Differentiation of Human Embryonic Stem Cells to the Pancreatic Lineage

A thesis submitted to the University of Sheffield for the degree of

Doctor of Philosophy

Nadia Yousif Murad

Department of Medicine
(Centre for Stem Cell Biology)

November 2008

To my great and beloved Mother, wonderful Husband
and lovely children, Omar and Ghaida

Acknowledgements

First of all I would like to thank Allah for his power and blessing without which I would never completed this work.

Secondly, I would like to express my gratitude to my supervisor Professor Harry Moore for his advice and expert guidance.

Big thanks go to my sister Badriya for helping me to get this scholarship and always being steady when I was feeling bad and lonely. She had a big heart when I called upon her, particularly when I encountered problems.

My deepest thanks and appreciation go to my husband Professor Adil Bahathiq for his love, assistance and support during my struggle to finish this work, He stood beside me at all times.

A special thanks to my sweeties, son Omar, for his friendship and for being with me all the time during this scholarship which encouraged me and filled me full of optimisms to get the degree, and later to my daughter Ghaida for giving me happiness in this life. They put up with the 'bad times', I talked to them when I felt frustrated, lonely and bored. I hope that 'good times' are ahead of us.

Warm thanks to all my sisters Majeda (and her kids), Amina, Hadeel, Hind, Salwa and Laila for their warm hearts.

A special word of thanks to Emeritus Ian Cooke and his wife Dr. Sheila for the effort that they made to support my thesis and for being so friendly, providing me with expert guidance, advice, help, encouragement and moral support.

Many thanks to my friend Hanan Al-Hazeem for her endless encouragement and support.

I would like to thank Professor William Ledger for his advice and concern.

I would like to express my sincere gratitude to my Mother, who suffered a lot in helping me to grow up to reach to this point and for being an active person in this world.

I am grateful to the government of Kuwait for giving me this opportunity.

Especial thanks go to The Public Authority for Applied Education and Training for their financial support for my scholarship and also for giving me the chance to met Adil to start my lovely life with him.

I would like to thank every one who stayed beside me during the difficult times I faced, supporting, encouraging and helping me in different ways, they gave me the strength to get this thesis finished.

Finally, sincerest thank to Allah for giving me such a nice Family, Adil, Omar and Ghaida.

ABSTRACT

Human embryonic stem (hES) cells have great therapeutic potential for the treatment of degenerative conditions such as Parkinson's disease, cardiac failure and type I diabetes. This potential is based on the ability of hES cells *in vitro* to self-renew and also differentiate to cells of all three germ layers; ectoderm, mesoderm and endoderm. Type I diabetes is due to an autoimmune disease destroying the insulin-secreting cells of the pancreas (β-cells) that regulate plasma glucose concentration. The pancreas develops from the endoderm lineage. To find a cure for type I diabetes based on the use of hES, it is essential to understand the differentiation process of ES cells into the endodermal, β-cell lineage. The aim of this study was to investigate the generation of insulin-secreting cells using hES cells *in vitro* and to compare such cells with those in the developing pancreas of the foetus.

The differentiation of hES cells was induced by their aggregation in culture to form embryoid bodies (EBs) using various combinations of growth factors. Peptide (C-peptide, insulin) and protein expression markers of cells were analysed by histochemical (dithizone stain) immunolocalisation, and immunosorbent assay (ELISA). mRNA expression (RT-PCR) was used to confirm the expression of some early and mature β-cells markers including Pancreatic duodenal homeobox 1 (*PDX-1*), Neurogenin 3 (*NEUROG 3; NGN3*), Glucokinase (*GCK, GK*), Glucose Transporter (GLUT) 1 and 2 (*SLC2A1* and *SLC2A2*) and insulin (*IDDM2*). Comparisons were made with pancreas recovered from human foetus between 8 - 12 weeks gestation and subjected to immunolocalisation and proteomic analysis.

The number of cells expressing markers of insulin-secreting cells in EB colonies varied with different growth factor/s combinations. Medium supplemented with

nicotinamide and activin A was the most successful in generating β-cell-like cells. However, EBs failed to secrete the pro-insulin peptide, C-peptide, in culture.

Immunocytochemistry of foetal pancreas indicated that islet β-cell development initiated at about week 10 of gestation and that consistent C-peptide secretion measured by ELISA was apparent in cells recovered from week 12 foetuses and cultured *in vitro*. The protein profile of the pancreas from week 10 and 12 foetuses revealed significant differences by proteomic analysis.

I conclude that *in vitro* culture processes of hES cells need to be further improved to achieve a higher efficiency and a full differentiation towards insulin-secreting cells that mimic those of foetal or mature islet β-cells.

Table of Contents

List of Tables

List of Figures

Abbreviations

Ab	Antibody solution
ADP	Adenosine diphosphate
AS	Adult stem
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
d	Days
dH_2O	Distilled water
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNase	Deoxyribonuclease
DTZ	Dithizone
DNA	Deoxyribonucleic acid
dNTP's	2'-deoxyribonecleoside-5'-triphosphate (N=any nucleotide G, C, T or A)
EBs	Embryoid bodies
EDTA	Ethylenediamine tetra-acetic acid
EG	Embryonic germ
ELISA	Enzyme linked immunosorbent assay
ES	Embryonic Stem
FA	Formaldehyde
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GK	Glucokinase
Glut-1	Glucose transporter 1
Glut-2	Glucose transporter 2
hEG	Human Embryonic germ
hES	Human embryonic stem

ICM	Inner cell mass
Kb	Kilobase pair
LIF	Leukaemia inhibitory factor
MEFs	Mouse embryonic fibroblasts
MMLV	Moloney Murine Leukaemia Virus
mRNA	Messenger ribonucleic acid
NaOH	Sodium Hydroxide
Ngn 3	Neurogenin 3
OD	Optical density
Oligo	Oligodeoxynucleotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdx-1	Pancreatic duodenal homeobox 1
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolution per minute
RT	Reverse transcriptase
SD	Standard deviation
SR	Serum replacement
TAE	Tris-acetic acid-EDTA
Taq	Thermus aquaticus
TRIS	Tris-(hydroxymethyl)-methlamine
UV	Ultra violet
V	Voltage
w	weeks
#	Number

CHAPTER ONE

INTRODUCTION (Literature Review)

INTRODUCTION

1. Diabetes

Carbohydrates are broken down to sugars and absorbed during digestion and when this sugar (mainly glucose) enters the bloodstream it can provide the body with energy for metabolic processes. Excess amount of glucose, which is not needed, is stored short-term in the liver or for long-term as fat deposits. Levels of sugar in the bloodstream are closely regulated by the hormone insulin, which is produced by the pancreas (Edlund, 2001; Ono, 2006). Insulin is synthesized and secreted by the β-cells, which reside in the pancreatic islet; the functional unit of specialized endocrine cells (Edlund, 2001; Peck *et al*, 2004; Gabrielle *et al*, 2005).

Diabetes is a chronic disease characterised by abnormally high levels (hyperglycemia) of glucose in the bloodstream (Baertschiger *et al*, 2006; Yang *et al*, 2006). Excessive amounts of free glucose can cause blindness, kidney failure,

heart disease, stroke, neuropathy and amputations (Efrat, 2002; Baertschiger *et al*, 2006; Yang *et al*, 2006).

There are two types of diabetes, type I, insulin-dependent, and type II, non-insulin dependent (Docherty, 2001; Edlund, 2001). Type I diabetes is characterised by loss of β-cells of the pancreas, generally as a result of an autoimmune reaction. That is, the body's own immune system destroys 90% or more of islet cells (in which β-cells reside) of the pancreas by attacking them as foreign bodies (Docherty, 2001; Peck *et al*, 2004; Trucco, 2006). In the absence of insulin, glucose accumulates in the blood creating the condition known as type I diabetes and patients must have regular insulin injections to control blood glucose. This condition is also referred to as juvenile diabetes as the disease often strikes the young (Efrat 2002; Peck *et al*, 2004). Although the exact cause of type I diabetes is not known, it is thought that genetic and environmental factors (e.g. viral infection, vitamin D levels) are involved. Recently, large-scale genetic fine mapping and genotype-phenotype associations implicated polymorphism in the interleukin 2 receptor alpha gene (*IL2RA*) as being important in whether individual developed type I diabetes (Lowe *et al*, 2007)

Type II, or adult-onset diabetes is the most common form of diabetes and occurs when the body either cannot use the insulin effectively because of the dysfunction of β-cells resulting in an inability to produce and secrete sufficient amounts of active insulin (Docherty, 2001; Edlund, 2001), or the cells of the body do not use insulin properly; 'insulin resistance'. In this case, more insulin than usual is required to keep the blood glucose levels at normal levels. Type II diabetic patients can usually control their blood glucose levels by regular diets,

exercises and oral medication. In type II diabetics the β-cells of the pancreas remain present in large numbers.

Binding of insulin to its specific receptor on the cell membrane, leads to the activation of the receptor and a phosphorylation of intracellular signaling pathways, which activate glucose absorption and utilization by the cell. Most cells have the insulin receptors on their cell membranes and are termed insulin-dependent. But some cells such as brain cells, kidneys cells, cells lining the digestive tract and red blood cells lack insulin receptors and are insulin-independent. These cells can absorb and utilize glucose without insulin stimulation (Rhodes *et al*, 1994; Dodson *et al*, 1998).

2. Islet Transplantation

Transplantation of whole pancreas or pancreatic islets is used as a fundamental treatment for type I diabetic patients by replacing the cells lost by disease. A pancreas transplant is surgery to implant a healthy pancreas from a donor into a patient thereby giving this person the chance to become independent (or less dependent) of insulin injections. However pancreas transplant is only offered to people who have severe diabetes due to the risks attached to the procedure. Since severe type I diabetes is often associated with chronic renal (kidney) failure, a person who needs a pancreas transplant also might need a kidney transplant. Therefore, three kinds of pancreas transplant operations are undertaken. These are (1) a combined kidney-pancreas transplant; (2) "pancreas after kidney" transplant, in which the pancreas is transplanted some time after a kidney has been transplanted; (3) pancreas transplant alone, for patients with functioning kidney (Peck *et al*, 2004; Pileggi *et al*, 2004; Ono, 2006; Urban *et al*,

2006). The first attempted transplant of both kidney and pancreas was carried out in 1967 by Kelly and her colleagues (Kelly *et al*, 1967 Hayek *et al*, 1997). Over the recent years, long-term success has improved and risks have decreased. One year after transplantation more than 95% of all patients are still alive and 80-85% of all pancreases are still functional. However, besides the surgical risks, there are other potential complications and transplantation patients need lifelong immunosuppression which increases the chances of acquiring various infections and cancer. The biggest restriction to the use of pancreas transplantation is the lack of suitable immune (histocompatibility) matched donors.

Sachs and Bonner-Weir considered that transplantation of pancreas as a single organ is complicated, and more effort was needed to find alternatives to insulin injections (Sachs *et al*, 2000). A more experimental treatment was therefore introduced which is the transplantation of purified pancreatic islets rather than the whole pancreas organ. This procedure reduces the need for the long and complicated surgery. The first successful islet transplantation was performed in 2000 by Shapiro and his colleagues for type I diabetic patients (Hayek *et al*, 1997; Amiel, 2001; Mathieu, 2001; Shapiro *et al*, 2006). Shapiro's group at the University of Alberta in Edmonton, Canada, have continued to use and refine the procedure called the Edmonton Protocol although there are still considerable problems with the technique.

In islets transplantation, the islets are isolated from the donor pancreas by using protease and collagenase enzymes. Because islets are fragile and cannot be preserved, transplantation must occur soon after they are removed. Usually a patient receives at least 10,000 islet "equivalents" per kilogram of body weight, extracted from two donor pancreases. Patients often require two transplants to

achieve insulin independence. The transplant of cells is often performed by a radiologist, who uses X-rays and ultrasound to guide a catheter (small plastic tube) through the upper abdomen and into the portal vein of the liver. The islets are then infused slowly through the catheter into the liver (Figure 1) (Hayek *et al*, 1997; Sachs *et al*, 2000; Mathieu, 2001; Shapiro *et al*, 2006). Some studies on mice, by Hayek and Beattie in 1997, showed that fetal endocrine islet-like cells if compared to adult islets are richer in generating insulin-producing cells (Hayek *et al*, 1997). After transplantation of adult islets, some β-cells were selectively lost but the reason for this is not clear (Hayek *et al*, 1997; Miszta-Lane *et al*, 2006).

The limited supply and low yield of islets from donor's pancreas prevents the wide-spread use of islet transplantation therapy (Ackermann *et al*, 2007) at present. In 2005, a 5-year follow-up of results for 65 patients who received transplants indicated that only about 10% of the patients remained free of the need for insulin injections. Most recipients returned to using insulin because the transplanted islets lost their ability to function over time. The researchers noted, however, that many transplant recipients were able to reduce their need for insulin and achieve better glucose stability. Like pancreas transplantation, patients require life long immunosuppression and therefore the procedure is not suitable for young patients. However, islet transplantation indicated that if alternative sources of insulin-producing cells could be generated then this approach might be a practical method of treatment for diabetic patients as the cell infusion was relatively simple.

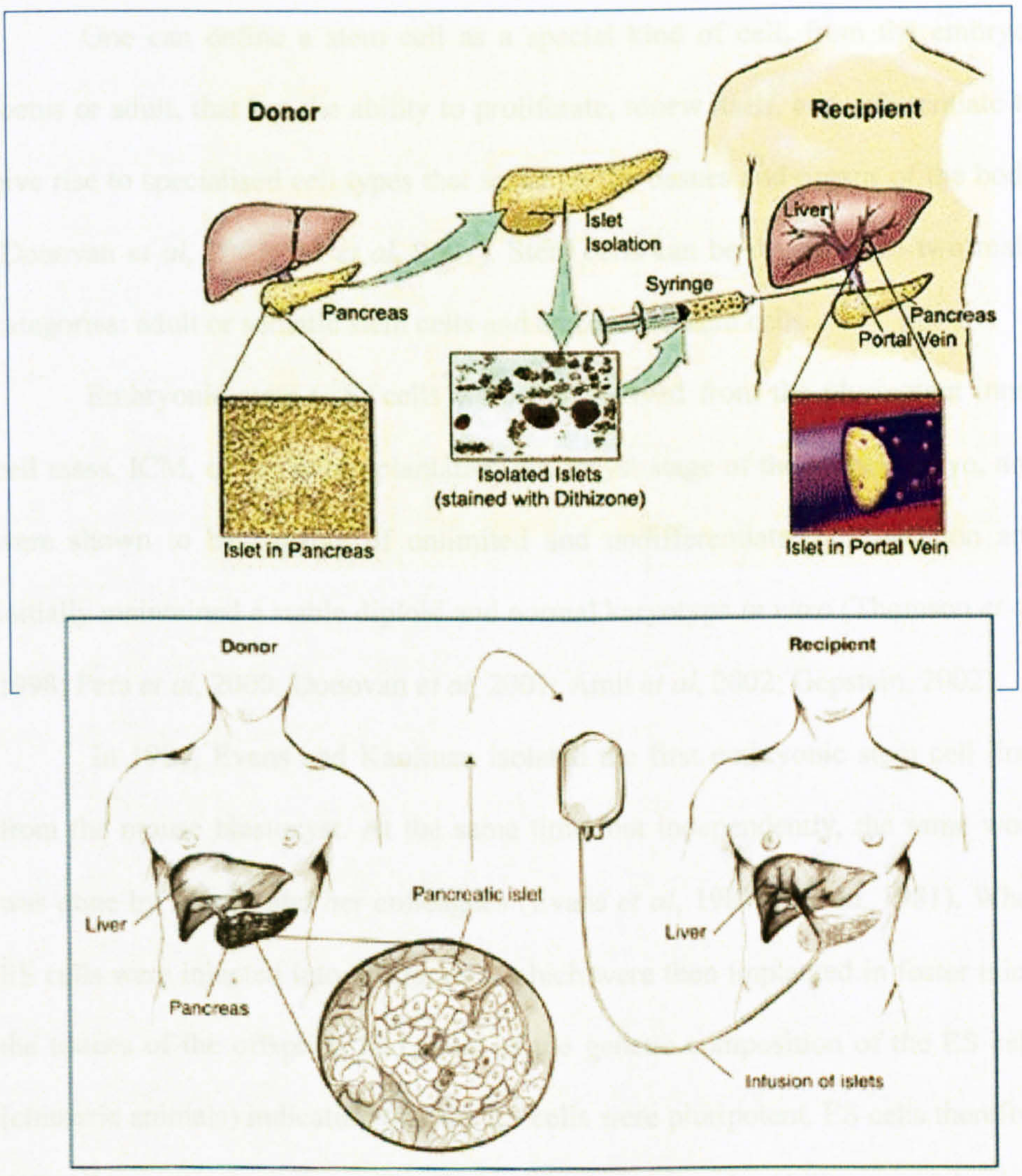


Figure 1 A diagram illustrating the principles of the islet transplantation process; the Edmonton Protocol. Islets are recovered and purified from one or two pancreases and then infused into recipient patient via the portal vein where the cells embed in liver (after Shapiro *et al*, 2001).

3. Embryonic Stem Cells

One can define a stem cell as a special kind of cell, from the embryo, foetus or adult, that has the ability to proliferate, renew itself, and differentiate to give rise to specialised cell types that make up the tissues and organs of the body (Donovan *et al*, 2001; Lü *et al*, 2007). Stem cells can be divided into two main categories: adult or somatic stem cells and embryonic stem cells.

Embryonic stem (ES) cells were first derived from the pluripotent inner cell mass, ICM, of the pre-implantation blastocyst stage of the early embryo, and were shown to be capable of unlimited and undifferentiated proliferation and initially maintained a stable diploid and normal karyotype *in vitro* (Thomson *et al*, 1998; Pera *et al*, 2000; Donovan *et al*, 2001; Amit *et al*, 2002; Gepstein, 2002).

In 1981, Evans and Kaufman isolated the first embryonic stem cell lines from the mouse blastocyst. At the same time, but independently, the same work was done by Martin and her colleagues (Evans *et al*, 1981; Martin, 1981). When ES cells were injected into blastocysts, which were then implanted in foster mice, the tissues of the offspring had some of the genetic composition of the ES cells (chimeric animals) indicating that the ES cells were pluripotent. ES cells therefore differ from all other types of cells in that they have the ability to retain their potential to generate derivatives of all the three germ layers, endoderm, mesoderm and ectoderm, and proliferate without differentiation in culture under appropriate conditions (Pera *et al*, 2000; Amit *et al*, 2002; Gepstein, 2002; Lü *et al*, 2007).

Mouse ES cells were shown to differentiate, *in vitro*, into a variety of cell types including cardiomyocytes, skeletal and smooth muscle cells, neural and glial cells, hematopoietic progenitor cells, adipocytes, endothelial cells, pancreatic cells (β-cells) and several other tissue types (Gepstein, 2002). However, the cells

remained undifferentiated *in vitro* if they were grown with leukaemia inhibitory factor, LIF, or if they were seeded on an inactivated feeder layer of cells of embryonic fibroblasts (Roche *et al*, 2005). A study was done by Williams and colleagues that demonstrated the importance of leukemia inhibitory factor, a member of the interleukin-6 related family of cytokines, in the maintenance of mouse ES cells from differentiation, *in vivo* and *in vitro* without the need of feeder cells (Williams *et al*, 1988).

Isolation of ES cells from the primate, rhesus and marmoset monkeys, were reported in 1995 by Thomson and coworkers where these cells remained undifferentiated in continuous passage for more than one year, and maintained their morphology, normal karyotype and expression of specific cell surface markers (Thomson *et al*, 1995; Reubinoff *et al*, 2000).

The first human embryonic stem (hES) cell lines were derived in 1998 (Thomson *et al*, 1998). In the human embryo, a hollow sphere of cells is formed 4-6 days after fertilization. This blastocyst, contains an outer cell layer and an inner cluster of cells which is known as the inner cell mass, ICM (Gepstein, 2002). The outer cell layer, the trophectoderm, forms part of the placenta and other supporting tissues, while the ICM creates all tissues of the developing foetus as well as foetal sacs. If the ICM is placed in culture then these cells can change their characteristic to become ES cells with indefinite self-renewal. In culture, it was found that hES cells like mouse ES cells required a feeder layer, to maintain the cells in an undifferentiated phenotype (Draper *et al*, 2003). On the other hand, experiments indicated that undifferentiated hES cells could not be maintained by LIF alone (Thomson *et al*, 1998). Xu and colleagues indicated that there were

undefined soluble factors released from the feeder layer which were important in maintaining an undifferentiated hES cell (Xu *et al*, 2003).

The similarities between human and rhesus ES cells, has allowed rhesus ES cells to provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety of ES cell- based therapies (Thomson *et al*, 1998; Furuya *et al*, 2003).

Since undifferentiated ES cells can be differentiated *in vitro* and *in vivo* to a specific cell lineage of ectoderm, mesoderm or endoderm potentially any disease resulting from the failure of specific cell types can be treated through the transplantation of differentiated cells derived from ES cells (Thomson *et al*, 1995; Meivar-Levy *et al*, 2006; Urban *et al*, 2006). Moreover, hES cells can be used for new drugs tests, such as in new medications tests, or for screening anti-tumor drugs for cancer (Meivar-Levy *et al*, 2006; Urban *et al*, 2006). The disadvantages of using ES cells for transplant therapy include tumors formation as undifferentiated ES cells are mitotically active and may result in the formation of teratomas (Draper *et al*, 2002). Also as hES cells produced from a blastocyst will not match the genetic composition of the patient a transplant with these cells will be rejected by the immune system unless immunosupression is used (Draper *et al*, 2002; Ono, 2006). To avoid immune rejection, derivation of the patient ES cell line by the use of somatic nuclear transfer technology has been suggested (Donovan *et al*, 2001; Gepstein, 2002).

In culture ES cells can form cluster of cells called an embryoid body (EB) which can then develop with differentiated cell types. The selection of a specific cell type from the mixed population of cells in an EB is important to have a relatively homogenous, pure, cell population (Gepstein, 2002; Calafiore *et al*,

2006). To do so, a tissue specific promoter can be utilized to derive a selectable marker as was used by Klug and coworkers (Klug *et al*, 1996) to obtain cardiomyocyte. An appropriate antibiotic and selection process of *in vitro* differentiated ES cells carrying a fusion gene resulted in 99% purity of cultured cardiomyocytes (Klug *et al*, 1996). A similar strategy was used to produce purified cultures of neurons and insulin secreting pancreatic β-cells that derived from murine ES cells (Gepstein, 2002).

ES cells can be used as a source of insulin-producing donor cells in type I diabetes cell therapy (Jiang *et al*, 2007). Derivation of insulin- producing cells from mouse ES cells was performed in 2000 by Soria's group (Soria *et al*, 2000). They used a simple genetic approach to generate insulin-producing cells, that normalizes blood glucose when transplanted into streptozotocin (STZ)-induced diabetic mice, which are able to maintain a stable glucose *in vivo*. Lumelsky, 2001, reported the successful differentiation of mouse ES cells into insulin-secreting structures, with similar topology and function as the pancreatic islets, by using an ES cell-based system (Lumelsky *et al*, 2001).

The derivation of human ES cells which differentiated into insulin-secreting cells, via EBs formation, was first performed by Assady and his colleagues in 2001 (Assady *et al*, 2001). They used undifferentiated hES cells to produce cells with the characterization of insulin producing β-cells (Assady *et al*, 2001).

Different researchers have tested different ways to produce β-cells from ES cells to be used for the transplantation purposes. The importance of the differentiation process of β-cells *in vitro* is summarized in the sensitivity of the

cells to the blood glucose and the quality and quantity of the insulin secreted from them (Ackermann *et al*, 2007).

Embryonic germ (EG) cells are another pluripotent type that have the ability to proliferate and differentiate to multiple types of somatic cells derived from all the three embryonic germ layers (Shamblott *et al*, 1998; Clark *et al*, 2007). These cells derived from primordial germ cells in the genital ridges of the developing embryos (Gepstein, 2002; Clark *et al*, 2007). EG cells, are like ES cells, and have the ability to differentiate *in vitro* to form embryoid bodies composed of mature cell types as well as proliferating progenitor cells. The first human embryonic germ (hEG) cells were isolated from a 5-10 week embryonic gonadal ridges in 1998 (Shamblott *et al*, 1998; Draper *et al*, 2002). Recently, glucose responsive insulin cells were produced from human embryonic germ (EG) cell derivatives (Clark *et al*, 2007).

Stem cells are also present in many tissues of adult animals (Donovan *et al*, 2001). Adult stem (AS) cells are undifferentiated cells that are found in a specific tissue or organ and have the ability to proliferate and differentiate to produce the specific cell types of the tissue that they are derived from; but they show limited proliferation *in vitro* compared to ES cells (Jonathan *et al*, 2003). The main function of AS cells is involved in tissue repair, in which they are found, and homeostasis (Donovan *et al*, 2001).

Expansion and differentiation of progenitor cells that might be derived from adult human pancreas, liver, spleen and bone marrow may help in producing insulin-secreting cells which can be used in the replacement therapies for diabetes and avoid immunorejection and formation of pluripotent cells (Gao *et al*, 2007; Lü *et al*, 2007). AS cells have been used to generate β-cells. AS cells from old

patients are likely to have less potential for differentiation and growth (Winston, 2001; Meivar-Levy *et al*, 2006; Urban *et al*, 2006). The advantage of using AS cells in transplantation is their histocompatibility with the donor for autotransplantation and this will not cause any rejection problems by the immune system (Miszta-Lane *et al*, 2006).

However, AS cells are very rare cells and hard to identify and it is not clear, to date, if they are present in all organs or only restricted to some specific organs. Furthermore, they are few in numbers and difficult to extract and grow in laboratory cultures (Williams *et al*, 1988; Winston, 2001). Some unexpected cells in the body were found producing insulin such as fat cells and liver in both (I and II) diabetic mice, but the number of cells and the amount of insulin produced were very small (Baylor College of Medicine, 2004).

In addition, some studies showed that insulin-producing cells can be generated from the mouse and human ductal cells, which form the tubes that carry the digestive enzymes from the pancreas to the duodenum (Bonner-Weir *et al*, 2000). However, the number of cells producing insulin in this experiment was too small for transplantation therapy in the future (Bonner-Weir *et al*, 2000).

Hence, stem cell biology is a new field that holds the promise for mass production of pancreatic β-cells if efficient methods can be devised for generating functional cells *in vitro* (Soria, 2001). This requires an understanding of the normal development of the human pancreas.

4. The Development of Human Pancreas

4.1 General Anatomy and Morphology

The pancreas is a gland situated in the upper part of the abdomen, posterior to the stomach, and connected to intestine by a fine tube called the bile duct (Figure 2) (Grapin-Botton *et al*, 2001; Fishman *et al*, 2002). It has two major functions. The first is the production of digestive enzymes, which are secreted by exocrine acinar cells which make up most of the organ, and the second is in the regulation of blood sugar, which is carried out by the endocrine cells of the islets of Langerhans (St-Onge *et al*, 1999; Grapin-Botton *et al*, 2001; Fishman *et al*, 2002; Murtaugh, 2007). Pancreas morphology was first described by Paul Langerhans in his thesis in 1869 (see Volk *et al*, 1985). At that time, the main function of the pancreas was not determined but it was suggested that it had a close connection with the nervous system (see Volk *et al*, 1985). The pancreas is innervated by sympathetic and parasympathetic fibres, in which the nerves in the islets of Langerhans follow the blood vessels and terminate within the pericapillary space (Felig *et al*, 2001). Moreover, the pancreas is composed of four different parts. The head which is a disc-shaped structure lying in the concavity of the second and third parts of the duodenum. The neck which is narrower than the head, connecting between the head and the body. The body which is triangular in shape with a flat posterior surface and finally the tail (Rogers, 1992).

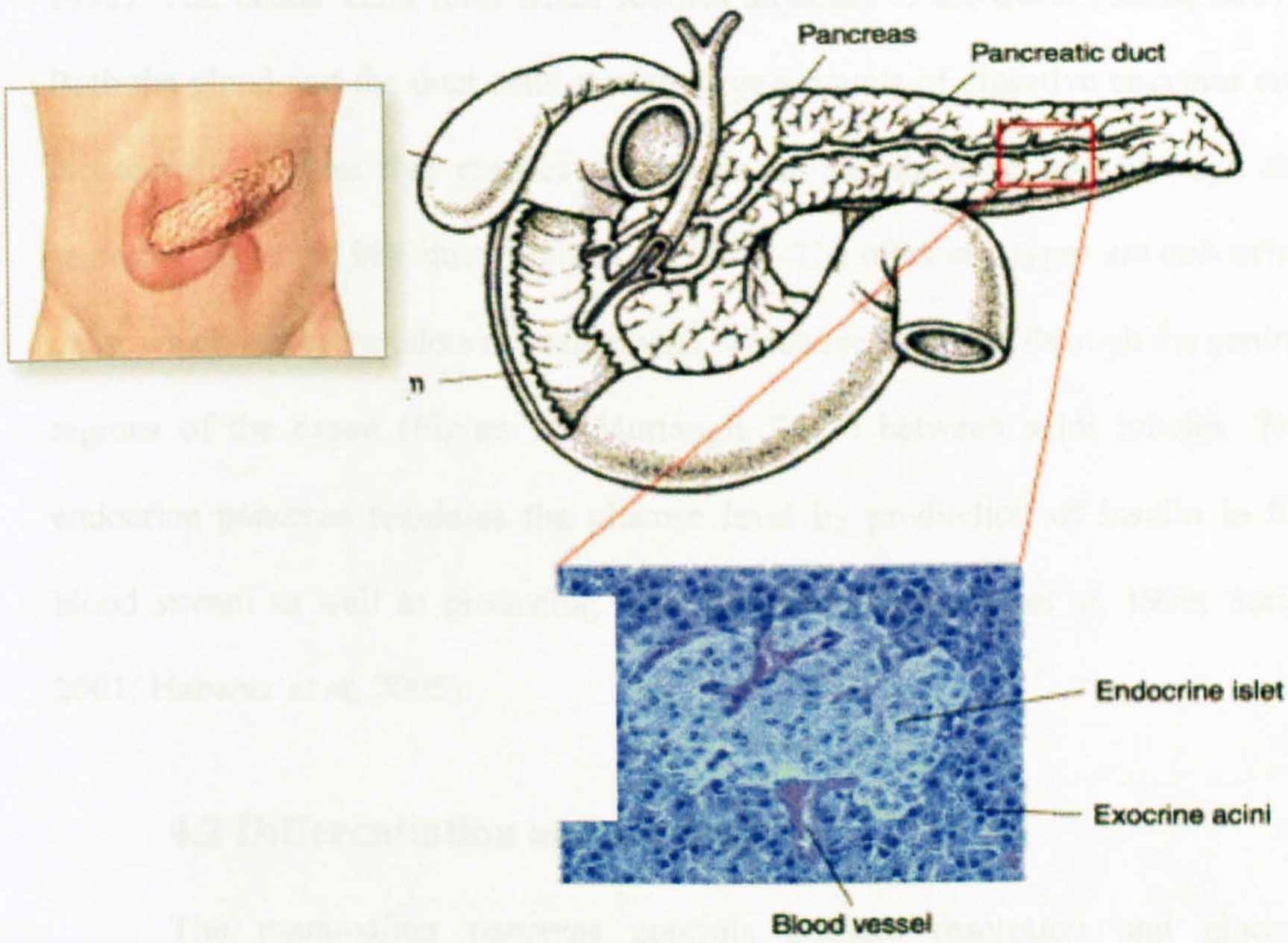


Figure 2 A diagram showing the gross morphology of the human pancreas and a micrograph histology section of the endocrine and exocrine regions (based on diagrams from www.Lifespan.org).

There are two types of secretory cells (Volk *et al*, 1985). About 99% of the pancreatic volume consists of clusters of gland cells, pancreatic acini, which are connected to the intestine through a highly branched ductal network (Rogers, 1992). The acinar cells form small lobules adjacent to the ducts (Soria, 2001). Both the gland and the duct cells secrete large amounts of digestive enzymes and bicarbonate mixture that reaches the lumen of the digestive tract through the secretory ducts and bile duct (Volk *et al*, 1985). The other cell types are endocrine cells, which are in the islets of Langerhans, which are scattered through the central regions of the organ (Figure 3) (Murtaugh, 2007) between acini lobules. The endocrine pancreas regulates the glucose level by production of insulin in the blood stream as well as producing other hormones (St-Onge *et al*, 1999; Soria, 2001; Habener *et al*, 2005).

4.2 Differentiation and Growth

The mammalian pancreas controls nutrient resorption and glucose metabolism by the various cell types in the islets, the exocrine acinar tissue and the ductules (Peter *et al*, 2000; Ramiya *et al*, 2000; Habener *et al*, 2005). Although all of the three structures have the same origin they differ in their functions (Peter *et al*, 2000).

The pancreas develops from the foregut of embryonic endoderm (Peters *et al*, 2000; Gu *et al*, 2002). During embryogenesis two pancreatic lobes arise as thickenings along the dorsal and ventral surfaces of the posterior foregut followed by the foregut development in later stage (Gradwohl *et al*, 2000; Murtaugh, 2007). These two lobes evaginate into the surrounding mesenchyme as dense epithelial buds, which then expand, branch and differentiate to a fully functional organ system prior to birth (Murtaugh, 2007). However, the two buds fuse together to

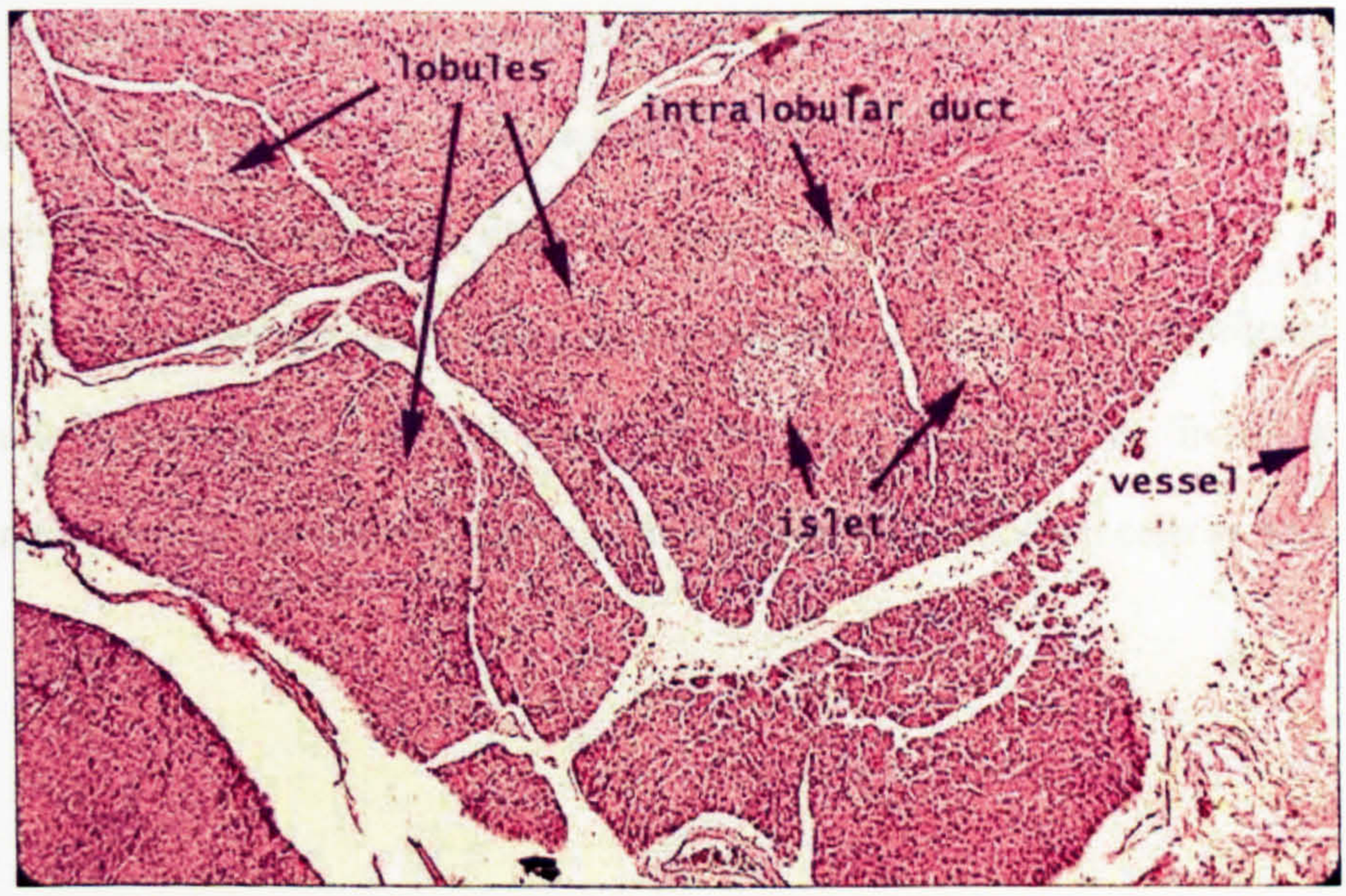


Figure 3 A low power section of adult human pancreas tissue showing the pancreatic islet of Langerhans surrounded by acinus cells and closer to the pancreatic intralobular duct (from www.udel.edu).

make a functional organ (Gradwohl *et al*, 2000; Grapin-Botton *et al*, 2001; Habener *et al*, 2005).

In the mouse which has been investigated the most, the pancreas firstly appears as an evagination on the dorsal surface of the primitive gut endoderm at early embryogenesis stage (Figure 4) (Docherty, 2001). Shortly after that, the ventral pancreatic bud arises. Rapid growth then takes place where the epithelial cells branch forming a network surrounded by mesenchymal tissue. Epithelial cells then develop into endocrine, ductal and acinar cells (Docherty, 2001).

Pancreas development requires signals originating from the notochord and pancreatic mesenchyme, where these signals are important in inducing proliferation and differentiation of pancreatic epithelial cells into islet cells as well

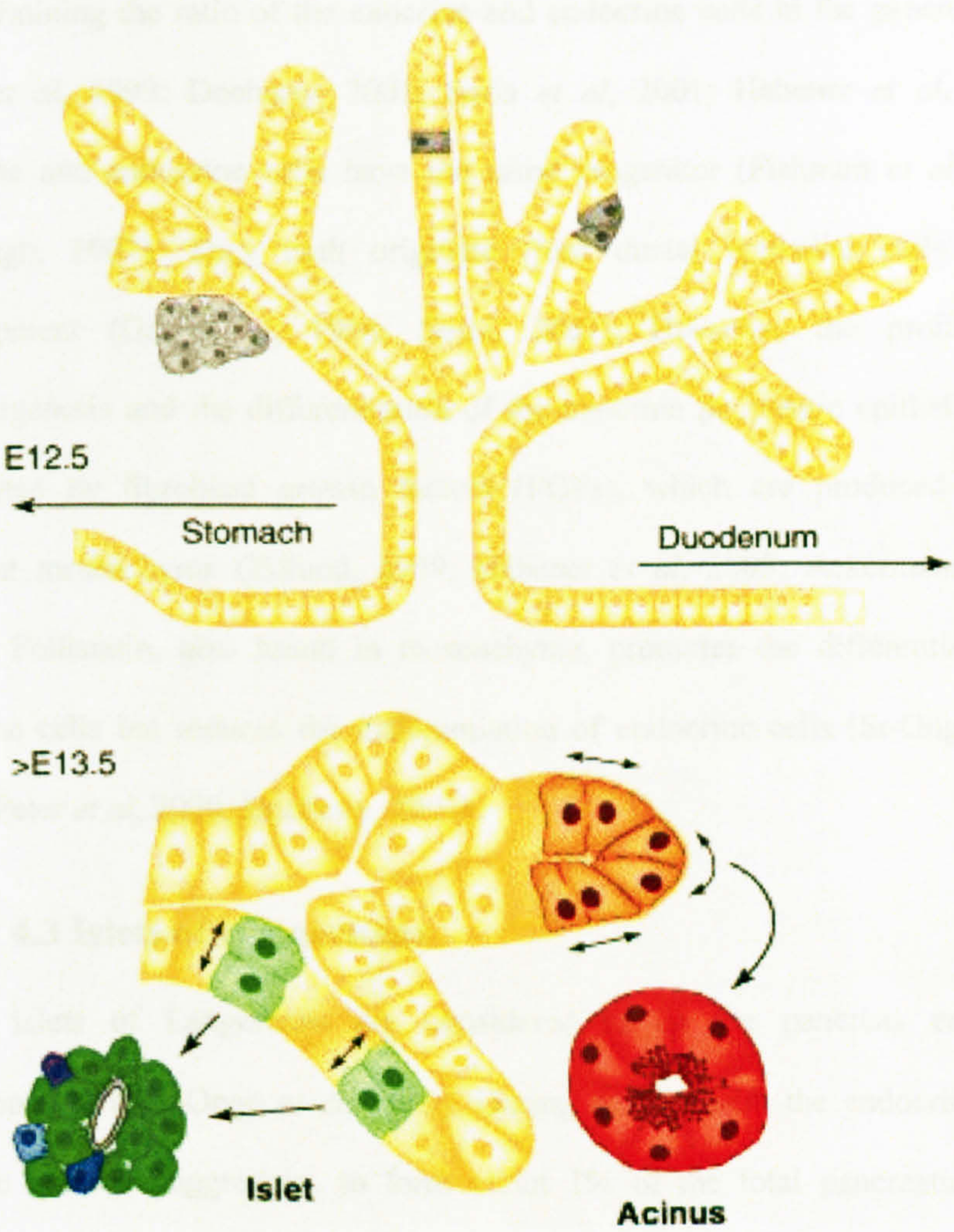


Figure 4 Diagram of early development of mouse pancreas. Definitive endoderm cells (at E 12.5 gestation) develop from the primitive gut tube to invaginate and then produce pancreatic endoderm at the posterior foregut. Ductal cells start to express β-cell markers and migrate into the mesenchyme to then aggregate and form the islets of Langerhans at gestational age E13.5 (from Homo-Delarche, 2004).

as determining the ratio of the exocrine and endocrine cells in the pancreas (St-Onge *et al*, 1999; Docherty, 2001; Soria *et al*, 2001; Habener *et al*, 2005). Exocrine and endocrine cells have the same progenitor (Fishman *et al*, 2002; Murtaugh, 2007). They both originate from ductal epithelial cells during development (Gu *et al*, 1994; Soria, 2001). However, the proliferation morphogenesis and the differentiation of the exocrine pancreatic epithelium are stimulated by fibroblast growth factors (FGFs), which are produced by the adjacent mesenchyme (Edlund, 1999; Habener *et al*, 2005; Ackermann *et al*, 2007). Follistatin, also found in mesenchyme, promotes the differentiation of exocrine cells but reduces the differentiation of endocrine cells (St-Onge *et al*, 1999; Peter *et al*, 2000; Docherty, 2001).

4.3 Islets of Langerhans

Islets of Langerhans are considered to be the pancreas endocrine functional unit (St-Onge *et al*, 1999). During development the endocrine cells, emerge as small aggregates to form about 1% of the total pancreatic tissue, migrate from the duct system network into the surrounding mesenchyme and aggregate around capillaries to form isolated clusters of cells, known as islets of Langerhans, which are scattered throughout the exocrine glandular tissue (Peter *et al*, 2000; Young, 2000; Docherty, 2001; Soria, 2001; Bertuzzi *et al*, 2006). In the human, each islet consists of about 930 cells (Bonner-Weir *et al*, 2000).

They are rounded clusters of cells condensed together particularly in the tail region of the pancreas (Figure 5; Stevens *et al*, 1997). Individual cells within the islets are smaller, paler than the exocrine cells and spherical in shapes (Stevens *et al*, 1997). Each islet is surrounded by a capillary network that contacts with each cell in the islet and carries hormones into the blood stream (Figure 5)

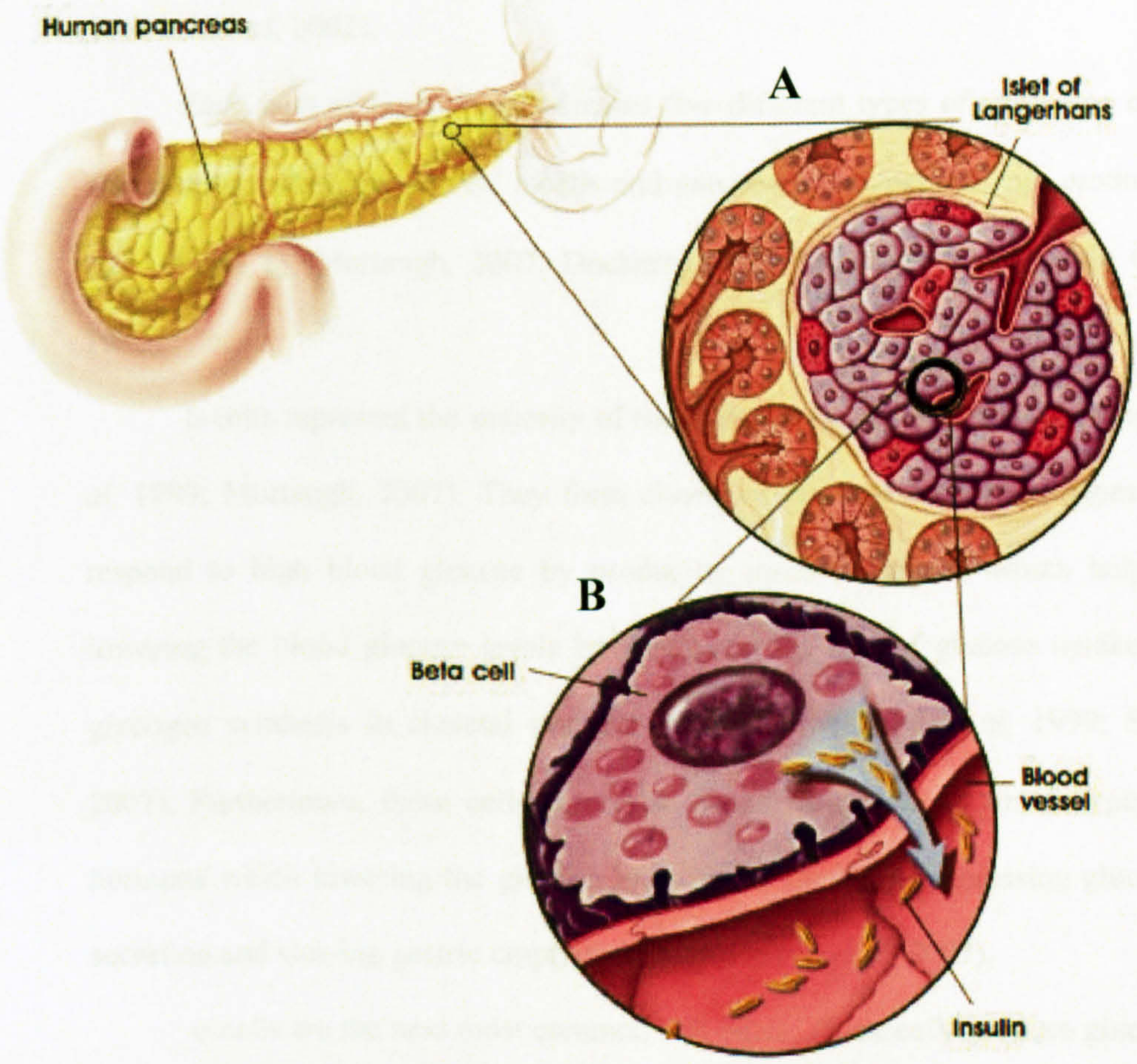


Figure 5 A diagram showing (A) the islet of Langerhans embedded in the exocrine region of the pancreas (B) the proximity of β-cells to blood vessels to enable a rapid secretion of insulin (National Institute of Health, USA).

(Stevens *et al*, 1997). Thus, the association of the pancreatic tissues with blood vessels is probably important for the formation of the insulin secreting cells (Fishman *et al*, 2002).

Each islet of Langerhans contains five different types of endocrine cells. These cells include β-, α-, δ-, ε-cells and pancreatic polypeptide, pp, producing cells (Table 1) (Murtaugh, 2007; Docherty, 2001; Gradwohl *et al*, 2000; Orci, 1982).

β-cells represent the majority of the endocrine cell population (St-Onge *et al*, 1999; Murtaugh, 2007). They form clusters connected by gap-junctions and respond to high blood glucose by producing insulin hormone which helps in lowering the blood glucose levels by increasing the rate of glucose uptake and glycogen synthesis in skeletal muscles and liver (St-Onge *et al*, 1999; Soria, 2001). Furthermore, these cells were also found to secrete amylin polypeptide hormone which lowering the glucose level in the blood by suppressing glucagon secretion and slowing gastric emptying (Singh-Franco *et al*, 2007).

α-cells are the next most common cell types. These cells produce glucagon which raises the blood glucose levels by increasing the rates of glycogen breakdown and glucose release by the liver (Murtaugh, 2007; Singh-Franco *et al*, 2007). They are silent at higher glucose concentrations but active when β-cells are silent (Soria, 2001).

δ-cells are cells produce somatostatin peptide hormone that suppresses the release of glucagon and insulin and slows the rates of food absorption and enzyme secretion along the digestive tract (Murtaugh, 2007). Finally, the pancreatic polypeptide cells that producing pp hormone which inhibits the gallbladder contr-

Table 1 The different cell types in the endocrine pancreas (Ross *et al*, 1995; Stevens *et al*, 1997; Klöppel *et al*, 2001; Toshinai *et al*, 2006).

Characteristic Features	α-cells	β-cells	δ-cells	PP-cells	ε-cells
Peptide Hormone	**Glucagon**	**Insulin**	**Somatostatin**	**Pancreatic Polypeptide**	**Ghrelin**
Location	**In the periphery of the islets**	**In the islet centre**	**In the periphery of the islets**	**In posterior lobe of pancreas, head, neck regions and scattered in duct wall**	**In the isle centre, acinar cel and ducta epitheliur**
Appearance	**Uniform in size and have dense granules compared to β-cells**	**Have polyhedral core and pale matrix**	**Have larger granules than α- & β-cells**	-	-
Molecular weight	**3500**	**5800**	**1500**	**4200**	**3314**
Number of amino acids	**29**	**51**	**14**	**36**	**28**
Total volume % in adult	**15-20**	**70-80**	**5-10**	**15-25**	**20**

actions and regulates the production of some pancreatic enzymes and help in controlling the rate of nutrient absorption by the digestive tract (Murtaugh, 2007).

Recently, ε-cells were identified as an endocrine cells found in the islets (Murtaugh, 2007). These cells produce ghrelin which is a gastric hormone synthesized in the epithelial cells lining the stomach, placenta, kidney, pituitary and hypothalamus (Shuto *et al*, 2001; Lugar *et al*, 2004; Toshinai *et al*, 2006; Murtaugh, 2007; Rindi *et al*, 2007). Ghrelin works as a stimulator of the growth hormone secretion, food metabolism, suppressor of fat utilization in the adipose tissue, stimulate gastric emptying and increase cardiac output (Shuto *et al*, 2001; Lugar *et al*, 2004; Toshinai *et al*, 2006; Rindi *et al*, 2007).

The distribution of endocrine cells in the islets is nonrandom. However, rodent β–cells are found in the core of the islets surrounded by the other three cell types α, δ and pp that containing glucagon, somatostatin and pp hormone respectively (Figure 6) (St-Onge *et al*, 1999). In contrast, human and primate islet appearance (Brissova *et al*, 2005). Islets are surrounded by fibroblasts and collagen fibres in an incomplete capsule (Felig *et al*, 2001).

All endocrine cells are derived independently during ontogeny from the foregut endodermal progenitors (Gradwohl *et al*, 2000; Herrera, 2000). The islets β-cells appear to be close to the duct-like structures during embryogenesis which indicates that they arise from the pancreatic duct (Edlund, 1999; Bonner-Weir *et al*, 2000). After a few weeks the primordial islets separate from the ducts where the non β-cells surround the β-cells. Later, the non β-cells start to extend to the centre of the islets forming the postnatal islets (Peter *et al*, 2000).

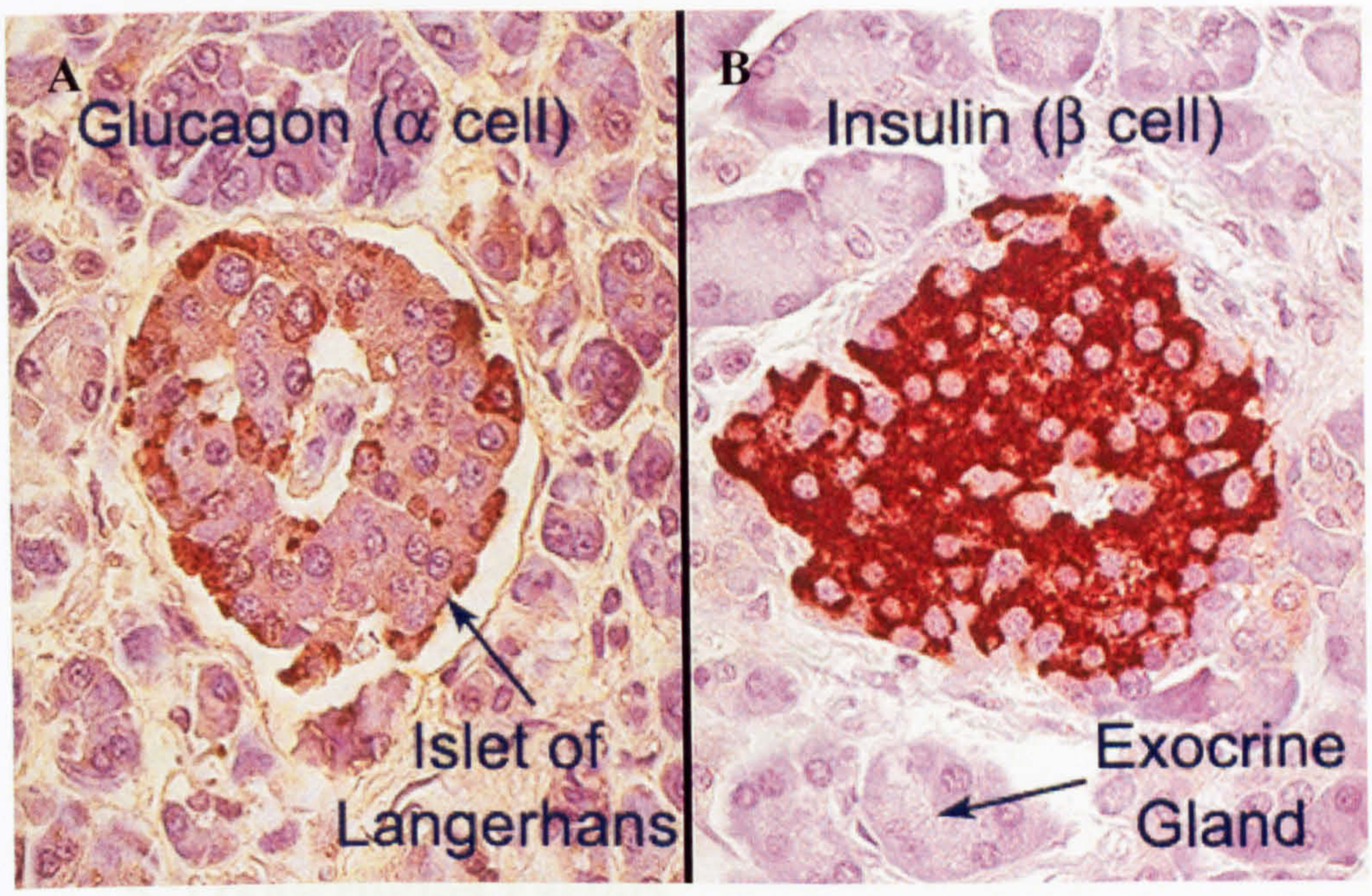


Figure 6 Distribution of α-cells and β-cells in the human islets of Langerhans. (A) glucagon secreting cells, cells stained brown, were found to be in the periphery of the islet, where as (B) insulin secreting cells, dark red stained, aggregated to the centre of the islet. Note capillary bed surrounding islet (University of Madison, Medicine).

The final stage of islet formation involves the migration of the endocrine progenitor cells out of the endodermal epithelium (Docherty, 2001). After birth there is a period during which the neonatal islet undergoes remodeling by β-cell replication and apoptosis, to balance the number of β-cells in the islets. Islet cells continue their growth to mature after birth, and with growth the islet mass of the organism may change according to the demands of the insulin (Docherty, 2001). It was found that human β-cells have low replication rate in late foetal and adult pancreas (Bonner-Weir *et al*, 2000).

Experiments to grow the islets of Langerhans in culture have not been successful, (Docherty, 2001). An understanding of the differentiation pathway of β-cell *in vivo* is requiredto increase the number of insulin-secreting cells successfully *in vitro* (Ackermann *et al*, 2007).

5. Transcription Factors

There are many homeodomain transcription factors that control and regulate the development of islet cells at different stages in mammals such as, Pdx-1, ngn3, Isl-1, Pax4, Pax6, NeuroD, Nkx2.2 and Hlxb9 (Table 2) (Edlund, 1999; Gradwohl *et al*, 2000). NeuroD, Nkx2.2, Pax4, Pax6 and Isl-1 are often referred to as a second group of regulatory genes because ngn3 regulate their function (Gradwohl *et al*, 2000). They are involved in pancreas morphogenesis, pancreatic endocrine cell proliferation, survival and differentiation and are found to be express during early stages of pancreas development (Edlund, 1999; Gradwohl *et al*, 2000).

Table 2 Some of the main transcription factors that are involved in pancreas development (revised from St-Onge *et al*, 1999).

Transcription Factor	Expression in adult pancreas
Pancreatic duodenal homeobox 1 (PDX-1)	β-cells
Isl lim homeobox 1 (Isl-1)	β-, α-, δ-, pp- cells dorsal mesenchyme
Paired box gene 4 (Pax4)	β-, δ-, pp- cells
Paired box gene 6 (Pax6)	β-, α-, δ-, pp- cells
NK2 HOMEOBOX 2 (Nkx2.2)	β-, α-, pp- cells
NeuroD1 (NeuroD/Beta2)	β-, α-, δ-, pp- cells

Several transcription factors are expressed in specific regions of the endoderm prior to organogenesis (Grapin-Botton *et al*, 2001). Some of the main transcription factors are discussed below.

5.1 Pancreatic duodenal homeobox 1 (PDX-1)

PDX-1, is the earliest gene expressed in the developing pancreas and a key transcriptional factor of pancreatic development and differentiation (Gu *et al*, 2002; Habener *et al*, 2005; Shiroi *et al*, 2005). It is proposed to be the master regulator of the pancreas at different development stages (Grapin-Botton *et al*, 2001). All the three types of pancreatic tissue arise from PDX-1 progenitor cells (Gu *et al*, 2002). PDX-1 plays an important role in pancreas formation in early embryonic stages as well as initiating islet cell differentiation, but does not complete it (Grapin-Botton *et al*, 2001; Noguchi, 2006). Pdx-1 is known also as insulin promoter factor, IPF-1, somatostatin transactivating factor 1, STF-1, or islet duodenal homeodomin protein, IDX-1, (Soria, 2001).

As the organogenesis progresses, Pdx-1 was found to be expressed during formation of the pancreas, stomach and duodenum (Gu *et al*, 2002). It acts at a later stage in pancreatic development in both dorsal and ventral regions of the pancreas but not for the evagination and initial bud formation (Edlund, 1998; Edlund, 1999). Deficiency of Pdx-1 in mice pancreas causes development blocking at the bud stage (Docherty, 2001; Gu *et al*, 2002).

After birth, Pdx-1 expression is found to be restricted to β-cells within the endocrine islets of pancreas, dorsal side of the stomach and all the mucosa cells in the duodenum (Gu *et al*, 2002; St-Onge *et al*, 1999). Furthermore, Pdx-1 is required for both regionalization of the primitive gut endoderm and in the maturation of the β-cells that produce insulin (Edlund, 1998; Grapin- Botton *et*

al,2001). It seems to be a key factor in insulin gene expression in the adult pancreas (Grapin-Botton *et al*, 2001; Soria, 2001; Noguchi, 2006). Pdx-1 is required for maintaining the hormone producing phenotype of the β-cells (Edlund, 1998; Habener *et al*, 2005). Thus, the expression of insulin and somatostatin was found to be regulated by Pdx-1 but not expressed in glucagon cells (Edlund, 1998; Soria, 2001; Habener *et al*, 2005).

Over expression of Pdx-1 leads endocrine cells to lose their elongation shape and enhance the ductal cells to secrete insulin instead of β-cells. In contrast cells expressing low level of Pdx-1 remain in the epithelium (Grapin-Botton *et al*, 2001; Noguchi, 2006). It has been shown that Pdx-1 regulates the expression of other islet-specific genes such as Glut-2, islet amyloid polypeptide and GK (Habener *et al*, 2005). Low activity of Pdx-1 may contribute with type II diabetes by causing stopping the expression of insulin (Edlund, 1998; Habener *et al*, 2005).

5.2 *Neurogenin 3* (Neurog 3; Ngn3)

Ngn3, is a transcription factor that is considered as a competence factor involved in the determination of neural precursor cells (Gradwohl *et al*, 2000; Shiroi *et al*, 2005). It can be found in a specific region in the nervous system as well as scattered within the embryonic pancreas (Gradwohl *et al*, 2000). However, it is first expressed in the developing pancreas (Edlund, 1999).

Ngn3 is a basic helix-loop-helix protein which is expressed in the developing pancreas specifically islet cell progenitors but not present in mature islets (Docherty, 2001; Gu *et al*, 2002; Habener *et al*, 2005). It is found within or adjacent to pancreatic ducts and was identified as a potential initiator of endocrine differentiation (Gradwohl *et al*, 2000; Grapin-Botton *et al*, 2001). It is expressed

in the islet precursor cells that have not start producing hormones and used as a marker for these cells (Gradwohl *et al*, 2000; Habener *et al*, 2005).

Moreover, this transcription factor is important for the survival of differentiated endocrine cells as well as for the generation and differentiation of endocrine and exocrine progenitor cells (Docherty, 2001; Gradwohl *et al*, 2000; Gu *et al*, 2002). In mouse, for example, it founds that Ngn3 is required for differentiation of all pancreatic endocrine lineages but when expressed alone before the formation of the bud, under the control of Pdx-1 promoter, it does not induce insulin cell differentiation (Grapin-Botton *et al*, 2001).

The inactivation of Ngn3, in mice, prevents all endocrine cells in the pancreas from differentiation as well as the islets of Langerhans which results in diabetes (Gradwohl *et al*, 2000; Habener *et al*, 2005). In contrast, over expression of Ngn3, in mice, may accelerate the differentiation of pancreatic endocrine cells, while decrease the pancreatic precursor cells (Edlund, 1999; Habener *et al*, 2005). Pdx-1 acts with Ngn3 to form the endocrine cells of the pancreas, in the islets of Langerhans (Fishman *et al*, 2002; Habener *et al*, 2005). Pax 6, Isl-1, NeuroD and Nkx2.2 are endocrine cell markers induced by Ngn3 (Grapin-Botton *et al*, 2001). Hes1 is an inhibitor of the endocrine cell differentiation which is promoted by Ngn3 and it plays a role in the maintenance of the pancreatic precursor cells during development (Habener *et al*, 2005; Soria, 2001).

5.3 Neurodifferentiation 1 (*NeuroD1*; NeuroD)

NeuroD is a basic helix-loop-helix late transcription factor promoted by Ngn3, that induces its expression (Docherty, 2001; Soria, 2001; Habener *et al*, 2005). The expression of NeuroD is important in regulating both β-cells of adult islets, as well as for the differentiation and maintenance of all endocrine cells, and

terminal differentiation of neurons (Edlund, 1998; Docherty, 2001; Soria, 2001; Habener *et al*, 2005). Loss of NeuroD expression occurs during endocrine cell differentiation (Docherty, 2001; Soria, 2001).

5.4 Isl lim homeobox 1 (Isl-1)

Isl-1 is a late expressed transcription factor of LIM-homeodomain protein, homeobox gene, which is expressed in all types of islet cells in the adult pancreas but in low level in β-cells (Edlund, 1998; Peter *et al*, 2000; Docherty, 2001; Habener *et al*, 2005). During early embryogenesis the expression of Isl-1 is important to determine which region of the gut endoderm that will form the pancreas (Edlund, 1999; Docherty, 2001). This homeodomain appears around the dorsal pancreatic bud only and it is important, later, for the differentiation of the islet cells (Edlund, 1998; Docherty, 2001; Peter *et al*, 2000; Habener *et al*, 2005).

Isl-1 has two functions. The first is in the development of the exocrine cells of the pancreas from the dorsal mesenchyme, whereas the second function is in differentiation of islet cells from the epithelial cells (Docherty, 2001; Soria, 2001). All endocrine cells depend on Isl-1 for their terminal differentiation (Edlund, 1998). Furthermore, Isl-1 acts upstream of Pax6 during pancreatic endocrine differentiation (Edlund, 1998).

5.5 Paired box gene 4 (Pax4) and Paired box gene 6 (Pax6)

These two homeobox genes are restricted to nervous system and endocrine cells in the developing pancreas (Gradwohl *et al*, 2000; Habener *et al*, 2005). They are a late expressed transcription factors (Docherty, 2001). Both genes expressed in the dorsal and ventral buds of the developing pancreas (Habener *et al*, 2005). They are essential for the generation of different cell types (Soria,

2001). In addition, Pax4 and Pax6 require Ngn3 for their expression (Gradwohl *et al*, 2000).

Pax4 is expressed in both buds of the pancreas but later in development it becomes restricted to β-cells (Edlund, 1998; Habener *et al*, 2005). However, Pax4 is important for the differentiation of insulin producing β-cells and somatostatin producing δ-cells (Edlund, 1998; Soria, 2001; Habener *et al*, 2005). On the other hand, Pax6 is required for the generation of glucagon producing α-cells (Peter *et al*, 2000; Soria, 2001; Habener *et al*, 2005). Furthermore, Pax6 is expressed in all endocrine cells during development (β-, δ-, α- as well as pp) (Edlund, 1998; Habener *et al*, 2005). Cells expressing both, Pax4 and Pax6, will develop into mature insulin producing β-cells (Peter *et al*, 2000; Soria, 2001). However, inactivation of both of them lead to the absence of mature β-cells (Gradwohl *et al*, 2000).

5.6 NK2 Homeobox 2; (NKX2-2; Nkx2.2) and NK Homeobox 6 (NKX6-1 Nkx6.1)

Nkx2.2 and Nkx6.1 are homeodomain genes that expressed in early pancreatic cells as well as the progenitor cells (Docherty, 2001; Shiroi *et al*, 2005). They are regulators of the differentiation of pancreatic endocrine cells (Habener *et al*, 2005).

Nkx2.2 is an important transcription factor that expressed in early development of pancreas and later in differentiation of β–cells. It was firstly found in the dorsal bud of the pancreas during early embryogenesis of mouse embryo (St-Onge *et al*, 1999; Habener *et al*, 2005; Shiroi *et al*, 2005). As differentiation proceeds scientists found that Nkx2.2 expression restricted to the β, α and pp-cells but not mature δ-cells (Edlund, 1998; St-Onge *et al*, 1999; Docherty, 2001;

Habener *et al*, 2005). Mutants of Nkx2.2 lead to hyperglycemia because of the lack of β-cells that produce insulin (Habener *et al*, 2005; Shiroi *et al*, 2005).

Nkx6.1 lies downstream of Nkx2.2 (Docherty, 2001). It is expressed in β-cells of adult islets (Edlund, 1998; Docherty, 2001; Habener *et al*, 2005). Moreover, Nkx6.1 is required for the termination of differentiation of β-cells to produce functional hormone producing cells (Edlund, 1998; St-Onge *et al*, 1999).

5.7 Homeobox HB9 (Hlxb9)

Hlxb9 is a homeobox transcription factor gene which expressed in the notochord and the gut endoderm (Edlund, 1999; Docherty, 2001). It is present also in β-cells of mature islets, where it is responsible for terminal differentiation and maturation of β-cells (Edlund, 1999; Docherty, 2001; Soria, 2001). Hlxb9 function is expressed in both early and late stages of pancreatic differentiation (Edlund, 1999). It controls the earlier step in morphogenesis of the dorsal pancreas (Edlund, 1999; Soria, 2001). Furthermore, Hlxb9 is found in regions of endoderm that give rise to the respiratory and digestive tubes as well as the dorsal and ventral pancreatic buds, before Pdx-1 (Edlund, 1999; Soria, 2001).

5.8 *p48* (P48)

P48 is the first exocrine transcription factor that has been discovered that controls the expression of genes in the exocrine pancreas (Edlund, 1998). This subunit factor is located downstream of Pdx-1 (Edlund, 1998). It is required for differentiation and proliferation of the exocrine cell lineage (Edlund, 1998). The expression of p48 mRNA is observed in both pancreatic buds (Edlund, 1998).

A number of other transcription factors are important in the field.

5.9 Octamer-binding Transcription Factor 4 (Oct 4) POU Domain, Class 5, Transcription Factor (POU5F1)

Oct4 is a transcriptional binding factor that is found in undifferentiated high proliferative cells (Gu *et al*, 2005; Tondreau *et al*; 2005). The expression of Oct 4 is important for maintaining the ICM and pluripotency of ES cells (Gu *et al*, 2005). It is specifically expressed in the germ line and pluripotent stem cells that play a role in the normal development of cells (Gu *et al*, 2005; Li *et al*, 2005; Marikawa *et al*; 2005).

Oct4 expression is regulated by proximal enhancer and promoter in the epiblast and distal enhancer and promoter in pluripotent cell lineage (Gu *et al*, 2005). Low expression of Oct4 indicate the initiation of differentiation of ES cells (Gu *et al*, 2005).

5.10 Glucose Trasporter 1 and 2 (Glut-1 and Glut-2)

Glucose transporters, Gluts, are membrane proteins that facilitate glucose uptake into cells. Gluts are important molecules for normal carbohydrate metabolism (Matsutani *et al*, 1990).

Immunolabeling techniques of pancreatic β-cells sectioned from human foetal and adult pancreas were used, by Richardson and colleagues, to identify the presence of Glut1, Glut2 and GK (Richardson *et al*, 2007). No expression of Glut1 was observed during early development of pancreas but it was detected in development, after 15 weeks gestation, when insulin positive cells increased. In contrast, Glut2 expression was detected during early development of the pancreas, at about 7 weeks gestation (Richardson *et al*, 2007).

Glut2 expression is regulated by transcription factors Pdx-1 and hepatocyte nuclear factor 1, Hnf-1α (Cerf *et al*, 2007). The early loss of Glut2

expression causes hyperglycemia and low insulin expression that leads to diabetes (Cerf *et al*, 2007).

High fat diet also causes reduction in the expression of Glut2 that inhibit glucose stimulation insulin secretion and affect β-cell mass and function (Cerf, 2007).

Humans differ from rodent in glucose transporter gene expression in the adult pancreas which is predominantly Glut-1 instead of Glut-2. The 100-fold lower glut 2 abundance in human versus rat β-cells is associated with a 10-fold slower uptake of alloxan, explaining their resistance to this rodent diabetogenic agent (De Vos *et al*, 1995).

5.11 Glucokinase (GCK, GK)

Glucokinase (GK) is a key enzyme that plays a role in glucose homeostasis which secretes from the liver (de la Iglesia *et al*, 1999; Cuesta-Munoz *et al*, 2000; Slosberg *et al*, 2001). However, mRNA of GK was first found in the liver parenchyma of neonatal rats (Moorman *et al*, 1991).

GK gene transcription initiates in the β-cells of the pancreatic islets, gut, and brain from promoter sequences upstream the liver sequences (Moates *et al*, 2003).

In β-cells, the expression of beta GK is controlled by Pdx-1 (Moates *et al*, 2003). Moreover, GK in the pancreatic β-cells functions as the glucose sensor determining the threshold for insulin secretion, while in liver it is essential for the regulation of glucose-responsive genes as well as facilitates the hepatic glucose uptake during hyperglycemia (Postic *et al*, 2001).

Mutations in the GK gene cause two different diseases: diabetes type II and hyperinsulinemic hypoglycemia of infancy (Postic *et al*, 2001; Cuesta-Munoz

et al, 2004). However, mice lacking GK in the pancreatic β-cell die within 3 days of birth with hyperglycemia. On the other hand, mice lacking hepatic GK are viable but are mildly hyperglycemic when fasted (Postic *et al*, 2001; Cuesta-Munoz *et al*, 2004). Glucokinase polymorphism is not a major determinant of type II diabetes (Laurino *et al*, 1994).

5.12 Notch Signaling

Notch signaling is an intercellular signaling molecule (Docherty, 2001). It is a transmembrane receptor that is stimulated by Ngn3 (Docherty, 2001). In the human, notch signaling is important for pancreatic development, in controlling both endocrine and exocrine fates (Edlund, 1999; Habener *et al*, 2005). The notch signaling pathway controls the differentiation of endocrine cells as well as the fate of endocrine and exocrine cells (Edlund, 1999). Lack of notch signaling results in high levels of Ngn3 which promote the differentiation of endocrine cells. On the other hand, cells with active notch signaling promote the differentiation of exocrine cells and keep the progenitor cells undifferentiated (Edlund, 1999).

6. Insulin Chemistry

Insulin is the polypeptide hormone produced by β-cells when glucose levels exceeded in blood more than the normal level of 4-7mmol/L, and regulates the metabolism of glucose, fats and proteins. Secretion of insulin is also stimulated by elevated levels of some amino acids, including arginine and leucine (Dodson *et al*, 1998). It was the first human gene to be cloned and the one of the first proteins to be isolated crystallized and sequenced (Bell *et al*, 1980).

Insulin has been found in all vertebrates and has a highly conserved structure (Dodson *et al*, 1998). It consists of two polypeptide chains, A and B,

connected by two disulfide bridges (Dodson *et al*, 1998). Another disulfide bridge is found within the A chain between the sixth and eleventh amino acids residues (Figure 7). In human there is a single copy of the insulin gene located on the short arm of chromosome 11 (Docherty *et al*, 1996). The complete insulin molecule contains 51 amino acids 21 amino acids in A chain and 30 amino acids in B chain with a 5,800 molecular weight (Dodson *et al*, 1998).

The insulin gene is composed of three exons and two introns, among different species (Dodson *et al*, 1998). Exon one is located in the translated region of the gene, while exon two contains sequences coding for the signal peptide, B chain and part of the C-peptide and exon three encodes the remainder of the insulin sequence (Bell *et al*, 1980). The N-terminal and C-terminal regions of the A chain and the hydrophobic residues of the B chain are highly conserved (Dodson *et al*, 1998).

Human insulin promoter region regulates the transcriptional response to glucose and other nutrients and limits transcription of the gene to the pancreatic β-cells (Docherty *et al*, 1996).

Insulin, like most secretory proteins, is synthesized as a precursor molecule, called preproinsulin that carries a single peptide (Rhodes *et al*, 1994). By the interaction of the signal recognition peptide with the signal recognition peptide receptor on the ribosome ensures that preproinsulin enters the secretory pathway in the rough endoplasmic reticulum (Rhodes *et al*, 1994). However, preproinsulin is a single polypeptide chain of 110 amino acids that permits correct alignment of the three pairs of disulfide bonds and with molecular weight of 11,500, as shown in figure 7, (Rhodes *et al*, 1994).

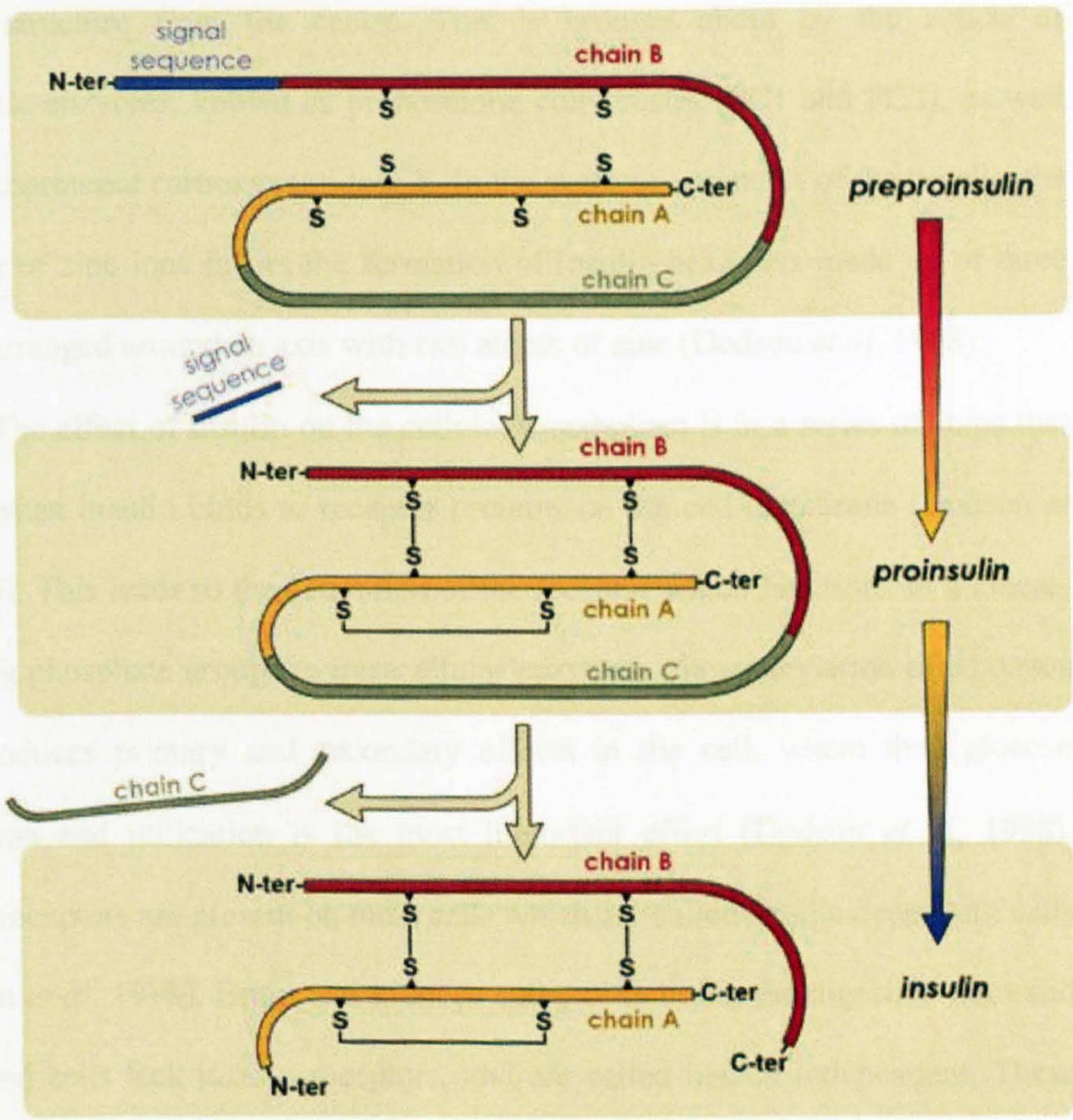


Figure 7 The diagram shows the active structure of insulin as processed. The two polypeptide chains of insulin, A and B, connected by two disulfide bridges. The other disulfide bridge is found within the A chain between the sixth and eleventh amino acids residues (Beta cell consortium, 2004).

Tertiary structure of the insulin, the active form, is derived from the proinsulin which is about 86 amino acids by cleavage of 31 amino acids of the C-peptide structure from the centre. This is brought about by the action of proteolytic enzymes, known as prohormone convertases (PC1 and PC2), as well as the exoprotease carboxypeptidase E. In the secretory granules of the β-cells, the presence of zinc ions favors the formation of insulin hexamers made up of three dimers arranged around an axis with two atoms of zinc (Dodson *et al*, 1998).

The effect of insulin on the cellular metabolism is in a series of steps that begins when insulin binds to receptor proteins on the cell membrane (Dodson *et al*, 1996). This leads to the activation of the receptor which functions as a kinase, attaching phosphate groups to intracellular enzymes. Phosphorylation of enzymes then produces primary and secondary effects in the cell, where then glucose absorption and utilization is the most important effect (Dodson *et al*, 1998). Insulin receptors are present on most cells which are called insulin dependent cells (Dodson *et al*, 1998). Brain and kidneys cells, cells lining the digestive tract and red blood cells lack insulin receptors, and are called insulin independent. These cells absorb and utilize glucose without insulin stimulation (Dodson *et al*, 1996).

7. Aims of this Study

In the present study, the goals were:

1. To induce insulin-secreting cells *in vitro* using ES cells (H7 cell line), derived from human embryonic stem cells, using different growth factors, and compare these cells with those produced by the foetal pancreas during early development.

2. To assess the functional output of any insulin-secreting cells generated from ES cells by measuring C-peptide secretion and gene expression.

3. To relate findings to development of the human pancreas during early organogenesis.

CHAPTER TWO

Immunoflurescent Localisation of Insulin- Containing Cells Derived from Human Embryonic Stem (hES) Cells

I. INTRODUCTION

The unique nature of hES cells to either self-renew or differentiate into most cell types, suggests that these cells could potentially supply an unlimited source of transplantable islet cells in the future. As discussed previously, the pancreatic islets of Langerhans originate from definitive endoderm and therefore the primary differentiation step that ES cells must undertake *in vitro* is along an endodermal pathway. However, the path from definitive endoderm to a mature hormone-producing islet cell phenotype is complex and involves sequential cell fate decisions, which include the formation of pancreatic endoderm, endocrine progenitors, and hormone-producing islet cell types, including β-cells that secrete insulin. Embryonic development of the pancreas is the result of several interacting mechanisms involving growth factors, epithelial-mesenchymal interactions and extracellular matrix that eventually regulate the expression of diverse transcription factors and genes (see chapter I). However, it is still unclear as to the nature of the initial signals in the

cascade of events that enables gut endoderm to develop to pancreas. Much remains to be learned about the extracellular cues that might be given in culture to specify, maintain, expand, and differentiate these endocrine progenitor cells. Thus, to control the differentiation of undifferentiated hES cells into functional effective pancreatic β-cells will be a challenging task.

As a first step we need to establish whether insulin-secreting cell types can be generated from hES cells in sufficient numbers for experimental investigation. Under non-adherent culture conditions embryonic stem cells can differentiate into aggregates called embryoid bodies (EBs). These EBs can be composed of different proportions of cells from all three layers, endoderm, mesoderm and an ectoderm (Roche *et al*, 2005; Trucco, 2005) and thereafter form a microenvironment for spontaneous embryonic development which may include endodermal-pancreatic developmental processes.

Studies have shown that insulin cells can be generated from mouse and human ES cells by the use of different experimental strategies (Keller, 1995; Klug *et al*, 1996; Soria *et al*, 2000; Assady *et al*, 2001; Amit *et al*, 2002) but mostly using EBs. The approach used most frequently is based on the findings that similar mechanisms operate to control the development of both the central nervous system and the pancreas. Specifically, embryonic stem cells have been induced to differentiate initially into nestin-producing neural precursors that are then expanded and exposed to conditions, which result in clusters of insulin-containing cells.

In the early steps of neural differentiation from mouse ES cells, some cells express nestin as a protein found in normal neuronal precursor cells (Lendahl *et al*, 1990; Lumelsky *et al*, 2001). Nestin was also found in immature, hormone negative pancreatic cells in culture, which produce glucagon initially and then insulin after

maturation (Lumelsky *et al*, 2001; Zulewski *et al*, 2001). For this reason, nestin was used as an early identifier of the differentiation of ES cells towards insulin-secreting cells (Lumelsky *et al*, 2001). However, it has also been found that nestin positive cells contribute to the intra- and extramicrovascular environment of the islet rather than directly to the hormone producing cells of the pancreas (Selander *et al*, 2002; Treutelaar *et al*, 2003) and therefore may only be a temporary marker. Thus, immunocytochemistry to localise nestin was used in this study to identify the putative progenitors cells for later identification of the insulin positive cells among the differentiated hES cells. Lumelsky (2001) used a working protocol that started with the enrichment of the nestin positive cell population derived from mouse EBs to generate cells expressing insulin and other pancreatic hormones (Lumelsky, 2001).

Nicotinamide has been used in a number of experiments to direct the differentiation of hES cells into insulin producing cells (Soria *et al*, 2000; Houard *et al*, 2003; Roche *et al*, 2005; Trucco, 2005). Nicotinamide can slow down the progression of type II diabetes by improving insulin and C-peptide secretion as well as preventing the death of β-cells in type I diabetes (Gale, 1996; Greenbaum *et al*, 1996; Hoorens *et al*, 1999; Kolb *et al*, 1999; Polo *et al*, 1998; Li *et al*, 2006). However, the activity of nicotinamide as a growth factor is not well understood, although it is thought to play a role in stimulating endocrine differentiation in the early development of the pancreas.

In two separate experiments, Soria and co-workers found that after incubation of mouse ES cells with nicotinamide for two weeks, or with nicotinamide in low glucose medium for five days, the differentiating cells increased their insulin secretion level (Soria *et al*, 2000; Roche *et al*, 2005). Thus, the Soria group indicated that addition of this growth factor played an important role in directing the

differentiation of ES cells into insulin secreting cells in all their protocols (Roche *et al*, 2005). In addition, Beattie and coworkers (1996) used nicotinamide to direct the differentiation and proliferation of progenitor cells from foetal pancreatic cells into EBs expressing insulin (Beattie *et al*, 1996; Trucco, 2005).

On the other hand, Schuldiner and co-workers succeeded in directing the differentiation of hES cells in culture into different cell types (ectoderm, mesoderm and endoderm) by using eight different growth factors, including FGF2 and activin A. These experiments indicated the importance of FGF2 in directing the differentiation of ES cells towards ectodermal and mesodermal cells, but not necessarily into endodermal cells after a ten day incubation period of hES cells in medium containing FGF2 (Schuldiner *et al*, 2000). However, Soria's group also used both FGF2 and activin A, with mouse ES cells to generate insulin-secreting cells (Schuldiner *et al*, 2000; Soria, 2001). It was also found that the insulin secretion from the cultured EBs, derived from mouse ES cells, remained stable from day seven until day 21 (Soria *et al*, 2000; Houard *et al*, 2003). Hart and co-workers (2000) have suggested that FGF2 signalling may be involved in β-cell maturation, terminal differentiation, and post natal expansion (Hart *et al*, 2000).

While growth factors have different effects on the differentiation of hES cells, none have the ability to direct the differentiation of ES cells into a single specific cell type (Schuldiner *et al*, 2000; Amit *et al*, 2002). From the literature it has been shown that most of the experiments used growth factors with EBs rather than using the ES cells directly in monolayer culture. Thus, growth factors were added to the EBs from the time of the earliest investigations (Perkins, 1998; Itskovitz-Eldor *et al*, 2000; Soria *et al*, 2000; Assady *et al*, 2001 Feraud *et al*, 2001; Lumelsky, 2001; Segev *et al*, 2004).

Other groups have derived insulin secreting cells *in vitro* from mouse ES cells by using the three step differentiation method: direct differentiation, cell lineage selection and maturation. The resulting cells were shown to express β-cell specific genes, but they could not respond to increased glucose concentration (Soria *et al*, 2000; Assady *et al*, 2001).

A number of markers have been used to detect insulin-secreting cells including the detection of insulin secretion into medium. However, the presence of insulin in many culture media as a growth factor complicates the interpretation of results. An alternative marker is C-peptide (connecting peptide), an active peptide hormone but which is also important for the synthesis of insulin, where it links the two chains of insulin, A and B, in the correct way that allows the formation of disulfide bridges between them (Figure 1) (Ido *et al*, 1997; Rigler *et al*, 1999; Wahren *et al*, 2000).

The structure, chain length and amino acid sequence of C-peptide varies in different species (Ohtomo *et al*, 1998). Human C-peptide, for example, has 31 amino acids with a molecular weight of 3020, but those of different species show differences in their physiological and biochemical structures (Ido *et al*, 1997; Rigler *et al*, 1999; Wahren *et al*, 2000).

Another marker which has been used in this study is dithizone (DTZ), which is a zinc chelating agent used as a marker that selectively stains the β-cells in the pancreas with a crimson red colour (Jiao *et al*, 1991; Fiedor *et al*, 1996; Shiroi *et al*, 2005; Priel *et al*, 2006). The depolarisation of β-cells lead to the influx of zinc into the β-cells through the L-type calcium channels (Priel *et al*, 2006). Zinc is required in secretory granules of pancreatic β-cells for packaging insulin (Figure 2) but needs to be chelated to prevent zinc toxicity (Priel *et al*, 2006). The Zinc transporter, ZnT8, is

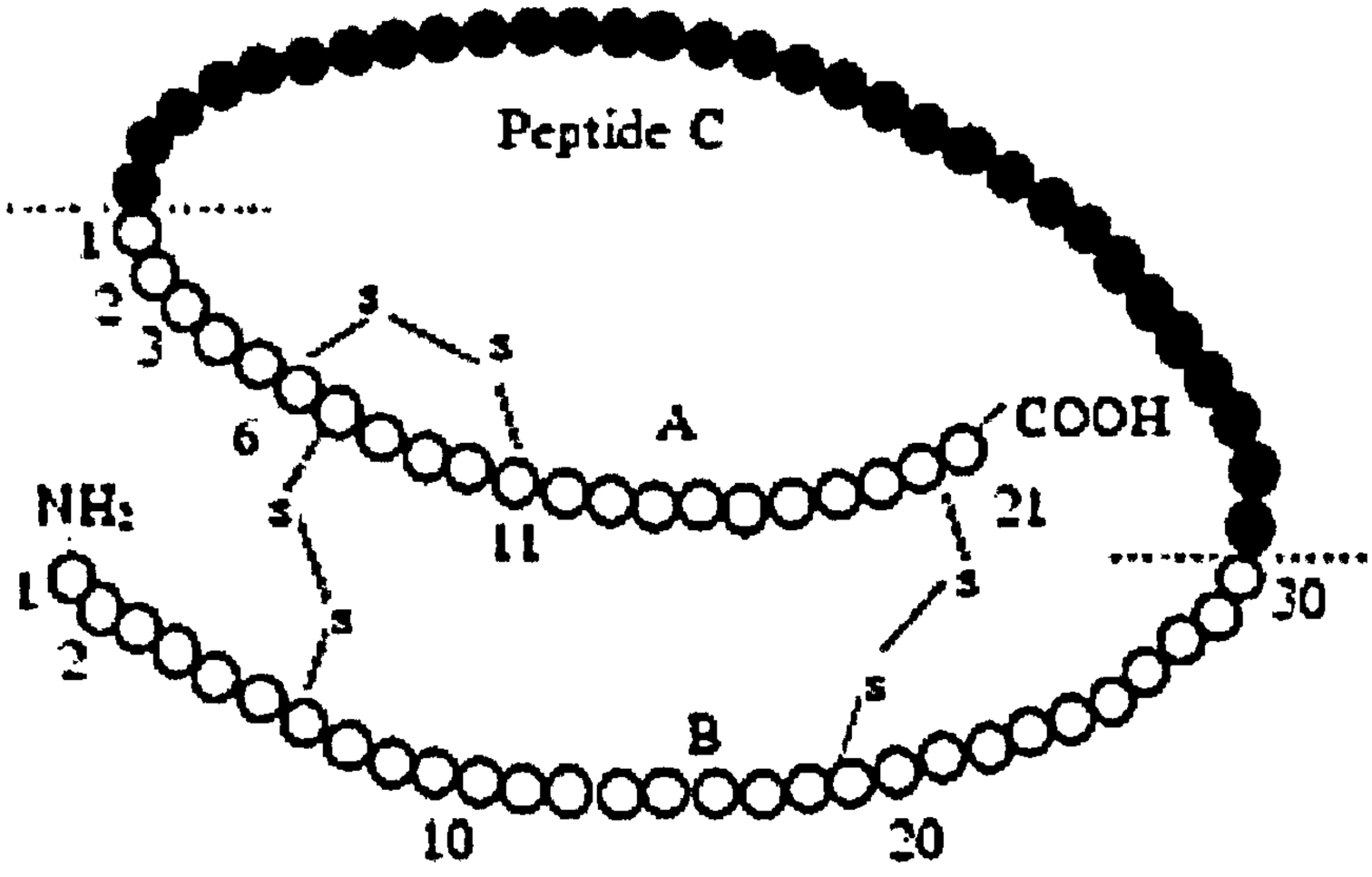


Figure 1 A diagram illustrating the human proinsulin and showing the place of C-peptide, which is located in the centre between the two chains of insulin, A and B. The disulphide bridge is located between the two amino acids in number 10 and between the two amino acids in number 20 of both chains A and B (www.pharmacorama.com/en/Sections/images/Insulin).

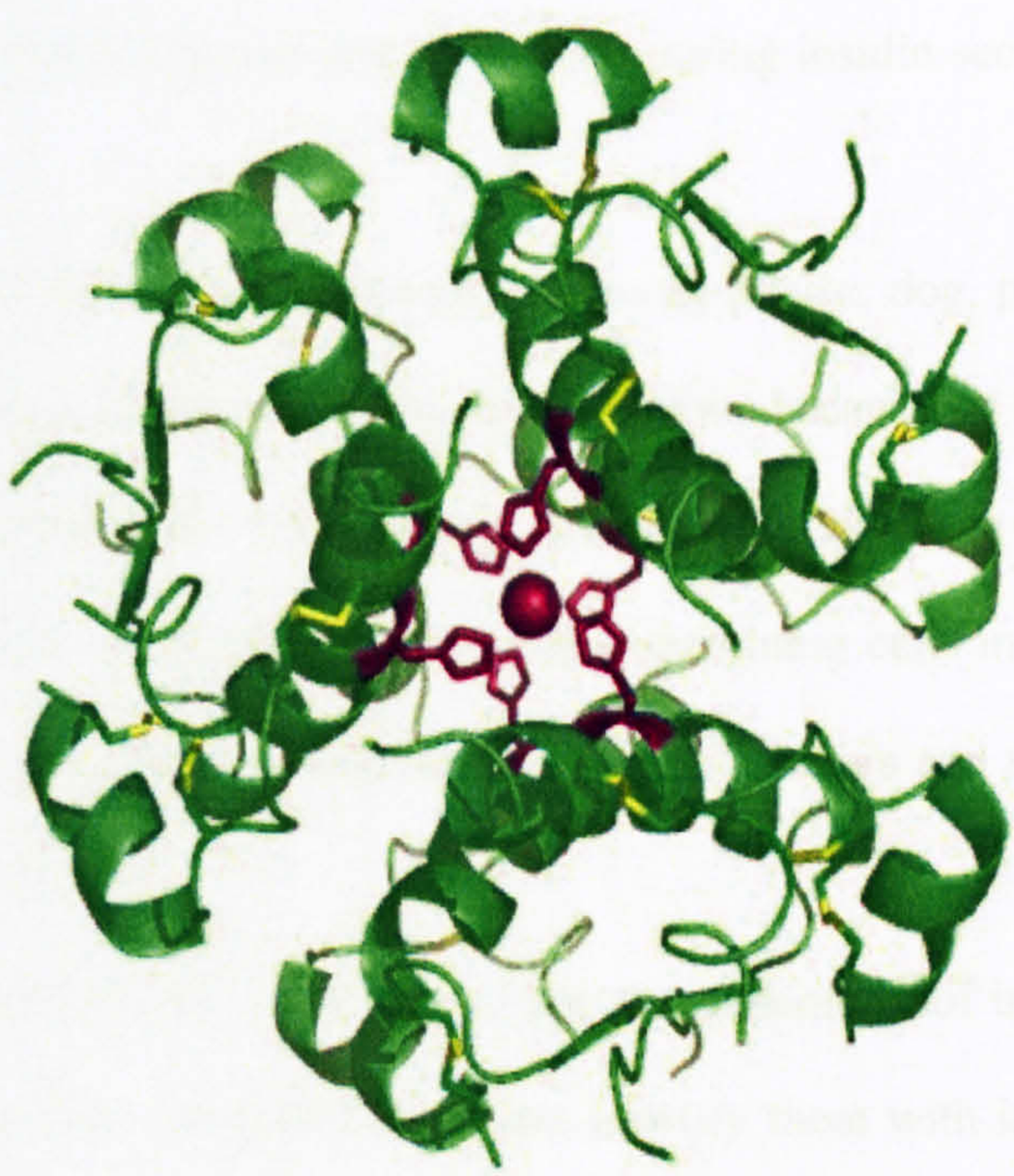


Figure 2 Tertiary structure of pro-insulin molecules (green) chelated with zinc (purple) in secretory granule of β–cell (beta cell consortium, 2004).

a major component for providing zinc to insulin during maturation and storage processes in β-cells (Fabrice *et al*, 2005). It is localised in the secretion vesicle membrane and enables accumulation of zinc into the vesicles from the cytoplasm (Fabrice *et al*, 2005).

Zinc is secreted to the out side of the cell during insulin secretion (Priel *et al*, 2006).

Pancreatic islets from such animal species as mouse, dog, pig and human are all known to be stained crimson red by its treatment, because of their much higher zinc contents compared with other tissues (Clark *et al*, 1994). In the present study DTZ was used to detect the presence of insulin-containing cells in EBs, which were derived from hES cells, grown using different growth factors and their combinations for two weeks *in vitro*.

In this chapter the aim is to investigate the generation of insulin secreting β-cell- like cells in culture using hES cells and identify them with immunofluorescent localisation.

II. MATERIALS & METHODS

1. Materials

All chemicals and media were from Sigma Aldrich (Poole, UK) or Invitrogen (Paisley, UK) unless otherwise stated. Culture flasks and other plasticware were from NUNC Germany (Thermofisher, UK) unless otherwise stated.

1.1 Glass Bead Preparation

The beads (3mm, Phillip Harris Scientific) were covered with concentrated HCl overnight in a beaker. The medium was neutralised by adding drops of 10M NaOH. The beads were rinsed copiously with tap water and then with sterile water, autoclaved and dried.

1.2 Preparation of stock medium

1.2.1 DMEM/FCS Medium Used for Mouse Embryonic Feeders

Dulbecco's Modified Eagle Medium (DMEM), with 4.5g/L D-Glucose, L-Glutamine without sodium pyruvate (DMEM) was prepared and supplemented with 10% foetal calf serum (FCS) and 0.6% (v/v) of gentamycin solution.

1.2.2 Human Embryonic Stem Cell (hES) Medium

To prepare hES medium, (80%) knockout DMEM was mixed with (20%) knockout SR, serum replacement, 1mM L-glutamine, 0.1mM β-mercaptoethanol solution, (1%) non-essential amino acid solution, 4ng/ml basic FGF (FGF2) and 0.6% gentamycin. The mixture was filtered in a 250ml 0.2μm cellulose acetate filtering unit. Medium was stored at 4°C and used within two weeks.

1.2.3 Embryoid Bodies (EBs) Medium

Prepared as hES, in section 1.2.2, but without the addition of basic FGF.

1.2.4 Mitomycin C Medium (10μg/ml)

Mitomycin C powder was dissolved in DMEM/FCS medium for a final concentration of 10μg/ml. The solution was sterilized by passing it through a 0.2μm cellulose acetate filtering unit and stored in a refrigerator, 4°C, for up to one month.

1.2.5 Ice Cold Freezing Medium

10% Dimethylsulfoxide (DMSO) was mixed with 90% FCS. The mixture was stored at 4°C.

1.2.6 RPMI 1640 Mix Medium

RPMI 1640 with L-glutamine was mixed with RPMI 1640 with L-glutamine without glucose in a ratio of 1:1, to have a final glucose concentration of 5.5g/L, and 0.6% gentamycin solution (v/v) added and the medium filter sterilized.

1.3 Preparation of Stock Solutions

1.3.1 Trypsin/EDTA Solution

Trypsin 0.25% (w/v) was mixed well with 1mM EDTA dissolved in 8.5g/L NaCl. The solution was stored at 4°C. 10X trypsin/EDTA solution was also used after dilution (1X).

1.3.2 Gelatin (0.1 %) Solution

Gelatin powder (0.2g) was dissolved in 200ml sterile (autoclaved) dH_2O, mixed well and stored at room temperature.

1.3.3 Nicotinamide (10mM) Solution

A 10mM solution was prepared by dissolving 0.1221g of nicotinamide in 100ml EB solution. The mixture was sterilized by passage through a 0.2µm syringe filter (Nalgene) and stored for no more than two weeks at 4°C.

1.3.4 Activin A (5ng/µl) Solution

Activin A (5µg) was diluted in 1ml autoclaved PBS. The solution was mixed by vortex and stored at -20°C. From this stock solution 50µl was added to 50ml EB medium, filter sterilized and stored at 4°C.

1.3.5 FGF4 (25ng/ml) Solution

FGF4 (1mg) was added to stock 0.1% bovine serum albumin solution mixed well by vortex, filter sterilized and stored at –20°C. From this stock solution 50µl was added to 50ml of EB medium.

1.3.6 bFGF (4ng/ml) Solution

Basic FGF (10µg) was dissolved in 5ml 0.1% BSA in PBS without Ca^{+2} and Mg^{+2}. 400µl aliquots were placed into 0.5ml Eppendorf tubes and stored at –20°C.

1.3.7 Collagenase IV Solution

Collagenase Type IV from *Clostridium histolyticum* was dissolved in DMEM/FCS and a 1mg/ml solution prepared. The solution was filter sterilized, stored at 4°C and used within two weeks.

1.3.8 Bovine Serum Albumin (BSA, 0.1%) Solution

BSA powder (0.1g) was dissolved in 100ml sterile PBS without Ca^{+2} and Mg^{+2}. The solution was stored at 4°C.

1.3.9 Antibody (Ab) Solution

Goat serum (10µg) was added to 0.1% Triton and dissolved in sterile PBS without Ca^{+2} and Mg^{+2}. The solution was incubated at 37°C to dissolve the Triton completely then stored at 4°C.

1.3.10 Stock Solution for Dithizone Staining

A stock solution of dithizone (DTZ) was prepared by mixing 50mg of DTZ with 5ml dimethylsulphoxide (DMSO). The mixture was stored at –80°C.

2. Methods

2.1 Mouse Embryonic Fibroblast Feeder Layers (MEFs)

Mouse embryonic fibroblast feeder layers (MEFs) were grown in culture on untreated tissue culture plastic flasks. MEF cells were passaged every 3 - 4 days, where cells in each flask were divided into 2 - 3 new untreated flasks until passage five. After passage five, MEF cells were inactivated by 10µg/ml mitomycin C (section 1.2.4) or with gamma irradiation (35 gray, Medical School secure source facility).

2.2 Passaging MEFs

Cells were washed once with PBS without Ca^{+2} and Mg^{+2} and incubated with trypsin/EDTA for 3-5 minutes in an incubator at 37°C in 5% CO_2 in air. DMEM/FCS medium was added and centrifuged at 1000rpm (400g) for 5 minutes at 4°C. The pellet was resuspended in DMEM/FCS medium, the cells divided between T25 flasks, at a concentration of $1.5 - 2 \times 10^6$ cells/flask and incubated at 37°C in 5% CO_2 in air.

2.3 Inactivation of MEFs

The inactivation process of MEFs was started by removing the DMEM/FCS medium (section 1.2.1) and covering the cells with 8ml mitomycin C medium. The cells were incubated with mitomycin C medium for about 2-3 hours at 37°C in 5% CO_2 in air. The cells were washed three times with 1X PBS and then covered with 0.25% trypsin (w/v): 1mM EDTA (section 1.3.1) and incubated for 3 minutes at 37°C in 5% CO_2 in air. Each flask was flicked to dissociate the clumps of cells into single

cells. Trypsin/EDTA was inactivated by DMEM/FCS and the whole solution centrifuged for 3 minutes at 400g. Medium was removed and the pellet dispersed and 10ml of fresh DMEM/FCS added. The number of cells was determined by haemocytometer with 7µl being taken from the cells and placed under the coverslip on the haemocytometer. Cells were reseeded on T25 tissue culture flasks pre-treated with 0.1% (w/v) gelatin solution (section 1.3.2) for about 1 - 2 hours. Inactivated cells were finally seeded at about 10^6 cells/ flask and used within two weeks.

2.4 Freezing MEFs

MEF cells were washed with PBS without Ca^{+2} and Mg^{+2} and incubated with 0.25% trypsin (w/v): 1mM EDTA solution (section 1.3.1) at 37°C in 5% CO_2 in air for 3 minutes. The solution was neutralised by adding DMEM/FCS and the mixture centrifuged at 400g for 3 minutes, the pellet resuspended in ice cold freezing medium (section 1.2.5) and finally transferred to cryovial tubes. The cryovials containing the cells were immediately immersed in ice and stored at –80°C. The next day cryovials were transferred to a liquid nitrogen tank for long term storage.

2.5 Thawing MEF Cells

The cryovial containing MEF cells was removed from the liquid nitrogen and thawed by immersing the bottom half of the cryovial in a 37°C water bath. After thawing, cryovials were cleaned from outside with alcohol to reduce contamination and cells immediately transferred to 10ml DMEM/FCS. Cells were centrifuged at 400g for 3 minutes, the supernatant removed and the pellet dispersed by flicking the tube. Fresh 10ml DMEM/FCS was added to the pellet, mixed well and transferred to an untreated T75 flask. The flask was incubated at 37°C in 5% CO_2 in air.

2.6 Inactivation of FCS

Foetal calf serum (FCS) was defrosted by putting it in a 37°C water bath and then incubated for 30 minutes in a 57°C water bath. The inactivated FCS was then ready to use.

2.7 Maintenance of Cell Lines

2.7.1 Passaging Human ES Cell Lines

Human ES cell lines H7 S6 and H7 S14 (WiCell, and obtained from Professor Peter Andrews, Centre for Stem Cell Biology, University of Sheffield) were cultured in hES medium (section 1.2.2). To maintain their undifferentiated status, ES cells were harvested by treatment with 2ml of 1mg/ml collagenase IV solution (section 1.3.7), for each T25 flask for 8 - 10 minutes at 37°C. ES cells were then dispersed by scraping with 3mm glass beads (section 1.1), centrifuged at 300g for 3 minutes, the supernatant removed and the pellet dissociated by flicking the tube and finally cells were seeded onto fresh MEFs which had been washed once with 1X PBS. 6ml of hES medium was added to each T25 flask and incubated at 37°C in 5% CO_2 in air.

Various passages number where used in this study from each cell line. For H7 S14 passages number 16, 18 and 20 were used, while H7 S6 passages number 37, 41, 44, 83, 88, 90, 92, 94, 95, 96 and 98 were used.

2.7.2 Freezing hES Cells

The hES cells were covered with collagenase IV solution (section 1.3.7) and incubated at 37°C in 5% CO_2 in air for 8 -10 minutes. The flask was gently scraped with sterile glass beads and transferred to a 15ml falcon tube, centrifuged at 400g for 3 minutes and the pellet washed by resuspending in hES medium and then replacing

the hES medium with ice cold freezing medium (section 1.2.5). The cells were transferred to cryovials, where one T25 flask of cells was divided into 2 cryovials, kept on ice and then transferred to a –80°C freezer. The following day all cryovials were transferred to liquid nitrogen.

2.7.3 Thawing hES Cells

The same method was used in thawing as for MEFs (section 2.5).

2.8 Differentiation of Human Embryonic Stem Cells

2.8.1 Formation of Embryoid Bodies (EBs)

Human H7 S6 and H7 S14 ES cells were differentiated after formation of embryoid bodies (EBs). hES cells were washed with (1X) PBS without Ca^{+2} and Mg^{+2}. Cells were incubated with collagenase IV solution at 37°C in 5% CO_2 in air for 10 - 15 minutes, the cells scraped with sterile 3mm glass beads and transferred to a falcon tube. The tube was centrifuged at 400g for 3 minutes, the pellet resuspended in 10ml hES medium and finally transferred to a sterile 10cm diameter non-treated bacterial grade Petri dish (Sterilin).

To induce insulin-secreting cells in the lab, both H7 S6 and H7 S14 cells were used and grown on MEFs as a feeder layer in hES medium. The EBs were grown using different growth factor media (see sections 1.3.3, 1.3.4, 1.3.5 and 1.3.6 of this chapter) and their combinations (Nicotinamide only, Nicotinamide + Activin A, Nicotinamide + FGF4, Nicotinamide + FGF2, Nicotinamide + Activin A + FGF4 + FGF2, Activin A only, Activin A + FGF4, Activin A + FGF2, FGF4 only, FGF2 only, FGF2 + FGF4 and Control) for the first week and normal EB medium for the

second week. The medium was changed every two days for each dish for the two weeks.

After the first week, some EBs from each Petri dish were plated out in a twelve - well culture plates, three wells for each sample, and left at 37°C in 5% CO_2 in air for two days with RPMI 1640 medium without FCS to spread out the EBs colonies to a monolayer making them easier to fix and undertake immunolocalisation analysis. At the end of the second week, the remaining EBs were spread out in twelve well plates. After the attachment and spreading of EBs in wells, some of them were stained for dithizone (section 1.3.10) while others were fixed and immunostained with different antibodies for C- peptide, insulin, glucagon and nestin.

These experiments were repeated fifteen times to assess reproducibility and consistency of the results obtained. To confirm the expression of insulin producing cells immunocytochemistry was obtained in parallel with dithizone staining.

2.9 Immunocytochemistry

HES cells were assessed regularly using stem cell specific markers [Tra-1-60, SSEA3, SSEA4 (undifferentiated cells positive) and SSEA1 (undifferentiated cells negative)]. Cells were washed three times with 1X PBS then once with 100% cold (5°C) methanol for 5-10 minutes at room temperature then washed twice with 1% antibody (Ab) solution (section 1.3.9). For EBs this was performed after attachment to culture plate.

To optimise the primary and secondary antibodies various dilution ratios were used (1:50, 1:100, 1:200 and 1:500) all with a 1% Ab solution. The different dilutions of primary antibodies used in this study are listed in the table below.

Primary antibody	Dilution	Supplier, Cat #
Tra-1-60 mouse monoclonal IgM	1:10	CSCB, hybridoma supn
SSEA4 ,mouse monoclonal IgG	1:10	CSCB, hybridoma supn
SSEA1 mouse monoclonal	1:10	CSCB, hybridoma supn
Mouse anti-human insulin monoclonal antibody	1:200	Abcam, ab7760
Rabbit anti-human glucagon	1:100	Dako, 0101A
Goat anti-human pro-insulin C-peptide	1:200	AutogenBioclear, AB94
Rabbit polyclonal to human nestin	1:100	Abcam, ab7659-100

The selected cells were covered with the primary antibody and incubated overnight at 4°C. The cells were washed twice with 1% Ab solution then twice with 1X PBS before secondary antibody was added.

The fluorescent conjugated secondary antibodies (using 1% Ab solution) used in this study and their dilutions are listed in the table below.

Secondary antibody	Dilution	Supplier, Cat #
Anti-mouse polyvalent immunoglobulin IgG+M (FITC or TRITC)	1:500	Sigma, F-1010
Anti-rabbit IgG (whole) (TRITC)	1:500	Sigma, T6778
Anti-mouse IgG (Fc specific) (FITC)	1:500	Sigma, F-5387
Anti-mouse goat (whole)- Cy3	1:500	Sigma, A5324
Anti-guinea pig IgG (whole molecule) (FITC)	1:500	Sigma, F-6261

Cells were covered with the secondary antibody and incubated for two hours. After the incubation time cells were washed twice with 1X PBS and examined under the microscope with UV optics. Sometimes double immunostaining was undertaken. No first antibody (PBS) and undiluted culture supernatant of the mouse myleoma P3X63 Ag8 were used as negative controls.

2.10 Transmission Electron Microscopy

This was done in conjunction with the Electron Microscope Facility (Department of Biomedical Sciences, University of Sheffield). EBs were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer and centrifuged at 1000g to form a pellet of aggregated EBs. This was then prepared for electron microscopy by post-fixation in 1% osmium tetroxide, dehydration through graded ethanol solutions and propylene oxide and embedding in epoxy resin. Ultrathin sections were stained with lead citrate and examined by transmission electron microscope (Phillips).

2.11 EBs Cells Stained for Dithizone

For staining cells, 10µl of the stock solution of DTZ were mixed with 1ml of serum-free RPMI 1640 mix medium. The staining solution was syringe filtered. Cells were covered with DTZ staining solution and incubated at 37°C for 3 - 4 hours. Cells were rinsed 3 times with 1X PBS and then examined under the microscope.

2.12 Determination of Dithizone Stained Cells

The total number of stained cells obtained after DTZ treatment was determined by counting the number of cells stained a red colour in each colony after trypsinisation of an EB with 0.25% trypsin (w/v): 1mM EDTA solution for about 7 minutes in a small Petri dish (3.5cm). The EBs stained with DTZ were selected as

being approximately the same size, with the biggest EB having about 4800 cells and the smallest about 3200 cells. Using a fine black marker pen, the dish lid was divided into four equal quarters. A mark was put on the dish bottom to align the lid. To determine the number of cells, the dish was scanned from the middle of each quarter outwards. The marks on the lid were out of focus but could still be clearly observed. Stained cells were determined in one quarter then multiplied by four to obtain the total number of stained cells on each plate.

The proportions (%) of red stained cells were calculated using the following formula:

$$\% \text{ of stained cells} = \frac{\text{The number of stained cells X 100}}{\text{Mean total number of cells of the (small/large) colony}}$$

III. RESULTS

1. Preparation of Mouse Embryonic Fibroblast Feeder Layers (MEFs)

The inactivated MEF cells, which were prepared prior to co-culture with hES cells were confluent, forming as a feeder layer and ready to passage hES cells on them the next day (Figure 3 A). They were pre-washed once with 1X PBS and covered with 6ml of hES medium in each T25 flask about one hour before seeding the hES cells. MEFs have to be used within a maximum of two weeks as they start dying after that and become unsuitable for passaging hES cells on them.

2. Maintenance of Cell Lines

2.1 Passaging Human ES Cell Lines

To maintain the hES cell lines H7 S6 and H7 S14 in an undifferentiated state, hES cells were seeded onto fresh MEFs every 4-5 days (Figure 3B).

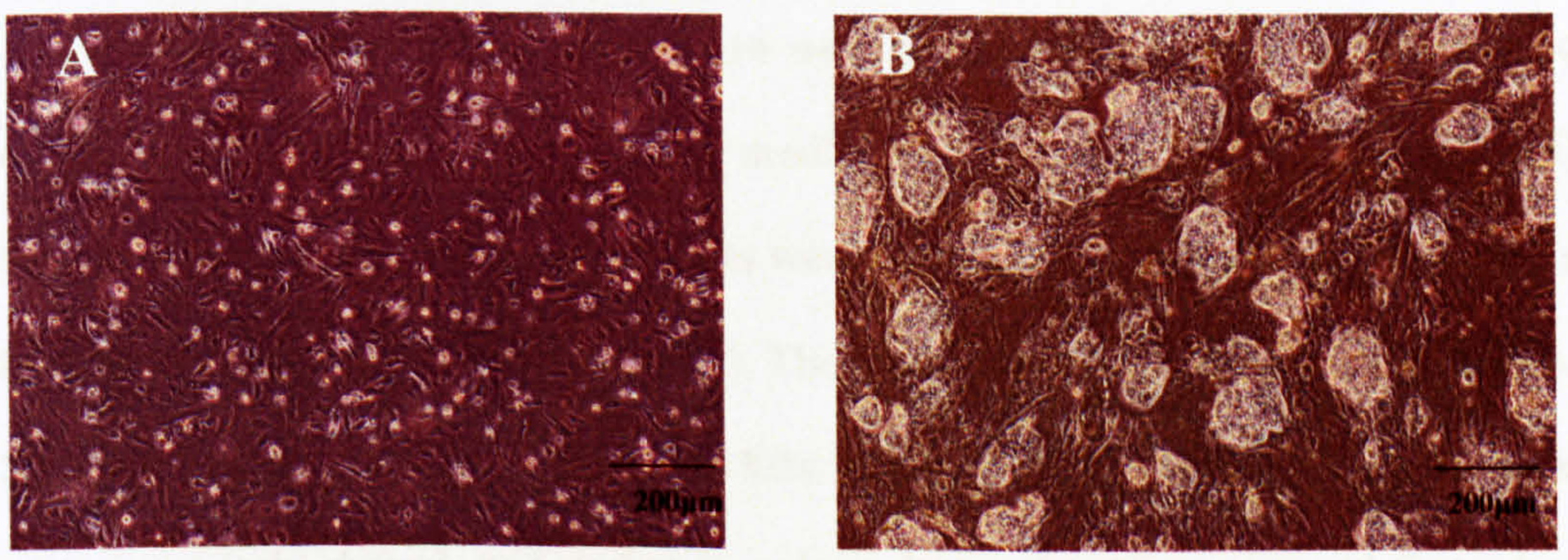


Figure 3 (A) Inactivated mouse embryonic fibroblast feeder layers (MEFs). (B) undifferentiated human ES cells H7 S6 maintained on inactivated mouse feeders.

Immunolocalisation with specific cell surface markers (Figure 4 Tra-1-60, SSEA1) was performed regularly to demonstrate characterisation.

3. Differentiation of Human Embryonic Stem Cells

3.1 Formation of Embryoid Bodies (EBs)

Both cell lines H7 S6 and H7 S14 were grown in Petri dishes, in the absence of MEFs, using different growth factor media (sections 1.3.3, 1.3.4, 1.3.5 and 1.3.6) and their combinations. When hES cells were cultured in suspension for two weeks they aggregated forming EBs (Figure 5). The EBs were three dimensional structures. Small, almost spherical and transparent EBs formed first but over time, these became more irregularly shaped, and darker in colour. It was therefore assumed that when observing cultures at any one time, recently formed EBs were small and clear while older mature EBs were irregular and large. The EBs which were differentiated from human H7 ES cells, S6 and S14, had normal karyotypes.

All ES cell lines in the laboratory were regularly karyotyped by a cytogeneticist (Mr Duncan Baker, Sheffield Children Hospital) using the G-banding technique. Both S6 and S14 had normal karyotype at passage number 44 and passage number 96 (end) of the study. Where as, S14 had normal karyotype at end of the study (passage 20).

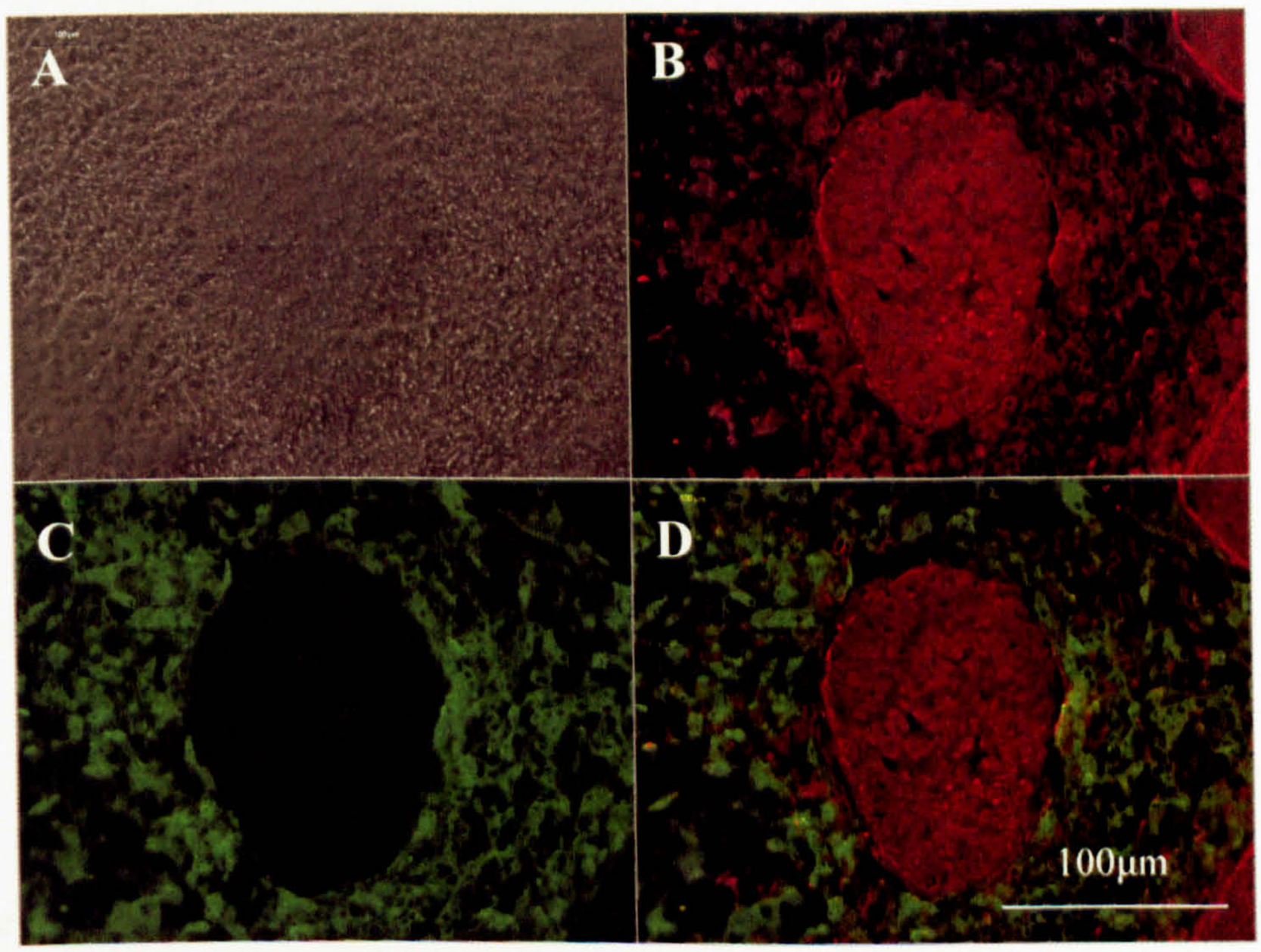


Figure 4 Immunolocalisation of hES cell markers with H7 cells in culture after 5 days. (A) phase-contrast of colony surrounded by differentiating cells; (B) Tra-1-60 localisation (TRITC secondary antibody) of undifferentiated cells; (C) SSEA1 localisation (FITC secondary antibody) of differentiated cells; (D) combined image.

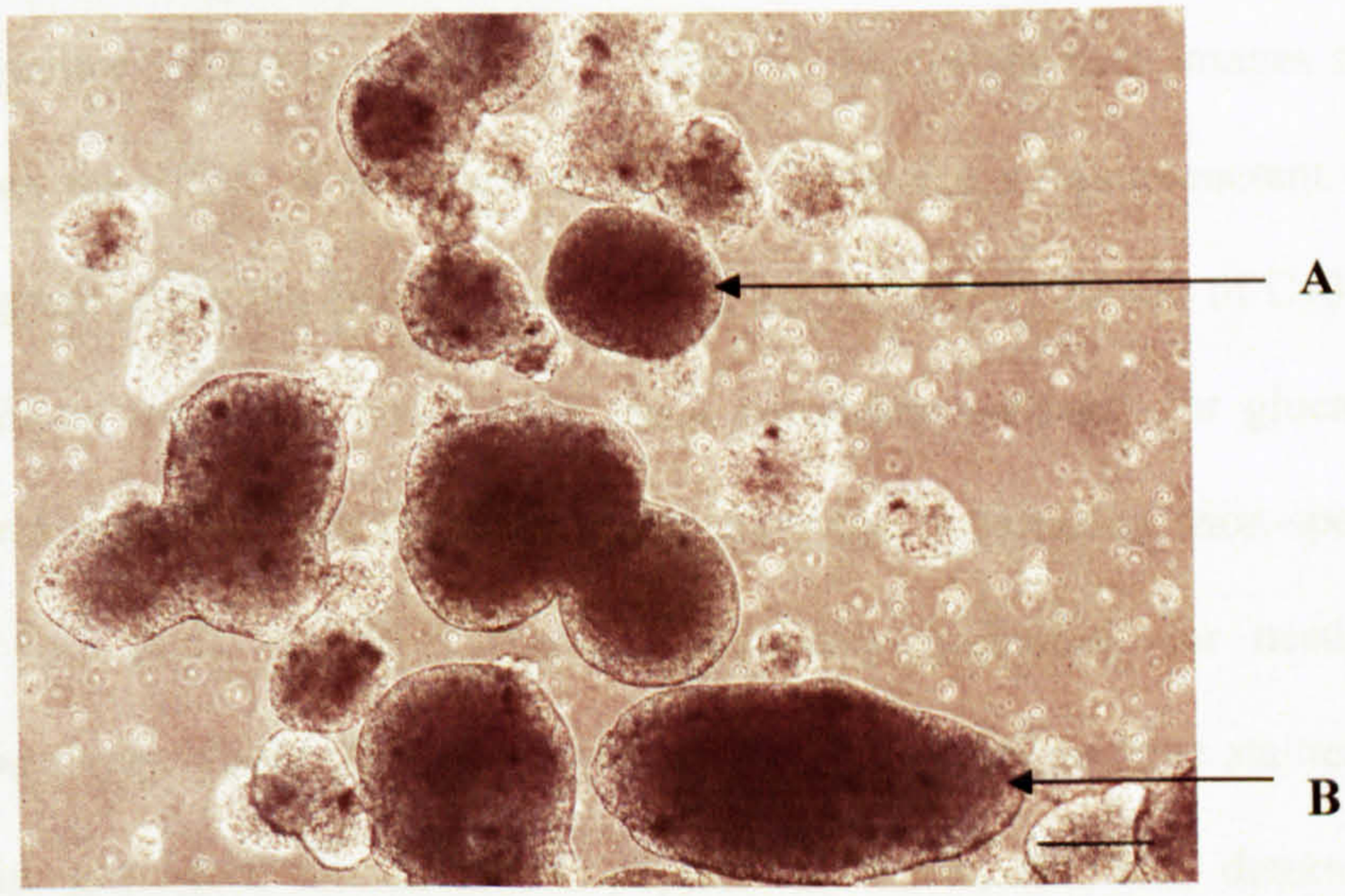


Figure 5 Embryoid bodies cultured in EB medium for about one week. The micrograph shows both types of EBs. (A) The recently formed EBs are almost spherical in shape and transparent compared to (B) which are more irregularly shaped, darker in colour and with outgrowth cells. Phase contrast. Scale bar = 300μm

4. Immunocytochemistry

4.1 The Expression of Nestin and Glucagon in Human EB Cells

Spread EBs cells showed positive nestin and glucagon immunolocalisation after both 7 and 14 days in EBs medium indicating the presence of potential pancreatic progenitor cells (Figures 6 and 7; Table 1). The bright field images show the cells without the effect of the fluorescent dye, while the immunoreactant cells stained red or green under the effect of the fluorescent dye due to the use of Cy3 as a secondary antibody for nestin and TRITC as a secondary antibody for glucagon. Negative control samples were incubated with myeloma supernatant (non-specific binding) and Cy3 (red) or TRITC (green) secondary antibodies for nestin or glucagon, respectively. Table 1 show the presence or absence of positive stained EB cells for nestin, glucagon, insulin and C-peptide. However, this was determined according to the stain density in EB colony grown with different growth factor/s, where +ve indicate slightly positive result obtained for stained cells, +++ve indicate the highly positive results, where most of cells in the EB colony were stained positive, and ++ve indicate the presence of some positive cells in the EB colony but less than +++ve EB colonies.

5. The Expression of Insulin and C-Peptide on Human ES Cells

To be sure of the presence of some insulin containing cells among the differentiated human EBs *in vitro*, anti-insulin and anti C-peptide antibodies were used (Table 1). When EBs cells, grown with different growth factors, were stained for insulin, a proportion of cells were stained and showed some immunoreactivity when examined under the fluorescent microscope (Figure 8). The brightfield image

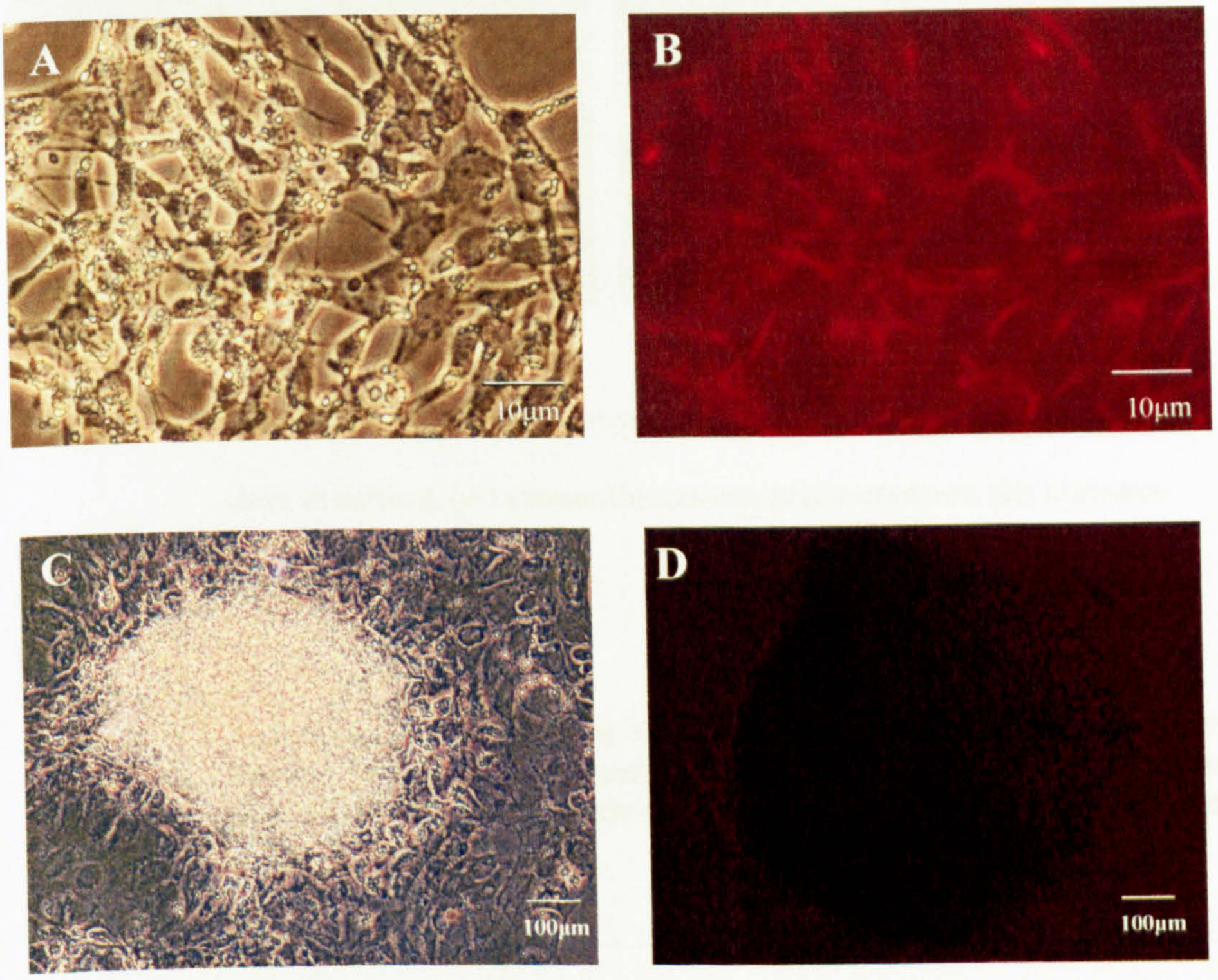


Figure 6 Immunolocalisation of nestin with spread human EB cells after 14 days in culture. (A) Phase-contrast; (B) Nestin localisation in monolayer cells from EB. (C) and (D) Phase-contrast and fluorescent low magnification image of control (Cy3).

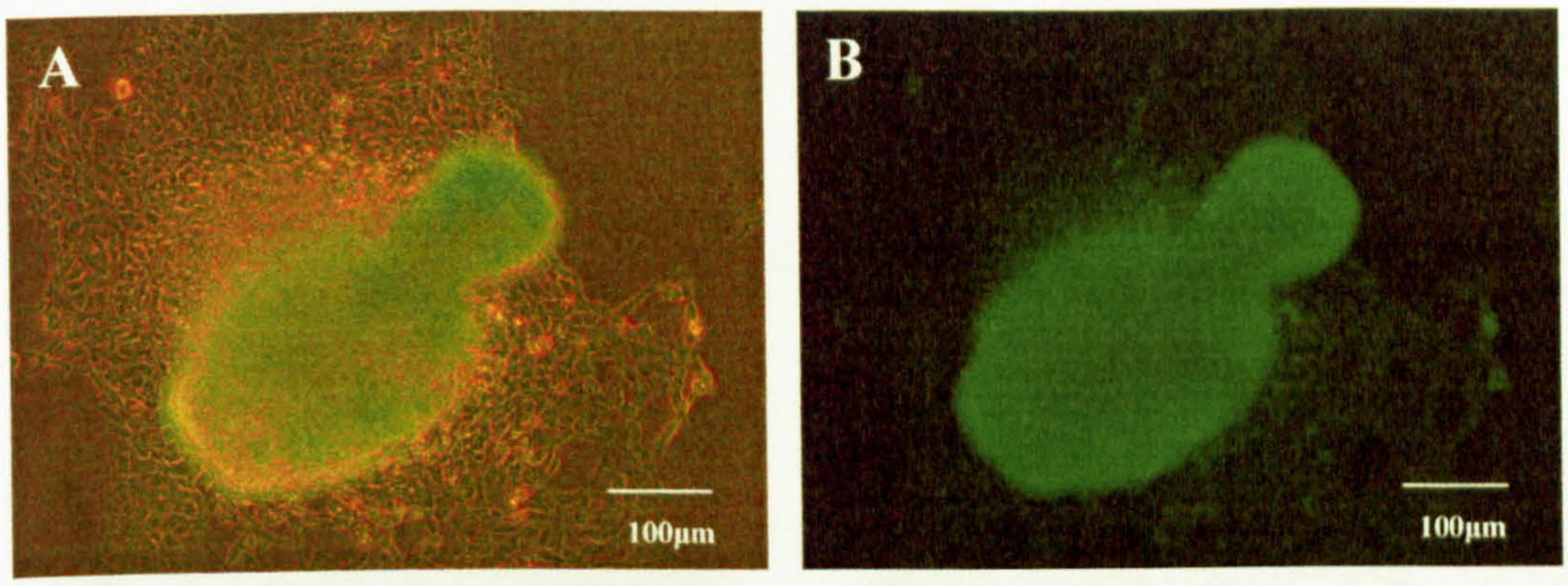


Figure 7 Immunolocalisation for glucagon with spread human EBs after 14 days in culture. (A) Immunfluorescent/phase-contrast; (B) Immuno-fluorescence alone.

Table 1 Indicating the presence or not of the positive stained EB cells for nestin and glucagons according to the stain density in each growth factor/s. +ve: slightly positive result, +++ve: highly positive result and ++ve: in between.

Growth Factors	7 days				14 days			
	Nestin	Glucagon	Insulin	C-peptide	Nestin	Glucagon	Insulin	C-peptide
Nicotinamide	+ve	-ve	++ve	-ve	+++ve	++ve	+++ve	++ve
Nicotinamide + Activin A	-ve	-ve	++ve	-ve	++ve	+ve	+++ve	++ve
Nicotinamide + FGF2	-ve	-ve	++ve	-ve	++ve	++ve	+++ve	++ve
Activin A + FGF2	-ve	+ve	+ve	-ve	++ve	+ve	++ve	++ve
Activin A	-ve	-ve	-ve	-ve	++ve	+ve	+ve	+ve
Control	-ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve

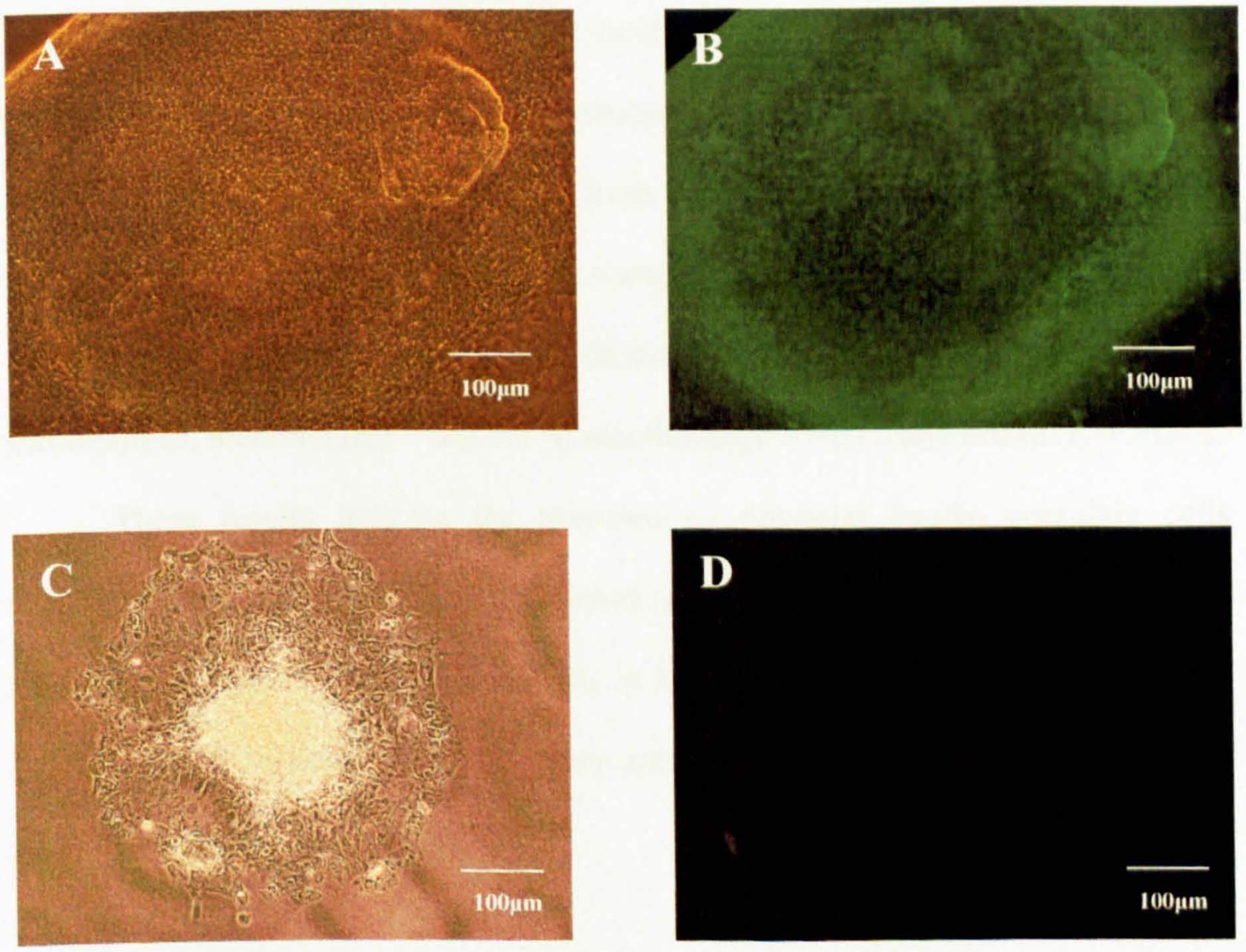


Figure 8 Immunolocalisation for insulin with spread human EBs after 14 days in culture. (A) Phase-contrast; (B) Immunofluorescence; (C and D) Phase-contrast and fluorescent low magnification of control.

the cells on day 14 without the effect of the fluorescent dye, while the immunoreactant cells stained green on day 14 under the effect of the fluorescent dye from the use of FITC as a secondary antibody for insulin. On the other hand, control samples did not show any reaction from incubation of the cells with FITC only (as a secondary antibody for insulin) after the second week in culture.

In addition, the results obtained from C-peptide immunostaining were found in small cell batches, positively stained, scattered throughout the EB colonies grown for 14 days with different growth factors and their combinations such as, nicotinamide, nicotinamide + activin A, nicotinamide + FGF2 and activin A + FGF2.

These results indicate the presence of potential insulin containig cells especially with cells grown in the presence of nicotinamide + activin A (Figure 9). From figure 9 some cells, in B and D, in human EBs were stained green for C-Peptide on day 14 using FITC secondary antibody, which indicated the presence of putative insulin producing cells.

6. EBs Cells Stained for Dithizone (DTZ)

Human EBs which were outgrowths from human ES H7 cells spread into a monolayer and after differentiation with growth factors in their various combinations some cells stained with DTZ. Most DTZ positive cells were found in clusters scattered throughout the colony rather than as isolated cells (Figure 10). The stained cells, were scattered within or outside the EBs colonies indicating the presence of some putative insulin producing cells in different proportions in each colony according to the growth factor used. In the control samples, the number of cells apparently stained faintly red with DTZ was minimal (0.1%) when compared with samples treated with various growth factors.

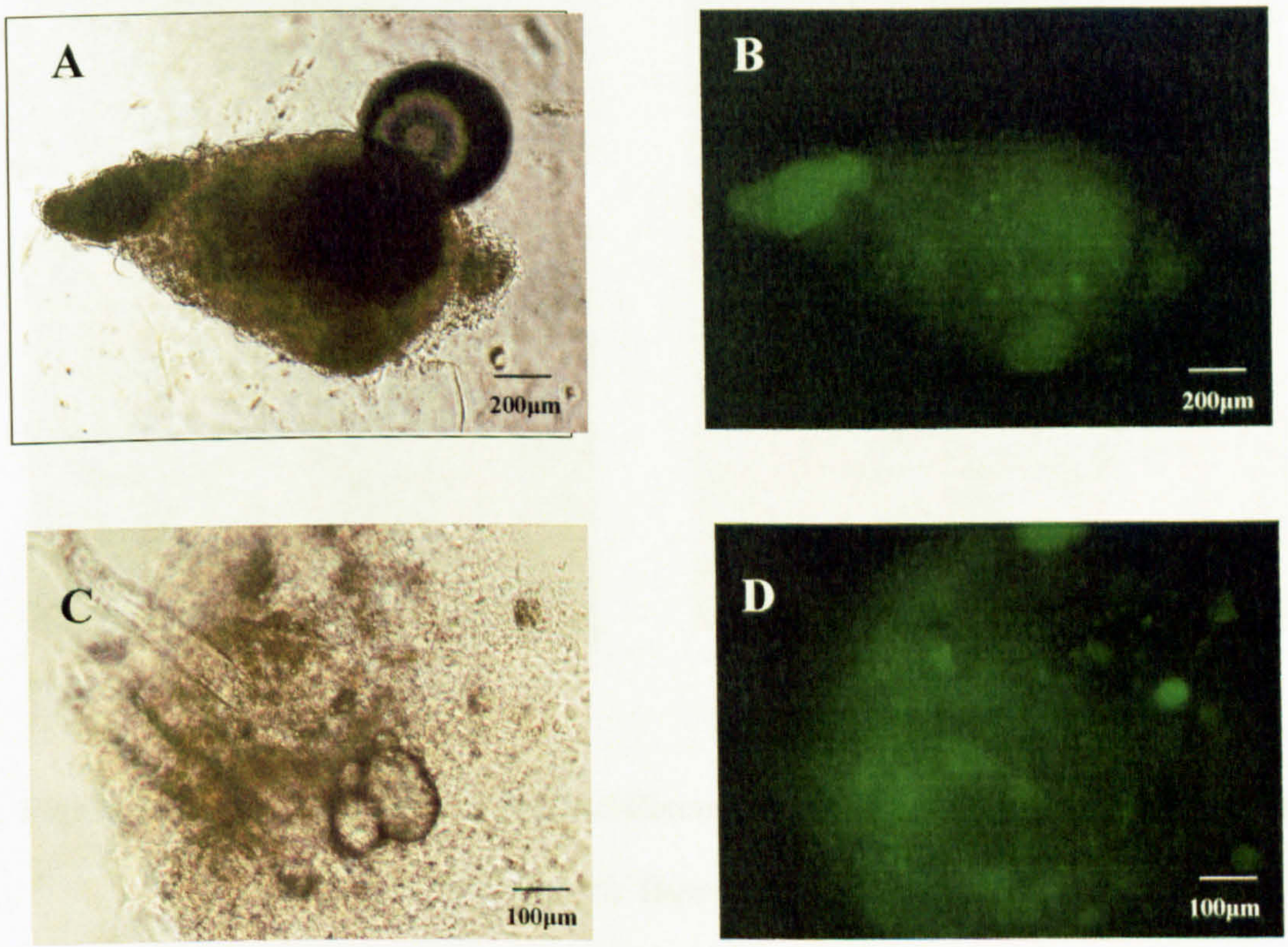


Figure 9 Immunolocalisation for C-Peptide with spread human EBs after 14 days in culture. (A) Phase-contrast; (B) Immuno-fluorescence for EBs supplemented with nicotinamide + activin A; (C) Phase-contrast; (D) Immunofluorescence for EBs supplemented with activin A + FGF2.

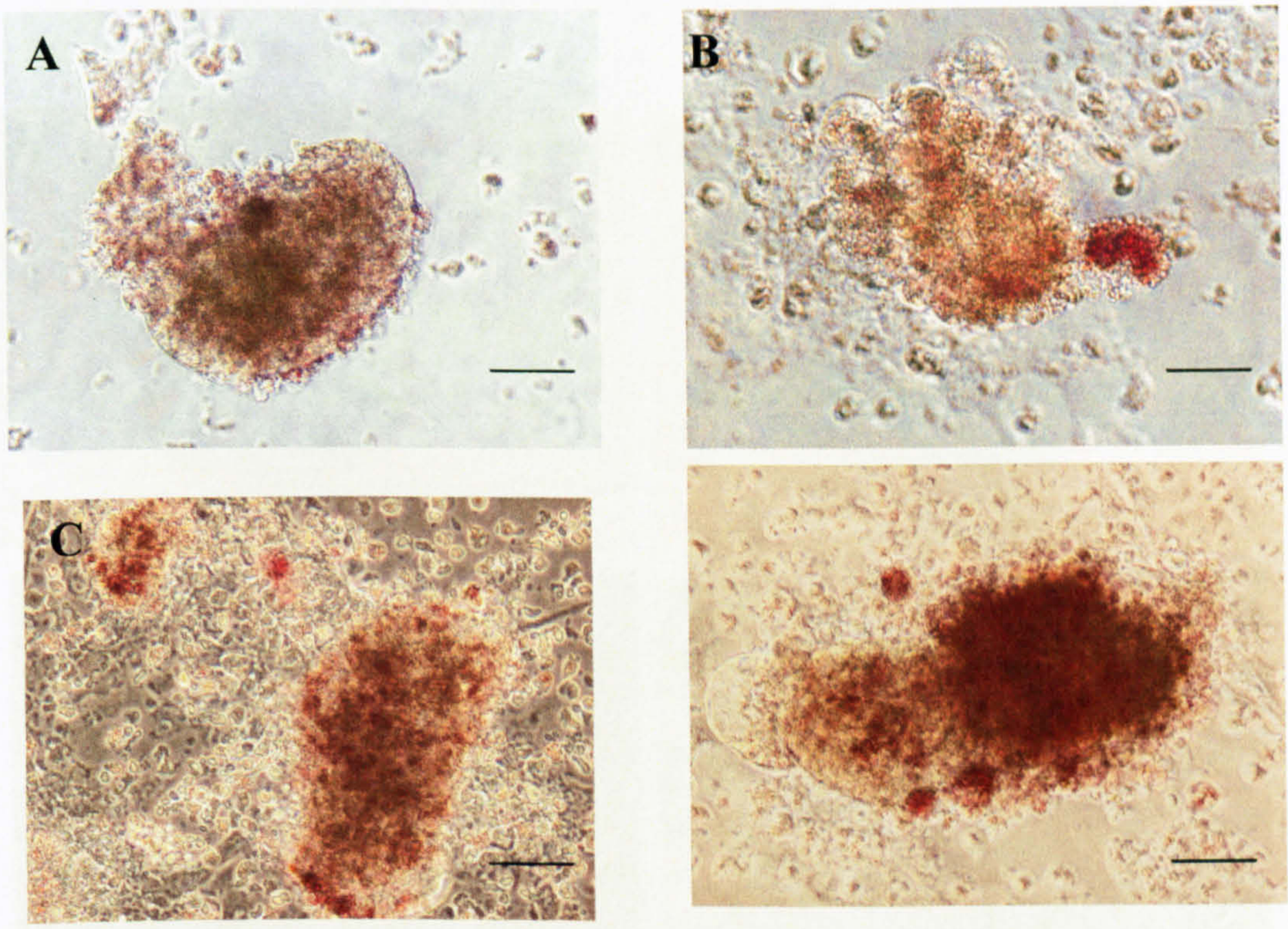


Figure 10 Human EBs cultured in different growth factor/s stained for DTZ. EBs were grown with growth factors for one week followed by another week with normal EBs medium and then stained with DTZ. (A) EB grown with Nicotinamide + Activin A; (B) EB grown with Nicotinamide + FGF4; (C) EB grown with Activin A + FGF2; (D) EB grown with Nicotinamide; (E) EB grown with Nicotinamide + FGF2 + Activin A + FGF4 (F) EB grown with Nicotinamide + FGF2 (G) Control. (E - G next page). (H) High magnification of DTZ stained aggregate (Nicotinamide + Activin A) from EB after 14 days in culture. Note morphology and resemblance to islet morphology in foetal pancreas. Scale bar = 100μm.

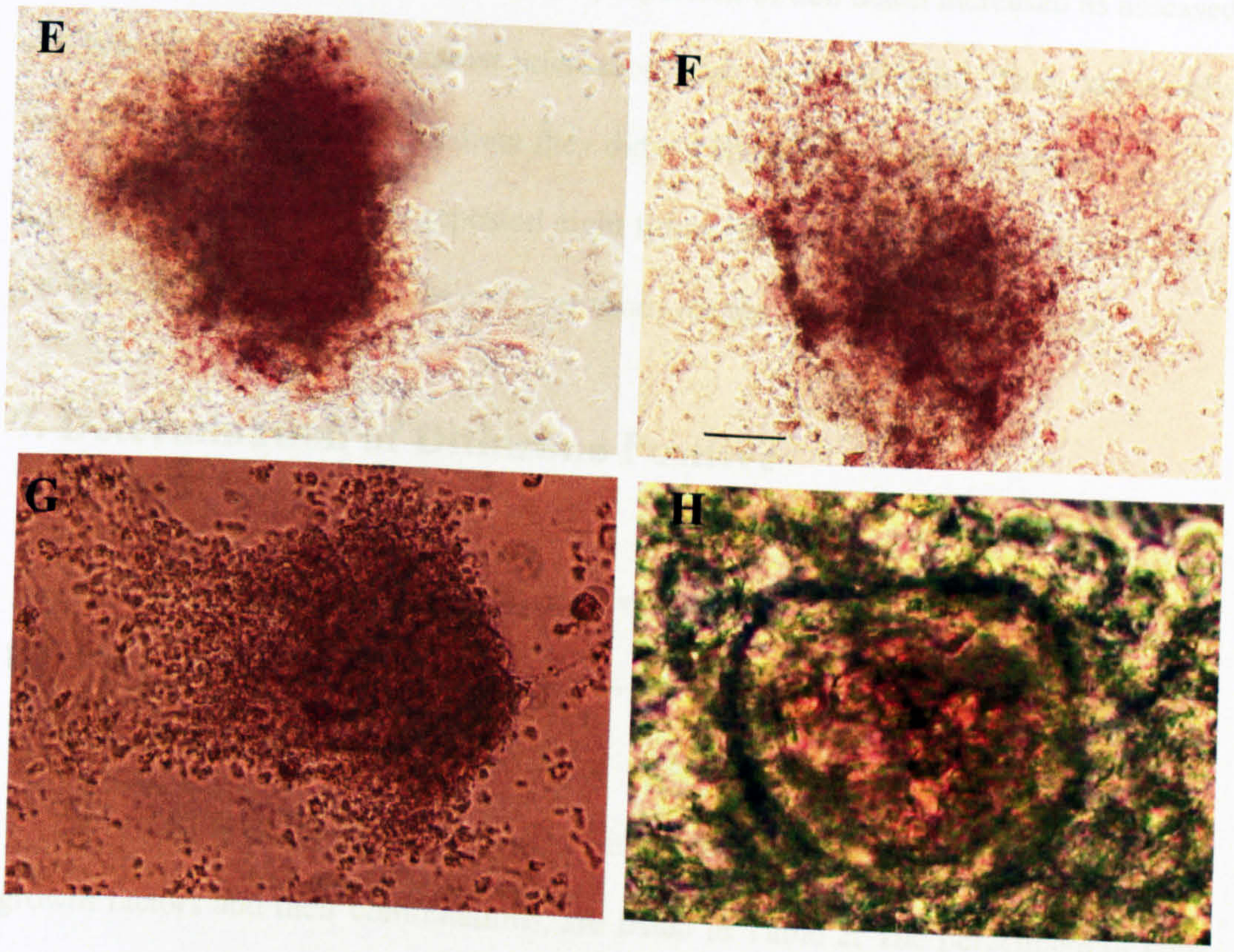


Figure 10 continued (see previous page).

When cells were incubated with DTZ after the long incubation time (of about two hours) in the absence of serum, the proportion of cell death increased as assessed subjectively under phase contrast microscopy (i.e. no bright halo). Thus, when cells were re-incubated with EB medium they did not show any obvious response.

The experiment was repeated eight times for each growth factor/s, four times for the small sizes EB colonies and four times for the large sizes EB colonies.

7. Determination of Dithizone Positive Stained Cells

The number of DTZ staining positive cells was counted after trypsinising the EB colony with 0.25% (w/v) trypsin for seven minutes to yield single cells (Figure 11) in small (3.5cm) Petri dish.

In addition, the percentage of positive cells, stained for DTZ, for different growth factors and their combinations, are listed in Table 2. The percentage of cells stained with DTZ in selected EBs varied among different growth factors and according to the size of EBs under examination. The highest percentage was obtained from the combination of nicotinamide + FGF2, about 31%, while the lowest, about 10%, resulted from the combination of both activin A + FGF2. Human EBs which were grown with activin A only and those grown with normal EBs medium in the control samples showed about 1% and 0.1%, respectively, which means that almost none of the cells in these EBs were producing insulin as assessed by this method.

The mean percentages, standard deviations (SD) and p values of the cells grown with different growth factors and stained positive for DTZ were determined.

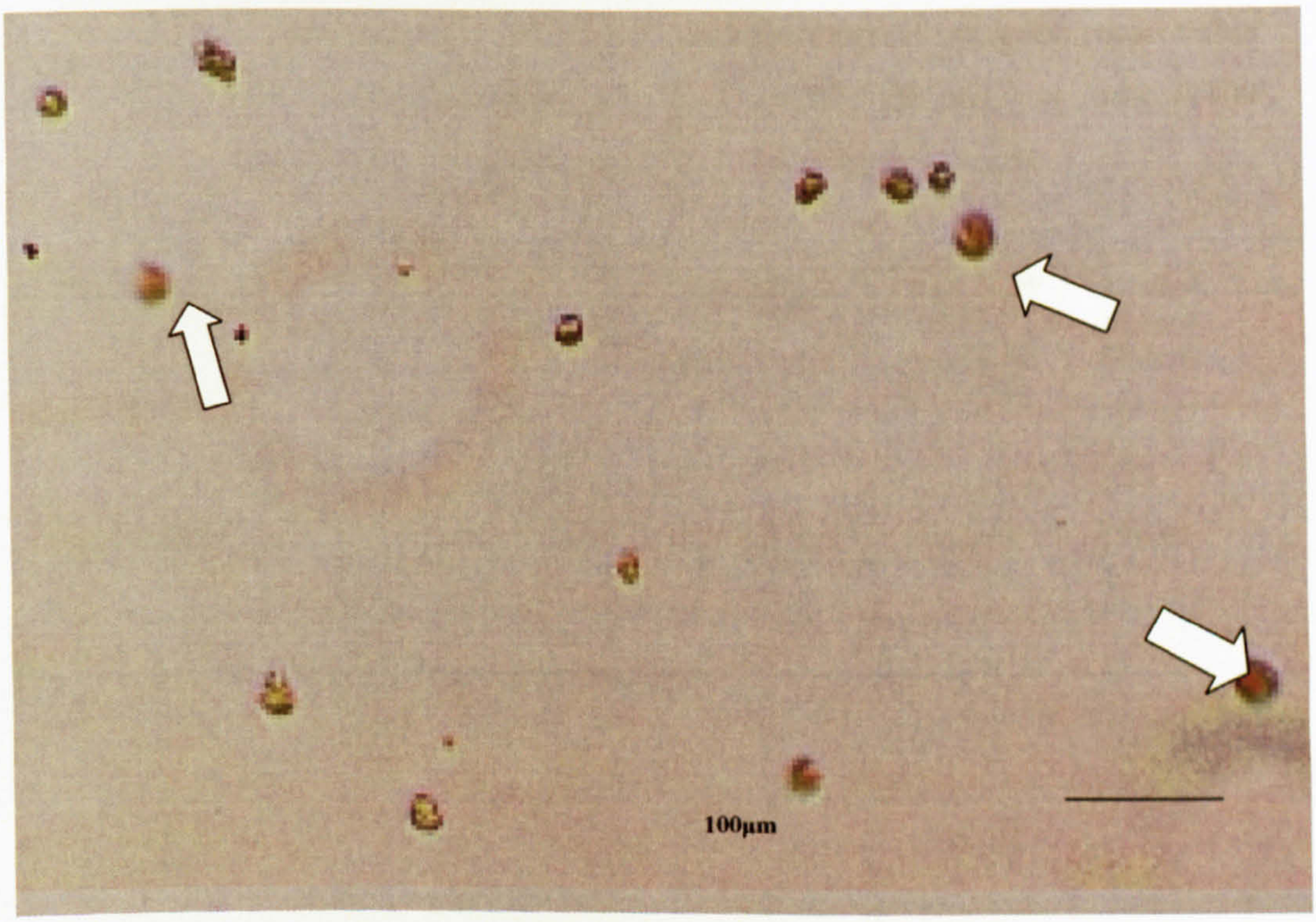


Figure 11 Phase-contrast micrograph of EB cells after trypsinisation with 0.25% (w/v) trypsin for seven minutes to make single cells. The arrow shows cells assessed to be stained red with dithizone. This was approximate magnification used when counting.

Table 2 The percentages of stained cells with Dithizone (DTZ) in EBs grown with different growth factors for 14 days. The number of positively stained cells was counted after trypsinisation of the selected EBs with 25% (w/v) trypsin for seven minutes in the plate to yield single cells. The proportion of stained cells was determined using the mean value for the total number of cells in a small (3200 cells) or large (4800 cells) colony. # = number and SD = standard deviations.

Growth Factors	# of Cells Stained for DTZ	Colony Size	Approximation of % of Cells Stained for DTZ	Mean % of Cells Stained for DTZ ± SD	P value against Control
Nicotinamide	802 483 450 600	Small Colonies (3200 cells)	25 15 14 19	**18 ± 4.6**	**<0.05**
	815 1189 620 800	Large Colonies (4800 cells)	17 25 13 17		
Nicotinamide + Activin A	420 901 660 615	Small Colonies	13 28 21 19	**22 ± 4.9**	**<0.05**
	1090 1110 998 1345	Large Colonies	23 23 21 28		
Nicotinamide + FGF2	1200 993 805 1150	Small Colonies	38 31 25 36	**31 ± 5.5**	**<0.05**
	1010 1500 1583 1594	Large Colonies	21 31 33 33		
Activin A + FGF2	220 435 210 108	Small Colonies	7 14 7 3	**10 ± 4.9**	**< 0.05**
	480 500 910 340	Large Colonies	10 10 19 7		
Activin A	19 10 24 13	Small Colonies	1 0 1 0	**1 ± 0.4**	**<0.05**
	38 66 7 19	Large Colonies	1 1 0 0		

Control	2 0 0 4	Small Colonies	0 0 0 0	**0.1 ± 0.07**	
	9 6 2 0	Large Colonies	0 0 0 0		

8. Data Analysis

T-tests, univariate analysis of variance and post-hoc tests were applied to the data from all cells stained with DTZ from EBs grown with different growth factors (appendix tables 3 A-F for T-test and table 4 for analysis of variance and post-hoc tests).

9. Transmission Electron Microsocopy (TEM) of EBs

This was carried out in conjunction with Dr Chris Hill, Department of Biomedical Sciences.

TEM was undertaken on EBs cultured with nicotinamide + FGF2. Generally, the majority cells showed a polarised morphology with many vacuoles. Cells were connected by tight junctional complexes, and extracellular matrix was present (Figure 12). However there were some cells containing secretory granules. These had a halo appearance characteristic of β- cell insulin granules but did not show a dark core (Figure 13).

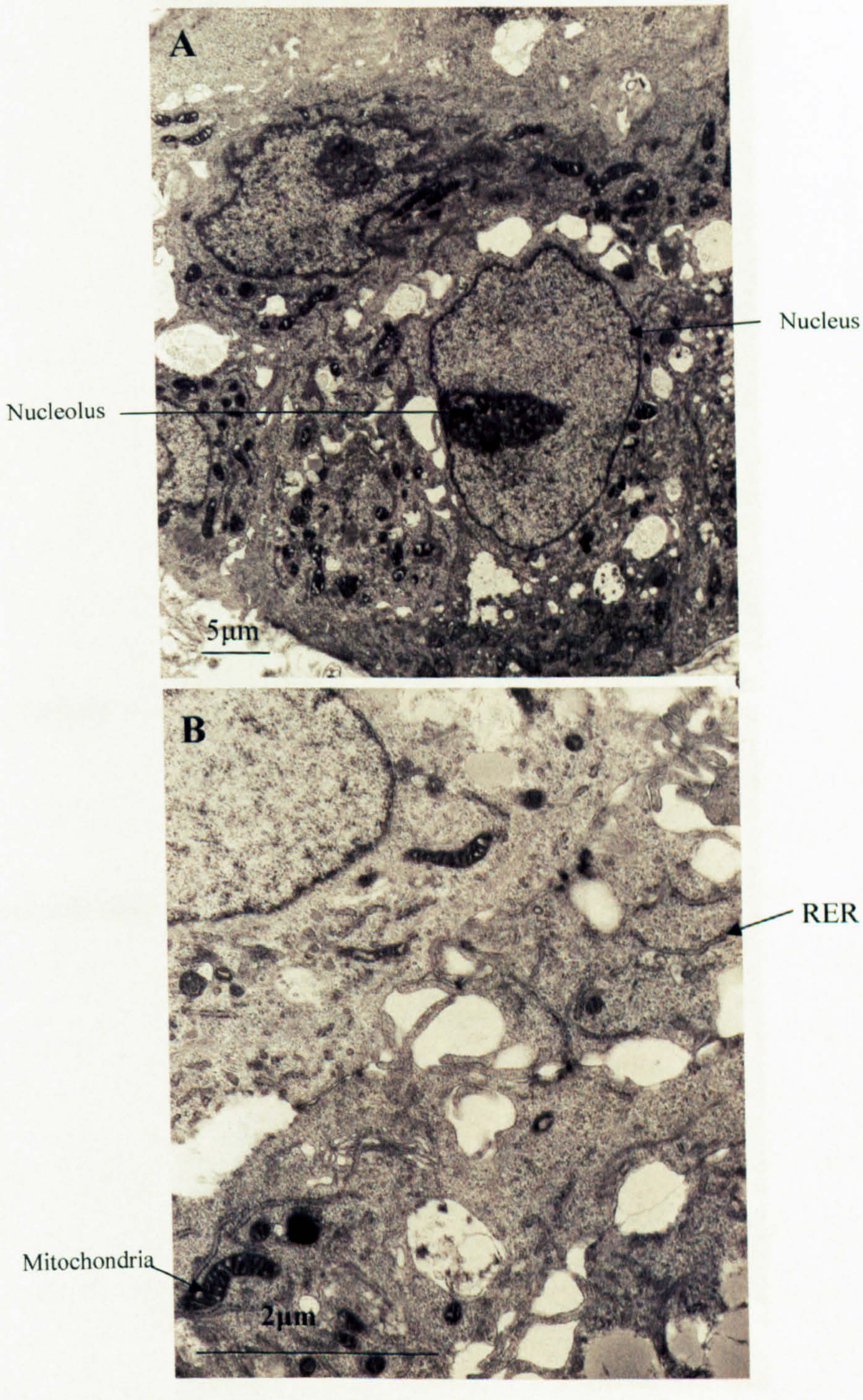


Figure 12 (A) Ultrastructure of polarised epithelial cells of EBs showing vacuoles. (B) higher magnification showing granules and tight junctions between cells.

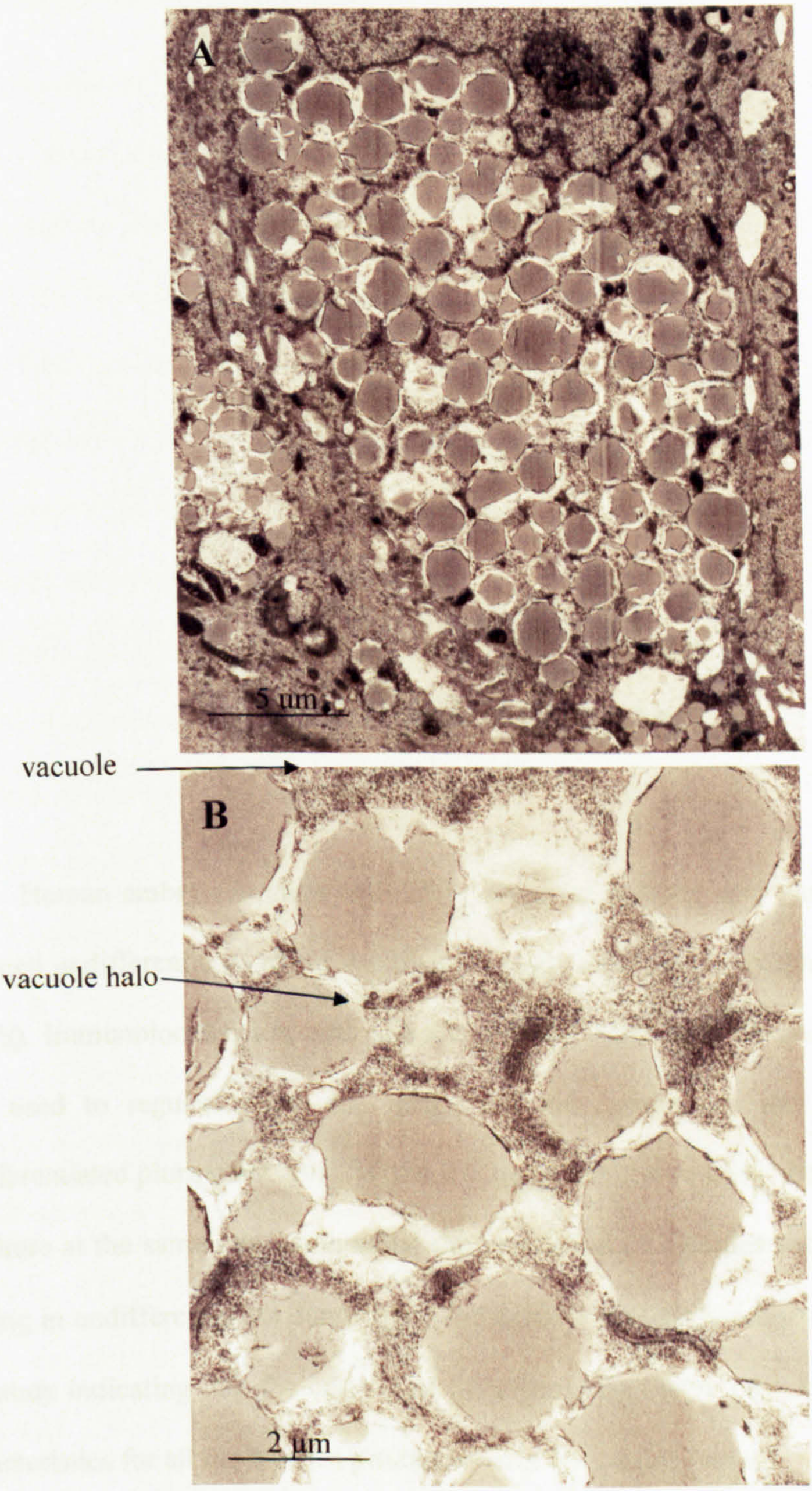


Figure 13 (A) Ultrastucture of cell of EB showing many secretory granules. (B) higher power of granules showing halo and contents.

IV. DISCUSSION

Human embryonic stem cells (H7) could be cultured and passaged so they remained undifferentiated using inactivated mouse embryonic fibroblast feeder cells (MEFs). Immunolocalisation with specific markers (Tra-1-60, SSEA4 and SSEA1) were used to regular assess the quality of the cultures so that they retained undifferentiated pluripotent cells. While it is quite difficult to keep hES cells growing in culture at the same rate continually, the proportion of colonies showing Tra-1-60 staining in undifferentiation conditions (about 70%) was fairly consistent throughout this study indicating that the cells used for experiments were probably of the same characteristics for all the batches produced (over 15 months period).

It was decided at the start of the project to only use one cell line to reduce variation in the starting cells. Although two subclones were used, there was no apparent difference. However, hES cell lines do show some variation in their characteristics as demonstrated by the comparing of lines (Adewuni *et al*, 2007).

1. Insulin Producing Cells

Edlund, in 2001, showed the importance of different growth factors at different stages of pancreatic development and especially those of β-cell function. She showed the role of FGF/s in the development of the ventral part of the pancreas (Edlund, 2001). Additionally, nicotinamide has been used as a growth factor to treat human foetal pancreas to increase the number of endocrine cells and consequently the insulin content (Hori *et al*, 2002).

Mouse ES cells were directed to differentiate into insulin producing cells by inhibiting the phosphoinositide 3-kinase, which is a member of a kinase enzyme superfamily involved in major signalling events in the cell including hES cells and that also mediates the effects of insulin on glucose and lipid metabolism. The cells produced accumulated in a way similar to that which occurs in pancreatic islets producing insulin when transplanted into diabetic mice (Shepherd *et al*, 1998; Fry, 2001; Hori *et al*, 2002).

In the present study, positive immunostaining was obtained for nestin and glucagon indicating the potential presence of progenitor immature insulin containing cells that differentiated from hES cells and with longer incubation period these cells in culture might have expressed mature insulin. The method used to direct the differentiation of human ES into insulin containing cells using different growth factors showed positive immunolocalisation, indicating the presence of some insulin containing cells in culture even although the amounts were relatively low in most cases.

Immunostaining cells for insulin have been shown to be a problem and subject to artefact because culture medium contains insulin which might be absorbed by cells. Rajagopal and coworkers (2003) reported that insulin expression in cells

derived from hES cells was due to insulin uptake by the cells rather than new synthesis (Rajagopal *et al*, 2003). Therefore, several methods need to be used to establish that true insulin production occurs. C-peptide localisation is particularly useful as this peptide is not part of the mature insulin molecule and therefore should discriminate between cells absorbing insulin from the culture and cells producing C-peptide as part of the pro-insulin molecule. Cells in some EBs were positive for C-peptide indicating probable insulin synthesis. Interesting, glucagon immunolocalisation was also obtained in some EBs suggesting α-cell development as well.

The different growth factors used in this study to direct the differentiation of hES cells towards insulin containing cells are listed as follows: nicotinamide, nicotinamide + FGF2, nicotinamide + activin A, nicotinamide + activin A + FGF2, nicotinamide + activin A + FGF4, activin A + FGF2, activin A, nicotinamide + FGF4, nicotinamide + FGF2 + FGF4, activin A + FGF4, activin A + FGF2 + FGF4, FGF4, FGF2, FGF2 + FGF4 and nicotinamide + activin A + FGF2 + FGF4. These growth factors were selected based on their importance in the process of the development of islets of the pancreas.

Experiments were repeated several times for each growth factor to check consistency, reproducibility and reliability of the results obtained from them. Some growth factors and their combinations have been avoided due to the negative results, by immunostaining or DTZ staining, they showed when they applied on the EBs samples.

From this study, it was noticed that some of the growth factors slowed the aggregation of cells to form EBs. These were nicotinamide + FGF4, nicotinamide + FGF2 + FGF4, activin A + FGF4, activin A + FGF2 + FGF4, FGF4, nicotinamide +

activin A + FGF2, nicotinamide + activin A + FGF4 and nicotinamide + activin A + FGF2 + FGF4. Therefore, FGFs may effect the rate of developmental process. However, there was always some cell death after hES transfer to non-treated Petri dishes in which cells were first single. If cells had not aggregated within a few days then they progressed to cell death. FGF2, FGF2 + FGF4 and FGF4 may have inhibited aggregation of cells leading to apoptosis. On the other hand, most of the positive results for putative insulin producing cells were obtained from the following growth factor combinations: nicotinamide, nicotinamide + FGF2, nicotinamide + activin A and activin A + FGF2. This suggests that nicotinamide with activin A had a positive influence.

2. EBs Cells Stained for Dithizone

As mentioned insulin immunolocalisation can give artefactual results due to insulin absorption from culture medium. Several studies showed that DTZ is a good tool to use for monitoring and identifying viable transplanted pancreatic islets. They showed the reliability of DTZ and that it fades quickly leaving islets cells undamaged (Jiao *et al*, 1991; Fiedor *et al*, 1995; Fiedor *et al*, 1996). Another group in China used DTZ to stain viable islets cells without damaging them (Jiang *et al*, 2002). Clark and his colleagues showed that prolonged exposure of islets to DTZ reduced insulin secretion and produced cell death (Clark *et al*, 1994). In a study in 1994, three different concentrations of DTZ, 2, 10 and 100μg/ml, were used on human and rat islets to evaluate the effect of DTZ on the islet secretion. However, the lowest concentration of DTZ, 2μg/ml, and the shortest period of incubation of the cells with DTZ were the best at maintaining the function of pancreatic islets (Conget *et al*, 1994).

Thus, because of the specificity and reliability of DTZ in staining islet cells, it was used in the present study to identify putative insulin-containing cells from EBs grown in culture from human ES cells. The different percentages of cells stained red in human EBs colonies were determined for all cells cultured with all growth factors and their combinations. Staining was quite clear after 2 hours although longer staining (6 hours) tended to kill the cells. Although liver and intestinal cells contain detectable levels of zinc, when assessed with immunostaining then the most likely phenotype is an insulin secreting β-cell type. In fact some outgrowths from EBs at 14 days of culture showed very similar morphology to an early foetal islet cluster with a group of DTZ stained cells surrounded by epithelial-like cells (see Figure 10H).

3. Electron Microscopy

Although most cells of EBs did not show β-cell morphology they were polarised and displayed vacuoles and tight junction of an epithelial morphology (Figures 12 and 13). Interestingly, some cells displayed secretory granules with a distinct halo which is a characteristic of pro-insulin secretory granules of β-cells. However, these secretory granules did not show the dark centre of granules of adult tissue and therefore could possibly be confused with lipid vesicles although these tend not to be granular in appearance. To verify that the cells were indeed pro-insulin secretory granules it would be necessary to carry out immunogold labelling.

In summary the differentiation of human ES cells was directed into insulin-containing cells, with β-cell-like phenotype based on immunolocalisation and DTZ staining. The number of these cells in an EB colony varied with different growth factors and their combinations. Some growth factors such as nicotinamide and

activin A encouraged the cultured cells to become more β-cell -like when compared to cells grown in other growth factors.

CHAPTER THREE

Measurement of C-Pepptide Secretion *In Vitro* from ES-Derived Cell and Foetal Pancreatic Cells

I. INTRODUCTION

Human pancreas development starts as two separate buds with the dorsal pancreatic bud appearing first, at about day 26 of gestation, as an evagination of about 300 cells in the posterior foregut. This is followed by the formation of the ventral pancreatic bud one day later on the opposite side to the dorsal pancreatic bud (www.ana.ed.ac.uk). The pancreas organ then forms at about six week of gestation when both buds fuse together. The ventral bud form the head of the adult pancreas while, the dorsal bud form all other parts of the pancreas, the neck, body and tail (www.ana.ed.ac.uk).

Previous studies have shown that hormone containing cells appear at about 8 week of gestation in the epithelial of ductal cells of the pancreas (Polak *et al*, 2000). At week 10 gestation α-cells start to differentiate followed by δ-cell which developed at about 11 weeks of gestation. β-cells were shown to be present at about 13 weeks gestation (www.ana.ed.ac.uk). At the end of week 17 β-cells start to secrete insulin

(www.ana.ed.ac.uk). Each type of cells initiated at very low number of cells (www.ana.ed.ac.uk).

In the human embryonic pancreas, glucagon producing cells, α-cells, are the first endocrine cells identified by their hormonal content after eight weeks gestation. Insulin-producing β-cells are detected in the ninth and tenth week of gestation by detecting either insulin or C-peptide (Peters *et al*, 2000). Both human insulin and C-peptide originate in the pancreatic β-cells as a single polypeptide chain known as pro-insulin with a molecular weight of 9000 (Ma *et al*, 2004). Proinsulin is cleaved proteolytically into mature insulin and C-peptide when released into the blood (Ma *et al*, 2004). Because C-peptide has the ability to persist in the plasma longer than insulin and has a longer half-life, it can be a more reliable indicator to reflect the secretion of pancreatic insulin (Ma *et al*, 2004; Risérus *et al*, 2004).

Enzyme-linked immunosorbent assay, ELISA, is a widely used method that allows rapid measurement of the concentrations of soluble components, mainly proteins, in plasma (Lindström *et al*, 2002; Sjöstrand *et al*, 2002; Risérus *et al*, 2004; Liu *et al*, 2005) and has been applied extensively for measurements of insulin levels in plasma.

A new strategy to generate progenitor pancreatic cells and early pancreatic endocrine cells without the need for any selective steps of identifying nestin or cytokeratin 19 expressing cells was created by Blyszczuk and coworkers (2004). The amount of insulin that was secreted from the differentiated cells *in vitro*, was determined by an insulin ELISA assay (Blyszczuk *et al*, 2004). A group in Japan succeeded in lowering the blood glucose in diabetic mice by implantation of primary cultured adipocytes, that secrete insulin. The plasma insulin concentration was dependent on the implanted cell number and the secretion of insulin and C-peptide from adipocytes was measured by an ELISA (Ito *et al*, 2005).

In addition, studies on plasma C-peptide collected from patients with type II diabetes mellitus, cord blood from newborn, from healthy human islets and from insulinoma patients have also been analysed by ELISA assay (Paoletta *et al*, 2002). A prospective study of plasma C-peptide and colorectal cancer in men was carried out in 2004 (Ma *et al*, 2004). The concentrations of plasma C-peptide, as a stable indicator of pancreatic β-cell function, were measured by ELISA (Ma *et al*, 2004).

Another clinical research study used different methods to measure different human proteins including leptin, insulin and C-peptide. The purpose of the study was to compare between different assays using human plasma (Liu *et al*, 2005).

C-peptide measurements are also useful for insulin therapy, especially for type II diabetes, monitoring the progress of pancreas or islet cell transplantation, insulinoma diagnosis and as a marker for residual pancreatic tissue after pancreatectomy (Ma *et al*, 2004; Rudovich *et al*, 2004; Ito *et al*, 2005; Liu *et al*, 2005).

The aim of this chapter is indirectly assess the amount of insulin secreted from human EBs into culture medium following various differentiation procedures using a C-peptide ELISA assay. The levels of C-peptide were compared to those produced by foetal pancreatic tissue collected from first trimester foetal samples (Table 1) and cultured *in vitro* and correlated with stages of pancreatic development as assessed by immunohistochemistry.

Table 1 A table indicating the number of foetal samples used at each gestational age for *in vitro* culture and histochemistry.

Age/week	Number of foetal samples for *in vitro* culture	Number of foetal samples for histochemistry
8	1	3
9	2	0
10	1	4
11	2	0
12	4	4

II. MATERIALS & METHODS

1. Materials

1.1 Human Foetal Sample Collection

Foetuses (first trimester) were obtained with full ethical approval (South Sheffield Research Ethics committee, Professor Harry Moore as person responsible). Tissue was obtained with informed patient consent after counselling by a research nurse independent from the research group and following the Polkinghorne guidelines. This was normally undertaken at the patient's first visit to clinic. Women underwent medical termination with Mefepristone and prostaglandin.

Following termination, foetal tissue were put into cold DMEM medium and transferred to the laboratory within 1 hour. Foetal age was determined by the last menstrual bleeding and subsequently confirmed by the gross anatomy of forearm digits

(Moore *et al,* 2000). Foetuses were obtained at different ages of development from 8 -12 weeks of gestation. In total 21 foetal samples were obtained for this study.

The foetal pancreas was immediately recovered by stereoscopic microscope dissection in a Class II laminar flow hood using gauge 19 needles attached to 1ml syringes. The foetus (usually intact) was removed from extra-embryonic membranes and placed in fresh cold medium in a Petri dish. The peritoneum was slit open and the contents removed by sliding a needle along the backbone and then carefully removing the entire internal alimentary canal and other associated organs including the liver. The pancreas was identified close to the liver by its distinctive lobular and granular appearance and removed intact. It was either prepared for culture *in vitro*, snapped frozen in liquid nitrogen and stored at $-70^{\circ}C$, or fixed for 1 hour in 4% paraformaldehyde for later processing for paraffin sections (3 x 8 weeks, 4 x 10 week and 4 x 12 weeks).

1.2 Culture of Foetal Pancreas

Using forceps and needle, the isolated pancreas was cut into small pieces under the dissecting microscope. The small pieces of pancreas were then washed with PBS without Ca^{+2} and Mg^{+2} and treated with 2 ml of 1mg/ml collagenase IV solution for 8-10 minutes at 37°C in 5% CO_2 in air. Tissue was also dispersed by scraping with 3mm glass beads, centrifuged at 200 g for 3 minutes, the supernatant removed and the pellet dissociated by moving them through the pipette up and down. Fresh RPMI 1640 media mixed with 10% FCS was added to the pellet and the cell suspension transferred to wells of 6-well culture plates and incubated at 37°C in 5% CO_2 in air.

Medium was changed every two days. Conditioned medium (0.5ml) was collected from different samples on various days to measure the amount of C-peptide

secreted (Table 2). Days of collecting media differed according to the availability of each sample. The collected media were stored at -80°C.

1.3 Culture of EBs

Conditioned media from cultured EBs, with different growth factors were collected after 7 and 14 days (see chapter II) post passage. Conditioned media from hES cells, growing on MEFs, were collected 2 - 3 days post passage (Table 2). The collected media were stored at –80°C.

1.4 Preparation of Washing Buffer

From the concentrated (10X) washing buffer (1X) dilution was prepared by mixing 100ml of (10X) concentrated washing buffer with 900 ml distilled water. The solution was then stored in the refrigerator.

2. ELISA Kit Reagents

The human C-Peptide ELISA (Linco 20K, Missouri, USA) displayed specificity for human C-Peptide and intact human proinsulin, but not human insulin, and was used to measure the C-peptide from the medium collected from different samples such as EBs grown with different growth factors, hES cells and human foetal pancreatic tissue at different ages in weeks (8w, 9w, 10w, 11w and 12w) as mentioned in tables 1 and 2. The ELISA kit contained the following reagents: a microtitre plate, adhesive plate sealer, 10X washing buffer concentrate of 50mM TBS buffer containing 0.05% Tween 20, human C-peptide standards (0.2, 0.5, 1, 2, 5, 10, and 20ng/ml), ELISA C- peptide quality controls 1 and 2, human C-peptide matrix solution, assay buffer of 0.05M PBS containing 0.025M EDTA, 1% BSA with 0.08% sodium azide (pH 7.4), a detection

Table 2 Foetal tissue samples of different ages were used to determine the amount of C-peptide secreted from them by ELISA method. Culture medium was collected on different days after the beginning of culture. Days of collecting media differed according to the availability and conditions.

Sample	Age/week	Days of collection after beginning culture
1	8	4, 8, 15
2	9	4, 8, 15, 25, 32, 34, 37
3	9	7
4	10	7, 9, 11, 14, 19, 25, 46
5	11	3, 5, 8, 12
6	11	3
7	12	3, 5, 9
8	12	4, 8
9	12	3
10	12	3, 8, 15

Table 3 The treatment of EBs and hES samples, used to measure the amount of C-peptide by the human C-peptide ELISA method.

Sample	EBs	EBs	EBs	EBs	hES
Growth Factor/s	Nicotinamide	Nicotinamide + FGF2	Nicotinamide + Activin A	Activin A + FGF2	-
Days Collecting media	7 and 14	7 and 14	7 and 14	7 and 14	2-3
Times Repeated	6	6	6	6	2

antibody of biotinylated anti-human C-peptide monoclonal antibody, enzyme solution of pre-titred streptavidin-horseradish peroxidase conjugate (SA-HRP), 3, 3`, 5, 5`-tetramethylbenzidine (TMB)and stop solution of 0.3M HCL.

3. Methods

3.1 Quantification Analysis of Human C-Peptide Using the ELISA Assay

3.1.1 Assay Procedure

Human C-peptide ELISA was applied to the media collected from growing EBs with different growth factors, hES cells and pancreatic tissue from human foetuses with different ages of 8, 9, 10, 11 and 12 weeks, to determine the concentration of C-peptide secreted by them into the media.

All kit reagents and medium samples were pre-warmed to room temperature. The microtitre assay plate was washed three times with 300µl 1X washing buffer. 20µl detection antibody was added to all wells followed by addition of 10µl assay buffer to the blank wells only. 10µl from each standard, 0.2, 0.5, 1, 2, 5, 10 and 20ng/ml, and 10µl from each experimental kit control, QC1 and QC2, were then added into wells in duplicate. DMEM/FCS medium, 10µl, was used in duplicate as a fresh control. Media samples, 10µl, were added to the rest of washed wells in triplicates. Serum matrix and assay buffer, 10µl and 40µl respectively, were added to the wells containing the blank, standards and experimental kit controls, while 50µl assay buffer was added to the wells containing the media samples. Moreover, the microtitre plate was then sealed and incubated at room temperature, 2- 5°C, while shaking on a microtitre plate shaker for about 2 hours.

Wells were washed five times with 300µl 1X washing buffer. 80µl enzyme solution was then added to all wells and the microtitre plate was incubated at room temperature shaking on the microtitre shaking plate for 30 minutes. The addition of 80µl substrate solution into all wells then took place after 5 washing steps with 300µl 1X washing buffer. Another incubation step for the microtitre plate at room temperature was carried out for 12-19 minutes. The reaction was then stopped by 80µl stopping solution followed by a gentle hand shake for the microtitre plate to mix the solution in each well. Finally, the reading was measured by spectrophotometer (DU 650, California, USA) at an absorbance of 450 nm.

The experiment was repeated six times in triplicate for each EB sample growing with different growth factors.

4. ELISA Assay Readings

The assay was repeated six times for most of the samples to show the consistency and reliability of the results that were obtained from the assay. The mean for each repeated sample was determined by adding the readings obtained for each sample divided by the total number of readings for the specific sample. The following formula was used:

$$\text{Mean for sample 1} = \frac{A + B + C + D + E + F}{N}$$ where,

A, B, C, D, E and F = readings obtained for the same sample at different times.

N = the total number of readings for a specific sample.

5. Immunohistochemistry

The pancreas was prepared from three foetuses at stages 8-9 week, 10-11 week and 12 week gestation. Tissue was fixed in 4% paraformaldehyde overnight and then dehydrated and infiltrated and embedded with paraffin before 5–10μm sections were cut and mounted on microscope slides (Pathology Department, Medical School). The sections were deparaffinized and dehydrated, blocked for endogenous peroxide activity and antigen enhanced by microwave treatment before immunolocalisation.

Slides were gently rinsed with PBS and incubated with four drops of normal rabbit serum (diluted 1:30) for 30 minutes at 37°C. The serum was blotted off and 50μl of primary antibody diluted (1:500) was used to cover the section which was incubated for 1 hour at 37°C. The slide was rinsed 3 times with PBS and then 50μl of enzyme-conjugated secondary antibody (horseradish peroxidase and alkaline phosphatase) applied to cover the section which was incubated for a further hour. The slide was then rinsed extensively with PBS before incubation with appropriate substrate/chromogen (diaminobenzidene, Naphthol-AS-B1-phosphate/fast red TR) solution for immunostaining. The slide was rinsed gently with distilled water, counterstained with haemotoxylin and eosin solution and mounted with coverslip. The primary antibodies used are shown in table 4.

Secondary horseradish peroxidase and alkaline phosphatase antibodies were all from Vector Laboratories. Control was with no primary antibody.

Table 4 The primary antibodies used for immunohistochemistry of foetuses samples.

Primary Antibodies	Raised in	Supplier
Polyclonal human anti-insulin	Rabbit	Zymed, USA
monoclonal human anti-glucagon	Mouse	Sigma Ltd
monoclonal human anti-CK19	Mouse	Novocastra Ltd
monoclonal human anti- C-peptide	Mouse	Abcam Ltd

III. RESULTS

1. Culture of Foetal Pancreas

The length of the foetal pancreas ranges from 1-2mm (8 - 9 weeks), 2-3mm (10 - 11 weeks) and 4-5mm (12 weeks). For comparison, the crown-rump of the 10 weeks foetus was about 25mm.

Ducts of the pancreas could be clearly seen by 8 weeks gestation (Figure 1A). The developing organ was surrounded by a membrane but collagenase digestion for about 10 minutes made it easier to dissect out pieces of ductal and stromal tissue for incubation (Figure 1B). Pancreas from 10–12 weeks had more membrane and required longer collagenase digestion. These pieces of tissue were placed in wells of six-well plates and rapidly adhered (within 10 minutes) to bottom of the well. In a few hours the ductal pieces rounded up (Figure 1C). When stained with DTZ within 24 hours of dissection, only a few cells stained red for tissue from 8 weeks gestation.

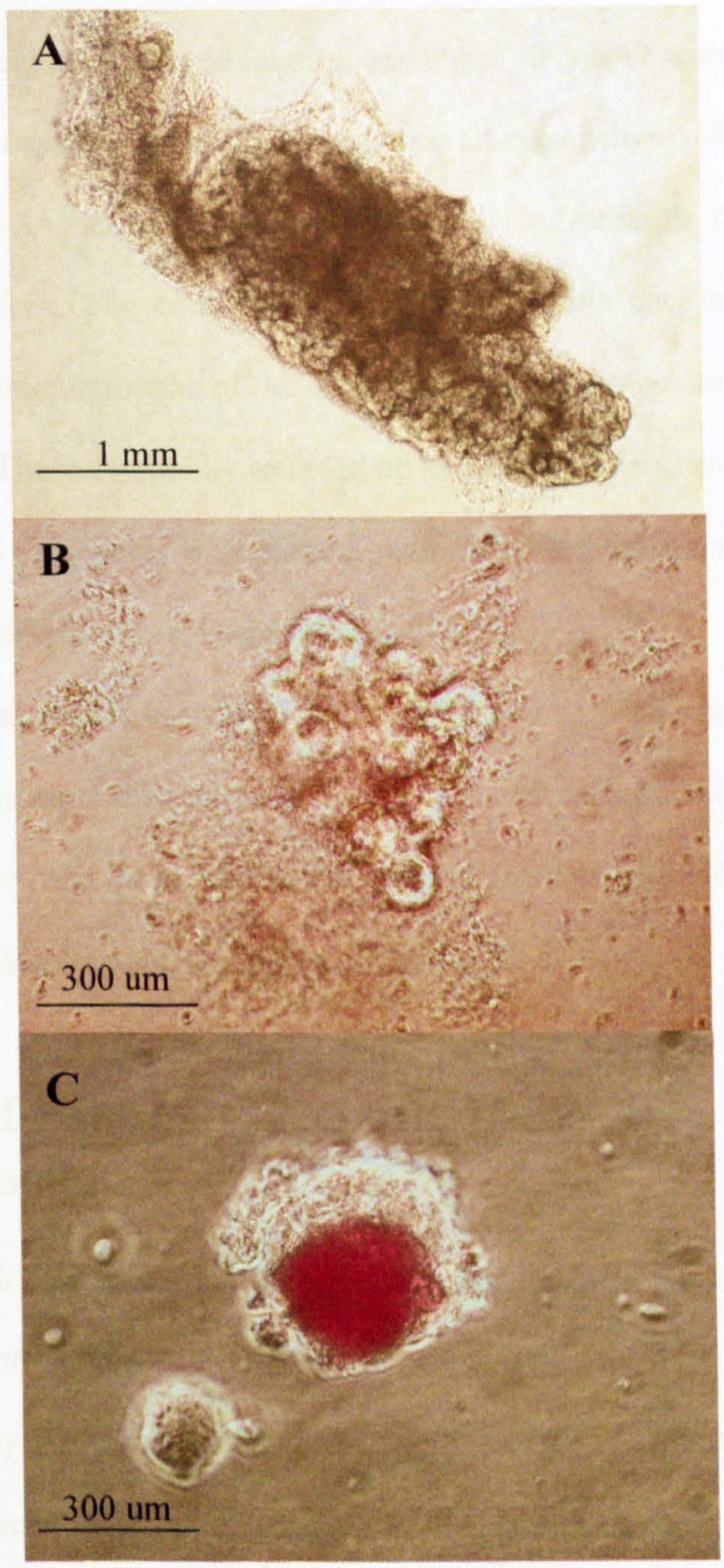


Figure 1 (A) Pancreatic tissue (8 weeks) after collagenase treatment showing ductal morphology. (B) A piece of tissue after needle dissection in culture. (C) Tissue after 24 hours in culture has rounded up. At 10 or 12 weeks some aggregates show intense DTZ staining. N=3.

However, some aggregates displayed intense staining at 10 and 12 weeks (Figure 1C).

Within a few days there was rapid outgrowth of mesenchymal-like cells from the tissue pieces (Figure 2A) and confluence of these cells surrounding the aggregate was achieved within a week. The cells displayed a characteristic cobblestone appearance (Figure 2B). Epithelial outgrowths of the ductal aggregates also occurred after few days (Figure 2A, arrow). These eventually rounded up into characteristic morphology (Figure 2B, arrows). If these aggregates were stained with DTZ then red staining was observed inside the epithelial outgrowths (Figures 2B and 2C).

While the flattened mesenchymal-like continued to proliferate throughout the culture period to was more difficult to ascertain if ductal cells proliferated. By the end of two weeks there was little indication of proliferation, therefore cultures were terminated for DTZ staining and C-peptide analysis.

2. Analysis of Human C-Peptide Using ELISA Assay

The quantities of C-peptide secreted into the medium from growing the EBs with different growth factors and foetal tissues were determined by using a human C-peptide ELISA assay. The Standard curve is shown in Figure 3, where the lower limit to be detected by the assay is 0.1ng/ml.

C-peptide readings obtained from the cultured EBs grown with different growth factors, after 14 days incubation, hES cells and the human foetal tissues at different gestational ages from 8 weeks to 12 weeks were shown in tables 5 and 6 respectively. Culture medium was recovered from foetal pancreas tissue cultures between 3 and 46 days of incubation depending upon the sample. In addition, means

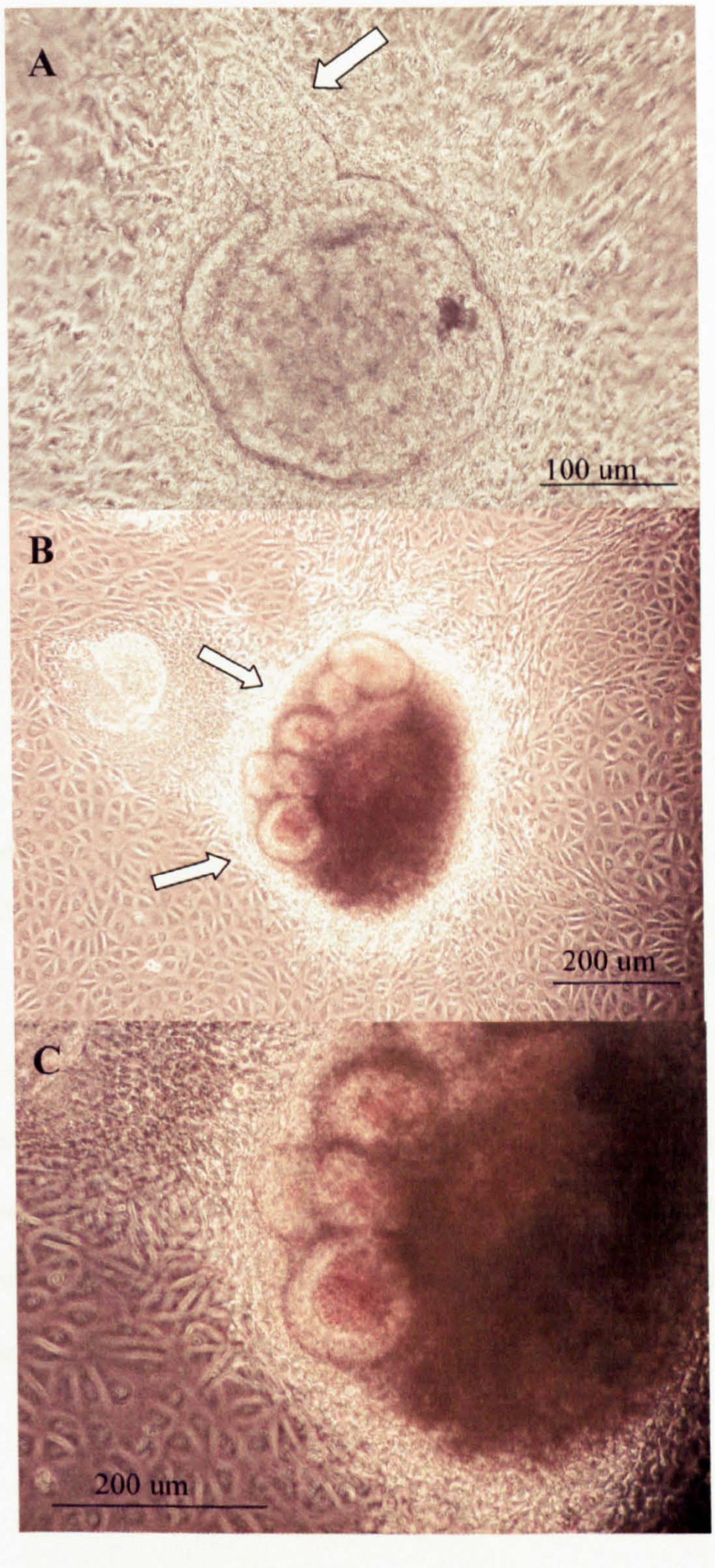


Figure 2 (A) Pancreas aggregate (10 week foetus) after 5 days in culture showing mesenchymal cells and ductal outgrowth (arrow). (B) At 10 days ductal outgrowth round up and stain red with DTZ. (C) higher magnification of (B). N=3.

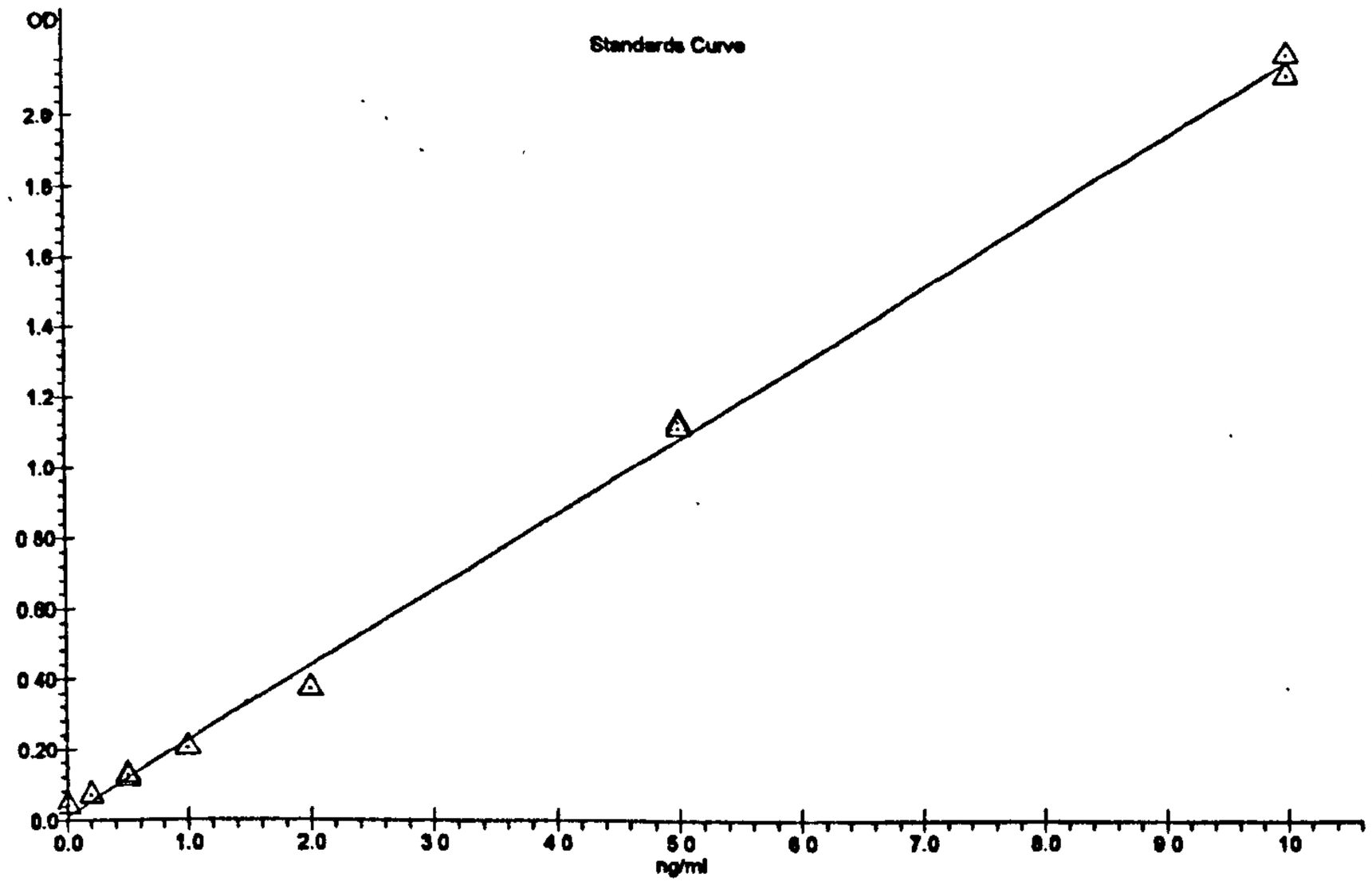


Figure 3 A graph showing the human C-peptide standards curve as given by 4 parameter logistic (4-PL) curve-fit software in the PC connected to the plate reader. Y axis = Optical Density, while X axis = the C-peptide concentrations (ng/ml). Proprietary software with the reader analysed the data and created the curve.

Table 5 The readings (ng/ml), means and standards deviations obtained from the C-peptide ELISA for cultured EBs with different growth factors: nicotinamide, nicotinamide + FGF2, nicotinamide + activin A, Activin A + FGF2, control sample and hES cells sample.

Sample	Reading 1	Reading 2	Reading 3	Reading 4	Reading 5	Reading 6	Mean ± SD
Control	0.047	0.042	0.046	0.049	0.044	0.042	0.045 ± 0.003
Nicotinamide	0.045	0.047	0.045	0.05	0.046	0.043	0.046 ± 0.002
Nicotinamide + FGF2	0.05	0.046	0.049	0.048	0.049	0.063	0.051 ± 0.006
Nicotinamide + Activin A	0.048	0.045	0.049	0.047	0.049	0.051	0.048 ± 0.002
Activin A + FGF2	0.046	0.048	0.044	0.049	0.057	0.055	0.05 ± 0.005
hES	0.052	0.049					0.051 ± 0.002

Table 6 The readings obtained from the human C-Peptide ELISA for foetal tissue samples of different ages (8, 9, 10, 11 and 12w), w = weeks and d = days.

Sample	Conc. (ng/ml)	Amount of C-Peptide Secreted in culture (ng/ml/hr)
Foetal 1 (8)w (4d)	0.053	Not detected
(8d)	0.055	Not detected
Foetal 2 (9)w (4d)	0.062	Not detected
(8d)	0.058	Not detected
(15d)	0.064	Not detected
Foetal 3 (9w) (7d)	0.08	Not detected
Foetal 4 (10)w (7d)	0.104	0.001
(9d)	0.054	Not detected
(11d)	0.083	Not detected
(14d)	0.081	Not detected
(19d)	0.051	Not detected
(25d)	0.055	Not detected
(46d)	0.051	Not detected
Foetal 5 (11)w (3d)	1.975	0.03
(5d)	2.189	0.02
(8d)	1.801	0.01
(12d)	2.355	0.008
Foetal 6 (11)w (5d)	0.63	0.005
Foetal 7 (12)w (3d)	0.245	0.003
(5d)	0.278	0.002
(8d)	0.047	Not detected
Foetal 8 (12)w (4d)	0.067	Not detected
(8d)	1.586	0.008
Foetal 9 (12)w (5d)	1.39	0.012

Foetal 10 (12)w (3d)	1.214	0.02
(8d)	1.901	0.01
(15d)	1.75	0.005

Continuation from the previous page of the readings obtained from the human C-peptide ELISA for foetal tissues samples of different ages.

and standards deviation of cultured EBs with different growth factors were also shown in table 5. Whereas C-peptide could not be detected readings obtained in EBs samples in culture for 7 days with different growth factor combinations.

The assay was repeated six times for each EB sample to check the reproducibility and reliability of the results obtained from the assay, as shown in table 3. In four experiments, using nicotinamide alone, when culture medium and cells were taken together from EBs samples at 14 days of culture and freeze-thawed twice to release intracellular content, 8-10 EB colonies, the concentration of C-peptide was 0.26 ± 0.21ng/ml (Table 7).

For foetal samples before week 11 C-peptide was absent from culture supernatant except for one medium sample from a week 10 tissue culture. However most samples of medium from week 11 and 12 tissue cultures showed positive levels of C-peptide.

3. Immunohistochemistry of Foetal Pancreas

By week eight of gestation pancreas displayed ductal epithelium surrounded by extensive stromal tissue and ductal cells exhibited clear cytokeratin 19 expression (Figure 4A and B). The amount of staining in individual ductal cells was variable at week 8 (Figure 4B), but the consistency and intensity increased by 10 (Figure 4C) and 12 weeks.

In two samples of pancreas at eight week gestation, insulin/C-peptide was either absent (Figure 5A), or only present in a few cells mainly of ductal origin rather than in the stroma (Figure 5B). It is possible that the pancreas showing no expression was from a growth retarded individual as crown-rump measurement was only 19mm

Table 7 The readings (ng/ml), means and standards deviations obtained from the C-peptide ELISA for 8-10 cultured EB colonies with nicotinamide only.

Sample	Reading 1	Reading 2	Reading 3	Reading 4	Mean ± SD
1	0.06	0.06	0.07	0.06	0.06 ± 0.005
2	0.14	0.14	0.14	0.14	0.14 ± 0.0
3	0.33	0.32	0.32	0.31	0.32 ± 0.008
4	0.53	0.53	0.53	0.53	0.53 ± 0.0

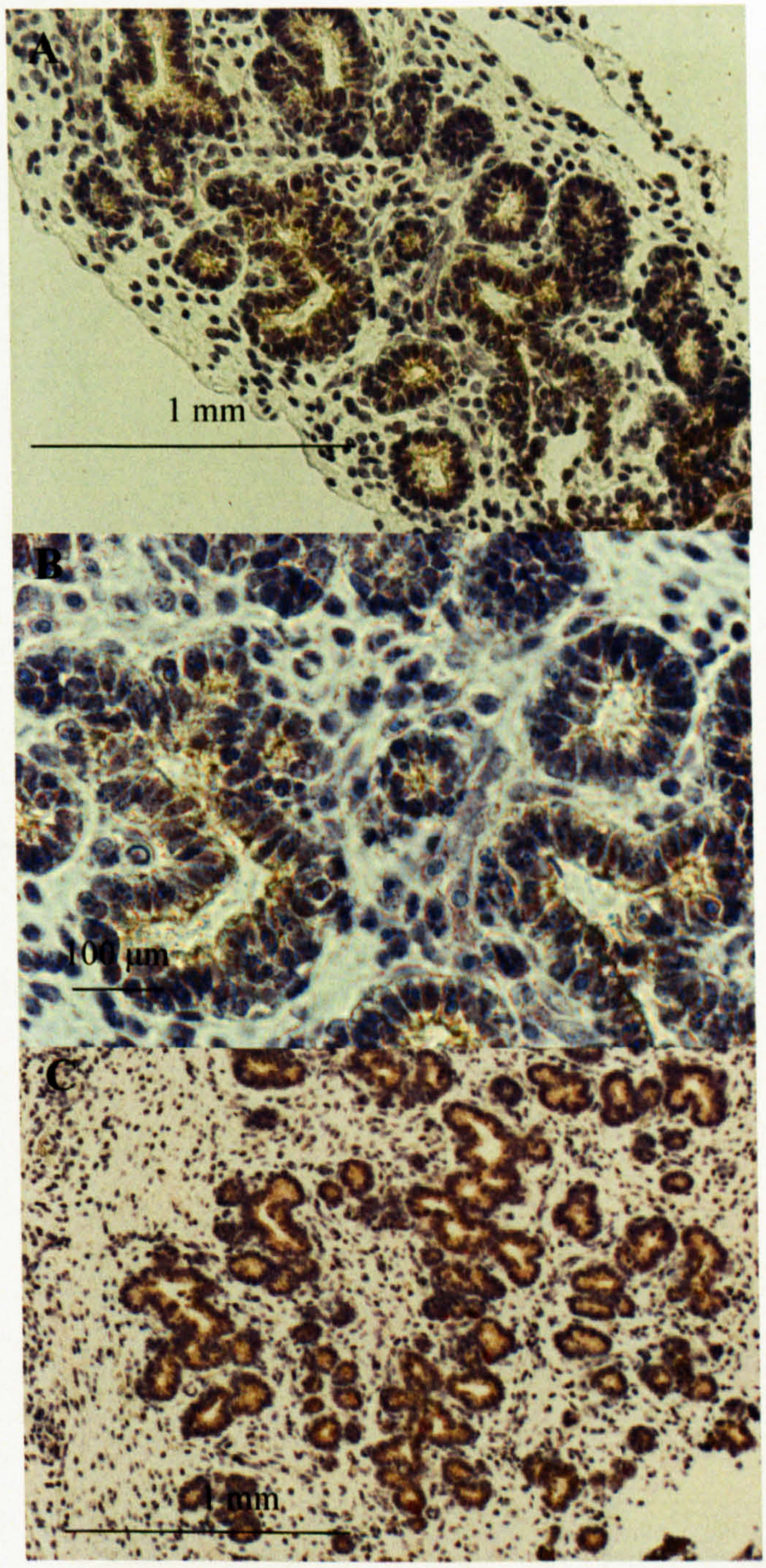


Figure 4 Cytokeratin 19 immunolocalisation in week 8 and 10 pancreas. (A) week eight pancreas showing ductal epithelium and surrounding stromal cells. CK19 staining is clearly detected but variable in ductal cells. (B) Higher magnification. (C) Week 10 pancreas shows increased intensity of CK19 staining and less variability. Brown stain was diaminobenzidine with haemotoxylin counterstain Controls showed no staining. N=3 and 4 respectively.

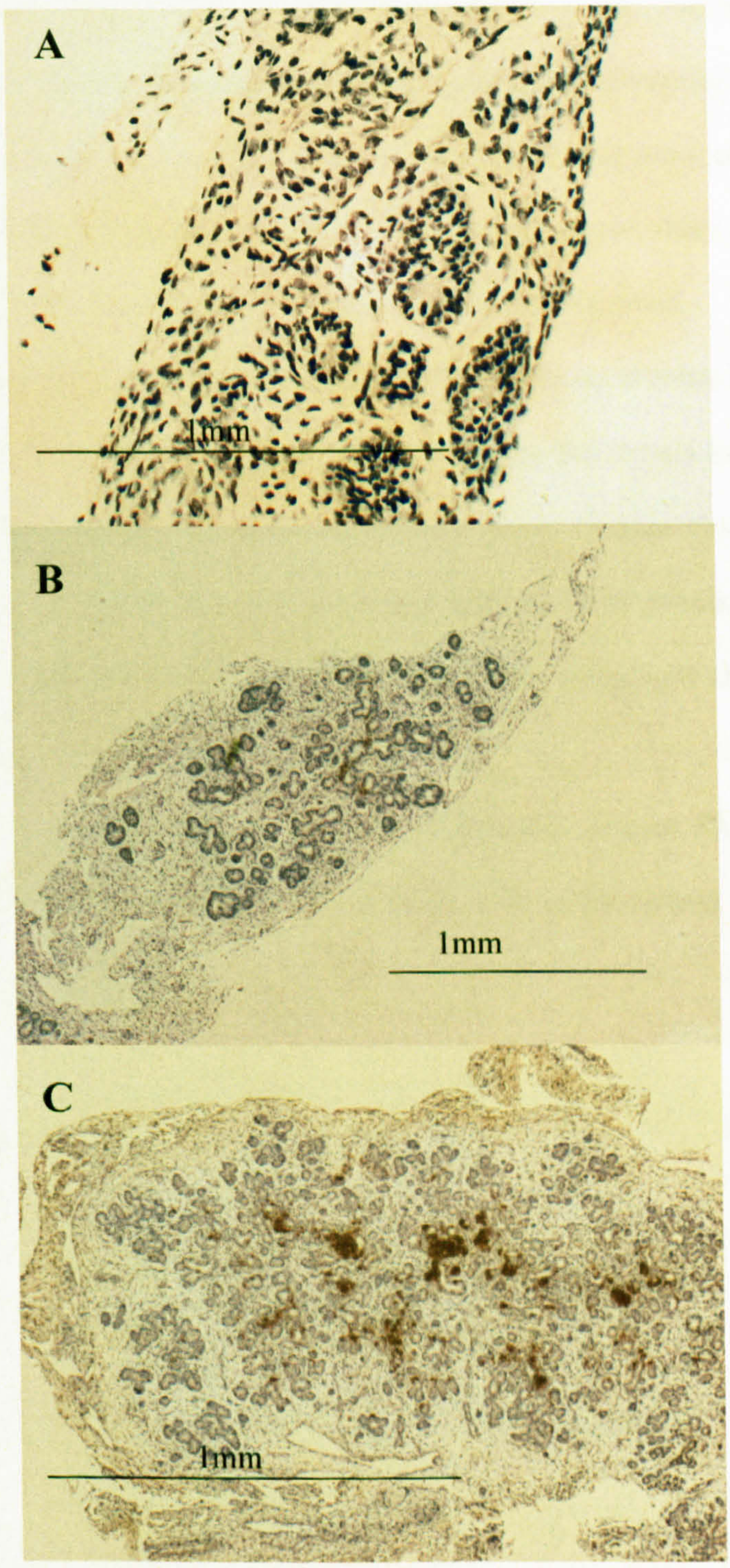


Figure 5 (A) A Pancreas section from most immature week 8 foetus. No insulin localisation. (B) Week 8 pancreas showing a small amount of insulin immunolocalisation. (C) Week 10 pancreas displaying insulin immunolocalisation. Controls showed no staining.

which was smaller than normal (usually 25-30mm) and gross morphology of the pancreas was immature. An apparently retarded foetus could be mis-classified as the length of pregnancy is determined from the last menstrual period and this method is not always accurate especially if menstrual cycles are irregular. However, there could also be other abnormalities of the foetus or placenta leading to retarded growth.

By 10 weeks insulin expression was greater and all samples displayed some expression (Figure 6A). There was indication of positive cells moving into the stroma as shown by insulin/CK19 co-immunolocalisation (Figure 6B and C). Week 12 pancreas expressed more extensive staining for insulin and there were clear indications of morphogenesis of islets of Langerhans (Figure 7). Individual cells in the ductal epithelium also showed intense insulin staining.

Glucagon expression was detected in week 10 pancreas (Figure 8A) in ductal epithelial cells. This increased by week 12 when some cells in the stroma in putative islets were observed (Figure 8B).

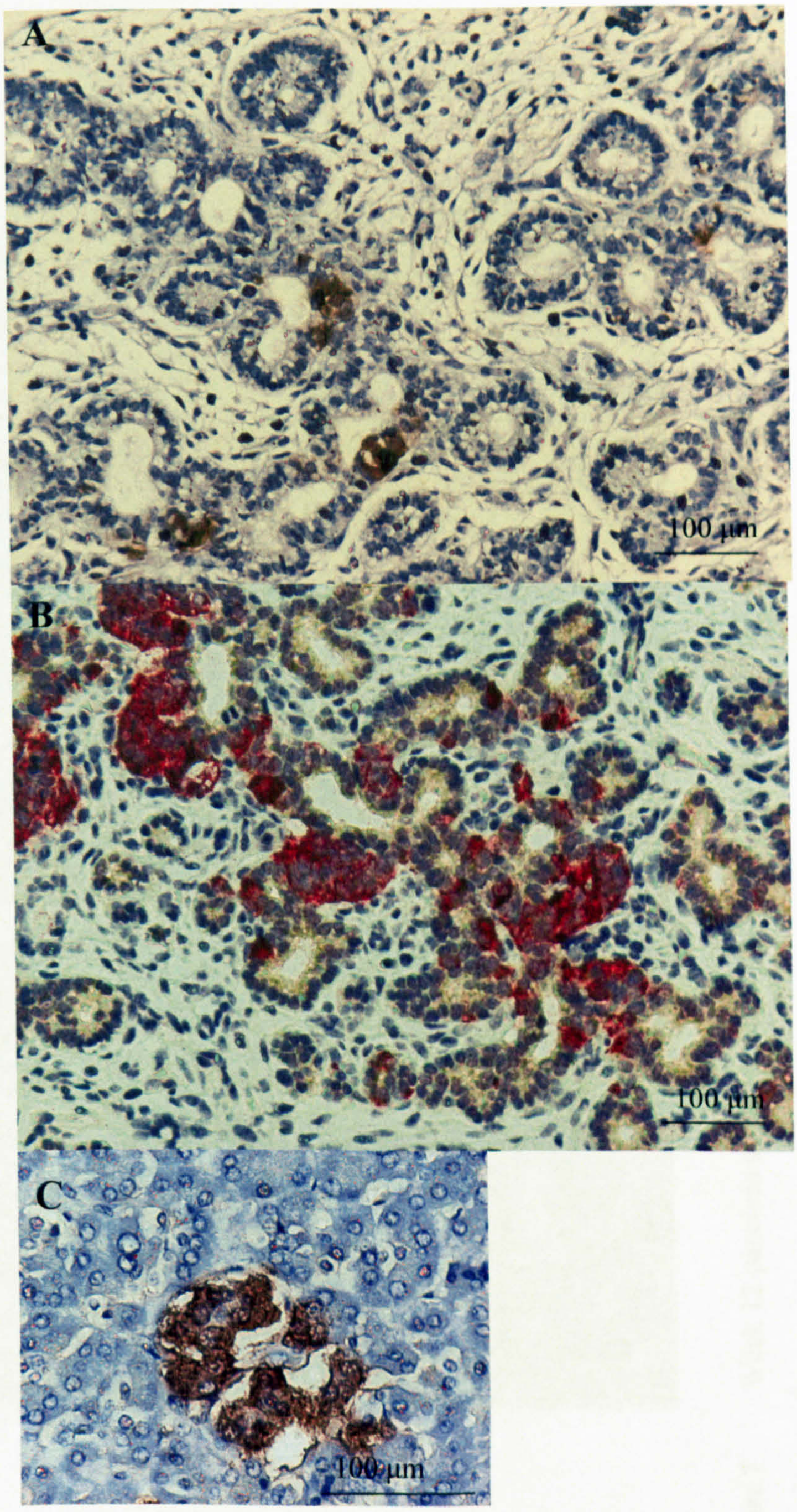


Figure 6 Higher magnification of 10 weeks pancreas insulin immunolocalisation. (A) insulin expression is mainly confined to ductal cells mainly. (B) co-localisation of CK19 (brown) and C-peptide (red-brown Naphol texas red). (c) the beginning of islet formation. N=4

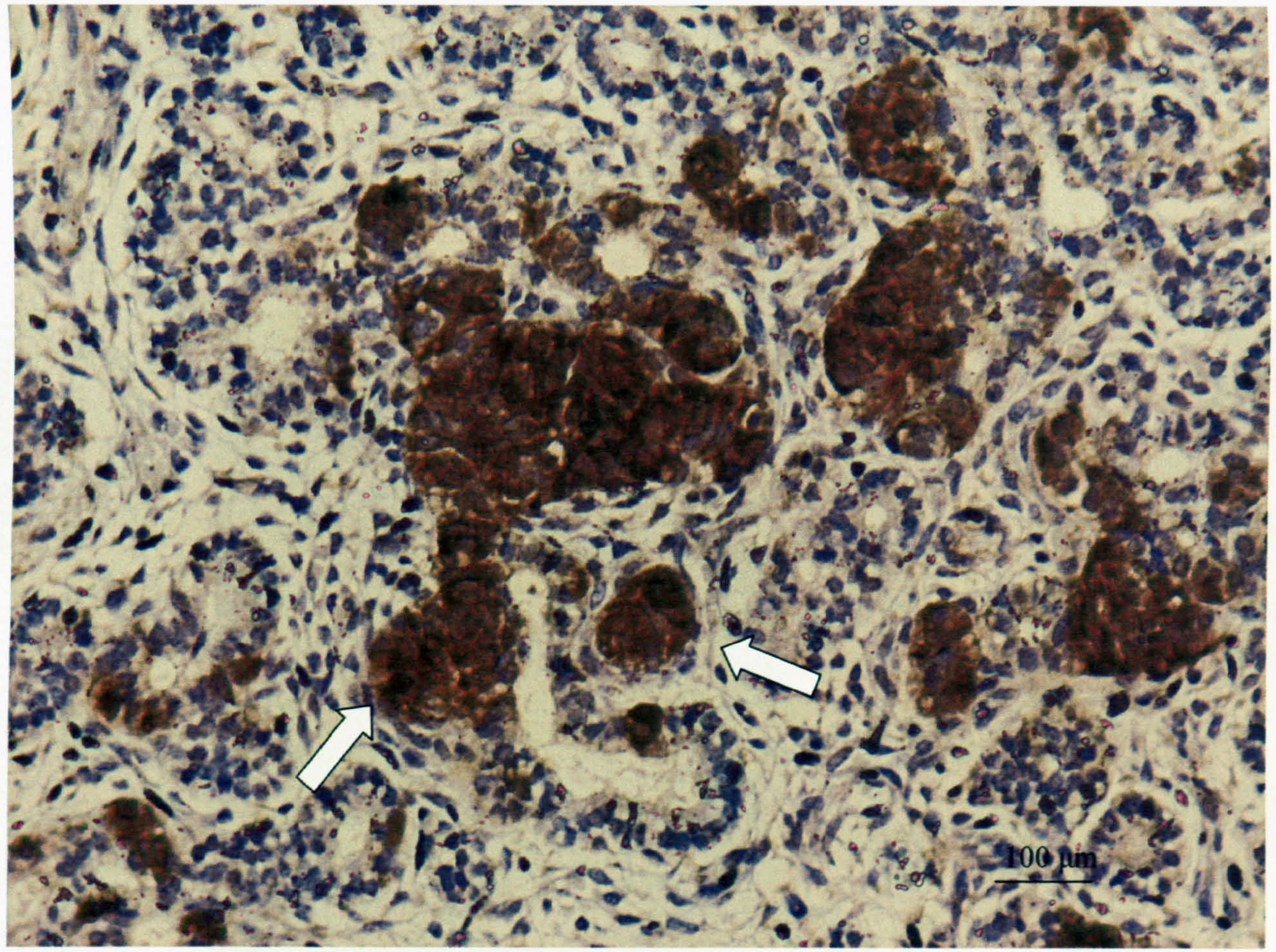


Figure 7 Week 12 pancreas showing insulin immunolocalisation and islet formation (arrows). Note that individual ductal cells express insulin. N=4

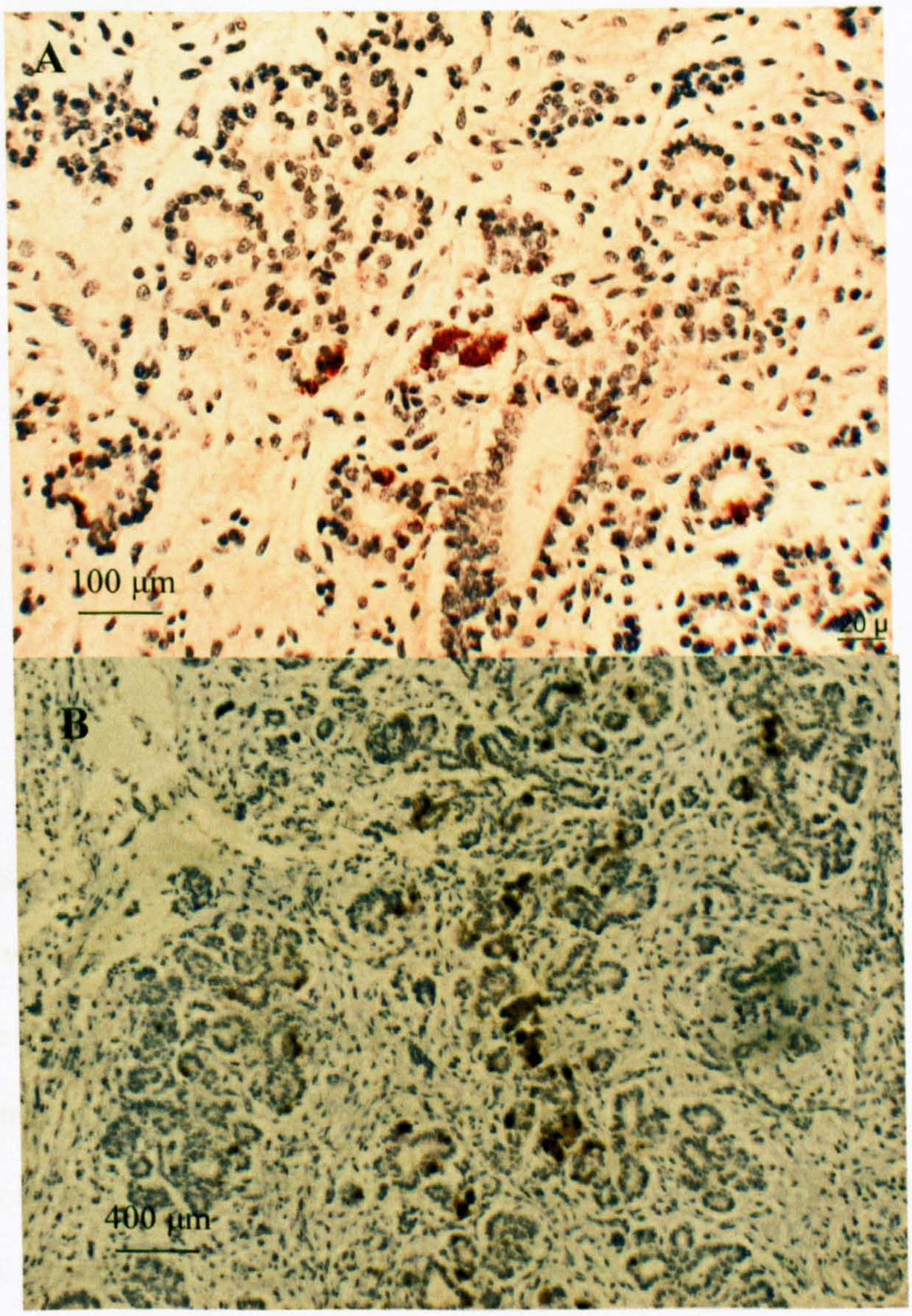


Figure 8 Glugagon immunolocalisation in (A) week 10 pancreas and (B) week 12 pancreas. N = 4.

IV. DISCUSSION

Because of the relation between C-peptide and insulin, a human C-peptide ELISA assay was used in the present study to measure the quantity of human C-peptide in cell culture medium. Nonetheless, the assay worked to capture the human C-peptide in the examined samples, EBs and foetal tissues, by monoclonal antibody which is found in the microtitre wells. All the unbound materials were then removed by a washing step. Finally, the reaction was stopped by adding acid to the samples which gives a colorimetric endpoint that can be detected spectrophotometrically at an absorbance of 450 nm.

Immunoreactivity for insulin, glucagon, somatostatin and pancreatic polypeptide (PP) was detected at 12-13 weeks gestation when immunohistochemical study of the development of endocrine cells within the human embryonic and early foetal pancreas was applied (Piper *et al*, 2004). However, insulin expression was detected closer to the end of the human embryonic period (Piper *et al*, 2004).

The results obtained from the human C-peptide ELISA method,(tables 5 and 6) showed that EBs samples which were grown in culture with different growth factors (nicotinamide, nicotinamide + FGF2, nicotinamide + activin A and activin A + FGF2) for 14 days, did not secrete C-peptide in the media with all readings below the assay limit, which was 0.1ng/ml or less. This may indicate that growing EB cells in culture with different growth factors may need more than 14 days which help the cells to have the ability to secrete more C-Peptide into the culture media.

Foetal pancreas tissue samples at 8 and 9 weeks were also incapable of secreting C-Peptide into culture media. However, the level of C-Peptide secreted from samples at 10 weeks gestation reached about 0.001ng/hr (1pg/hr) with levels increasing to reach about 0.01ng/hr at 11 weeks. The amount of C-Peptide secreted from human foetal tissues varied between different wells of samples of the same age. However, this may due to the differences in the number of cells in each sample. Each well contained different sizes of pancreatic human foetal tissue and the number of cell secreting C-Peptide may have differed accordingly. In addition, isolation, dissection and incubation steps of the sample tissue in culture may have led to cell death.

During the incubation period of the foetal pancreas tissue in culture the secretion of insulin decreased week by week and this may be due to the environmental differences that are found between *in vitro* and *in vivo* conditions.

Moreover, the results obtained from the foetal tissues indicated that, even if the human foetal pancreas developed completely morphologically, the β-cells will not secrete huge amounts of insulin until later in development i.e. at more than 12 weeks' gestation.

In conclusion, the EBs which were grown in culture with different growth factor/s did not secrete C-peptide into the culture media as detected by ELISA. This

may have been due to the short incubation period of the EB cells in culture or the method that used to direct their differentiation from ES cells toward insulin secreting cells was not successful and needed improving. On the other hand, foetal tissues were shown to secrete C-peptide after 9 weeks gestation.

CHAPTER FOUR

Pattern of mRNAs Expression Related to β-Cell Development from hES Cells

I. INTRODUCTION

As discussed in chapter I, the expression of a number of genes are important for pancreas and islet formation *in vivo* and therefore their expression in culture of differentiating embryonic stem cells may indicate development down a pathway towards an insulin-secreting cell. A profile of gene expression can therefore be used to characterise cell cultures (D'Amour *et al*, 2006).

Initially, PDX-1, defines a distinct compartment of the endodermal foregut that specifies the dorsal and ventral pancreas. Another gene expressed early in pancreatic development is the LIM-domain protein Isl-1 (ISL-1) which is expressed in all types of islet cell in the adult. Isl-1 may serve two functions. First, in the development of the dorsal mesenchymal cell, the presence of which is necessary for development of the exocrine pancreas, and second, in epithelial cells where its expression is necessary for differentiation of islet cells. Nestin has also been identified as a marker of a progenitor stem cell population. Early markers of islet differentiation

include genes such as Nkx2.2 (*NKX2-2*) and Nkx6.1 (*NKX6-1*) which produce homeodomain proteins. Nkx2.2 is expressed in α, β and PP cells of islets, whereas Nkx6.1 expression is believed to be restricted to the β cells and is therefore a useful marker of this cell type. Ngn3 (*NGN3*) is also important and leads to increases in endocrine cell differentiation factors (such as NeroD1) that push the cell towards a differentiated endocrine cell lineage. NeuroD1 (*NEUROD1*) is expressed in the developing endocrine pancreas, the small intestine, and the nervous system. NeuroD1 has been shown to act downstream of ngn3 and Isl-1. The expression of Pax 4 (*Pax4*) is also important at this stage. Finally insulin-secreting cells should express genes related to their function including glucose transporter genes (Glut-1 (*SLC2A1*) and 2), glucokinase (*GK*) and insulin (*IDDM2*) (D'Amour *et al*, 2006).

Therefore, various combinations of genes related to the pancreatic and β-cell pathway have been used to characterise embryonic stem cells induced to differentiate to insulin-secreting cell types (D'Amour *et al*, 2006; Fukazawa *et al*, 2006). Such cells differentiated *in vitro* should have the ability to transcribe and process insulin, which can be detected by the reverse transcriptase polymerase chain reaction, RT-PCR (Segev *et al*, 2004). Hori and coworkers, in 2005, used RT-PCR to show the expression of some essential genes for pancreatic endocrine insulin-producing cells which were differentiated from human neural progenitor cells (Hori *et al*, 2005).

Islet cells differentiation, which was derived from mouse, expressed insulin C-peptide, Glut-2 and Pdx-1 when RT-PCR was applied on the RNA extracted from them. However, differentiated mouse ES cells showed changes in the levels of mRNA in the studied genes (Vaca *et al*, 2006). Islet-like clusters, which were differentiated from bone marrow stem cells showed an endocrine genes expression similar to that of the pancreatic β-cells. These genes include Pdx-1, insulin I, glucagon, insulin II,

somatostatin, Glut-2 and Pax-4 which were expressed by PCR (Tayaramma *et al*, 2006).

The chemistry of PCR depends on the complementary nature of the DNA bases (Murad *et al*, 2004). When a DNA molecule is heated to a certain temperature the hydrogen bonds that held the two strands together will break down into single strands. In addition, when the same solution cools down the hydrogen bonds between the bases of the DNA strands will re-nature to form the original DNA double helix form (Murad *et al*, 2004). PCR method includes three main steps. These steps starts with denaturation and ending with extension, with an in between annealing step. The three steps repeated in number of cycles between 20-45 cycles, and after each cycle the amplified target doubled (Murad *et al*, 2004). The denaturation step, first step, required an incubation of the DNA in a high temperature that can break down the hydrogen bonds between the double helix into single strands. The oligonucleotide primers then, bind to the specific region, to be amplified, on the template (Murad *et al*, 2004). In the second, annealing, step the temperature is reduced, to allow the primers to anneal to their complementary target sequences. However, the oligo primer anneals toward the 5` end of the template RNA and extend it with DNA polymerase to form the complementary cDNA. The best method to generate cDNA is reverse transcription of total RNA using Moloney Murine Leukaemia Virus reverse transcriptase, MMLV-RT, enzyme with gene specific primers or oligo (dTs) (Freeman *et al*, 1999; Ginzinger, 2002; Nishimura *et al*, 2006). These oligo primers can be either gene specific or not specific. On the other hand, oligo (dT) anneal to the RNA 3` end and extend it (Freeman *et al*, 1999).

The extension step is the third step in the PCR method. This step requires an intermediate temperature, where the polymerase binds to each oligo primer and

extension occurs. Following the RT reaction, the cDNA is ready to be amplified by PCR (Freeman *et al*, 1999; Murad *et al*, 2004).

In this study, some of the genes expressed during pancreatic development were analysed (Figure 1) for expression in embryoid bodies cultured with various growth factors (Chapter II).

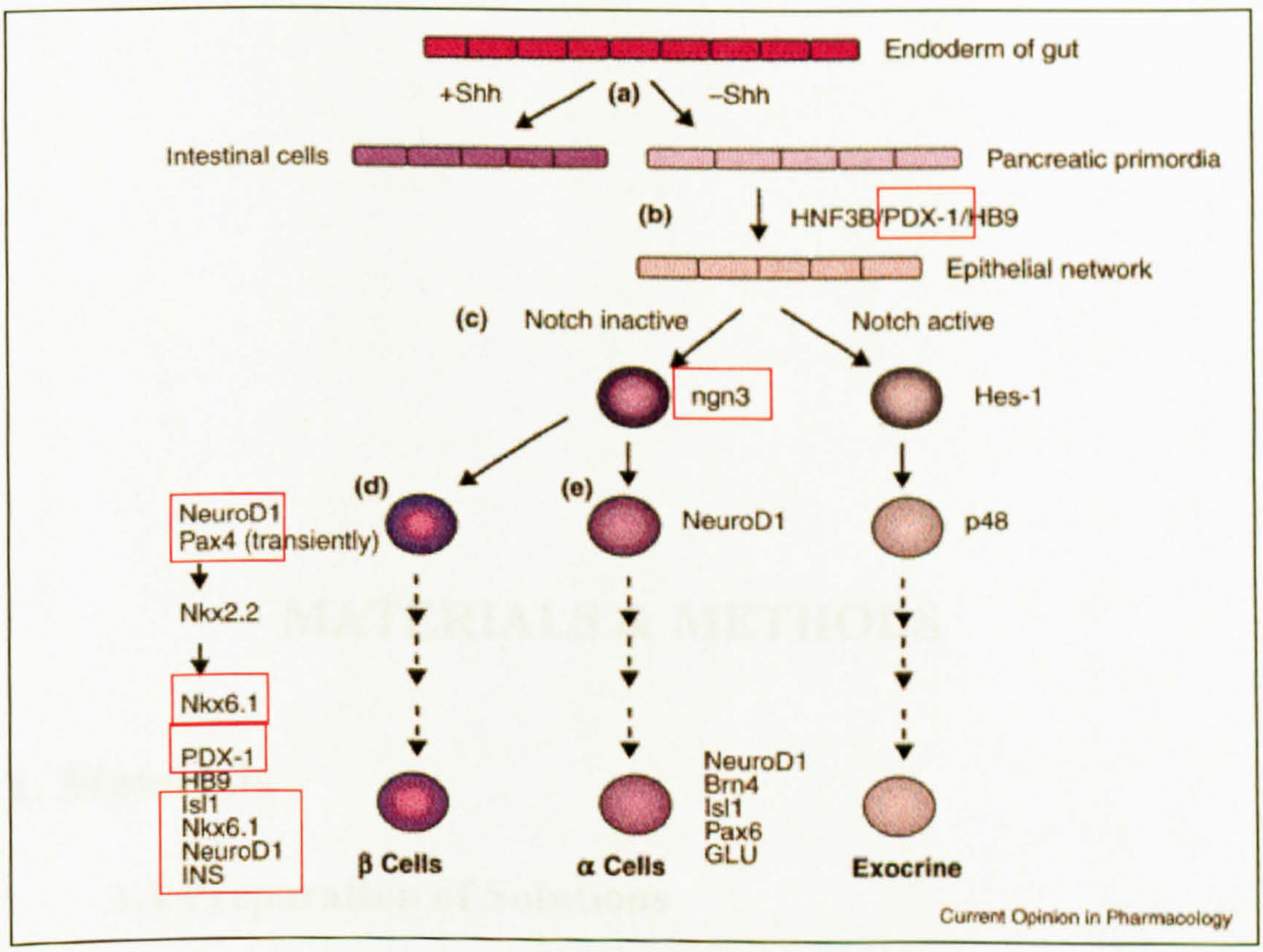


Figure 1 Some of genes expressed during pancreatic development. Genes surrounded by red box analysed in this study. Also analysed Oct 4, nestin, glucokinase, Glut 1 and 2 and β-actin (developed from Docherty 2001).

MATERIALS & METHODS

1. Materials

1.1 Preparation of Solutions

1.1.1 TAE (Tris-Acetate-EDTA) Buffer (1X)

To 10ml of TAE (10X) solution was added 90ml of RNase-free water. The mixture was shaken well and stored at room temperature. Fresh (1X) TAE was used for each electrophoresis run.

1.1.2 Formaldehyde Gel Running Buffer (1%)

In a 1000ml measuring cylinder, 100ml of (10X) formaldehyde gel buffer was mixed with 20ml of (37%) formaldehyde and 880ml RNase-free water.

2. Methods

2.1 RNA Extraction Using TRIzol

After incubating EBs, derived from hES H7 S6, in culture for 14 days with different growth factors, cells were washed with (1X) PBS without Ca^{+2} and Mg^{+2} then transferred to a centrifuge tube. Cells pellet were collected by centrifugation at 12000g for 3 minutes. TRIzol reagent was added to the pellet, 2ml per 2 x 10^6 cells, and the suspension was passed through the syringe several times to disrupt the pellet. The sample was incubated at room temperature for 5 minutes. Chloroform was added to the sample as 0.2ml/ 1ml TRIzol reagent and the tube was shaken for about 1 minute. Another incubation time for 2 minutes and 30 seconds at room temperature took place. The tube was centrifuged at 12000g for 15 minutes to remove cellular debris. The aqueous phase was removed and an equal volume of isopropanol was added to precipitate the RNA. After an incubation time of 15 minutes at room temperature, centrifugation was performed at 12000g for 15 minutes. The pellet was left to dry at room temperature for few minutes and finally re-suspended in RNase-free water.

2.2 DNase Treatment of Extracted RNA

To the extracted RNA, 0.1 volume of (10X) DNase I buffer (Ambion) was mixed with 1µl DNase I (2 units) (Ambion). The mixture was incubated at 37°C for 30 minutes then 5µl of DNase inactivation reagent (Ambion) was added to the tube. The tube was incubated at room temperature for 2 minutes then centrifuged at 10000g for 1 minute to pellet the DNase inactivation reagent. The aqueous layer was removed and the steps were repeated again twice to clean up the RNA from any DNA residue.

2.3 Agarose Gel Preparation

Agarose gel was prepared by adding agarose powder (1%, 1.2%, 1.5% and 2% w/v) to (1X) TAE buffer. The mixture was heated in the microwave oven until no granules of agarose powder were visible. The molten agarose was allowed to cool down to about 65°C then 0.2µg/ml of ethidium bromide (10mg/ml) was mixed with it and poured into the gel tray. The gel was allowed to solidify for about 30 - 40 minutes. Approximately two litres of fresh (1X) TAE buffer were poured into the tank to cover the gel surface to about 2cm above the gel.

2.4 Formaldehyde Gel Preparation (1.2%)

Agarose gel powder (0.6g) was dissolved in 5ml (10X) formaldehyde gel buffer then RNase-free water added to the mixture to a total of 50ml. The mixture was boiled in a microwave oven until no agarose granules were present then left to cool to about 65°C. Formaldehyde (37%) was added to the gel, mixed and poured to the gel tray to solidify.

2.5 Preparation of Loading Buffer

Loading buffer for RNA samples was prepared in a ratio of 1:2, RNA sample to loading buffer, by mixing 3µl of extracted RNA sample with 3 µl RNase-free water and 6µl loading buffer. The mixture was heated to 65°C for 10 minutes then chilled on ice for few seconds. Samples were, finally, loaded into appropriate wells.

2.6 Electrophoresis

The electrophoresis apparatus was placed on a flat surface and leveled using the in-built adjustable legs.

The samples were taken out from the freezer, thawed then centrifuged in a microcentrifuge at 12000g for few seconds. From each sample, 1µl of cDNA were mixed with 2µl loading dye and 7µl RNase-free water then loaded into the sample well. Into one well, 2µl of both high molecular weight (1 Kb) DNA marker (Bioline) and (100bp) DNA ladder (Fermentas), used to estimate the size of cDNA fragments, was mixed with 2µl loading dye and 6µl RNase-free water then loaded into the well. The electrophoresis tank was closed and connected to a power supply. The samples migrate from the cathode, negative electrode, to the anode, positive electrode. Electrophoresis was carried out at a constant voltage of 100 V with a current of 250 MA for about 60 minutes or as required.

The electrophoresis was monitored, by following the migration of the loading dye. When the dye reached to about the edge of the gel, the power supply was switched off. The gel tray was removed and placed on the surface of an ultraviolet transluminator 254 nm (Genius). The gel was then photographed using a video camera connected to a digital graphic printer (Sony).

2.7 Synthesis of First Strand cDNA

In a nuclease- free microcentrifuge tube, 2µl oligo (dT) of 0.5µg/µl was added to 5µg of total RNA and up to 29µl of RNase-free water as a final volume. The tube was heated at 70°C for 8 minutes then quick chilled on ice for 10 minutes. During this time a master mix was prepared in a separate tube on ice for a final volume of 40µl by mixing 8µl Moloney Murine Leukaemia Virus reverse transcriptase, MMLV RT, (5X) buffer (Promega), 1µl MMLV RT enzyme (Promega), 2µl dNTP's mix (25mM; Promega) and 1µl RNase inhibitor (Promega).

The sample tube was removed from the ice, mixed by vortex and centrifuged by pulse. The master mix was added to the sample tube and the mixture incubated at 37°C for 2 - 3 hours. The reaction was then stopped by heating at 95°C for 2 minutes.

2.8 Analysis of the Extracted RNA

2.8.1 Quantification of RNA by Spectrophotometer

The quantity of extracted RNA was determined by a spectrophotometer (Beckman DU 650). RNA samples were diluted with RNase-free water in a final volume of 100µl in a cuvette and readings were taken.

The optical density (OD) of each RNA sample was measured at two wavelengths, 260nm and 280nm in triplicates. The 260 nm/ 280 nm ratio was calculated to determine the purity of RNA, where the desired range of ratios was between 0.9 - 1.5. The RNA concentration was calculated in µg/µl using the following formula:

1OD = 40µg (for single stranded RNA)/ µl

therefore, RNA concentration (µg/µl) = OD@260 X 40 X Dilution Factor (100X)

The diluted RNA was stored at –80°C.

2.8.2 Analysis of the Quality of Extracted RNA

The quality of the extracted RNA was analyzed by agarose gel elecrophoresis in order to evaluate the suitability of the RNA for PCR and to ensure that the extracted RNA is of high molecular weight and with minimal degradation.

2.9 RT-PCR Technique

RT-PCR was used in the present study and was performed in a T gradient PCR machine (Biometra). For the optimized procedures a gradient PCR were used.

In a final volume of 30µl aliquots the following was added:

Chemicals	Suppliers
10Xbuffer	Ambion
(dTTP, dATP, dGTP and dCTP)	Ambion
50mM $MgCl_2$	Ambion
100pm/µl forward primer	Metabion
100pm/µl reverse primer	Metabion
0.3units Taq polymerase	Ambion
cDNA template	

RNase-free water up to 30µl. In control samples RNase-free water was used instead of cDNA template, where no positive controls were used.

The PCR conditions used was as follows:

Process	Temp.	Time	
Initial Denaturation	95°C	3 min	
Denaturation	95°C	30 seconds	(22-40) Cycles
Annealing	*(X) °C	1 min	
Extension	72°C	1 min	

Final extension	72°C	5 min
Hold	4°C	for as long as required

*(X) = the annealing temperature selected according to the primers in use.

The amplified products were then loaded into the agarose gel wells, electrophoresis was then applied, gel visualized on a UV transluminator and photographed.

2.10 Primers Selection

Primers which were selected in this study where chosen according to their importance in the development of endocrine progenitor pancreas. The genes used to apply in this study were selected according to their importance in the development of pancreas (Table 10).

All the primers used in this study had been validated by colleagues in the laboratory for in other investigations..

2. 11 Primers Design

All primers in this study were designed to have about 45-55% GC content and with at least two GC base pairs at the last 3' end. To prevent the formation of secondary structure no more than three of the same bases were repeated through each primer. In addition, there was no more than two degree difference between the annealing temperature for each primers pairs, forward and reverse.

By using Genbank database (http//:www.ncbi.nlm.nih.gov) specific gene DNA sequences were obtained to determine the highly conserved regions and homologous genes. Primer pairs and the predicted products were BLAST searched to exclude nonsp-

Table 10 The human primers used in PCR and characterization. Tm: annealing temperature, bp: base pair and #: number.

Gene of interest	Accession Number	Primers Sequences (5`____3`)	Product size (bp)	Tm	# of Cycles
Pax-4	NM_006193	F-ATG AAC CAG CTT GGG GGG CTC T R-CTC CTT CCC ACT CCC TGC CTC A	1161	58	30
β-actin	NM_001101	F-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG R-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC	838	60	28
Ngn-3	NM_020999	F-TAG AAA GGA TGA CGC CTC A R-GAA GCA GAA GGA ACA AGT G	768	52	38
Nkx6.1	NM_006168	F-AAC AAT ATC CTG AGC CGG CC R-CGA GTC CTG CTT CTT CTT GG	629	55	38
GK	NM_000162	F-CTG TGA CTG AAC CTC AAA CCC C R-AAG GAG AAG GTG AAG CCC AGG	621	58	38
Oct-4	NM_00270	F-CGA CCA TCT GCC GCT TTG AG R-CCC CCT GTC CCC CAT TCC TA	576	60	28
Isl-1	NM_002202	F-GGC CAA TAT TTC CCA CTT AGC R-GGC CCT CTC CAC CAC ATC	520	55	35
Nestin	NM_006617	F-CCG CTA AGG TGA AAA GGG GTG TG R-AGC GGA GGC ATT ACT TTA TTC AGG	416	58	28
Glut-1	NM_006516	F-CAT GTG CTT CCA GTA TGT GG R-GTC AGG TTT GGA AGT CTC AT	312	52	28
Glut-2	NM_ L09674	F-GCA GCT GCT CAA CTA ATC AC R-TCA GCA GCA CAA GTC CCA CT	265	60	38
Pdx-1	NM_000209	F-CCC ATG GAT GAA GTC TAC C R-GTC CTC CTC CTT TTT CCA C	262	52	35
Insulin	NM_000207	F-GCC TTT GTG AAC CAA CAC CTG R-GTT GCA GTA GTT CTC CAG CTG	261	58	35

ecific primers and products. All the primers were obtained from metabion (Germany).

The presence of genomic DNA, gDNA, was checked in all extracted RNA samples, between the DNase treatment and cDNA synthesis.

2.12 Optimization Procedure

By using a T gradient PCR machine (Biometra), the annealing temperatures for each primer pairs were optimized to obtain good PCR products.

III. RESULTS

1. RNA Extraction

25 - 30 EB colonies were generated by culture conditions with each different growth factor treatment (Activin A + FGF2, Activin A, Nicotinamide + FGF2, Nicotinamide + Activin A and Nicotinamide) (chapter II) for 14 days and used for extraction of RNA.

The extracted RNA was of good yield and high quality as determined, from the readings measured, by spectrophotometer. In addition, the purity was also determined by the 260 nm/280 nm absorbance ratio which was ranged between 1.5 and 1.8.

The final concentration of extracted RNA was diluted to 5μg/μl. The extracted RNA showed minimal degradation as visualized on 1.2% formaldehyde agarose gel as well as on 0.7% agarose gel (Figures 2 and 3).

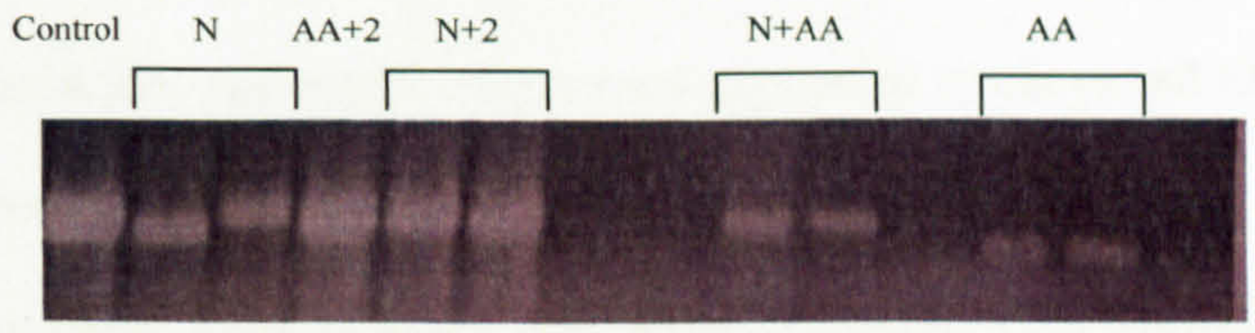


Figure 2 Formaldehyde agarose gel (1.2%) shown the quality of RNA extracted from different EBs grown with different growth Factors. N = Nicotinamide, AA = Activin A and 2 = FGF2. This experiment was repeated four times for each EB sample grown with different growth factor/s.

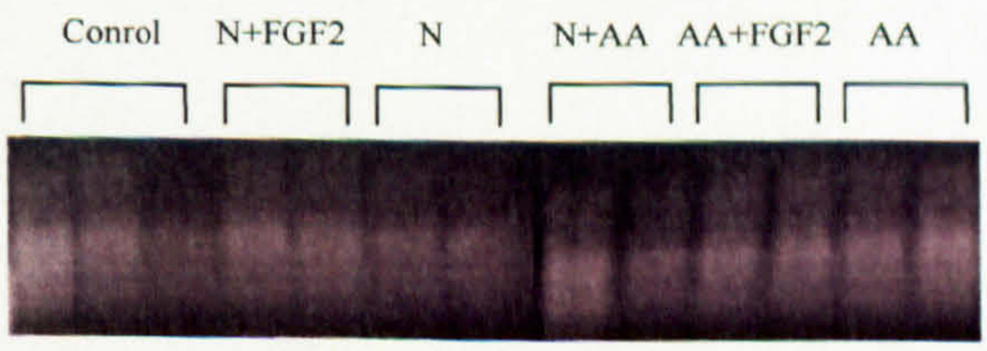


Figure 3 An agarose gel (0.7%) showing the RNA extracted from different EBs grown with different growth factors. N = Nicotinamide, AA = Activin A. The experiment was repeated ten times for each EB sample grown with different growth factor/s.

2. RT-PCR Technique

The RNA samples which were amplified with RT-PCR technique, using all the primers which were listed in Table 1, indicated positive expression for some genes such as *Pdx-1*, insulin, Pax-4, GK and nestin which were expressed in almost all EB samples grown with the different growth factor combinations.

On the other hand, Glut-2, Ngn3 and Nkx6.1 showed only slightly positive expression with all EB samples grown with different growth factors, while Glut-1 , Oct-4 and Isl-1 showed negative results, no bands were expressed, in all EB samples grown with different growth factors (Figure 4).

The PCR experiments were repeated at least four times for each gene to obtain consistentand accurate results.

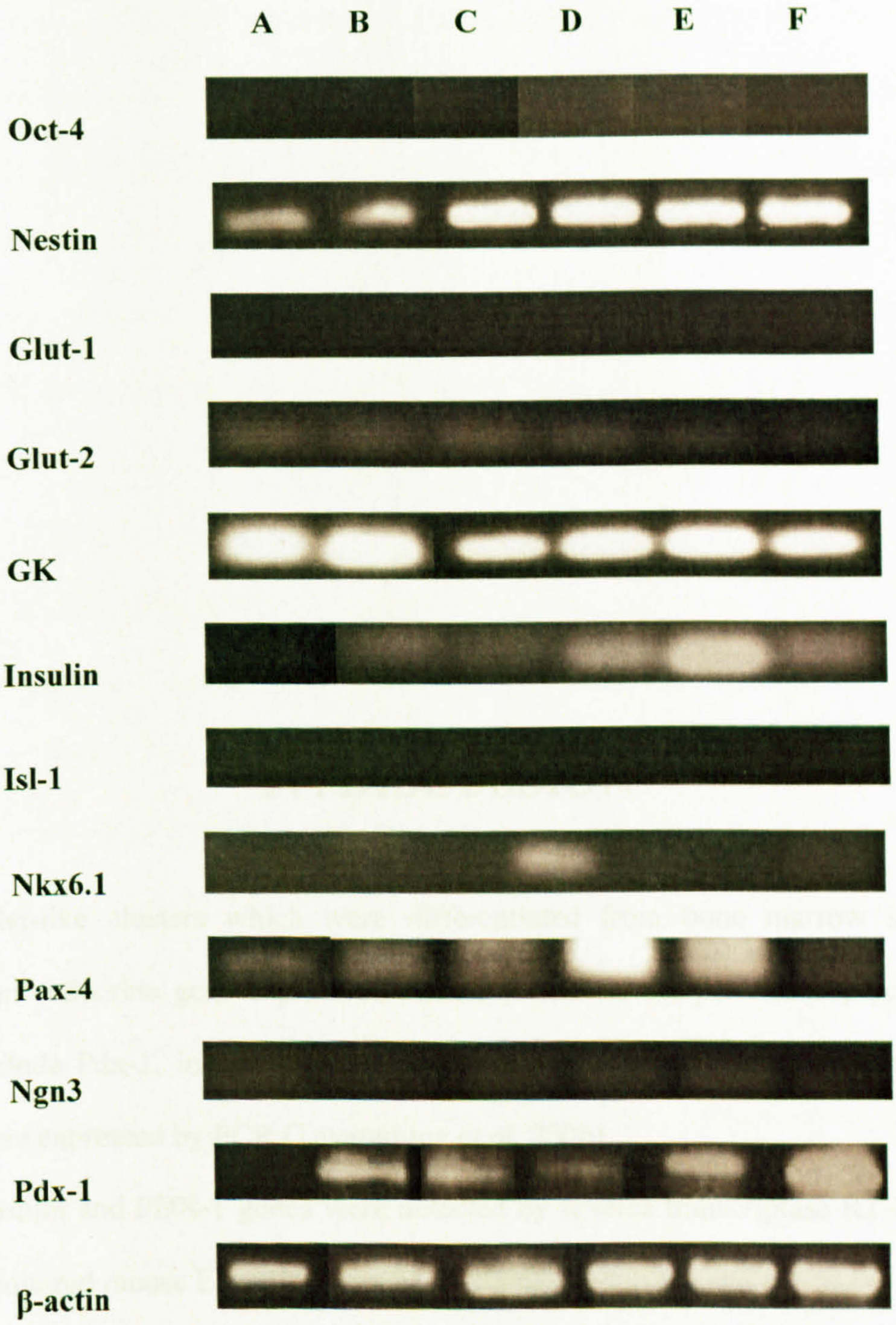


Figure 4 PCR products from different EBs, H7 S6, grown with different growth factors. (A) Control (EBs grown in EBs medium, no growth factor/s was added, for the 14 days) (B) Activin A + FGF2, (C) Activin A, (D) Nicotinamide + FGF2, (E) Nicotinamide + Activin A and (F) Nicotinamide.

IV. DISCUSSION

Islet-like clusters which were differentiated from bone marrow stem cells showed an endocrine gene expression similar to that of the pancreatic β-cells. These genes include Pdx-1, insulin I, glucagon, insulin II, somatostatin, Glut-2 and Pax-4 which were expressed by PCR (Tayaramma *et al*, 2006).

Insulin and PDX-1 genes were detected by reverse transcriptase RT-PCR from 14 days cultured mouse ES cells (Vaca *et al*, 2006).

RT-PCR study by Fujikawa and colleagues was used to generate insulin-producing cells from rat ES cells using a modified protocol of nestin positive cells. By using RT-PCR method they showed that transcriptional factors found in endocrine and pancreatic lineages such as PDX1, NKX2.2, NKX6.1, PAX6, nestin and insulin I and II were expressed, *in vitro,* in the rat ES cells. However, the undifferentiated ES cells did not express insulin message (Fujikawa *et al,* 2005).

Moreover, RT-PCR was used to detect pancreatic genes from murine thymus (Palumbo *et al*, 2006). Isolation of proinsulin-producing epithelial cells which were derived from cultured murine thymus expressed *Pdx-1, Glut-2* and insulin I and II as shown by RT-PCR (Palumbo *et al*, 2006).

The expression of endocrine hormone genes and proteins of human islet-like structures from pancreatic precursor cells was analyzed in culture by RT-PCR (Bodnar *et al*, 2006). The amplified genes included insulin, glucagon, somatostatin, pancreatic polypeptide (Bodnar *et al*, 2006).

In addition, RT-PCR was performed to assess the expression of some genes involved in the β-cell differentiation pathway which were differentiated from hES cells such as *GK, PDX-1, ISL-1, NKX6.1, GLUT-1* and 2 and *PAX-4* (Baharvand *et al*, 2006).

Human foetal pancreases between gestational ages 8-21 weeks were shown to express *PDX-1*, insulin, cytokeratin 19, *NGN3*, glucagon, somatostatin, *ISL-1*, *NEUROD1*, *NKX2.2*, *NKX6.1* and *PAX6* during early development of the pancreas (Jiang *et al*, 2007; Gannon *et al*, 2008; Lyttle *et al*, 2008). While Sarkar and coworkers showed expression of insulin, glucagon, somatostatin, ghrelin and pp in the development of human foetal pancreases between gestational ages of 9-23 weeks when they used gene expression profiling (Sarkar *et al.*, 2008). Moreover, *PDX-1*, *GLUT-2(SLC2A2)* and *GK* were implicated in playing an important role in the development of human foetal pancreases (Kaneto *et al*, 2007).

In the present study *PDX-1* gene was found in all EBs samples grown with different growth factors, this may indicated that all the growth factors which were selected in this study were encouraging the EB cells toward insulin containing cells but after maturation, especially with cells grown in nicotinamide where they display higher intensity of PCR product.

On the other hand, *NGN-3* and nestin genes are mostly expressed in neural precursor cells as well as in progenitor cells. *NGN-3* regulates the function of both *ISL-1* and *PAX-4* genes in early stages of pancreas development. Thus, the presence of *NGN-3*, even if it was very little as showed from the bands in all EB samples, may indicate that the chance for expression of more *ISL-1*, which is always found in adult pancreas, *PAX-4* and insulin later in development.

NKX6.1 gene was not expressed in all EB samples grown with different growth factors except EBs with nicotinamide + FGF2. *NKX6.1* gene is usually expressed at the termination of pancreas development.

Insulin was expressed in about all EB samples especially in those cells grown with nicotinamide + FGF2 that showed a sharp band when repeating the experiment four times. This may be in accordance with the presence of some developed cells to become insulin-like cells, especially in nicotinamide + FGF2, that start secreting small amounts of insulin but with incubating all EBs cells, longer than two weeks, in culture they may secrete more amount of insulin.

Oct-4 gene was not expressed in any EB samples grown with different growth factors. Oct-4 is expressed in undifferentiated cells and while the EBs cells start expressing other genes this means that the cells were differentiated towards a cell line so there was no Oct-4 in these differentiated cells.

Glut-1 and Glut-2 glucose transporter genes may not be expected to be expressed in immature insulin-like cells, and only in cells that become glucose responsive, which are mature insulin secreting β-cells.

On the other hand, the similarities in gene expression patterns observed in the developing pancreas and central nervous system (CNS) can confuse conclusions about the differentiation of real insulin-secreting cells from ES cells when the only assay is

the expression of gene markers. For example, somatostatin, pancreatic polypeptide, Nkx6.1, and Glut-2 all have been detected in differentiated cells by RT-PCR, microarray analysis, or immunocytochemistry, from which it was concluded that insulin -like cells have been formed. Although these genes are indeed expressed in pancreatic endocrine cells, they are also expressed in the CNS.

In conclusion, as most of the 14 days cultured EBs, which were derived from H7 S6 hES cells, showed positive expression for some genes e.g. *Pdx-1*, Ngn3, Pax4, Nkx6.1, Insulin and GK this may indicate the presence of insulin-like cells in culture but they may remain immature.

To improve the amount of mature insulin-containing cells in culture, a longer incubation, for more than 7 days, with different growth factor/s may be recommended, while this may give the possibility to more cells to differentiated towards insulin containing cells.

CHAPTER FIVE

Protein Expression Patterns During Development of the Foetal Pancreas Between 10 and 12 Weeks Gestation

I. INTRODUCTION

The proteome is the expression of the total protein profile of a cell. Proteomics is the large-scale study of proteins, particularly their structures and functions. The development of proteomic technology gives the chance to determine genetic variants at the protein level within cells or tissue (Valmu *et al*, 2006) and it gives a much better understanding of an organism than genetic analysis alone. Firstly, the level of transcription of a gene gives only an approximate indication of level of expression as a protein. The mRNA might be produced but degraded rapidly or translated inefficiently in both cases resulting in a small amount of protein. Also many proteins undergo post-translational modifications that can affect their activity or composition and transcripts give rise to more than one protein, through the process of alternative splicing. Proteomics is also important for identifying biomarkers, such as markers that indicate a particular disease or for identifying stages of development.

To determine the number of proteins in a sample and their composition and identity they must be first extracted from the sample and then individually separated. Protein separation can be performed using two-dimensional gel electrophoresis (2D PAGE), which separates proteins first by their isoelectic point (PI) and then by their molecular weight. Protein spots in a gel can be visualized using a variety of chemical stains (or fluorescent markers) and can be semi-quantified by the intensity of the stain. Image analysis software can be used to make comparisons between gels of two different samples.

Once proteins are separated and quantified, they can be analysed to determine their precise composition. Individual spots are cut out of the gel and cleaved into peptides with proteolytic enzymes. The peptides can then be identified by mass spectrometry. However, spots may contain a mixture of proteins. Protein mixtures can also be analyzed without prior separation and are often injected onto a high pressure liquid chromatography column (HPLC) that separates peptides based on hydrophobicity. HPLC can then be coupled directly to a time-of-flight mass spectrometer using electrospray ionization. It is very useful in producing ions because it overcomes the problem of molecular fragmentation when macromolecules are ionized. John Bennett Fenn was awarded a Nobel prize in 2002 for development of electrospray ionization. Peptides eluting from the column can be identified by tandem mass spectrometry (MS/MS). The first stage of tandem MS/MS isolates individual peptide ions, and the second breaks the peptides into fragments and uses the fragmentation pattern to determine their amino acid sequences. A particular type of MS/MS is called a Q-Tof (quadrupole time of flight) and has two analysers. The first is a quadrupole analyser that is used as an ion guide in MS mode, but as a resolving analyser in MS/MS mode. The second analyser is a reflectron time-of-flight analyser placed at an angle to

the quadrupole. The final detector is a microchannel plate detector for high sensitivity. In MS/MS mode, the two analysers are used together for structural studies by monitoring fragmentation patterns in molecules. The fragmentation patterns of peptides are particularly well documented and the information of an unknown peptide can be compared with databases.

There are a number of proteomic studies on the human adult pancreas (Hu, *et al* 2004; Shen *et al*, 2004) and on the human adult islets (Ahmed, *et al* 2005). But there is no proteomic study on the differentiate protein expression between two different ages of foetal pancreas. By studying the developmental stages of the pancreas will increase our knowledge of pancreatic development and perhaps lead to a greater understanding in how the basic mechanism of endocrine homeostasis is maintained and to how a disease state may arise when one or more of the mechanisms are disrupted.

The aim of this chapter was to determine the proteome profile of the human foetal pancreas between weeks 10 and 12 when islet formation was shown to first occur (Chapter III).

MATERIALS & METHODS

1. Materials

1.1 Foetal Pancreas

Collection of foetal pancreas was as described in Chapter III. The age of the foetus was estimated from last menstrual bleeding and then determined by forearm digit morphology. The foetal pancreas was recovered by microscopic dissection. A small sample was prepared for immunohistochemistry as described in chapter III and the rest placed on aluminum foil and snapped frozen in liquid nitrogen and then stored at $-70^{\circ}C$.

2. Methods

These experiments were carried out in conjunction with Dr Chi Wong, Proteomics Facility, Centre for Stem Cell Biology.

2.1 Two-Dimensional Gel Electrophoresis

The isoelectric point (pI) is the pH at which a molecule or surface carries no net electrical charge. A protein at its pI will therefore not migrate in an electric field at this point and molecules of the same pI will become focused. When combined with separation according to the size of the molecule, molecular weight, MW, (2^{nd} stage MW separation) a spot of protein with molecules of the same pI and MW occurs. This process is called two dimensional gel electrophoresis (2D-PAGE).

2.1.1 Sample Preparation

Foetal pancreas was cut into small pieces and proteins were extracted using the Grinding kit (Amersham Biosciences) in lysis solution (6M urea, 2M thiourea, 40mM DTT, 2% w/v CHAPS, 0.5% v/v IPG buffer, protease inhibitor cocktail (Sigma)). After grinding for 1 minute the sample was centrifuged at 500g for 3 minutes and the solution transferred to a fresh microcentrifuge tube. PlusOne 2D Clean up kit (Amersham Biosciences) was used according to the manufacturer's instructions to desalt and remove DNA/RNA contaminants from the lysis solution. The final protein pellet was resuspended in 6M urea in 2% CHAPS, and the protein concentration was determined by the BCA (Bicinochoninic acid) assay (Smith *et al*, 2007).

2.1.2 Isoelectric Focusing

Samples (200μg of total proteins) were passively hydrated into 18cm Immobiline DryStrips pH 4-7 (Amersham Biosciences, UK), overnight at room temperature. The proteins were resolved under the following conditions: 500Vhr

gradient, 1000Vhr gradient, 8000 V step-n-hold, for a total of 32500kVhr using the IPGphor system (Amersham Biosciences). The resolved proteins were reduced in 10ml of equilibration buffer (6M urea, 30% v/v glycerol, 2% w/v SDS and 0.25% w/v bromophenol blue in 50mM Tris-HCl pH 8.8) containing 100mM DTT for 15 minutes followed by alkylation for 15 minutes in 10ml of equilibration buffer containing 350 mM iodoacetamide.

2.1.3 2nd Dimension Gel Electrophoresis

The Ettan DALT 6 electrophoresis system (Amersham Biosciences) was assembled according to manufacturer's instructions. Focused and equilibrated pancreatic protein strips were placed on top of a pre-cast 12.5% homogenous SDS-PAGE gel (24 x 24 cm, Amersham Biosciences), sealed with 2% molten agarose and ran at 25°C at 5 W/gel for 30 minutes, then at 18 W/gel for 4 hrs. These gels were fixed overnight with 40% methanol in 7% acetic acid. The fixed gel were stained with Brilliant Blue Colloidal concentrate (Sigma, UK) for 2 hours, destained for 1 minutes with 25% methanol in 10% acetic acid and washed in 25% methanol until the gel background was clear.

2.2 Gel Analysis

Stained gels were scanned with the flat bed ImageMaster Scanner and LabScan software (Amersham Biosciences). Gel images were normalized and differential protein expression were analysis using the ImageMaster 2D Platinum software (Amersham Biosciences).

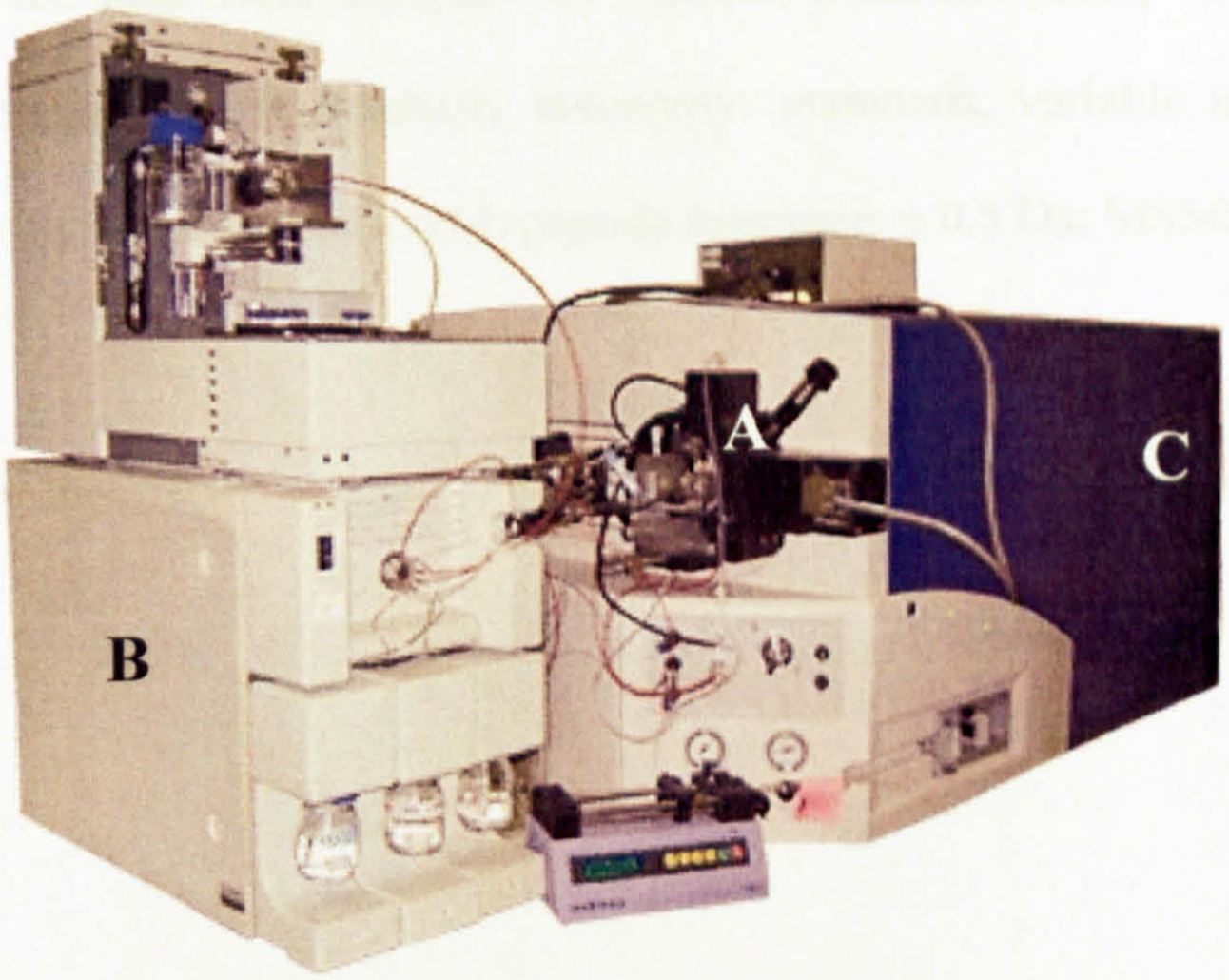
Gel spots were cut and transferred to a 0.5ml siliconised tube using the GelPal spot cutter (Genetix, UK). Excess water was removed and spots destained with 300µl of 50mM NH_3HCO_3 containing 25% acetonitrile (ACN) were added to the gel spot

for 30 minutes at 37°C. The gel pieces were dried under vacuum in a speed Vac (Eppendorf) for 15 minutes, rehydrated in 20µl of 200ng/ml modified trypsin solution (Promega, UK) in 50mM NH_3HCO_3 solution and incubated overnight at 37°C. Peptides were recovered by transferring the solution to appropriately labeled fresh microcentrifuge tubes. To recover the peptides from the gel pieces a four stage extraction procedure was used. Firstly, 15µl of 25mM NH_3HCO_3 solution were added to the gel pieces, incubated for 15 minutes at 37°C and transferred to labeled tubes. Secondly, 20µl of ACN was added to the gel pieces and incubated at 37°C for 15 minutes followed by drying for 3 minutes by vacuum centrifugation. The dehydrated gel pieces were rehydrated with 40µl of 5% formic acid, incubated for 15 minutes at 37°C and then transferred to labeled tubes. The second step was then repeated for the final peptide extraction. The pooled peptide extractions were dried under vacuum centrifuge for 4 hours, resuspended in 12µl of 3% ACN containing 0.1% formic acid and transferred to recovery vials.

2.3 MSMS Analysis

Recovery vials containing peptides were placed into the liquid chromatography system, CapLC (Waters, UK). Peptides were first desalted through a 5mm C18 PepMap pre-column (Dionnex, LC Packing, UK) and then separated through a 15cm C18 PepMap column (Dionnex, LC Packing, UK) under the following conditions: 5% - 85% B (95% ACN and 0.5% formic acid) over 45 minutes, 85% B for 2 minutes, 85%-5% B for 1 minutes, 5% B for 12 minutes. The eluent from the reverse phase column directly sprays into the QTOFµ mass spectrometer (Waters, UK). The Q-TOFµ was controlled by MassLynx v4 (Waters),

parent ions were acquired and analyzed by data dependent analysis (DDA) that saves the data as peak list.



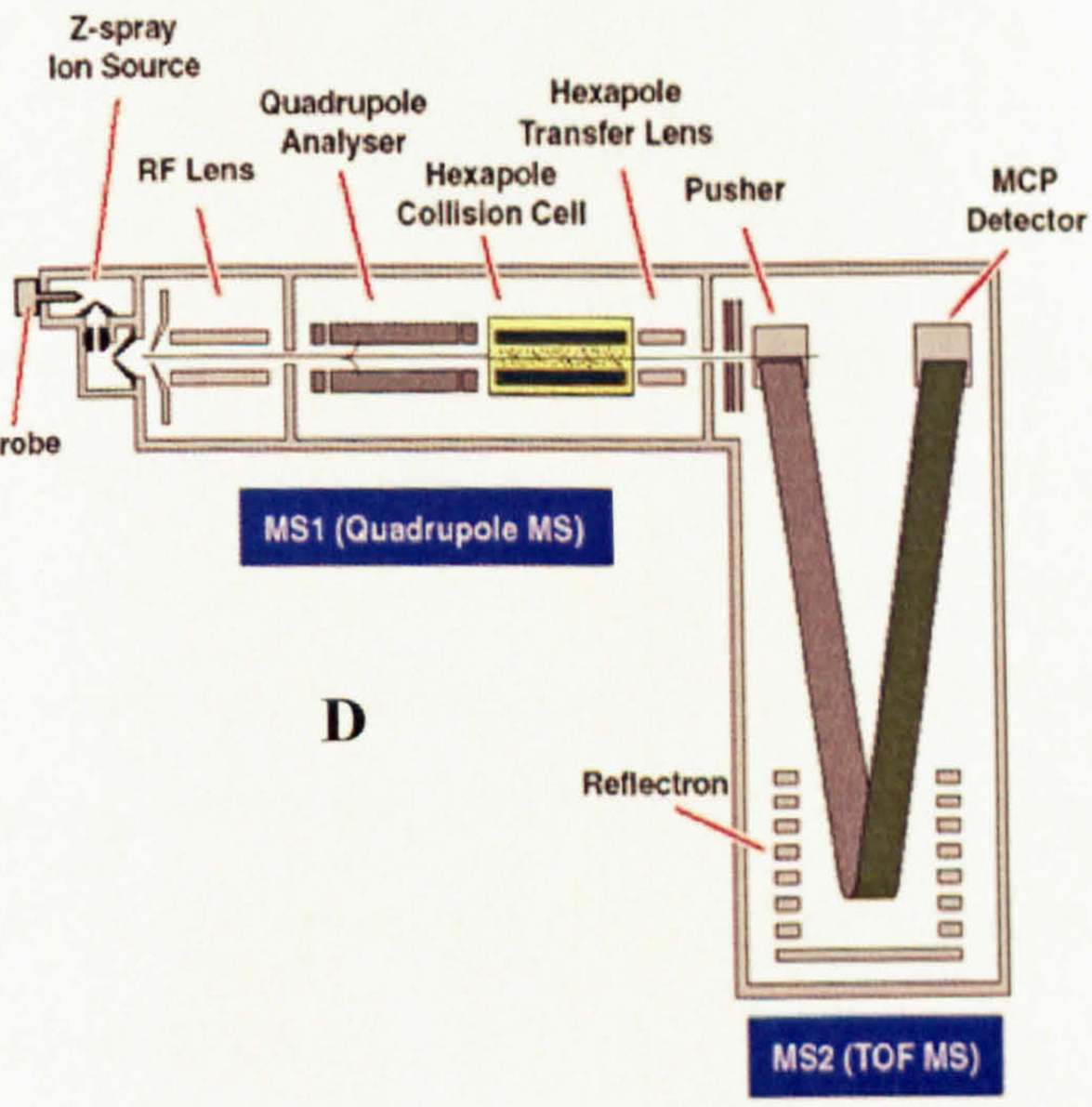


Figure 1 (A) Electrospray: the ion source (B) CapLC (Waters Coporation) liquid chromatography system: mass analyser and (C) Q-TOF micro (Waters Corporation) MS/MS analyzer: detector system (D) Diagram of mass spectrometer.

Peak list data were analyzed by Mascot (Matrixsciences, UK) under the following criteria: MSDB database, taxonomy: mammals, variable modifications: carbamidomethyl C and oxidation M; peptide tolerance: ± 0.5 Da; MSMS tolerance: ± 0.5 Da.

III. RESULTS

Immunohistochemistry of insulin and glucagons showed the same pattern as described in chapter III indicating the samples were of the developmental age expected.

Pancreas from at least three week 10 foetus were required for obtaining sufficient protein for spot resolution on the 2D PAGE for MS analysis. Two pancreases from week 12 samples were sufficient. 2D PAGE gels of pooled protein samples of week 10 and week 12 foetuses are shown in Figures 2 and 3. As expected the protein profiles showed differences.

Expression of 81 and 132 proteins from week 10 and 12 foetal pancreas, respectively were determined and analysed. Of these, 13 proteins were found only in week 10 foetal pancreas (Table 1), and 75 proteins found only in week 12 pancreas (Table 1). However, the samples did share common proteins such as house keeping proteins (heat shock proteins, alcohol dehydrogenase [NADP+]) and structural protein

Figure 2 2D PAGE gel of protein extracted from week 10 foetuses. Obtained from three foetal pancreases of 10 weeks gestation which were pooled together.

pH 4 **pH 7**

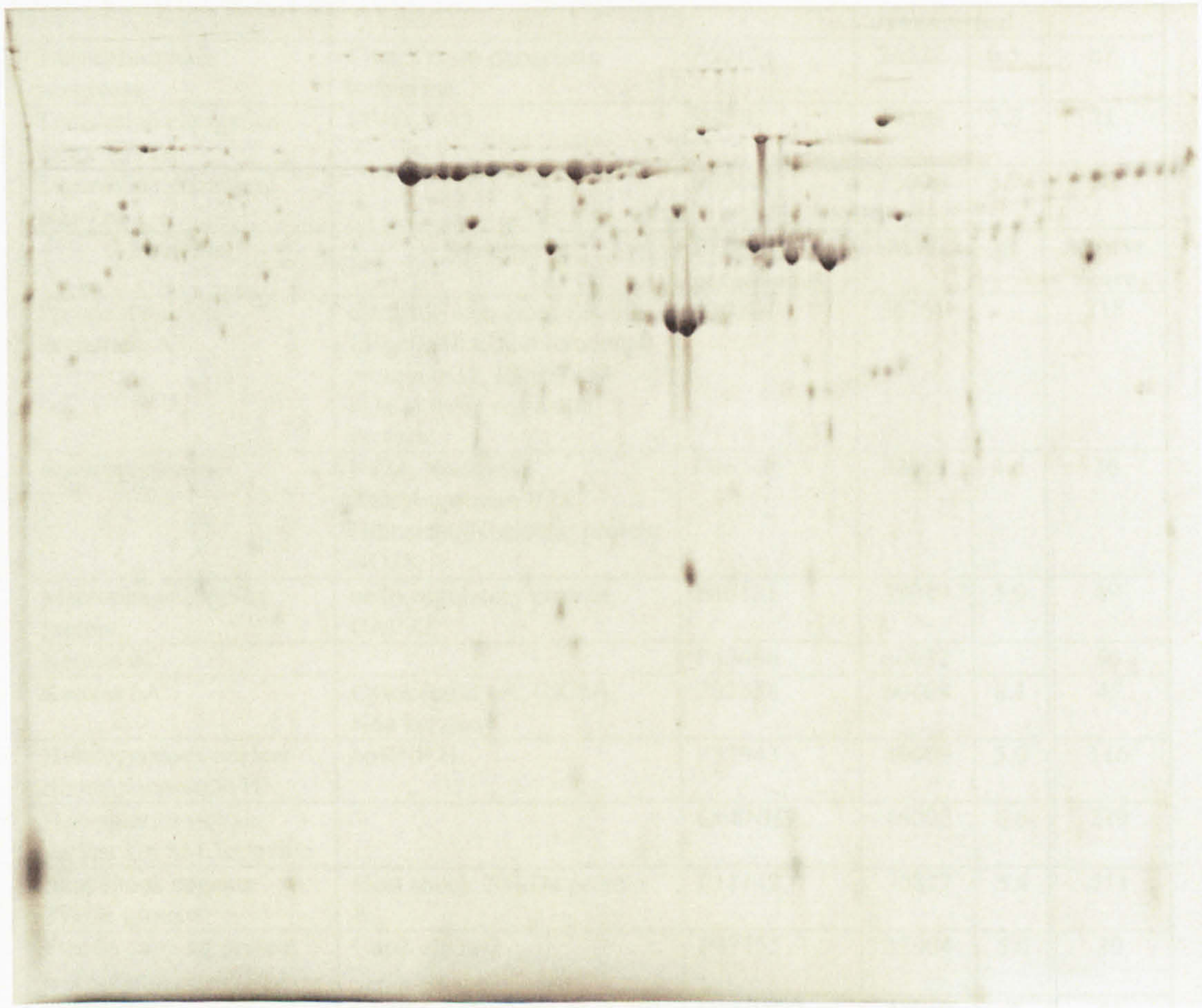


Figure 3 2D PAGE gel of protein extracted from week 12 foetuses. Obtained from two foetal pancreases of 12 week gestation which were pooled together.

Table 1 Differential expression of proteins between foetal pancreas at week 10 and week 12. Individual ions scores > 40 indicate identity or extensive homology ($p<0.05$). MW: molecular weight, pI: isoelectric focusing point, Mowse Score: molecular weight search, and #: number.

			Theoretical		
Triosephosphate isomerase	TIM, Triose-phosphate isomerase.	P60174	26522	6.5	67
Translation elongation factor EF-Tu	EF-Tu, P43.	S62767	49509	7.3	71
Superoxide dismutase [Cu-Zn]		P00441	15926	5.7	86
Proteins	**Synonyms**	**Primary accession #**	**MW**	**pI**	**Mowse Score**
Protein disulfide isomerase A3	disulfide isomerase ER-60, ERp60, 58 kDa microsomal protein, p58, ERp57, 58 kDa glucose regulated protein.	P30101	56761		218
Nucleophosmin	NPM, Nucleolar phosphoprotein B23, Numatrin, Nucleolar protein NO38.	P06748	32603	4.6	36
Macrophage capping protein	actin-regulatory protein CAP-G.	P40121	39949	5.9	49
Keratin 6C		P48666	60032		56
Keratin 6A	Cytokeratin 6A, CK 6A, K6a keratin.	P02538	60008	8.1	45
Heterogeneous nuclear ribonucleoprotein H	hnRNP H.	P31943	49099	5.9	116
Hemoglobin γ-chain variant Hb.M-Circleville		Q68NH9	16092	6.6	219
Heat shock cognate 71kDa protein	Heat shock 70 kDa protein 8.	P11142	70827	5.4	511
F-actin capping protein α-2 subunit	CapZ alpha-2.	P47755	32904	5.6	39
Calreticulin	CRP55, calregulin, HACBP, ERp60, grp60.	Q9UDG2	47019	7	41

Continuation from the previous page of the differential expression of proteins between foetal pancreas at week 10 and week 12. Individual ions scores > 40 indicate identity or extensive homology ($p<0.05$).

Proteins	Synonyms	Primary accession #	Theoretical MW	pI	Mowse Score
Annexin A5	Annexin V, lipocortin V, endonexin II, calphobindin I CBP-I,placental anticoagulant protein I, PAP-I, PP4, thromoboplastin inhibitor, vascular anticoagulant- α, VAC-α, anchorin CII.	P08758	35787	4.9	262
Annexin A6	Annexin VI, lipocortin VI, P68, P70, protein III, chromobindin 20, 67 kDa calelectrin, calphobindin-II, CPB-II.	P08133	75724	5.5	179
Collagen α-2 (VI) chain precursor		P12110	108269	5.8	56
Proliferation associated protein 2G4	Cell cycle protein P38-3G4 homolog, hG4-1.	Q9UQ80	43787	7.1	136
14-3-3 protein γ	Protein kinase C inhibitor protein -1, KCIP-1.	P61981	28171	4.8	209
Peroxiredoxin 2	Thioredoxin peroxidase 1, thioredoxin-dependent peroxide reductase 1, thiol-specific antioxidant protein, TSA, PRP, natural killer cell enhancing factor B, NKEF-B.	P32119	21873	5.7	114
Heat shock protein beta-1 (need more analysis)	Heat shock 27 kDa protein, HspB1, HSP 27, stress-responsive protein 27, SRP27, estrogen-regulated 24 kDa protein, 28 kDa heat shock protein.	P04792	22768	6.0	84
Iron responsive element binding protein 1	Citrate hydrolyase, aconitase.	P21399	98398	6.2	52
Heat shock protein HSP90-β	HSP 84, HSP 90.	P08238	98398	6.2	330
Elongation factor 1-δ	EF-1-δ, antigen NY-CO-4.	P29692	31203	4.9	53
Lamin B1	LMNB1, LMN2, LMNB.	P20700	66368	5.1	617
Acylamino acid releasing enzyme	AARE, Acyl-peptide hydrolase, APH, Acylaminoacyl-peptidase, oxidized protein hydrolase, OPH, DNF15S2 protein.	P13798	81274	5.3	54
Gelsolin, plasma	Actin-depolymerizing factor, ADF, grevin, AGEL.	P06396	85679	5.9	88
Caldesmon	CDM.	Q05682	93232	5.6	90

Continuation from the previous page of the differential expression of proteins between foetal pancreas at week 10 and week 12. Individual ions scores > 40 indicate identity or extensive homology ($p<0.05$).

Proteins	Synonyms	Primary accession #	Theoretical MW	pI	Mowse Score
Aldehyde dehydrogenase 1A1	Aldehyde dehydrogenase, cytosolic,.ALDH class 1, retinal dehydrogenase 1, ALHDII, ALDH-E1.	P00352	54712	6.3	695
T-complex protein 1, α–subunit	TCP-1-α, CCT-α.	P17987	60325	5.8	507
T-complex protein 1, γ-subunit	TCP1 ring complex protein TriC5, TCP-1- γ, CCT- γ.	P49368	60402	6.1	74
T-complex protein 1, ε-subunit	TCP-1-ε, CCT-ε.	P48643	59653	5.4	427
Dihydropyrimidinase related protein-2 (2 diff spots)	DRP-2, collapsing response mediator protein 2, CRMP-2, N2A3.	Q16555	62275	5.9	123
Dihydropyrimidinase related protein-3	DRP-3, Unc-33-like phosphoproteins, ULIP protein, collapsing response mediator protein 4, CRMP-4	Q14195	61945	6.4	204
Bifunctional purine biosynthesis protein PURK	OK/SW-cl.86, phosphoribosylaminoimidazolecarboxamide formyltransferase, EC 2.1.2.3, AICAR transformylase, IMP cyclohydrolase, EC 3.5.4.10, inosinicase, IMP synthetase, ATIC.	P31939	64447	6.8	250
Fibrinogen beta chain precursor	Fibrinopeptide B	P02675	55910	8.7	184
α-internexin	α-Inx, 66 kDa neurofilament protein, neurofilament-66, NF-66.	Q16352	55390	5.3	281
Serine/threonine protein phosphatase 2A	65 kDa regulatory subunit A, alpha isoform, (PP2A, subunit A, PR-alpha isoform), (PP2A, subunit A, R1-alpha isoform), medium tumor antigen-associated 61 kDa protein.	P30153	65092	5.0	53
Probable protein disulfide isomerase A3	Disulfide isomerase ER-60, Erp60, 58 kDa microsomal protein, p58, Erp57, 58 kDa glucose regulated protein.	P30101	56764	6.0	798
HSC70-interacting protein	Hip, putative tumor suppressor ST13, progesterone receptor-associated p48 protein.	P50502	41313	5.2	98
Heterogeneous nuclear ribonucleoprotein F	hnRNP F.	P52597	45653	5.5	104
Peripherin	PRPH.	P41219	53860	5.4	144

Continuation from the previous page of the differential expression of proteins between foetal pancreas at week 10 and week 12. Individual ions scores > 40 indicate identity or extensive homology ($p<0.05$).

Proteins	Synonyms	Primary accession #	Theoretical MW	pI	Mowse Score
Cytokeratin 8	Keratin 8, cytokeratin 8, K8, CK8.	P05787	53525	5.5	648
Cytokeratin 19	Cytokeratin 19, keratin 19, K19, CK19.	P08727	45870	5.2	549
α -centractin	Centratin, centrosome-associated actin homolog, actin-RPV,ARP1.	P61163	42595	6.2	98
NG,NG-dimethylarginine dimehtylaminohydrolase 2	Dimethylargininase 2, dimethylarginine dimethylaminohydrolase 2, DDAHII, G6a, S-phase protein.	O95865			
Rho GDP-dissociation inhibitor 2	Rho GDI 2, Rho-GDI beta, Ly-GDI.	P52566			
Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCH-L1, Ubiquitin thiolesterase L1, neuron cytoplasmic protein 9.5, PGP 9.5, PGP9.5.	P09936			
>2 fold increase expression in week 12					
*Alpha-2-Heremans Schmid glycoprotein	Fetuin A, α-2-Z-globulin, Ba-α-2-glycoprotein, PRO2743.	P02765	39306	5.6	144
*Serum albumin		P02768	69348	5.9	

such as actin and tubulin, and channel proteins such as chloride intracellular channel protein 1.

There were significant differences in the relative proportion of signalling and structural proteins between pancreas at week 10 and week 12 (Figure 4). The proteins were grouped into functional categories based on the information provided in the UniProt Knowledge base (European Bioinformatics Institute, UK).

Proteins found in both week10 and week12 pancreases are also present in the adult human islet protein database (Ahmed *et al*, 2005). They include calreticulin, superoxide dismutase (Cu-Zn), translation elongation factor EF-Tu, protein disulfide isomerase A3 precursor, triosephosphate isomerase, Tumor rejection antigen (TRA1 protein), transitional endoplasmic reticulum ATPase, heat shock protein HSP 90-β, heat shock cognate 71 kDa, 78 kDa glucose-regulated protein precursor, heat shock 70 kDa protein 1, 60 kDa heat shock protein, keratin 8, keratin 18, keratin 19, aldehyde dehydrogenase1A1, ATP synthase β-chain, Tubulin α-1, brain specific, protein disulfide isomerase A6 precurosor, α-enloase, β-actin, elongation factor 1-δ, guanine nucleotide binding protein β-subunit, annexin A5, and glutathione S-transferase P.

Specific examples of differences in protein expression patterns of 2D-PAGE are shown in Figure 5A-E. The protein/peptide spot or isoforms of the protein varied between week 10 and 12 samples. For example, serotransferrin isoforms increased in expression in week 12 with the protein caldesmon completely absent in week 10 samples (Figure 5A). Likewise, the alpha-2-Hereman Schmid (HS) glycoprotein, had a number of isoforms that were expressed at elevated levels in week 12 as compared to week 10 while other isoforms were relatively unchanged (Figure 5B). Cytokeratin proteins also displayed differences between week 10 and 12 samples. Cytokeratin 19 and 8 were absent at week 10 but present at week 12 while other proteins of similar

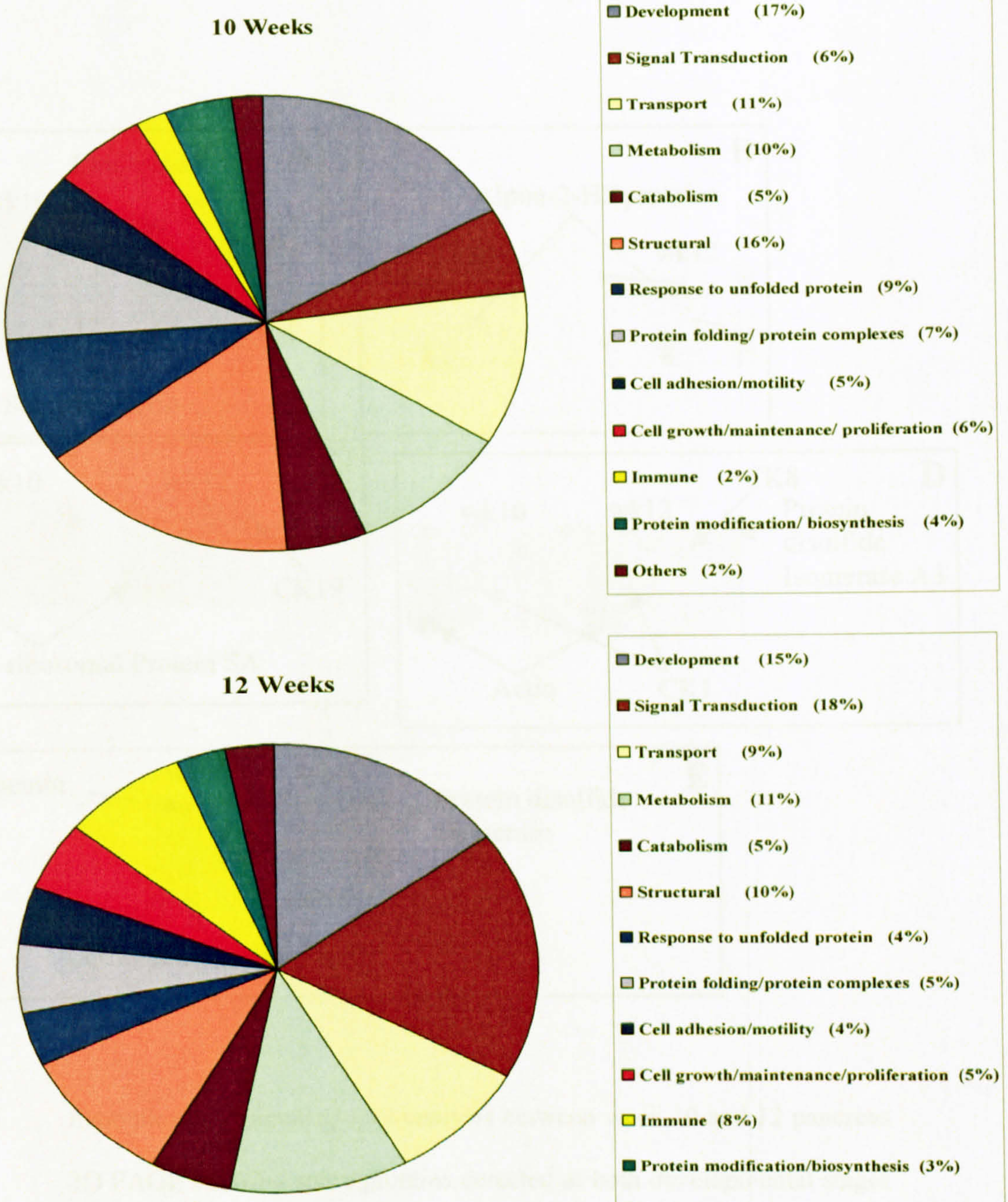


Figure 4 Pie chart of proportion of the different functions of proteins/peptides detected by mass spectroscopy in pooled samples of 10 and 12 week foetal pancreas after 2D-PAGE analysis. Based on the peptide sequence, the function of the protein was assigned according to the information provided by Uniprot knowledgebase database, European Bioinformatic Institute (www.ebi.ac.uk).

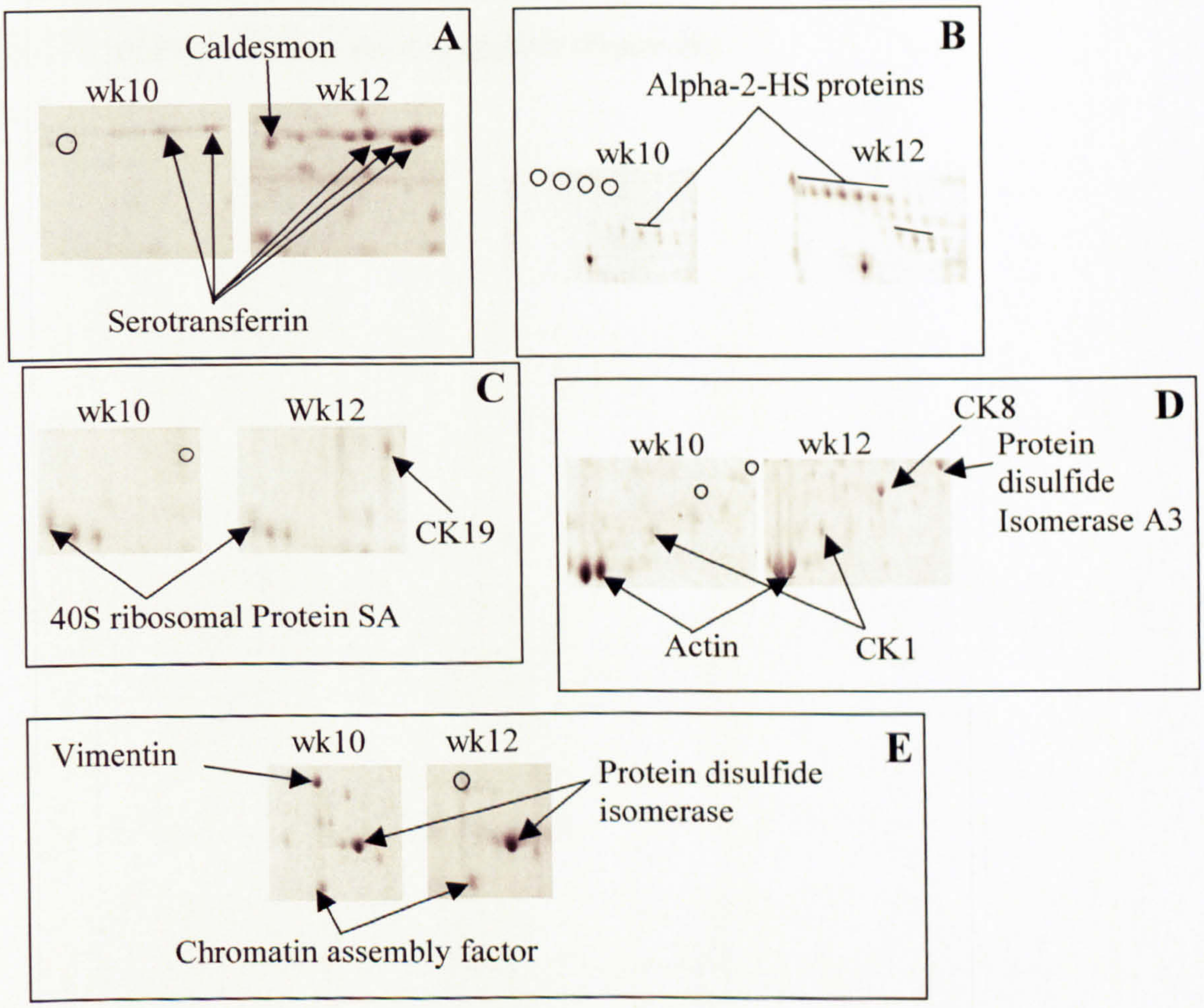


Figure 5 Examples of differential spot analysis between week 10 and 12 pancreas 2D PAGE showing some proteins detected at both developmental stages and proteins present or absent (open circle for position). The whole gel was closely examined for differences and spots recovered for analysis. (A) Caldesmon and serotransferrin (B) Alpha-2-HS proteins (C) CK19 (D) CK1, CK8, actin and protein disulfide isomerase A3 (E) Vimentin.

molecular weight were not significantly different including cytokeratin 1 and actin (Figure 5C and D). In contrast vimentin was present in week 10 sample but absent at week 12 compared with surrounding proteins (Figure 5E).

IV. DISCUSSION

Proteomics is the study of the protein expression profile of cells/tissues at the time of sampling. To date a number of proteomic studies on adult human pancreas (Hu *et al*, 2004; Shen *et al*, 2400) and adult islets (Ahmed *et al*, 2005) have been performed. The results on foetal human pancreas here have shown that there are a number of proteins that were also found in the adult pancreas, indicating that the use of proteomics is a proven technique in the study of foetal tissues.

There were significant differences in the relative proportion of signalling and structural proteins between pancreas at week 10 and 12 (Figure 4). This may reflect that the foetal pancreas at week 10 comprises mainly structural proteins, while at week 12 there were significant increases in signalling proteins and these may correspond to the increase in islet development and activity.

The use of the proteomics technique has shown that it can be used in the identifying differences between the foetal developmental stages. It has shown common

proteins between all different stages of pancreas as well as compared with the adult pancreatic proteome. A number of these proteins including calreticulin, superoxide dismuatase (Cu-Zn), Translation elongation factor EF-Tu, protein disulfide isomerase A2, triosephosphate isomerase, TRA1 protein, translational ER ATPase, heat shock protein 90-α, heat shock protein cognate 71 kDa protein, 78 kDa glucose regulated protein, cytokeratin 10, heat shock 70 kDa protein 1, 60 kDa heat shock protein, aldehyde dehydrogenase 1A1, ATP synthase b-chain, protein disulfide isomerase A6, cytokeratin 8, cytokeratin 18, α-enloase, cytokeratin 19, actin, EF1-δ, annexin A5 and GST-P were all expressed in adult human islets (Ahmed *et al*, 2005). It is unclear to whether all these proteins are involved in insulin synthesis and secretion.

No pancreatic enzymes were found in the foetal pancreas and may be due to their relatively low concentration in week 10 and week 12 foetal pancreases.

The protein, alpha-2-Hereman Schmid (HS) glycoprotein, has a number of isoforms that were expressed at elevated levels in week 12 as compared to week 10 (Figure 5b). The function of α-HS glycoprotein is uncertain but is found to be a natural inhibitor of insulin stimulated insulin receptor tyrosine kinase activity (IR-TK). The insulin receptor is found on cell surface and may play a physiological role in regulating insulin sensitivity, a condition of type II diabetes. Alpha-HS glycoprotein has been shown to inhibit autophosphorylation of the insulin receptor (Matthews *et al*, 2000) indicating that it may play a role in the regulation of insulin signaling and has been implicated in insulin resistance (Maegawa *et al*, 1991). It is interesting there was an increase in alpha HS glycoprotein corresponds with the insulin secretion and activity in week 12 pancreas.

Keratins 1, 6a, 6C, 8, 9, 10, 18 and 19 were found in the pancreatic tissues between the two samples. While keratins 1 and 10 were most likely to be contaminants

because they showed greater similarity to bovine keratins so they might get it from the media (FCS), keratins 6, 8, 9, 18 and 19 may play an important role in pancreatic development.

Keratins are usually expressed in pairs, for example, keratin 1 and 10, keratin 8 and 18/19, and can be tissue-specific and differentiation-specific manner (Bouwens, 1998). The intermediate filament protein, cytokeratin 19 (CK19), is also of interest as it has been associated with pancreatic stem cells (Kang *et al*, 2005). Bouwens et al have shown, by immunohistochemistry, that CK19 is present in human foetal pancreatic epithelial cells and islet cells from week 12 to 16 (Bouwens *et al*, 1997). They have also shown that acinar and islet cells progressively losses the CK19 expression but the islet cells remains weakly antigenic until the end of gestation. Immunohistostaining showed that CK19 was also expressed on epithelial cells lining the ductal structures of week 7 to week 12 foetal pancreas (Chapter III) and by week 10 cells around the ductal region start to synthesize insulin and appear to migrate away from the ductal structures by week 12 (Chapter III). However, the week 10 2D gels failed to identify CK19, but this may be due to smaller amount of the ductal structures in week 10 as compared to week 12 foetal pancreas.

When the proteins were categorized according to their functions, it revealed that there were little change in the relative proportions of proteins involved in transport, metabolism, catabolism, protein folding/protein complexes, cell adhesion/motility, cell growth/ maintenance/ proliferation, protein modification and others (Figure 4 A and B). There were a relatively high proportion of structural proteins in week 10, which were expected as the major proportion of the pancreas consists of supporting and not specialized cell types.

Comparing the foetal protein data with those of the adult pancreas revealed that a lot of the foetal pancreatic proteins were not found in the adult pancreas. This may indicate that the proteins in the foetal pancreas may be involved in pancreatic development whilst those of the adult may be involved in pancreatic maintenance (Ahmed *et al*, 2005).

CHAPTER SIX

GENERAL DISCUSSION

GENERAL DISCUSSION

1. Immunofluorescent Localisation of Insulin-Containing Cells Derived from Human Embryonic Stem (hES) Cells

A principal aim of this study was to generate insulin-secreting cells from human embryonic stem (hES) cells. If this process could be made efficient and robust then such cells might be used in the future for regenerative cell therapies to treat type I diabetes. In this disease, insulin-secreting cells of the islets of Langerhans are progressively lost from the pancreas due to a degenerative auto-immunity. Various growth factors were used to direct the differentiation of hES cells towards insulin-containing cells in a defined tissue culture protocol involving embryoid body (EB) formation. The factors were selected on the basis of their importance in the process of pancreatic differentiation in mammals and on the findings of previous studies *in vitro*. EBs formation were considered as a good step to start

the differentiation of cells, where cells in EBs can mimic those in the islets by way of cell to cell communication (Segev *et al*, 2004).

Some spontaneous differentiation of hES cells in culture towards insulin producing cells was observed, however the number of cells expressing insulin was very low. A significantly greater number of cells apparently expressing insulin was obtained when culture medium was supplemented with nicotinamide alone, nicotinamide + FGF2, nicotinamide + activin A and activin A + FGF2 compared with medium without growth factor supplements or various other combinations of growth factors (Chapter II). These findings are supported by a number of reports indicating that nicotinamide, FGF2 and activin A all have a positive effect on the generation of insulin-secreting cells from ES cells.

The addition of nicotinamide as a growth factor to the cultured ES cells has been reported to be important in the differentiation of cells into insulin producing cells (Santana *et al*, 2006) although there are few studies of using nicotinamide alone as a supplement with culture medium. In the mouse model, Vica and co-workers (2007) showed that undifferentiated mouse ES cells treated with nicotinamide alone exhibited an 80% decrease in cell proliferation but produced a progenitor cell phenotype that co-expressed insulin, C-peptide and Glut-2. These cells after transplantation into diabetic mice, normalized blood glucose levels over a 7-week period. But exactly how nicotinamide exerts an action on ES cells to influence insulin-secreting cell differentiation remains unclear. Nicotinamide can act to remove free radicals that can attack cell membranes; is a component of the coenzyme NAD; and may act on DNA repair pathways (Knip *et al*, 2000). However, none of these processes are thought to be specific to pancreatic differentiation or ES cells. High doses of

nicotinamide seem to have a protective effect on β-cell survival and function in animal and during *in vitro* studies (Knip *et al*, 2000), and of relevance to the present study, nicotinamide stimulated formation of cultured foetal human islet-like cell clusters (Sandler *et al*, 1989). This protective action of nicotinamide suggests that it may prevent degeneration or apoptosis of insulin-expressing cells once they have formed rather than directly acting on hES differentiation. Thus nicotinamide may increase the number of cells surviving in culture and in this way indirectly support FGF2 and activin A.

FGF2 play a role during definitive endodermal development to pancreas and therefore is more likely to have a specific action on hES cell differentiation. However, it may not necessarily improve the survival of cells expressing insulin once they are generated. For example, Segev and colleagues demonstrated that nicotinamide and FGF2 together increased the insulin content in culture of differentiated β-cell-like cells from mouse ES cells after the formation of EBs (Segev *et al*, 2004).

As hedgehog signaling inhibits tissue morphogenesis and cell differentiation during early stages of pancreas organogenesis, it is required to maintain adult endocrine cell functions (Hebrok, 2002). However, FGF2 has the ability to repress endodermal hedgehog signals and allows the expression of pancreatic Pdx-1 and insulin genes (Hebrok *et al*, 1998; Dilorio *et al*, 2003).

Transforming growth factor β, TGFβ, signaling pathway is involved in many cellular processes like cell growth, proliferation, differentiation apoptosis and cellular homeostasis (Riedy *et al*, 1999; Florio *et al* 2000). Activin A is a member of TGFβ proteins. Furthermore, TGFβ and activin A are important proteins during the development of human embryo (Riedy *et al*, 1999; Florio *et al* 2000).

Activins are required for the specialization of pancreatic precursors and inactivation of activins enhance expansion of epithelial pancreatic cells and decrease the number of differentiated β-cells (Zhang *et al*, 2004).

Activin A was also included as a supplement in the present experiments to enhance the differentiation of hES cells towards insulin secreting β-cell-like cells. Activin A may act to prevent self - renewal of hES cells and promote differentiation particularly to endoderm. Recently, it was shown by D'Amour and colleagues that activin A has the ability to direct the differentiation of hES cells towards endocrine progenitor cells even if they are in low numbers (D'Amour *et al*, 2006). However unlike these investigators, EB culture was used instead of an adherent culture method. It has been postulated that for efficient endoderm differentiation of hES cells it is better to avoid EB formation, as this may hinder uniform exposure of cells on the interior of the aggregates to medium and factors (D'Amour *et al*, 2006). This may be one reason why the proportion of insulin expressing cells in EB culture was relatively low compared to the D'amour protocol.

In the current investigation, dithizone (DTZ) was used to check the presence of insulin containing cells in culture, which had been differentiated from hES cells. While this stain is not specific to insulin-secreting cells (as it detects zinc content rather than insulin) it still remains a good method of monitoring putative β-cells because of its ease of use. The results obtained from DTZ staining showed different percentages of cells stained red in human EBs colonies with the various growth factors and their combinations. Comparable cultures were then followed by immunostaining to confirm the presence of cells expressing insulin in culture. A similar procedure was used by others to detect insulin –secreting cells (Shiroi *et al*, 2005; D'Amour *et al*, 2006). However it must be taken into account that

insulin containing cells may result from insulin absorption from culture medium and it is important to also detect C-peptide or hormone for other endocrine cells (i.e glucagon). Blyszczuk concluded that nestin and C-peptide expression was important throughout the pathway of development of β-cell-like cells from ES cells to establish the presence of endocrine progenitor cells (Blyszczuk *et al*, 2004). Some investigators contend that differentiation of ES cells into insulin-secreting cells need development through a neuronal-like differentiation with cells expressing nestin followed by insulin later in differentiation (Brolén *et al*, 2005; Santana *et al*, 2006).

Thus, DTZ staining was used in this study as a preliminary check for the presence of potential insulin-producing cells in EBs colonies. This was followed by immunostaining for nestin, glucagon (to detect progenitor immature insulin-producing cells), C-peptide and insulin (to detect mature insulin producing cells) to confirm the differentiation of hES cells towards an endocrine lineage.

In summary some cells expressed the characteristics of insulin-expressing cells after hES differentiation to EBs but incubation periods longer than 14 days may be required in future to produce cells with greater insulin content and secretory capacity.

2. Measurement of C-Peptide Secretion *In vitro* from ES-Derived Cell and Foetal Pancreatic Cells

To quantify the amounts of insulin secreted from the cultured hES cells grown with different growth factors, a human C-peptide ELISA was used and comparisons made with cultures from foetal pancreatic tissue recovered from 8-12 weeks gestation. Although the ELISA assay was reproducible and consistent, it was not very sensitive, so that even when

C-peptide was detectable it was at the lower end of the standard curve. A similar ELISA assay was used by D'Amour and colleagues, who differentiated hES cells into endocrine precursor cells and measured C-peptide levels (D'Amour *et al*, 2006).

After various incubation periods, foetal pancreatic cultures exhibited islet-like cell clusters, which were very similar to those described previously by others for both foetal and adult pancreatic tissue (Sandler *et al*, 1989; Gao *et al*, 2003). Clusters of cells budded from tissue outgrowths showing ductal epithelial morphology and staining relatively intensely with DTZ stain. However these foetal cultures showed very limited proliferative capacity. The amount of C-peptide detected in culture supernatants was generally greater in foetal tissues of older gestational age indicating that pancreatic tissue before weeks 11-12 of foetal age secreted very little insulin. This observation was consistent with the immuno-histological staining of pancreatic tissue of equivalent developmental age (chapter III), indicating far fewer C-peptide/insulin positive cells present before 10 weeks while more insulin positive islet clusters by 12 weeks of age. However, prolonged incubation of tissue also resulted in a decrease of C-peptide in the culture supernatant when removed at regular intervals. This demonstrated a gradual failure of C-peptide/insulin secretion by cells in culture possible due to the environmental differences between *in vitro* and *in vivo* conditions. Several factors may be important for developing and maintaining islet –like cell buds *in vitro*. In the present study, culture medium was supplemented with foetal calf serum, which promoted cell attachment and outgrowths. However, it had been reported previously that serum suppresses islet buds in culture of adult pancreas tissue samples (Gao *et al*, 2003). Extracellular matrix components may also be important since Matrigel aided adult pancreatic cultures (Gao *et al*, 2003).

While immunostaining indicated that some cells that differentiated from hES cells into EBs under various growth factor conditions expressed C-peptide and were ß cell-like it was unclear what quantity of C-peptide/insulin they might secrete. Hence by using the ELISA technique it was considered it might be possible to determine the amounts of C-peptide such differentiated cells secreted in comparison with equivalent amounts of foetal pancreatic tissue in culture. However the amounts of C-peptide in supernatants of EBs were undetectable (not significantly different from zero control).

In conclusion, it was possible to detect a proportion of cells exhibiting C-peptide/ insulin expression that differentiated from human EBs in culture, but in comparison to foetal tissue there was no indication of C-peptide in culture supernatant suggesting that hormone secretion was absent. On the other hand C-peptide levels in foetal tissue cultures generally increased with the gestational age of the sample.

To ensure of the presence of some insulin like cells from the EBs cells, derived from hES cells, grown with different growth factors, endocrine gene expression by RT-PCR for some selected genes like Pdx-1, Ngn3, GK, Glut-1 and 2 and insulin was applied.

3. Pattern of mRNAs Expression Related to β-cell Development from hES Cells

After differentiation of hES cells in culture it was important to show that these cells expressed genes that would be present in cells of the pancreatic cell lineage and ß- cells including insulin, glucagon, somatostatin, pancreatic polypeptide, ghrelin, Pdx-1, Ngn-3, Nkx2.2 and Pax-4 (D'Amour *et al*, 2006).

During human foetal pancreas development, Pdx-1 expression decreased slowly from the early development of pancreatic progenitors until they reached mature β-cells,

where the expression of this gene increased again (Brolén *et al*, 2005; D'Amour *et al*, 2006). In the present study, after incubation of hES cells in culture for two weeks, Pdx-1 and insulin genes were observed as concentrated sharp bands in almost all examined samples grown with different growth factors, especially nicotinamide and nicotinamide + FGF2, respectively. These results may indicate that all the growth factors selected in this study encouraged the EB cells toward insulin-containing cells.

On the other hand, other genes like Ngn-3, Nkx6.1 and Pax-4 were also found to be expressed during early stages of the development of the pancreas as well as in the progenitor endocrine cells (D'Amour *et al*, 2006; Shiroi *et al*, 2005). However, as these genes expressed in the progenitor cells of the pancreas there were selected to be examined in the cultured EBs grown with different growth factors for 14 days in the present study. The expression of these genes in the cultured EBs confirms the pathway of differentiation of the hES cells from endocrine progenitor cells towards later mature β-cell-like cells.

4. Protein Expression Patterns During Development of the Foetal Pancreas Between 10 and 12 weeks Gestation

Protein analysis was applied in this study to compare human foetal pancreases at two different gestational ages, 10 and 12 weeks, where this may help in understanding the pancreatic development at the protein level. However, to date there is no similar work that uses proteomic profiles to explain the development of the pancreas.

The development of proteomic technology gives the chance to express genetic variants at the protein level (Valmu *et al*, 2006). Recently, proteomic profiling by using serum markers was used to diagnose pancreatic cancer at early stage (Chen *et al*, 2007; Pappas *et al*, 2007).

Quantitative protein analysis of some pancreatic proteins extracted from pancreatic cancer patients and normal control was detected (Chen *et al*, 2006). With proteomic technology used by Chen group for the diagnosis of early pancreatic cancer 116 differentially expressed proteins were detected from human patients (Chen *et al*, 2007).

Proteomic analysis was employed to identify cellular proteins that interact with the core protein of Hepatitis C virus (HCV) (Kang *et al*, 2005). Cytokeratin 8, cytokeratin 19 and cytokeratin 18 were identified as intermediate microfilament proteins that involved in the infection processes of the HCV disease (Kang *et al*, 2005).

In the present study, proteins which where expressed in 10 weeks foetuses where more likely to be structural proteins, while the ones expressed in 12 weeks foetuses seems to be signalling proteins and this is may be due to the developmental and activity of the pancreas at late stages.

However, it has been found that there were proteins which displayed expression in 12 weeks foetuses, but not 10 weeks, and were found in adult pancreas as well (Ahmed *et al*, 2005). There were no pancreatic enzymes in the foetal pancreas and this is may be due to their relatively low concentration in week 10 and week 12 foetal pancreas.

By comparing foetal proteins with those of adult pancreas it has been indicated that most of the foetal pancreas proteins do not appear in the adult pancreas. Thus, most of foetal pancreas proteins are involved in the development of pancreas where in adult they more likely to be involved in the maintenance of pancreas function.

By using a proteomics approach in the future it may be possible to identify proteins that can characterise each stage of pancreatic development.

6. Conclusion

The EB, a three dimensional structure, , has been used in many different studies to direct the differentiation of ES cells towards different lineages (Perkins *et al*, 1998; Soria *et al*, 2000; Assady *et al*, 2001; Lumelsky, 2001; Conley *et al*, 2004; Kim *et al*, 2007; Peiffer *et al*, 2007). In the EB colony, cells have the chance to communicate and react with each other as they do *in vivo*.

Differentiation of ES cells by forming EB towards endothelial cells was done by Kim group (Kim *et al*, 2007). The purpose of their study was to compare between endothelial cells isolated from the centre of the EB colony and the cells isolated from the outgrowth of the EB colony. They reached to the conclusion that cells which were isolated from the centre of the EB colony showed higher number of endothelial cells than the cells isolated from the outgrowth of the EB colony (Kim *et al*, 2007).

Differentiation of the ES cells and human pancreatic β-cells towards different cell lineages has also been carried out by the formation of a monolayer differentiated cells (Schuldiner *et al*, 2000; Lachner *et al*, 2004; Karner *et al*, 2007). However, in this case all the cultured cells have the chance to be exposed to the same amount of growth factor/s in use, CO_2 and light during their incubation period in culture.

Using different growth factor/s may help in directing the differentiation of ES cells into a specific cell line (Schuldiner *et al*, 2000; Loebel *et al*, 2003; Cho *et al*, 2008). In addition, these growth factors can be used at different stages during the incubation of the cells *in vitro* (Hori *et al*, 2002; Bai *et al*, 2005; Beattie *et al*, 2005). For example, in the present study the growth factors were used from day one of the formation of EB colony and continued to the end of day 7 when media were changed to a normal EB medium (Chapter

II section 1.2.3) for another 7 days. These growth factors can also be used after few days of the differentiation, the formation of EB colony (Houard *et al*, 2003; Segev *et al*, 2003).

On the other hand, a selection/purification process by selecting specific cells from the cultured differentiated cells may generate a pure pool of one cell type of differentiated cells. Moreover, selected cells can then be encouraged, by using different growth factor/s, to keep their differentiation until they reach to the maturation step (Blyszczuk *et al*, 2003; Baharvand *et al*, 2006).

In summary, the differentiation *in vitro* of human ES cells into insulin containing, β-cell-like has been successfully achieved. Moreover, the growth factors, nicotinamide FGF2 and activin A were shown to significantly increase the proportion of such cells in culture. However, cultures displayed an absence of C-peptide in the supernatant, indicating that these β-like cells fail to secrete insulin. By comparison cultures of an equivalent numbers of cells from foetal pancreatic tissue exhibited detectable levels of C-peptide in culture supernatant at later gestational ages (week 10-12). Overall these findings suggest that β-like cells generated from hES cells are non-functional. Further studies are required to determined their precise phenotype and further optimize the culture conditions to generate true β-cells from hES cells.

REFERENCES

REFERENCES

Ackermann, A. and Gannon, M. (2007). Molecular Regulation of Pancreatic β-cell Mass Development, Maintenance and Expantion. Journal of Molecular Endocrinology; 38: 193-206.

Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P., Beighton, G., Bello, P., Benvenisty, N., Berry, L., Bevan, S., Blum, B., Brooking, J., Chen, K., Choo, A., Churchill, G., Corbel, M., Damjanov, I., Draper, J., Dvorak, P., Emanuelsson, K., Fleck, R., Ford, A., Gertow, K., Gertsenstein, M., Gokhale, P., Hamilton, R., Hampl, A., Healy, L., Hovatta, O., Hyllner, J., Imreh, M., Itskovitz-Eldor, J., Jackson, J., Johnson, J., Jones, M., Kee, K., King, B., Knowles, B., Lako, M., Lebrin, F., Mallon, B., Manning, D., Mayshar, Y., McKay, R., Michalska, A., Mikkola, M., Mileikovsky, M., Minger, S., Moore, H., Mummery, C., Nagy, A., Nakatsuji, N., O'Brien, C., Oh, S., Olsson, C., Otonkoski, T., Park, K., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M., Piekarczyk, M., Pera, R., Reubinoff, B., Robins, A., Rossant, J., Rugg-Gunn, P., Schulz, T., Semb, H., Sherrer, E., Siemen, H., Stacey, G., Stojkovic, M., Suemori, H., Szatkiewicz, J., Turetsky, T., Tuuri, T., van den Brink, S., Vintersten, K., Vuoristo, S., Ward, D., Weaver, T., Young, L., Zhang, W. (2007). Characterization of Human Embryonic Stem Cell Lines by the International Stem Cell Initiative. Nature Biotechnology; 25(7): 803-816.

Ahmed, M., Forsber, J. and Bergsten, P. (2005). Protien Profiling of Human Pancreatic Islets by Two- Dimensional Gel Electrophoresis and Mass Spectrometry. Journal of Proteome Research; 4: 931-940.

REFERENCES

D'Amour, K., Bang, A., Eliazer, S., Kelly, O., Agulnick, A., Smart, N., Moorman, M., Kroon, E., Carpenter, M. and Baetge, E. (2006). Production of Pancreatic Hormone-Expressing Endocrine Cells from Human Embryonic Stem Cells. Nature Biotechnology, 23.

Amiel, S. (2001). Islet Transplantation. Diabetic Medicine; 18: 77.

Amit, M. and Itskovitz-Eldor, J. (2002). Derivation and Spontaneous Differentiation of Human Embryonic Stem Cells. Journal of Anatomy; 200: 225-232.

Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. and Tzukerman, M. (2001). Insulin Producing by Human Embryonic Stem Cells. Diabetes, 50: 1691-1697.

Baertschiger, R., Morel, P. and Berney, T. (2006). Islets of Langerhans or Pancreas Transplantation in the Treatment of Type 1 Diabetes. Rev Med Suisse; 2(68): 1462-1466.

Baharvand, H., Jafary, H., Massumi, M. and Ashtiani, S. (2006). Generation of Insulin-Secreting Cells from Human Embryonic Stem Cells. Development, Growth & Differntiation; 48: 323-332.

Bai, L., Meredith, G. and Tuch, B. (2005). Glucagon-like peptide-1 enhances production of insulin in insulin-producing cells derived from mouse embryonic stem cells. Journal of Endocrinology; 186: 343-352.

Beattie, G., Rubin, J., Mally, M., Otonkoski, T. and Hayek, A. (1996). Regulation of Proliferation and Differentiation of Human Fetal Pancreatic Islet Cells by

Extracellular Matrix, Hepatocyte Growth Factor, and Cell-Cell Contact. Diabetes; 45(9): 1223-1228.

Bell, P., Wood, R., Peters, M. and Nash, J. (1980). Comparison of Various Methods of Chemical Immunosuppression in Islet Cell Transplantation. Transplantational Proceedings; 12(2): 291-293.

Bennett, N. (2002). Monitoring Techniques for Diabetes Mellitus in the Dog and the Cat. Clinical Techniques in Small Animal Practice; 17(2): 65-69.

Bertram, R. and Sherman, A. (2004). Filtering of Calcium Transients by the Endoplasmic Reticulum in Pancreatic β-Cells. Biophysical Journal; 87: 3775-3785.

Bertuzzi, F., Marzorati, S. and Secchi, A. (2006). Islet Cell Transplantation. Current Molecular Medicine; 6(4): 369-374.

Blyszczuk, P., Asbrand, C., Rozzo, A., Kania, G., ST-Onge, L., Rupnik, M. and Wobus, A. (2004). Embryonic Stem Cells Differentiate into Insulin-Producing Cells without Selection of Nestin-Expressing Cells. International Journal of Developmental Biology; 48: 1095-1104.

Bodnar, C., Sen, A., Kallos, M., Behie, L., Petropavlovskaia, M. and Rosenberg, L. (2006). Characterization of Human Islet-Like Structures Generated from Pancreatic Precursor Cells in Culture. Biotechnology and Bioengineering; 93(5): 980-988.

Boeuf, V., Jublot, D., Loizon, S., Barale, J., Akanmori, B., Mercereau-Puijalon, O and Behr, C. (2005). CyProQuant-PCR: a Real Time RT-PCR Technique for Profiling Human Cytokines, Based on External RNA Standards, Readily Automatable for Clinical Use. BMC Immunology; 6: 5.

REFERENCES

Bonner-Weir, S., Taneja, M., Weir, G., Tatarkiewicz, K., Song, K., Sharma, A. and O'Neil, J. (2000). *In Vitro* Cultivation of Human Islets from Expanded Ductal Tissue. PNAS; 97 (14): 7999-8004.

Böttcher, R. and Niehrs, C. (2005). Fibroblast growth factor signaling during early vertebrate development. Endocrinology Revision; 26(1): 63-77.

Bouwens, L. (1998). Transdifferentiation Versus Stem Cell Hypothesis for the Regeneration of Islet Beta-Cells in the Pancreas. Journal of Pathology. 184: 234-239.

Bouwens, L., Lu, W. and De Krijger, R. (1997). Prolifiration and Differentiation in the Human Fetal Endocrine Pancres. Diabetologia; 40(4): 398-404.

Brissova, M., Michael, J., Fowler, T., Nicholson, W., Chu, A., Hirshberg, B., Harlan, D. and Powers, A. (2005). Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy .Journal of Histochemistry and Cytochemistry; 53(9): 1087-1097.

Brolén, G., Heins, N., Edsbagge, J. and Sembl, H. (2005). Signals from the Embryonic Mouse Pancreas Induce Differentiation of Human Embryonic Stem Cells into Insulin-Producing-Cell–Like Cells. Diabetes; 54: 2867-2874

Calafiore, R., Basta, G., Luca, G., Lemmi, A., Racanicchi, L., Mancuso, F., Montanucci, M. and Brunetti P. (2006). Standard Technical Procedures for Microencapsulation of Human Islets for Graft into Nonimmunosuppressed Patients with Type 1 Diabetes Millitus. Transplantation Proceeding.; 38(4): 1156-7.

REFERENCES

Cerf, M. (2006). High Fat Diet Modulation of Glucose Sensing in the Beta-Cell. Medical Science Monitor; (1)13: 12-17.

Cerf, M., Muller, C., Du Toit, D., Louw, J. and Wolfe-Coote, S. (2007). Hyperglycaemia and Reduced Glucokinase Expression in Weanling Offspring from Dams Maintained on a High-Fat Diet. Biochemical and Biophysical Research Communications; (4)326: 699-702.

Chen, R., Brentall, T., Pan, S., Cooke, K., White Moyes, K., Crispin, D., Goodlett, D., Aebersold, R. and Bronner, M. (2007). Quantitative Proteomic Aanalysis Reveals that Proteins Differentially Expressed in Chronic Pancreatitis are also Frequently Involved in Pancreatic Cancer. Molecular and Cellular Proteomics; 6(8): 1331-1342.

Chen, R., Pan, S., Yi, E., Donohoe, S., Bronner, M., Potter, J., Goodlett, D., Aebersold, R. and Brentnall, T. (2006). Quantitative Proteomic Profiling of Pancreatic Cancer Juice. Proteomics; 6(13): 3871-3879.

Cho, Y.; Lim, J.; Yoo, D.; Kim, J.; Chung, S.; Park, S.; Kim, T.; Oh, S.; Choi, Y.; Moon, S.; Park, K. and Lee, H. (2008). Betacellulin and nicotinamide sustain *PDX1* expression and induce pancreatic β-cell differentiation in human embryonic stem cells. Biochem Biophys Res Commun; 366(1): 129-34.

Clark, G., Yochem, R., Axelman, J., Sheets, T., Kaczorowski, D. and Shamblott, M. (2007). Glucose Responsive Insulin Production from Human Embryonic Germ

REFERENCES

(EG) Cell Derivatives. Biochemical and Biological Research Communications; 356: 587-593.

Clark, S., Borland, K., Sherman, S., Rusack, T. and Chick, W. (1994). Staining and *In vitro* Toxicity of Dithizone with Canine, Porcine and Bovine Islets. Cell Transplantation; 3(4): 299-306.

Conget, J., Sarri, Y., Gonzalez-Clemente, J., Casamitjana, R., Vives, M. and Gomis, R. (1994). Deleterious Effect of Dithizone-DMSO Staining on Insulin Secretion in Rat and Human Pancreatic Islets. Pancreas; 9(2): 157-160.

Cuesta-Muñoz, A., Huopio, H., Otonkoski, T., Gomez-Zumaquero, J., Näntö-Salonen, K., Rahier, J., López-Enriquez, S., García-Gimeno, M., Sanz, P., Soriguer, F. and Laakso, M. (2004). Severe Persistent Hyperinsulinemic Hypoglycemia Due to a De Novo Glucokinase Mutation. Diabetes; 53(8): 2164-2168.

DiIorio, P., Alexa, K., Choe, S., Etheridge, L., Sagerström, C. (2003). TALE-Family Homeodomain Proteins Regulate Endodermal Sonic Hedgehog Expression and Pattern the Anterior Endoderm. Developmental Biology; 304 (1): 221-231.

Docherty, K. (2001). Growth and Development of the Islets of Langerhans: Implications for the Treatment of Diabetes Mellitus. Current Opinion in Pharmacology; 1: 641-650.

Docherty, K. (1996). Transgenic animals. Proceedings of the Nutrition Society.; 55(1B): 613-8.

Dodson, G. and Steiner, D. (1998). The Role of Assembly in Insulin's Biosynthesis. Current Opinion in Structural Biology, 8: 189-194.

REFERENCES

Dodson, S., Bernard, G., Kenney, E. and Carranza, F. (1996). *In vitro* Comparison of Aged and Young Osteogenic and Hemopoietic Bone Marrow Stem Cells and Their Derivative Colonies. Journal of Periodontology; 67(3): 184-96.

Donovan, P. and Gearhart, J. (2001). The End of the Beginning for Pluripotent Stem Cells. Nature; 414: 92-97.

Draper, J. and Victoria, F. (2003). Human Embryonic Stem Cells: Multilineage Differentiation and Mechanisms of Self-Renewal. Hematopoietic Stem Cells; 34(6): 558-564.

Draper, J. and Andrews, P. (2002). Embryonic Stem Cells: Advances toward Potential Therapeutic Use. Current Opinion in Obstetrics and Gynecology; 14: 309-315.

Draper, J. and Victoria, F. (2003). Human Embryonic Stem Cells: Multilineage Differentiation and Mechanisms of Self-Renewal. Hematopoietic Stem Cells; 34(6): 558-564.

Edlund, H. (1999). Pancreas: How to Get There from the Gut. Current Opinion in Cell Biology; 11: 663-668.

Edlund, H. (2001). Factors Controlling Pancreatic Cell Differentiation and Function. Diabetologia; 44 (9): 1071-1079.

Efrat, S. (2002). Cell Transplant Therapy for Type 1 Diabetes. TRENDS in Molecular Medicine; 8(7): 334-339.

Evans, A. and Kaufman, M. (1981). Establishment in Culture of Pluripotential Cells from Mouse Embryos. Nature; 292(5819): 154-6.

Felig, D. and Geuder, J. (2001). Gastric Outlet Obstruction Due to a Large Duodenal Tubulovillous Adenoma. Gastrointestinal Endoscopy; 53(3): 340-1.

REFERENCES

Feraud, O. and Vittet, D. (2001). Embryonic Stem Cell-Derived Embryoid Bodies Developed in Collagenase Gels Recapitulates Sprouting Angiogenesis. Laboratory Investigation; 81(12): 1669-1681.

Fiedor, P., Oluwole, S. and Hardy, M. (1996). Localization of Endocrine Pancreatic Islets. World Journal of Surgery; 20: 1016-1023.

Fiedor, P., Rowinski, W., Licinska, I., Mazurek, A. and Hardy, M. (1995). The Survival Identification of Pancreatic Islets of Langerhans: *In vitro* and *in vivo* Effects of Two Dithizone Preparations on Staining of Rat and Human Islets of Langerhans-Preliminary Study (Part I). pharmaceutical Engineering; 52(5): 431-436.

Fishman, J., Kubak, B. (2002). Clinical Cases in Transplantation. Transplant Infectious Disease; 4(3): 62-66.

Florio, P., Luisi, S., Marchetti, P., Lupi, R., Cobellis, L., Falaschi, C., Sugino, H., Navalesi, R., Genazzani, A. and Petraglia, F. (2000). Activin A Stimulates Insulin Secretion in Cultured Human Pancreatic Islets. Journal of Endocrinol. Invest; 23 (4): 231-4.

Freeman, W., Walker, S. and Vrana, K. (1999). Quantitative RT- PCR: Pitfalls and Potential. BioTechniques; 26 (1): 112- 125.

Fry, M. (2001). Phosphoinositide 3-Kinase Signaling in Breast Cancer: How Big a Role Might it Play?. Breast Cancer Research; 3: 304-312.

Fukazawa, T., Matsuoka, J., Naomoto, Y., Nakai, T., Durbin, M., Kojima, I., Lakey, J. and Tanaka, N. (2006). Development of a Novel β-Cell Specific Promoter System for the Identification of Insulin-Producing Cells in I*n vitro* Cell Cultures. Experimental Cell Research; 312: 3404-3412.

REFERENCES

Fujikawa, T., Oh, S., Pi, L., Hatch, H., Shupe, T. and Petersen, B. (2005). Teratome Formation Leads to Failure of Treatment for Type I Diabetes Using Embryonic Stem Cell-Derived Insulin-Producing Cells. American Journal of Pathology; 166 (6): 1781-1791.

Furuya, M., Yasuchika, K., Mizutani, K., Yoshimura, Y., Nakatsuji, N. and Suemori, H. (2003). Electroporation of Cynomolgus Monkey Embryonic Stem Cells. Genesis; 37: 180-187.

Gabrielle, G., Tysma, O., Bergen, C., Ossendorp, F., Veelen, P., Keymeulen, B., Drijfhout, D. and Roep, B. (2005). Autoreactive CD8 T Cells Associated with β Cell Destructi Diabetes. PNAS; 102(51): 18425-18430.

Gale, E. (1996). Molecular Mechanisms of β-Cell Destruction in IDDM: The Role of Nicotinamide. Hormone Research; 1: 39-43.

Gannon, M., Ables, E., Crawford, L., Lowe, D., Offield, M., Magnuson, M. and Wright, C. (2008). Pdx-1 Function is Specifically Required in Embryonic Beta Cells to Generate Appropriate Numbers of Endocrine Cell Types and Maintain Glucose Homeostasis. Developmental Biology; 314(2): 406-17.

Gao, R., Ustinov, J., Pulkkinen, M., Lundin, K., Korsgren, O. and Otonkoski, T. (2003). Characterization of Endocrine Progenitor Cells and Critical Factors for Their Differentiation in Human Adult Pancreatic Cell Culture. Diabetes; 52: 2007-2015.

Gepstein, L. (2002). Derivation and Potential Applications of Human Embryonic Stem Cells. Circulation Research; 91(10): 866-886.

REFERENCES

Ginzinger, D. (2002). Gene Quantification Using Real time- Quantitative PCR: An Emerging Technology Hits the Mainstream. Experimental Hematology; 30: 503-512.

Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, G. (2000). Neurogenin 3 is Required for the Development of the four Endocrine Cell Lineages of the Pancreas. Proceedings of the National Academy of Sciences. 97 (4): 1607-1611.

Grapin-Botton, A., Majithia, A. and Melton, D. (2001). Key Events of Pancreas Formation are Triggered in Gut Endoderm by Ectopic Exprission of Pancreatic Regulatory Genes. Genes and Development; 15 (4): 444-454.

Greenbaum, J., Kahn, S. and Palmer, J. (1996). Nicotinamide's Effects on Glucose Metabolism in Subjects at isk for IDDM. Diabetes; 45 (11): 1631-1634.

Gu, P., Goodwin, B., Chung, A., Xu, X., Wheeler, D., Price, R., Galardi, C., Peng, L., Latour, A., Koller, B., Gossen, J., Kliewer, S. and Cooney, A. (2005). Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development .Molecular Cell Biology; 25(9): 3492-505.

Gu, P., LeMenuet, D., Chung, A., Mancini, M., Wheeler, D. and Cooney, A. (2005). Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation .Molecular Cell Biology; 25(19): 8507-19.

Gu, G., Dubauskaite, J. and Melton, D. (2002). Direct Evidence for the Pancreas Lineage: $NGN3^{+}$ Cells are Islet Progenitors and are Distinct from Duct Progenitors. Development; 129: 2447-2457.

REFERENCES

Habener, J., Kemp, D. and Thomas, M. (2005). Minireview: Transcriptional Regulation in Pancreatic Development. Endocrinology; 146(3): 1025-1034.

Hart, A., Baeza, N., Apelqvist, Å., Edlund, H. (2000). Attenuation of FGF Signaling in Mouse ß-cells leads to diabetes. Nature; 408:864–868.

Hayek, A. and Beattie, G. (1997). Experimental Transplantation of Human Fetal and Adult Pancreatic Islets. The Journal of Clinical Endocrinology and Metabolism; 82 (8): 2471- 2475.

Hebrok, M., Kim, S. and Melton, D. (1998). Notochord Repression of Endodermal Sonic hedgehog Permits Pancreas Development. Mechanisms of Development; 12 (11): 1705-1713.

Hebrok, M. (2002). Hedgehog Signaling in Pancreas Development. Diabetes; 3 (2): 331-333.

Herrera, P. (2000). Adult Insulin- and Glucagon-Producing Cells Differentiate from Two Independent Cell Lineages. Development; 127: 2317-2322.

Homo-Delarche, F. (2004). Neuroendocrine Immuno-Ontogeny of the Pathogenesis of Autoimmune Disease in the Nonobese Diabetic (NOD) Mouse. ILAR Journal; 45(3): 237-258.

Hoorens, A. and Pipeleers, D. (1999). Nicotinamide Protects Human β-Cells Against Chemically-Induced Necrosis, But Not Against Cytokine-Induced Apoptosis. Diabitologia; 42 (1): 55-59.

REFERENCES

Hori, Y., Gu, X., Xie, X. and Kim, S. (2005). Differentiation of Insulin-Producing Cells from Human Neural Progenitor Cells. PLoS Medicine; 2(4): e103.

Hori, Y., Rulifson, I., Tsai, B., Heit, J., Cahoy, J. and Kim, S. (2002). Growth Inhibitors Promote Differentiation of Insulin-Producing Tissue from Embryonic Stem Cells. PNAS; 99(25): 16105-16110.

Houard, N., Rousseau, G. and Lemaigre, F. (2003). HNF-6-Independent Differentiation of Mouse Embryonic Stem Cells into Insulin-Producing Cells. Diabetologia; 46: 378-385.

Hu, L., Evers, S., Lu, Z., Shen, Y. and Chen, J. (2004). Two-Dimensional Protein Database of Human Pancreas. Electrophoresis 25, 512-518.

Ido, Y., Vindigni, A., Chang, K., Stramm, L., Chance, R., Heath, W., DiMarchi, R., Di Cera, E. and Williamson, J. (1997). Prevention of Vascular and Neural Dysfunction in Diabetic Rats by C-peptide. Science; 277: 563-566.

de la Iglesia, N., Veiga-da-Cunha, M., Van Schaftingen, E., Guinovart, J. and Ferrer, J. (1999). Glucokinase Regulatory Protein is Essential for the Proper Subcellular Localisation of Liver Glucokinase. FEBS Letters; 456(2): 332-338.

Ito, M., Bujo, H., Takahashi, T., Arai, M. and Tanaka, I. (2005). Implantation of Primary Cultured Adipocytes that Secrete Insulin Modifies Blood Glucose Levels in Diabetic Mice. Diabetologia; 48: 1614-1620.

Itskovitz-Eldor, J. (2000). Differentiation of Human Embryonic Stem Cells into Embryoid Bodies Comprising the Three Embryonic Germ Layers. Molecular Medicine; 6(2): 88-95.

REFERENCES

Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G. and Majumdar, A. (2007). Generation of Insulin-producing Islet-like Clusters from Human Embryonic Stem Cells. Cellular and Molecular Neurobiology; 1573-6830. Stem Cells; 10: 1549-4918.

Jiang, T., Su, H. and Zhou, Z. (2002). Isolation and Purification of Islets in Non-Obese Diabetic Mice. Hunan Yi Ke Da Xue Xue Bao; 27(1): 85.

Jiao, L., Gray D., Gohde, W., Flynn, G. and Morris, P. (1991). *In vitro* Staining of Islets of Langerhans for Fluorescence-Activated Cell Sorting. Transplantation; 52(3): 450-452.

Kaneto, H., Miyatsuka, T., Shiraiwa, T., Yamamoto, K., Kato, K., Fujitani, Y. and Matsuoka, T. (2007). Crucial Role of PDX-1 in Pancreas Development, beta-Cell Differentiation, and Induction of Surrogate beta-Cells. Curr Med Chem.; 14(16): 1745-52.

Kang, S., Shin, M., Kim, J. and Oh, J. (2005). Proteomic Profiling of Cellular Proteins Interacting with the Hepatitis C Virus Core Protein. Proteomics; 5(8): 2227-2237.

Kärner, E., Unger, C., Sloan, A., Ahrlund-Richter, L., Sugars, R. and Wendel, M. (2007). Bone matrix formation in osteogenic cultures derived from human embryonic stem cells *in vitro*. Stem Cells Dev.;16(1): 39-52.

Keller, G. (1995). *In vitro* Differentiation of Embryonic Stem Cells. Current Opinion in Cell Biology; 7: 862-869.

Keller, G., Snodgrass, R. (1990). Life Span of Multipotential Hematopoietic Stem Cells *In vivo*. The Journal of Experimental Medicine; 171(5): 1407-18.

REFERENCES

Kelly, W., Lillehei, R. and Merkel, F. (1967). Allotransplantation of the Pancreas and Duodenum Along with the kidney in Diabetic Nephropathy. Surgery; 61: 827- 837.

Kim, J., Moon, S., Lee, S., Lee, D., Koh, G. and Chung, H. (2007). Effective isolation and culture of endothelial cells in embryoid body differentiated from human embryonic stem cells. Stem Cells Dev; 16(2): 269-280.

Klöppel, G. and Kosmahl, M. (2001). Cystic Lesions and Neoplasms of the Pancreas. The Features are Becoming Clearer. Pancreatology; 1: 648-55.

Klug, M., Soonpaa, M., Koh, G., Field, L. (1996). Genetically Selected Cardiomyocytes from Differentiating Embryonic Stem Cells from Stable Interacardiac Grafts. Journal of Clinical Investigation; 98: 216-224.

Knip, M., Douek, I., Moore, W., Gillmor, H., McLean, A., Bingley, P., Gale, E. and European Nicotinamide Diabetes Intervention Trial Group. (2000). Safety of high-dose nicotinamide: a review. Diabetologia; 43(11): 1337-45.

Kolb, H. and Burkart, V. (1999). Nicotinamide in Type II Diabetes. Diabetes Care; 2: B16-B20.

Laurino, C., Bertolini, S. and Cordera, R. (1994). Linkage Analysis Does Not Support a Role for Glucokinase Gene in the Aetiology of Type 2 Diabetes Mellitus Among North Western Italians. Molecular Cell Endocrinology; 104(2): 147-151.

Lavoir, M., Conaghan, J. and Pedersen, R. (1998). Culture of human embryos for studies on the derivation of human pluripotent cells: a preliminary investigation.Reproduction Fertility and Development; 10 (7-8): 557-61.

REFERENCES

Lechner, A; Nolan, A; Blacken, R. and Habener, J. (2004). Redifferentiation of insulin-secreting cells after *in vitro* expansion of adult human pancreatic islet tissue. American Journal of Physiology & Endocrinology Metabolism; 284: 259–266.

Lendahl, U., Zimmerman, L. and McKay, R. (1990). CNS Stem Cells Express a New Class of Intermediate Filament Protein. Cell; 60 (4): 585-95.

Li, T., Zheng, J., Xie, Y., Wang, S., Zhang, X., Li, J., Jin, L., Ma, Y., Wolf, D., Zhou, Q. and Ji, W. (2005). Transplantable Neural Progenitor Populations Derived from Rhesus Monkey Embryonic Stem Cells. Stem Cells; 23(9): 1295-303.

Li, F., Chong, Z. and Maiese, K. (2006). Cell Life Versus Cell Longevity: The Mysteries Surrounding the NAD+ Precursor Nicotinamide. Current Medical Chemistry; 13 (8): 883-895.

Lindström, T., Hedman, C. and Arnqvist, H. (2002). Use of A novel Double-Antibody Technique to Describe the Pharmacokinetics of Rapid_Acting Insulin Analogs. Diabetes Care; 25(6): 1049- 1054.

Liu, M., Xydakis, A., Hoogeveen, R., Jones, P., Smith, E., Nelson, K. and Ballantyne, C. (2005). Multiplexed Analysis of Biomarkers Related to Obesity and the Metabolic Syndrome in Human Plasma, Using the Luminex-100 System. Clinical Chemistry; 51(7): 1102-1109.

Loebel, D., Watson, C., Young, R. and Tam, P. (2003). Lineage choice and differentiation in mouse embryos and embryonic stem cells. Developmental Biology; 264(1): 1-14.

Lowe, C., Cooper, J., Brusko, T., Walker, N., Smyth, D., Bailey, R., Bourget, K., Plagnol, V., Field, S., Atkinson, M., Clayton, D., Wicker, L. and Todd, J. (2007). Large-

Scale Genetic Fine Mapping and Genotype-Phenotype Associations Implicate Polymorphism in the IL2RA Region in Type 1 Diabetes. Nature Genetics; 39(9): 1074-1082.

Lü, P., Liu, F., Yan, L., Peng, T., Liu, T., Yao, Z. and Wang, C. (2007). Stem Cells Therapy for Type I Diabetes. Diabetes Research and Clinical Practice; 3820: 3827.

Lugar, C., Clay, M., Lindstrom, T., Woodson, A., Smiley, D., Heiman, M. and Dodge, J. (2004). Synthesis and biological evaluation of an orally active ghrelin agonist that stimulates food consumption and adiposity in rats. Bioorganic & Medicinal Chemistry Letters; 14(23): 5873-5876.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. and McKay, R. (2001). Differentiation of Stem Cells to Insulin- Secreting Structures Similar to Pancreatic Islets. Science; 292: 1389-1394.

Lyttle, B., Li, J., Krishnamurthy, M., Fellows, F., Wheeler, M., Goodyer, C. and Wang, R. (2008). Transcription Factor Expression in the Developing Human Fetal Endocrine Pancreas. Diabetologia; 51(7): 1169-1180.

Ma, J., Giovannucci, E., Pollak, M., Leavitt, A., Tao, Y., Gaziano, J. and Stampfer, M. (2004). A prospective Study of Plasma C-peptide and Colorectal Cancer Risk in Man. Journal of the National Cancer Institute; 96(7): 546-553.

Maegawa, H., Shigeta, Y., Egawa, K. and Kobayashi, M. (1991). Impaired Autophosphorylation of Insulin Receptors from Abdominal Skeletal Muscles in Nonobese Subjects with NIDDM. Diabetes, 40, 815-819.

REFERENCES

Marikawa, Y., Fujita, T. and Alarcón, V. (2005). Heterogeneous DNA Methylation Status of the Regulatory Element of the Mouse Oct4 Gene in Adult Somatic Cell Population. Cloning Stem Cells; 7(1): 8-16.

Martin, G. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc .Nat. Acad. Sci USA, 78: 7634-7638

Martini, W., Chinkes, D. and Wolfe R. (2004). The Intracellular Free Amino Acid Pool Represents Tracer Precursor Enrichment for Calculation of Protein Synthesis in Cultured Fibroblasts and Myocytes. Journal of Nutrition; 134(6): 1546-50.

Mathieu, C. (2001). Current Limitations of Islet Transplantation. Elsevier Science Inc. New York. 1707: 1708.

Matsutani, A., Koranyi, L., Cox, N. and Permutt, M. (1990). Polymorphisms of GLUT2 and GLUT4 genes. Use in Evaluation of Genetic Susceptibility to NIDDM in Blacks. Diabetes; 39(12): 1534-1542.

Matthews, S., Chellam, N., Srinivas, P., Cintron, V., Leon, M., Goustin, A. and Grunberger, G. (2000). Alpha2-HSG, A Specific Inhibitor of Insulin Receptor Autophosphorylation, Interacts with the Insulin Receptor. Molecular Cell Endocrinology; 164(1-2): 87-98.

McClenaghan, N., Barnett, C., Ah-Sing, E., Abdel-Wahab, Y., O'Harte, F., Yoon, T., Swanston-Flatt, S. and Flatt, P. (1996). Characterization of a novel glucose-responsive insulin-secreting cell line ,BRIN-BD11, produced by electrofusion. Diabetes; 45: 1132-1140 .

McClenaghan, N., Gray, A., Barnett, C. and Flatt, P. (1996). Hexose Recognition by Insulin-Secreting BRIN-BD11 Cells .Biochemical and Biophysical Research Communications; 223(3): 724-728.

Meivar-Levy, I. and Ferber, S. (2006). Regenerative Medicine: Using Liver to Generate Pancreas for Treating Diabetes. Israel Medical Association Jornal; 8(6): 430-434.

Miszta-Lane, H., Mirbolooki, M., James, M., Shapiro, A. and Lakey, J. (2006). Stem cell Sources for Clinical Islet Transplantation in Type 1 Diabetes: Embryonic and Adult Stem Cells. Medical Hypotheses; 67(4): 909-913.

Moates, J., Nanda, S., Cissell, M., Tsai, M. and Stein, R. (2003). BETA2 Activates Transcription from the Upstream Glucokinase Gene Promoter in Islet Beta-Cells and Gut Endocrine Cells. Diabetes; 52(2): 403-408.

Moore, R. (2000). Color Atlas of Clinical Embryology. Second edition, Eds.Pp: 300.

Murad, N., Alnaqeeb, M., Al-Bustan, S. (2004). Effect of Urban Industrial Expansion on the Genetic Biodiversity of Two Desert Animal Species in Kuwait Determined by RAPD-PCR. Kuwait Journal of Science & Engineering; 31 (1): 15-32.

Murtaugh, L. (2007). Pancreas and Beta-Cell Development: from the Actual to the Possible. Development; 134: 427-438.

Neville, H. (2006) .(Physiological regulation of the pancreatic ß-cell: functional insights for understanding and therapy of diabetes .Experimental Physiology; 92(3): 481-496.

Nishimura, M., Koeda, A., Suzuki, E., Kawano, Y., Nakayama, M., Satoh, T., Narimatsu, S. and Naito, S. (2006). Regulation of mRNA Expression of MDR1, MRP1, MRP2

and MRP3 by Prototypical Microsomal Enzyme Inducers in Primary Cultures of Human and Rat Hepatocytes. Drug Metabolism Pharmacokinetics; 21 (4): 297-307.

Noguchi, H. (2006). Stem Cells for the Treatment of Diabetes. Endocrinology Journal; 54(1): 7-16.

Odobel, F. and Zabri, H. (2005). Preparations and Characterizations of bichromophoric systems composed of a ruthenium polypyridine complex connected to a difluoroborazaindacene or a zinc phthalocyanine chromophore. Inorg Chem; 44(16): 5600-11.

Ohtomo, Y., Bergman, T., Johansson, B., Jörnvall, H. and Wahren, J. (1998). Differential Effects of Proinsulin C-Peptide Fragments on Na^+, K^+-ATPase Activity of Renal Tubule Segments. Diabetologia; 41: 287-291.

Ono, J. (2006). Treatment of Type 1 Diabetics by Transplantation of Isolated Pancreatic Islets. Rinsho Byori; 54(4): 379-385.

Orci, L. (1982). Macro- and Micro- Domains in the Endocrine Pancreas. Diabetes; 31: 538-565.

Overbergh, G., Valckx, D., Bouillon, B. and Mathieu, A. (2003). The use of Real-Time Reverse Transcriptase PCR for the Quantification of Cytokine Gene Expression. Journal of Bimolecular Techniques; 14: 33–43

Palumbo, M., Levi, D., Chentoufi, A. and Polychronakos, C. (2006). Isolation and Characterization of Proinsulin-Producing Medullary Thymic Epithelial Cell clones. Diabetes; 55: 2595-2601.

REFERENCES

Paoletta, M., Kahn, E. and Halban, P. (2002). Truncated (des-[27-31]) C-peptide is not A major Secretory Product of Human Islets. Diabetologia; 45: 1523-1527.

Pappas, S., Federle, M., Lokshin, A. and Zeh, H. (2007). Early Detection and Staging of Adenocarcinoma of the Pancreas. Gastroenterology Clinics of North America; 36(2): 413-429.

Peck, A. and Ramiya, V. (2004). *In vitro*-Generation of Surrogate Islets from Adult Stem Cells. Transplant Immunology; 12: 259-272.

Peiffer, I., Belhomme, D., Barbet, R., Haydont, V., Zhou, Y., Fortunel, N., Li, M., Hatzfeld, A., Fabiani, J. and Hatzfeld, J. (2007). Simultaneous differentiation of endothelial and trophoblastic cells derived from human embryonic stem cells. Stem Cells Dev; 16(3): 393-402.

Pengfei, L., Andrew, E., Gail, M. and Zena, W. (2006) Mosaic analysis reveals essential function of FGF receptor 2 in mammary gland branching morphogenesis. Developmental Biology; 295(1): 335-336.

Pedersen, M., Bertram, R. and Sherman, A. (2005). Intra- and Inter-Islet Synchronization of Metabolically Driven Insulin Secretion. Biophysical Journal; 89(1): 107-119.

Pera, M., Reubinoff, B. and Trounson, A. (2000). Human Embryonic Stem Cells. Journal of cell science; 113: 5-10.

Perkins, A. (1998). Enrichment of Blood from Embryonic Stem Cells *In vitro*. Reproduction, Fertility and Development; 10: 7-8.

Peter, J., Jűrgensen, A. and Klöppel, G. (2000). Ontogeney, Differentiation and Growth of the endocrine Pancreas. Virchows Arch; 436: 527-538.

Petropavlovskaia, M. and Rosenberg, L. (2002). Identification and Characterization of Small Cells in the Adult Pancreas: Potential Progenitor Cells?. Cell and Tissue Research; 310(1): 51-58.

Pileggi, A., Ricordi, C., Kenyon, N., Froud, T., Baidal, D., Kahn, A., Selvaggi, G. and Alejandro, R. (2004). Twenty Years of Clinical Islet Transplantation at the Diabetes Research Institute-University of Miami. Clinical Transplantation; 177-204.

Piper, K., Brickwood, S., Turnpenny, L., Cameron, I., Ball, S., Wilson, D. and Hanley, N. (2004). Beta Cell Differentiation During Early Human Pancreas Development. Journal of Endocrinology; 181: 11–23.

Polo, V., Saibene, A. and Pontiroli, A. (1998). Nicotinamide Improves Insulin Secretion and Metabolic Control in Lean Type 2 Diabetic Patients with Secondary Failure to Sulphonylureas. Acta Diabetology; 35 (1): 61-64.

Postic, C., Shiota, M. and Magnuson, M. (2001). Cell-Specific Roles of Glucokinase in Glucose Homeostasis. Recent Progress in Hormone Research.; 56: 195-217.

Priel, T., Hershfinkel, M. and Sekler, I. (2006). The ways in which zinc enters beta cells in the pancreas and the influence of zinc blockers on the development of type 1 diabetes. Society of Endocrinology; 11:411.

Rajagopal, J., Anderson, W., Kume, S., Martinez, O. and Melton, D. (2003). Insulin Staining of ES Cell Progeny from Insulin Uptake. Science; 299(5605): 363.

Rasschaert, J., Flatt, P., Barnett, C., McClenaghan, N. and Malaisse, W. (1996). d -Glucose Metabolism in BRIN-BD11 Islet Cells .Biochemical and Molecular Medicine; 57(2): 97-105.

Riedy, M., Brown, M., Molloy, C. and Turner, C. (1999). Activin A and TGF-β Stimulate Phosphorylation of Focal Adhesion Proteins and Cytoskeletal Reorganization in Rat Aortic Smooth Muscle Cells. Development; 132, 1273-1282

Reubinoff, B., Pera, M., Fong, C., Trounson, A. and Bongso, A. (2000). Embryonic Stem Cell Lines from Human Blastocysts: Somatic Differentiation In Vitro. Nature Biotechnology; 18: 399-404.

Rhodes, C. and Alarcón, C. (1994). What Beta-Cell Defect Could Lead to Hyperproinsulinemia in NIDDM? Some Clues from Recent Advances Made in Understanding the Proinsulin-Processing Mechanism. Diabetes; 43(4): 511-517.

Richardson, C., Hussain, K., Jones, P., Persaud, S., Löbner, K., Boehm, A., Clark, A. and Christie, M. (2007). Low levels of glucose trasporters and K+ATP channels in human pancreatic beta cells early in development.diabetologia; 50(5): 1000-1005.

Rigler, R., Pramanik, A., Jonasson, P., Kratz, G., Jansson, O., Nygren, P., Stahl, S., Ekberg, K., Johansson, B., Uhlén, S., Uhlén, M., Jörnvall, H. and Wahren, J. (1999). Specific Binding of Proinsulin C-Peptide to Human Cell Membranes. PNAS; 96(23): 13318-13323.

Rindi, G., Torsello, A., Locatelli, V. and Solcia, E. (2007). Ghrelin expression and actions: a novel peptide for an old cell type of the diffuse endocrine system. Mechanisms of Aging and Development; 128(2): 182-186.

Risérus, U., Vessby, B., Arner, P. and Zethelius, B. (2004). Supplementation with Trans10cis12-Conjugated Linoleic Acid Induces Hyperproinsulinaemia in Obesc

Men: Close Association with Impaired Insulin Sensitivity. Diabetologia; 47: 1016-1019.

Roche, E., Reig, J., Campos, A., Paredes, B., Isaac, J., Lim, S., Calne, R. and Soria, B. (2005). Insulin-Secreting Cells Derived from Stem Cells: Clinical Perspectives, Hypes and Hopes. Transplant Immunology; 15: 113-129.

Rogers, A. (1992). Text Book of Anatomy. Churchill Livingstone, London.

Ross, A., Miller, G., Moss, T., Kahn, D., Warner, N., Sweet, D., Louie, K., Schneidermann, E., Pecora, A. and Meagher, R. (1995). Immunocytochemical Detection of Tumor Cells in Bone Marrow and Peripheral Blood Stem Cell Collections from Patients with Ovarian Cancer. Bone Marrow Transplant; 15(6): 929-33.

Rudovich, N., Rochlitz, H. and Pfeiffer, A. (2004). Reduced Hepatic Insulin Extraction in Response to Gastric Inhibitory Polypeptide Compensates for Reduced Insulin Secretion in Normal-Weight and Normal Glucose Tolerant First-Degree Relatives of Type 2 Diabetic Patients. Diabetes; 53: 2359-2365.

Sachs, D. and Bonner-Weir, S. (2000). New Islets from Old Nature Medicine; 6 (3): 250-251.

Sandler, S., Andersson, A., Korsgren, O., Tollerman, T., Petersson, B., Groth, C. and Hellerstrom, C. (1989). Tissue culture of human fetal pancreas. Effects of nicotinamide on insulin production and formation of islet-like cell clusters, *Diabetes* 38: 168–171.

Santana, A., Enseñat-Waser, R., Arribas, M., Reig, J. and Roche, E. (2006). Insulin-Producing Cells Derived from Stem Cells: Resent Progress and Future Directions. Journal of Cellular Molecular Medicine; 10(4): 866-883.

REFERENCES

Sarkar, S., Kobberup, S., Wong, R., Lopez, A., Quayum, N., Still, T., Kutchma, A., Jensen, J., Gianani, R., Beattie, G., Jensen, J., Hayek, A. and Hutton, J. (2008). Global Gene Expression Profiling and Histochemical Analysis of the Developing Human Fetal Pancreas. Diabetologia; 51(2): 285-97.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, DA. and Benvenisty, N. (2000). Effects of Eight Growth Factors on the Differentiation of Cells Derived from Human Embryonic Stem Cells. PNAS; 97 (21): 11307-11312.

Segev, H., Fishman, B., Ziskind, A., Shulman, M. and Itskovitz-Eldor, J. (2004). Differentiation of Human Embryonic Stem Cells into Insulin-Producing Clusters. Stem Cells; 22: 265-274.

Selander, L., Edlund, H. (2002). Nestin is Expressed in Mesenchymal and Not Epithelial Cells of the Developing Mouse Pancreas. Mechanisms of Development; 113: 189-192.

Shamblott, M., Axelman, J., Wang, S., Bugg, E., Littlefield, J., Donovan, P., Blumenthal, P., Huggins, G. and Gearhart, J. (1998). Derivation of Pluripotent Stem Cells from Cultured Human Primordial Germ Cells. Proceedings of the National Academy of Sciences; 95: 13726-13731.

Shapiro, A. and Merani, S. (2006). Current Status of Pancreatic Islet Transplantation. Clinical Science (Lond); 110(6): 611-625.

Shen, J., Wang, R., Zhang, C., Liu, M. and Guo, F. (2004). Protein Expression Profiles in Pancreatic Adenocarcinoma Compared with Normal Pancreatic Tissue and Tissue

Affected by Pancreatitis as Detected by Two-Dimensional Gel Electrophoresis and Mass Spectrometry. Cancer Research; 64: 9018-9026.

Shepherd, P., Withers, D. and Siddle, K. (1998). Phosphoinositide 3-kinase: The Key Switch Mechanism in Insulin Signalling. Biochemical Journal; 333(3): 471-490.

Shiroi, A., Ueda, S., Ouji, Y., Saito, K., Moriya, K., Fukui, Y., Ishizaka, S. and Yoshikawa, M. (2005). Differentiation of Embryonic Stem Cells into Insulin-Producing Cells by Nkx2.2 Gene Transfer. World Journal of Gastroenterology; 11(27): 4161-4166.

Shuto, Y., Shibasaki, T., Wada, K., Parhar, I., Kamegai, J., Sugihara, H., Oikawa, S. and Wakabayashi, I. (2001). Generation of polyclonal antiserum against the growth hormone secretagogue receptor (GHS-R): Evidence that the GHS-R exists in the hypothalamus, pituitary and stomach of rats. Life Sciences; 68(9): 991-996.

Singh-Franco, D., Robles, G. and Gazze, D. (2007). Pramlintide acetate injection for the treatment of type 1 and type 2 diabetes mellitus. Clinical Therapeutics; 29(4):535-62.

Sjöstrand, M., Gudbjörnsdottir, S., Holmäng, A., Lönn, L., Strindberg, L. and Lönnroth, P. (2002). Delayed Transcapillary Transport of Insulin to Muscle Interstitial Fluid in Obese Subjects. Diabetes; 51: 2742-2748.

Slosberg, E., Desai, U., Fanelli, B., St Denny, I., Connelly, S., Kaleko, M., Boettcher, BR. and Caplan, SL. (2001). Treatment of Type 2 Diabetes by Adenoviral-Mediated Overexpression of the Glucokinase Regulatory Protein. Diabetes; 50(8): 1813-1820.

REFERENCES

Smith, J., Lambert, J., Elisma, F. and Figeys, D. (2007). Proteomics in 2005/2006: Developments, Applications and Chalenges. Analytical Chemistry; 79(12): 4325-4343.

Soria, B. (2001). *In vitro* Differentiation of Pancreatic β-Cells. Differentiation; 68(4-5): 205-19.

Soria, B., Roche, E., Bernà, G., Leon-Quinto, T., Reig, J. and Martín, F. (2000). Insulin-Secreting Cells Derived from Embryonic Stem Cells Normalize Glycemia in Streptozotocin-Induced Diabetic Mice. Diabetes; 49: 157-162.

Stevens, A. and Lowe, J. (1997). Human Histology. Mosby; London.

St-Onge, L., Wehr, R. and Gruss, P. (1999). Pancreas Development and Diabetes. Current Opinion in Genetics & Development; 9: 295-300.

Tayaramma, T., Ma, B., Rohde, M. and Mayer, H. (2006). Chromatin-Remodeling Factors Allow Differentiation of Bone Marrow Cells into Insulin-Producing Cells. Stem Cells; 24(12): 2858-67.

Thomson, J., Kalishman, J., Golos, T., Durning, M., Harris, C., Becker, R. and Hearn, J. (1995). Isolation of a Primate Embryonic Stem Cell Line. Proceedings of the National Academy of Sciences; 92: 7844-7848.

Thomson, J., Itskovitz-Eldor, J., Shapiro, S., Waknitz, M., Swiergiel, J., Marshall, V. and Jones, J. (1998). Embryonic Stem Cell Lines Derived from Human Blastocysts. Science; 282: 1145-1147.

Tondreau, T., Meuleman, N., Delforge, A., Dejeneffe, M., Leroy, R., Massy, M., Mortier, C., Bron, D. and Lagneaux, L. (2005). Mesenchymal Stem Cells Derived from

CD133-Positive cells in Mobilized Peripheral Blood and Cord Blood: Proliferation, Oct4 Expression, and Plasticity. Stem Cells; 23(8): 1105-1112.

Treutelaar, M., Skidmore, J., Dias-Leme, C., Hara, M., Zhang, L., Simeone, D., Martin, M. and Burant, C. (2003). Nestin-lineage Cells Contribute to the Microvasculature But Not Endocrine Cells of the Islet. Diabetes; 52(10): 2503-2512.

Trucco, M. (2006). Is Facilitating Pancreatic Beta Cell Regeneration a Valid Option for Clinical Therapy. Cell Transplantation; 15(1): S75-84.

Trucco, M. (2005). Regeneration of the Pancreatic β-Cell. The Journal of Clinical Investigation; 115 (1): 5-12.

Urban, S., Kiss, J., Vas, V., Kovacs, J. and Uher, F. (2006). Stem Cell Therapy for Diabetes Mellitus: Progress, Prospects and Challenges. Orv Hetil; 147(17): 791-797.

Vaca, P., Martín, F., Vegara-Meseguer, J., Rovira, J., Berná, G. and Soria, B. (2006). Induction of Differentiation of Embryonic Stem Cells into Insulin-Secreting Cells by Fetal Soluble Factors. Stem Cells; 24: 258-265.

Valmu L., Paju A., Lempinen M., Kemppainen E. and Stenman U. (2006). Application of Proteomic Technology in Identifying Pancreatic Secretory Trypsin Inhibitor Variants in Urine of Patients with Pancreatitis. Clinical Chemistry; 52(1): 73-81.

Volk, B. and Wellman, K. (1985). Historical Review in Volk BW, Arquilla ER (eds) The Diabetic Pancreas. New York: Plenum, pp. 1-16.

A De Vos, H., Quartier, E., Huypens, P., Bouwens, L., Pipeleers, D. and Schuit, F. (1995). Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. Journal of Clinical Investigation; 96(5): 2489-2495.

REFERENCES

Wahren, J., Ekberg, K., Johansson, J., Henriksson, M., Pramanik, A., Johansson, B., Rigler, R. and Jörnvall, H. (2000). Role of C-Peptide in Human Physiology. American Journal of Physiology and Endocrinology Metabolism; 278: E759-E768.

Wang, Q., Fang, Z., Jin, F., Lu, Y., Gai, H. and Sheng, H. (2005). Derivation and Growing Human Embryonic Stem Cells on Feeders Derived from Themselves. Stem Cells; 23: 1221-1227.

Weinhaus, A., Tabiin, M., Poronnik, P., Palma, C, Cook, D. and Tuch, B. (2003). Insulin Secretagogues, But Not Glucose, Stimulate an Increase in $[Ca^{2+}]_i$ in the Fetal Human and Porcine β-Cell. The Journal of Clinical Endocrinology and Metabolism, 88(6): 2753-2759.

Wiesli, P., Brändle, M., Zapf, J., Seiler, H., Zwimpfer, C., Spinas, GA. and Schmid, C. (2004). Assessment of Hyperinsulinaemia at the Termination of the Prolonged Fast. Clinica Chimica Acta; 432: 227-231.

Williams, R., Hilton, J., Pease, S., Willson, TA, Stewart, C., Gearing, D., Wagner, E., Metcalf, D., Nicola, N. and Gough, N. (1988). Myeloid Leukemia Inhibitory Factor Maintains the Developmental Potential of Embryonic Stem Cells. Nature; 336(15): 684-687.

Winston, R. (2001). Embryonic Stem Cell Research. Nature Medicine; 7(4): 396-399.

Yang, Y, Santamaria, P. (2006). Lessons on Autoimmune Diabetes from Animal Models. Clinical Sceinces (Lond); 110(6): 627-639.

Young, B. and Heath, J. (2000). Wheater's Functional Histology. Churchill Livingstone; Edinburgh.

REFERENCES

Yamamoto, T., Fukumoto, H., Koh, G., Yano, H., Yasuda, K., Masuda, K., Ikeda, H., Imura, H. and Seino, Y. (1991). Liver and Muscle-Fat Type Glucose Transporter Gene Expression in Obese and Diabetic Rats. Biochemical and biophysical research communications; 175(3): 995-1002.

Yamamoto, T., Katakabe, K., Akiyoshi, K., Kan, K., Asano, T. and Okumura, M. (1990). Topical Application of the Hypoglycemic Agent Glibenclamide and Changes in Blood Glucose, Plasma Insulin (IRI) Levels and Plasma Concentration of Glibenclamide in Normal Rats: Diabetes Research & Clinical Practice; 8(1): 19-22.

Zhang, Y., Cleary, M., Si, Y., Liu, G., Eto, Y., Kritzik, M., Dabernat, S., Kayali, A. and Sarvetnick, N. (2004). Inhibition of Activin Signaling Induces Pancreatic Epithelial Cell Expansion and Diminishes Terminal Differentiation of Pancreatic ß-Cells. Diabetes; 53: 2024-2033.

Zou, C., Suen, P., Zhang, Y., Wang, Z., Chan, P., Leung, P. and Zhang, Y. (2006). Isolation and *In vitro* Characterization of Pancreatic Progenitor Cells from the Islets of Diabetic Monkey Models. The International Journal of Biochemistry & Cell Biology; 38: 973-984.

Zulewski, H., Abraham, E., Gerlach, M., Daniel, P., Moritz, W., Müller, B., Vallejo, M., Thomas, M. and Habener, J. (2001). Multipotential Nestin-Positive Stem Cells Isolated from Adult Pancreatic Islets Differentiate Ex *vivo* into Pancreatic Endocrine, Exocrine, and Hepatic Phenotypes. Diabetes; 50: 521-533.

www.ana.ed.ac.uk

www.Baylor College of Medicin

REFERENCES

www.betacellconsortium

www.diabetes.org.uk

www.en.wikipedia.org/wiki/Baylor College of Medicine

www.Lifespan.org

www.Parmacorama.com/en/Sections/images/Insulin

www.Udel.edu

APPENDICES

Table 3 t-tests between small and large EBs colonies for each growth factor of cells stained with Dithizone (DTZ). The tabulated t value of 2.45 ($p = 0.05$) rises to a tabulated value of 5.96 ($p = 0.001$).

A.

Nicotinamide	**Small colonies**	**Large colonies**	
Replicate 1	802 (643204)	815 (664225)	
Replicate 2	483 (233289)	1189 (1413721)	
Replicate 3	450 (202500)	620 (384400)	
Replicate 4	600 (360000)	800 (640000)	
Sum of x	2335	3424	
n (repeated times)	4	4	Total (= sum of the 4 replicate values)
Mean (total/n)	583.75	856	
Sum x^2 (Σx^2)	1438993	3102346	Sum of the squares of each replicate value
$(\Sigma x)^2$	5452225	11723776	Square of the total (Σx).
$\frac{(\Sigma x)^2}{n}$	1363056.25	2930944	
Σd^2	4089168.75	171402	$\Sigma d^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$
σ^2	1363056.25	57134	$\sigma^2 = \Sigma d^2 / n-1$
$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$	340764.062 + 14283.5 = 355047.562		σ_d^2 is the variance of the difference between the means
σ_d	595.859		$= \sqrt{\sigma_d^2}$ (the standard deviation of the difference between the means)
$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$	-0.457		

B.

Nicotinamide + Activin A	**Small colonies**	**Large colonies**	
Replicate 1	(176400) 420	1090 (1188100)	
Replicate 2	901 (811801)	1110 (1232100)	
Replicate 3	660 (435600)	998 (996004)	
Replicate 4	615 (378225)	1345 (1809025)	
Sum of x	2596	4543	
n (repeated times)	4	4	Total (= sum of the 4 replicate values)
Mean (total/n)	649	1135.75	
Sum x^2 (Σx^2)	1802026	25864078	Sum of the squares of each replicate value
$(\Sigma x)^2$	6739216	20638849	Square of the total (Σx).
$\frac{(\Sigma x)^2}{n}$	1684804	5159712.25	
Σd^2	117222	20704365.8	$\Sigma d^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$
σ^2	39074	6901455.27	$\sigma^2 = \Sigma d^2 / n-1$
$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$	9768.5 + 1725363.82 = 1735132.32		σ_d^2 is the variance of the difference between the means
σ_d	1317.244		$= \sqrt{\sigma_d^2}$ (the standard deviation of the difference between the means)
$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$	-1.478		

C.

Nicotinamide + FGF2	**Small colonies**	**Large colonies**	
Replicate 1	1200 (1440000)	1010 (1020100)	
Replicate 2	(986049) 993	1500 (2250000)	
Replicate 3	805 (648025)	1583 (2505889)	
Replicate 4	1150 (1322500)	1594 (2540836)	
Sum of x	4148	5687	
n (repeated times)	4	4	Total (= sum of the 4 replicate values)
Mean (total/n)	1037	1421.75	
Sum x^2 (Σx^2)	4396574	8316825	Sum of the squares of each replicate value
$(\Sigma x)^2$	17205904	32341969	Square of the total (Σx).
$\frac{(\Sigma x)^2}{n}$	4301476	8085492.25	
Σd^2	95098	231332.75	$\Sigma d^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$
σ^2	31699.33	77110.92	$\sigma^2 = \Sigma d^2 / n-1$
$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$	7924.83 + 19277.73 = 27202.56		σ_d^2 is the variance of the difference between the means
σ_d	164.93		$= \sqrt{\sigma_d^2}$ (the standard deviation of the difference between the means)
$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$	-2.333		

D.

Activin A + FGF2	**Small colonies**	**Large colonies**	
Replicate 1	220 (48400)	580 (336400)	
Replicate 2	435 (189225)	510 (260100)	
Replicate 3	210 (44100)	910 (828100)	
Replicate 4	108 (11664)	352 (123904)	
Sum of x	973	2352	
n (repeated times)	4	4	Total (= sum of the 4 replicate values)
Mean (total/n)	243.25	588	
Sum x^2 (Σx^2)	293389	1548504	Sum of the squares of each replicate value
$(\Sigma x)^2$	946729	5531904	Square of the total (Σx).
$\frac{(\Sigma x)^2}{n}$	236682.25	1382976	
Σd^2	-56706.75	165528	$\Sigma d^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$
σ^2	18902.25	55176	$\sigma^2 = \Sigma d^2 / n-1$
$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$	4725.563 + 1382976 = 1387701.56		σ_d^2 is the variance of the difference between the means
σ_d	1178.01		$= \sqrt{\sigma_d^2}$ (the standard deviation of the difference between the means)
$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$	-1.1		

E.

Activin A	**Small colonies**	**Large colonies**	
Replicate 1	19 (361)	(1444) 38	
Replicate 2	10 (100)	66 (4356)	
Replicate 3	24 (576)	7 (49)	
Replicate 4	13 (169)	19 (361)	
Sum of x	1206	130	
n (repeated times)	4	4	Total (= sum of the 4 replicate values)
Mean (total/n)	301.5	32.5	
Sum x^2 (Σx^2)	1206	6210	Sum of the squares of each replicate value
$(\Sigma x)^2$	1454436	16900	Square of the total (Σx).
$\frac{(\Sigma x)^2}{n}$	363609	4225	
Σd^2	-362403	1985	$\Sigma d^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$
σ^2	-120801	661.67	$\sigma^2 = \Sigma d^2 / n-1$
$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$	-30200.25 + 165.417 = -30034.833		σ_d^2 is the variance of the difference between the means
σ_d	173.31		$= \sqrt{\sigma_d^2}$ (the standard deviation of the difference between the means)
$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$	1.55		

F.

Control	**Small colonies**	**Large colonies**	
Replicate 1	2 (4)	9 (81)	
Replicate 2	0	6 (36)	
Replicate 3	0	2 (4)	
Replicate 4	4 (16)	0	
Sum of x	6	17	
n (repeated times)	4	4	Total (= sum of the 4 replicate values)
Mean (total/n)	1.5	4.25	
Sum x^2 (Σx^2)	20	121	Sum of the squares of each replicate value
$(\Sigma x)^2$	36	289	Square of the total (Σx).
$\frac{(\Sigma x)^2}{n}$	9	72.25	
Σd^2	4	48.75	$\Sigma d^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$
σ^2	1.33	16.25	$\sigma^2 = \Sigma d^2 / n-1$
$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$	0.333 + 4.063 = 4.396		σ_d^2 is the variance of the difference between the means
σ_d	2.097		$= \sqrt{\sigma_d^2}$ (the standard deviation of the difference between the means)
$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$	-1.31		

Table

Based on observed means.

* The mean difference is significant at the .05 level.

	(I) Growth Factor	(J) Growth Factor	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Upper Bound	Lower Bound
Tukey HSD	Nicotinamide	Nicotinamide+Activin A	-3.8750	2.10489	.454	-10.2077	2.4577
		Nicotinamide + FGF2	-12.8750(*)	2.10489	.000	-19.2077	-6.5423
		Activin A +FGF2	8.5000(*)	2.10489	.003	2.1673	14.8327
		Activin A	17.6250(*)	2.10489	.000	11.2923	23.9577
		control	18.1250(*)	2.10489	.000	11.7923	24.4577
	Nicotinamide+Activin A	Nicotinamide	3.8750	2.10489	.454	-2.4577	10.2077
		Nicotinamide + FGF2	-9.0000(*)	2.10489	.002	-15.3327	-2.6673
		Activin A +FGF2	12.3750(*)	2.10489	.000	6.0423	18.7077
		Activin A	21.5000(*)	2.10489	.000	15.1673	27.8327
		control	22.0000(*)	2.10489	.000	15.6673	28.3327
	Nicotinamide + FGF2	Nicotinamide	12.8750(*)	2.10489	.000	6.5423	19.2077
		Nicotinamide+Activin A	9.0000(*)	2.10489	.002	2.6673	15.3327
		Activin A +FGF2	21.3750(*)	2.10489	.000	15.0423	27.7077
		Activin A	30.5000(*)	2.10489	.000	24.1673	36.8327
		control	31.0000(*)	2.10489	.000	24.6673	37.3327
	Activin A +FGF2	Nicotinamide	-8.5000(*)	2.10489	.003	-14.8327	-2.1673
		Nicotinamide+Activin A	-12.3750(*)	2.10489	.000	-18.7077	-6.0423
		Nicotinamide + FGF2	-21.3750(*)	2.10489	.000	-27.7077	-15.0423
		Activin A	9.1250(*)	2.10489	.001	2.7923	15.4577
		control	9.6250(*)	2.10489	.001	3.2923	15.9577
	Activin A	Nicotinamide	-17.6250(*)	2.10489	.000	-23.9577	-11.2923
		Nicotinamide+Activin A	-21.5000(*)	2.10489	.000	-27.8327	-15.1673
		Nicotinamide + FGF2	-30.5000(*)	2.10489	.000	-36.8327	-24.1673
		Activin A +FGF2	-9.1250(*)	2.10489	.001	-15.4577	-2.7923
		control	.5000	2.10489	1.000	-5.8327	6.8327
	control	Nicotinamide	-18.1250(*)	2.10489	.000	-24.4577	-11.7923
		Nicotinamide+Activin A	-22.0000(*)	2.10489	.000	-28.3327	-15.6673
		Nicotinamide + FGF2	-31.0000(*)	2.10489	.000	-37.3327	-24.6673
		Activin A +FGF2	-9.6250(*)	2.10489	.001	-15.9577	-3.2923
		Activin A	-.5000	2.10489	1.000	-6.8327	5.8327
Games-Howell	Nicotinamide	Nicotinamide+Activin A	-3.8750	2.37876	.595	-11.6797	3.9297
		Nicotinamide + FGF2	-12.8750(*)	2.56653	.002	-21.3329	-4.4171
		Activin A +FGF2	8.5000(*)	2.40071	.031	.6211	16.3789
		Activin A	17.6250(*)	1.65224	.000	11.4110	23.8390

		control	18.1250(*)	1.64140	.000	11.9050	24.3450
	Nicotinamide+Activin A	Nicotinamide	3.8750	2.37876	.595	-3.9297	11.6797
		Nicotinamide + FGF2	-9.0000(*)	2.61861	.038	-17.6114	-.3886
		Activin A +FGF2	12.3750(*)	2.45631	.002	4.3181	20.4319
		Activin A	21.5000(*)	1.73205	.000	14.9815	28.0185
		control	22.0000(*)	1.72171	.000	15.4757	28.5243
	Nicotinamide + FGF2	Nicotinamide	12.8750(*)	2.56653	.002	4.4171	21.3329
		Nicotinamide+Activin A	9.0000(*)	2.61861	.038	.3886	17.6114
		Activin A +FGF2	21.3750(*)	2.63857	.000	12.7034	30.0466
		Activin A	30.5000(*)	1.98206	.000	23.0286	37.9714
		control	31.0000(*)	1.97303	.000	23.5233	38.4767
	Activin A +FGF2	Nicotinamide	-8.5000(*)	2.40071	.031	-16.3789	-.6211
		Nicotinamide+Activin A	-12.3750(*)	2.45631	.002	-20.4319	-4.3181
		Nicotinamide + FGF2	-21.3750(*)	2.63857	.000	-30.0466	-12.7034
		Activin A	9.1250(*)	1.76208	.010	2.4920	15.7580
		control	9.6250(*)	1.75191	.007	2.9862	16.2638
	Activin A	Nicotinamide	-17.6250(*)	1.65224	.000	-23.8390	-11.4110
		Nicotinamide+Activin A	-21.5000(*)	1.73205	.000	-28.0185	-14.9815
		Nicotinamide + FGF2	-30.5000(*)	1.98206	.000	-37.9714	-23.0286
		Activin A +FGF2	-9.1250(*)	1.76208	.010	-15.7580	-2.4920
		control	.5000	.18898	.202	-.2161	1.2161
	control	Nicotinamide	-18.1250(*)	1.64140	.000	-24.3450	-11.9050
		Nicotinamide+Activin A	-22.0000(*)	1.72171	.000	-28.5243	-15.4757
		Nicotinamide + FGF2	-31.0000(*)	1.97303	.000	-38.4767	-23.5233
		Activin A +FGF2	-9.6250(*)	1.75191	.007	-16.2638	-2.9862
		Activin A	-.5000	.18898	.202	-1.2161	.2161