J. R., INGRAM, R. S., TILGHMAN, S. M. & BARTOLOMEI, M. S. 1995. A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. *Nat Genet*, 9, 407-13.
- TRIPATHI, A., KUMAR, K. V. & CHAUBE, S. K. 2010. Meiotic cell cycle arrest in mammalian oocytes. *J Cell Physiol*, 223, 592-600.
- TROMBLY, D. J., WOODRUFF, T. K. & MAYO, K. E. 2009. Roles for transforming growth factor beta superfamily proteins in early folliculogenesis. *Semin Reprod Med*, 27, 14-23.
- TSUTSUMI, M., FUJIWARA, R., NISHIZAWA, H., ITO, M., KOGO, H., INAGAKI, H., OHYE, T., KATO, T., FUJII, T. & KURAHASHI, H. 2014. Age-related decrease of meiotic cohesins in human oocytes. *PLoS One*, 9, e96710.
- TSUTSUMI, O., SATOH, K., TAKETANI, Y. & KATO, T. 1992. Determination of enzyme activities of energy metabolism in the maturing rat oocyte. *Mol Reprod Dev*, 33, 333-7.

- TSUZUKI, K., YAMASHITA, K., IZUMISAWA, Y. & KOTANI, T. 2008. Microstructure and glycosaminoglycan ratio of canine cornea after reconstructive transplantation with glycerin-preserved porcine amniotic membranes. *Vet Ophthalmol*, 11, 222-7.
- TUBMAN, M., MAJUMDAR, S. R., LEE, D., FRIESEN, C. & KLASSEN, T. P. 2005. Best practices for safe handling of products containing concentrated potassium. *BMJ*, 331, 274-7.
- TUNSTER, S. J., MCNAMARA, G. I., CREETH, H. D. J. & JOHN, R. M. 2016. Increased dosage of the imprinted *Ascl2* gene restrains two key endocrine lineages of the mouse Placenta. *Dev Biol*, 418, 55-65.
- URA, H., NASHIRO, K., KIKUCHI, K., IMAKADO, S., MATSUKAWA, A. & FURUE, M. 1997. Significance of mitotic cells or clumping cells in p53 immunopositivity of Bowen's disease. *Dermatology*, 194, 87-8.
- URNER, F. & SAKKAS, D. 1999a. Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte. *Biol Reprod*, 60, 973-8.
- URNER, F. & SAKKAS, D. 1999b. A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. *Biol Reprod*, 60, 733-9.
- UYSAL, F., OZTURK, S. & AKKOYUNLU, G. 2017. Superovulation alters DNA methyltransferase protein expression in mouse oocytes and early embryos. *J Assist Reprod Genet*.
- UZBEKOVA, S., SALHAB, M., PERREAU, C., MERMILLOD, P. & DUPONT, J. 2009. Glycogen synthase kinase 3B in bovine oocytes and granulosa cells: possible involvement in meiosis during in vitro maturation. *Reproduction*, 138, 235-46.
- VACCARI, S., HORNER, K., MEHLMANN, L. M. & CONTI, M. 2008. Generation of mouse oocytes defective in cAMP synthesis and degradation: endogenous cyclic AMP is essential for meiotic arrest. *Dev Biol*, 316, 124-34.
- VAN BLERKOM, J. 1991. Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proc Natl Acad Sci U S A*, 88, 5031-5.
- VAN BLERKOM, J. 2000. Intrafollicular influences on human oocyte developmental competence: perifollicular vascularity, oocyte metabolism and mitochondrial function. *Hum Reprod*, 15 Suppl 2, 173-88.
- VAN BLERKOM, J. 2009. Mitochondria in early mammalian development. *Semin Cell Dev Biol*, 20, 354-64.
- VAN BLERKOM, J. 2011. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. *Mitochondrion*, 11, 797-813.
- VAN BLERKOM, J. & DAVIS, P. 2006. High-polarized ($\Delta\Psi_m$ (HIGH)) mitochondria are spatially polarized in human oocytes and early embryos in stable subplasmalemmal domains: developmental significance and the concept of vanguard mitochondria. *Reprod Biomed Online*, 13, 246-54.
- VAN BLERKOM, J., DAVIS, P. & ALEXANDER, S. 2000. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. *Hum Reprod*, 15, 2621-33.
- VAN BLERKOM, J., DAVIS, P. W. & LEE, J. 1995. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod*, 10, 415-24.
- VAN DEN HURK, R. & ZHAO, J. 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology*, 63, 1717-51.
- VAN SOOM, A., YSEBAERT, M. T. & DE KRUIF, A. 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophoctoderm in in vitro-produced bovine embryos. *Mol Reprod Dev*, 47, 47-56.
- VAN WISSEN, B., BOMSEL-HELMREICH, O., DEBEY, P., EISENBERG, C., VAUTIER, D. & PENNEHOAT, G. 1991. Fertilization and ageing processes in non-divided human

- oocytes after GnRHa treatment: an analysis of individual oocytes. *Hum Reprod*, 6, 879-84.
- VANDESOMPELE, J., DE PAEPE, A. & SPELEMAN, F. 2002. Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR. *Anal Biochem*, 303, 95-8.
- VANHOUTTE, L., NOGUEIRA, D. & DE SUTTER, P. 2009. Prematuration of human denuded oocytes in a three-dimensional co-culture system: effects on meiosis progression and developmental competence. *Hum Reprod*, 24, 658-69.
- VANHOUTTE, L., NOGUEIRA, D., GERRIS, J., DHONT, M. & DE SUTTER, P. 2008. Effect of temporary nuclear arrest by phosphodiesterase 3-inhibitor on morphological and functional aspects of in vitro matured mouse oocytes. *Mol Reprod Dev*, 75, 1021-30.
- VARANI, S., ELVIN, J. A., YAN, C., DEMAYO, J., DEMAYO, F. J., HORTON, H. F., BYRNE, M. C. & MATZUK, M. M. 2002. Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Mol Endocrinol*, 16, 1154-67.
- VASKIVUO, T. E., OTTANDER, U., ODUWOLE, O., ISOMAA, V., VIHKO, P., OLOFSSON, J. I. & TAPANAINEN, J. S. 2002. Role of apoptosis, apoptosis-related factors and 17beta-hydroxysteroid dehydrogenases in human corpus luteum regression. *Mol Cell Endocrinol*, 194, 191-200.
- VENDOLA, K. A., ZHOU, J., ADESANYA, O. O., WEIL, S. J. & BONDY, C. A. 1998. Androgens stimulate early stages of follicular growth in the primate ovary. *J Clin Invest*, 101, 2622-9.
- VERONA, R. I., MANN, M. R. & BARTOLOMEI, M. S. 2003. Genomic imprinting: intricacies of epigenetic regulation in clusters. *Annu Rev Cell Dev Biol*, 19, 237-59.
- VERROTTI, A. C., THOMPSON, S. R., WREDEN, C., STRICKLAND, S. & WICKENS, M. 1996. Evolutionary conservation of sequence elements controlling cytoplasmic polyadenylation. *Proc Natl Acad Sci U S A*, 93, 9027-32.
- VIRANT-KLUN, I., KNEZ, K., TOMAZEVIC, T. & SKUTELLA, T. 2013. Gene expression profiling of human oocytes developed and matured in vivo or in vitro. *Biomed Res Int*, 2013, 879489.
- VOET, D. & VOET, J. G. 2011 *Biochemistry* (4th ed.). John Wiley & Sons.
- VOGT, E., KIRSCH-VOLDERS, M., PARRY, J. & EICHENLAUB-RITTER, U. 2008. Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. *Mutat Res*, 651, 14-29.
- WADE, D. P., PUCKEY, L. H., KNIGHT, B. L., ACQUATI, F., MIHALICH, A. & TARAMELLI, R. 1997. Characterization of multiple enhancer regions upstream of the apolipoprotein(a) gene. *J Biol Chem*, 272, 30387-99.
- WAI, T., AO, A., ZHANG, X., CYR, D., DUFORT, D. & SHOUBRIDGE, E. A. 2010. The role of mitochondrial DNA copy number in mammalian fertility. *Biol Reprod*, 83, 52-62.
- WALKER, S. K., LAMPE, R. J. & SEAMARK, R. F. 1989a. Culture of sheep zygotes in synthetic oviduct fluid medium with different concentrations of sodium bicarbonate and HEPES. *Theriogenology*, 32, 797-804.
- WALKER, S. K., SMITH, D. H., ANCELL, P. & SEAMARK, R. F. 1989b. Time of ovulation in the South Australian Merino ewe following synchronization of estrus. 2. Efficacy of GnRH treatment and its relevance to insemination programs utilizing frozen-thawed semen. *Theriogenology*, 31, 555-64.
- WALSH, C., GLASER, A., FUNDELE, R., FERGUSON-SMITH, A., BARTON, S., SURANI, M. A. & OHLSSON, R. 1994. The non-viability of uniparental mouse conceptuses correlates with the loss of the products of imprinted genes. *Mech Dev*, 46, 55-62.
- WAN, H., ZHAO, Z., QIAN, C., SUI, Y., MALIK, A. A. & CHEN, J. 2010. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. *Anal Biochem*, 399, 257-61.

- WANG, D., PASCUAL, J. M., YANG, H., ENGELSTAD, K., MAO, X., CHENG, J., YOO, J., NOEBELS, J. L. & DE VIVO, D. C. 2006. A mouse model for Glut-1 haploinsufficiency. *Hum Mol Genet*, 15, 1169-79.
- WANG, E., MILLER, L. D., OHNMACHT, G. A., LIU, E. T. & MARINCOLA, F. M. 2000. High-fidelity mRNA amplification for gene profiling. *Nat Biotechnol*, 18, 457-9.
- WANG, H. L., SUI, H. S., LIU, Y., MIAO, D. Q., LU, J. H., LIANG, B. & TAN, J. H. 2009a. Dynamic changes of germinal vesicle chromatin configuration and transcriptional activity during maturation of rabbit follicles. *Fertil Steril*, 91, 1589-94.
- WANG, L. M., FENG, H. L., MA, Y., CANG, M., LI, H. J., YAN, Z., ZHOU, P., WEN, J. X., BOU, S. & LIU, D. J. 2009b. Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. *Anim Reprod Sci*, 114, 99-108.
- WANG, Q., CHI, M. M., SCHEDL, T. & MOLEY, K. H. 2012. An intercellular pathway for glucose transport into mouse oocytes. *Am J Physiol Endocrinol Metab*, 302, E1511-8.
- WANG, Q. & MOLEY, K. H. 2010. Maternal diabetes and oocyte quality. *Mitochondrion*, 10, 403-10.
- WANG, Q., RATCHFORD, A. M., CHI, M. M., SCHOELLER, E., FROLOVA, A., SCHEDL, T. & MOLEY, K. H. 2009c. Maternal diabetes causes mitochondrial dysfunction and meiotic defects in murine oocytes. *Mol Endocrinol*, 23, 1603-12.
- WANG, S., HUANG, W., SHI, H., LIN, C., XIE, M. & WANG, J. 2010. Localization and expression of peroxiredoxin II in the mouse ovary, oviduct, uterus, and preimplantation embryo. *Anat Rec (Hoboken)*, 293, 291-7.
- WANG, Y. K., LI, X., SONG, Z. Q. & YANG, C. X. 2017. Methods of RNA preparation affect mRNA abundance quantification of reference genes in pig maturing oocytes. *Reprod Domest Anim*, 52, 722-730.
- WATSON, A. J. 1992. The cell biology of blastocyst development. *Mol Reprod Dev*, 33, 492-504.
- WATSON, A. J., DE SOUSA, P., CAVENEY, A., BARCROFT, L. C., NATALE, D., URQUHART, J. & WESTHUSIN, M. E. 2000. Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. *Biol Reprod*, 62, 355-64.
- WEBB, R. J., BAINS, H., CRUTTWELL, C. & CARROLL, J. 2002. Gap-junctional communication in mouse cumulus-oocyte complexes: implications for the mechanism of meiotic maturation. *Reproduction*, 123, 41-52.
- WEI, L. N., LI, L. L., FANG, C., HUANG, R. & LIANG, X. Y. 2013. Inhibitory effects of controlled ovarian stimulation on the expression of GDF9 and BMP15 in oocytes from women with PCOS. *J Assist Reprod Genet*, 30, 1313-8.
- WHITE, Y. A., WOODS, D. C., TAKAI, Y., ISHIHARA, O., SEKI, H. & TILLY, J. L. 2012. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med*, 18, 413-21.
- WICKRAMASINGHE, D., EBERT, K. M. & ALBERTINI, D. F. 1991. Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev Biol*, 143, 162-72.
- WILCOX, A. J., WEINBERG, C. R. & BAIRD, D. D. 1998. Post-ovulatory ageing of the human oocyte and embryo failure. *Hum Reprod*, 13, 394-7.
- WILDING, M., COPPOLA, G., DALE, B. & DI MATTEO, L. 2009. Mitochondria and human preimplantation embryo development. *Reproduction*, 137, 619-24.
- WILDING, M., DALE, B., MARINO, M., DI MATTEO, L., ALVIGGI, C., PISATURO, M. L., LOMBARDI, L. & DE PLACIDO, G. 2001. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod*, 16, 909-17.
- WILDING, M., DI MATTEO, L. & DALE, B. 2005. The maternal age effect: a hypothesis based on oxidative phosphorylation. *Zygote*, 13, 317-23.

- WILDING, M., FIORENTINO, A., DE SIMONE, M. L., INFANTE, V., DE MATTEO, L., MARINO, M. & DALE, B. 2002. Energy substrates, mitochondrial membrane potential and human preimplantation embryo division. *Reprod Biomed Online*, 5, 39-42.
- WILL, M. A., CLARK, N. A. & SWAIN, J. E. 2011. Biological pH buffers in IVF: help or hindrance to success. *J Assist Reprod Genet*, 28, 711-24.
- WILLIAMS, S. A., BLACHE, D., MARTIN, G. B., FOOT, R., BLACKBERRY, M. A. & SCARAMUZZI, R. J. 2001. Effect of nutritional supplementation on quantities of glucose transporters 1 and 4 in sheep granulosa and theca cells. *Reproduction*, 122, 947-56.
- WIMSATT, W. A. 1975. Some comparative aspects of implantation. *Biol Reprod*, 12, 1-40.
- WIRTU, G., BAILEY, T. L., CHAUHAN, M. S., PARKER, N. A., DASCANIO, J. J., GWAZDAUSKAS, F. C. & LEY, W. B. 2004. Xenogenous fertilization of equine oocytes following recovery from slaughterhouse ovaries and in vitro maturation. *Theriogenology*, 61, 381-91.
- WITTWER, C. T. & GARLING, D. J. 1991. Rapid cycle DNA amplification: time and temperature optimization. *Biotechniques*, 10, 76-83.
- WITTWER, C. T., RIRIE, K. M., ANDREW, R. V., DAVID, D. A., GUNDRY, R. A. & BALIS, U. J. 1997. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques*, 22, 176-81.
- WOLFFE, A. P. & HAYES, J. J. 1999. Chromatin disruption and modification. *Nucleic Acids Res*, 27, 711-20.
- WOODS, D. C. & TILLY, J. L. 2013. Isolation, characterization and propagation of mitotically active germ cells from adult mouse and human ovaries. *Nat Protoc*, 8, 966-88.
- WRENZYCKI, C., HERRMANN, D., KESKINTEPE, L., MARTINS, A., JR., SIRISATHIEN, S., BRACKETT, B. & NIEMANN, H. 2001. Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Hum Reprod*, 16, 893-901.
- WRIGHT, R. J., HOLLY, J. M., GALEA, R., BRINCAT, M. & MASON, H. D. 2002. Insulin-like growth factor (IGF)-independent effects of IGF binding protein-4 on human granulosa cell steroidogenesis. *Biol Reprod*, 67, 776-81.
- WU, B., IGNOTZ, G., CURRIE, W. B. & YANG, X. 1997. Dynamics of maturation-promoting factor and its constituent proteins during in vitro maturation of bovine oocytes. *Biol Reprod*, 56, 253-9.
- WU, G. 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids*, 37, 1-17.
- WU, L., SHEN, F., LIN, L., ZHANG, X., BRUCE, I. C. & XIA, Q. 2006. The neuroprotection conferred by activating the mitochondrial ATP-sensitive K⁺ channel is mediated by inhibiting the mitochondrial permeability transition pore. *Neurosci Lett*, 402, 184-9.
- WU, X., VIVEIROS, M. M., EPPIG, J. J., BAI, Y., FITZPATRICK, S. L. & MATZUK, M. M. 2003. Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat Genet*, 33, 187-91.
- WU, Y., QI, S. B., WU, F. F., ZHANG, X. C., LI, M., WU, J. & CHAN, A. S. 2011. Synthesis of beta-amino acid derivatives via copper-catalyzed asymmetric 1,4-reduction of beta-(acylamino)acrylates. *Org Lett*, 13, 1754-7.
- WYNN, P., PICTON, H. M., KRAPEZ, J. A., RUTHERFORD, A. J., BALEN, A. H. & GOSDEN, R. G. 1998. Pretreatment with follicle stimulating hormone promotes the numbers of human oocytes reaching metaphase II by in-vitro maturation. *Hum Reprod*, 13, 3132-8.
- XIE, H. L., WANG, Y. B., JIAO, G. Z., KONG, D. L., LI, Q., LI, H., ZHENG, L. L. & TAN, J. H. 2016. Effects of glucose metabolism during in vitro maturation on cytoplasmic maturation of mouse oocytes. *Sci Rep*, 6, 20764.
- YADAV, B. R., KATIYAR, P. K., CHAUHAN, M. S. & MADAN, M. L. 1997. Chromosome configuration during in vitro maturation of goat, sheep and buffalo oocytes. *Theriogenology*, 47, 943-51.
- YAMADA, C., CAETANO, H. V., SIMOES, R., NICACIO, A. C., FEITOSA, W. B., ASSUMPCAO, M. E. & VISINTIN, J. A. 2007. Immature bovine oocyte cryopreservation: comparison of

- different associations with ethylene glycol, glycerol and dimethylsulfoxide. *Anim Reprod Sci*, 99, 384-8.
- YAN, C., WANG, P., DEMAYO, J., DEMAYO, F. J., ELVIN, J. A., CARINO, C., PRASAD, S. V., SKINNER, S. S., DUNBAR, B. S., DUBE, J. L., CELESTE, A. J. & MATZUK, M. M. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol*, 15, 854-66.
- YAN, J., ZHANG, L., WANG, T., LI, R., LIU, P., YAN, L. & QIAO, J. 2014. Effect of vitrification at the germinal vesicle stage on the global methylation status in mouse oocytes subsequently matured in vitro. *Chin Med J (Engl)*, 127, 4019-24.
- YANG, S., LUO, A., HAO, X., LAI, Z., DING, T., MA, X., MAYINUER, M., SHEN, W., WANG, X., LU, Y., MA, D. & WANG, S. 2011. Peroxiredoxin 2 inhibits granulosa cell apoptosis during follicle atresia through the NFKB pathway in mice. *Biol Reprod*, 84, 1182-9.
- YANIV, K. & YISRAELI, J. K. 2002. The involvement of a conserved family of RNA binding proteins in embryonic development and carcinogenesis. *Gene*, 287, 49-54.
- YAO, H. B., SHAW, P. C., WONG, C. C. & WAN, D. C. 2002. Expression of glycogen synthase kinase-3 isoforms in mouse tissues and their transcription in the brain. *J Chem Neuroanat*, 23, 291-7.
- YASEEN, M. A., WRENZYCKI, C., HERRMANN, D., CARNWATH, J. W. & NIEMANN, H. 2001. Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different in vitro systems. *Reproduction*, 122, 601-10.
- YEO, C. X., GILCHRIST, R. B., THOMPSON, J. G. & LANE, M. 2008. Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice. *Hum Reprod*, 23, 67-73.
- YOSHIDA, Y., MIYAMURA, M., HAMANO, S. & YOSHIDA, M. 1998. Expression of growth factor ligand and their receptor mRNAs in bovine ova during in vitro maturation and after fertilization in vitro. *J Vet Med Sci*, 60, 549-54.
- YOSHIMURA, T., TOYODA, S., KURAMOCHI-MIYAGAWA, S., MIYAZAKI, T., MIYAZAKI, S., TASHIRO, F., YAMATO, E., NAKANO, T. & MIYAZAKI, J. 2009. Gtsf1/Cue110, a gene encoding a protein with two copies of a CHHC Zn-finger motif, is involved in spermatogenesis and retrotransposon suppression in murine testes. *Dev Biol*, 335, 216-27.
- YOU, J., LEE, J., HYUN, S. H. & LEE, E. 2012. L-carnitine treatment during oocyte maturation improves in vitro development of cloned pig embryos by influencing intracellular glutathione synthesis and embryonic gene expression. *Theriogenology*, 78, 235-43.
- YOUNG, J. M. & MCNEILLY, A. S. 2010. Theca: the forgotten cell of the ovarian follicle. *Reproduction*, 140, 489-504.
- YOUSAF, M. R. & CHOCHAN, K. R. 2003. Nuclear morphology, diameter and meiotic competence of buffalo oocytes relative to follicle size. *Reprod Fertil Dev*, 15, 223-9.
- YU, Y., DUMOLLARD, R., ROSSBACH, A., LAI, F. A. & SWANN, K. 2010. Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. *J Cell Physiol*, 224, 672-80.
- YUAN, Y., IDA, J. M., PACZKOWSKI, M. & KRISHER, R. L. 2011. Identification of developmental competence-related genes in mature porcine oocytes. *Mol Reprod Dev*, 78, 565-75.
- YUE, G. H., ZHU, Z. Y., WANG, C. M. & XIA, J. H. 2009. A simple and efficient method for isolating polymorphic microsatellites from cDNA. *BMC Genomics*, 10, 125.
- YUE, M. X., FU, X. W., ZHOU, G. B., HOU, Y. P., DU, M., WANG, L. & ZHU, S. E. 2012. Abnormal DNA methylation in oocytes could be associated with a decrease in reproductive potential in old mice. *J Assist Reprod Genet*, 29, 643-50.
- ZANDER-FOX, D., CASHMAN, K. S. & LANE, M. 2013. The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development. *J Assist Reprod Genet*, 30, 107-16.

- ZAROS, L. G., COUTINHO, L. L., SIDER, L. H., DE MEDEIROS, H. R., NEVES, M. R., BENVENUTI, C. L., DO CARMO NAVARRO, A. M. & DA SILVA VIEIRA, L. 2010. Evaluation of reference genes for real-time PCR studies of Brazilian Somalis sheep infected by gastrointestinal nematodes. *Genet Mol Biol*, 33, 486-90.
- ZENG, H. T., REN, Z., GUZMAN, L., WANG, X., SUTTON-MCDOWALL, M. L., RITTER, L. J., DE VOS, M., SMITZ, J., THOMPSON, J. G. & GILCHRIST, R. B. 2013. Heparin and cAMP modulators interact during pre-in vitro maturation to affect mouse and human oocyte meiosis and developmental competence. *Hum Reprod*, 28, 1536-45.
- ZENG, H. T., REN, Z., YEUNG, W. S., SHU, Y. M., XU, Y. W., ZHUANG, G. L. & LIANG, X. Y. 2007. Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in in vitro matured human oocytes. *Hum Reprod*, 22, 1681-6.
- ZENG, H. T., RICHANI, D., SUTTON-MCDOWALL, M. L., REN, Z., SMITZ, J. E., STOKES, Y., GILCHRIST, R. B. & THOMPSON, J. G. 2014. Prematuration with cyclic adenosine monophosphate modulators alters cumulus cell and oocyte metabolism and enhances developmental competence of in vitro-matured mouse oocytes. *Biol Reprod*, 91, 47.
- ZENG, H. T., YEUNG, W. S., CHEUNG, M. P., HO, P. C., LEE, C. K., ZHUANG, G. L., LIANG, X. Y. & O, W. S. 2009. In vitro-matured rat oocytes have low mitochondrial deoxyribonucleic acid and adenosine triphosphate contents and have abnormal mitochondrial redistribution. *Fertil Steril*, 91, 900-7.
- ZHANG, K., RAJPUT, S. K., LEE, K. B., WANG, D., HUANG, J., FOLGER, J. K., KNOTT, J. G., ZHANG, J. & SMITH, G. W. 2015. Evidence supporting a role for SMAD2/3 in bovine early embryonic development: potential implications for embryotropic actions of follistatin. *Biol Reprod*, 93, 86.
- ZHANG, M., SU, Y. Q., SUGIURA, K., WIGGLESWORTH, K., XIA, G. & EPPIG, J. J. 2011a. Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro. *Endocrinology*, 152, 4377-85.
- ZHANG, M., SU, Y. Q., SUGIURA, K., XIA, G. & EPPIG, J. J. 2010. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*, 330, 366-9.
- ZHANG, W., LIU, Y., AN, Z., HUANG, D., QI, Y. & ZHANG, Y. 2011b. Mediating effect of ROS on mtDNA damage and low ATP content induced by arsenic trioxide in mouse oocytes. *Toxicol In Vitro*, 25, 979-84.
- ZHANG, X., TAKANO, T. & LIU, S. 2006. Identification of a mitochondrial ATP synthase small subunit gene (RMtATP6) expressed in response to salts and osmotic stresses in rice (*Oryza sativa* L.). *J Exp Bot*, 57, 193-200.
- ZHENG, P. & DEAN, J. 2009. Role of Filia, a maternal effect gene, in maintaining euploidy during cleavage-stage mouse embryogenesis. *Proc Natl Acad Sci U S A*, 106, 7473-8.
- ZHENG, P., VASSENA, R. & LATHAM, K. E. 2007. Effects of in vitro oocyte maturation and embryo culture on the expression of glucose transporters, glucose metabolism and insulin signaling genes in rhesus monkey oocytes and preimplantation embryos. *Mol Hum Reprod*, 13, 361-71.
- ZHOU, J. & BONDY, C. 1993. Anatomy of the human ovarian insulin-like growth factor system. *Biol Reprod*, 48, 467-82.
- ZHOU, J., CHIN, E. & BONDY, C. 1991. Cellular pattern of insulin-like growth factor-I (IGF-I) and IGF-I receptor gene expression in the developing and mature ovarian follicle. *Endocrinology*, 129, 3281-8.
- ZHOU, J., KUMAR, T. R., MATZUK, M. M. & BONDY, C. 1997. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Mol Endocrinol*, 11, 1924-33.
- ZHU, K., YAN, L., ZHANG, X., LU, X., WANG, T., YAN, J., LIU, X., QIAO, J. & LI, L. 2015. Identification of a human subcortical maternal complex. *Mol Hum Reprod*, 21, 320-9.
- ZIELKE, H. R., OZAND, P. T., TILDON, J. T., SEVDALIAN, D. A. & CORNBATH, M. 1976. Growth of human diploid fibroblasts in the absence of glucose utilization. *Proc Natl Acad Sci U S A*, 73, 4110-4.

- ZIPPER, H., BRUNNER, H., BERNHAGEN, J. & VITZTHUM, F. 2004. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res*, 32, e103.
- ZUCCOTTI, M., GIORGI ROSSI, P., MARTINEZ, A., GARAGNA, S., FORABOSCO, A. & REDI, C. A. 1998. Meiotic and developmental competence of mouse antral oocytes. *Biol Reprod*, 58, 700-4.
- ZUCCOTTI, M., PICCINELLI, A., GIORGI ROSSI, P., GARAGNA, S. & REDI, C. A. 1995. Chromatin organization during mouse oocyte growth. *Mol Reprod Dev*, 41, 479-85.
- ZUCCOTTI, M., PONCE, R. H., BOIANI, M., GUIZZARDI, S., GOVONI, P., SCANDROGLIO, R., GARAGNA, S. & REDI, C. A. 2002. The analysis of chromatin organisation allows selection of mouse antral oocytes competent for development to blastocyst. *Zygote*, 10, 73-8.
- ZUELKE, K. A. & BRACKETT, B. G. 1993. Increased glutamine metabolism in bovine cumulus cell-enclosed and denuded oocytes after in vitro maturation with luteinizing hormone. *Biol Reprod*, 48, 815-20.

APPENDICES

APPENDIX I: SUPPLIERS ADDRESSES

Advanced Instruments Inc., Two Technology Way, Norwood MA 02062, USA.

Affymetrix UK Ltd., Voyager, Mercury Park, Wycombe Lane, Wooburn Green, High Wycombe HP10 0HH, UK.

Agilent Technologies UK Ltd., Lakeside, Cheadle Royal Business Park, Stockport SK8 3GR, UK.

Alpha Laboratories, 40 Parham Drive, Eastleigh, Hampshire SO50 4NU, UK.

Applied Biosystem Ltd., Lingley House, 120 Birchwood Boulevard, Warrington WA3 7QH, UK.

Becton Dickinson, Danby Building, Edmund Halley Road, Oxford Science Park, Oxford OX4 4DQ, UK.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maxted Road, Hemel Hempstead HP2 7DX, UK.

BioGene Ltd., BioGene House, 6 Business Centre, Harvard Way, Kimbolton, Cambs PE28 0NJ, UK.

Bioline Reagents Ltd., Unit 16 Edge Business Centre, Humber Road, London NW2 6EW, UK.

Bostik Ltd., Ulverscroft Road, Leicester LE4 6BW, UK.

Carl Zeiss Ltd., 509 Coldhams Lane, Cambridge CB1 3JS, UK.

Chromacol Ltd., 3 Mundells Industrial Centre, Welwyn Garden City, Herts AL7 1EW, UK.

Clontech Laboratories, Inc., 1290 Terra Bella Ave., Mountain View, CA 94043, USA.

Drummond Scientific Co., 500 Parkway, Box 700, Broomall PA 19008, USA.

ELGA Process Water, Marlow International, Parkway, Marlow SL7 1YL, UK.

Eppendorf UK Ltd., Endurance House, Vision Park, ChiversWay, Histon, Cambridge CB24 9ZR, UK.

Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK.

GE Healthcare Life Sciences, Amersham Place, Little Chalfont HP7 9NA, UK.

Genus Breeding Ltd., Alpha Building, London Road, Nantwich, Cheshire CW5 7JW, UK.

Gilson UK, 20 Charles Street, Luton LU2 0EB, UK.

Grant Instruments Ltd., Shepreth, Cambridgeshire SG8 6GB, UK.

GraphPad Software Inc., La Jolla, CA, USA.

Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK.

John Penny and sons Abattoir, 40 Leeds Road, Rawdon, Leeds LS19 6NU, UK.

K-Systems®-Kivex Biotec Ltd., Klintehoj Vaenge 3-5, DK-3460 Birkerod, DA.

Leica Microsystems GmbH, Ernst-Leitz-Strasse 17-37, 35578 Wetzlar, DE.

Leica Microsystems Ltd., Davy Avenue Knowlhill, Milton Keynes MK5 8LB, UK.

Leo Laboratories Ltd., Princes Risborough, Bucks HP27 9RR, UK.

Leo Labs, Princes Risborough, Bucks HP27 9RR, UK.

Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK

Medichem International, PO Box 237, Sevenoaks, Kent TN15 0ZJ, UK.

Millipore UK Ltd., Suite 3 & 5 Building 6, Croxley Green Business Park, Watford WD18 8YH, UK.

MTG Medical Technology Vertriebs-GmbH, 9 Dr.Pauling Street, Bruckberg D-84079, DE.
Nanodrop, 3411 Silverside Rd, Bancroft Building, Wilmington, DE 19810, USA.
New Brunswick-Eppendorf UK Ltd., Endurance House, Vision Park, Chivers Way, Cambridge CB24 9ZR, UK.
New England Biolabs UK Ltd., 75/77 Knowl Piece, Wilbury Way, Hitchin, Herts SG4 0TY, UK.
New England Small Tube Corporation, Litchfield Technology Park, 480 Charles Bancroft Highway, Litchfield NH 03052, USA.
Nunclon Kamstrupvej 90, Postbox 280, DK-4000, Roskilde, DA.
Olympus UK Ltd., (Cameras), 2-8 Honduras Street, London EC1Y 0TX, UK.
Olympus UK Ltd., (Microscopes), Great Western Industrial Park, Dean Way, Southall, Middlesex UB2 4SB, UK.
Pall Life Sciences, 5 Harbournate Business Park, Southampton Road, Portsmouth PO6 4BQ, UK.
Phenomenex UK, Queens Avenue, Hurdsfield Ind. Est., Macclesfield, Cheshire SK10 2BN, UK.
Philip Harris - Findel Education Ltd., 2 Gregory Street, Hyde SK14 4TH, UK.
Photon Technology International, M1, Rudford Estate, Ford Road, West Sussex BN18 0BF, UK.
Planer Plc, 110 Windmill Road, Sunbury-On-Thames, Middlesex TW16 7HD, UK.
Qiagen Ltd., Skelton House, Lloyd Street North, Manchester M15 6SH, UK.
Real Time Primers, LLC, 7304 Mountain Ave, Elkins Park, PA 19027, USA.
Research Instruments Ltd., Bickland Industrial Park, Falmouth, Cornwall TR11 4TA, UK.
Roche Diagnostics Ltd., Charles Avenue, Burgess Hill, West Sussex RH15 9RY, UK.
Scientific Laboratory Supplies Ltd., Wilford Industrial Estate, Ruddington Lane, Nottingham NG11 7EP, UK.
SciQuip Ltd., 2 & 3 Merrington Hall Farm, Merrington, Shrewsbury, Shropshire SY4 3QJ, UK.
Sigma-Aldrich Co. Ltd., The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT, UK.
SPSS Inc., Chicago, IL, USA.
Starlab Ltd., Unit 4 Tanners Drive, Blakelands, Milton Keynes MK14 5NA, UK.
Swann Morton Ltd., Owlerton Green, Sheffield S6 2BJ, UK.
Takara Bio Europe/Clontech, 2 Av. du President Kennedy, Saint Germain en Laye 78100, FR.
Terumo UK Ltd., Tamesis 1, The Causeway, Egham, Surrey TW20 9AW, UK.
Thermo Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough LE11 5RG, UK.
Tokai Hit CO.Ltd., 306-1, Gendoji-cho,Fujinomiya-shi, Shizuoka-ken 418-0074, JP.
VWR International Ltd., (BDH), Merck House, Poole, Dorset BH15 1TD, UK.

APPENDIX II: PREPARATION STOCKS FOR CULTURE

Sodium Pyruvate Stock

To make up the stock, 0.0517g of sodium pyruvate was dissolved in 30 ml universal tube containing 10 ml tissue culture grade water. This composition will contribute 47mM sodium pyruvate stock as a final solution. Then the stock was dispensed through a 0.2 µm disposable disc filter (Nalgene) and kept in a sterile, sealable container for further use.

20X Bovine Serum Albumin (BSA) Stock

The 500ml BSA stock was prepared by dissolving 10g BSA (Sigma, A9418, 100g) into a volumetric flask containing 500ml Minimum Essential Medium Eagle HEPES Modification (HEPES-MEM) (Sigma, M7278-500ml). The 20 mg/ml BSA solution was sterilised by filtration through a 0.2 µm disposable disc filter (Nalgene) and then was divided into 25ml aliquots in 30 ml universal containers. The ready to use stock will be kept frozen and stored at -20°C. Thawing is performed whenever it is needed for medium preparation.

Penicillin-Streptomycin Stock

The sterile-filtered, stabilized Penicillin-Streptomycin Solution (Sigma, P4333) contained with 10,000 units' penicillin and 10 mg streptomycin/ml was prepared into multiples of 5ml aliquot in sterile 5 ml universals. The stock will be kept frozen in freezer at -20°C until the use is required.

Sodium Bicarbonate Stock

The 250mM stock of sodium bicarbonate was prepared in 50 ml volumetric flask by mixing 1.05 g and three drops of 0.5% phenol red in PBS (Sigma, P0290) into up to 100 ml of tissue culture grade water. The dissolved solution was filtrated to sterilize and kept in a sterile 60 ml flask at 4°C up to two weeks.

HEPES Stock

The 100ml HEPES stock was prepared in 100 ml volumetric flask by mixing HEPES (>99.5% (w/v) titration free acid; Sigma, H6147), HEPES sodium salt (Sigma, 3784) and three drops of 0.5% phenol red in PBS (Sigma, P0290) into up to 100 ml tissue culture grade water. Then pH was adjusted to 7.4 by adding 1M sodium hydroxide or 5M hydrochloric acid which is appropriate. The final solution was sterilized by filtration through a 0.2 µm disposable disc filter (Nalgene) and the filtrate was kept in a sterile 250 ml universal at 4°C up to two months.

L-Glutamine Stock

The 100 mM L-glutamine stock was prepared by making 1:1 dilution of a 100 ml of 200 mM L-glutamine solution (Sigma, G7513) into 100 mL tissue culture grade water (Sigma, W3500). The mixed-well solution was dispensed into 200 μ l and 1 ml aliquot in sterile microfuge tubes for single use. The stock was stored in -20°C freezer until needed.

Bovine holo-transferrin stock

The 5 mg/ml bovine holo-transferrin was prepared by dissolving/adding 100 mg of transferrin (Sigma, T1283) into 20 ml of prepared α -MEM basic solution-1, which was made up by mixing 0.020 g BSA (Sigma, A6003), 200 μ l of Penicillin/Streptomycin Stock with 20 ml α -MEM (Sigma, M4526). Then the transferrin stock was dispensed into 20 μ l aliquot in sterile microfuge tubes for single use. These were stored in -20°C freezer until needed.

Sodium Selenite Stock

The 50 μ g/ml sodium selenite stock was prepared by dissolving 1 mg of sodium selenite (Sigma, S9133) into 20 ml of prepared α -MEM basic solution-1 as described in Bovine holo-transferrin stock preparation. The stock solution was thoroughly mixed and then dispensed into 20 μ l aliquot in sterile microfuge tubes for single use. These were stored in -20°C freezer until needed.

Bovine Insulin Stock

The 10 mg/ml bovine insulin stock was prepared by adding 10 ml of acidified water, which was made of mixing 750 μ l glacial acetic acid with 24.25 ml tissue culture water, into a 100 μ g vial of bovine insulin (Sigma, I1882). The stock solution was thoroughly mixed, and then dispensed into 10 μ L aliquot in sterile microfuge tubes for single use. These were stored in -20°C freezer until needed. Upon thawing to make 1:1000 dilution, 1 μ l of the stock solution was added into 999 μ L of prepared α -MEM basic solution-1.

R3 IGF1 Stock

The 1 mg/ml stock was prepared by dissolving 100 μ g Long R3 IGF1 (Sigma, I1271) in 100 μ l of acidified water. Then the solution was further diluted with 900 μ l of α -MEM basic-10 in order to make final concentration of 100 μ g/ml. For single use purpose, it was aliquot 10 μ l into microfuge tubes and stored at -20°C freezer until needed.

Ovine FSH stock

The 2 IU/mL FSH stock was prepared by dissolving 50 IU ovine FSH (Sigma, F8174) into 25 ml of α -MEM basic-10. For single use purpose, it was aliquot 20 μ l into microfuge tubes and stored at -20°C freezer until needed.

Ovine LH stock

A 2 IU/ml LH stock was prepared by dissolving 25 IU ovine LH (Sigma, L5269) in 12.5 ml of α -MEM basic-10. For single use purpose, it was aliquot 10 μ l into microfuge tubes and stored at -20°C freezer until needed.

DABA Stock

A 50mM DABA stock was prepared by dissolving 51.56 mg of DABA into 10 mL of tissue culture water and then thoroughly mixed using a sonicator bath. For single use purpose, it was aliquot 20 μ l and 150 μ l into microfuge tubes and stored at -20°C freezer until needed.

Cilostamide stock

A 50 μ M DABA stock was prepared by dissolving 5 mg of cilostamide (Sigma, C7971) into 250 μ l of DMSO. If other concentration needed, diluted stock with certain amount of DMSO immediately before use. Stock solution was kept light off and stored at 4°C up to 6 months.

Hyase solution

A working solution 80IU/ml hyaluronidase stock was prepared by dissolving 12.12 mg hyaluronidase type I-S from bovine testes in 50 ml of H199+ medium. Then the solution was filtrated and dispensed into 1 ml Eppendorf for single use. The stock was stored at -20°C freezer until required.

APPENDIX III: STOCK PREPERATIONS FOR GPL ASSAYS

10X KSOM stock A

The reagents in the following table listed were mixed in a sterile 100ml volumetric flask. For sterilization, the solution was filtered and stored at 4°C for up to 3 months.

Reagent	MW (g/mol)	Amount	Stock Conc (mM)
NaCl	58.44	5.55 g	949.69
KCl	74.55	186 mg	24.95
KH ₂ PO ₄	136.09	48 mg	3.53
MgSO ₄ • 7H ₂ O	246.47	49 mg	2
EDTA Tetrasodium Salt	372.24	4 mg	0.11
Tissue Culture Grade H ₂ O	-	Up to 100 ml	-

10X KSOM stock B

A 250mM sodium bicarbonate (NaHCO₃) stock was prepared by dissolving 1.05 g of NaHCO₃ (MW=84.01g/mol) into tissue culture grade H₂O to make up a total volume of 50ml in a volumetric flask. The solution was thoroughly mixed, and filter-sterilised before stored at 4°C for up to 4 weeks.

100X KSOM stock C

A 20mM sodium pyruvate (C₃H₃NaO₃) stock was prepared by dissolving 220mg of sodium pyruvate (MW=110g/mol) into tissue culture grade H₂O to make up a total volume of 100 ml in a volumetric flask. The solution was thoroughly mixed, filtered-sterilised and stored at 4°C for up to 1 week.

100X KSOM stock D

A 170mM calcium chloride (CaCl₂) stock solution was prepared by dissolving 252mg of CaCl₂ • 2H₂O (MW=147.02g/mol) into 10ml of tissue culture grade H₂O. The solution was mixed, filter-sterilised and stored at 4°C for up to 3 months.

100X KSOM Glucose stocks

A 20mM Glucose stock solution was prepared by dissolving 36mg of D (+) Glucose (MW=180.16g/mol) were mixed into 10ml of tissue culture grade H₂O in a volumetric flask before the solution was filter-sterilised and stored at 4°C for up to 3 months.

EPPS buffer (N-2-hydroxyethyl piperazine-N'-3-propanesulfonic acid)

A 1.26g of minimum 99.5% titration EPPS were dissolved in 75ml in a 100ml glass beaker. The solution was mixed thoroughly by stirring with the aid of a magnetic flea and a magnetic stirrer and adjusted pH to 8.0 by adding 4M NaOH solution. Then volume of the solution was made up to 100ml with tissue culture H₂O in a volumetric flask before being filter-sterilised into a sterile flask and stored at 4°C for up to 6 weeks.

Glycine hydrazine buffer

Hydrazine sulphate powder was weighted and added to a 150ml universal with lid containing 50ml of tissue culture grade H₂O in an extractor hood due to toxicity of hydrazine powder. After that the reagents in the following table were added before the solution was well mixed with the aid of a flea and a magnetic stirrer while me adjusting the pH to 9.4 by using a 4M NaOH solution. In a volumetric flask, the volume of the solution was made up to 100ml with tissue culture grade H₂O before being filter-sterilised into a sterile flask and stored at 4°C for up to 6 weeks.

Reagent	MW (g/mol)	Amount
Tissue Culture Grade H ₂ O	-	50ml
Hydrazine Sulphate	-	5.2g
1M Glycine	75.07	7.5g
5.26 mM EDTA	380.2	0.2g

*Adjust pH to 9.4 by adding 4M NaOH before add more H₂O to 100ml****

4mM NaOH solution

A 4M sodium hydroxide solution was prepared by dissolving 8.0016g of NaOH (MW=40.01g/mol) into a 100ml glass beaker containing 50ml of tissue culture grade H₂O, before thoroughly mixed using a magnetic flea and stirrer. Extra precautions were alert as this can make a very corrosive reaction.

Reagent	Amount (μ l)
Lysis Buffer (Dynabeads mRNA DIRECTTM kit, Life Technologies Ltd.)	910 μ l
RNA Later Solution (Ambion®, Life Technologies Ltd)	50 μ l
SDS 20% Solution (Ambion®, Life Technologies Ltd)	20 μ l
IGEPAL CA-630	20 μ l

Dithiothreitol, MgSO₄, ATP, NAD(P) and NADH stocks

Stocks of dithiothreitol (DTT), MgSO₄, ATP, NAD(P) and NADH were made just before use as the preparation in the following table for using as the composition in glucose, pyruvate and lactate reaction mixtures.

Reagent	MW (g/mol)	Amount (mg)	Tissue Culture Grade H ₂ O (ml)	Stock Concentration (mM)
DTT	154.3	15.4	20	5
MgSO ₄ • 7 H ₂ O	246.48	91.2	10	37
ATP	551.14	27.6	5	10
NADP disodium salt	787.4	39.37	5	10
NADH disodium salt	709.4	35.5	10	5
NAD free acid	663.4	80	2	60

Stocks for glucose, pyruvate and lactate standards and quality controls

The standards of known concentration of glucose, pyruvate and lactate were required for the standard curve construction in section 4.2.3. Therefore, the serial dilutions were made up the stocks required for the preparation of standards and quality controls.

Reagent	MW (g/mol)	Amount	Tissue Culture Grade H ₂ O (ml)	Stock Conc (mM)
D (+) Glucose	180.16	99 mg	10	50
50 mM Glucose Stock		800 μ l	3.2	10
10 mM Glucose Stock		2 ml	2	5
5 mM Glucose Stock		1 ml	4	1
Sodium Pyruvate	110.04	110.04 mg	10	100
100 mM Sodium Pyruvate		2 ml	18	10
10 mM Sodium Pyruvate		2 ml	18	1
60% (v/v) DL-Lactate Syrup	1.32g/ml	186.8 mg	10	DL:100 (L only:50)
50 mM L-lactate		1 ml	9	5
5 mM L-lactate		1 ml	4	1

APPENDIX IV: STOCK PREPARATIONS FOR MOLECULAR ANALYSIS

1X PBS

Sterile 1xPBS was prepared by diluting 1 ml of 10X PBS with 9 ml of DNase, RNase-free H₂O and solution was aliquot and kept in clean 2.0 ml nuclease-free microfuge tubes. Then each tube will be used once to prevent the contamination.

10mM dNTP mix

A ready-to-use 100mM mixed dNTP containing dATP, dCTP, dGTP and dTTP (Bioline Reagents Ltd., London, UK) was diluted in UltraPure™ DNase/RNase-Free (Life Technologies Ltd) distilled H₂O to produce a 10mM dNTP mix. The solution was thoroughly mixed and aliquoted into 0.5ml clean PCR tubes and stored at -20°C until use.

10μM Primer stocks

All primers were purchased from Life Technologies Ltd. Primer vials contained ~15-30nmols of desalted primers. The required volume (~150-300μl) of UltraPure™ DNase/RNase-free distilled H₂O was added to each vial to make up a 10 pmoles/μl working stock. The solution was thoroughly mixed and a few aliquots were dispensed into thin walled PCR tubes for everyday use and stored at -20°C. The rest of the solution was also kept at -20°C.

1X TBE buffer (Trisaminomethane, borate, EDTA buffer)

3 reagents including 108g of Tris, (Trisaminomethane), 55g of Boric acid and 7.4g of EDTA were thoroughly mixed together in a 10l plastic container by the aid of a magnetic stirrer and flea.

Lysis buffer

The reagents used for lysis buffer preparation as the following table, each one of the reagents as the list was equilibrated at room temperature, before mixing into a RNase, DNase free 1ml microfuge tube, using filtered tips. The solution was vortexed, dispensed into 200μl aliquots in thin walled PCR tubes and stored at -20°C until required.