The University Of Sheffield.

School of Medicine &Biomedical Sciences

The department of Oncology & Metabolism, Maternal and development unit

The role of matrix metalloproteinases and relaxin hormone in trophoblast-endometrium interactions during implantation

Jamela Sarb

MBChB (Libya), MMedSci (UK)

Supervisors

Prof. Dilly OC Anumba and Dr. Craig Murdoch

Thesis submitted to the University of Sheffield

For the Degree of Doctor of Philosophy

July 2017

Dedication

This thesis is dedicated to:

The memory of my mother

(Gammer Sreab)

My father Prof. Daw Ahmed Sarreb

My sister, Rebah Sarreb

My Brothers, Ahmed, Tarek, Waled and Mohamed

Thank you…..you always offered me with constant love and understanding….

without your love, I could not go so far

Acknowledgment

First, I would like to express my sincerest gratitude to Allah for his help and blessing without which I would never have completed this work. I would also like to recognise and thank Libyan government for the research grant which made this project a possibility.

I have been extremely privilege to able to carry out my postgraduate studies within the Department of Oncology & Metabolism, Maternal and Developmental Unit at University of Sheffield.

My sincerest and heartfelt thanks go to my primary supervisor Professor Dilly Anumba, whose expert knowledge and leadership assisted me greatly, and learned a lot from his exceptional idea for this project, thoughtful comments, knowledgeable guidance, encouragement, and support during my research study and writing were invaluable.

A huge gratitude is sent to my second supervisor Dr. Craig Murdoch for his professional guidance and inspirational ideas and excellent training in many laboratory techniques. He provided scientific input into the design and interpretation of my experiments and invaluable advice on the writing of my PhD.

I would like to express my appreciation to Professor Martina Daly and Dr Colby Eaton for their great help and support.

All the thanks to all the participants who kindly take part in this research, and all thanks to the staff nurses for their help in arranging for collection of blood from patients.

Finally, this thesis would not have been completed without the support and encouragement of my family, many friends and colleagues, and to all of them I convey sincere thanks indeed.

Sponsorship and scholarship

My PhD was sponsored by scholarship granted by the Libyan government.

Publications and presentations as a result of research work reported in this thesis

Sarb, J. Murdoch, C. Anumba, D.O.C. The effect of RLX-2 and tissue inhibitors of matrix metalloproteinases (TIMPs) on the expression levels of matrix metalloproteinase-2 (MMP-2) by co-incubation of endometrial stromal and trophoblast cell lines. Proceeding of the 63nd Annual Scientific Meeting. Society for Reproductive Investigation (SRI), Montreal, QC, Canada, 16-19 March 2016, Poster.

Sarb, J. Murdoch, C. Anumba, D.O.C. Using 3D models to investigate the role of tissue inhibitors of matrix metalloproteinases (TIMPs) and relaxin (RLX) in trophoblast-endometrial interactions Proceeding of the 62nd Annual scientific Meeting, Society for Reproductive Investigation (SRI), San Francisco, California, March 25-28, 2015, Poster.

Sarb, J. Murdoch, C. Anumba, D.O.C. Functional expression of tissue inhibitors of matrix metalloproteinases (TIMPs) and human relaxin (RLX) hormone during early pregnancy in women at risk of miscarriage. The 1st Scientific Symposium for Libyan Students in the UK, 5-6 June, London, UK.

Sarb, J. Murdoch, C. Anumba, D.O.C. Investigating the potential role of tissue inhibitors of matrix metalloproteinases (TIMPs) and human relaxin (RLX) in trophoblast-endometrial interactions in various pregnancy states and using an in vitro model. Medical School Annual Research Meeting, 3rd Year Graduate Presentation Day, Sheffield, UK: 10th of June 2013; oral presentation.

Sarb, J. Murdoch, C. Anumba, D.O.C. Functional expression of tissue inhibitors of matrix metalloproteinases (TIMPs) and human relaxin (RLX) hormone during early pregnancy in women at risk of miscarriage. Medical School Annual Research Meeting, 2rd Year Graduate poster Day, Sheffield, UK: 10th of June 2013; poster.

Sarb, J. Murdoch, C. Anumba, D.O.C. Functional expression of tissue inhibitors of matrix metalloproteinases (TIMPs) and human relaxin (RLX) hormone during early pregnancy in women at risk of miscarriage. Medical School Annual Research Meeting, 1st Year Graduate Presentation Day, Sheffield, UK: 9th of September 2011 oral presentation.

Sarb, J. Murdoch, C. Anumba, D.O.C. Investigating the role of tissue inhibitors of matrix metalloproteinases (TIMPs) and human relaxin (RLX) in trophoblast-endometrial interactions in various pregnancy states. 3rd Academic Unit of Reproductive & Developmental Medicine Annual Symposium. University of Sheffield, UK, 22nd of November 2013, presentation.

Sarb, J. Murdoch, C. Anumba, D.O.C. Investigating the potential role of tissue inhibitors of matrix metalloproteinases (TIMPs) and relaxin (RLX) in trophoblast-endometrial interactions. 4th Academic Unit of Reproductive & Developmental Medicine Annual Symposium. University of Sheffield, UK, 21st of November 2014, three minutes' presentation.

Sarb, J. Murdoch, C. Anumba, D.O.C. Dysregulation in the molecular events of early implantation stages may lead to failure of implantation. 5th Academic Unit of Reproductive & Developmental Medicine Annual Symposium. University of Sheffield, UK, 20th of November 2014, three minutes' presentation.

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Abbreviations

ABC	Avidin-biotin complex
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
βhCG	Beta-human Chorionic Gonadotrophin
BMI	Body Mass Index
8-Br-cAMP	8-Bromoadenosine 3′,5′-cyclic monophosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cm	Centimetre
COSHH	Control of Substances Hazardous to Health
CO_2	Carbon dioxide
CRH	Corticotrophin-releasing hormone
CV	Coefficient of variation
DAB	3,3′-Diaminobenzidine
DDT	Dithiothreitol
DF	Dilution factor
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPX mounting	Distyrene, a plasticizer, and xylene mounting
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked ImmunoSorbant assay
FCS	Fetal calf serum

EVT	Extra villous trophoblast
FBS	Fetal bovine serum
FN	Fibronectin
g	Gram
H&E	Haematoxylin and Eosin
HCG	human chorionic gonadotropin
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IF	Implantation failure
IL	Interleukin
ITS+ Premix	Insulin-Transferrin-Selenium (ITS)+ Premix
IUGR	Intrauterine growth retardation
KDa	Kilo--Dalton
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LIF	Leukaemia inhibitory factor
µg	Microgram
µl	Microliter
µm	Micrometre
MDD	Minimal detected dose
MEM	Modified Eagle's Medium
MFR	Monthly fecundity rate
mm	Millimetre
M	Molar
MMPs	Matrix metalloproteinases

mRNA	Messenger RNA
MT1-MMP	Membrane type1 MMP
NaCl	Sodium chloride
NEAA	Non-Essential Amino Acids
Ng	Nano gram
NHS	National Health Service
C°	Celsius degrees
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
RLX	Relaxin
RM	Recurrent miscarriage
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TEPs	Trophoectodermal projections
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
T-HESCs	Human endometrial stromal cells
TIMPs	Tissue inhibitors of matrix metalloproteinases
VT	Villous cytotrophoblast
WHO	World Health Organization

Abstract

Several modulatory molecules interact at the trophoblast-endometrial interface to generate a favourable environment for blastocyst implantation. Successful implantation requires a delicate balance between the tissue inhibitor matrix metalloproteinases (TIMPs) and activators of matrix metalloproteinases (MMPs) which are involved in the synthesis and degradation of extracellular matrix. The tissue hormone relaxin (RLX) has been reported to stimulate MMPs activity in various species including human, pig and rat. In this study we hypothesised that trophoblast-endometrial interactions are influenced by the functional expression of MMPs, TIMPs and human RLX during early implantation. The aims of the study are to determine whether RLX-2, MMPs and TIMPs levels would be clinically useful predictive markers of impending pregnancy loss, or other adverse pregnancy outcomes, and to explore the trophoblast-endometrial interactions under the influence of altered expression levels of RLX-2, MMP-2 and TIMP-2.

We conducted both *in vivo* and *in vitro* studies. *In vivo*: Four cohorts of pregnant women were recruited. These were a) pregnant women with a viable pregnancy and a history of recurrent miscarriage (RM), b) pregnant women, matched for gestational age with no history of RM (as controls), c) women seen in early pregnancy presenting with threatened miscarriage and d) healthy controls also matched for gestational age. Serum RLX-2, MMP-2, MMP-9 and TIMP-2 levels were determined in each of these cohorts by ELISA and compared for corresponding study gestations.

In vitro: studies involved co-incubation of T-HESCs and JAR cell lines with increasing concentrations of RLX-2, TIMP-2 and Batimastat to the cultures, Production of MMP-2 in the supernatant was evaluated by ELISA. In vitro 2D and 3D endometrial models, and JAR spheroids were developed and employed to study attachment and invasion stages of implantation and determine how these processes were affected by MMP-2, MMP-9, TIMP-2 and RLX-2.

Results: *In vivo* study: There was a significant increase in the serum expression of TIMP-2, and a significant decreased in the serum expression of MMP-2, MMP-2/TIMP-2 complex and RLX-2 hormone in RM compared to their controls with no history of RM.

In vitro study: Stimulation of T-HESCs with RLX-2 hormone showed increased secretion of TIMP-2. The data also showed that RLX-2 increased MMP-2 expression by JAR trophoblast cells in a dose-dependent manner, with significant differences being observed compared to unstimulated cells.

Cultured T-HESCs and JAR with TIMP-2 showed a biphasic effect. Both showed decreased levels of MMP-2 in the medium after 48h in comparison to unstimulated controls, this was followed by increase in MMP-2 levels compared to the day before. While the effect Batimastat on the levels of secreted MMP-2 by both T-HESCs and JAR cells showed a dramatic decline in secreted MMP-2 levels in both a time- and dose-dependent manner.

The kinetics of attachment between endometrial epithelial and/or endometrial stromal monolayers with the JAR spheroids under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation indicated that TIMP-2 significantly decreased the attachment rate of JAR spheroids to endometrial stromal cell line in a time-dependent manner of co-incubation. The expansion of the trophoblast cells over the endometrial stromal cells was promoted mainly by MMP-2 and inhibited by a Batimastat inhibitor. It was surprising that the Batimastat inhibitor showed a significant decrease in expansion whereas recombinant TIMP-2 did not.

In conclusion, it appears that MMP-2, TIMP-2 and RLX-2 hormone among other proteins play a key role in early human embryo implantation stages. The *in vivo* data suggest that any dysregulation of any of these proteins may play a role in the pathogenesis of implantation and recurrent pregnancy loss.

The *in vitro* data also suggests that MMP-2 and TIMP-2 appear to be implicated in this process. However, there is a need to optimise a cell culture model to promote and assist in understanding the potential mechanism and the signalling pathways of trophoblast attachment and invasion during implantation. Such a model could provide a platform for further studies to

reduce implantation failure and recurrent pregnancy, thereby improving pregnancy success rate following both spontaneous and assisted conception.

Chapter 1 Introduction

1.1. Anatomy of the female reproductive system

The anatomy of the female reproductive system undergoes considerable growth and development from intrauterine life to menopause. The female reproductive system includes external genitalia and internal genitalia. The anatomy of the female reproductive is reviewed extensively in (Stevens and Lowe, 2005, Campbell and Monga, 2006, Patton and Thibodeau, 2010).

1.1.1. External genitalia

The external female genitalia is called the vulva. It includes the mons pubis, labia minora, labia majora, and the vestibule that includes the clitoris, vaginal orifice, urethral orifice. The size and shape of the vulva depend on the age, race, and gravidity.

1.1.1.1. Mons pubis

This is the triangular area overlying the symphysis pubis, and includes the pad of fatty tissue underlying the skin. At the onset of sexual maturity, the mons pubis is covered with coarse and curly pubic hair arising from hair follicles.

1.1.1.2. Labia majora

The labia majora are the extensions of the mons pubis on the posterior-lateral side of the vaginal introitus forming the outer lips of the valve. They contain subcutaneous fat and smooth muscle. The labia majora are rich in sebaceous glands and apocrine glands. The outer skin of the labia majora is pigmented and characterised by the appearance of coarse hair while the inner surface is smooth and without hair.

1.1.1.3. Labia minora

Labia minora are two thin, hairless skin flaps just medial to the labia majora. The labia minora is divided into two parts to enclose the clitoris anteriorly, uniting to form the fourchette posteriorly. The vestibule is the area between the two labia minora.

1.1.1.4. Clitoris

The clitoris is an erectile vascular organ. It is covered by a thin epidermis that is rich in sensory nerves and receptors. The clitoris is situated below the mons pubis behind the junction of the labia minora. It contains two corpora cavernosa running side-by-side covered by a fibrocollagenous sheath and two corpora spongiosum.

1.1.2. Internal genitalia

The internal female genitalia includes the vagina, ovaries, fallopian tubes and uterus as illustrated in figure 1.1.

1.1.2.1. Vagina

The vagina is a distensible muscular canal that extends from the introitus to the cervix. The vagina has anterior and posterior walls; both walls are in apposition. The anterior wall is around 7 cm long while the posterior wall is about 9 cm long. The vaginal wall is formed of three layers; the inner layer is the mucosal layer lined with stratified squamous non-keratinised epithelium. The middle layer is the muscular layer that contains an inner circular smooth muscle and an outer longitudinal smooth muscle. Finally, the outer layer is adventitia that is formed of fibrocollagenous tissue.

1.1.2.2. Ovaries

The paired ovaries are situated on the right and the left side of the uterus. The size of the ovary varies with age and function. In women of reproductive age, it is approximately 30 mm x15 mm x10 mm. The microscopic structure of the ovary includes medulla, cortex and outer tunica albuginea.

The medulla is the internal part of the ovary that contains supportive fibromuscular tissue, blood vessels, nerves and lymphatics. The cortex contains a supporting stroma and the functional part of the ovarian cortex that is the ovarian follicles in varying phases of development. The outermost layer is the tunica albuginea that surrounds the ovarian cortex. The two main functions of the ovaries are the formation of mature ova (oogenesis) and production of steroid hormones including oestrogen and progesterone. The

hormones play an essential role in the regulation of the reproductive function in women.

1.1.2.3. Fallopian tubes

The human fallopian tubes are seromuscular structure attached to the lateral aspects of the uterine fundus and end close to the ovary from the lateral side. Each fallopian tube is 10-12 cm long and is divided into four portions termed, from medial to lateral, the intramural part, the isthmus, the ampulla and the infundibulum.

The intramural or interstitial segments lie within the uterine myometrium wall of the uterus and opens into the cavity of the uterus, this part is the narrowest part of the tube. The isthmus is characterised by a thick muscular wall and narrow lumen. The ampulla is the widest and longest segment of the oviduct. The distal part is the infundibulum from where the fimbria arise giving it a funnel-shaped appearance with finger-like projections.

1.1.2.4. Uterus

The uterus is a pear-shaped muscular organ. In a woman who has no history of pregnancy it measures approxiamtely 75 mm x 50 mm x 30 mm, and weighs about 70 gm. The normal uterus is anteverted and anteflexed. The uterus is located in the pelvic cavity posterior to the urinary bladder and anterior to the rectum. The uterus has four main parts: the fundus, the corpus, the isthmus and the cervix. The fundus is the upper end of the uterus, to which both fallopian tubes are attached laterally in a portion called the cornu. The corpus or body is the middle part of the uterus, and the lower part is the cervix.

The uterine wall is made up of three layers: the outermost layer is the serous layer (peritoneum). The middle layer is the muscular layer (myometrium), it is a thick layer and contains three smooth muscle layers. The layers extend in different directions, oblique, transverse and longitudinal. The muscular layer is thick at the fundus and thin at the cervix. The third layer is the innermost layer of the uterus, the endometrium.

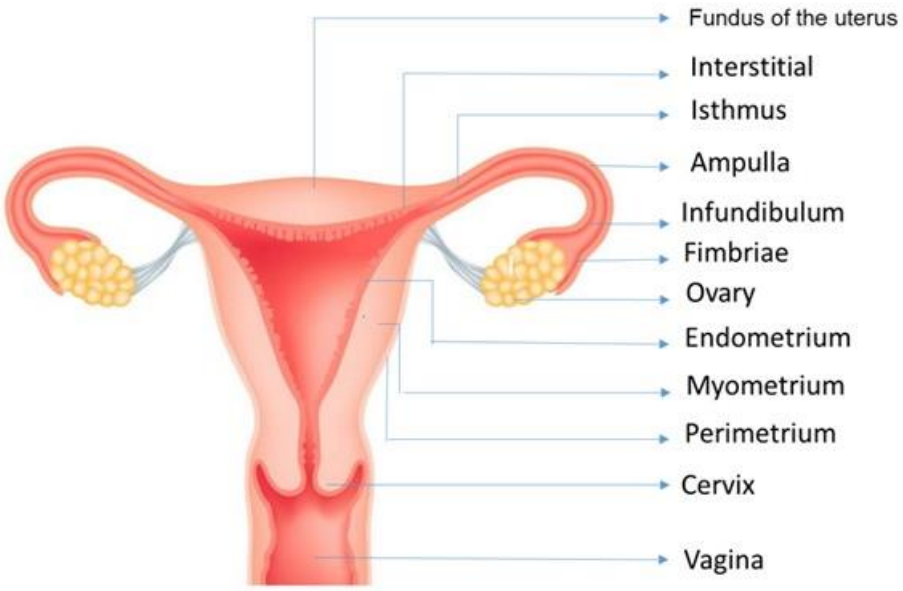

Figure 1.1. The anatomy of the internal female reproductive tract

Adopted from http://www.gynreform.com/

1.1.2.5. The endometrium

The endometrium lines the inner uterine cavity and contains three layers. The stratum compactum is the thin surface layer of partially ciliated simple columnar epithelium. The stratum spongiosum is the middle layer of loose connective tissue and the stratum basale is the deep layer that overlies underlying myometrium. The stratum compactum and the stratum spongiosum slough off after delivery and during menstruation.

The endometrium is a dynamic structure considered an endocrine organ secreting numerous hormones, cytokines and growth factors. The key role of the endometrium is to allow a viable embryo to implant during the time of receptivity by providing an appropriate local environment for the establishment of a successful pregnancy (Johnson, 2000).

In response to a cyclic variation in the expression of progesterone and oestrogen, the endometrium undergoes repeated proliferation, differentiation and tissue breakdown during the menstrual cycle (Salamonsen et al., 2003). The principal function of the endometrium is to orchestrate the processes that result in fertilization, embryo implantation and pregnancy.

1.1.2.6. The cervix

The cervix is the narrowest part of the uterus, it is about 3 cm long. The vaginal part of the cervix projects into the vagina to form the fornices. The upper two-thirds of the cervix is lined by columnar epithelium, whilst the lower third comprises stratified squamous epithelium. The junction between the columnar and stratified epithelium is known as the transformation zone.

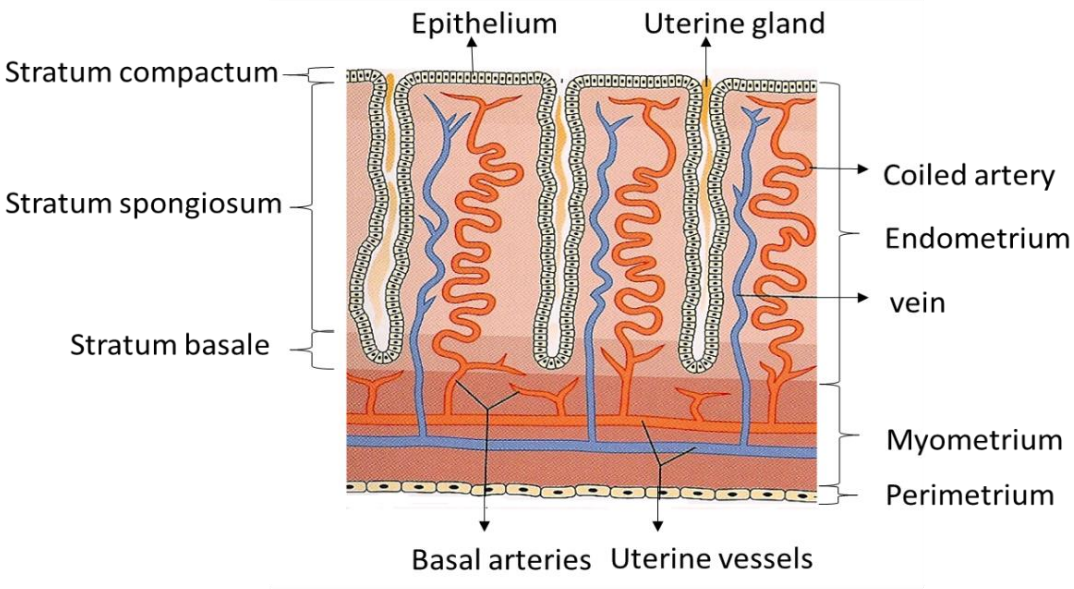

Figure 1.2. Histological structure of the endometrium

Adopted from (Richard and Kristin, 2006)

1.1.3. Cyclic changes of the endometrium (endometrial cycle)

Unlike other endometrial mucosal surfaces, the human endometrium undergoes cyclic changes during the reproductive phase, in response to steroid hormones, locally expressed growth factors and cytokines, to prepare the endometrium to host the embryo (Lass et al., 2001, Salamonsen et al., 2002).

1.1.3.1. Menstrual phase

During this phase, and in the absence of fertilisation, shedding of the superficial layer of the endometrium results in menstruation. The secretion of progesterone and oestrogen decline due to the corpus luteum degeneration (Salamonsen et al., 2002). There are a molecular and cellular alterations including release of matrix metalloproteinases, cytokines and chemokines.

1.1.3.2. The proliferative phase

The proliferative phase begins immediately after the menstrual phase and lasts till ovulation. This phase is characterised by proliferation and angiogenesis of the endometrium (Strowitzki et al., 2006). After the menstruation period, the thickness of the endometrium is about 1-2 mm. Ovarian oestradiol is the main hormone which promotes growth and proliferation of the endometrium. The proliferative changes include the elongation of endometrial glands, the stromal cells acquire spindle shape and the epithelial cells become columnar with basal nuclei (Palter and Olive, 2002, Loke et al., 1995). Moreover, the stroma become dense compact layer, the basal layer spiral blood vessels become elongated and form a network of loose capillary (Gambino et al., 2002). In the early proliferative phase these vessels are uncoiled and unbranched. There is extensive proliferation of the glands, endothelial cells and stromal cells by day 8-10 of the cycle. During this phase the height of the endometrium increases to 3.5-5 mm (Speroff and Fritz, 2005).

1.1.3.3. Secretory phase

This phase is after the period of ovulation, from day 14 to day 28 of the cycle. The endometrium is mainly under the effect of progesterone and oestrogen hormone secreted by corpus luteum (Palter and Olive, 2002). During the secretory phase, all endometrial cells are differentiated and infiltration of a variety of immune cells into the endometrium (Strowitzki et al., 2006). During the time of implantation, the thickness of the endometrium is approximately 10-14 mm, the endometrial stroma become oedematous (Gambino et al., 2002).

1.2. Implantation

1.2.1. Physiology of implantation

The implantation process is considered as a very critical step during pregnancy and involves a complicated chain of events (Makrigiannakis et al., 2006, Kennedy, 1997). For many years, implantation physiology has been studied extensively but ethical limitations have hampered human studies such that most studies report animal implantation (Kennedy, 1997). Much of the mechanism of implantation remains ill-understood. An outline is provided here of the processes involved in implantation.

1.2.2. Preimplantation and Decidualisation

Decidualisation is defined as “the process of transformation of the endometrium into the morphologically and functionally distinct decidual tissue, involving the differentiation of endometrial cells and infiltration by large numbers of lymphoid cells” (Salamonsen et al., 2003). For successful implantation, the endometrium has to convert to decidua. Decidual cells play an essential role in implantation and provide the optimum nutritional environment for the embryo, it produces factors that appear to key roles in regulating trophoblast invasion whilst also protecting the embryo from “immune rejection” (Fazleabas and Strakova, 2002). Unlike in other species, the decidualisation process in humans starts in the late secretory phase and is not dependent on the presence of the blastocyst (Lunghi et al., 2007) (King, 2000).

At the time of uterine receptivity, cellular morphological changes occur, involving endometrial stromal cells that change to decidual cells and assume large and round appearances. Furthermore, the secretory endometrial glands undergo active growth transformation (Dekel et al., 2010).

Decidua development continues throughout pregnancy, and appears to regulate trophoblast invasion and placental development by modifying the expression of regulatory molecules including MMPs, surface integrins, major histocompatibility complex molecules and cytokines (Lunghi et al., 2007). Proteolysis is crucial for decidua remodelling, migration of cells and

vascularisation during human embryo implantation. This involves several matrix metalloproteinases whose activity is modulated by growth factors, steroid hormones and cytokines (Plaisier, 2011).

Decidua, as a specialised tissue, is thought to provide an environment to control and regulate trophoblast invasion. If embryo implantation occures, the decidua form three regions. The first layer lies beneath the placenta called decidua basalis, formed of both maternal tissue and extravillous trophbalsts. The seconed layer overlayes the embryo called decidua capsularis and the last layer lines the uterus away from the area of implantation site and known as decidua parietalis (Loke et al., 1995). As a result, if implantation occurs in extrauterine sites or where there is a deficiency in decidua development, for example, a fallopian tube or a previous Caesarean scar, there is a more significant degree of trophoblast invasion than usual (Loke et al., 1995).

1.2.3. Embryo-endometrial synchronization

The implantation process is an interaction between the embryonic trophoblast and the endometrium (Dimitriadis et al., 2010). This requires synchronization of the processes involved in embryo development and the molecular and cellular events that occur in the endometrium of the uterus. It is regulated by paracrine and autocrine hormonal mechanisms (Staun-Ram and Shalev, 2005). The interaction takes place at specific time-points called the “window of receptivity” which depends on a number of complex molecules including cytokines, hormones and growth factors (Nikas, 1999, Singh et al., 2011). In humans, the window of receptivity is between days 20-24 of the menstrual cycle (Lunghi et al., 2007). The synchronization process is regulated by steroid hormones and facilitated by molecular events and secretions from both the embryo and reproductive tissue (Vigano et al., 2003) as illustrated in figure 1.3. Any dysregulation in the window of natural embryo selection or the embryo quality may lead to miscarriage (Teklenburg et al., 2010).

In human, the monthly fecundity rate (MFR) is defined as the probability of achieving a pregnancy within one menstrual cycle, it is about 20%–30%. In contrast, other mammalian species, the MFR is as high as 90% in rabbits and 80% in baboons. It has been estimated that human conceptions failure to progress to an ongoing pregnancy is approximately 50%. However, about 15% of clinically recognized pregnancies are ended by miscarry. The theory of the discerning endometrium and the high rate of embryo implantation failure that characterizes reproduction of the human is thought to be due to two key features of human embryos: their high incidence of chromosomal abnormalities and their intrinsic invasiveness (Macklon and Brosens, 2014).

1.2.4. Implantation stages

The human embryo implantation process involves four phases: hatching, apposition, adhesion and invasion (Achache and Revel, 2006). However, two factors are essential for successful implantation, the quality of the embryo and endometrial receptivity (Cavagna and Mantese, 2003).

1.2.4.1. Hatching stage

Before implantation the blastocyst need to hatch out of the zona pellucida (acellular glycoprotein coat). Blastocyst hatching begins with formation of a nick in the zona pellucida, as a result of hydrostatic pressure exerted the increase of the blastocyst expansion. Blastocyst hatching is a crucial step in implantation phenomena and any dysregulation in the process results in embryo implantation failure.

Hatching stage is regulated by both cellular and molecular factors, the exact process is still ill-understood. However, from the perspective of cellular biology aspect trophoectodermal projections (TEPs) are thought to play an essential role in hatching. On the other hand, the molecular regulators including cytokines, proteases and transcription factors are fundamental for blastocyst hatching (Seshagiri et al., 2009).

1.2.4.2. Apposition stage

Orientation or apposition of the blastocyst inside the uterine cavity begins at day 6 after the mid-cycle luteinizing hormone surge (day LH+6) (Lim, 2003). In this phase, the blastocyst comes in close contact with the epithelial cells of the endometrium (Bischof and Campana, 1996). Apposition is also considered as an unstable linkage between the blastocyst and the receptive epithelial endometrium (Cakmak and Taylor, 2010) through the micro protrusions located on the top of epithelium identified as pinopodes (Singh et al., 2011). The early signals of the blastocyst and uterine epithelium during this stage are illustrated in figure 1.3.

1.2.4.3. Adhesion stage

This stage is characterized by a precise transient adhesive molecular interaction between the trophoblast and uterine epithelium (van Mourik et al., 2009). The molecular events engaged in cell adhesion activate cytoplasmic signals that play a significant role in the next step (Kimber and Spanswick, 2000). This stage is regulated by cell adhesion molecules such as (integrins, cadherins, selectins and kisspeptins), cytokines and immune cells (van Mourik et al., 2009).

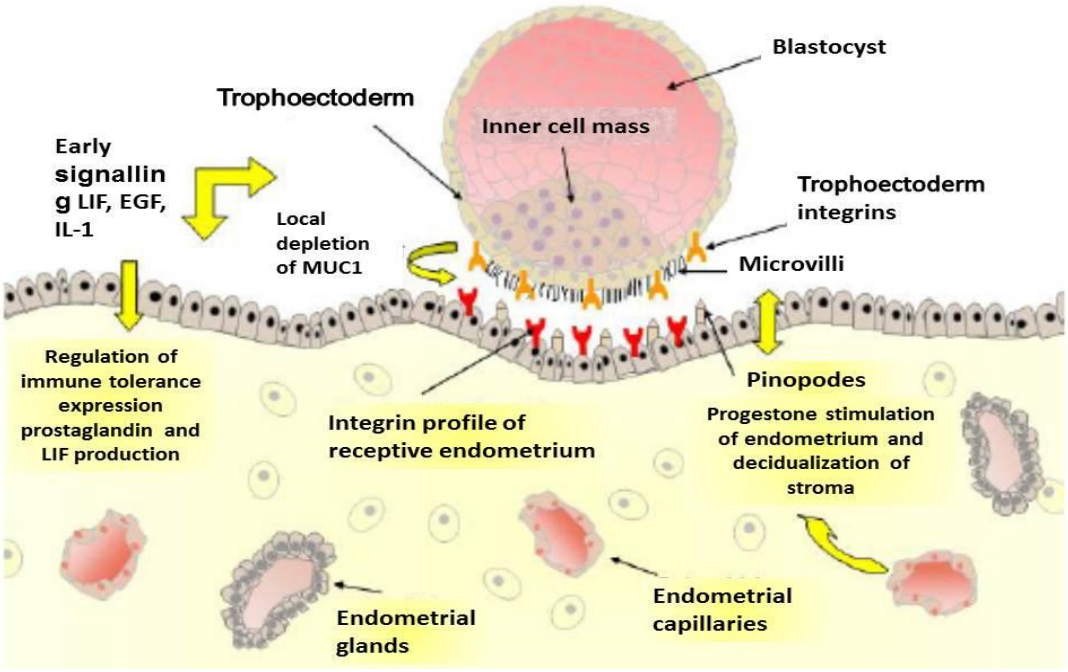

Figure 1.3. Early signals of blastocyst and receptive endometrium before the stage of adhesion

The figure represents the integrins, integrin receptors and formation of the pinopodes. Adapted from (Staun-Ram and Shalev, 2005).

1.2.4.4. Invasion phase

The last stage is the invasion phase, within a few hours of adhesion, the embryo penetrate the endometrial epithelium to invade the stroma thereby creating a vascular network between the embryo and the mother (Singh et al., 2011). The invasion process is the longest phase of implantation. It begins with degradation of the basement membrane and the extracellular matrix (ECM) where the matrix metalloproteinases (MMPs) play a vital role in the control of the tissue inhibitors of matrix metalloproteinases (TIMPs) to prevent under or over activity of invasion (Lim, 2003). The cellular, hormonal and molecular interactions are illustrated in figure 1.4. This stage is critical although it is not explicitly defined (Helige et al., 2008), it is under tight regulation (Lala and Graham, 1990), and any imbalance may lead to pathological conditions. Excessive trophoblast invasion may lead to placenta increta, percreta and accreta, while inadequate invasion may lead to intrauterine growth restriction, preeclampsia and miscarriage (Lim, 2003).

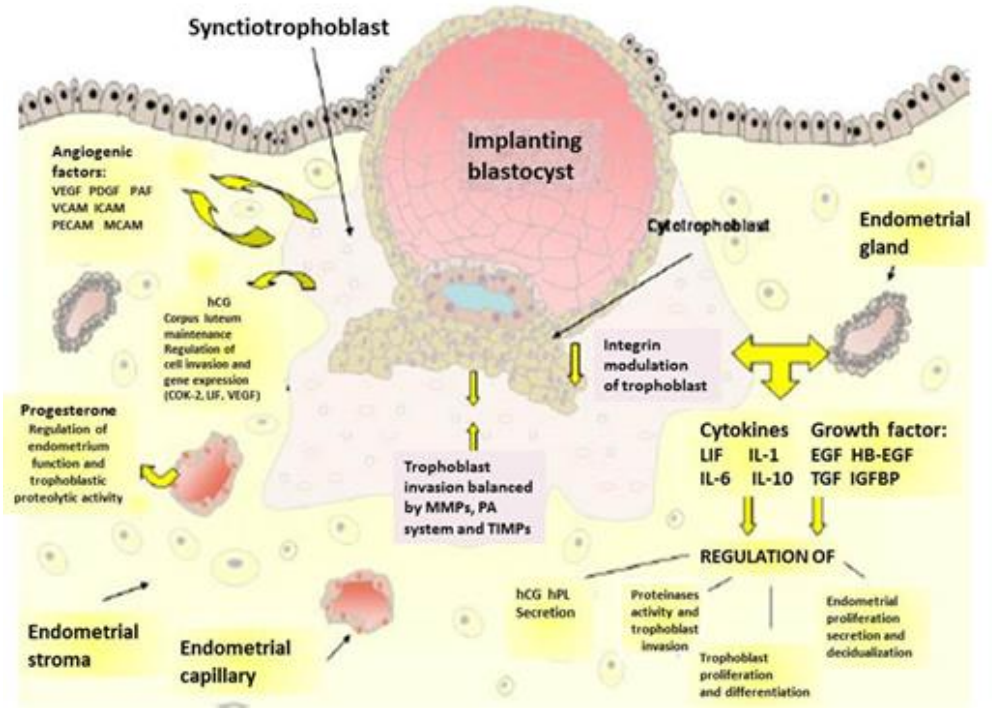

Figure 1.4. The blastocyst-endometrium interaction including cellular and molecular event during invasion phase

Adapted from (Staun-Ram and Shalev, 2005)

1.3. Placenta

The placenta is a fetal organ that acts as a transporter and secretory unit throughout all its development stages (Huppertz, 2008). The placenta and its vascular development are crucial to the development and growth of the fetus (Reynolds et al., 2010). The importance of sufficient blood flow and vascular development of the placenta have been reviewed by many investigators. A recent report has emphasized that angiogenesis in the placenta is a critical determinant of placental blood flow and consequently affects fetal growth (Reynolds et al., 2010). Co-ordination between the development of the embryo and placenta is essential, as dysregulated processes can result in pregnancy complications such as miscarriage, intrauterine growth retardation, preeclampsia and stillbirth (Chaddha et al., 2004).

1.3.1. Placenta development

After the blastocyst attaches to the uterine endometrium, it penetrates its epithelium cell layer, the basement membrane and the stroma. These events are under hormonal (Aplin, 1991) and molecular influence, involving angiogenic growth factors, cytokines, adhesion molecules, growth factors and proteinases (Regnault et al., 2002). The phases of early placental development have been described as follows and illustrated in figure 1.5.

1.3.1.1. Preimplantation phase

During days 6 to 7 after conception, blastocyst need to hatch out of the zona pellucida, this is followed by the attachment of the blastocyst to the epithelial layer of the uterus, this step is considered as the first step in placenta development. After the morula stage and before the stage of the blastocyst, the first cell type to differentiate is the trophoblast. The inner cell mass of the trophoblast is covered with one layer of mononucleated trophoblast. This layer surrounds the embryoblast, blastocoel and blastocyst cavity (Huppertz, 2008). The placenta and the fetal membrane are derived from the trophoblast layer while the embryo, placental mesenchyma and the umbilical cord are derived from the inner cell mass.

1.3.1.2. Prelacunar phase

The inner cell mass is the part that is covered with trophoblast cells and forms the polarity of the blastocyst, and trophoblast cells that covered this part are the cells that attach the uterine epithelium (Huppertz, 2008). Following the attachment of the blastocyst to the uterine epithelium, differentiation of the polar trophoblast to cytotrophoblast and syncytiotrophoblast takes place (Enders and Blankenship, 1999). Cytotrophoblast cells are comprised of mononucleated cells while the syncytiotrophoblast consists of multinucleated cells (Huppertz, 2008).

During the prelacunar stage, the cytotrophoblast divides rapidly and fuse with the syncytiotrophoblast resulting in development and expansion of syncytiotrophoblast. The syncytiotrophoblast cells act as an invasive phenotype to the uterine epithelium, which are the only cells to make contact with the maternal cells directly. Any defect in the attachment of the polar part of the trophoblast due to rotary motion of the blastocyst during this period may lead to implantation failure or umbilical cord placing defect (Huppertz, 2008).

1.3.1.3. Lacunar phase

During this phase (8 days after conception) the syncytiotrophoblast develops fluid-filled spaces called lacunae. The tissue masses around these lacunae are called trabeculae. The trabeculae play a significant role in the development of the placental villus tree. The syncytiotrophoblast invades and penetrates the endometrium where it makes contact with maternal capillaries as well as the endometrial superficial venous plexuses. It is at this point that the first maternal blood cells appear in the syncytiotrophoblast lacunae (Huppertz, 2008).

At day 12 post conception, the cytotrophoblast cells start to invade and penetrate the syncytiotrophoblastic mass, this makes contact with the placenta from the maternal side by day 15 post conception (Frost and Moore, 2010, Huppertz, 2008). The cytotrophoblast cells differentiate into the extra villus cytotrophoblast (Gude et al., 2004) which penetrates the stroma of the endometrium (Chaddha et al., 2004) between capillaries and glands. It

invades the spiral artery wall and ends in the vessel cavities (Huppertz, 2008).

1.3.1.4. Villous phase

The development of this stage is illustrated in figure 1.5. Throughout this phase, the trabeculae develop branches, with central cytotrophoblast enclosed by the syncytiotrophoblast, these branches are the primary villi. The cells of the extraembryonic mesoderm infiltrate into the trabeculae but do not make contact with the maternal tissue, forming trophoblastic cell columns (Huppertz, 2008). Next, the primary villi are invaded by the mesodermal cells to form a mesenchymal core. These structures are called secondary villi (Chaddha et al., 2004) or chorionic villi (Mihu et al., 2009). However, the tertiary villi are formed when the first placental vessels develop (Huppertz, 2008).

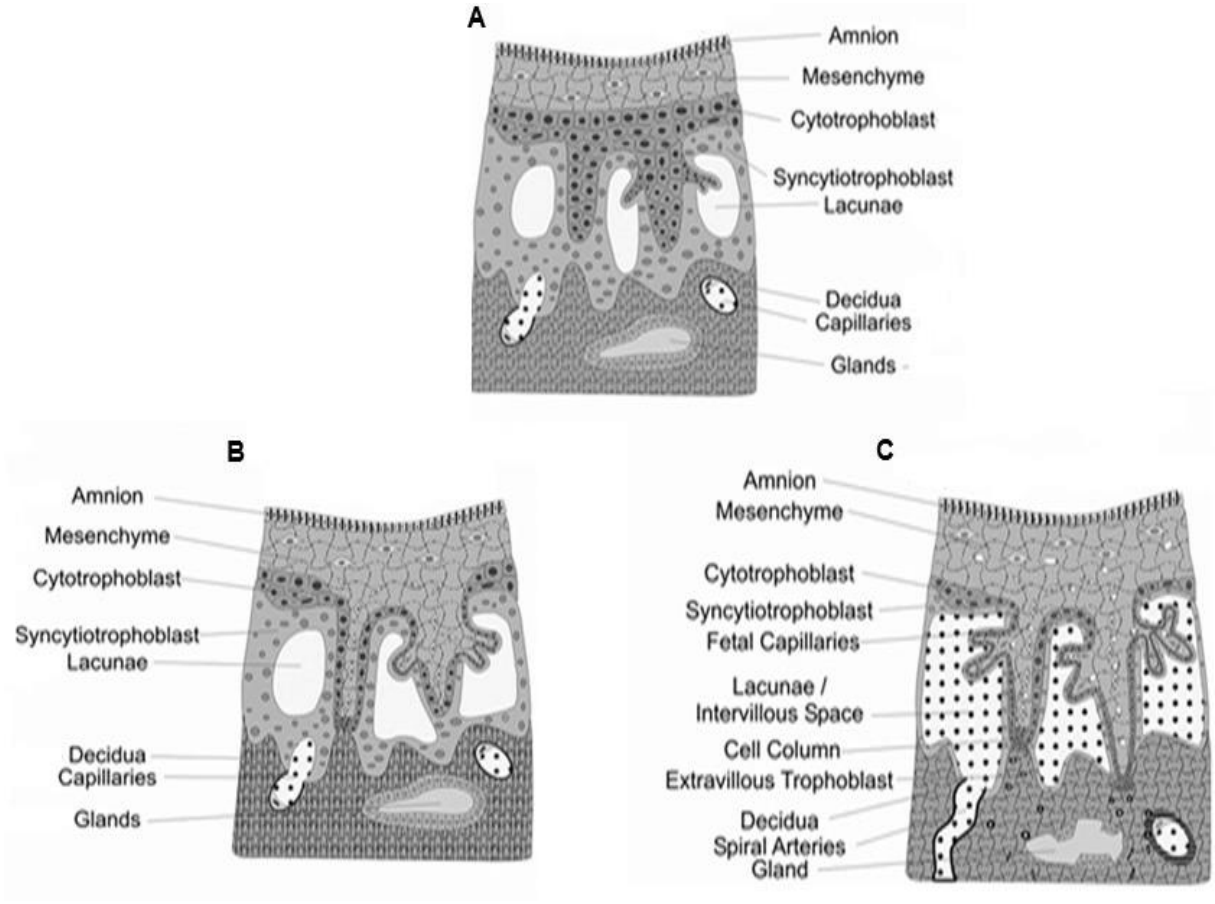

Figure 1.5. Phases of early placental development

(**A**) From days 12–15 post-conception formation of primary villi from cytotrophoblast cells in the core and enveloped by a layer of syncytiotrophoblast cells. (**B**) From days 15–21 post-conception the extra-embryonic mesenchyma penetrate the trophoblast column leads to development of secondary villi (**C**) From day 18 post-conception until term, formation of tertiary villi by formation of the placental blood vessels. The tertiary villi lead to the formation of the placenta villus trees. Adapted from (Chaddha et al., 2004).

1.4. Role of hormones in implantation

The hormonal endocrine system plays a fundamental role in the human implantation process. Hormones are involved in the expression, stimulation and inhibition of different cytokines, growth factors, cell adhesion factors and decidual proteins (Sunder and Lenton, 2000) during implantation. The precise role and the exact mechanism are still poorly understood, although numerous studies have shown that disturbance in any hormonal function may result in impaired embryo implantation (Makrigiannakis et al., 2006). However, understanding the molecular and hormonal background of embryo implantation can help to diagnose pregnancy loss or subfertility precisely and may enable better clinical management (Makrigiannakis et al., 2006). The hormones that appear to have functional roles in implantation include steroids, human chorionic gonadotropin (HCG) and corticotrophin-releasing hormone (CRH).

1.4.1. Steroids

It is accepted that the uterine endometrial cells are under the direct control of ovarian steroids and the indirect control of different growth factors and cytokines (Makrigiannakis et al., 2006). Ovarian progesterone and oestrogen regulate several modulatory molecules to enhance uterine receptivity for successful embryo implantation (Singh et al., 2011). The progesterone concentration increases during the luteal phase of the menstrual cycle and is crucial for the secretory differentiation changes in the uterine endometrium to facilitate blastocyst attachment (Sengupta and Ghosh, 2000).

Progesterone plays an essential role in the decidualisation process, with cAMP being the principal intracellular mediator (van Mourik et al., 2009). It has been suggested that progesterone is a potent stimulator of calcitonin mRNA induction in the uterine glandular epithelial cell during the implantation window (uterine receptivity). It is also considered as an important suppressant factor of leukaemia inhibitory factor (LIF) in endometrium cells (Sunder and Lenton, 2000).

The role of the oestrogen hormone in the implantation process is to promote and express progesterone receptors on endometrial cells. With progesterone, it acts to prepare the blastocyst for implantation by promoting endometrial cell differentiation (Aplin and Kimber, 2004). However, both ovarian steroids are the dominant modulators and play a pivotal role in endometrial development, they prepare endometrium with an appropriate functional and morphological state to facilitate the blastocyst attachment (Singh et al., 2011). Therefore, progesterone deficiency during the luteal phase is a recognized association with female infertility (Sengupta and Ghosh, 2000).

1.4.2. Human chorionic gonadotropin (hCG)

Human chorionic gonadotropin plays a role in the decidualisation process and modulates the expression of growth factors and cytokines during embryo implantation. It maintains corpus luteum progesterone function until the placenta is developed and able to take over (Kodaman and Taylor, 2004). During pregnancy, the invading embryo produces hCG and prevents menstruation by rescuing the corpus luteum that continue to secret progesterone (Fritz and Speroff, 2011). At 10 weeks of gestation, hCG reaches its peak and is continued to be secreted throughout pregnancy, but its role in progesterone secretion only lasts for the first 3-4 weeks after embryo implantation (Cole, 2010).

1.4.3. Corticotrophin releasing hormone (CRH)

The corticotrophin-releasing hormone is a glycoprotein hormone that is synthesized by the decidua and trophoblast of the placenta (Staun-Ram and Shalev, 2005). Recent studies have described the role of corticotrophin-releasing hormone in the implantation phenomena. They suggested that CRH promotes decidualisation and acts as a stimulatory factor for progesterone. They also demonstrated its role in decidualisation by regulating the local modulators, such as stimulation of inhibitor interleukin-1 (IL-1), inducer interleukin-6 (IL-6) and the inhibition of enhancer prostaglandin E2 (Makrigiannakis et al., 2006).

1.5. Molecular and cellular biology of implantation

Numerous studies have attempted to explain the molecular biology of implantation. However, it is still not well defined (Singh and Aplin, 2009). The implantation process involves a complex sequence of events starting with changes in the uterine epithelial cells organization (Heneweer et al., 2005) and endometrial components, these include endometrial glands, stromal cells and vessels and the ECM to prepare and drive changes in the endometrium for successful embryo implantation (Duc-Goiran et al., 1999). Nevertheless, dysregulation in the molecular and cellular events at different stages of the implantation process including pre-implantation, implantation and post-implantation may lead to an increase in the rate of implantation failure (Dey et al., 2004). Successful implantation and placentation involves factors which act on the endometrium to make it receptive to the embryo and other factors which render the endometrium resistant to implantation. The implantation window relies on a critical balance between these two opposing series of events (Tabibzadeh, 1998).

1.5.1. Pinopodes

Pinopodes are cellular organelles which appear as protrusions located on the surface epithelium of the endometrium. They are considered to be progesterone-dependent, their life span is about forty-eight hours. Their appearance is from day 19 to 21 of the menstrual cycle, corresponding to the time of the implantation window (Sunder and Lenton, 2000). Even though this has not been confirmed *in vivo* it seems that the presence of endometrial pinopodes throughout the mid-secretory phase may be considered as a useful marker of endometrial receptivity to enhance the rate of implantation (Achache and Revel, 2006).

There is a controversy in the literature about the physiological role of pinopodes as well as their function and timing. Whilst they are thought by many to be markers of implantation (Quinn and Casper, 2009), a recent review has queried this assumption, highlighting the lack of evidence in humans as well as mice (Quinn and Casper, 2009). Although the function of

pinopodes is not clearly defined, it seems that blastocyst attachment at the stage of embryo endometrial interactions has more tendency to occur in the area of pinopodes, suggesting that their surface may contain receptors that are necessary for blastocyst attachment (Achache and Revel, 2006).

1.5.2. MUC-1

MUC-1 is a complex glycoprotein layer (Staun-Ram and Shalev, 2005) with high molecular weight more than 250 kDa (Meseguer et al., 2001). It is continuously expressed at the apical surface of endometrial luminal epithelium during the menstrual cycle and the level increases in the secretory phase (Aplin, 2006). MUC-1 prevents the adhesion between trophoblast and uterine epithelial cells (Aplin, 1997), acting as a protective agent against infection (Aplin and Kimber, 2004). It is thought to act as a barrier by inhibiting the binding of integrin receptors to their ligands (Staun-Ram and Shalev, 2005).

Unlike other species, there is an increase in the level of MUC-1 during the apposition stage and the presence of the blastocyst in humans. At the stage of adhesion, the blastocyst produces an enzyme that metabolises MUC-1 in the area of implantation thereby removing its repulsive anti-implantation properties (Dey et al., 2004). Moreover, the presence of a large quantity of desmosomes in the lateral sides of the epithelial surfaces is another anti-adhesion factor that impairs blastocyst implantation (Lunghi et al., 2007).

1.5.3. Integrins

Integrins are transmembrane glycoproteins, cell surface receptors (Sunder and Lenton, 2000), consisting of α and β subunits that bind to different ECM substances as well as cell adhesion molecules (Staun-Ram and Shalev, 2005). Each unit contains three domains: extracellular, transmembrane and intracellular (Singh and Aplin, 2009). The expression of integrins on the endometrium is under the control of steroids and cytokines such as IL-1 (van Mourik et al., 2009). It is suggested that integrins have an important role in fertilization and implantation, including blastocyst attachment and invasion through the ECM as well as cell to cell signalling (Carson et al., 2000,

Sunder and Lenton, 2000). At the time of implantation, integrins are expressed by both the uterine endometrium and human trophoblast. They each bind to a particular ECM element including osteopontin produced by the uterine epithelium and oncofetal fibronectin (FN) which is generated by the trophoblast. This binding provides embryonic adhesion in the form of a sandwich model (Achache and Revel, 2006). Integrins participate in other physiological processes by cell-matrix and cell–cell adhesion, such as wound healing, thrombosis, haemostasis, oncogenic transformation and immune and non-immune defence mechanisms (Achache and Revel, 2006).

1.5.4. Cytokines

Various cytokines and cytokine receptors are expressed in both the embryo and uterine endometrium and have a role in the different phases of embryo implantation (Dey et al., 2004). Cytokines secreted by the embryo and the uterus play a role in embryo-maternal interaction, and in promoting receptivity of the endometrium, by regulating the activity of adhesion and anti-adhesion proteins (Cavagna and Mantese, 2003). In addition, it is suggested that they have an essential role in the regulation of the activity of MMPs and TIMPs (Lim, 2003). Cytokines that appear to participate in human implantation include Interleukin-1,6 (IL-1, IL-6) and Leukaemia inhibitory factor (LIF) (van Mourik et al., 2009). However, a range of other cytokines are also expressed during this process including IL-11 (Dimitriadis et al., 2005), IL-10 and IL-5 which are reported as an autocrine regulator in trophoblast invasion (Bischof and Campana, 2000).

IL-1 is secreted by trophoblast cells and stromal endometrial cells. It stimulates the secretion of some MMPs that act as extracellular remodelling factors essential for implantation and placentation (Dimitriadis et al., 2005). Moreover, IL-1 stimulates LIF production by the endometrium and enhances the expression of integrin β3, an adhesive molecule crucial for apposition and adhesion (van Mourik et al., 2009). Nonetheless, it has been emphasised that IL-1 stimulates the production of human chorionic gonadotropin by the embryo (Dimitriadis et al., 2005).

IL-6 is another cytokine that is suggested to contribute in implantation and placentation in humans. Moreover, it regulates a variety of aspects of immunity reaction (Dimitriadis et al., 2005). Throughout the luteal phase IL-6 is highly expressed in the epithelium and glandular cells more than the stromal cells (Achache and Revel, 2006).

Leukaemia inhibitory factor is a glycoprotein produced by the endometrial glands in the uterus and considered to play a significant but as yet ill-defined role in implantation (Singh et al., 2011). LIF and its receptors are highly expressed between six and nine days after the luteinizing hormone (LH) surge and this rise corresponds to the appearance of pinopodes (Makrigiannakis et al., 2006) while the uterine endometrium is under the influence of progesterone (Aghajanova, 2004). Investigators have demonstrated that LIF regulates gelatinases and urokinase plasminogen activator production, both of which play a vital role in trophoblast invasion of the endometrial stroma during implantation (Sunder and Lenton, 2000). LIF acts as both a stimulator and a mediator in the implantation process and pregnancy establishment (Aghajanova, 2004), it has been shown that LIF stimulates tissue inhibitors of MMPs in fibroblast (Bischof and Campana, 2000).

1.5.5. Growth factors

Various growth factors are expressed in early pregnancy as well as during the menstrual cycle. As a result, they are considered essential to implantation (Dey et al., 2004). Growth factors are suggested to participate in all stages of implantation including apposition, attachment and invasion (Giudice, 1999).

Epidermal growth factor (EGF) is expressed in the endometrium during the secretory and proliferative phases of the menstrual cycle, especially from the uterine decidua (Bischof and Campana, 2000). It has been found that EGF promotes the invasion of the endometrium by cytotrophoblast (Staun-Ram and Shalev, 2005), possibly by the direct stimulus of MMP-1 and MMP-3 (Bischof and Campana, 2000). However, these observations remain controversial as the receptors of the epidermal growth factor are expressed

in proliferative but not invasive cytotrophoblast (Bischof and Campana, 2000) suggesting that EGF plays a vital role in the cytotrophoblast proliferation (Staun-Ram and Shalev, 2005) as well as the formation of the syncytiotrophoblast (Bischof and Campana, 2000).

Transforming growth factor beta (TGF-β) is expressed in human uterine epithelium, stroma (Makrigiannakis et al., 2006) as well as the cells of the deciduae, and has been localized in cytotrophoblast and syncytiotrophoblast (Bischof and Campana, 2000). Studies have shown that TGF acts as an inhibitory factor for both proliferation and invasion of trophoblast (Staun-Ram and Shalev, 2005) through promoting MMP-2 and MMP-9 secretion and inhibiting TIMPs, activities (Bischof and Campana, 2000). Therefore, dysregulation in their expression and activity may result in partial or complete embryo implantation failure (Singh et al., 2011).

Several studies have shown that many growth factors play fundamental roles in implantation and placentation, such as heparin-binding epidermal growth factor, it is expressed in uterine epithelium and stroma and is thought to regulate blastocyst implantation (Staun-Ram and Shalev, 2005). Insulin-like growth factor binding protein is the most significant endometrial decidual secretion (Bischof and Campana, 2000), it is found to be involved in the trophoblast migration and invasion by stimulating the gelatinolytic effect of trophoblast (Staun-Ram and Shalev, 2005).

1.6. Extracellular matrix (ECM)

The ECM is a three-dimensional complex, non-cellular scaffold. All organs have an ECM with a unique structure that is created in early embryonic phases (Frantz et al., 2010). The ECM is a compound active network of many components that provides structural support to the tissues in the form of cartilage, bone and tendon. These components also play profound roles in many biological activities such as cellular proliferation and differentiation (Matrisian, 1990).

Beyond their structural-mechanical functions, ECM molecules control diverse cellular roles, and play a significant role in various physiological and pathological processes, such as embryonic development, haemostasis, fibrosis of several organs, wound healing and inflammation, tumour growth and metastasis and angiogenesis (Bonnans et al., 2014).

1.6.1. Components of extracellular matrix

There are two main kinds of ECM: the interstitial connective tissue matrix, which surrounds cells and is responsible for structural support for tissues and the basement membrane, which separates the epithelium from the adjacent stroma (Bonnans et al., 2014). The interstitial matrix is essentially composed of collagen type I and fibronectin, elastin, proteoglycans, glycosaminoglycan and tenascin-C. The basement membrane is mainly composed of collagen type IV, laminins, heparin sulphate proteoglycan, entactin and nidogen (Bonnans et al., 2014). They are organised as a complex and dynamic meshwork for cell adhesion (Adams and Watt, 1993). The ECM acts as a barrier or a filter for soluble molecules, and as a structural supporter for cells. The cells may be surrounded by an ECM from all sides as in chondrocytes, or it may be on one side like the epithelial and endothelial cells (Adams and Watt, 1993). They also influence cell migration and adhesion process as well as tissue morphogenesis (Matrisian, 1990). There are cell receptors that mediate adhesion of cells to the ECM. These receptors belong to the family of integrins (Loke et al., 1995).

In the endometrium, collagen, laminin and fibronectin are the main components of ECM (Skrzypczak et al., 2007a). The human endometrium contains collagen type I, III, IV, V and VI. Collagen types I, III and V are responsible for the integrity of the structure and tissue strength (Adams and Watt, 1993). Collagen type IV, which is present in the basement membrane, promotes trophoblast invasion. Several enzymes contribute to the degradation of ECM and basement membrane, but the MMPs are considered as the most essential factors in this event, and their functions are highly regulated (Hulboy et al., 1997).

MMPs and TIMPs govern trophoblast invasion of ECM (Jokimaa et al., 2002). In the first trimester of pregnancy, both fibronectin and laminin are plentiful in the decidual stroma (Loke et al., 1995). Although the function of decidual fibronectin and laminin have not yet been clearly identified, one possible role is preserving tissue structure of the decidual stroma (Burrows et al., 1996).

1.7. Matrix metalloproteinases (MMPs)

Proteases family can be categorised into cysteine proteases, aspartate proteases, serine proteases and matrix metalloproteases. The matrix metalloproteases are members of a unique family which are responsible for degradation and remodelling of the basement membrane and ECM components (Hulboy et al., 1997).

1.7.1. Structure of the matrix metalloproteinases

The mammalian family of MMPs comprises about twenty-six members (Cohen et al., 2006). They are zinc-dependent enzymes and have the fundamental multidomain feature: it contains pro-domain, a catalytic domain, a hemopexin domain and a hinge domain as illustrated in figure 1.6. They function extracellularly at a neutral pH (Amalinei et al., 2007). However, they are divided into six groups with respect to their structural and functional properties: collagenases, gelatinases, matrilysins, stromelysins, membrane type and heterogeneous group (Amalinei et al., 2007). All the six groups of

MMPs share common functional domains and activation methods. (Goldman and Shalev, 2003) (Table 1.1).

They are frequently secreted into the ECM as proenzymes or inactive enzymes except the MT-MMPs (Nagase and Woessner, 1999). The proenzymes need proteolytic activation by proteases including plasmin, elastase, tryptase and activated MMPs as illustrated in figure 1.7 (Salamonsen and Nie, 2002). However, cell behaviour is regulated by MMPs in several ways including cell-cell and cell-matrix interactions, stimulation or inhibition of paracrine and autocrine signalling molecules and receptors on the cell surface (Staun-Ram and Shalev, 2005). The regulation of MMPs activity occurs at different levels including regulation of gene transcription, secretion, activation, inactivation and degradation (Cohen et al., 2006).

The function of MMPs are under the regulation of Tissue Inhibitors of Matrixmetaloproteinases (TIMPs). TIMPs inhibit MMPs through Binding of their inhibitory domain the amino-terminal domain to the active site of the MMPs to form tight 1:1 complexes (section 1.8) (Murphy, 2011). Their dysregulated metabolism has been implicated in several pathological conditions such as tumour invasion, endometriosis, abnormal wound healing amongst others (Amalinei et al., 2007), nephritis, arthritis, fibrosis and chronic ulcer (Nagase et al., 2006). They are also involved in bone development, corneal repair and cartilage remodelling (Curry and Osteen, 2003). The trophoblast implantation aggressiveness is similar to that in a tumour cell invasion. The fundamental dissimilarity is that the invasion process of the trophoblast is tightly controlled by MMPs and TIMPs (Salamonsen, 1999).

Table 1.1. Summary of members of the Matrix metalloproteinase family of enzymes expressed in human tissue.

Metalloproteinase	MMP	kDa	Substrates	Source
Collagenase	MMP-1	43	Collagen type (I, II, III)	Fibroblast, endothelial cells, keratinocytes, hepatocytes, macrophages, chondrocytes and blasts
	MMP-8	58	Collagen type (I, II), nonmatrix proteins for instance serpins, angiotensin I, bradykinin and substance P	Activated macrophages, chondrocytes, endothelial cells and smooth muscle cells.
	MMP–13	55	Collagen (II, IV, IX, X and XIV) , fibronectin, laminin, aggrecan core protein, fibrillin–1 and serine proteinase inhibitors	
Gelatinases	MMP–2	72	Collagen type IV, the main BM component	Fibroblasts, keratinocytes, endothelial cells, chondrocytes, osteoblasts and monocytes
	MMP–9	92	Collagens (IV, V, VII, X and XIV) gelatin entactin, elastin, fibronectin, Aggrecan, plasminogen, osteonectin and MB componenet	Invading trophoblasts, polymorphonuclear leukocytes, keratinocytes, osteoclasts and normal alveolar macrophages
Stromelysins	MMP–3, MMP–10 MMP–11	46 46 44	Collagen type (IV, V, IX and X), proteoglycans, fibronectin, gelatin, laminin and fibrillin-1	Fibroblastic cells,
Matrilysins	MMP-7	20	Fibronectin, laminin, collagen type IV and proteoglycans	Glandular epithelial cells in the endometrium, parotid, mammary gland, liver, pancreas, small intestinal crypts, prostate, skin and conducting airways in the lung.
	MMP-26			Uterus, kidney and placenta

Membrane-type MMPs	MT1–MMP	54	Collagen type (I, II and III), gelatin, laminin–1, fibronectin, cartilage proteoglycans and vitronectin.	Dermal fibroblasts and osteoclasts.
	MT2–MMP	61	of laminin, fibronectin and tenascin	Human placenta, heart and brain.
	MT3–MMP	55	Collagen type III, gelatin, fibronectin and casein	Placenta, ovary, lung, kidney, prostate, intestine, spleen, skeletal muscle and heart
	MT4–MMP	54	Fibrinogen and fibrin	Ovary, testis, leukocytes, colon and brain.
	MT5–MMP		Unknown	Kidney, brain, pancreas and lung
	MT6–MMP (MMP–25)		Collagen type 4, fibronectin, fibrin and gelatin	Leukocytes
Other MMPs	MMP–12	45	Type IV collagen, elastin, type I gelatin, laminin, fibronectin, vitronectin and proteoglycans	Placenta
	MMP–18		unknown	Placenta, ovary, mammary gland, lung, pancreas, thymus, small intestine, colon, spleen, testis, prostate and heart.
	MMP–19		unknown	Leukocytes and in a variety of human tissues including placenta, ovary, mammary gland, lung, pancreas, thymus, small intestine, colon, spleen, testis, prostate, heart, skeletal muscle, kidney, liver and brain
	MMP–20		Amelogenin	Dental tissues, the developing human teeth in the secretory enamel.
	MMP–21		Unknown	Placenta, ovary, liver, kidney, brain, lung and peripheral blood leucocytes
	MMP–22 MMP–23		Unknown	unknown
	MMP–27			Testis, intestine, lung and skin
	MMP–28		Casein	Human placenta

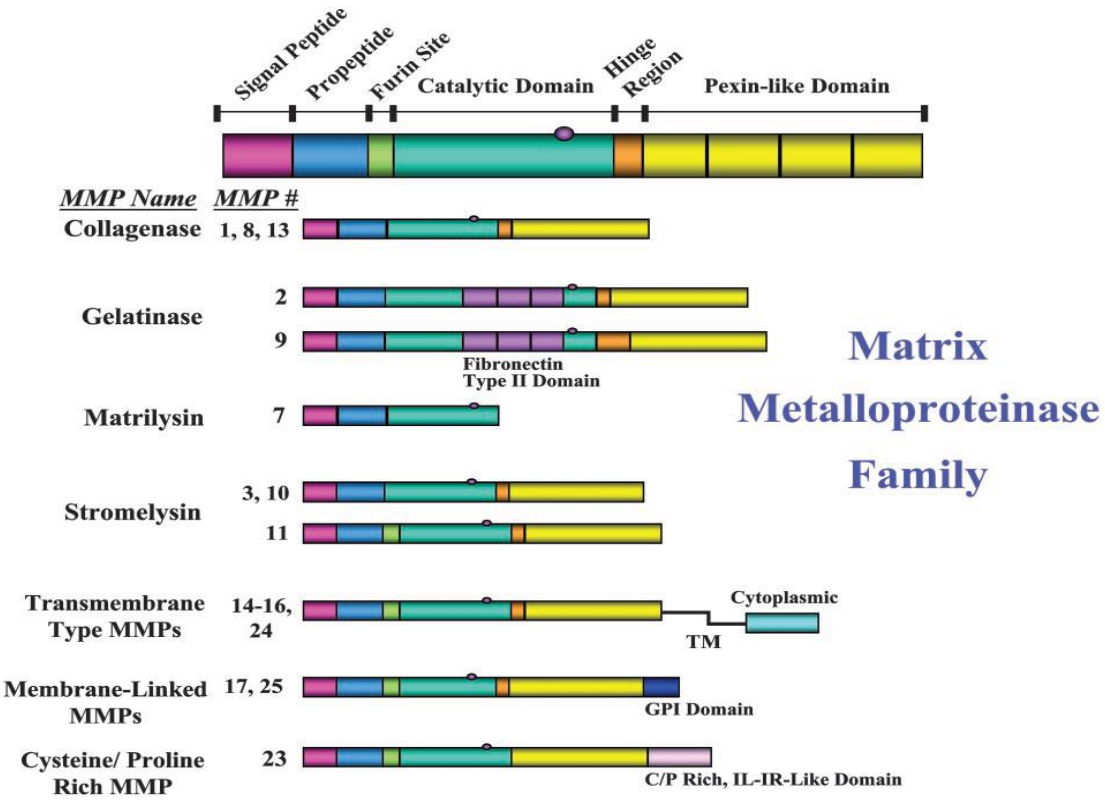

Figure 1.6. MMPs family

MMPs family contains a single peptide, pro-peptide domain, catalytic domain with the zinc-binding site and hemopexin-like domain. Some of the MMPs (gelatinases) have a fibronectin domain while other MMPs hold a furin-susceptible site. The membrane-linked MMPs have a glycophosphatidyl inositol domain while transmembrane type matrix metalloproteinases possess a transmembrane linker domain. Adapted from (Curry and Osteen, 2003).

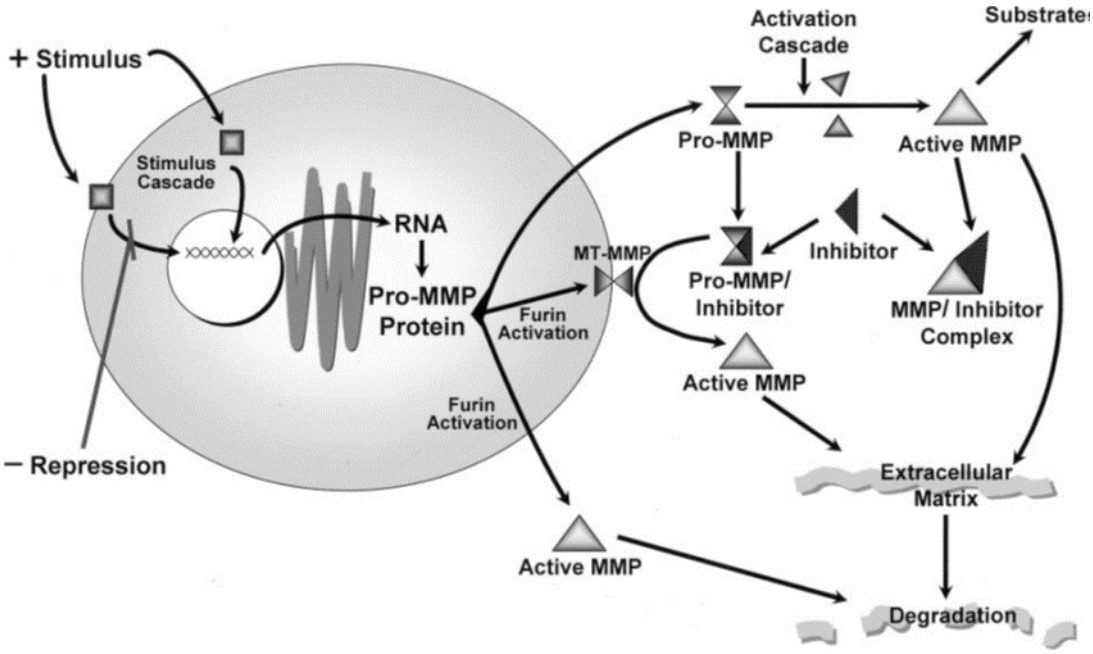

Figure 1.7. MMPs system regulation mechanism

The stimulation process acts via membranous or intracellular receptors. Intracellular stimulation cascades lead to the release of the MMP mRNA that is translated into the pro-MMP protein. Specific MMPs are activated inside the cell for instance MT-MMPs and MMP-11 while most of the MMPs are produced in an inactive form and need activation by other MMPs, serine proteinases. Active MMPs act on ECM leading to extracellular degradation or react with tissue inhibitor matrix metalloproteinase leading to suppression of matrix metalloproteinase activity. Adapted from (Curry and Osteen, 2003).

1.7.2. Role of matrix metalloproteinase in implantation

Even though numerous studies have investigated the role of MMPs in early embryo implantation, its precise role is still unclear. MMPs are suggested to play a crucial role in embryogenesis, ovulation, implantation and menstruation (Amalinei et al., 2007). The first step of successful placentation is the implantation of the blastocyst into the endometrium, where a considerable transformation of the uterine endometrium happens in the area of implantation (Amalinei et al., 2007). MMPs are the main mediators in the process of remodelling and degradation of ECM (Cohen et al., 2006). They have a potential action related to the bioactivity regulation of the molecules that are likely to play a vital role in the trophoblast-endometrial interaction. These molecules include cytokines, growth factors, vasoactive factors, angiostatic and angiogenic factors. The activation is either by releasing these factors from ECM stores or by activating the molecules by degradation or cleavage of the binding protein. Matrix metalloproteinases also modulate the stimulation of the other factors such as angiogenesis inhibitor factors and endostatin (Salamonsen and Nie, 2002).

The regulation of MMPs and TIMPs activity appears to be critical during the fetal-maternal interface for a successful implantation and placentation. Trophoblast cells express MMPs and therefor invasive by nature. Various studies using animal models, found that most MMPs are produced not only by trophoblast cells, but also by endometrial stromal cells and natural killer cells within the maternal uterus tissues. They suggest that decidual stromal cells have higher expression levels of MMP than trophoblast cells, and the decidua susceptibility to invasion appears to be increased in the presence of cytotrophoblast cells (Zhu et al., 2012).

Differences in MMPs production have also been founded. For instance, production of MMP-2 and MMP-9 has been localized in during early pregnancy most strongly than other MMPs. At 6–8 weeks the pro-MMP-2 expression is elevated over MMP-9 with subsequent decreasing concentrations, while the expression of pro-MMP-9 increases from 8 to 11 weeks, this lead to conclude that MMP-2 has a key role during implantation and MMP-9 during invasion (Espino et al., 2017). However, with the

progression of pregnancy the expression of pro-MMP and active MMPs by trophoblast are expressed with deferent variations, differential MMP production has also been confirmed before and after delivery. Therefore, dysregulation of the controlled process of trophoblast invasion can lead to adverse pregnancy outcomes. Excessively shallow invasion may result in fetal intrauterine growth retardation (IUGR) and preeclampsia. Proper trophoblast invasion is therefore essential for maternal health and adequate fetus growth and development (Kim and Kim, 2017).

The roles of MMP-2 and MMP-9 in implantation and placentation have been described (Xu et al., 2000). During the first trimester when invasiveness is maximal, human trophoblast secretes both gelatinases MMP-2 and MMP-9 (Hulboy et al., 1997). However, accumulating evidence has showed that MMP-9 is the more potent mediator compared to MMP-2 (Cohen et al., 2006). Both MMP-2 and MMP-9 degrade collagen IV, the main basement membrane collagen (Staun-Ram and Shalev, 2005). They also degrade other ECM proteins including collagen I, V, VII, IX, laminin, fibronectin, vitronectin and elastin (Cohen et al., 2006).

In humans, throughout the first trimester of pregnancy, MMP-2 is produced by extra villous trophoblast while MMP-9 is produced by villous cytotrophoblast (Cohen et al., 2006). MMP-2 is expressed mainly in early first trimester between 6-8 weeks while MMP-9 is expressed the main gelatinase later, between 9-12 weeks (Staun-Ram and Shalev, 2005).

Other MMPs involved in the trophoblast invasion process include MMP-3, MMP-11, MT1-MMP as well as MT2-MMP (Curry and Osteen, 2003). MT1-MMP and MT2-MMP are expressed in the tissue of human placenta during the 1st trimester. Apart from the degradation of laminin, fibronectin and gelatine, it has been found that these MMPs play a role in the activation process of other MMPs especially MMP-2 (Cohen et al., 2006).

Hurskainen et al studied five placentae obtained from legal abortions cases between 8-10 weeks of pregnancy and employed *in situ* hybridization. They showed that membrane type1-MMP (MT1-MMP) may have a role in the cytotrophoblast invasion and tissue organization of early placenta, because

of its abundantly expressed during trophoblast invasion, MT1-MMP might have a key in this process (Hurskainen et al., 1998). In support of these observations, another study indicated that MT1-MMP expressed in the uterine endometrium and decidua takes part in the adhesion and invasion of the blastocyst into the endometrium, and in the degradation and remodelling of the ECM (Nakano et al., 2001).

1.8. Tissue inhibitor of matrix metalloproteinases

Tissue inhibitor of matrix metalloproteinases (TIMPs) are a family of protein MMP inhibitors consisting of four members (Table.1.2). They act as key regulators of MMPs. The MMPs bind to TIMPs through the zinc binding site. The inhibitory effect of TIMPs on MMPs involves interaction between the active site of the MMPs, which is found in the catalytic domain and the TIMPs N-terminal domain (Curry and Osteen, 2003) as illustrated in figure 1.8.

TIMP-1, secreted by most cells (Skrzypczak et al., 2007a), acts as an inhibitory factor for most MMPs except MT1-MMPs and MMP-2 (Amalinei *et al.*, 2007). TIMP-2, secreted by endothelial and fibroblast cells (Skrzypczak et al., 2007a), it acts as an inhibitory factor for most of the MMPs. TIMP-3 inhibits MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13, which is expressed as a result of mitogenic stimulation. TIMP-4 is expressed mainly in the heart and following vascular injury and wounds, it acts as an inhibitor to MMP-2, MMP-9 and MMP-7 (Amalinei et al., 2007). However, some studies have observed that the TIMP-4 is also located in reproductive tissues, and it is suggested to have a mode of action similar to TIMP-2 (Curry and Osteen, 2003). In addition, to inhibition of MMPs, TIMPs have other biological activities such as erythroid potentiating activity, inhibition of endothelial cell proliferation and angiogenesis (Visse and Nagase, 2003). Furthermore, they also stimulate proliferation of cells and development of the embryo (Staun-Ram and Shalev, 2005). TIMP-1 and TIMP-2 act as antiapoptotic while TIMP-3 acts as proapoptotic factors (Amalinei et al., 2007).

TIMPs and MMPs are expressed during human embryo implantation. TIMP-1, TIMP-2 and TIMP-3 are secreted by trophoblast cells and decidual tissues during pregnancy (Staun-Ram and Shalev, 2005). TIMP-2 is also secreted by fibroblast cells and endothelial cells as a soluble protein and has high affinity to active or inactive MMP-2 (figure 1.8) (Skrzypczak et al., 2007b). Furthermore, TIMP-2 plays a significant role in the metabolism of MMP-2 and MT1-MMP (Staun-Ram and Shalev, 2005)

Table 1.2. Human tissue inhibitors of MMPs (TIMPs) family.

TIMPs	Other names	Molecular mass (Da)	Location of gene	MMP substrate
TIMP-1	MMP inhibitor 1 erythroid potentiating activity fibroblast collagenase inhibitor collagenase inhibitor	23 171	Chromosome X	MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -16
TIMP-2	MMP inhibitor 2, CSC-21K	24 399	Chromosome 17	MMP-1, -2, -3, -7, -8, -9, -10, -13, -14, -15, -16, -19
TIMP-3	MMP inhibitor 3, MIG-5 protein	24 145	Chromosome 22	MMP-1, -2, -3, -7, -9, -13, -14, -15
TIMP-4	MMP inhibitor 4	25 502	Chromosome 3	MMP-1, -2, -3, -7, -9

Adapted from (Cohen et al., 2006).

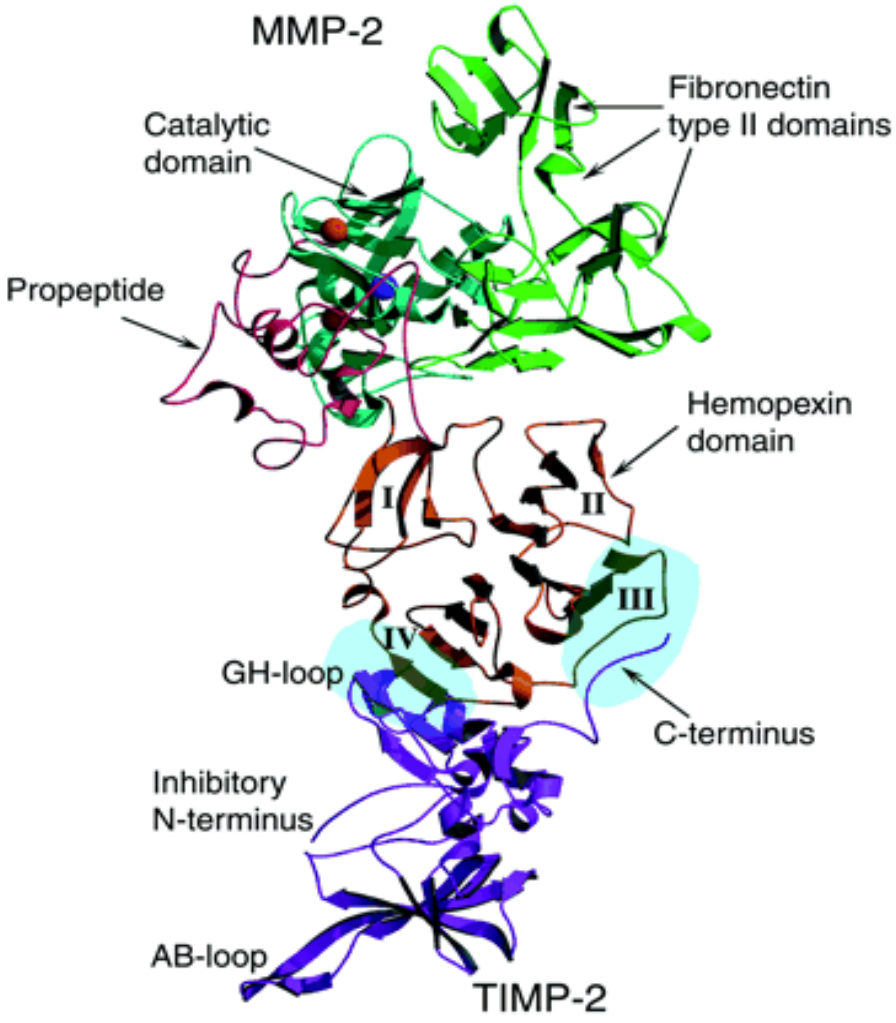

Figure 1.8. Structure of the proMMP-2/TIMP-2 complex

The figure shows the interaction of MMP-2 and TIMP-2 through their C-terminal domains. The MMP-2 catalytic site and TIMP-2 the inhibitory active site are turned away from each other. Catalytic, structural Zn2+ ions, Ca2+ ion, the hemopexin domain numbered from I to IV and number III and IV indicate two areas of interaction between proMMP-2 and TIMP-2 molecules.

1.9. Relaxin

Relaxin is a 6 kDa peptide hormone derived from the family of insulin-like growth factor (Sunder and Lenton, 2000). It was discovered by Dr Frederick Hisaw in the 1920s (Bathgate et al., 2013). There are three relaxin genes in humans: relaxin-H1, relaxin-H2 and relaxin-H3. In 1991, scientists determined the crystal structure of human relaxin that is found to be similar to that of insulin as illustrated in figure 1.9 (Ivell and Einspanier, 2002). Human relaxin-H1 and H2 genes are located on chromosome 9 and situated at a single locus while relaxin H3 gene is located on chromosome 19. Relaxin is secreted as a preprohormone and then converted by tissue convertases to relaxin hormone by cleavage of the c-domain (Hayes, 2004) and development of the disulfide bridge between A and B domains (Wilkinson et al., 2005).

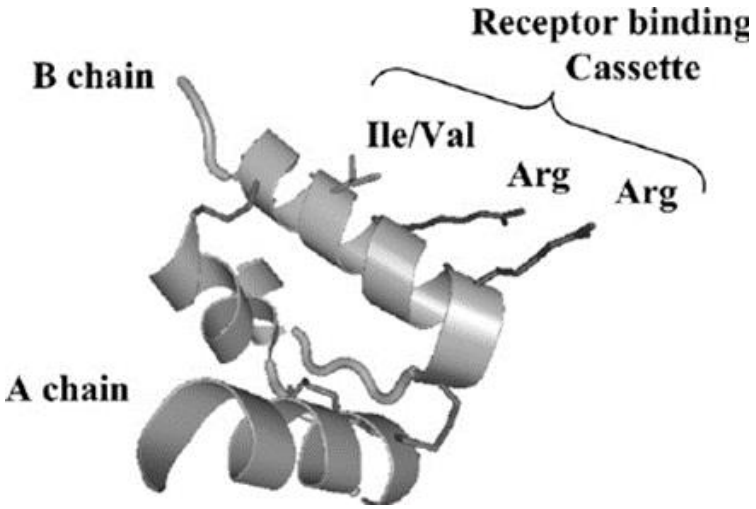

Figure 1.9. Crystal structure of RLX-2 hormone

Representation of the structure of H2 relaxin based on the crystal structure the separate A and B chains and the relaxin receptor binding motif. Adopted from (Samuel et al., 2006).

1.9.1. Reproductive functions of Relaxin

Relaxin is a hormone that has several effects in a variety of tissues, It has both reproductive and non-reproductive functions (Wilkinson et al., 2005). The major circulating form secreted and stored in human is Relaxin-H2 (RLX-2). It is produced mainly by the corpus luteum (Bogic et al., 1997, Vogel et al., 2006a) and considered as the predominant subtype in the human circulation (Wilkinson et al., 2005). During pregnancy, it is secreted in large amounts (Bathgate et al., 2013). The pattern of relaxin secretion in human pregnancy is different from other species. In human pregnancy, the peak serum levels are attained between 10-12 weeks gestation, followed by a gradual decline toward 24 weeks when levels remain steady until the end of gestation (Vogel et al., 2002), attaining maximum concentrations of only 1 ng/ml (Anand-Ivell and Ivell, 2014). In contrast, in other species such as rats, pigs and mice, the concentrations are higher and increasing throughout pregnancy with maximum levels about 100-200 ng/ml (Anand-Ivell and Ivell, 2014), declining just prior to parturition (Bathgate et al., 2013). However in women with ovarian failure who lack corpus luteum and become pregnant by IVF, circulating relaxin is undetectable (Bathgate et al., 2013) consistent with a primarily ovarian source in early pregnancy. Relaxin is also expressed in amniotic fluid in increasing concentrations from 10 to 14 weeks of pregnancy (Johnson et al., 1994).

In pregnancy, relaxin has endocrine functions (Bathgate et al., 2013) and plays a vital function in early pregnancy implantation, embryogenesis and placentogenesis (Anumba et al., 2009). Investigators have found that relaxin hormone enhances the uterine endometrial growth, including endothelial cell proliferation at the distal end of maternal spiral arteries. This suggest a role in endometrial preparation for implantation (Sherwood, 2004). It is an extreme stimulatory factor of a variety of growth factors and hormones including prolactin and insulin-like growth factor binding protein (Bonnans et al., 2014). Relaxin regulates the biochemical process of the ECM remodeling of the cervix and vagina throughout pregnancy (Parry and Vodstrcil, 2007).

Relaxin is suggested to act as a supportive factor for embryo implantation (Ivell and Einspanier, 2002). It has been demonstrated that endometrial receptors interact with RLX, therefore, they promote endometrial stromal cells differentiation in the decidualisation process which is important for embryo implantation (Ivell and Einspanier, 2002, Telgmann and Gellersen, 1998). Furthermore, it has been suggested that relaxin hormone acts as human chorionic gonadotropin by the reflection of the corpus luteum function. Therefore, it could be suggested that the low concentration of the serum relaxin in early pregnancy may indicate corpus luteum dysfunction that results in either spontaneous miscarriage or placental dysfunction (Vogel et al., 2006b).

1.10. Implantation failure (IF)

Unsuccessful implantation can result from abnormalities of the conceptus or the uterus. The rate of implantation failure is estimated to range between 30% and 70% of conceptus, although many women may not be aware that they were pregnant (Kennedy, 1997). Approximately 50% of IF are probably due to genetic defects in the conceptus while the others are of unknown aetiology (Gagnon, 2003). Failure of implantation may result in miscarriage, preeclampsia, Intrauterine growth retardation (IUGR), stillbirth or preterm birth (King and Loke, 1994).

1.10.1. Placenta dysfunction

The mechanism of the placental pathology that leads to the placental deterioration is still unidentified (Gagnon, 2003). Insufficient placentation may lead to progressive placental functional decline that results in fetal growth restriction due to a decrease in the transfer of nutrition and oxygen from the placenta toward the fetus (Fox, 1976). Placental dysfunction is one of the serious obstetric problems which should be considered carefully, as it has a significant influence on the fetus and the placenta. Moreover, placental insufficiency can result in longer term conditions in surviving fetuses such as cardiovascular, metabolic and neurological complications (Gagnon, 2003).

The Doppler flow velocity in the umbilical artery is an indirect measure of placental insufficiency, if abnormal it reflects an abnormal increase in umbilical placental vascular resistance which results in decreased umbilical blood flow. The placental pathology that leads to a reduction in umbilical blood flow can be due to abnormal function or structure of the primary unit of the placenta (Gagnon, 2003). Numerous studies reveal that defects in the decidua, placenta or its vascular bed may result in pre-eclampsia, fetal growth retardation, placenta abruption and preterm birth (Rasmussen et al., 1999).

1.10.2. Intrauterine growth retardation (IUGR)

Fetal growth restriction (FGR) is defined as “the rate of fetal growth that is below normal in light of the growth potential of a specific infant as per the race and gender of the fetus” (Sharma et al., 2016), and also defined as “the failure of fetus to reach its growth potential for various reasons, leading to multiple perinatal complications and adult diseases of fetal origins” (Tang et al., 2017). Fetal growth restriction is the second commonest cause of perinatal death after prematurity. Up to 4-6% of all pregnancies can be complicated by intrauterine growth restriction, 60% of which are due to placental insufficiency (Gagnon, 2003) and impaired growth of the feto-maternal placental unit. This problem is attributed to defective interaction between embryo trophoblast cells and maternal luminal uterine cells (King and Loke, 1994, Barut et al., 2010). Fetal well-being at term coordinates with normal development of the placenta. However the abnormal growth of fetal placenta may contribute to serious adverse pregnancy outcomes including pregnancy loss, preeclampsia, placenta abruption and intrauterine growth restriction (Kingdom et al., 2000). Several studies concluded that intrauterine growth restriction is related to the high incidence of abnormalities in adulthood. These include metabolic, endocrine and cardiovascular disorders amongst others (Fowden et al., 2006).

A recent study demonstrated that lower level of MMP-1 in the extra villous trophoblast might be associated with defective trophoblast invasion in pregnancies complicated by intrauterine growth restriction and preeclampsia (Lian et al., 2010). Another study analysed the amniotic fluid samples from 44 pregnant women with intrauterine growth restriction and 98 pregnant women with appropriate intrauterine fetal growth between 15-17 weeks’ gestation by PCR. They concluded that pregnancies at high risk for intrauterine growth restriction were associated with fetal expression of the MMP-2-1306 single nucleotide polymorphism genes, while MMP-9-1562 single nucleotide polymorphism genes and MMP-9 microsatellite showed no association (Gremlich et al., 2007).

1.10.3. Stillbirth

The rate of stillbirth in the UK is estimated to be one in every two hundred infants (Smith and Fretts, 2007). It is defined as *in utero* fetal death after 20 weeks gestation (Cnattingius and Stephansson, 2002). Smith et al reported that threatened miscarriage in the first trimester is associated with high risk of such adverse pregnancy outcomes as stillbirth, pre-eclampsia, preterm birth, IUGR and placental abruption (Smith et al., 2004). However, further research is required to understand the cellular and molecular basis of placentation (Smith and Fretts, 2007). To our knowledge, there are no studies that have investigated pregnant women with stillbirth and the expression of relaxin, MMPs or TIMPs in early pregnancy.

1.10.4. Premature birth

Preterm birth is described as delivery before 37 weeks of gestation. It affects 10% of all pregnancies (Cockle et al., 2007). The perinatal mortality and morbidity in preterm birth are 70% which is more among infants deliver prior to 32 weeks gestation, and with a weight less than 1500g (Cockle et al., 2007). Over the last three decades, preterm birth has been investigated by many scientists seeking to unravel the principal mechanism of the initiation of labour, which remains ill-understood (Dubicke et al., 2008). The key point in the protection of the fetal membrane strength and tensity is the ECM homeostasis (Vadillo-Ortega and Estrada-Gutierrez, 2005). Remodelling of the ECM during the softening of the cervix is associated with expression of several mediators including hormones and cytokines (Dubicke et al., 2008). In a recent study done by Anumba and his colleagues found that a strong association between high serum MMP-3 levels during the 20 weeks gestation and women who delivered preterm (Anumba et al., 2010).

A prospective cohort study showed that women delivering preterm had a different expression pattern of serum RLX-2 throughout pregnancy. In early pregnancy, they expressed lower serum RLX-2 and later in pregnancy they tend to have a high level of serum RLX-2 compared with term deliveries because it reduced slowly in women delivering preterm than women delivering at term. To determine the time of intersection, they need at least

two serum RLX-2 measurement in early pregnancy to define the accurate intersection time and this was the main limitation of this study (Vogel et al., 2006b).

1.10.5. Miscarriage

Early pregnancy loss or miscarriage is counted as the most frequent problem during pregnancy. It is defined by World Health Organization (WHO) as “the expulsion or extraction from its mother of an embryo or fetus weighing 500g or less” (Garcia-Enguidanos et al., 2002). The incidence of miscarriage among clinically-recognised conceptions is 15%, and it is increased if we consider the early reproductive losses which are near to 50% (Rai and Regan, 2006). Spontaneous miscarriage is sub-categorised into missed miscarriage, complete miscarriage, incomplete miscarriage, inevitable miscarriage, septic miscarriage, threatened miscarriage and recurrent miscarriage depending on the clinical feature (Griebel et al., 2005). This review will concentrate on threatened and recurrent miscarriage as they are the primary subject of the research.

1.10.5.1. Threatened miscarriage

A threatened miscarriage is defined as first-trimester vaginal bleeding in women with a viable pregnancy (Griebel et al., 2005). Approximately 25% of all conceptions have vaginal bleeding in the first trimester (Wijesiriwardana et al., 2006). Half of them end in spontaneous miscarriage, whereas the remaining half continue their pregnancy without complications (Wieringa-de Waard et al., 2002). It has been reported in a large retrospective cohort study that, primigravida women with a history of the first trimester bleeding are at higher risk of adverse pregnancy outcomes compared with those without a history of early pregnancy vaginal bleeding (Wijesiriwardana et al., 2006). The retrospective nature of the study considered as one of its drawbacks, another point is that data on the amount of bleeding were not gathered, as a result, they were not able to study the relationship between severity of bleeding and the adverse pregnancy outcomes. Most of the studies in the literature regarding threatened miscarriage investigate the relationship of early pregnancy vaginal bleeding and risk of obstetric complications. To our

knowledge, there are no studies that have investigated women with threatened miscarriage and the expression of RLX-2, MMPs or their tissue inhibitors of MMPs in early pregnancy.

1.10.5.2. Recurrent miscarriage

Recurrent miscarriage is defined as three or more consecutive spontaneous miscarriages. It has an incidence rate of 1% of women (Mtiraoui et al., 2004). Recurrent pregnancy loss is a heterogeneous situation with multifactorial causes. Although our understanding of recurrent miscarriage aetiology is limited, approximately 50% of cases have no known aetiology (Prakash et al., 2005, Laird et al., 2003, Li, 1998). Many cases are associated with impaired uterine receptivity (Dekel et al., 2010). However, unexplained recurrent miscarriage has received more attention from both researchers and clinicians and considered as a tough challenge.

The aetiology of recurrent pregnancy loss can be attributed to two main factors: embryological factors that are mainly due to abnormal karyotype and maternal factors including endocrine disorders, autoimmune defects, coagulation disorders and endometrial defects that may affect the endometrium, placenta or both (Laird et al., 2003). The risk of pregnancy loss is 30% in women with a history of two recurrent miscarriage while it is 33% in women with a history of three recurrent miscarriage and no history of live birth (Ford and Schust, 2009). Women with a history of recurrent miscarriage whose pregnancy progressed further than 24 weeks are at risk of adverse pregnancy outcomes. They have a high incidence of preterm delivery, intrauterine growth restriction and risk of caesarean section suggesting closer surveillance during pregnancy (Jivraj et al., 2001).

A number of serum molecules are changed in some women with a history of recurrent miscarriage although no one of these markers reliably predicts recurrent pregnancy loss (Anumba et al., 2010). Anumba and his colleagues, have recently found in a longitudinal prospective case-controlled study that pregnant women with a history of recurrent pregnancy loss have lower serum human RLX-2 and higher serum TIMP-2 in comparison with pregnant women with no history of recurrent miscarriage, while no differences in the

expression of MMP-1, MMP-3, MMP-9 and TIMP-1 between the two groups, suggesting a role for these two molecules (RLX-2 and TIMP-2) in implantation and angiogenesis (Anumba et al., 2010). They suggested that dysfunction of RLX-2 and TIMP-2 at the implantation stage may contribute to repeat pregnancy loss, perhaps by inhibiting endometrial and placental angiogenesis. This was the first study that investigated serum levels MMPs and TIMPs throughout human pregnancy. It has been suggested that the lower serum relaxin levels are likely to reflect reduced expression in reproductive tract tissue (Anumba et al., 2009). In a follow-on study, assessing TIMP2 levels in the same cohort of women. They also demonstrated that between 10 and 12 weeks gestation, a ratio of RLX-2: TIMP-2 < 0.1 was best associated with a history of recurrent miscarriage (Anumba et al., 2010).

The main limitation of these observations was the small sample size that limited the ability of the study to detect any association between serum marker levels and adverse clinical outcomes such as: stillbirth, premature delivery, repeat miscarriage and placental abruption. Furthermore, the study measured systemic serum marker levels rather than the production of RLX-2 and TIMP-2 from local reproductive tract tissue. This limited the conclusions that could be drawn from their observations since various non-reproductive tracts may contribute to the systemic expression of the serum markers studied (Anumba et al., 2010).

To explore these observations further, a prospective study of four cohorts of pregnant women is planned: pregnant women with a viable pregnancy with a history of recurrent miscarriage, normal pregnant group with no history of recurrent miscarriage with the same matching gastitional age, and women being seen in early pregnancy with a history of threatened miscarriage and their healthy normal pregnant group with no history of recurrent miscarriage with the same matching gastitional age. We will investigate whether serum RLX-2, MMP-2 and TIMP-2 levels have predictive potential for the following adverse pregnancy outcomes: repeat miscarriage, fetal growth restriction or premature delivery. We will also study *in vitro* endometrial-trophoblast cell line interactions and the effect on matrix protein and relaxin expression at

that interface. We will employ cell lines validated in previous studies most likely to mirror the *in vivo* situation.

Hypothesis

Trophoblast-endometrial interactions are affected by altered expression of TIMP-2, MMP-2 and human RLX-2 during early implantation.

Research question

1- Is there an association between the serum levels of RLX-2, MMP-2 and TIMP-2 and the timing of pregnancy loss in the index pregnancy?

2- Could these proteins (RLX-2, MMP-2 and TIMP-2) be employed as useful clinical markers for adverse pregnancy outcomes?

Objectives

1- To determine whether RLX-2, MMP-2 and TIMP-2 levels could be clinically useful markers of impending pregnancy loss during the current pregnancy.

2- To identify any associations between the serum levels of RLX-2, MMP-2 and TIMP-2 with adverse pregnancy outcomes including miscarriage, spontaneous preterm birth, fetal growth restriction, placental abruption and preeclampsia.

3- To determine whether dysregulation of the metabolism of RLX-2, MMP-2 and TIMP-2 characterises the endometrial implantation site prior to conception.

4- To explore, using appropriate cell lines and 3D endometrial model, trophoblast-endometrial interactions and the effect of RLX-2, MMP-2 and TIMP2 functional expression levels on implantation stages.

Chapter 2 Materials and Methods

2.1. *In vivo* study

2.1.1. Health and safety

The requirements and precautions recommended by the Control of Substances Hazardous to Health (COSHH) were followed before starting the laboratory work. All blood samples, materials and reagents were handled according to University Health and Safety regulations. Laboratory coats and gloves were worn during work in the laboratory.

2.1.2. Ethical approval

Ethical approval for this study was granted by South Yorkshire Research Ethics Committee (06/Q2305/128). Each participant was provided with a detailed information sheet about the study and written informed consent was obtained from all participants.

2.1.3. Study subjects

2.1.3.1. Recruitment

Women who fulfilled the inclusion criteria were recruited from the antenatal booking clinic, recurrent miscarriage clinic and the Early Pregnancy Assessment Unit at the Jessop Wing, Royal Hallamshire Hospital, Sheffield Teaching Hospitals, NHS Foundation Trust. Potential participants were approached by their doctor or midwife and provided with an information leaflet. If they agreed to take part in the study, a meeting was arranged with the researcher who explained what the study entails and written informed consent was obtained. Four cohort groups of women were recruited into this study.

2.1.3.2. Recurrent miscarriage women

The first group included women with a history of recurrent pregnancy loss; defined as three or more consecutive miscarriages. Women attending for their first antenatal (booking) visit were recruited from the recurrent miscarriage clinic at the Jessop Wing, Royal Hallamshire Hospital, Sheffield Teaching Hospitals, NHS Foundation Trust.

Inclusion criteria:

1. Maternal age between 20-40 years.
2. Pregnant women between 10-13 weeks' gestation
3. Pregnant women with a history of recurrent miscarriage.

Exclusion criteria:

1. Women who may have difficulty in understanding the protocol or in giving informed written consent.
2. Women with recurrent pregnancy loss attributed to uterine tumour, uterine malformation, or proven cervical weakness.
3. Women who are smokers.

2.1.3.3. Healthy pregnant women between 10-13 weeks' gestation (Control)

The women in the control group were recruited from the routine antenatal clinics at the Jessop Wing, Royal Hallamshire Hospital, Sheffield Teaching Hospital, NHS Foundation Trust. Women were attending for their first antenatal (booking) visit.

Inclusion criteria:

1. Maternal age between 20-40 years.
2. Pregnant women between 10-13 weeks' gestation.
3. Pregnant women without a history of recurrent miscarriage or infertility.

Exclusion criteria:

1. Women who may have difficulty in understanding the protocol or in giving informed written consent.
2. Pregnant women with a history of recurrent miscarriage, threatened miscarriage or infertility
3. Women who are smokers.

2.1.3.4. Threatened miscarriage women

The third group comprised women in early pregnancy with threatened miscarriage, between 6-10 weeks gestation. These women were recruited from an Early Pregnancy Assessment Unit at the Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Foundation Trust.

Inclusion criteria:

1. Maternal age between 20-40 years.
2. Pregnant women between 6-10 weeks gestation presented with early vaginal bleeding and viable pregnancy
3. Pregnant women without a history of recurrent miscarriage or infertility.

Exclusion criteria:

1. Women who may have difficulty in understanding the protocol or in giving informed written consent.
2. Women who are smokers.

2.1.3.5. Healthy pregnant women between 6-10 weeks' gestation (control)

The fourth group included healthy pregnant women from an Early Pregnancy Assessment Unit at the Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Foundation Trust

Inclusion criteria:

1. Maternal age between 20-40 years.
2. Pregnant women between 6-10 weeks gestation with out a history of recurrent miscarriage, threatened miscarriage or infertility .

Exclusion criteria:

1. Women who may have difficulty in understanding the protocol or in giving informed written consent.
2. Pregnant women with a history of recurrent miscarriage, threatened miscarriage or infertility.
3. Women who are smokers.

2.1.3.6. Blood samples

Investigations were carried out on subjects not exceeding 13 weeks gestation. 15 ml of peripheral venous blood was collected from consenting subjects and divided between two tubes: a serum test tube (BD Biosciences, USA) and a vacutainer tube with K2 EDTA (BD Biosciences, USA). The samples were transported to the lab immediately and were centrifuged (MSE Mistral 2000, DJB Lab care, UK) within 30 minutes of collection at 1300 g for 10 minutes to obtain serum and plasma. Serum and plasma samples were each aliquoted into three vials and stored at -80°C (Revco, USA) before analysis.

2.1.3.7. Outcome measure

The primary clinical outcome was miscarriage. Secondary clinical outcomes included, the birth of a small infant for gestation age (birth weight less than the 10^{th} customized centile for gestation), premature delivery before 34 weeks gestation, stillbirth, placental abruption and the development of pre-eclampsia.

2.1.4. Enzyme-linked ImmunoSorbant Assay

Enzyme-linked ImmunoSorbant assay (ELISA), is an immunoassay used to quantify the amount of test substance in a sample. ELISA is based on antibody specificity for a target antigen. The capture antibody is coated onto the surface of a well, then captures the antigen from the test solution if it is present. A secondary antibody is used (detection antibody) that binds to the same antigen but at a different site on the molecule. The detection antibody is usually conjugated with an enzyme that can covert a substrate into a colour product the optical density of which can be determined spectrophotometrically. The amount of colour product formed is directly related to the amount of test antigen in the sample. This amount can be quantified using a standard curve.

In this project, ELISA was used to measure the amount of RLX-2, MMP-2, MMP-9, TIMP-2 and MMP-2/TIMP-2 complex in the serum of the four groups of subjects studied. The main advantage of ELISA is high sensitivity and

specificity, it can be used to investigate a large numbers of samples at one time, and it is a safer alternative to radio-immunoassay (RIA) that uses radioactive labels.

The ELISA kits used in this study were as follows:

1. Human RLX-2 immunoassay (R&D Systems, Minneapolis, USA) the minimal detected dose (MDD) of human RLX-2 is between 0.26-4.57 pg/ml. The kit is highly specific for human RLX-2, as insulin, IGFs, relaxin-1 and relaxin-3 showed no significant cross-reactivity. The detection range of the kit is between 7.8 - 500 pg/ml.

2. Human MMP-2 ELISA assay (DuoSet development kit, R&D Systems, Minneapolis, USA). This kit is highly specific for human pro-MMP-2 and active MMP-2, but does not recognise MMP-2/TIMP-2 complex. There is no detectable cross-reactivity with other MMPs and TIMPs, but there is cross-reactivity with TIMP-2 at a concentration of more than 1.56 ng/ml. The detection range of the kit is between 0.6 - 20 ng/ml.

3. Human MMP-9 ELISA assay (DuoSet development kit, R&D Systems, Minneapolis, USA). This kit is highly specific for human pro-MMP-9 and active MMP-9. There is no detectable cross-reactivity with other MMPs and TIMPs, but there is cross reactivity with recombinant human TIMP-1 at a concentration of more than 1.56 ng/ml. The detection range of the kit is between 31.2 - 2,000 pg/ml.

4. Human TIMP-2 ELISA assay (DuoSet development kit, R&D Systems, Minneapolis, USA). This kit is highly specific for human TIMP-2. There is no detectable cross-reactivity or interference with any other TIMPs. The detection range of the kit is between 31.2 - 2,000 pg/ml.

5. Human MMP-2/TIMP-2 complex ELISA assay (DuoSet development kit,R&D Systems, Minneapolis, USA). This kit is highly specific for human MMP-2/TIMP-2 complex. There is no detectable cross-reactivity with other MMPs and TIMPs, but there is cross reactivity with MMP-2/TIMP-3 at a concentration of more than 12.5 ng/ml. The detection range of the kit is between 125 - 8,000 pg/ml.

6. βhCG ELISA Assay, this assay allows for the quantitative detection of βhCG (Abcam, Cambridge, UK) and was used to measure βhCG released by JAR spheroids. The assay was performed according to a manufacturers protocol, the intra-assay coefficient of variation (CV) was calculated at <10%, and the inter-assay was <12%. The mean minimal detected dose was 50 pg/ml. The kit is highly specific for βhCG and shows no cross-reactivity with the following cytokines including IL-1α, IL-1β, IL-2, IL-3, other interleukins, Leptin, TIMP-1, TIMP-2, TNF-α, TNF-β and others. The detection range of the kit is between 54.87 - 40000 pg/ml.

2.1.4.1. General sandwich ELISA protocol

The concentration of human RLX-2, MMP-2, MMP-9, TIMP-2 and MMP-2/TIMP-2 complex was measured in the supernatents and the serum blood samples of the four groups according to the manufacturers' protocol.

The capture antibody was diluted to the working concentration using PBS. Then a high binding 96-well plate was immediately coated with 100 µl of the recommended diluted capture antibody in each well. The plate was sealed and incubated overnight at room temperature. The following day, the coating solution was removed and the plate was washed with 400 µl wash buffer three times. Complete removal of the liquid after each wash was important. Following the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean, absorbent paper towels. Non-specific binding sites were blocked using 300 µl reagent diluent to each well. The plate was incubated for a minimum one hour at room temperature before wells were rinsed with wash buffer three times as previously described. At this point, the plate was ready for addition of standards and samples.

One hundred microliters of standard solutions or samples (serum/ supernatant) were added to each well to bind to the capture antibody. The standards were run in duplicate while the serum/supernatant samples were run in triplicate. The plate was covered with an adhesive strip and incubated at room temperature for two hours after which the solutions were discarded and wells washed with wash buffer following the same step as mentioned previously. After the detection antibody was diluted in a reagent diluent, 100

µl of the diluted detection antibody was added to each well and incubated for two hours at room temperature. The solutions were then discarded and washed with wash buffer as previously described.

Streptavidin-HRP was diluted to working concentration using a reagent diluent, and 100 µl were added to each well, the plate covered to avoid direct light and incubated at room temperature for 20 minutes. After plate washing 100 µl of the substrate solution [1:1 mixture of colour reagent one A (H_2O_2): colour reagent B (tetramethylbenzidine)] was added to each well and incubated at room temperature in the dark for 20 minutes. Finally, 50 µl of stop solution was added to each well to stop the enzyme reaction. The plate was gently tapped to ensure mixing and the optical density of the solution in the wells measured at 450 nm with a wavelength correction set to 540 nm using a spectrophotometer (Infinite 200 PRO, Tecan, Reading, UK). DeltaSoft Microplate Analysis Software (BioMetallics, Inc) was used to analyse the data. Details of capture and detection antibodies, standards and solutions used in the procedures are illustrated in (Table 2.1) and (Table 2.2).

Table 2.1. ELISA kits

Elisa kit	Capture antibody	Detection Antibody	Standard
Human MMP-2	-360 µg/ml -Mouse anti-human MMP-2 -Working concentration 2 µg/ml	-90 µg/ml -Mouse anti-human MMP-2 -Working concentration 500 ng/ml	-1920 ng/ml -High Standard: 20 ng/ml
Human MMP-9	-180 µg/ml -Mouse anti-human MMP-2 -Working concentration 1 µg/ml	-54 µg/ml -Gout anti-human MMP-9 -Working concentration 300 ng/ml	-95 ng/ml -High Standard: 2000 pg/ml
Human TIMP-2	-360 µg/ml -Mouse anti-human MMP-2 -Working concentration 2 µg/ml	-9 µg/ml -Gout anti-human TIMP-2 -Working concentration 50 ng/ml	-95 ng/ml -High Standard: 2000 pg/ml
Human MMP-2 /TIMP-2 complex	-288 µg/ml -Goat anti-human MMP-2 -Working concentration 1.6 µg/ml	-72 µg/ml - Goat anti-human TIMP-2 -Working concentration 400 ng/ml	-160 ng/ml -High Standard: 8000 pg/ml

Table 2.2. Solutions used in the ELISA procedure

Solution	Ingredients	Company	Catalog no.
PBS	137 mM NaCl 2.7 mM KCl 8.1 mM Na2HPO4 1.5 mM KH2PO4	R&D Systems	DY006
Wash buffer	0.05% Tween 20 in PB	R&D Systems	WA126
Reagent Diluent	1% BSA (Bovine Serum Albumin) in PBS	R&D Systems	DY995
Substrate solution	1:1 mixture of Colour Reagent A (H2O2) and Colour Reagent B (Tetramethylbenzidine)	R&D Systems	DY999
Stop solution	2 N H2SO4	R&D Systems	DY994

2.1.4.2. Human Realxin-2 and βhCG ELISA protocol

The assay procedure for Human Realxin-2 ELISA started by preparing all reagents, working standard and samples as directed by manufacture instruction. Then 100 µl of the Assay diluent RD1-19 were added to each well. Fifty microliters of standard solution and serum sample or supernatant were added to each well. The standards were run in duplicate while the serum/ supernatant samples were run in triplicate. The plate was covered with an adhesive strip and incubated at room temperature for two hours, after which the solutions were discarded and wells washed with wash buffer following the same step as mentioned previously. The rest of the steps are the same as mentioned in the previous protocol. Two hundred microliter of RLX-2 conjugate was added to each well. The plate was covered with new adhesive strip and incubated for 2 hours at room temperature. The washing step was repeated as mentioned in the previous protocol. The rest of the steps are similar to the previous general ELISA protocol (section: 2.1.4.1).

The assay procedure for βhCG ELISA started by adding 100 µl of standard solution and sample supernatant to each well. The standards were run in duplicate while the supernatant samples were run in triplicate. The plate was covered with an adhesive strip and incubated at room temperature for two hours after which the solutions were discarded and wells washed with wash buffer following the same step as mentioned previously. The rest of the steps are the same as mentioned in the previous general ELISA protocol (section: 2.1.4.1).

2.1.5. Data collection and management

Data regarding adverse pregnancy outcomes including miscarriage, spontaneous preterm birth, fetal growth restriction, placental abruption and pre-eclampsia were collected using a predesigned proforma, from the study subjects directly and/or from their notes and entered into a computer database for analysis. The computer on which data was entered was secured by encryption and password protected, and the forms were securely kept in a locked cabinet in a locked office that was accessible only to the lead investigator.

2.1.6. Statistical analysis

Both parametric and non-parametric tests were employed as appropriate to compare means or medians. The Student's t-test or the Mann-Whitney U test or ANOVA with multiple comparisons for data containing a number of groups was used to compare the serum levels of RLX-2, MMP-2, MMP-9, TIMP-2 and MMP-2/TIMP-2 complex.

2.2. *In vitro* study

2.2.1. Health and safety

Materials and reagents were handled as stated by the University of Sheffield Health and Safety Regulations. The precautions and requirements mentioned in Control of Substances Hazardous to Health (COSHH) were followed before starting the laboratory work. Laboratory coats and gloves were worn at all times during laboratory work.

2.2.2. General cell culture

All cell culture procedures were performed under sterile conditions in a biological safety class II laminar flow cabinet (Walker Safety Cabinets Ltd, Glossop, UK). Equipment and solutions were sterilized by autoclaving (MP 25 Control, Rodwell Scientific Instruments, UK) for 20 minutes at 121°C. All equipment that could not be sterilised by autoclaving were sterilised using 70% ethanol. Cells were cultured according to manufacturers instructions, and incubated at 37°C with an atmosphere of 5% CO_2, 95% humidity (Galaxy R Plus CO_2 incubator, Scientific Laboratory Supplies, Nottingham).

2.2.3. Cell lines and their complete growth media

2.2.3.1. HEC-1-A

An endometrial epithelial cell line derived from a 71-year-old female (Catalogue no. HTB-112) was obtained from American Type Culture Collection (ATCC, Manassas, VA). The characteristics of the HEC-1-A are human epithelial endometrium adenocarcinoma, with adherent growth properties (Hannan et al., 2010). HEC-1-A cells were grown in McCoy's 5A medium supplemented with 1.5 mM L-glutamine, 2200 mg/L sodium bicarbonate (ATCC, Manassas, VA) 10% fetal bovine serum (FBS) (ATCC, Manassas, VA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Poole, UK) (Kuramoto et al., 1972).

2.2.3.2. RL95-2

An endometrial epithelial cell line that was originally derived from a 65-year-old female (ATCC, catalogue no. CRL-1671). The characteristics of the RL95-2 are a human epithelial endometrium carcinoma with adherent growth properties (Hannan et al., 2010). RL95-2 cells were grown in a 1:1 volume of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 medium supplemented with 2.5 mM L-glutamine, 0.5 mM Sodium pyruvate, 15 mM HEPES, 1200 mg/L sodium bicarbonate, 0.005 mg/ml insulin (ATCC, Manassas, VA), 10% FBS (ATCC, Manassas, VA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Poole, UK) (Way et al., 1983).

2.2.3.3. Ishikawa cell line

An endometrial epithelial cell line derived from a 39-year-old female (Sigma-Aldrich, Poole, UK, catalogue no.99040201). The characteristics of the Ishikawa cell line are human epithelial endometrium adenocarcinoma with adherent growth properties (Hannan et al., 2010). Ishikawa cells were grown in a Modified Eagle's Medium (MEM) supplemented with 2mM Glutamine, 1% Non-Essential Amino Acids (NEAA) (Sigma-Aldrich, Poole, UK), 5% FBS (ATCC, Manassas, VA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Poole, UK) (Castelbaum et al., 1997).

2.2.3.4. JAR cell line (trophoblast cell line)

A trophoblast cell line derived from a male foetus (ATCC, catalogue no. HTB-144). The characteristics of the human epithelial choriocarcinoma are adherent growth properties. JAR cells were grown in a RPMI-1640 medium (ATCC, Manassas, VA) supplemented with 10% FBS (ATCC, Manassas, VA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Poole, UK) (Sasaki et al., 1982).

2.2.3.5. Endometrial stromal cell (T-HESCs)

Fibroblast cells immortalized with hTERT that was derived from an adult female (ATCC, catalogue no. CRL-4003™). The characteristics are of human endometrial fibroblasts with culture adherent growth properties. T-HESCs were grown in a DMEM/F12 at a 1:1 v/v ratio (without phenol red)

supplemented with 3.1 g/L glucose, 1mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 1% Insulin-Transferrin-Selenium (ITS)+ Premix (BD Cat no. 354352), 500 ng/mL puromycin, 10% charcoal/dextran-treated FBS (HyClone Cat no. SH30068.03), 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Poole, UK) (Krikun et al., 2004).

2.2.4. Cell culture

Cell lines, frozen in 1 ml vials stored in liquid nitrogen, were thawed in a 37°C water bath for 2-3 minutes, the water was kept lower than the O-ring and the cap. After the vial contents, had thawed, the vial was removed from the water bath and decontaminated by spraying with 70% ethanol. In a class II hood, the contents of the vial were transferred to a universal centrifuge tube and diluted with 9 ml of the recommended pre-warmed complete growth medium. The tube was centrifuged at 200 x g for 5 minutes. The supernatant was removed and the cells pellet resuspended in 10 ml of the recommended growth medium. Cells were added to a 75 cm^2 tissue culture flask (Nunclone, Fisher Scientific UK, Loughborough, UK) at the desired concentration and incubated at 37°C with an atmosphere of 5% CO_2, 95% humidity. Complete growth medium was renewed every other day. In all the experiments, decidualisation of T-HESCs was performed in DMEM/F12 by treatment with 0.5 mM 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) (Sigma-Aldrich, Poole, UK) for five days (Gonzalez et al., 2011). Cells were washed twice with PBS before used.

2.2.5. Cell line subculture

Once the cells had reached 70%-90% confluence, cells were subcultured. The growth culture medium was removed and discarded and cells briefly rinsed twice with 10 ml phosphate-buffered saline (PBS) (Sigma-Aldrich, Poole, UK). This step is necessary to remove any traces of serum that would suppress the activity of the trypsin. The cell monolayer was completely covered with 5 ml 0.25% Trypsin-EDTA solution (Sigma-Aldrich, Poole, UK) for 5-10 minutes. Cells were incubated at 37°C to facilitate cell detachment. Then the cells were gently aspirated and added to 5 ml of complete medium

and the mixture centrifuged (Sigma 3-18K, Scientific laboratory supplies, UK) at 200 x g for 5 minutes. The supernatant was removed and the cell pellet re-suspended in 10 ml recommended complete growth medium. The cell suspension was diluted to the appropriate seeding volume to obtain the required seeding density and reseeded in fresh 75 cm^2 culture flasks. Cells were incubated at 37°C with an atmosphere of 5% CO_2, 95% humidity. Complete growth medium was renewed every 2-3 days.

2.2.6. Cryopreservation of cell lines

Cryopreservation of cells by freezing maintains cell reserves. Cell lines were removed from the surface of flasks as described in (section: 2.2.5.). Cell pellets were resuspended in the recommended complete culture medium supplemented with 5% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Poole, UK). One ml of the suspension was transferred to labelled cryovials (Greiner Bio-one, Germany) and stored in a Nalgene cryo-freezing container (Nalgene, USA) at -80ºC with a steady decrease in temperature. After 24 hours, all cryovials were transferred to liquid nitrogen until required.

2.2.7. Local expression of MMP-2, MMP-9, TIMP-2, RLX-2, endometrial epithelial markers and endometrial stromal cell markers by all cell lines using immunohistochemistry

2.2.7.1. Agarose/formaldehyde for fixing cell lines for wax processing

After 80% confluency all cell lines were detached from cell culture flasks as described in (section: 2.2.5.). After centrifugation the cell supernatants were discarded and cell pellets washed three times with PBS. Excess PBS was removed and cells were fixed with 10% PBS-buffered formalin for at least 2 hours. Following centrifugation excess fixative was removed and 100 µl agarose/formaldehyde [2g Agarose (Sigma, A-9539), 90 ml distilled water and 10 ml 40% formaldehyde (BDH/Merk, 28421)] was carefully added to make sure the cell pellets was not disturbed. The agarose was allowed to set for 20 minutes, and then the agarose containing cells pellet was gently removed and placed in small plastic cassette for processing, and then this was wax-embedded as described in (section: 2.2.8.2).

2.2.7.2. Principles of immunohistochemistry

Immunohistochemistry, a procedure used to identify and localize antigens. Antibodies are used to detect the target antigens based on an antibody-antigen reaction. The process involves locating proteins in cells and tissue specimens through the use of a visual marker. Immunohistochemistry methods are divided into two techniques, direct and indirect technique. The direct technique involves a single conjugated antibody reacting directly with the antigen in tissue sections. However, this technique is often seen as insensitive due to small signal amplification. The indirect method involves identification of antigen by a primary antibody with signal amplification provided by conjugated secondary antibodies that are raised against the primary antibody.

Immunohistochemistry requires several steps. The first step is fixation of samples and is considered to be a critical step as any inadequate fixation will lead to unsuccessful or impaired Immunostaining. The substances used for immunohistochemistry fixation should maintain the morphology of the cell

and tissue and the target epitopes antigenicity. The most common fixative substance used is 10% neutral buffered formalin although other fixatives include paraformaldehyde, ethanol, methanol and Boulin solution. Fixation with formalin leads to masking of antigens by generating cross-linking methylene bridges between basic amino acids residues in tissue samples. Samples are often embedded in paraffin after fixation, sectioned using microtomes to approximately 5 microns, then mounted onto tissue-adhesive-coated slides and left at room temperature overnight to remove excess water or dehydration.

The second step is deparaffinization and rehydration of tissue sections. This is an essential step before Immunostaining as inadequate removal of paraffin can lead to poor staining of sections because antibodies cannot react or reach with the target epitope. Deparaffinization is performed using xylene and tissues rehydrated using serial dilutions (100%, 90%, 70% and 50%) of alcohol. Next, antigen sites are unmasked using various forms of antigen retrieval such as heating via microwave oven, pressure cooker, autoclave heating, steam heating or water bath heating in a citrate, Tris-buffered or EDTA solution. Enzymes (trypsin, pronase etc) are sometimes used to unmask antigens. Endogenous peroxidase in tissues/cells is then quenched using a hydrogen peroxide solution followed by protein blocking (usually BSA or serum) to avoid high background staining and a false positive result due to antibodies binding nonspecifically to proteins.

Two types of antibodies are used to detect the antigen: the first type is polyclonal antibodies and the second type is monoclonal antibody. Polyclonal antibodies are heterogeneous antibodies that identify multiple epitope while monoclonal antibodies are homogeneous and specific for a single epitope. Consequently, monoclonal antibodies are more specific to the target epitopes than the polyclonal antibodies. Unconjugated primary antibodies react with the antigen of interest, whereas secondary antibodies reacted with the primary antibody. The secondary antibody is commonly conjugated to a linker molecule, such as biotin, which then recruits reporter molecules such as enzyme-conjugated avidin often termed the avidin-biotin complex (ABC).

There are several techniques used to identify the antigen of interest; the most common methods are the chromogenic and fluorescent techniques. The chromogenic technique identifies the target antigen by relying on labelled enzyme that binds to a substrate to form coloured end product that can be investigated with an ordinary light microscope. Horseradish peroxidase and alkaline phosphatase are the most common enzymes used, whilst DAB and Vector Red are common substrates. Moreover, the fluorescent technique that identifies the target antigen by the fluorophore that conjugated with primary or secondary antibody and it is analysed by fluorescent microscopy. Tissue and cells are often counterstained with haematoxylin to provide contract to the immunostain. Sections are then dehydration, in serial alcohol concentrations and dehydrated in xylene before being mounted to visualize the sections under the microscope.

2.2.7.3. Immunohistochemical staining in wax-embedded endometrial epithelial cell lines and T-HESCs

Immunohistochemistry was used to identify antigens in sections from the wax-embedded cell lines and tissues. The technique was optimized for each individual antigen of interest. All steps were performed at room temperature unless otherwise stated. Paraffin-embedded endometrial cell lines (5 µm) were de-waxed with xylene and rehydrated through ethanol (5 minutes each concentration) and then washed in distilled water twice for five minutes. Antigen retrieval was performed by immersing the slides into a heat resistant plastic staining dish containing citrate buffer (10 mmol/l) and microwaved on high power for 8 minutes. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 20 minutes after rinsing the slides briefly in PBS for five minutes. Slides were washed twice in Tris-buffered saline and Tween 20 (TBS+ 0.1% tween-20) for five minutes and then samples were circled with a solvent resistant pen. Slides were blocked with 5% serum from the animal species of the primary antibody in PBS for 30 minutes at room temperature. After washing, slides were incubated with 200 µl of primary antibody diluted in (5% serum + PBS) as illustred in table 2.3, and incubated overnight at 4°C. Optimisation to find the best concentration of the antibody was performed using serial dilutions of the antibody (table 2.3)

to obtain minimal background staining. The negative control slides had the primary antibody omitted to control for non-specific binding of the detection system. Slides were removed from the refrigerator and washed twice for five minutes with PBS. After washing, 200 µl the secondary biotinylated antibody solution was added to each slide and incubated in humidified chamber for 30 minutes at room temperature. The slides were then rinsed for 5 minutes in PBS twice. The ABC reagent was prepared as described in the manufacturer's instructions and the solution was allowed to stand for 30 minutes before use. 200 µl of ABC reagent was added to each slide then incubated for 30 minutes at room temperature, then slides were rinsed and incubated for 5 minutes twice in PBS. Antibody binding to target antigens of interest was visualised using DAB (3, 3'- diaminobenzidine) stain. The DAB reagent was mixed well and added to slides and incubated for 5 minutes at room temperature until the stain had developed to the required intensity. Slides were rinsed for 5 minutes in distilled water and placed in distilled water until counterstained with hematoxylin using a Shadon linear stainer. Finally, slides were mounted in Distyrene, a plasticizer, and xylene mounting (DPX) and covered with glass slide.

Table 2.3. Details of antibodies used in immunohistochemistry

Antibody	Clonality	Clone	DF	Company	Cat no
Galectin 9	Rabbit polyclonal		1:250	Abcam, Cambridge, UK	ab69630
Cytokeratin 7	Mouse monoclonal	RCK105	1:1000	Abcam, Cambridge, UK	ab9021
Vimentin	Mouse monoclonal	V9	1:1000	Abcam, Cambridge, UK	ab8069
CD10	Mouse monoclonal	56C6	1:25	Abcam, Cambridge, UK	ab951
Relaxin-2	Rabbit monoclonal	EPR14205	1:500	Abcam, Cambridge, UK	ab183505
TIMP-2	Mouse monoclonal	3A4	1:25	Abcam, Cambridge, UK	ab1828
MMP-2	Mouse monoclonal	4D3	1:25	Abcam, Cambridge, UK	ab2462
MMP-9	Rabbit polyclonal		1:100	Abcam, Cambridge, UK	ab744277
hCG	Rabbit monoclonal	EPHCGR2	1:100	Abcam, Cambridge, UK	Ab131170

DF = Dilution factor

2.2.8. Expression of MMP-2 and MMP-9 secreted from cell lines using gelatin zymography

2.2.8.1. Basic principles of zymography

Matrix metalloproteinases are important for protein catabolism. The family of MMPs contains at least 26 members that have been divided into subgroups based on the molecular structure and the substrate preference (Cohen et al., 2006). MMPs are secreted as proenzymes (inactive form) (Snoek-van Beurden and Von den Hoff, 2005) and converted to an active form by cleavage. MMPs play a fundamental role in extracellular remodelling in a wide range of physiological processes such as implantation, embryogenesis and angiogenesis. On the other hand, they also contribute to the pathological process of many diseases such as inflammation, tumour metastasis, arthritis and a number of cardiovascular pathologies such as heart failure, ischemic heart disease and atherosclerosis. The activity of matrix metalloproteinases is controlled by tissue inhibitors of the matrix metalloproteinases that are a group of four antagonists that inhibit proteases by binding to the catalytic site (Snoek-van Beurden and Von den Hoff, 2005).

MMPs can be identified by a number of techniques, however, the most common used is zymography. This assay detects MMPs based on their molecular weight and on the degradation of their preferred substrate such as gelatin, albumin, casein, etc. Gelatin zymography is the technique mainly used for the identification of MMP-2, MMP-9 (often termed gelatinases) in cells and conditioned medium samples (Snoek-van Beurden and Von den Hoff, 2005).

Gelatin zymography is an electrophoretic method, it is a simple, powerful, quantifiable and functional technique to identify the latent and activated forms of MMPs. The gelatin zymography method depends on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and a gelatin substrate that is copolymerised within a polyacrylamide matrix. Samples are prepared using the standard SDS-PAGE buffer in the absence of heating or reducing agents such as dithiothreitol (DDT) or mercaptoethanol. When the electrophoresis is completed, the SDS is

removed from the zymogram gel. The movement of the molecule through the gel rely on many factors including the size, the shape, the net charge of the molecule, the field strength, the ionic strength and the medium properties through which the molecules migrate such as pore size and viscosity (Snoek-van Beurden and Von den Hoff, 2005, Claudia M. d'Avila-Levy, 2012). After electrophoresis the gel was incubated in non-ionic detergent (Triton X-100) to renature the peptidases through removing the denaturing SDS, subsequently the gel was incubated in an appropriate activation buffer which reactivates enzyme activity allowing the MMPs to cleave the gelatin in the gel. After the gel is stained areas of enzyme activity are distinguished, these appear as clear bands on a blue stained background due to Coomassie staining (Claudia M. d'Avila-Levy, 2012).

2.2.8.2. Gelatin zymography

Gelatin Zymography gel was made with the use of gels (10% polyacrylamide containing 0.1% gelatin. Before starting the procedure, all solutions were prepared as mentioned in (Table 2.4). All materials were purchased from Sigma, Poole, UK unless otherwise stated.

Table 2.4. Preparation of gelatin zymography solutions

Solution	Volume
2X Sample loading buffer	
0.5 M Tris-HCL, pH 6.8	2.5 ml
10% SDS	4.0 ml
Glycerol	2.0 ml
0.1% Bromophenol Blue	0.5 ml
Distilled water	To 10 ml
10% Ammonium persulfate solution (Mw=228.2 gm)	
ammonium persulfate	500mg

Distilled water	5 ml
1 % Gelatine substrate	
Gelatine	1g
Distilled water	100 ml
10 % SDS solution	
SDS	10 g
Distilled water	100ml
Coomassie blue stain stock	
Coomassie blue stain R-250	2.0 g
Distilled water	200 ml
Coomassie blue stain	
Coomassie blue stain stock	62.5 ml
Methanol	250 ml
Acetic acid	50 ml
Distilled water	200 ml
Destain Solution	
Methanol (50%)	500 ml
Acetic acid (10%)	100 ml
Distilled water (40%)	400 ml
1.5 M Tris base pH 8.8	
Tris base	23.64 g
Distilled water	100 ml
0.5 M Tris base pH 6.8	
Tris-HCl	7.88 g
Distilled water	100 ml
1X Gel running buffer (pH=8.3)	
Tris base	2.9 g
Glycine	14.4 g
SDS	1.0 g

Distilled water	Up to 1 L
1X Renaturing buffer	
Triton X-100	12.5 ml
Distilled water	500 ml
10X Developing Buffer (pH=7.5)	
0.5 M Tris	60.57 g
2M NaCl (Mw=58.44)	116.88 g
50 mM CaCl2.2H2O (Mw=147.01)	7.35 g
1% Triton X-100	10 ml
Distilled water	Up to 1L

Preparation of separating (resolving) gel:

The separating gel was prepared first with a final 10% acrylamide concentration according to order as mentioned in (table 2.5) for detecting MMP-2 and MMP-9.

Table 2.5. Separation gel

Resolving Gel	Volume
40% Acrylamide	5 ml
Resolving gel buffer 1.5 M Tris (pH=8.8)	5 ml
Distilled water	7.66 ml
1% Gelatin substrate	2 ml
10% SDS	200 µl
10% Ammonium persulfate	100 µl
TEMED	20 µl

The SDS was used without delay after addition of Tetramethylethylenediamine (TEMED) as the polymerization process is relatively quick. A pipette was used to add the resolving gel solution into sandwich plates slowly. Isopropanol was added to the top of the gel to remove bubbles. The resolving gel was allowed to polymerise for approximately 30 minutes. Once polymerised the alcohol was decanted, and the gel was rinsed with distilled water to remove any remaining alcohol. Any remaining distilled water on top of the gel was drained from the unit with a small thin strip blotting paper.

While the resolving gel was polymerised, the stacking gel was prepared accordingly as mentioned in (table 2.6).

Table 2.6. Stacking gel

Stacking gel	Volume
40% Acrylamide	720 µl
Stacking gel buffer 1.5 M Tris (pH=6.8)	2.4 ml
Distilled water	6.28 ml
10% SDS	100 µl
10% Ammonium persulfate	100 µl
TEMED	10 µl

The stacking gel was mixed and poured onto the separating gel. The well comb (10 combs) was inserted and the gel was allowed to polymerise for another 30 minutes. The protein sample (cell supernatant or cell lysate) was prepared using the 2 x zymogram loading buffer (20 µl sample + 20 µl loading dye) and incubated at room temperature for 30 to 60 minutes, samples were not boiled and no reducing agents were used.

Once the stacking gel had polymerised, the well combs were removed, then the gels inserted into the electrophoresis apparatus. The apparatus was filled with running buffer (both the upper and lower container). Samples were loaded with the equivalent amount into the wells using fine tipped pipette tips. The molecular weight marker was loaded into the first lane and a positive control in the last lane and the electrophoresis run at 125 V, for approximately 70 minutes until the blue dye had run off the end of the gel. Once the electrophoresis was completed the plates were removed from the apparatus and opened carefully to remove the gel, which was then inserted into transparent plastic box and washed with Renaturing buffer on a shaker at room temperature for 1 hour. This was repeated four times with fresh buffer each time (15 minutes for each wash). The Renaturing buffer was decanted and replaced with 1X developing buffer and incubated for 24 hours at 37°C.

After 24 hours, the developing buffer was removed and the gel was stained with coomassie stain and placed on the rotator for 3 hours at room temperature. The gel then was destained with destaining solution for 1 hour to reveal transparent bands representing an area of protease activity against a dark blue background.

2.2.9. Expression of MMP-2, MMP-9 and TIMP-2 secreted from T-HESCs and JAR cell line treated with various concentrations of RLX-2

At 80% confluency in a 75 cm² flask, T-HESCs and JAR cells were trypsinised, centrifuged and seeded at about (2 x 10^5 cells/ml and 4x 10^5 cells/ml) respectively into 6 well plate (each contained 1 ml) and allowed to adhere overnight. Cells were treated with recombinant RLX-2 human (1 to 100 ng) (Sigma-Aldrich, Poole, UK), or medium alone as a control in triplicate. Cell culture conditioned medium (section: 2.2.3.4 and 2.2.3.5) was collected at 24, 48 and 72 hours, centrifuged at 10,000 x g for 10 minutes and stored at -80°C for further analysis by ELISA.

2.2.10. Expression of MMP-2 and MMP-9 secreted from T-HESCs and JAR cell line treated with various concentrations of TIMP-2 and Batimastat.

At 80% confluency in a 75 cm² flask, T-HESCs and JAR cells were trypsinised, centrifuged and seeded at about (2 x 10^5 cells/ml and 4x 10^5 cells/ml) respectively into 6 well plate (each contained 1 ml) and allowed to adhere overnight. Cells were treated with increasing dose of either recombinant TIMP-2 human, broad-spectrum matrix metalloproteases inhibitor (Batimastat) (Sigma-Aldrich, Poole, UK) (1 to 100 ng) or medium alone as a control in triplicate. Cell culture conditioned medium (section: 2.2.3.4 and 2.2.3.5) was collected at 24, 48 and 72 hours, centrifuged at 10,000 x g for 10 minutes and stored at -80°C for further analysis by ELISA.

2.2.11. Preparation of tissue engineered 3D uterine endometrial models

To produce a tissue engineered 3D uterine endometrial model, the following components listed in (Table 2.6.) were mixed on ice in 50 ml conical tubes. All materials were purchased from Sigma, Poole, UK unless otherwise stated.

Table 2.7. Reagents for three gels

10 gels	Volume (µl)
10x DMEM (13.48g of DMEM powder in 100ml distilled water)	940
Reconstitution Buffer (2.2g sodium bicarbonate, 5.2g HEPES, 0.24g NaOH)	940
Fetal calf serum	780
L-Glutamine	96
5 mg/ml rat-tail collagen (Trevigen, Gaithersburg, US)	6060

The mixture was acidic (determined by the colour-yellow), and so a few drops of 1N NaOH were added to neutralise it and the tube was inverted to mix (the colour then became light pink). T-HESCs were added to the mixture, with each culture made to contain 1 x 10^6 T-HESCs. The mixture was inverted gently several times to mix the solution. Seven hundred µl of the cell mixture was added into each cell culture transwell insert (Nunc serving the life science, Denmark). The inserts were then transferred gently to 6-well cell culture plates (Greiner Bio-one, Germany) and incubated at 37°C, 5% CO_2 in a humidified atmosphere for two hours until the mixture was solidified. The gels were then submerged with endometrial epithelium growth medium from outside and inside the inserts and incubated at 37°C, 5% CO_2 in a humidified atmosphere. After 24 hours, the T-HESCs growth medium was aspirated

from the inserts, and endometrial epithelial cells were seeded over the gels (1 x 10^6 cells diluted in 400 µl medium for each insert). Sterile forceps were used to place the inserts on top of the three metal stands with 0.3 cm^2 size and 0.15 cm height stood inside each well. The endometrial epithelium growth medium was gently added to each well outside the inserts, and the gel surfaces were kept dry as an air-to-liquid interface for cell differentiation. The cell culture plate was transferred carefully to an incubator and incubated at 37°C, 5% CO_2 in a humidified atmosphere. The medium was replaced every other day for two weeks.

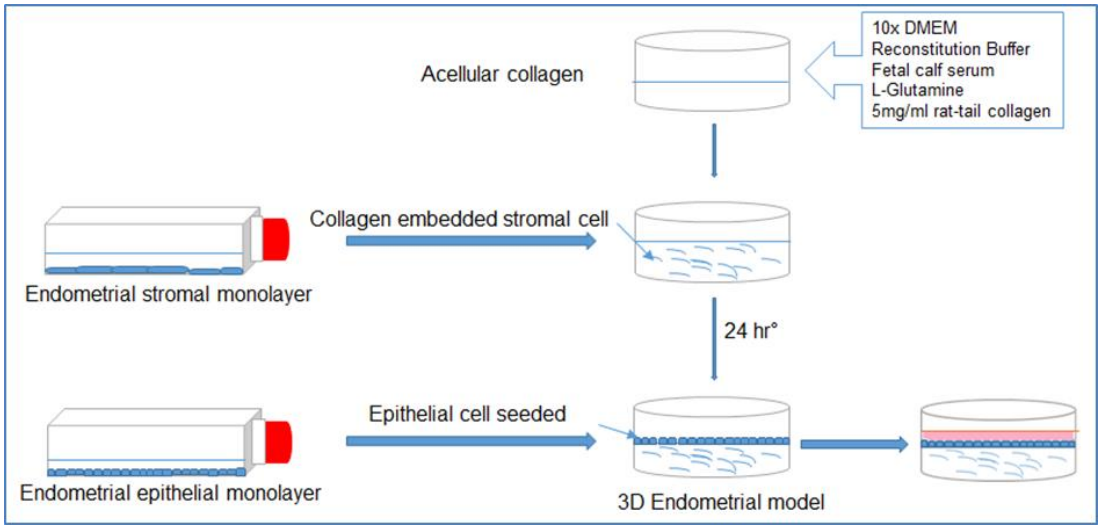

Figure 2.1. Procedure outlines of 3D endometrial models with monolayer epithelial

2.2.12. Preparation of 3D models for histology

2.2.12.1. Chemical fixation with 10% formalin, processing, embedding and sectioning samples.

3D models were fixed with 10% formalin overnight. Each model was then placed into a processing/embedding cassette and further soaked in buffered 10% formalin to preserve cells and tissues from degradation and to maintain cellular and subcellular structure. Tissues were processed through alcohol gradients to xylene and paraffin wax using a Shandon Citadel 2000 tissue processing machine (GMI, USA). Each biopsy model was cut into two pieces

from the middle and placed in the embedding moulds (the cut edges were faced down). External embedding by wax was achieved using the Histoncentre 2 external embedding unit (Thermo Shandon, UK). The tissue was then covered with the cassette and kept for 15-20 minutes to form hardened blocks. Paraffin wax blocks containing tissue were sectioned using a microtome machine (Leica RM 2235, Germany) at 5 µm thickness. The tissue sections were transferred to a water bath and mounted on slides for staining.

2.2.12.1. Staining

Haematoxylin and Eosin (H&E) staining was performed automatically by a *Shandon Linistain GLX Linear Stainer* machine (Thermo Scientific, UK). Slides were mounted using DPX mounting media (*Raymond A Lamb,* East Sussex, UK) and cover-slipped ready for histology examination. Slides were examined under a light microscope (Olympus BX51, Germany) and sections imaged using Olympus imaging solution GmbH Camera (Olympus, Germany).

2.2.13. Tissue engineered 3D models with monolayer epithelium

To improve the model and make it resemble the endometrium *in vivo*, the epithelium layer needs to be a monolayer. To achieve this, after the epithelial cells were seeded, the model was incubated for two hours at 37°C, 5% CO_2 in a humidified atmosphere to enhance epithelium attachment. Gently, 1.5 ml of fibroblast growth medium was added to the wells outside the inserts and incubated for 24 hours. Then the medium was replaced with endometrial epithelium growth medium inside and outside the inserts and incubated for a further 2-3 days until the cells became confluent (Csibra et al., 2000). The epithelial layer of the model was kept covered with endometrial epithelium growth medium instead of airlifting in order to control the differentiation of the cells. The six-well plate was incubated at 37°C, 5% CO_2 in a humidified atmosphere for 5-7 days, the medium being replaced every 2-3 days**.** After one weeks, the models were ready for histology study.

2.2.14. Attachment Assay of Human Choriocarcinoma JAR spheroids to the endometrial epithelium monolayer cell lines (RL95-2, HEC-1-A and Ishikawa) and T-HESCs.

2.2.14.1. Optimisation of JAR spheroids

In order to mimic trophoblasts, spheroids from JAR trophoblast cells were produced on the basis of a method defined by Korff *et al,.* (Korff et al., 2004) Initial experiments were performed to optimise the kinetics of JAR spheroids growth. JAR monolayers were trypsinization washed with PBS and harvested as mentioned in (section: 2.2.5). The cells pellet was suspended with recommended growth media and cells counted and resuspended at 1 x 10^6 cells/ml. Increasing JAR cell concentrations per 100 µl RPMI-1640 media (100, 250, 500, 1000, 5000 and 10,000 cells) were added to wells of a non adherent U-bottom 96 well plates (Greiner Bio-one, Germany, catalogue No.: 650970) and centrifuged in a swinging bucket rotator at 300 x g for 3 minutes. Plates were then transferred to an incubator and cultured at 37°C, 5% CO_2 in a humidified atmosphere to determine the ideal JAR spheroids size, which is from 150 µm to 250 µm. Spheroids were measured every day for nine days using the 4X objective. Media were changed every other day, the supernatant collected every other day and stored at -80C° for further ELISA analysis.

2.2.14.2. Measurement of βhCG secreted by JAR spheroids

JAR spheroids of two different sizes from 150-250 µm and from 1500 - 2000 µm were cultured for 8 days and the conditioned medium from these were collected on days 2, 4, 6 and 8 and stored at -80°C for further βhCG ELISA analysis, refer to (section: 2.1.4.)

2.2.15. Attachment of JAR spheroids to endometrial cell lines

The RL95-2 cell line was used alongside HEC-1-A, Ishikawa and T-HESCs cell lines to establish an *in vitro* attachment assay. However, after the establishment of the attachment assay, only RL95-2, Ishikawa cell line and endometrial stromal cell line were used for further experiments. All cell lines

used were grown until 80% confluent in T75 flasks. Subsequently, cells were harvested using trypsin/EDTA as outlined in (section: 2.2.5). All cell lines were cultured in a 6 well plate and incubated until confluent. Confluent monolayers of Ishikawa, HEC-1-A, RL95-2 and T-HESCs were co-cultured with 20 JAR spheroids that were gently picked and delivered to each well using a wide pipette tip. Each cell line was co-cultured in the recommended media. Tissue culture plates were transferred to the tissue culture incubator at 37°C, 5% CO_2 in a humidified atmosphere. Adhesion of spheroids was measured at (1, 6, 24 and 48 hours). The non-adherent spheroids were removed from the well by washing gently twice using Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (Sigma-Aldrich, Poole, UK). Each wash lasted for 5 minutes and the well were completely filled with Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} and covered with adhesive tape to dislodge the loosely adherent and non-adherent spheroids. The final number of JAR spheroids was counted and the results expressed as the percentage of spheroids attached to the monolayer from the initial total number of spheroids added.

2.2.16. The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 treatment on JAR spheroids co-cultured with endometrial monolayers.

In a 6-well plate cell monolayers grew in complete growth media (section: 2.2.3), JAR spheroids were co-cultured and treated at the same time with RLX-2 (10 ng/ml), MMP-2 (10 ng/ml), MMP-9 (10 ng/ml) and TIMP-2 (10 ng/ml) all obtained from (Sigma-Aldrich, Poole, UK). Untreated cells were used as controls. Twenty JAR spheroids were added onto the top of the different monolayer cell lines (Ishikawa, RL95-2 and T-HESCs) using a sterile Pasteur pipette. All JAR spheroids were between 200 and 300 µm. After each co-culture time (2, 6 and 24 hours) the wells were washed twice using Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (Sigma-Aldrich, Poole, UK) to remove the non-adherent JAR spheroids and the per cent adherent spheroids calculated as mentioned before (section: 2.2.15).

2.2.17. The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 treatment on JAR spheroids co-cultured with 3D endometrial model

To study the attachment of JAR spheroids to the 3D endometrial model, the 3D endometrial culture system was set up in 4-well plate (Nunc, Roskilde, Denmark), and then cultured for 4-5 days before starting the assay. Twenty JAR spheroids were transferred onto the top of each 3D model using a Pasteur pipette. For each four wells a different treatment was applied including control (without treatment), RLX-2 (10 ng/ml), MMP-2 (10 ng/ml), MMP-9 (10 ng/ml) and TIMP-2 (10 ng/ml). After each co-culture time (6 and 24 hours), models were washed twice using Ca^{2+} and Mg^{2+} Dulbecco's phosphate-buffered saline to remove the loosely adherent JAR spheroids as described in (section: 2.2.15). The final number of JAR spheroids was calculated and the results were expressed as the percentage of spheroids attached to the initial total number of spheroids added.

2.2.18. Invasion study using 3D matrix invasion assay and JAR spheroids under the effect of of RLX-2, MMP-2, MMP-9 and TIMP-2.

An invasion assay kit (Trevigen, Gaithersburg, US) was used to study the invasion of JAR spheroids in the 3D matrix. JAR spheroids were produced as in (section: 2.2.15.1). Breast cancer MDA-MB-231 cells were used as a positive control. All cells were cultured, trypsinised, harvested and counted as previously described. A single cell suspension was prepared with 1 x spheroid formation extracellular matrix (basement membrane extract (BME)). The 10 x spheroid formation ECM was thawed overnight at 4°C, diluted with the complete growth media at 4°C and mixed by pipetting up and down using a sterile pipette. Cells were re-suspended in 1 x spheroid formation ECM and 50 µl of the cell suspension added to each well of the 96 well plate at approximately 100-150 cells per well. The 96 well plate was centrifuged at 200 xg for 3 minutes in a swinging bucket rotator at room temperature. The plate was incubated at 37°C in a tissue culture incubator for 72 hours to promote JAR spheroids formation.

After 72 hours, the invasion matrix was thawed at 4 C° overnight and centrifuged at 300 x g for 5 minutes at 4°C to avoid bubbles and the 96 well

spheroids formation plate was placed on ice for 15 minutes to cool the wells. Working on ice, 50 µl of invasion matrix was added to each well. The 96 well plate was centrifuged at 300 x g for 5 minutes at 4C° in a swinging bucket rotator to avoid bubbles and make the spheroids within the invasion matrix locate in the middle of the well. The plate was then incubated at 37°C for 1 hour to promote the invasion matrix to form a gel. After 1 hour, 100 µl of warm (37°C) cell culture media was added to each well. Treatments included: control (no treatment), RLX-2 (10 ng/ml), MMP-2 (10 ng/ml), MMP-9 (10 ng/ml) and TIMP-2 (10 ng/ml) performed in quadruplicate. Then the 96 well plate was incubated at 37°C in a tissue culture incubator for 5 days. Spheroids in each well were photographed every 24 hours using the 4x objective and images were analysed using Image J software (NIH, USA).

2.2.19. Spheroid expansion assay under the effect of RLX-2, MMP-2, MMP-9, TIMP-2 and Batimastat.

T-HESCs was plated in 12 well plates at a density of 1 x 10^5 cells/ml complete growth media DMEM/F12 (section: 2.2.3.5). When the cells reached 80% confluent, JAR spheroids were added to the T-HESCs monolayer in 20 µl culture media, one spheroid per well. Treatments applied were: control (without treatment), RLX-2 (10 ng), TIMP-2 (10 ng), MMP-2 (10 ng), MMP-9 (10 ng) and Batimastat (10 ng). The 12 well plates were incubated at 37°C in a tissue culture incubator for 4 days. Using a digital camera, spheroids in each well were photographed every 24 hours with a 4x objective, and this was counted as time point d0, d2, d3 and d4 respectively. Finally, all spheroid areas were measured using Image J software (NIH, USA). Expansion of individual spheroid over the 4 time points was calculated, each time relative to the previous time point. Each treatment was carried out on triplicate wells and each experiment was repeated 3 times.

Chapter 3 Expression of MMP-2, MMP-9, TIMP-2 and RLX-2 in the serum of pregnant women with and without a history of recurrent miscarriage

3.1. Introduction

Recurrent miscarriage is defined as three or more repeated spontaneous miscarriage and occurs with a rate of 1% of all women (Mtiraoui et al., 2004). However, recurrent miscarriage is a heterogeneous condition that may be as result of multifactorial fundamental causes. In approximately 50% of cases the aetiology is unidentified (Prakash et al., 2005, Laird et al., 2003, Li, 1998), although many recurrent miscarriage cases are related to impaired uterine receptivity (Dekel et al., 2010).

Implantation is the most critical process during the establishment of conception. The study of the molecular basis of embryo implantation has widely expanded and has not only been explored in animals but also now in humans. Human implantation starts with the invasion of the epithelium of the uterus and underlying stroma by embryonic trophoblast cells. This process is complicated and involves a series of molecular and cellular interactions (Burrows et al., 1996). Failure of implantation may cause many clinical pathological problems such as early miscarriage, preeclampsia, intrauterine growth restriction (IUGR), stillbirth and preterm birth (King and Loke, 1994).

A number of serum molecules are altered in some women with recurrent miscarriage, although no single one of these markers can reliably predict recurrent miscarriage (Anumba et al., 2010). Recently, in a longitudinal prospective case-controlled study Anumba and colleagues demonstrated that women with a history of recurrent miscarriage have lower serum human RLX-2 and higher serum TIMP-2 levels, suggesting roles for these molecules in implantation and angiogenesis. These authors suggested that dysfunction of RLX-2 and TIMP-2 at the implantation stage may contribute to repeat pregnancy loss, attributable in part, to inhibition of endometrial and placental angiogenesis (Anumba et al., 2009, Anumba et al., 2010).

To explore these observations further, a prospective study of four cohorts of pregnant women was planned: normal pregnant women with no history of recurrent miscarriage, pregnant women with a viable pregnancy but who have a history of recurrent miscarriage, and women being seen in early pregnancy with a history of threatened miscarriage and their controls. The

idea was to evaluate whether serum RLX-2, MMP-2, MMP-9 and TIMP -2 levels have the predictive potential for the following adverse pregnancy outcomes: miscarriage, foetal growth restriction or premature delivery (Anumba et al., 2009, Anumba et al., 2010).

Hypothesis

Trophoblast-endometrial interactions are affected by altered expression of MMP-2, TIMP-2 and human RLX-2 during early implantation.

Objectives

To determine whether RLX-2, MMP-2 and TIMP-2 levels could be clinically useful markers of impending pregnancy loss during the current pregnancy. Furthermore, to identify any associations between the serum levels of RLX-2, MMP-2 and TIMP-2 with adverse pregnancy outcomes including miscarriage, spontaneous preterm birth, fetal growth restriction, placental abruption and preeclampsia.

3.2. Materials Methods

3.2.1. Subjects and samples

Frozen aliquots of serum from 22 pregnant women between 10-13 weeks gestation (first trimester) with a history of recurrent miscarriage, and a control cohort of 23 gastitional age-matched pregnant women between 10-13 weeks gestation without history of recurrent miscarriage. The third group comprised 13 women in early pregnancy between 6-10 weeks gestation with threatened miscarriage (who presented with vaginal bleeding and a viable pregnancy confirmed on ultrasound) from an Early Pregnancy Assessment Unit. The fourth group comprised a healthy control group of 9 pregnant women with no vaginal bleeding between 6-10 weeks gestation, recruited from an Early Pregnancy Assessment Unit, Royal Hallamshire Hospital, University of Sheffield, UK. The samples were stored at -80°C and assayed for MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 and RLX-2 using ELISA.

3.2.2. Enzyme-linked ImmunoSorbant Assay (ELISA)

ELISA kits were used to identify the serum levels of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 and RLX-2 provided by (R&D Systems, Minneapolis, USA). The procedures were done according to the manufacturer instructions. (For more details refer to (section: 2.1.4.1).

3.2.3. Statistical analysis

The statistical analysis was performed using Graph Pad Prism version 6.0. Both parametric and non-parametric tests were employed as appropriate to compare means or medians between the groups. The Student t-test or the Mann-Whitney U test were used to compare the serum levels of RLX-2, MMP-2, MMP-9 and TIMP-2 levels. $P<0.05$ were considered to be significantly different.

3.2.4. Outcome measure

Serum concentrations of RLX-2, MMP-2, MMP-9, TIMP-2 and TIMP-2/MMP-2 complex, were determined and their individual associations with the following clinical outcomes tested: spontaneous miscarriage, premature delivery (before 37 weeks gestation), stillbirth, placental abruption and small for gestational age (fetal estimated birth weight less than the 10th customized centile).

3.3. Results

3.3.1. Clinical results

3.3.1.1. Recurrent miscarriage group and their controls

Table 3.1 summarises the general clinical details of the recurrent miscarriage group and their healthy controls and the outcomes of their pregnancies. There was no significant difference in the rate of miscarriage, preterm birth and pre-labour spontaneous rupture of foetal membrane. However, there was a significant increase in the rate of caesarean section and the rate of post-delivery blood loss in the recurrent miscarriage group.

Table 3.1. Subjects (recurrent miscarriage and controls) clinical details and pregnancy outcomes

Clinical variables	Recurrent miscarriage group	Control group	P value
n	22	23	
Mean (SE) Body mass index (BMI)	25.84 (1.19)	24.60 (1.20)	0.47
Median (range) age (years)	33.5 (19-44)	29 (22-39)	
Mean (SE) gestational age at delivery (weeks)	38+6 (0.41)	40+2 (0.29)	0.01
Delivery before 37 weeks, n (%)	1 (4.5%)	0 (0%)	
Mean (SE) birth weight (g)	3198 (114.5)	3493 (114)	0.08
Pre-labour spontaneous rupture of fetal membrane, n (%)	1 (4.5%)	1 (4.3%)	
Infant birth weight < 2500g, n (%)	2 (9%)	1 (4.3%)	0.5
Still births, n (%)	0 (0%)	0 (0%)	
Miscarriage, n (%)	2 (9%) (11 and 13) weeks	0 (0%)	0.15
Pregnancy induced hypertension (PIH)	1 (4.5%)	0 (0%)	
Emergency cesarean section (EMCS)	8 (36%)	1 (4.3%)	0.009
Mean (SE) Post-delivery blood loss	577.5 (52.63)	361.9 (36.99)	0.0017

3.3.1.2. Threatened miscarriage group and their controls

Table 3.2 summarises the general clinical details of the threatened miscarriage group and their healthy controls and the outcomes of their pregnancies. There was no significant difference in the rate of miscarriage, preterm birth, pre-labour spontaneous rupture of fetal membrane, rate of caesarean section and post-delivery blood loss between the both groups.

Table 3.2. Subjects (threatened miscarriage and controls) clinical details and pregnancy outcomes

Clinical variables	Threatened miscarriage group	Control group	P value
n	13	9	
Mean (SE) Body mass index (BMI)	25.85 (1.43)	25.89 (2.20)	0.99
Median (range) age (years)	27 (22-41)	31 (19-34)	
Mean (SE) gestational age at delivery (weeks)	39.59 (0.66)	38.86 (0.48)	0.42
Delivery before 37 weeks, n (%)	1 (7.7%)	1 (11%)	0.7
Mean (SE) birth weight (g)	3445 (149.2)	3283 (225.4)	0.54
Pre-labour spontaneous rupture of fetal membrane, n (%)	1 (7.7%)	0 (0%)	0.4
Infant birth weight < 2500g, n (%)	1 (7.7%)	1 (11%)	0.7
Still births, n (%)	0 (0%)	0 (0%)	
Miscarriage, n (%)	3 (23%) (9,11 and 13 weeks)	1 (11%) (9 weeks)	0.4
Pregnancy induced hypertension (PIH)	0 (0%)	0 (0%)	
Emergency cesarean section (EMCS)	0 (0%)	2 (22%)	0.08
Mean (SE) Post-delivery blood loss	538.9 (158.3)	400.0 (62.68)	0.47

3.3.2. Laboratory analysis

3.3.2.1. Serum expression of TIMP-2

TIMP-2 levels in serum from the recurrent miscarriage group were higher than those in normal healthy pregnant women, and the data was statistically significant (p value < 0.0001) as illustrated in figure 3.1.

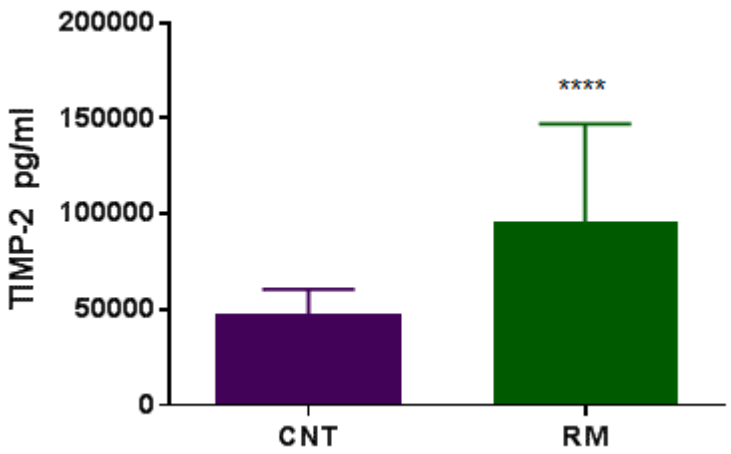

Figure 3.1. Comparison of serum TIMP-2 expression in pregnant women with history of recurrent miscarriage and healthy pregnant controls.

(**** p< 0.0001)

There was no significant difference in TIMP-2 serum levels between women with threatened miscarriage and normal healthy pregnant group with the same matching gestational age (p value = 0.3109) as illustrated in figure 3.2.

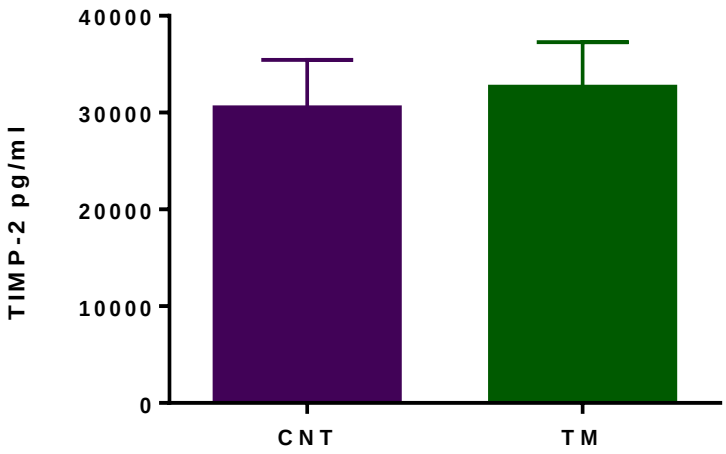

Figure 3.2. Comparison of serum expression of TIMP-2 in threatened miscarriage group and healthy pregnant controls

(p value = 0.3109)

3.3.2.2. Serum expression of MMP-2

There was a significant decrease in the expression of MMP-2 in the serum samples of pregnant women with a history of recurrent miscarriage group compared to their healthy controls matched for gestational age (p value = 0.0009) as illustrated in figure 3.3.

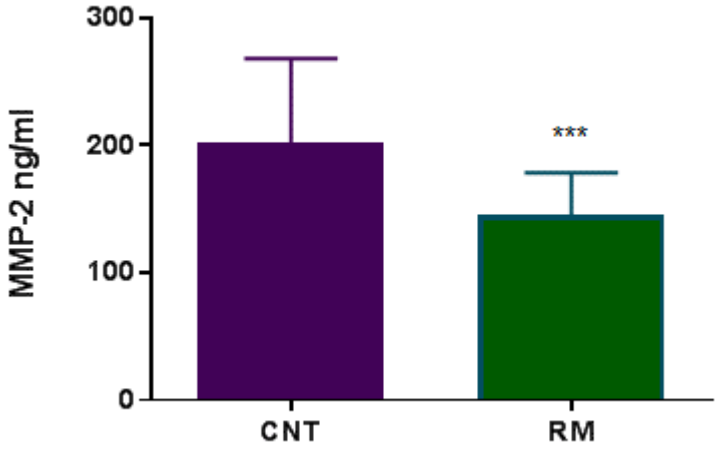

Figure 3.3. Comparison of serum expression of MMP-2 in pregnant women with history of recurrent miscarriage and healthy pregnant controls

(p value = 0.0009)

However, there was no significant difference in MMP-2 levels between the threatened miscarriage group and the normal healthy pregnant group matched for gestational age (p value = 0.9113) as illustrated in figure 3.4.

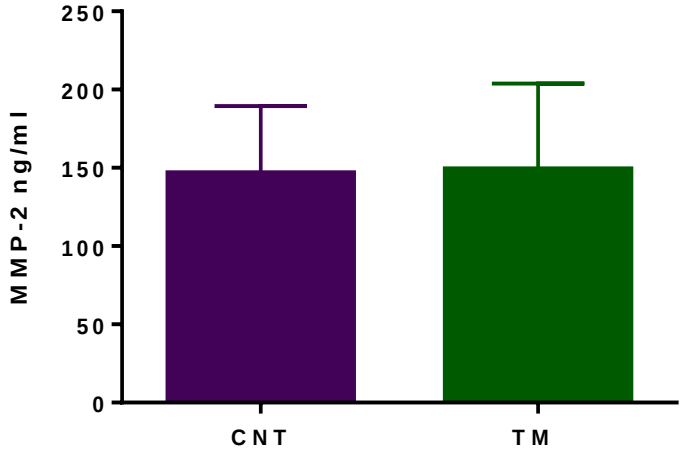

Figure 3.4. Comparison of serum expression of MMP-2 in threatened miscarriage group and healthy pregnant controls

(p value = 0.9113)

3.3.2.3. Serum expression of MMP-9

The expression of MMP-9 in the serum samples showed no significant difference between recurrent miscarriage group and their healthy controls with the same matching gestational age (p value = 0.0608) as illustrated figure 3.5.

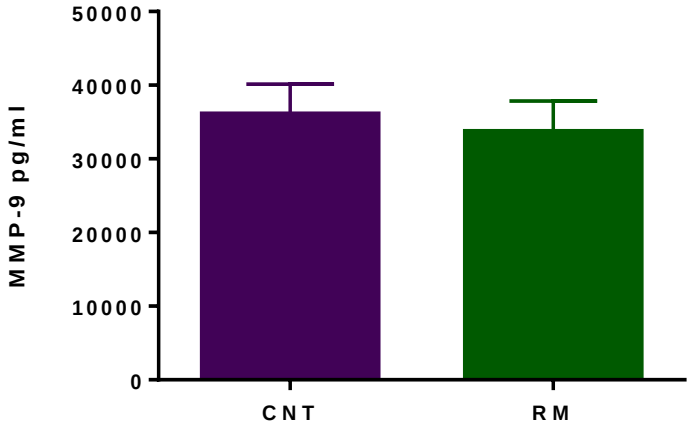

Figure 3.5. Comparison of serum expression of MMP-9 in pregnant women with history of recurrent miscarriage and healthy pregnant controls

(p value = 0.0608)

The expression of MMP-9 in the serum samples shows no significant difference between threatened miscarriage group and their normal healthy pregnant group with the same matching gestational age (p value = 0.3109) as illustrated in figure 3.6.

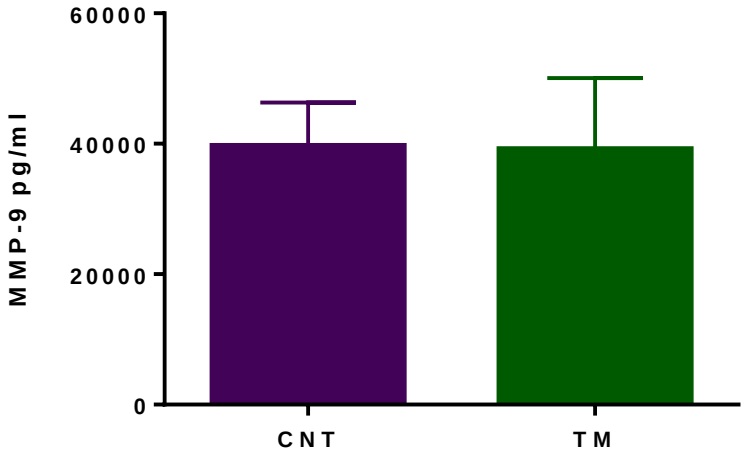

Figure 3.6. Comparison of serum expression of MMP-9 in threatened miscarriage group and healthy pregnant controls

(p value = 0.9113)

3.3.2.4. Serum expression of MMP-2/TIMP-2 Complex

The serum levels of MMP-2/TIMP-2 Complex from the recurrent miscarriage group were significantly lower than those in normal healthy pregnant women (p value = 0.0014) as illustrated in figure 3.7.

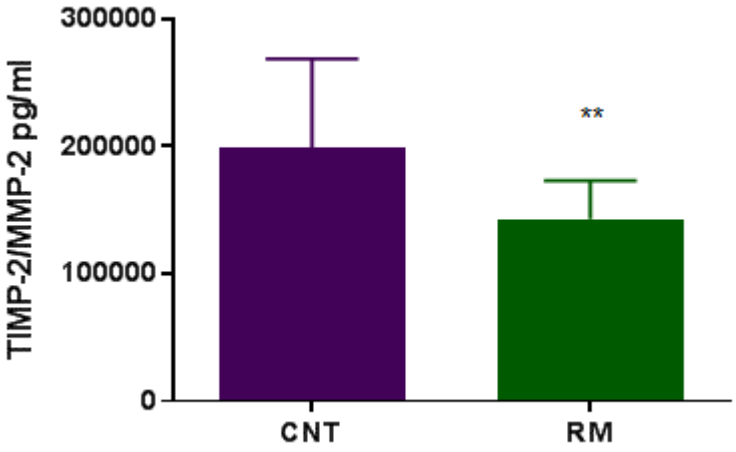

Figure 3.7. Comparison of serum expression of TIMP-2/MMP-2 Complex in pregnant women with history of recurrent miscarriage and healthy pregnant controls

(p value = 0.0014)

There was no significant difference in the MMP-2/TIMP-2 complex serum levels between threatened miscarriage group and their normal healthy pregnant group with the same matching gestational age (p value = 0.3197) as illustrated in figure 3.8.

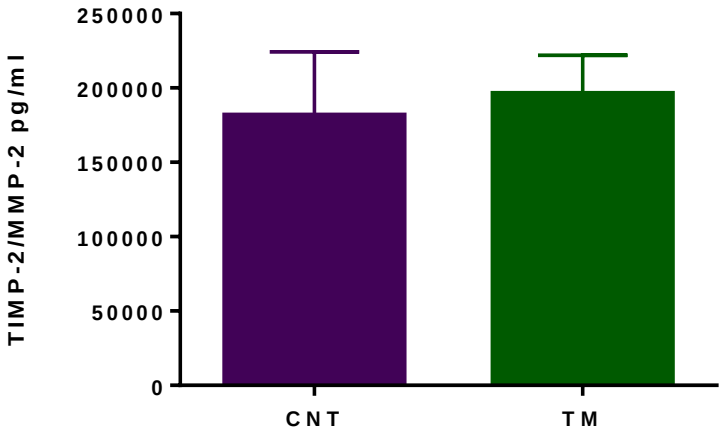

Figure 3.8. Comparison of serum expression of TIMP-2/MMP-2 Complex in threatened miscarriage group and healthy pregnant controls

(p value = 0.3197)

3.3.2.5. Serum expression of RLX-2

Serum RLX-2 levels from the recurrent miscarriage group were significantly lower than those in normal healthy pregnant women (p value = 0.0190) as illustrated in figure 3.9.

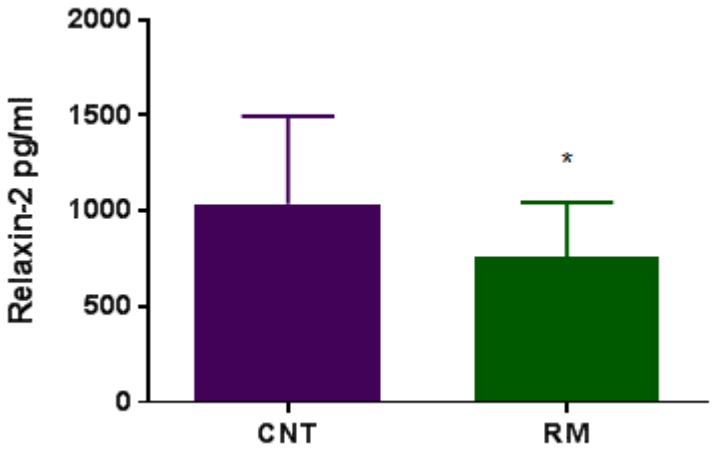

Figure 3.9. Comparison of serum expression of RLX-2 in pregnant women with history of recurrent miscarriage and healthy pregnant controls

(p value = 0.0190)

There is no significant difference in the RLX-2 serum levels between the threatened miscarriage group and their normal healthy pregnant group with the same matching gestational age (p value = 0.9508) as illustrated in figure 3.10.

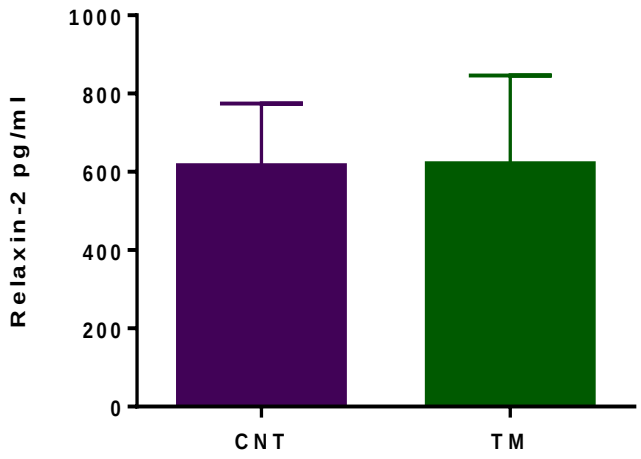

Figure 3.10. Comparison of serum expression of RLX-2 in threatened miscarriage group and healthy pregnant controls

(p value = 0.9508)

3.3.2.6. The expression trends of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 complex and RLX-2 in the healthy control groups at 6-10 weeks and 10-14 weeks.

The figures in 3.11 show the trend of the different proteins measured for the healthy pregnant women with no history of recurrent or threatened miscarriage at two different gestational age time points (at 6-10 weeks and at 10-14 weeks of gestation). It is clear that the expression levels of all the proteins rise with in serum with increasing gestational age attaining statistical significance for MMP-2 (A), TIMP-2 (C) and RLX-2 (E) (p value = 0.03, 0.001 and 0.02 respectively). However, the expression of MMP-9 decreased slowly toward 10-14 weeks of gestation but this did not attain statistical significance (p value = 0.0702).

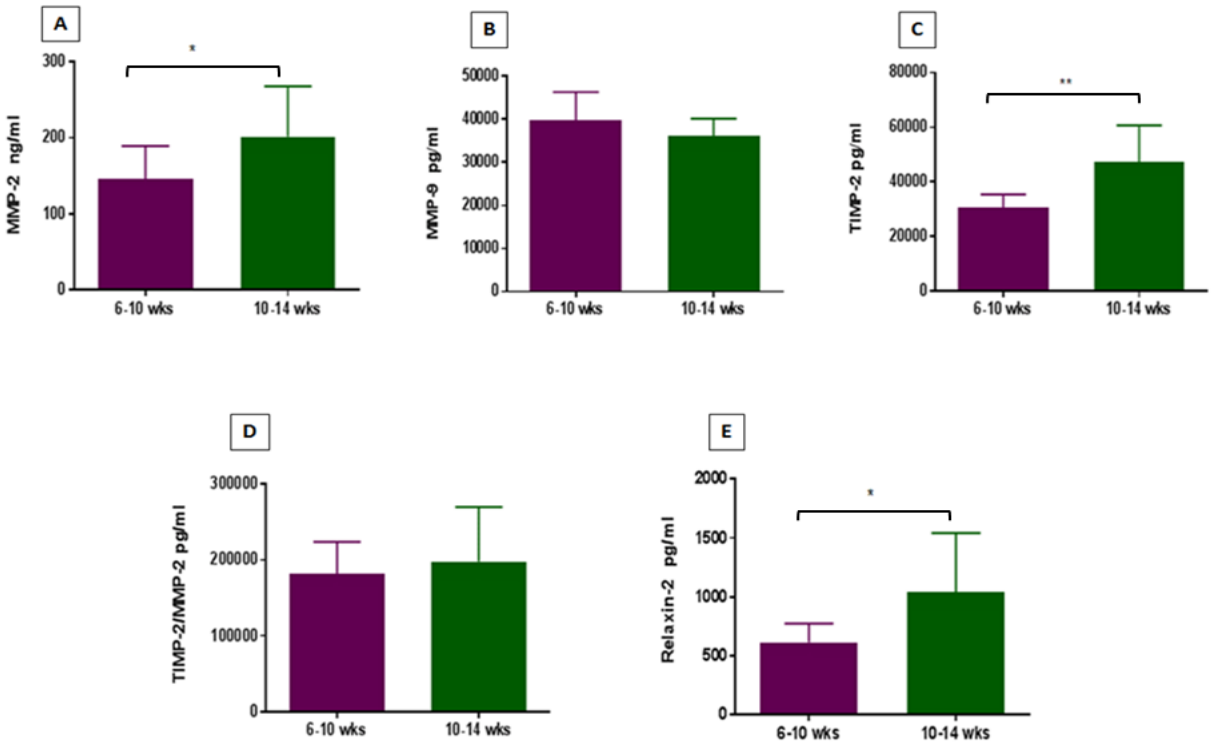

Figure 3.11. The expression of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 complex and RLX-2 in the healthy control groups at 6-10 weeks and 10-14 weeks

3.4. Discussion

The clinical data results of this study indicate that there was no significant difference in the rate of miscarriage, preterm birth and pre-labour spontaneous rupture of foetal membrane between the recurrent miscarriage group and their healthy controls, while there was a significant increase in the rate of caesarean section and the rate of post-delivery blood loss in the recurrent miscarriage group compared to their controls. Despite the limited sample sizes to draw a firm conclusion, there was no significant difference in the rate of miscarriage, preterm birth, pre-labour spontaneous rupture of foetal membrane, rate of caesarean section and post-delivery blood loss between the threatened miscarriage group and their healthy controls.

Consistent with data in a previous study, Jivraj and colleagues found that women with recurrent miscarriage had a high risk of caesarean section. They also found that women with recurrent miscarriage had a high risk of small for gestational age, preterm delivery and perinatal mortality (Jivraj et al., 2001).

Wijesiriwardana *et al*, in a large epidemiology study about the pregnancy outcomes in women with threatened miscarriage, found that women with threatened miscarriage had a high risk of caesarean section, preterm delivery and antepartum hemorrhage (Wijesiriwardana et al., 2006), while our study did not determine any difference in the pregnancy outcomes. Moreover, Ahmed, S.R. and his colleagues also suggested that patients having threatened miscarriage had a significantly increased risk of complete miscarriage, preterm labour and low birth weight infants. Other pregnancy adverse outcomes such as preeclampsia, IUGR, PROM and caesarean section rates were relatively increased (Ahmed et al., 2012). Results from another study confirm findings from other authors, that threatened miscarriage is not only associated with miscarriage but also with an increased risk of pregnancy outcomes such as preterm labour, placental abruption, PPROM and IUGR (Dadkhah et al., 2010). However, the number of women in our study is too small for any recognized statistical analysis or conclusions to be made, and limited the ability of the study to detect any

association between serum marker levels and adverse clinical outcomes such as: miscarriage, stillbirth, premature delivery and placental abruption.

Reproductive tract tissue is the primary site for relaxin hormone secretion (Goldsmith and Weiss, 2009, Parry and Vodstrcil, 2007). The relaxin plays a role in stimulating placental growth factor and vascular endothelial growth factor, these factors promote neo-vascularisation to increase blood flow to the endometrium, placenta and other reproductive tract tissue (Unemori et al., 1999). Thus, a dysregulation in relaxin activity is likely to contribute to the pathogenesis of implantation.

In this study two groups were compared, the first group was pregnant women with viable pregnancy and history of recurrent miscarriage compared to their healthy pregnant women controls, and the second group was pregnant women with a viable pregnancy presenting with threatened miscarriage compared to healthy pregnant controls, both groups being matched for age and gestation. This study shows that there is a significant increase in the serum expression of TIMP-2 in pregnant women with history of recurrent miscarriage compared to their healthy controls and low expression of MMP-2 and MMP-2/TIMP-2 complex and RLX-2 hormone. In contrast, there were no significant differences in the expression of TIMP-2, MMP-2, MMP-2/TIMP-2 complex and RLX-2 between the threatened miscarriage group and their healthy controls, while there were no significant differences in the serum expression of MMP-9 in both groups. The results suggest that the matrix metalloproteinases and their inhibitors and RLX-2 hormones may play a key role in early pregnancy development, and that dysregulation may result in implantation failure by affecting remodeling of the endometrial matrix. It is plausible that altered angiogenesis may influence the occurrence of adverse pregnancy outcomes.

The findings observed in this study regarding the serum expression of TIMP-2 and RLX-2 in the recurrent miscarriage group are consistent with previous work undertaken by members of the Anumba group (Anumba et al., 2009, Anumba et al., 2010). In a longitudinal study, they reported for the first time, that pregnant women with a viable pregnancy with history of recurrent miscarriage have a significant increase in the expression level of TIMP-2

compared to healthy pregnant women without history of recurrent miscarriage, while there was no significant difference in the levels of MMP-1, MMP-3 and MMP-9 between two groups during the first trimaster. They also reported a significant decrease in the level of serum RLX-2 hormone in recurrent miscarriage groups compared to healthy pregnant women (Anumba et al., 2010). Although one study has suggested that the serum expression levels of RLX-2 were low in women during miscarriage and ectopic pregnancy compared to those with healthy early pregnancy (Petersen et al., 1995), we have not been able to confirm these observations in our series. In contrast, Jiang and Qi determined that MMP-9 and TIMP-3 mRNA expression levels in the villi of pregnant women undergoing early spontaneous miscarriage was higher than that of the normal pregnant women requesting abortion at the same gastational age (Jiang and Qi, 2015). However, to our knowledge, this is the first study investigated the serum expression levels of MMPs, TIMPs and RLX-2 in pregnant women with viable pregnency and thretened miscarriage.

The Matrix mettaloproteinaes family of proteins are key in the remodelling of the extracellular matrix of the endometrium throughout implantation and pregnency (Hulboy et al., 1997, Woessner, 1991). MMPs are suggested to have numerous potential roles in stimulating the growth of reproductive tissue, including altering the integrity of the basement membrane to prepare tissue for expansion and growth factor activation. The ability of RLX-2 to promote tissue growth and remodlling suggest a regulatory interaction between RLX-2 and MMPs. In a recent study Mi Kang *et al* (2017) reported that RLX-2 inhibits excessive extracellular matrix synthesis by reducing the expression of its components, such as fibronectin, phosphorylated Smad2 and a-SMA, through increasing the levels of MMPs, whilst simoultaneously decreasing the levels of TIMPs (Kang et al., 2017). In another study by Lenhart (2001), it was suggested that RLX-2 stimulates MMP-2 and MMP-9 secretion in uterine flushes during growth and remodlling of the uterus and cervix of the pig (Lenhart et al., 2001).

One of the weakness of this study is that the serum results represent the systemic levels of the serum markers as several tissues contribute to this measurement and the local expression may show a difference from one tissue to another. Although we studied the systemic expression of the proteins rather than the local expression of MMPs, TIMPs and relaxin hormone, profound local changes associated with implantation and placentation could well have been undetected. Nevertheless, the significant difference in the serum expression of these proteins suggest that these molecules have a role in the biology of human reproduction. However, a larger sample size will be required to confirm if the serum marker can be used as a clinical predictive value for adverse pregnancy outcomes.

In summary, this study has demonstrated that women with history of recurrent miscarriage have a high expression of TIMP-2 and low expression levels of MMP-2, MMP-2/TIMP-2 complex and RLX-2. These data suggest that any dysregulation of any of these proteins may play a role in the implantation pathogenesis and pregnancy loss. Further studies with a large sample size are required to confirm and explain these data. However, an *in vitro* study is needed to explore if there is any interaction between RLX-2, MMPs and their inhibitors, and the effect of all these molecules on the early implantation stages to support or refute these *in vivo* observations.

Chapter 4 The potential effect of RLX-2 on MMP-2, MMP-9 and TIMP-2 expression by T-HESCs and JAR cells, and the effect of TIMP-2 on MMP-2 and MMP-9 expression by T-HESCs and JAR cells

4.1. Introduction

During pregnancy, growth of the uterus and cervix are a result of cellular proliferation and connective tissue remodelling to facilitate tissue expansion. The tissue remodelling process is a fine balance between breakdown and remodelling of the ECM that is highly coordinated. Some of the main factors involved in this process are matrix metalloproteinases (MMPs) enzymes that belong to a family of extracellular proteinases that target the extracellular matrix and are vital for tissue remodelling (Hulboy et al., 1997). MMPs expression and production are regulated by various stimulatory factors, including growth factors, cytokines and hormones. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are MMP subclasses that mediate basement membrane degradation in the early embryo implantation stages of the cellular invasion process and tissue expansion, and therefore they are essential for growth and remodelling of the reproductive tissue (Staun-Ram et al., 2004).

Several modulatory molecules interact at the trophoblast-endometrial interface to generate a favourable environment for blastocyst implantation. Successful implantation requires a delicate balance between the activators and inhibitors of MMPs that control the synthesis and degradation of the ECM. Relaxin has been reported to stimulate MMPs activity in various species including humans, pigs and rats (Lenhart et al., 2001).

Numerous studies have shown that during pregnancy, the hormone relaxin stimulates growth of the uterus and increases its collagenous framework distensibility that is essential for foetal accommodation. The primary target for relaxin in the reproductive tract is the connective tissue, it has an important collagenolytic role in the remodelling the connective tissue matrix structure to prompt ripening and dilation of the cervix in preparation for parturition. These observations point to the significant association between relaxin and enzyme activity within the connective tissue (Lenhart et al., 2001).

Hypothesis

RLX-2 regulates the expression of MMP-2, MMP-9 and TIMP-2 secreted from trophoblast and endometrium cells during early implantation stages.

Aims

The experiments performed in this chapter aim to define the impact of RLX-2 on the production and activity of MMP-2, MMP-9 and TIMP-2 from endometrial stromal (T-HESCs) and trophoblast (JAR) cells, and also to determine the effect of MMP inhibitors on the expression of MMP-2 and MMP-9.

4.2. Materials Methods

In this chapter, the expression of MMP-2, MMP-9, TIMP-2, RLX-2, endometrial epithelial markers and endometrial stromal cell markers in all the cell lines (Ishikawa, RL95-2, JAR and T-HESCs) were examined using immunohistochemistry (see chapter 2 for details, section: 2.2.7). In addition, the secretion of MMP-2, MMP-9 and TIMP-2 was determined from T-HESCs and JAR cells upon stimulation with increasing concentrations of RLX-2 over time using ELISA. The effect of TIMP-2 and broad spectrum matrix metalloproteases inhibitor (Batimastat) on the production and activity of MMP-2 and MMP-9 was also assessed (see chapter 2 for details, section: 2.2.9 and 2.2.10). The data described in this chapter are from 4 independent experiments, with each experiment performed in triplicate. The expression levels for each group of samples were compared with each other using ANOVA with post-hoc tests within Graph Pad Prism version 6.02 statistical software.

4.3. Results

4.3.1. Immunohistochemistry

4.3.1.1. Haematoxylin and eosin H&E staining

All the cell lines were cultured, fixed with agarose/formaldehyde for wax processing, sectioned and stained with haematoxylin and eosin (H&E). It is a routine histological stain used for examining and localising the cell pallets in the section. Figure 4.1 shows H&E stained sections of (RL95-2, Ishikawa, JAR and endometrial stromal cell lines (T-HESCs)), the cytoplasm stained red/pink and the nuclei stained blue. Cells were present as individual cells scattered throughout the agarose. The sections showed typical phenotypes for epithelial cells with large nuclei surrounded by cytoplasm.

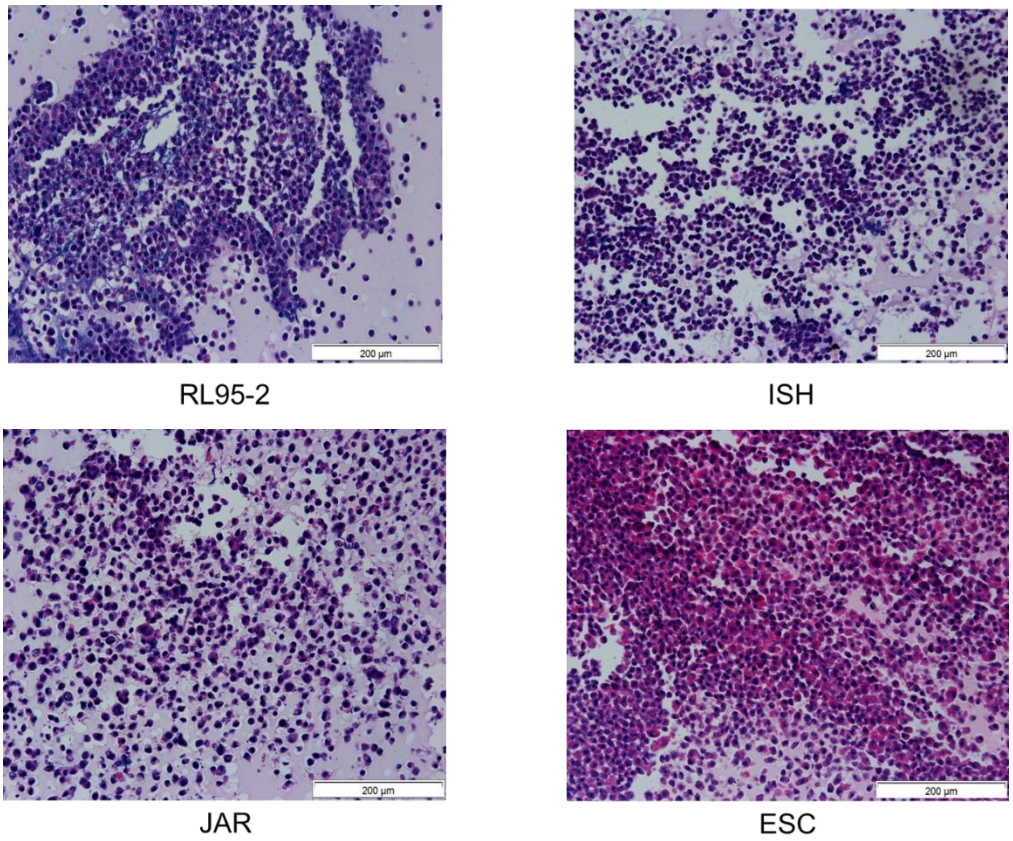

Figure 4.1. Haematoxylin and eosin (H&E) stained sections of (RL95-2, Ishikawa, JAR and endometrial stromal cell lines).

Scale bar = 200 µM.

4.3.1.2. Galectin-9

Wax embedded cell lines were processed for immunohistochemical staining. DAB staining was performed to visualise the expression of galectin-9 in the endometrial cell lines and JAR cell line. Images of Immunostaining demonstrated a strong immunoreactivity for galectin-9 in the endometrial epithelial, stromal cell lines and the JAR cell line as illustrated in figure 4.2. No immune-reactivity was observed in the negative control where the primary antibody was omitted to assess the antigen dependent binding. Placental tissue was used as a positive control as it is known to express galectin-9.

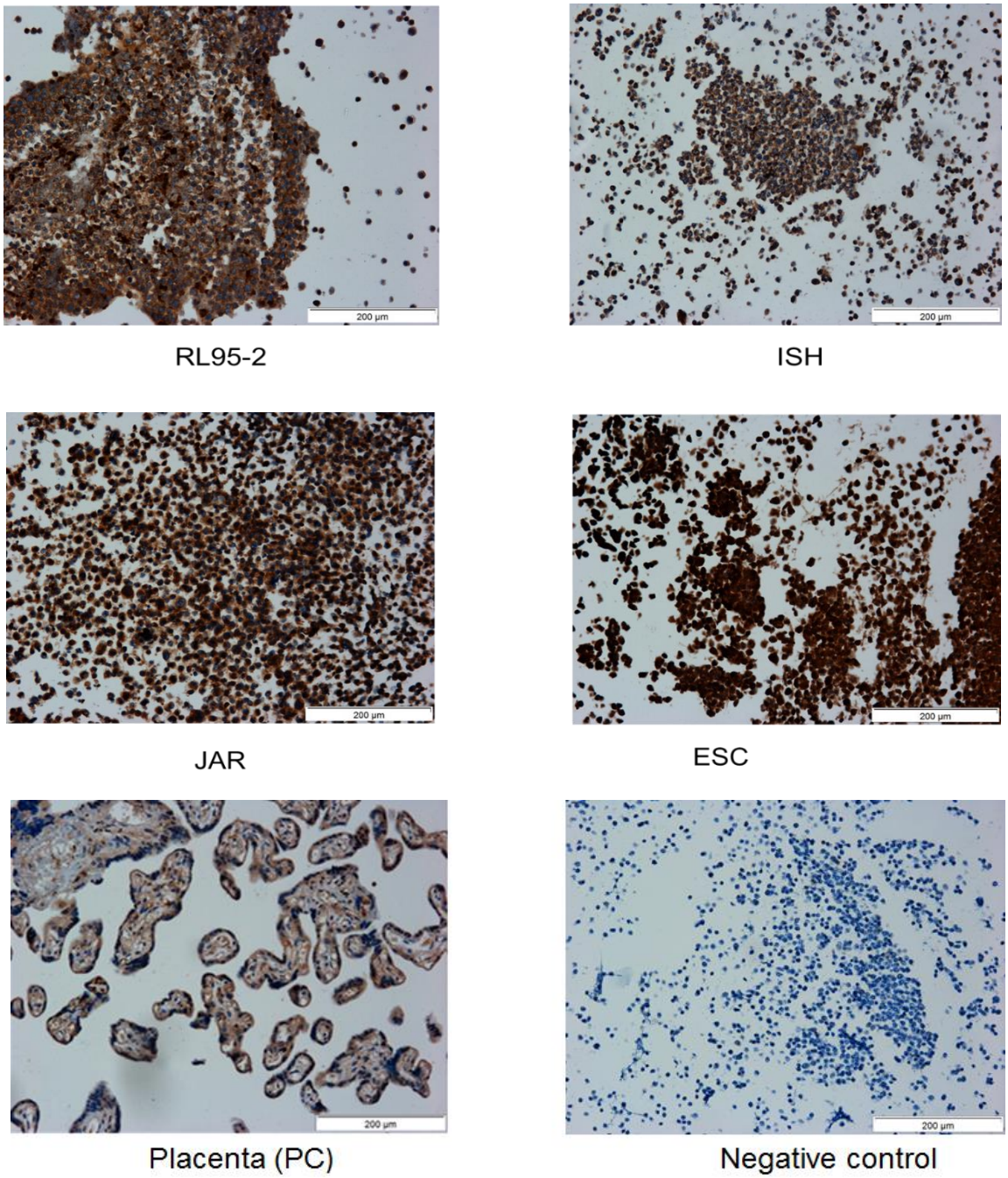

Figure 4.2. Immunostaining for Gelactin-9

Tissue sections of endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR trophoblast cells. Endometrial tissue and placenta were used as positive controls. Scale bar = 200 µM.

4.3.1.3. Vimentin

Figure 4.3 shows examples of immune-reactive staining for vimentin in endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and the JAR cell line. Immunostaining demonstrated a strong reactivity for vimentin in the endometrial stromal cell line. No immune-reactivity was observed in the endometrial epithelial cell lines (RL95-2 and Ishikawa), whereas JAR cell line displayed a subpopulation of immune-positively stained cells. Immunoreactivity was not observed in the negative control where the primary antibody was omitted to investigate the antigen-dependent binding, whereas strong immune-positive staining was seen in placental tissue that was used as a positive control.

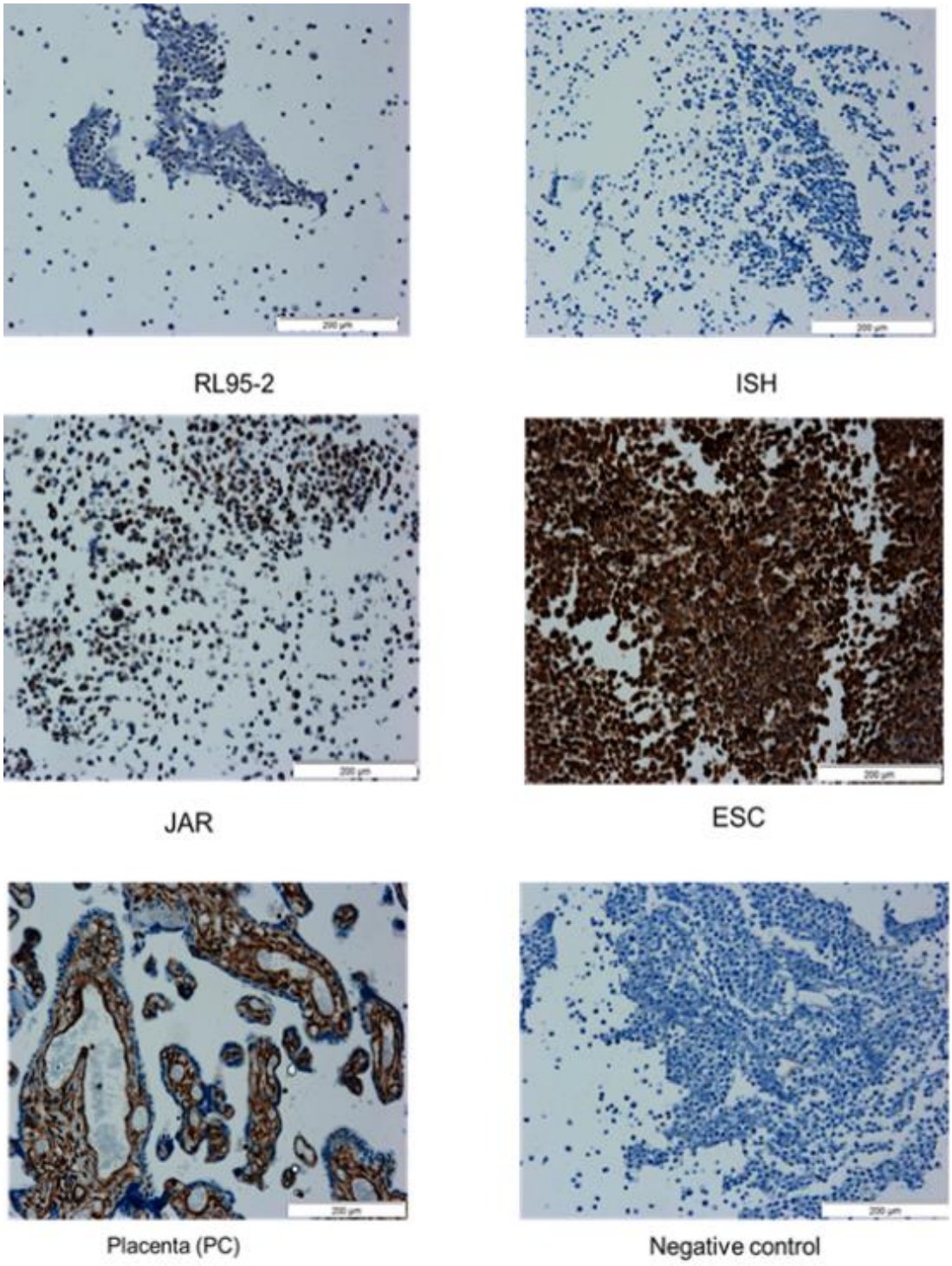

Figure 4.3. Immunostaining for vimentin

Endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR cell line. Omission of primary antibody showed no Immunostaining (negative control), whereas strong immune-reactive staining was observed in the positive control (placenta). Scale bar = 200 µM

4.3.1.4. CD10

Expression of CD10 was examined in the endometrial cell lines, stromal cells and the JAR cell line using a specific CD10 antibody followed by DAB staining. Strong immune-reactive staining for CD10 was observed in the endometrial stromal cell line. However, no immune-reactivity was observed in the endometrial epithelial cell lines (RL95-2 and Ishikawa) and the JAR cell line. Absence of immunoreactivity in the negative control where the primary anti body was omitted confirmed antibody specificity as did immune-positive staining in the placental tissue as a positive control, as illustrated in figure 4.4.

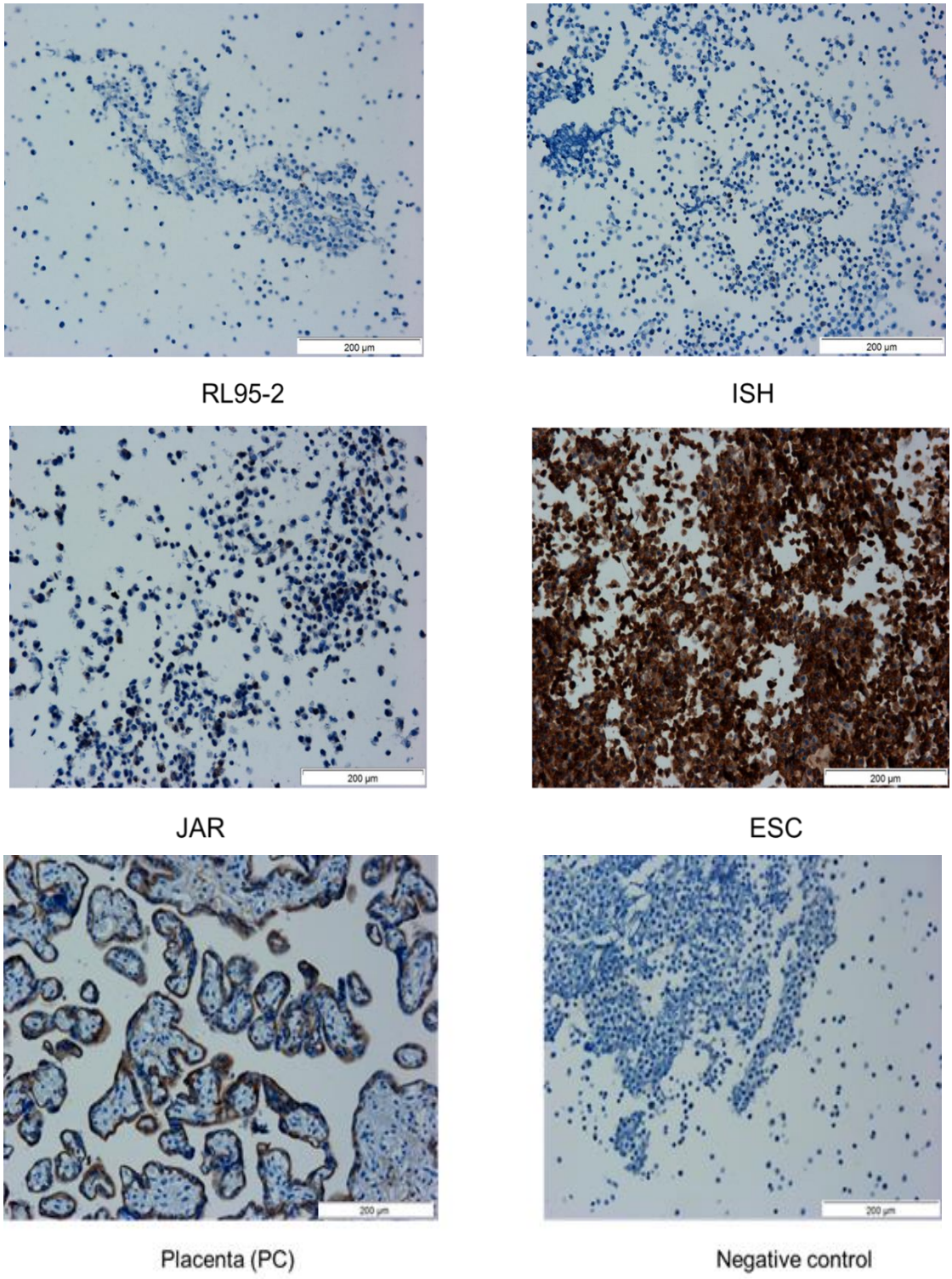

Figure 4.4. Immunostaining for CD10

Tissue section of endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR trophoblast cells. Omission of primary antibody showed no Immunostaining (negative control), whereas strong immune-reactive staining was observed in the positive control (placenta). Scale bar = 200 µM.

4.3.1.5. TIMP-2

DAB staining was performed to localise the expression of TIMP-2 in the endometrial cell lines and JAR cell line. Images of Immunostaining demonstrated a strong reactivity for TIMP-2 in the endometrial epithelial cell line RL-95-2, the stromal cell line and JAR cell line, where staining was strong in all cells. However, staining in the Ishikawa cell line was not widespread but was evident in a subpopulation of cells. No immune-reactivity was observed in the negative control where the primary antibody was omitted to assess the antigen-dependent binding. As expected, positive–Immunostaining was observed in human placental tissue as illustrated in figure 4.5.

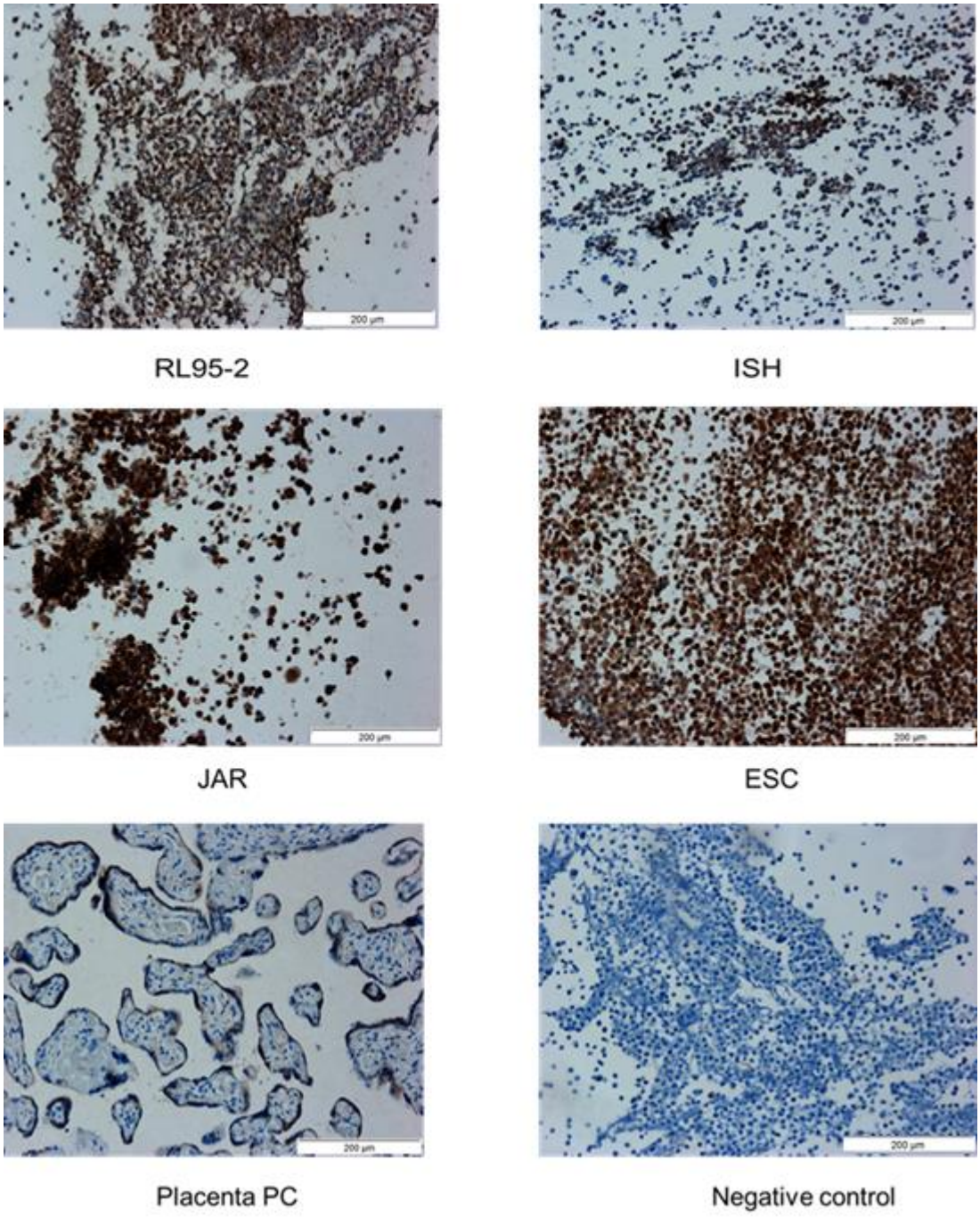

Figure 4.5. Immunostaining for TIMP-2

Tissue section of endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR trophoblast cells. Omission of primary antibody showed no Immunostaining (negative control), whereas strong immune-reactive staining was observed in the positive control (placenta) and in the epithelium of human endometrial tissue. Scale bar = 200 μM.

4.3.1.6. RLX-2

Expression of RLX-2 was observed in all cell lines tested and in each case, strong immuno-reactivity was evident. Strong expression of RLX-2 and Ishikawa was observed in the epithelium of human endometrial tissue, endometrial stromal cells and JAR cell line. No immune-reactivity was observed in the negative control where the primary antibody was omitted to assess the antigen dependent binding as illustrated in figure 4.6.

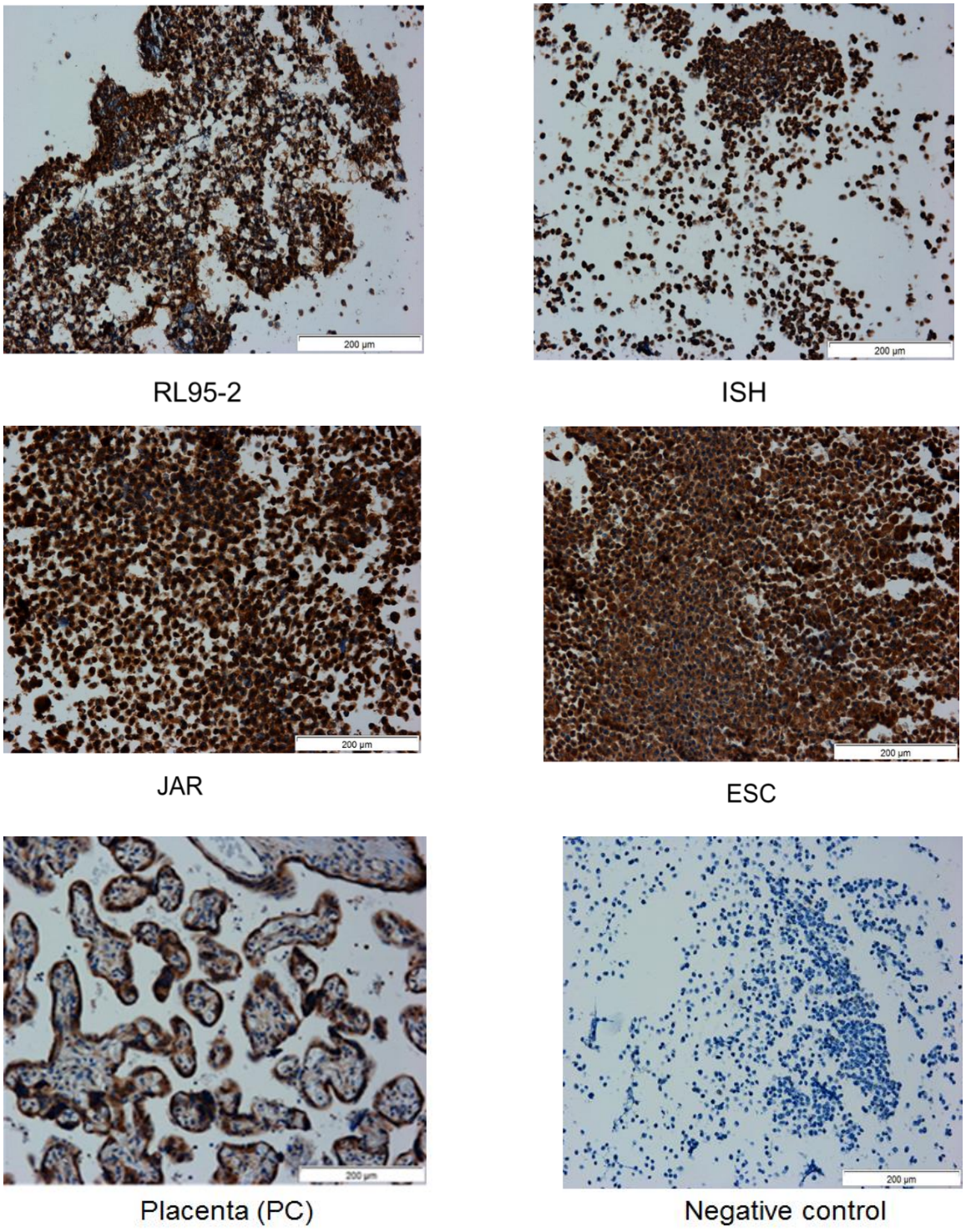

Figure 4.6 Immunostaining for RLX-2

Tissue section of endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR trophoblast cells. Omission of primary antibody showed no Immunostaining (negative control), whereas strong immune-reactive staining was observed in the positive control (placenta) and in the epithelium of human endometrial tissue. Scale bar = 200 µM.

4.3.1.7. MMP-2 and MMP-9

Figure 4.7 and figure 4.8 show examples of Immunostaining for MMP-2 and MMP-9 in endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR cell line respectively. Images of Immunostaining demonstrated a strong reactivity for MMP-2 in the endometrial stromal cells and RL-95-2. Weaker expression was observed with Ishikawa and JAR cells. Weak MMP-2 expression was detected in the positive control placenta tissue and no immune-reactivity was observed in the negative control where the primary anti body was omitted to assess the antigen dependent binding as illustrated in figure 4.7. For MMP-9, strong immuno-reactive staining was observed for all cell lines tested as well as the positive control placental tissue whereas no immuno-reactive staining was seen in the negative control tissue as illustrated in figure 4.8.

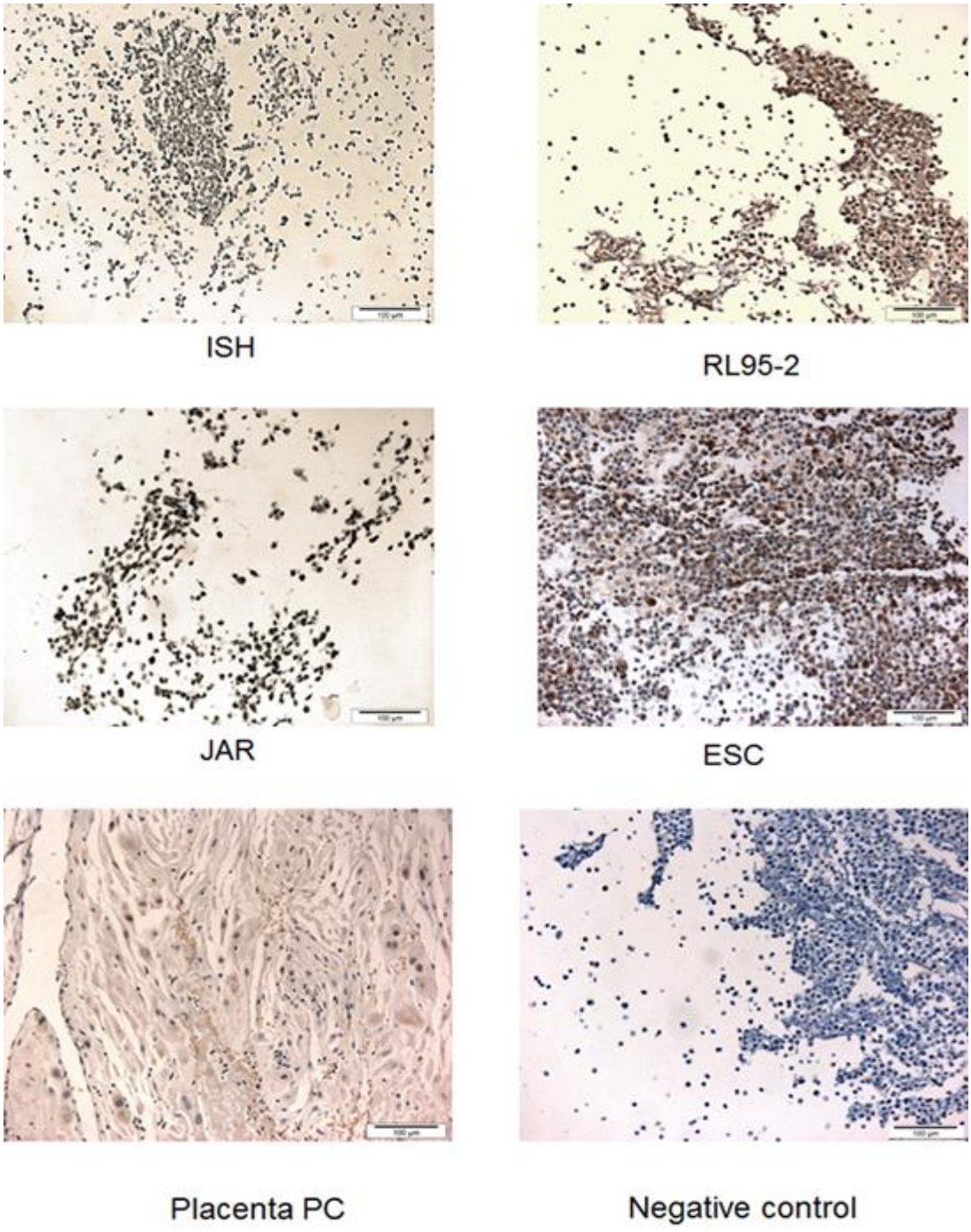

Figure 4.7. Immunostaining for MMP-2

Tissue section of endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR trophoblast cells. Omission of primary antibody showed no Immunostaining (negative control), whereas immuno-reactive staining was observed in the positive control (placenta). Scale bar = 200 µM.

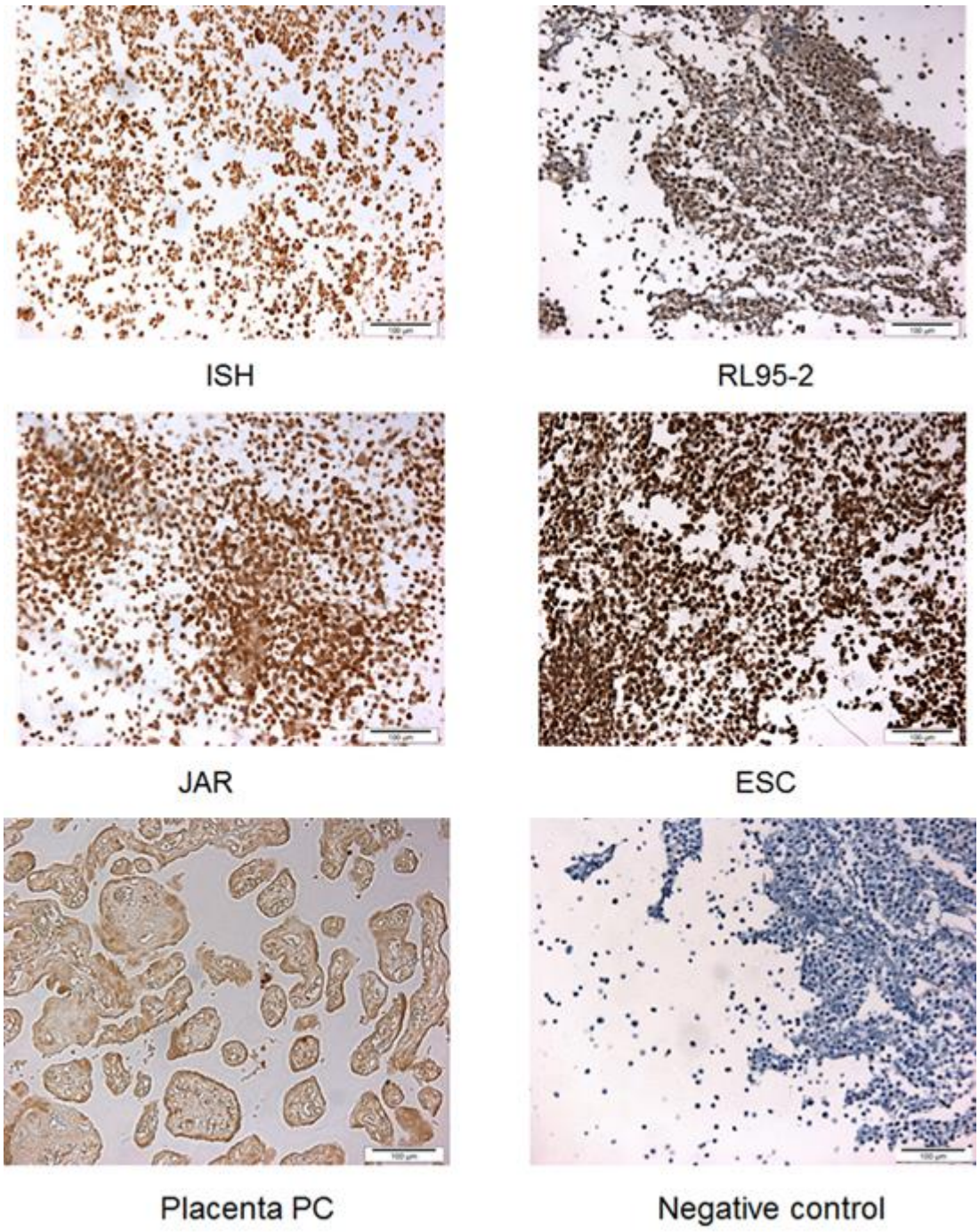

Figure 4.8 Immunostaining for MMP-9

Tissue section of endometrial epithelial cell lines (RL95-2 and Ishikawa), endometrial stromal cells and JAR trophoblast cells. Omission of primary antibody showed no Immunostaining (negative control), whereas immuno-reactive staining was observed in the positive control (placenta). Scale bar = 200 µM.

4.3.2. Measurement of MMP-2 and MMP-9 secretion of different cell lines by zymography

Conditioned medium from all the cell lines were analysed using zymography to determine the secreted levels of MMP-2 and MMP-9, as described in chapter 2 (section: 2.2.8). Gelatin zymography identified the amount of enzyme activity attributed to both pro and active forms of the enzymes. Figure 4.9 shows a representative image of a zymogram from the conditioned medium, media of the different cell line used: RL95-2, Ishikawa, HEC-1-A, JAR and T-HESCs. Conditioned medium from JAR cells and T-HESCs shows stronger intensity of gelatinolytic bands for the pro-enzymatic forms of MMP-2 (72 kDa) when compared with the positive control supernatant from (PMA-treated HeLa cells), and the slightly lower molecular weight activate form of MMP-2 enzyme was expressed from the T-HESCs (62 kDa). Conditioned medium from endometrial epithelial cells (RL95-2, Ishikawa and HEC-1-A) showed weak intensity bands. Much smaller intensity bands corresponding to the pro-MMP-9 (92 kDa) were observed for all cell lines and the active form of MMP-9 (82 kDa) could only be detected with JAR cells as illustrated in figure 4.9.

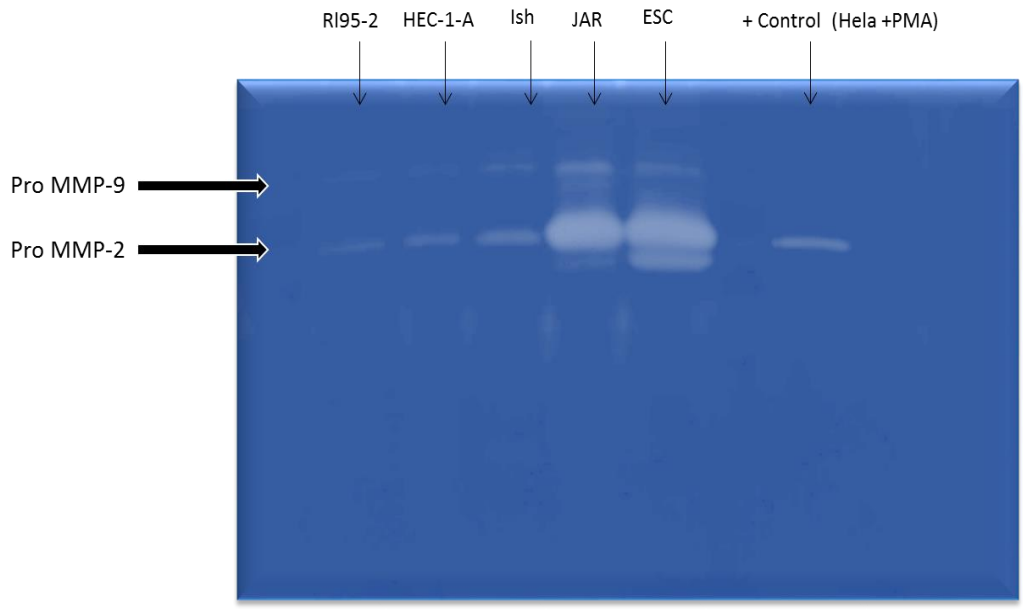

Figure 4.9. Panel of representative gelatin zymogram of cell lines supernatant (RL95-2, Ishikawa, HEC-1-A, JAR and T-HESCs)

T-HESCs and JAR cells expressed stronger intensity of gelatinolytic bands for pro-enzymatic forms MMP-2 (72 kDa) and the lower molecular weight activate enzyme (62 kDa) was expressed from the T-HESCs. Expression bands for pro-MMP-9 (92 kDa) were detected in all cell lines but their intensity was much lower than for MMP-2. PMA-treated HeLa cells were used as a positive control.

4.3.3. Evaluation of the effect of various RLX-2 concentration on MMP-2, MMP-9 and TIMP-2 production.

As described in chapter 2 (section: 2.2.9) functional studies were carried out to evaluate the effect of three different concentrations of RLX-2 at three different time-points on the production of MMP-2, MMP-9 and TIMP-2 by cultured T-HESCs and JAR cells.

4.3.3.1. Expression of MMP-2 from endometrial stromal (T-HESCs) cells treated with RLX-2.

Cultured T-HESCs were treated with increasing concentrations of RLX-2 (1, 10 and 100 ng/ml) for a duration of 24, 48 and 72 hours (section: 2.2.9). Medium alone was used as controls. Figure 4.10. shows a time-dependent increase in the secretion of MMP-2 for all cultures of T-HESCs irrespective of treatment with RLX-2. Levels of MMP-2 were significantly increased (p value < 0.05) in all samples tested from 24 to 48 hours in culture. However, levels of MMP-2 began to plateau at 72 hours, and with the exception of controls, the levels of MMP-2 at this time point were not significantly different from those measured at 48 hours. Importantly, stimulation of T-HESCs with RLX-2 did not alter the secretion of MMP-2 compared to unstimulated cells for any concentrations of RLX-2 and at any time point tested.

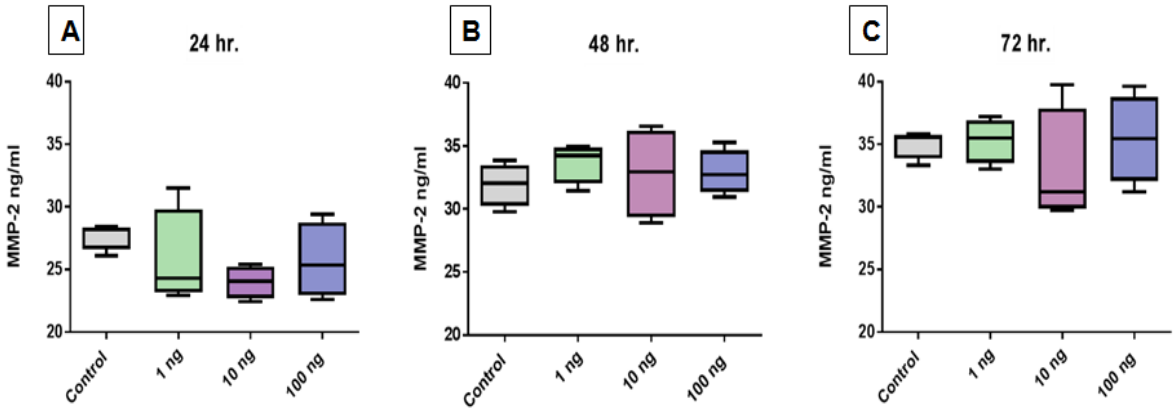

Figure 4.10. Comparison of MMP-2 levels secreted by T-HESCs treated with increasing concentrations of RLX-2

$2X10^5$ T-HESCs were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml RLX-2 for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml RLX-2) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA. The secretion of RLX-2 increased in a time dependent manner in both treated and untreated samples. There were no significant differences between RLX-2 -treated and untreated controls at any time point tested.

4.3.3.2. Expression of MMP-2 from JAR cells treated with RLX-2

Cultured JAR cells were treated with increasing concentrations of RLX-2 (0, 1 and 100 ng/ml) for a duration of 24, 48 and 72 hours (section: 2.2.9). There was a significant increase (p value = 0.016) in the levels of MMP-2 secreted by JAR cells in response to 100 ng/ml RLX-2 compared to cells cultured with medium alone and to treatment with 1 ng/ml (p value = 0.039) over a 24 hours period. After 48 hours, no significant difference was detected in the levels of MMP-2 when JAR cells were treated with any concentration of RLX-2 compared to medium alone controls. However, at 72 hours there was a significant increase in the secreted levels of MMP-2 when JAR cells were treated with 10 and 100 ng/ml but not 1 ng/ml RLX-2 concentrations, compared to medium alone control samples (p value = 0.0003 and 0.0002 respectively), and when the 10 and 100 ng/ml treated cells were compared to the 1ng/ml treated cells (p value = 0.005 and 0.003 respectively) as illustrated in figure 4.11.

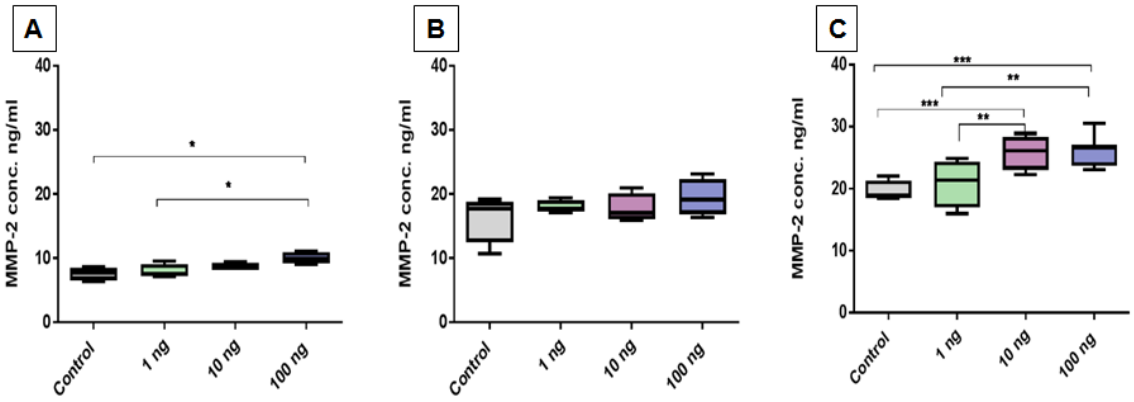

Figure 4.11. Comparison of MMP-2 levels secreted by JAR cells treated with increasing concentrations of RLX-2

$4X10^5$ ES cells were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml RLX-2 for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml RLX-2) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

4.3.3.3. Expression of TIMP-2 from T-HESCs treated with RLX-2

Cultured T-HESCs were treated with increasing concentrations of RLX-2 (0, 1 ng, 10 ng/ml and 100 ng/ml) for 24, 48 and 72 hours (section: 2.2.9). After 24 hours, there was an increase in the expression levels of TIMP-2 secreted by T-HESCs in response to 1 and 100 ng/ml RLX-2, compared to untreated control samples (p value = 0.029 and 0.034 respectively). However, after 48 hours no significant differences were detected in the TIMP-2 expression levels in any of the RLX-2 treated cells compared to controls. At 72 hours, there was significant increase in the expression levels of TIMP-2 secreted by T-HESCs treated with 10 ng/ml (p value = 0.03) RLX-2 compared to the control samples in which RLX-2 treatment had been excluded, and to 1 ng/ml RLX-2 treated cells (p value = 0.04). However, TIMP-2 levels secreted from cells treated with (100 ng/ml) RLX-2 were not significantly different from controls or other RLX-2 treated cells as illustrated in figure 4.12.

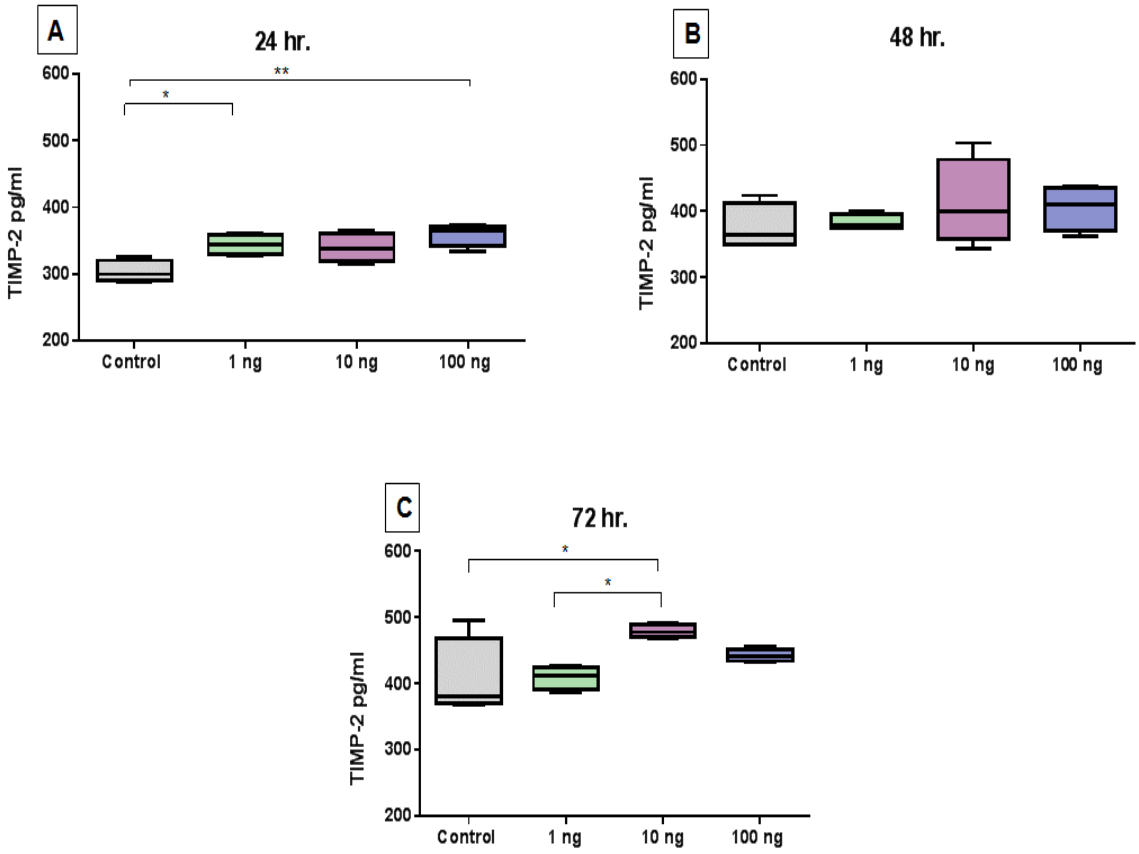

Figure 4.12. Comparison of TIMP-2 levels secreted by T-HESCs treated with increasing concentrations of RLX-2

2X10^5 T-HESCs were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml RLX-2 for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml RLX-2) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

4.3.3.4. Expression of TIMP-2 from JAR cells treated with RLX-2

Cultured JAR cells were treated with increasing RLX-2 concentrations (0, 1 ng/ml, 10 ng/ml and 100 ng/ml) for 24, 48 and 72 hours (section: 2.2.9). There was no significant difference detected in the expression levels of TIMP-2 between JAR cells treated with the different doses of RLX-2 at any of the three time points analysed as illustrated in figure 4.13.

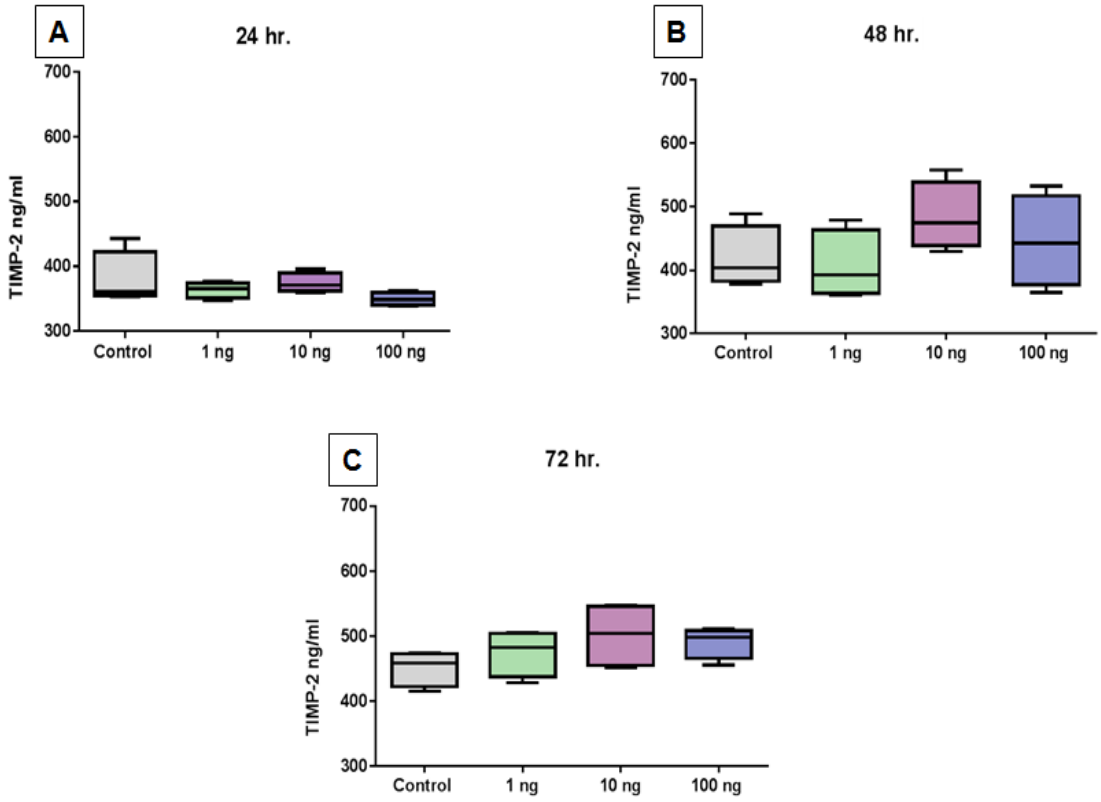

Figure 4.13. Comparison of TIMP-2 levels secreted by JAR cells treated with increasing concentrations of RLX-2

$4X10^5$ JAR cells were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml RLX-2 for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml RLX-2) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

4.3.3.5. Expression of MMP-9 from T-HESCs and JAR cells treated with RLX-2

Both T-HESCs and JAR cells treated with increasing concentration of RLX-2 did not express MMP-9 at any time points tested as the levels of MMP-9 were below the detection limit of the ELISA test.

4.3.4. Evaluation of the effect of various TIMP-2 and Batimastat concentrations on the expression level of MMP-2 and MMP-9

As described in chapter 2 (section: 2.2.10), studies were performed to evaluate the effect of increasing TIMP-2 and Batimastat concentrations on cultured T-HESCs and JAR cells at three different time points and the secretion of MMP-2 and MMP-9 was measured.

4.3.4.1. Expression of MMP-2 from endometrial stromal cells (T-HESCs) treated with TIMP-2

Cultured T-HESCs were treated with increasing concentrations of TIMP-2 (0, 1, 10 and 100 ng/ml) for 24, 48 and 72 hours (section: 2.2.10). No significant changes were detected in the MMP-2 secreted levels when T-HESCs were treated with increasing doses of TIMP-2 compared to each other or controls after 24 hours of culture. In contrast, after 48 hours there was a significant dose-dependent decrease in the levels of MMP-2 detected in the conditioned medium derived from T-HESCs in response to 1, 10 and 100 ng/ml TIMP-2 concentrations compared to cells treated with medium alone (p value = 0.0014, 0.0001 and 0.0001 respectively). After 72 hours of incubation of T-HESCs with TIMP-2, there was a recovery in the secretion of MMP-2 and a dose-dependent increase in MMP-2 levels was observed, and a statistically significant difference was achieved upon incubation with 100 ng/ml TIMP-2 as illustrated in figure. 4.14.

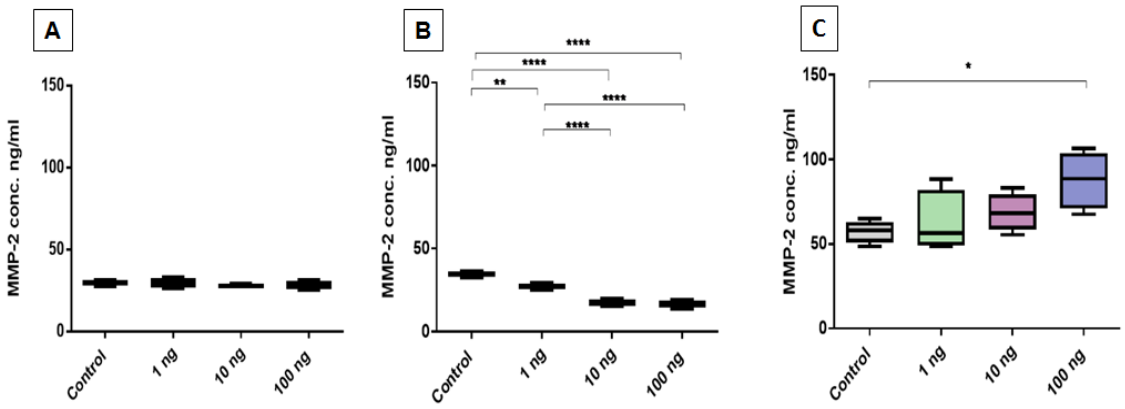

Figure 4.14. Comparison of MMP-2 levels secreted by T-HESCs treated with increasing concentrations of TIMP-2

2X10^5 T-HESCs were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml RLX-2 for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml TIMP-2) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

4.3.4.2. Expression of MMP-2 from JAR cells treated with TIMP-2

Similar to T-HESCs, no significant differences were detected in the levels of MMP-2 in the conditioned medium of JAR cells were these cells were treated with increasing concentrations of TIMP-2 after 24 hours. However, there was a significant dose-dependent decrease in the levels of MMP-2 detected after 48 hours of incubation with TIMP-2 at 1, 10 and 100 ng/ml concentrations compared to untreated controls (p value = 0.0023, 0.0001 and 0.0001 respectively). Furthermore, there was a significant decrease in the expression levels of MMP-2 from JAR cells in response to 10 and 100 ng/ml TIMP-2 treatment compared to the 1 ng/ml TIMP-2 treated cells (p value = 0.039 and 0.042 respectively), where the content of MMP-2 in these cultures was reduced to almost undetectable levels. After 72 hours, the overall levels of MMP-2 were increased in both TIMP-2 treated and untreated cells, and there were no significant differences detected in the expression levels of the MMP-2 when JAR cells were treated with increasing doses of TIMP-2 as illustrated in figure. 4.15.

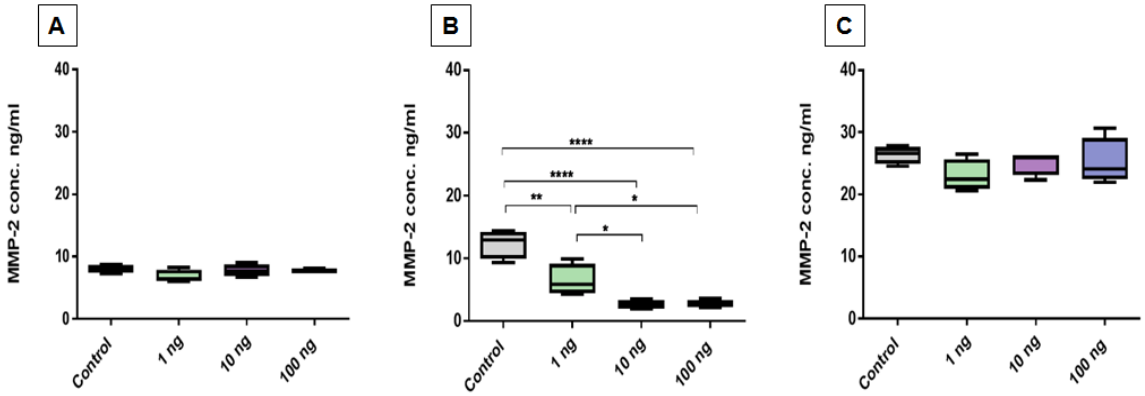

Figure 4.15. Comparison of MMP-2 levels secreted by JAR cells treated with increasing concentrations of TIMP-2

4X10^5 JAR cells were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml TIMP-2 for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml TIMP-2) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

4.3.4.3. Levels of MMP-2 in the conditioned medium of T-HESCs treated with Batimastat.

Cultured T-HESCs were treated with increasing concentrations (0 to 100 ng/ml) of Batimastat, a broad spectrum MMPs inhibitor for 24, 48 and 72 hours (section: 2.2.10). There was a dose dependent decrease in the levels of MMP-2 within the conditioned medium of T-HESCs upon treatment with Batimastat. A significant decrease in MMP-2 was observed when cells were treated with 100 ng/ml Batimastat compared to untreated control samples after 24 hours (p value = 0.017). Levels of MMP-2 continued to decline in a dose-dependent manner the medium of Batimastat-treated cells after 48 hours, and significant differences between levels of MMP-2 in cells treated with 1, 10 and 100 ng/ml of Batimastat compared to untreated cells were observed (p value = 0.001, 0.0004 and 0.0009 respectively). The reduction in secreted levels of MMP-2 in Batimastat-treated cells was even more pronounced at 72 hours with significant decreases in the levels of secreted MMP-2 by T-HESCs treated with Batimastat concentrations (1, 10 and 100 ng/ml) compared to controls ($p < 0.0001$) for all treated samples as illustrated in figure. 4.16.

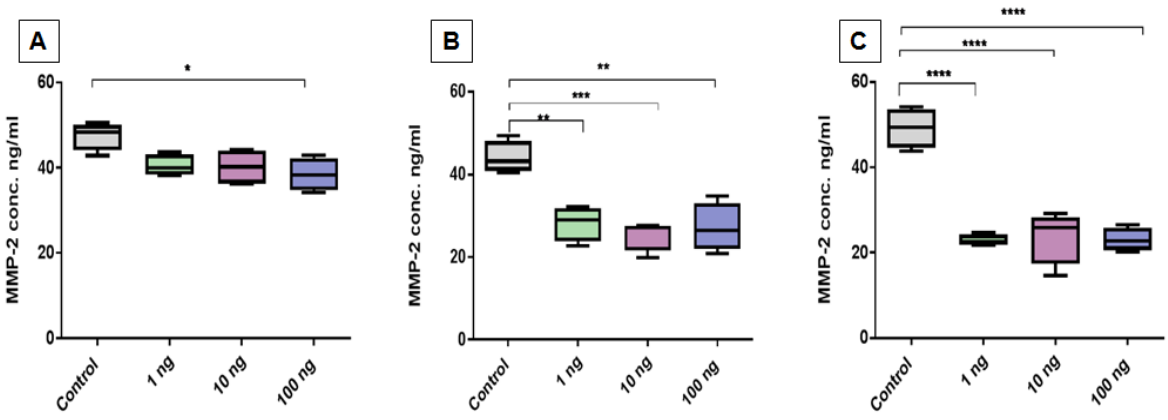

Figure 4.16. Comparison of MMP-2 levels secreted by T-HESCs treated with increasing concentrations of Batimastat

2X10^5 T-HESCs were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml Batimastat for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml Batimastat) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

4.3.4.4. Expression of MMP-2 from JAR cells treated with Batimastat

Cultured JAR cells were treated with different Batimastat concentrations (0, 1 ng, 10 ng/ml and 100 ng/ml) for 24, 48 and 72 hours (section: 2.2.10). After 24 hours, no significant changes in the levels of MMP-2 in the conditioned medium of JAR cells treated with different doses of Batimastat concentrations (1ng/ml, 10 ng/ml and 100 ng/ml) were detected. However, a significant dose-dependent decrease in the levels of MMP-2 was seen after 48 hours of co-incubation with JAR cells with all Batimastat concentrations tested compared to controls (p value = 0.02, 0.0004 and 0.0001 respectively). Moreover, the levels of MMP-2 in the conditioned medium of Batimastat-treated JAR cells were decreased further at 72 hours with levels of MMP-2 at the lower detection limit and significantly decreased compared to untreated controls for all concentrations tested (p value < 0.0001) for all Batimastat concentrations, as illustrated in figure 4.17.

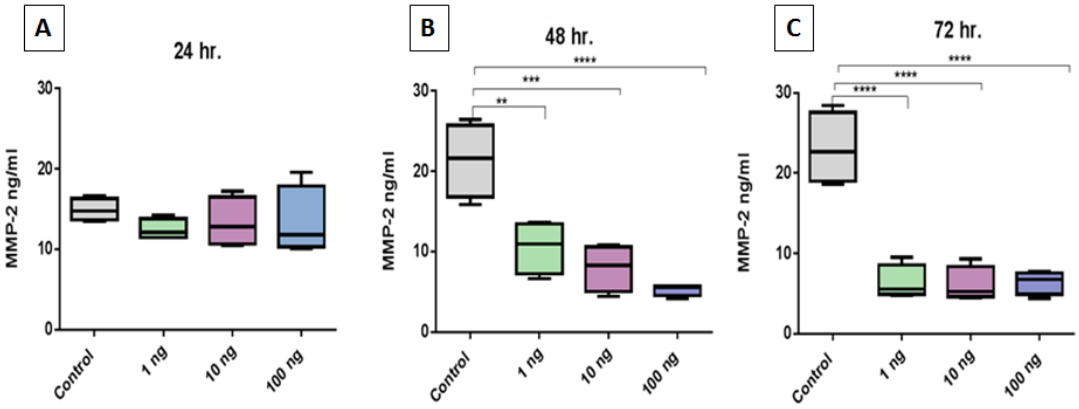

Figure 4.17.Comparison of MMP-2 levels secreted by JAR cells treated with increasing concentrations of Batimastat

$4X10^5$ JAR cells were cultured in 6 well plates and stimulated with 1, 10 or 100 ng/ml Batimastat for (A) 24, (B) 48 and (C) 72 hours. Medium alone (0 ng/ml Batimastat) was used as a control and MMP-2 was measured by ELISA. Data are the median and interquartile ranges from 4 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA

4.3.4.5. Expression of MMP-9 from T-HESCs and JAR cells treated with TIMP-2 and Batimastat.

Both T-HESCs and JAR cells treated with increasing concentration of TIMP-2 or Batimastat did not express MMP-9 over 24, 48 and 72 hours. The values recorded for MMP9 secretion were below the detection limit of the ELISA.

4.4. Discussion

MMPs are involved in remodelling the ECM of the reproductive tissues during the menstrual cycle, embryo implantation, placentation and parturition. Numerous roles for MMPs in stimulating reproductive tissue growth have been suggested, including promoting tissue expansion by changing basement membrane integrity and release of growth factors from the ECM (Lenhart et al., 2001, Curry and Osteen, 2003, Amalinei et al., 2007).

IHC studies were performed to characterise the cells for markers examined in this study. Results showed that endometrial epithelial cells, stromal cells and JAR cells were all galectin-9 positive as expected. As with other stromal cells like dermal fibroblasts (Driskell et al., 2013), endometrial stromal cells (T-HESCs) were positive for the stromal marker vimentin and CD10 whereas the endometrial epithelial cells and JAR cells were negative, confirming the phenotype of these cells. Although not quantifiable, immunohistochemical staining also showed that both endometrial epithelial, stromal and JAR trophoblasts expressed TIMP-2, RLX-2, MMP-2 and MMP-9. The stromal cells and JAR cells appeared to express more TIMP-2 than the endometrial epithelial cells. Zymography was used to provide a semi-quantitative measurement of both pro- and active MMP-2 and MMP-9 production by these cells and these experiments showed that the stromal and JAR cells constitutively expressed markedly more MMP-2 than the endometrial epithelial cells. In contrast MMP-9 levels were very low for all cell types suggesting that of the two MMPs tested, MMP-2 is the most abundant.

RLX-2 has been illustrated to have a role in promoting uterine growth and placentation, as well as stimulating cervical remodlling and ripening at parturition at the end of pregnancy (Lenhart et al., 2001). Although RLX-2 is an essential factor in the remodelling of connective tissue of the reproductive tract (Palejwala et al., 2001), little is known regarding the effects of relaxin on the expression of MMPs in humans. Given the ability of RLX-2 to promote tissue remodelling and growth, it was hypothesised that a regulatory interaction exists between relaxin and MMPs.

In this chapter, endometrial stromal (T-HESCs) and trophoblast (JAR) cells were used to investigate the effect of RLX-2 on levels of MMP-2, MMP-9 and TIMP-2. Various studies suggest that RLX-2 can up-regulate MMPs expression. Data presented by Lenhart et al (2001) showed that MMP-2 and MMM-9 were significantly increased in uterine flushes from relaxin-treated pigs. They also observed that the expression levels of MMP-2 and MMP-9 were lower locally in both the cervix and the uterus after treatment with RLX-2 (Lenhart et al., 2001). Palejwala *et al* demonstrated that RLX-2 had a positive effect on the expression of MMP-1 and MMP-3 by fibroblasts in the lower uterine segments in human tissue at both the mRNA and protein level (Palejwala et al., 2001). In addition, the same authors showed that RLX-2 increased secretion of MMP-2 by the cervix as well as up-regulating the expression of various cytokines secreted by leucocytes and other cells in the cervix known to stimulate MMPs expression (Palejwala et al., 2001).

In this study T-HESCs secreted MMP-2 constitutively and the levels of secreted MMP-2 increased in the medium over time with cell proliferation. Stimulation of T-HESCs with RLX-2 did not significantly alter secreted levels of MMP-2 when compared to unstimulated controls in both a time- and dose-dependent manner. Moreover, T-HESCs did not express MMP-9 with or without treatment with RLX-2. Other studies found that RLX-2 negatively regulates endometrial MMPs expression (Goldsmith et al., 2004). Goldsmith *et al* (2004) found that RLX-2 had a negative effect on endometrial MMPs expression, where levels of both proMMP-1 and proMMP-3 were significantly lower in relaxin-treated rhesus monkey. These contradictory effects of RLX-2 on MMPs may be due to experimental design (*in vitro* versus *in vivo*). For example, in an *in vivo* system, MMPs activity may be regulated locally by a number of tissue inhibitors of matrix metalloproteinases that may not be available in the *in vitro* environment (Lenhart et al., 2001). The data presented in this thesis strongly suggest that RLX-2 does not affect the expression of MMP-2 or MMP-9 by T-HESCs. Although there was no effect on MMP-2 activity in response to RLX-2 treatment this does not exclude the role of RLX-2 in extracellular remodelling in early implantation as alternate pathways utilising other proteases such as MMP-1/3 could be involved.

In contrast to MMP-2, relaxin-stimulated T-HESCs showed increased secretion of TIMP-2, this was significant but minimal at 24 hours, the difference increased by 72 hours when stimulated with 10 but not 100 ng/ml, suggesting that activation is reached at a maximal dose. These data are in agreement with Goldsmith *et al* who showed that TIMP-2 expression levels were significantly elevated in relaxin-treated animals compared to controls (Goldsmith et al., 2004). In contrast, Palejwala *et al* previously showed that RLX-2 negatively affected the expression of TIMP-2 in human uterine fibroblasts (Palejwala et al., 2001) although this difference may be due to tissue differences in these stromal cells.

The invasion of trophoblast into the decidua is an essential step for early placenta development and early pregnancy maintenance. For successful embryo implantation, the invasion of trophoblast into the decidua is under a tight control and MMPs play a major role in this process. The production and action of RLX-2 hormone during pregnancy and parturition are highly species dependent. In human, the production of RLX-2 at the maternal-fetal interface is mainly by the placenta, decidua and foetal membranes (Maruo et al., 2007). While there are numerous studies on the effect of RLX-2 as a positive regulator of MMPs in term placenta, there is little data regarding the interaction between RLX-2 and MMPs in early development of the placenta. The data presented in this chapter show that RLX-2 increased the secretion of MMP-2 by JAR trophoblast cells in a dose-dependent manner with significant differences being observed compared to unstimulated cells after 72 hours in culture. These data are in agreement with Maruo *et al* who were the first to describe the effects of RLX-2 on MMP-2, MMP-3 and MMP-9 in early extra villous trophoblast cultured *in vitro*. These authors demonstrated that RLX-2 up-regulated gene expression of both MMP-2 and MMP-9 whilst down-regulating expression of TIMP-2. Gene expression of MMP3 was not altered by RLX-2 in the cultured early placental extra villous trophoblast (Maruo et al., 2007). In contrast, this study showed that relaxin-stimulated JAR cells did not show increased secretion of TIMP-2 and did not express MMP-9. Differences in the data provided in this study and that of Maruo *et al* could be due to the use of human early placental extra villous trophoblasts

compared to JAR trophoblasts and the method of analysis; qPCR (gene expression) compared to ELISA (protein) used in this study.

Culture of T-HESCs and JAR cells with TIMP-2 showed a biphasic effect. The T-HESCs showed decreasd levels of MMP-2 in the conditioned medium after 48 hours in comparison to unstimulated controls. This was followed by almost a threefold increase in MMP-2 levels by 72 hours compared to MMP-2 levels at 48 hours. Likewise, JAR cells displayed decreased levels MMP-2 at 48 hours in comparison to unstimulated controls followed by an almost five-fold increase at 72 hours compared to MMP-2 levels at 48 hours. The likely explaination is that at 48 hours there was enough TIMP-2 to bind to and reduce the levels of MMP-2 in the medium (the MMP-2 is present but complexed with the TIMP-2, so the antibodies in the ELISA are blocked from binding and therfore can not detect it). However, at 72 hours the cells have proliferated and so there are more cells secreting MMP-2 into the medium. At this point MMP-2 is in excess of the exogenous TIMP-2 so there is more MMP-2 in the medium that can be bound by the antibidoes in the ELISA. Moreover, recombinant TIMP-2 has a limited half-life and so at 72 hours it is likely that the inhibitory effects of TIMP-2 are more limited.

The effect of a broad spectrum MMPs inhibitor (Batimastat) on the levels of secreted MMP-2 was also investigated. Both T-HESCs and JAR cells treated with Batimastat showed a dramatic decline in secrted MMP-2 levels at both 48 and 72 hours but not at 24 hours. Data that is not disimilar to use of recombinant TIMP-2. The prolonged inhibitor affect seen with Batimastat is likely due to the high concentrations of inhibitor used or due to the high affinity of Batimastat for MMP-2 (Rasmussen and McCann, 1997). In this instance Batimastat binds to and saturates all the MMP-2 in the medium so that the MMP-2 is not detected by the ELISA at 48 hours. Even when more MMP-2 is secreted at 72 hours the inhibitor is still present at saturating concentrations so any MMP-2 present is still complexed and is not detected. TIMP-2 regulates the activity of MMP-2 so any dysregulation of this relationship could lead to alterations in the remodelling of the ECM that may potentially cause failure of implantation. It is interesting to note that addition of either endogenous TIMP-2 or Batimastat did not affect MMP-2 levels after

24 hours, while MMP-2 levels were significantly affected at 48 hours. Inhibitors usually bind quickly to their ligands and so further experiments are required to determine why this was the case for both protein and synthetic MMP inhibitors.

T-HESCs and JAR cells did not express MMP-9 with or without treatment by both ELISA and zymogram which could be due to the presence of α2-macroglobulin in FCS which binds and inhibits the activity of MMPs. However, numerous investigators used supernatant including FCS from different cell lines to detect MMPs by ELISA and they were detected including MMP-9 (Barille et al., 1997, Sobrin et al., 2000, Verma et al., 2010), on other hand other investigator used serum free media to detect MMPs (Yu and Stamenkovic, 2000), therefor, a further investigations are required to compare the results obtained from same cell line cultured with serum free media and complete growth media including FCS.

In summary this chapter has illustrated that endometrial cells, and in particular, T-HESCs and JAR cells express TIMP-2 and MMP-2. RLX-2 increased the secretion of TIMP-2 by T-HESCs but has no significant effect on MMP-2 production. Conversely, RLX-2 increases the secretion of MMP-2 by JAR trophoblast cells but has no affect on TIMP-2 production. Taken together these data suggest a role for RLX-2, MMP-2 and TIMP-2 in trophoblast-endometrial/stromal interactions that may affect trophoblast implantation. The next chapter will investigate the potential effect of RLX-2, MMP-2 and TIMP-2 on trophoblast-endometrial interactions using 2D and 3D endometrial *in vitro* models to study attachment and invasion stages.

Chapter 5 The effect of RLX-2 hormone, MMP-2, MMP-9 and TIMP-2 on trophoblast-endometrial interactions in vitro

5.1. Introduction

The implantation of the embryo to the wall of the uterus is an intricate process and a pivotal step for a successful pregnancy. Two key components are required for successful implantation; embryo quality and a receptive endometrium. However, a considerable transformation of the uterine lining is necessary to generate a favourable environment for embryo implantation, a process that is under the tight control of a complex network of molecules. A delicate balance exists between the molecules that promote endometrial receptivity and those that prevent it and any imbalance can lead to implantation failure, which is associated with several adverse pregnancy outcomes.

The molecular mechanisms of early embryo implantation remain poorly understood. Studies of the process have been mainly descriptive and the information derived from *in vivo* studies are insufficient. Studying embryo implantation *in vivo* is a complicated area of research because of practical and ethical implications. As a result, scientists have developed numerous approaches or models to explore and investigate human embryo implantation. *In vivo* animal studies have led to important developments in the understanding of the process of embryo implantation. However, due to the differences in the reproductive system between the animal models used and humans, knowledge gained from these functional animal studies cannot always be directly translated to humans. In addition, for *in vitro* experiments, the use of primary cells derived from human endometrial tissue has limitations including, patient to patient variation, short cell life span and cell differentiation. Consequently, there is a need to establish multi-cellular *in vitro* models to investigate human embryo implantation (Weimar et al., 2013).

In recent years, several studies using *in vitro* tissue culture models have shown a promising way forward to understand the embryo-endometrium cross-talk at early implantation stages and the pathophysiology of implantation failure.

Immortalized cell lines derived from the endometrium at different phases of the menstrual cycle that keep their cell behaviour and chracteristics have been generated. These include cells of the receptive endometrium (Ishikawa, EEC-1 and HES cell lines), non-receptive endometrium (HEC-1A) and glandular epithelium (Ishikawa and RL95-2) (Hannan et al., 2010). Similarly, human embryos used in IVF procedures have a very restricted access for research. As a result several different trophoblast cell lines derived from the human placenta have been established for research purposes. These have different migration and adhesion (AC1M-88 and HTR-8/Svneo), syncytialization (BeWo) and invasion (JAR, JEG-3, HTR-8/Svneo and BeWo) characteristics. (Hannan et al., 2010).

Due to the difference in the developmental stages of endometrial and trophoblast cells, the selection of the appropriate cell line for experiments is considered fundamental (Hannan et al., 2010). In the present study, we investigated the trophoblast-endometrial interaction using an *in vitro* model of early human embryo implantation. The endometrium was simulated by the use of Ishikawa, RL95-2 and HEC-1A cell lines. RL95-2 and Ishikawa cell lines are highly adhesive to trophoblast cell lines compared to HEC-1A cells, and so these cells are considered a more appropriate model for receptive endometrial epithelial cells (Hannan et al., 2010). The human embryo was simulated using JAR trophoblast cell line, which was previously reported to have the ability to attach in a short time of co-incubation with endometrial epithelial cells (Thie et al., 1998).

To complement the experiments performed *in vivo* in chapter three, 2D and 3D endometrial *in vitro* models were optimised to study the trophoblast-endometrial interaction. The attachment and the invasion stage under the stimulation of human RLX-2 hormone, MMP-2, MMP-9 and TIMP-2 was investigated to determine if stimulation of endometrial epithelial cells and endometrial stromal cells with these factors could interfere with attachment of trophoblast spheroids to different endometrial cell lines.

Hypothesis

Altered expression of RLX-2 hormone, TIMP-2, MMP-2 and MMP-9 affect trophoblast-endometrial interaction during early human implantation.

Aims

The aims of this chapter are to gain insight into the role of RLX-2 hormone, MMP-2, MMP-9 and TIMP-2 during trophoblast-endometrial interaction, using an *in vitro* implantation model to measure the adhesion of JAR spheroids to an Ishikawa, RL95-2 or T-HESCs monolayer. Additionally, to explore the effect of recombinant human of RLX-2 hormone, MMP-2, MMP-9 and TIMP-2 during trophoblast-endometrial interaction in the *in vitro* co-culture model.

5.2. Materials Methods

Refer to chapter 2 from (section: 2.2.11 to 2.2.20).

5.3. Results

5.3.1. Optimisation of JAR spheroids

In order to establish an attachment assay, JAR spheroid size was optimised (150 - 250 µm to represent a trophoblast) so this could be produced *in vitro*. JAR spheroids were generated using the method of Korff et al with minor modifications (Korff et al., 2004) see (section: 2.2.14.1). JAR spheroids were produced in a cell density dependent manner ranging from an initial seeding density of (100, 250, 500, 1000, 5000 and 10,000 cells/100 µl) Figure 5.1 shows the mean diameter of each spheroid group (6 spheroids per group). The size of spheroids increased gradually over time, from day one to day nine. Cultures starting with 100 cells /100 µl media was the ideal number of cells at the start of the culture period in order to obtain spheroids with a diameter of 150 to 250 µm (the desired size) at the fourth to the fifth day of culture.

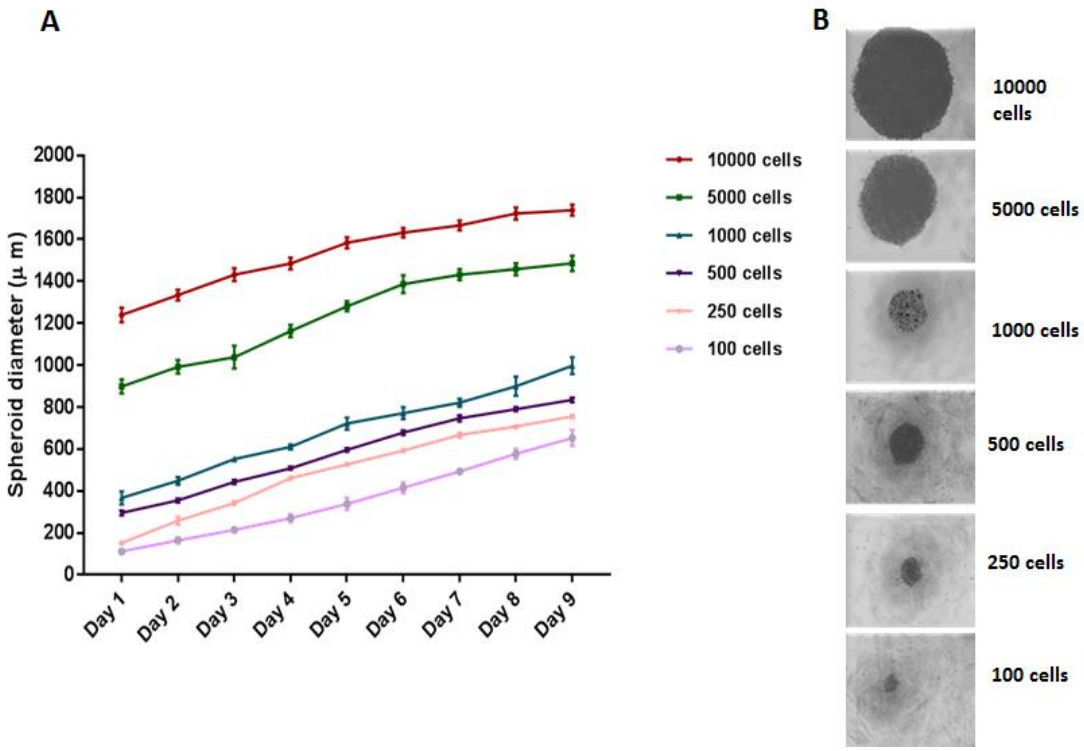

Figure 5.1. Optimization of JAR spheroids

(A) Shows the mean diameter of each spheroids group over nine days, each group with different number of cells was used to form the spheroids (100, 250, 500, 1000, 5000 and 10,000 cells/100 µl). (B) Figures of JAR spheroids each represent a number of cells/well

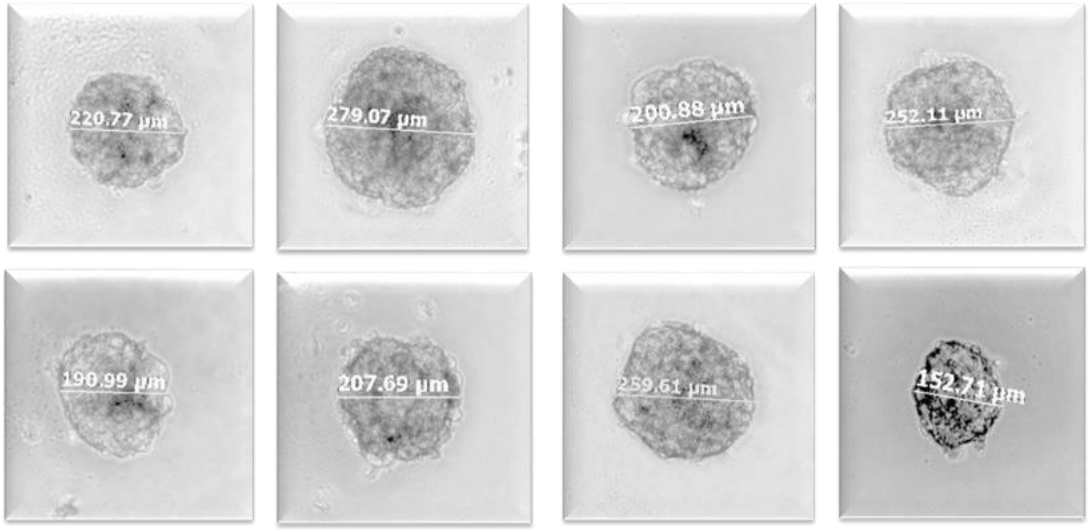

Figure 5.2. JAR spheroids with the mean diameter from (150 µm to 250 µm).
Starting with 100 JAR cells/ 100 µl media was the ideal cell number to start with to develop spheroids with a 150 to 250 µm diameter by the fourth day.

5.3.2. Production of β-hCG by JAR spheroids

To evaluate the functional properties of the JAR trophoblast spheroids, ELISA was used to measure the secreted hormone β-hCG in the spheroid conditioned medium over 8 days at 48 hours' intervals. Figure. 5.3 shows that JAR spheroids secreted β-hCG in a cell density-dependent manner. However, the amount of β-hCG secreted every 48 hours per spheroid did not increase over time (Fig. 5.3).

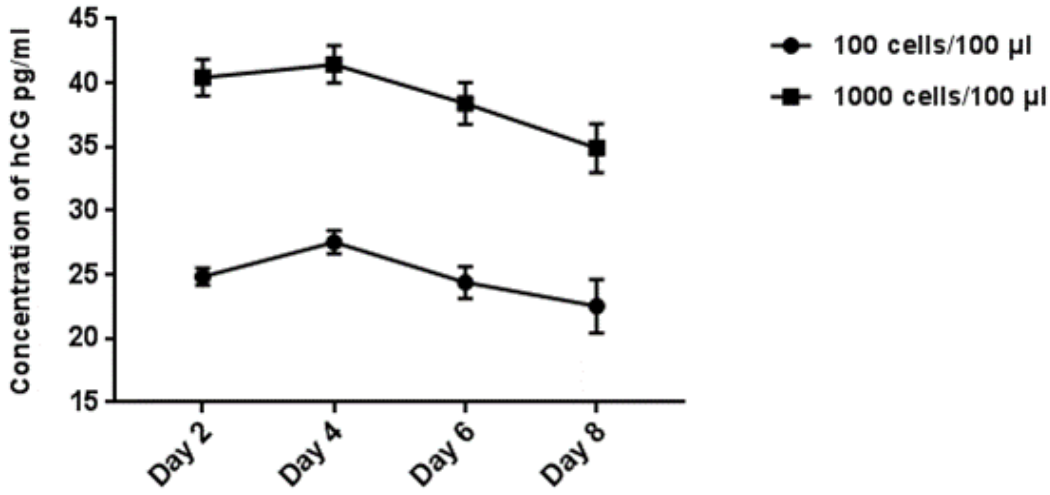

Figure 5.3. Secretion of β-hCG by JAR spheroids of human trophoblast cells.
The secretion of β-hCG represented as pg/ml of β-hCG per spheroid in 1 ml medium at day 2, 4, 6 and 8. Two different of spheroids were used to investigate the effect JAR spheroids on β-hCG secretion. The conditioned medium was analysed every 48 hours. Data are representative of 4 independent experiments.

5.3.3. *In vitro* adhesion assay of JAR spheroids to endometrial epithelial and stromal monolayer cell lines

Figure 5.4 identified the attachment kinetics of JAR spheroids to monolayers of endometrial epithelial cell lines and an endometrial stromal cell line. The adhesion of JAR spheroids to the different monolayers increased over time. However, experiments with Ishikawa, RL95-2 and endometrial stromal cells (T-HESCs) showed a markedly higher attachment rate than the HEC-1-A cell line. Therefore, HEC-1-A cell line was excluded from further experiments, and the Ishikawa and RL95-2 cell lines were used as a receptive endometrium to study the attchment and inavsion stages.

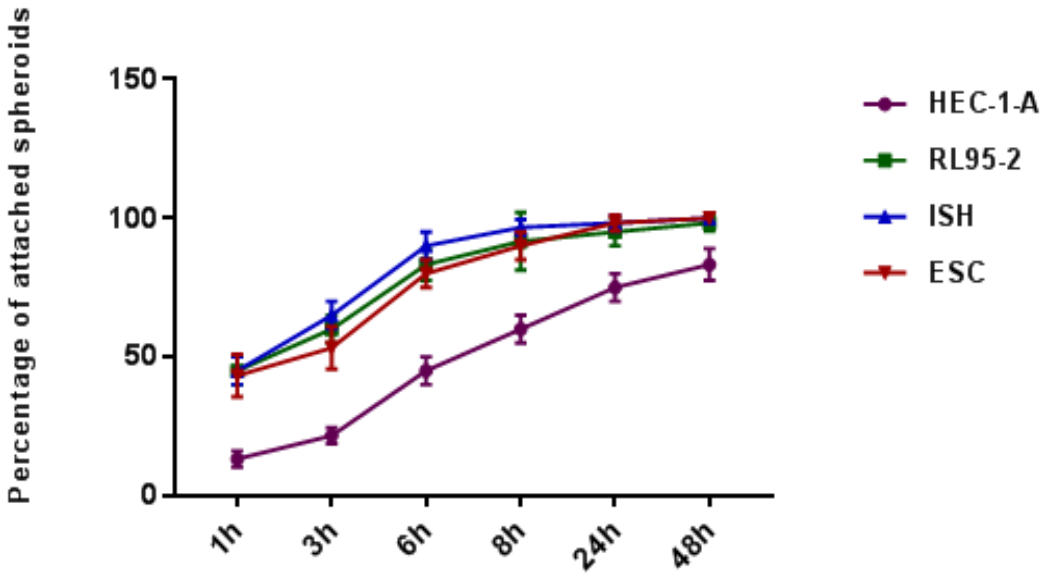

Figure 5.4. Attachment of 20 JAR trophoblast spheroids to epithelium endometrium monolayers (RL95-2, Ishikawa and HEC-1-A cell lines) and endometrial stromal cells over 48 hours

Data are representative of 3 independent experiments.

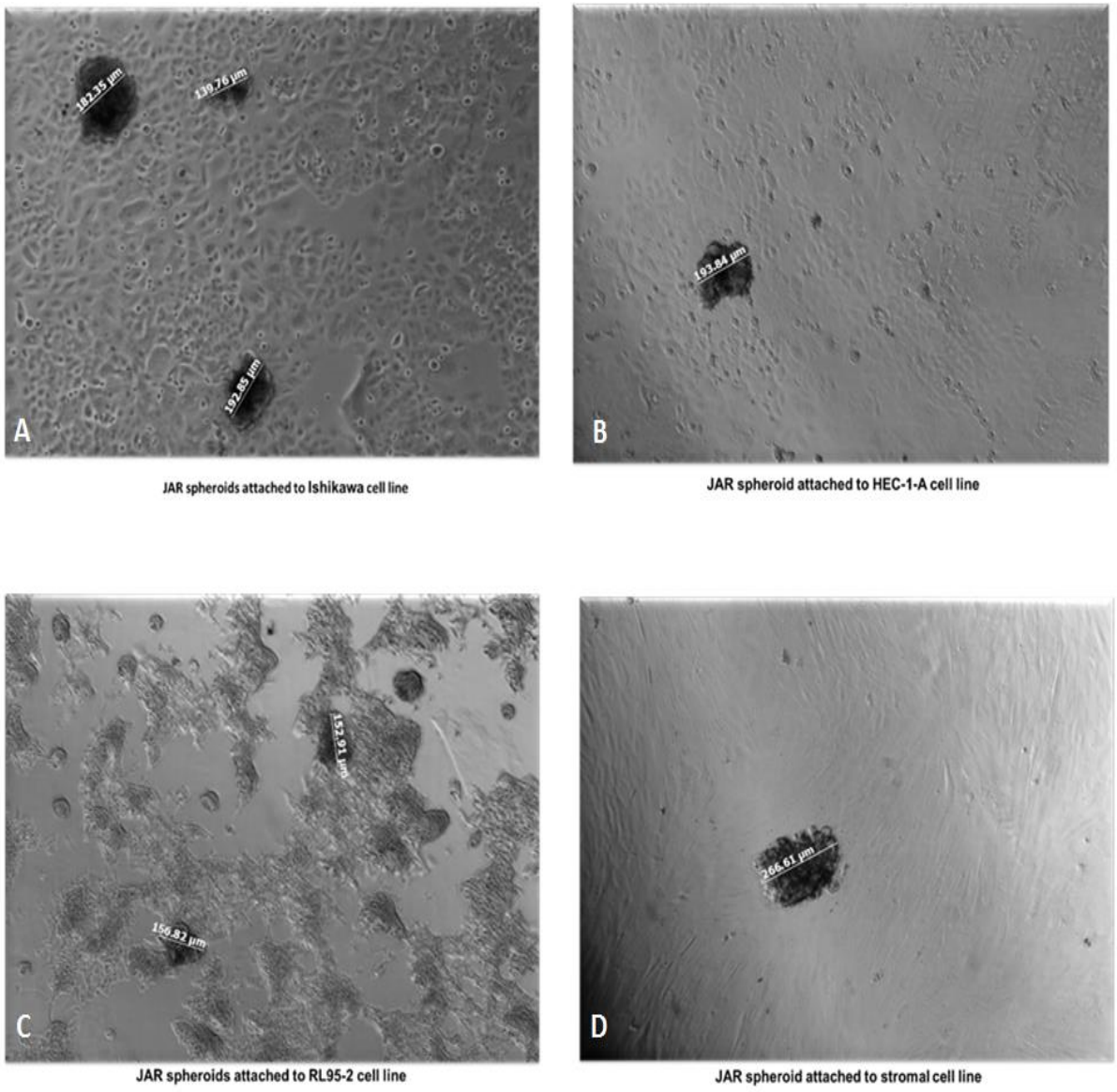

Figure 5.5. Representative images showing the attachment of JAR trophoblast spheroids to epithelium endometrium monolayers

(**A**) Ishikawa, (**B**) HEC-1-A, (**C**) RL95-2 endometrial epithelium cell lines and (**D**) endometrial stromal cells

5.3.4. The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation on the adhesion of JAR spheroids to endometrial epithelial and stromal cell monolayers

To examine the potential effects of RLX-2, MMP-2, MMP-9 and TIMP-2 on the binding of JAR spheroids (20 per treatment) to the endometrial epithelial (Ishikawa and RL95-2) and stromal cells (T-HESCs) monolayers. An adhesion assay was performed in the absence of treatment (control) and under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 treatment over 24 hours (section: 2.2.16). In figure 5.6 the attachment rate of JAR spheroids to Ishikawa cell line monolayer after 2 and 6 hours shows no significant difference between the control (without treatment) and the attachment of JAR spheroids under the effect of different treatments. A similar response was also observed after 24 hours with all treatments with the exception of treatment with TIMP-2 that resulted in a significant reduction (p value = 0.0413) in spheroid attachment compared to un-treated controls.

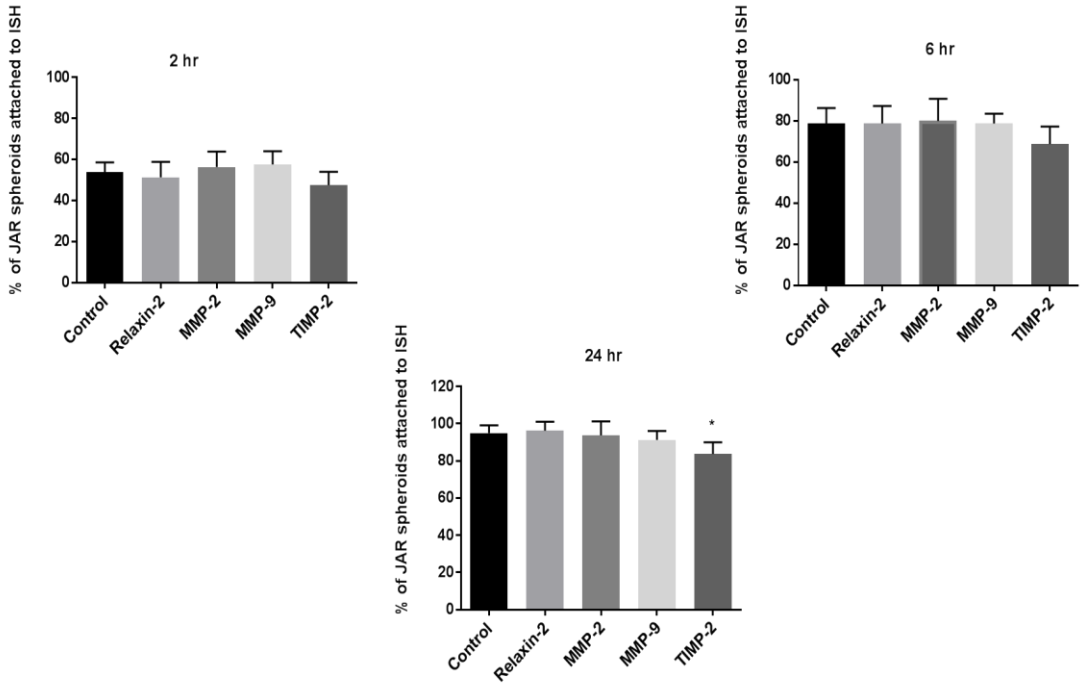

Figure 5.6. Attachment of 20 JAR spheroids to Ishikawa cell line monolayer under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 and over 24 hours

Data are the mean and standard deviations (SD) from 3 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 on the binding of 20 JAR spheroids to the RL95-2 shows no significant differences when compared to untreated controls at any of the time points examined (Figure 5.7).

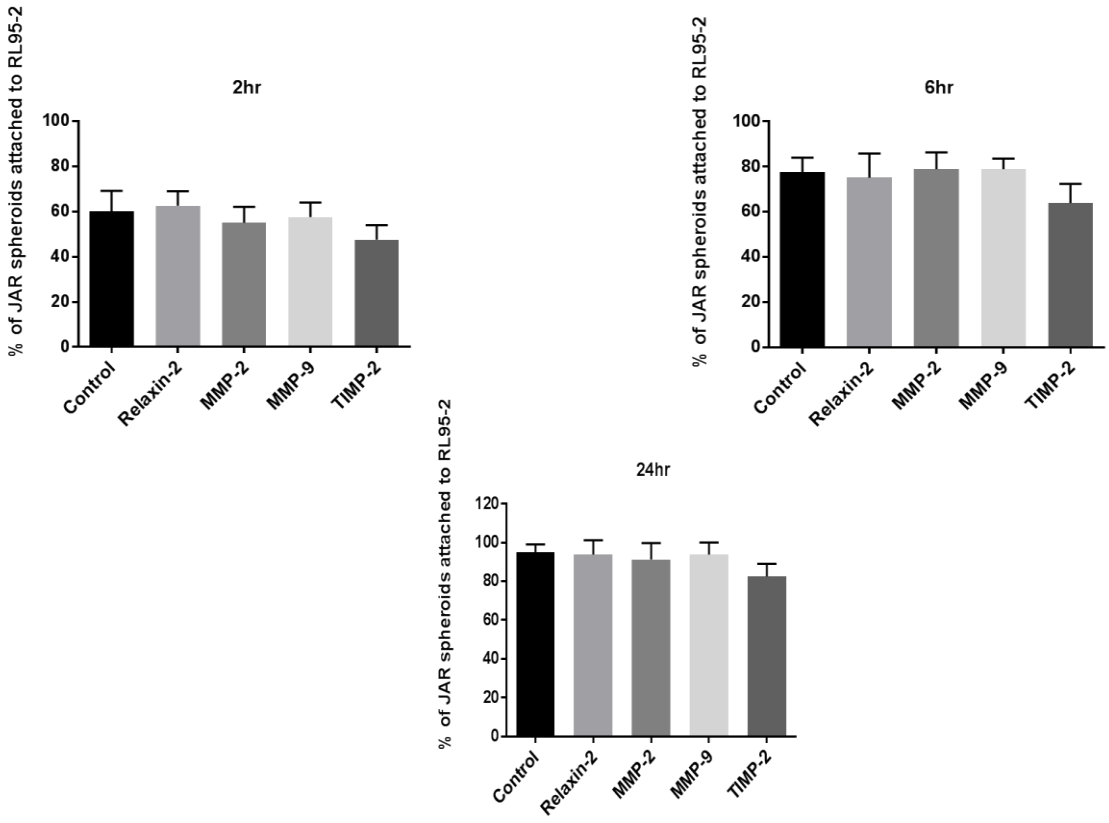

Figure 5.7. Attachment of 20 JAR spheroids to RL95-2 cell line monolayer under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 and over three different time points (2, 6 and 24 hours)

Data are the mean and standard deviation (SD) from 3 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

In figure 5.8 the attachment of 20 JAR spheroids to T-HESCs monolayer was significantly decreased when the T-HESCs were treated with TIMP-2 at (6 and 24 hours) compared to untreated control (p value = 0.0033 and 0.0016 respectively). However, there was no significant difference between JAR spheroids attachment to the untreated control group or JAR spheroids under the effect of different treatments at any other time point tested.

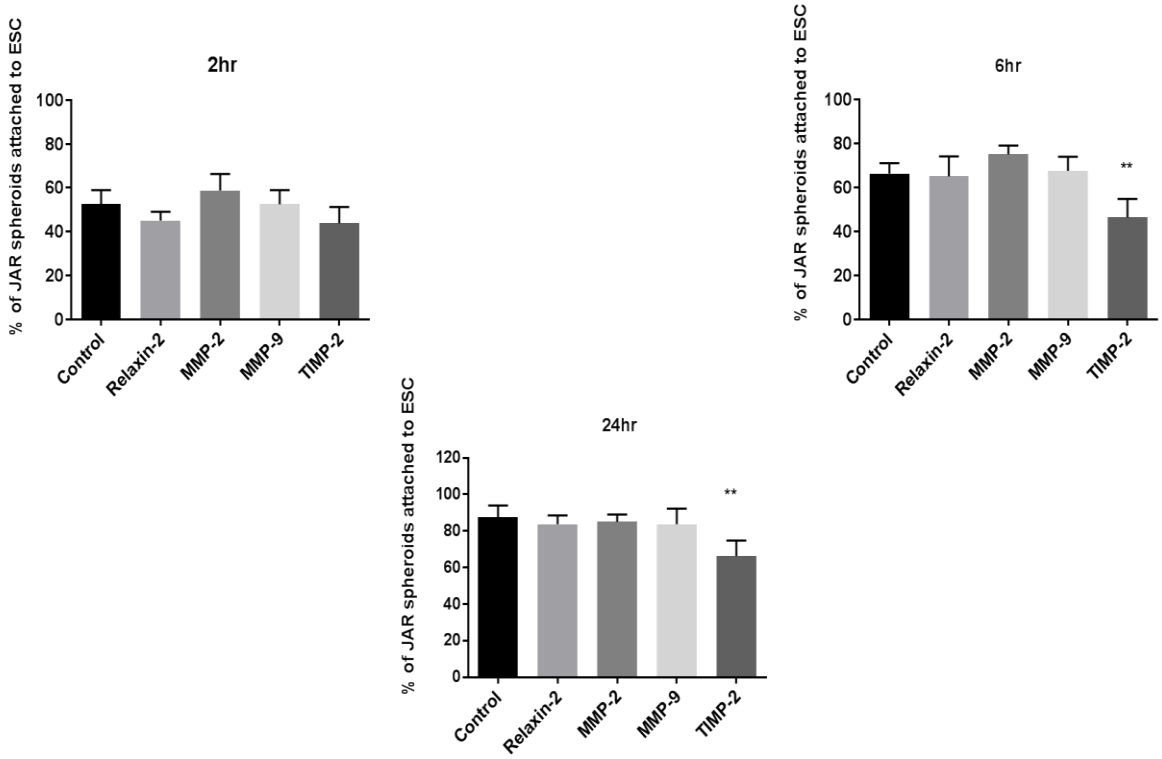

Figure 5.8. Attachment of 20 JAR spheroids to ESC cell line monolayer under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 and over three different time points (2, 6 and 24 hours)

Data are the mean and standard deviation (SD) from 3 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA.

5.3.5. Characterisation and optimisation of 3D endometrial model

For *in vitro* studies, the endometrial environment and early embryo implantation stages have, for a long time, been modelled by simple monolayer culture of endometrial epithelial cells. However, with the advancement of tissue engineering, an *in vitro* 3D tissue model was established in an attempt to mimic the endometrial tissue and to represent the attachment and invasion stages in early embryo implantation process.

Tissue engineered 3D endometrial model was based on oral mucosal model previously described by Dongari-Bagtzoglou *et al* (2006) with minor modifications (Dongari-Bagtzoglou and Kashleva, 2006b) see (section: 2.2.12 and 2.2.13). The 3D models were produced by culturing endometrial stromal cells at the air-to-liquid interface on a rat-tail type I collagen populated with endometrial epithelial cell line RL95-2 or Ishikawa cell line. The 3D models were fixed, sectioned and stained with haematoxylin and eosin (H&E) for histological analysis. Figure 5.9 shows H&E stained sections of 3D endometrial model cultured with Ishikawa cells. In the figure the nuclei are stained blue and the cytoplasm and connective tissue stained pink. The epithelium generated was multiple-layered, however, this does not represent the native phenotype of the tissue that is generally single layered. To make the 3D model similar to native endometrium the models were not cultured at the air-to-liquid interface but were kept submerged in the culture media. Figure 5.10 shows (H&E) stained sections of 3D endometrial models cultured with Ishikawa and RL95-2 cell lines respectively. Here, epithelial cells formed a single cell monolayer on the top of the 3D model with endometrial stromal cells incorporated into the collagen hydrogel, a phenotype more characteristic of native endometrium.

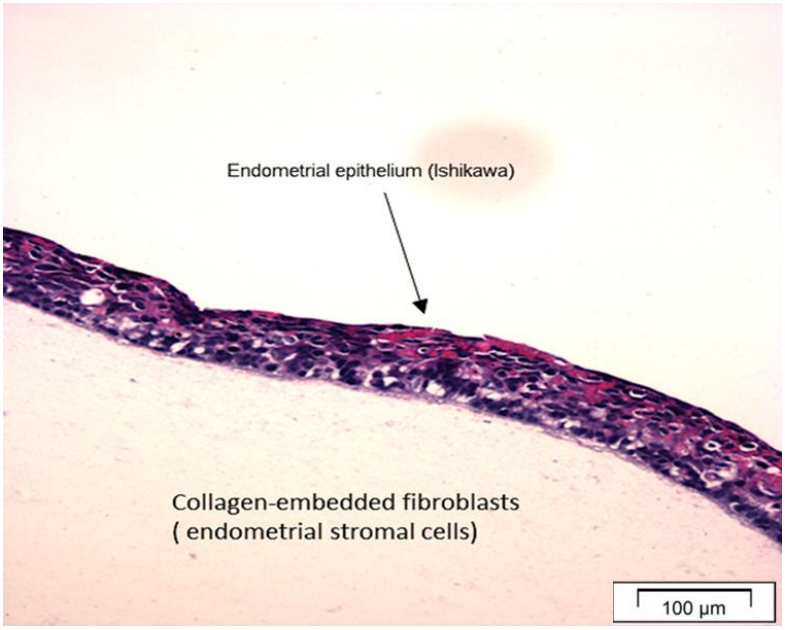

Figure 5.9. Representative images of Haematoxylin and Eosin (H&E) stained 3D endometrial models.

Ishikawa cell line was seeded onto the surface of a rat-tail type I collagen populated with endometrial stromal cells, and then the culture raised to an air-to-liquid interface and cultured for 7-10 days. Models were fixed, paraffin embedded, sectioned and H&E stained.

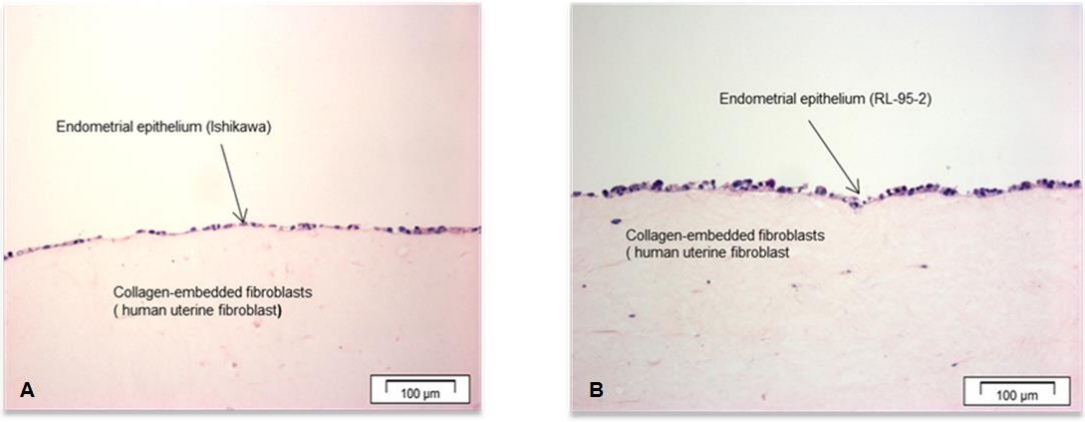

Figure 5.10. Representative images of Haematoxylin and Eosin (H&E) stained 3D endometrial models.

To culture the endometrial models, endometrial stromal cells were suspended in rat-tail type I collagen, after 24 hours (A) Ishikawa and (B) RL95-2 cell lines respectively were seeded onto the surface and were left completely submerged in culture media. Following 5-7 days in culture the models were fixed, paraffin embedded, sectioned and H&E stained.

5.3.5.1. Immunohistochemistry

To verify the comparability of the endometrial 3D models with the native endometrial tissue, immunohistochemistry was conducted to detect the known endometrial markers galectin 9 and cytokeratin 7. Figure 5.11 shows diffuse immunostaining throughout the whole epithelium for galectin 9 that was comparable with the positive staining detected in native endometrium. In contrast, cytokeratin 7 showed a very week positive Immunostaining in the 3D endometrial model and the same expression profile for the marker was observed with native endometrial tissue. No immune-reactivity was observed in the negative controls where the primary antibody was omitted to assess the antigen dependent binding.

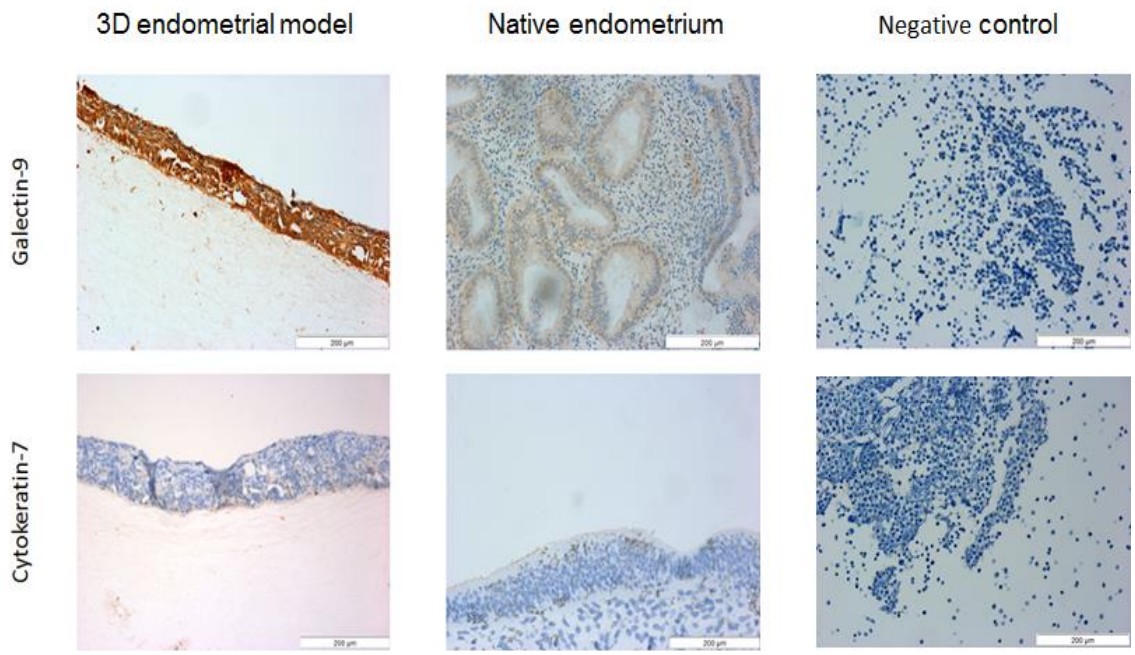

Figure 5.11. Endometrial epithelial markers (galectin 9 and cytokeratin 7) expressed in the 3D endometrial models and native human endometrial tissue visualised by immunohistochemistry

5.3.5.2. Histology of JAR spheroids attachment to the 3D endometrial model

The attachment of JAR spheroids to the 3D culture system was investigated after 24 hours co-culture. Figure 5.12 shows H&E staining at 24 hours co-culture (A, B and C) of the attachment of JAR spheroids to 3D culture system. JAR spheroids attached to the epithelial surface at random sites on the 3D model. Histological examination showed that the JAR spheroids were intimately attached and in some instances, had fused with the surface epithelium of the endometrial model (Fig. 5.12 A&B) and this occurred with models comprised of either RL-95-2 or Ishikawa epithelial cells.

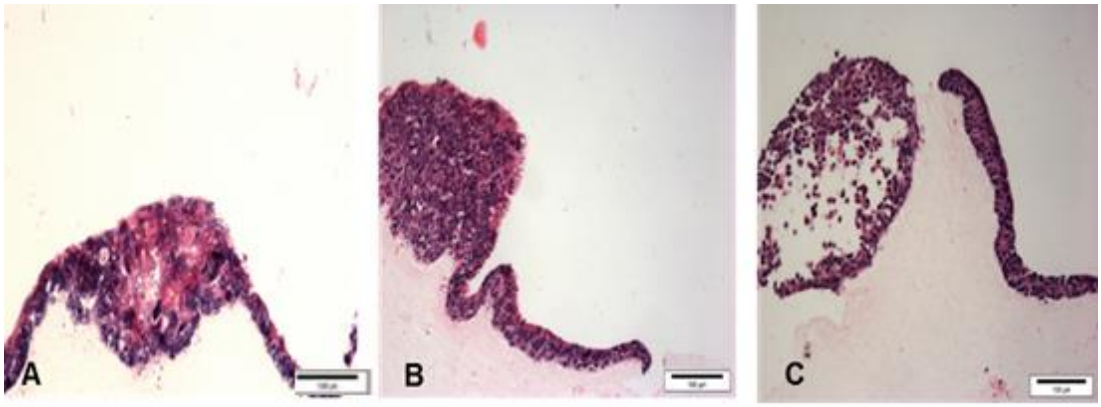

Figure 5.12. Representative images of JAR trophoblast spheroids attachment to the 3D endometrial models

(**A**) JAR spheroid attaching to RL95-2/ T-HESCs 3D culture model, (**B** and **C**) JAR spheroid attaching to Ishikawa / T-HESCs 3D culture model after 24 hours of implantation. The models were fixed, paraffin embedded, sectioned and H&E stained.

5.3.5.3. Immunohistochemical staining of βhCG as a functional marker of JAR trophoblast spheroids

To investigate the properties of JAR spheroids, the expression of βhCG was evaluated by immunohistochemistry in the 3D trophoblast/model co-culture. Figure 5.13. Shows JAR spheroid immunostained with an antibody to βhCG, 20-30% of cells within the JAR spheroid were positively stained for βhCG.

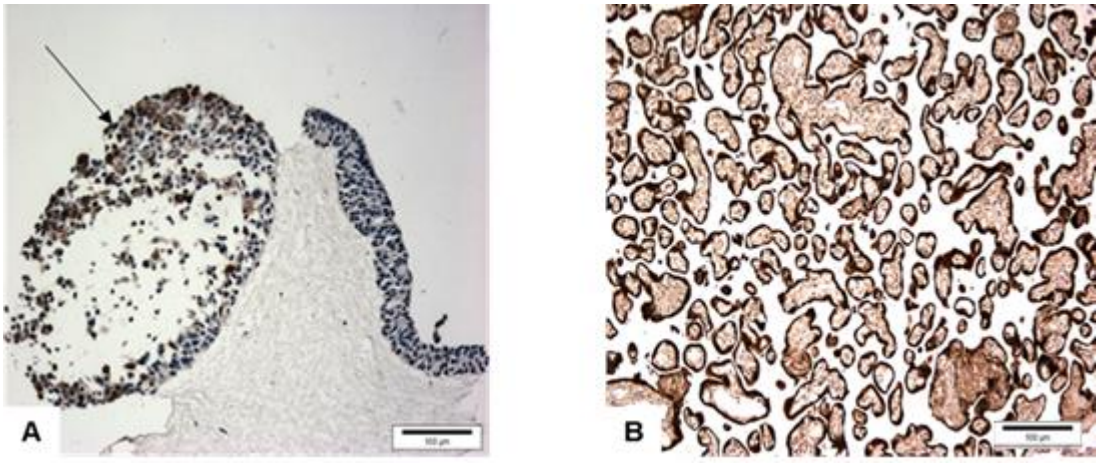

Figure 5.13. JAR spheroids express βhCG in 3D co-culture models

(A) Immunohistochemical staining for βhCG in trophoblast cells of JAR spheroid implanted over 3D culture system after 24 hours co-incubation (Brown, indicated by arrow). (B) Placental tissue was used as a positive control, scale bar 100 µm.

5.3.6. The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation on the adhesion of JAR trophoblast spheroids to 3D culture system

The attachment of JAR spheroids to the 3D culture system using Ishikawa epithelial cells was investigated at 6 and 24 hours co-culture see (section: 2.2.17). Figure 5.14 shows the percentage of JAR spheroids attached to the 3D endometrial model under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 at 6 and 24 hours. There was a significant decrease in the attachment rate of JAR spheroids to the endometrium when the models were treated with TIMP-2 at 6 and 24 hours (p value = 0.0193, and 0.0368 respectively), compared to untreated controls. In contrast, there was no significant difference detected when models were treated with RLX-2, MMP-2 and MMP-9 compared to untreated controls.

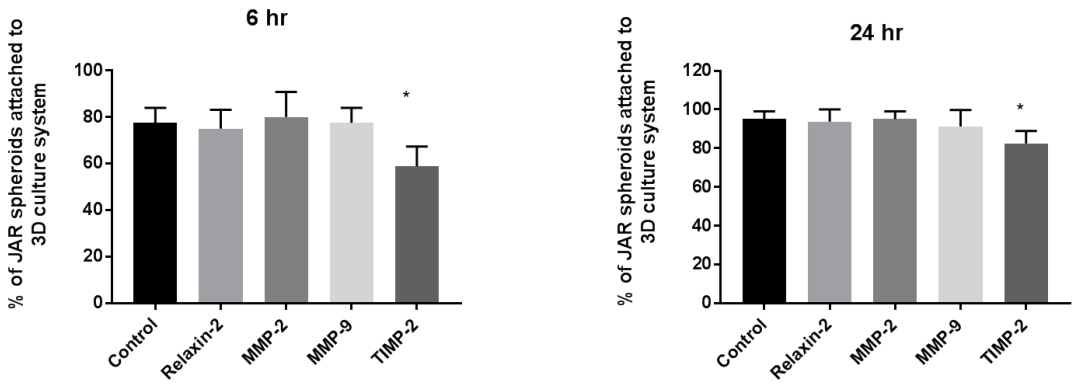

Figure 5.14. Attachment of 20 JAR spheroids to the 3D culture system under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation at 6 and 24 hours.

Data are the mean and standard deviation (SD) from 3 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA (*p<0.05).

5.3.7. The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation on the invasion of JAR trophoblast spheroids into an invasion matrix

A commercially available invasion assay was performed to examine the invasion on JAR spheroids into a tissue matrix and whether RLX-2, MMP-2, MMP-9 or TIMP-2 had an effect on invasion. MDA-MB-231 invasive breast cancer cells were used as a positive control as recommended by the manufacturer protocol, see (section: 2.2.18). The morphological results of the 3D cell invasion over a four-day co-incubation showed that both JAR spheroids and the control MDA-MB-231 breast cancer spheroids do not invade into the invasion matrix but rather persisted as cell aggregates in the matrix. (Fig. 5.15, Fig. 5.16) shows the morphology of the 3D JAR trophoblast and MDA-MB-231 (as invasive cells control) spheroids invasion under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 at day 1, 2, 3 and 4.

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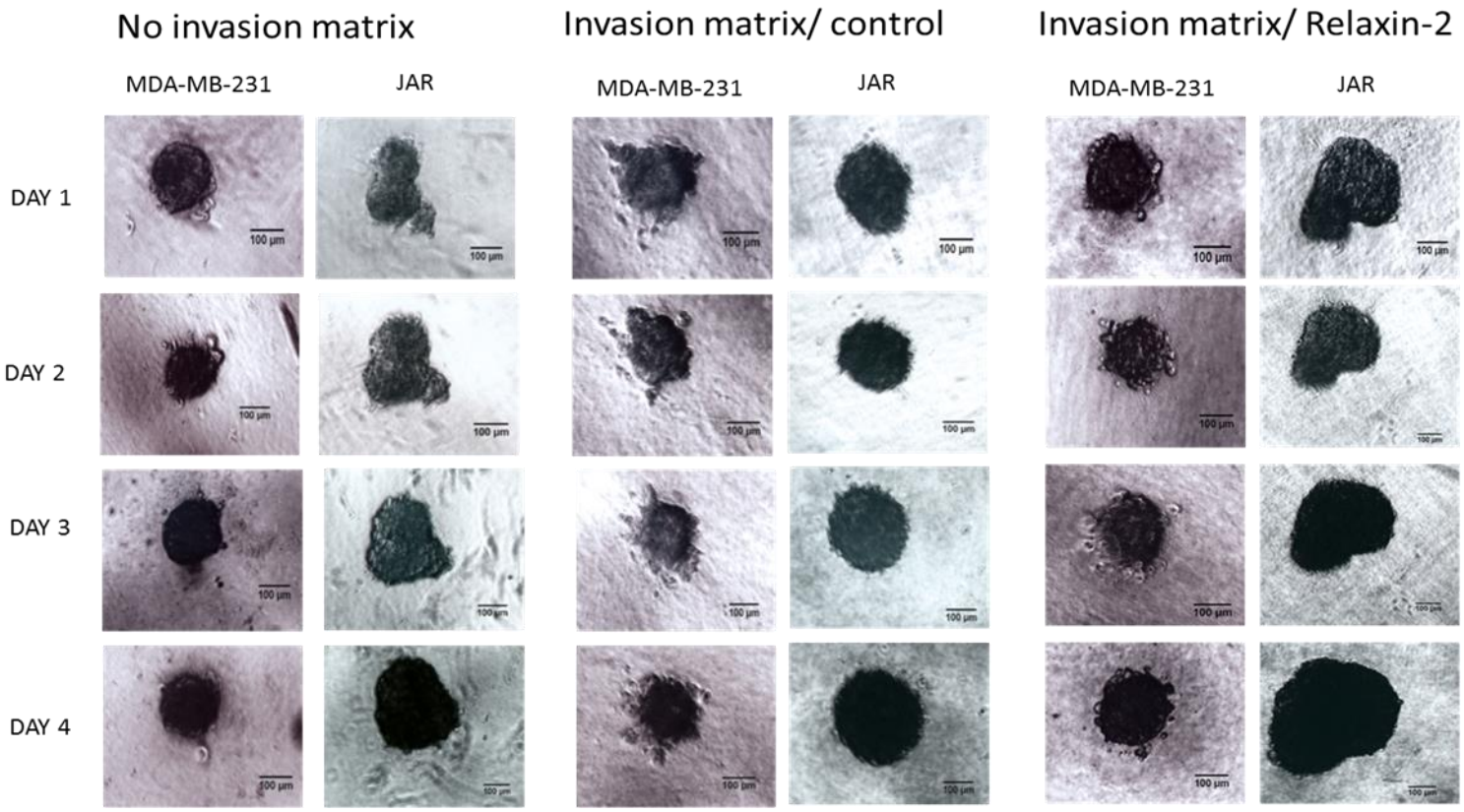

Figure 5.15. Morphology of the 3D JAR trophoblast and MDA-MB-231 (as invasive cells control) spheroids invasion under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 at day 1, 2, 3 and 4.

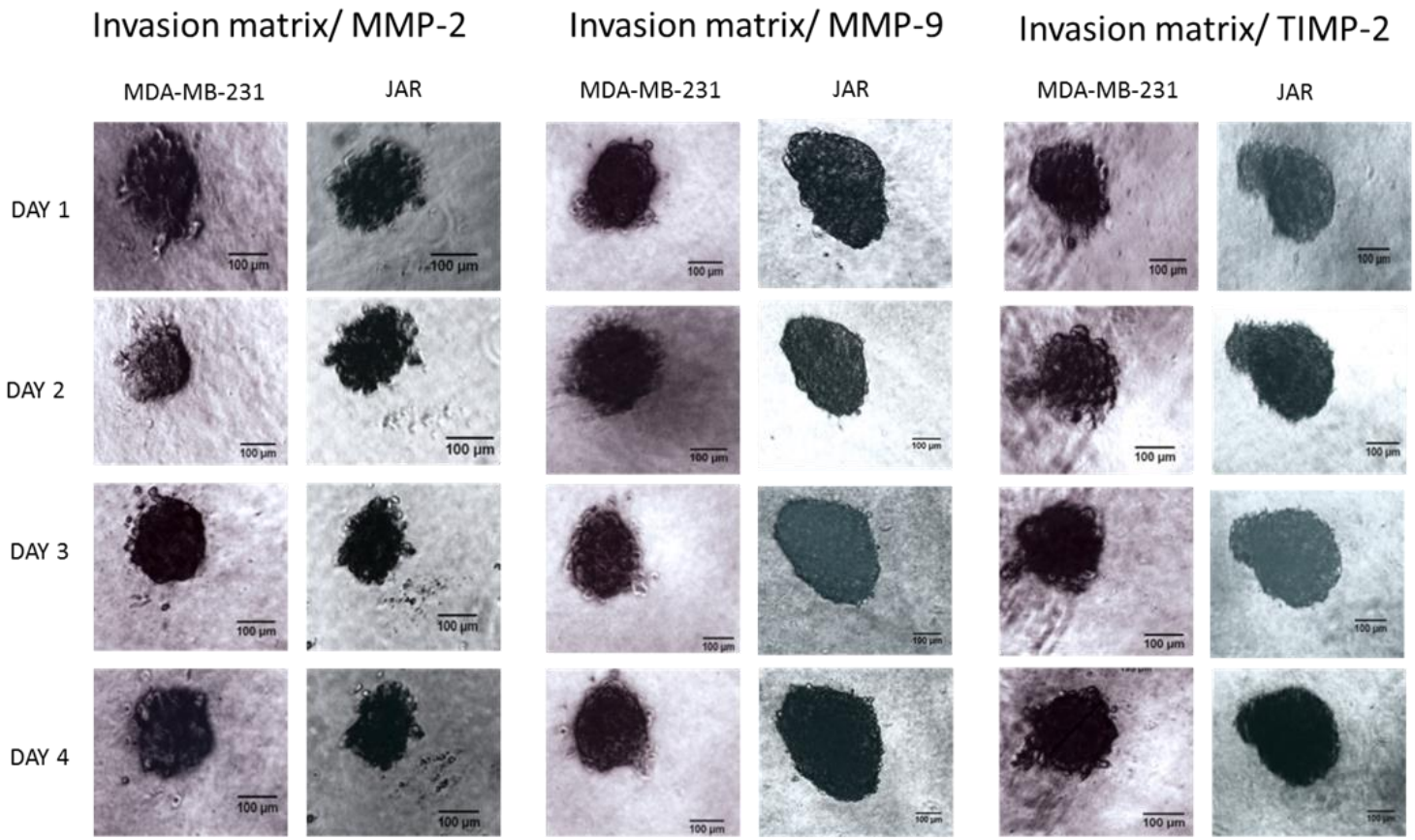

Figure 5.16. Morphology of the 3D JAR trophoblast and MDA-MB-231 (as invasive cells control) spheroids invasion under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 at day 1, 2, 3 and 4.

5.3.8. The effect of RLX-2, MMP-2, MMP-9, TIMP-2 and Batimastat co-incubation on the expansion of JAR trophoblast spheroids on endometrial stromal cells

A co-culture 2D model was established to mimic trophoblast endometrial interaction in early implantation stages. Figure 5.17 (A) shows the effect of RLX-2, MMP-2, MMP-9, TIMP-2 and Batimastat (broad spectrum MMPs inhibitor) on JAR spheroids expansion on endometrial stromal cells after one-day co-incubation, the expansion was significantly enhanced by the presence of MMP-2 and MMP-9 (p value = 0.0021 and 0.0052 respectively) compared to the untreated control, and significantly inhibited by co-incubation with Batimastat (p value = 0.0203) compared to the no treatment control. Figure 5.17 (B) shows that the expansion was significantly enhanced (p value = 0.048) in the presence of MMP-2 co-incubation compared to the control after two days of co-incubation, while there was no significant difference with other treatments compared with the control. On the third day of co-incubation as illustrated in figure 5.17 (C), the spheroid expansion was significantly enhanced by the presence of MMP-2 and MMP-9 (p value = 0.0409 and 0.0164 respectively) compared to the untreated control, and significantly inhibited by co-incubation with Batimastat (p value = 0.0125) compared to the untreated control. However, by day four of co-incubation as illustrated in figure 5.17 (D), a significant increase in the expansion rate was observed when the co-culture was treated with MMP-2 (p value = 0.0374) and a significant decrease in the expansion rate was observed when co-incubated with Batimastat (p value = 0.0008) compared to untreated controls. Co-incubation with RLX-2, MMP-9 and TIMP-2 showed no significant difference compared to the controls at all-time points tested. Figure 5.18 shows the expansion of JAR trophoblast spheroids on endometrial stromal cells under the effect of RLX-2, MMP-2, MMP-9, TIMP-2 and Batimastat at day 1, 2, 3 and 4.

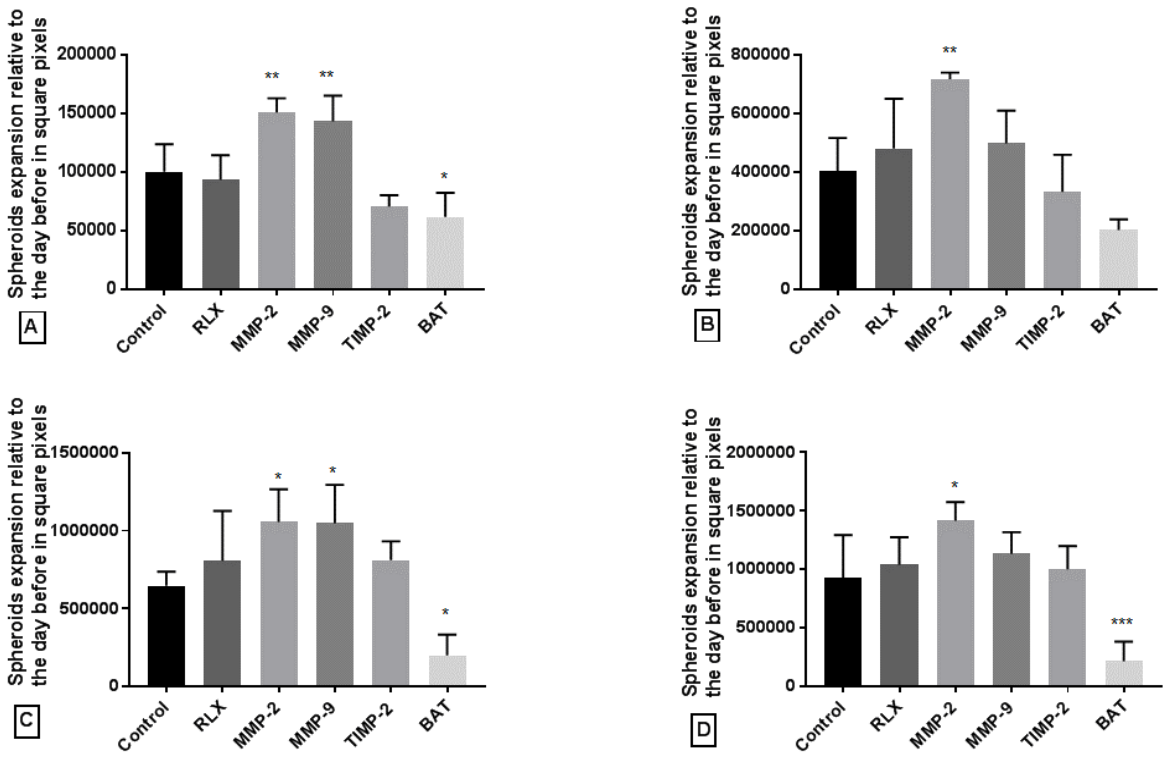

Figure 5.17. Expansion of JAR trophoblast spheroids on endometrial stromal cells under the effect of RLX-2, MMP-2, MMP-9, TIMP-2 and Batimastat (BAT)

Day 1 (**A**), day 2 (**B**), day 3(**C**) and day 4 (**D**). The area covered by the JAR spheroids is expressed relative to the day before. The results are expressed relative to JAR spheroids expansion in the present of each treatment compared to the control in square pixels. Data are the mean and standard deviations (SD) from 3 independent experiments, with each experiment performed in triplicate. Differences between groups and time-points were performed using ANOVA ($p<0.05$=*, $p<0.01$=**, $p<0.001$=***).

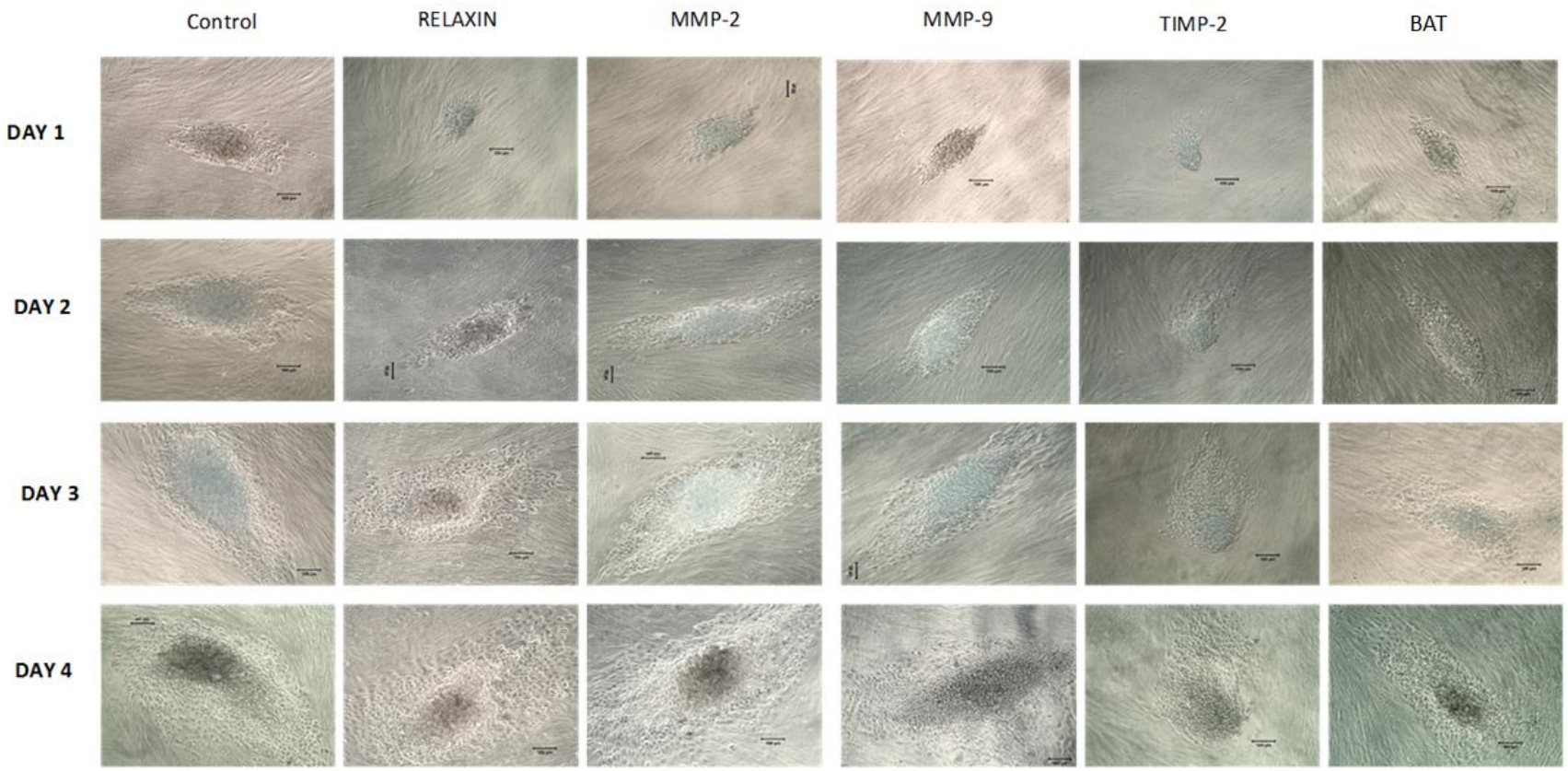

Figure 5.18. Representative images showing the expansion of JAR trophoblast spheroids on endometrial stromal cells under the effect of RLX-2, MMP-2, MMP-9, TIMP-2 and Batimastat (BAT) at day 1, 2, 3 and 4. The area covered by the JAR spheroids was measured using ImageJ.

5.4. Discussion

Embryo implantation is a critical process initiated by the attachment of the trophoblast to the endometrium epithelial, followed by trophoblast invasion into the uterine stroma. Successful implantation requires two major factors, a receptive endometrium and a good quality of embryo. However, dysregulation during this process including inappropriate levels of hormones, adhesion factors, cytokines and proteases and their inhibitors may lead to failure of implantation (Singh et al., 2011). The aim of this chapter was to explore the effect of the hormone RLX-2, the matrix metalloproteinases MMP-2, MMP-9 and their inhibitor TIMP-2 on trophoblast-endometrial interaction. *In vitro* studies rely on model systems that mimic early implantation stages when the blastocyst has directly attached to and breached the epithelium and then the trophoblast invades the decidua cells.

The experiments performed in this chapter represent important stages of implantation (attachment and invasion). The use of 2D models, in terms of JAR spheroids attachment to endometrial epithelial or endometrial stromal cells grown as monolayers, and a multi-cellular 3D culture system provide a means to investigate early adhesion and implantation events in detail. Although, it is difficult to evaluate precisely how well these human *in vitro* models represent what happens in human *in vivo*, they do provide the closest possible alternative to the native *in vivo* environment.

The endometrial epithelial cell lines (RL95-2, Ishikawa and HEC-A-1) used in the experiments have different receptivity characteristics. RL95-2 and Ishikawa cell lines are highly receptive endometrial epithelial cells, while HEC-A-1 is considered as non-receptive endometrial epithelium cell line (Hannan et al., 2010). In order to study the attachment and invasion stages under the stimulation of human RLX-2, MMP-2, MMP-9 and TIMP-2, an experiment was performed to confirm the receptivity character of the three different endometrial epithelial cell lines (RL95-2, Ishikawa and HEC-A-1) along with endometrial stromal cells.

The attachment rate of different endometrial epithelial cell lines has been well recognised using different cell line monolayers and trophoblast spheroids (Heneweer et al., 2005). However, the results presented here demonstrated that Ishikawa and RL95-2 cell lines were more receptive to JAR spheroids than HEC-A-1 cell line, that is consistent with the data from previous studies (John et al., 1993, Thie and Denker, 2002, Heneweer et al., 2005, Wang et al., 2012).

The JAR cell line was chosen as the trophoblast model as this cell line combines a characterisation of invasion extra villous trophoblast (EVT) and villous cytotrophoblast (VT) (Hannan et al., 2010). The multicellular JAR spheroids used as a model for trophoblast attachment and invasion were confirmed by positive staining for the functional trophoblast marker β-hCG, and the cultured media displayed positive β-hCG secretion by the cells with an increased secretion in a cell number dependent manner. The immunohistochemistry results for JAR spheroids showed that approximately 20-30% of cells were positively stained for β-hCG. This result is consistent with previous studies, which demonstrated that approximately 25% of cells displayed positive Immunostaining for β-hCG (White et al., 1988, Wang et al., 2012)

The effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation on the adhesion of JAR spheroids to the endometrial epithelial and stromal cells monolayers indicated that TIMP-2 significantly decreased the attachment rate of JAR spheroids to endometrial stromal cell lines after 6 and 24 hours of co-incubation. However, co-incubation of RL95-2, Ishikawa cell line and endometrial stromal cell line with RLX-2, MMP-2 and MMP-9 showed no significant difference in the attachment rate of JAR spheroids.

The contribution of the TIMPs to the cell adhesion process either via direct interaction with cell adhesion molecules or with ECM components is much less studied. Ki-Kyung Jung (2006) and his colleagues demonstrated that TIMP-1 engaged with its cellular receptor, CD63, a member on the tetraspanins known to interact with cell adhesion molecules including integrins, modulating cell adhesion process (Jung et al., 2006).

It was also confirmed that TIMP-1 interacts with β1 integrin on the mammary cell membrane surface in a CD63-dependent manner, leading to inhibition of apoptosis. Dong-Wan Seo (2003) and his group confirmed that endothelial cell growth *in vitro* was inhibited by TIMP-2 by stimulation with endothelial growth factors, VEGF-A or FGF-2. The recognised mechanism is independent of TIMP-2 mediated MMP inhibition. It was also shown that extracellular TIMP-2 and Ala+2TIMP-2 (TIMP-2 mutant without MMP inhibitory activity) interact with integrin α3β1 located on the surface of tumour and endothelial cells, prompting an integrin-dependent signalling reaction (Seo et al., 2003). Dimitra Bourboulia (2010) and colleagues confirmed that TIMP-2 also stimulated RECK expression resulting in loss of cell migration. RECK is a protein on the plasma membrane that suppress a number of MMPs including MMP-2, MMP-9, MT1-MMP and ADAM10 (Bourboulia and Stetler-Stevenson, 2010).

Studies have also attempted to investigate the effect of the MMPs on cell adhesion. Early on, it was shown that MMP-2 interacts with cell membrane bound components, directly affecting the properties of cell adhesion of human melanoma A2058 cells *in vitro*. They confirmed that, altering the production of TIMP-2 modulates the adhesion and spreading properties of the cells, not only proteolysis of the ECM. These effects of TIMP-2 appear to be mediated by inhibition of MMP-2 activity (Ray and Stetler-Stevenson, 1995).

To our knowledge, there are no previous studies that have explored the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation on the trophoblast-endometrium interaction *in vitro.* However, further investigations are required to explore the interaction of TIMP-2 and cell adhesions molecules to identify the mechanism of inhibition of cell to cell adhesion.

To investigate the attachment and invasion stages it was decided to develop a 3D culture system, based on oral mucosal models that have been used to mimic the oral mucosa (Dongari-Bagtzoglou and Kashleva, 2006a). These models are constructed of a connective tissue matrix comprised of type1 collagen populated with fibroblasts and topped with oral keratinocytes. In our experiment, we developed a 3D endometrial model based on the oral

mucosa with modifications that make it imitate the native endometrial tissue by using type1 collagen populated with endometrial stromal cells and topped with endometrial epithelial cells. It is necessary to characterise these models histologically to validate their structure to native tissue. When cultured at an air-to-liquid interface the endometrial epithelium was several layers thick as seen with oral mucosal models (Dongari-Bagtzoglou and Kashleva, 2006a). However, this is not characteristic of native endometrial tissue that is only 1-2 epithelial layers thick (Stevens and Lowe, 2005). When the endometrial 3D models were covered in medium, cell stratification did not occur and so models remained at 1-2 cell layers in thickness that was more representative of the native tissue. Similar observations were observed by Pinnock et al for oral mucosal models (Pinnock et al., 2014).

Ishikawa cell line was selected as the endometrial epithelium for the 3D culture, as it represents a good model for trophoblast-endometrial interactions (Heneweer et al., 2005). Indeed, data from the 2D assays showed that Ishikawa was receptive and bound JAR spheroids rapidly. Moreover, models made from Ishikawa cells were positive for galectin-9, a specific marker for endometrial cells *in vivo* (Popovici et al., 2005). Furthermore, these cells are able to polarise and have the capacity for apical attachment for trophoblast cells *in vitro* (Heneweer et al., 2005). The potential effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation on the adhesion rate of JAR spheroids to 3D Ishikawa culture system indicated that TIMP-2 significantly decreased the adhesion of JAR spheroids to the endometrium epithelium.

This study is the first *in vitro* study to explore the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 on trophoblast-endometrial interaction using trophoblast spheroids and endometrial cell lines or a 3D endometrial model. The role of TIMP-2 and its negative effect on cell/spheroid adhesion is poorly understood and needs further investigation. Therefore, in addition to their function as inhibitors of metalloproteinases, it is essential to investigate whether TIMPs function to directly affect cell attachment and the potential mechanisms for these effects. A study conducted by Ray and Stetler-Stevenson (1995) specified that altering the balance of MMPs and TIMP-2 by

genetic influence on the expression of TIMP-2 effects not only the proteolysis of the extracellular matrix but also cell attachment to the extracellular matrix, and alteration of TIMP-2 expression effects cell migration and attachment.

Using the endometrial 3 D model, we were able to identify and histologically confirm attachment of the JAR spheroids to the endometrium. The cell adhesion and spreading of the JAR spheroids onto the endometrium were observed by histological analysis. However, invasion of JAR spheroids into the 3D endometrial matrix after implantation was not observed, and it seems that JAR spheroids failed to invade the stroma even after 24-hours co-culture. This could be attributed to many reasons, one suggestion is that it could be due to the not using the correct collagen (rat tail type 1collagen), which is not similar to the native endometrium. However Wang, H. (2012) and his group developed a 3D endometrial model to investigate the attachment and invasion stages, and the 3D cultured system were developed with fibrin-agarose as a matrix (Wang et al., 2012). The 3D model performed in this study was constructed with rat tail type1 collagen to examine the role of MMPs and TIMPs and their potential effect on attachment and invasion so it was thought that collagen was the most appropriate matrix to use as scaffold as these enzymes are collagenases. However, the use of collagen/laminin or other matrix combinations may be more appropriate in future experiments.

It was decided to develop an alternative model to study the invasion of JAR spheroids under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation using a 2D assay. The endometrial stromal cell monolayer and JAR spheroids were used as an alternative invasion model. By measuring the expansion of JAR spheroids over the stromal cells monolayer every day for four days, adhesion and invasion were measured. These results indicated that the expansion of the trophoblast spheroids over the endometrial stromal cells was promoted mainly by MMP-2, and inhibited by Batimastat inhibitor. RLX-2 and TIMP-2 showed no significant effects during all time points of co-incubation. It was surprising that the Batimastat inhibitor showed a significant difference in expansion whereas recombinant TIMP-2 did not. This could be due to other MMPs being involved in the process that are inhibited by the

Batimastat inhibitor and not by TIMP-2. An alternative explanation could be the half-life and relative affinity for the Batimastat inhibitor compared to recombinant TIMP-2. For example, recombinant TIMP-2 has a relatively short half-life and affinity compared to the Batimastat inhibitor and so its action may be limited over a 24 hours assay period. Indeed, at day 1 in figure 5.17 it appears that TIMP-2 and the Batimastat inhibitor display equal potency for preventing adhesion. However, thereafter the inhibition of TIMP-2 is lost whereas that of the chemical Batimastat inhibitor continues.

To our knowledge, this study is the first to investigate the expansion of JAR spheroids over endometrial stromal cells under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2. A previous study (Gonzalez et al., 2011) showed that the expansion of trophoblastic spheroids is enhanced by decasualised endometrial stromal cells, interleukin-1β and heparin-binding epidermal growth factor-like growth factor. Furthermore, Staun-Ram et al., 2004 and colleagues suggested that MMP-2 is the key-enzyme for JAR trophoblast invasion (Staun-Ram et al., 2004). The data presented in this chapter appear to suggest that the balance between MMP-2 and its inhibitor is heavily implicated in the adhesion and potentially invasion of trophoblasts to the endometrium. Although further experimentation is required, RLX-2 appears not to be involved in this process.

Adding MMP-2 and MMP-9 directly into a culture media containing FCS and therefore α2-macroglobulin which binds and inhibits the activity of MMPs. However, our results showed that although the MMP-2 and MMP-9 were added to culture media containing FCS there was a significant increase in the expansion of JAR trophoblast spheroids on endometrial stromal cells under the effect of MMP-2 over four days compared to the untreated controls. MMP-9 shows a significant increase in the expansion of JAR trophoblast spheroids on endometrial stromal cells on 1st and 3rd of co-culture, while there was no significant difference on 2nd and 4th day. In our experiment the complete growth media containing FCS used because of the long-time experiments, and using serum free media could effect on the growth and the physiology of the cell lines.

The main limitation of this study is that the cell culture models lack *in vivo* authenticity, and do not fully represent the *in vivo* trophoblast-endometrial interaction during early human embryo implantation. There is the issue of the concentrations of agents used in the different treatment regimens to study their potential effect of both attachment and invasion assays. This is difficult to determine, as there is no data available on the localised expression of MMP-2, MMP-9 and TIMP-2. For this reason, the concentrations used were based on the data obtained from the *in vivo* study. Even if the appropriate concentrations were identified, the expression pattern of the enzymes and hormone are likely to be quite different *in vitro* compared to *in vivo*.

The early embryo implantation into the endometrium is a critical step in the establishment of pregnancy. This process is under a tight control of a very complicated network of molecules including cytokines, growth factors, hormone, cell adhesion molecules and proteases and their inhibitors, and it is clear that dysregulation of one of these molecules may lead to failure of implantation. The data in this chapter suggests that an imbalance of the MMP-2/TIMP-2 axis is implicated in this dysregulated process.

In conclusion, there are huge difficulties in exploring human embryo implantation *in vivo* due to poor tissue accessibility and so there is a challenge for researchers to continue to search for an ideal *in vitro* cell culture model. In this chapter a cell culture model of attachment and invasion was used to mimic stages of implantation and also provide data for the first time on the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 on trophoblast endometrium interaction. It appears that MMP-2 and TIMP-2 are implicated in this process; however, there are limitations. An optimal cell culture model will promote and assist in understanding the potential mechanism and the signalling pathways of trophoblast attachment and invasion during implantation, and may provide a platform for future opportunities to improve pregnancy successful rate in both spontaneous and assisted conception.

Chapter 6 Discussion

6.1. Summary of finding

The maintenance of normal pregnancy is a vital process between the embryo and the mother. It is a complex phenomenon that starts with the attachment of embryo to the endometrial epithelial cells, followed by trophoblast invasion into the stroma. Successful pregnancy depends on two essential factors, a proper receptive endometrium and a good quality embryo. Any dysregulation in the intricate interaction including hormones, proteases, adhesion factors, growth factors and cytokines may lead to failure of embryo implantation (Merviel et al., 2009, Lim, 2003). The endometrial receptivity is a term used to refer to the time period when the endometrium undergoes morphological and molecular changes to favour the implantation of the embryo. These changes among others include proliferation of endometrial epithelial cells and stromal cells, alteration of cell morphology and polarization of cell adhesion molecules expressed on the apical cell membrane (Achache and Revel, 2006, Bentin-Ley et al., 1999).

During implantation, trophoblast cells invade the uterine stroma. In order to perform that, they must degrade both ECM and basement membrane. Therefore, enzymes that target ECM and basement membrane components are required, mainly MMPs (Hulboy et al., 1997). On the other hand, large quantities of TIMPs are produced to control trophoblast invasion and provide a protective role (Salamonsen, 1999). The integrity of the endometrial connective tissue needs a precise balance between the action of tissue inhibitors of MMPs that control the activity of the MMPs, and the MMPs that extract the ECM. Relaxin hormone has been identified to stimulate MMPs activity in various species including human, pig and rat (Goldsmith and Weiss, 2009).

Over the past decade the knowledge of feto-maternal communication and decidualisation mechanism has developed extensively, however, knowledge of this complex dynamic process has not translated into predictive tests and useful interventions to improve pregnancy outcomes. Furthermore, RM is still poorly understood and therefore its management remain very challenging. This thesis has examined the effect of altered expression of MMPs, TIMPs

and RLX-2 on trophoblast-endometrium interaction. The hypotheses of the project were derived from clinical evidence presented previously in our research group, they identified an association between recurrent miscarriage and altered expression of proteinases and their inhibitors as well as RLX-2 hormone (Anumba et al., 2009, Anumba et al., 2010).

In chapter three, the *in vivo* study, we confirmed these observations and explored the expression levels of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 complex and RLX-2 hormone in women with a history of recurrent miscarriage and healthy pregnant controls matched for gestational age. We also compared the concentrations of these peptides in women with threatened miscarriage and controls with healthy pregnancies, again matching for gestational age. Our data showed that there is a significant increase in the serum expression of TIMP-2 in recurrent miscarriage compared to their healthy controls (consistent with previous findings) and low expression of MMP-2 and MMP-2/TIMP-2 complex and RLX-2 hormone. In contrast, there were no significant differences in the expression of TIMP-2, MMP-2, MMP-2/TIMP-2 complex and RLX-2 between the threatened miscarriage group and their healthy controls, while there was no significant difference in the serum expression of MMP-9 in both groups. Given these findings, and the paucity of clinically applicable markers, more research is now needed to define more accurate predictive biomarkers of endometrial receptivity and adverse pregnancy outcomes that can be used in prenatal period in a clinical setting (Edgell et al., 2013). Development of biomarkers to predict adverse pregnancy outcomes could have clinical utility in managing women with a history of RM management, and for predicting the course and outcomes of pregnancy complications such as pre-eclampsia and preterm.

Several studies have stressed that MMPs are involved in remodelling the extracellular matrix of the reproductive tissues during the menstrual cycle, embryo implantation, placentation and parturition. The potential roles for MMPs in stimulating reproductive tissue growth, including promoting tissue expansion by remodelling basement membrane integrity and stimulation of growth factors has been variously noted (Lenhart et al., 2001, Curry and Osteen, 2003, Amalinei et al., 2007). RLX-2 hormone has been shown to

have a role in stimulating uterine growth and placentation (Lenhart et al., 2001), although it is an important factor in the remodelling of connective tissue of the reproductive tract (Palejwala et al., 2001), little is known regarding the effects of RLX-2 on the expression of MMPs in humans. Given the ability of RLX-2 to stimulate tissue remodelling and growth, it was hypothesised that a regulatory interaction exists between RLX-2 and MMPs.

In chapter four, *in vitro* data indicated that stimulation of T-HESCs with RLX-2 hormone did not significantly alter secreted levels of MMP-2 when compared to unstimulated controls in both a time- and dose-dependent manner. Furthermore, T-HESCs did not express MMP-9 with or without treatment with RLX-2, while relaxin-stimulated T-HESCs showed increased secretion of TIMP-2. Although there was no effect on MMP-2 activity in response to RLX-2 treatment this does not exclude the role of RLX-2 in extracellular remodelling in early implantation as alternate pathways could be involved. The data showed that RLX-2 increased MMP-2 expression by JAR trophoblast cells in a dose-dependent manner with significant differences being observed compared to unstimulated cells. In contrast, relaxin-stimulated JAR cells did not show increased TIMP-2 expression and did not express MMP-9.

As detailed in the thesis, proteases and their inhibitors are thought to be important in the implantation process. The dynamic balance between MMPs and their inhibitors within certain levels is critical for cytotrophoblast cell invasion into the maternal endometrium. This maintains the dynamic balance of MMPs/TIMPs within a certain range, and subjects the invasion of cytotrophoblast cells into the maternal body to certain restrictions.

Culture of T-HESCs and JAR with TIMP-2 showed a biphasic effect. Both showed decreased levels of MMP-2 in the medium after 48 hours in comparison to unstimulated controls, this was followed by increase in MMP-2 levels by 72 hours compared to unstimulated cells. While the effect of a broad spectrum MMPs inhibitor (Batimastat) on the levels of secreted MMP-2 by both T-HESCs and JAR cells showed a dramatic decline in secreted MMP-2 levels in both a time- and dose-dependent manner.

The study of trophoblast endometrium interaction in early human implantation has always been challenging, it is difficult to perform *in vivo* due to strict ethical reasons to bypass these challenges during the study of implantation and reproductive development, other approaches have been used such as animal models and the use of trophoblast cell lines (mostly choriocarcinoma) and endometrial cell lines (Hannan et al., 2010).

In chapter five, the kinetics of attachment between endometrial epithelial and/or endometrial stromal monolayers with the JAR spheroids under the effect of RLX-2, MMP-2, MMP-9 and TIMP-2 co-incubation indicated that TIMP-2 significantly decreased the attachment rate of JAR spheroids to endometrial stromal cell lines in a time dependent manner up to 6 and 24 hours of co-incubation. However, co-incubation of RL95-2, Ishikawa cell line and endometrial stromal cell line with RLX-2, MMP-2 and MMP-9 showed no significant difference in the attachment rate of JAR spheroids.

As identified in our results, we have demonstrated that the expansion of the trophoblast cells over the endometrial stromal cells was promoted mainly by MMP-2, and inhibited by a Batimastat inhibitor. RLX-2 and TIMP-2 showed no significant difference during all time points of co-incubation. It was surprising that the Batimastat inhibitor showed a significant decrease in expansion whereas recombinant TIMP-2 did not. This could be due to other MMPs being involved in the process that are inhibited by the Batimastat and not by TIMP-2. An alternative explanation could be the half-life and relative affinity for the Batimastat inhibitor compared to recombinant TIMP-2. The data presented in this chapter appear to suggest that the balance between MMP-2 and its inhibitor is heavily implicated in the adhesion, and potentially the invasion of trophoblast to the endometrium. Although further experimentation is required, RLX-2 appears not to be involved in this process.

6.2. Future work

Local expression of the MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 complex and RLX-2 *in vivo*

The results of the in vivo data in this work identified that, there is a significant increase in the serum expression of TIMP-2 in women with a history of recurrent miscarriage compared to their controls with no such history, and also low expression of MMP-2, MMP-2/TIMP-2 complex and RLX-2 hormone. Altogether, this *in vivo* study was designed to highlight observations that may be examined by definitive functional studies. A large sample size in each study group will be required and will be more informative to clarify the question.

The serum results represent the systemic levels of the serum markers as several tissues contribute in this measurement and the local expression may show a difference from one tissue to another. It would be interesting to study the local expression levels of MMP-2, MMP-9, TIMP-2, MMP-2/TIMP-2 complex and RLX-2 hormone in reproductive tract tissue such as endometrium derived from women with history of RM and healthy controls during the late secretory phase to examine the expression of the markers locally, it may be that the local expression of these markers may explain the changes observed in the serum. It would also be necessary to mount functional experiments that seek to explore the signalling mechanisms of these hormones and peptides at the endometrial trophoblast interface.

Relaxin mechanisms that may be involved in the regulation of matrix metalloproteinases and their inhibitors

To understand how RLX-2 hormone could be implicated in the regulation of matrix metalloproteinases and their inhibitors a further work is required to clarify the molecular mechanisms explaining these interactions. Altogether, our study has highlighted observations that provide insights and options for further investigations to explore the signalling pathways of RLX-2 in regulation of matrix metalloproteinases and their inhibitors.

Inhibitory pathway of TIMP-2 on trophoblast endometrial interaction

Given the findings in this work, it would be interesting to explore the potential inhibitory effect of TIMP-2 on trophoblast-endometrial interaction. Understanding the mechanism whereby cells adhesion is inhibited by TIMP-2 is crucially important as it may uncover potential therapeutic targets. Moreover, there is no obvious interpretation of these findings through either dependent and/or independent MMP-inhibitory activity. Further investigation on the interaction of TIMP-2 and cell adhesion molecules are required.

The effect of absence of TIMP-2, MMP-2, MMP-9 and RLX-2 on trophoblast endometrium interaction

In chapter five we studied the effect of RLX-2, TIMP-2, MMP-2 and MMP-9 in general using different assays including attachment, invasion and expansion assays of JAR spheroids on endometrial epithelial and stromal cell. Based on the in vivo observations where the group with a history of RM showed low expression of MMP-2, MMP-2/TIMP-2 complex and RLX-2 hormone, further in vitro investigations could explore the effect of the deficiency or absence of the mentioned protein employing knockout cell lines lacking receptors to MMP-2, MMP-9, TIMP-2 and RLX-2 hormone genes to clarify their role in the trophoblast endometrium interaction process.

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Appendices

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Study Number:
Patient Identification Number for this study:

CONSENT FORM

Changes in serum relaxin levels and maternal and fetal blood flow in pregnant women with or without a history of recurrent miscarriage.

Researchers:

Dr Dilly Anumba MD, MRCOG — Senior Lecturer/Consultant Obstetrician & Gynaecologist
Dr S El Gelany MBBS — Honorary Clinical Research Associate

Please initial box

1. I confirm that I have read and understand the information sheet dated June 2006 ☐ for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, ☐ without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study. ☐

_____ Name of Patient	_____ Date	_____ Signature
_____ Name of Person taking consent (if different from researcher)	_____ Date	_____ Signature
_____ Researcher	_____ Date	_____ Signature

Volunteer **Information** sheet – Normal pregnant controls

Changes in serum relaxin levels and maternal and fetal blood flow in pregnant women with or without a history of recurrent miscarriage.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. You may obtain more information regarding the conduct of clinical research from the CERES web site at www.ceres.org.uk

1. What is the purpose of the study?

Pregnancy to women who have a history of more than 3 consecutive miscarriages (called “recurrent miscarriage”) may be more complicated than pregnancy to women who do not have this history. “Relaxin” is a protein found in blood which plays a role in normal pregnancy and fetal growth. In this study, we want to find out whether we can use the blood levels of “relaxin” during pregnancy to predict those women who will develop complications. We will compare relaxin levels in women with a history of recurrent miscarriage to those who have no such history. Using ultrasound, we will also check the growth of the baby, and the flow of blood through the womb and through the baby. We will then determine whether relaxin has any relationship with the blood flow results that we obtain, and whether these results can help us predict pregnancy problems in order to prevent them.

2. Why have I been chosen?

You have never had recurrent miscarriage and we would like to compare your group to another group of women who have had recurrent miscarriage.

3. Do I have to take part?

No. It is up to you to decide whether or not to take part. You are free to decline to take part, or to withdraw at any time after you have agreed to take part, without your decision affecting your care or that of your baby in any way. You can simply tell us by telephone or in writing that you no longer wish to take part.

4. What will happen to me if I take part?

You will be given this information sheet to keep, and asked to sign a consent form. The study will involve taking a small sample of blood from your vein several times during your pregnancy in addition to several scans to assess blood flow through your womb and your baby. Both procedures are safe and carry no risks to you or your baby. The study will involve a total of 4 visits to the hospital during which you will receive your normal antenatal checks. You will be reimbursed your travel expenses for attending to take part in this research study.

5. What do I have to do?

You simply need to attend your antenatal clinic appointments when, in addition to your routine check, a blood sample will be taken and a scan performed. These hospital visits will fall in with when you would normally attend for an antenatal check-up and when you would normally require a blood test or a scan for your care.

6. What are the side effects of any treatment received when taking part?

You will receive no medication as part of the study. There are no side effects. Scans are safe throughout pregnancy, and the blood tests pose no risks to you additional to the slight pain and inconvenience that you normally experience when you have a blood test.

7. What are the benefits of taking part?

The study will not be able to prevent miscarriage. However, there is the possibility that the extra scans and visits may help us keep a closer eye on your pregnancy, and identify any problems early enough to treat them.

8. What will happen when the research study stops?

We hope to determine whether any of our observations during this study may predict problems or assist carers look after women whose pregnancies may be at risk of complications or impaired fetal growth. This study will in part be presented as a thesis for an educational qualification. We will also publish our results in medical journals and present our findings at conferences. You will not be identified in any report/publication. We will not send you the results of the study unless you ask us to.

9. Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The data that we obtain from you in relation to this study is kept anonymised so that no one can trace the information to any individual study participant.

10. What will happen if I do not want to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. Any stored blood samples that can still be identified as yours will be destroyed if you wish. You will receive the same quality of clinical care even if you withdrew from the study.

11. What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will arrange for your care to continue. On receiving new information, your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is discontinued for any other reason, you will be told why and your continuing care will be arranged.

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12. Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the South Sheffield Research Ethics Committee.

13. What if there is a problem?

No problem is envisaged from your taking part in this study. If you are harmed by your participation in the study, there are no special compensation arrangements. You can decide to take legal action. You are covered in the same way as for your ordinary treatment. If you have a concern about any aspect of this study, you should ask to speak with or write the researchers who will do their best to answer your questions. The Principal Investigator is Mr Anumba, Consultant Obstetrician, Jessop Wing, Sheffield on telephone number 0114 2268172. If you wish to complain formally, you may address any complaints to Professor Chris Welsh, Medical Director, Sheffield Teaching Hospitals NHS Foundation Trust, 8 Beech Hill Road, Sheffield.

Patient Information sheet - history of recurrent miscarriage

Changes in serum relaxin levels and maternal and fetal blood flow in pregnant women with or without a history of recurrent miscarriage.

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No. It is up to you to decide whether or not to take part. You are free to decline to take part, or to withdraw at any time after you have agreed to take part, without your decision affecting your care or that of your baby in any way. You can simply tell us by telephone or in writing that you no longer wish to take part.

4. What will happen to me if I take part?

You will be given this information sheet to keep, and asked to sign a consent form. The study will involve taking a small sample of blood from your vein several times during your pregnancy in addition to several scans to assess blood flow through your womb and your baby. Both procedures are safe and carry no risks to you or your baby. The study will involve a total of 4 visits to the hospital during which you will receive your normal antenatal checks. You will be reimbursed your travel expenses for attending to take part in this research study.

5. What do I have to do?

You simply need to attend your antenatal clinic appointments when, in addition to your routine check, a blood sample will be taken and a scan performed. These hospital visits will fall in with when you would normally attend for an antenatal check-up and when you would normally require a blood test or a scan for your care.

6. What are the side effects of any treatment received when taking part?

You will receive no medication as part of the study. There are no side effects. Scans are safe throughout pregnancy, and the blood tests pose no risks to you additional to the slight pain and inconvenience that you normally experience when you have a blood test.

7. What are the benefits of taking part?

The study will not be able to prevent miscarriage. However, there is the possibility that the extra scans and visits may help us keep a closer eye on your pregnancy, and identify any problems early enough to treat them.

8. What will happen when the research study stops?

We hope to determine whether any of our observations during this study may predict problems or assist carers look after women whose pregnancies may be at risk of complications or impaired fetal growth. This study will in part be presented as a thesis for an educational qualification. We will also publish our results in medical journals and present our findings at conferences. You will not be identified in any report/publication. We will not send you the results of the study unless you ask us to.

9. Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The data that we obtain from you in relation to this study is kept anonymised so that no one can trace the information to any individual study participant.

10. What will happen if I do not want to carry on with the study?

If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. Any stored

blood samples that can still be identified as yours will be destroyed if you wish. You will receive the same quality of clinical care even if you withdrew from the study.

11. What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will arrange for your care to continue. On receiving new information, your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study is discontinued for any other reason, you will be told why and your continuing care will be arranged.

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12. Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the South Sheffield Research Ethics Committee.

13. What if there is a problem?

No problem is envisaged from your taking part in this study. If you are harmed by your participation in the study, there are no special compensation arrangements. You can decide to take legal action. You are covered in the same way as for your ordinary treatment. If you have a concern about any aspect of this study, you should ask to speak with or write the researchers who will do their best to answer your questions. The Principal Investigator is Mr Anumba, Consultant Obstetrician, Jessop Wing, Sheffield on telephone number 0114 2268172. If you wish to complain formally, you may address any complaints to Professor Chris Welsh, Medical Director, Sheffield Teaching Hospitals NHS Foundation Trust, 8 Beech Hill Road, Sheffield.

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Human trophoblast function during the implantation process

Elsebeth Staun-Ram[1,2] and Eliezer Shalev*[1,2]

Address: [1]Laboratory for Research in Reproductive Sciences, Department of Obstetrics and Gynecology, Ha'Emek Medical Center, 18101, Afula, Israel and [2]Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

Email: Elsebeth Staun-Ram - elsebeth@tx.technion.ac.il; Eliezer Shalev* - shaleve@tx.technion.ac.il

* Corresponding author

Published: 20 October 2005

Reproductive Biology and Endocrinology 2005, 3:56 doi:10.1186/1477-7827-3-56

Received: 01 August 2005
Accepted: 20 October 2005

This article is available from: http://www.rbej.com/content/3/1/56

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E-JOURNAL
ISSN: 1091-6490
Publication year(s): 1914 - present
Author/Editor: National Academy of Sciences (U.S.)
Publisher: NATIONAL ACADEMY OF SCIENCES
Language: English
Country of publication: United States of America
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