The Molecular and Cellular Biology
Of
Pancreatic β Cell Differentiation *In Vitro*

A Thesis submitted for the degree of Ph.D.

By

Patrick Gammell (B.Sc.)

November 2002

The work in this thesis was carried out under the supervision of

Prof. Martin Clynes and Dr. Lorraine O' Driscoll

National Cell and Tissue Culture Centre
School of Biotechnology
Dublin City University
Glasnevin
Dublin 9

I Certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D is entirely my own work and has not been taken from the work of others and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ ID No. 98970348

Date: 5.11.02

This thesis is dedicated to the memory of
Noel Michael Gammell
who we still miss every day.

Acknowledgements

To Martin Clynes for his support and enthusiasm for the project over the last four years, I really appreciate it.

To Lorraine O' Driscoll, a fantastic supervisor, a proof-reader par excellence but more importantly, a good friend. Thanks for everything.

To all the gang in the Diabetes lab, Rasha, Irene, Eadaoin and Elaine, thanks for letting me hog all the equipment in those final crazy days and for the post-viva balloons and champagne!

To Niall and Carmel a special mention for the help with the molecular biology and arrays, thanks a million. Thanks also to Dee for the help with the arrays, cheers.

The thesis would not have been completed without the afternoon tea sessions with Brendan (Backpacker magazine playmate of the year 2001) and Finbar (Safari) O' Sullivan cheers lads.

To the rest of the centre staff and post grads, I have asked for help from most of you over the years and I appreciate the fact that it was always available, thank you. Thanks especially to Yvonne, Carol and of course Joe.

To TJ, for the madness, lets have us a couple of mint juleps this Christmas. GWF

To my family for all the support (financial and the other kind!) over the years. Thanks to Chrissann, Elaine, Philip, Eoin, Aisling, Alan, Stephen, Cian and all the 'outlaws'!

Mom & Dad, there is nothing I can say really that could possibly convey my gratitude for all the support and help over the years. A lot of the credit for this is all yours. Thanks a million (will be off your back and out of your wallet soon!).

Finally, last but not least, Elaine, I have always said you can do better but you chose to stick it out with me. You were there at the beginning and there at the very end. I am truly the luckiest man I know to have you in my life. Thanks for everything babe.

Table of Contents **Page**

Abstract

Previous studies have revealed that embryonic stem (ES) cells are capable of differentiating spontaneously towards pancreatic β cells. The use of growth factors and specific agents, such as nicotinamide, can further enhance this effect in a process known as directed differentiation. *In vivo* monitoring of pancreatic development indicates that the pancreas is derived from an endodermal lineage. This thesis investigated the differentiation of an embryonic carcinoma cell line, F9, which is characterised as having minimal spontaneous differentiation, towards endoderm and from there, on to pancreatic lineages.

Treatment of monolayer F9 cells with 10^{-7} M retinoic acid (RA) or a combination of retinoic acid and 10^{-3} M dibutyryl cyclic adenosine monophosphate (db cAMP) for 72 hours generates both primitive and parietal endodermal types. Culture of F9 cells in suspension leads to the formation of embryoid bodies (EBs). Treatment of these aggregates with RA generates visceral endoderm on the outer surface of the EB. These endodermal subtypes were found to express the full catalogue of cytokeratins (CKs) associated with the pancreatic islets i.e. CKs 8, 18, 7 & 19.

The F9-derived endoderm was then treated with agents that have been shown to promote endocrine differentiation from progenitor cells i.e. activin A, betacellulin hepatocyte growth factor (HGF), nicotinamide and sodium butyrate. Certain treatments were found to increase the levels of important pancreatic mRNAs i.e. a combination of HGF and activin A increased pancreatic and duodenal homeobox gene 1 (PDX1) and preproinsulin (PPI) expression in RA-treated F9 monolayers. Sodium butyrate was shown to have positive effects on the expression of somatostatin and pancreatic polypeptide (PP) mRNAs. Other β cell genes detected in differentiating F9 cells include Pax6 and indian hedgehog (Ihh).

To further study the role of the transcription factor PDX1 in pancreatic development, cell trapping was employed. In this system, a specialised construct was designed with the zeocin resistance gene (Sh *ble*) under the control of the PDX1 promoter region. This construct was shown to be capable of selectively maintaining the viability of F9 cells with the capability to drive the PDX1 promoter, in the presence of zeocin. The cells purified using this technique were found to exhibit increased levels of PDX1 mRNA and in certain cases PPI also, even though PDX1 protein was not detected.

Further evidence for the presence of functional PDX1 in cell trapped lines was provided by the observation that these cell lines co-expressed somatostatin, which is known to be transactivated by PDX1.

Upon culturing β cells for prolonged periods *in vitro* it has been noticed that these cells lose the ability to secrete insulin in response to environmental glucose signals (glucose stimulated insulin secretion, GSIS), in a process sometimes referred to as de-differentiation. Upon culturing the murine β cell line, Min6, for 40 passages similar effects were noticed. Analysis of the gene expression profiles in continuously cultured Min6 cells via DNA array led to the discovery that a number of genes associated with rapidly proliferating primitive type cells were upregulated in the high passage Min6 cells (e.g. the polycomb group gene EED & p55CDC). This analysis also revealed that a number of genes critical for the regulated secretion of insulin were downregulated, including; nucleobindin, secretogranin II, secretogranin V, chromogranin B and prohormone convertase 2 (PC2). These observations and the associated reduction/loss of terminal differentiation markers such as PDX1, glucagon and somatostatin were all consistent with de-differentiation or with overgrowth of the culture by a more poorly differentiated subpopulation.

The human insulin gene has been successfully expressed in a number of cell lines as a possible method for 'artificial β cell delivery'. BHK-21 cells are characterised as a safe cell line to implant into humans in a suitably encapsulated format. Expression of the human PPI gene in BHK-21 cells resulted in large amounts of proinsulin production. There was no evidence of proinsulin processing to mature insulin and this was associated with an inability to store the protein properly. This also resulted in the continuous secretion of proinsulin at a steady rate of 0.12 pmol proinsulin/hr/10^5 cells. These cells were found to be unable to regulate proinsulin secretion in response to glucose. Overexpression of glucokinase resulted in a moderate GSIS but overexpression of GLUT2 and glucokinase resulted in the total loss of insulin secretion. This may be due to a deleterious increase in the metabolic burden in the cells. Proinsulin secreting BHK-PPI-C16 cells were also capable of increasing proinsulin secretion upon treatment with cAMP and it is proposed that this increase in secretion is due to a general increase in intracellular proinsulin i.e. the rate of secretion was determined by the availability of the protein.

Abbreviations

Ab	-	Antibody
Act	-	Activin A
ACTH	-	Adrenocorticotropic hormone
ADP	-	Adenosine Diphosphate
ALS	-	Amyotropic Lateral Sclerosis
Amp	-	Ampicillin
ATCC	-	American Tissue Culture Collection
ATP	-	Adenosine Triphosphate
BCIP	-	5 Bromo-4chloro-3-indolyl-phosphate,4 toluidine salt(X-phosphate, 4 toluidine salt)
BETA2	-	β-cell E-box transacticator 2
bHLH	-	basic Helix Loop Helix
BrdU	-	Bromodeoxyuridine
BSA	-	Bovine Serum Albumin
BTC	-	Betacellulin
CAD	-	Cadherin
CAM	-	Cell Adhesion Molecule
cAMP	-	cyclic Adenosine Monophosphate
CAT	-	Chloramphenicol Acetyl Transferase
cDNA	-	complimentary Deoxyribonucleic Acid
CK	-	Cytokeratin = Keratin
C-peptide	-	Connecting peptide
CNS	-	Central Nervous System
CRE	-	cAMP Responsive Element
CREB	-	CRE Binding Protein
db cAMP	-	Dibutyryl cAMP
DLK	-	Delta-like Homologue 1
DMEM	-	Dulbecco's Minimal Essential Medium
DMSO	-	Dimethyl Sulphoxide
DNA	-	Deoxyribonucleic Acid
DNase	-	Deoxyribonuclease
dNTP	-	Deoxynucleotide triphosphate (N= A,C, T, G)
EB	-	Embryoid Body
EC	-	Embryonic Carcinoma
ECM	-	Extracellular Matrix
EDTA	-	Ethylene diamine tetraacetic acid
EGF	-	Epidermal Growth Factor
EIA	-	Enzyme Immuno Assay
ES	-	Embryonic Stem
EtOH	-	Ethanol
FCS	-	Fetal Calf Serum
FGF	-	Fibroblast Growth Factor
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
GCK	-	Glucokinase

GLP	-	Glucagon Like Peptide
GLUT	-	Glucose Transporter
GSIS	-	Glucose Stimulated Insulin Secretion
HDAC	-	Histone Deacetylase
HEPES	-	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HGF	-	Hepatocyte Growth Factor
HSC	-	Hematopoietic Stem Cell
IAPP	-	Islet Amyloid Polypeptide
IBMX	-	3-isobutyl-methylxanthine
ICC	-	Islet-like Cell Cluster
IDDM	-	Insulin Dependent Diabetes Mellitus
IDX1	-	Islet Duodenal Homeodomain Protein
IEF1	-	Insulin Enhancer binding Factor 1
Ig	-	Immunoglobulin
Ihh	-	Indian Hedgehog
IMS	-	Industrial Methylated Spirits
IPCs	-	Islet Progenitor Cells
IPF1	-	Insulin Proximial Factor 1
IUF1	-	Insulin Upstream Factor 1
kDa	-	kilo Daltons
k_m	-	Michaelis Constant
KRB	-	Krebs Ringers Buffer
NAD	-	Nicotinamide Adenine Dinucleotide
NPY	-	Neuropeptide Y
MEM	-	Minimal Essential Medium
Min	-	Minute
MMLV-RT	-	Moloney Murine Leukemia Virus-Reverse Transcriptase
MODY	-	Mature Onset Diabetes of the Young
mRNA	-	messenger RNA
mV	-	Milivolts
MW	-	Molecular Weight
NBT	-	Nitro Blue Tetrazolium Salt
ODC	-	Ornithine Decarboxylase
PBS	-	Phosphate Buffered Saline
PC	-	Prohormone Convertases
PCR	-	Polymerase Chain Reaction
PDX1	-	Pancreatic and Doudenal Homeobox gene 1
PE	-	Parietal Endoderm
PEPCK	-	Phosphenolpyruvate carboxykinase
PP	-	Pancreatic Polypeptide
PPI	-	Preproinsulin
PrE	-	Primitive Endoderm
PYY	-	Peptide Tyrosine Tyrosine
RA	-	Retinoic Acid
RAR	-	Retinoic Acid Receptors
RER	-	Rough Endoplasmic Reticulum

RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
RNasin	-	Ribonuclease Inhibitor
Rpm	-	Revolutions per Minute
RT-PCR	-	Reverse Transcriptase-PCR
s	-	Seconds
SB	-	Sodium Butyrate
SDS	-	Sodium Doedecyl Sulphate
SFM	-	Serum Free Medium
Shh	-	Sonic Hedgehog
STF1	-	Somatostatin gene Transactivating Factor 1
TBE	-	Tris-boric acid-EDTA buffer
TBS	-	Tris Buffered Saline
TE	-	Tris-EDTA
TEMED	-	N', N', N', N' – Tetramethyl-Ethylenediamine
TGF	-	Transforming Growth Factor
TGN	-	trans-Golgi network
UHP	-	Ultra high pure water
VE	-	Visceral Endoderm
v/v	-	volume/volume
w/v	-	weight per volume

1.0 INTRODUCTION.

1.0 General Introduction

The pancreatic islets of Langerhans secrete glucoregulatory hormones in response to metabolic fuels. In the case of glucose, its transport into the pancreatic β cells and its subsequent metabolism lead to the controlled release of the polypeptide hormone insulin. Following the discovery of insulin, and advances in the study of metabolism, it was discovered that higher mammals could avoid continuous food consumption due to the presence of a regulated system of energy storage. The main regulator in this system is insulin, levels of which rise post-prandially and fall again during fasting. Insulin is absolutely required to maintain normoglycemic conditions in the blood, this and its unique role as an anabolic peptide hormone means it is regulated in a controlled fashion to allow it to respond to blood glucose levels rapidly. When this system is impaired i.e. through autoimmune destruction of β cells as in the case of insulin dependent diabetes mellitus, (Type I Diabetes), serious complications and illness may result.

Polyuric states (passing large amounts of urine), resembling diabetes mellitus, have been observed since before 1000 BC. Diabetes was discovered to be a disease of the pancreas in the 19th century (discovered when a pancreatectomy induced diabetes in dogs (Mering & Minkowski, 1890)). Early therapeutic strategies were based on pancreatic replacement and some of these trials caused a reduction in the hyperglycemic state. Others began searching for the pancreatic extracts that were responsible for the control of glycemia in the blood. In 1922 a collaborative effort between F. Banting, C. Best, J. Collip, and J.J.R. Mc Leod resulted in the discovery of a pancreatic extract, that saved the life of a 14-year old boy dying of diabetes. This was the proposed 'insulin' (it was named prior to discovery, and refers to the Latin root for the islet cells in the pancreas) (Banting & Best, 1922).

Insulin dependent diabetes mellitus (IDDM), is generally synonymous with type I diabetes and is a result of massive autoimmune destruction of β-cells. In the initial stages of IDDM, patients usually experience severe thirst and polyuria. Extreme tiredness, weight loss and muscle cramps are also symptoms of IDDM. Following diagnosis, patients are prescribed insulin. A remission period of up to two years may then occur, this is due to improved β-cell function as a result of lowered blood

glucose. This allows for very low insulin requirements. This respite is temporary however and eventually a stage is reached when the quantity of β-cells available becomes so low that regular insulin injections are required to survive.

While insulin is a life saving drug, there are problems associated with injecting insulin. These supplements do not mimic the way the normal β-cells constantly regulate circulating glucose levels in the body. Complications that can occur include, kidney failure, blindness, atherosclerosis in large vessels and nerve damage leading to pain or numbness in the extremities (peripheral neuropathy). All of these are basically due to the presence of too much glucose in the blood (hyperglycemia), however increased insulin doses can lead to confusion, shakes and blackouts, due to blood glucose being lowered too far (hypoglycemia). As well as the obvious adverse effects on the quality of life for the diabetic, these complications are very expensive for health services/patients to maintain i.e., IDDM has a prevalence of about 0.4% therefore ~1 million Americans have the disorder (Mitanchez *et al.*, 1997).

Alternative therapies are currently under investigation. The transplantation of pancreatic material including β-cells (as whole pancreata or isolated islets) is expensive and limited due to the scarcity of donated material. The most promising methods for the future may employ gene technology and undifferentiated stem cells to produce artificial β-cells, which on implantation would, in theory, regulate blood glucose constantly. Before these therapies are possible however, it is necessary to have a full understanding of the *in vivo* processes such as the development of fetal pancreata, specification of islet cell types and regulation of insulin synthesis and secretion in response to metabolic signals.

The purpose of this study is to investigate the possibility of generating endocrine tissue from undifferentiated embryonic carcinoma (EC) cells (F9), and to monitor the events dictating cell fate determination. Also the generation of human insulin secreting cell lines from BHK21 cells through direct genetic manipulation is examined. It is hoped that the results outlined here will add to the understanding of the issues surrounding the generation of artificial β cells and thus may lead to the development of cells that could maintain normoglycemia following transplantation into a diabetic host.

1.1 Development of Fetal Pancreata

The pancreas is a pinkish tan organ and is divided in to four regions: the head, neck, body and tail (Bockman, 1993). Study of the development of the pancreas is of interest due to its unusual property of being both an exocrine and endocrine gland, and also, to understand the events surrounding its organogenesis. This knowledge will be necessary to generate fully functional replacement tissue for diabetes treatment in the future. To achieve this, a full understanding of the extrinsic and intrinsic factors employed in β cell specification is required, and also the identification of a catalogue of markers to correctly distinguish β cell-producing stem cells.

1.1.1 The Origins of the Fetal Pancreas

Embryonic cells arrange themselves in to three 'layers' known as the primary germ layers, the ectoderm, the mesoderm and the endoderm. The digestive tract of the embryo originates from the endodermal germ layer (Edlund, 2001; Kim & Melton, 1998; Madsen *et al.*, 1996). Within the developing embryo the dorsal mesenchyme condenses and evagination of the foregut occurs on about day 11 of gestation (20-22 somites) (Githens, 1993; Kim *et al.*, 1997). This region of the embryonic gut is composed of two 'buds' known as the dorsal and ventral buds. These buds grow, branch and later fuse to form the vertebrate pancreas (Edlund, 2002). The vertebrate pancreas consists of exocrine acinar cells that synthesise digestive enzymes, and the branched ductal epithelium which secretes the enzymes in to the intestine. The endocrine portion of the pancreas is much smaller (Bishop & Polak, 1991; Madsen *et al.*, 1996), and consists mainly of four types of hormone secreting cells (α, β, δ and PP cells) in clusters called islets (the Islets of Langerhans) (Bishop & Polak, 1991).

1.1.2 Initiation of Pancreatic Organogenesis

The early commitment of the region of the embryonic gut to initiate dorsal and ventral budding is not well understood, i.e. how a single layer of epithelial cells in the foregut is instructed to commence organogenesis. It is known that the region of the gut that subsequently becomes the pancreas expresses the transcription factor PDX1

(discussed separately in Section 1.1.3.5.1), and that mice and humans lacking this gene do not form a pancreas (Jonsson *et al.*, 1994; Stoffers *et al.*, 1997). However this factor must act downstream of the initial commitment signal as apancreatic mice ($PDX1^{-/-}$) still form the pancreatic buds and the appearance of early pancreatic markers is unaffected (Kim *et al.*, 1997; Edlund, 1999). The observation that *in vitro* culture of embryonic endodermal tissue with exogenous mesenchymal tissue can lead to the formation of dorsal buds (Ahlgren *et al.*, 1997), infers that there are intrinsic determinants within this region of the gut for its eventual fate.

1.1.2.1 Hedgehog Signalling and Initiation of Pancreatic Organogenesis

Sonic Hedgehog (Shh) and Indian Hedgehog (Ihh) are expressed in regions of the gut endoderm except where the dorsal and ventral buds form and can almost describe a molecular boundary between pancreas and stomach/duodenal analgen. Repression of Shh expression, using the steroid alkaloid teratogen cyclopamine, can lead to ectopic pancreatic development in the stomach and intestine (Kim & Melton, 1998). The repression of hedgehog gene expression can also be used to explain why only a restricted region of PDX1 expressing foregut actually goes on to form the dorsal and ventral buds.

1.1.2.2 Implications for Notochord Repression in Pancreatic Development

The region of the foregut destined to become the dorsal pancreatic bud is in direct contact with the notochord (the notochord is a flexible structural organ that runs the length of the body. This is the organ from which vertebrae evolve), at the period when pancreatic commitment occurs (Kim *et al.*, 1997). Removal of the notochord from embryonic mice just prior to the time when pancreatic fate is specified prevents expression of PDX1 and Isl1 (crucial for dorsal mesenchyme formation, (Ahlgren *et al.*, 1997); Section 1.1.3.5.4), and the dorsal bud does not form. The ventral bud is unaffected by this change (Kim *et al.*, 1997). The notochord appears to function through the release of notochord factors such as activin βB and FGF2. These factors were found to repress Shh expression thus allowing normal morphogenesis (Section 1.1.2.1). Removal of the notochord allowed Shh expression and prevented induction

of the genes required for pancreatic differentiation. Similarly, culture of midline endoderm from the pre-pancreatic region of the gut with isolated notochord resulted in the generation of cells expressing PDX1 and insulin (Hebrok *et al.*, 1998). These experiments were carried out in chick endoderm; however, in similar experiments using murine tissue, recombination of dorsal endoderm with exogenous notochord generated PDX1 expressing cells only (i.e. insulin expression was not present) (Edlund, 2002). This may reflect either differing mechanisms between chick and mouse differentiation or contamination of the chick cultures with endothelial cells which would have supported the differentiation of insulin expressing cells (Lammert *et al.*, 2001). While the notochord related effects on dorsal bud formation are clear, it is not known how Shh is repressed in the pre-ventral bud region. In fact FGF2 had a negative effect on endocrine development in the ventral bud (Deutsch *et al.*, 2001). Therefore, ventral bud differentiation and dorsal bud differentiation appear to involve different initiation signals.

1.1.2.3 Mesenchymal Effects on Pancreatic Organogenesis in the Developing Embryo

Mesenchymal tissue surrounding the dorsal pancreatic bud expresses Isl1 (a LIM homeodomain protein (Section 1.1.3.5.4)). Isl-$1^{-/-}$ null mutants fail to form dorsal mesenchyme and thus do not form the dorsal pancreatic bud. The expression of PDX1and Beta2 in $Isl1^{-/-}$ mice (Ahlgren *et al.*, 1997), however, indicates that Isl1 expression and thus mesenchymal factors are not the initiator signals for embryonic foregut commitment and should be considered as permissive factors required to facilitate proper development.

Mesenchymal effects on pancreatic dorsal bud formation are effected through the release of soluble factors. This was demonstrated by the observation that mesenchyme could induce differentiation across a millipore filter (Golosow & Grobstein, 1962). These mesenchymal secretions appear to be involved in regulating the relative proportions of endocrine versus exocrine tissue in the developing pancreas by repressing endocrine development. Follistatin could duplicate these effects and has been shown to be present in embryonic pancreatic mesenchyme (Miralles *et al.*, 1998).

HGF secretion has also been implicated in the mesenchymal effects on developing islets and the HGF receptor (the c-met protooncogene) is expressed in both developing and mature β cells. HGF has been shown to have mitogenic action on developing β cells. However, this was accompanied by a decrease in insulin content (Otonkoski *et al*, 1996).

1.1.2.4 Homeobox Hlxb9 and Pancreatic Development

Although the effects of Shh expression and signalling through the notochord and mesenchymal layer are understood, the early events in the morphogenesis of the pancreatic buds are still unclear. Hlxb9 (encodes Hb9 protein), is a homeobox gene that is transiently expressed in the regions of the gut that commit to pancreatic morphogenesis and is later restricted to the β cells (Li *et al.*, 1999). These investigators noted that mice lacking this gene did not form a dorsal pancreas and although the ventral pancreas was present, its organisation was disrupted. Phenotypic comparisons of Hlxb9 and PDX1 deficient mice do indicate that the role of Hb9 is upstream from PDX1. While the actual role of Hb9 is unknown there are three possible explanations as to its function and distribution in the developing pancreas:

(I) Expression of Hlxb9 confers a competence to that region of the foregut to respond to subsequent signals to initiate pancreatic morphogenesis.

(II) The ventral function of Hb9 is compensated by a gene product with similar activity.

(III) Expression of Hlxb9 in the gut epithelium is not as important as its reported expression in the notochord at an early stage, thus it is possible that the actions of Hb9 may be to regulate notochord secretions involved in activating/repressing pancreatic determinants. However, this is unclear following observations that Hlxb9 mutants exhibit normal Ihh/Shh restrictions; thus, Hb9 does not appear to affect this aspect of developmental control (Li *et al.*, 1999)).

(Edlund, 1999).

1.1.2.5 HNF 3β/Foxa2 Expression and the Developing Pancreas

HNF-3β or Foxa2, a member of the forkhead/winged family of transcription factors, is expressed in the notochord and gut of mouse embryos and knockout animals die *in utero* due to the lack of a notochord which leads to defects in the patterning of the neural tube (Monaghan *et al.*, 1993; Ang & Rossant, 1994; Weinstein *et al.*, 1994). Foxa2 is expressed throughout the fetal gut (including the region that evaginates into the pancreatic buds) and is widely expressed in islet and acinar cells (Monaghan *et al.*, 1993; Cockell *et al.*, 1995; Wu *et al.*, 1997). The importance of Foxa2 is that it is involved in the regulation of pancreatic genes such as α amylase (Cockell *et al.*, 1995) and, more significantly, PDX1 where it also contributes to tissue specific expression (Sharma *et al.*, 1997; Wu *et al.*, 1997). Due to the lethality of $Foxa2^{-/-}$ mutants, when the cre-lox system was employed to disrupt its expression in pancreatic β cells the animals had significantly reduced PDX1 levels (Lee *et al.*, 2002). This places Foxa2 upstream of PDX1 in pancreas development which infers that this is the key regulator of pre-pancreatic gut development.

1.1.2.6 Pancreatic Stem Cells?

The adult pancreas does have limited regenerative ability following partial pancreatectomy or injury (Lehv & Fitzgerald, 1968; Pearson *et al.*, 1977; Bouwens, 1998b). The regeneration appears to stem from activation of cells within the ductular region of the pancreas (Wang *et al.*, 1995; Bonner-Weir *et al.*, 2000). This suggests that cells exist within the pancreas that can be called upon to differentiate when needed, these cells would have to be able to propagate *in vivo* in the undifferentiated state until required. Such proliferating cell lines have been generated from duct epithelial cells (Tsao & Duguid, 1987). However there is also the possibility that the regenerated tissue is as a result of transdifferentiation of fully differentiated cells within the pancreatic ducts (Bouwens, 1998b; Rosenberg, 1995). Differentiation of the embryonic pancreas has many similarities to neuronal differentiation (Edlund, 1998) and critical transcription factors involved in endocrine differentiation are also required for neuronal differentiation e.g. Isl1, NeuroD1, Nkx2.2 and Pax6 (Edlund, 1999). This seems to imply a common origin for pancreatic and neuronal tissue. This

theory is somewhat validated following a study whereby the SV40 T antigen under the insulin gene regulatory control was found to be transiently expressed in the neural tube and crest of embryonic transgenic mice (Alpert *et al.*, 1988). Insulin II mRNA transcripts have also been identified in the developing rat CNS (Devaskar *et al.*, 1993). Thus it was unsurprising to discover the presence of nestin (an intermediate filament protein expressed in undifferentiated neuronal progenitor cells during the development of the CNS) in a subset of hormone negative cells of the pancreas (Zulewski *et al.*, 2001). These nestin positive cells were found to be capable of differentiating towards endocrine, exocrine and hepatic phenotypes. In a similar study by Lumelsky *et al.*, (2001) a protocol to promote and purify nestin expressing cells from undifferentiated stem cells was used and these cells could be differentiated in to insulin expressing cells. It is unlikely however, that these ES derived cells are truly representative of pancreatic stem cells due to their lack of PDX1 expression. Ramiya *et al.*, (2000) identified duct epithelial cells (in mice) as pancreatic stem cells and generated islet-like structures following long term cultures of these cells. However, these 'adult stem cells' are not necessarily the same cells as those initial epithelial cells that derived all the pancreatic cell types and probably represent a population of pre-committed cells.

Thus the identity or even existence of pancreatic stem cells in the developing gut epithelium remains unclear.

1.1.3 Development of the Pancreatic Endocrine Cell Types

The pancreas contains exocrine, endocrine and ductal cells which are all originally derived from the same region of gut epithelium in the developing embryo. The development of the individual cell types and the order of their appearance is of major importance for the understanding of developmental biology and also for implementation of this information for the generation of replacement tissue for diabetes treatment. This study is mostly concerned with the development of the endocrine pancreas thus the development of the four main cell types of endocrine islets are more closely examined.

1.1.3.1 The Cell Types Within the Islet

The endocrine islets comprise only 1-3% of the whole pancreatic mass (Bishop & Polak, 1991; Madsen *et al.*, 1996). There are four main endocrine cell types within the pancreatic islet i.e. the α cells, which secrete glucagon and comprise 15-20% of the cells in the islet; insulin secreting β cells (β cells also secrete small amounts of IAPP) account for 60-80 % of the islet cell mass; the δ cells (5-10% of the islet) synthesise somatostatin (and some IAPP); and the pancreatic polypeptide producing PP cells make up < 2% of the total cell mass (Edlund, 2002). Insulin is released following feeding and subsequent increases in blood sugar, the insulin released causes the uptake of blood sugar by the tissues of the body e.g. muscles. Insulin also inhibits glucose production in the liver (Hardie & Cohen, 1991). Glucagon is released in conditions of low glucose and promotes gluconeogenesis. Somatostatin and pancreatic polypeptide are inhibitors of endocrine and exocrine secretion (Kreymann & Bloom, 1991).

1.1.3.2 Chronological Appearance of the Islet Cell Types

Numerous studies have been carried out on the development of islet cell types with an aim to discovering the progenitor cells that give rise to mature endocrine cells, especially β cells. The data obtained suggests that β cell turnover (especially following injury) is facilitated by the differentiation of a precursor cell-type, due to the very low rate of proliferation of β cells (Bouwens *et al.*, 1997; Polak *et al.*, 2000).

RT-PCR analysis of microdissected mouse embryos indicates that the induction of the hormone gene expression occurs even before evagination of the foregut to form the dorsal and ventral buds. In this study, Gittes & Rutter (1992), discovered that somatostatin RNA was initially detectable, followed by glucagon and insulin. PP transcripts were not detected until the dorsal and ventral buds were undergoing fusion to form the pancreas.

Immunohistochemical studies have revealed that during islet development the pancreas evolves from a position of hormone-negativity to the eventual secretion of

the four endocrine hormones. The order of appearance of immunoreactive hormone protein is different however from the appearance of their mRNA transcripts. Using immunohistochemistry, insulin and glucagon expressing cells are the initial endocrine cells detected and, while exclusive glucagon expressing cells are present at this stage (embryonic day 9.5; E9.5), all of the initial insulin expressing cells co-express glucagon (Teitelman *et al.*, 1993). Somatostatin is not detected until much later and PP is only immunohistochemically detectable after birth (possibly due to the fact that there are fewer cells of this type in the developing pancreas, therefore the making the protein more difficult to detect). Earlier reports of PP expression prior to the other islet hormones (Herrera *et al.*, 1991) is likely to be as a result of cross reaction of the PP antibody with PYY (Upchurch *et al.*, 1994) which is expressed early in the developing islet (Section 1.1.3.3).

1.1.3.3 Co-expression of Endocrine Hormones During Islet Cell Development

Peptide YY (PYY) is a pancreatic and intestinal hormone that can be found in glucagon expressing cells in pancreatic islets. Studies using the SV40 large T antigen under the regulatory control of the PYY gene revealed that this peptide is co-expressed in all four lineages of the developing mouse pancreas. In fact it was hypothesised that the earliest endocrine cell in the pancreas is a $PYY^{+}/Insulin^{+}/Glucagon^{+}$ expressing cell (Upchurch *et al.*, 1994). In mature pancreata, PYY is only found in a subset of α cells, δ cells and PP cells. Similar data was presented for mice by Teitelman *et al.* (1993); however this study suggested that neuropeptide Y (NPY) was co-expressed in the early endocrine progenitors. It is likely that the NPY antibody cross reacted with the closely related PYY peptide. As already indicated, the earliest insulin expressing cells co-express glucagon, studies using human fetal islets demonstrated that these cells also co-express somatostatin (measured at 8 weeks of development). Within one week however, the co-expression of hormones was much reduced and cells expressing only insulin could be detected (Polak *et al.*, 2000).

Mature islet cells do not tend to co-express any of the other four main islet hormones. This co-expression at the early stages of development is likely to be a result of the

initial activation of key regulatory factors resulting in non-specific hormone expression. As the pancreas differentiates, selective activation/inactivation of regulatory molecules leads to mature endocrine cells expressing a single hormone (Madsen *et al.*, 1996). It has been suggested that cells co-expressing multiple hormones represent progenitor cells that can segregate into the individual endocrine cell types (Alpert *et al.*, 1988, Teitelman *et al.*, 1993). This hypothesis is disputed by a number of observations including the fact that the insulin/glucagon/PYY co-expressing cells were found to have a high level of DNA synthesis while newly derived insulin cells do not (Jackerott *et al.*, 1996). The multihormonal cells were also found largely negative for the homobox proteins PDX1 and Nkx6.1 which are crucial for β cell development (Larsson, 1998).

1.1.3.4 The Origins of β Cells in the Developing Pancreas

Over time, typical islets develop to a mature state consisting of a rim of glucagon expressing cells surrounding the insulin positive cells. The co-expressing glucagon/insulin cells are typically found to be more associated with the glucagon expressing cells and become far fewer in number as the islet develops. (It is possible that these cells are progenitors of α cells rather than β cells (Larsson, 1998). However Herrera (2002) presented data whereby it appeared that glucagon expressing α cells in the developing mouse arose from PDX1 expressing, hormone-negative cells. This disputes other observations whereby disruption of PDX1 expression in cultured rat islet cells led to increased numbers of α cells (Wang *et al.*, 2001)). The appearance of the insulin-only expressing cells occurs during a phase known as the secondary transition of pancreatic development. These insulin$^{+/+}$ cells initially appear in the parenchyma in the vicinity the glucagon and glucagon/insulin expressing cells. It is postulated that the insulin-only cells are as a result of differentiation of the hormone negative parenchymal cells (which do however express critical β cell transcription factors e.g. PDX1 and Nkx6.1). Potential signalling molecules to induce this transition include the TGFβ superfamily ligand activin, which together with mitogens such as betacellulin or HGF (Larsson, 1998), has been shown to be capable of inducing β cell neogenesis *in vitro* from hormone negative AR42J cells (Mashima *et al.*, 1996a,b).

GLUT2 is a critical component in the glucose sensing apparatus of β cells and although it has widespread expression in fetal and adult tissues, it is restricted to β cells in the pancreas. Immunohistochemical monitoring of GLUT2 expression in the developing pancreas indicates that exclusive insulin expression occurs initially within GLUT2 expressing cells in the ductal network. These GLUT2/Insulin cells were found to aggregate in to the β cell masses of the islets of Langerhans (Pang *et al.*, 1994).

1.1.3.5 Endocrine Cell Determination Through the Expression of Transcription Factors

β cell neogenesis is a complex process and direct monitoring of immunoreactive hormones has often led to confusion and contradictory data. As already indicated in Section 1.1.2, transcription factors such as PDX1 play an important role in the determination and maturation of the islet cells of the pancreas. A selection of the most significant (usually determined by the effects in knockout mice) are discussed below. The cascades and interrelationships of the transcription factors are summarised at the end in Figure 1.1.1

1.1.3.5.1 PDX1 Expression in the Developing Pancreas

PDX1 was initially identified as a β cell transcription factor in studies with mouse β and α cell tumours, when it was shown to be present only in the β cells (Ohlsson *et al.*, 1991). PDX1 was first cloned by Ohlsson *et al.*, (1993), (who called it IPF1, Insulin Promoter Factor 1). The rat version was cloned soon after by two groups and called somatostatin transactivating factor (STF1) (Leonard *et al.*, 1993) or islet duodenal homeodomain protein (IDX1) (Miller *et al.*, 1994). PDX1 (pancreatic duodenal homeodomain protein) has been proposed as the official term for this gene in mice by the International Committee on Standardisation of Genetic Nomenclature for Mice and is used throughout this thesis.

As indicated earlier (Section 1.1.2.2), FGF2 and activin βB are secreted from the notochord in contact with the region of the gut epithelium destined to evaginate in to

the dorsal pancreas (Hebrok *et al.*, 1998; Kim & Melton, 1998). This facilitates PDX1 expression through the concomitant repression of Shh expression. In early development, PDX1 is expressed in the ductular regions of the pancreas, the exocrine cells and in the endocrine cells. As the pancreas develops however its expression becomes more restricted to β cells and to a portion of δ cells (10-20%) (Ohlsson *et al.*, 1993; Madsen *et al.*, 1996; Carty *et al.*, 1997; Serup *et al.*, 1995; Øster *et al.*, 1998).

The critical importance of PDX1 to pancreatic development is clear from clinical and knockout studies. Pancreatic agenesis (lack of a pancreas) in a patient homozygous for a point mutation in the PDX1 coding region was attributed to the lack of expression of functional PDX1 (Stoffers *et al.*, 1997). Transgenic mice homozygous for a targeted mutation of the PDX1 gene lack a pancreas; although the mutation is not lethal *in-utero*, the pups died soon after birth (Jonsson *et al.*, 1994). Using the CRE-Lox system, Ahlgren and collegues (1998) specifically disrupted PDX1 in the β cells. These pups were normal at birth and remained so for 3-5 months. At this point all animals were overtly diabetic. Analysis revealed that 17% of the insulin positive cells still co-expressed PDX1, however the total number of insulin positive cells was 60% lower than in normal animals (Ahlgren *et al.*, 1998). Further analysis revealed that the number of α cells was increased and that there were insulin/glucagon co-expressing cells in the mutant pups. Using clonal populations of the rat INS-1 cell line it was shown that to promote α cell differentiation in the transitory Ins1αβ population, PDX1 expression had to be eliminated. Overexpression of the α cell differentiation marker Brain4 did not have the same effect (Wang *et al.*, 2001). Thus PDX1 appears to be a fate determinant between α and β cells in the pancreas. Expression of β cell specific genes is also dependent on PDX1 levels, e.g. pancreatic GLUT2 expression was non-existent when PDX1 was specifically knocked out of β cells. The role of PDX1 in GLUT2 transcription is dose-dependent as shown by GLUT2 levels in heterozygous ($PDX1^{+/-}$) animals and thus may be also implicated in the poor glucose sensing associated with Type II diabetes (Ahlgren *et al.*, 1998; Ramirez & Raskin, 1991).

In summary there appears to be two related but separate functions of PDX1 in the developing pancreas, i.e. (I) to aid in determination of which region of the gut

epithelium will evolve in to a pancreas (global activity) and (II) as a β cell determinant through positively regulating β cell genes such as insulin and GLUT2. The specific β cell role of PDX1 in determining β cell fate follows preliminary cell fate determination which is controlled by the Notch signalling pathway (reviewed separately Section 1.1.3.5.2). It is interesting to note that while monitoring PDX1 expression, another β cell transcription factor was identified that had very similar expression patterns i.e. Nkx6.1. PDX1 was seen to activate Nkx6.1 (Øster *et al.*, 1998; Ahlgren *et al.*, 1998) and it is suggested that Nkx6.1 may mediate some of the effects of PDX1. Transfection experiments have shown that although PDX1 expression can stimulate insulin gene expression (and thus direct a cell towards a β cell type), it is not always the case and depends on the co-expression of Beta2 to exert its effects (Serup *et al.*, 1996; Madsen *et al.*, 1996; Madsen *et al.*, 1997). Based on this it is likely that the effects of PDX1 are mediated through, or at least require, the co-operation of other gene products such as Beta2, Nkx6.1and Pax6 (Madsen *et al.*, 1997).

1.1.3.5.2 Ngn3 Expression and Notch Signalling in Specifying Pancreatic Cell Fate

The development of individual cell types from a relatively homogenous initial cell population can be executed through a process known as 'lateral specification' (Edlund, 1999), which is controlled by the Notch signalling pathway. The appearance of scattered differentiated endocrine cells within a field of progenitor cells is indicative of lateral specification (Apelqvist *et al.*, 1999), thus the Notch signalling pathway appears to dictate cell fate following initial pancreatic determination. Notch is a transmembrane receptor and Delta is its transmembrane ligand. Ngn3 is a bHLH protein that stimulates expression of Delta and pushes cells towards an endocrine fate through its effects on β cell differentiation factors such as Beta2 (Huang *et al.*, 2000). Stimulation of Notch by a Delta-expressing neighbouring cell causes the expression of Hes1 (another bHLH protein), which inhibits Ngn3 (Lee *et al.*, 2001). Thus a cell that is undergoing endocrine fate specification through Ngn3 actively inhibits its neighbours from taking the same fate (Edlund, 1999; Mc Kinnon & Docherty, 2001). As differentiated endocrine cells form they are seen to migrate into the adjacent mesenchyme. This removal of differentiated (Delta expressing) cells thus allows

further differentiation of endocrine cells through a decrease in Notch signalling and facilitates the formation of islet structures (Edlund, 2001).

Notch signalling in endocrine differentiation can be studied through the disruption of the signalling by creating transgenic mice deficient in Delta-like gene 1 (Delta) or similarly through overexpressing Ngn3 (by placing it under the control of the PDX1 promoter). These transgenic mice were found to have poorly developed pancreatic buds primarily consisting of differentiated endocrine cells. The islet cells produced were predominantly α cells, which may indicate that this is the default pathway in endocrine differentiation (Schwitzgebel *et al.*, 2000; Ritz-Laser *et al.*, 2002). The poor development and small size of the dorsal and ventral buds was as a result of premature differentiation of the progenitor cell population to the slower proliferating endocrine cells. Exocrine differentiation was totally disrupted in these animals (Apelqvist *et al.*, 1999).

These observations regarding Ngn3 led to interest in $Ngn3^{-/-}$ knockout mutants, which died from diabetes due to the total lack of endocrine cells in the pancreas. Ngn3 is present transiently in hormone negative cells just prior to the appearance of endocrine cells, and it is proposed that Ngn3 is a likely candidate for a marker of the endocrine progenitor cell (Gradwohl *et al.*, 2000; Schwitzgebel *et al.*, 2000). These $Ngn3^{+}$ cells also co-express the proliferation marker Ki-67 which is in keeping with their proposed role as self-replicating progenitor cells (Jensen *et al.*, 2000).

1.1.3.5.3 Beta2 Expression in Developing Islet Cells

Insulin enhancer factor 1 (IEF1) (Section 1.2.1.2) was discovered due to its enhancement of insulin gene transcription in rats (Walker *et al.*, 1990). IEF1 was found to be a heterodimer of Beta2 (also known as NeuroD1, and is a novel bHLH protein (Naya *et al.*, 1995)) and the E2A gene product, the ubiquitous bHLH proteins E12/E47. This complex is important for insulin gene transcription (Dumonteil *et al.*, 1998; Glick *et al.*, 2000). Beta2 expression is detected early in the developing pancreas and is one of the first genes induced by Ngn3 following notch based entry to endocrine differentiation (Huang *et al.*, 2000; Jensen *et al.*, 2000; Gradwohl *et al.*,

2000; Apelqvist *et al.*, 1999). Transgenic mice with disrupted Beta2 genes died perinatally from severe diabetes due to a significant reduction in β cell numbers and improperly formed islets. These results indicate that Beta2 plays a role in islet morphogenesis (Naya *et al.*, 1997). Islet morphogenesis has been found to be dependent on the presence of neural cell adhesion molecule (N-CAM), as mice deficient in this molecule have disrupted islets (Esni *et al.*, 1999). However, Naya and collegues report that N-CAM expression is not affected in Beta2-disrupted mutants. Thus there may be novel CAMs that are affected by the loss of Beta2 or possibly other known CAMs, it is known for instance that Ep-CAM expression is also implicated in islet organisation in the developing pancreas (Cirulli *et al.*, 1998).

Thus Beta2 expression facilitates correct spatial organisation of the developing islet. It is also important in specifying β cell-type determination, i.e. PDX1 mediated induction of insulin gene expression, and subsequent transcription of the insulin gene, is impaired without the presence of Beta2 (Serup *et al.*, 1995; Serup *et al.*, 1996; Madsen *et al.*, 1996; Ohneda *et al.*, 2000).

1.1.3.5.4 Isl1 Expression in Developing Islet Cells

The insulin gene transcription factor/endocrine determinant Isl1 (Islet 1), is a LIM homoedomain protein and is involved in lineage determination of pancreatic endocrine cells (Karlsson *et al.*, 1990). Isl1 is expressed in the mesenchymal cells that surround the dorsal bud of the embryonic gut. Mice engineered with a disrupted Isl1 gene were found to lack dorsal mesenchymal cells and therefore did not form a dorsal pancreas. The ventral exocrine cells were found to be present, however there was a complete lack of differentiated endocrine tissue. Exocrine differentiation in the dorsal bud was recoverable by exposing the dorsal evagination to wild type mesenchymal cells (Ahlgren *et al.*, 1997). The importance of mesenchymal secretions in the developing pancreas has already been discussed in Section 1.1.2.3, and it is noteworthy that fetal mesenchyme can cause islet neogenesis when co-cultured with pancreatic ductal epithelium or when they are co-transplanted into nude mice (Dudek & Lawerence, 1988; Dudek *et al.*, 1991).

Isl1 has been shown to act downstream of Beta2 in the transcription factor cascade towards endocrine cells (Ahlgren *et al.*, 1997; Jensen *et al.*, 2000).

1.1.3.5.5 Pax6 Expression in Developing Islet Cells

Pax genes are members of the paired-box family of genes which encode nuclear transcription factors. Pax genes are involved in the development of a number of organs in the body including the eyes, brain, kidney, immune system and the pancreas and a number of genetic disorders are as a result of Pax gene mutations (Dohrmann *et al.*, 2000). During mouse embryogenesis, Pax6 is widely expressed in developing embryos including the eyes, nose, CNS and pancreas.

Pax6 expression can be initially detected in a subset of cells throughout the development of the dorsal and ventral buds. Pax6 is restricted to the islets in newborn animals where it is expressed in the four main pancreatic cell types. Knockout animals ($Pax6^{-/-}$) were found to have a similar phenotype to the naturally occurring small eye mutant animals, Sey^{Neu} (these animals lack eyes due to point mutations in the Pax6 gene). All islet hormones were expressed with the exception of glucagon and the hormone producing cells were disorganised (St-Onge *et al.*, 1997). Thus Pax6 is critical in α cell differentiation and has a role in the organisation of the islets. A study of homozygous mutants found that all Sey^{Neu} animals had decreased numbers of all four hormone producing cell types, however α cells were the most seriously affected (Sander *et al.*, 1997). Again there were structural abnormalities in the organisation of the islet; however the mechanism for this disruption was unclear. The N-CAM promoter is a known target of Pax6. N-CAM levels, however, were unaffected in Sey^{Neu} animals (Sander *et al.*, 1997). This is similar to results obtained from Beta2 disrupted animals (Section 1.1.3.5.3) and again raises the possibility of a novel cadherin/CAM involved in structural organisation of the islet e.g. E-cadherin (E-CAD) has been shown to be important in spatial organisation of Min6 generated pseudoislets (Hauge-Evans *et al.*, 1999). The reduction in hormone levels in Sey^{Neu} animals may be due in part to transcriptional effects. Pax6 binding sites are present on the promoter sequences of insulin, glucagon and somatostatin (Sander *et al.*, 1997).

1.1.3.5.6 Pax4 Expression in Developing Islet Cells

Pax 4 is also a member of the paired box family of transcription factors. The expression of Pax4 is far more restricted than that of Pax6 in the embryo (i.e. Pax4 has been detected in a few cells in the ventral spinal cord and in the developing pancreas) (Dohrmann *et al.*, 2000). Mice with disrupted Pax4 genes were found to lack insulin- and somatostatin-expressing cells and the animals died within a few days. The knockout mice had an abundance of α cells, however (Sosa Pineda *et al.*, 1997). This suggests that Pax4 may be important for pushing cells towards β and δ lineages and away from what may be possibly the 'default' α cell differentiation pathway (Schwitzgebel *et al.*, 2000; Ritz-Laser *et al.*, 2002). When ectopically expressed in α cells, the mode of action of Pax4 in diverting the default α cell differentiation may be related to its inhibition of glucagon gene expression through competitively binding Pax6 sites within the promoter region (Ritz-Laser *et al.*, 2002). This action restricts glucagon expression to α cells in the pancreas.

Interestingly a double knockout of Pax4 and Pax6 resulted in the complete lack of endocrine hormone production (St-Onge *et al.*, 1997).

1.1.3.5.7 Nkx6.1 Expression and Islet cell Determination

Nkx6.1 is a homeodomain transcription factor (a member of the NK2 homeobox transcription factor family) that is specifically expressed in β cells in the endocrine pancreas (Jensen *et al.*, 1996). Nkx6.1 is initially detected with PDX1 in parenchymal cells during early development (however this only represents a subset of $PDX1^+$ cells), and as the pancreas develops both factors become progressively more islet/β cell specific. In fact the PDX1/Nkx6.1 co-expressing parenchymal cells have been identified as possible β cell precursors within the developing pancreas (Øster *et al.*, 1998; Larsson, 1998).

Preliminary data indicates that Nkx6.1 knockout mice lack any insulin expressing cells (Edlund, 1998).

1.1.3.5.8 Nkx2.2 Expression and Islet cell Determination

Nkx2.2 is also a member of the NK2 family of transcription factors present in the developing pancreas (and in the developing CNS). Nkx2.2 expression can be found in the developing dorsal pancreatic bud and it is widespread in the early stages of the pancreas. As the pancreas specialises and develops distinct endocrine cells and exocrine cells, Nkx2.2 expression can be found associated with most endocrine cells. In fact Nkx2.2 is present in all insulin producing cells, 80 % of α cells and most PP cells. But it is completely absent from δ cells. Knockout studies (Nkx2.2 $^{-/-}$) resulted in mice that developed severe diabetes within a few days of birth due to the total lack of β cells. α cell numbers were also reduced as were PP cells, δ cells were unaffected by the absence of Nkx2.2. There were large numbers of hormone negative cells expressing β cell markers (IAPP and PDX1) in the Nkx2.2 knockout mice (Sussel *et al.*, 1998). From this it appears that Nkx2.2 is not directly involved in the early stages of β cell development (due to the expression of other β cell markers e.g. Nkx6.1), but that its absence prevents insulin expression possibly due to a role in final maturation of the β cell. PDX1 expression was present in reduced amounts in the knockout mice (Sussel *et al.*, 1998) and this may account for the lack of final maturation of the β cells.

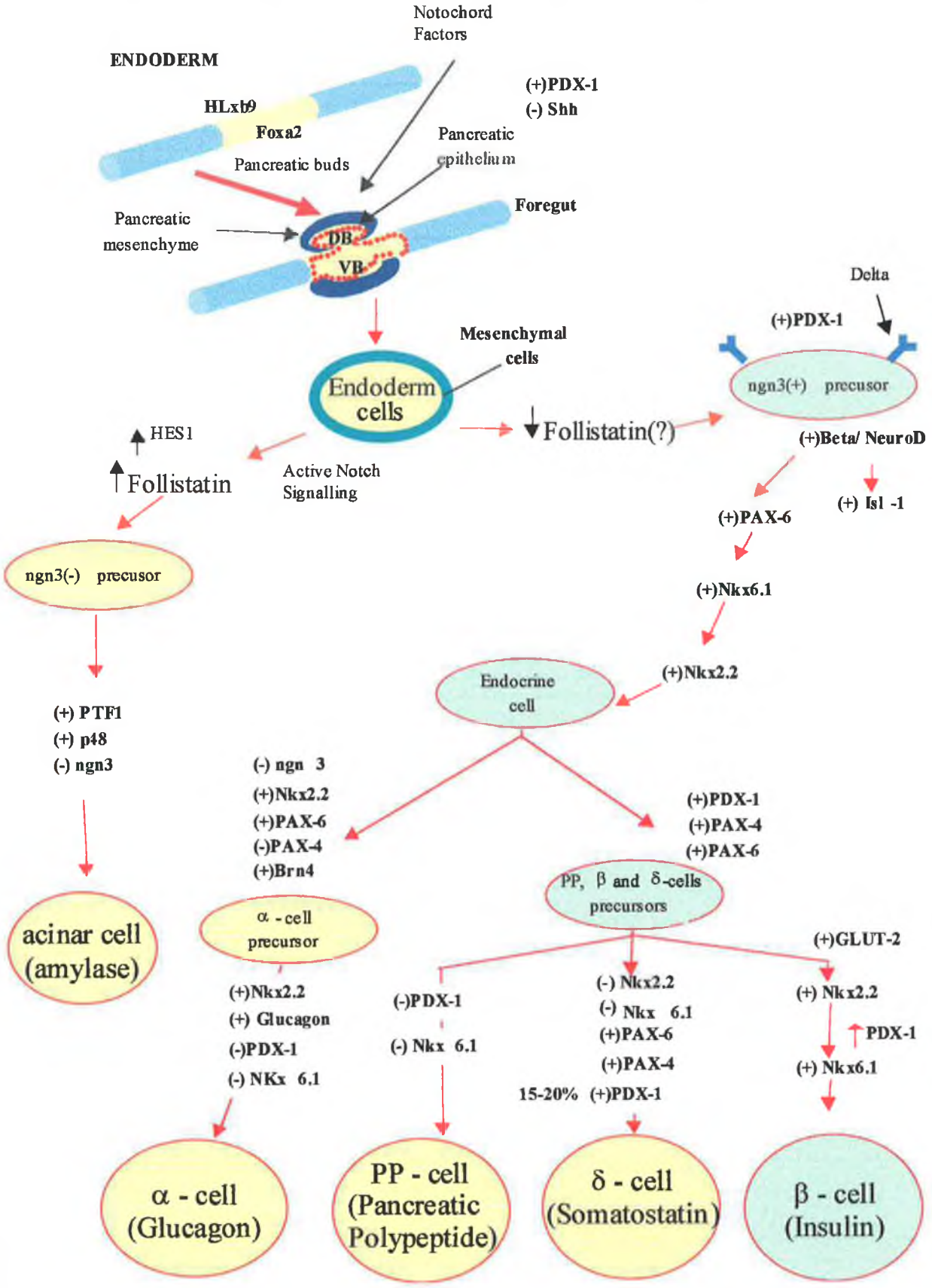


Figure 1.1.1 Summary of the transcription factor involvement in islet cell specification and the order in which they appear. (+) indicates that a factor is 'on' and (-) indicated it is 'off'.

1.1.4 Monitoring Pancreatic Differentiation Through Cytokeratin Expression

Cytokeratins are classed among the intermediate-sized filaments of the eukaryotic cytoskeleton and show cell-type specific expression. There are more than 20 types of cytokeratins and they are classed as type I (9-20) and type II (1-8). As the expression of pairs of type I and type II cytokeratins is relatively tissue specific, this makes them a useful tool in the study of differentiating systems. Cytokeratins (CKs) are immunocytochemical markers of epithelial cells (Bouwens, 1998a).

The pancreas is composed of simple epithelial cells that generally show two combinations of CK expression. The exocrine ducts usually contain CK's 7 and 19 (Bouwens *et al.*, 1994), while CK's 8 and 18 are present in the exocrine acinar cells, duct cells and in the endocrine islets of Langerhans (Bowens, 1998a). Rat pancreata also contain CK20 as a potent marker for precursor cells but CK20 is absent in mature endocrine cells (Bowens *et al.*, 1994; Bowens & De Blay, 1996).

Specific immunohistochemical CK markers for duct, endocrine and mesenchymal cells have been employed in studies to follow islet morphogenesis in developing pancreata. In the human fetus all epithelial cells initially express CK19 and CK7 (Bouwens *et al.*, 1997; Kasper *et al.*, 1991). The loss of expression of these CKs is progressive with increasing differentiation, leading to a stage where the islets do not express these markers and the ducts stain positively for them. The expression of these markers is paralleled by proliferation marker expression (e.g. Ki-67) demonstrating that while the ducts retain their ability to proliferate and grow, the islets have limited proliferative ability (Bouwens *et al.*, 1997).

Cytokeratin-based identification of tissues must be considered as preliminary and requires additional knowledge of other markers for positive identification, e.g., in rats the liver has a similar cytokeratin profile to the pancreas i.e., the bile ducts are positive for CKs 7, 19 & 20 while the hepatocytes express CKs 8 & 18 (Bouwens, 1998a). (This observation is the basis for the proposal of a common progenitor cell-type for the liver and the pancreas. It has also been observed that pancreatic progenitor epithelial cells can differentiate in to hepatocytes upon transplantation in to the liver (Dabeva *et al.*, 1997) or after treatment of regenerating pancreatic cells with

N-nitrosobis(2-oxopropyl)amine (Scarpelli & Rao, 1981)). However despite these limitations, cytokeratin profiling can reveal on-going activities within the pancreas when combined with other markers e.g., in duct ligated rat pancreata and low dose streptozotocin treated rats (both stimulate β cell neogenesis) one can observe the appearance of individual β cells and small aggregates of β cells. Transient co-expression of CK20 within these cells indicates that the new β cells are of ductal origin. Reg expression was also present in these cells (Reg expression can be seen in regenerating islets in de-pancreatized rats, and is linked to DNA synthesis in the regenerating cells) (Wang *et al.* 1995; Anastasi *et al.*, 1999; Rosenberg, 1995). This is in agreement with findings that during rat pancreatic morphogenesis, aggregates of CK20-expressing duct cells form, and over time the cells at the center of these aggregates were found to be insulin^{+}/CK20^{-}. Thus these aggregates were termed 'islet forming units' (Bouwens & De Blay, 1996). Similar β-cell units were observed in donated pancreata of humans (Bouwens & Pipeleers, 1998).

Thus the use of double immunofluorescence/immunohistochemistry for pancreatic hormones and cytokeratins is a useful method of 'tracking' differentiating cells and can reveal the location and identity of the progenitor populations when used in combination with carefully chosen differentiation markers.

1.2 The β Cell

As discussed in Section 1.1 the origins of the β cell are complex and highly regulated. Furthermore the β cell itself is complex and extensive research of this cell and its specialised functions have been reported.

1.2.1 The Insulin Gene

β cells are the only cells in the body that actively express the insulin gene for the purposes of glucose homeostasis; many reviewers regard β cells as the only cells in the body that express this gene however there is evidence of insulin expression in the CNS (Alpert *et al.*, 1988; Devaskar *et al.*, 1993) and retinas (Budd *et al.*, 1993). The human insulin gene is located on the short arm of chromosome 11 and contains three exons and two introns. In rats and mice there are two insulin genes, and although the mRNAs of these genes are quite similar, they are differentially regulated e.g. there is a preferential expression of rat Insulin I under stimulatory conditions (Kakita *et al.*, 1982; Wentworth *et al.*, 1986). The duplicate genes are non-allelic and in the case of mouse are on two different chromosomes (6 & 7). The ancestral gene is Insulin II and Insulin I arose due to a retro-transposition which resulted in a 6 bp deletion from the mouse insulin I gene (Wentworth *et al.*, 1986). Transcription of the insulin gene yields a 600 nucleotide mRNA and this is translated in to an 11.5 kDa polypeptide. Tissue specific regulation and the regulation of transcription rates are controlled by the 5' region of the gene.

1.2.1.1 Tissue Specific Regulation of the Insulin Gene

As already mentioned (Section 1.2.1) expression of the insulin gene is under the control of a tissue specific promoter. The extreme importance of the insulin gene to human wellbeing and health prompted many studies to elucidate the factors that restrict the expression of this gene. Most studies on the insulin promoter have been carried out on the rat insulin I promoter region. Targeted deletions identified a cell-specific enhancer in the region –103 to –333 relative to the transcriptional start site (Walker *et al.*, 1983; Edlund *et al.*, 1985). Mutational analysis of this region identified

critical regions for insulin gene transcription which, when combined together restrict insulin gene transcription. Other negative response elements also serve to restrict insulin gene expression in non-islet cell types (Nir *et al.*, 1986; Boam *et al.*, 1990; Clarke *et al.*, 1995).

1.2.1.2 E Boxes Within the Insulin Gene Enhancer

Synthetic oligonucleotides spanning the 5' region of the gene led to the discovery of two major regulatory sequences in the 5' region of the promoter between –104 to –112 and –233 to –241 (Karlsson *et al.*, 1987). These are illustrated in Figure 1.2.1. Individual disruption of these sequences led to a reduction in insulin gene transcription and disruption of both led to abolishment of transcriptional activity. These regions have been termed IEB1 and IEB2 boxes; FAR and NIR boxes; and E4 and E5 boxes; respectively, but are more conveniently termed the E1 and E2 boxes (Philippe, 1991; Mc Kinnon & Docherty, 2001). These regions were found to contain an 8 bp homologous sequence GCCATCTG which was found to interact with the same protein i.e. IEF1. As already discussed (Section 1.1.3.5.3) IEF1 was discovered to be a heterodimer of the ubiquitous E2A gene product(s) E12/E47 and the novel bHLH protein Beta2 (Naya *et al.*, 1995).

1.2.1.3 A Boxes Within the Insulin Gene Enhancer

The A boxes (or CT boxes as they were originally known) are present at positions –77 to –84, -210 to –217 and –313 to –320 relative to the transcriptional start site (Figure 1.2.1). These A boxes can bind several homeodomain transcription factors such as Isl1, HNF1 and Lmx1.1 and contain the sequence TAAT (Mitanchez *et al.*, 1997; Karlsson *et al.*, 1990; Emens *et al.*, 1992; Ohneda *et al.*, 2000). Electro shift mobility assays (EMSAs) identified another factor, IUF1, that strongly bound to A boxes (Boam & Docherty, 1989). One of these sites i.e. the CT2 site, resembled a site in the rat insulin I promoter that although void of activity, potentiated the activity of the E2 box close by (known as the E2A3/4 minienhancer (Ohneda *et al.*, 2000)). IUF1 is now better known as the PDX1 transcription/differentiation factor (Section 1.1.3.5.1 & Section 1.2.1.3.1).

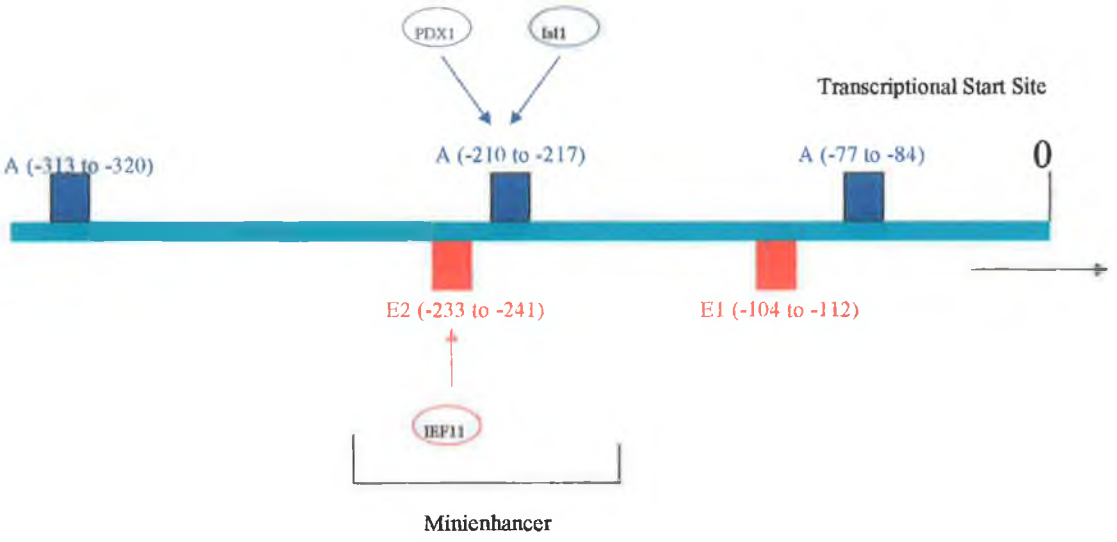


Figure 1.2.1 Summary of the layout of the 5' region of the insulin gene and the control elements within it. A boxes are in blue while E boxes are in orange. All positions referenced as the nucleotide position upstream from the transcriptional start site (0).

1.2.1.3.1 PDX1 and Insulin Gene Transcription

PDX1 (Section 1.1.3.5.1) is a homobox transcription factor protein with a predicted molecular weight of 31kDa. PDX1 is present in almost all (~91%) of β cells and a subpopulation of δ cells. In the presence of glucose, PDX1 is activated through phosphorylation and translocates to the nucleus of the β cell where it exerts its effects on the transcription of insulin, glucokinase, GLUT2 and IAPP (Mac Farlane *et al.*, 1999; McKinnon & Docherty, 2001; Serup *et al.*, 1996). The loss of insulin gene transcription in continuously cultured HIT-T15 cells (hamster β cell line) is at least partially due to the loss of PDX1 expression (Olson *et al.*, 1995; Moran *et al.*, 1997). Transfection of PDX1 cDNA in to high passage HIT-T15 cells however, can lead to partial restoration of insulin promoter activity (Harmon *et al.*, 1998). The ability of PDX1 to activate transcription, is dependent on the co-operation of other transcription factors especially the IEF1 or Beta2-E12/E47 heterodimer combination (Serup *et al.*, 1996; Madsen *et al.*, 1996; Harmon *et al.*, 1998). In fact, the design of the promoter region and the close association of the E and A boxes within the E2A3/A4 minienhancer (Figure 1.2.1) indicate that the co-activation properties of PDX1 and Beta2/E47 are due to co-operative DNA binding (Ohneda *et al.*, 2000). The

requirement for the presence of Beta2 expression was clearly seen in the activation of endogenous insulin expression in the glucagonoma cell line MSL-G-AN following overexpression of PDX1 (Serup *et al.*, 1996). Three subdomains exist within the N terminal region of PDX1 that are conserved between PDX1 and the related *Xenopus* homoeprotein, XIHbox8. These subdomains are required for the synergistic transactivation of the insulin gene (Peshavaria *et al.*, 1997; Peshavaria *et al.*, 2000). A recent study has in fact demonstrated that the homoedomain of PDX1 acts as to facilitate protein-protein interaction and recruitment to form an activation complex (Ohneda *et al.*, 2000). The recruitment of other β cell specific proteins and arrangement of the insulin gene enhancer contribute to the tissue specific expression of this gene (Peshavaria *et al.*, 2000).

PDX1 appears to be present in large excess in β cells as an 86% reduction in PDX1 levels (achieved through antisense technology) did not result in a significant reduction in insulin gene transcription (Kajimoto *et al.*, 1997). Overexpression of PDX1 in insulin expressing RIN1046-38 cells and HIT-T15 cells increased insulin promoter activity in a dose-dependent manner until a point was reached where the overexpression began to repress insulin transcription, again in a dose dependent manner (Seijffers *et al.*, 1999). PDX1 expression is not entirely necessary for basal insulin gene transcription however, as shown by the presence of insulin expression in PDX1-negative cells in the developing pancreas (Øster *et al.*, 1998); the presence of insulin positive cells in mutant mice with disrupted PDX1 expression in their β cells (Ahlgren *et al.*, 1998); and the low level insulin gene transcription noted in βTC3 cells with mutant (non-functional) PDX1 (Peshavara *et al.*, 1997).

1.2.1.4 Hedgehog Signalling and Insulin Gene Transcription

In Section 1.1.2.1 the role of hedgehog signalling in pancreas development was discussed, however it appears as if hedgehog signalling may also be implicated in insulin gene transcription. Indian hedgehog (Ihh) and Desert hedgehog (Dhh) were both shown to positively regulate insulin gene transcription in cultured INS1 and Min6 cell lines, and administration of the hedgehog inhibitor, cyclopamine, caused a decrease in insulin promoter activity (Thomas *et al.*, 2000).

1.2.1.5 Glucose Regulation of the Insulin Gene

Short term exposure of insulin secreting cells to stimulatory levels of glucose leads to significant increases in insulin gene transcription in HIT-T15 β cells and in isolated islets within 60-90 minutes (Leibiger *et al.,* 1998a). Prior to this report it was believed that the short term effects of glucose were predominantly at the post-transcriptional level such as stabilisation of insulin mRNA (Welsh *et al.,* 1985; Skelly *et al.,* 1996; Docherty *et al,* 1996). Glucose was observed to be capable of direct interaction with the insulin gene enhancer through the 'Z element' located –292 to –243 in 5' region of the human insulin gene. This was shown to function through the binding of the ZaI complex (identified in the nuclear extracts of fetal and adult islets), the binding of which was increased significantly upon exposure of the islets to glucose (Sander *et al.,* 1998).

Glucose also affects insulin gene transcription rates through the action of intermediates such as cAMP. cAMP levels increase upon glucose metabolism in β cells (Charles *et al.,* 1975; Nielsen *et al.,* 1985; Hammonds *et al.,* 1987; Inagaki *et al.,* 1992). There are four cAMP responsive elements between the 5' enhancer region of the insulin gene and within the gene itself, which additively contribute to cAMP induction of insulin transcription (Inagaki *et al.,* 1992). The interaction of cAMP with these elements is mediated by a cAMP responsive element binding protein (CREB) (Philippe & Missotten, 1990). Thus cAMP could almost be described as a form of second messenger for glucose activation of the insulin gene. These effects of cAMP were negatively regulated by the protooncogene c-jun which, is increased in conditions of low glucose. The direct effects of cAMP may be demonstrated through the exposure of cells to membrane permeable cAMP (dibutyryl cAMP) or through the use of activators of the adenylate cyclase cascade such as cholera toxin or forskolin (Nielsen *et al.,* 1985; Hammonds *et al.,* 1987).

1.2.1.5.1 Glucose Regulation of Insulin Gene Expression via Modulation of PDX1 Binding

The metabolism of glucose causes the phosphorylation of PDX1 through a cascade of factors including phosphatidylinositol 3-kinase (PI 3-kinase) and a stress activated kinase p38/SAPK2. This causes the activation of PDX1 and its molecular weight is seen to increase (from 31to 46 kDa). This is followed by translocation of PDX1 to the nucleus (Macfarlane *et al.*, 1997; Macfarlane *et al.*, 1999). The activation of PDX1 was mediated through phosphorylation but it is unknown if the phosphorylation alone is responsible for the increase in molecular weight (Macfarlane *et al.*, 1999). As well as mediating its transport in to the nucleus, glucose increases the DNA binding ability of PDX1 thus facilitating its role as a transactivator of the insulin gene (Petersen *et al.*, 1998; Wu *et al.*, 1999). PDX1 has also been shown to be involved in the glucose responsiveness of another β cell gene i.e. the GAD(67) isoform of the β cell autoantigen glutamic acid decarboxylase (Pedersen *et al.*, 2002).

1.2.1.6 Regulation of the Insulin Gene by Hormones

Regulation of the insulin gene is complex and occurs at a number of levels within the cell, including hormonal regulation. Decoding of the glucagon gene revealed two other peptides encoded in the proglucagon molecule (precursor to glucagon) called glucagon like peptide I and II (GLP I & GLP II). The shorter of these, GLP-1 can stimulate cAMP levels within the β cell causing an increase in insulin gene transcription (Drucker *et al.*, 1987). Epinephrine and somatostatin both suppress insulin gene transcription (Redmon *et al.*, 1994), while insulin may positively affect its own transcription through an autocrine action which may involve the activation of PDX1 (Leibiger *et al.*, 1998b; Wu *et al.*, 1999; McKinnon & Docherty, 2001).

1.2.2 Insulin Biosynthesis and Processing

Glucose stimulates initiation of insulin translation and causes increased transfer of initiated ribosome bound RNA from the cytoplasam to the rough endoplasmic reticulum (Welsh *et al.*, 1987).

Insulin is synthesised as the precursor protein preproinsulin (PPI). This 11.5 kDa polypeptide is rapidly discharged into the rough endoplasmic reticulum where it is converted into proinsulin via the action of proteolytic enzymes. Proinsulin is a 9 kDa polypeptide containing the A and B chains of insulin and the C-peptide (connecting peptide) fragment. Within the Golgi apparatus disulphide bond formation occurs. Proinsulin is then passed through the Golgi cisternae to the trans-Golgi network (TGN). Here the proinsulin is packaged into immature secretory granules. Proinsulin is very similar to insulin in structure. The C-peptide aligns the proinsulin molecule for correct cleavage to mature insulin (Holwell, 1991; Docherty, 1997).

At this point the immature secretory granules can either form part of the constitutive pathway or the regulated pathway of protein secretion (regulated and constitutive methods of secretion are discussed in Section 1.2.4.1). During constitutive secretion, the immature granules are transported immediately to the β cell membrane and a mixture of proinsulin and insulin is released. On the regulated pathway, the proinsulin is stored in secretory granules where it is converted to mature insulin via the action of two endoproteases and carboxypeptidase. The two endoproteases PC2 and PC1 (also called PC3; PC = prohormone convertase) are members of a subtilisin-like family of calcium-dependent enzymes that are restricted to neuroendocrine cells (Smeekens *et al.*, 1991; Bennet *et al.*, 1992; Docherty, 1997). PC3 cleaves the Arg^{31}-Arg^{32} sequence at the B-chain C-peptide junction and PC2 cleaves at Lys^{64}-Arg^{65} at the A-chain C-peptide junction. These two intermediate molecules are converted to mature insulin and C-peptide via the action of carboxypeptidases (Figures 1.2.2 & 1.2.3).

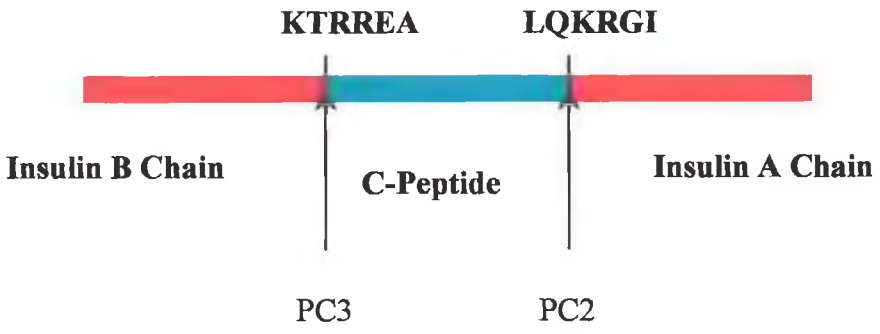


Figure 1.2.2 Cleavage Points for PC2 and PC3

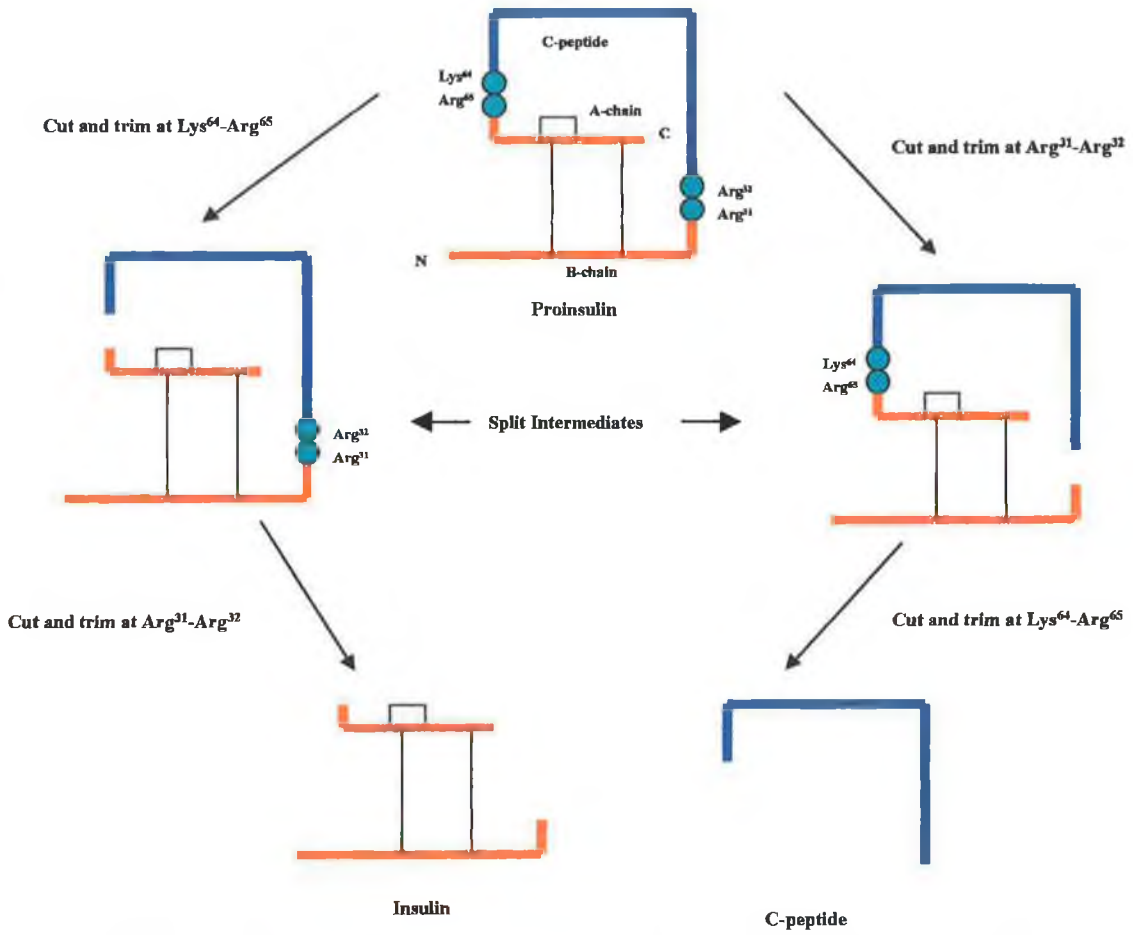


Figure 1.2.3 Processing of Proinsulin to insulin. Following initial cleavage by either PC2 or PC3, trimming of the residual C-terminal residues is carried out by the action of ubiquitously expressed carboxypeptidases.

Insulin has a lower solubility than C-peptide and thus co-operates with zinc ions to form microcrystals within the secretory granule. The precipitation is facilitated by the low pH within the granule which is due to the excision and loss of basic amino acids during proteolytic conversion (Howell, 1991). From this it is apparent that insulin and C-peptide will be released from the β cell in eqimolar amounts by exocytosis upon stimulation by glucose. The advantage of storing proteins in storage granules is that having such a large concentration of the hormone/protein in the granules facilitates the release of large amounts upon stimulation by a secretagogue (Halban & Irmanger, 1994).

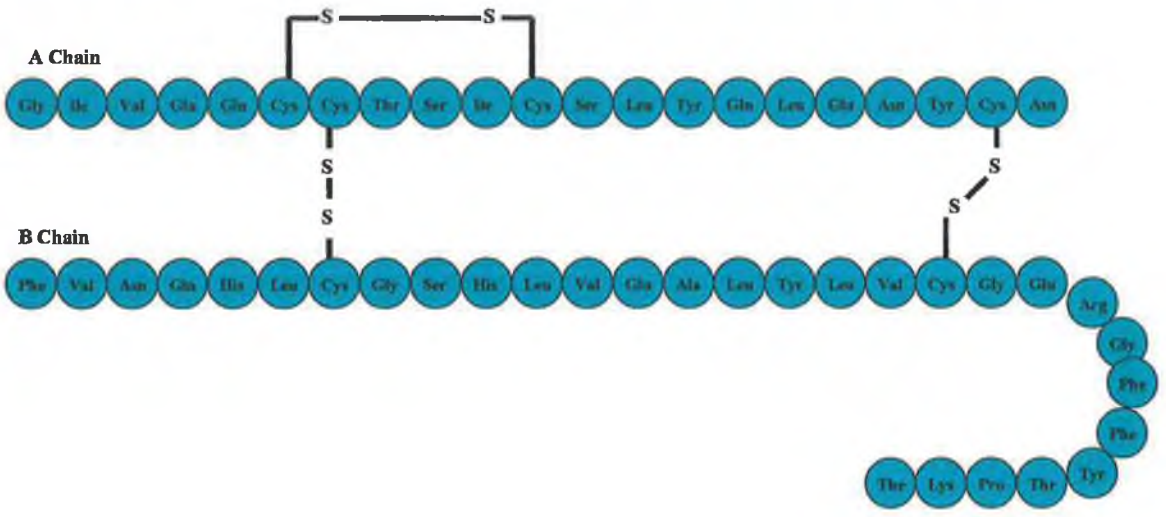


Figure 1.2.4 The Primary Structure of Human Insulin. The primary structure refers to the amino acid sequence of the peptide.

1.2.3 Glucose Sensing Within the β Cell

To correctly control blood glucose levels, the β cell needs to accurately determine how much glucose is present in the blood stream. Understanding of glucose sensing is important for both non-insulin dependent diabetes mellitus (NIDDM), where the

glucose stimulated insulin secretion (GSIS) may be impaired (Ramirez & Raskin, 1991), and for the generation of 'artificial β cells'. The level of glucose is determined from the metabolism of the blood glucose and the subsequent changes in glucose metabolites (Ashcroft, 1980; German, 1993). Thus the most important components of the 'glucose sensor' are (I) the transport mechanism allowing glucose in to the cell i.e. GLUT2 and (II) the initial rate limiting step in the metabolism of glucose i.e. glucokinase (Hexokinase family member IV) (Figure 1.2.5). GSIS requires that the metabolism of glucose can be increased within the β cell over the entire physiological range. This is facilitated by the fact that both GLUT2 and glucokinase have k_ms above the physiological range of glucose (Marshall *et al.*, 1993; Ishihara *et al.*, 1994). The relative importance of the two components of the glucose sensor were compared by the overexpession of their low k_m counterparts i.e. GLUT1 and hexokinase I in the Min6 cell line. The presence of GLUT1 did not affect the GSIS ability of the cells; however, the expression of hexokinase I did increase glucose utilisation and release at concentrations below 10 mM (Ishihara *et al.*, 1994). In similar experiments with rat islets, the expression of hexokinase I abolished GSIS over the entire physiological range (German, 1993). Patients with maturity onset diabetes of the young (MODY) have impaired GSIS and this is due to heterozygous point mutations in the glucokinase gene. Similarly, disruption of the glucokinase gene in transgenic mice leads to the same phenotypic effects (Bali *et al.*, 1995). While this data indicates that the critical portion of the glucose sensor is glucokinase, GLUT2 is still relevant. Mutations in the GLUT2 gene totally abolished glucose transport and thus the ability to 'sense' glucose over the physiological range in a patient with NIDDM, while the expression of GLUT2 antisense led to diabetes in transgenic mice (Mueckler *et al.*, 1994; Valera *et al.*, 1994). RINm5F insulinoma cells (Section 1.5.1.1) were insensitive to external glucose levels until GLUT2 was transfected in to this cell line. Following this transfection, insulin content and glucose uptake were improved and the RINm5F-GLUT2 cells were found to have functional GSIS (Tiedge *et al.*, 1993). Similarly in glucokinase expressing cell lines (AtT20 & HEP G2) transfected with the PPI cDNA, these cells were not capable of responding to the external glucose levels unless GLUT2 was transfected also. (Hughes *et al.*, 1992; Simpson *et al*, 1997). Thus, while the presence of glucokinase is the main rate limiting glucose sensor, the combination of GLUT2 and glucokinase is required for effective glucose sensing.

This was again seen in the case of the Min7 insulinoma cells that were isolated at the same time as Min6 cells. Min7 cells were found to be glucose insensitive due to a combination of high levels of hexokinase I and GLUT1 (Miyazaki *et al.*, 1990; Ishihara *et al.*, 1993).

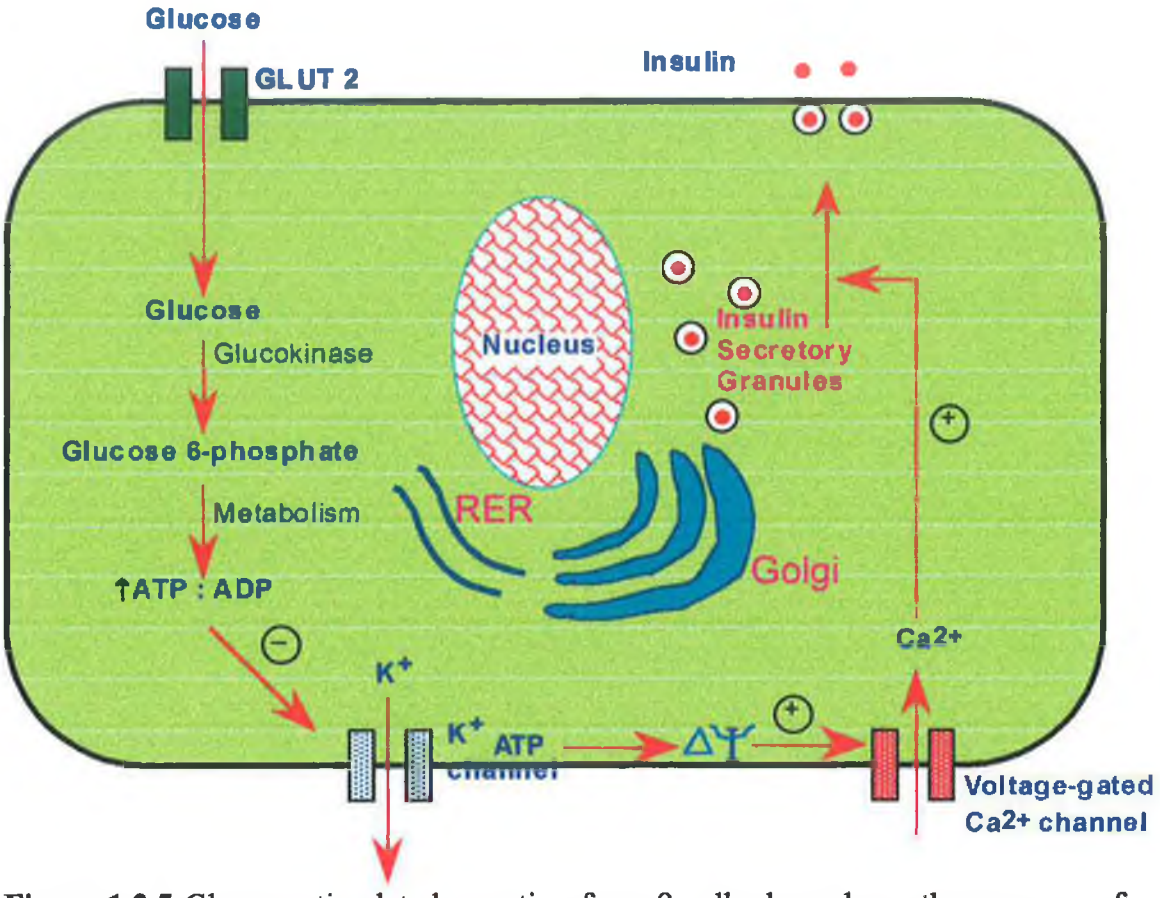


Figure 1.2.5 Glucose stimulated secretion from β cells depends on the presence of correct glucose sensing, insulin storage and regulated secretion pathways.

1.2.4 Stimulated Insulin Secretion from β Cells

The main difference between the maintenance of glucose homeostasis through the action of fully functional β cells and insulin injections is the fact that insulin injections do not provide the minute to minute control of blood glucose levels in the same way as β cells. This temporal control is facilitated by proportional release of stored insulin depending on the blood glucose level. Thus insulin secretion pathways are highly regulated.

1.2.4.1 Regulated Pathways of Protein Secretion

Most of the secretory proteins from eukaryotic cells share a common origin in the rough endoplasmic reticulum (RER) from where they are transferred to the Golgi apparatus. It is here that the protein either enters a regulated pathway of secretion or the more common constitutive pathway of secretion. The sorting of constitutive proteins from regulated proteins occurs in the trans-Golgi network (TGN) (Kelly, 1985).

Secretion via the regulated pathway allows the release of the protein to be controlled via secretagogues. The action of the secretagogue occurs at the level of exocytosis and allows the discharge of protein from mature secretory granules at an appropriate moment. The constitutive pathway results in continuous exocytosis meaning that protein availability is the main modulator of this pathway and thus is only regulated at the level of biosynthesis (Halban & Irminger, 1994). This pathway of secretion is typically used to transfer membrane proteins, viral glycoproteins and other rapidly secreted proteins to the cell surface (Docherty, 1997).

The regulated pathway of secretion requires secretory granules. The earliest form of the granule is clatherin coated although the reason for this is unknown. It is in this clatherin-coated granule that the bulk of prohormone conversion occurs as the granule matures. The maturation of the granule continues with the eventual loss of the clatherin coat. While it has been well established that the sorting of the regulatory and constitutive proteins occurs in the TGN it is unknown how the processing of constitutive from regulated proteins is carried out (Halban & Irminger, 1994).

When cDNA encoding proinsulin was transfected into a mouse cell line secreting ACTH via the regulated pathway, storage, processing and secretion of the proinsulin on stimulation occurred (Moore *et al.*, 1983; Motoyoshi *et al,* 1998). Thus it seems that regulated proteins have structural determinants, which cause them to be sorted in to the regulated pathway. In a series of experiments, it was demonstrated that constitutive proteins will be secreted via the constitutive pathway even in cells containing a regulated pathway of protein secretion, but regulated proteins from one cell will be regulated again in the new cell type. Interestingly it was observed that

when a constitutive viral protein was fused to the C-terminal domain of a regulated protein, that the new hybrid protein was released in a regulated manner, (Halban & Irminger, 1994).

1.2.4.2 Glucose Stimulated Insulin Secretion (GSIS) from β Cells

Insulin secretagogues can be divided in to two groups i.e. initiators and potentiators (Howell, 1991). Initiators are capable of stimulating secretion on their own and include glucose, and the sulphonurea drugs while potentiators such as L-Arganine can increase insulin secretion when present with an initiator. Glucose is the most important initiator of insulin secretion. β cells are sensitive to changes in glucose from around 5-8 mM to 20 mM (Knaack *et al.*, 1994; Poitout *et al.*, 1996; Best, 2000) i.e., there is no stimulation of secretion below 5 mM glucose and the maximum glucose stimulated insulin secretion (GSIS) occurs at approximately 20 mM.

Studies have shown that stimulation by glucose triggers characteristic electrical responses at the β cell membrane (Ullrich *et al.*, 1996). These changes in membrane potential are due to the knock-on effects resulting from the metabolism of glucose. β cells can monitor increases in blood glucose via the 'glucose sensing system' (Section 1.2.3). Increased blood glucose leads to increased glucose metabolism, which determines the extent of the insulin response. The simplest model of GSIS is that the direct result of increased glucose metabolism is an increase in the ATP/ADP ratio within the β cell. This causes the closure of ATP sensitive potassium channels on the β cell membrane. In resting β cells these channels are open and allow a steady flow of K^+ ions outward, this holds the membrane potential of the cell at a negative level of – 70 mV to –80 mV (Ashcroft and Gribble, 1999; Best, 2000; Ullrich *et al.*, 1996). There are also voltage gated (L-type) calcium channels in the β cell membrane. At the resting potential – 70 mV they are closed, however once the ATP sensitive K^+ channels are closed membrane depolarisation occurs i.e. the cell becomes less negative as a result of the build up of K^+ ions. Membrane depolarisation results in the opening of the voltage gated Ca^{2+} channels, thus allowing Ca^{2+} ions to flow in to the β cell. The increase in intracellular calcium results in the translocation of the insulin

storage granules to the β cell membrane and facilitates release of the stored insulin (Ashcroft & Gribble, 1999; Howell, 1991). This is outlined in Figure 1.2.5.

Evidence for an alternative Ca^{2+} independent mode of secretion has been presented also. Evidence for this was provided by the observation that the adenylate cyclase activator forskolin (increases intracellular cAMP) did not cause an increase in intracellular calcium ($[Ca^{2+}]i$) but did result in increased secretion in Min6 cells. This and other observations involving glucose, leucine and arganine in Ca^{2+}-free systems indicated that there is another mechanism of secretion that involved cAMP and cellular alkalinization (Sakuma *et al.*, 1995). This was also shown by the use of analogues of forskolin that did not activate the adenylate cyclase pathway (and therefore did not increase cAMP) and thus did not stimulate insulin secretion (Ullrich *et al.*, 1996). It has also been observed that although tolbutamide causes significant membrane depolarisation (and therefore Ca^{2+} influx) it does not increase insulin secretion in INS1 cells (Ullrich *et al.*, 1996). The relative contribution of the Ca^{2+} independent pathway was not found to be of major significance to the physiological regulation in mouse islets however (Sato *et al.*, 1998).

The response of β cells to glucose is heterogeneous in nature both at the level of insulin biosynthesis and secretion (Moitoso de Vargas *et al.*, 1997; Giordano *et al*, 1993). It was discovered that the GSIS response from β cells is heterogeneous due to differing β cell size, and not as a result of individual cell metabolism of glucose. The release of insulin in response to glucose is also dependent on cell to cell contacts within the intact islets (Section 1.2.4.2.2).

1.2.4.2.1 Implications for the Cell-Matrix Interactions in GSIS

Islets cultured *in vitro* show altered functions compared to their *in vivo* counterparts, which is partly due to altered cell-cell interactions and altered cell matrix interactions. Culture of primary rat β cells on 804G matrix (produced by rat carcinoma cell line) was found to increase cell spreading, the percentage of insulin secreting cells and the amount of insulin secreted. This effect was mediated at least in part, through α6β1 integrin (Bosco *et al.*, 2000). Integrins are involved in the attachment of cells to

various matrices including fibronectin and laminin. They are dimers of an α and a β subunit, subgroups of integrin families share their β subunit and the α subunit seems to confer the ligand specificity (Burdsal *et al.*, 1994). Integrin subunits α3, α5, and αV are also important in maintaining β cell function in culture (Wang & Rosenberg, 1999).

1.2.4.2.2 Implications for Connexin Expression and Gap Junctions in GSIS

Gap junctions are cell to cell channels which allow direct transfer of ions and metabolites e.g. cAMP, IP_3 and calcium between cells. Channels are formed by the interaction of hemichannels on one cell with the open channel of another. These hemichannels consist of 6 protein subunits, the connexins which are coded for by a multigene family (Willecke, *et al.*, 1991). Over 15 different types of connexins are known for mammals and they are characterised by molecular weight.

When the insulin levels, secretion rates and GSIS capabilities of intact islets, dispersed islet cells and re-aggregated islet cells were compared, it was found that the single cells and the aggregates had a reduced response to glucose signals in perfusion assays. This was considered to be, at least partly, due to disruption of cell-to-cell signalling due to loss of gap junctions (Linzel *et al.*, 1988). In fact it has been shown that homologous contact between β cells rather than with other islet cell types is critical to effective GSIS (Bosco *et al.*, 1989). Connexin 43 (Cx43) was identified as a critical component in stimulation of insulin secretion in gibenclamide treated rats (Meda *et al.*, 1991). Expression of Cx43 in INS1, to levels comparable with those in native islets, led to increased insulin content and preserved GSIS. Overexpression of Cx43 in INS1 resulted in diminished insulin content and GSIS (Vozzi *et al.*, 1995). Cx36 has also been implicated in insulin secretion from β cells (Calabrese *et al.*, 2001).

1.2.4.3 cAMP Mediated Insulin Secretion from β Cells

As discussed previoulsy cAMP levels are implicated in Ca^{2+} independent secretion from β cells (Section 1.2.4.2). Compounds that increase cAMP through activation of adenyl cyclase (cholera toxin) or through inhibition of cAMP phosphodiesterase (theophylline and 3-isobutyl-methylxanthine (IBMX)) have been shown to increase secretion from insulin secreting cells (Nielsen *et al.*, 1985; Ullrich *et al.*, 1996; Hammonds *et al.*, 1987; Simpson *et al.*, 1997; Lu *et al.*, 1998). Overexpression of cAMP degrading phosphodiesterase (converts cAMP to cGMP) led to significant decreases in insulin secretion and GSIS was reduced by 40-50 %. The incretin effect of GLP1 on insulin secretion (mediated through cAMP (Drucker *et al.*, 1987)), was also affected (Harndahl *et al.*, 2002).

1.2.4.4 Insulin Secretion Through the Action of the Sulphonurea Drugs

The main factor in common between initiators of insulin secretion is the closure of the ATP dependent potassium channels. While the metabolism of glucose can cause this by increasing the ATP/ADP ratio, other drugs can have the same effect by direct interaction with the channel. The most common drug type employed to close potassium channels are the sulphonurea drugs. These drugs are used by patients with Type II diabetes to enhance insulin secretion (Ashcroft & Gribble, 1999).

The ATP dependent potassium channel (K_{ATP}) found in β cells is composed of two different types of subunits i.e. the pore forming unit (Kir6.2) and the regulatory subunit (SUR1). These subunits assemble with a 4:4 stoichiometry. Kir 6.2 is a member if the inwardly rectifying K^+ channel family and is the subunit that ATP interacts with to close the channel. Kir 6.2 is unlike other members of this family in that it is unable to form functional channels in the absence of the sulphonurea receptor (SUR), due to the role of SUR as a chaperone protein allowing the surface expression of Kir 6.2. The sulphonurea receptors are members of the ABC-transporter superfamily, which also includes the cystic fibrosis transmembrane receptor (CFTR) and p-glycoprotein (a membrane pump implicated in multidrug resistance in cancer). (See review by Ashcroft & Gribble (1999)).

The sulphonurea drugs interact with the β cell K_{ATP} channel via a low affinity site on the Kir 6.2 unit and a high affinity site on SUR1 (Gros *et al.*, 1999). Glibenclamide is the most potent sulphonurea drug employed to stimulate insulin secretion and is active within a narrow operating range 50 – 200 nM, which corresponds to a very low oral dose (Luzi & Pozza, 1997).

1.2.4.5 Insulin Secretion Mediated by Amino Acids and Vitamins

The amino acids L-Arganine and L-Leucine have been found to be potentiators of GSIS. L-Arganine is a cationic amino acid and induces secretion via membrane depolarization of β cells allowing for the opening of the voltage gated Ca^{2+} channels. This facilitates Ca^{2+} mediated secretion as outlined above (Section 1.2.4.2). L-leucine, on the other hand, causes cellular alkalinization (Sakuma *et al.*, 1995). L-leucine is also a metabolizable β cell substrate, which generates high-energy compounds such as ATP (Welsh *et al*, 1987). An increase in ATP levels within cells as a result of leucine metabolism may result in closure, or partial closure, of the K_{ATP} channels allowing for a potentiating effect in the presence of glucose.

The regulation of insulin secretion by Vitamin A has also been observed. Vitamin A deficient perfused islets had impaired insulin secretion. Furthermore vitamin A deficient rats had an impaired acute insulin response to glucose stimulation, (Chertow *et al.*, 1987).

1.3 Differentiation of Cells Towards a β Cell Lineage for Diabetes Treatment

Replacement of correctly functioning β cells is the ultimate goal for treatment of IDDM. However, due to shortages of donor tissue this cannot be carried out unless a pool of self-replicating tissue could be isolated, from which β-like cells could be generated for transplant. Such sources of replicating cells include embryonic stem (ES) cells, and pancreatic precursor cells.

1.3.1 Stem Cells

Tissues that become differentiated to carry out certain roles in the body are subject to turnover, however the differentiation of these cells is often accompanied with a loss of proliferative ability. Thus to match replacement with loss, progenitor cells must differentiate to fill the void. These proliferating progenitor cells are a type of stem cell (Bach *et al.*, 2000). Embryonic stem (ES) cells are pluripotent cells isolated from the inner cell mass of pre-implantation embryos and theoretically represent totally undifferentiated cells with unlimited differentiation potential. A sub class of the ES cells are embryonic carcinoma (EC) cells, which are generally derived from testicular or ovarian tumors and exhibit similar properties to ES cells (Dinsmore *et al.*, 1998). The isolation and culture of the first human stem cell line H9 (Thompson *et al.*, 1998) was a breakthrough that has heralded a new branch of diabetes research aimed at the identification of the factors and signals required to generate insulin producing β cells (or even better, replicating β cell precursors) for diabetes treatment.

1.3.1.1 Determination of Stem Cell Fate

To culture a cell to a desired type the conditions that precede this event *in vivo* should be known. This is more straightforward for simple organisms such as *Drosophila* it is quite difficult for larger animals and humans. Stem cell fate determination follows relatively standard rules however.

1.3.1.1.1 Internal Factors Involved in Determining Stem Cell Fate

Internal factors control division and gene expression in developing stem cells. The asymmetric pathway of stem cell division involves the generation of a stem cell and a differentiated progeny from a mother cell therefore the stem cell population can maintain itself. This has been shown in *Drosophila* (Morrison *et al.*, 1997; Jan & Jan, 1998). In higher mammals, symmetric division takes place i.e. either the stem cell divides to give two stem cells or differentiation signals (combination of internal and external), result in the generation of two differentiated progeny. Sometimes a combination of asymmetric and symmetric cell division occurs (Morrison *et al.*, 1997; Wagers *et al.*, 2002). Transcription factors also control stem cell fate, e.g., SCL/Tal-1 which is essential for the formation of blood cell lineages in the mouse (Watt & Hogan, 2000) and, as already discussed, the expression of Ngn3 can lead to cell fate determination in developing β cells (Section 1.1.3.5.2).

1.3.1.1.2 External Factors Influencing Stem Cell Fate

Secreted factors e.g., growth factors (GFs) are classical determinants of cell fate. These have however, both instructive and selective/enabling mechanisms of action. The instructive mode involves a specific GF instructing a cell to take a specified lineage (Shah *et al.*, 1996), whereas the selective mechanism involves the promotion of survival of pre-committed cells from a population, and driving them into their more differentiated compartments (Faribairn *et al.*, 1993; Gandarillas & Watt, 1997). Instructive and selective-like differentiation has been observed in the developing pancreas where mesenchymal secretions actively select for exocrine cell survival and differentiation while instructively repressing endocrine cell differentiation (Section 1.1.2.3).

Cell-cell contact is an external stimulus that can influence differentiation. Notch and its ligand, Delta, are involved in *Drosophila* for sensory organ fate determination (Artavanis-Tsakonas, 1999) and in developing endocrine cells from proliferating precursors (Section 1.1.3.5.2).

The interaction of cells with their extracellular matrix via surface proteins can influence stem cell lineage determination e.g. the matrix can render the cells competent to respond to other signals by allowing them to assume a particular spatial organisation. The extracellular matrix can also potentially sequester and modulate the local concentrations of secreted differentiation agents available within the stem cell niche (Watt & Hogan, 2000). Integrins have a critical role in this level of stem cell determination. β1 integrin is required at high levels to maintain epidermal cells in the stem cell compartment. Expression of a dominant negative β1 integrin subunit (CD8β1) in proliferating keratinocytes led to an exit from the stem cell compartment (Zhu *et al.*, 1999). Selective differentiation of epidermal stem cells through overexpression of c-myc was accompanied with a reduction in β1 integrin also (Gandarillas & Watt, 1997).

1.3.2 Differentiation of Stem Cells to an Endocrine Lineage

For clarity the generation of β cell-like cells through differentiation of ES cells and adult 'stem' cells/progenitors will be discussed separately, as the cells involved are quite different, i.e. the ES cells are pluripotent while the adult stem cells/progenitor cells are likely to be more committed. It is still relevant to refer to both cell types as stem cells as the definition of a stem cell is a cell that has the ability for long-term proliferation with the capacity to produce at least one type of highly differentiated descendent (Watt & Hogan, 2000).

1.3.2.1 Differentiation of ES cells in to β Cells

The H9 ES cell line (Thomson *et al.*, 1998) can be grown in an undifferentiated state through careful culture, however spontaneous differentiation is easy to induce by simply allowing the cells to form aggregates called embryoid bodies (EBs) in suspension culture. Previous studies had demonstrated that following EB formation, pluripotent stem cells were found to differentiate and express markers associated with neural, vascular, hematopoietic, and endodermal lineages (Vogel, 1999). Schuldiner *et al.* (2000) investigated the effects of different differentiation agents on the EBs but surprisingly it was found that formation of EBs alone led to the expression of insulin

and PDX1. Similar reports regarding the differentiation of EBs are present in the literature (Gerrish *et al.*, 2000; Shiroi *et al.*, 2002). Endodermal markers such as alfafeto protein (AFP) and HNF3β were also discovered in spontaneous EBs (Shamblott *et al.*, 2001). As pancreatic origins are known to be endodermal (Section 1.1.1), spontaneous EBs from human ES cells were probed for immunoreactive insulin, and a number of cells were found to express the protein. These cells were also able to secrete immunoreactive insulin in to the medium in a differentiation related manner, and the increased secretion was correlated with expression of β cell markers (Assady *et al.*, 2001). This was an important study of functionality as the constitutive expression of random differentiated cell markers in stem cells is not unknown (Wagers *et al.*, 2002).

A purified population of insulin secreting cells has already been generated from mouse stem cells. These cells were differentiated as EBs in the presence of low glucose and nicotinamide (Section 1.3.4.4) and the insulin expressing cells were purified from the population as a whole using the 'cell trapping' method of Klug *et al.* (1996). In this system the undifferentiated cells were transfected with a construct whereby the transfected cells could be selected through acquired resistance to a constitutively expressed antibiotic resistance gene. The gene encoding another resistance gene (neomycin) was also present under the regulatory information of the insulin gene. Thus, following differentiation, the cells resistant to neomycin were selected, resulting in a purified population of insulin secreting cells i.e. these cells contained the cellular machinery to drive the insulin promoter (Soria *et al.*, 2000a). These cells were capable of GSIS and normalised the blood glucose of streptozotocin induced diabetic mice. These implanted cells did not become tumorigenic and maintained normoglycemia for at least 12 weeks.

As discussed already the differentiation of pancreatic islets and that of neurons have a number of features in common (Section 1.1.2.6). Neuron turnover in the adult is facilitated by multipotential cells e.g. subventricular zone astrocytes (Doetsch *et al.*, 1999). The intermediate filament protein nestin has been identified as a neural stem cell (NSC) marker (Lendahl *et al.*, 1990; McKay 1997; Bjornson *et al.*, 1999; Momma *et al.*, 2000). Culture of ES cells in bFGF and serum free conditions results in a

population of proliferating nestin rich cells that can be further differentiated in to neuronal cells by mitogen withdrawal (Okabe *et al.*, 1996). Multipotential nestin-positive cells have been isolated from the ducts of rat pancreata that could differentiate towards endocrine, exocrine and hepatic lineages when cultured *in vitro* (Zulewski *et al.*, 2001). Nestin positive cells were purified from partially purified ES cells using supplemented serum free medium (Okabe *et al.*, 1996; Lee *et al.*, 2000) and expanded in the presence of bFGF. Removal of the bFGF mitogen and addition of nicotinamide resulted in clusters of cells of which, approximately 30 % were insulin positive (Lumelsky *et al.*, 2001). These cells were found to have an intact GSIS response, and they had a very low proliferation index.

1.3.2.2 Differentiation of Precursor Tissue to Functional β Cells

1.3.2.2.1 Differentiation of Ductular Tissue

There is convincing evidence for the existence of a self-renewing population of cells within the adult pancreas that are capable of differentiating/transdifferentiating into β cells upon injury or other such stimulus (Tsao & Duguid, 1987; Wang *et al.*, 1995; Rosenberg, 1995; Bouwens & DeBlay, 1996; Bouwens & Pipeleers, 1998; Anastasi *et al.*, 1999).

When isolates from the pancreatic ducts were digested and cultured as a monolayer of epithelial-type cells, islet cell masses budded from these monolayers. These proliferating islet cell masses (islet progenitor cells, IPCs) were found to contain low levels of insulin at their core and were also positive for glucagon and somatostatin. Following exposure to nicotinamide, the IPCs were found to secrete large amounts of insulin and to have a functional GSIS. Implantation of the immature IPCs into diabetic mice led to differentiation of the IPCs and to reversal of diabetes (Cornelius *et al.*, 1997; Ramiya *et al.*, 2000). The advantage of this system over some of the ES cell to β cell systems in Section 1.3.2.1 is that there is a possibility for constant self-renewal of progenitor material. Also the presence of α, β and δ cells in an islet-like arrangement may offer a co-ordinated insulin response like that obtained in normal pancreata (Soria, *et al.*, 2000b). The human pancreatic duct cell line Capan-1 was

differentiated in to insulin producing cells via treatment with Exendin-4 (an agonist of the incretin hormone GLP-1). This differentiation was facilitated by the expression of important transcription factors such as PDX1 and HNF3β/Foxa2 (Section 1.1.2.5). In this system similar effects were noted by overexpression of PDX1 in the Capan-1 cell line (Zhou, *et al.*, 2002).

1.3.2.2.2 Nestin Expressing Progenitors

As already discussed (Section 1.3.1.2.1) nestin expressing cells have been located within the ducts of adult pancreas which differentiated upon reaching confluency in culture and were found to express and secrete insulin (Zulewski *et al.*, 2001). These do not appear to be ductal cells, however, as they lack CK19 expression which is a ductal cell marker in the pancreas (Bouwens *et al.*, 1994). Treatment of these cells with GLP-1 leads to differentiation to insulin secreting cells through the activation of PDX1. In fact the differentiation of cultured nestin positive cells to insulin secreting cells may be due to GLP-1 expression in those cultures, therefore resulting in autocrine-controlled differentiation (Abraham *et al.*, 2002).

1.3.2.2.3 Epithelial Precursors

As already outlined in Section 1.1.1 the pancreas generates from epithelial cells of the endoderm in the developing embryo. The mesenchyme is very important in dictating the extent of endocrine/exocrine differentiation from these epithelial cells and does so by the release of soluble secreted factors such as follistatin (Miralles *et al.*, 1998). Removal of pancreatic epithelial tissue from rats and culturing in the absence of mesenchyme led to the development of insulin secreting cells expressing β cell terminal differentiation factors e.g., Nkx6.1 (Miralles *et al.*, 1999). The intestinal epitheloid cell line IEC-6 could also be induced to adopt a β cell-like fate. These cells were found to express a number of β cell markers following transfection of PDX1, and follow up betacellulin treatment or upon transplantation under the renal cap of a rat, these cells synthesised and secreted insulin in a non-regulated fashion (Yoshida *et al.*, 2002).

1.3.2.2.4 Acinar Cells (AR42J)

The pancreatic acinar cell line AR42J possesses both exocrine and neuroendocrine properties in that they secrete amylase and γ-aminobutyric acid, while expressing such markers as neurofilament (Christophe, 1994). Upon treatment with dexamethasone they resemble acinar cells (Logsdon *et al.*, 1985; Guthrie *et al.*, 1991). Treatment of AR42J cells with the TGFβ family member activin A resulted in neuronal-like differentiation (Ohnishi *et al.*, 1995). This alone was not significant as neuronal differentiation appears to be a 'default' mechanism of differentiation (Vogel, 1999). However, these cells also displayed what appeared to be both the sulphonurea receptors and calcium channels associated with β cells. Closer examination of activin A treated AR42J cells revealed that pancreatic polypeptide expression and GLUT2 were also expressed. Thus, in a wider study a range of growth factors and differentiation agents were applied to these cells and it was found that betacellulin could induce 4% of the cells to express insulin. A combination of activin A and betacellulin resulted in 10% of the treated cells expressing insulin (Mashima *et al.*, 1996a). These insulin secreting cells did not have a functional GSIS but did respond to tolbutamide, indicating the presence of the functional potassium and calcium channels (Ohnishi *et al.*, 1995). Combining activin A with HGF produced similar results (Mashima *et al.*, 1996b). This is in keeping with the theory that these compounds are involved in β cell specification in the developing pancreas (Larsson, 1998). As with the ductal cells and nestin expressing precursor cells (Zhou, *et al.*, 2002; Abraham *et al.*, 2002), extendin-4 and GLP-1 also caused differentiation of AR42J cells to insulin secreting cells.

The differentiation of AR42J cells was mediated by the mitogen activated protein kinase pathway (MAPK) through which the expression of a number of other genes is controlled (Mashima *et al.*, 1999). Besides the genes affected directly by the MAPK pathway, some more characteristic determinants of β cells were affected in differentiating AR42J cells, including Ngn3 and Pax4. Overexpression of Ngn3 had similar effects to activin A treatment (Zhang *et al.*, 2001). It has also been reported that Nkx2.2 expression can induce PP expression while the expression of other

transcription factors PDX1, Nkx6.1, or Isl1 had no effect (alone or in combination) (Palgi *et al.*, 2000).

1.3.3 Transdifferentiation of Hepatocytes to β Cells

Transdifferentiation of one cell type to another is not uncommon, in fact upon changing environment cells may respond to different environmental factors and take up new lineages. Neuronal cells can differentiate to form smooth muscle (Shah *et al.*, 1996), and haematopoietic cells can differentiate in to neuronal (Eglitis & Mezey, 1997) and muscle cells (Ferrari *et al.*, 1998). Pancreatic epithelial cells are also capable of differentiating into hepatocytes following transplantation in to the liver (Dabeva *et al.*, 1997). Adult rat hepatic 'oval' cells (hepatic oval cells may be hepatic stem cells (Petersen *et al.*, 1999)), have been found to be capable of differentiating in to glucose-sensitive insulin secreting cells which express a number of β cell markers upon culture in a high glucose environment (Yang *et al.*, 2002). α cell tumor derived cells can also be differentiated in to insulin expressing cells through the over-expression of PDX1 and the treatment of the transfected cells with betacellulin (Watada *et al.*, 1996). This is not as significant as the transdifferentiation of hepatic cells to β cells as α and β cells are developmentally related and the expression of PDX1 alone stimulates IAPP expression in transformed α cells (Serup *et al.*, 1996; Watada *et al.*, 1996)

1.3.4 Common Agents Employed in the Differentiation of Cells Towards and Endocrine Lineage

While ES cells can be seen to spontaneously differentiate in to insulin secreting cells, it can be seen that the addition of nicotinamide promoted a larger proportion of the cells to adopt a β cell fate. This is one of a few common agents employed in the directed differentiation towards β cells which are discussed individually below.

1.3.4.1 Activin A

Activin A is a member of the transforming growth factor beta (TGFβ) family of secreted proteins. It was successful in experiments with AR42J cells where it initiated endocrine differentiation (Section 1.3.2.2.4). ES cell derived EBs were found to express the activin A receptor (Activin R II B) and generated mesodermal cells upon treatment with activin A (Schuldiner *et al.*, 2000). Activin A has been implicated in the differentiation of endocrine precursor cells in the developing pancreas (Larsson, 1998; Demeterco *et al.*, 2000) where it has shown to be expressed (Furukawa *et al.*, 1995). Activin A was shown to inhibit DNA synthesis in cultured INS1 cells while at the same time increasing insulin content by almost three-fold (Huotari *et al.*, 1998).

1.3.4.2 Betacellulin

Betacellulin is the most recently identified member of the epidermal growth factor (EGF) family of growth factors, and it was initially discovered in the conditioned medium of β tumors (Shing *et al.*, 1993). It has mitogenic activity on Balb/c 3T3 fibroblasts (Watanabe *et al.*, 1994) and on fetal pancreatic tissue in culture (Demeterco *et al.*, 2000). It was shown to have potent effects on the proliferation of cultured β cells (INS1), with no effect on insulin levels (Huotari *et al.*, 1998). Betacellulin is capable of differentiating small percentages (< 5%) of AR42J cells into insulin secreting cells (Mashima *et al.*, 1996a). PDX1-transfected epithelial cells and α cell derived lines only expressed insulin following betacellulin treatment (Watada *et al.*, 1996; Yoshida *et al.*, 2002). Injections of recombinant betacellulin have led to the generation of islet-like cell clusters in the ducts of glucose intolerant mice after 8 weeks. These clusters were found to be predominantly composed of β cells (Yamamoto *et al.*, 2000). Betacellulin is reported to interact with the EGF receptor (EGFR/c-erb B1) and c-erb B4 (Watanabe *et al.*, 1994; Riese *et al.*, 1996). Another 190 Kd receptor has been isolated that seems to interact specifically with betacellulin in AR42J cells (Ishiyama *et al.*, 1998), however its identity is unknown.

1.3.4.3 HGF

Hepatocyte growth factor (HGF), also known as the scatter factor (SF), was identified as a mitogen for hepatocytes in a screen to identify factors involved in liver regeneration. HGF exerts its mitogenic effects through its receptor c-met (Michalopouls & DeFrances, 1997). The c-met receptor is present in the fetal pancreas, it is preferentially expressed in developing β cells, and it is restricted to the β cells of adults. This distribution reflects the importance of mesenchymal derived HGF to the growth of pancreatic β cells (Otonkoski *et al.*, 1996). The formation of islet-like cell clusters (ICCs) from human fetal tissue was increased 2-3 fold in the presence of HGF with an associated insulinotropic activity (Otonkoski *et al.*, 1994). The increase in β cell mass within fetal pancreatic epithelial tissue was accompanied however, with an associated reduction of insulin content (Otonkoski *et al.*, 1996). HGF was found to have no effect on the proliferation of cultured INS1 cells (Huotari *et al.*, 1998) and this may reflect its role in differentiation of more undifferentiated tissue. The action of HGF on AR42J cells was very similar to betacellulin (Mashima *et al.*, 1996b).

1.3.4.4 Nicotinamide

Nicotinamide is a metabolite of the vitamin niacin. Nicotinamide was shown to be a potent inducer of β cell regeneration following partial pancreatectomy in rats, this appears to be due to its role in inhibiting poly(ADP-ribose) synthetase (Yonemura *et al.*, 1984). Experimental states of diabetes can be instigated by administration of streptozotocin to cause breaks in the DNA strands of β cells which induces poly(ADP-ribose) synthetase, this results in reduced NAD, thus causing inhibition of β cell functions (Yamamoto & Okamoto, 1980). ICCs isolated from the pancreata of fetal pigs are of limited use as they secrete low amounts of insulin, however upon culture in nicotinamide containing medium these clusters synthesised and secreted more insulin (Sjoholm *et al.*, 1994; Otonkoski *et al.*, 1999). The increase in β cell number and insulin content could be correlated with increased polyamine levels (i.e., spermidine, putrescine and spermine) and the effects of nicotinamide were blocked by inhibitors of polyamine synthesis (Sjoholm *et al.*, 1994). This is consistent with the

observations regarding the requirement of polyamines for β cell function (Sjoholm *et al.*, 1990; Welsh & Sjoholm, 1988).

Nicotinamide has been found to have a potent insulinotropic effect on fetal pancreatic tissue (resulting in β cell outgrowths) (Otonkoski *et al.*, 1993), and on cultured β cells (Ohgawara *et al.*, 1995; Huotari *et al.*, 1998). Direct administration of nicotinamide following partial pancreatectomy in to the OLTEF (Otsuka Long-Evans Tokushima fatty strain of rat) rat model (impaired ability to regenerate β cells) resulted in significant increases in β cell mass and ameliorated hyperglycemia (Shima *et al.*, 1998).

1.3.4.5 Sodium Butyrate

Sodium butyrate is a short chain aliphatic acid that occurs naturally in the colon through fermentation of dietary fiber (Otonkoski *et al.*, 1999). Sodium butyrate was identified as a differentiation agent for insulinoma cell lines in a study relating cessation of proliferation with differentiation (Philippe *et al.*, 1987). It was found that sodium butyrate increased the levels of insulin and glucagon in cultured RIN cell lines through the recruitment of hormone negative cell lines to differentiate (Powers *et al.*, 1988). Combinations of sodium butyrate and nicotinamide resulted in significant increases in the insulin content of INS1 cells and in porcine fetal ICCs (Huotari *et al.*, 1998; Otonkoski *et al.*, 1999), but this effect was associated with increased apoptotic cell death (Otonkoski *et al.*, 1999). Sodium butyrate is a member of a class of compounds known as histone deacetylase (HDAC) inhibitors, this inhibition leads to increased histone acetylation. Highly transcribed genes are associated with highly acetylated core histones (Marks *et al.*, 2000; Wade *et al.*, 1997). This possibly infers the mechanism by which sodium butyrate operates.

1.4 F9 Cell Biology

Human ES cell lines have only been available for the last two to three years thus most of the research to date has been carried out using mouse ES and EC cell lines. EC (Embryonic Carcinoma) cells are derived from testicular or ovarian tumors and exhibit similar properties to those of ES cells (Lethonen *et al.*, 1989). They make an excellent model for study due to their ease of culture and the large cell numbers that can be generated, however their applications in therapy may be limited due to their tumor forming abilities (Dinsmore *et al.*, 1998).

The F9 line was isolated from the OTT6050 teratocarcinoma in 1973 (Bernstein *et al.*, 1973) and are characterised by their lack of spontaneous differentiation and thus are referred to as being *nullipotent* (Martin & Evans, 1975; Alonso *et al.*, 1991). F9 cells have a short doubling time (8 hours) during the exponential phase of growth due to a very short G1 phase. Differentiation of the cells leads to lengthening of the G1 phase and a reduction in the proliferative ability of the cells (Linder *et al*, 1981). It is speculated that the short G1 phase of F9 cells prevents the expression of the genes required for differentiation (Lethonen *et al.*, 1989). This would be consistent with observations that inhibitors of DNA synthesis can induce differentiation of F9 cells (Nishimune *et al.*, 1983; Griep & De Luca, 1986a).

1.4.1 Differentiation of F9 cells

1.4.1.1 Spontaneous Differentiation

Although characterised as being nullipotent, F9 cells have been observed to show some low level differentiation in normal culture over time, e.g. low levels of Ihh expression (mRNA and protein) has been detected in untreated F9 cells after 8 days culture (Becker *et al*, 1997). Spontaneous differentiation has also been discovered in low-density cultures of F9 cells i.e., F9 cells plated at a density of 12 cells/cm^2 gave rise to neuroblast cells as determined by a silver staining method specific for colonies differentiating neuroblasts (Zakany *et al*, 1984). Mummery *et al.* (1990) identified low level spontaneous differentiation in F9 cultures due to the presence of transcripts

for the cytokeratin Endo A (Cytokeratin A now known as Cytokeratin 8 (Moll *et al.*, 1982)) and tissue plasminogen activator (tPA).

1.4.1.2 Differentiation Through the Action of Differentiation Agents

F9 differentiation studies have generally employed retinoic acid or a combination of retinoic acid and di butyryl cyclic AMP and are described below. Other data concerning differentiation agents with roles in β cell differentiation are discussed also. There do not appear to be any references to any previous F9 studies involving betacellulin, HGF or nicotinamide.

1.4.1.2.1 RA and db cAMP

Treatment of the F9 cells with Retinoic acid (RA) alone or with dibutyryl cAMP (db cAMP) leads to the differentiation of F9 cells and is associated with a number of changes in differentiation markers. The action of RA on genes is most likely mediated by the RA receptors (RARs) and retinoid X receptors (RXR) that are present on the nuclei of most cells. Cytoplasmic proteins such as cellular retinoic acid binding protein I or II (CRABP I or II) mediate the binding of RA to the receptors (Means *et al.*, 2000). This leads to the activation of the receptors to RA responsive elements in the promoter regions of certain genes e.g. laminin (Vasios *et al.*, 1991; Manglesdorf *et al.*, 1991).

Upon differentiation with RA or RA/db cAMP, F9 cells are characterised as having increased synthesis of tissue-type plasminogen activator, laminin, collagen type IV, cytokeratins (Alonso, 1991; Lehtonen *et al.*, 1989; Mummery *et al.*, 1990; Clairmont *et al.*, 1996). Alkaline phosphatase activity is reduced and there is a disappearance of the stage-specific embryonic antigen (Moore *et al.*,1986). All of these are indicators of parietal endoderm (Alonso *et al*, 1991). Differentiated F9 cells adopt a number of morphologies dependent on the mode and duration of the differentiation. Treatment of the cells with RA alone commits the cells to irreversible differentiation after only 8 hours and brings the cells to a primitive cell type which is then capable to adopting

either parietal (PE) or visceral (VE) endoderm (Hogan *et al.*, 1981; Lethonen *et al.*,1989).

1.4.1.2.2 Sodium Butyrate

Sodium Butyrate is a HDAC inhibitor (Section 1.3.4.5) and was found to inhibit RA mediated differentiation of F9 cells if added within the initial critical 8 hour period of RA-induced differentiation (most changes in gene expression occur during this period see Section 1.4.1.4) (Levine *et al.*, 1984). Sodium butyrate treated F9 cells demonstrate morphological changes as well as changes in protein and gene expression, including increased expression of metallothionen genes, tissue and urokinase type plasminogen activator and endo A (Andrews & Adamson, 1987; Kosaka *et al.*, 1991; Takeda *et al.*, 1992; Miyashita *et al.*, 1994). Differentiation using sodium butyrate was found to inhibit F9 proliferation (Takana *et al.*, 1999). Sodium butyrate induced differentiation was largely found to be reversible upon removal from the culture medium (Kosaka *et al.*, 1991; Shimada *et al.*, 2001). The effect of sodium butyrate on endo A expression was as a result of changes in the chromatin structure (Miyashita *et al.*, 1994), which suggests its mechanism of action in differentiation.

1.4.1.2.3 Activin A

F9 cells were found to express Activin βB, levels of which dropped dramatically upon differentiation towards endoderm. This was seen to be consistent with the proposed role of activin in mesoderm formation (Albano *et al.*, 1993). F9 cells were found to express 3500-20,000 activin receptors per cell, including activin receptor II B. This gene was found to be transcriptionally induced following RA treatment (Kondo *et al.*, 1989; Wan *et al.*, 1995).

1.4.1.2.4 DMA and BRDU

Differentiation of F9 cells has been carried out via N'-N'-dimethylacetamide (DMA) and 5-bromodeoxyuridine, (Moore *et al*, 1986). The observations made during this differentiation indicate a similar differentiation pathway for RA and BrdU, however

the differentiated phenotype as a result of DMA treatment appeared to be unique suggesting a novel pathway. Also like the action of RA and dbcAMP it was found that the action of BrdU and DMA seemed irreversible.

1.4.1.3 Formation of F9 Embryoid Bodies *in vitro*

When EC cells are cultured *in vitro* in the absence of feeder layers/support matrix (e.g., gelatin) they multiply rapidly for several days and form tight rounded aggregates which can be maintained indefinitely. If the EC cell line is pluripotent then the outer layers of the aggregates (Embryoid Bodies) will differentiate to endodermal-type cells without the aid of differentiation agents. It was also found that nullipotent EC lines also formed embryoid bodies, however these exhibit a smooth outer surface of undifferentiated cells (Martin & Evans, 1975). Subsequent findings demonstrated that nullipotent cell lines such as F9 cells could form differentiated embryoid bodies in the presence of RA, with the outer layers being composed of predominantly alpha-fetoprotein expressing visceral endoderm (Hogan *et al,* 1981; Burdsal *et al.,* 1995; Miki *et al.,* 1999). Embryoid bodies are interesting because they resemble a 5-6 day-old embryo. Plating of embryoid bodies to certain types of substratum (e.g. gelatin) leads to the outgrowth of the differentiated outer layer. The endodermal cells migrate outwards from the bodies forming a 'halo' which can give rise to such cells as fibroblasts, cartilage, adipose tissue, beating heart muscle and neural tissue (Martin & Evans, 1975). It has been discovered that the maturation of the visceral endoderm outer layer in RA primed embryoid bodies of F9 cells is dependent on the size of the embryoid body. Embryoid bodies with diameters of about 180 μm or larger resulted in expression of AFP (Miki, 1999).

1.4.1.4 Differentiation and Gene Expression

Differentiation is mediated by the alteration of gene expression, however it is difficult to determine the genes that are the effectors of differentiation rather than genes that are expressed differently as a result of differentiation. RNA polymerase I based transcription was found to be reduced upon parietal differentiation of F9 cells (Alzuherri & White, 1999). The first 8 hours of RA treatment are considered to be the

commitment stage of differentiation in F9 cells (Levine *et al.*, 1984; Alonso *et al*, 1991), thus this is the crucial period in which to monitor the initial changes in gene expression. A recent study identified 109 differentially expressed genes following only 6h differentiation (Harris & Childs, 2002).

The level of c-myc oncogene expression in F9 cells is seen to decrease sharply after RA treatment, i.e. expression is at 50% of normal within 3 hours of exposure and decreased by 90% within 12 hours (Greip and DeLuca, 1986). This study also demonstrated that other oncogenes were differentially expressed in RA treated F9 cells, e.g., c-erbB mRNA increased 4 fold between 6 and 9 hours following RA treatment (this had been noticed previously by Adamson & Rees, 1981). The regulation of the c-myc expression following differentiation was linked to methylation of sites within the c-myc gene (Griep & DeLuca, 1986b). Expression of the c-myc antisense sequence in F9 cells resulted in differentiation similar to that of mediated by RA, similarly overexpression of c-myc resulted in F9s resistant to differentiation by RA (Griep & Westphal, 1988). Other authors have been unable to reproduce these results so they remain controversial (Nishikura *et al,* 1990).

A similar experiment was reported whereby RA-like differentiation was duplicated via the transfer of the c-fos proto-oncogene into F9 cells (Muller & Wagner, 1984). These results concur with the study where antisense to c-fos was transfected into F9 cells leading to inhibition of endodermal differentiation, (Edwards *et al,* 1988).

Table 1.4.1 outlines a number of genes that have been altered in F9 differentiation experiments.

1.4.1.4.1 Hedgehog gene expression in Differentiating F9 Cells

It has been shown that Ihh is upregulated dramatically in the differentiation of F9 cells from EC to an endodermal (visceral *or* parietal) fate (Becker *et al.*, 1997; Grabel *et al.*, 1998). Its role is active and not just structural as determined by transfection of the full length cDNA for Ihh (under an inducible promoter) resulting in morphological and other changes indicative of endodermal differentiation including the expression of alpha fetoprotein and tissue type plasminogen activator (tPA) (Becker, *et al.*, 1997).

1.4.1.4.2 Negative Regulation of Viral Promoters

While discussing gene expression in F9 systems it should be pointed out that undifferentiated F9 cells appear to possess a negatively acting factor(s) which prevents/reduces the transcription of viral promoters. This effect is more pronounced in retroviral promoters. This block is absent in differentiated F9 cultures (Kelly and Condamine, 1982). Studies have demonstrated that heterologous genes can be driven at high efficiency by the GRP78 promoter in undifferentiated F9 cells (Kim *et al.*, 1990), possibly providing an alternative to constitutive viral promoters for F9 studies. Nowling *et al.* (2002) have recently presented protocols on highly efficient transfection of F9 cells via liposome mediated gene transfer.

Genes with altered expression levels in differentiated F9 cells

Gene	F9 (Untreated)	Differentiated F9 Cells
Alkaline Phosphatase	+	-
Apolioprotein E	+	-/++*
Bmp2#	+	++
Bmp4#	++	+
CRABP1***	++	-/+
Cytokeratins 8 and 18	-	+
EGF Receptors	+	++
Enactin	-	+
Era-1	-	+
Fetomodulin	-	+
c-fos	-	++
GLUT3****	++	+/-
Indian Hedgehog**	-	++
Laminin	+	++
c-myc	+	+/-
Retinol Binding Protein	-	+
Sparc	-	+
TGFβ2	-	+
Transferrin Receptors	+	++
Type IV Collagen	-	+

Table 1.4.1 A selection of genes with altered expression levels as a result of differentiation of F9 EC cells. (*= varying levels). Table adapted from Alonso *et al.* (1991) and references therein.

(**Becker *et al.*, 1997)

(***Means *et al.*, 2000)

(****Faria *et al.*, 1998)

(#Grabel, *et al.*, 1998)

1.4.2 Monitoring F9 Differentiation

1.4.2.1 Monitoring of F9 Differentiation by Intermediate Filament Expression

Intermediate filament expression patterns are classic markers for the differentiation-state of cells. Undifferentiated EC lines express only vimentin (Paulin *et al.*, 1982). Overexpression of vimentin in F9 cells leads to aberrant vimentin structures and the collapse of K8/K18 filaments in the differentiated phenotype (Andreoli & Trevor, 1995). The differentiation of F9 cells to PE via RA and RA/db cAMP, respectively, results in the cells expressing large amounts of cytokeratin filaments (8 & 18) and a reduction in vimentin expression (Kurki *et al.*, 1989; Paulin *et al.*, 1982; Ramaekers *et al.*, 1984). Using a monoclonal antibody against keratin 18 it was shown that this was induced in at least 50% of F9 cells differentiated with RA and cAMP (Ramaekers *et al*, 1984).

1.4.2.2 Integrin expression in Differentiating F9 Cells

β1 integrin expression has been found to be extremely important to F9 differentiation. When the β1 integrin genes were disrupted (F9 cells maintain three copies of the gene, so triple knockout lines (TKO) had to be generated), the parental cells showed a disrupted morphology and demonstrated poor adhesion to typical β1 integrin substrates such as laminin or fibronectin. More interesting however was the observation that the triple knockout (TKO) cell lines did not differentiate normally when exposed to RA and/or db cAMP. The morphology of RA treated cells was altered. However it was also discovered that the TKO cells, while not appearing to be properly differentiated, did express the correct tissue specific markers one would expect from the above treatments (Stephens *et al*, 1993). Following RA induced differentiation of F9 cells there is a switch in the expression levels of different integrin subunits, namely there is a reduction in α5β1and a massive increase in the α3β1 subunit (Burdsal, *et al*, 1994). The β1 integrin subunit was also shown to be up-regulated in a study of RA mediated differentiation of F9 cells (Ross *et al.*, 1994).

1.4.2.3 Gap Junctions and Connexin Expression in Differentiating F9 Cells

F9 EC cells express connexins 26, 32 and 37 while the differentiated primitive and parietal endoderm cells express connexins 26,32, 37 and 43. Connexin43 is the most abundant and thus more relevant in F9 communication (van der Heyden *et al*, 2000). External agents that alter the differentiation-state of the cell regulate cell-to-cell communication, i.e. tumor promoting factors tend to down regulate communication with differentiating agents tending to promote it (Clairmont *et al,* 1996). This group discovered that RA (10^{-7} M) can increase cell-cell communication due to increases in connexin43 (Cx43) mRNA and protein. The change in Cx43 appears to be incidental to the differentiation of F9 cells i.e. it does not have an active role in the process.

1.4.3 Pluripotency of F9 Cells

As discussed, the F9 cell line shows some low-level spontaneous differentiation and is typically induced to differentiate with agents such as RA. The cell types generated by this treatment have been limited thus F9 cells were considered unsuitable for the study of overall development. However in a study where the action of RA was retarded by conditioned medium from STO fibroblasts or by using 10^{-8} M RA rather than 10^{-7} M, it was found, that low density cultures of F9 cells expressed the differentiation markers of a number of cell types, such as parietal and visceral endoderm, adipocytes, neurons, fibroblasts and trophoblasts (Koopman & Cotton, 1987). F9 cells were also found to be capable of expressing neuronal markers following RA/db cAMP treatment, and if nerve growth factor was also applied the adrenergic neuronal marker tyrosine hydroxylase was expressed (Liesi *et al.*, 1983). Zakany *et al.* (1984) identified neuronal markers in spontaneously differentiated F9 cells generated from single cells, merocyanine-540 incorporation also indicated the presence of electrically excitable membranes. Certain neuronal markers were found to be constitutively present within undifferentiated F9 cultures and a subclone of F9, D9L2, was shown to be capable of extending neurites and expressing several neuronal antigens under conditions of serum depravation (Murtomaki *et al.*, 1999).

1.5 Engineering β Cells for Replacement Therapy in IDDM

β cells have been used in the treatment of diabetes in the form of pancreas or islet transplants (this procedure is typically carried out with or after a kidney transplant). Although this therapy has shown some success, there is a problem with the availability of donor tissue, thus the regulated expression of the human insulin gene has been examined in non-β cell lines. These cell lines would represent a continuous source of replacement material. Somatic cell therapy has been proposed as another option i.e. whereby cells would be isolated from a patient, transfected with the human insulin gene, purified characterised and replaced in to the body (Bailey *et al*, 1999). This would overcome immunological considerations as the cells would not be considered as foreign. Finally insulinoma cell lines have been generated through transformation of β cells, these cells already have the correct machinery for proinsulin biosynthesis and processing and can be expanded to large numbers in culture.

1.5.1 Insulinoma Cell Lines as a Treatment for IDDM

Cell lines derived from insulinoma tumors have been generated and are of interest due to the fact that these cells have the cellular machinery required for GSIS, however thus far these cell lines have mostly been derived from rodents.

1.5.1.1 RIN 1046-38 (RIN-38)

The rat derived RIN cell line was one of the first isolated from radiation induced rat tumors (Gazdar *et al.*, 1980). These cells were found to show a 5-9 fold increase in secretion upon glucose stimulation sub-physiological levels. As the passage number increased the cells were seen to show decreased cellular insulin and they lost GSIS capability (Clark *et al.*, 1990). RIN-38 cells transfected with the human insulin gene, glucokinase and GLUT2 and the human insulin gene with glucokinase alone, demonstrated increased cellular insulin and a higher fold response that in the parental population. These RIN-derived cells had a maximal glucose stimulation at 25 μM glucose due to the expression of low k_m hexokinases (Hohmeier *et al.*, 1997) thus

limiting thier usefulness for therapy unless those enzymes were knocked out by molecular means.

1.5.1.2 β-Tumor-Cell (βTC) Cell Lines

βTC cell lines are derived from transgenic mice expressing the SV40-T-antigen oncogene under the control of the insulin promoter. This results in expression of the T-antigen (Tag) in β cells and immortalization of the cell line (Soria *et al.*, 2000b). Although the cells exhibit GSIS, the phenotype is unstable and can be lost through serial passaging. When single cell clones were isolated from the BTC6 cell line however, two of the resultant cell lines were found to maintain GSIS for over a year in culture. This was due to the fact that these clones contained high glucokinase/hexokinase ratios and no GLUT1 expression (only GLUT2) (Knaack *et al.*, 1994).

1.5.1.3 INS-1

INS-1 cells were derived from an x-ray-induced insulinoma. The continued growth of these cells was found to be dependent on the presence of 2-mercaptoethanol. INS-1 cells secrete insulin in response to glucose signals and were shown to demonstrate approximately 2.2-fold stimulation of insulin secretion upon glucose stimulation (Asfari *et al.*, 1992). Other researchers have noted 4 -13 fold increases in insulin secretion from INS1 upon stimulation with glucose (Hohmeier *et al.*, 2000). The cells proliferate extremely slowly (doubling time ~ 100h) and have maintained stable glucose responsiveness for 2 years (80 passages) (Asfari *et al.*, 1992). This cell line is an ideal model for the study of β cells due to its differentiated nature.

1.5.1.4 Min6

Min6 cells were isolated from insulinomas from SV40 T antigen expressing mice and were shown to exhibit an increase in insulin secretion of almost 10-fold upon stimulation with 25 mM glucose (Miyazaki *et al.*, 1990). However literature data varies from 2-fold to 10-fold increase in secretion between 3 and 25 mM glucose

(Kayo *et al.*, 1996; Ishihara *et al.*, 1993, Zhao & Rutter, 1998). The reported stability of Min6 in culture also varies. Min6 cells are described as being fully GSIS capable at passage 30–40 (Miyazaki *et al.*, 1990; Ishihara *et al.*, 1995), however Kayo *et al.* (1996) identified a mere 1.2 fold increase in secretion at passage 35 (from 5 mM to 25 mM glucose). Loss of GSIS in Min6 cells was accompanied with a decrease in the proinsulin processing enzymes PC1 (PC3) and PC2 with a concomitant increase in furin that, was linked to Min6 cells adopting a de-differentiated state with increasing passage number (Kayo *et al.*, 1996). Min6 cells are also known to express glucagon, somatostatin and IAPP (Ohgawara *et al.*, 1995; Kanatsuka *et al.*, 1992). Culture of Min6 in an agarose-PVMA-collagen matrix led to the formation of islet like cell clusters (ICCs). In the presence of nicotinamide the ICCs maintained GSIS and were in a better state of preservation than ICCs cultured without nicotinamide. These ICCs were implanted into chemically induced diabetic mice (in a diffusion chamber) and maintained normoglycemia for up to 12 weeks (Ohgawra *et al.*, 1995). Min6 cells inoculated subcutaneously into the backs of an obese-diabetic-model mouse (C57BL/KsJ-db/db) formed a vascularised tumor and effected a return to normoglycemia for 100 days, although the obesity was unchanged (Inada *et al.*, 1996). Min6 cells cultured on gelatin coated flasks can form 3-D structures with good cell- cell contacts, Formation of these 'pseudoislets' relied upon the expression of the Ca^{2+} dependent adhesion molecule E-cadherin (E-CAD). Pseudoislets generated from non-responsive Min6 cells (passage 43-53) were seen to have restored glucose and secretagogue stimulated insulin secretion (Hauge-Evans *et al.*, 1999). Thus the Min6 ICCs or pseudoislets would be ideal for IDDM therapy if they were engineered to express the human insulin gene, and if the endogenous insulin genes could be disrupted as these may illicit an immunological response in a human.

1.5.2 Engineering of non-β Cell Lines to Synthesise and Secrete Human Insulin

Most of the work regarding replacement therapies for IDDM has centred on the engineering of non-β cell lines to secrete insulin. The advantage of non-β cells is that these cells do not express the antigenic determinants that are targeted in the autoimmune destruction of β cells in IDDM. Ideally the cells should have some of the features of β cell lines which would allow them to regulate the synthesis and secretion

of insulin (Mitanchez *et al*, 1997). No such complete cell line exists so existing cell lines have been modified via gene transfer to resemble functional β cell lines. The main cell types investigated are discussed below.

1.5.2.1 Neuroendocrine Cells as Artificial β Cells

A variety of neuroendocrine cell lines contain secretory granules to allow for storage and regulated secretion of peptide hormones e.g., the adrenocorticotropic hormone (ATCH) secreting cell-type AtT20 from the anterior pituitary (Newguard, *et al.*, 1997). These cells have other features which make them suitable for the regulated expression and secretion of insulin such as the expression of the PC1 (PC3) and PC2 prohormone convertases (Smeekens *et al.*, 1991; Bennet *et al.*, 1992) and the high k_m glucose phosphorylating enzyme glucokinase, even though this was not accompanied by GLUT2 expression (Hughes *et al.*, 1991). Proinsulin synthesis, processing and secretion upon stimulation following transfection into AtT20 cells was reported by Moore *et al.* (1983). The insulin-expressing $AtT20_{ins}$ cells were found to be glucose responsive between 0 and 2.5 mM glucose i.e. below the physiological range of glucose but demonstrated large secretagogue stimulated secretion using agents known to increase intracellular cAMP, such as db cAMP and 8-bromo-cAMP (Moore *et al.*, 1983; Hughes *et al.*, 1991). Transfection of $AtT20_{ins}$ cells with GLUT2 resulted in a large increase in the insulin content of $AtT20_{ins}$ cells and to a more significant GSIS, however this was maximal at 10 μM glucose which is far below the point at which β cells respond to glucose (4-5 mM). It was discovered that although the $AtT20_{ins}$ cells expressed glucokinase, the predominant glucose-phosphorylating enzyme in the cell was its low k_m counterpart hexokinase I (Hughes *et al.*, 1992). Previous studies have shown that overexpression of hexokinase I can abolish GSIS in β cells (German, 1993). Similar results were obtained in another study whereby GLUT2 and insulin were co-transfected in to AtT20 cells. These researchers noted maximal GSIS at around 50 μM, also the hexokinase I inhibitor 2 deoxy-glucose caused an increase of insulin secretion at all glucose concentrations up to 10 mM glucose. Thus it was theorised that the glucokinase:hexokinase I ratio was too low to achieve proper GSIS in AtT20 cells (Davies *et al.*, 1998). This was confirmed by the overexpression of glucokinase, GLUT2 and insulin in AtT20 cells which resulted in GSIS over the

whole physiological range. These cells also demonstrated glucose potentiation upon stimulation of the cells with agents that cause closure of the potassium channels e.g., glibenclamide (Motoyoshi *et al.*, 1998).

Although this cell line appears to have addressed all the requirements for an artificial β cell line, the inherent secretion of ACTH is undesirable. ACTH secretion leads to adrenal glucocorticoid secretion which can cause insulin resistance (Stewart *et al.*, 1994). Following implantation if insulin secreting $AtT20_{ins}$ cells to streptozotocin diabetic mice, overgrowth of the cells led to tumor-like growths with a periphery of healthy cells surrounding a necrotic core (Stewart *et al* 1994., Davies *et al.*, 1998). This highlights the requirement for physical containment to prevent implant-overgrowth which would also provide a barrier to prevent immunological rejection.

1.5.2.2 Hepatocytes as Artificial β Cells

Hepatocytes are possibly the only other cell type to have the same glucose sensing system as β cells i.e. GLUT2 and glucokinase (Mitanchez *et al.*, 1997). Initial experiments with rat hepatoma cells (FAO cells) demonstrated that proinsulin processing was present in these cells (albeit inefficient) despite the fact that these cells secreted the transfected protein constitutively (Vollenweider *et al.*, 1992). The human hepatoma cell line HEP G2 was also shown to be capable of processing transfected insulin and the mature form of the peptide was preferentially stored while there was constitutive release of the unprocessed proinsulin. These cells were observed to exhibit secretagogue stimulated insulin secretion (SSIS) following stimulation with agents leading to increased intracellular cAMP such as 8-bromo-cAMP or theophylline. Mature-processed insulin was predominantly secreted as a result of SSIS and these cells were not capable of GSIS (Simpson *et al.*, 1995). Cultured HEP G2 cells do not express the high capacity glucose transporter GLUT2 (Permutt *et al.*, 1989) and are therefore missing a critical component of the glucose sensing system. Overexpression of GLUT2 in the insulin secreting HEP G2 cell line (HEP G2ins) resulted in GSIS capability over the entire physiological range, although initial minimal stimulation occurred at ~ 2 mM glucose which is lower than that the minimal stimulation level in β cells (4-5 mM) (Simpson *et al.*, 1997).

Efficient processing of insulin in rat hepatoma cells (FTO-2B) was achieved by genetically engineering the PC1/PC2 cleavage sites so that they were susceptible to cleavage by the ubiquitous endoprotease furin as described by Groskerutz *et al.*, (1994) (Figure 1.5.1). Expression of the mutant proinsulin resulted in 90% conversion of proinsulin to mature insulin (Gros *et al.*, 1997).

The liver has also been considered a viable target for direct gene therapy for diabetes. Studies have already been carried out on transgenic mice expressing wild type and furin cleavable proinsulin in their livers and also on mice infected with adenoviral vectors carrying these forms of insulin (Mitanchez *et al.*, 1998; Auricchio *et al.*, 2002).

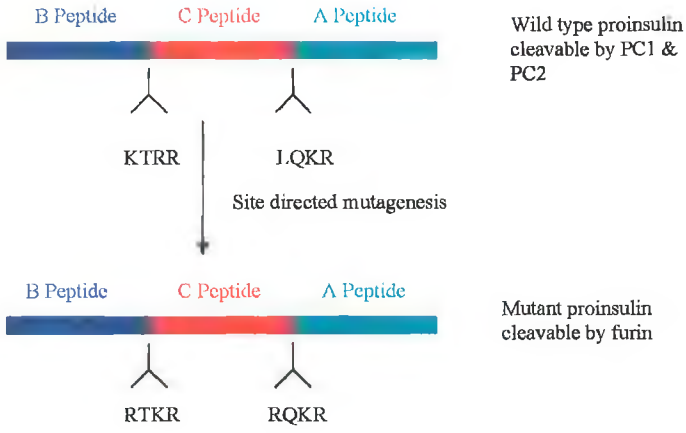


Figure 1.5.1 Site directed mutagenesis of wild type proinsulin to render it susceptible to cleavage by the ubiquitous endoprotease furin.

1.5.2.3 Engineering Fibroblasts as Artificial β Cells

Fibroblasts would not be considered as the ideal choice for an artificial β cell due to the fact that they only posses constitutive protein secretion pathways and do not have the capacity to store and process proteins (Kelly, 1985). However, fibroblasts would be considered an excellent option for *ex-vivo* somatic cell therapy. This would involve the removal of a convenient cell type (without causing trauma to the patient), transfection of the insulin gene (and any extra genes to aid processing, etc.), selection

and characterisation of transfected cells, cell stock generation and re-implantation of the patients own cells (thus avoiding immunological rejection) (Bailey *et al.*, 1999). Temporary reversal of diabetes in mice has already been demonstrated by transplanting primary human fibroblasts expressing furin cleavable proinsulin (Falqui *et al.*, 1999).

Initial work with mouse Ltk^- fibroblasts led to the generation of insulin secreting cells Ltk^+Ins cells which were characterised as secreting proinsulin constitutively. Implantation of these cells into diabetic mice led to normoglycemia after two weeks (proinsulin is 8-20% as effective as insulin (Taniguchi *et al.*, 1997; Selden *et al.*, 1987; Falqui *et al.*, 1999)), however these animals eventually died due to implant overgrowth and the resulting hypoglycemia (Selden *et al.*, 1987). This also occurred in a study by Kawakami *et al.* (1992). This group also designed a system whereby the transplanted cells could be specifically removed by the co-transfection of an antigenic determinant that could be specifically targeted by a monoclonal antibody. Regulated secretion of proinsulin from fibroblasts was achieved through an inducible promoter system (which regulates the secretion at the level of protein availability) (Kawakami *et al.*, 1992). Encapsulation of Ltk^+Ins cells in 5% agarose prevented overgrowth of the proinsulin secreting fibroblasts while allowing proinsulin secretion unimpeded. These encapsulated cells were capable of growing for up to 80 days in culture and 2 x 10^7 encapsulated cells maintained normoglycemia in mice for up to 30-50 days (Taniguchi *et al.*, 1997). The constitutive release of endogenous insulin is reported to significantly aid in control of IDDM in patients and may prevent some diabetes-associated complications (Falqui *et al.*, 1999). In an attempt to confer GSIS to fibroblast cells, Vero cells were co-transfected with furin cleavable proinsulin, GLUT2 and glucokinase. Although the cells did manage to process 62 % of the proinsulin formed to mature insulin, the cells were not rendered glucose sensitive by the presence of GLUT2 and glucokinase (the GLUT2 expression was predominantly nuclear, which may have affected glucose sensing) (O'Driscoll *et al.*, 2002).

1.5.3 BHK-21 Cells as Artificial β Cells

BHK-21 cells have not as yet been transfected with the human insulin gene and were considered a suitable cell line for research due to the fact that the cells have already been used in a cell-therapy based clinical trial for amyotropic lateral sclerosis (ALS) (Aebischer *et al.*, 1996). BHK cells were engineered to constitutively secrete human ciliary neurotrophic factor (hCNTF). The cells were encapsulated and implanted in a tethered device intrathecally, which allowed for retrieval of the implanted cells. Thus these cells have already been considered safe for human therapy. Also BHK cells can grow in serum free media (Merten *et al.*, 1999), which is advantageous for a therapeutic cell line.

1.6 Aims of this Thesis

Previous work by other researchers has focused on the generation of β-like cell lines from ES cells, but there are difficulties with maintaining these cell lines in culture in an undifferentiated state and they require expensive medium supplements and/or feeder cell layers. EC lines e.g. F9 cells, have not been reported in any such studies as of yet. F9 cells exhibit low spontaneous differentiation as long as the cells are maintained in the active growing phase (Nishimune *et al.*, 1983; Griep & De Luca, 1986a) and are easy to culture and maintain. Although F9 cells were characterised as differentiating towards a number of cell types, the methods used were essentially random (Section 1.4.3). Thus it was undertaken to design a 'directed differentiation' protocol to push F9 cells towards an endocrine lineage using the following steps:

1. Generation of partially differentiated parietal (PE) and visceral (VE) from F9 cells.
2. Treatment of these resultant cell types with endocrine differentiation agents and monitoring crucial endocrine differentiation markers via RT-PCR and immunological techniques (where applicable).
3. To purify PDX1 expressing sub-populations from F9/ differentiated F9 cells using cell trapping (Klug *et al.*, 1996; Soria *et al.*, 2000a; Section 1.3.2.1) and to characterise the resultant cells.
4. To investigate the effects of overexpression of genes believed to be relevant to pancreatic development in partially differentiated F9 cells.

The stability of cultured insulinoma cell lines with regard to the maintenance of GSIS is an important issue if such cells are to be used for therapeutic applications. Min6 cells had been characterised as almost adopting a de-differentiated phenotype with increased passage number (Kayo *et al.*, 1996).

Thus this system afforded a useful model system in which to investigate the changes occurring in the de-differentiating β cells. This was considered to be important as it may identify important markers/junction points which could aid in directed differentiation of F9 cells towards β cells. It also allowed the opportunity to

investigate why the cells were loosing their specialised function during their time in culture.

This study was addressed using DNA microarrays and RT-PCR to identify the differences between the GSIS-capable and GSIS-deficient Min6s.

Finally, much research has been carried out on the generation of artificial β cells through the direct genetic modification of non-β cell lines. As BHK-21 cells had already been employed in an approved human clinical trial (Aebischer *et al.*, 1996), it was proposed that these cells may be a suitable target for such modification. In carrying out this work it was hoped to obtain a suitable therapeutic cell line but also to examine the factors involved in regulated protein expression and secretion.

2.0 MATERIALS AND METHODS.

2.1 Cell Culture Methods

2.1.1 Water

Ultrapure water was used in the preparation of all media and solutions. Initially the water was pre-treated which involved activated carbon, pre-filtration and anti-scaling. This water was then purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12 - 18 MΩ/cm resistance.

2.1.2 Treatment of Glassware

All solutions for use in cell culture and maintenance were prepared and stored in sterile glass bottles. Bottles (and lids) and all other glassware used for any cell-related work were prepared as follows: - all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1-hour. This is a deproteinising agent, which removes proteineous material from the bottles. Glassware was scrubbed and rinsed several times in tap water, the bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving.

2.1.3 Sterilisation

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121°C for 20 minutes (min) under pressure of 1bar. Thermolabile solutions were filtered through a 0.22 μm sterile filter (Millipore, millex-gv, SLGV-025BS). Low protein-binding filters were used for all protein-containing solutions.

2.1.4 Media Preparation

Medium was routinely prepared and sterility checked by Mr. Joe Carey (technician) as in SOP NCTCC 003-02. The basal media used during routine cell culture were prepared according to the formulations shown in Table 2.1.1. 10x media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7.45 - 7.55 using sterile 1.5M NaOH and 1.5M HCl. The media were then filtered through sterile 0.22μm bell filters (Gelman, 121-58) and stored in 500ml sterile bottles at 4°C. Sterility checks were carried out on each 500ml bottle of medium as described in Section 2.2.8.

The basal media were stored at 4°C up to their expiry dates as specified on each individual 10x medium container. Working stocks of culture media was prepared as 100ml aliquots, supplemented with L-glutamine (Gibco, 25030-024) and fetal calf serum as required. This was stored for up to 2 weeks at 4°C, after which time, fresh culture medium was prepared.

	DMEM (Gibco, 12501-029)	**Hams F12** (Gibco, 21700-109)	**MEM** (Gibco, 21430-020)
10X Medium	500ml	Powder	500ml
Ultrapure H_20	4300ml	4700ml	4300ml
1M HEPES* Sigma , H-9136	100ml	100ml	100ml
7.5% $NaHCO_3$ BDH, 30151	45ml	45ml	45ml

* HEPES = N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

Table 2.1.1 Preparation of basal media

F9 EC cell lines were cultured in DMEM supplemented with 10% FCS (Sigma, F7524, lot No.-010K3361). and 4 mM L-Glutamine. Min6 cells were routinely grown in DMEM supplemented with 20% FCS (heat inactivated for 35 minutes at 56 ^{0}C). For DLKP, ATCC medium (Ham's F12/ DMEM (1:1)) was supplemented with 5% FCS and 2mM L-glutamine (Gibco, 25030-024). BHK-21 cells and clones were cultured in MEM supplemented with 5% FCS, 2mM L-glutamine, 1X NEAA (Gibco, 11140-035) and 1X sodium pyruvate (Gibco, 11360-039).

2.2 Methods Used in Maintaining Cell Lines

2.2.1 Safety Precautions

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work, which involved toxic compounds, was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet. Each cell line was assigned specific media and waste bottles and only one cell line was used at a time in the cabinet, which, was allowed to clear for 15min between different cell lines. The cabinet itself was cleaned each week with industrial detergents (Virkon, Antec. International; TEGO, TH.Goldschmidt Ltd.), as were the incubators.

The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2.2.1.

2.2.2 Culture of Adherent Cell Lines

The cell lines employed during the course of this research are listed in Table 2.2.1, these cells were generally maintained in non-vented 25 cm^2 (Costar, 3050) and 75 cm^2 flasks (Costar, 3075) or vented 25 cm^2 (Costar, 3056), 75 cm^2 (Costar 3376) and 175 cm^2 flasks (Nunclon, 178883). F9 cells were maintained on a layer of 0.1 % gelatin (Sigma G1890). Coating of flasks was carried out by adding enough gelatin to cover the tissue culture surface of the flask, these were then placed at 4 ^{o}C for at least 2 hours (usually overnight). The flasks were then rinsed X3 in sterile UHP. These coated flasks were stored at room temperature until required.

Cell Line	Source of Cell Line	Medium	Cell Type	CO_2 Requirement
F9	ATCC (CRL-1720)	DMEM	Undifferentiated Murine EC Line	5% CO_2
Min6	Dr. Per Bendix Jeppesen	DMEM	Murine β Cell Line	5% CO_2
BHK-21	NCTCC	MEM	Hamster Fibroblast Cell Line	5% CO_2
DLKP	NCTCC	ATCC Medium	Poorly Differentiated Human Squamous Carcinoma	No CO_2 Requirement

Table 2.2.1 Cell lines used in this thesis including sources, growth conditions and type.

2.2.3 Subculture of Adherent Cell Lines

During routine sub-culturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment. The following protocol outlines the methods used in subculturing F9, BHK and DLKP cell lines.

Waste medium was removed from the flasks and rinsed with a pre-warmed (37°C) trypsin/EDTA (TV) solution (0.25% trypsin (Gibco, 25090-028), 0.01% EDTA (Sigma, E5134) solution in PBS (Oxoid, BR14a)). The purpose of this was to eliminate any naturally occurring trypsin inhibitor, which would be present in residual serum. Fresh TV was then placed on the cells (1ml/25cm^2 flask or 2ml/75cm^2 flask) and the flasks were incubated at 37°C until the cells were seen to have detached (5 min). The trypsin was deactivated by addition of an equal volume of growth medium (*i.e.* containing serum). The entire solution was transferred to a 30ml sterile universal tube (Greiner, 201151) and centrifuged at 1,000 rpm for 5 min (F9 cells were centrifuged at 125 x g for 10 minutes). The resulting cell pellet was re-suspended in pre-warmed (37°C) fresh growth medium, counted (Section 2.2.5) and used to re-seed a flask at the required cell density or to set up an assay.

2.2.3.1 Subculture of Min6 Cell Lines

Min6 flasks were emptied of conditioned medium and rinsed in pre-warmed 1X Ca^{2+}/Mg^{2+}- free PBS (Gibco 14200-067). Trypsin/EDTA was then added (1 ml/ 25 cm^2 flask and 2 ml/ 75 cm^2 flask) and incubated at 37°C for 2 minutes exactly. The flask was tapped sharply to dislodge the cells and then 5 ml of culture medium was added to inhibit the trypsin. The cell suspension was aspirated gently and with care to avoid separating the cells in to a single cell suspension. Following this the cells were centrifuged at 900 rpm for 5 minutes. The resulting pellet was re-suspended in pre-warmed culture medium, counted (Section 2.2.5) and re-seeded to flasks or assay plates. Min6 cells were never re-seeded back in to the same flask.

2.2.4 Subculture of Suspension Embryoid Bodies

Culture of F9 cells at a cell density of 1 x 10^5 cells/ml in mini bacteriological-grade petri dishes (Greiner, 627102), resulted in the formation of aggregates called embryoid bodies (EBs). These cells grew in suspension and thus did not require enzymatic detachment. Subculture was effected by gentle removal of the cells to a universal using a Pasteur pipette (Copan, 200CS01). The EBs were separated from the medium using gentle centrifugation at 100 x g for 10 minutes. Following centrifugation the universals were treated gently as the pellet was quite loose and the waste medium removed carefully using a Pasteur pipette. The pellet was gently re-suspended in pre-warmed medium and the EBs were transferred to new petri dishes.

2.2.5 Cell Counting

Cell counting and viability determinations were carried out using a tryphan-blue (Gibco, 15250-012) dye exclusion technique.

An aliquot of tryphan-blue was added to a sample from a single cell suspension in a ratio of 1:5. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically. An average per corner grid was calculated with the dilution

factor being taken into account and final cell numbers were multiplied by 10^4 to determine the number of cells per ml (volume occupied by sample in chamber is 0.1cm x 0.1cm x 0.01cm *i.e.* $0.0001cm^3$ therefore cell number x 10^4 is equivalent to cells per ml). Non-viable cells were those, which stained blue while viable cells excluded the tryphan-blue dye and remained unstained.

2.2.6 Cell Freezing

To allow long term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below –180°C. Once frozen properly, such stocks should last indefinitely.

BHK-21 and DLKP were frozen using the standard freezing method as follows. Cells to be frozen were harvested in the log phase of growth (*i.e.* actively growing and approximately 60-70% confluent) and counted as described in Sections 2.2.5. Pelleted cells were re-suspended in serum and an equal volume of a DMSO/serum (1:9, v/v) freezing solution. The freezing solution was slowly added (drop-wise) to the cell suspension (as DMSO is toxic to cells). A final concentration of at least $5x10^6$ cells/ml was generated. The suspension was then aliquoted into cryovials (Greiner, 122 278) which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -80°C). After 2.5 to 3.5 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required.

2.2.6.1 Freezing F9 EC Cells

F9 Cells were trypsinised as outlined previously (Section 2.2.3), while the cells were being trypsinised, freezing medium (a filter sterilised mixture of DMSO/culture medium (1:19 v/v)), was pre-chilled on ice as were labelled cryovials. Upon isolation of the cell pellet, the cells were re-suspended in the freezing medium to give approximately 1-5 x 10^6 cells/ml and aliquoted in to the pre-chilled cryovials. These cryovials were placed in the centre of an enzyme box (Boehringer Mannheim), (i.e. with Styrofoam surrounding the cryovial on all sides) and placed at – 80°C. This method of freezing ensures an approximate cooling rate of 1°C/min. The cells could be stored like this for long periods but to spread out the cell stocks, some vials were stored in liquid nitrogen (after at least 12 hours at –80°C).

2.2.6.2 Freezing of Min6 Cells

Prior to trypsinisation of Min6 cells, a freezing medium containing DMSO and FCS was prepared (1:4) and filter sterilised. The freezing medium was then placed on ice until ready. The cryovials were also placed on ice at this point. Min6 cells were trypsinised as outlined previously (Section 2.2.3.1). The pellet was re-suspended in culture medium to give a cell density of $5 \times 10^6 - 1 \times 10^7$ cells/ml. To this suspension, an equal volume of freezing medium was added, dropwise, with stirring on ice. This suspension was aliquoted to cryovials (also on ice). The cryovials were cooled on ice for 10 minutes, and then transferred to a –20°C freezer in a Boehringer Mannheim enzyme box for 1 hour. Following this, the cells were placed at –80°C for at least 12 hours. The cells could be stored at this temperature for long periods, reserve stocks were placed in liquid nitrogen containers however.

2.2.7 Cell Thawing

For thawing Min6, BHK-21 cells and DLKP, very similar thawing protocols were employed. Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing 5 ml growth medium was prepared. This allowed for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution (it is toxic at room temperature). The cryovial was partially thawed and its contents were transferred to the universal. The suspension was centrifuged at 1,000 rpm for 5 min (900 rpm for Min6), the DMSO-containing supernatant was removed and the pellet was then re-suspended in fresh growth medium. Viability counts were carried out (Section 2.2.5) to determine the efficacy of the freezing/ thawing procedures. A sample was also taken for sterility analysis (Section 2.2.8). Thawed cells were placed into tissue culture flasks with the appropriate volume of medium (10ml/25cm^2 flask and 15ml/75cm^2 flask) and allowed to attach overnight. Min6 medium was pre-warmed and conditioned in a vented flask in the presence of 5% CO_2 for at least 30 minutes prior to thawing. 24 hours after thawing, the cells were re-fed with fresh medium to remove any residual traces of DMSO.

2.2.7.1 Thawing F9 EC Cells

Prior to thawing F9 cells, 14 ml of culture medium was placed in a gelatin coated 25 cm^2 flask (vented) and conditioned for temperature and pH in a CO_2 incubator for at least 30 minutes. The frozen F9 cryovial was then thawed and the contents were transferred directly in to the medium. A sample was taken for cell counting, viability (Section 2.2.5) and for sterility checks (Section 2.2.8). This medium was changed 12-24 hours later.

2.2.8 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture. Samples of basal media were inoculated into Columbia (Oxoid, CM331) blood agar plates, Sabauraud (Oxoid, CM217) dextrose and Thioglycollate (Oxoid, CM173) broth's which should between them detect most contaminants including bacteria, fungus and yeast. Growth media (*i.e.* supplemented with serum and L-glutamine) were sterility checked at least 3 days prior to use by incubating samples at 37°C. These were subsequently examined for turbidity and other indications of contamination. Freshly thawed cells were also subjected to sterility checks.

2.2.9 *Mycoplasma* Analysis

Mycoplasma examinations were carried out routinely (at least every 3 months) on all cell lines used in this study. These analyses were performed by Dr. Mary Heenan, Ms. Bojana Cumpf, and Mr. Michael Henry.

2.2.9.1 Indirect Staining Procedure

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells i.e. these cells were incubated with supernatant from test cell lines and then examined for *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present. A

Mycoplasma infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells.

NRK cells were seeded onto sterile coverslips in sterile Petri dishes (Greiner, 633 185) at a cell density of $2x10^3$ cells per ml and were allowed to attach overnight at 37°C in a 5% CO_2, humidified incubator. 1ml of cell-free (cleared by centrifugation at 1,000 rpm for 5 min) supernatant from each test cell line was then inoculated onto a NRK petri dish and incubated as before until the cells reached 20 - 50% confluency (4-5 days). After this time, the waste medium was removed from the petri dishes, the coverslips (Chance Propper, 22 x 22 mm) were washed twice with sterile PBS, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid:methanol-1:3) for 10 min. The fixative was then removed and after air drying, the coverslips were washed twice in deionised water and stained with 2ml of Hoechst 33258 stain (BDH) (50ng/ml) for 10 min.

From this point on, work proceeded in the dark to limit quenching of the fluorescent stain.

The coverslips were rinsed three times in PBS. They were then mounted in 50% (v/v) glycerol in 0.05M citric acid and 0.1M disodium phosphate and examined using a fluorescence microscope with a UV filter.

2.2.9.2 Direct Staining

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid, CM403) - supplemented with 20% serum, 10% yeast extract (Oxoid L21, 15% w/v) & 10% stock solution (12.5g D-glucose, 2.5g L-arganine & 250 mls sterile-filtered UHP). This medium was designed to optimise the growth of any contaminants and was incubated at 37°C for 48 hours. Samples of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had also been supplemented as

above and the plates were incubated for 3 weeks at 37°C in a CO_2 environment. The plates were viewed microscopically at least every 7 days and the appearance of small, "fried egg" -shaped colonies would be indicative of a mycoplasma infection.

2.3 Specific Cell Culture Techniques Employed in this Thesis

2.3.1 Differentiation Studies

Differentiation Studies were carried out using the differentiation agents listed in Table 2.3.1.

Differentiation Agent	Supplier	Stock Concentration	Working Concentration
All trans Retinoic Acid (RA)	Sigma (R2625)	10^{-3} M	10^{-7} M
Di Butyryl cAMP (db cAMP)	Sigma (D0260)	100 mg/ml	10^{-3} M
Activin A	Sigma (A4941)	20 µg/ml	2 nM
Betacellulin (BTC)	Sigma (B3670)	2 µg/ml	1 nM
Hepatocyte Growth Factor (HGF)	R & D systems (294-HG)	5 µg/ml	100 pM
Nicotinamide (Nico)	Sigma (N0636)	10 mM	10 mM
Sodium Butyrate (Sod But)	Sigma (B5887)	1 mM	1 mM

Table 2.3.1 Differentiation agents used in the course of this work

2.3.1.1 Reconstitution of Differentiation Agents

RA was reconstituted in 95% EtOH to a final concentration of 10^{-3}M. RA was aliquoted and wrapped in foil as it is light sensitive, it was routinely stored at –80°C. db cAMP was re-suspended in UHP (pH adjusted to exactly 7.0) to a final concentration of 100 mg/ml. The resulting solution was aliquoted, wrapped in foil (it is also light sensitive) and stored at –20 °C. Immediately prior to differentiation assays, cAMP stocks were diluted by a factor of 203.5 in complete medium to achieve the correct working concentration (10^{-3}M) . This was then filter sterilised. The stock solutions of the recombinant proteins activin A, BTC and HGF were made up in PBS-0.1% BSA to the desired concentration. These were aliquoted in working volumes and stored at –20 °C until required. Immediately prior to differentiation assays, these stock solutions were diluted in complete medium to the working concentration and filter sterilised using a 0.22 µm filter. Both nicotinamide and sodium butyrate were

supplied in stable powder form and were reconstituted to the correct working concentrations immediately prior to any differentiation experiments. The reconstitution was carried out in complete medium and they were dissolved at 37°C, after which, they were filter sterilised.

2.3.1.2 Differentiation Assays

2.3.1.2.1 Stage 1 (Endodermal) Differentiation

All differentiation assays were carried out on F9 monolayers or on F9 EBs (Section 2.2.4). Monolayer experiments were carried out in 6 well plates (pre-coated in 0.1% gelatin) while EB experiments were carried out in mini-petri dishes. Monolayers were seeded at 1 x 10^4 cells/well and to generate EBs, F9 cells were seeded at 1 x 10^5 cells/ml in mini petri dishes (2 ml/ dish). Monolayer cells were allowed to attach overnight, and the following day, were treated with RA (10^{-7} M) or RA in combination with db cAMP (10^{-3} M). EBs were treated with RA (10^{-7} M) only. The cells were exposed to these compounds for 72 hours at 37°C in a 5% CO_2 atmosphere. The plates and dishes were wrapped in foil as RA and db cAMP are light sensitive.

2.3.1.2.2 Stage 2 (Endocrine) Differentiation

For second stage (endocrine differentiation), cells from Stage 1 differentiation were fed directly in their wells with the agents listed in Table 2.3.1, combinations of the individual agents with activin A were also used. On day 3, the wells were fed again with the endocrine differentiation agents. Re-feeding of the EBs has already been outlined (Section 2.2.4). On day 5 the wells and EB dishes were subcultured to prevent overconfluence. Monolayer wells were rinsed in 1 ml PBS and then exposed to 200 µl/ trypsin-EDTA per well. The trypsin reaction was incubated for 5 min at 37°C. To stop the trypsin action, 1 ml of complete medium was added/well. The cell suspensions were then centrifuged at 125 x g for 10 minutes and 1/3rd of the each pellet was re-seeded in to a new well. On this day also, the EBs were subcultured as outlined (Section 2.2.4) but each pellet (representing the EBs from 1 dish) was re-suspended in enough differentiation medium for 2 mini petri dishes. The treatments were again carried out on day 7-8 and the experiments were sampled on day 10 (Section 2.3.1.3).

2.3.1.3 Sampling Differentiation Assays

2.3.1.3.1 Fixing Cells for Immunofluorescence/Immunocytochemistry

Upon reaching the end of stage 1 or stage 2 differentiation, monolayer samples were rinsed 3X in PBS. 2 ml of ice cold methanol was added to each well and the plates were incubated at –20°C for 7 minutes and removed. The plates were allowed to air-dry, wrapped in foil and stored at –20°C. EB samples were seeded on to gelatin coated wells for 48 hours. This allowed the outgrowth of the differentiated outer layer prior to fixing and storage.

Immunofluorescence and immunocytochemistry are described in Section 2.5.2.2.

2.3.1.3.2 Taking Samples for Western Analysis

Upon reaching the end of stage 1 differentiation, monolayer wells were trypsinised as indicated in Section 2.3.1.2.2, the resultant cell pellets (in universals) were washed in PBS (X2). PBS washes were followed by centrifugation at 1000 rpm (to ensure a firm pellet). The excess PBS was allowed to drain off and the pellets were stored at –80°C until required for protein extraction and western analysis (Section 2.5.2.1). EB pellets were generated by directly centrifuging the EBs from solution as during subculture (Section 2.2.4) and then washing the resulting pellets in PBS (2X) using 1000 rpm spins. These pellets were drained and stored at –80°C until required.

2.3.1.3.3 Taking Samples for RT-PCR Analysis

Samples for RT-PCR were taken in exactly the same manner as in obtaining cell pellets for western blotting except the pellets were then dissolved in TriReagent™ (Sigma, T9424). These samples were then stored in pre-autoclaved eppendorf tubes (Eppendorf, 0030 121 023) at –80°C until required for RNA isolation (Section 2.5.1.2.1).

2.3.2 Miniaturised *In Vitro* Toxicity Assays

In vitro toxicity assays were carried out to determine the lethal ranges of antibiotics prior to transfection experiments and to determine the effectiveness of transfected constructs (e.g. Section 3.3.1.2).

2.3.2.1 Assay Procedure

Cells in the exponential phase of growth were trypsinised as outlined in Section 2.2.3 and were then seeded in to 96 well plates (Costar, 3599). The seeding density reflected the growth rate of the cell line being used i.e. cell lines with high growth rates were seeded at lower densities (Table 2.3.2). The seeding density was chosen to allow a healthy growing cell line to achieve 80-90% confluence in 7 days. When carrying out toxicity assays on F9 cells, the plates needed to be coated with 0.1% gelatin prior to use. This was carried out by adding 50 µl 0.1%gelatin/well for 2 hours at 4°C followed by 3X UHP rinses (100 µl/well/rinse). All rinsing and seeding work with 96 well plates was carried out using a multichanell pipette.

Cell Line	Seeding Density For 96 Well Plates
BHK-21	5×10^2/well
F9	5×10^2/well
Min6	5×10^3/well-1×10^4/well

Table 2.3.2 96 well plate seeding densities for *in vitro* toxicity assays.

The cells were seeded to the wells and mixed gently to give an even spread of cells and to prevent clumping. The plates were incubated overnight at 37°C to allow the cells to attach. The following day the culture medium was removed and 100 µl aliquots of antibiotic containing medium was added to each well. The antibiotic concentrations were arranged in an ascending fashion and untreated cells were included as controls. The plate was laid out so that each concentration of antibiotic corresponded to an entire row (i.e. the plate is arranged in an 8 x 12 grid) therefore each concentration of the antibiotic was exposed to 8 individual wells (i.e. n=8 for each concentration point used). After 6 days culture (or when the control wells were

80% confluent) the effects of the antibiotic were calculated using the acid phosphatase assay (Section 2.3.2.2).

2.3.2.2 Assessment of Cell Number via Acid Phosphatse Assay

Following the *in vitro* toxicity assay the media was removed from the plates and the plates were rinsed 3X in PBS (100 µl/well). Upon removal of the PBS the cells were incubated (100 µl/well) in freshly prepared acid phosphatase substrate (10 mM *p*-nitrophenol phosphate (Sigma 104-0) in 0.1 M sodium acetate (Sigma, S8625), 0.1% triton X-100 (BDH, 30632), pH 5.5). This incubation was carried out for 2 hours at 37^0C and the plates were wrapped in foil due to the light sensitive nature of the *p*-nitrophenol phosphate. The reaction was stopped by addition of 50 µl 1M NaOH to each well. The plates were read at 405 nM using a Spectramax plus plate-reader (Molecular Devices) and the data was processed using specialised software (SoftMax Pro).

2.3.3 Measurement of Cellular Proliferation

2.3.3.1 Proliferation Assay Experimental Protocol

Min6 cells were seeded at a density of 5 x 10^4 cells/well in 24 well plates (Costar, 3524). The cells were allowed to attach overnight at 37°C in a 5% CO_2 incubator. On each day following initial seeding, the cells were trypsinised (Section 2.2.3.1) and counted (Section 2.2.5). Each well was counted on both counting chambers of a Hemocytometer and three wells were counted each day. Thus n=6 for the count per day. The proliferation assay was carried out for a total of 7 days (168 hours).

2.3.4 Transfection of Mammalian Cell Lines

2.3.4.1 Optimisation of Transfection

Prior to any transfection experiments, the transfection protocol was optimised for the plasmid, cell line and the transfection agent available. This was done using a reporter plasmid, pCH110 (Invitrogen), which encodes β galactosidase activity.

Cells were seeded to wells at different concentrations and allowed to attach and initiate growth overnight. The following day, the cells were transfected with pCH110, using different amounts of plasmid (1-2 μg) and transfection agent. The cells were generally transfected in the presence of serum for 24-48 hours. In the case of F9 cells, the differentiation-state of the cells was also altered to investigate if transfection efficiency could be further increased. Following the transfection, the cells were rinsed 2X in cold PBS and fixed for 5 min in 'fixing solution' (2% Formaldehyde, (Sigma, F8775)). This was followed by 2X PBS washes. Staining was carried out in x-gal medium (1M $MgCl_2$ (Sigma, M8266), 5 M NaCl (MERCK, K1880814), 0.5 M Hepes (Sigma, H9136), 30 mM Potassium Ferrocyanide (Sigma, P9387), 30 mM Potassium Ferricyanide (Sigma, P8131), 2% X-gal (Sigma B4252)). Positively transfected cells were identified by blue staining, thus the transfection method with the highest blue : unstained ratio was chosen for future transfection experiments.

2.3.4.2 Transient Transfection of F9 EC lines with Nuclear Transcription Factors

F9 cells were found to have very low transfection efficiency upon transfection of undifferentiated cells, thus, prior to transient transfections, cells were differentiated using stage 1 differentiation (Section 2.3.1.2.1) in the presence of RA and db cAMP. On the day of the assay the liposome transfection agent Fugene6 (Boehringer Mannheim, 1 814 443) was mixed with the DNA to be transfected in a 3:1 ratio and allowing 2 μg DNA/ well. The components were mixed in 100 μl serum free medium (SFM) per well to be transfected. The Fugene6 was added to the SFM and care was taken not to allow it to touch the sides of the eppendorf. The DNA was then added. The components were mixed by gentle tapping and allowed to sit for 25 minutes at room temperature. During this period the medium was changed on the wells to be transfected and on the control wells. After 25 minutes had elapsed 100μl of the

transfection mix/well was added to the relevant wells in a drop-wise fashion with constant swirling. The transfections were carried out over 48 hours and then the wells were sampled for immunofluorescence (Section 2.3.1.3.1) or RT-PCR (Section 2.3.1.3.3).

2.3.4.3 Generation of Stably Transfected Cell Lines

To generate stably transfected BHK-21 cells expressing PPI, glucokinase and GLUT2 (the plasmids used are described in Section 2.4.1) four flasks were set up per transfection, i.e. three for transfection and one as a control. The cells were seeded at 3 x 10^5 cells/flask and allowed to attach overnight. The transfection was carried out using the Lipofectamine Plus™ system (Gibco BRL, 10964-013). For each plasmid 2.5 μg (in 1 μl) was added to 9 μl Lipofectamine Plus solution, and this was then added to 190 μl SFM. Simultaneously 12 μl Lipofectamine was added to 188 μl SFM. These solutions were allowed to sit at room temperature for 15 min before being added to 5.6 ml complete medium (MEM). This was added to the three 25 cm^2 flasks for transfection (2ml/flask). The flasks were incubated overnight at 37°C and at 5% CO_2. The following day, the flasks were rinsed 2X in PBS to remove residual Lipofectamine reagents and were re-fed with complete medium. Antibiotic selection commenced 48 hours after transfection and the relevant cells and their controls were selected with initial concentrations of 10 μg/ml for zeocin (Invitrogen, 46-0509) and hygromycin (Roche, 843 555) and 200 μg/ml G418 (Sigma, G9516). The concentration of the selection agents were incrementally increased over time until the contents of the control flasks were all dead. The final selection concentrations reached in the BHK-21 system were 150 μg/ml (Hygromycin), 100 μg/ml (zeocin) and 1 mg/ml (G418).

2.3.4.3.1 Generation of Clonal Populations From Stably Transfected Mixed Populations

To generate clonal populations from stably transfected BHK-PPI cells, the cells were diluted and plated out in 96 well plates, such that, the probability was that one cell would be found in every third well (i.e. a plating suspension of 3.3 cells/ml). These plates were incubated overnight at 37°C and at 5% CO_2. 12 hours after plating, the

plates were examined and wells containing single cells were highlighted and monitored. Up to 20 clones were usually isolated and gradually expanded to 12 well plates, 6 well plates and eventually flasks.

2.3.5 Insulin Secretion Assays for Monolayer Cells

2.3.5.1 Constitutive Insulin Secretion Assay

To determine the constitutive secretion rate for proinsulin secreting BHK-PPI-C16 cells, 2 x 10^5 cells/well were seeded to 6 well plates and allowed to attach in complete medium (MEM) overnight. The next day, the cells were rinsed three times in PBS (pre-warmed) and then subsequently incubated in glucose-free medium for two separate 15-minute periods. The cells were then exposed to serum-free MEM containing 0.1% BSA for 0, 1, 2, 4, 8 and 24 hours of incubation. At each time point the medium was collected and stored at –20°C for later analysis (Section 2.5.2.3) and the cells were lysed in lysis buffer (20 mmol Tris pH 8.0, 2 mmol ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1 % Triton X-100, 10% glycerol, 1.5 mmol $MgCl_2$ 137 mmol NaCl, 1 mmol, Na_3VO_4, 1X protease inhibitor cocktail (Boehringer Mannheim 169 7498), 5% BSA). These lysates were also stored at –20 °C for insulin analysis (Section 2.5.2.3).

2.3.5.2 Glucose Stimulated Insulin Secretion (GSIS) Analysis Using BHK-Derived Clones

For GSIS analysis insulin secreting BHK cells were seeded in to 6 well plates at a cell density of 2 x 10^5 cells/well. The cells were allowed to attach overnight and in the morning were pre-treated in the same manner as the cells used for constitutive secretion assays (Section 2.3.5.1). For GSIS, the cells were exposed to serum-free MEM supplemented with 0.1% BSA and increasing amounts of glucose (Sigma, G6152) (i.e. to generate media containing 0, 5, 10, 15 and 20 mmol/l glucose) for 2 hours. The conditioned medium was removed after the assay and either directly analysed (Section 2.5.2.3) or stored at –20 °C for later analysis.

2.3.5.3 GSIS Analysis of Cultured Min6 Cells

Due to the sensitive nature of Min6 cells and their slow growth, Min6 cells were handled differently to maximise their GSIS response. Min6 cells of passage 16-23 were used for GSIS during the course of this work.

Min6s were seeded at a cell density of 2 x 10^5 cells/well in a 24 well plate (taking care not to generate a single-cell suspension (Section 2.2.3.1)). These cells were allowed to grow for 72 hours prior to the GSIS assay. On the day of the assay 1X KRB was prepared from a aliquot of frozen 10X stock (36.525g NaCl, 2.2g KCl, 0.941g $CaCl_2.2H_2O$, 1.22g $MgCl_2.6H_2O$, 29.8g HEPES dissolved in 500 ml H_2O). BSA was added to a final concentration of 0.1%. The KRB-BSA was pH adjusted to 7.36 exactly with 1 M NaOH. This was heated to 37°C and the pH was maintained in a 37°C incubator with a 5% CO_2 atmosphere (30 min). Glucose concentrations of 0, 3.3, 10, 16.7 and 26.7 mmol/l were constituted in the conditioned 1X KRB and were then placed at 37 °C and 5% CO_2 for 30 min. After this, the conditioned medium was removed carefully from the Min6 wells and the wells were rinsed (X2) in 1X KRB. The Min6 cells were then equilibrated at 3.3 mM glucose for 30 minutes at 37°C. After equilibration, the 3.3 mM glucose was removed, and the glucose containing stimulation media were administered (1ml/well). GSIS was carried out over 60 minutes and was stopped by placing the entire plate on ice. 500 µl of conditioned medium was removed from each well and placed in an ice-cold eppendorf. These were spun at 2500 rpm for 5 minutes and 200 µl of the supernatant was taken and stored at –20°C until analysis (Section 2.5.2.3). The attached cells were lysed in 0.1 M NaOH and stored at –20°C for later protein analysis (Section 2.5.2.1.2).

2.4 Plasmid DNA Manipulation

2.4.1 Plasmids Used During the Course of this Thesis

cDNA	Plasmid	Source
β Galactosidase	PCH110	Invitrogen
Cell Trap Construct	PGK-TKCAT-Zeo/pBS	Cytomyx (Cambrige)
PDX1	PcDNA3-PDX-1(FLAG)	Y. Kajimoto (Japan)
Beta2	PCR3.1-Beta2	J. Tsai (Baylor Inst.)
Ngn3	PcDNA3-Ngn3	I. Kojima (Japan)
Nkx2.2	PBAT12.shNkx2.2	M. German (UCSF)
PPI*	PT-PPI	Cytomyx (Cambrige)
Glucokinase**	PcDNA3.1/Hyg/GCK	Cytomyx (Cambrige)
GLUT2**	PcDNA3.1/Zeo/GLUT2	Cytomyx (Cambrige)

* The human PPI cDNA in pBMGneo-hInsulin was kindly donated by Prof. H. Nakauchi (University of Tsukuba, Japan).

** Glucokinase and GLUT2 supplied as phIGLK2 and P-7-GLUT2 by Prof. M. A. Permutt (Washington School of Medicine, St Louis, MO).

Upon receipt of plasmids bacteria were transformed with the plasmid to create 'Mini Preps' of the plasmid for analysis, upon confirmation of plasmid integrity further glycerol bacterial stocks were prepared and 'Maxi Preps' of plasmid DNA were prepared.

2.4.2 Transformation of Bacteria

100 µl of competent JM109 bacterial cell suspension (Promega, L2001) was mixed with 1-50 ng DNA (in a volume not greater than 10 µl) in a pre-chilled eppendorf and mixed by flicking. The tubes were placed on ice for 10 minutes. Following this, the tubes were heat shocked at exactly 42°C for 50 seconds after which they were returned to the ice for 2 minutes. 900 µl of cold LB medium (10g Tryptone (Oxoid, L42), 5g Yeast Extract (Oxoid, L21), 10g NaCl (Merck, K1880814), made up to 1 L with UHP, pH adjusted to 8.0 with NaOH and autoclaved) was added to the JM109 cell suspension. The JM109 cells were then incubated at 37°C for 60 min with shaking

(~ 225 rpm). Following this, the cell suspension was diluted 1/10 and 1/100 and all three were plated out on LB agar plates (4 g Tryptone, 2g Yeast Extract, 2g NaCl, 6 g Agar (Sigma, A5054) mixed in 400 ml UHP and pH adjusted to 7.5. The agar was then autoclaved. To pour the plates, the agar was melted gently in an autoclave) containing 100 μg/ml Ampicillin (Sigma, A9393) (Ampicillin was added to the molten agar once it had cooled to ≤ 55°C). These plates were incubated overnight at 37°C. Single colonies were isolated the next day and streaked on fresh LB-Agar plates.

2.4.3 DNA Miniprep of Plasmids

Single colonies were isolated from LB/Amp plates and incubated in universals containing 5 mls LB/Amp. These were shaken at 180-225 rpm for 16-24 hours. Following this, 1.5 ml was taken from each tube, placed in an eppendorf and centrifuged at 8500 rpm on a bench-top microfuge. The supernatant was decanted and another 1.5 ml of culture was added and spun down as before. The samples were mini-prepped using the Stratagene Clearcut™ system (Stratagene, 400732). The cell preps were re-suspended in 105 μl solution 1. To this, 125 μl solution 2 was added and the tube was mixed by inversion. Finally, 125 μl solution 3 was added and mixed by inversion, the eppendorf was then placed on ice for 5 minutes. The tubes were then spun at 8500 rpm for 5 minutes and the supernatants were carefully removed. Also supplied with the kit was a specific DNA binding resin that was re-suspended by vortexing. 15 μl of this resin was added to the supernatant from the previous step and mixed-in by inversion. The resin-supernatant mixes were loaded on to the provided 'spin cups' and centrifuged at 13,000 for 30 s. These cups allowed the resin and its bound DNA to be preferentially retained. 400 μl of the supplied wash buffer (generated by diluting a concentrate solution 1:2 with 100% EtOH) was then passed through the spin cup by centrifuging at 13,000 for 30 s (this step was carried out twice). The DNA was eluted by adding 50 μl UHP to the spin cup and placing it in a clean, autoclaved eppendorf which was then spun at 13,000 for 30 s. The eluted DNA could be stored at –20 until required.

2.4.3.1 Quantification of DNA Using a UV Spectrophotometer

The plasmid DNA was quantified using a UV spectrophotometer (Molecular Devices) at 260 nm. By simultaneous measurement of the OD at 280 nm the relative purity of the sample could be ascertained. The amount of DNA was calculated from the following formula:

$$OD_{260} \times 50 \times \text{Dilution factor}/1000 = \text{DNA amount } (\mu g/\mu l)$$

The relative purity could be calculated using:

$$\text{Purity} = OD_{260}/OD_{280}$$

Purity values of 1.8 – 2 were considered suitable for transfection and PCR purposes.

2.4.4 Restriction Enzyme Digestion of Plasmid DNA

All plasmids isolated were electrophoresed on 1% agarose gels (Section 2.5.1.5.3) to check for degradation, upon confirmation of undegraded plasmid DNA, the plasmids were digested using restriction enzymes (REs) to confirm the plasmid contained the correct fragments in the correct orientation. The standard RE digestion mix is outlined below:

Component	**Volume per Reaction (µl)**
H_2O (UHP)	11
RE Buffer	2
BSA (1 mg/ml)	2
DNA (0.5 µg/µl)	1
RE # 1	2
RE # 2	2
Total Volume	20 µl.

Specific REs are more efficient in particular RE buffers, in cases where two REs were optimised for different buffers, a compromise was made to maximise cutting efficiency for both. This information can be obtained in the Promega Catalogue.

The digests were carried out at 37°C in a water bath for 1 hour.

Digests were examined by the addition of 2 µl 6X electrophoresis loading buffer (Section 2.5.1.5.3) to 10 µl of the digestion mix and electrophoresed on 1% agarose gels with an appropriate size marker. The banding pattern was then examined for the expected fragments.

2.4.5 Large Scale Plasmid Preparation

A single colony of transformed bacteria (Section 2.4.2) was inoculated in to 1 ml of LB broth containing 100 µg/ml Ampicillin. This was allowed to grow for at least 8 hours at 37°C with shaking (~225 rpm). After 8 hours the 1 ml inoculum was transferred under sterile conditions to 400 ml (LB-Amp) and grown overnight at 37°C with shaking. This culture was then split between two centrifuge buckets and spun at 6,000 x g in a Beckman centrifuge (JA-14 rotor) for 15 minutes at 4°C. For purification of the plasmid DNA from these transformed bacterial pellets, The Quiagen Maxi (100) kit was used (Quiagen, 12165). The pellets were re-suspended in 10 ml buffer P1 (ensuring that RNase A solution (provided) had been added to a final concentration of 100 µg/ml) until no cell clumps remained (this was to ensure efficient lysis). Once the pellets had been re-suspended, 10 ml of buffer P2 was added to each re-suspended pellet, and gently mixed by inversion, these solutions were allowed to incubate at room temperature for 5 minutes. 10 ml of buffer P3 was then added and immediately mixed by inversion (gently). The resultant solutions were centrifuged at 20,000 x g for 30 min at 4 °C (JA-14 rotor) and the plasmid containing supernatant was immediately removed to pre-washed, autoclaved, polypropylene tubes. The supernatant was mixed gently and immediately re-centrifuged at 20,000 x g at 4°C for 15 minutes (JA-17 rotor). The DNA extraction columns (Quiagen-tip 500) to be used were equilibrated during this step. This was done by adding 10 ml of buffer QBT to the tip and allowing it to drain through via gravity. Once the tip was pre-equliabrated, the re-centrifuged supernatant was loaded on to the tip and drained

by gravity. Once the supernatant had passed completely through the Quiagen tip it was washed twice in the provided wash buffer (QC). The DNA was finally eluted using 15 ml buffer QF per tip. Isolation of the DNA was carried out by adding 10.5 ml room temp isopropanol to the eluted DNA/QF-containing solution with mixing. The precipitated DNA was isolated by centrifuging the tubes at 15,000 x g for 30 minutes at 4 °C. The DNA pellet was washed (X2) using 5 ml 100% EtOH followed by spins of 15,000 x g for 10 minutes. The washed DNA pellet was air-dried for 5-10 min and finally dissolved in 100-200 µl TE buffer (10mM Tris, 1 mM EDTA). The DNA was quantified as described (Section 2.4.3.1) and run out on a 1% agarose gel to confirm plasmid integrity and size (Section 2.5.1.5.3).

2.5 Analytical Techniques and Assays

2.5.1 RNA Analysis

2.5.1.1 Preparation for RNA Analysis

RNA is easily degraded by RNase enzymes which are ubiquitous, thus the following precautions were taken prior to RNA work.

All solutions for RNA related work, i.e. that would come in to contact with the RNA were prepared from sterile UHP that had been treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma, D5758) before autoclaving. Solutions for RNA work were made in bottles that had been baked at 180°C for 8 hours or more. All eppendorfs PCR tubes used etc. were RNase free and pre-autoclaved prior to use as were Gilson pipette tips. Disposable nitrile gloves were worn at all times during RNA work (to protect the operator and to prevent RNase degradation). The gloves were changed frequently during RNA manipulation. For a full review of the precautions to be taken while working with RNA then it is advised to refer to O' Driscoll *et al.* (1993).

2.5.1.2 RNA Isolation

2.5.1.2.1 RNA Isolation using TriReagent

RNA was extracted from cultured cells, cells following differentiation treatment and transfected cells from a variety of sources including flasks, plates and petri dishes. In all cases the cells were initially trypsinised (Section 2.2.3) to pellet form, lysed in TriReagent (Section 2.3.1.3.3) and stored at –80 °C until required.

The following protocol outlines the method whereby pure RNA was isolated from TriReagent. The frozen TriReagent samples were allowed to thaw at room temperature and upon thawing, were allowed to sit for at least 5 minutes to ensure complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per ml of TriReagent in the sample. This was shaken vigorously for 15 s and the samples were allowed to stand for 15 minutes at room temperature. The sample was

then centrifuged at 13,000 rpm for 15 minutes at 4 °C. Following centrifugation, the sample separated in to three layers, the upper aqueous layer containing the RNA, the interphase (DNA) and the red layer containing the protein. The upper layer was thus carefully removed to a fresh eppendorf and 0.5 ml of isopropanol (Fluka, 59304) was added to the aqueous RNA solution per ml of TriReagent used initially. The tubes were mixed well and allowed to stand at room temperature (15-20 minutes). This effected the precipitation of the RNA, which was then recovered by centrifugation at 13,000 rpm for 10 min. The RNA was washed (2X) in 75% EtOH and air dried for 5-10 minutes. The recovered RNA pellet was then dissolved in 12-20 µl DEPC treated H_2O with repeated pipetting. To aid re-suspension of the RNA, the sample was also heated to 55°C for 10 minutes followed by cooling on ice. RNA was aliquoted and stored at –80°C until required.

2.5.1.2.2 RNA Isolation for DNA Array Analysis

2.5.1.2.2.1 RNA Isolation Using RNeasy Spin Columns

For reproducible results using DNA arrays the initial RNA had to be of extremely high quality, thus the RNeasy (Quiagen 74104) system was used. Cell pellets for RNA extraction (pellets had been flash frozen in liquid nitrogen upon harvesting and stored at –80°C.) were re-suspended in 1.2 ml of buffer RLT (which was supplemented with 10 µl/ml of β mercaptoethanol) which was then vortexed to loosen the pellet. The lysed pellet was aspirated a number of times and vortexed until the sample was completely homogenised. 1 volume (1.2 ml) of 70% EtOH was added to the homogenised sample and mixed well by pipetting. This mixture was then loaded in 700 µl aliquots on to an RNeasy mini column which was placed in a collection tube and centrifuged at 8,000 x g for 15 seconds (i.e. this was continued until the entire mixture had been passed through the column). Once all the homogenised cells had been passed through the column, the washes were carried out. Initially 700 µl RW1 was loaded on to the column and centrifuged at 8,000 x g for 15 s. This was closely followed by 2 x washes in buffer RPE (also followed by centrifuging at 8,000 rpm for 15 s). To completely dry the spin column, it was placed in a fresh collection tube and centrifuged at full speed for 1 minute. The RNA was eluted by passing two lots of 25 µl RNase free water (supplied) through the column by centrifuging it at 8,000 rpm for

1 minute. The eluted RNA was then quantified (Section 2.5.1.3) and carried on for DNase I treatment.

2.5.1.2.2.2 DNase I Treatment of RNA for DNA Array Analysis

Any DNA isolated with the RNA during the extraction procedure would affect the results in DNA array analysis thus the samples were treated with DNase I to remove any contaminating DNA. The DNase I used is characterised as being RNase free (Promega, M6101). The typical DNase I reaction components are listed below:

RNA (100 µg)	X µl
10X DNase I Buffer (supplied)	20 µl
DNase I	10 µl
H_2O	(170-X) µl
Final Volume	200 µl

The samples undergoing DNase I digestion were incubated for 30 minutes at 37 °C. The reaction was terminated by the addition of 20 µl Termination mix. The DNase I was inactivated by heating the reaction mix for 10 minutes at 65 °C.

The RNA was re-purified from the DNase I digestion mix using RNeasy spin columns again, in brief, 100 µl of sample was mixed with 350 µl RLT buffer (including 10 µl/ml β mercaptoethanol). 250 µl 100% EtOH was then added and mixed by pipetting. The sample was applied to an RNeasy spin column and centrifuged at 8,000 x g for 15 s. The collection tube and the flow-through were discarded at this point. The spin column was transferred to a new collection tube and washed twice in buffer RPE. The second wash was eluted by centrifuging at 8,000 x g for 2 minutes. To ensure that all the RPE was eluted, the column was placed in a fresh collection tube and centrifuged at full speed for 1 minute. The purified RNA was eluted by adding 25 µl RNase-free H_2O to the spin column and centrifuging it in to a fresh collection tube for 1 minute at 8,000 rpm. This step was repeated once more to ensure all the RNA was isolated. The RNA was quantified (Section 2.5.1.3) and electrophoresed on an

agarose gel to determine that it was of sufficient quality for array analysis (Section 2.5.1.4).

2.5.1.2.2.3 Purification of Poly $(A)^+$ RNA from Total RNA

Poly A purification was carried out using the Atlas™ Pure Total RNA Labelling System (Clontech, PT3231-1). The methods used are outlined below.

The streptavidin magnetic beads (supplied) were mixed by inverting and tapping the tube. 15 µl beads per reaction were transferred to a new tube. These beads were separated from their supernatant using a magnetic particle separator (Clontech) and were washed 4 times with binding buffer 1 (after each wash the beads were separated using the magnetic separator). The beads were eventually re-suspended in 15 µl 1X binding buffer. 45 µg of total RNA was placed in a sterile eppendorf tube (if the volume was found to be less than 45 µl, deionized water was added to a final volume of 45 µl). 1 µl of biotinylated oligo(dT) was added and mixed thoroughly by pipetting. These samples were then placed at 70 °C in a preheated thermal cycler. Following this, the tube was cooled at room temperature for 10 minutes. Once the sample was cool, 45 µl 2X binding buffer was added and mixed by pipetting. The washed beads were re-suspended and 15 µl was added to the RNA sample. The beads were mixed continuously through the RNA sample for 25-30 minutes on a vortex shaker set to 1,500 rpm. The beads were isolated using the magnetic separator and washed twice in 50 µl 1X wash buffer and then re-suspended in 1X reaction buffer. The beads were magnetically isolated once more and re-suspended in 3 µl H_2O. The poly $(A)^+$ RNA was then taken directly to the probe synthesis reaction for DNA arrays (Section 2.5.1.6.1).

2.5.1.3 RNA Quantification

RNA (like DNA) was quantified using a UV spectrophotometer. The OD_{260} was used to quantify the RNA in the sample using the following equation:

$$OD_{260} \times 40 \times \text{Dilution Factor}/1000 = \text{RNA content } (\mu g/\mu l)$$

By simultaneously measuring the OD_{280} the purity of the sample could be estimated.

$$\text{Purity} = OD_{260}/OD_{280}$$

This was typically in the range of 1.8-2.0. A ratio of <1.6 indicated that the RNA may not be fully in solution. The RNA was diluted to 1 μg/μl stocks for reverse transcription (RT-reaction).

2.5.1.4 Gel Electrophoresis of RNA to Determine Quality

All solutions to be used for the gel electrophoresis (covered extensively in Section 2.5.1.5.3) of RNA were prepared in DEPC treated water (Section 2.5.1.1) including the electrophoresis buffer and the gels. The samples (containing approximately 5 μg RNA/well) were run on 0.8% agarose gels. To sink the RNA in to the wells, pre-autoclaved glycerol was mixed with the RNA to a final concentration of 10% (v/v).

2.5.1.5 Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis

2.5.1.5.1 Reverse Transcription of RNA (cDNA Synthesis)

To form cDNA the following components were mixed in a 0.5 ml eppendorf tube (Eppendorf, 0030 121 023) and heated to 70 °C for 10 minutes followed by cooling on ice.

1 µl Oligo dT 12-18 primers (0.5 µg/µl)

1 µl RNA (1 µg/µl)

3 µl DEPC H_2O

This step gets rid or RNA secondary structure and allows the oligo dT to bind the poly $(A)^+$ tail of the RNA.

As this mixture was heating, the following reaction mix was generated (all volumes listed in master mix assume 1 µg total RNA).

4 µl 5X buffer (Sigma, P2317)

2 µl 100 mM DTT (Sigma, D6059)

1 µl RNasin (40 U/µl) (Sigma, R2520)

1 µl dNTPs (10 mM each) (Sigma, DNTP-100)

6 µl DEPC H_2O

1 µl MMLV-RT (200U/µl) (Sigma, M1302)

Once the RNA mixture had cooled (~2 minutes) 15 µl of the master mix was added and mixed by flicking. The resultant mixture was given a rapid centrifuge to collect the material in the bottom of the tube and then incubated at 37°C for 1 hour. The resultant cDNA was stable at 4°C but for prolonged storage was maintained at –20°C.

2.5.1.5.2 Polymerase Chain Reaction (PCR)

The cDNA was then analysed for the expression of genes of interest by PCR.

The standardised PCR mix is listed below and did not change significantly with any of the PCRs carried out in this thesis.

2.5 µl cDNA was added to the following reaction components.

12.25 µl H_2O

2.5 µl 10X PCR buffer (Sigma, P2317)

1.5 µl 25 mM $MgCl_2$ (Sigma, M8787)

4 µl 1.25 mM dNTP

0.5 µl each of the forward and reverse primers (250 ng/µl) for the target gene (Oswel DNA Service, Southhampton)

0.5 µl each of the forward and reverse primers to the housekeeping gene of interest i.e. β Actin (25 ng/µl) or GAPDH (25 ng/µl), (also synthesised by Oswel DNA Service).

0.25 µl Taq Polymerase (5U/µl) (Sigma, D4545).

The samples were mixed and centrifuged before being placed on the thermocycler (Biometra).

A typical PCR protocol is outlined below however annealing temperatures can vary from primer set to primer set therefore a full list of the primers used in this thesis and the annealing temperatures are listed in Table 2.5.1

95 °C for 3 minutes (Denaturation step)

25-30 cycles of :

95 °C for 30 s (Denaturation)

52-60 °C for 30 s (Annealing)

72 °C for 30 s (Extension)

and

72 °C for 7 minutes (Extension)

PCR products were stored at 4 °C until they were analysed by gel electrophoresis.

2.5.1.5.3 Gel Electrophoresis of PCR Products

Typically 2% agarose (Sigma, A9539) gels were used for PCR gel electrophoresis, these gels were prepared and run in 1X TBE (10.8g Tris base, 5.5 g Boric Acid, 4 ml 0.5M EDTA and made up to 1L with UHP) and were melted in a laboratory microwave. Upon cooling, the gel was supplemented with 5 µl ethidium bromide (10 mg/ml) (to allow visualisation of the DNA. The gel was then poured in to the electrophoresis unit (Biorad) and allowed to set. By placing a comb in to the top of the gel prior to hardening, sample wells were formed.

To run the samples, 2 µl of 6X loading buffer (50% Glycerol, 1 mg/ml bromophenol blue, 1 mM EDTA) was added to 10 µl PCR product and loaded to the gel with an appropriate size marker (Sigma, D0672). The gels were electrophoresed at 120-150 mV for 1-2 hours (depending on size of the target gene, i.e. to get adequate separation). Once the internal control and target bands were seen to have migrated to the required extent, then the gel was taken to the gel analyzer (an EpiChemi II Darkroom, UVP Laboratory Products), photographed and densitometrically analysed using Labworks software (UVP).

Gene	Sequences (all sequences listed 5' to 3')	Annealing Temp (°C)	Fragment size (bp)
PPI	agcgtggcttcttctacacacc ggtgcagcactgatccacaatg	54	158
PDX1	ggtggagctggcagtgatgtt accgcccccactcggggtcccgc	60	125
HGLUT2 (Human)	tggcagctgctcaactaatcac aaacaggtttgctgataccagc	52	759
MGLUT2 (Mouse)	cacactctctgaagacgccagga gaggcatcgactgagcagaaggtc	52	729
GCK	gatgctggatgacagagccaggatg agatgcactcagagatgtagtcga	54	392
Pax4	tggcttcctgtccttctgtgagg tccaagacacctgtgcggtagtag	52	242
Pax6	aagagtggcgactccagaagt accatacctgtattcttgcttcagg	60	545
Ihh	aaggcccacgtgcattgctct tgtccgcaatgaagagcaggtg	60	298
Shh	gctgatgactcagaggtgcaa agtcgaaacctgcttccacag	60	227
PP	aggatggccgtcgcatactg gagctgcactccaggaagtc	60	249
Somatostatin	ccgtcagtttctgcagaagt cagggtcaagttgagcatcg	60	356
Glucagon	actcacagggcacattcacc ccagttgatgaagtccctgg	60	353
Ngn3	tggcgcctcatcccttggatg cagtcacccacttctgcttcg	54	160
Isl1	acgtctgatttccctgtgtgttgg tcgatgtggtacaccttagagcgg	60	276
Nkx2.2	cggagaaaggtatggaggtgac ctgggcgttgtactgcatgtgctg	60	188
Beta2	cttggccaagaactacatctgg ggagtagggatgcaccgggaa	60	229
β Actin (Long)	gaaatcgtgcgtgacattaaggagaagct tcaggaggagcaatgatcttga	Variable*	383
β Actin (Short)	tggacatccgcaaagacctgtac tcaggaggagcaatgatcttga	Variable*	142
GAPDH	catgaccacagtccatgccatc caccctgttgctgtagccgtattc	Variable*	451

Table 2.5.1 Primer sequences used during the course of this work.

* These internal standard primers were used at a range of temperatures from 52-60°C depending on the target gene being examined.

2.5.1.6 DNA Array Analysis of RNA Expression

2.5.1.6.1 cDNA Probe Synthesis

For cDNA probe synthesis for DNA arrays highly purified poly $(A)^+$ RNA was used (Section 2.5.1.2.2.3).

As the arrays involved the comparison of two different RNAs, the probes were generated simultaneously to reduce variation between reactions. Thus, the following probe synthesis reaction mix is a 2.5X master mix. These components were all supplied in the Atlas™ Pure Total RNA Labelling System (Quiagen, PT3231-1).

The following labelling reaction master mix was prepared in a 0.5 ml tube at room temperature:

10 µl 5X Reaction Buffer
5 µl dNTP Mix (minus dATP)
12.5 µl [α^{33}P]dATP (>2,500 Ci/mmol, 10 µCi/µl) (Amarsham, BF1001)
1.25 DTT (100 mM)

A PCR thermal cycler was heated to 65°C. While this was heating, 4 µl CDS primer mix was added to the re-suspended poly $(A)^+$ RNA-containing beads (Section 2.5.1.2.2.3). This mixture was heated on the 65°C block for 2 minutes, when the time was up, the temperature of the block was reduced to 50 °C and the tubes were incubated for another 2 minutes. During this phase 5 µl MMLV-RT enzyme was added to the labelling master mix and mixed by pipetting. When the beads and primer mix had been at 50 °C for 2 minutes, 13.5 µl of the labelling reaction master mix was added to each reaction tube and mixed well by pipetting. This reaction was allowed to incubate for 25 minutes at 50 °C. To end the reaction, 2 µl termination mix was added. The probe was then purified using column chromatography (Section 2.5.1.6.2).

2.5.1.6.2 Purification of Radioactive cDNA Probes Using Column Chromatography

The materials for column chromatography were all included with the Atlas™ Mouse 1.2 Array (Clontech, 7853-1).

The probe synthesis reactions (Section 2.5.1.6.1) were diluted to 200 µl by addition of buffer NT2 and were mixed well by pipetting. A NucleoSpin column was placed in a 2 ml collection tube and the sample was placed on the column. This was then centrifuged at 14,000 rpm for 1 min. The collection tube and radioactive contents were discarded appropriately. The column was placed in a fresh tube and washed with buffer NT3 (diluted in EtOH) three times. Each time the wash solution was eluted by a spin of 14,000 rpm and the collection tube was changed on each occasion. The purified probe was eluted using buffer NE, which was allowed to soak in to the column for 1 minute, and then centrifuged at 14,000 for 1 minute to elute the purified probe. The probes were checked for radioactivity by taking 2 µl of probe and mixing it in to 5 ml scintillation fluid with vortexing. The counts were multiplied by a factor of 50 to account for the dilution. Probes generated from pure poly $(A)^+$ RNA typically had counts of 2-10 x 10^6 CPM.

2.5.1.6.3 Hybridization of cDNA Probes to Arrays

15 ml of hybridization solution (ExpressHyb) was pre-warmed to 68°C (in the hybridization oven), during this period 1.5 mg of sheared salmon testes DNA was heated to 95°C on a PCR thermocycler for 5 minutes followed by cooling on ice. The heat denatured salmon testes DNA was then added to the hybridization fluid and maintained at 68°C until required. The hybridization bottles were filled to the brim with UHP and the array membranes placed inside carefully. By pouring off the UHP the array remained attached to the sides of the bottle with no air bubbles. At this point 7 ml heated hybridization fluid was added to each bottle and the arrays were pre-hybridized for 30 minutes with continuous rotation at 4-7 rpm. During pre-hybridization the probe was prepared by adding 5 µl C_0t-1 DNA to the pool of each labelled probe (Section 2.5.1.6.2). The C_0t-1 DNA 'spikes' the array to generate guide spots for subsequent alignment and analysis. The probes were denatured at 100°C for

2 minutes followed by cooling for 2 minutes on ice. Upon cooling, the probes were then added to their respective hybridization bottles (they were added to the hybridization fluid and swirled, raw probe was not allowed to touch the array membrane) and hybridization was carried out overnight at 68 °C with rotation at 4-7 rpm.

The following day the arrays were washed four times in pre-warmed wash solution 1 (2X SSC, 1 % SDS) and once in pre-warmed wash solution 2 (0.1X SSC, 0.5% SDS). Washing was carried out at 68°C with continuous agitation. The array membranes were then rinsed well in 2X SSC and wrapped in cellophane (still damp, to prevent drying). The wrapped array membranes were fixed to a developing cassette and exposed to phosphoimager screens for 4-10 days at room temperature.

2.5.1.6.4 Developing the Arrays and Subsequent Analysis

After the required period, the phosphoimager screens were taken and placed on the drum of phosphoimager (Cyclone, Packard) and visualised using OptiQuant software (Cyclone, Packard). The array image could then be manipulated to minimise background and highlight the spots. The array data was processed using AtlasImage™ (Clontech) software.

2.5.1.6.5 Stripping Arrays for Re-Use

After the arrays had been analysed they were stripped for re-use (nylon arrays can be re-used 3-4 times). The arrays were stripped by boiling each membrane in 500 ml 0.5% SDS for 5-10 minutes. The solution was allowed to cool (~10 minutes) and then rinsed in wash solution 1. At this point the array membrane was checked for residual radioactivity. If the array was fully stripped then it was either re-used immediately or wrapped in cellophane and placed in storage at –20 °C until needed.

2.5.2 Protein Analysis

2.5.2.1 Western Blotting analysis

2.5.2.1.1 Sample Preparation

Cell pellets were taken from flasks of cells in culture and following differentiation experiments or transfection experiments. These pellets were usually washed X2 in PBS, dried and stored at -80°C until needed. Cell pellets were lysed in NP-40 lysis buffer (62.5 mM Tris-HCl pH 6.8, 12.5% glycerol, 2 % Nonidet P40 (Sigma, N6507), 2.5 mM PMSF (Sigma, P7626), 1.25 mM EDTA, 12.5 μg/ml leupeptin (Sigma, L2884), 116 μg/ml aprotinin (Sigma, A1153)). Lysis was carried out on ice for 30 minutes followed by sonication in a Labsonic U (Braun) unit. The cells were pulsed 3-5 times on ice using a repeating duty cycle of 0.3s. Once 60-80% of the cells were determined to be lysed (by checking a sample of lysate under a microscope), the sonication was stopped. The sonicated samples were then centrifuged at 2,500 rpm for 5 minutes on a benchtop microfuge to remove cellular debris. The supernatant was carefully removed and aliquoted to eppendorf tubes. Protein samples were stored at –20°C until required for quantification or analysis (Section 2.5.2.1.2).

2.5.2.1.2 Protein Quantification by DC Protein Assay

Protein quantification could be carried out using the DC protein assay (Biorad, 500-0116) which is based on the Lowry protein assay. To quantify the protein extracted, serial dilutions of the protein were prepared in duplicate (all sample and standard dilutions were made in lysis buffer) and a standard curve was generated using serial dilutions of a 1mg/ml BSA (Sigma, A9543) stock. 5 μl of standards and samples were loaded on into a clean 96 well plate. 25 μl of solution A (provided) was added to each well followed by 200 μl solution B. The plate was mixed gently and the colour was allowed to develop for 15 minutes. The plates were quantified using a Spectra max plus plate reader (Molecular Devices) at 750 nm and the data was processed using Soft max Pro software.

2.5.2.1.2.1 Protein Quantification by Bradford Protein Assay

The Bradford assay based Bio-Rad protein assay (Bio-Rad; 500-0006) was also used as a method for quantifying protein during the course of this thesis. A standard curve was prepared from the stock 1 mg/ml BSA using lysis buffer as the diluent (Section 2.5.2.1.2). 10 µl of each standard was then added to clean, dry eppendorf tubes. 10 µl of each sample (appropriately diluted 1/10-1/20 with lysis buffer) were also added to clean eppendorf tubes. The BioRad stock solution was pre-filtered using 3 mm filter paper (Schleicher & Schuell, 311647) and diluted 1/5 with UHP. 490 µl of diluted Biorad solution was added to each standard and sample eppendorf tube and the tubes were mixed by vortexing. 100 µl aliquots of these samples and standards were then added in triplicate to a clean, dry, 96 well plate. These plates were analysed on a Spectra max plus plate reader (Molecular Devices) at 570 nm and the data was processed using Soft max Pro software.

2.5.2.1.3 Acrylamide Gel Electrophoresis

Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS PAGE). The resolving and stacking gels were prepared as outlined in Table 2.5.2. The gels were poured in to clean 10 cm x 8 cm gel cassettes consisting of an alumina plate and a glass plate separated by 0.75cm plastic spacers. The resolving gel was poured initially, allowed to solidify, and overlayed with the stacking gel. Before the stacking gel was set, a comb was inserted to generate sample wells. Gels were generally used immediately, but could be stored at 4°C overnight if wrapped well in foil. For most applications 50 µg of protein was loaded to each well. Samples were mixed with 5X loading buffer (6.25 ml 1.25 M Tris-HCl pH 6.8, 2.5 g SDS, 14.5 ml glycerol, .025% bromophenol blue, this was made up to 50 ml with H_2O). The samples were boiled for three minutes prior to loading and were run in parallel with protein size markers (Isis, P77085). The electrophoresis conditions were 250 V and 45 mA. The gels were run for 1-1.5 hours (time depends on the size of the protein to be studied, i.e. larger proteins were run for longer).

Component	Resolving Gel (7.5%)	Resolving Gel (10%)	Stacking Gel (5%)
30 %Acrylamide Stock (Sigma, E344-500ML-C)	3.8 ml	5 ml	0.8 ml
UHP	8 ml	6.8 ml	3.6 ml
1.875 M Tris-HCl (pH 8.8)	3 ml	3 ml	-
1.25 M Tris-HCl (pH 6.8)	-	-	0.5 ml
10 % SDS	150 µl	150 µl	50 µl
10 % Ammonium Persulphate (Sigma, A1433)	60 µl	60 µl	17 µl
TEMED (Sigma, T8133)	9 µl	9 µl	6 µl

Table 2.5.2 Preparation of Acrylamide Gels

2.5.2.1.4 Western Blotting

Following electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, 192 mM Glycine (Sigma, G7126) pH 8.3-8.5 without adjustment) for 15 minutes with agitation. Protein gels were transferred to Hybond ECL nitrocellulose membrane (Amarsham, RPN 2020D) using semi-dry electroblotting (using a semi-dry transfer cell (Biorad)). For this, 8 sheets of Whatman 3 mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus. Excess air was removed from between the filters by rolling a glass pipette over the filter paper. Nitrocellulose, cut to the same size of the gel, was soaked in transfer buffer and placed over the filter paper, making sure there were no air bubbles. The acrylamide gel was placed over the nitrocellulose and 8 more sheets of pre-soaked filter paper were placed on top of the gel. Excess air was again removed by rolling the pipette over the filter paper. The proteins were transferred from the gel to the nitrocellulose at a current of 34mA at 15V for 20-25 min.

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus (Stovall, Bellydancer) to ensure even exposure of the nitrocellulose blot to all reagents.

The nitrocellulose membranes were blocked for 1-2 hours at room temperature with fresh, filtered, 5% non-fat dried milk (when probing for glucokinase 2.5% Marvel was used) (Cadburys; Marvel skimmed milk) in TBS (8.76 g NaCl, 6.09 g Tris-HCL in 1L UHP)/ 0.1% Tween (Sigma P1379) pH 7.5.

After blocking, the membranes were rinsed with TBS-tween and incubated with primary antibody (Table 2.5.3) overnight at 4^0C. The primary antibody was removed and the membranes rinsed 3 times with TBS/ 0.1% Tween. The membranes were then washed for 15 min, and then twice for 5 minutes in TBS/ Tween. Bound antibody was detected using enhanced chemiluminescence (ECL).

Following chemiluminescent detection, blots were again washed and blocked as described and re-probed for an internal standard e.g. β actin. This was carried out as before.

2.5.2.1.5 Enhanced Chemiluminescence Detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions.

Secondary antibody (diluted appropriately, Table 2.5.3) was added to the blots with shaking for 1 hour at room temperature. The secondary antibody was removed and the membranes were washed as before. A sheet of cellophane was flattened over a smooth surface, *e.g.* a glass plate, making sure all air bubbles were removed. The membrane was then placed on the cellophane, and excess fluid removed. 1.5ml of ECL detection reagent 1 and 1.5ml of reagent 2 were mixed and covered over the membrane. Charges on the cellophane ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane wrapped in cellophane. The membrane was exposed to autoradiographic film (Kodak; X-OMAT S, 500 9907) in an autoradiographic cassette for various times (depending on the level of signal). The autoradiographic film was then developed.

The exposed film was developed for 5min in developer (Kodak, LX24) diluted 1:6.5 in water. The film was briefly immersed in water and transferred to a Fixer solution (Kodak, FX-40) diluted 1:5 in water, for 5min. The film was transferred to water for 5 min and then air-dried.

2.5.2.1.6 Antibodies Used for Western Blotting

Antibody	Host	Supplier	Dilution *
β1 Integrin	Rabbit	Chemicon (AB1952)	1/500
Activin R II B	Mouse	NeoMarkers (MS-677-P0)	1/200
c-met	Goat	R & D Systems (AF527)	1/500
Acetylated Histone H3	Rabbit	Upstate Biotechnology (06-599)	1/800
GLUT2	Rabbit	Chemicon (AB1342)	1/2,500
Glucokinase	Goat	Santa Cruz (GCK N-19, SC-1980)	1/100
β actin	Mouse	Sigma (A5441)	1/10,000
α tubulin	Mouse	Sigma (T5168)	1/2,000
Mouse IgG	Sheep	Sigma (A6782)	1/1,000
Rabbit IgG	Goat	Sigma (A4914)	1/5,000
Goat IgG	Rabbit	Sigma (A5420)	1/1,000-1/5,000

Table 2.5.3 Antibodies used in for western blotting

*All antibodies were diluted in TBS.

2.5.2.2 Immunofluorescence/Immunocytochemical Analysis.

The fixation of samples for immunocytochemical and immunofluorescent analysis is already outlined in Section 2.3.1.3.1. This method was found to be compatible with all analyses carried out

2.5.2.2.1 Immunofluorescence Analysis

2.5.2.2.1.1 Basic Immunofluorescence

Basic immunofluorescence was found to be adequate for immunofluorescence for keratins and other cytoplasmic and membrane proteins. For this, frozen 6 well sample plates were removed from the –20°C freezer and allowed to equilibrate to room temperature (~10 minutes). Grease circles (DAKO pen, DAKO Cat S2002) were then drawn within the wells (to contain the solutions used in the subsequent analysis). The cells were re-hydrated using 1X TBS for 5 minutes. This was tapped off and the relevant serum (listed in Table 2.5.4) (diluted 1/5) was added as a 'blocker ' for 20 minutes at room temperature. The serum was removed at this point and the primary antibody was applied (antibodies and dilutions are listed in Table 2.5.4). This was incubated overnight at 4°C in a moist environment. The following day, the primary antibody was removed and the wells were washed three times in TBS-0.1% tween at 5 minutes per wash. The fluorescent secondary antibodies were prepared in the dark room under dim conditions and were coated in foil upon dilution with TBS (They are light sensitive). The secondary antibodies (Table 2.5.4) were incubated for 60 minutes and the plates were wrapped in foil to maintain dim conditions. All work from this point onwards was carried out in the dark to prevent 'quenching' fluorescent signal. After 60 minutes incubation the antibodies were removed and the plates were washed three times in TBS-0.1% tween. The wells were then mounted using fluorescent mounting medium (Dako, S3023) and covered with coverslips (Chance Propper, 22 x 22 mm). Fluorescent staining was visualised using a fluorescent microscope (Nikon) and the appropriate filters, i.e. for FITC labelled mouse IgG (Dako, F0261) the B2 filter was used and for TRITC labelled rabbit IgG (Dako, R0156) the G2A filter was used.

2.5.2.2.1.2 Immunofluorescence for Nuclear Transcription Factors

To visualise the nuclear transcription factors PDX1, Ngn3, Beta2 and Nkx2.2 more clearly the immunofluorescence procedure was different and is outlined below.

As before the plates were removed from storage and grease circles were drawn. In this case however the cells were pre-incubated in 50 mM NH_4Cl (Riedel de Haen, 31107) for 30 minutes. Following this the cells were incubated in 1X TBS for 5 minutes followed by blocking in blocking buffer (5% swine serum (Dako, X0901) diluted in PBS-1%BSA). Blocking was carried out for 1 hour. The primary antibodies were diluted to the required level in the dilution buffer (PBS-1%BSA+0.1% Triton X-100). These antibodies were allowed to incubate overnight at 4°C in a moist environment. The following day, the wells were washed 3X in TBS-0.1% tween and the labelled secondary antibodies were applied (again these antibodies were prepared in the dark and they were also diluted in the dilution buffer listed above). The secondary antibodies were incubated in the dark at room temperature for 1 hour and mounted etc. as described in Section 2.5.2.2.1.1.

2.5.2.2.2 Immunocytochemical Analysis

The avidin-biotin complex (ABC) immunoperoxidase technique combined with the diaminobenzidine (DAB) visualisation procedure was used in all immunocytochemistry experiments. The ABC method involves application of a biotin-labelled secondary antibody to cells probed with a primary antibody, followed by the addition of avidin-biotin-peroxidase complex which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains peroxidase molecules. The peroxidase enzyme then reacts with a DAB solution to give an insoluble, brown-coloured precipitate. The formation of this brown precipitate-coloured precipitate is indicative of primary antibody reactivity.

The procedure used is as follows:
Cell preparations on 6-well tissue culture plates (which had been previously fixed in methanol and frozen at -20^{0}C) were allowed to thaw and equilibrate at room temperature. A grease pen (DAKO, S2002) was used to encircle cells in the tissue culture plates to contain the various solutions involved during the procedure. The

cells were incubated for 5 minutes with a 3% H_2O_2 solution to quench any endogenous peroxidase activity that may be present in the cells and could lead to false positive results. The cells were then rinsed with UHP and placed in TBS for 5 minutes. The plates were incubated for 20 minutes at room temperature (RT) with an appropriate serum diluted 1:5 in TBS to block non-specific binding. This was removed and 30-50µl of optimally diluted primary antibody was administered. The tissue-culture plates were placed on a tray containing moistened tissue paper and incubated at 37^0C for 2 hours. The primary antibodies used in these studies are listed in Table 2.5.4. The wells were then rinsed in TBS/ 0.1% Tween, x3 for 5 min each, and then incubated for 30 min with a biotinylated secondary antibody diluted in TBS (Table 2.5.4). The wells were rinsed as before and incubated with strepABComplex/ Horse Radish Peroxidase (HRP) (DAKO, K377) for 30 min at RT, after which they were rinsed x3 in TBS/ 0.1% Tween. The cells were then incubated with a DAB solution (DAKO, S3000) for 7-10 min. Excess DAB solution was then rinsed off with UHP water. The wells were then mounted using a commercial mounting solution (DAKO, S3023).

Antibody	Host	Supplier	Dilution	Serum for Blocking
CK8	Mouse	Chemicon (MAB3228)	1/300	Rabbit (Dako, X092)
CK18	Mouse	Chemicon (MAB3234)	1/100	Rabbit
CK7	Mouse	Chemicon (MAB3226)	1/200	Rabbit
CK19	Mouse	Dako (M0772)	1/50	Rabbit
AFP	Rabbit	NeoMarkers (RB-315-R7)	Ready for Use	Swine (Dako, X0901)
Proinsulin	Mouse	Biogenesis (5330-3369)	1/300	Rabbit
Somatostatin	Rabbit	DAKO (A0566)	1/400	Swine
PP	Rabbit	DAKO (A0619)	1/600	Swine
PDX1	Rabbit	Gift from C.V.E. Wright Vanderbilt, TN	1/500	Swine
Beta2	Rabbit	Chemicon (AB5686)	1/25-1/50	Swine
Ngn3	Rabbit	Chemicon (AB5684)	1/25	Swine
Nkx2.2	Mouse	Developmental Hybridoma Bank (74.5A5)	Neat Serum	Swine
Anti Mouse (FITC)	Rabbit	Dako (F0261)	1/30	N/A
Anti Rabbit (TRITC)	Goat	Dako (R0156)	1/40	N/A
Anti Mouse (Biotin)	Rabbit	Dako (E0354)	1/300	N/A
Anti Rabbit (Biotin)	Goat	Dako (E0432)	1/300	N/A
Anti Goat (Biotin)	Rabbit	Dako (E0466)	1/300	N/A

Table 2.5.4 Antibodies used in Immunofluorescent/Immunocytochemical studies including the labelled secondary antibodies.

2.5.2.3 ELISA Analysis for Insulin and Proinsulin

Insulin secretion studies have already been described (Section 2.3.5). These analyses were carried out using ELISA kits i.e. a proinsulin kit (Mercodia, 10-1124-10) and a specific human insulin kit for processed insulin (Mercodia, 10-1113-10). The kits were fully inclusive and all reagents, standards etc. were supplied. The generalised protocol for their use is outlined below. Samples from insulin secretion studies were generally diluted (1/10 – 1/20 depending on the application) with PBS and mixed well by vortexing. 25 µl of the standards and samples were added directly to the test plate (in duplicate). 50 µl conjugate solution (stock conjugate diluted 1+ 10 in conjugate buffer) was then applied to the samples and standards. The plate was wrapped in foil (to prevent debris falling in to the wells) and incubated with shaking at room temperature for 2 hours on a belly dancer set to maximum speed (human insulin plates were incubated for 1 hour only). Following this, the conjugate/sample mixes were tapped off in to a sink and the wells were washed X6 using the washing solution (stock diluted 1 + 20 in UHP prior to use). Washing was carried out using a wash bottle with a wide bore nozzle and care was taken not to overflow wells during washing (to prevent cross contamination between samples). After washing, the plate was tapped firmly on tissue to remove the excess wash solution. At this point 200 µl peroxidase substrate was added to each well. This reaction was allowed to develop in the dark (the plate was re-wrapped in foil) for 15 minutes after which, it was stopped by adding 50 µl stop solution per well. The plates were mixed gently and any bubbles were burst manually. The plates were read at 450 nm on a plate reader (Labsystems Multiskan Ex) and the data was processed using Genesis™ software.

2.5.2.4 Alkaline Phosphatase Determination

Alkaline phosphatase determination was carried out on fresh cells cultured on 6 well plates or on frozen fixed samples (Section 2.3.1.3.1). Alkaline phosphatase staining was carried out by adding the assay components (16.5µl BCIP (Roche, 1383 221) (5 Bromo-4chloro-3-indolyl-phosphate, 4 toluidine salt (X-phosphate, 4 toluidine salt) and 33 µl NBT (Roche, 1383, 213) (nitro blue tetrazolium chloride)) to 5ml alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$ in 100 ml UHP). In this system, BCIP was used as a substrate for alkaline phosphatase. The 5 bromo-4

chloro-3 indoxyl formed generates an insoluble purple dye. Nitro blue tetrazolium chloride was used as the electron acceptor in the reaction rather than O_2 to enhance the colour formed.

The staining solution was applied directly to the test wells and the colour was allowed to develop. Colour development depended on the application and ranged from 30 minutes to 8 hours. Once the colour had developed the wells were washed X3 in UHP and the plates were photographed.

3.0 RESULTS.

3.1. Initial differentiation of F9 cells

F9 cells are characterised by the inability to differentiate spontaneously and thus are termed a *nullipotent* cell line (Alonso *et al.* 1991). Differentiation of F9 cells using agents such as Retinoic Acid (RA) or a combination of RA and dibutyryl cyclic adenosine monophosphate (db cAMP) is well established (Section 1.4.1.2.1). This differentiation is typically accompanied by a number of phenotypic changes as the cells assume different lineages. Monitoring the changes that occur can indicate the cell types being generated within the population and these changes may in certain cases be exploited to purify certain desirable cells from the population as a whole. In this experiment F9 cells were differentiated as monolayers and EBs in the presence of RA and db cAMP to initiate endodermal differentiation (Section 2.3.1.2.1).

3.1.1 Morphological Changes in Monolayer F9 Cells Grown in the Presence of Retinoic Acid and db cAMP

From Figure 3.1.1 the effects of RA (10^{-7} M) treatment carried out over 72 hours can be seen. RA treated F9 cells (F9-RA), (Figure 3.1.1 (C&D)) are seen to be larger than the untreated cells (Figure 3.1.1 (A&B)). The RA treated cells are separated from one another and pointed cell processes extend from the differentiated cells. Granular structures are present within the RA treated cells also (Figure 3.1.1 (D)).

Treatment of F9 cells with a combination of RA (10^{-7} M) and db cAMP (10^{-3} M) (RA/db cAMP) over 72 hours leads to the cells becoming more rounded in appearance (Figure 3.1.1 (E&F)) and some cellular processes emerge which are indicative of neural processes (Arrow Figure 3.1.1 (F)). Both RA/db cAMP and the RA treatment of the cells resulted in reduced cell numbers, suggesting that the differentiation is accompanied by reduction in the growth rate. The effect was most apparent in RA/db cAMP treated cells (F9-RAC).

These results show that, despite the short duration of the differentiation protocol (72 hours), the partially differentiated F9 cells are already displaying the morphological changes normally associated with RA and db cAMP (Darrow *et al.,* 1990; Moore *et al.,* 1986).

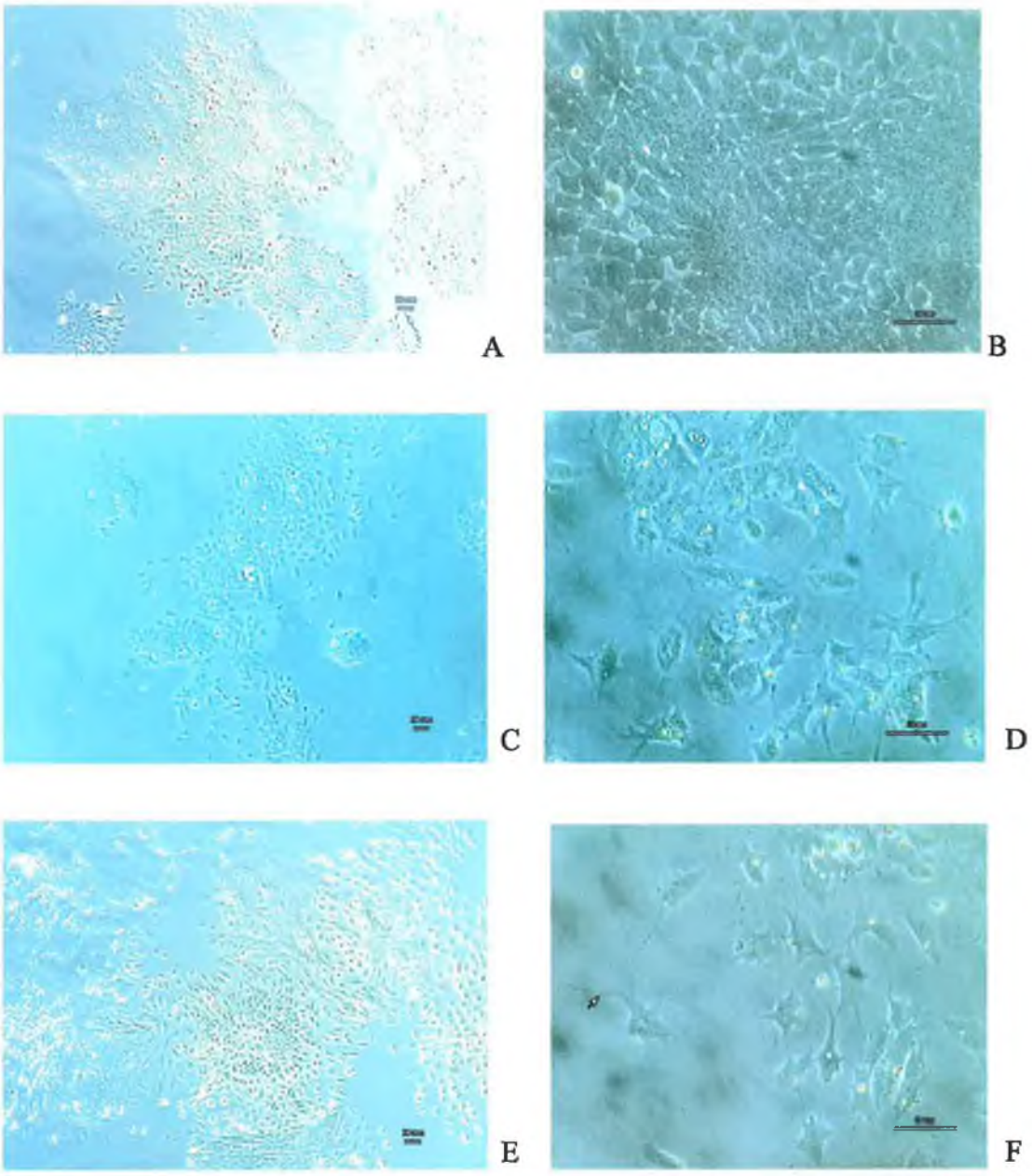


Figure 3.1.1 The morphological changes that occur upon differentiation of F9 cells (A&B) using RA (C&D) or RA/db cAMP (E&F) over 72 hours. Magnification = 10 X (A, C & E) or 40 X (B, D & F). Bar = 50 μm in all cases.

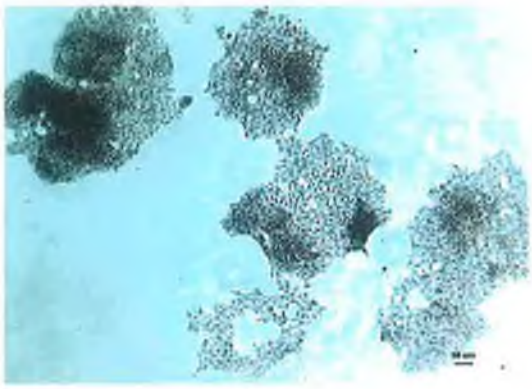
3.1.2 Investigation of the Expression of Differentiation Markers in Treated Monolayer F9 Cells

The differentiation of F9 cells is associated with changes in expression levels of a number of proteins, including tissue-type plasminogen activator and extracellular matrix proteins such as laminin and collagen type IV. For subsequent experiments it is important to be able to monitor the differentiation of the F9 cells via methods other than morphology, thus differentiation markers were chosen to visualise the development of F9 cells following 72 hours differentiation. The markers chosen were selected not only to monitor continuing differentiation, but to also provide information as to the lineages emerging within the differentiating population.

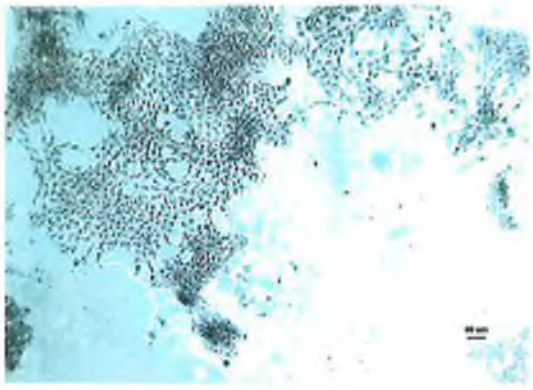
3.1.2.1 Alkaline Phosphatase Expression in Differentiating F9 cells

Alkaline phosphatase expression is a characteristic marker of stem cells and has been used as a marker to identify undifferentiated stem cells in similar applications where the differentiated progeny are of scientific interest (Lumelsky *et al.*, 2001). Reduction in the expression of alkaline phosphatase therefore is indicative of increasing differentiated state. Thus alkaline phosphatase levels were investigated in differentiating F9 cells as outlined in Section 2.5.2.4

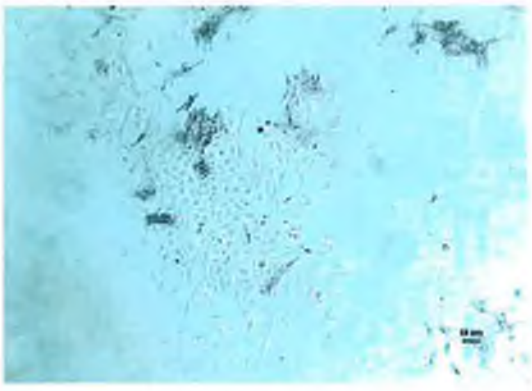
Undifferentiated F9 cells stained intensely for alkaline phosphatase (Figure 3.1.2 (A)). Following 72 h RA treatment the intensity of staining is greatly reduced (Figure 3.1.2 (B)), and is almost completely absent in the RA/db cAMP treated cells (Figure 3.1.2 (C)). Residual staining can be seen in both differentiated types and may reflect sub-populations of cells that were resistant to differentiation. These differentiation resistant cells (i.e. the stained cells within the differentiated population in Figure 3.1.2 (B)) are of similar morphology to the undifferentiated cells in Figure 3.1.2 (A) and may represent differentiation resistant cells, i.e. subpopulations of cells that are refractory to the action of RA or db cAMP.



(A)



(B)



(C)

Figure 3.1.2 Alkaline Phosphatase expression in differentiating F9 cells. Undifferentiated cells (A) show intense blue/black staining, while differentiation using RA (B) or RA/db cAMP (C) leads to a progressive loss of this staining. Magnification = 10X in all cases. Bar = 50 μm.

3.1.2.2 The Expression of Cytokeratins in Differentiating F9 cells

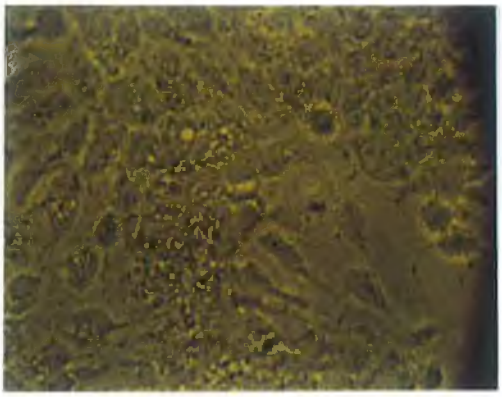
Cytokeratins are characteristically expressed in epithelial cells. The expression of cytokeratins has been used in a number of studies to monitor the differentiation state of developing cells. Exocrine acinar and endocrine cells in the pancreas has been found to contain the paired cytokeratins 8 and 18, while the less differentiated tissues of the ducts are found to contain cytokeratins 7 and 19 (Bouwens, 1998). Cytokeratins are also indicators of parietal endoderm. Immunofluorescence studies were conducted to investigate the pattern of cytokeratin expression in F9 cells following RA or RA/db cAMP mediated differentiation (ref. Sections 2.3.1.2.1 & 2.5.2.2.1.1).

Untreated F9 cells do not express any of the cytokeratins examined. Cytokeratins 8 and 18 are clearly expressed in approximately 20 % of RA treated cells (Figure 3.1.3 (B&D)). Expression of cytokeratins 7 and 19 was difficult to detect at 72 hours but these cytokeratins could be detected following 7 days of RA treatment and were expressed in 10% and 5-10% of treated cells respectively. In general, cytokeratins were found to be present in the cells demonstrating stretched morphology as opposed to the large rounded cells that are also observed following RA treatment.

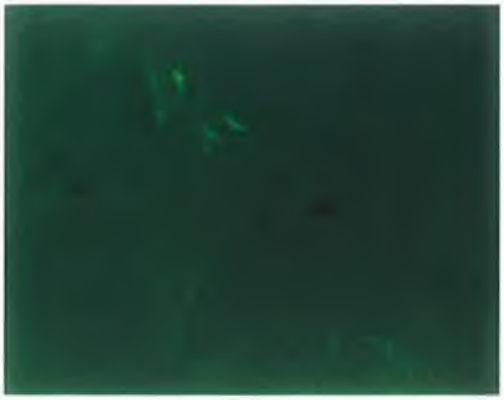
F9 cell populations treated with RA/db cAMP are also found to be positive for cytokeratins 8 and 18 (Figure 3.1.3 (F&H)). Positive cells account for up to 50 % of the entire cell population. RA/db cAMP treated F9 monolayers also contain small clumps of pointed cells that express high levels of cytokeratin 7 (Figure 3.1.3 (J)). Cytokeratin 19 expression was extremely low as measured by this method. Again, as with RA treatment, the expression of all cytokeratins studied was found to be more intense following 7 days of RA/db cAMP treatment.

These results indicate that that the partially differentiated monolayer cells can express the catalogue of cytokeratins associated with the pancreatic islets (Section 1.1.4).

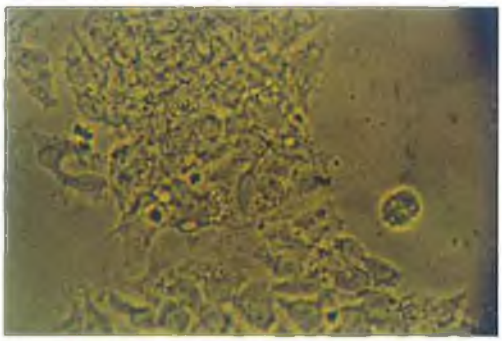
For all the immunofluorescence carried out, negative controls were included where the primary antibody was not applied. All of these controls were negative for fluorescence thus demonstrating that the fluorescence displayed is not due to non-specific binding of the labelled secondary antibody.



A



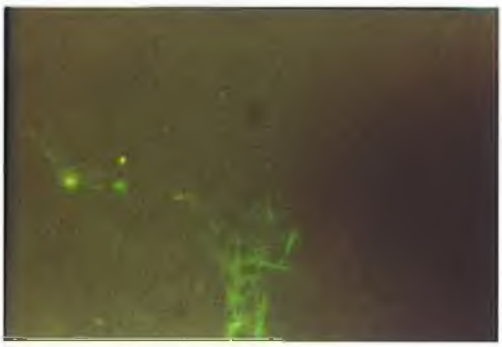
B



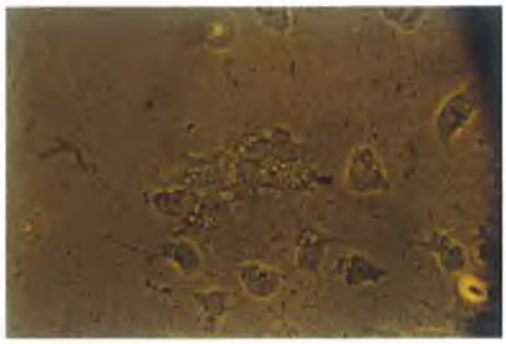
C

D

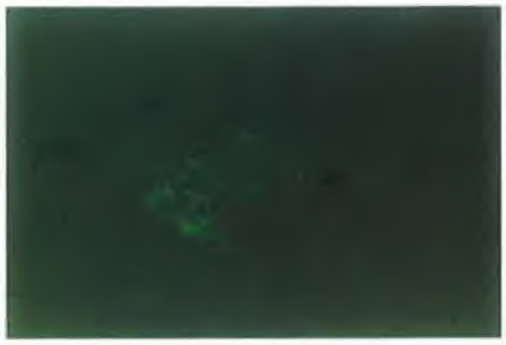
E



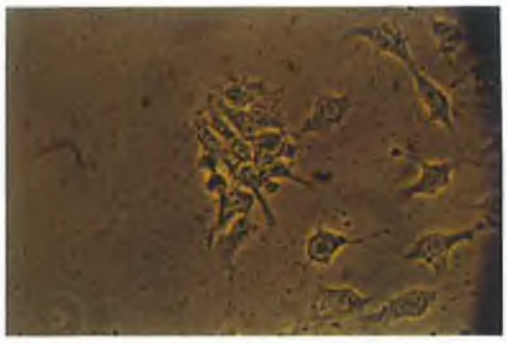
F



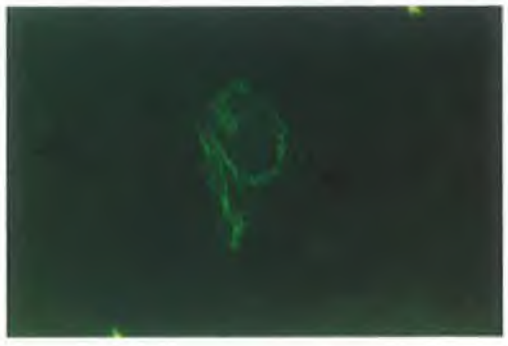
G



H



I



J

Figure 3.1.3 Cytokeratin expression profiles of F9 cells differentiated in the presence of RA or RA/db cAMP over 72 hours. The figures presented indicate the phase contrast view of the labelled cells (A, C, E, G & I) and the corresponding fluorescent image depicting cytokeratin expression (B, D, F, H & J). RA treated cells (A-D) expressed CK8 (B) and CK18 (D), while RA/db cAMP treated cells (E-J) were found to be positive for CK8 (F), CK18 (H) and CK7 (J). Magnification = 40X.

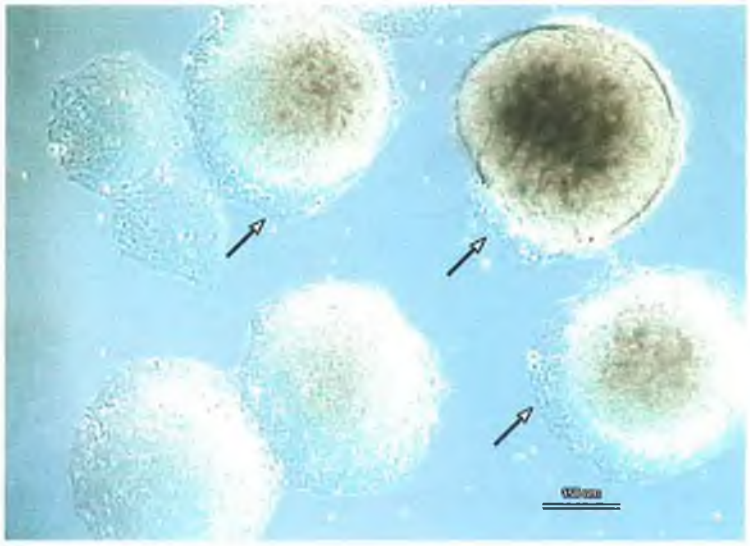
3.1.3 Morphological Changes during the Culture of F9 Embryoid Bodies in the Presence of RA (10^{-7} M)

Embryoid bodies (EBs) were generated by seeding $1x10^5$ cells/ml in mini bacteriological grade petri dishes (to a final volume of 2 ml), and incubating overnight (Section 2.2.4). EBs cultured for 72 hours in normal DMEM were generally spherical with a smooth outer layer of undifferentiated cells. Embryoid bodies cultured in the presence of RA (10^{-7} M) also displayed a general spherical shape, although distorted spheres were found which had irregular outer layers of protruding (differentiated) cells.

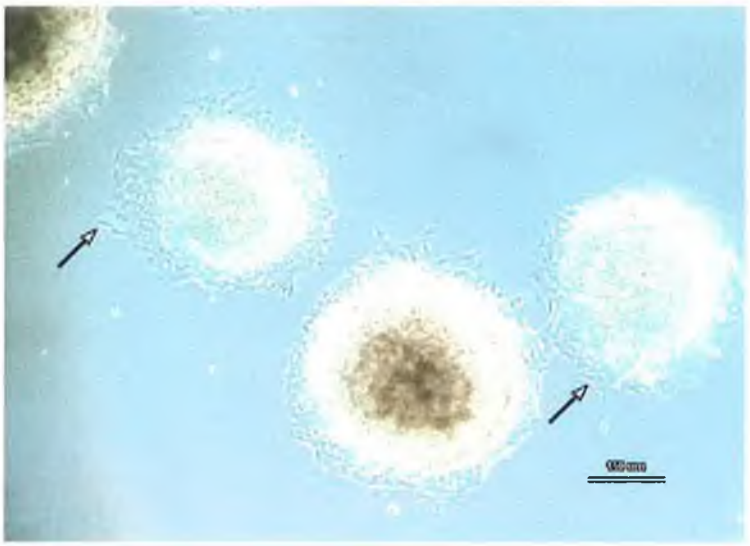
The embryoid bodies shown in Figure 3.1.4 were plated onto gelatin coated plates for 48 hours. This resulted in the outgrowth of the outer layer of the EB onto the gelatin, forming a 'halo' around the anchored EB. Undifferentiated embryoid bodies retained their spherical shape and the cells that were observed growing out from the bodies resembled the undifferentiated F9 cells as seen in Figure 3.1.1 (A). These cells also demonstrate similar growth patterns to those seen in undifferentiated F9 cells, i.e. the cells grow in tight clusters within the halo.

The cells growing out from the differentiated EBs were larger than the 'halo' cells from undifferentiated EBs and were similar in morphology to the differentiated cells presented in Figure 3.1.1 (C). These cells were also seen to be more distinct from one another i.e., the cells were spread out over a larger area than in the undifferentiated case.

The undifferentiated embryoid bodies were found to have an average diameter of 332.23 μM whereas the differentiated bodies had an average diameter of 244.27 μM, which presumably reflects the reduction in growth rate found to be associated with increased differentiation state.



(A)



(B)

Figure 3.1.4 Morphological changes in embryoid bodies associated with differentiation in the presence of RA. Untreated EBs (A) are more spherical and are surrounded by a halo of tightly compacted cells (indicated by arrows). RA treated EBs (B) however, are distorted spheres and the component cells of the halo are elongated and stretched (indicated by arrows). Magnification = 10X Bar = 150 μm.

3.1.4 Investigation of the Expression of Differentiation Markers in F9 Embryoid Bodies

EB differentiation is unique as within it are 3-D cell-cell contacts present that are absent in cell monolayers, thus EBs have been compared to early (5-6 day) embryonic masses. The expression of differentiation markers in EBs following RA differentiation was investigated to determine the possible lineages that were evolving.

3.1.4.1 Alkaline Phosphatase Expression in Differentiating F9 cells

From Figure 3.1.5 (A&B) it was observed that the central mass of the EBs stained intensely for alkaline phosphatase. The halo of cells surrounding the undifferentiated EB also stained for the enzyme. The cells within the halo growing out from the EB treated with RA showed little or no staining and displayed the elongated morphology typically observed following treatment of F9 cells with RA (indicated by arrows in Figure 3.1.5 (B)).

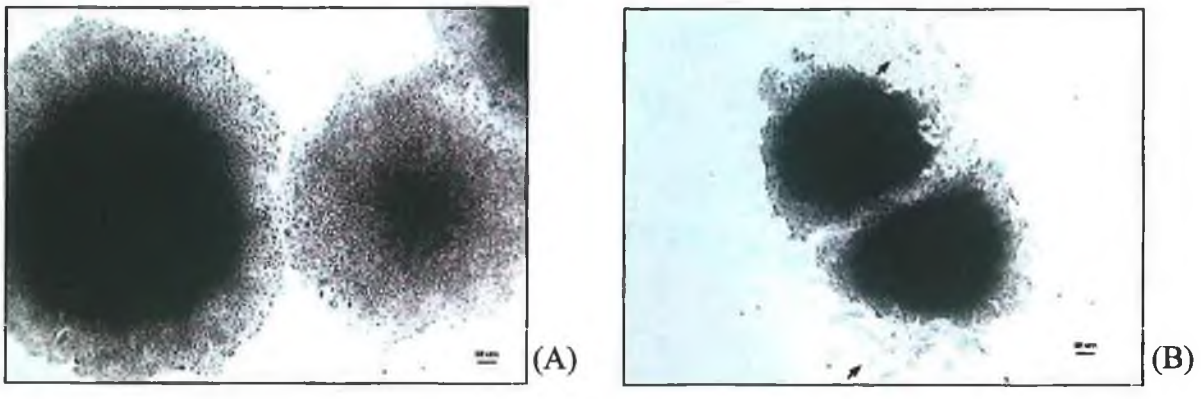

(A) (B)

Figure 3.1.5 Alkaline phosphatase activity during embryoid body differentiation. (A) Untreated EBs grown for 72 h in DMEM and cultured for 48 h on gelatin to promote halo formation. Following this the cells were fixed and stained. (B) EBs cultured in the presence of RA for 72 hours prior to plating on gelatin, fixing and staining. Magnification = 10X. Bar = 50 μm.

3.1.4.2 Cytokeratin Expression in Differentiating Embryoid Bodies

The paired cytokeratins 8 and 18 were expressed in the cells growing out from both differentiated and undifferentiated EBs (Figure 3.1.6 (B&D) & Figure 3.1.7 (B&D)). The staining in the undifferentiated EBs was of lesser intensity than that in the RA treated EBs and far less widespread. Staining for cytokeratins in RA treated EBs was predominantly observed in the 'halo' surrounding the EB and the isolated cells that had broken off from the halo. The low intensity staining in the untreated EBs is noticed within the small number of cells that were seen to exhibit differentiated morphology including those cells that had broken off from the halo (Figure 3.1.6 (A&B)).

The paired cytokeratins 7 and 19 were also expressed in the outgrowths from the undifferentiated (Figures 3.1.8 & 3.1.9 (B)) and differentiated EBs (Figures 3.1.8 & 3.1.9 (D)). Contrary to the observations with cytokeratins 8 & 18, it was found that the intensity of staining was similar (cytokeratin 7) or slightly greater (cytokeratin 19) in untreated EBs. Staining was predominantly noted in the periphery of the EBs and within the surrounding halo.

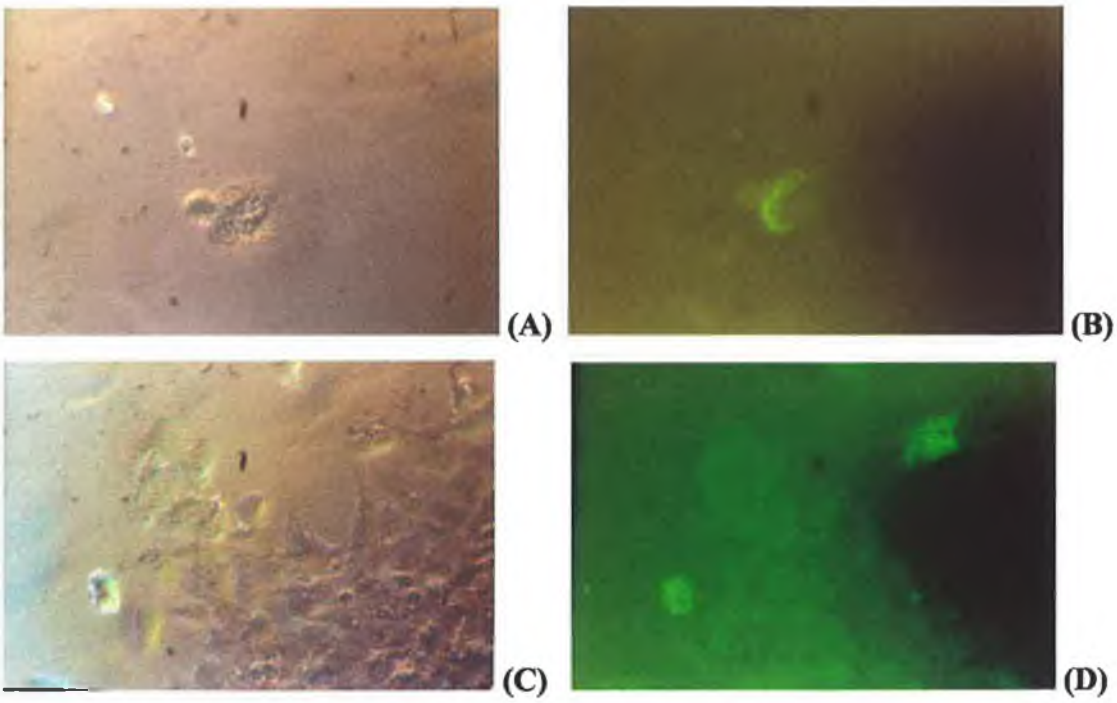


Figure 3.1.6 Cytokeratin 8 expression in undifferentiated EBs (A&B) and RA treated EBs (C&D) including phase contrast views of the fixed EBs (A&C). The cytokeratin staining was noted in the halo region surrounding the EB and was more intense in the RA treated EBs. Magnification = 40X

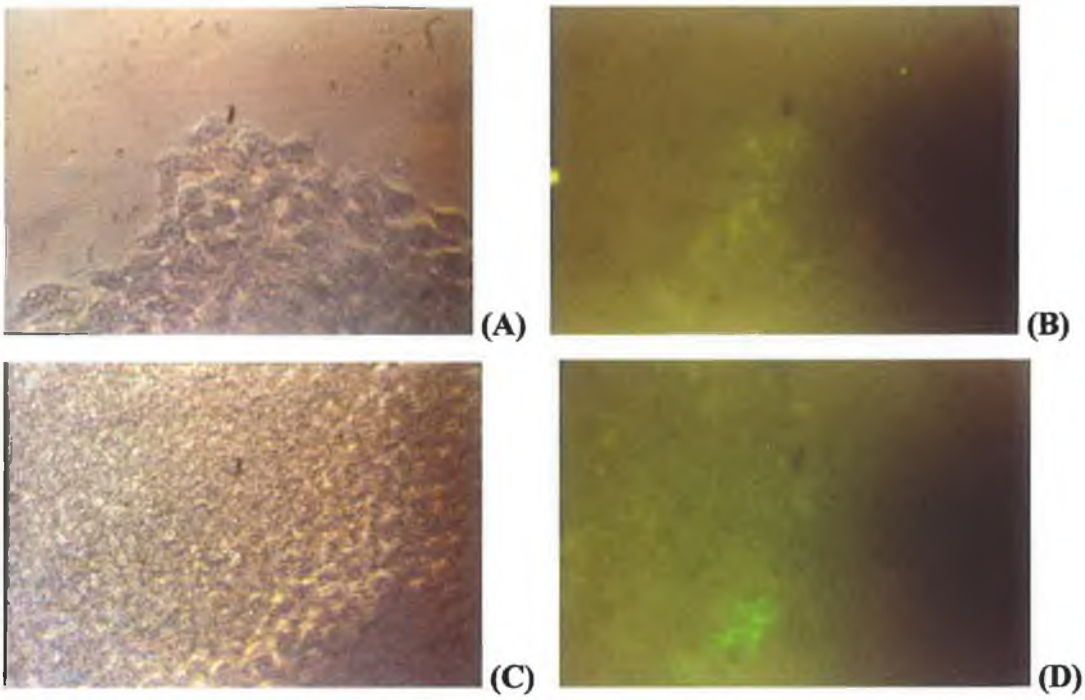


Figure 3.1.7 Cytokeratin 18 expression in undifferentiated EBs (A&B) and RA treated EBs (C&D) including phase contrast images of the fixed EBs (A&C). Staining was most intense around the periphery of the EBs and into the halo surrounding the central region. Magnification = 40X

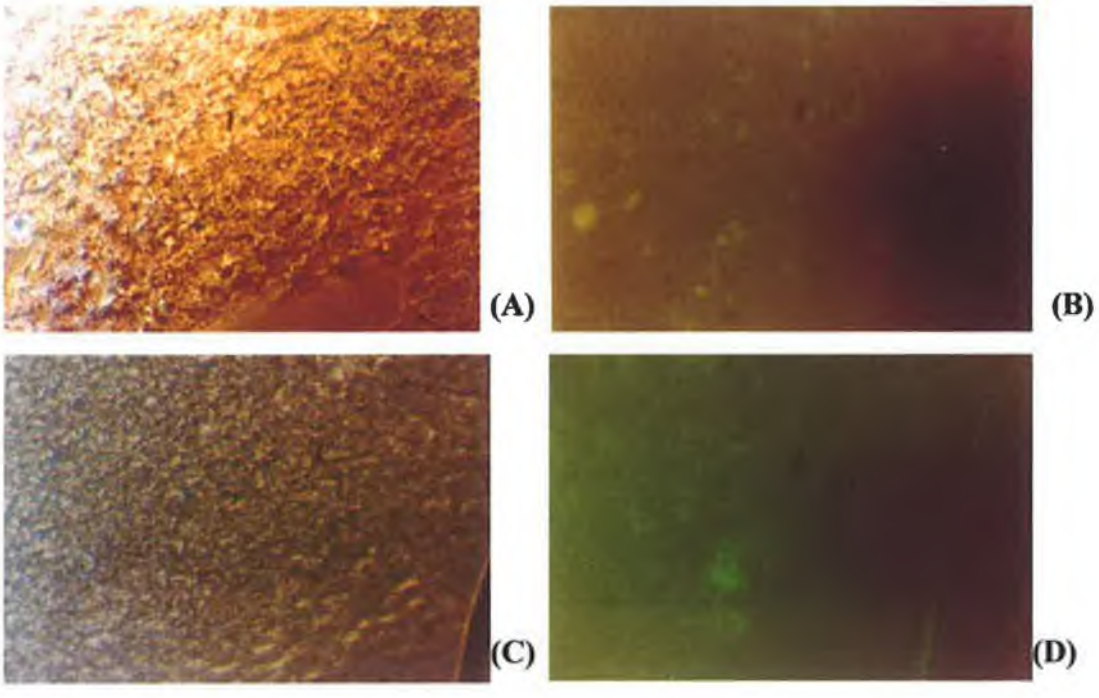


Figure 3.1.8 Cytokeratin 7 expression in undifferentiated EBs (A&B) and in RA treated EBs (C&D) including phase contrast images of the fixed EBs (A&C). Staining was in the periphery of the EBs and was of similar intensity in both untreated EBs and RA treated EBs. Magnification = 40X

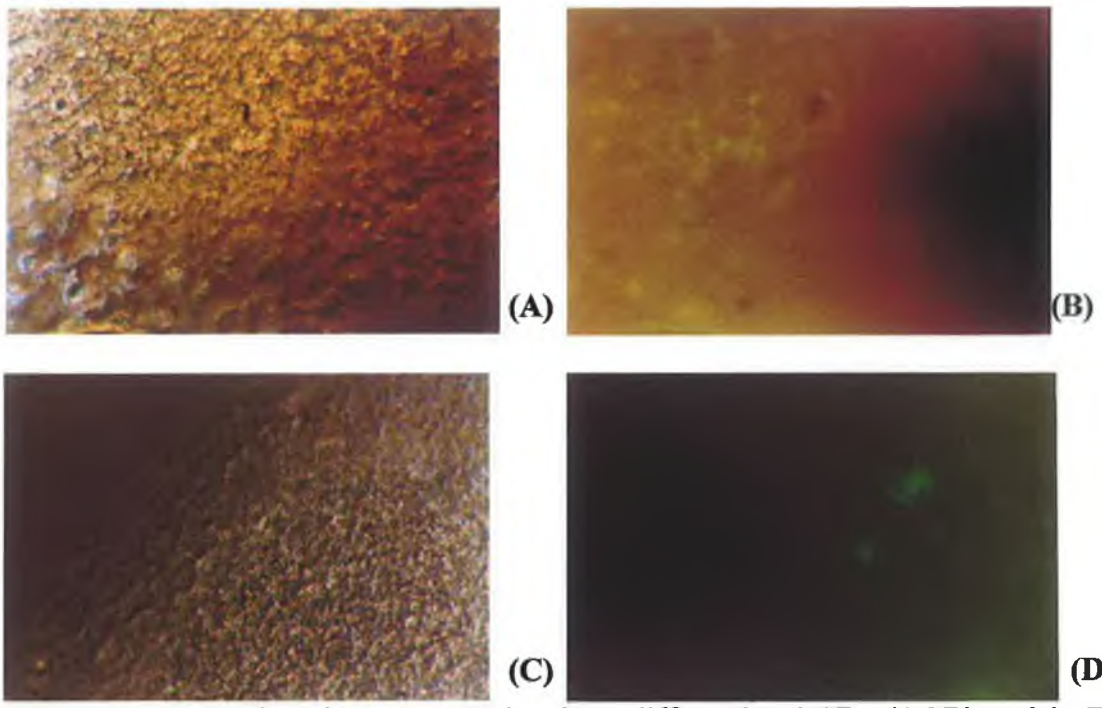


Figure 3.1.9 Cytokeratin 19 expression in undifferentiated EBs (A&B) and in RA treated EBs (C&D) including phase contrast images of the fixed EBs (A&C). Staining was in the periphery of the EBs. Magnification = 40X.

3.1.4.3 Alpha Fetoprotein Expression in Differentiating EBs

Alpha fetoprotein (AFP) expression is a characteristic of visceral endoderm. The actual endodermal origins (visceral or parietal) of β cells is unknown; thus AFP expression levels were investigated in differentiating EBs. From Figure 3.1.10 it is clear that AFP expression was present in the periphery of the EBs treated with RA. Untreated EBs did not have detectable levels of the protein using this method.

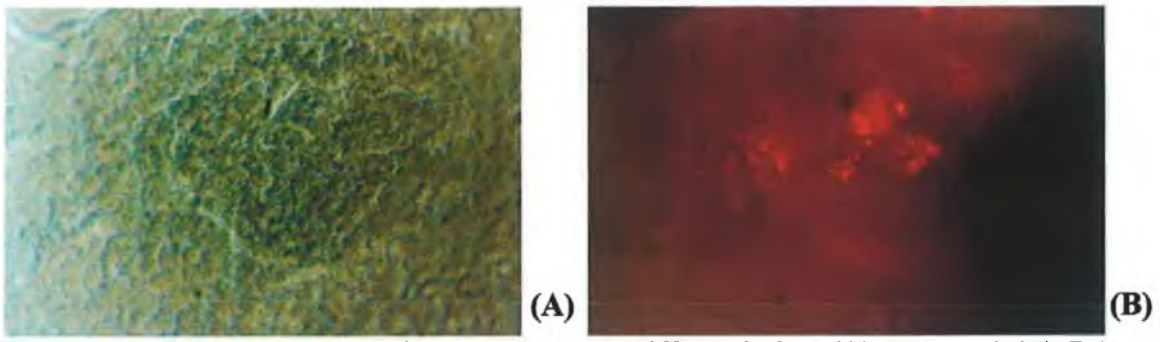


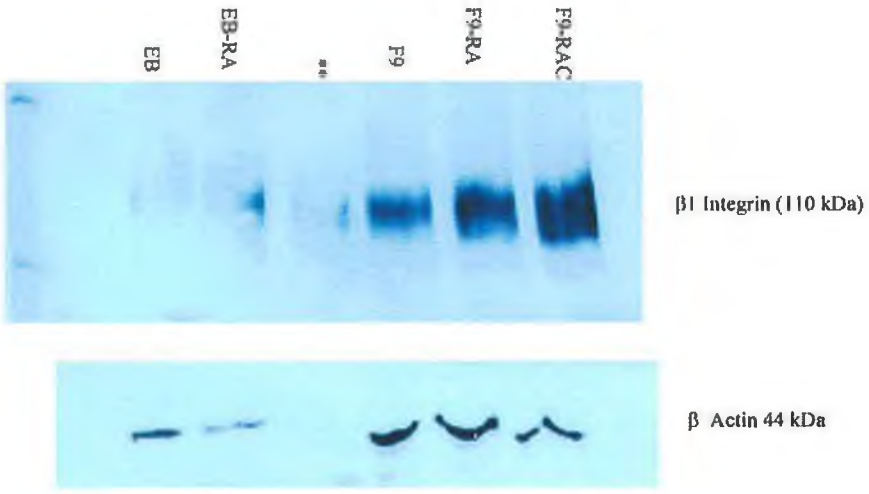
Figure 3.1.10 Alphafeto protein expression in differentiating (A) EBs and (B) RA treated EBs. Staining is predominantly in the periphery of the EB. Magnification = 40X.

3.1.5 β1 Integrin Expression in Differentiating F9 cells and Embryoid Bodies

β1 Integrin gene expression is critical to F9 differentiation and its role appears to be in the morphological changes that occur during differentiation of F9 cells (Stephens *et al.*, 1993).

As shown in Figure 3.1.11 it is clear that β1 Integrin protein expression increased significantly upon differentiation of F9 cells and EBs. There was a 9-fold increase in expression upon differentiation of EBs and a 2-3 fold increase when F9 monolayer cells were treated with RA and RA/db cAMP respectively.

(A)



(B)

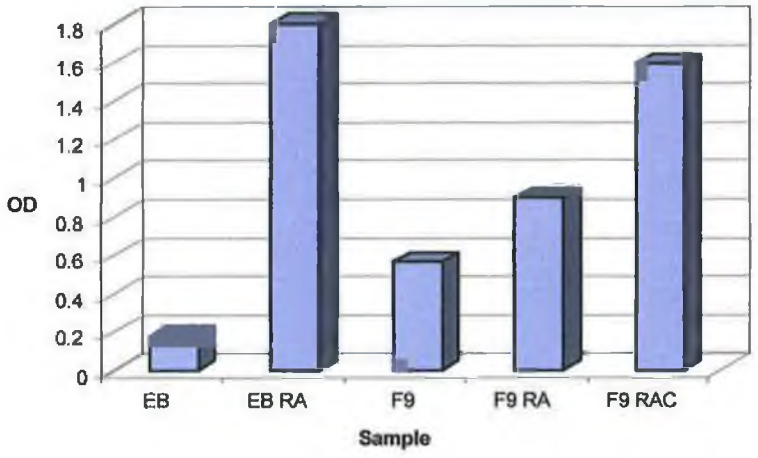


Figure 3.1.11 Western Blot analysis of β1 Integrin expression in differentiating F9 monolayers and EBs. The blot presented (A) is representative of at least three independent experiments. β-actin was used as an endogenous control for subsequent densitometry (B).

** This faint banding in the β1 Integrin western blot is due to overflow from the EB-RA well.

3.2 Second Stage Differentiation of Endodermal cells Generated from F9 Cells

From Section 3.1 it was seen that both visceral and parietal endoderm could be generated from F9 cells by differentiating the monolayers/EBs in the presence of RA or RA/db cAMP. β cells are generally accepted to be of endodermal origin, thus the immature endodermal cells generated during the initial stages of F9 differentiation were considered an ideal starting point from which to generate endocrine cells. Previous studies have identified various differentiation factors and growth factors which are critical in pancreatic neogenesis in the developing embryo (Section 1.1.3.4), these agents and others have been exploited to generate endocrine tissue in *in-vitro* situations (Section 1.3.4). The protocols employed for secondary differentiation and sampling are as outlined in Section 2.3.1.2.2

3.2.1 Identification of Receptors for Critical Second Stage Differentiation Factors

It is known that the betacellulin (BTC) receptor (Epidermal Growth Factor Receptor; EGFR) increases upon endodermal differentiation of F9 cells (Alonso *et al.,* 1991). However the effects of differentiation on levels of Activin A and HGF receptors were unknown. Antibodies were sourced for these receptors and western blotting experiments were carried out (Section 2.5.2.1).

3.2.1.1 Activin RII B expression in Differentiating F9 Cells

Activin A receptors are classified as type I, II or III, the type II receptors are further composed of A and B categories. The role of Activin RII B in development has been shown in experiments with *Xenopus* embryos. In differentiating EBs and monolayer F9 cells there is a slight increase in Activin RII B levels upon differentiation with RA or RA/db cAMP (Figure 3.2.1). β actin was employed as an endogenous control in all blots for denstiometric analysis. This result confirms the presence of receptor expression for subsequent secondary treatments.

3.2.1.2 HGF Receptor (c-Met) Levels in Differentiating F9 Cells

From Figure 3.2.2 it can be seen that levels of c-met increase upon differentiation of F9 cells and EBs. Levels are low in undifferentiated F9 cells but there are appreciable levels of the receptor in undifferentiated EBs. β actin levels were used as an endogenous control for densitometric analysis. As with the investigation of Activin RII B levels, the increase in protein levels is not as important as the qualitative data, i.e. that there are receptors present for HGF treatments. As indicated there is a large increase in c-met expression in untreated EBs compared to untreated F9 cells and this has been observed previously in other ES cell systems (Schuldiner *et al.,* 2000).

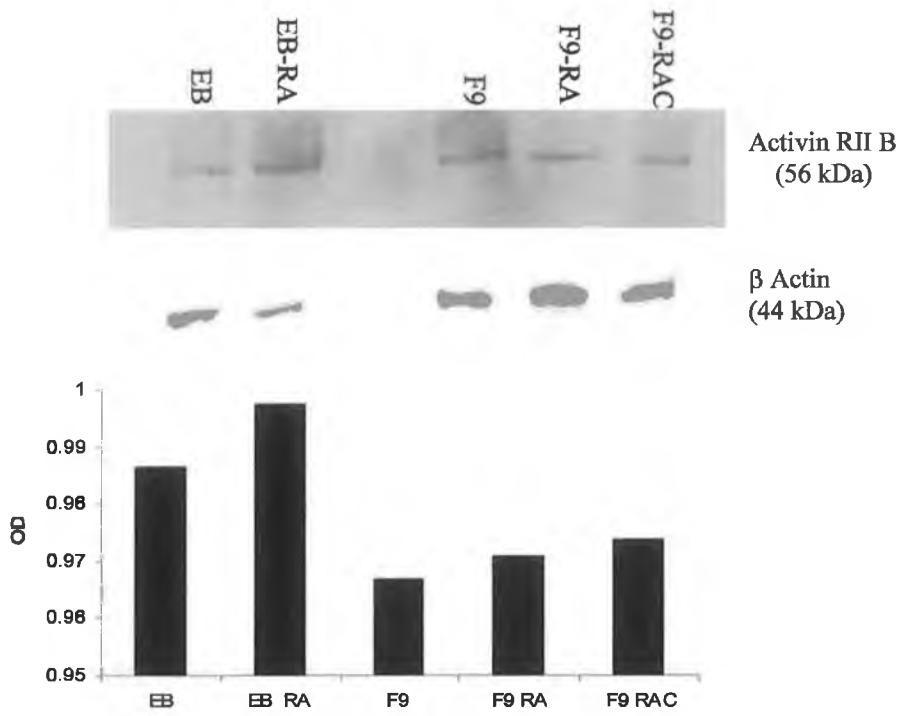


Figure 3.2.1 Levels of Activin RII B in differentiating F9 monolayers and EBs. F9 cells were differentiated with RA (F9-RA) or RA/ db cAMP (F9-RAC) for 72 hours. EBs were generated overnight and then treated with RA (EB-RA) for 72 hours.

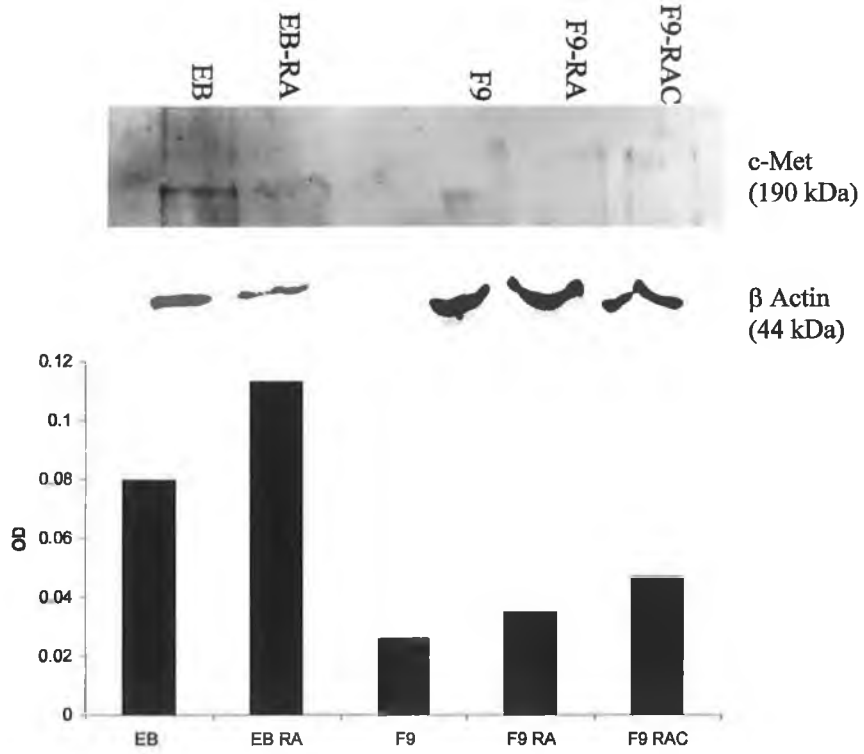


Figure 3.2.2 Levels of c-Met in differentiating F9 monolayers and EBs. F9 cells were differentiated with RA (F9-RA) or RA/ db cAMP (F9-RAC) for 72 hours. EBs were generated overnight and then treated with RA (EB-RA) for 72 hours.

3.2.2 Differentiation of RA Treated F9 Monolayers (F9-RA) with Endocrine Differentiation Agents

Treatments	Concentration
Activin A (Act) {Sigma}	2 nM
Betacellulin (BTC) {Sigma}	1 nM
Hepatocyte Growth Factor (HGF) {R&D Systems}	100 pM
Nicotinamide (Nico) {Sigma}	10 mM
Sodium Butyrate (Sod But) {Sigma}	1 mM

Table 3.2.1 Endocrine differentiation agents employed in the secondary differentiation of F9 derived endodermal cells. Combinations of all the above agents with activin A were also carried out using the same working concentrations used for single application. Reconstitution of these agents to working stocks is described in Section 2.3.1.1.

3.2.2.1 PDX1 Expression in F9-RA Monolayers

From Figure 3.2.3 it can be seen that low-level PDX1 transcription was present in the untreated F9 cell population. PDX1 levels were found to increase almost two-fold after exposure of the cells to RA for 10 days. Subsequent treatments did not cause an increase in PDX1 mRNA levels, with the exception of the combination of HGF and activin A which resulted in a 2.5 fold increase in PDX1 expression when compared to untreated F9s. Despite these increases, PDX1 levels were still far lower in treated F9-RA cells than in Min6 β cells.

3.2.2.2 PPI Expression in F9-RA Monolayers

Untreated F9 cells were found to have low level PPI gene expression which can be clearly seen in Figure 3.2.4. Treatment of the cells with RA increased levels of PPI mRNA in the cells. Subsequent treatment with endodermal differentiation agents resulted in increased PPI mRNA levels especially in the case of the combination

treatment of HGF and activin A which caused a 3-4 fold increase in PPI mRNA levels (compared to untreated F9 cells). This treatment coincides with an increase in PDX1 (Section 3.2.2.1).

3.2.2.3 Pax6 Expression in F9-RA Monolayers

F9 cells were found to express high levels of Pax6 mRNA. Large increases in Pax6 levels were noted following treatment with nicotinamide or a combination of nicotinamide and activin A. Pax 6 levels were decreased by a factor of two following activin A and betacellulin combination treatment and likewise following HGF and activin A exposure (Figure 3.2.5). It is interesting that this treatment also results in the highest expression of PPI and PDX1 and may represent a possible inhibitory role of Pax6 overexpression in β cell development.

3.2.2.4 Indian hedgehog (Ihh) Expression in F9-RA Monolayers

As shown in Figure 3.2.6, Indian hedgehog (Ihh) mRNA levels increased 10 fold upon differentiation with RA, while all subsequent treatments involving pancreatic differentiation agents caused Ihh levels to decrease to approximately basal levels. Treatments involving nicotinamide, sodium butyrate and the combination of sodium butyrate and activin A increased levels of Ihh compared to the other treatments and untreated F9 cells. The wide variation in Ihh levels possibly indicates the development of different cell lineages with differing requirements for hedgehog signalling.

3.2.2.5 Pancreatic Polypeptide (PP) Induction in F9-RA Monolayers

F9 cells were found to be negative for pancreatic polypeptide (PP) mRNA as were F9-RA cells. However, subsequent treatment of F9-RA monolayers with sodium butyrate or the combination of sodium butyrate and activin A resulted in the appearance of PP mRNA (Figure 3.2.7). As sodium butyrate or activin A have not had any substantial effects on any other genes examined so far, it seems that these treatments are acting directly on the PP gene. Min6 cells were also found to contain significant levels of PP transcripts which does not appear to have been reported before.

3.2.2.6 Somatostatin Gene Induction in F9-RA Monolayers

Large increases in somatostatin mRNA levels were noted in F9-RA monolayers treated with either sodium butyrate or a combination of sodium butyrate and activin A and to a lesser extent following treatment with activin A, betacellulin or HGF (Figure 3.2.8). There was a slight increase in somatostatin expression in the presence of both sodium butyrate and activin A that may represent a slight additive effect (i.e. as activin A had moderate effects on somatostatin mRNA levels when used alone). As with PP mRNA levels, the data here indicates that the substantial increases in somatostatin appear to be due to a direct effect of sodium butyrate on the somatostatin gene.

3.2.2.7 Other Genes Examined in F9-RA Monolayers

The expression levels of a number of other pancreatic genes were examined in F9-RA monolayers however these genes were not found to be expressed in F9 cells and were not induced as a result of any of the treatments. The other genes investigated were:

- GLUT2
- Glucokinase
- Pax4
- Sonic Hedgehog (Shh)
- Glucagon

Min6 cells were used as a positive control in each case.

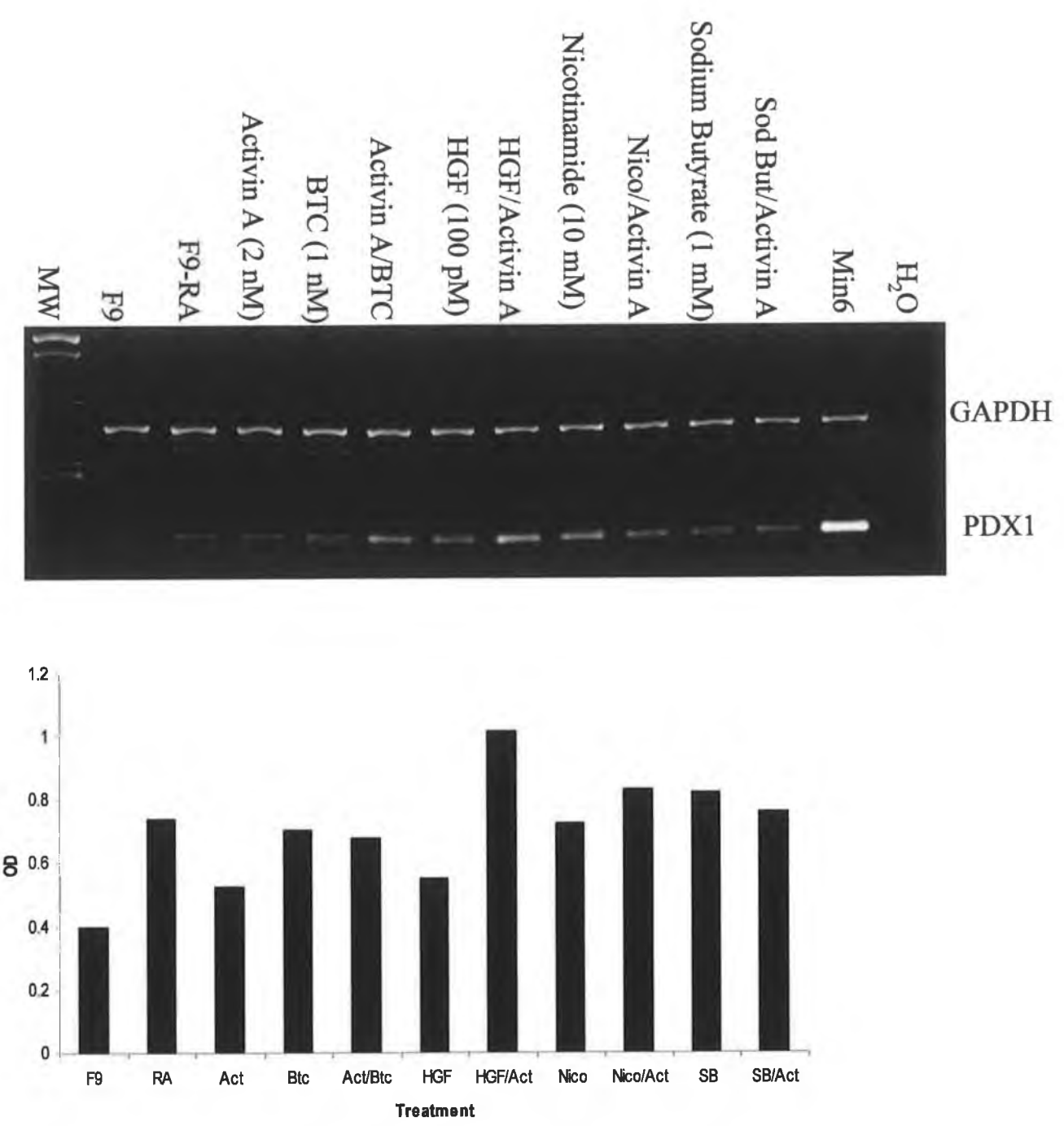
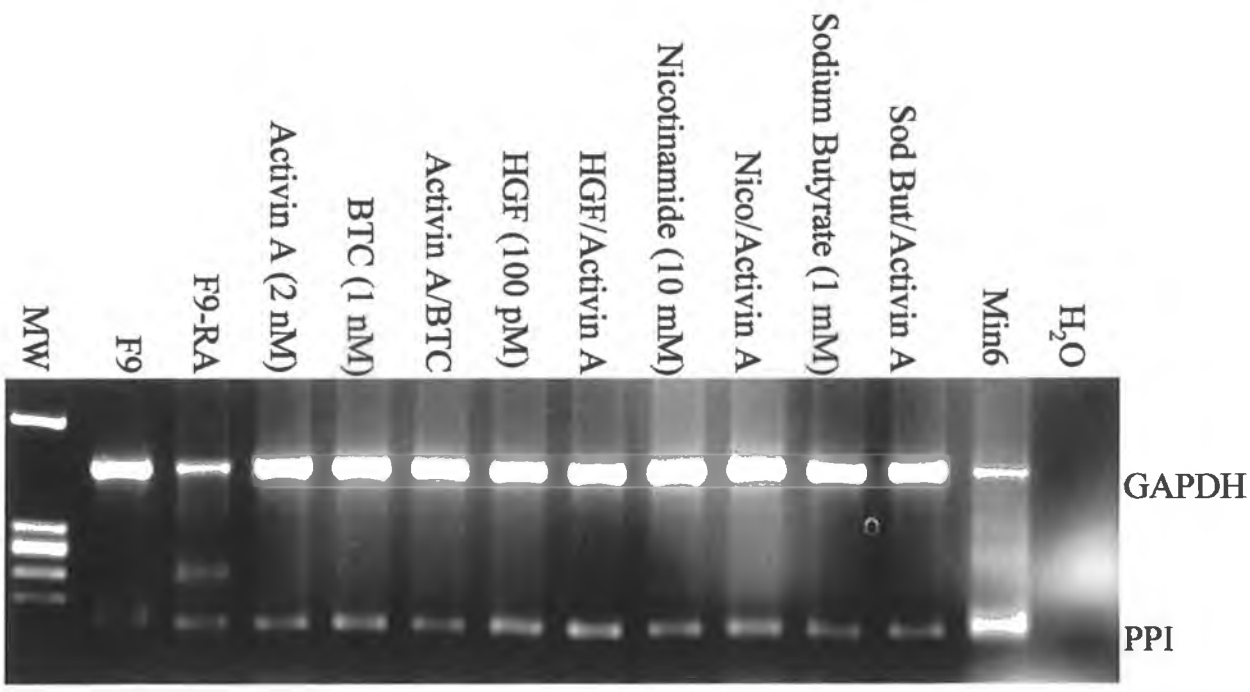


Figure 3.2.3 PDX1 expression in F9-RA cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were employed as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



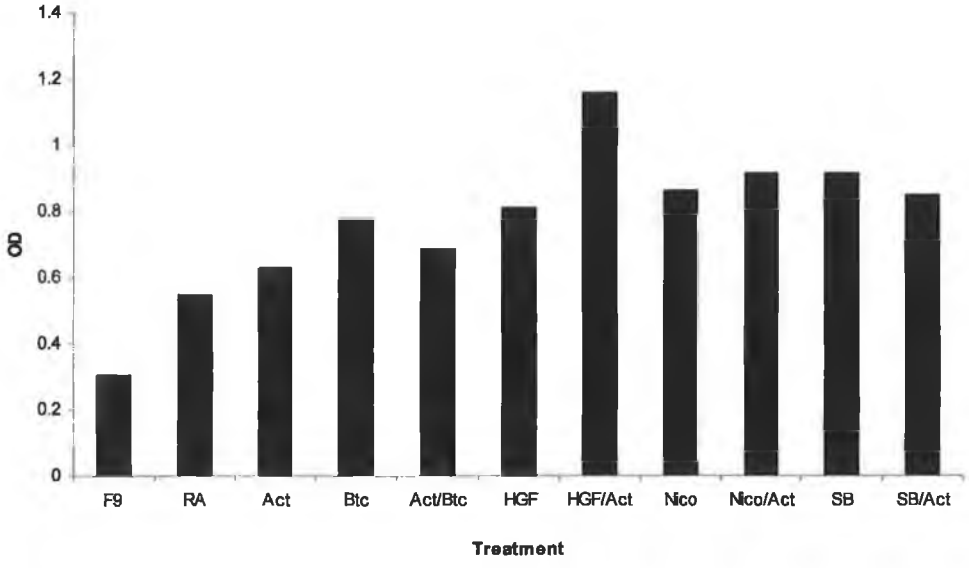
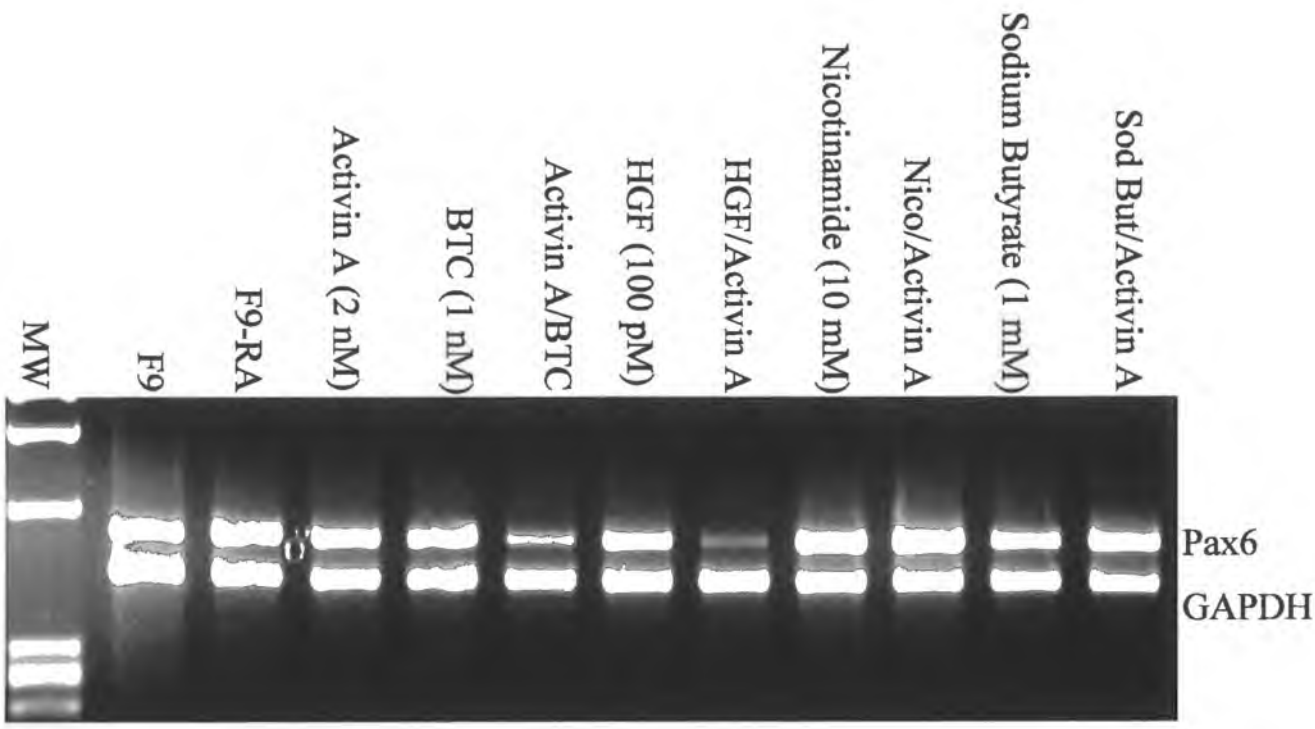


Figure 3.2.4 PPI expression in F9-RA cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



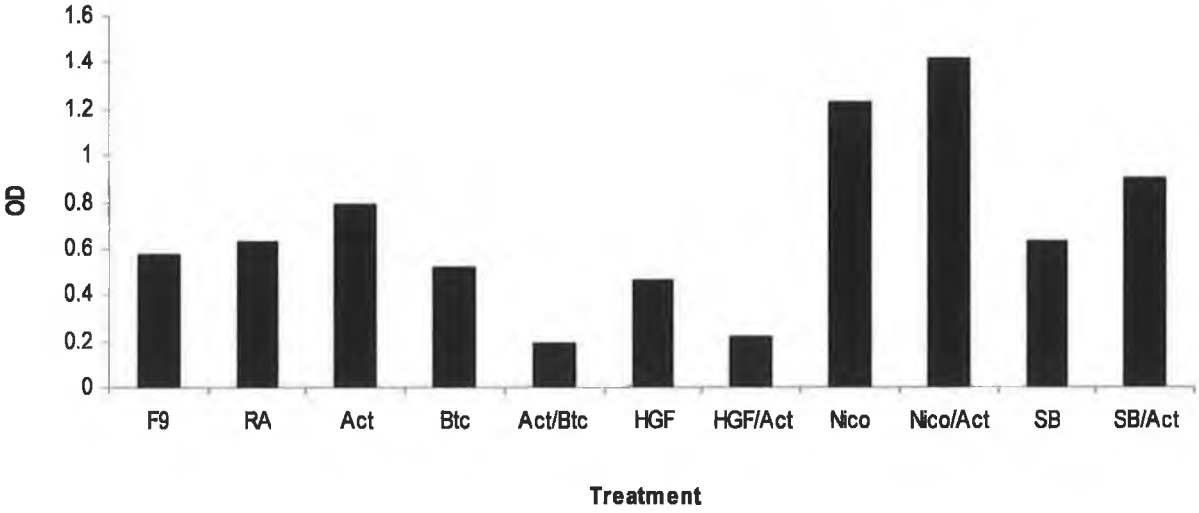


Figure 3.2.5 PAX6 expression in F9-RA cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. The results presented are representative of repeat gels from two individual treatment experiments.

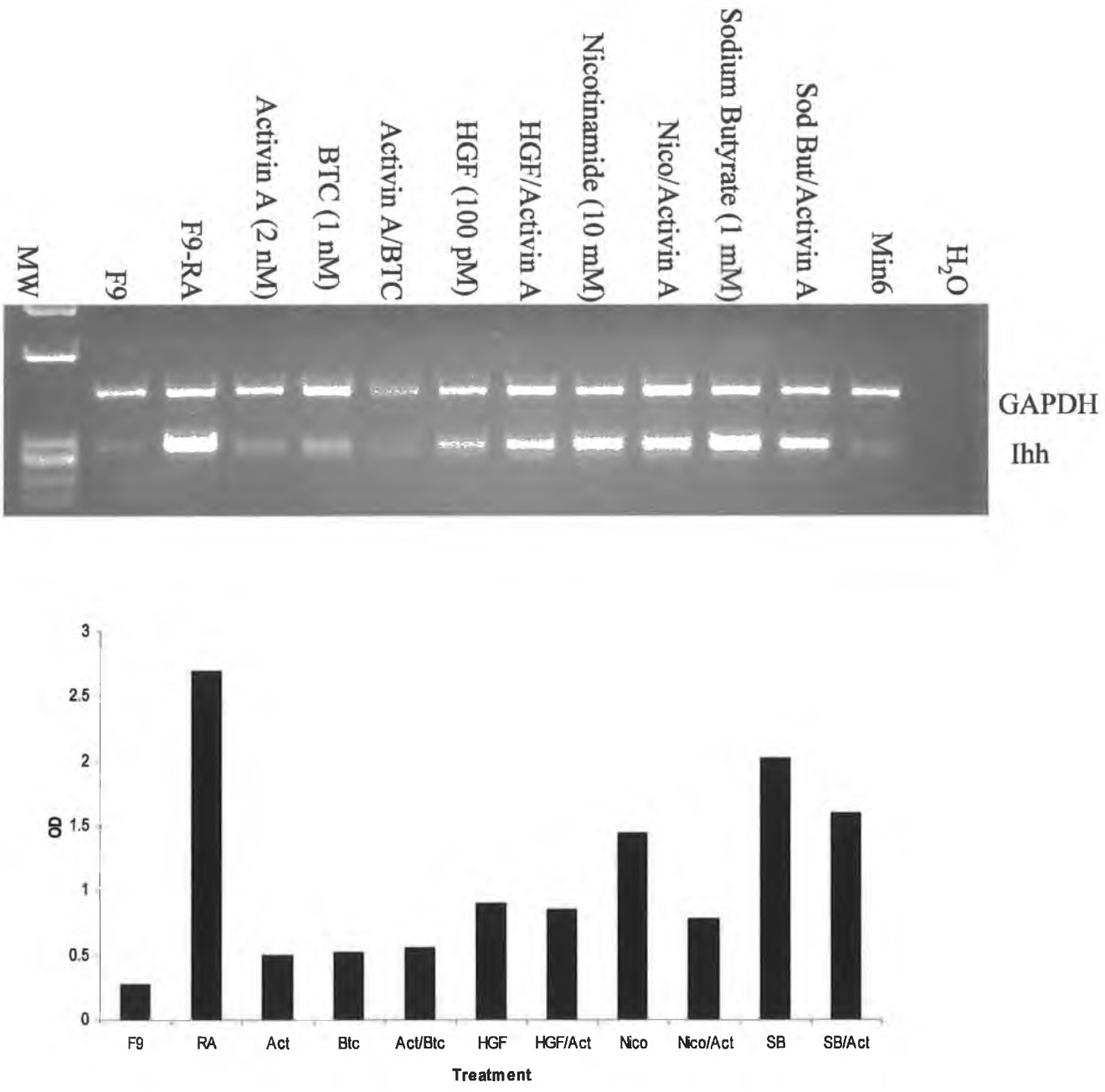


Figure 3.2.6 Indian hedgehog (Ihh) expression in F9-RA cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.

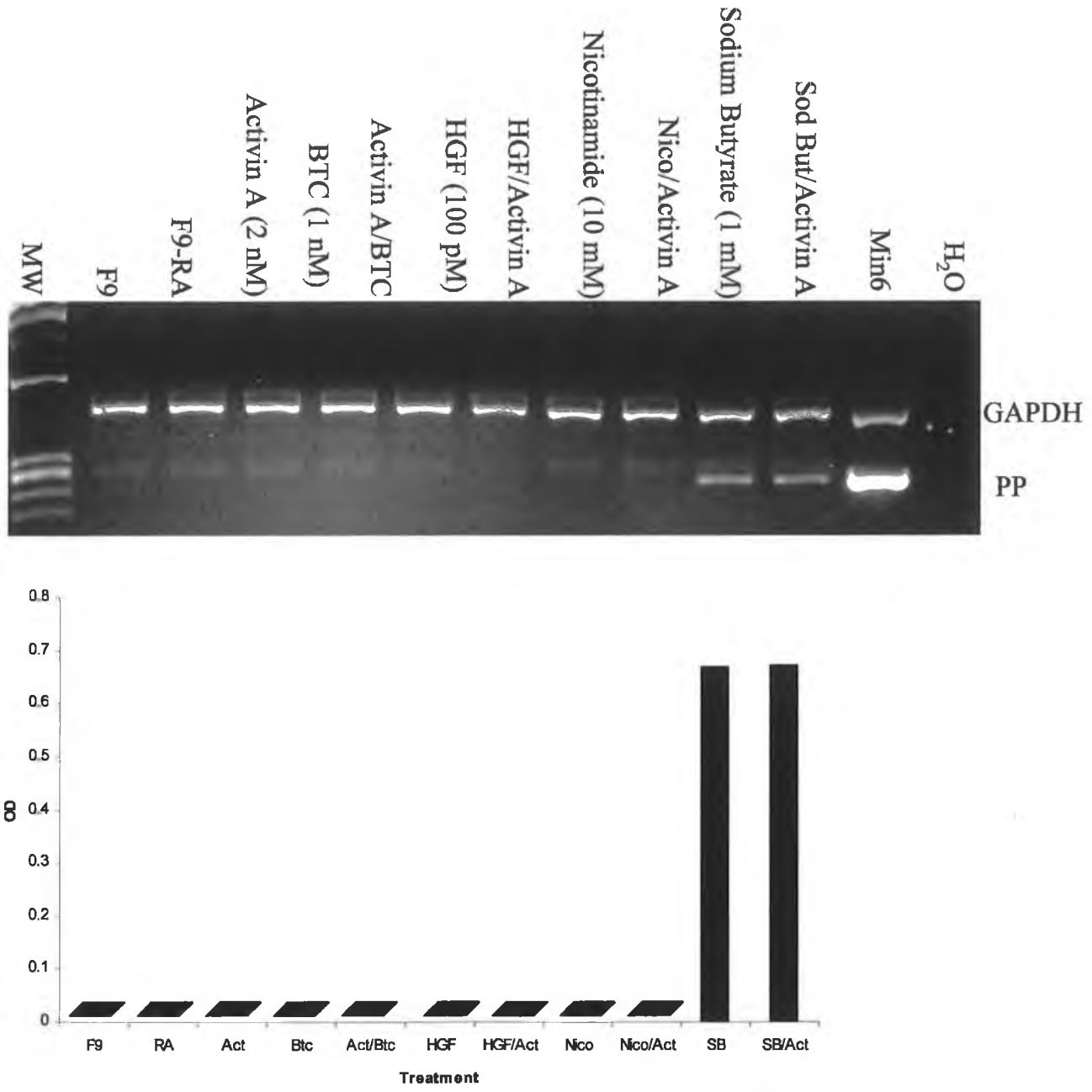
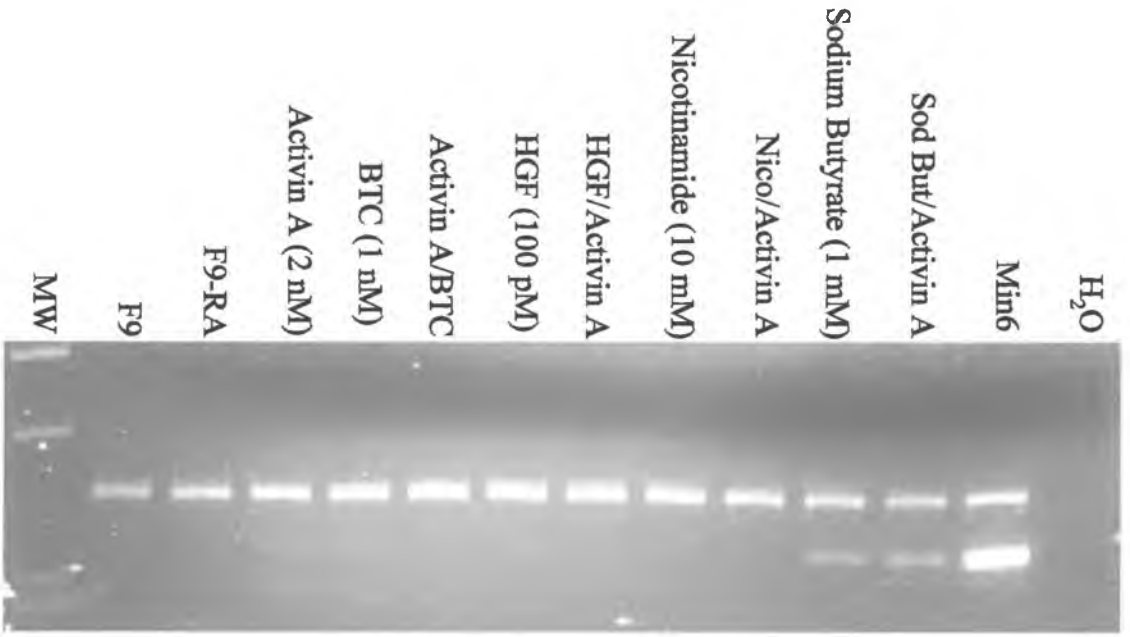


Figure 3.2.7 Pancreatic Polypeptide (PP) induction in F9-RA cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



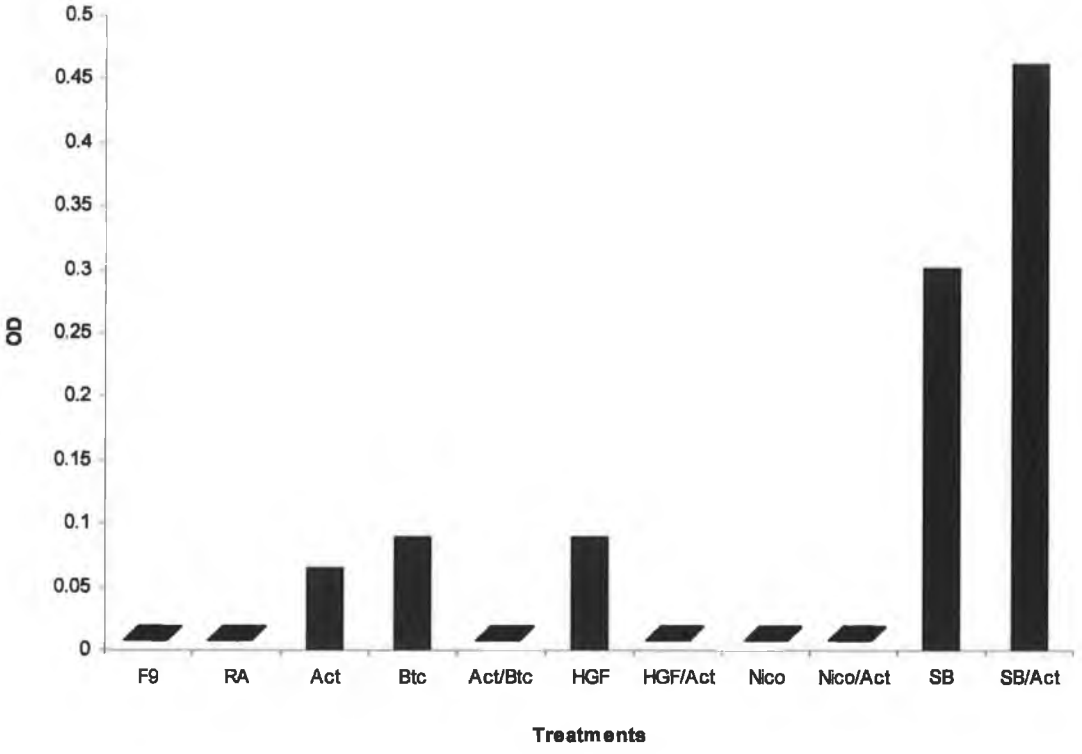


Figure 3.2.8 Somatostatin gene induction in F9-RA cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.

3.2.3 Differentiation of RAC treated Monolayers

As outlined previously, (Section 3.1) RAC monolayers were generated over 72 hours by exposing F9 cells to a combination of RA (10^{-7} M) and db cAMP (10^{-3} M). These cells were then treated with the endocrine differentiation agents listed in Table 3.2.1.

3.2.3.1 PDX1 Expression in F9-RAC Monolayers

PDX1 gene expression was present at low level in F9 cells and increased upon treatment with RA and db cAMP. There were no substantial increases in PDX1 mRNA levels after any of the other treatments although expression was found to be slightly reduced following activin A treatment (Figure 3.2.9).

3.2.3.2 PPI Expression in F9-RAC Monolayers

PPI gene expression was detected in F9 cells (as in Figure 3.2.4) and was present in all treatments examined. The levels of PPI did not change substantially with any treatment although there was a slight (< 2-fold) increase in expression upon treatment of the cells with sodium butyrate or the combination of sodium butyrate and activin A (Figure 3.2.10). This increase in PPI levels did not correspond with similar increased levels in PDX1 expression but did coincide with slightly decreased levels of Pax6 expression (Figure 3.2.11), which is similar to observations in F9-RA monolayers (Section 3.2.2.3).

3.2.3.3 Pax6 Expression in F9-RAC Monolayers

From Figure 3.2.11 it can be seen that there are high levels of expression of Pax6 mRNA in F9 cells. Levels of Pax 6 were found to decrease upon treatment of the cells with the combination RA/db cAMP however the endocrine differentiation agents resulted in an increase in Pax6 transcripts (not as obvious following the treatments involving sodium butyrate). The highest levels of Pax6 were found following treatment of F9-RAC cells with nicotinamide or the combination of nicotinamide and activin A which is similar to findings with F9-RA monolayers (Figure 3.2.5) and EBs (Figure 3.2.17).

3.2.3.4 Indian Hedgehog (Ihh) Expression in F9-RAC Monolayers

Indian hedgehog (Ihh) expression was present at low level in F9 and F9-RAC cells after 10 days treatment. This indicates that strong differentiation in the presence of db cAMP can be inhibitory to Ihh expression as Ihh was present at high levels following long term RA treatment of F9 cells (Figure 3.2.6), and following short term treatment with RA and db cAMP (Figure 3.3.27). Ihh mRNA levels increased upon endocrine treatments, however there were no substantial differences in expression levels between the treatments, other than slightly decreased expression following betacellulin treatment (i.e. compared to the other treatments) (Figure 3.2.12).

3.2.3.5 Pancreatic Polypeptide (PP) Induction in F9-RAC Monolayers

Pancreatic polypeptide (PP) mRNA transcripts were observed in F9-RAC cells following treatment with sodium butyrate and sodium butyrate combined with activin A (Figure 3.2.13). mRNA expression levels were similar between the two treatments however sodium butyrate alone did result in slightly higher expression of PP. As with F9-RA monolayers (Figure 3.2.7), the data indicates that the effect is mediated by sodium butyrate alone and is a direct effect.

3.2.3.6 Somatostatin Gene Induction in F9-RAC Monolayers

Somatostatin gene transcripts were found to be present in F9-RAC cells that were exposed to sodium butyrate or a combination of sodium butyrate and activin A for 10 days (Figure 3.2.14). Expression levels (even following the treatments) were very low and difficult to detect. The slight additive effect of activin A is again noted here despite that it had no effect on its own. However the data again suggests that the appearance of somatostatin mRNA transcripts is more closely linked to the action of sodium butyrate.

3.2.3.7 Other Genes Examined in F9 Monolayers

The expression levels of a number of other pancreatic genes were examined in F9-RAC monolayers. However these genes were not found to be expressed in either F9 cells and were not induced as a result of any of the treatments. The other genes investigated were:

- GLUT2
- Glucokinase
- Pax4
- Sonic Hedgehog (Shh)
- Glucagon

Min6 cells were used as a positive control in each case

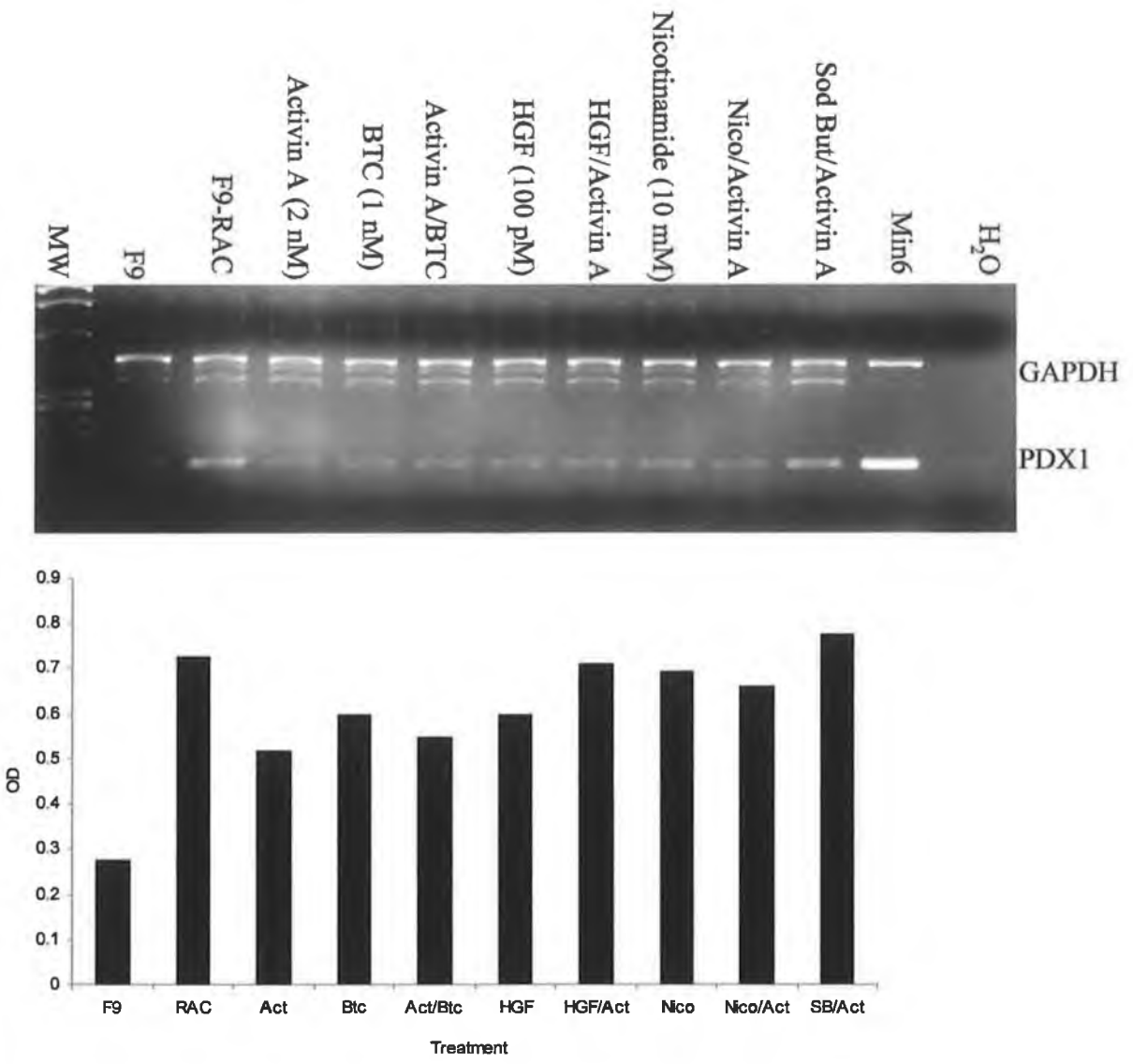
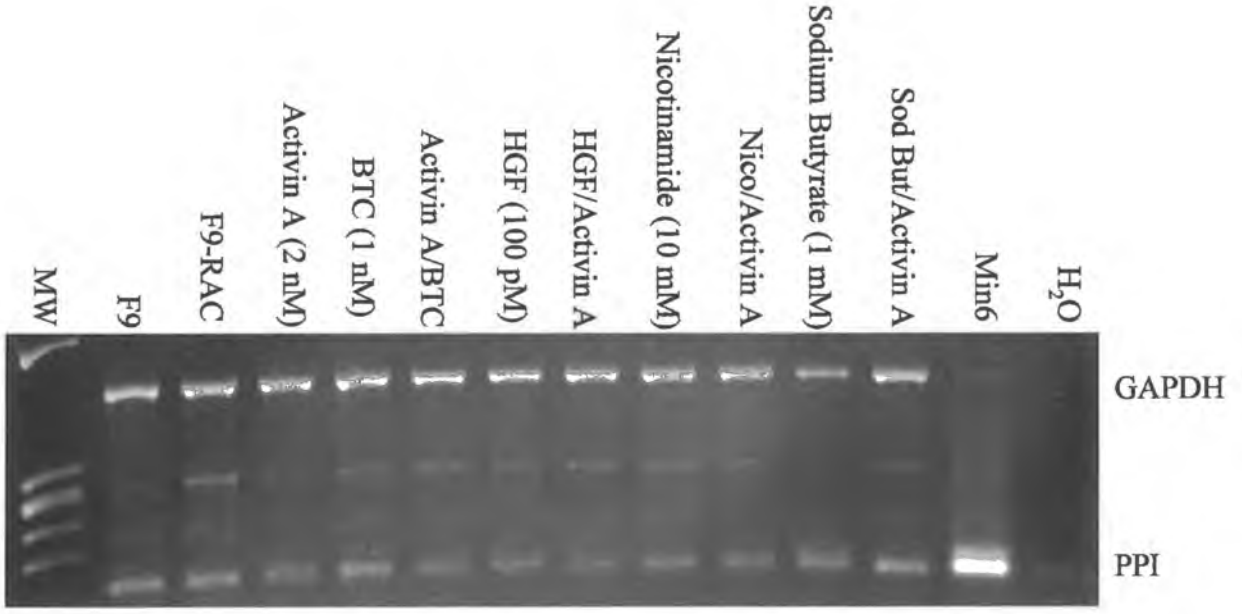


Figure 3.2.9 PDX1 expression in F9-RAC cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



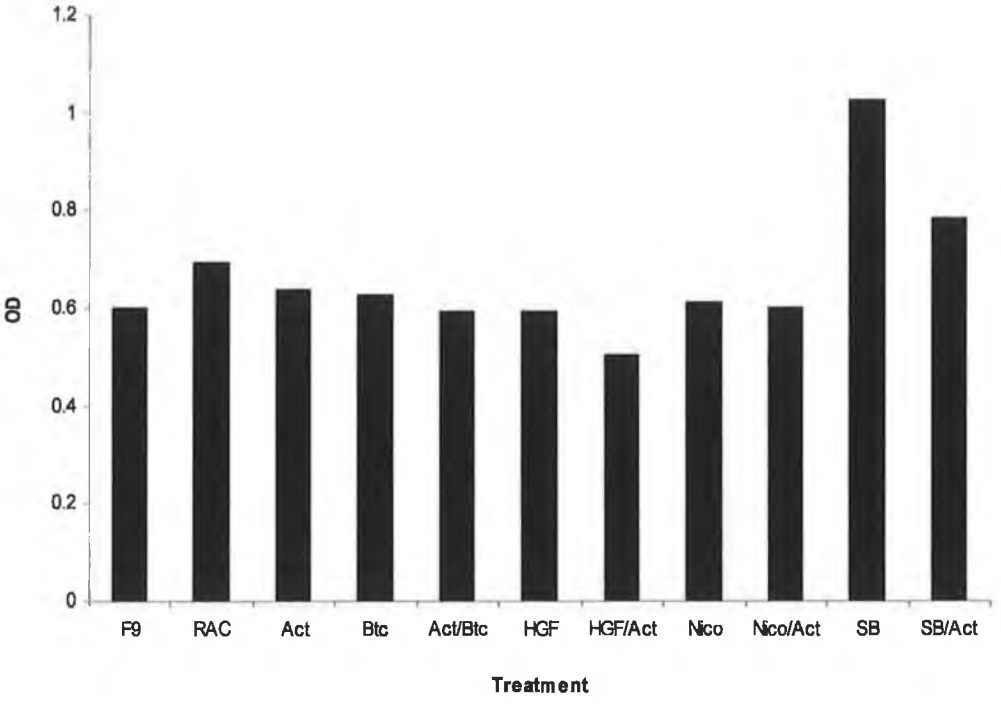
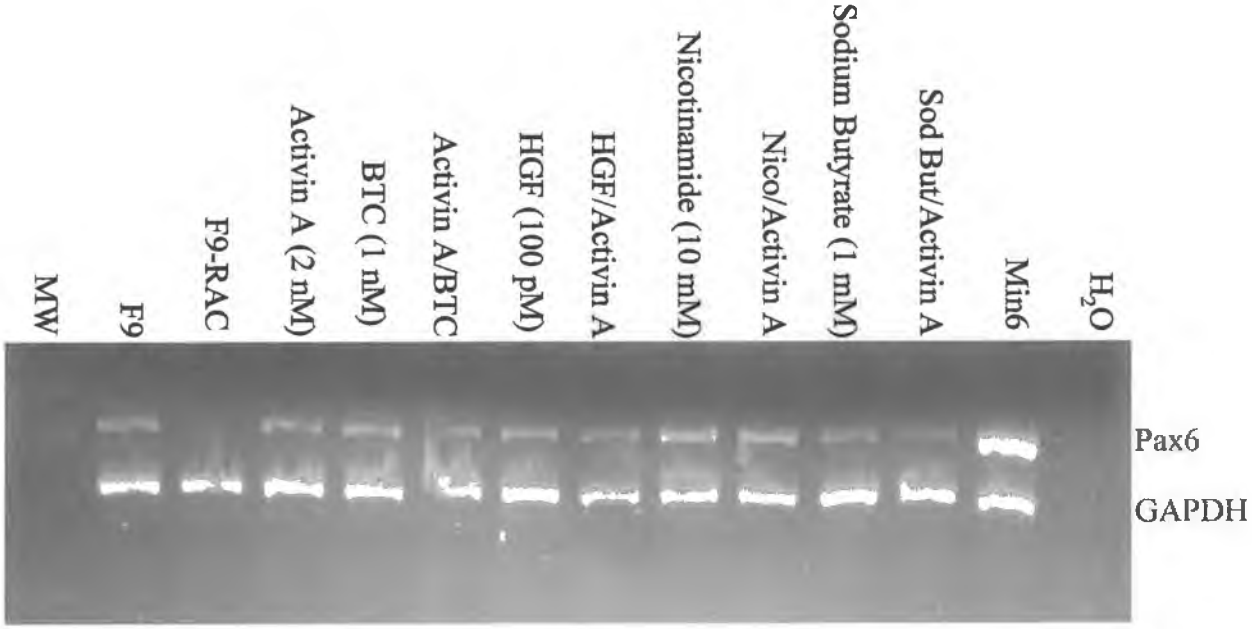


Figure 3.2.10 PPI expression in F9-RAC cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



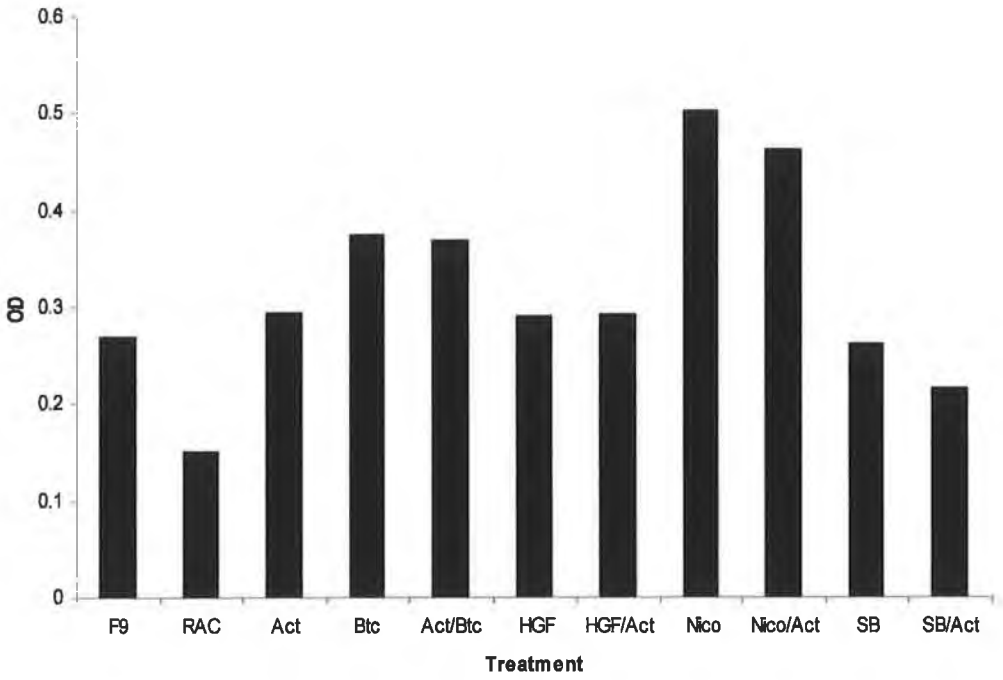
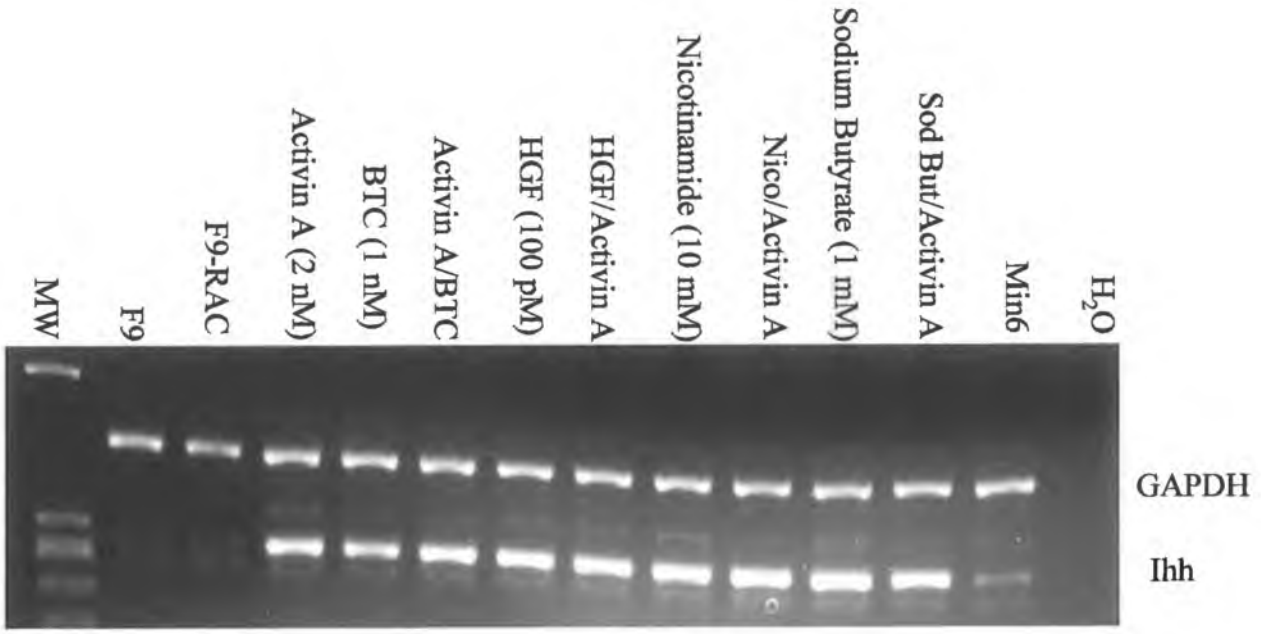


Figure 3.2.11 Pax6 expression in F9-RAC cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



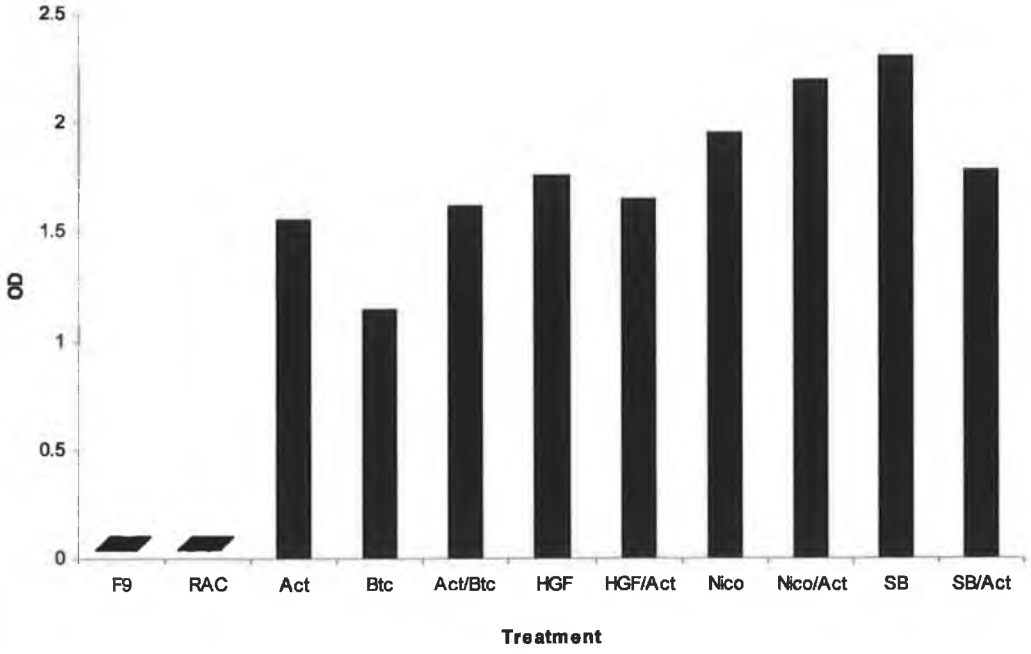
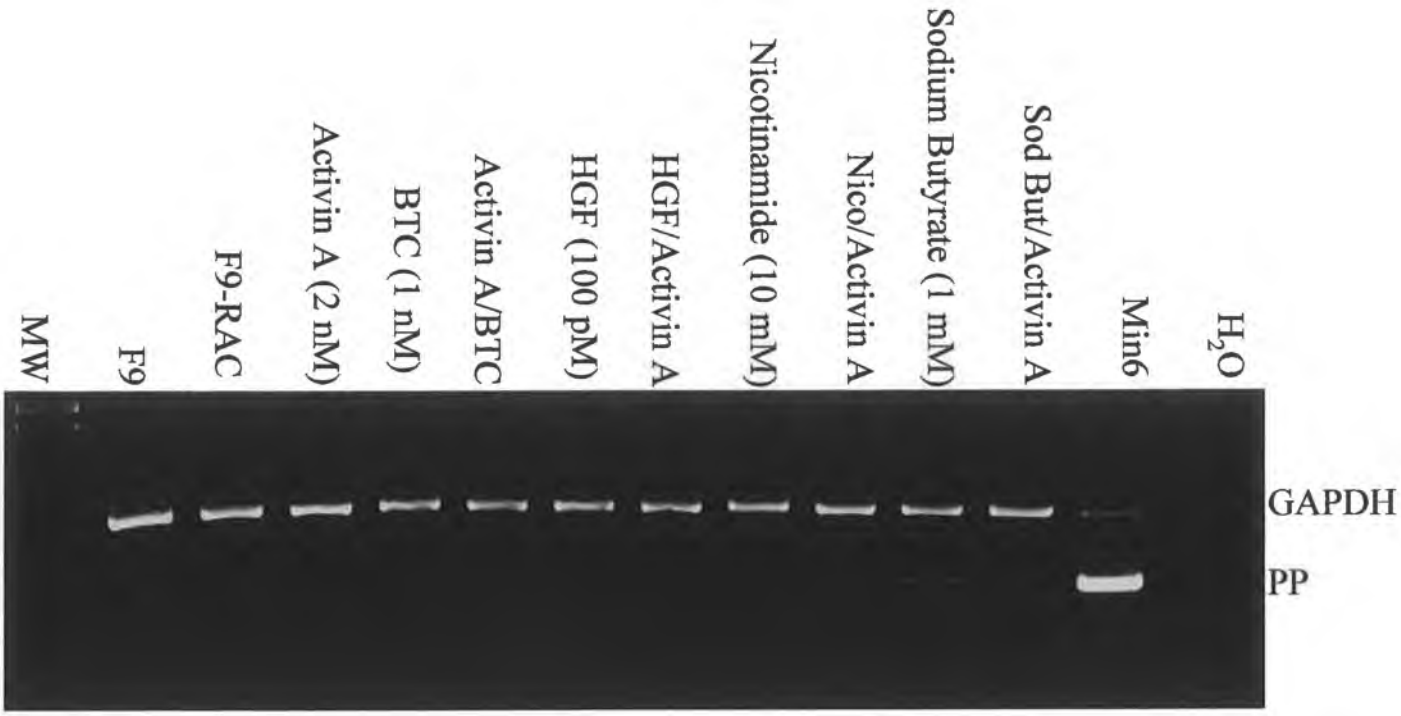


Figure 3.2.12 Indian hedgehog expression in F9-RAC cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



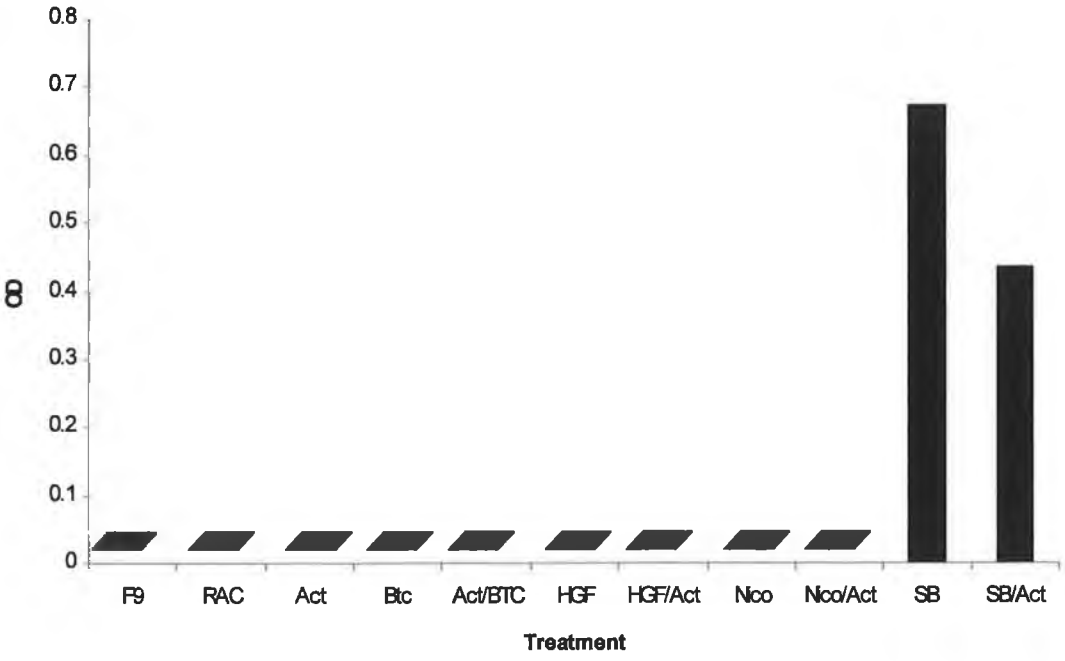


Figure 3.2.13 PP expression in F9-RAC cells treated with endocrine differentiation agents. Untreated F9 cells are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.

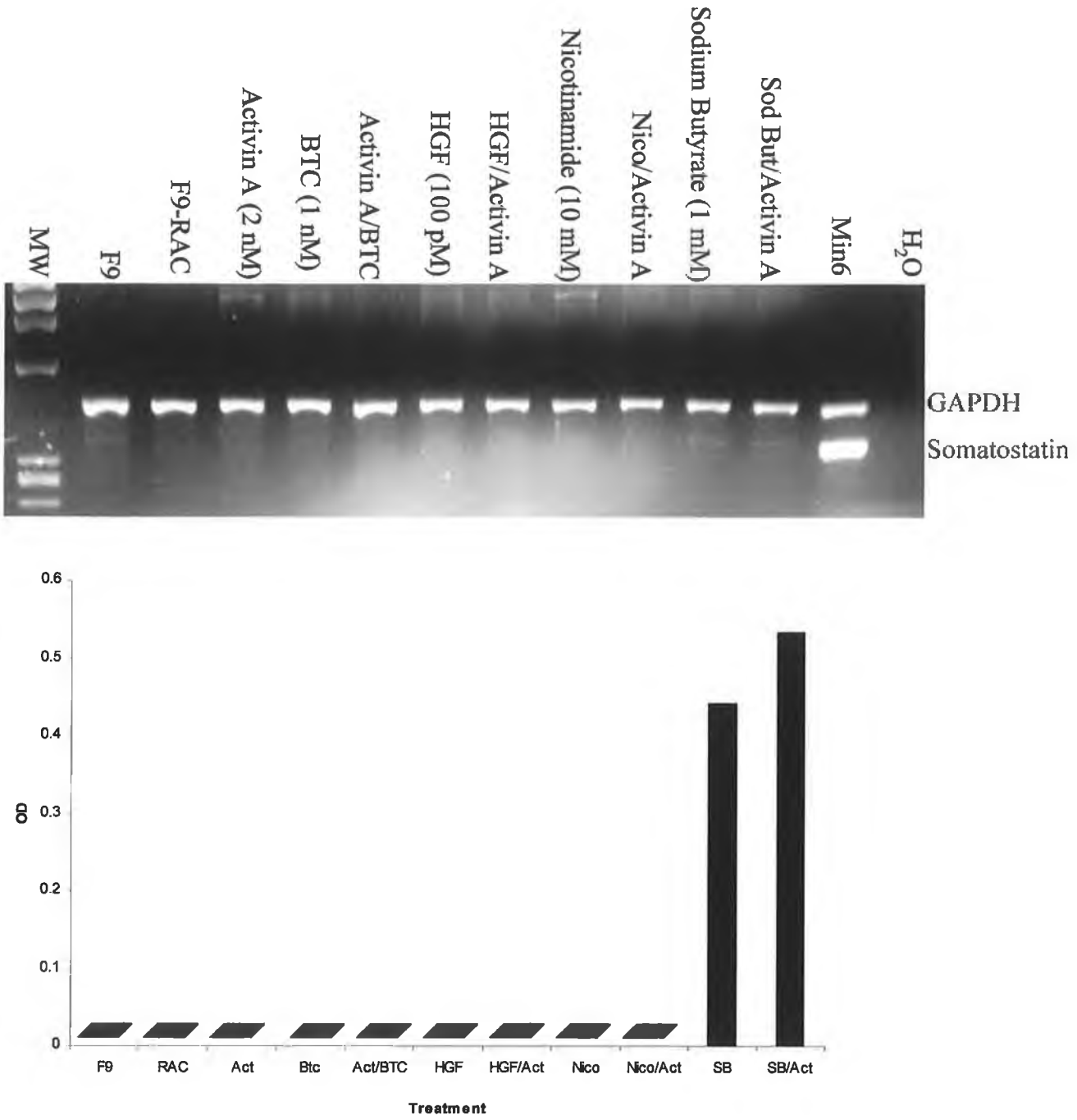


Figure 3.2.14 Somatostatin expression in F9-RAC cells treated with endocrine differentiation agents. Untreated F9 cells (F9) are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.

3.2.4 Differentiation of RA Treated Embryoid Bodies (EBs) with Endocrine Differentiation Agents

EBs were generated as already described (Section 2.2.4) and treated for 72 hours with RA. These EBs were then treated using the differentiation agents outlined in Table 3.2.1 for a period of 10 days in accordance with the secondary differentiation protocol (Section 2.3.1.2.2).

3.2.4.1 PDX1 Expression in EB-RA Bodies

From Figure 3.2.15 it can be seen that PDX1 mRNA was present in appreciable levels in the untreated EBs and levels of the transcription factor were not substantially changed as a result of any of the endocrine differentiation treatments. The highest level of PDX1 expression was found to be as a result of sodium butyrate treatment or following a combination of nicotinamide and activin A. The levels of PDX1 in the untreated EBs is much higher than in untreated F9 cells (Figure 3.2.3 or Figure 3.2.9), which is similar to findings by Gerrish *et al.,* (2000) and Schuldiner *et al.,* (2000), upon generating EBs from ES cells.

3.2.4.2 PPI Expression in EB-RA Bodies

PPI mRNA was found to be present in untreated EBs and appeared to be unaffected by RA treatment. All of the endocrine differentiation agents caused an increase in PPI gene transcription levels of approximately 1.5-2 fold, but the treatments did not differ from one another to any notable extent (Figure 3.2.16).

3.2.4.3 Pax6 Expression in EB-RA Bodies

EBs were found to contain Pax6 transcripts and expression of Pax6 was upregulated following RA treatment (~2 fold). Nicotinamide/activin A treatment resulted in further upregulation of Pax6 expression (almost 3 fold greater than untreated EBs) and this is similar to findings with F9-RA (Figure 3.2.5) and F9-RAC (Figure 3.2.11), monolayers. Pax6 expression was decreased following treatment of EB-RA bodies

with HGF, sodium butyrate and the combination of sodium butyrate and activin A (Figure 3.2.17).

3.2.4.4 Indian Hedgehog (Ihh) Expression in EB-RA Bodies

Ihh expression in EBs was increased following RA treatment. None of the endocrine differentiation agents caused any further increase of Ihh expression however. Activin A, and the combination of activin A with betacellulin or HGF all caused Ihh levels to fall below those in untreated EBs (Figure 3.2.18). The observed increase in Ihh levels upon RA treatment of F9-RA monolayers (Figure 3.2.6), the increase observed here and the lack of Ihh expression following 10 days RAC treatment indicates preferential expression of Ihh in primitive endoderm and visceral endoderm types rather than parietal endoderm.

3.2.4.5 Pancreatic Polypeptide (PP) Expression in EB-RA Bodies

PP mRNA expression was found to be negligible in EBs. Low levels of PP transcripts were detected in EB-RA bodies, EB-RA bodies treated with activin A, HGF and also following a combination treatment of nicotinamide and activin A. More substantial expression was induced following treatment with sodium butyrate (whether alone or in combination with activin A) (Figure 3.2.19). The discovery of PP transcripts in a wider range of treatments confirms that the cells generated from EB-based differentiation are unique compared to either of the monolayer populations generated.

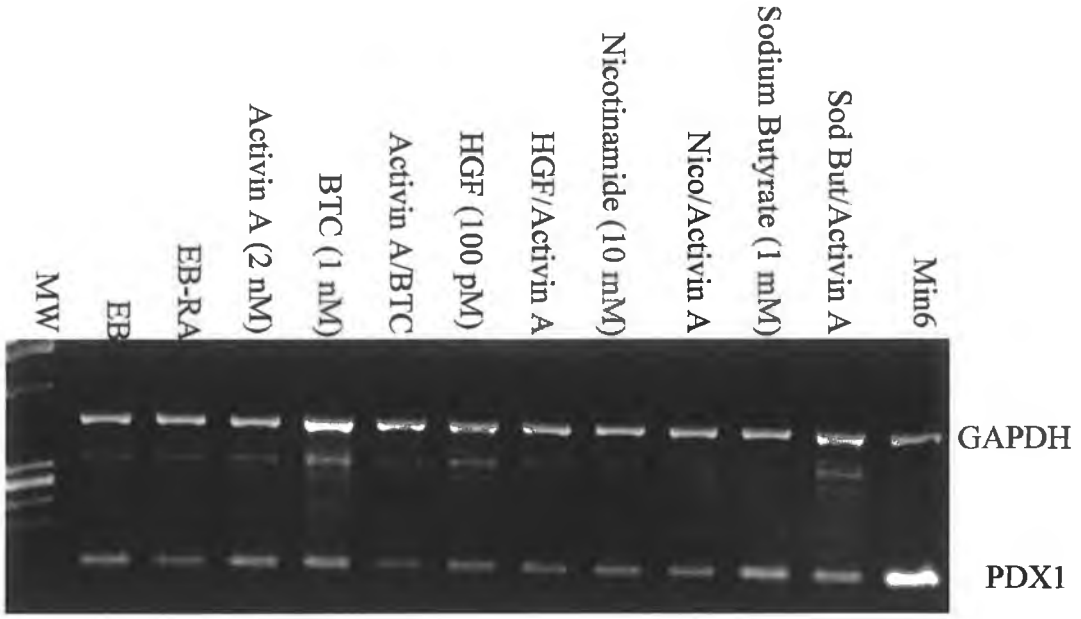
3.2.4.6 Somatostatin Gene Expression in EB-RA Bodies

From Figure 3.2.20 it can be seen that untreated EBs were found to contain low levels of somatostatin transcripts; these levels were not significantly altered in EB-RA cells. EB-RA cells treated with betacellulin contained low levels of somatostatin gene transcripts while combination treatments of activin A with HGF or sodium butyrate resulted in further increases in mRNA levels. In previous experiments activin A has had minor effects on somatostatin expression in monolayers (Figure 3.2.8 & Figure 3.2.14), but the data presented in Figure 3.2.20 suggests that it has a more positive role in inducing levels of somatostatin mRNA.

3.2.4.7 Other Genes Examined in Differentiated EBs

The expression levels of a number of other pancreatic genes were examined in EB-RA bodies, however these genes were not found to be expressed in either F9 cells and were not induced as a result of any of the treatments. The other genes investigated were:

- GLUT2
- Glucokinase
- Pax4
- Sonic Hedgehog (Shh)
- Glucagon



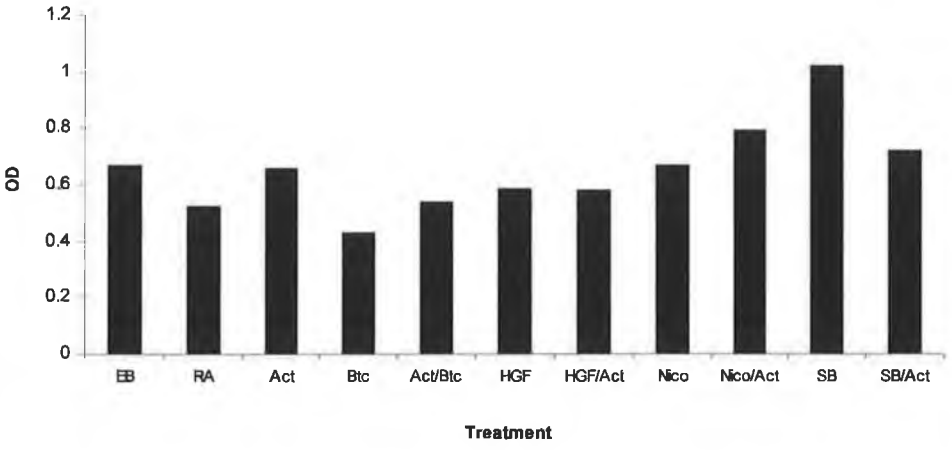
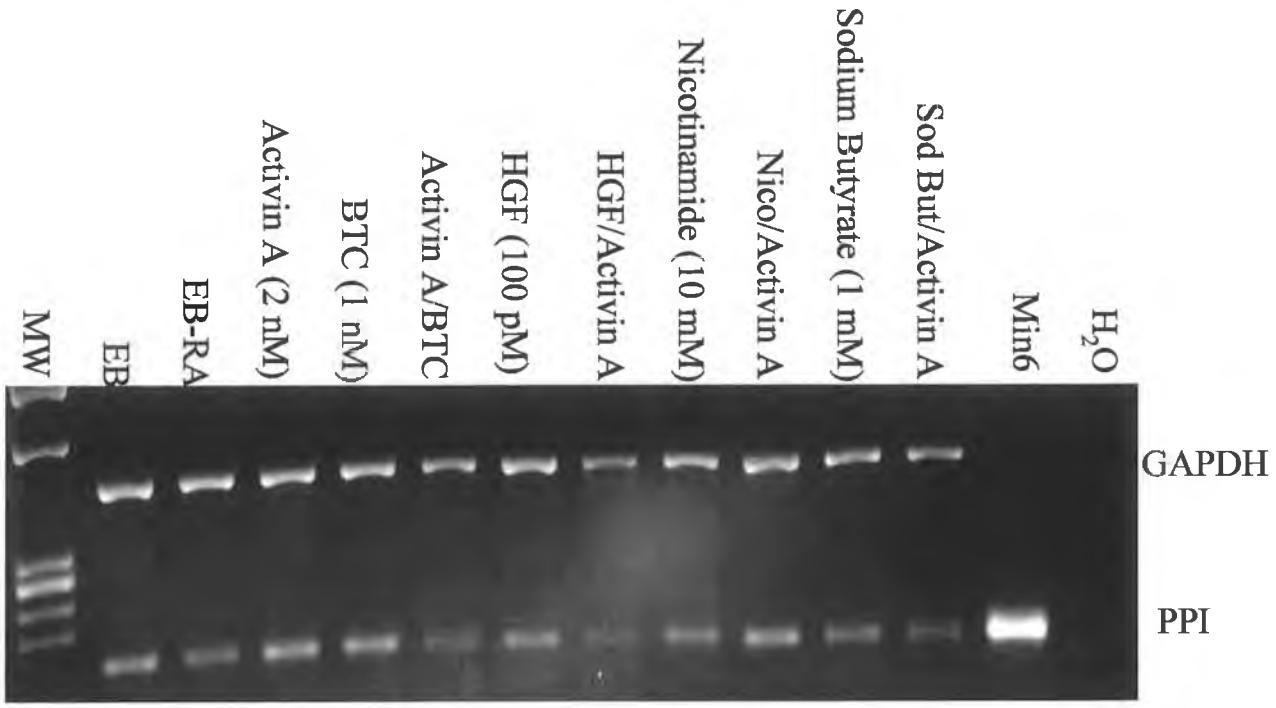


Figure 3.2.15 PDX1 expression in EB-RA cells treated with endocrine differentiation agents. Untreated EBs are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



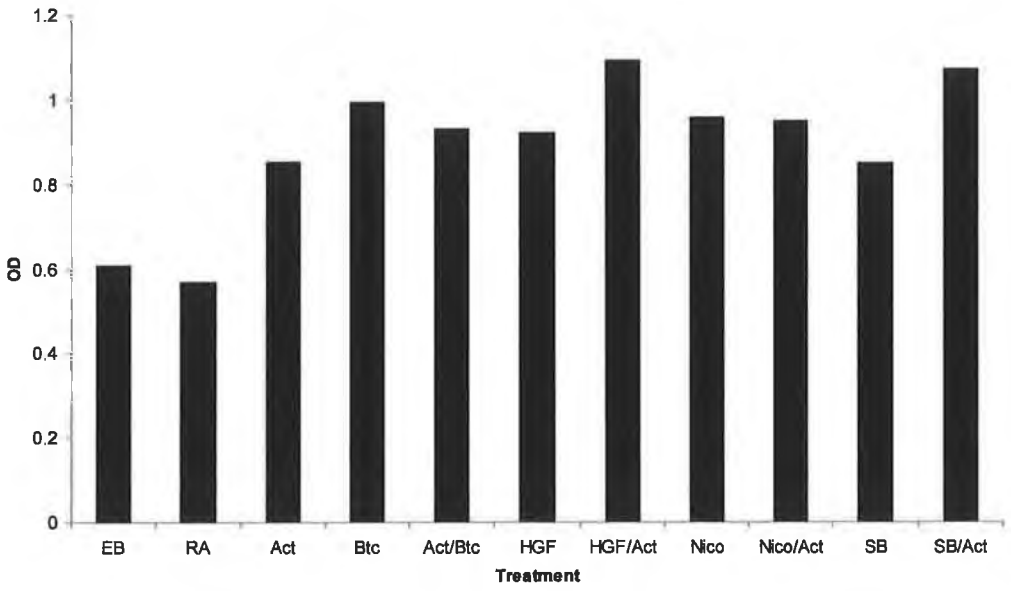
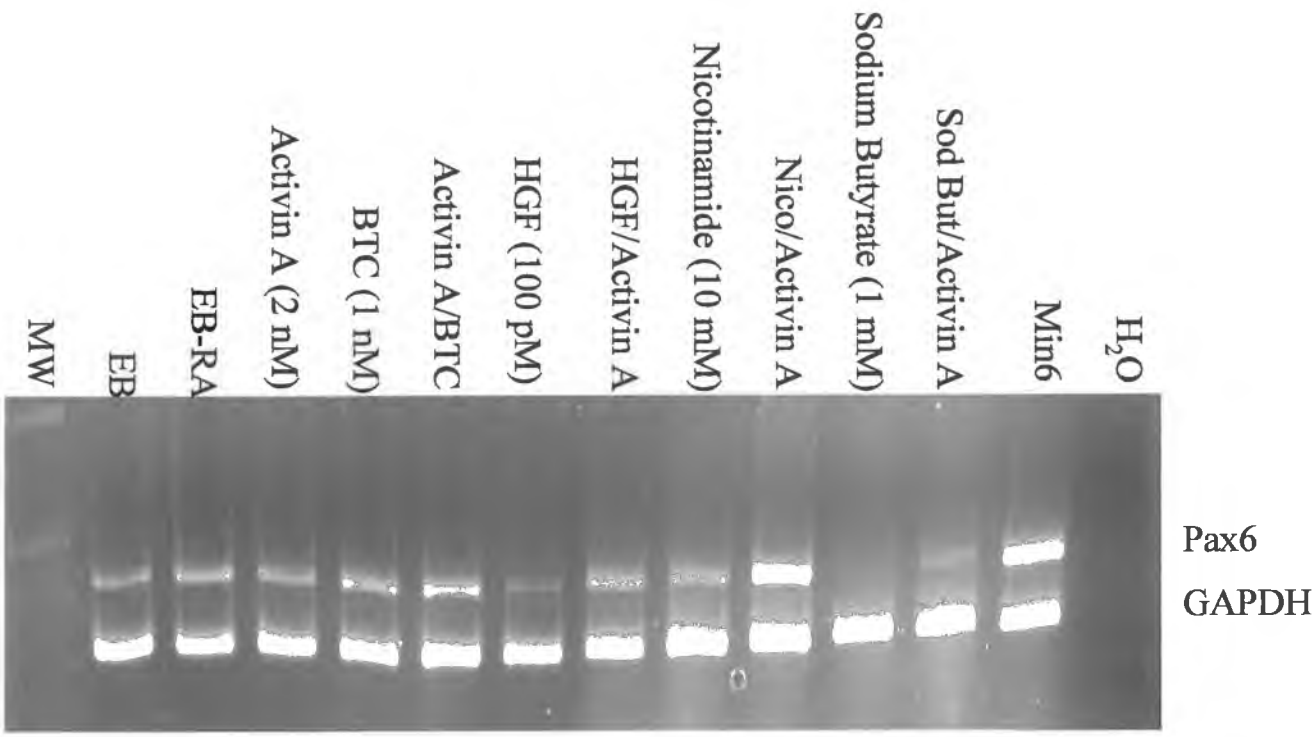


Figure 3.2.16 PPI expression in EB-RA cells treated with endocrine differentiation agents. Untreated EBs are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



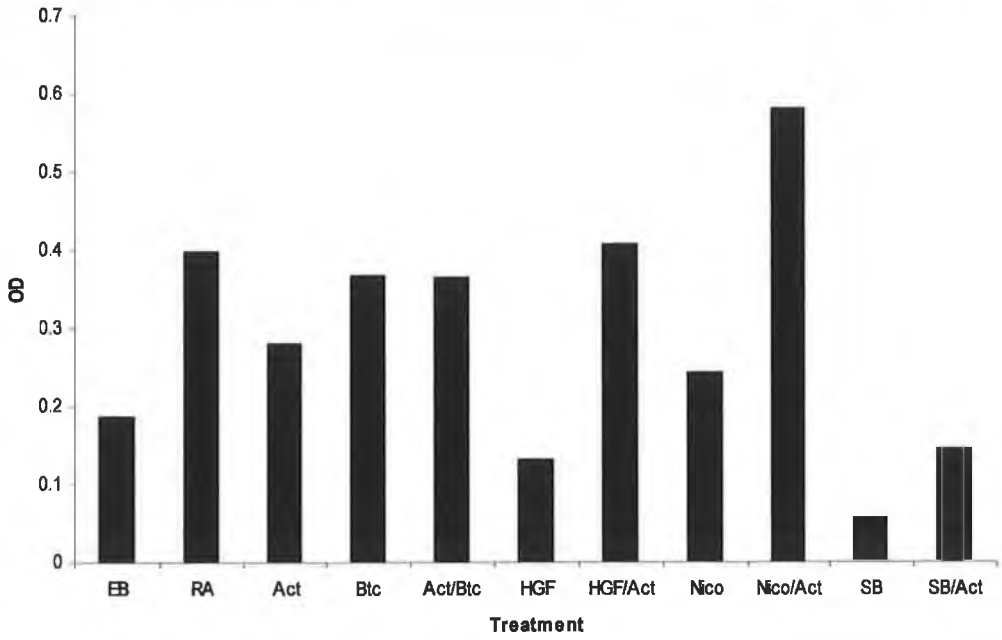


Figure 3.2.17 Pax6 expression in EB-RA cells treated with endocrine differentiation agents. Untreated EBs are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.

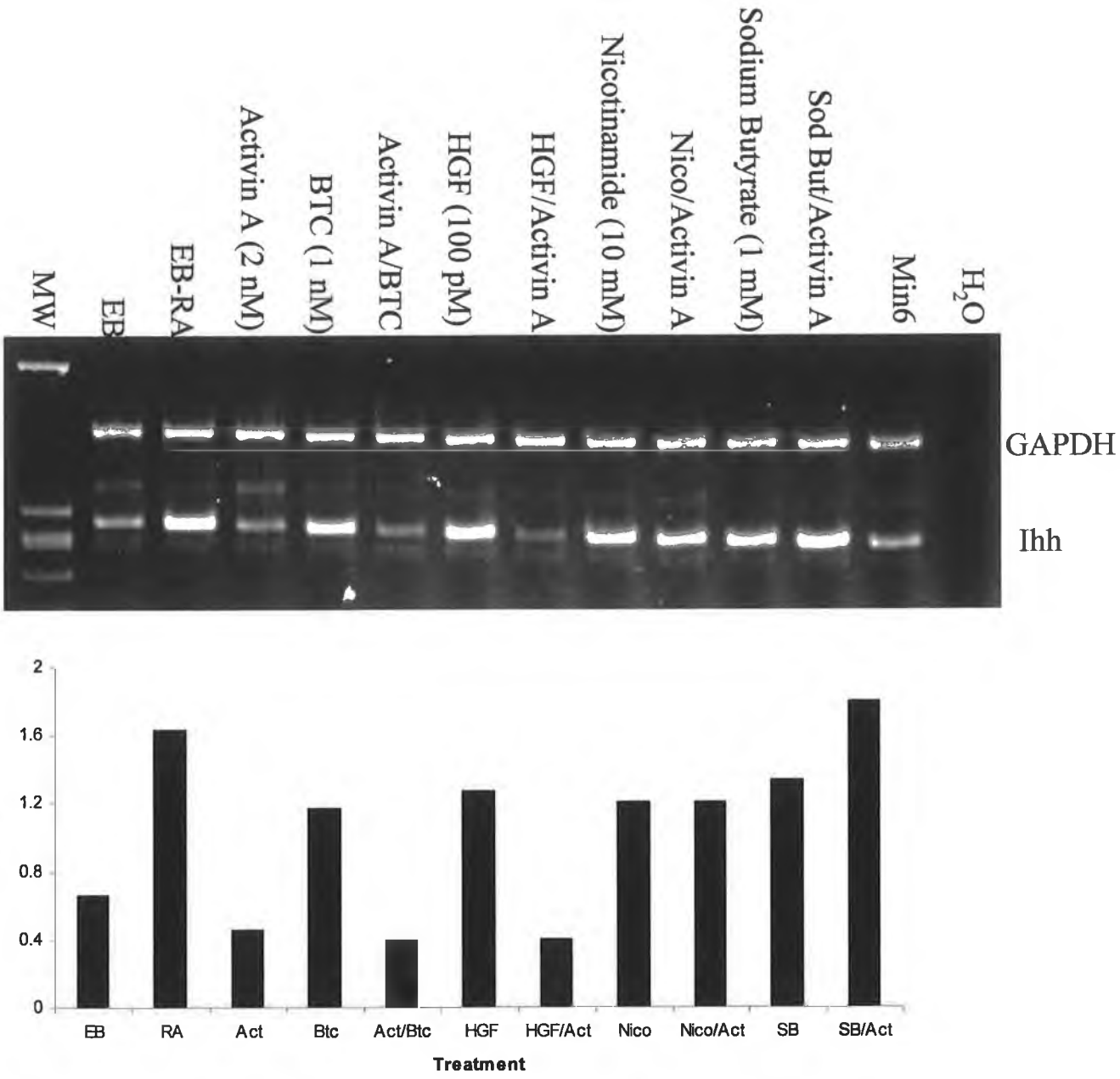
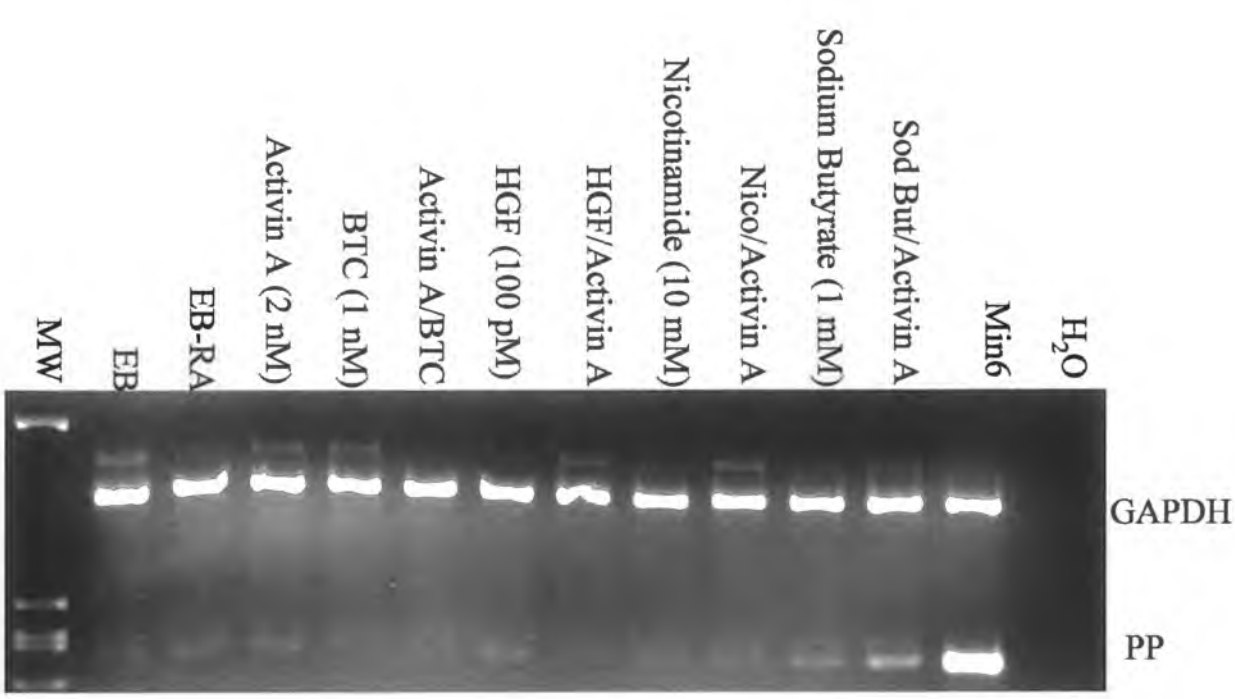


Figure 3.2.18 Indian hedgehog (Ihh) expression in EB-RA cells treated with endocrine differentiation agents. Untreated EBs are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



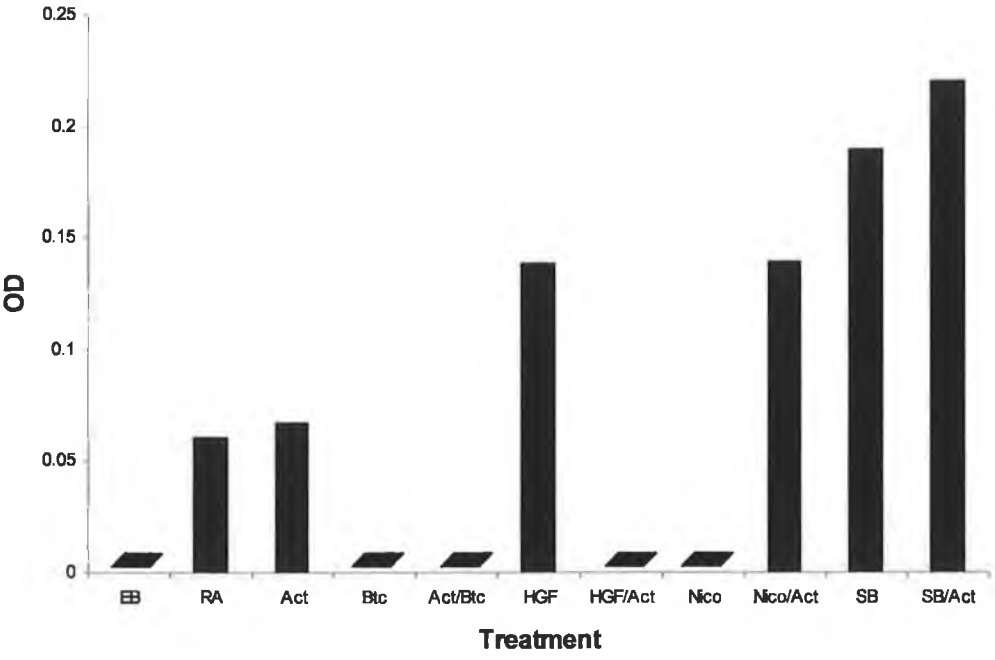
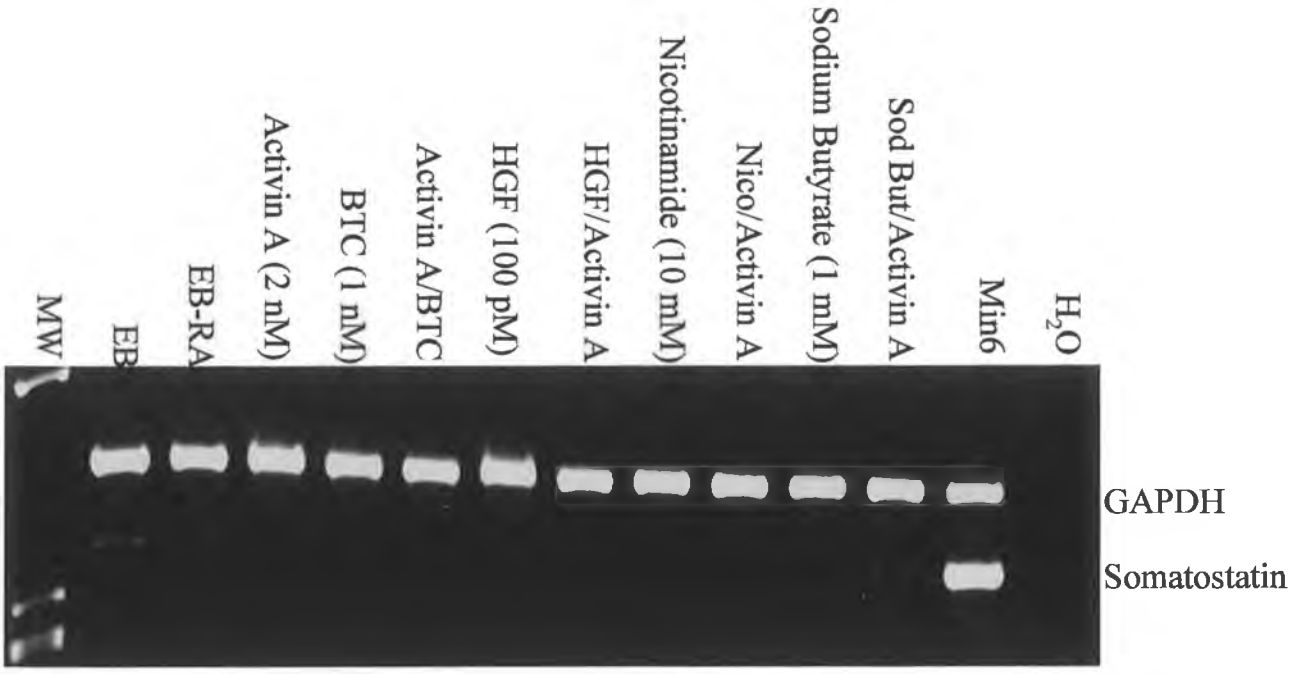


Figure 3.2.19 Pancreatic polypeptide (PP) expression in EB-RA cells treated with endocrine differentiation agents. Untreated EBs are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.



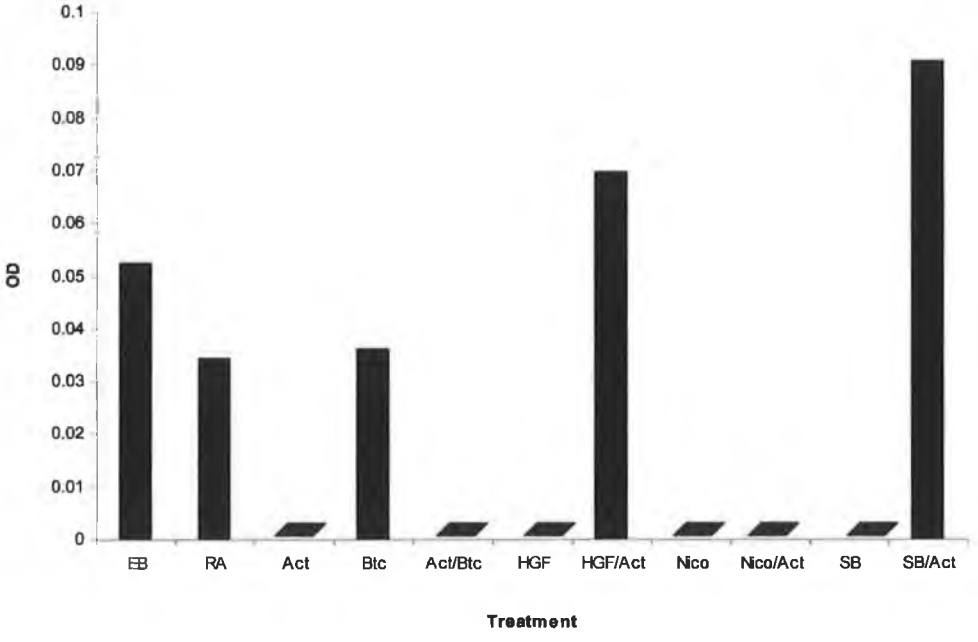


Figure 3.2.20 Somatostatin expression in EB-RA cells treated with endocrine differentiation agents. Untreated EBs are included for comparison. All treatments were carried out over 10 days. Min6 cells and water were used as positive and negative controls, respectively. The results presented are representative of repeat gels from two individual treatment experiments.

	F9-RA		F9-RAC		EB-RA	
Gene	**Highest Level of Expression**	**Lowest Level of Expression**	**Highest Level of Expression**	**Lowest Level of Expression**	**Highest Level of Expression**	**Lowest Level of Expression**
PDX1	HGF/Activin A	N/A*	SB/Activin A	N/A*	SB**	BTC**
PPI	HGF/Activin A	N/A*	SB**	HGF/Activin A	HGF/Activin A ***	N/A*
Pax6	Nicotinamide (Nico) or Nico/ Activin A	Activin A/ Betacellulin or HGF/Activin A	Nicotinamide or Nico/ Activin A	RAC	Nico/ Activin A	HGF, SB or SB/Activin A
Ihh	RA	N/A*	Sodium Butyrate***	N/A*	RA or SB/Activin A	Activin A/ Betacellulin or HGF/Activin A
PP	Sodium Butyrate (SB) or SB/Activin A	N/A*	Sodium Butyrate or SB/Activin A	N/A*	Sodium Butyrate or SB/ Activin A	N/A*
Somato-Statin	Sodium Butyrate (SB) or SB/Activin A	N/A*	Sodium Butyrate (SB) or SB/Activin A	N/A*	SB/Activin A	A Number of Treatments Showed no PP Expression
GLUT2	Negative	Negative	Negative	Negative	Negative	Negative
Glucokin-ase	Negative	Negative	Negative	Negative	Negative	Negative
Pax4	Negative	Negative	Negative	Negative	Negative	Negative
Shh	Negative	Negative	Negative	Negative	Negative	Negative
Glucagon	Negative	Negative	Negative	Negative	Negative	Negative

Notes:

N/A* : indicates no reduction below level of untreated cells

** Treatment shows largest induction but is not significantly (>1.5 fold) higher than untreated cells.

*** Treatment shows highest levels of mRNA, but many other treatments similar.

Table 3.2.2 Summary of treatments resulting in highest/lowest levels of expression of the genes studied in the three differentiation systems i.e. F9-RA, F9-RAC and EB-RA cells.

3.2.5 Protein Expression Following Secondary Differentiation of F9 Monolayers and Embryoid Bodies

As the expression levels of a number of genes were increased/induced following secondary differentiation of F9 monolayers and EBs, it was decided to investigate the expression of these genes at the level of protein expression. Levels of PPI, PDX1, PP and somatostatin protein were investigated using immunofluorescence /immunocytochemistry (and EIA in the case of PPI). PDX1 and PPI protein were found to be negligible (even in cases where transcript levels were found to be significantly upregulated e.g. F9-RA monolayers in the presence of HGF and activin A, Figures 3.2.3 & 3.2.4). Somatostatin and PP levels were investigated in F9-RA cells treated with sodium butyrate and the combination of sodium butyrate and activin A (these treatments demonstrated the greatest induction levels of these mRNAs (Figures 3.2.7 & 3.2.8) and the protein expression levels are displayed below (Section 3.2.5.1 & Section 3.2.5.2).

Acetylated Histone H3 protein levels were also investigated in sodium butyrate treated cells to investigate a possible mechanism for the induction of these genes at an epi-genetic level.

3.2.5.1 Cellular Localisation of Pancreatic Polypeptide (PP) Protein in F9-RA cells Treated with Sodium Butyrate and Activin A

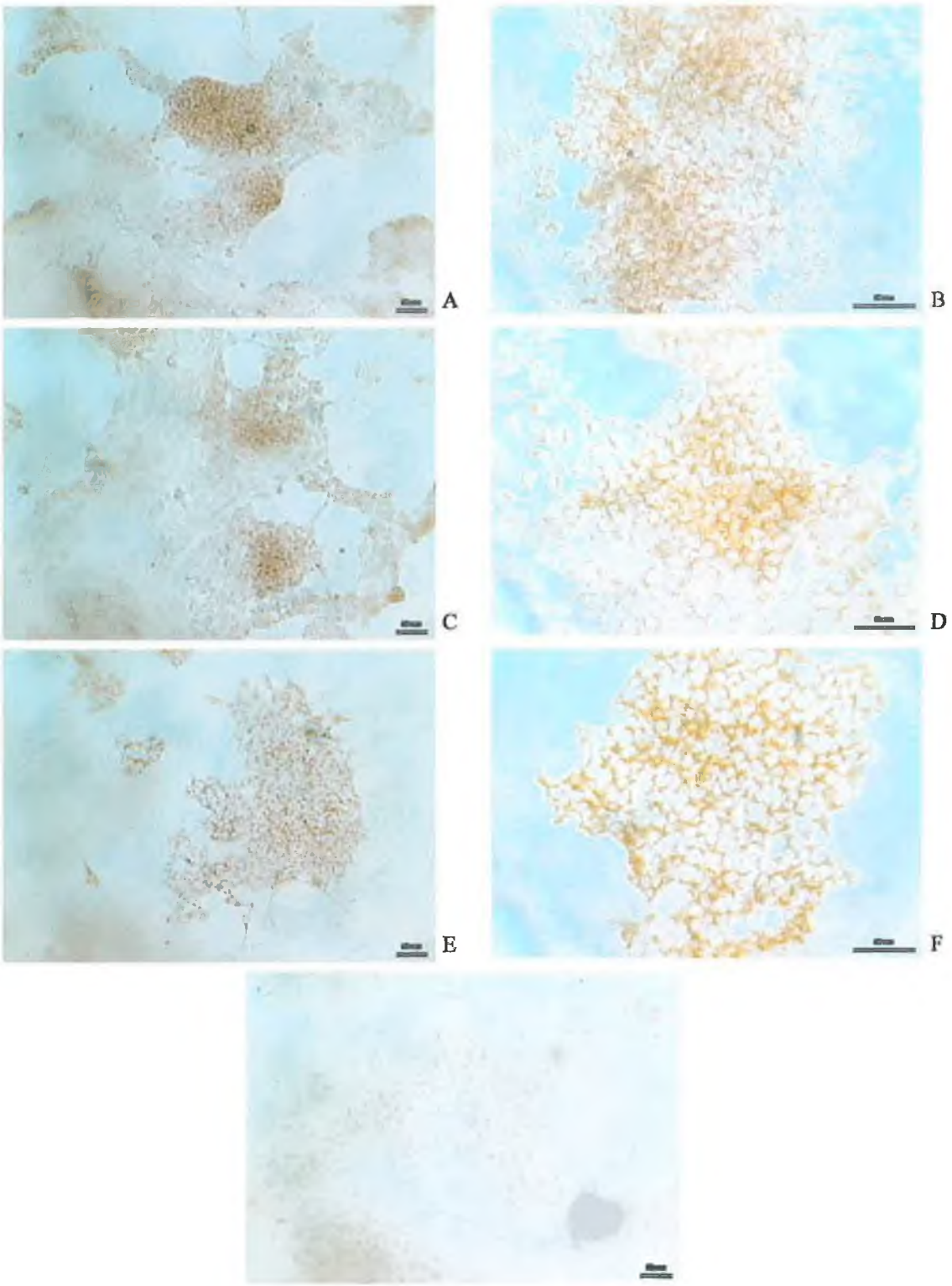


Figure 3.2.21 Pancreatic polypeptide expression in F9-RA cells treated with Sodium Butyrate (A & B) and a combination of Sodium Butyrate and Activin A (C & D). Min6 cells were included as positive controls (E & F) while untreated F9 cells were used as a negative control (G). PP staining is present in localised clumps of treated F9 cells whereas it is widespread throughout the Min6 cell population. Staining is diffuse and is predominantly cytoplasmic. Magnification = 20 X (A, C, E & G) and 40 X (B, D & F). Bar = 50μM in all cases.

3.2.5.2 Somatostatin Localisation within F9 cells treated with Sodium Butyrate and Activin A

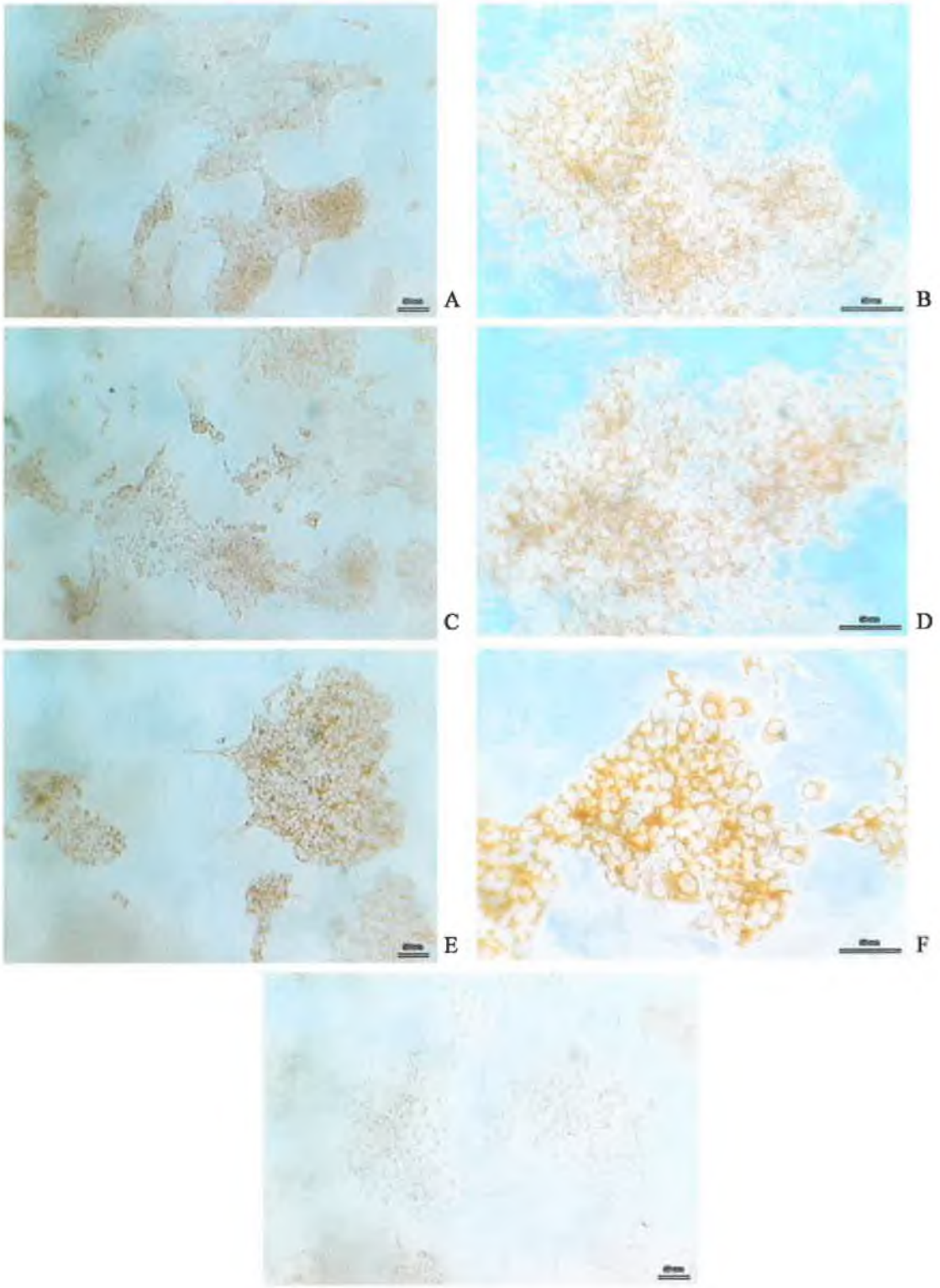


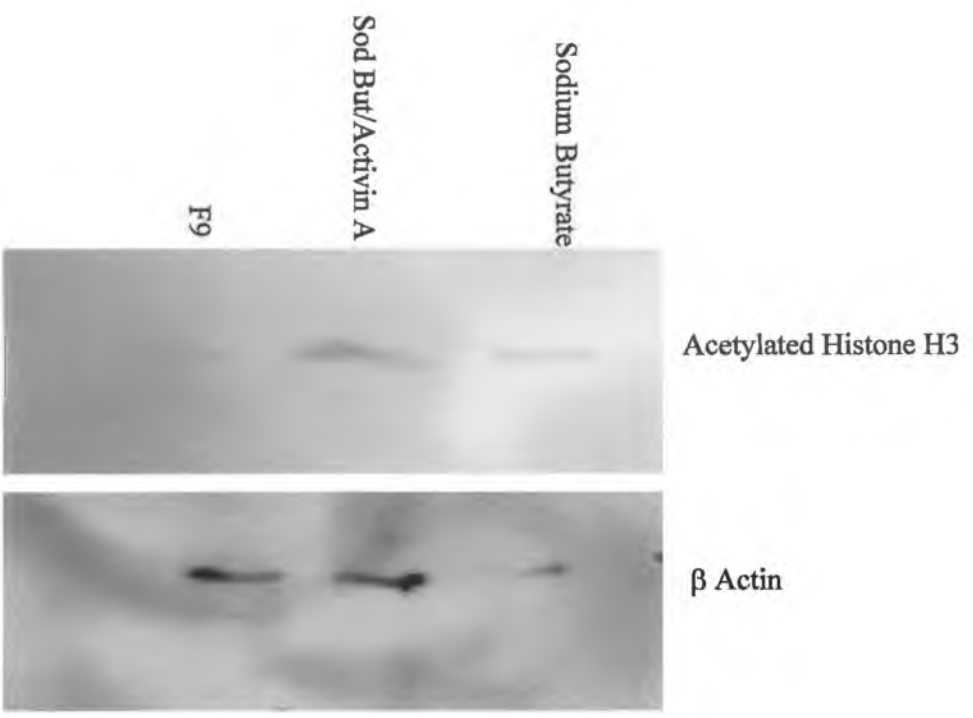
Figure 3.2.22 Somatostatin staining can be clearly be identified in localised clumps of cells in F9-RA cells treated with Sodium Butyrate (A & B) or a combination of Sodium Butyrate and Activin A (C & D). Min6 cells have been used as positive (E & F) and untreated F9 cells (G) as a negative controls respectively. Staining is diffuse in the treated F9 cells and is predominantly cytoplasmic. Magnification = 20 X (A,C, E & G) and 40 X (B, D & F). Bar = 50 μM in all cases.

3.2.5.3 Acetylated Histone H3 Expression levels in F9-RA Monolayers Treated with Sodium Butyrate and Activin A

Sodium butyrate treatment alone (and in combination with activin A) led to expression of PP and Somatostatin both at the transcriptional and translational levels. n-Butyrate is a naturally occurring short chain fatty acid produced in the colon through fermentation of dietary fibre and has already been identified as a potent agent in β cell differentiation (Otonkoski *et al*, 1999). n-Butyrate is a member of a class of compounds known as histone deacetylase (HDAC) inhibitors, a group of compounds known to be inducers of growth arrest and differentiation. By inhibiting HDAC, histone acetylation (and in certain cases, protein acetylation) is increased. Actively transcribed genes are associated with chromatin fractions enriched in highly acetylated core histones. Thus to determine if sodium butyrate was having such an effect on F9-RA cells, levels of acetylated histone H3 were examined using western blotting.

Preliminary results from western blot analysis indicate that sodium butyrate caused an increase in the levels of acetylated histone H3. Densitometric analysis indicates that the level of acetylated histone H3 increase 2-3 fold when F9-RA cells were treated with Sodium Butyrate and a combination of Sodium Butyrate and Activin A respectively (Figure 3.2.23).

This western blotting was carried out by Dr. Finbar O' Sullivan.



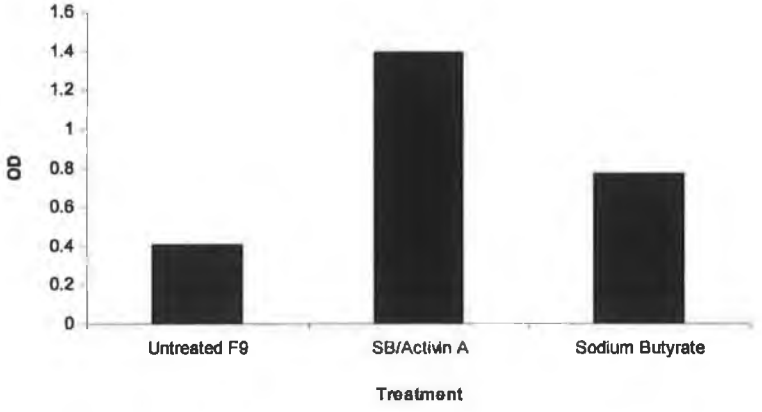


Figure 3.2.23 Comparison of acetylated histone H3 expression levels between untreated F9 cells and F9 cells exposed to two-step differentiation involving RA (10^{-7} M) , Sodium Butyrate (1 mM) and Activin A (2nM).

3.3 Differentiation of F9 cells using Gene Transfer Technology and Endocrine Differentiation Treatments

Transfection of the c-fos proto-oncogene in to F9 cells has been shown to duplicate some of the effects of RA differentiation (Muller & Wagner, 1984). Transcription factor expression has also been used in directed differentiation experiments with AR42J cells to generate insulin secreting cell lines, thus it is clear that directed differentiation can be modified and refined by direct overexpression of relevant genes. To investigate such effects in F9 cells, a construct was generated to enable isolation of PDX1 overexpressing cells from transfected cell populations, following secondary endocrine differentiation. It was hoped that these cells would provide an interesting model of β cell differentiation.

To investigate the effects of overexpression of other genes/transcription factors believed to be essential for pancreas/islet cell development, expression plasmids containing PDX1, Beta2, Ngn3 and Nkx2.2 cDNAs were obtained. Transient transfection protocols were designed to investigate the effects of their expression on endogenous gene(s) and pancreatic marker(s) expression in partially differentiated monolayers.

3.3.1 The Cell Trap Construct

The cell trap construct is designed with two antibiotic resistance genes, one of which is driven by a constitutive promoter and the other under the control of a tissue specific promoter. The initial transfectants, i.e. the cells that have integrated the construct can be isolated and purified using the antibiotic under the constitutive promoter. Cells that assume appropriate lineages that enable them to express the tissue specific gene of interest (i.e. PDX1 in this case) can subsequently be selected due to their acquired resistance to the antibiotic encoded by the resistance gene under the control of that promoter.

The cell trap construct employed in these experiments (PGK-TKCAT-Zeo/pBS) was designed from cDNAs available in our laboratory and material donated from an

outside source (see * below). The component plasmids and the design specifications were sent to Cytomyx (Cambridge), who constructed the final vector. The plasmids used and the sequence of steps in building the construct are outlined in Figure 3.3.1. The construct was generated by initially digesting the PGK-Neo plasmid with XhoI/KpnI, the 2 kb fragment containing the G418 resistance gene under the control of the constitutive pGK promoter was then subcloned into the multiple cloning site (MCS) in the expression plasmid pBluescript. The cDNA encoding the PDX1 promoter region was excised from the PstBstII[Sense]pTKCAT(An) plasmid (*generously supplied by Roland Stein, Vanderbilt Medical Centre, Tennessee) using HindIII and BamHI, and subsequently cloned in to the pBS-pGK-Neo plasmid. The gene encoding resistance against zeocin was copied from the pcDNA3.1/Zeo plasmid using PCR. The primers in this reaction were also designed to generate a BamHI site at the 5' end of the product and a NotI site at the 3' end. This product was then subcloned into these sites in the pBS-pGK-TKCAT plasmid to complete the construct.

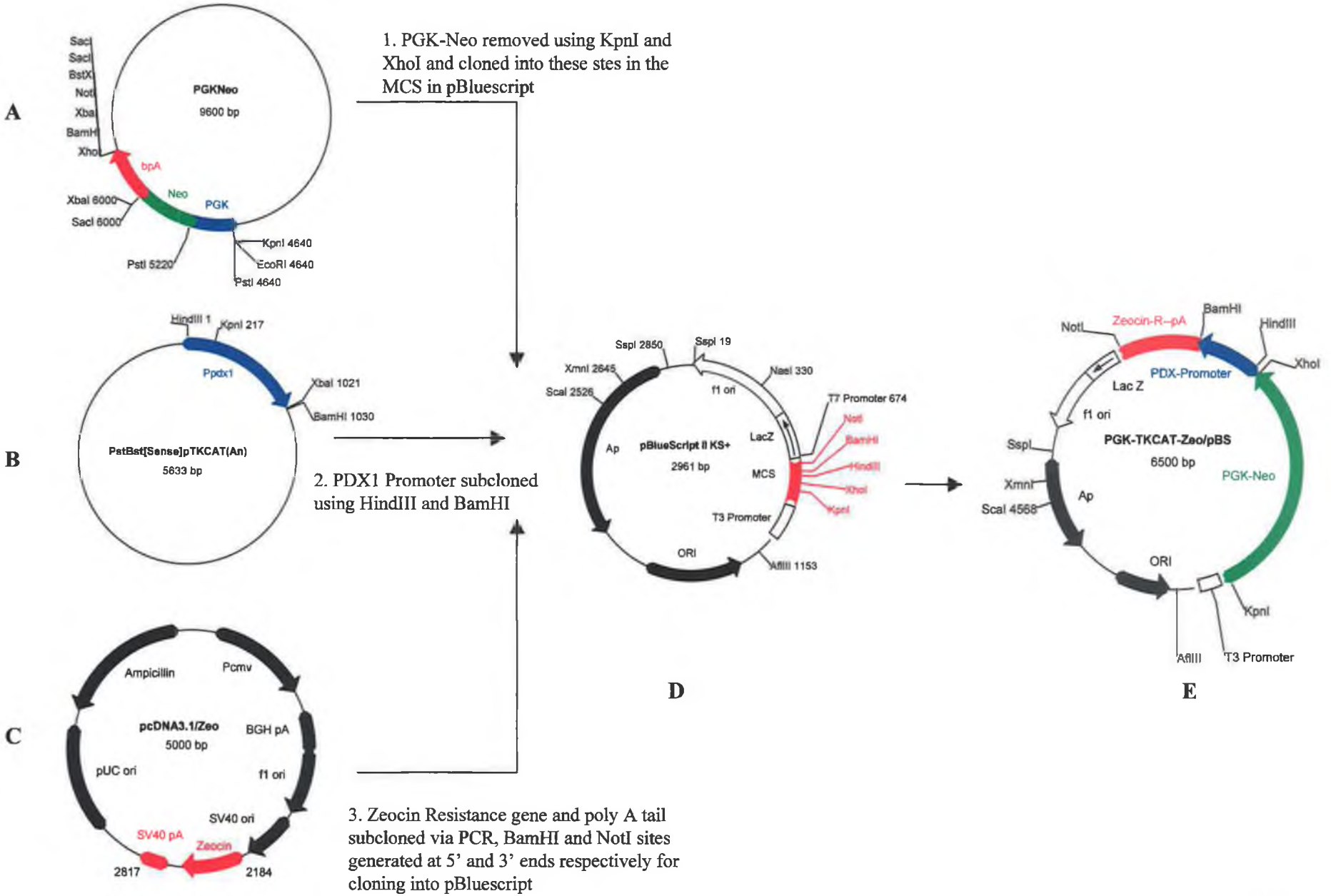


Figure 3.3.1 Generation of the cell trap construct PGK-TKCAT-Zeo/pBS (E) using PGK-Neo (A), PstBst[Sense]pTKCAT(An) (B), pcDNA3.1/Zeo (C) and pBluescript II KS+ (D).

3.3.1.1 Restriction Analysis of the Completed Construct

To verify that the PGK-TKCAT-Zeo/pBS construct was correctly assembled, restriction analysis was carried out (Section 2.4.4). The digests are discussed below and refer to Figure 3.3.2

NotI/KpnI: This cut was designed to remove all of the subcloned DNA from the pBluescript backbone (2.9 kb). The presence of three bands is due to a second KpnI site within the PDX1 promoter region (Figure 3.3.1 (B)). Thus the two lower bands represent (I) a fragment NotI-KpnI containing the zeocin resistance gene and most of the PDX1 promoter region, and (II) a KpnI-KpnI fragment containing mostly the G418 resistance gene with a small portion of the PGK promoter.

BamHI/NotI: This digest was designed to remove the zeocin resistance gene (652 bp) from the construct. The bands of 652 bp and 5.9 kb are present as expected.

BamHI/HindIII: The 1 kb fragment corresponds to the PDX1 promoter region being excised from the construct. The remainder of 5.5.kb is correct.

Based on the data from these restriction digests it is clear that the plasmid was generated to the exact specifications.

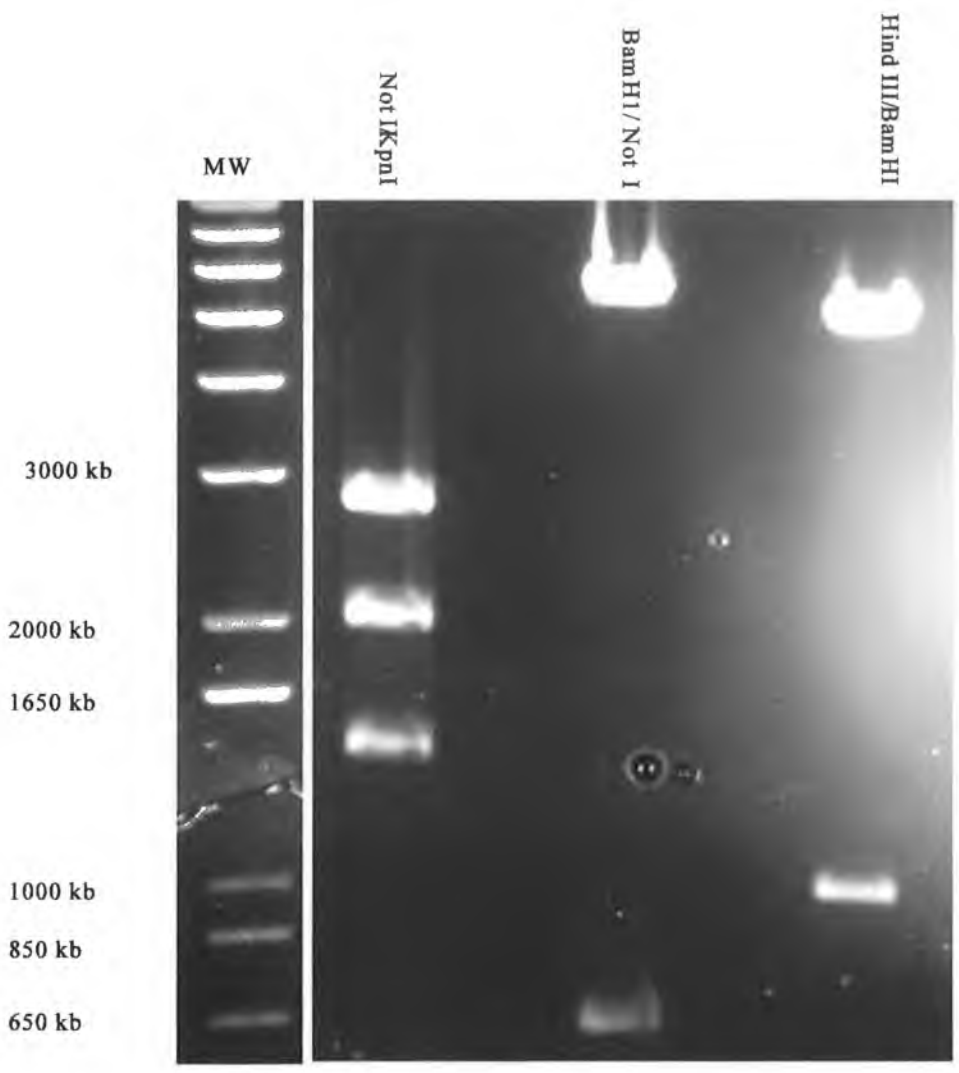


Figure 3.3.2 Restriction digests of the cell trap construct received from Cytomyx. Restriction digests were carried out for 1 hour at 37 °C in a water bath. 10 µl of digested product was mixed with 2 µl of 6X loading dye and subsequently loaded on to an agarose long gel (0.8 %).

3.3.1.2 Validation of the Cell Trap Construct

The cell trap construct PGK-TKCAT-Zeo/pBS presented should impart immediate G418 resistance upon transfection in to any cell line. Zeocin resistance will depend on the cells ability to drive the PDX1 promoter. Thus experiments were designed to validate the construct using suitable cell lines, i.e. cells with and without PDX1 expression.

The cell trap construct was transfected into Min6 ($PDX1^{+/+}$) and BHK-21($PDX1^{-/-}$) cells (Figure 3.3.3). Following this transfection, cells were exposed to zeocin at levels of 100 μg/ml for BHK-21 and 500 μg/ml for Min6 (levels were chosen from preliminary toxicity assays (Section 2.3.2) to determine sensitivities of the cells to zeocin). A significant number of transfected Min6 cells (Min6CT) cells survived while non-transfected Min6 were wiped out. All BHK-CT and BHK-21 cells were killed. To clarify that the PDX1 expressing cells transfected with PGK-TKCAT-Zeo/pBS had actually become resistant to zeocin while those without PDX1 had not, toxicity assays were carried out using non-transfected cells as controls. Thus, re-transfected Min6CT and BHK-CT cells were selected using G418 (400 μg/ml Min6-CT, 1000 μg/ml BHK-CT) to obtain homogenous populations of cells expressing the PGK-TKCAT-Zeo/pBS for the toxicity assays.

All transfected CT cells were found to show total resistance to G418 (Figure 3.3.4 (A&C)), as expected, due to the constitutive nature of the G418 resistance gene. Only Min6CT cells however demonstrated increased resistance to zeocin (Figure 3.3.4 (B)). This result indicated that the cells are expressing at least some level of the zeocin resistance gene (Sh *ble*) and this would be expected as Min6 cells express PDX1. The effect is less than was anticipated however, i.e. when comparing parental Min6 cells to Min6CT cells. The level of resistance also appears to be lower in the stable Min6CT cell lines than in the freshly transfected mixed population tested directly with zeocin. It is presumed that during the serial passaging of the Min6CT cells, while generating the stocks for toxicity assays (approximately 8-9 passages), that PDX1 levels were reduced (an effect noted in other experiments, see Figure 3.4.11(A)). Impaired ability to drive the PDX1 promoter would therefore result in less zeocin

resistance. The level of zeocin resistance of Min6CT cells observed was consistently higher than that of Min6 in a number of repeat experiments. BHKCT cells were resistant to G418 (Figure 3.3.4 (C)) but showed no resistance to zeocin (Figure 3.3.4 (D)). Thus, the cell trap can only confer zeocin resistance to $PDX1^{+/+}$ cell lines.

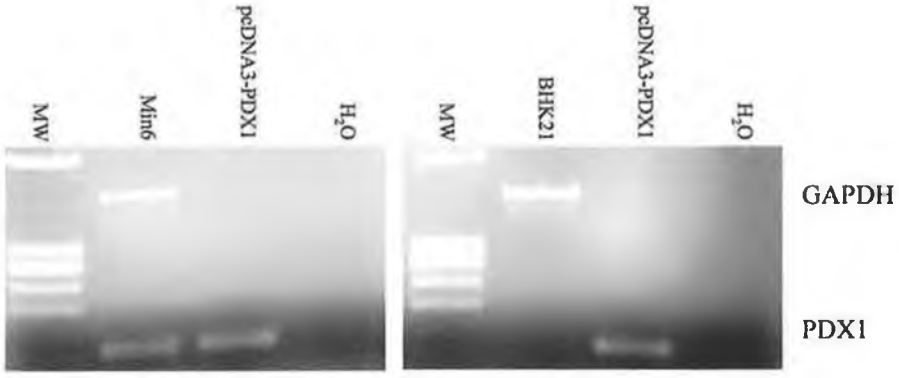


Figure 3.3.3 PDX1 expression levels in Min6 and BHK-21. PDX1 expression is present in the murine β cell line Min6 while the fibroblast line BHK-21 is negative for PDX1 transcripts. 1 ηg PDX1 cDNA in the pcDNA3-PDX1 plasmid and H_2O were used as positive and negative controls respectively.

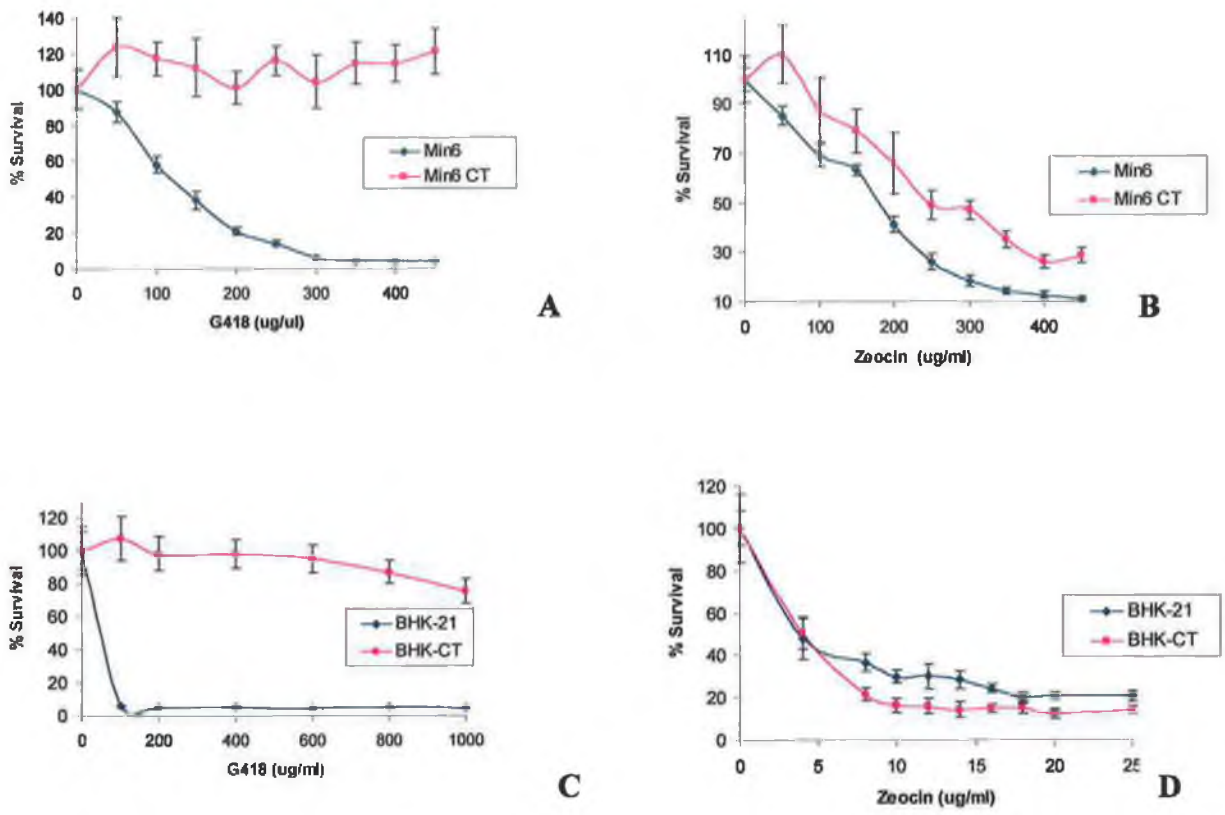


Figure 3.3.4 Toxcicity Assays on Min6 and BHK-21 cells with and without the cell trap construct PGK-TKCAT-Zeo/pBS. The toxicity profiles of G418 (A&C) and Zeocin (B&D) were compared in each case.

3.3.1.3 Generation of Cell Trap Cell Lines

Initially the PGK-TKCAT-Zeo/pBS construct was transfected in to undifferentiated F9 monolayer cells and after 24 hours these cells were selected with increasing levels of G418 (to a maximum of 1000 μg/μl). Stocks of these F9-CT cells were prepared. F9-CT cells were set up as described in Section 2.3.1.2.1 i.e. as F9-RA monolayers and EB-RA bodies. These cells were then either immediately selected with zeocin (25 μg/ml) or treated with a number of agents known to increase PDX1 levels for 5-10 days (Table 3.3.1) prior to zeocin selection (to a final selection concentration of 100 μg/ml). Cells that were differentiated as EBs were grown on gelatin during the selection process.

F9-CT-RA	EB-CT-RA
HGF/Act	Sodium Butyrate
Sodium Butyrate	Nicotinamide/ Act
RA	RA

Table 3.3.1 Treatments carried out on F9-CT and EB-CT cells prior to zeocin selections.

3.3.1.3.1 Zeocin Resistant Cell Lines Isolated

A number of the treatments resulted in cell lines that were initially resistant to 100 μg/ml zeocin; however these cell lines eventually died through gradual cessation of growth as opposed to cytotoxicity. Four monolayer cell lines (two of which originated from the differentiated outer layer of EBs) and 2 suspension EB cell types were found to proliferate normally in 100 μg/ml zeocin. The cell lines generated are tabulated below in Table 3.3.2

Cell Line	Origin
Zeocin Resistant Cells Grown as Monolayers	
F9-CT	PGK-TKCAT-Zeo/pBS expressing F9 cells (untreated before zeocin selection)
F9-CT-RA	F9-CT cells treated for 72 hours in RA (10^{-7} M) before zeocin selection.
EB-CT-N/A_5	EBs grown in DMEM followed by 5 days exposure to a combination of 10 mM nicotinamide and 2 nM activin A. Selection was carried out on gelatin coated wells.
EB-CT-N/A_10	EBs grown in DMEM followed by 10 days exposure to a combination of 10 mM nicotinamide and 2 nM activin A. Selection was carried out on gelatin coated wells.
Zeocin Resistant Cells Grown as Suspension EBs	
EB-CT-RA-N/A(10)	EBs grown in RA (10^{-7} M) followed by 10 days exposure to a combination of 10 mM nicotinamide and 2 nM activin A. Selection was carried out on gelatin coated wells, however these cells were found to grow better as suspension EBs.
EB-CT-N/A(5)	EBs grown in RA (10^{-7} M) followed by 5 days exposure to a combination of 10 mM nicotinamide and 2 nM activin A. Selection was carried out on gelatin coated wells, however these cells were found to grow better as suspension EBs.

Table 3.3.2 Cell lines expressing PGK-FKCAT-Zeo/pBS construct that were selected following treatment and capable of proliferating in the presence of 100 μg/ml zeocin.

3.3.1.3.2 Characterisation of the Isolated CT Cell lines

The cell lines isolated (and the two suspension EB-CTs) were subjected to RT-PCR, immunofluorescence and proinsulin EIA. It was found that although the CT cell lines had increased PDX1 (and in some cases PPI mRNA), that there was no detectable protein expression. In certain cases the level of zeocin used in the selection medium was increased to determine if highly resistant sub populations of cells could be isolated, these cells would then presumably have increased levels of PDX1. In these cases zeocin was employed at 200 μg/ml and 400 μg/ml. However this did not result in further increases in PDX1 mRNA. Two of the monolayer cell lines which were found to express the greatest levels of PDX1 were selected for further analysis; F9-CT-RA and EB-CT-N/A-10. The PDX1 level in F9-CT-RA was slightly higher than that following RA treatment of F9 cells (Figure 3.3.5(A)), (when comparing both to F9 parent cells), however the PDX-1 level in EB-CT-N/A-10 was similar to that of N/A treated EBs (Figure 3.2.15). In the CT cell lines, however, the differentiation agent was no longer present in the medium and the PDX-1 levels were maintained by

the selective pressure of the zeocin. These cell lines were also found to have elevated levels of PPI gene transcripts (Figure 3.3.6), similar to those observed in treated F9 cells and EBs (Figures 3.2.4. & 3.2.16).

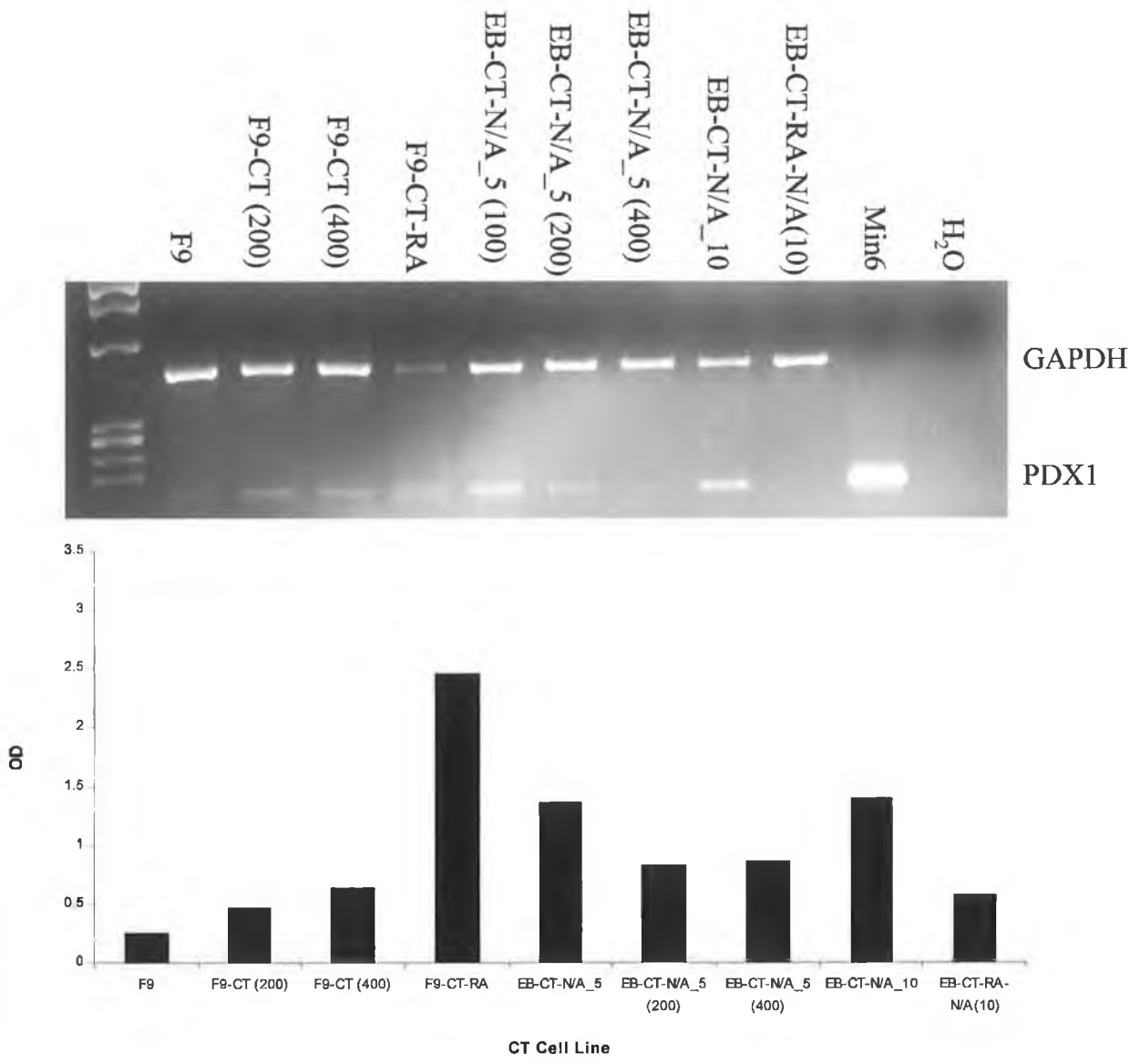


Figure 3.3.5 Characterisation of PDX1 mRNA levels in F9-CT cell lines and differentiated variants. Where the zeocin concentration used in the selection process was in excess of the typical selection concentration of (100 μg/ml) the concentration used is indicated in parentheses after the cell line name. F9 cells were included for comparison. Min6 cells and water were employed as positive and negative controls, respectively.

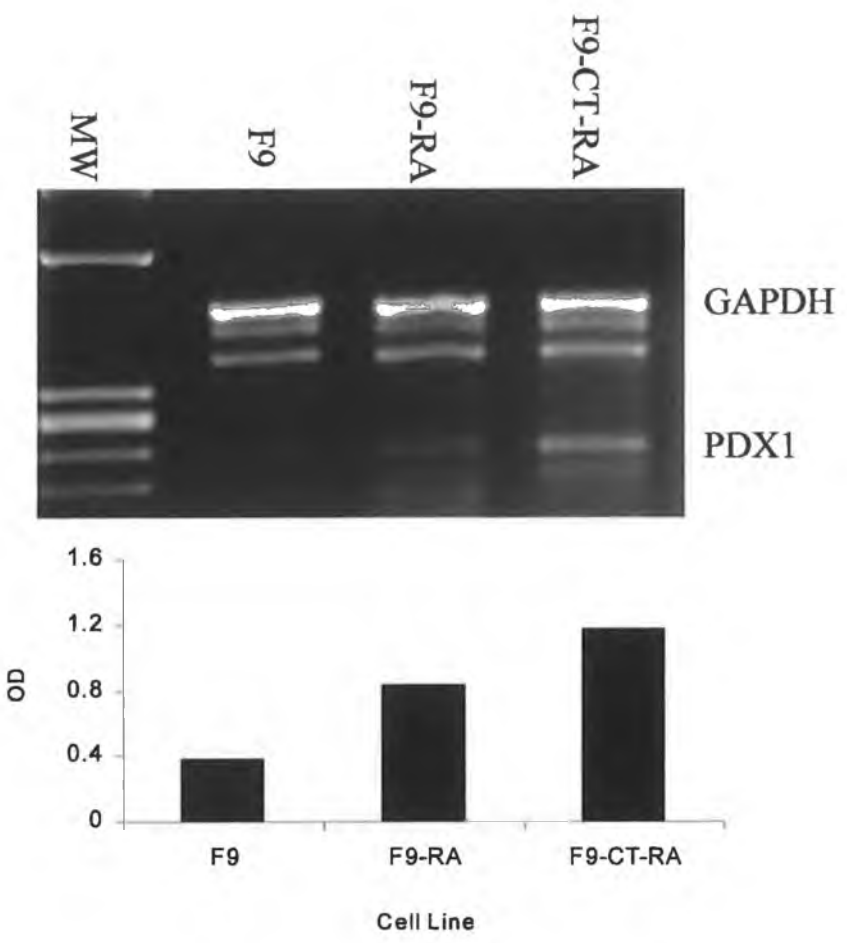


Figure 3.3.5(A) Comparison of PDX1 levels in F9, RA treated F9 cells and in RA-treated F9 cells subsequently passed through cell trapping.

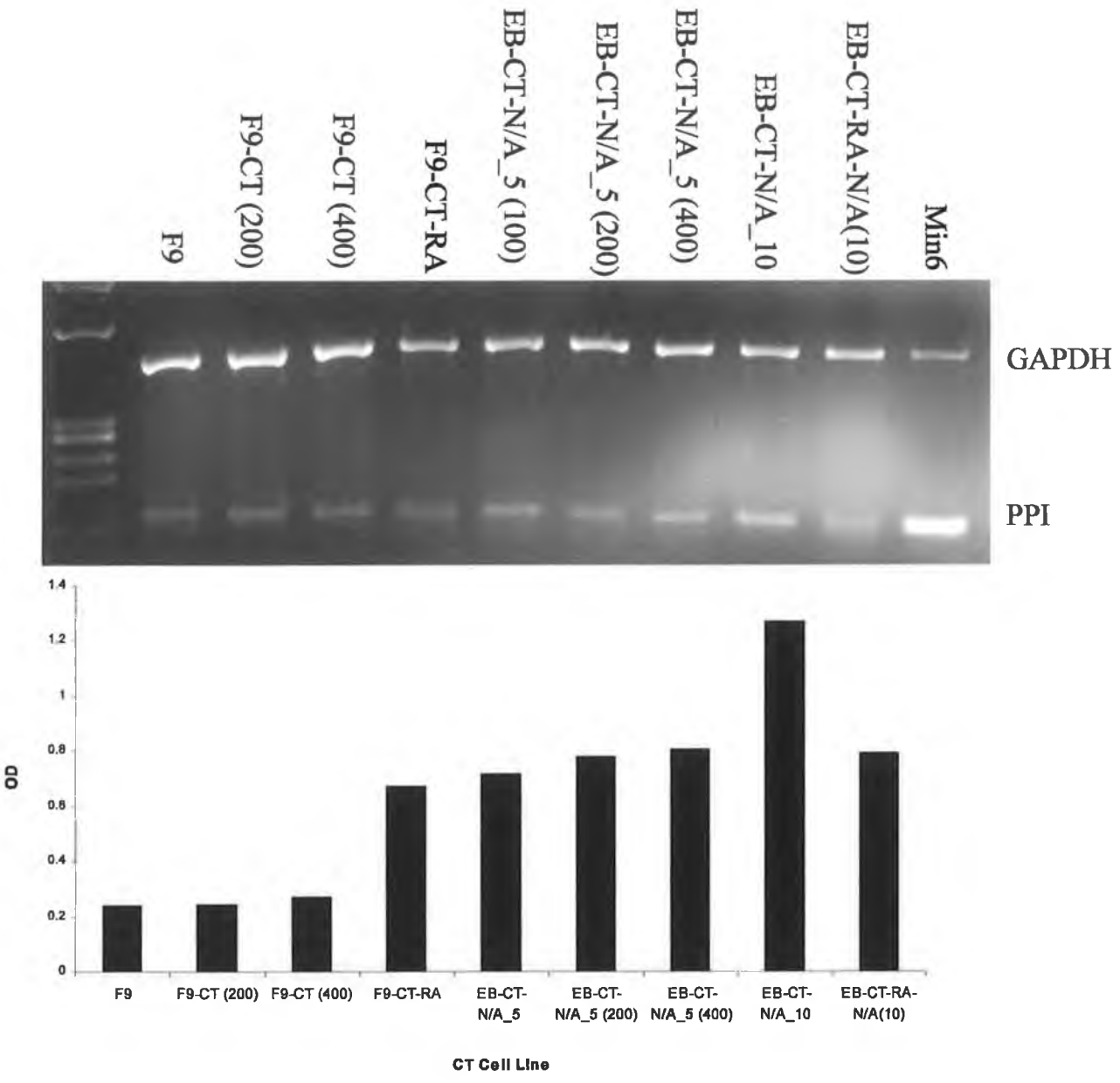


Figure 3.3.6 Characterisation of PPI levels in F9-CT cell lines and differentiated variants. Where the zeocin concentration used in the selection process was in excess of the typical selection concentration of (100 μg/ml) the concentration used is indicated in parentheses after the cell line name. F9 cells were included for comparison. Min6 cells and water were employed as positive and negative controls, respectively.

3.3.1.4 Secondary Differentiation of Selected Cell Trap Cell Lines

As shown in Section 3.3.1.3.2 the cell trapping protocol resulted in overexpression of PDX1 at the transcriptional level, which in turn caused an increase in PPI expression. However as the cells did not synthesise or secrete insulin (when analysed by immunofluorescence or EIA), it was decided to treat the with agents already established during the course of this study as differentiating F9 monolayers towards an endocrine fate (e.g. in Section 3.2 treatments involving sodium butyrate and activin A were shown to result in somatostatin and PP expression). As already indicated the cell lines selected for further analysis were F9-CT-RA and EB-CT-N/A_10, the treatments used are listed below:

- RA (10^{-7} M)
- HGF (100 pM)/Activin A (2 nM)
- Sodium Butyrate (1 mM)
- Sod But (1 mM) /Activin A (2 nM)

All treatments were preceded by 72 hours initial differentiation with 10^{-7} M RA, the subsequent secondary differentiation was carried out over 10 days.

3.3.1.4.1 PDX1 Expression in Treated CT Cells

As shown in Figure 3.3.7, PDX1 expression was increased upon RA treatment of both F9-CT-RA and EB-CT-N/A_10. The other treatments carried out did not alter the level of PDX1 expression compared to untreated F9-CT-RA cells or EB-CT-N/A_10. Thus to clarify, the untreated cells on Figure 3.3.7 are untreated F9-CT-RA cells and untreated EB-CT-N/A_10, respectively. Parental F9 cells are not represented on these gels (Figures 3.3.7-3.3.10 inclusive).

3.3.1.4.2 PPI Expression in Treated CT Cells

From Figure 3.3.8 it is clear that the expression of the PPI gene was increased following RA treatment in F9-CT-RA. There was a slight increase in PPI expression in EB-CT-N/A_10 following RA treatment; however it was not as large.

3.3.1.4.3 PP Expression in Treated CT Cells

As with the other monolayer cell lines examined, PP expression was absent in the CT cells and was only induced following sodium butyrate treatment (either with or without the presence of activin A) (Figure 3.3.9). PP transcript levels were similar in CT cell lines treated with sodium butyrate alone, and in combination with Activin A.

3.3.1.4.4 Somatostatin Expression in Treated CT Cells

From Figure 3.3.10 it is clear that somatostatin expression was present in untreated F9-CT-RA cells and also in untreated EB-CT-N/A_10 cells. Somatostatin transcripts were not found to be present following RA treatment of F9 cells (Figure 3.2.8) or following Nico/Act treatment of EBs (Figure 3.2.20), and it is known that somatostatin is not expressed in F9 cells (Figures 3.2.8 & 3.2.14). Thus it appears that the PDX1-rich CT cells co-express somatostatin at detectable levels. RA treatment of F9-CT-RA cells resulted in the loss of somatostatin expression, all other treatments did not increase expression levels above those already present in the untreated cells. The proposed mechanism of somatostatin activation in CT cells is discussed in Section 4.4.3.2.

3.3.1.5 Proinsulin Synthesis in Treated CT Cell Lines

EIA analysis and immunofluorescence microscopy failed to detect any proinsulin in either of the treated cell lines, even though the cells were induced to increase PPI transcript levels though the combination of the cell trapping protocol and the subsequent treatments.

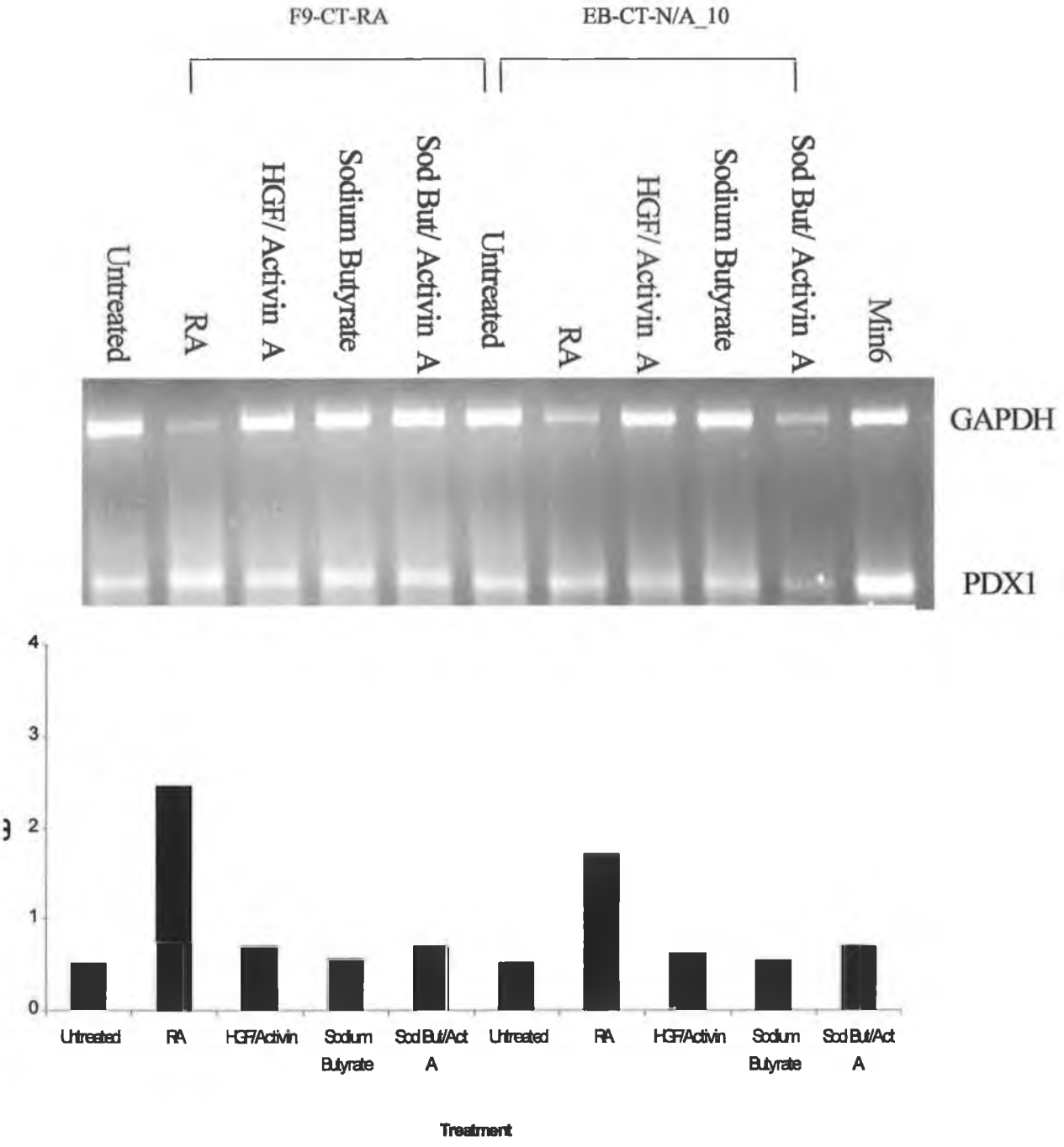


Figure 3.3.7 PDX1 expression in CT cell lines treated with a selection of secondary endocrine differentiation agents. Min6 cells were used as a positive control. The result presented is representative of a number of repeat gels.

GAPDH
PPI
Min6
Sod But/Activin A
Sodium Butyrate
HGF/Activin A
RA
Untreated
Sod But/Activin A
Sodium Butyrate
HGF/Activin A
RA
Untreated
EB-CT-N/A_10
F9-CT-RA
2
16

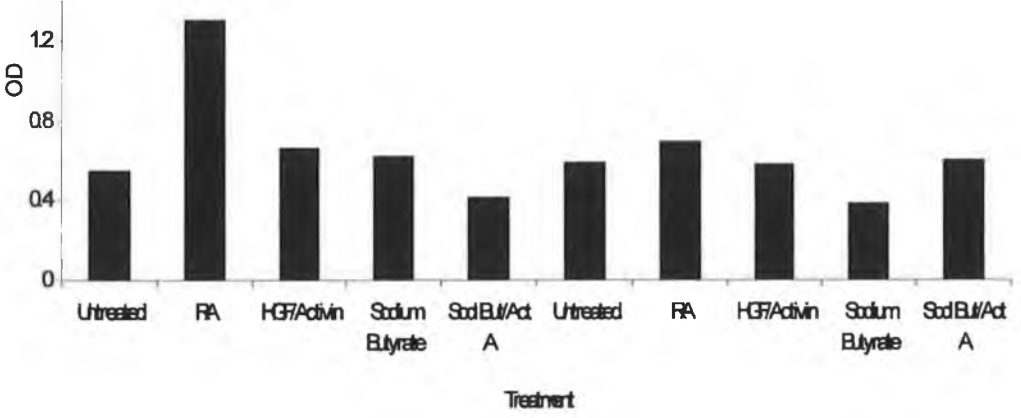


Figure 3.3.8 PPI expression in CT cell lines treated with a selection of secondary endocrine differentiation agents. Min6 cells were used as a positive control. The result presented is representative of a number of repeat gels.

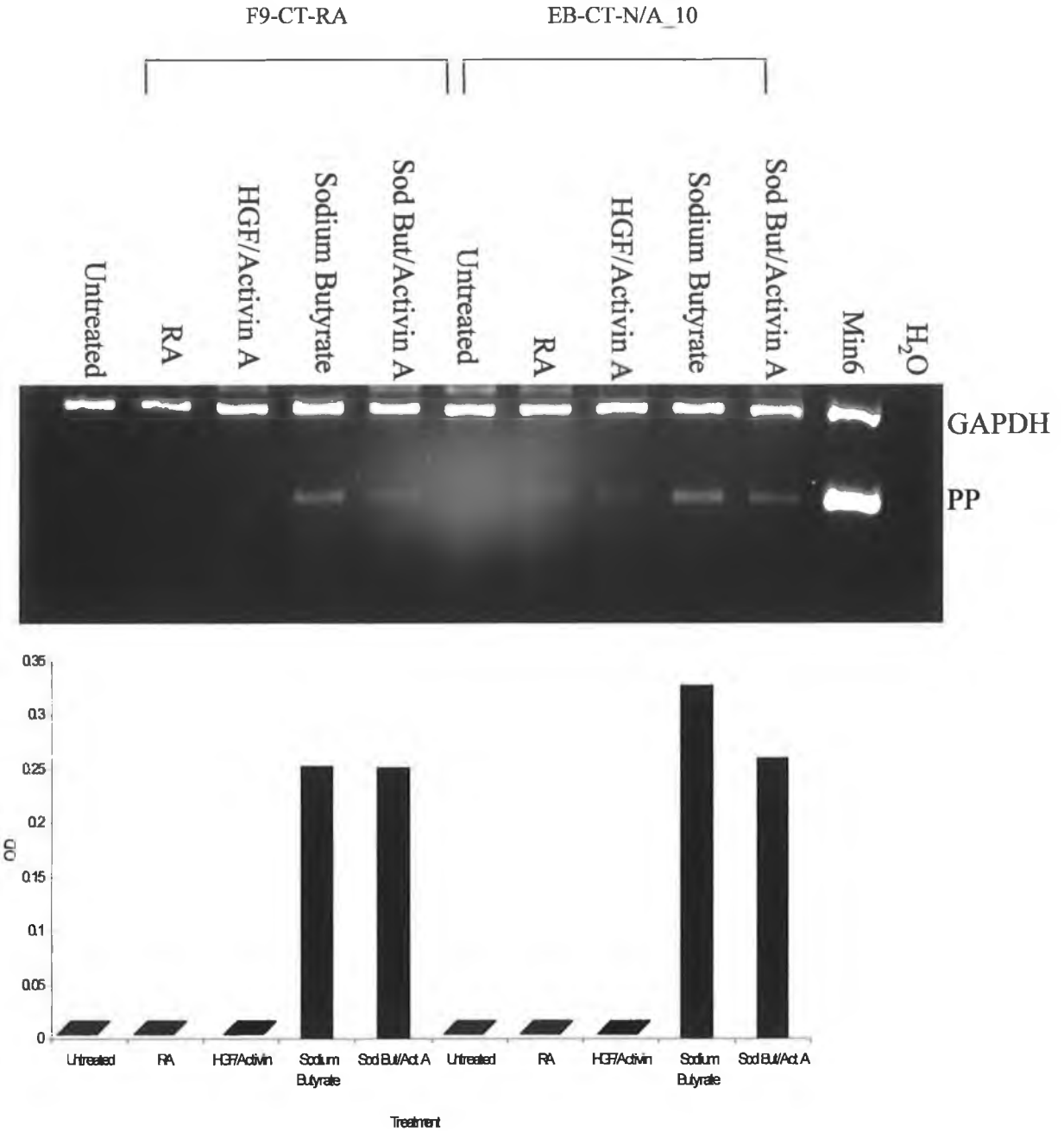


Figure 3.3.9 PP expression in CT cell lines treated with a selection of secondary endocrine differentiation agents. Min6 cells and H_2O were used as positive and negative controls respectively. The result presented is representative of a number of repeat gels.

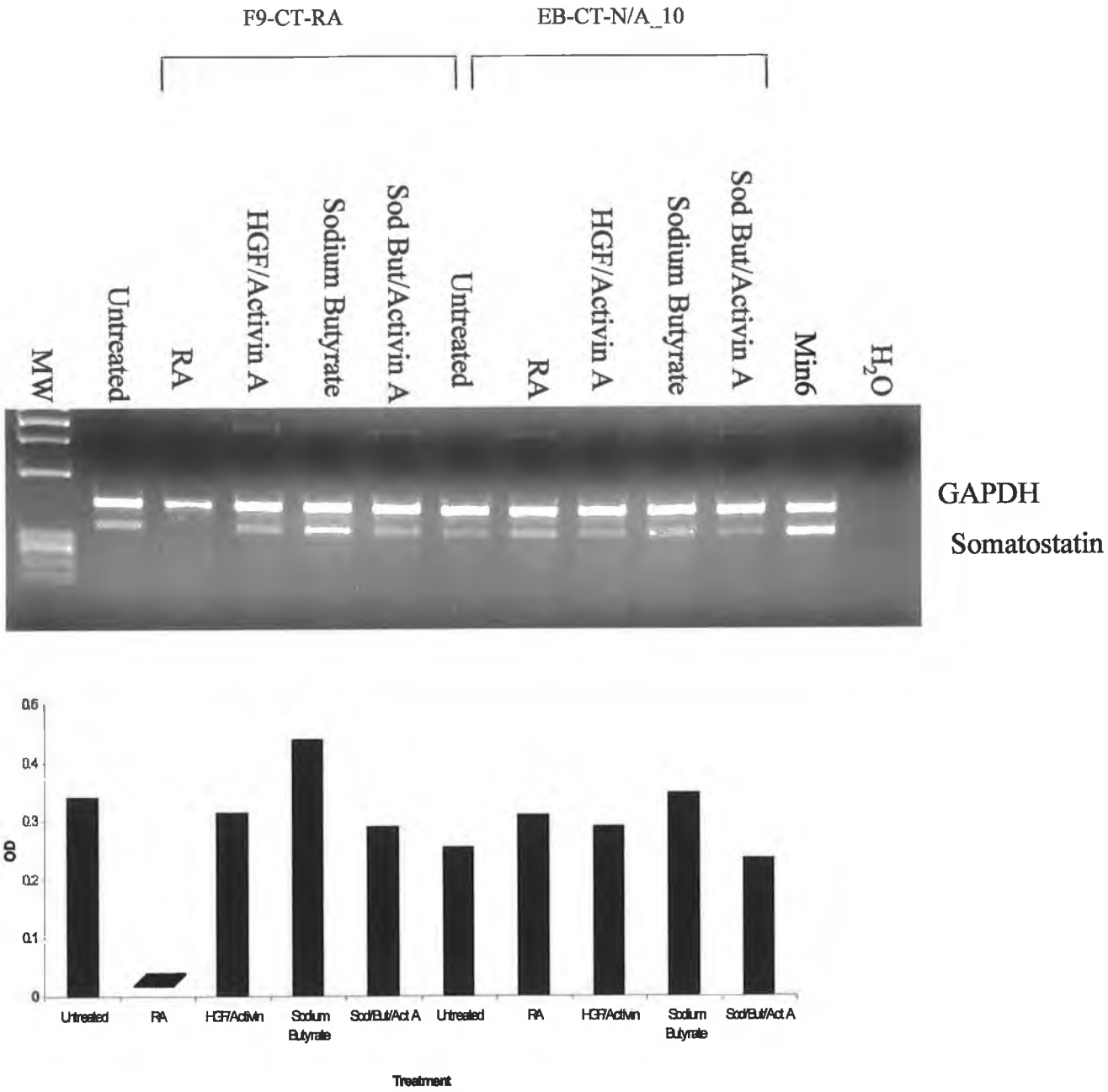


Figure 3.3.10 Somatostatin expression in CT cell lines treated with a selection of secondary endocrine differentiation agents. Min6 cells and H_2O were used as positive and negative controls respectively. The result presented is representative of a number of repeat gels.

3.3.2 The Effects of Transient Expression of Beta Cell Transcription Factors in F9 Cells

Previous overexpression studies with F9 cells have generally employed stable transfections (Griep & Westphal, 1988; Edwards *et al.*, 1988). This is at least partly due to the difficulty in overexpressing viral promoters in undifferentiated F9 cells (Kelly & Condamine, 1982). Optimisation studies using the lipid based reagents Lipofectamine Plus, Fugene and β Gal expressing plasmid pCH110, were performed as described (Section 2.3.4.1). As shown in Figure 3.3.11, Fugene is by far the superior transfection reagent for the purpose of this study and it was observed to be less cytotoxic to the F9 cells than Lipofectamine Plus. The ratio of Fugene to DNA was found to be critical in these transfections. Even in the most successful case however (3:1 Fugene/DNA ratio using 2μg DNA), the transfection efficiency was less than 1%.

3.3.2.1 Optimisation of Transfection Efficiency in Undifferentiated F9 Cells

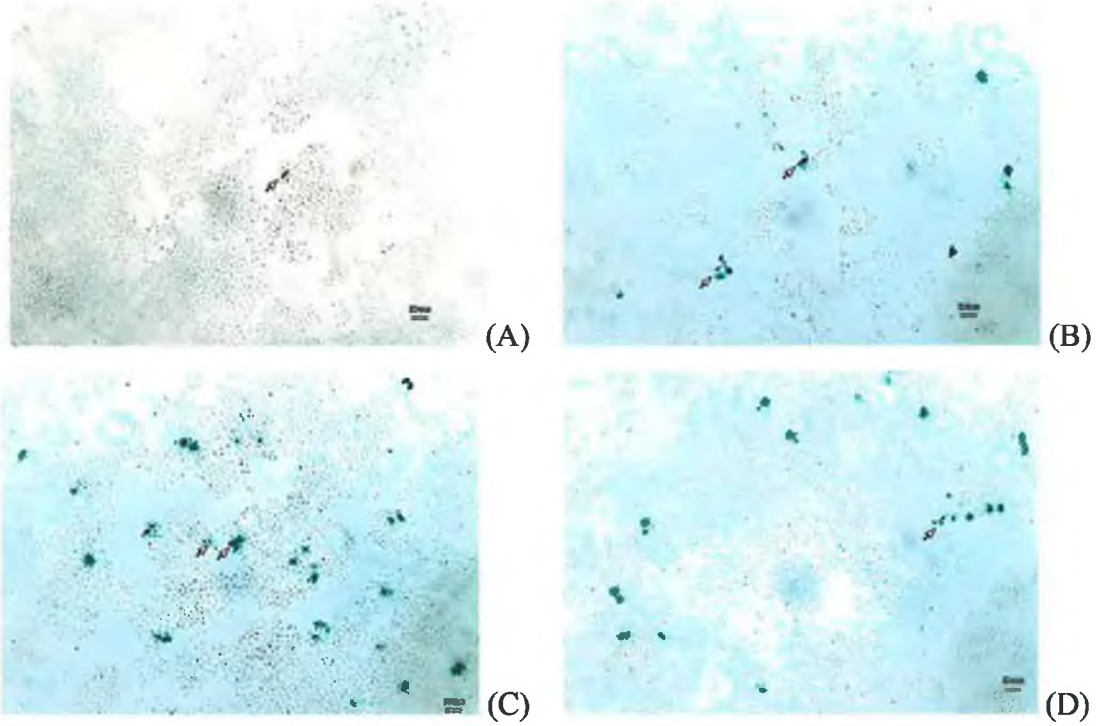


Figure 3.3.11 Initial optimization studies on F9 transfection using pCH110. (A) Transfection of 1 μg pCH110 using Lipofectamine Plus. (B) Transfection of 1 μg pCH110 using Fugene in 3:1 ratio (μl Fugene: μg DNA). (C) Transfection of 2 μg pCH110 using Fugene in 3:1 ratio. (D) Transfection of 2 μg pCH110 using Fugene in 6:1 ratio. Magnification = 10X. Bar = 50 μM in all cases.

3.3.2.2 Optimisation of pCH110 DNA Transfection into Differentiated F9 cells

Due to the poor transfection efficiency in undifferentiated F9 cells, it was decided to differentiate the cells prior to transfection. It was discovered that differentiating the cells using RA alone caused a dramatic increase in the number of cells found to be expressing the pCH110 plasmid. It was clear that it was predominantly differentiated cells that were expressing the gene (as determined from morphology). Combining RA with db cAMP further increased the transfection efficiency. It was found here also that it was predominantly differentiated cells that were expressing pCH110. The relative percentage expression was increased not only due to the greater number of cells expressing pCH110, but also as a result of the reduced growth rate, thus reducing the overall cell count.

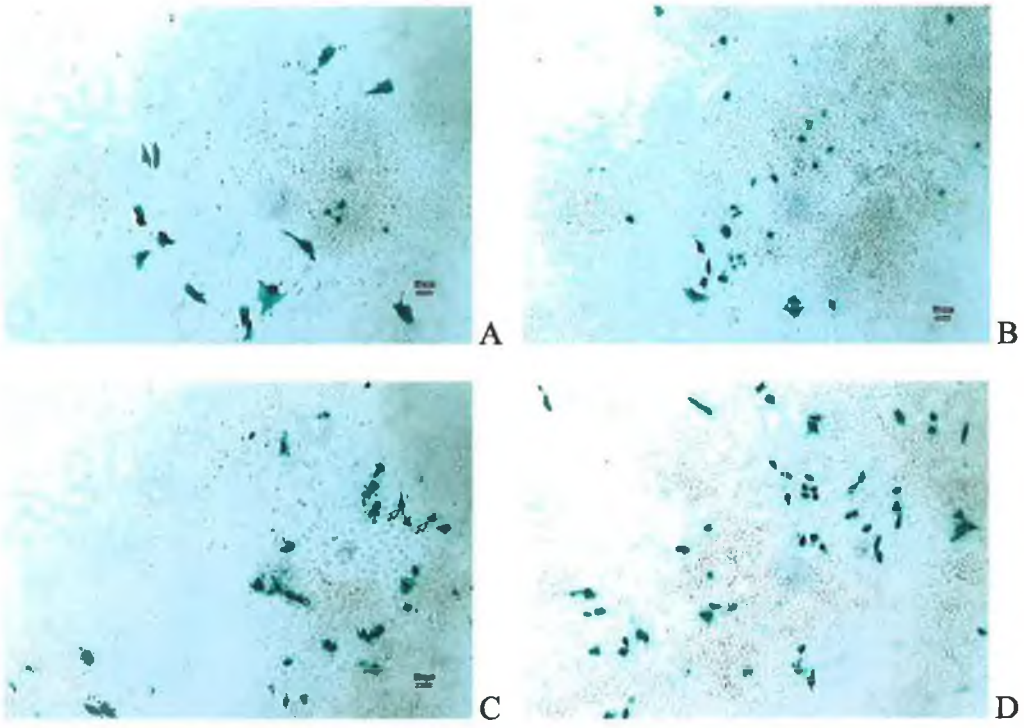


Figure 3.3.12. Transfection efficiency studies using differentiated F9 cells. F9 cells differentiated over 72 hours using RA (A&B) or RA + db cAMP (C&D). 2 μg of pCH110 cDNA was transfected in each case employing a Fugene/DNA ratio of 3:1. Magnification = 10X. Bar = 50 μM in all cases.

3.3.2.3 Transient Overexpression of Pancreatic Transcription Factors in F9 cells

The cDNAs for a number of critical pancreatic differentiation factors were obtained (sources and plasmids listed in Table 3.3.3) and transfected in to partially differentiated F9 cells (F9-RAC). A list of the cDNAs transfected and combinations of transcription factors is listed in Table 3.3.4. The co-transfections were chosen where it was suspected that synergistic/co-operative effects may be observed (Figure 3.3.13 summarises the importance and interrelationships of the transcription factors chosen). Overexpression at the mRNA level was achieved in each case. It was observed that untransfected F9 cells had low level expression of each of the genes examined. The levels of these genes, prior to transfection, were largely unaffected by the combination of the RA and db cAMP.

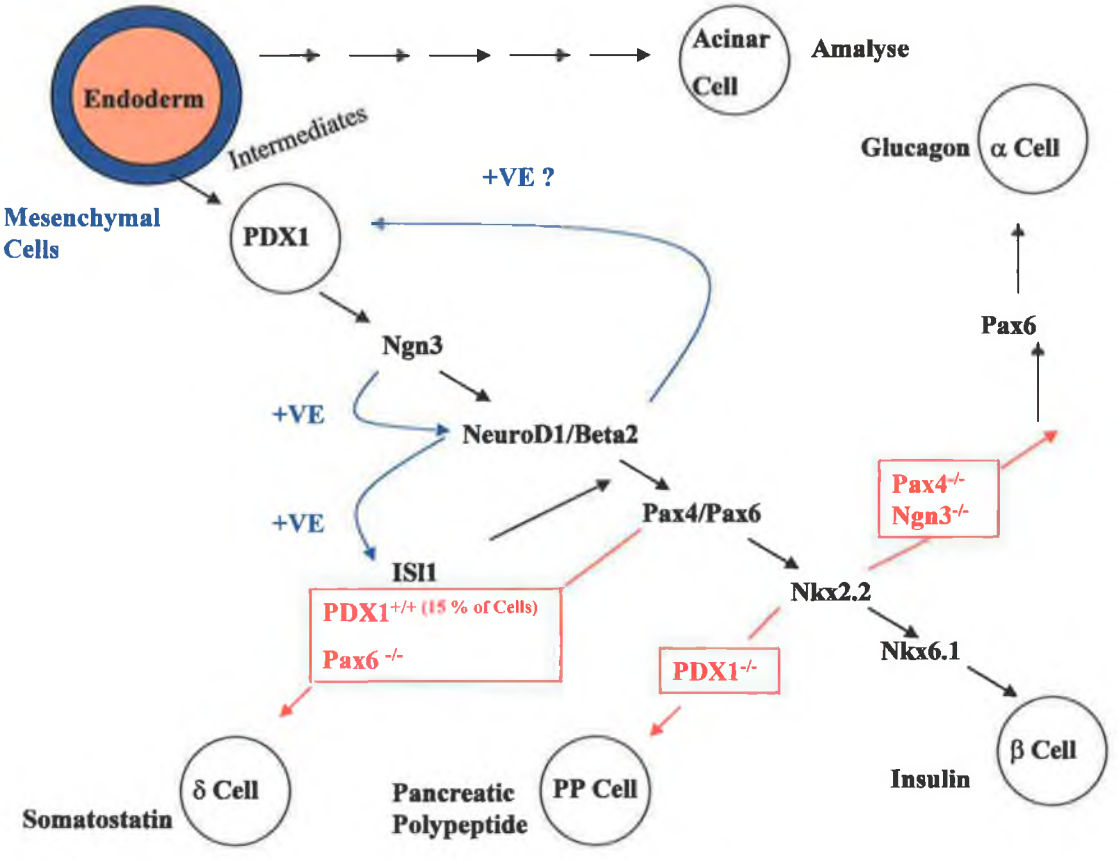


Figure 3.3.13 Transcription factor expression in developing endocrine cells and their interrelationships.

Plasmid Name (cDNA)	Supplier
PcDNA3-PDX-1(FLAG) (PDX 1)	Yoshita Kajimoto (Japan)
PCR3.1-Beta2 (Beta2)	J. Tsai (Baylor Institute, Texas)
PcDNA3-Ngn3 (Ngn3)	Itaru Kojima (Japan)
PBAT12.shNkx2.2 (Nkx2.2)	M. German (UCSF, San Francisco)

Table 3.3.3 Vectors containing pancreatic developmental genes for use in transient transfections of F9-RAC cells and their sources.

Transfection	**Figure**
PDX1	3.3.14
Beta 2	3.3.15
Ngn3	3.3.16
Nkx2.2	3.3.17
PDX1/Beta2	3.3.14/3.3.15
PDX1/Ngn3	3.3.14/3.3.16

Table 3.3.4 Transfections and co-transfections carried out on F9-RAC cells.

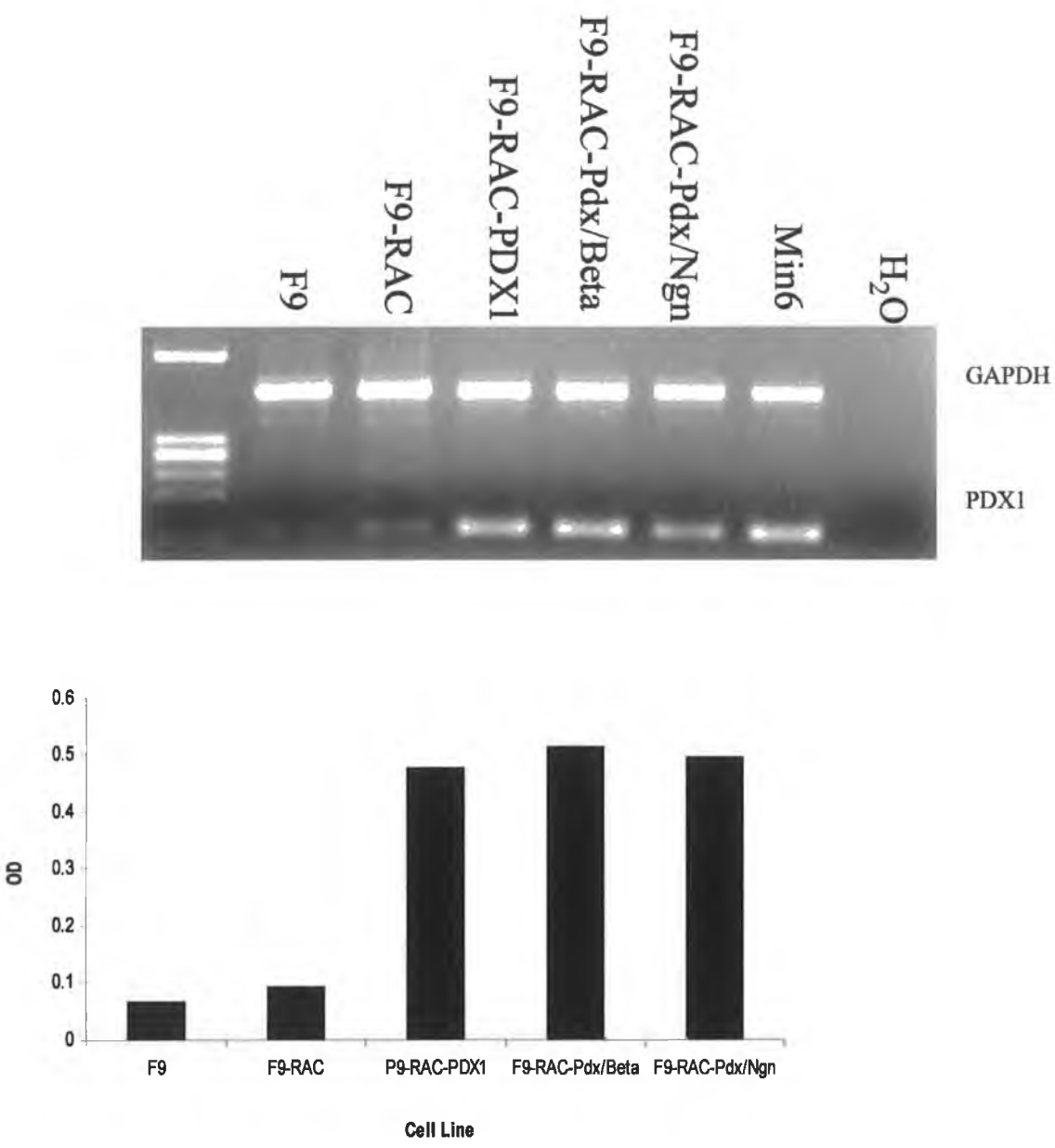


Figure 3.3.14. RT-PCR analysis demonstrating overexpression of PDX1 in F9 cells following the transfection of the pcDNA3-PDX1 plasmid. Overexpression was also achieved following co-expression of the PDX1 cDNA with Beta2 (PCR3.1-Beta2) or Ngn3 (pcDNA3-Ngn3). PDX1 was overexpressed at a similar level in all cases. H_2O and the murine β cell line Min6 were used as negative and positive controls, respectively. GAPDH was used as an endogenous control for RNA quality and quantity. The result presented is representative of repeat gels from independent experiments.

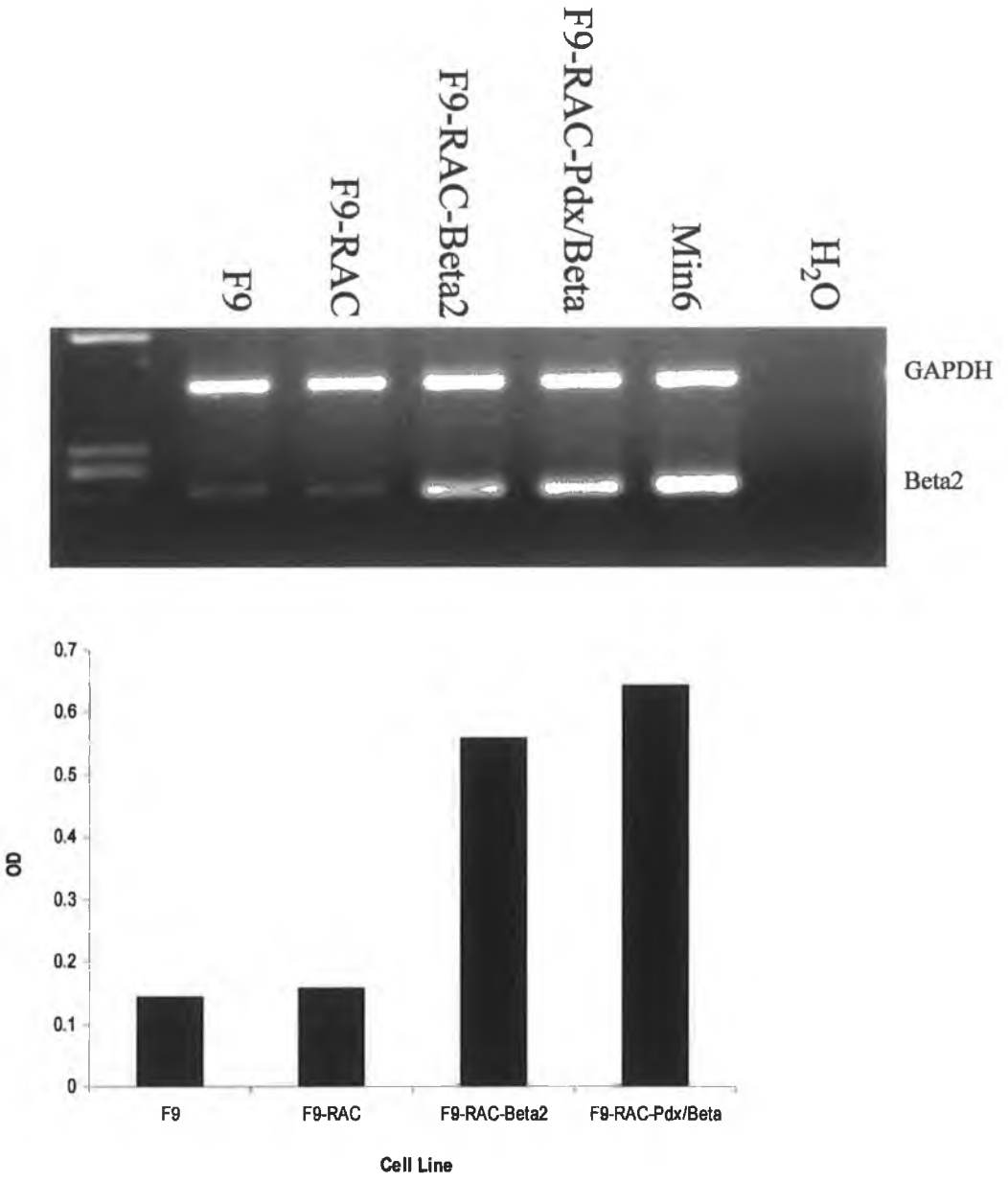


Figure 3.3.15. RT-PCR analysis of Beta2 expression following transfection of PCR3.1-Beta2 in to F9 cells. Overexpression was also achieved following co-transfection with the PDX1 cDNA (pcDNA3-PDX1). Beta2 was overexpressed at a similar level in both transfected cell lines. H_2O and the murine β cell line Min6 were used as negative and positive controls, respectively. GAPDH was used as an endogenous control for RNA quality and quantity. The result presented is representative of repeat gels from independent experiments.

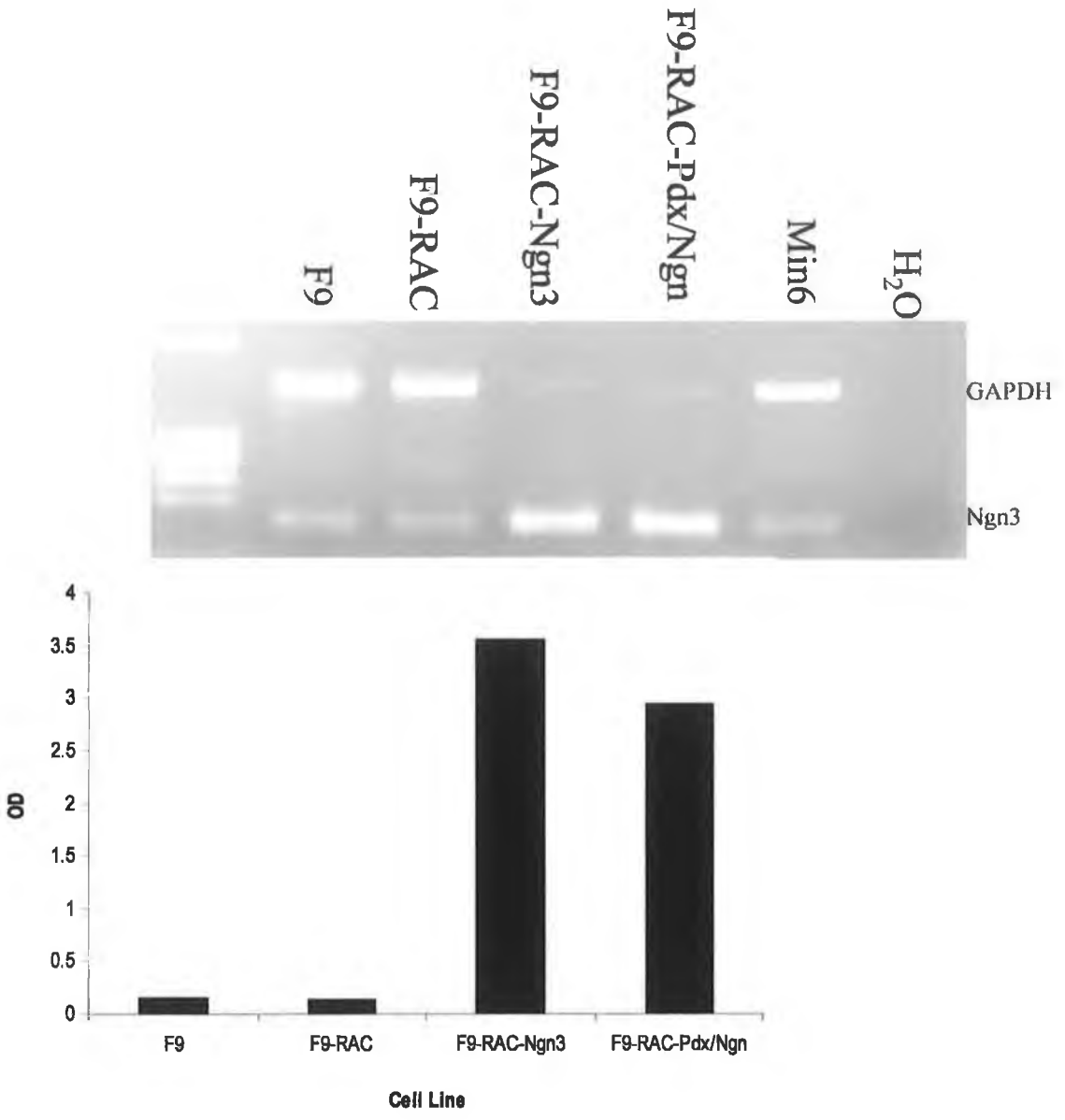


Figure 3.3.16 Overexpression of Ngn3 in F9 cells following transfection of pcDNA3-Ngn3. Overexpression was also achieved following co-expression of the Ngn3 cDNA with the PDX1 cDNA plasmid (pcDNA3-PDX1). Ngn3 was expressed at a similar level in both transfected cell lines. H_2O and the murine β cell line Min6 were used as negative and positive controls, respectively. GAPDH was used as an endogenous control for RNA quality and quantity. The result presented is representative of repeat gels from independent experiments.

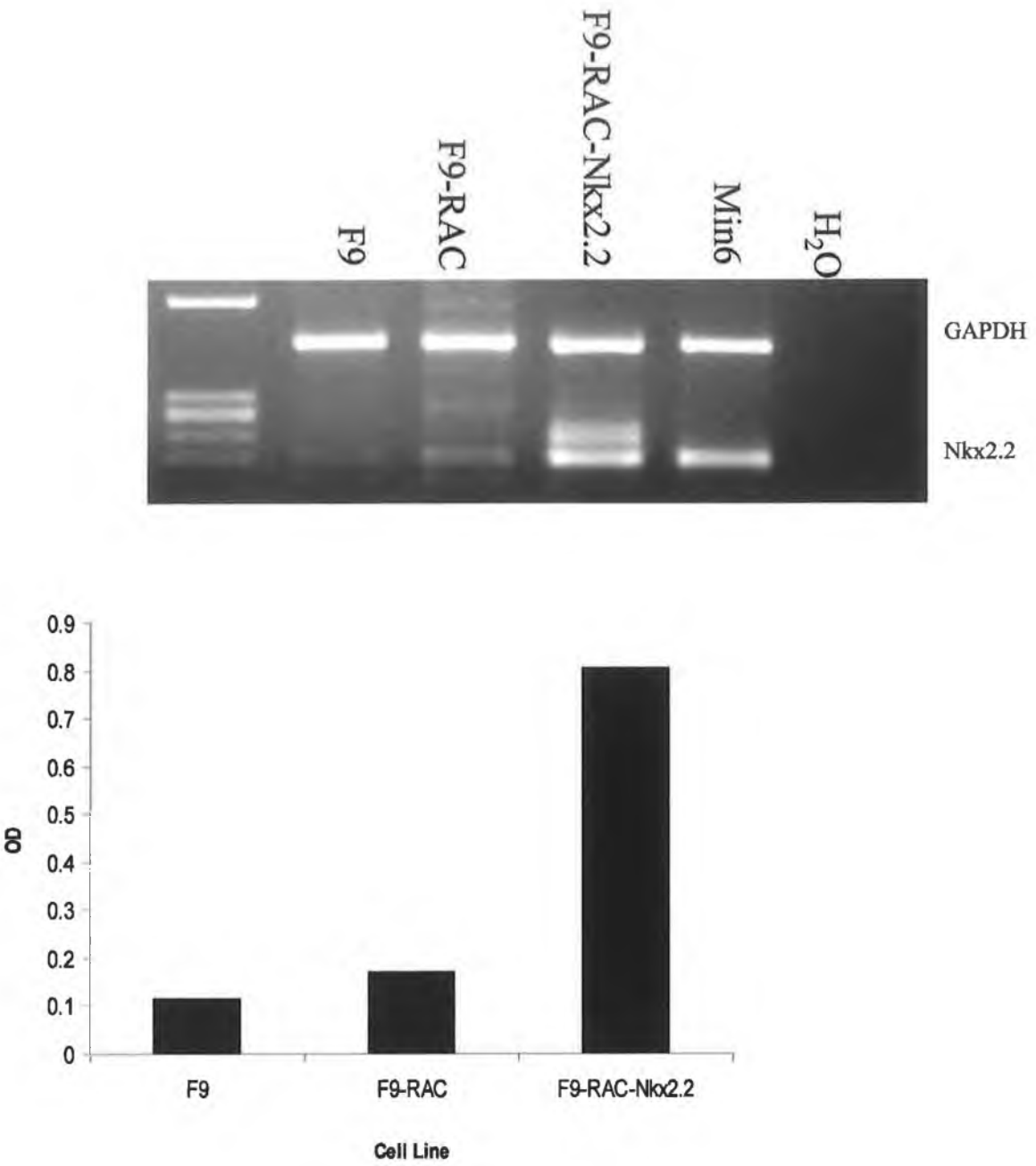


Figure 3.3.17 Overexpression of Nkx2.2 in F9 cells following transfection of the Nkx2.2 expression vector pBAT12.shNkx2.2. H_2O and the murine β cell line Min6 were used as negative and positive controls, respectively. GAPDH was used as an endogenous control for RNA quality and quantity. The result presented is representative of repeat gels from independent experiments.

3.3.2.4 Immunofluorescent Localisation of Transfected Transcription Factors Within the Cells

PDX1 was expressed in the nuclei of both transfected F9-RAC cells (Figure 3.3.18 (A)) and Min6 (Figure 3.3.18 (C)). Overall there was increased staining in the transfected F9-RAC-PDX1 population while there was only faint background staining in untransfected F9-RAC cells probed as controls. The levels of over-expression ranged from the intense nuclear staining shown to fainter nuclear-associated staining. There also appears to be low-level cytoplasmic staining.

Beta2 expression was clearly present in the nuclei of transfected F9-RAC cells and Min6 cells (Figure 3.3.19 (A&C)). Staining was seen to be associated with cells displaying differentiated morphology, this is in keeping with Section 3.3.2.2 where it was found that differentiated cells preferentially expressed transfected cDNAs. Staining in the Min6 cells appears to be nuclear membrane associated (Figure 3.3.9 (C)).

Ngn3 staining, although faint, was clearly expressed in the nuclei of the cells probed (Figure 3.3.20 (A&C)), likewise Nkx2.2 was selectively expressed in the nuclei of transfected cells (Figure 3.3.21 (A)).

Negative controls included non-transfected F9-RAC cells and transfected cells where the primary antibody was not applied. The non-transfected F9-RAC cells displayed diffuse background staining that was extremely faint i.e. there was none of the intense nuclear staining noticed in the transfected cells presented. In experiments where the primary antibody was omitted there was little staining, demonstrating that the fluorescence presented here is not due to non-specific binding of the labelled secondary antibody. Min6 cells were used in all cases as positive controls with the exception of Nkx2.2. It was found that Min6 cells did not cross react well with this antibody. The cDNA transfected (pBAT12.shNkx2.2) encodes hamster Nkx2.2, thus perhaps the antibody is less suitable for a murine antigen especially considering the antibody was raised in a mouse.

All photographs were taken at 40X magnification, the slight differences in cell sizes in the following images are due to differences in subsequent scanning of the photographs.

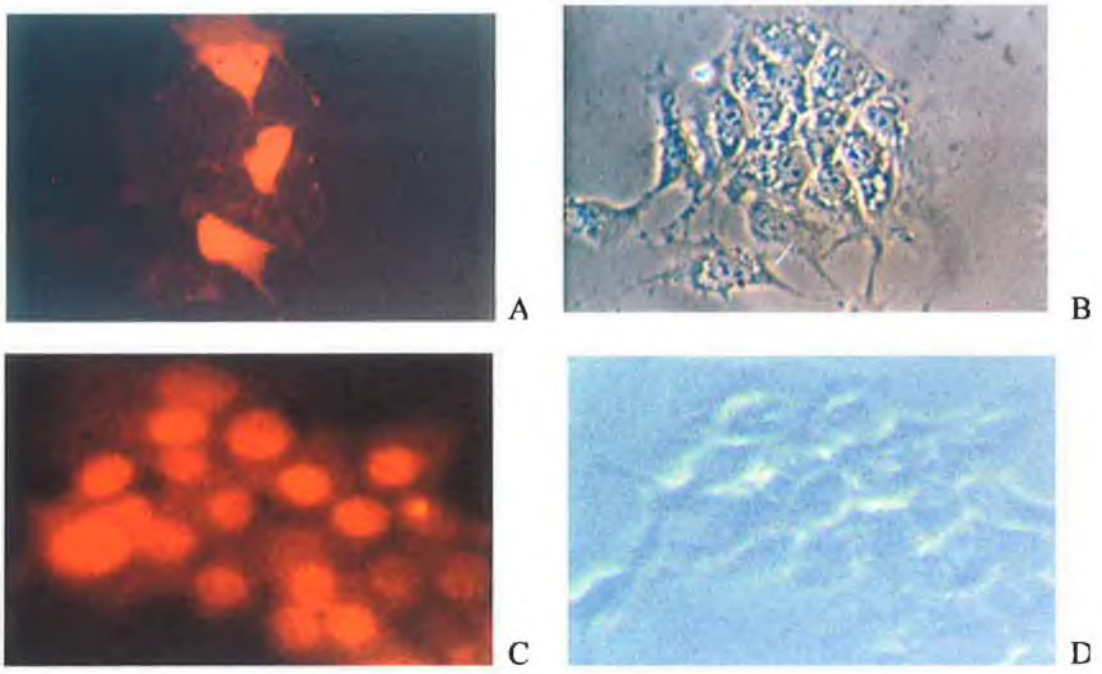


Figure 3.3.18 PDX1 expression in F9-RAC-PDX1 (A). Min6 cells were used as a positive control (C). Also included are the relevant phase contrast images (B&D). Magnification = 40X in all cases.

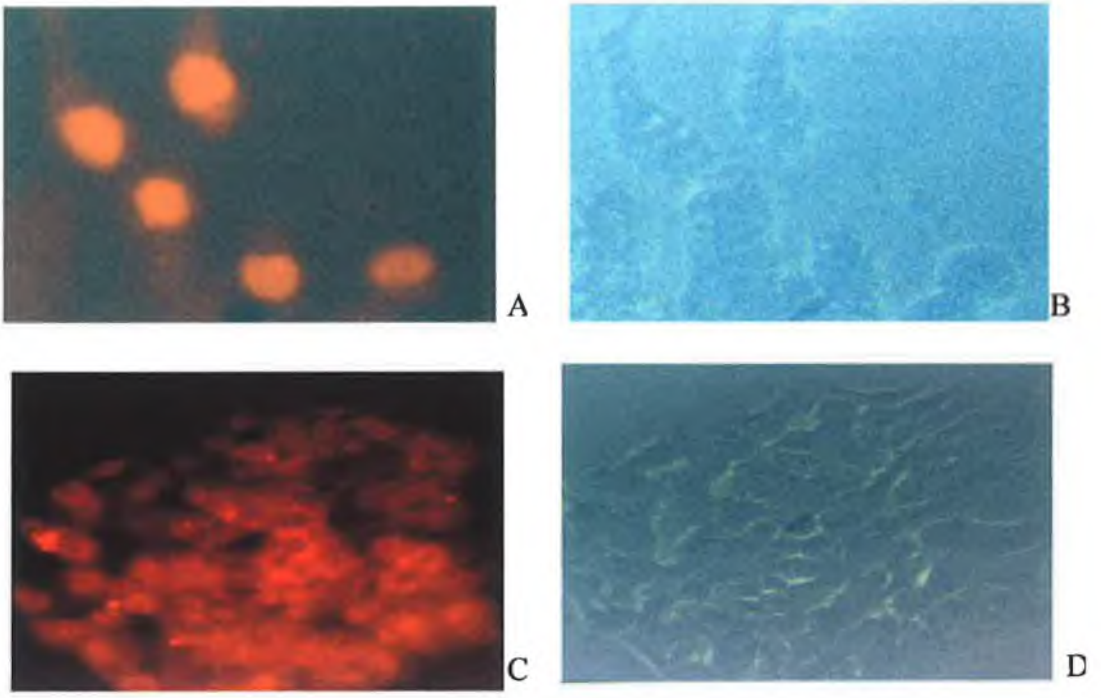


Figure 3.3.19 Beta2 expression in F9-RAC-Beta2 (A). Min6 cells were used as a positive control (C). Also included are the relevant phase contrast images (B&D). Magnification = 40X in all cases.

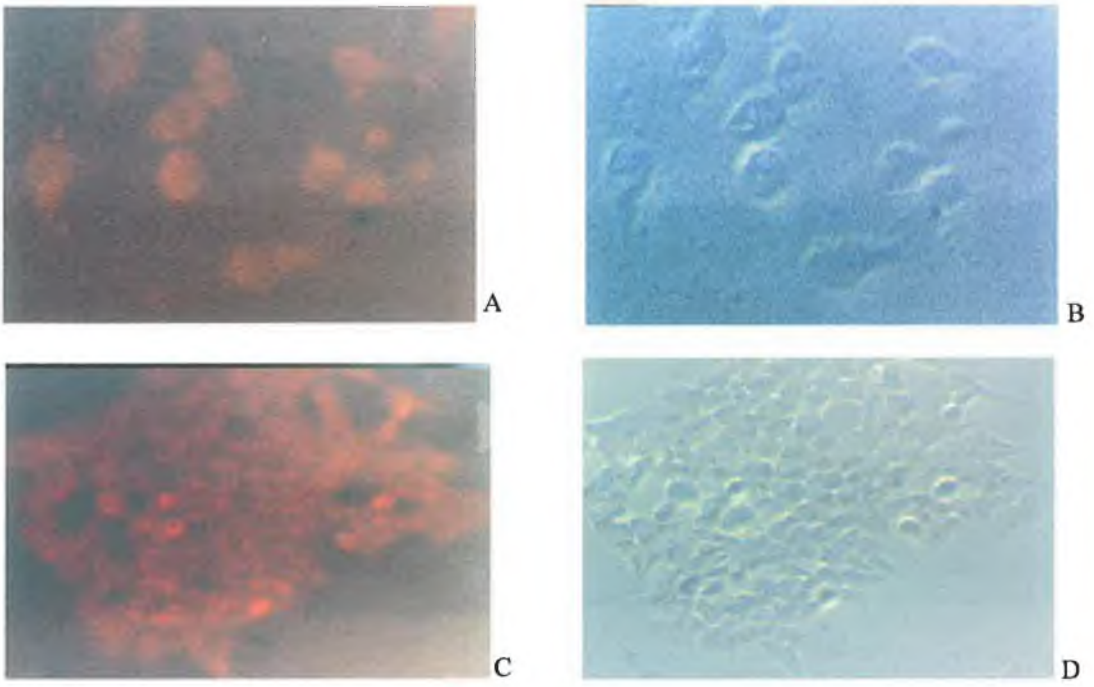


Figure 3.3.20 Ngn3 expression in F9-RAC-Ngn3 (A). Min6 cells were used as a positive control (C). Also included are the relevant phase contrast images (B&D). Magnification = 40X

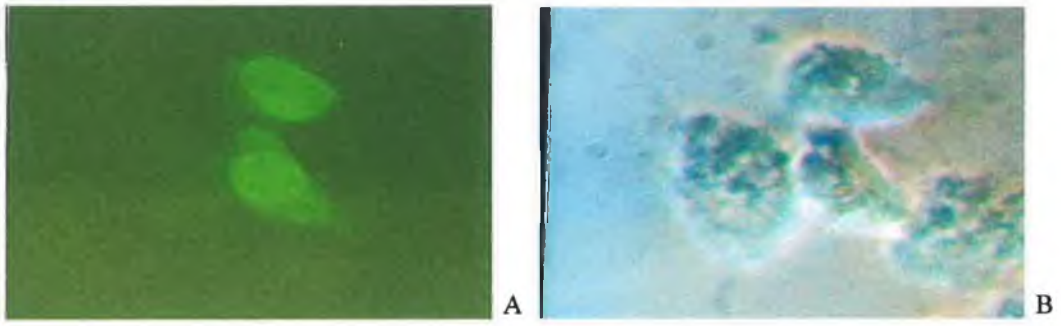


Figure 3.3.21 Nkx2.2 expression in F9-RAC-Nkx2.2. This antibody did not cross react well with Min6 cells. The relevant phase contrast image is also presented (B). Magnification = 40X

3.3.2.5 Endogenous Gene Expression in Cells Transiently Transfected with Pancreatic Transcription Factors

3.3.2.5.1 Endogenous PDX1 Levels in Transfected F9-RAC Cells

Endogenous PDX1 expression levels were increased approximately 2 fold following RA/db cAMP treatment. Transient expression of Beta2 and Nkx2.2 in F9-RAC cells reduced endogenous PDX1 expression back to the level seen in untreated F9 cells, there is no explanation for this as these factors should act 'downstream' of PDX1 (Figure 3.3.13). Ngn3 expression had no effect on the levels of PDX1 i.e. PDX1 mRNA levels were comparable to those in F9-RAC cells (Figure 3.3.22).

3.3.2.5.2 Endogenous PPI Gene Levels in Transfected F9-RAC Cells

PPI gene expression increased 1.5-2 fold following initial RAC treatments, however subsequent overexpression of pancreatic transcription factors generally reduced PPI mRNA back to levels found in untreated cells (Figure 3.3.23).

3.3.2.5.3 Endogenous Beta2 Levels in Transfected F9-RAC Cells

Beta2 levels were quite similar in F9 cells and the RA/db cAMP treated cells (F9-RAC). However, in all cases where pancreatic transcription factors were expressed, Beta2 levels decreased by almost 2-fold (Figure 3.3.24). There is no obvious explanation for this observation

3.3.2.5.4 Endogenous Isl1 Levels in Transfected F9-RAC Cells

Isl1 levels in F9 cells increased upon treatment with RA/db cAMP, further slight increases in endogenous expression were noticed following expression of PDX1 or Beta2, which are both 'upstream' of Isl1 (Figure 3.3.13). All other transfections had little effect on Isl1 levels, with the exception of Nkx2.2 which caused a reduction in Isl1 to untreated F9 levels (Figure 3.3.25)

3.3.2.5.5 Endogenous Pax Gene Levels in Transfected F9-RAC Cells

Pax 4 was not expressed in any of the transfected populations or in any of the control populations (F9 & F9-RAC). Pax 6 expression however was abundant and its levels did not vary with treatment or transfection. There was a slight negative effect observed with Nkx2.2 expression (Figure 3.3.26).

3.3.2.5.6 Endogenous Indian hedgehog (Ihh) Gene Levels in Transfected F9-RAC Cells

As shown in Figure 3.3.27, Ihh transcripts were present at low-level in the parent F9 population, however the RAC treated population showed a large increase in Ihh levels. Transient overexpresion of the selected transcription factors did not lead to any effects on Ihh expression.

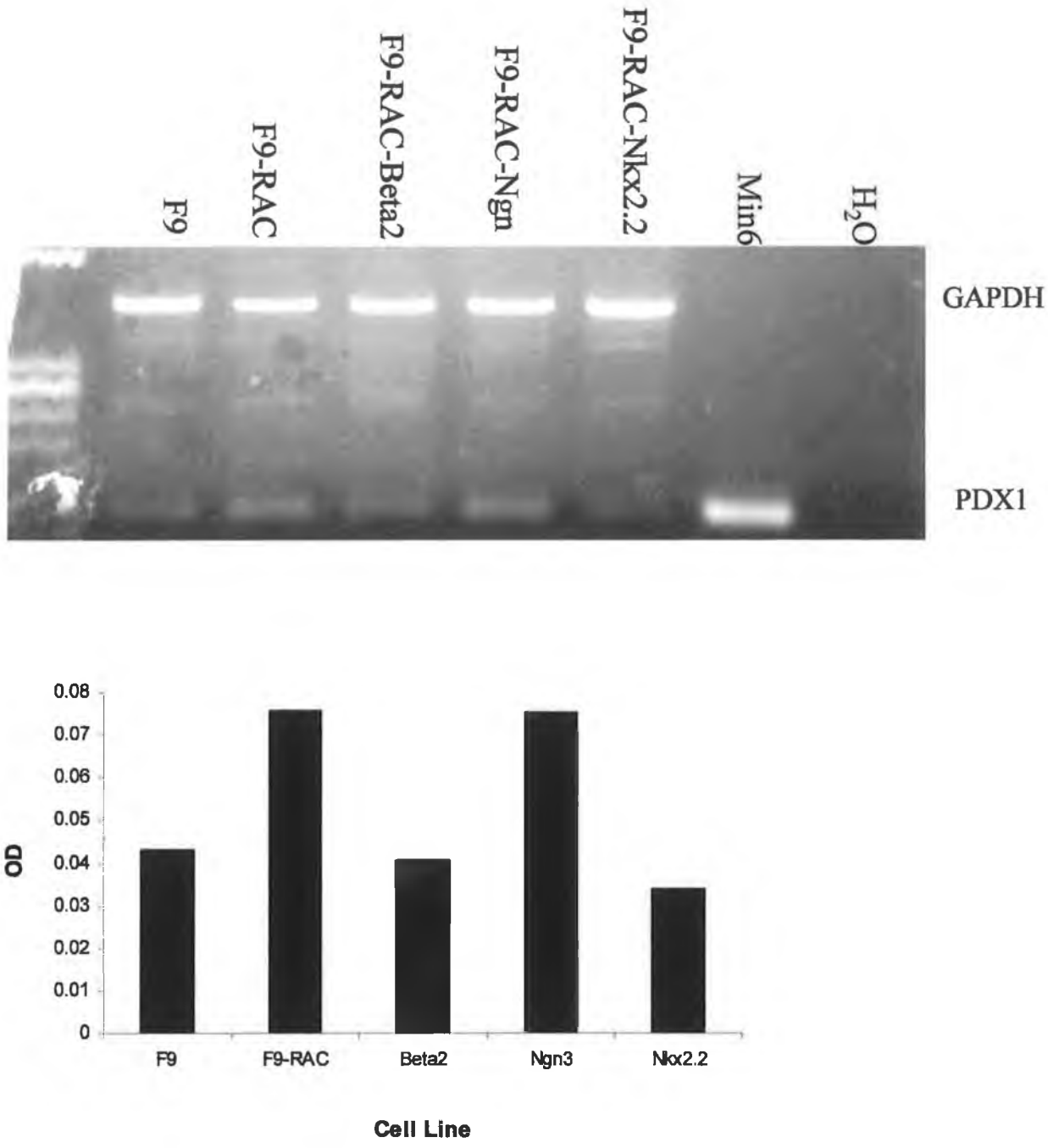


Figure 3.3.22 Endogenous PDX1 expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. Min6 and water were used as positive and negative controls, respectively. The result shown is a representative of repeat gels from two independent experiments.

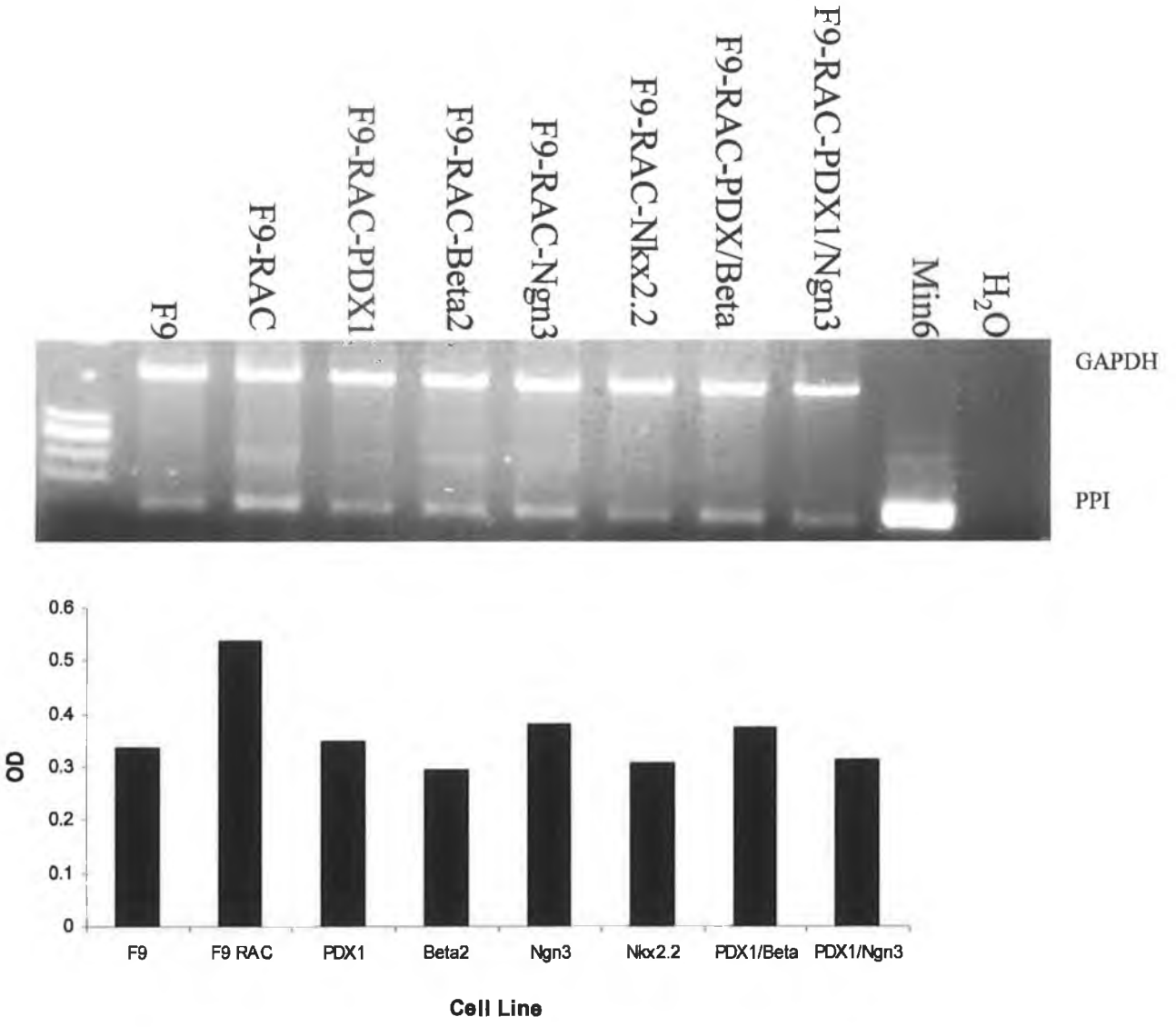
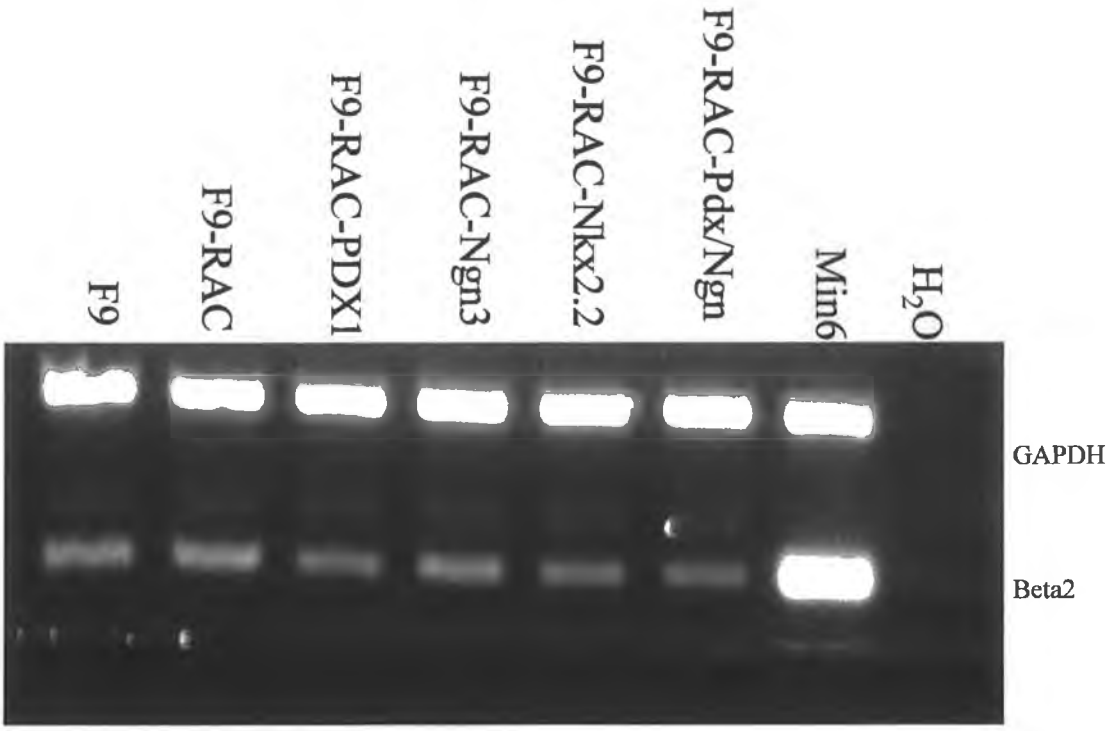


Figure 3.3.23 Endogenous PPI gene expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. Min6 and water were used as positive and negative controls, respectively. The result shown is a representative of repeat gels from two independent experiments.



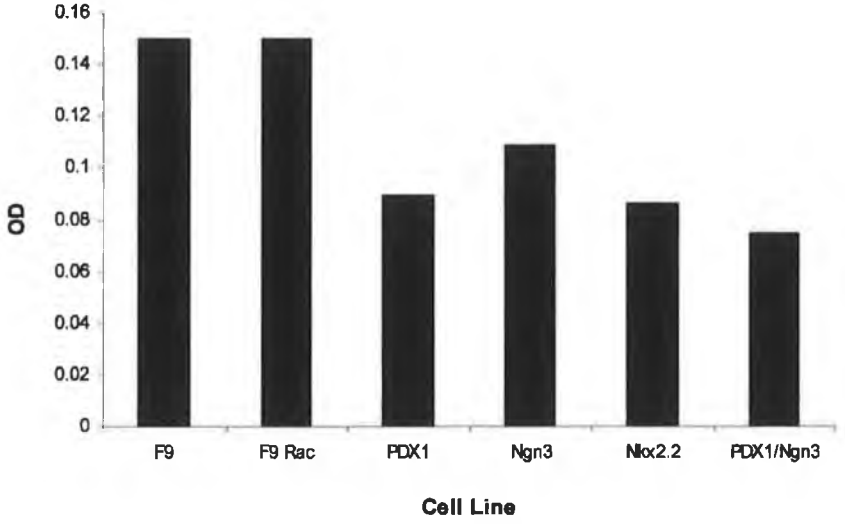


Figure 3.3.24 Endogenous Beta2 expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. Min6 and water were used as positive and negative controls, respectively. The result shown is a representative of repeat gels from two independent experiments.

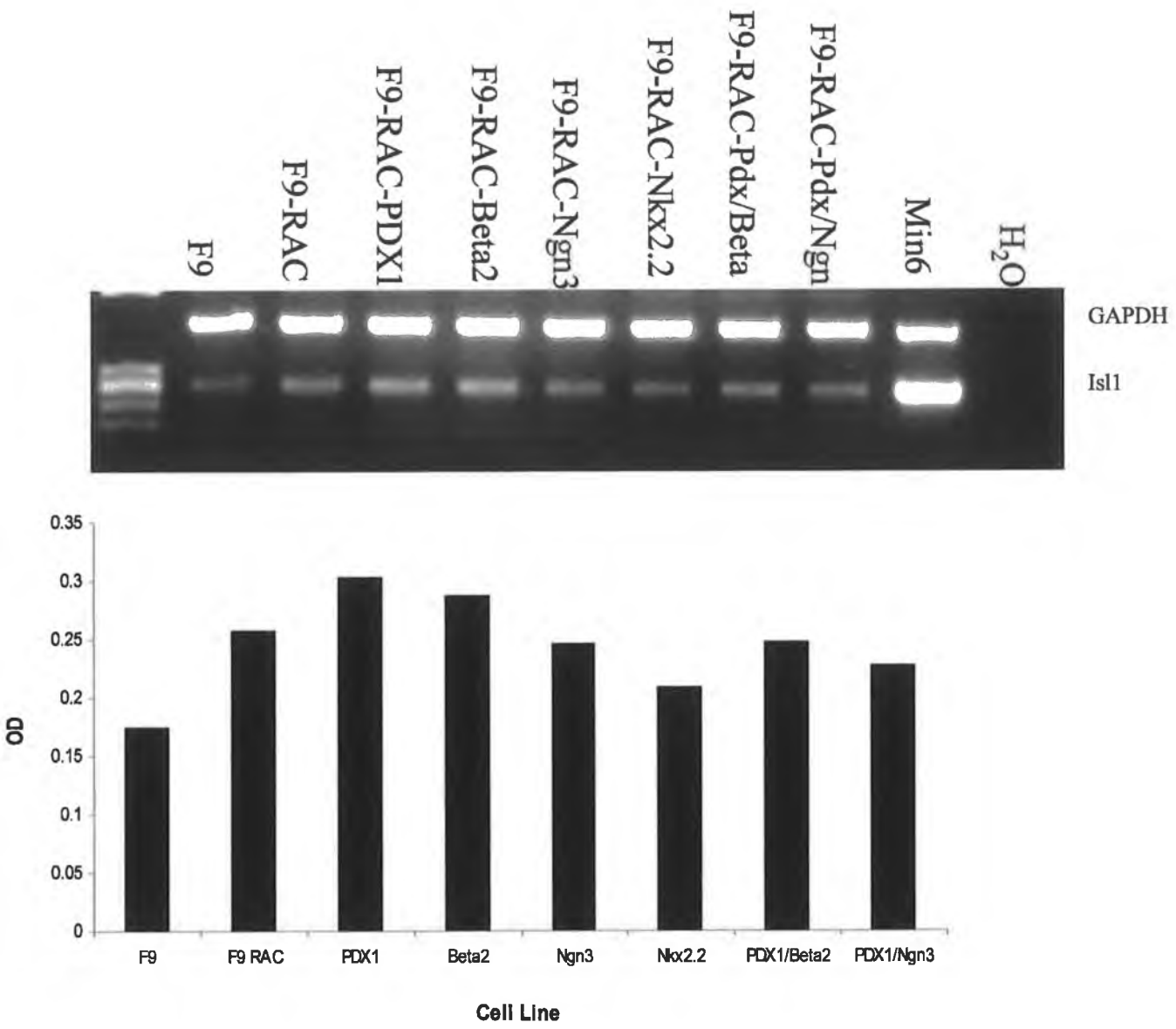


Figure 3.3.25 Endogenous Isl1 expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. Min6 and water were used as positive and negative controls, respectively. The result shown is a representative of repeat gels from two independent experiments.

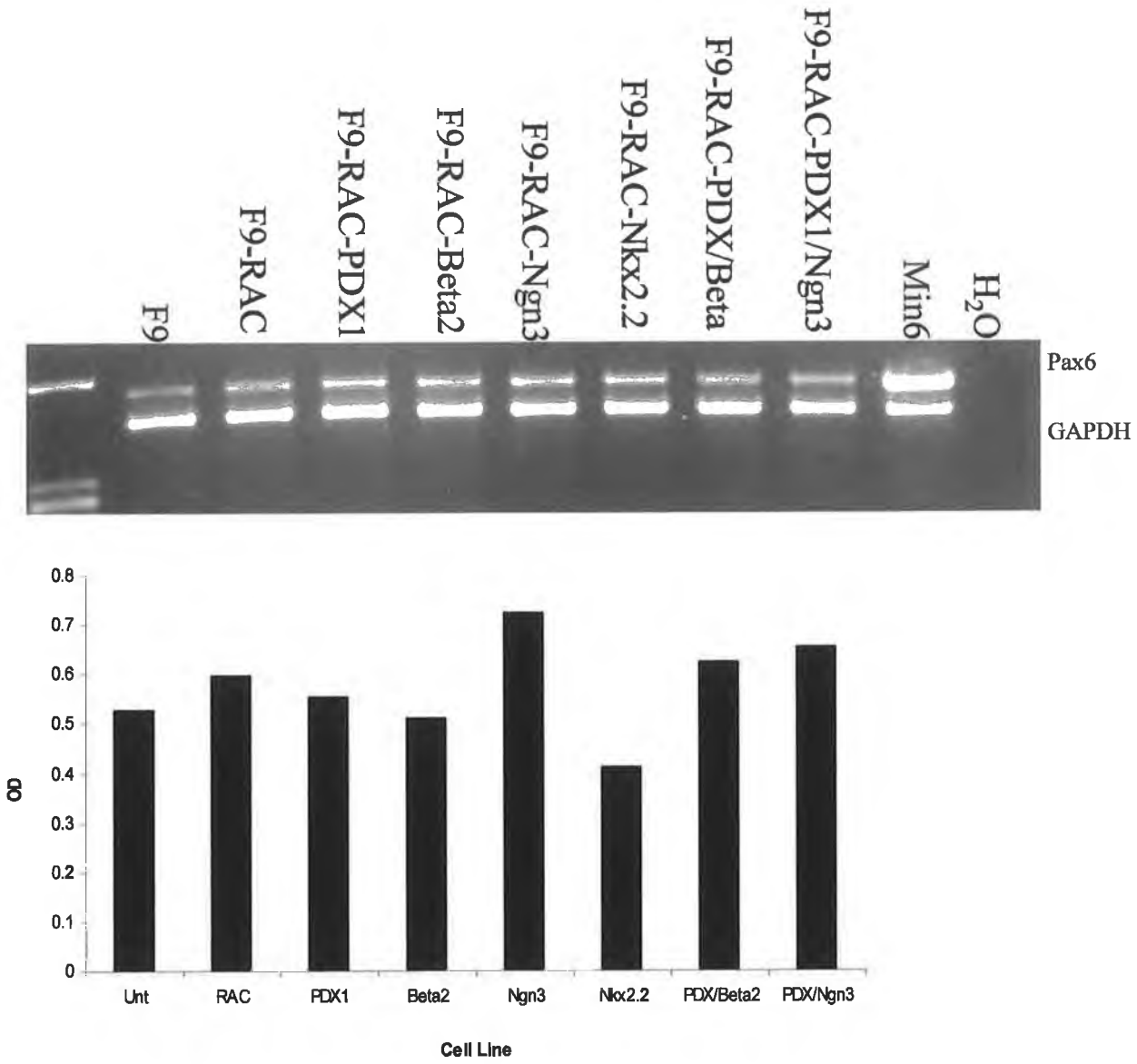


Figure 3.3.26 Endogenous Pax6 expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. Min6 and water were used as positive and negative controls, respectively. The result shown is a representative of repeat gels from two independent experiments.

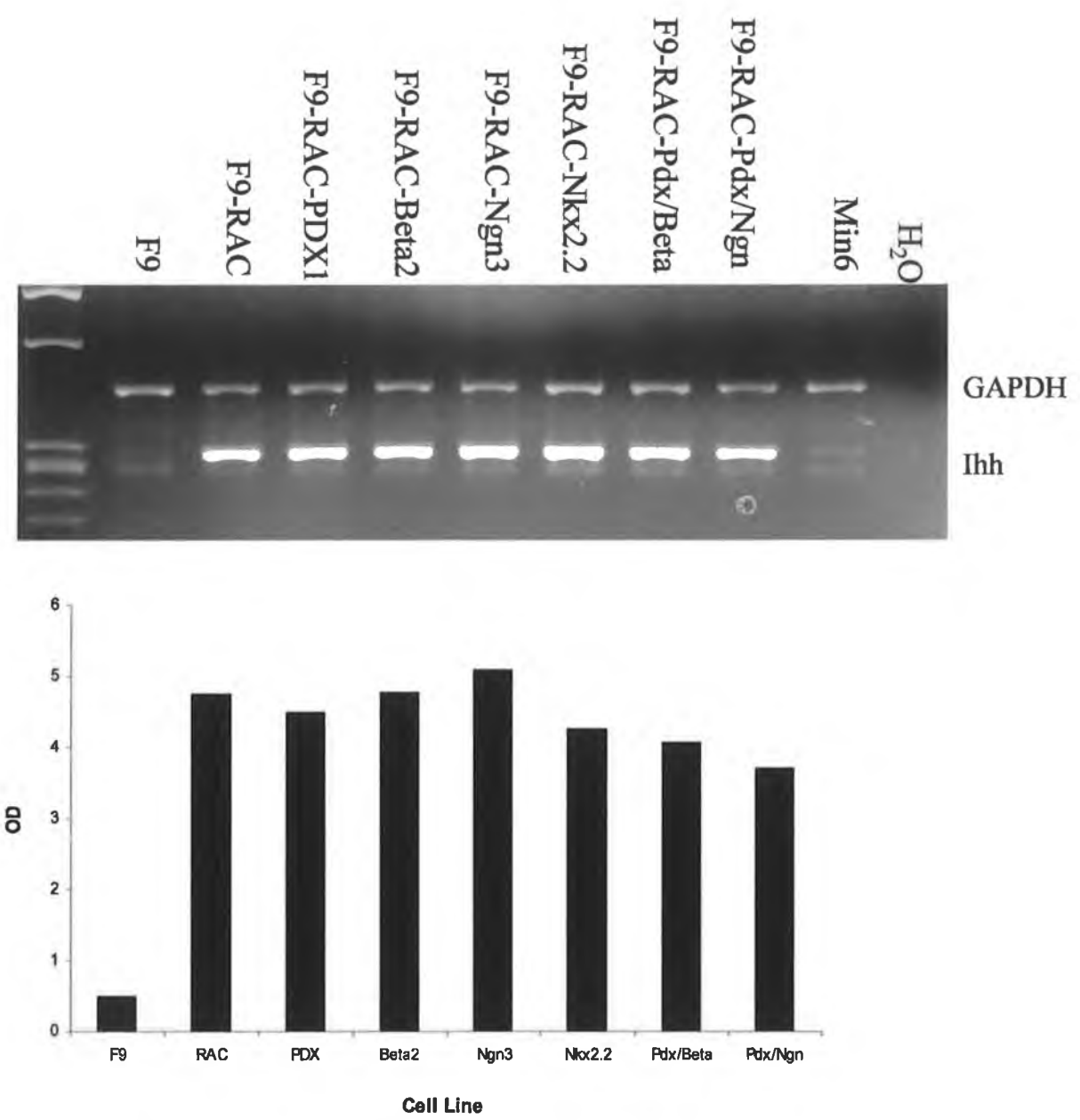


Figure 3.3.27 Endogenous Indian hedgehog (Ihh) expression in F9-RAC cells transiently expressing pancreatic transcription factors. F9 and F9-RAC cells are shown for comparison. Min6 and water were used as positive and negative controls, respectively. The result shown is a representative of repeat gels from two independent experiments.

3.4 Loss of β cell Phenotype Occurs Upon Continuous Culture of Min6 cells

Min6 cells have been used throughout this thesis as a positive control for experiments ranging from insulin secretion studies (Section 3.5) to RT-PCR analysis of β cell specific markers (Section 3.2). It was observed (and has been documented by others see Section 1.5.1.4), that as the cells were continuously cultured beyond passage 30 that they were seen to lose the ability to secrete insulin in response to environmental glucose levels (i.e. glucose stimulated insulin secretion, GSIS). This effect was accompanied with morphological changes and an increase in the growth rate of the cells. It was considered that the loss of such specialised β cell function and the associated morphological and proliferation changes may be due to a de-differentiation mechanism and thus this was investigated using EIA (Section 2.5.2.3), RT-PCR (Section 2.5.1.5) and DNA Array technology (Section 2.5.1.6).

Such a 'de-differentiating' system would be a useful tool for the identification of crucial pathways and markers involved in maintaining β cell function intact while in culture and also may provide useful information relevant to the production of insulin secreting β cell-like cell lines via the differentiation of stem cells. There are at least two possible mechanisms for the de-differentiation of the Min6 cells. One mechanism involves a gradual change in the gene expression profile of the cells over time in culture leading to a de-differentiated phenotype. It is also possible that the overgrowth of the differentiated cells by a less differentiated sub population with greater proliferative capability could occur over time and result in a less differentiated overall population, these issues are addressed later (Section 4.5.4).

3.4.1 Morphological Changes Associated with Continuous Culture of Min6 Cells

The initial modification in Min6 cells that indicated a change in the nature of the cells was an alteration in the typical Min6 cell morphology and growth patterns. Low passage Min6 (passages 13-23) cells were found to grow in localised clumps, i.e. they did not form an even monolayer over the tissue culture surface of the flask/well. The cells had pointed extensions but were mostly round in appearance (Figure 3.4.1 (A)). As the passage number was increased beyond passage 30, the cells no longer associated in clumps and were found to form even monolayers. The individual cells appeared to be slightly smaller than lower passage cells. The cells were also seen to have lost the round appearance of the lower passage cells and were pointed in appearance (Figure 3.4.1 (B)).

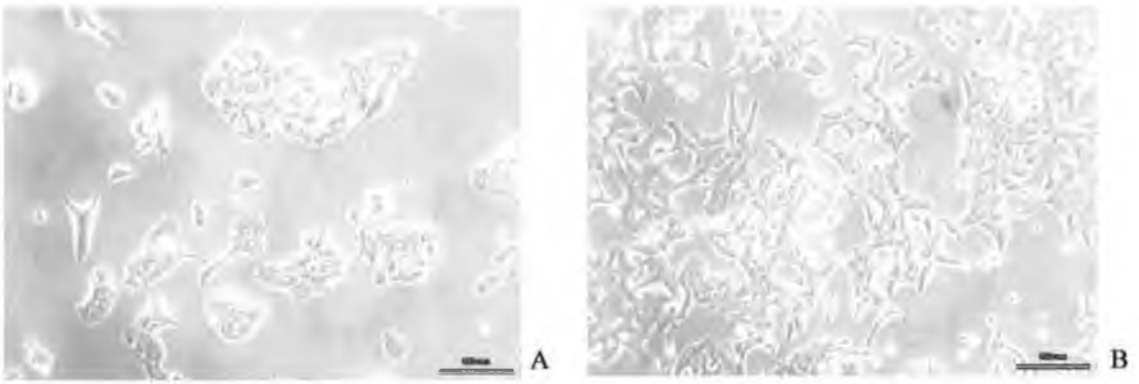


Figure 3.4.1 Min6 cells at passage 17 (P17) associate in to rounded clumps of cells which can be seen to grow 'upwards' as well as outwards (A). At Passage 40 however the cells are observed to have formed a monolayer over the flask and no longer form discrete colonies (B). In this experiment, Min6 P17 and Min6 P40 were seeded at 2 x 10^6 cells/flask and were grown for 1 week with 1 re-feed. Magnification = 20X and bar = 100 μ M.

3.4.2 Proliferation Rates of Min6 Cells at Low and High Passages

As Min6 cells were passaged beyond P35 there was a noticeable increase in proliferation rates, in Figure 3.4.1 it is clear that there are far more cells in the P40 flask even though the cells were seeded at the same density. Thus proliferation experiments (Section 2.3.3), were carried out to demonstrate the increase in proliferation more clearly. As shown in Figure 3.4.2 the rate of proliferation at the higher passages (P40-P49) is approximately double that observed in the low passage cells tested (P17-P22). The high passage cells continued to proliferate after 7 days (168 hours); however the lower passage cells were found to cease proliferation after approximately 6 days (144 hours) as the 'clumps' had reached maximal size.

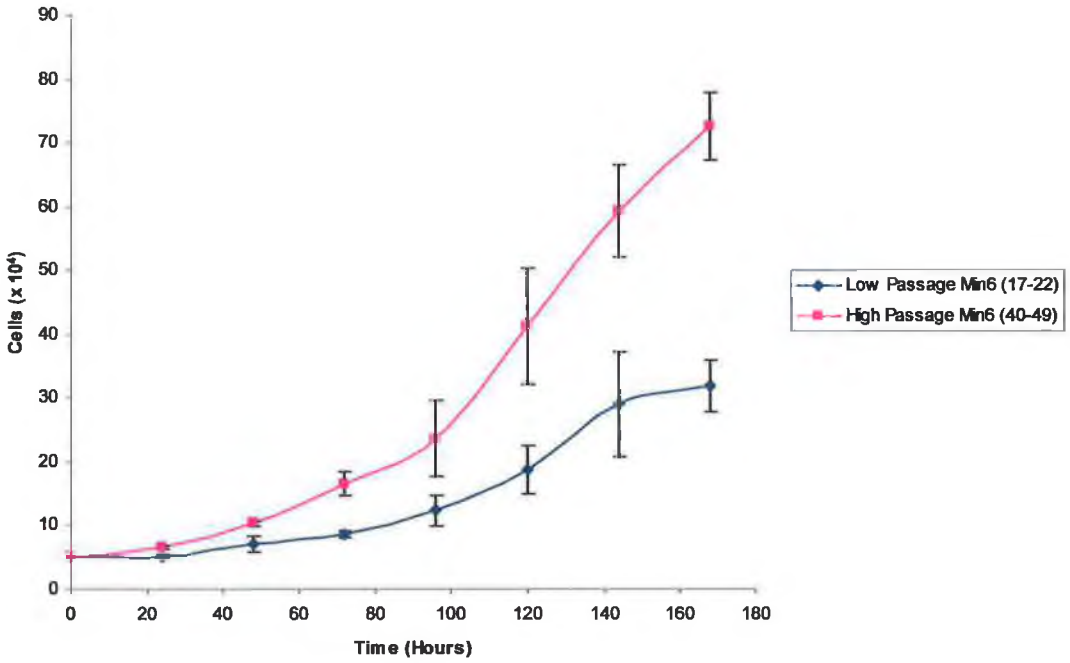


Figure 3.4.2 Proliferation data comparing low (P17-P22) to high (P40-P49) passage Min6 cells. Cells were seeded at 5 x 10^4 cells/well in 24 well plates and 3 wells were counted each day. Each well was counted using two sections of a Neubauer™ counting chamber, thus each well was counted twice. The experiment was repeated for at least two independent cell populations at low and high passage numbers. The data presented equals mean cell number ± SD.

3.4.3 Glucose Stimulated Insulin Secretion (GSIS) in Min6 cells Continuously Passaged in Culture

The critical observation concerning high passage Min6 cells was that the ability to regulate the secretion of insulin in response to external glucose signals (GSIS) was lost. Figure 3.4.3 clearly demonstrates this loss of function. Basal proinsulin secretion in high passage Min6 cells is higher than lower passage Min6 cells, however this basal secretion rate level does not change significantly despite increasing environmental glucose levels. Low passage Min6 cells increased proinsulin secretion approximately 6-8 fold over the glucose range tested (0 – 26.7 mM).

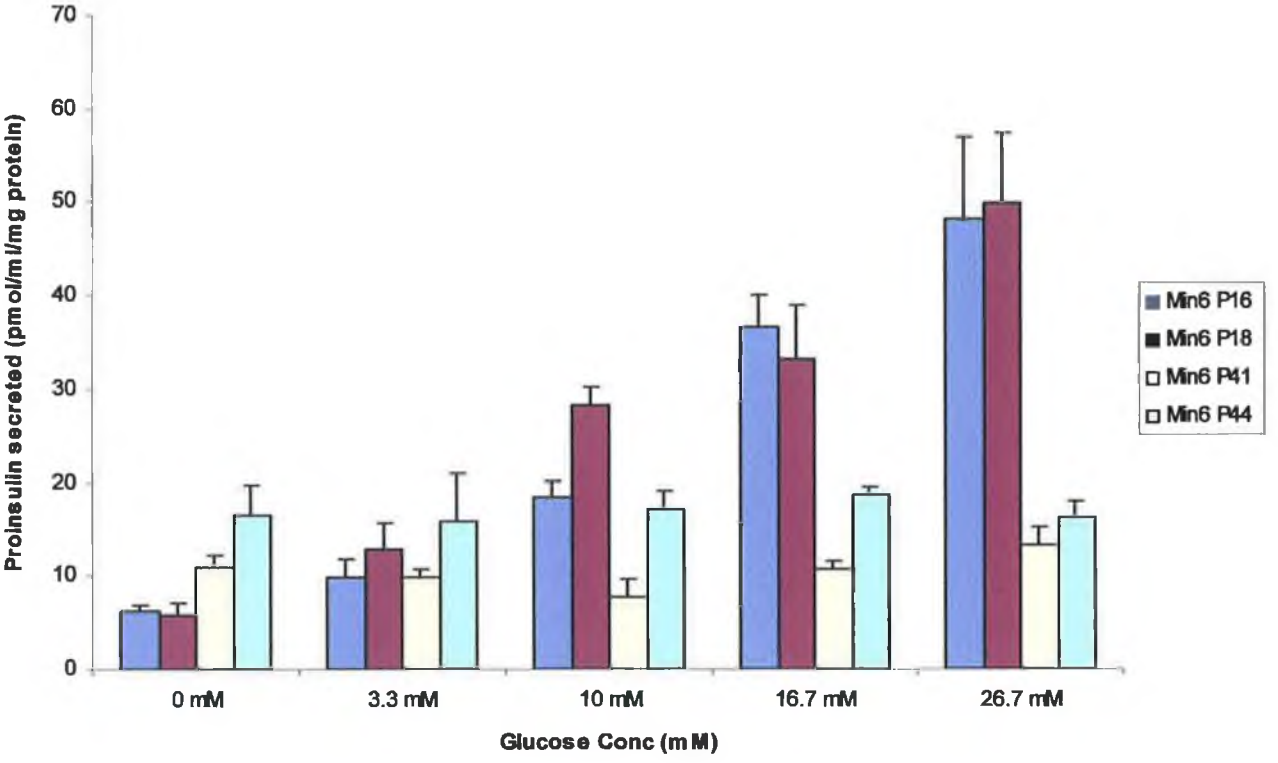


Figure 3.4.3 Proinsulin secretion profiles with increasing glucose concentrations in low passage Min6 cells (P16 & P18) and high passage Min6 cells (P41 & P44). Readings are given in pmol/ml/mg protein to allow for the increased growth rate of high passage Min6 cells. Data is presented as means ± SD from at least three experiments.

3.4.4 Alkaline Phosphatase Expression in Min6 cells Passaged Continuously in Culture

As already indicated in Section 3.1.2.1, alkaline phosphatase activity is a characteristic of undifferentiated stem cells and can be seen to decrease following differentiation of the ES cells (Lumelsky *et al.,* 2001; Sukoyan *et al.*, 2002). The decrease in alkaline phosphatase expression with differentiation of EC cells has already been demonstrated with F9 cells (Figure 3.1.2 & Figure 3.1.5), thus the levels of alkaline phosphatase activity were examined in Min6 cells. As before, BCIP was used as the substrate for alkaline phosphatase (Section 2.5.2.4).

Figure 3.4.4 clearly shows that alkaline phosphatase staining is far more widespread in the higher passage Min6 cells (P51) than in the lower passage Min6 (P19). At higher magnifications (Figure 3.4.4 B&D) it is clear that the intensity of the staining is also increased in the high passage cells.

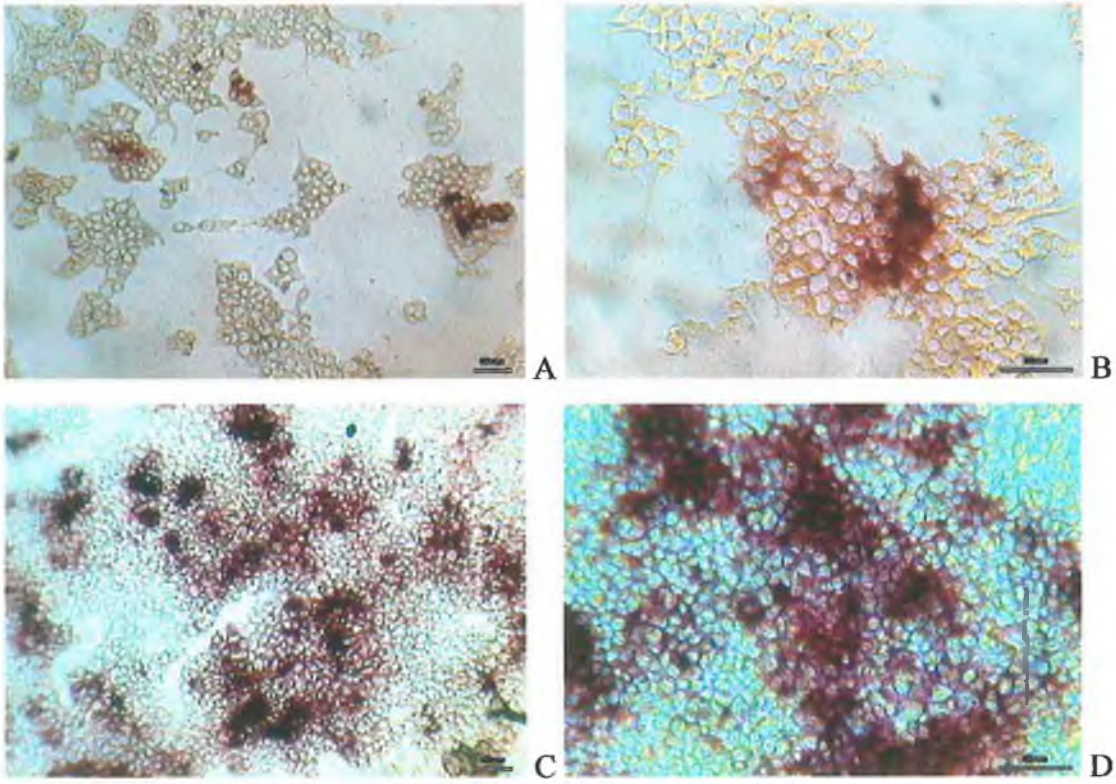


Figure 3.4.4 Min6 cells stained for alkaline phosphatase expression, Min6 P19 cells (A & B) were found to have much less staining than Min6 cells at passage 51 (C & D). Images were taken at 20X (A & C) and 40X (B & D). Bar = 50μm in each case.

3.4.5 Analysis of Serially Passaged Min6 cells by DNA Array

From the results already presented, it is clear that there is a significant change in the properties of Min6 cells as they are serially passaged in culture, most notably that the cells lose their ability to secrete insulin in response to increasing environmental glucose levels (Section 3.4.3). The loss of regulated secretory function and the observed changes such as increased proliferation (Section 3.4.2) and increased alkaline phosphatase expression (Figure 3.4.4), indicates that the high passage Min6 cells are less differentiated than low passage Min6 cells with a resultant loss of specialised cell function.

RNA was extracted from Min6 cells at passages 18 and 19 (GSIS responsive), 47 and 48 (incapable of GSIS) using RNeasy spin columns (Qiagen) (Section 2.5.1.2.2.1). This RNA was Dnase treated (Promega) and re-purified on RNeasy columns (Section 2.5.1.2.2.2). The RNA quality was confirmed by OD 260/280 readings and by gel analysis (Sections 2.5.1.3 & 2.5.1.4) (Figure 3.4.5). Poly $(A)^+$ RNA was purified from the total RNA using 'The total RNA labelling system' (Clontech). This poly $(A)^+$ RNA was used to generate P^{33} labelled cDNA probes for hybridization to the Mouse 1.2 array (Clontech 7853-1). Aliquots of this RNA were also used in subsequent RT-PCR experiments to examine other genes and confirm results obtained in the array. The expression profiles of four different RNA extracts were examined on four different membranes to examine the reproducibility of the technique (Figure 3.4.6).

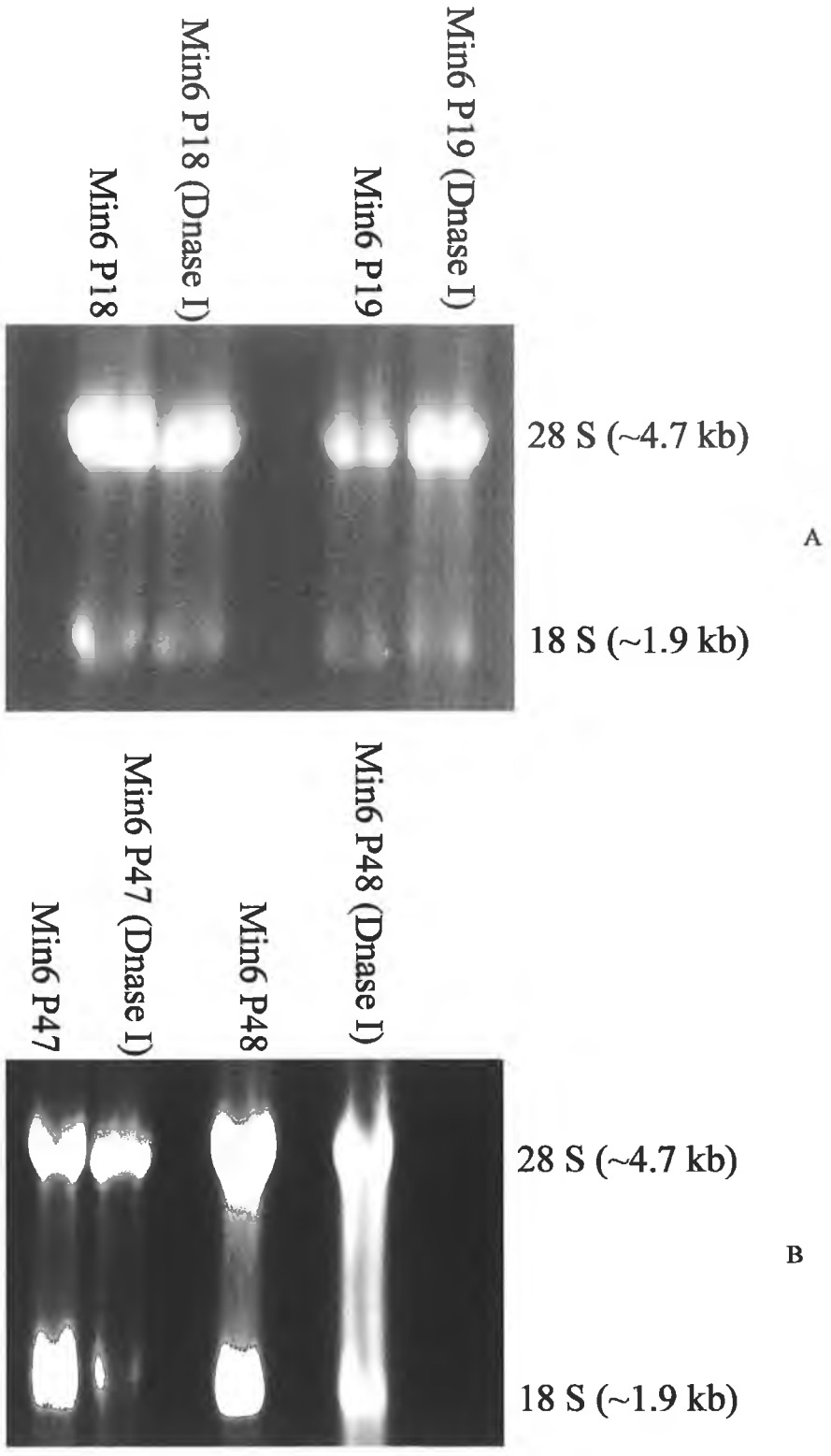
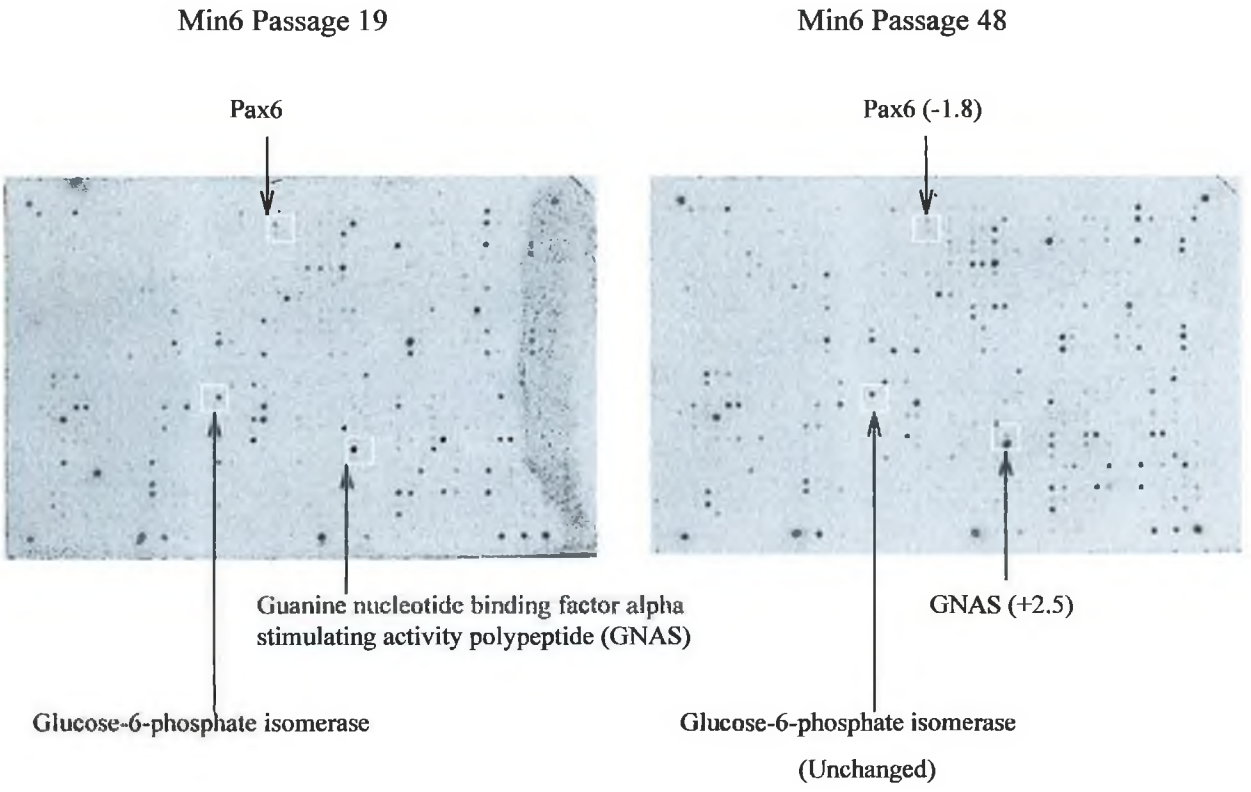
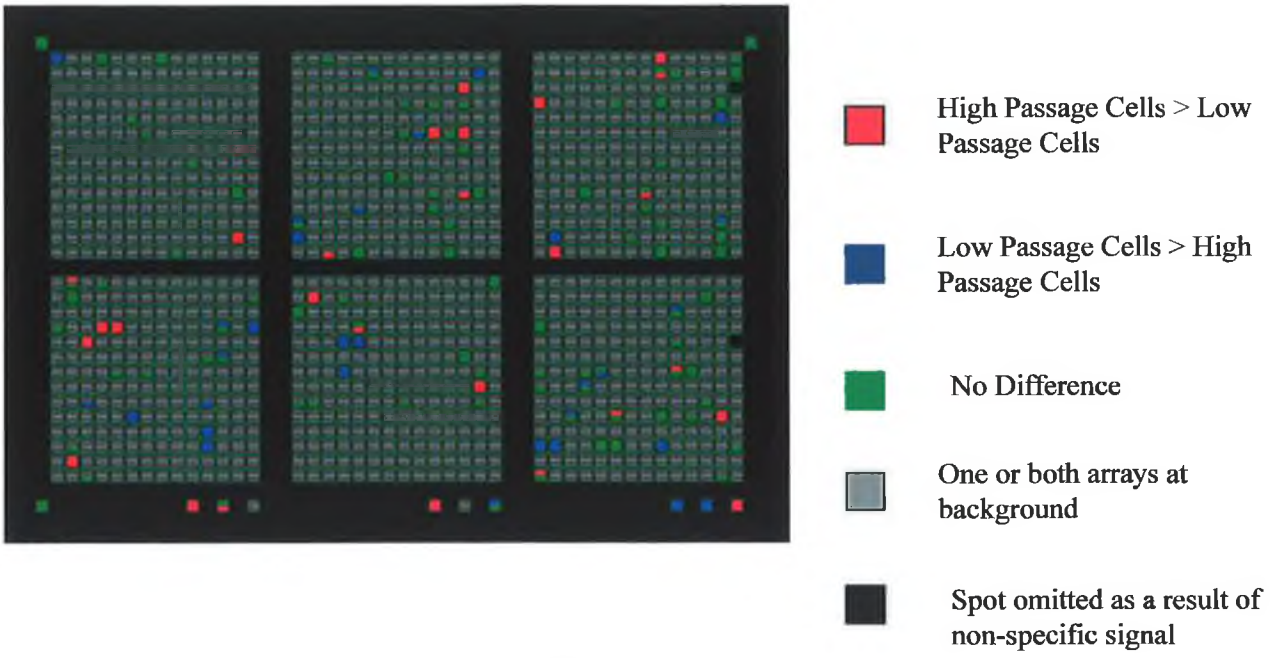


Figure 3.4.5 RNA extracted from (A) low and (B) high passage Min6 cells was run out on a 0.8% agarose gel to determine the quality of the RNA. 2-5 μg was run out (depending on amounts available). All gel boxes and combs etc. were soaked in 0.5M NaOH for 1 hour prior to running the gel to prevent RNase degradation of the samples. Similarly, the gel and TBE electrophoresis buffer were prepared using DEPC treated H_2O. Total RNA and RNA that had been DNase treated were examined for degradation.



(A)



(B)

Figure 3.4.6 Array membranes from Array experiment 2 (Array 2) comparing Min6 passage 19 with Min6 passage 48 (A) arrows indicate examples of altered and unchanged genes. (B) A comparative view of the array membranes superimposed and colour coded as indicated.

3.4.5.1 Summary of Results Obtained from Array Analysis of Low and High Passage Min6 Cells

The 1.2 array contains 1,173 cDNAs immobilised on to a nylon membrane This corresponds to approximately 3.5% of the mouse genome (Wada, 2001). A total of 129 genes were positively detected over the two separate array experiments. All of these 129 genes were detected in the second array experiment (i.e. Array 2; comparing Min6 P19 and P48) as a longer exposure time was used (10 days compared to 4 for Array 1). The shorter duration Array 1 experiment generated 80 positive genes. All of the genes detected using the short incubation were again detected in the second experiment (i.e. 80 out of the total of 129 genes detected in that experiment). Of the 80 'common genes' detected between the repeat array experiments, 5 results were significantly contradictory and were mostly associated with apoptosis related genes (Table 3.4.1), (i.e. in one array experiment these genes appeared to either up-regulated or downregulated upon passaging Min6 cells while on the repeat experiment the result was reversed). A further 7 genes appeared to be regulated differently between the two array experiments, but in these cases the differences were <1.5 and therefore considered insignificant. Therefore of the 80 gene changes detected between the two array experiments 68 produced similar results using different RNA and membranes, corresponding to a reproducibility level of 85%.

Based on the results obtained it was decided to set a difference of 1.5 fold as the lower limit of significance thus, 57 genes were found to be upregulated/induced in the high passage Min6 cells (Section 3.4.5.3) and 35 genes were downregulated/silenced as a result of the continuous culture (Section 3.4.5.4).

Gen Bank Accession No.	Gene Name	Classification	Array 1	Array 2
U02098	Purine-rich element binding protein A(PURA)	Stress Response Protein	Down	Up
AF020185	Cytoplasmid dynenin light chain 1	Apoptosis Associated	Down	+2.5
U83628	Defender against cell death 1(DAD1)	Apoptosis Associated	-1.52	1.65
U171162	BCL2-associated athanogene (BAG1)	Apoptosis Associated	Down	+1.59
X67083	Growth Arrest and DNA damage inducible protein 153 (GADD153)	Apoptosis Associated	Up	Down

Table 3.4.1 Genes where significant differences were noted between Array experiment 1 and Array experiment 2. The low passage Min6 membrane was the reference in each case. Thus the case where a gene is described as 'Up' refers to incidents where genes were expressed in the high passage Min6 cells but not at the lower passages. Similarly 'Down' refers to cases where genes which were present in lower passages were silenced in the higher passage cells.

3.4.5.2 Patterns of Gene Expression Changes Observed after the Serial Passage of Min6 cells

The Upregulated/Downregulated genes were examined separately under the following classifications (Section 3.4.5.3 & Section 3.4.5.4):

- Transcription associated
- Cell Cycle
- Oncogenes and Tumor Suppressors
- Stress associated
- Apoptosis
- Growth Factors and Receptors (including ligand receptors)
- Signalling and Communication
- Housekeeping Proteins
- Neuropeptides and Hormones
- Protein Turnover
- Miscellaneous (including unclassified proteins, proteins involved in motility, carrier proteins surface antigens, etc.)

3.4.5.3 Genes Found to be Induced or Upregulated in Min6 cells Serially Passaged in Culture and Unresponsive to Environmental Glucose Levels

Genbank Accession No.	Gene Name	Array 1	Array 2
Transcription Associated			
M60523	DNA-Binding Protein ID3	NP	Up
S81932	Distal-less homeobox Protein 3	NP	Up
U89876	Transcriptional Co-Activator of AML-1 & LEF-1	Up	Up
U69270	LIM Domain-Binding Protein 1	Up	Up
L21671	EGFR Kinase Substrate EPS8	Np	Up
X53476	Non-histone Chromosomal Protein HMG-14	+3.4	Up
U78103	Embryonic Ectoderm Development Protein (EED)	Np	Up
U29762	D-Binding Protein (DPB)	Np	Up
X72310	Transcription factor E2F dimerisation Partner 1	Np	Up
L12140	Groucho gene-related protein (GRG)	1.9	2.2
Cell Cycle			
Z26580	G2/M Specific Cyclin A2 (CCNA2)	Np	Up
X75888	G1/S Specific Cyclin E1 (CCNE1)	Np	Up
L01640	Cell Division Protein Kinase 4 (CDK4)	Up	Up
AA289122	Cyclin-dependent kinases regulatory subunit 2 (CKS2)	Np	Up
U05341	P55CDC	Up	Up
D78382	Tob Antiproliferative Factor	Np	Up
AF068780	Geminin	Up	Up
D86725	MCM2 DNA replication licensing factor	Np	Up
X56135	Prothymosin alpha (PTMA)	3.4	2.9
X66032	G2/M-specific cyclin B2 (CCNB2)	Up	2
D26090	MCM5 DNA replication licensing factor	2.3	1.8
U43918	Proliferation associated protein 1 (PLFAP)*	1.4	1.6
Oncogenes and Tumor Suppressors			
X60671	Ezrin	Np	Up
Z50013	H-ras proto-oncogene	Np	Up
M94335	akt proto-oncogene	Np	Up
U57311	14-3-3 protein eta	1.6	1.6
X83974	Transcription termination factor 1 (TTF1)*	1.1	1.9
Stress Associated			
L40406	Heat Shock 105 kDa Protein	Np	Up
U40930	Oxidative Stress-induced protein	Np	Up
M36829	Heat shock 84 kDa protein 1 (HSP84-1)	1.8	2.3

Apoptosis

U77714	Survival Motor Neuron (SMN)	Np	Up
U39643	Fas-associated factor 1(FAF1)	Np	Up
AF033353	Sentrin	Np	Up
U44088	T-cell death gene 51 (TDAG51)	Up	2.8

Growth Factors and Receptors (including ligand receptors)

S53216	Tyrosine-Protein Kinase ryk	Np	Up
AF028242	Calcitonin gene-related peptide receptor component	Np	Up
X81582	Insulin-like growth factor binding protein 4 precursor	Np	Up
M89797	Wingless related MMTV integration site 4 protein	Np	Up
AB006191	Cornichon-like protein	Np	Up
AF020738	Fibroblast growth factor 12 (FGF12)	Np	Up
X97818	Semaphorin G	1.7	2.2
M85078	Granulocyte-macrophage colony-stimulating factor Receptor low affinity subunit precursor (GM-CSF-R)	Np	1.5

Signalling and Communication

J00612	Pro-opiomelanocortin-alpha	Np	Up
M36777	Guanine nucleotide binding Protein	Np	Up
U34960	Transducin beta-2 Subunit	Np	Up
Y00703	Guanine Nucleotide binding Protein alpha stimulating activity polypeptide (GNAS)	1.7	2.5

Housekeeping Proteins

L31609	40S Ribosomal protein S29 (RPS29)	2	1.8
X51703	Ubiquitin	2.3	1.6
M32599	GADPH	1.7	1.5
D78647	Phospholipase A*	1.9	1.1

Miscellaneous

U97327	Calcyclin binding protein	Up	Up
X59379	Alzheimer's disease amyloid A4 protein homologue	Np	Up
S50213	Structure Specific recognition protein 1	Up	Up
M64292	Anti-proliferative B-cell translocation gene 2 (BTG2)	Np	Up
U95736	Frataxin (Friedreich ataxia protein; FRDA)	Np	Up
AF021031	DiGeorge Syndrome chromosome region 6 protein (DGCR6)	Up	2
X02165	Neurofilament triplet L protein (NEFL)*	1.2	2

3.4.5.4 Genes Found to be Silenced or Downregulated in Min6 cells Serially Passaged in Culture and Unresponsive to Environmental Glucose Levels

		Array1	Array2
Transcription			
M20157	Early Growth Response Protein 1 (EGR1)	Down	Np
U71208	Eyes absent homologue 2 (EYA2)	Np	Down
M96823	Nucleobindin	Down	Down
L47650	Stat6	Down	Down
L12721	Delta like homologue 1 (DLK1)	-13	-9
X63963	Paired box protein 6 (PAX6)	Down	-1.8
M94087	Activating transcription factor 4*	-1.1	-1.9
Oncogenes and Tumor Suppressors			
U28423	58 kDa inhibitor of RNA-activated protein kinase	-4.8	-3.6
Apoptosis			
J04696	Glutathione S-transferase mu 2 (GSTM2)	Down	Down
L08235	Clusterin (CLU)	Down	Down
Neuropeptides and Hormones			
AF026537	Beta-neoendorphin-dynorphin	NP	Down
X15830	7B2 neuroendocrine protein; secretogranin V	-3.5	-6
Z46845	Preproglucagon	-7	-4.1
U22516	Angiogenin	Down	-3.2
X68837	Secretogranin II*	-1.1	-4.1
Growth Factors and Receptors (including ligand receptors)			
M13177	Transforming growth factor beta 1 (TGF-beta 1)	Np	Down
X80992	Bone morphogenetic protein 6 (BMP6)	Down	Down
X59520	Cholecystokinin	Down	-65.8
J04806	Osteopontin (OP)	Down	-7
X96618	Recombination activating protein 1 gene activation protein	-1.8	-1.9
X16353	Prothmyosin beta 4	NP	-1.8
L24755	Bone morphogenetic Protein 1 (BMP1)	NP	-1.6
Signalling and Communication Proteins			
X53028	Chromogranin B (CHGB)	Down	-36
X95403	Ras-related protein RAB2*	-2	-1.4

Housekeeping genes

U45977	45 kDa calcium binding protein (CAB45)	-4.2	-4.1
M10624	Ornithine decarboxylase (ODC)	Down	-1.8

Protein Turnover

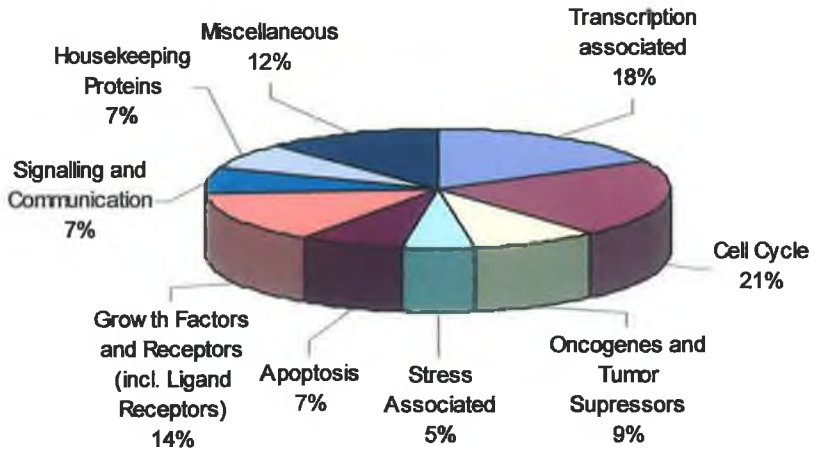
J05261	Carboxypeptidase C (Cathepsin A)	Down	-2.9
X53337	Cathepsin D	Down	-2.1
M55669	Neuroendocrine convertase 2		
	Prohormone convertase 2 (PC2)	-3.3	-1.8
D00611	Basigin Precursor*	-2.2	-1.4

Miscellaneous

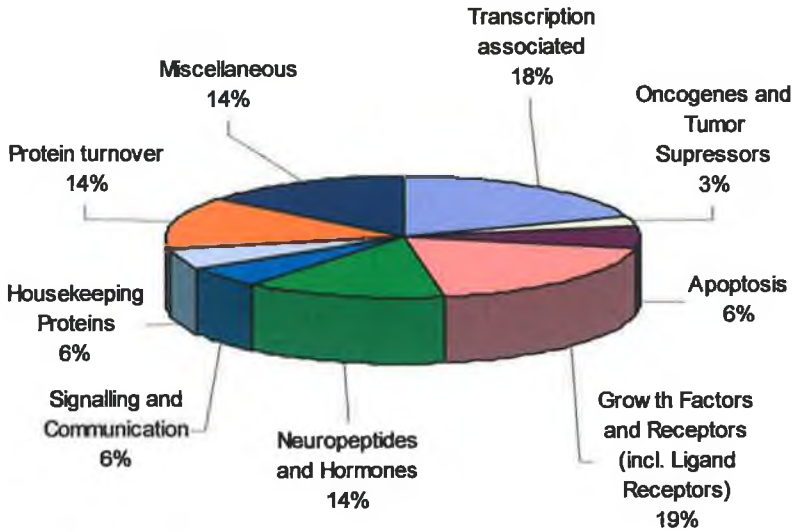
M14222	Cathepsin B1	Down	Np
U13705	Plasma glutathione peroxidase (GSHPX-P)	Down	Down
D89076	Tranthyretin (TTR); Prealbumin	-5	-10
X17320	Brain Specific Polypeptide	Down	-2.9
X05640	Neurofilament triplet M protein*	Down	-1.2

Notes:

- Array 1 refers to the experiment comparing Min6 P18 to Min6 P47, Array 2 refers to the comparison of Min6 P19 and Min6 P48. These experiments were performed on different mRNAs and using different array membranes.
- NP indicates that a particular gene was not detected in that Array Experiment
- * In these cases there was a significant change in expression in one array experiment (> 1.5) but not in the other but the average of the two results is > 1.5.
- When a gene is detected on one membrane and not the other numerical comparison is impossible, therefore if a gene is described as 'UP' then it is only present in high passage Min6 cells and if it is 'Down' then is can only be detected on the low passage membrane.



(A)



(B)

Figure 3.4.7 Comparison of (A) positively and (B) negatively affected categories of genes in serially passaged Min6 cells.

3.4.5.5 RT-PCR Confirmation Analysis of Genes Detected on Array

Primers were available for three of the genes detected using the Atlas™ array membranes, thus RT-PCR analysis was carried out to determine if the results obtained on the array could be reproduced. RT-PCR analysis was carried out for glucagon, Pax6 and Nkx2.2. β actin was used as the internal standard for cDNA quality and quantification as its levels were found to be unchanged at higher passages unlike GAPDH which showed increased mRNA expression (Section 3.4.5.3). Table 3.4.2 below compares the results obtained using the array and RT-PCR analysis. Glucagon mRNA levels were decreased in both cases however the decrease was far more noticeable in the array experiments. Pax6 mRNA was reproducibly downregulated in both arrays, however in the RT-PCRs carried out there was no change in the levels of this gene. Nkx2.2 levels were not altered as a result of passaging in either the array or the RT-PCR.

Gene Name	Fold Change Observed Using Atlas Array	Fold Change Observed Using RT-PCR	RT-PCR Results
Glucagon	-4 to -7	-2	3.4.8
Pax6	-1.8	-1.1 (Unchanged)	3.4.9
Nkx2.2	- 1.4 (Unchanged)	+1.2 (Unchanged)	3.4.10

Table 3.4.2 Comparison of data from Atlas array and RT-PCR results obtained for the same genes.

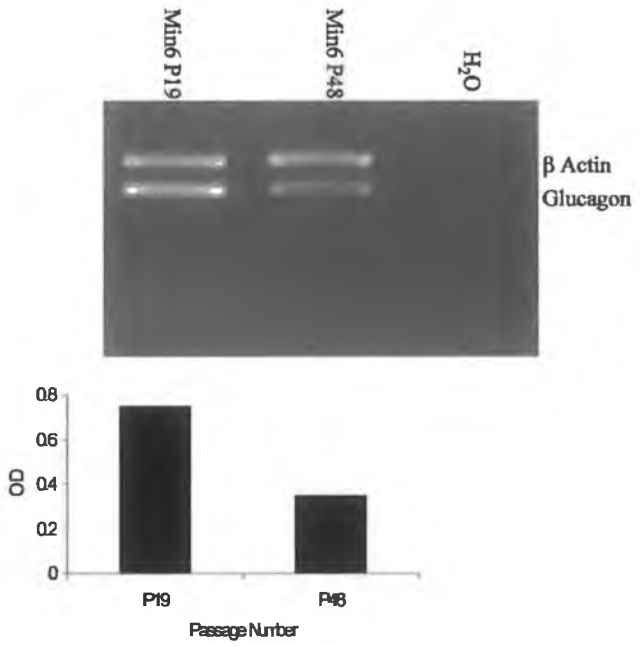


Figure 3.4.8 RT-PCR analysis of glucagon expression in low (P19) and high (P48) passage Min6 cells. Water was included as a negative control. The result presented is representative of repeat gels from two individual experiments.

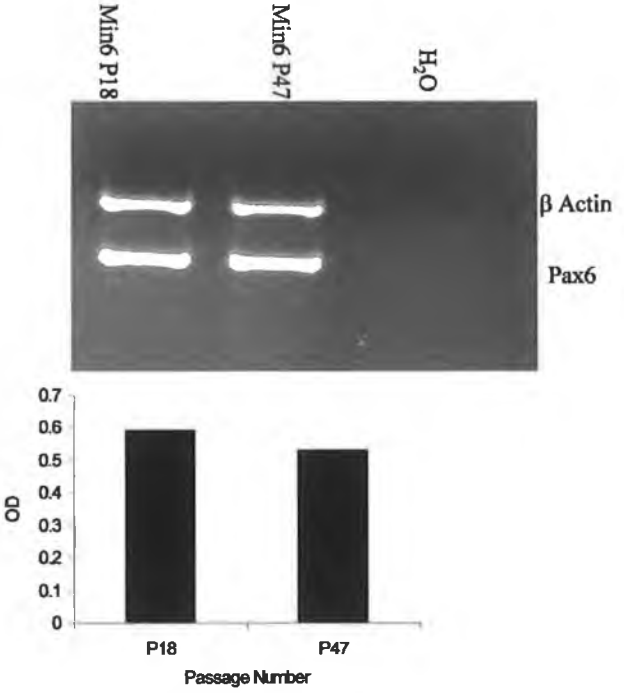


Figure 3.4.9 RT-PCR analysis of Pax6 expression in low (P18) and high (P47) passage Min6 cells. Water was included as a negative control. The result presented is representative of repeat gels from two individual experiments.

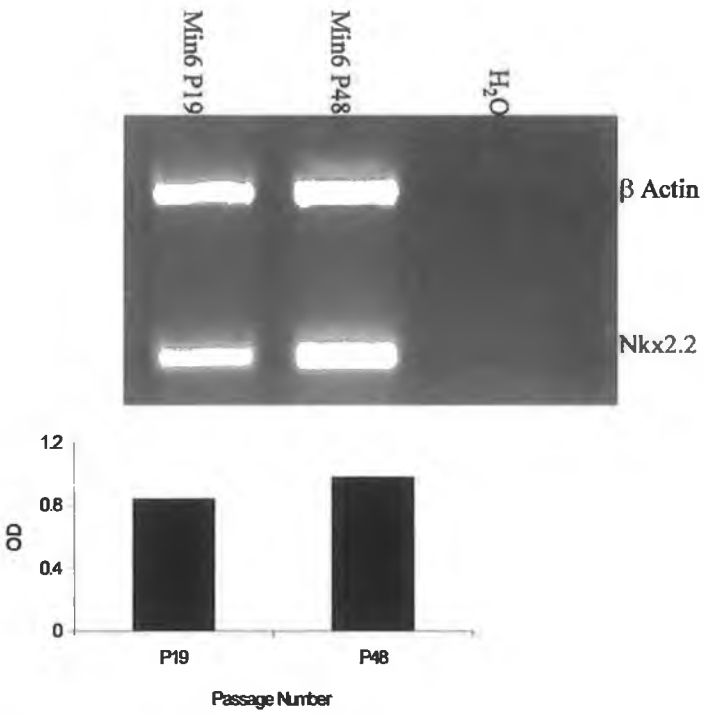


Figure 3.4.10 RT-PCR analysis of Nkx2.2 expression in low (P19) and high (P48) passage Min6 cells. Water was included as a negative control.

3.4.6 RT-PCR Analysis of Pancreatic Markers in Low and High Passage Min6 Cells

The Atlas™ array (Section 3.4.5) revealed many of the changes in gene expression associated with the increased passage number of Min6 cells. However few pancreatic markers were detected even though a number of these were represented on the array membrane e.g. PDX1 and GLUT2. Thus it was decided to carry out RT-PCR analysis to investigate if the continuous culture led to changes in the expression of these genes.

3.4.6.1 PDX1 Expression in Low and High Passage Min6 Cells

PDX1 is a major transcription factor involved in PPI gene transcription and it levels are implicated in regulating GLUT2 and glucokinase transcript levels also. PDX1 mRNA levels were found to be over two fold lower in the high passage Min6 cells than in the lower passage cells (Figure 3.4.11 (A)).

3.4.6.2 PPI Expression in Low and High Passage Min6 Cells

Earlier results (Figure 3.4.3) have shown that the high passage Min6 cells can secrete proinsulin and in fact secrete proinsulin at a greater rate than the lower passage cells tested. As Figure 3.4.11 (B) demonstrates, PPI gene transcript levels are not significantly altered in the high passage cells despite the reduction in PDX1.

3.4.6.3 GLUT2 Expression in Low and High Passage Min6 Cells

GLUT2 mRNA levels were five fold greater in the high passage Min6 cells that were GSIS unresponsive than in the low passage GSIS-capable cells (Figure 3.4.12 (A)). This may be related to the cells increased growth-rate and thus their higher requirement for glucose.

3.4.6.4 Glucokinase Expression in Low and High Passage Min6 Cells

Glucokinase mRNA levels were largely unaffected by the continuous passaging of the Min6 cells to a point where the ability to respond to external glucose had been lost (Figure 3.4.12 (B)).

3.4.6.5 Pancreatic Polypeptide Expression in Low and High Passage Min6 Cells

Min6 cells were found to contain high levels of PP transcripts in as shown in Section 3.2 and from Figure 3.4.13 (A) it is clear that the serial passaging did not affect the expression of this gene.

3.4.6.6 Somatostatin Expression in Low and High Passage Min6 Cells

Min6 cells are already characterised as possessing the ability to express somatostatin (Ohgawara *et al.,* 1995) and this was also demonstrated in Section 3.2 of this thesis. Somatostatin expression was found to be eradicated after the serial passage of Min6 cells to passage 47-48 (Figure 3.4.13 (B)).

3.4.6.7 Beta2 Expression in Low and High Passage Min6 Cells

Beta2 is a crucial transcription factor which is required with PDX1 to ensure efficient transcription of the PPI gene (Section 1.2.1.3.1). As shown in Figure 3.4.14 (A) Beta2 levels were unaffected by the serial passage of Min6 cells through 47 passages and above.

3.4.6.8 Isl1 Expression in Low and High Passage Min6 Cells

Isl1 is another transcription factor that binds to the 5′ region of the PPI gene to facilitate transcription. Isl1 transcript levels were found to be unchanged over 48 passages (Figure 3.4.14 (B)).

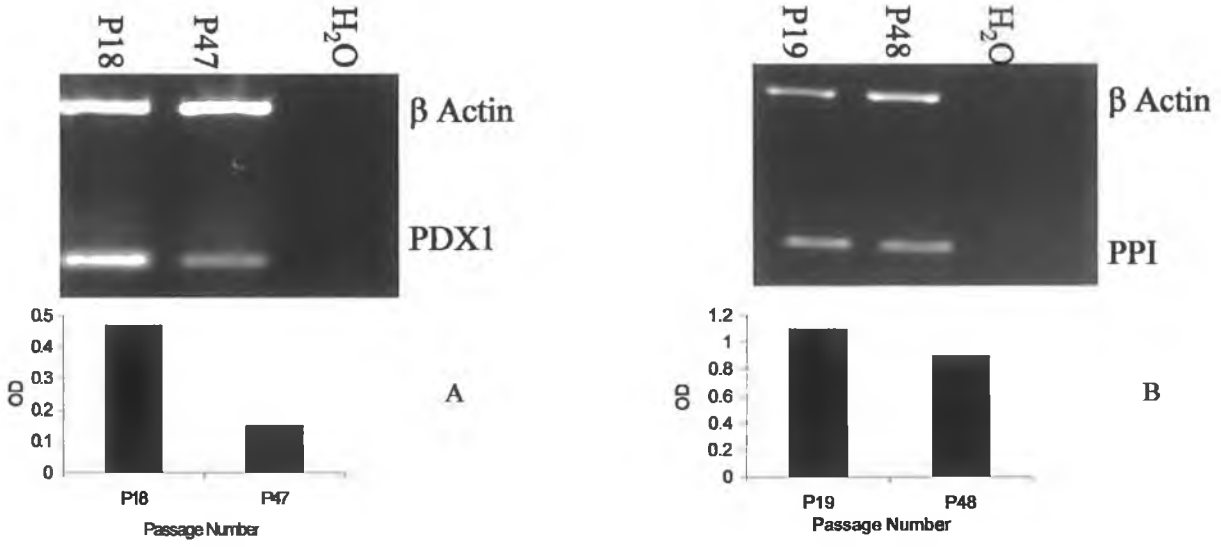


Figure 3.4.11 RT-PCR analysis of (A) PDX1 and (B) PPI in serially passaged Min6 cells. Water was included in all PCR reactions as a negative control. The results presented are representative of repeat gels from two independent experiments.

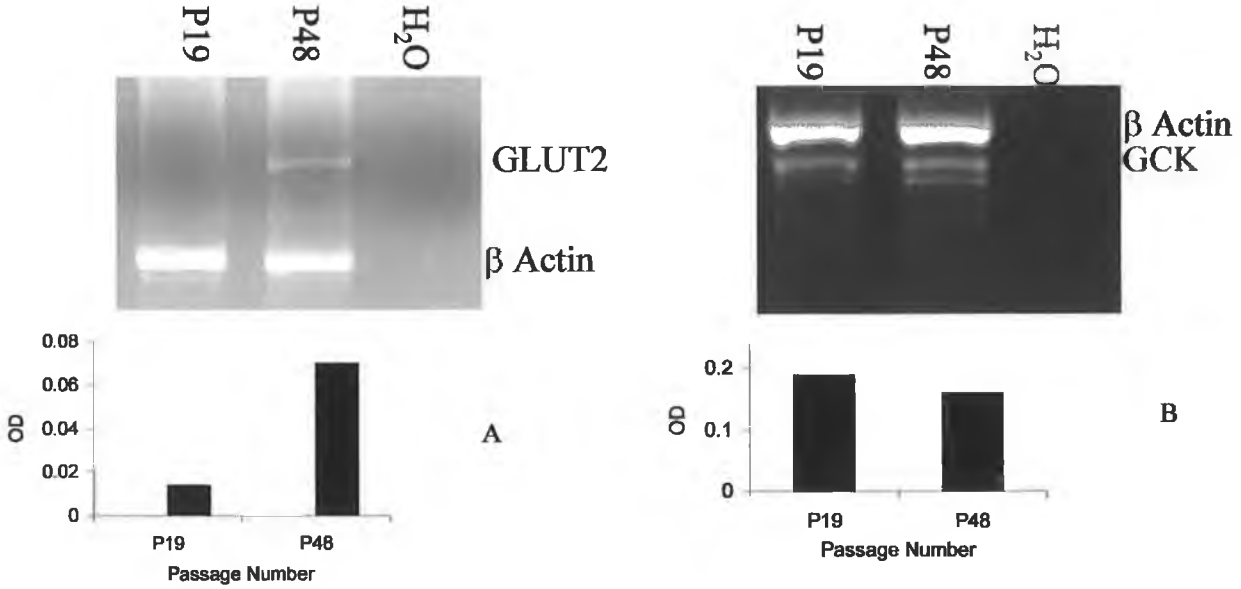


Figure 3.4.12 RT-PCR analysis of (A) GLUT2 and (B) Glucokinase (GCK) levels in serially passaged Min6 cells. Water was included in all PCR reactions as a negative control. The results presented are representative of repeat gels from two independent experiments.

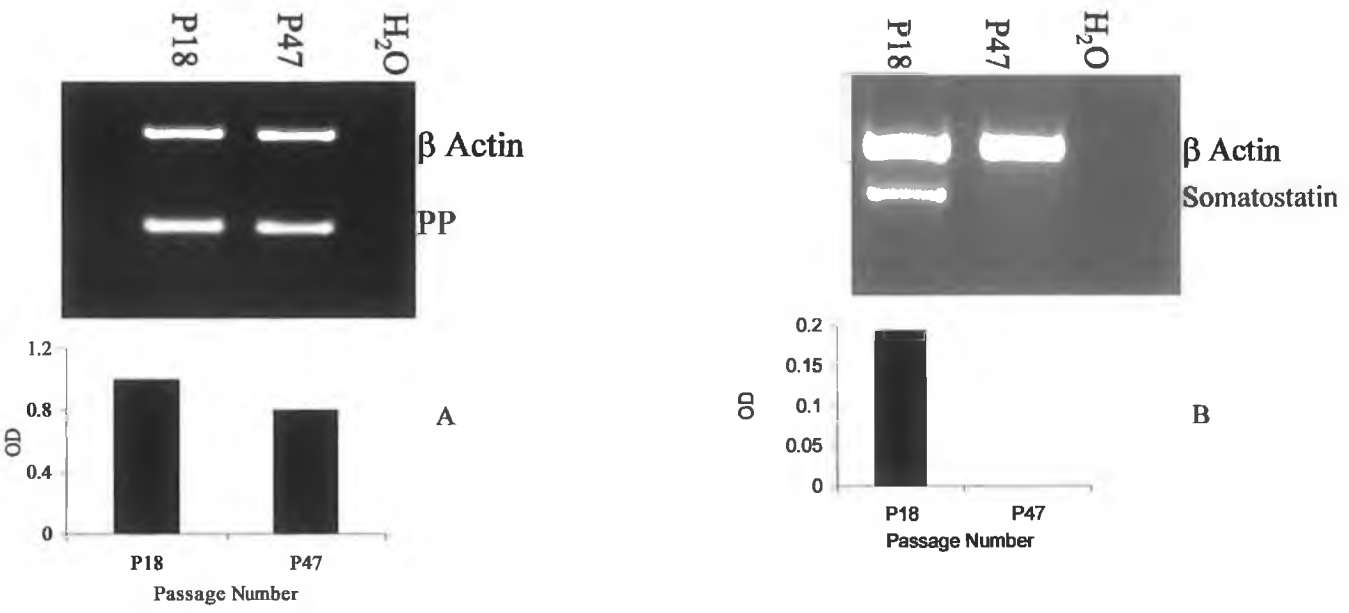


Figure 3.4.13 RT-PCR analysis of (A) PP and (B) somatostatin levels in serially passaged Min6 cells. Water was included in all PCR reactions as a negative control. The results presented are representative of repeat gels from two independent experiments.

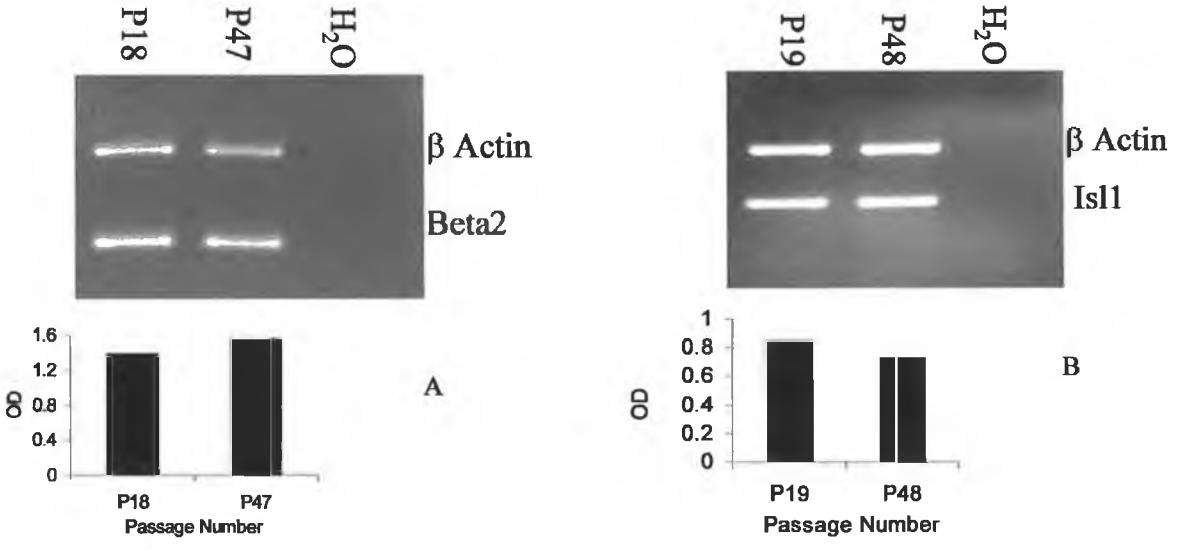


Figure 3.4.14 RT-PCR analysis of (A) Beta2 and (B) Isl1 levels in serially passaged Min6 cells. Water was included in all PCR reactions as a negative control. The results presented are representative of repeat gels from two independent experiments, with the exception of Isl1 which is a preliminary result.

3.5 Characterisation of the Proinsulin Expressing Clone BHK-PPI-16

BHK21 cells were transfected with human preproinsulin (PPI) cDNA in the pT-PPI construct, which contains the human PPI cDNA. 20 cell lines resistant to 1 mg/ml geneticin were isolated. Once stocks of the clones had been created, initial characterisation was carried out, that led to the selection of the clone expressing and secreting the greatest levels of proinsulin. This clone (BHK-PPI-C16) was further characterised for β cell properties.

3.5.1 Human Preproinsulin mRNA Transcription in BHK-PPI-C16

Expression of PPI in the selected clone, BHK-PPI-C16, was confirmed by reverse transcriptase PCR (RT-PCR). From Figure 3.5.1 it is clear that there is expression of PPI at the mRNA level, while the parental line BHK-21 does not transcribe the PPI gene. The plasmid pT-PPI and the β cell line Min6 were used as positive controls in this reaction.

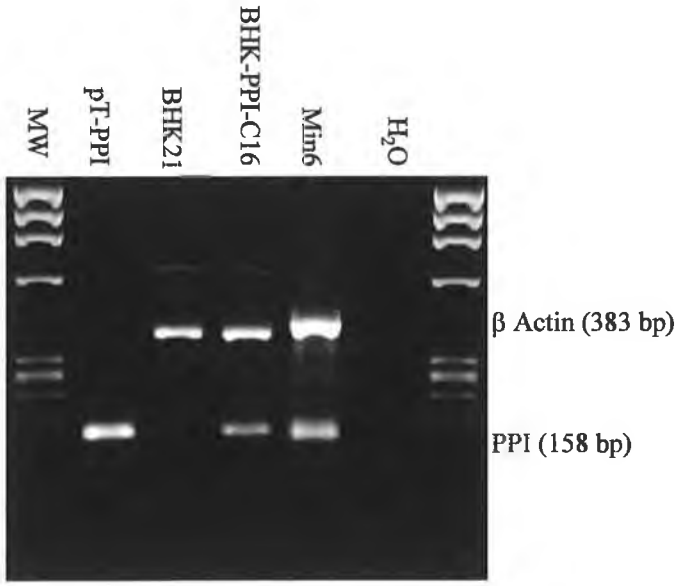


Figure 3.5.1 RT-PCR analysis of human PPI expression in BHK-21 and in the transfected clone BHK-PPI-C16. β-actin was employed as an endogenous control in the reaction. MW refers to molecular weight marker.

3.5.2 Immunocytochemistry for Proinsulin in BHK-PPI-C16

Figure 3.5.2 shows the expression of proinsulin within BHK-PPI-C16 fibroblasts. The cells were stained with a polyclonal antibody for proinsulin (Biogenesis). There is strong staining for proinsulin in the transfected clone whereas there is little or no staining in the parental cell line. Expression of the protein is diffuse within the cytoplasm and there is no evidence of discrete storage granules that are present in the mature β-cells, Min6 (Figure 3.5.2 (C)). The staining in this cell line is of a more granular nature, although the magnification is greater in this case and may allow granules to be seen.

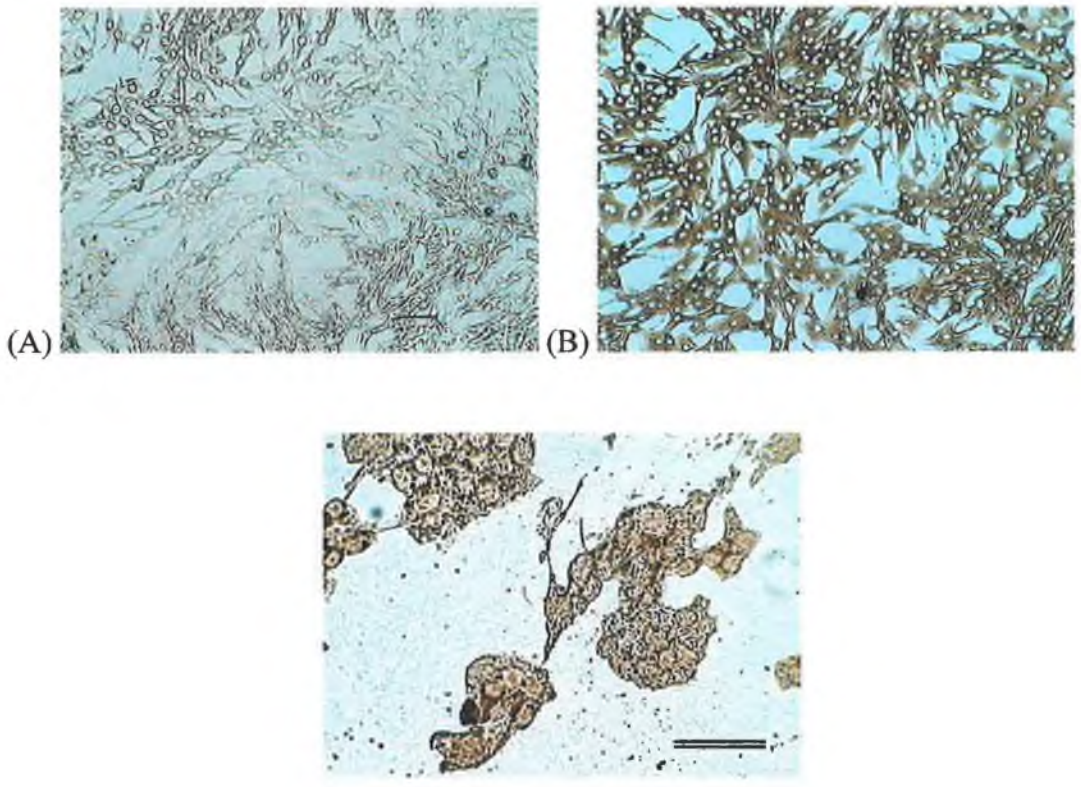


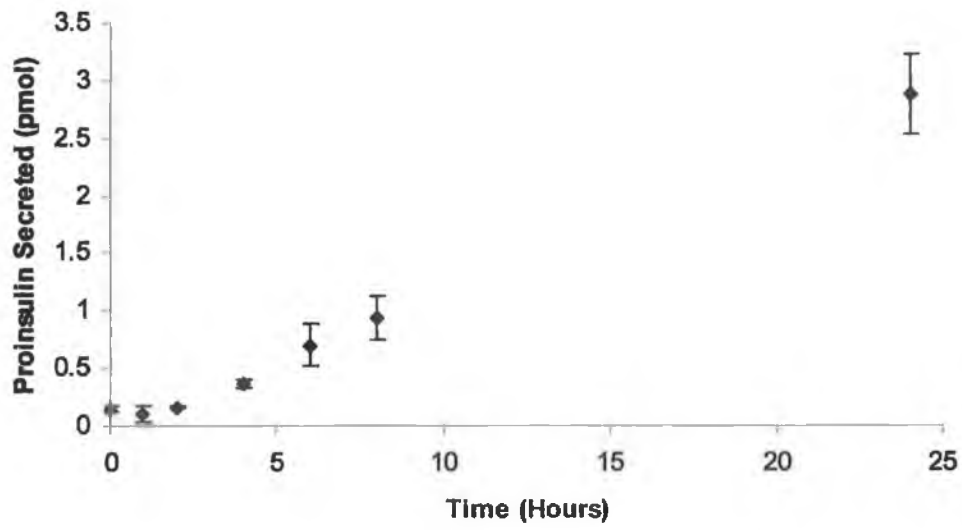
Figure 3.5.2 Immunocytochemistry for Proinsulin expression in (A) BHK21, (B) BHK-PPI-C16 and (C) Min6 cells. The brown DAB staining indicates the presence of PPI in both BHK-PPI-C16 and the positive control cell line, Min6. Magnification = 20 X for BHK21 and BHK-PPI-C16 and is at 40 X for Min6 due to the small size of the cells. Bar = 50 μm.

3.5.3 Proinsulin Secretion from BHK-PPI-C16

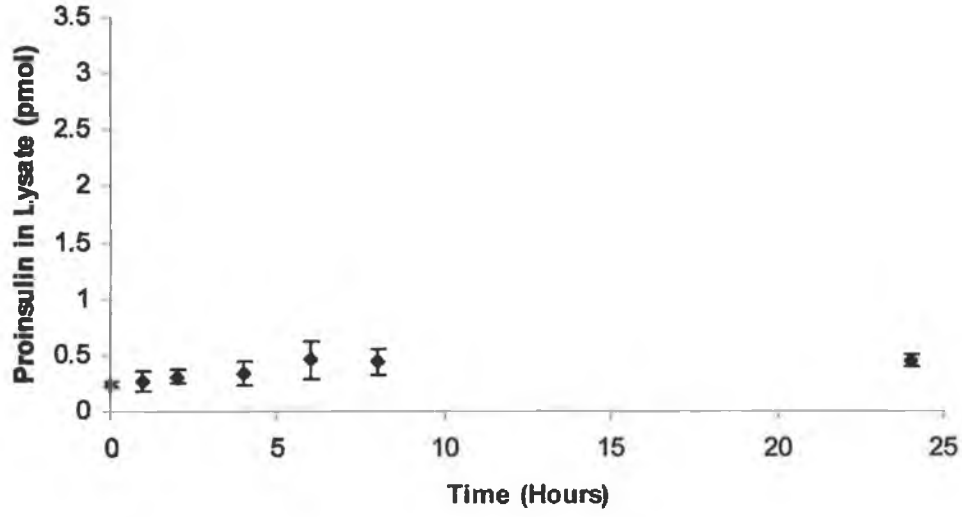
Previous attempts to generate artificial β-cell lines from immortalised non β-cell lines have been hampered by the high basal secretion of proinsulin from the cells (Selden *et al.*, 1987; Kawakami *et al.,* 1992). Preliminary experiments to determine the constitutive proinsulin release from BHK-PPI-C16 were carried out at 5.6 mM glucose. This is approximately the resting physiological glucose concentration in the blood and it is the glucose concentration to which the cells are typically exposed to while in culture.

Proinsulin was measured using a proinsulin EIA (Mercodia). It was found that the cells released proinsulin at a steady rate of 0.12 pmol/hour/$1x10^5$ cells. By lysing the cells at each time point, it was possible to measure the amount of proinsulin within the cells. The proinsulin levels within the cells remained relatively constant over the course of the experiment (24 hours), and was measured at 0.36 pmol ± 0.04. Thus the cells secrete approximately 34% of their proinsulin into the medium every hour (Figure 3.5.3).

Using a specific EIA kit (Mercodia) with a monoclonal antibody for mature human insulin, it was found that the cells do not secrete any processed insulin nor do they store any mature insulin within the cell.



(A)



(B)

Figure 3.5.3 Constitutive Secretion of Proinsulin from BHK-PPI-16. The above graphs represent (A) the linear increase in proinsulin in the medium as a result of constitutive secretion and (B) the unchanging cellular proinsulin levels over the same 24 hours. The data is presented as means ± SD of at least three individual experiments.

3.5.3.1 Regulatable Proinsulin Secretion From BHK-PPI-C16 in Response to Glucose

Fibroblast cell lines have previously been described as possessing constitutive secretion pathways. Cells of this type can not be stimulated to secrete increased amounts of protein in response to extracellular signals; in fact the only modulator of secretion is protein availability (Halban and Irminger, 1994). Cells constitutively expressing and secreting insulin have been used in animal trials for diabetes (Taniguchi *et al.* 1997). The goal for an artificial β-cell, however, is that it would be responsive to glucose concentrations similar to those in the blood. It would be similarly desirable that the cells would respond to other secretagogues that stimulate the release of insulin from mature β-cells, e.g. the sulphonylurea drugs.

3.5.3.2 Investigation of the Response of BHK-PPI-16 to Glucose Concentrations Covering the Physiological Range

The physiological range for blood glucose is in the region 4-5 mM to 20 mM. For a cell to be a truly artificial β cell it should only begin active secretion of insulin once the glucose levels in its external environment exceed 4-5 mM. From Figure 3.5.4 it is clear that there is no significant response of the BHK-PPI-C16 cells to glucose over the physiological range. Secretion was constant regardless of external glucose concentrations and the cells secreted proinsulin only. No processed insulin was detected in the conditioned medium. Conversely, when cultured murine β cells (Min6) were stimulated for 30 minutes in conditions of increasing glucose there was a concomitant increase in proinsulin secretion which correlated with the increasing glucose concentration (Figure 3.5.5).

3.5.3.3 Investigation of the Response of BHK-PPI-16 to Glucose Concentrations Below the Physiological Range

Studies by Hughes *et al.* (1992) and Hohmeier *et al.* (1997) have generated cell lines expressing proinsulin that show GSIS at glucose levels < 1 mM. Thus it was decided to determine if similar subphysiological GSIS could be achieved for BHK-PPI-C16. As indicated in Figure 3.5.6, proinsulin secretion rates were not influenced by glucose levels below the physiological range.

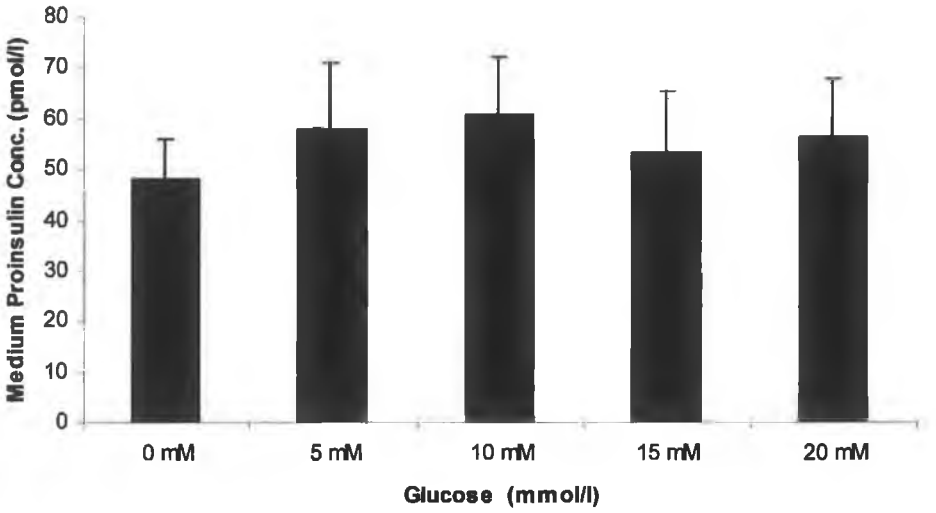


Figure 3.5.4: The effect of physiological glucose levels on BHK-PPI-C16. The figure presented represents the average concentration of proinsulin secreted by 5 x 10^4 cells over two hours. The data presented is of mean values ± SD of at least three independent experiments.

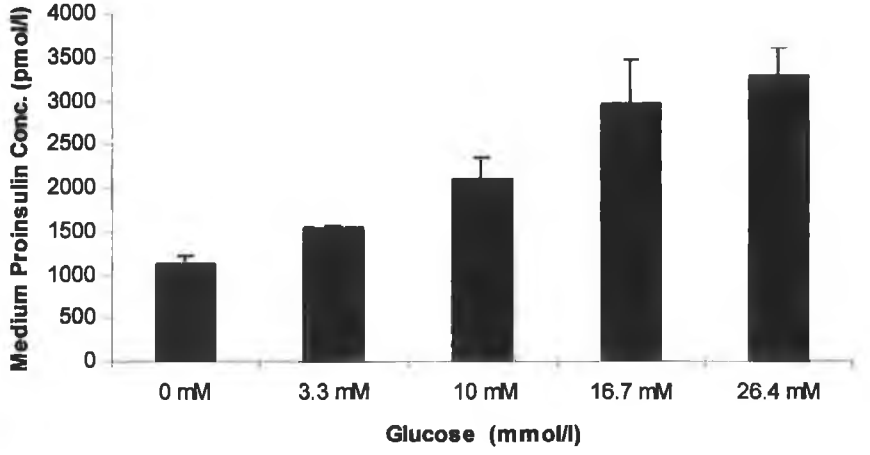


Figure 3.5.5: The graph above represents the level of stimulation displayed by Min6 cells after 30 minutes exposure to increasing levels of glucose. The Min6 cells were seeded at 2 x 10^5 cells/well in 24 well plates for 72 hours prior to the stimulation (Section 2.3.5.3). The data presented is of mean values ± SD, representative of at least three independent experiments.

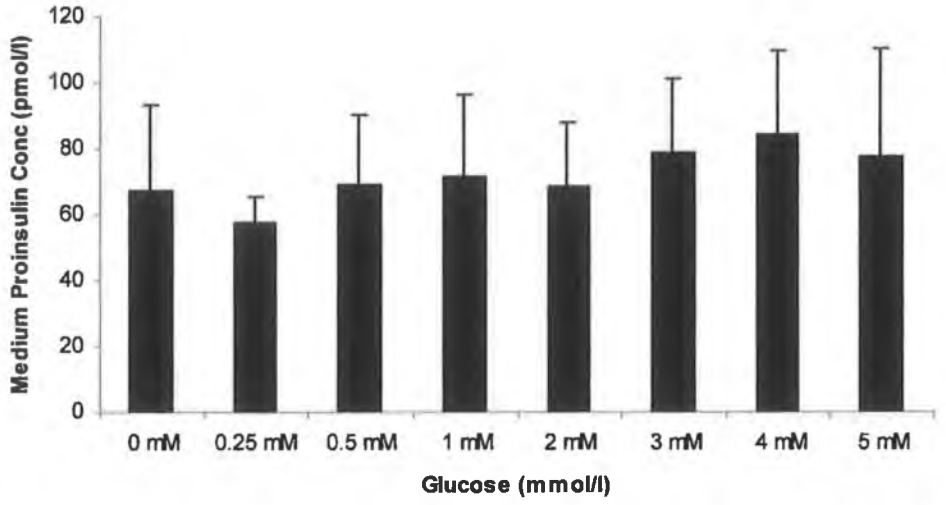


Figure 3.5.6 The effect of subphysiological glucose levels on the secretion of proinsulin from BHK-PPI-C16. This figure represents the concentration of proinsulin secreted by 5 x 10^4 cells over two hours. The data presented is of mean values ± SD of at least three independent experiments.

3.5.4 Expression Levels of the Glucose Sensing Proteins GLUT2 and Glucokinase (GCK) in BHK-PPI-C16

As the proinsulin expressing cell line BHK-PPI-C16 showed such general insensitivity to glucose, it was decided to determine if the components of the glucose sensing system were present, i.e. glucose transporter type 2 (GLUT2) and glucokinase (GCK). Levels of these proteins were determined by immunocytochemistry.

As shown in Figure 3.5.7 (A), it is clear that BHK-PPI-C16 does express the high km glucose transporter, GLUT2, but that expression levels are relatively low when compared to the β cell line, Min6.

The BHK-PPI-C16 cell line was also screened for the presence of the other component of the glucose sensing system i.e. GCK. From Figure 3.5.7 (E) it is again clear that low level GCK expression is present in the cytoplasm of the BHK-PPI-C16 cells. Min6 cells were used as a positive control for GCK in this experiment.

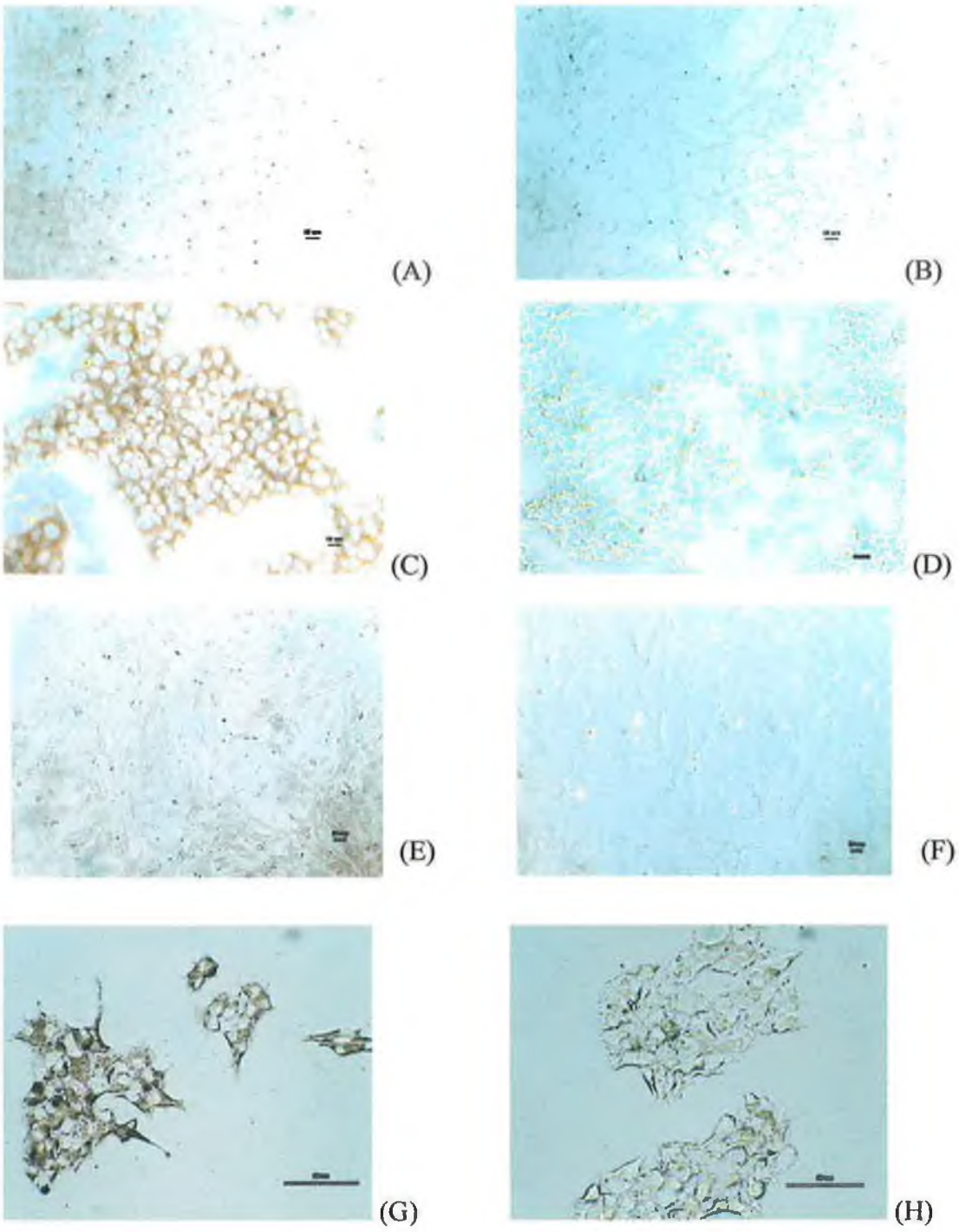
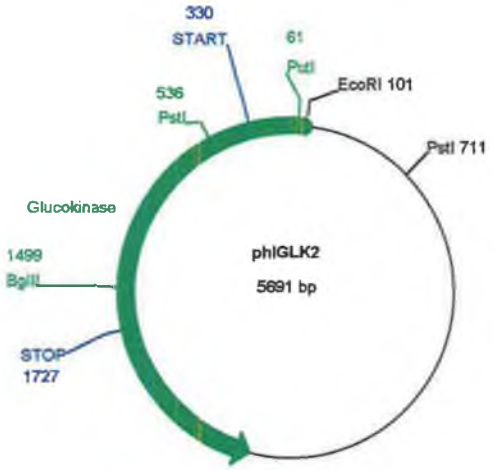


Figure 3.5.7 Immunocytochemical analysis demonstrating GLUT2 expression in BHK-PPI-C16 (A) and in the positive control cell line Min6 (C). GCK expression was also found to be present in BHK-PPI-C16 (E) and again Min 6 was used as a positive control (G). Negative controls whereby the primary antibody was omitted were carried out in each case and are presented above (B, D, F & H). Magnification was 10 X for BHK-PPI-C16 but was increased to 20X (C&D) and 40 X for Min6 (G&H) due to the small size and localised growth of the cells. Bar = 50 μm.

3.5.5 Transfection of Glucose Sensing Genes in to BHK-PPI-C16

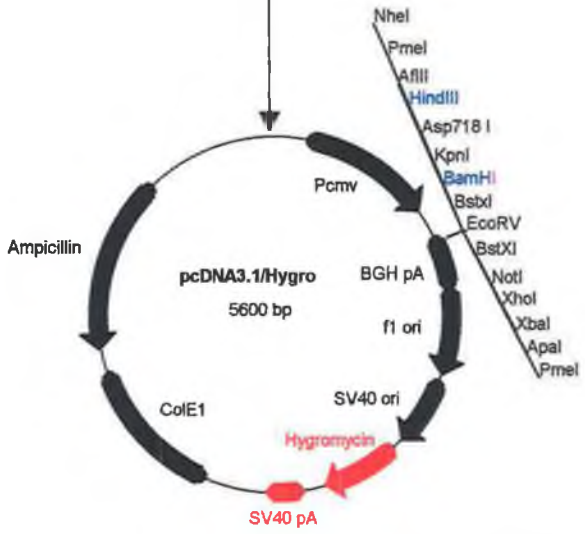
The presence of low-level GLUT2 and GCK expression was confirmed in BHK-PPI-C16 in Section 3.5.4. It was decided to investigate the effect of over-expression of the glucose sensing proteins in the insulin-expressing clone BHK-PPI-C16.

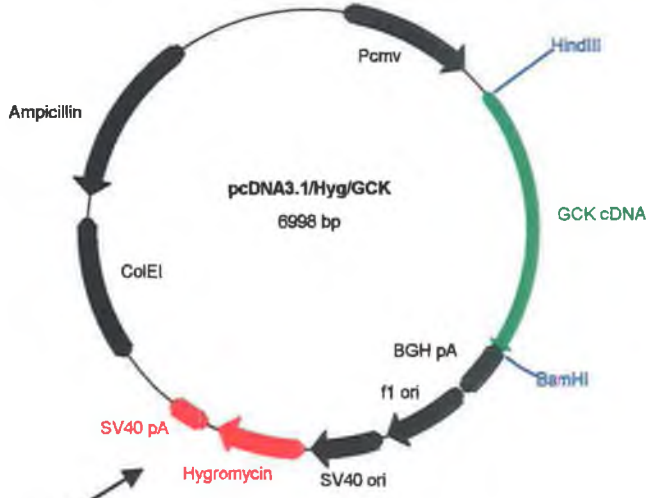
The glucose sensing genes, GLUT2 and glucokinase, were transfected in to BHK-PPI-C16 in two mammalian expression vectors. Glucokinase was transfected initially in the expression vector pcDNA3.1/Hygromycin (Invitrogen, Cat V870-20). GLUT2 was subsequently transfected in the pcDNA3.1/Zeocin construct (Invitrogen, Cat V870-20). The generation of these expression vectors was carried out by Cambridge Biosciences according to specifications, outline diagrams describing the vectors can be seen in Figure 3.5.8 and 3.5.9. Upon transfection in to the cells, co-selection was carried out at levels of 150 μg/ml hygromycin and 100μg/ml zeocin.



R.E Sites within the GCK coding region are positioned relative to the 5' end of the cDNA whereas RE sites on the plasmid are relative to an arbitrary zero position on the plasmid.

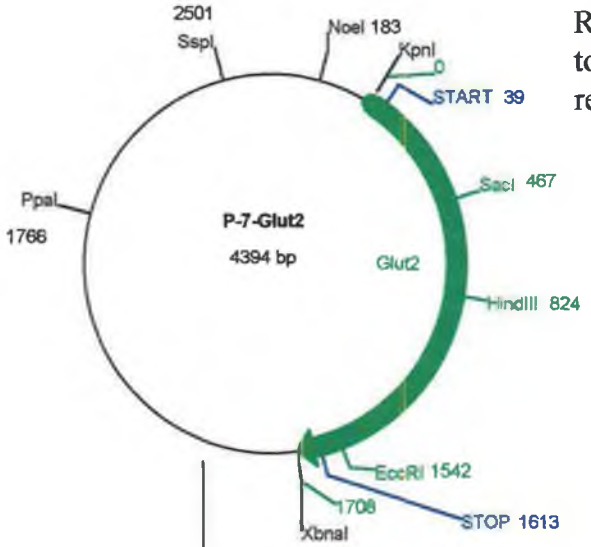
1. PCR Primers were generated for the GCK coding sequence. The resulting PCR product was digested with HindIII/BamHI and gel Purified.



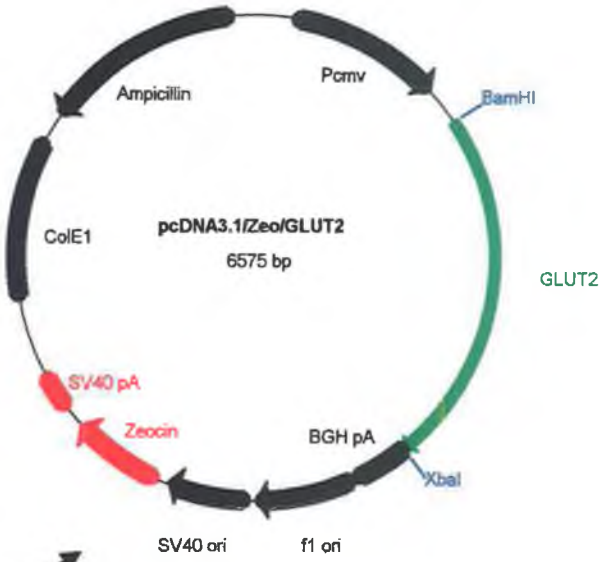


2. The purified coding sequence was ligated in to PcDNA3.1/Hygro digested with HindIII/BamHI.

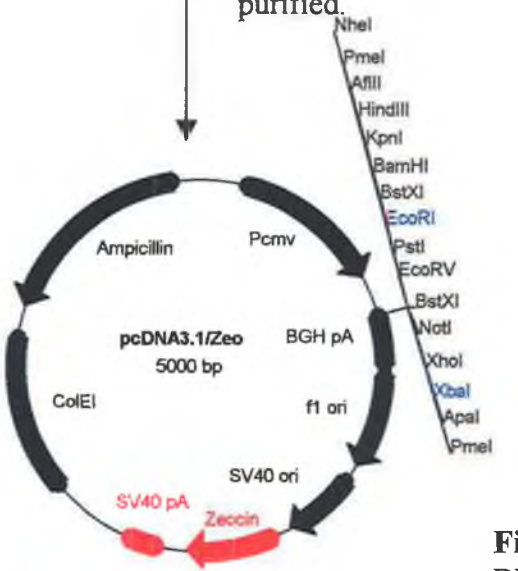
Figure 3.5.8 Generation of Glucokinase cDNA expression plasmid for transfection in to BHK-PPI-C16 cells



R.E Sites within the GLUT2 coding region are positioned relative to the 5' end of the cDNA whereas RE sites on the plasmid are relative to an arbitrary zero position on the plasmid.



1. Primers were designed specific for the GLUT2 coding sequence (a kozak sequence was included before the ATG initiation codon). The 1575 product was then digested with BamHI/XbaI and gel purified.



2. The gel purified coding sequence was then ligated in to pcDNA3.1/Zeo digested with BamHI/XbaI

Figure 3.5.9 Generation of GLUT2 cDNA expression plasmid for transfection in to BHK-PPI-C16

3.5.5.1 RT-PCR Analysis of GCK Expression in Transfected BHK cell lines

From Figure 3.5.10 it can be clearly seen that overexpression of GCK was achieved in the PPI expressing cell line BHK-PPI-C16 following GCK transfection alone (BHK-PPI-C16_GCK) or along with GLUT2 (BHK-PPI-C16_GCK_GLUT). The low level GCK expression in BHK-21 identified by immunocytochemistry (Section 3.5.4) is confirmed here. This low-level expression is unaffected by the presence of the PPI gene transcripts. The plasmid pcDNA3.1-GCK was used as a positive control in the PCR reaction, while β-actin was employed as an endogenous control.

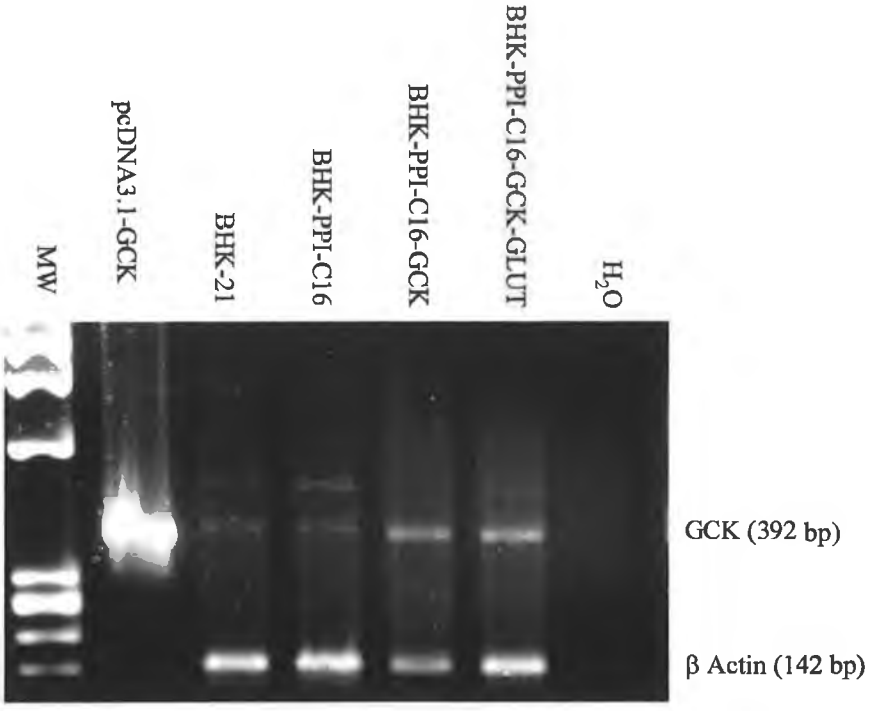


Figure 3.5.10 RT-PCR analysis of GCK expression in BHK-21 cell lines clearly demonstrating the overexpression of the transfected GCK cDNA in the two transfected populations i.e. BHK-PPI-C16_GCK and BHK-PPI-C16_GCK_GLUT.

3.5.5.2 Immunocytochemical analysis of GCK expression in transfected BHK cell lines

Using immunocytochemical analysis to investigate GCK expression in the cell lines transfected with GCK cDNA, it can be seen that there is no apparent change in GCK protein expression (Figure 3.5.11 (C)) when compared to the non-transfected population (Figure 3.5.11 (A)). Intensely stained cells however can be seen within the GCK transfected population and are highlighted with arrows, Figure 3.5.11 (C).

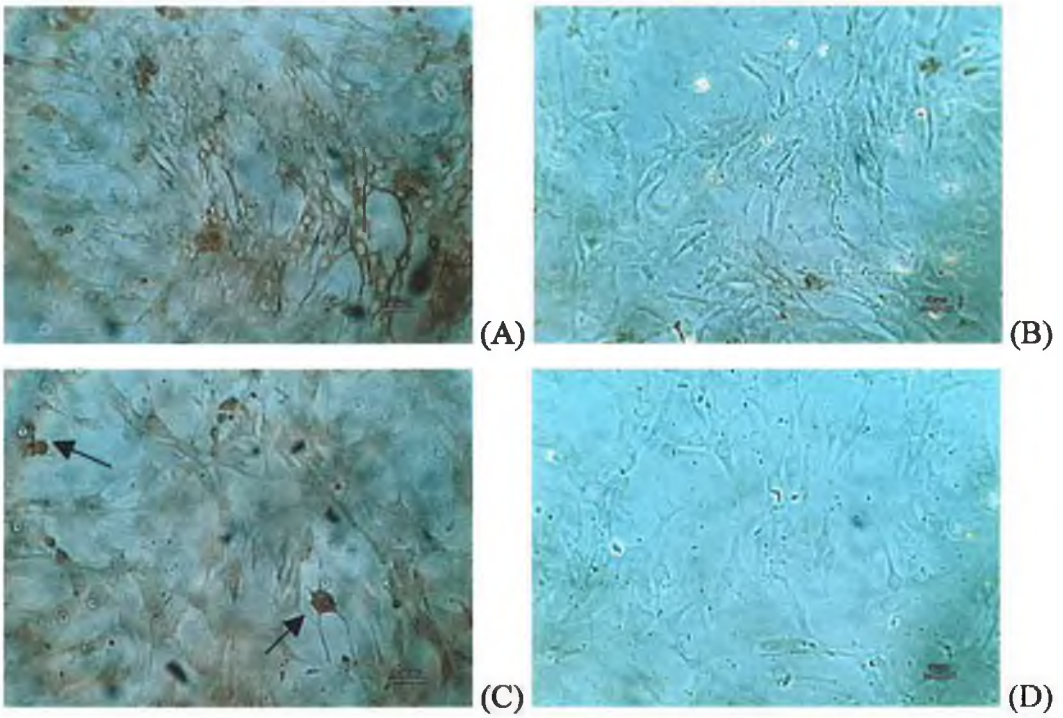


Figure 3.5.11 Immunocytochemical analysis of GCK expression in (A) BHK-PPI-C16 and in (B) the transfected population BHK-PPI-C16_GCK. There is no noticeable increase in GCK expression overall but intensely stained cells are present in the transfected population (indicated by arrows). Negative controls where the primary antibody was omitted were also included to demonstrate that the staining was not due to non-specific binding of the secondary antibody (B&D). Magnification = 20 X. Bar = 50 μm.

3.5.5.3 Western Blot analysis of GCK expression in BHK-PPI-C16

As immunocytochemistry is typically regarded as a qualitative rather than a quantitative technique, western blotting was carried out to determine if there was a noticeable up-regulation of GCK protein as a result of the transfection of the GCK cDNA. From Figure 3.5.12 it is clear that there is little or no up regulation of GCK protein. Densitometric analysis confirmed this by normalising the GCK bands against the levels of the housekeeping protein β-actin. The poorly differentiated cell human lung carcinoma cell line, DLKP, was employed as a positive control in this experiment, this has already been characterised as expressing GCK (O' Driscoll *et al.,* 2002).

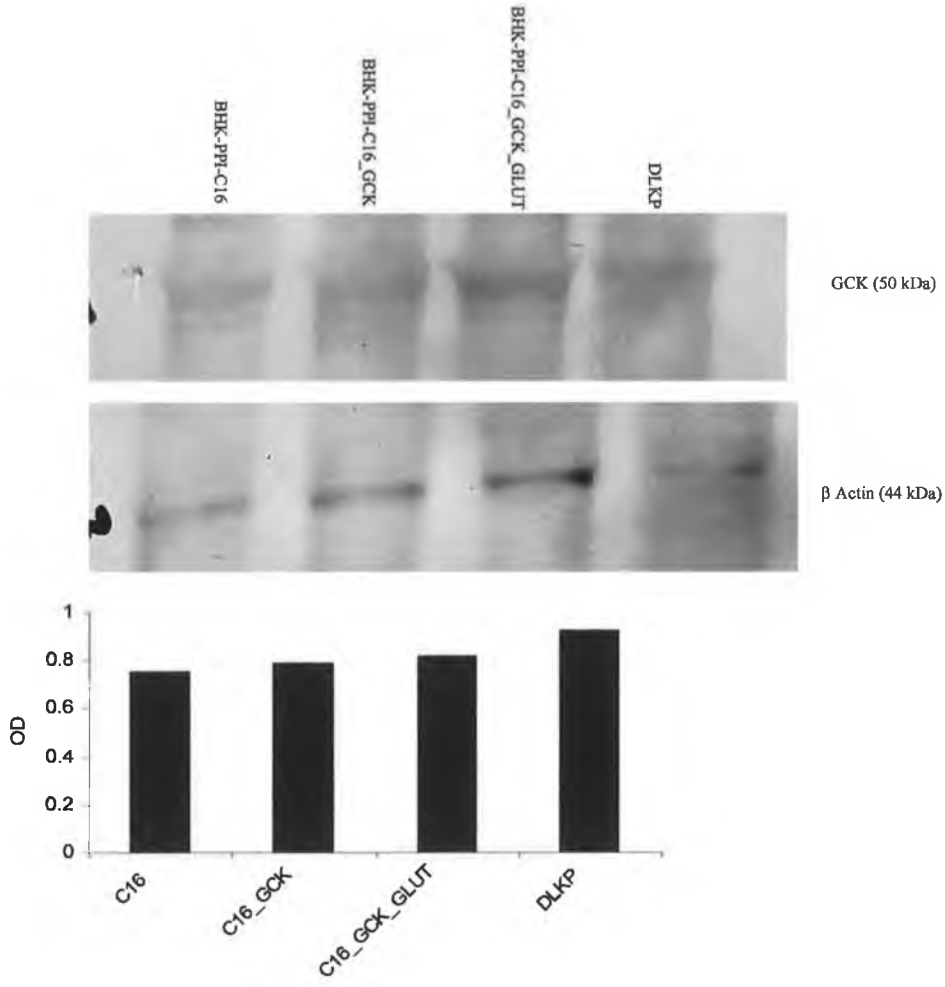


Figure 3.5.12 Glucokinase expression in BHK-21 cell lines. β-actin was used as an endogenous control for densitometry. DLKP was used as a positive control for GCK expression.

3.5.5.4 RT-PCR Analysis of GLUT2 expression in GLUT2 Transfected BHK-PPI-C16 Cell Lines

From Figure 3.5.13 it is clear that the transfection of GLUT2 into the BHK-PPI-C16 cell line was successful and that GLUT2 over-expression was achieved. Faint traces of GLUT2 bands are clear in the BHK-21 and BHK-PPI-C16 cell lines which correlates with the immunocytochemical analysis in Section 3.5.4, (Figure 3.5.7 (A)). Expression of the PPI gene had no effect on the level of GLUT2 transcripts present.

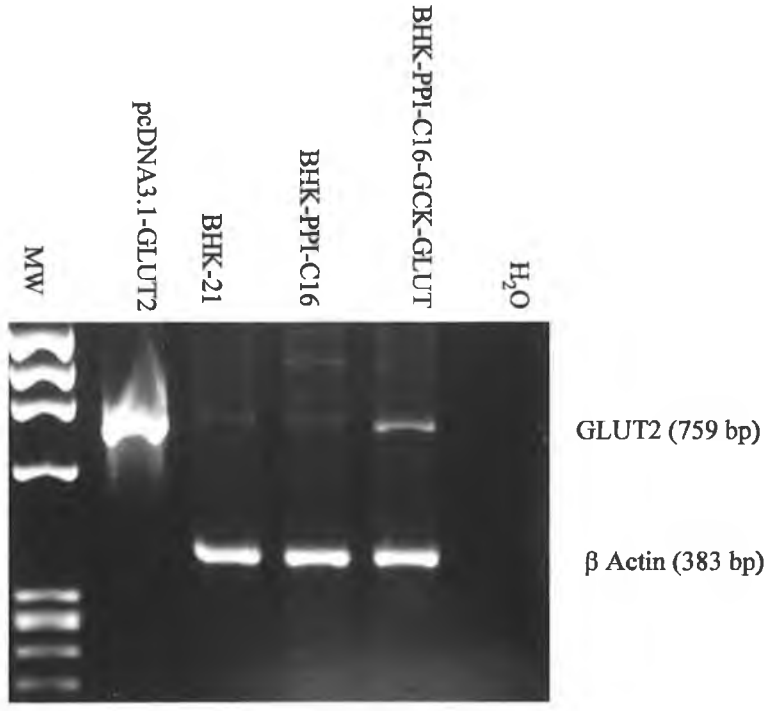


Figure 3.5.13 RT-PCR analysis of GLUT2 in BHK-21 cell lines clearly demonstrating the overexpression of the GLUT2 cDNA in the transfected population BHK-PPI-C16_GCK_GLUT.

3.5.5.5 Immunocytochemical analysis of GLUT2 overexpression in BHK-PPI-C16 cells

The immunocytochemical analysis presented in Figure 3.5.14 appears to indicate that increased expression of GLUT2 was achieved as a result of the transfection of GLUT2 cDNA in to BHK-PPI-C16 cells. It is clear that the population is not homogenous, i.e. there are cells within the population that are overexpressing GLUT2 to a greater degree than others.

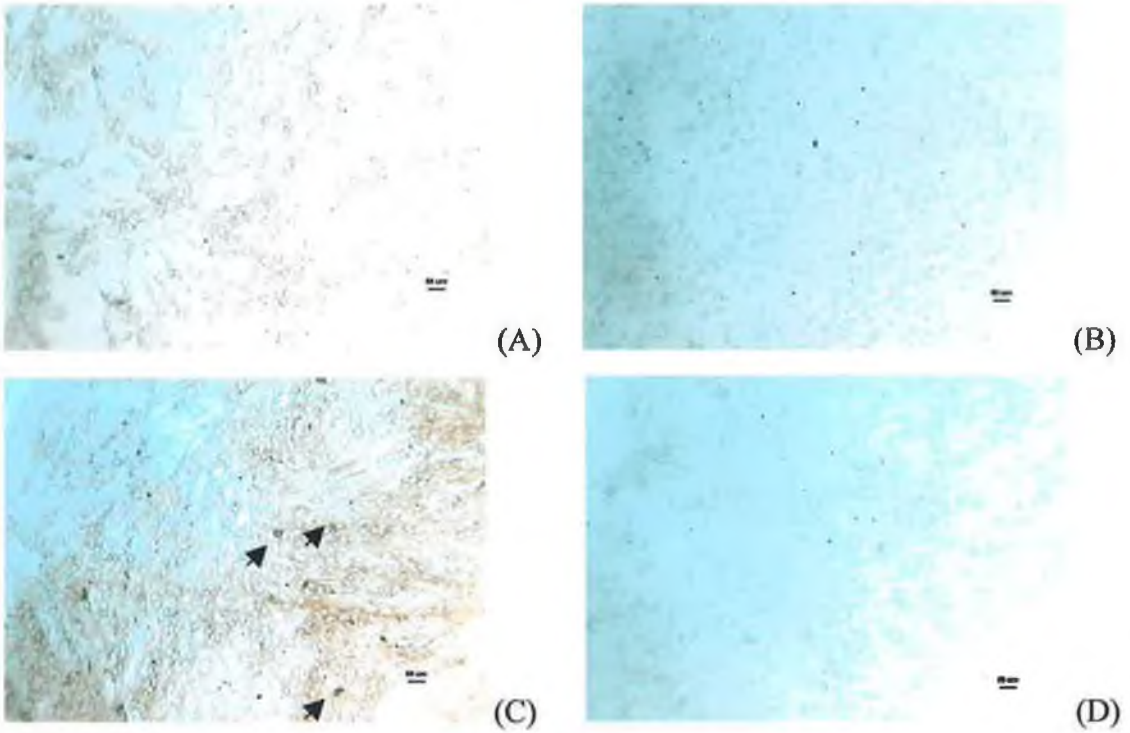


Figure 3.5.14 Immunocytochemical analysis of GLUT2 expression in (A) BHK-PPI-C16 and (C) in the transfected population BHK-PPI-C16_GCK_GLUT. Arrows indicate intensely stained cells within the BHK-PPI-C16_GCK_GLUT population. Also included are negative controls (B&D) where the primary antibody was omitted. This demonstrates that staining is not due to non-specific binding of the labelled secondary antibody. Magnification = 10X. Bar = 50 μm.

3.5.5.6 Western analysis of GLUT2 Overexpression in BHK Cells

To provide quantitative information regarding the expression of GLUT2 protein in BHK-PPI-C16_GCK_GLUT cells, western blotting was carried out. From Figure 3.5.15 it is clear that the effect of the transfection was minor at the protein level. Densitometric analysis confirms that there is a slight up regulation of GLUT2 but it is not significant. Expression of GCK in BHK-PPI-C16_GCK had no significant effect on the endogenous GLUT2 levels. The poorly differentiated human lung carcinoma DLKP was employed as a positive control. This cell line has previously been assessed as being positive for GLUT2 (O' Driscoll *et al.* 2002).

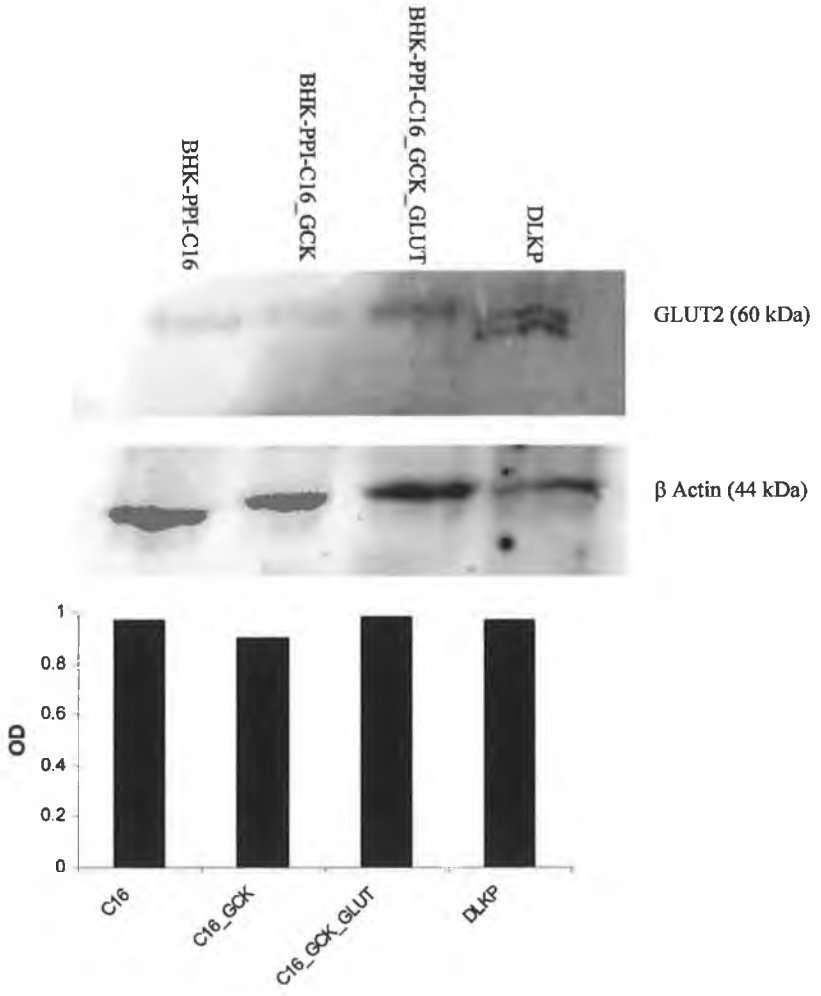


Figure 3.5.15 GLUT2 expression in BHK Cell Lines. β-actin was used as an endogenous control for densitometric analysis. DLKP was included as a positive control for GLUT2 expression.

3.5.5.7 Glucose Stimulation of the Cell Lines Transfected with the Glucose Sensing Genes

As illustrated in Figure 3.5.16, it appears as if there is an increase in proinsulin secretion in the population expressing glucokinase (BHK-PPI-C16_GCK), up to a level of 2-5 mM glucose. At these glucose concentrations there was a maximal (2-fold) stimulation in insulin secretion over basal levels. Full GSIS over the entire physiological range was not achieved, however, as the increase did not continue in a dose dependent fashion with increasing glucose; in fact there was a fall-off in secretion at higher glucose levels. Proinsulin secretion was negligable in the doubly transfected line BHK-PPI-C16_GCK_GLUT.

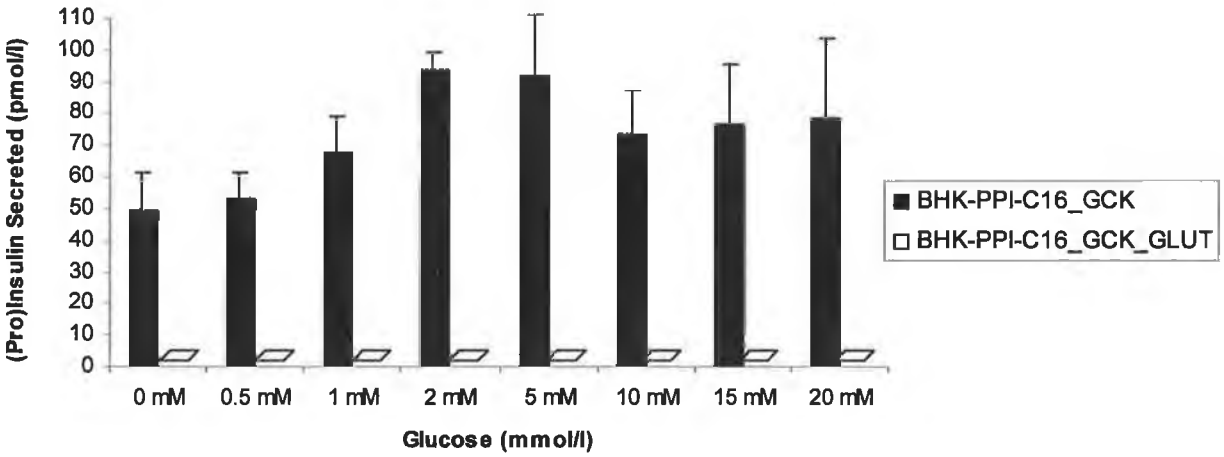


Figure 3.5.16 Secretion of Proinsulin from BHK-PPI-C16_GCK and BHK-PPI-C16_GCK_GLUT upon stimulation with increasing glucose levels. The figures presented represents the concentration of proinsulin secreted by 5 x 10^4 cells over two hours. The data above represents means ± SD of at least three independent experiments.

3.5.5.8 Impaired Protein Synthesis in Transfected BHK-PPI-C16 Cells

Proinsulin secretion from BHK-PPI-C16_GCK_GLUT cells was completely absent following the transfection of the two components of the glucose sensor (GLUT2 and GCK), despite the fact that there were significant levels of PPI mRNA in these cells (Figure 3.5.17). The problem appeared to be at the level of protein synthesis; this, and the observation that the cells exhibited a reduced growth rate indicated a negative effect due to increased transfection. The levels of two 'housekeeping proteins' (β actin and α tubulin) expressed in equal cell numbers were examined and, as it can be seen in Figure 3.5.18, there is a large reduction in expression levels of these proteins associated with the increasing number of transfected genes. Biorad analysis (Bradford based protein assay; Section 2.5.2.1.2.1) of pellets of equal cell number confirmed that cellular protein levels were 2-fold lower in the BHK-PPI-C16_GCK_GLUT cells when compared to the BHK-PPI-C16 cell line (Figure 3.5.19).

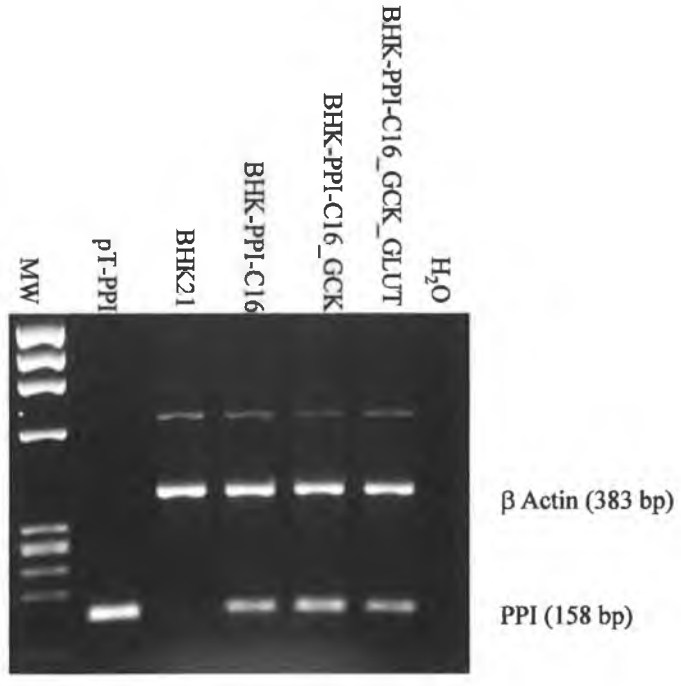


Figure 3.5.17 PPI gene expression in BHK-PPI-C16 and derivative cell populations. The PPI cDNA vector pT-PPI and H_2O were used as positive and negative controls, respectively and β actin was employed as an endogenous control.

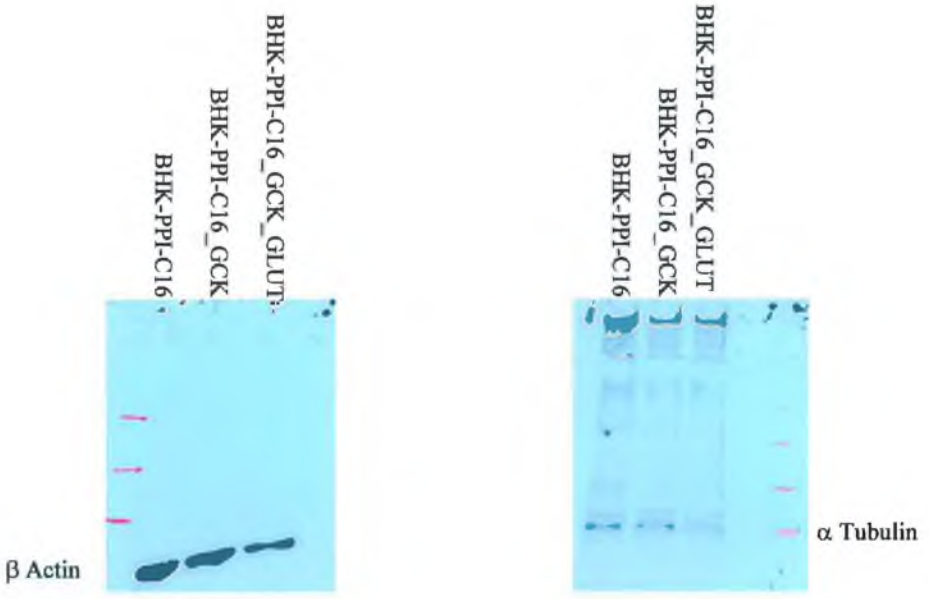


Figure 3.5.18 Housekeeping protein levels in BHK-PPI-C16 and derivative cell populations. Pellets of 4 x 10^5 cells were lysed on ice in 50 µl NP-40 lysis buffer. 50 µl of 2X loading buffer was added to lysate after 30 minutes. Following 3 minutes boiling, 10 µl of the lysate was loaded on to the acrylamide gel. Thus each lane contained the equivalent of 4 x 10^4 cells.

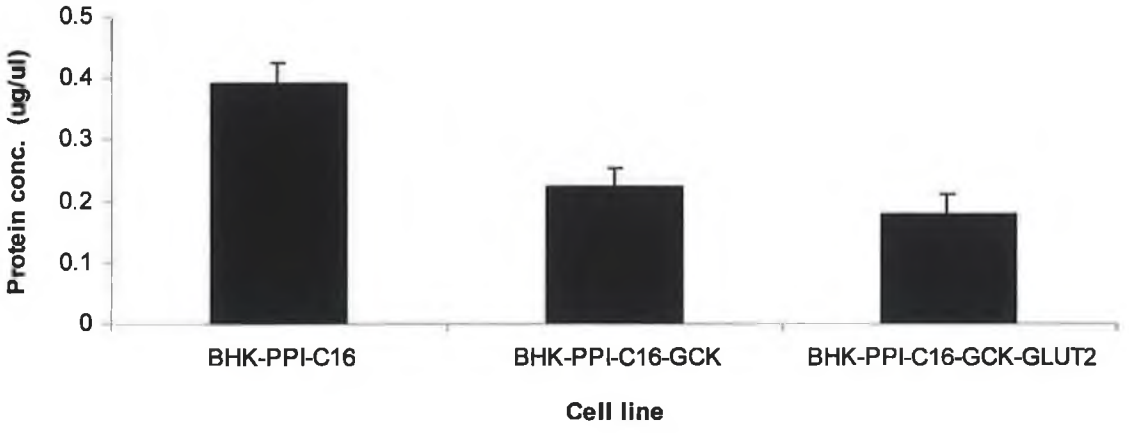


Figure 3.5.19 Bradford protein analysis of BHK-PPI-C16 cells and derivative cell populations. The above data is based on protein levels in 4 x 10^4 cells. The data is based on protein extracted from at least three different pellets and assayed in triplicate.

3.5.6 Stimulated Secretion Mediated by Increasing Intracellular cAMP

cAMP inducing agents have previously been shown to increase secretion of insulin from β cells e.g. forskolin (activates adenyl cyclase) and theophylline (inhibits cAMP phosphodiesterase) (Ullrich *et al.* 1996; Simpson *et al.* 1997). From Figure 3.5.20 it is clear that these agents result in an increase in the levels of secreted proinsulin. To validate the results, two other agents known to increase intracellular cAMP were chosen for repeat stimulation assays. Glucagon is an adenyl cyclase activator and 3-isobutyl-1-methylxanthine (IBMX) is a cAMP phosphodiesterase inhibitor, i.e. these compounds are similar in action to those initially mentioned. Table 3.5.1 summarises the stimulatory effects on proinsulin secretion due to the different agents. The data presented indicates an approximately 1.5-2 fold maximal secretion upon stimulation of BHK-PPI-C16 with agents that increase intracellular cAMP.

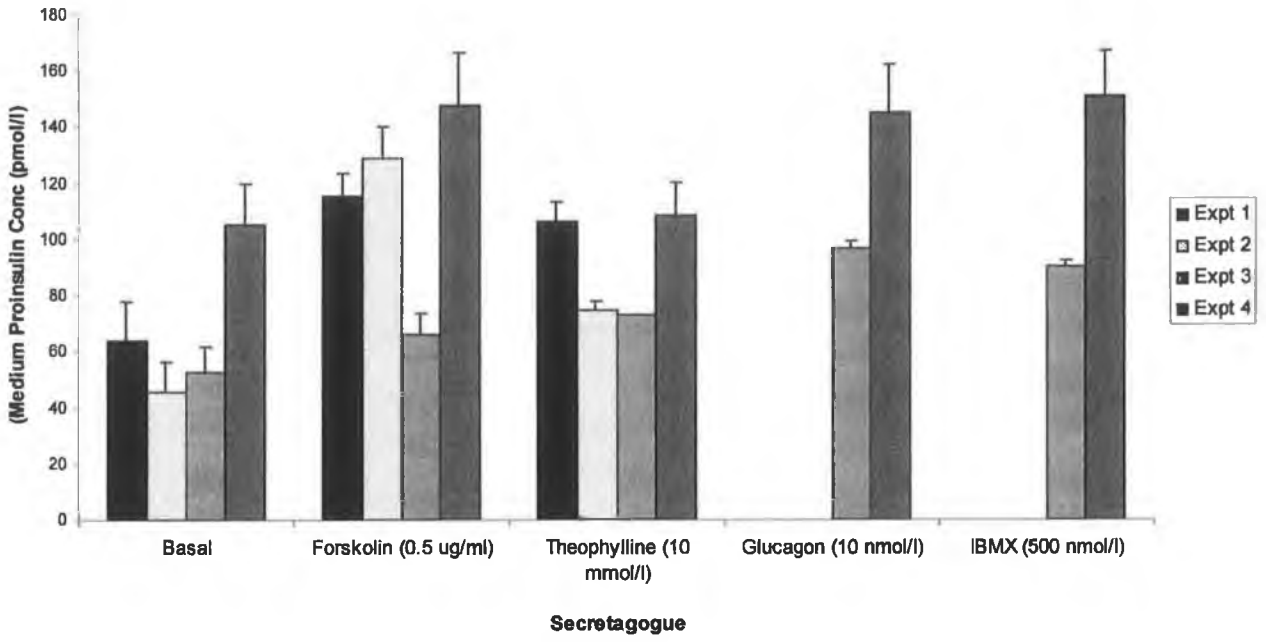


Figure 3.5.20 Secretion of Proinsulin from BHK-PPI-C16 in the presence of agents that increase intracellular cAMP. This figure represents the concentration of proinsulin secreted from 5 x 10^4 cells over two hours. All treatments were carried out in triplicate and repeated 4 times (n=12) in the case of forskolin and theophylline, and repeated twice (n=6) in the case of glucagon and IBMX. (Basal = basal medium without addition of cAMP stimulating agents). All data presented represent means ± SD.

Medium Type	Fold Stimulation over Basal Secretion
Forskolin	1.8
Theophylline	1.4
Glucagon	1.6
IBMX	1.6

Table 3.5.1 Summary of stimulation of proinsulin secretion upon exposure to cAMP inducing agents.

4.0 DISCUSSION.

4.1 General Introduction.

IDDM is a chronic illness and conventional treatments do not preserve the level of normoglycemia maintained by a functioning pancreas, thus efforts are being made to generate functional replacement tissue from cultured cell lines. These cell lines are being derived through differentiation of multipotent tissue or through direct gene transfer into established cell lines.

It has been previously shown that ES cells can be differentiated into insulin secreting cells via spontaneous or directed methods (Schuldiner *et al.*, 2000; Soria *et al.*, 2000a; Assady *et al.*, 2001; Lumelsky *et al.*, 2001). EC cells are known to exhibit a number of similarities to ES cells and in many cases are easier to culture *in vitro*. F9 cells have been characterised as a pluripotent cell line with the capacity to express multi-lineage markers upon careful differentiation (Koopman & Cotton, 1987). Directed differentiation of F9 cells towards an endocrine fate had not been attempted prior to this study despite the ease at which these cells adopt endodermal lineages (Hogan *et al.*, 1981; Moore *et al.*, 1986; Becker *et al*, 1997; Miki *et al.*, 1999), which is where the pancreas derives from in the developing gut (Madsen *et al.*, 1996; Kim & Melton, 1998; Edlund, 2001). Thus the F9 cell line was considered an excellent model in which to study islet neogenesis via directed differentiation.

The cultured β cell line Min6 has been observed to lose its specialised functions e.g., GSIS, while in culture for long periods, and it was postulated that this may be due to de-differentiation (Kayo *et al.*, 1996) although overgrowth of an already present poorly differentiated subpopulation remains a possibility. Thus Min6 cells were considered a potentially useful model in which to study β cell differentiation in reverse.

Finally, BHK-21 cells have been used in a human somatic cell therapy trial for ALS (Aebischer *et al.*, 1996), thus these cells may represent a useful recipient for transfer of a functional human insulin gene to diabetic patients.

4.2 Initial Differentiation of F9 Cells

The pancreatic islets develop from primitive endoderm within the developing gut (Section 1.1.1), thus in designing a directed differentiation protocol for EC cells, endodermal differentiation was considered to be the logical first step. F9 EC cells are characterised as differentiating to endodermal subtypes upon a straightforward one-step differentiation. F9 cells treated with RA differentiate to a primitive endoderm (PrE) stage that may be differentiated towards visceral endoderm (VE), or parietal endoderm (PE). Culture of F9 aggregates in suspension (EBs), in the presence of RA leads to predominantly VE in the outer layer (Hogan *et al.*, 1981; Moore *et al.*, 1986; Becker *et al*, 1997; Miki *et al.*, 1999). The addition of db cAMP modulates the RA differentiation of F9 monolayers and enhances the generation of PE (Strickland *et al.*, 1980 Lethonen *et al.*, 1989; Alonso *et al.*, 1991). As already mentioned, the pancreas is of endodermal origin, but it is unclear if it is derived from PE or VE. Recent work with ES cells however demonstrates the associated expression of VE markers, e.g. AFP and GATA4, during differentiation of these cells towards β cells (Schuldiner *et al.*, 2000; Lumelsky *et al.*, 2001). Thus F9 differentiation serves as a useful model to study the emergence of the different types of endoderm and to possibly assign lineages to the differentiated progeny.

4.2.1 Short Term Treatment With RA and RA/db cAMP Induce Differentiation in F9 Cells

Initial differentiation protocols were carried out over 72 hours (Section 2.3.1.2.1), which is shorter than most RA or RA/db cAMP based differentiation protocols. Previous reports indicate that strong differentiation in the presence of 10^{-7} RA eventually leads to purely PE thus reducing the number of possible lineages (Koopman & Cotton, 1987). To avoid this, the following system, involving a 72-hour differentiation procedure, was designed to capitalise on all the possible endodermal permutations (Figure 4.2.1). Following these initial 'partial-differentiations', the expression levels of differentiation markers were examined to investigate if they had similar features to the strongly induced differentiated cells described earlier (Section 1.4.2).

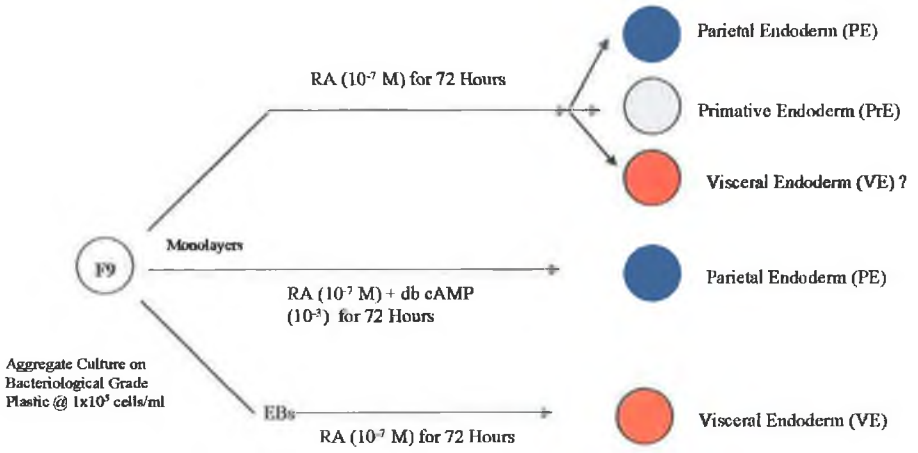


Figure 4.2.1 First stage differentiation of F9 cells to maximise endodermal types generated for stage two directed differentiation.

4.2.1.1 RA and RA/db cAMP Induce Morphological Changes in F9 Monolayers

When the undifferentiated F9 cell population (as evidenced by morphological features i.e., the typical polygonal cells in tightly packed three dimensional colonies) were exposed to 10^{-7} M RA (RA treatment), or a combination of 10^{-7} RA and 10^{-3} M db cAMP (RAC treatment), for 72 hours, induction of a differentiated phenotype was indicated by morphological changes. RA treated F9 cells were observed to exhibit at least two sub populations with different morphologies, including large flattened cells that contained cytoplasmic vacuoles and more structured cells with stretched processes emanating outwards from the cell. Both cell types within RA treated F9 monolayers were quite granular. The RAC treated cells were more homogenous in nature and the cells were morphologically similar to the smaller, structured cells observed within the RA treated population. Based on the similarities between the structured cells in RA and the RAC treated F9 cells, it was considered possible that this represents a sub-population of PE within the RA treated cells. These results are similar to those obtained by Moore *et al.* (1986), when differentiating F9 cells. Those researchers however, considered the RAC treated cells as a unique cell-type (i.e. not a homogenous population of the PE-like cells seen in the RA treated population).

The morphological changes during the differentiation are accompanied by a reduction in growth rate as indicated by the lower degree of confluency in differentiated cultures. Alternatively, it could be considered that the differentiated phenotype observed may be as a result of the reduction in growth rate which allows differentiation to occur (Lethonen *et al.*, 1989).

4.2.2 Morphological Changes in the Outer Layer of RA Treated EBs

Culture of F9 cells at a density of $1x10^5$ cells/ml on bacteriological-grade plasticware leads to the formation of evenly sized aggregates known as embryoid bodies (EBs). Culture of EBs in the presence of RA results in the outer layer differentiating to form predominantly VE (Hogan *et al.*, 1981). Differentiated EBs in culture are surrounded by an uneven layer of differentiated cells, whereas untreated EBs are smooth. To facilitate examination of the outer layers, EBs were cultured on gelatin (resulting in them attaching to the surface) thus allowing the outer later of cells to grow outwards and form a 'halo' around the central core of the EB. Cells growing out from the RA treated EB are long and stretched with pointed processes, whereas the cells growing out from untreated EBs resemble normal F9 cells. The undifferentiated EBs were generally of a larger size than those formed in the presence of RA (on average 36% larger), and this presumably reflects the faster growth rate of undifferentiated cells. There is no morphological evidence for any spontaneous differentiation in the outer layers in untreated EBs. Low-level spontaneous differentiation has previously been observed in the outer layers of F9 EBs (Becker *et al.*, 1997). The lack of obvious differentiation may be due to the shorter differentiation period employed here (72h compared to 7 days).

4.2.3 Induction of Differentiation Marker Expression by RA and RA/db cAMP Treatments

Morphological analysis, as described in Section 4.2.2, clearly indicated that differentiation was occurring. The nature of the differentiation is unclear however unless the presence of differentiation markers is established.

4.2.3.1 Differentiation of EC Cells is Correlated with a Decrease in Alkaline Phosphatase Expression and a Concomitant Increase in Cytokeratin Expression

Alkaline phosphatase is a typical marker of stem cells and has been previously reported as a method of monitoring stem cell differentiation (Lumelsky *et al.*, 2001; Draper *et al.*, 2002). Alkaline phosphatase staining was observed to decrease upon treatment of F9 cells with RA, however within the RA population there are still regions of closely grouped cells that exhibit intense staining. These cells correspond to sub-populations within the F9 population that are characterised as being refractory to the action of RA (Alonso *et al.*, 1991), and thus do not differentiate. The cells at the periphery of the cell clusters show much less staining and are similar in morphology to the RAC treated cells. The RAC cells are observed to be almost entirely free of alkaline phosphatase staining. These observations collectively indicate that the cells within the RA population which are morphologically similar to the RAC treated cells are not the same, however, as they still have residual alkaline phosphatase activity. This would agree with these cells being a transitory population (as proposed by Moore *et al.*, (1986)) towards the fully parietal cells seen following the RAC treatment. The outer 'halo' of differentiated EBs also demonstrated much reduced alkaline phosphatase staining while the central, inner portion of the EB stains intensely (Figure 3.1.5). This is similar to observations with EBs taken directly from the transplantable teratocarcinoma OTT 6050 (Paulin *et al.*, 1982).

The importance of cytokeratin expression in differentiating systems is discussed with regard to monitoring pancreatic islet development in Section 1.1.4. Differentiation of F9 cells in the presence of RA or RAC is known to induce cytokeratin expression and cytokeratins are present in both VE and PE. CKs 8 and 18 have already been identified in differentiated F9 populations (Ramaekers *et al.*, Kurki *et al.*, 1989; Lethonen *et al.*, 1989). The full cytokeratin profile for F9 cells is unknown however. Immunofluorescence studies failed to detect any cytokeratin expression in undifferentiated F9 monolayers but there was evidence for low-level cytokeratin expression in undifferentiated EBs. Specifically CK8, CK18, CK7 & CK19 (Section 3.1.4.2), were detected in the periphery/halo region of the EBs. Spontaneous differentiation of untreated EBs was not indicated by either the morphology of the outer layer or by alkaline phosphatase staining, however, as the level of spontaneous

differentiation appears to be low, it is likely that it required the increased sensitivity of immunofluorescence to be detected.

Treatment of F9 monolayers with RA for 72 hours led to the induction of cytokeratins 8 and 18 in around 20 % of the cells which agrees with the work of Kurki *et al.* (1989). Cytokeratins 7 and 19 were also examined but were not readily detected at this point (they were detectable however after 7 days treatment with RA). These results demonstrate the capability of RA treated F9 cells to express the critical endodermal cytokeratins associated with the pancreas (Bouwens *et al.*, 1994; Bouwens, 1998a). Cytokeratin staining is more associated with the PE-like cells rather than the PrE like cells in the RA treated population, and this is confirmed by the level and intensity of cytokeratin staining in RAC treated F9 cells. CK7 was also readily detectable after 72 hours RAC treatment. As with RA treated cells, CK19 was present following 7 days RAC treatment. The role of cAMP in RA mediated differentiation is unknown and there are conflicting reports regarding its function, i.e. whether or not it can illicit an effect on its own (Darrow *et al.*, 1990; Alonso *et al.*, 1991).

RA treated EBs also demonstrated low-level cytokeratin staining and, as mentioned, had already displayed low-level spontaneous differentiation. There was slightly intensified CK8 and 18 staining in RA treated EBs but not in the case of CK7 and CK19. The restriction of cytokeratin expression to the periphery of EBs has been observed in a similar situation by Paulin *et al.* (1982).

4.2.3.1.1 Implications of Cytokeratin Expression in Differentiated F9 Cells

Cytokeratins are markers for epithelial cells which show defined tissue distribution allowing for putative epithelial tissue characterisation based on the cytokeratin profile (Section 1.1.4). Simple epithelia of the liver and gallbladder are enriched in CKs 8, 18 and to a lesser extent 7 and 19 as are tracheal epithelium and complex glands (Moll *et al.*, 1982). However as already discussed (Section 1.1.4), cytokeratin-based characterisation is purely speculative (especially considering the 'promiscuity' of certain cytokeratins e.g. CK19 (Hatzfeld & Franke, 1985)). Thus, all that can be inferred from the cytokeratin profiles of the three partially differentiated F9 types is that they exhibit a similar cytokeratin expression profile to that of the pancreas.

Cultured cells are very different to the *in vivo* situation however, and cytokeratin profiles of cells often bear limited resemblance to their tissue of origin. CKs 8 & 18 have also been shown to be very common in cultured cells (Moll *et al.*, 1982).

4.2.3.2 Alpha fetoprotein is Expressed in Differentiated EBs

Alpha-fetoprotein (AFP) expression is a marker of VE (Hogan *et al.*, 1981), and was detected only at low levels on the periphery/halo of RA treated EBs (in accordance with the work of Hogan *et al.*, (1981)). The low-level nature of the staining was surprising as RA treated EBs are characterised as being rich in VE, however the short duration of the treatment (72 hours) may not have resulted in enough protein to be detected easily. In a study by Becker *et al.* (1997), AFP expression was only detectable after 5-6 days treatment with RA. AFP expression was not found in RAC or RA monolayers.

4.2.3.3 β1 Integrin Expression Increases Upon Differentiation of F9 Cells

β1 integrin facilitates the morphological changes occurring in differentiating F9 cells (Stephens *et al.*, 1993), thus it was not surprising to find large changes in the β1 integrin subunit following all three types of differentiation (Figure 3.1.11). Integrins facilitate information transfer from the ECM. This may serve to explain the low-level β1 integrin expression in EBs i.e., they are in suspension and have no contact with the ECM. The observed increase in β1 integrin level upon RA treatment is in keeping with observations by Stephens *et al.*, (1993). The role of cAMP in enhancing β1 integrin expression following RAC treatment appears to be modulatory as cAMP alone was shown to have no effect on β1 integrin levels in F9 cells (Ross *et al.*, 1994). The correct expression of β1 integrin is crucial to the terminal differentiation of a number of cell types and is implicated in the differentiation of the neural crest (Bronner-Fraser, 1986). Differentiation of the CNS is closely related to that of the developing pancreas (Section 1.1.2.6). Correct integrin expression is also crucial for maintaining β cell function in culture (Section 1.2.4.2.1).

4.2.4 Overview of F9 Cells Differentiated Towards An Endodermal Lineage

Examination of the various endodermal types via morphology and differentiation markers clearly indicates that the cells are differentiating despite the short duration of the initial differentiation protocol. The differentiation markers that are being expressed are relatively common, in that the lineages evolving cannot be definitively characterised. It is clear, however, that there are similarities in protein expression between the tissue derived from these experiments and pancreatic tissue. These are listed in Table 4.2.1. The main point is however, that these cells clearly resemble primitive endodermal cells and thus are differentiating in the correct 'direction' towards pancreatic β cells.

Marker	F9	F9-RA	F9-RAC	EB-RA*	Pancreas (β Cells)
Alkaline Phosphatase	+++	+	+/-	+/-	+/- (β Cells) (Figure 3.4.4)**
K8	-	+ (20%)	+ (50%)	+ (<20%)	Endocrine Islets
K18	-	+ (20%)	+ (50%)	+ (<20%)	Endocrine Islets
K7	-	U	+ (20%)	+ (<20%)	Exocrine Ducts
K19	-	U	U	+ (<20%)	Exocrine Ducts
β1 Integrin	-/+	+	++	++	β Cells
Alpha Fetoprotein	-	-	-	+ (<20%)	-

Table 4.2.1 Comparison of markers found to be expressed in partially differentiated F9 cells with pancreatic markers. (+) indicates the presence of a marker while (-) denotes its absence. (U) denotes markers that were undetectable after 72 hours differentiation but were detectable after longer periods (6-7 days). Approximate percentages of cells expressing the proteins studied are included in parentheses where applicable.

Notes

* This column corresponds to the outer halo of differentiated cells surrounding the EB when it is cultured on 0.1% gelatin.

** Fully differentiated functional Min6 cells have low levels of alkaline phosphatase activity, such that colour development took up to 8 hours.

4.3 Secondary Differentiation of Endoderm with Differentiation Agents

The differentiation protocols discussed in Section 4.2 resulted in the generation of PE, VE and some PrE endodermal types, and, due to the short duration of the initial treatments, it was expected that the cells would retain plasticity/multipotency. Thus it was hoped that within one of these populations, cells would be present that are similar in nature to the fetal endoderm from which all pancreatic cell types originate. These three heterogeneous populations were considered an ideal starting point from which to investigate the effects of endocrine differentiation agents outlined in Section 1.3.4. The relatively long duration of the secondary treatments were based in part on a similar set of experiments involving the treatment of ES cells with various agents (Schuldiner *et al.*, 2000). It has also been found that longer periods in culture (22-35 days) result in greater chance of multiple-lineage differentiation in RA treated F9 cells (Koopman & Cotton, 1987).

The secondary differentiation agents (Section 1.3.4) were carefully chosen based on their reported involvement in islet cell neogenesis *in vivo* and also due to previously characterised actions on cultured cells. In the case of activin A and HGF, the effects of the initial differentiation step on the levels of their receptor proteins was unknown, thus these proteins were investigated via western blotting (Section 2.5.2.1). The actions of the agents on the endodermal cells from stage 1 were examined using RT-PCR (Section 2.5.1.5) and, where applicable, immunofluorescence and immunocytochemistry (Section 2.5.2.2). The experiment is outlined in Figure 4.3.1.

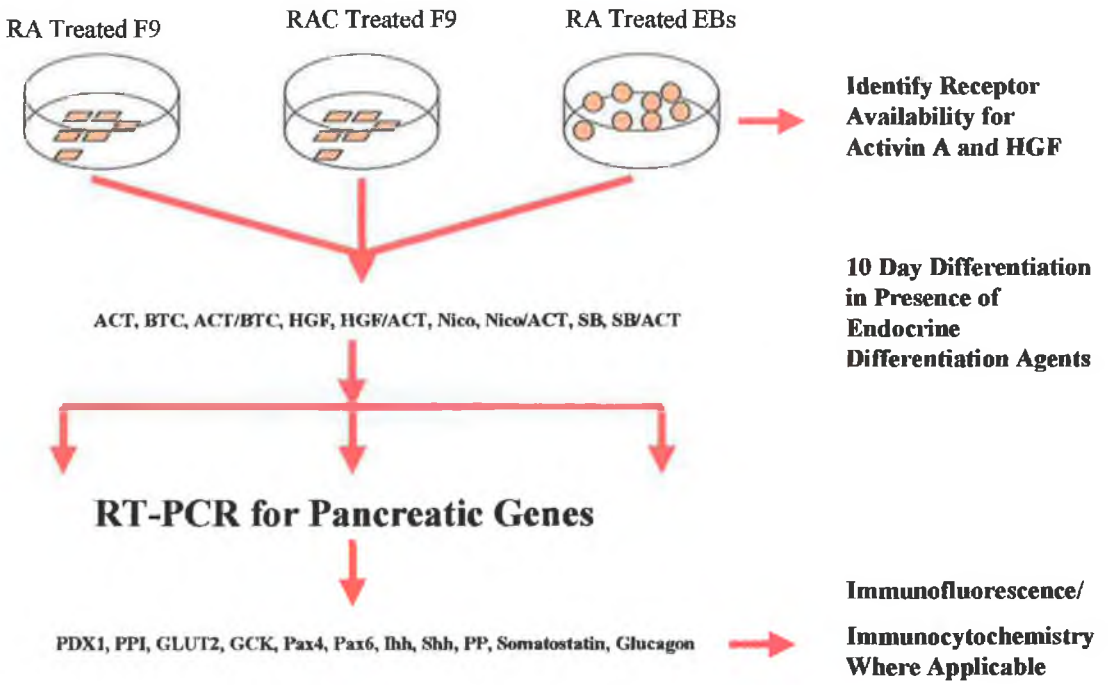


Figure 4.3.1 Endodermal-like cells from stage 1 differentiation were examined for the presence of appropriate receptors and subsequently differentiated using endocrine promoting agents (Section 1.3.4). The analysis is predominantly RT-PCR-based with immunological protein detection methods employed where applicable.

4.3.1 Analysis of Receptor Expression Following Stage 1 Differentiation

F9 cells are known to express activin A receptors including activin RII B (Kondo *et al.*, 1989; Wan *et al.*, 1995). Activin type II receptors have been shown to be the critical component in mediating the response of endothelial cells to activin A and binding of activin A is limited by the availability of this receptor (McCarthy & Bicknell, 1994). It was unknown however if the stage 1 differentiation would affect activin RII B protein levels (it is transcriptionally upregulated in differentiated F9 cultures, Section 1.4.1.2.3). The moderate increase in protein levels following all three modes of differentiation is far less than the increases noted in the transcript levels (Wan *et al.*, 1995), or in spontaneously differentiating EBs generated from ES cells (Schuldiner *et al.*, 2000). Activin RII B mRNA levels were not examined as this was a qualitative exercise to confirm the presence of receptor protein expression prior

to treatment with activin A, thus it is not known if activin RII B mRNA levels were upregulated following the 72 hour differentiation carried out here.

The HGF receptor (the c-met protooncogene), similarly, shows a slight increase upon RA/RAC mediated differentiation, however there is a >2-fold increase between the levels of c-met in F9 monolayers and in EBs. This was also observed upon EB formation from multipotent H9 ES cells (Schuldiner *et al.*, 2000). As with the activin RII B result, the increases and decreases were considered secondary to the fact that a receptor had been identified for the second stage differentiation using HGF.

Betacellulin is known to interact at least partly through the EGFR and this has already been shown to increase upon F9 differentiation (Greip & De Luca, 1986). Betacellulin has also been shown to interact with another protein in AR42J cells. This 190 kDa protein was identified by immunoprecipitating I^{125} labelled betacellulin that had bound its receptors on the AR42J cells (Ishiyama *et al.*, 1998). It was not possible to screen differentiated F9 cells for this protein however as its identity is unknown.

4.3.2 RT-PCR Analysis Reveals Changes in Expression of Islet Markers Following Secondary Differentiation of RA-Monolayers

The changes in gene expression observed as a result of secondary differentiation of F9-RA monolayers are summarised in Table 4.3.1

Treatment → / Gene ↓	F9 (Untreated)	RA	Act	BTC	Act/ BTC	HGF	HGF/ Act	Nico	Nico/ Act	SB	SB/ Act	**Min6**
PDX1	+/-	+	+	+	+	+	++	+	+	+	+	+++
PPI	+/-	+	+	++	+	++	+++	++	++	++	++	+++
Pax6	++	++	++	+	+/-	+	+/-	+++	+++	++	++	++
Ihh	+/-	++++	+	+	+	++	++	+++	++	+++	+++	+/-
PP	-	-	-	-	-	-	-	-	-	+	+	++++
Somatostatin	-	-	+/-	+/-	-	+/-	-	-	-	++	++	++++

Table 4.3.1 Summary of the changes in gene expression following secondary treatment of F9-RA monolayers. For each mRNA, the level of expression in the parental F9 population is used as the reference level. Min6 cells are included for comparison. The relative expression levels were determined from densitometric analysis.

As shown in Table 4.3.1, undifferentiated F9 cells were shown to express a number of tissue specific genes at low levels. This so called 'leaky' expression of lineage-specific transcription factors is not uncommon and has also been noticed in hematopoietic stem cells (HSCs) where subsequent commitment is associated with the loss of expression of genes that are unrelated to the intended lineage (Wagers *et al.*, 2002). Treatment of F9 monolayers with RA alone resulted in increased levels of PDX1, PPI and Ihh (Section 3.2.2). It is known that increased Ihh gene expression is implicated in enhancement of PPI gene expression (Thomas *et al.*, 2000). Ihh levels are much lower in HGF/Act treated F9 RA cells, however, whereas PPI expression is almost 2-fold higher than in RA treated F9 cells. These differences do not indicate a direct Ihh-mediated mechanism for PPI stimulation in the F9 system. Increases in PDX1 and PPI gene expression following HGF/Act treatment of AR42J cells were also observed by Mashima *et al.* (1996b). These researchers proposed that the action

of HGF was similar to that of BTC in combination with activin A (Mashima *et al.*, 1996a). In this study, Act/BTC did not result in the same level of PPI or PDX1 up-regulation as HGF/Act. A possible explanation for this may involve a novel BTC receptor discovered on AR42J cells (see Section 4.3.1). This receptor seemed far more important to the differentiation-related actions of BTC than does EGFR (Ishiyama *et al.*, 1998). If this protein were absent from F9 cells then it is possible that the effects of BTC would be less marked.

Immunofluorescence analysis did not detect any PDX1 or PPI expression in either untreated F9 or any of the F9-RA cultures treated with differentiation inducing agents. It is possible that expression levels of these proteins were too low for protein detection (mRNA levels were much lower than in Min6, which had clearly detectable PPI and PDX1 protein). When attempting to determine the order of appearance of the islet hormones in the developing pancreas, the data differs upon comparing RT-PCR analysis and immunohistochemical data (Gittes & Rutter, 1992; Herrera *et al.*, 1991; Teitelman *et al.*, 1993; Upchurch *et al.*, 1994). The order of appearance of the immunoreactive islet hormones correlates with the proportion of each cell type, i.e. insulin and glucagon expressing cells are present in high numbers in islets and these proteins were detected first, while PP cells account for 1-2% of the islet cell mass and PP was the last protein detected. Thus, immunological methods may not be sensitive enough to detect the levels of PPI and PDX1 present in treated F9 cells, even following the elevated mRNA levels induced by such treatments as HGF/Activin A. It is also possible that the F9 cells are not translating these mRNAs.

The Pax6 gene is highly expressed at the mRNA level in F9 cells. Its levels were increased in the nicotinamide treatments, but this was not correlated with any other major changes in gene expression. Pax6 expression was very low in the HGF/Act treatments, however, and this treatment was associated with increased levels of PPI and PDX1. Pax6 expression is present in all four endocrine types in the islet, but it is far more important for α cell formation than that of β cells (St-Onge *et al.*, 1997; Sander *et al.*, 1997). Pax4 expression is considered crucial for β cell expression *in vivo* and it acts, at least partly, by competitive binding with Pax6 thus inhibiting Pax6 and α cell development (Smith *et al.*, 1999; Ritz-Laser *et al.*, 2002). Pax4 was not

detected in F9 cells, either prior to or following any treatment, thus it was apparently not present to modulate the effects of Pax6. HGF/Act treatment resulted in reduced Pax6 expression and may have substituted for Pax4 effects. Some Pax6 expression is required for pancreatic hormone gene expression and also for efficient transcription of PDX1 (Sander *et al.*, 1997; Samaras *et al.*, 2002).

The other treatments all showed increased PPI gene expression and were not markedly different from one another in repeat experiments. Changes such as increased PPI expression upon RA treatment (compared to parental F9), and HGF/Act treatment, were consistent over a number of experiments. Minor changes in expression between treatments i.e., <1.5 (as judged from densitometry) were not considered significant and were therefore regarded as being unchanged from one to another.

Shh was not found to be expressed in F9 or in PE differentiated F9 cells which is in agreement with work by Becker *et al.* (1997), and it was not induced by any of the treatments. RT-PCR of a positive control indicated that the primers were suitable and the procedure was conducted successfully.

4.3.2.1 Sodium Butyrate Leads to Expression of PP and Somatostatin mRNAs in F9 RA Monolayers

The expression of the mRNAs for all four islet hormones was investigated following the secondary treatment of F9 RA monolayers. It was found that both somatostatin and PP were expressed at high level following sodium butyrate treatment or sodium butyrate treatment with activin A. The results indicate that the effect is being mediated directly by sodium butyrate, as activin A has no effect on PP alone and only a minor effect on somatostatin expression. Unlike the situation with PPI, the induction of PP and somatostatin expression was observed at the protein level also (Section 3.2.5.1 & 3.2.5.2).

The expression of these pancreatic islet hormones was encouraging as it indicated that the differentiation protocol did induce endocrine differentiation even if insulin was not expressed. Lumelsky *et al.*, (2001) also generated somatostatin expressing cells

during their differentiation of ES cells towards insulin secreting cells. This would indicate that the protocols employed here might generate insulin secreting cells with more modification or possibly using different cells.

Min6 cells were used as positive controls in all RT-PCRs for islet hormones. It was already known that Min6 express IAPP, glucagon and somatostatin (Kanatsuka *et al.*, 1992; Ohgawara *et al.*, 1995) but there do not appear to be any previous reports in which Min6 cells were shown to express PP.

4.3.2.2 Sodium Butyrate Causes an Increase in Acetylated Histone H3 Levels in F9-RA Monolayers

Sodium butyrate is a member of the HDAC (histone deacetylase) inhibitor family, and it has been shown to promote β cell differentiation (Section 1.3.4.5). It has effects on a number of genes within F9 cells (Section 1.4.1.2.2), including endo A (CK A/ CK 8) expression. The action of sodium butyrate on endo A is related to histone acetylation (Myiashita *et al.*, 1994). Examination of sodium butyrate treated F9 cells showed that levels of acetylated histone H3 protein were increased, and the presence of activin A had an additive effect. Upon closer examination of Figures 3.2.8, 3.2.14 & 3.2.20 it can be seen that in the case of the monolayers the presence of activin A did appear to have a slight additive effect on somatostatin mRNA levels, whereas activin A was required with sodium butyrate for somatostatin expression in EBs. Histone H3 methylation is already known to be crucial for the active expression of genes (Santos-Rosa *et al.*, 2002), which confirms the importance of histone modifications and chromatin structure in gene transcription. Histone modifications are reported to act in concert, i.e. the combination of acetylation/deacetylation and methylation, etc., may serve to either bind or prevent binding of transcriptional complexes (Sartorelli & Puri, 2001). Acetylation is also linked to the expression of genes in the developing pancreas, and defective acetyltransferase-dependent transcription has been linked to mature onset diabetes of the young (MODY) (Sartorelli & Puri, 2001).

4.3.2.3 Summary of Secondary Treatment of F9-RA Monolayers

Secondary treatment of F9 monolayers resulted in altered expression of a number of genes and in certain cases possible cascades, or fragments of cascades may have been identified (examples of which are illustrated in Figure 4.3.2). However, while individual treatments may result in some common effects e.g. decreasing Pax6, the subsequent 'knock on' events may be altered due to the differing effects of the individual treatments on other genes not examined during this experiment. This leads to difficulty in assigning mechanisms to F9 endocrine differentiation, except in cases like that of sodium butyrate, which appears to act independently of all of the genes examined in its enhancement of somatostatin and PP mRNA expression.

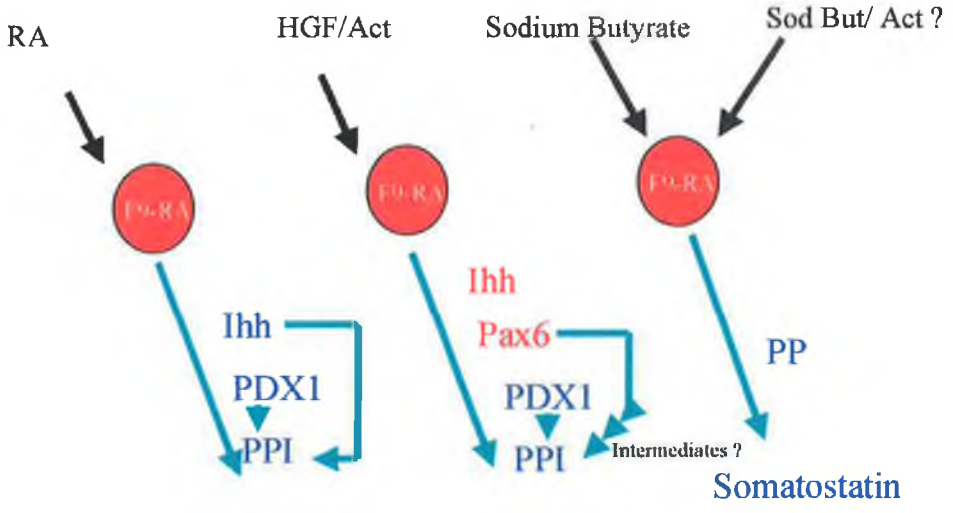


Figure 4.3.2 Summary of main changes in hormone gene expression following endocrine differentiation of F9-RA cells. Genes in blue were shown to be increased at the mRNA level following the indicated treatment, while those in red were downregulated. Green arrows indicate 'knock on' effects of treatments e.g. down regulation of Pax6 as a result of HGF/Act treatment may allow β cell differentiation and therefore increase PPI gene expression.

4.3.3 Secondary Endocrine Treatments of RAC Monolayers

The levels of the different genes examined following secondary differentiation of F9 cells are summarised in Table 4.3.2.

Treatment → / Gene ↓	F9 (Untreated)	RAC	Act	BTC	Act/ BTC	HGF	HGF/ Act	Nico	Nico/ Act	SB	SB/ Act	**Min6**
PDX1	+/-	+	+	+	+	+	+	+	+	+	+	+++
PPI	+	+	+	+	+	+	+	+	+	++	++	+++
Pax6	+	+/-	+	++	++	+	+	+++	+++	+	+	+++
Ihh	+/-	+/-	+	+	+	+	+	++	++	++	+	+/-
PP	-	-	-	-	-	-	-	-	-	+	+	++++
Somatostatin	-	-	-	-	-	-	-	-	-	+	+	++++

Table 4.3.2 Summary of the changes in gene expression following secondary treatment of F9-RAC monolayers. For each mRNA, the level of expression in the parental F9 population is used as the reference level. Min6 cells are included for comparison. The relative expression levels were determined from densitometric analysis.

The expression of PDX1 is increased following initial RAC treatment however the levels of PPI were not similarly affected. Sodium butyrate treatment alone, and with activin A leads to increases in PPI mRNA levels. These treatments all coincide with slightly lowered levels of Pax6 mRNA, which may be implicated in β cell differentiation (as already discussed in Section 4.3.2). Ihh expression is not induced following 10 days treatment in RAC medium. This is interesting as Ihh was strongly induced following 3 days RAC treatment (Figure 3.3.27). Becker *et al.* (1997) found significant levels of Ihh protein in RAC-derived PE after 5 days treatment. It was reported that Ihh expression immediately preceded the VE marker AFP and was expressed at the same time as the PE marker α6β1 integrin during F9 differentiation. Transfection studies indicated that Ihh could induce the expression of markers of VE, and to a lesser extent PE, in the absence of RA (Section 1.4.1.4.1). It is possible that

Ihh action promotes the formation of a transitory endodermal cell which can then go on to PE or preferentially VE. Ihh expression is also preferentially maintained in visceral endoderm *in vivo.* It is therefore possible that 10 days continuous culture in the presence of RAC could have generated a homogenous culture of highly differentiated PE with little or no PrE or VE, resulting in the loss of Ihh expression.

4.3.3.1 Expression of PP and Somatostatin Following Sodium Butyrate Treatment

As with the RA generated monolayers, sodium butyrate induced PP and somatostatin gene expression and again activin A did not modulate this activity to any considerable extent. PP expression was approximately equivalent in sodium butyrate treated RAC monolayers and RA monolayers. Somatostatin expression however was not as strong in this case which suggests that db cAMP may have some effect that inhibits somatostatin mRNA levels. If it can be assumed that VE is the lineage from which the islet cells originate, then islet hormone gene expression may be harder to induce in the more homogenous PE population resulting from RAC treatment.

4.3.3.2 Summary of Secondary Differentiation of F9-RAC Monolyers

The changes that occurred during the differentiation of F9-RAC do not reveal any obvious mechanistic actions for pancreatic development from F9 cells and it is possible that the treatments are favouring other cell types. The poor induction of somatostatin may suggest that the endoderm derived from F9-RAC cells might be too committed to PE and thus might not be as open to directed differentiation towards an endocrine lineage.

4.3.4 Secondary Differentiation of RA Treated EBs

For ease of reference, the levels of the genes expressed in secondary differentiated EBs are summarised in Table 4.3.3.

Treatment → / Gene ↓	EB (Untreated)	EB (RA)	Act	BTC	Act/ BTC	HGF	HGF/ Act	Nico	Nico/ Act	SB	SB/ Act	**Min6**
PDX1	+	+	+	+	+	+	+	+	+	++	+	+++
PPI	+	+	++	++	++	++	++	++	++	++	++	++++
Pax6	+	++	++	++	++	+/-	++	+	+++	+/-	+/-	+++
Ihh	+	+++	+/-	++	+/-	++	+/-	++	++	++	+++	+
PP	-	+/-	+/-	-	-	+	-	-	+	++	++	++++
Somatostatin	+	+	-	+	-	-	+	-	-	-	++	++++

Table 4.3.3 Summary of the changes in gene expression following secondary treatment of RA treated EBs. For each mRNA, the level of expression in untreated EBs is used as the reference level. Min6 cells are included for comparison. The relative expression levels were determined from densitometric analysis.

There are considerable levels of PDX1 and PPI expression in untreated EBs. Previous studies with ES cells have already noted that EB formation alone led to the expression of PPI and PDX1 (Gerrish *et al.*, 2000; Schuldiner *et al.*, 2000; Shiroi *et al.*, 2002). Sodium butyrate had a slight effect on PDX1 expression (compared to RA treated EBs), and all secondary treatments resulted in increased PPI mRNA transcripts. There were no differences between the individual treatments however. Unfortunately the increased PPI gene expression in all the secondary treated EBs does not coincide with any other sweeping changes in gene expression. Pax6 expression was increased following nicotinamide and activin A treatment, which is similar to results obtained using both the RA and RAC monolayers. Pax6 expression was also increased following RA treatment of EBs but it was unchanged following RA treatment of F9 monolayers. These differences highlight the fact that the three different initial differentiation steps resulted in three different cell types with heterogeneous responses to external stimuli and signals. Similarly the Ihh expression profiles are different.

These differences in the expression levels of the different genes make it difficult to identify common differentiation mechanisms and pathways.

4.3.4.1 PP and Somatostatin Expression in Secondary Differentiated EBs

PP and somatostatin expression are widespread in treated EBs. It is hard to relate the expression profiles of PP and somatostatin to any of the other genes examined, but it is possible that the naturally elevated levels of PDX1 in EBs may encourage somatostatin expression (Section 4.4.3.2). It is also possible that the curved nature of the cells or the cell-to-cell contacts in EBs could alter gene expression profiles compared to those of monolayers. Miki (1999), postulated that the curved nature of the cells on the outer layer of an EB may have added to the differentiation capacity of those cells and that the underlying cells were not as important.

For both PP and somatostatin, the highest levels of expression resulted from the combination treatment of sodium butyrate and activin A. In the case of somatostatin, sodium butyrate alone was ineffective. So far it has appeared that the expression of somatostatin has depended on sodium butyrate only, but it has been shown that the presence of activin A resulted in increased levels of acetylated histone H3 (Figure 3.2.23). There also appeared to be a slight additive effect of activin A on sodium butyrate action as shown in Figures 3.2.8 & 3.2.14. Thus, activin A may play a more active role in PP and somatostatin gene expression, but it may be specific to certain situations e.g., in EB-derived endoderm in our system. In RA-derived endoderm, somatostatin was moderately induced by activin, betacellulin and HGF. EBs also show widespread expression of both somatostatin and PP. Again this may infer that the development of islet hormone cells is linked to VE, i.e. in RAC treated F9 cells (predominantly PE) PP and somatostatin expression were restricted to sodium butyrate-containing treatments only.

4.3.4.2 Summary of Secondary Treatment of RA Treated EBs

The initial high levels of PPI and PDX1 in untreated EBs and the expression of PP and somatostatin following treatments suggest that EB derived endoderm is the best cell type (i.e. VE) from which to differentiate pancreatic cells. This is in keeping with

the association of VE markers in stem cells differentiating towards insulin secreting cells e.g. as shown by Lumelsky *et al.* (2001).

4.3.5 Somatostatin and Pancreatic Polypeptide Transcription

The somatostatin gene is positively regulated by cAMP (Montminy *et al.*, 1996) and PDX1 (Schwartz *et al.*, 2000). More significantly, however, it has been shown that the somatostatin gene can be regulated by proteins involved in histone methylation. Coactivator-associated arganine methyltransferase 1 (CARM1) is a methyl transferase that methylates three Arg residues on histone H3. Methylation through the action of CARM1 can inhibit CREB (cAMP responsive binding element binding protein) signalling *in vivo*. This results in decreased somatostatin expression (Xu *et al.*, 2001). This is a precedent for the involvement of epigenetic-associated control in somatostatin gene transcription. There is little known about the biological activities of pancreatic polypeptide except that it stimulates secretion of enzymes from the exocrine pancreas and relaxes the gall bladder. Its secretion is normally stimulated by fasting and hypoglycemia (Kreymann & Bloom, 1991). PP expression is upregulated in human pancreatic islet tumors, but studies on the PP gene have not linked this to differences in the structure of the gene (Leiter *et al.*, 1985).

The evidence in this thesis points to an epigenetic mechanism for the induction of PP and somatostatin gene expression in sodium butyrate-treated F9-derived endoderm-like cells. Confirmation of this mechanism would involve further analysis of the chromatin itself e.g. by DNase I digestion, i.e., histone acetylation results in chromatin modifications that can render it more susceptible to DNase I digestion (see Section 5.2.7).

4.3.6 Overall Summary of Secondary Endocrine Differentiation

F9 cells were found to express a number of islet marker genes in their undifferentiated state. There are numerous reports in the literature indicating that many genes linked to differentiation may be expressed at low level, or in subpopulations of, ES and EC cells (e.g Murtomaki *et al.*, 1999). The secondary treatments resulted in altered gene expression, especially in the case of developmental transcription factors Pax6 and Ihh.

However, the effects on the terminal differentiation markers (PPI and PDX1) were less obvious. This may infer the cells are adopting lineages other than pancreatic, e.g. treatment of ES cell-derived EBs with activin or HGF resulted mostly in mesodermal marker expression with only some endodermal differentiation (Schuldiner *et al.*, 2000). Directed differentiation of cultured ES cells is not an exact science at this time, e.g. studies where ES cells are differentiated via the action of powerful neuronal differentiation factors often result in only approximately 50% neurons. This is likely to be due to the lack of proper tissue architecture and cell signalling in cultured cells (Vogel, 1999).

This study has again addressed the issue of F9 pluripotency, and has revealed that F9 EC cells can be differentiated towards pancreatic-like cell types with ease in a simple two-step differentiation protocol. Comparison of F9-RA, F9-RAC and EB-RA cells taken through secondary differentiation does not reveal any obvious patterns and this presumably reflects the different nature of the cells generated as a result of stage 1 differentiation. Examples of the kind of putative mechanisms that can be generated from the data obtained here are illustrated in Figure 4.3.2. Table 4.3.4 compares the mRNAs that were found to be expressed during the course of this experiment with those found in pancreatic islets. It is clear that F9 cells and their differentiated progeny do share a number of genes in common with islet cells.

The main similarity between the three endodermal populations generated was the role of sodium butyrate in effecting PP and somatostatin expression. As sodium butyrate did not have major effects on any of the other genes examined and as it had a similar effect in F9-RA, F9-RAC and EB-RA cells, it is suggested that sodium butyrate is acting directly on the promoter regions of somatostatin and PP. This is in accordance with previous observations on the action of sodium butyrate and with the noted increase in acetylated histone H3 levels demonstrated here.

Expansion of the mRNA screening beyond those genes involved in pancreatic development may provide a more 'global' view regarding the mechanisms of differentiation of the agents employed in this study. Thus, such genes as albumin (liver), neurofilament (CNS) or enolase (muscle) could be examined (see Section 5.2.1).

Gene	F9 Cell Type	Islet Cell Type
PPI	All	β
Glucagon	Negative	α
Somatostatin	Sodium butyrate treated cells & EBs (treated and untreated)	δ
PP	Sodium butyrate treated cells and some treated EBs	PP
PDX1	All	β & δ
Glucokinase	Negative	β
GLUT2	Negative	β
Pax4	Negative	β, δ & PP
Pax6	All (varying levels, nicotinamide and nicotinamide with activin A generally increased mRNA levels)	α, β, δ & PP
Ihh	All (except F9-RAC)	β
Shh	Negative	Negative

Table 4.3.4 A comparison of the genes expressed in F9 cells (treated and untreated) and in pancreatic islets. In certain cases (e.g. PP), only the treatments resulting in high levels of expression are listed.

4.4 F9 Differentiation Through the use of 'Cell Trapping'

Cell trapping was initially used as a method for purification of differentiating cells by Klug *et al.* (1996). In this system, differentiated cardiomyocytes were specifically purified from ES cells due to their ability to transcribe aminoglycoside phosphotransferase (G418 resistance gene), under the control of the α-cardiac myosin heavy chain promoter. The resultant cardiomyocyte population was > 99 % pure. The use of cell trapping to isolate insulin secreting cells has also been successful (Soria *et al.*, 2000), and is described in more detail in Section 1.3.2.1.

In this study, the ability to drive the PDX1 promoter region was employed as the purification criterion. Low-level PDX1 expression has already been observed in the undifferentiated F9 population (e.g. Figure 3.2.3), and it is known that PDX1 expression can be increased through RA treatment or through the formation of EBs. The critical importance of PDX1 to β cell development and function has already been discussed (Section 1.1.3.5.1 & 1.2.1.3.1). Based on this, it was decided that the isolation of purified cells expressing PDX1 would be an excellent method of investigating the role of PDX1 in developmental biology and its effects on gene transcription. The cell trap (CT) derived cells could potentially resemble early islet progenitors or the more terminally differentiated cell types expressing PDX1 i.e., β cells and certain δ cells.

4.4.1 Analysis of PGK-TKCAT-Zeo/pBS Demonstrate Its Fitness for use in Cell Trapping

The cell trap construct PGK-TKCAT-Zeo/pBS was generated from component plasmids available in our laboratory and most critically, the PDX1 tissue specific promoter region (PstBstII[Sense]pTKCAT(An)), which was supplied by Prof. Roland Stein (Vanderbilt Medical Centre, Tennessee). This region of the PDX1 promoter was initially described by Wu *et al.* (1997). Following construct design in our laboratory, the plasmid was generated by Cytomyx (Cambridge, UK) who also carried out restriction and orientation analysis of the final construct (outlined in Figure 3.3.1).

Upon receiving the plasmid, repeat restriction analysis confirmed that the construct had been generated in accordance with our specifications.

Prior to any lengthy differentiation experiments the construct had to be validated for function, i.e. did the construct specifically confer zeocin resistance to PDX1 expressing cell lines. Min6 and BHK-21 cells were chosen as positive and negative controls respectively as they have been characterised as being $PDX1^{+/+}$ and $PDX1^{-/-}$ (Kajimoto *et al.*, 1997; Wu *et al.*, 1997), which was again confirmed prior to these experiments (Figure 3.3.3). Following initial transfection of the construct into BHK-21 and Min6, initial selection was carried out in zeocin containing medium. Cytotoxicity assays had already shown that Min6 cells were more resistant to the effects of zeocin than BHK-21, so the Min6-CT cells were exposed to higher levels of zeocin. (The increased resistance to zeocin may be as a result of the slower growth rate of Min6 cells, since zeocin works best on actively dividing cells (Invitrogen, Technical Support Data Sheet)). This selection resulted in the death of all BHK-21, BHK-CT and Min6 cells; the Min6-CT wells were, however, seen to contain surviving colonies of cells. This is what was expected as the Min6-CTs should have been driving the PDX1 promoter and therefore the zeocin resistance gene. The transfection efficiency was quite low so there were only a few small surviving colonies. To obtain a more representative picture of the functioning construct, stable Min6-CT and BHK-CT cells were generated via transfection of PGK-TKCAT-Zeo/pBS and selection using the constitutive G418 promoter (Figure 3.3.1). This experiment proved difficult as the efficiency of transfection for Min6 was extremely low using the liposome based Fugene (Roche). This transfection method was used because F9 transfections had been optimised using this system and this was a control experiment. Following isolation of stable mixed populations, toxicity assays were carried out to test the functioning construct. Min6-CT and BHK-CT were resistant to G418 (as expected), but only Min6-CT showed increased zeocin resistance (compared to Min6). The level of increased resistance in Min6-CT was lower than expected but this may be explained by the initial low transfection efficiency. Following selection, small numbers of isolated transfected cells survived. These cells grew very slowly and in clumps. To expand this population, the cells needed to be trypsinised regularly to separate the clumps and to seed new wells (of 6 well plates). Over a number of weeks the cell number had reached a point where the cells could be seeded to flasks for

generation of stocks and cytotoxicity assays. However it has been shown that PDX1 expression in Min6 is passage dependent and reduces over time in culture (Figure 3.4.11(A)). Cell contact is also critical for the correct maintenance of Min6 cells (Dr. Per Bendix Jeppesen, personal communication), and this was lost at the initial stages of the selection process. The increased resistance in the stable Min6-CT population was reproducibly present over a number of experiments and the effect was specific to the β cell population, thus the construct was considered fit for use in cell trapping differentiating cells.

4.4.2 Differentiated F9-CT Cells Show Resistance to Zeocin

Stable PGK-TKCAT-Zeo/pBS expressing F9-CT cells were generated and selected as described (Section 3.3.1.3). These cells were morphologically similar to the parental population and exhibited the same rapid proliferation. Thus it was assumed that the CT construct did not induce differentiation. F9-CT and EB-CT cells were differentiated as indicated in Table 3.3.1 (following initial endodermal differentiation, Figure 4.2.1). The treatments were chosen on the basis of two criteria i.e., that they had increased PDX1 (see Section 3.2), but also because these treatments had not been harsh on the differentiating cells (an important consideration for subsequent antibiotic selection). Following the differentiation, antibiotic selection was initiated at 25 μg/ml zeocin and increased up to 100 μg/ml.

Initially there were a number of differentiated zeocin resistant cell lines in culture, but several of these gradually ceased proliferation. This post-differentiation senescence can be a feature of F9 cells and is usually followed by the outgrowth of differentiation resistant cells which are often still present (Alonso *et al.*, 1991). This did not occur here due to the nature of cell trap purification i.e., the differentiation-resistant cells did not survive selection with zeocin. A few CT-cell lines did continue to proliferate and grow however, i.e., they did not terminally differentiate (see Table 3.3.2). It is interesting to note that a small proportion of undifferentiated F9-CT cells survived the zeocin selection and presumably represents a subpopulation of cells expressing enough PDX1 to effectively drive the zeocin resistance gene. F9 cells have previously been shown to contain subpopulations within the undifferentiated parental population

(Murtomaki *et al.,* 1999). The selection of low density clonal populations would also have facilitated spontaneous differentiation (Zakany *et al.*, 1984), and due to the selective pressure in the environment (100 μg/ml zeocin), this would have favoured the differentiation of PDX1 expressing cells.

4.4.2.1 Zeocin Resistant Cell Lines Show Increased Levels of PDX1 mRNA Transcripts

RT-PCR analysis demonstrated that the CT cell lines selected in zeocin showed increased levels of PDX1 when compared to the parental F9 population (Figures 3.3.5 & 3.3.5(A)). This was important as it demonstrates again that the PGK-TKCAT-Zeo/pBS was functioning correctly, and that the zeocin resistant cells were not as a result of spontaneous mutations. The levels of PDX1 in CT cells vary in that in certain cases the level of PDX1 is higher than that achieved following treatment alone (F9-CT-RA) and in others are similar to the levels of PDX1 following secondary differentiation. However in the CT system, the cells grow continuously without the presence of the differentiation agents and the levels of PDX1 are always maintained. This is critical for F9 cells as it has been shown that continuous culture of F9 cells for long periods can lead to enhanced differentiation (Koopman & Cotton, 1987). Attempts to generate cells expressing very high levels of PDX1, by increasing the level of zeocin in the selection medium from 100 μg/ml to 200 and 400 μg/ml, did not result in similarly large increases in PDX1 expression. Presumably the level at which the PDX1 promoter was functioning in selected cells was sufficient to endow resistance to a wide range of zeocin concentration.

4.4.2.1.1 The Mechanism of Zeocin Resistance in Transfected Mammalian Cell Lines

Zeocin is the commercial name for phleomycin D1, which is a member of the bleomycin family of antibiotics. These antibiotics can bind and break the DNA strand at low concentrations. Resistance to zeocin is conferred by expression of the bacterial Sh *ble* gene, and this resistance is a result of reversible binding of Sh *ble* protein (14 kDa) to zeocin, thus preventing DNA cleavage (Gatignol *et al.*, 1988). This protein

binds zeocin with high affinity in a one-to-one ratio, thus conferring effective resistance to stably transfected mammalian cells. This implies that a cell expressing Sh *ble* to a level where the cell is resistant to 100 μg/ml, will be resistant to higher levels of zeocin also.

4.4.2.2 Cell Trap Lines Selected for High Levels of PDX1 Also Show Enhanced PPI mRNA Levels

To further characterise the isolated CT cell lines generated, RT-PCR was carried out for PPI, and it was found that a number of the CT cell lines showed increased PPI gene transcripts. The effects of PDX1 on PPI transcription have been outlined in Section 1.2.1.3.1, so an increase in PPI mRNA was expected (Figure 3.3.6). This result infers that PDX1 is being activated properly and is crossing the nuclear membrane to exert its effect (Macfarlane *et al.*, 1999). This could not be confirmed, however, due to difficulties in detecting PDX1 via western blot. PDX1 is unable to stimulate transcription of PPI alone and requires the presence of Beta2, which has also been confirmed to be expressed in F9 cells (Figure 3.3.24).

4.4.2.3 Cell Lines Expressing High Levels of PDX1 and PPI mRNA do not have Detectable Levels of Either Protein

Despite showing overexpression of PDX1 and PPI in certain lines, the CT lines derived from F9 cells did not appear to translate equivalent levels of these proteins. Immunofluorescence and insulin EIAs did not detect any increases in the protein level. This could imply a translational block or translational repression of some kind. A similar repression has been seen in this laboratory where the poorly differentiated lung cell line DLKP was shown to have transitionally repressed keratin expression. In that system, increasing the expression of eIF4E (one of the initial factors to interact with RNA in translation initiation) through treatment with BrdU, was able to overcome this (Derek Walsh, PhD Thesis, 1999). The CT population may possibly contain a translational repressor; however, this would have to be identified. PDX1 has a 5' UTR (untranslated region) of 281 bp, whereas 90% of vertebrate mRNA UTRs are between 10 and 200 bp long (Willis, 1999). Growth related 5' UTRs tend to be

long with complex structures that impede the progress of the scanning ribosome and renders these RNAs suceptible to translational regulation (van der Velden & Thomas, 1999). The PDX1 5' UTR is moderately GC rich (61% GC) and this can also be associated with translational regulation (Willis *et al.*, 1999).

The glucose concentration is another important factor in this system, as already shown in the development of insulin secreting cells via cell trapping (Soria *et al.*, 2000). In that study it was found that the insulin content of the 'trapped' cells was significantly reduced and that there was no GSIS present if the final stage differentiation had been carried out at high glucose (30 mM). These cells would still have been able to express the PPI gene, however, as otherwise they would not have made it through the cell trapping procedure. High glucose levels have a wide range of effects on β cells in culture and *in vivo* including glycogen deposition, increased insulin secretion at low glucose, hypertrophy and loss of β cell differentiation (Roche *et al.*, 1997; Roche *et al.*, 1998; Jonas *et al*, 1999). This 'glucose-effect' may have impaired PPI expression at a protein level.

Observations that PPI levels were increased and that somatostatin can be induced in CT cell lines (Section 4.4.3.2), indicate the presence of functional PDX1. It is possible that the reason PDX1 protein has not been detected is that, despite overexpression at mRNA level compared to the parental F9 cells, that its levels are still too low to detect by immunofluorescence. It may, however, be present at levels high enough to exert its effect over time.

4.4.3 Differentiation of Selected Cell Trap Lines Does Not Result in Translation of PDX1 & PPI

F9-CT-RA and EB-CT-N/A_10 were selected for further analysis as the cells expressed high levels of PDX1 and PPI mRNA, respectively. It was decided to differentiate the cells using a selection of the agents already employed in Section 3.2. The agents selected have already been shown to increase PDX1 expression and in the case of sodium butyrate to effect δ-like and PP cell-like differentiation, thus it was hoped that the increased levels of PDX1 in the starting population would lead to more

committed β cell differentiation. In both CT cell lines, RA alone increased levels of PDX1 and PPI gene expression but did not lead to the expression of these proteins. Again, it is possible that the glucose level (25 mM) used to culture F9s and employed during differentiation may adversely affect the biosynthesis of insulin.

These cells were not found to have detectable levels of the glucose sensing genes, glucokinase and GLUT2, either even though PDX1 expression is reported to positively regulate their expression (McKinnon & Docherty, 2001).

4.4.3.1 F9-CT-RA and EB-CT-N/A_10 Express PP Upon Treatment with Sodium Butyrate

As with the monolayers and EBs described in Section 3.2, PP gene expression was noted following treatment with sodium butyrate (and the combination of sodium butyrate and activin A). There were no substantial differences in PP expression levels between the partially differentiated F9 endoderm and the F9-CT cells, indicating that increased PDX1 is not implicated in PP induction. This is additional evidence that sodium butyrate is having a direct effect on the gene itself rather than working through the transcription factor cascades.

4.4.3.2 F9-CT-RA and EB-CT-N/A_10 Constitutively Express Somatostatin

Both of the cell trap cell lines selected were found to express the somatostatin gene constitutively. It has already been shown that parental F9 cells do not show any somatostatin expression and RA treatment alone did not induce any somatostatin mRNA (Section 3.2.2.6). Parental EBs were found to contain low-level somatostatin; however, EBs treated with nicotinamide and activin A did not (Section 3.2.4.6) and this was the treatment employed in generating the EB-CT-N/A_10 cell line (Section 3.3.1.3.1).

Treatment of the cells with sodium butyrate resulted in a slight increase in somatostatin gene expression in both CT lines, but the combination of sodium butyrate and activin A did not have any such effect. RA treatment of F9-CT-RA

abolished somatostatin expression but reasons for this are unknown other than this treatment may have promoted PE-like differentiation in cells that had already differentiated in the presence of RA.

One of the early reports describing PDX1 referred to it as STF1 (somatostatin transactivating factor 1), where it was shown to stimulate somatostatin expression in islets (Leonard *et al.*, 1993). This was confirmed in embryonic neural cells by Schwartz *et al*, (2000). The promoter region of the somatostatin gene has at least four regions with a common TAAT core motif (this motif is also present in the A boxes of the PPI gene to which PDX1 binds), these regions are referred to as UE, T1, T2 and T3 (Leonard *et al.*, 1993; Schwartz *et al.*, 2000; McKinnon & Docherty, 2001). PDX1 preferentially binds to these regions during activation of the somatostatin gene (Figure 4.4.1).

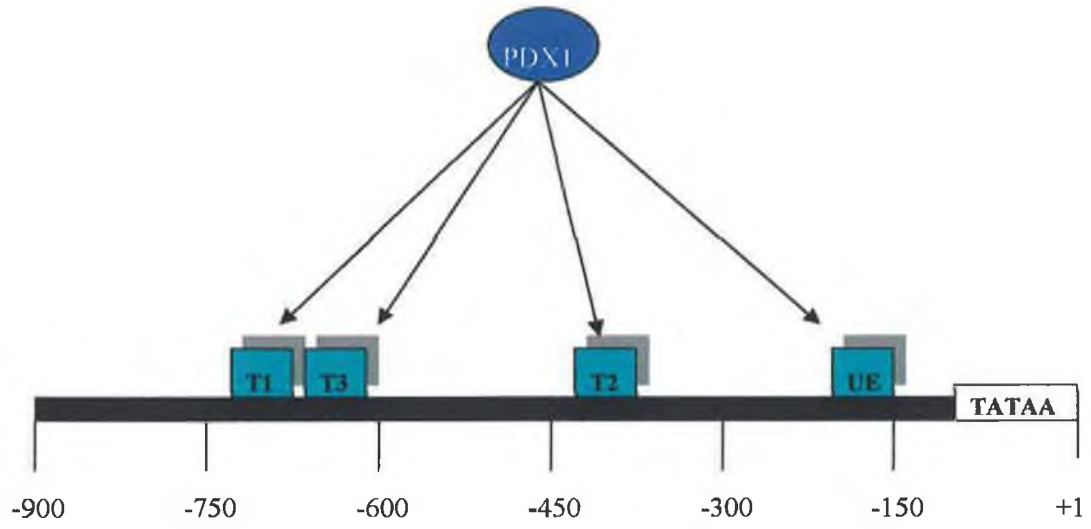


Figure 4.4.1 The 5' region of the somatostatin promoter identifying the regions to which PDX1 preferentially binds.

Thus the continuously elevated levels of PDX1 in the CT cells lines could have facilitated binding of PDX1 to the somatostatin promoter. PDX1 levels are induced in untreated EBs (Figure 3.2.15) and somatostatin expression is found to be present after 10 days culture in normal medium. However there are obviously more factors involved in this situation as sodium butyrate treated EBs have high levels of PDX1

but do not have active somatostatin expression. In the selective pressure based environment of the cell trap system, PDX1 expression is continuously favoured, thus it appears when PDX1 is continuously present for binding to the somatostatin promoter it can effect transcription of that promoter. As already discussed, F9 cells cultured for long periods (22-35 days) can differentiate towards multiple lineages when carefully differentiated (Koopman & Cotton, 1987). Thus it is suggested that the continuous presence of PDX1 in CT cells could, over time, lead to further changes within the CT cells, including constitutive expression of somatostatin mRNA. These results are in agreement with a recent study where overexpression of PDX1 in an islet-derived cell line caused 10-100 fold overexpression of the somatostatin gene (Itkin-Ansari *et al.*, 2000).

The induction of somatostatin following cell trapping i.e., via PDX1, is considered to be separate to that observed following sodium butyrate treatment as sodium butyrate did not enhance PDX1 expression (Section 3.2).

4.4.3.3 An Overview of Cell Trapping of F9 Cells

Cell trapping of F9 resulted in continuously proliferating cell lines that were resistant to high levels of zeocin due to their ability to drive the PDX1 promoter. Concomitantly, these cells expressed PDX1 at levels equivalent to, and above, those obtained during secondary differentiation (Section 3.2). Although the PDX1 protein was not detected via immunological methods there was evidence for the presence of functional PDX1 protein, through the observed increases in PPI and somatostatin mRNA. Prior to these experiments, somatostatin mRNA expression was limited to a few treatments e.g. the direct action of sodium butyrate. Thus purification of F9 cells continuously expressing PDX1 appears to promote endocrine differentiation of F9 cells towards δ cells.

4.4.4. High Efficiency Transfection of F9 Cells Depends on Transfection System and on Differentiation State of the Cells

Prior to studying the effects of transient overexpression of critical transcription factors on the differentiation-state and the gene expression profile of F9 cells, protocols had to be optimised to increase transfection efficiency. Initial transfection studies with the plasmid pCH110 (Section 2.3.4.1) demonstrated extremely low transfection efficiencies even when using Fugene (Roche). This is a reported feature in undifferentiated F9 cells and represents a transcriptional 'block' on viral promoters. Previous reports indicated that this could be overcome by differentiation of the cells (Darrow *et al.*, 1990; Alonso *et al.*, 1991), and this is clearly shown in Figure 3.3.12. In fact, the X-gal staining clearly highlights that it is the morphologically differentiated cells that are preferentially expressing the transfected plasmid.

4.4.5 Expression of Critical Transcription Factors Does Not Activate Endogenous Genes in F9-RAC Cells

F9 cells have been differentiated by direct transfection in the past e.g. through the expression of c-myc antisense and through c-fos overexpression (Griep & Westphal, 1988; Muller & Wagner, 1988). The overexpression of pancreatic transcription factors has also resulted in number of 'knock-on' effects in various systems (Table 4.4.1). PDX1, Beta2, Ngn3 and Nkx2.2 were all transfected into F9-RAC separately and PDX1 was co-transfected with Beta2 and Ngn3. As discussed, the effects of PDX1 are critically dependent on the presence of Beta2 (Serup *et al.*, 1996; Madsen *et al.*, 1996; Harmon *et al.*, 1998), and Ngn3 is known to induce Beta2 expression (Huang *et al.*, 2000). Despite overexpression of all of the genes at mRNA level and nuclear localisation of the transfected proteins, none of the β cell determinants examined were induced, in fact in the case of endogenous PDX1, levels were decreased following Beta2 or Nkx2.2 transfection. There appears to be no explanation for this as both of these factors are indicated to act downstream of PDX1 in pancreatic development (Naya *et al.*, 1997; Sussel *et al.*, 1998). Immunofluroescence analysis for PPI confirmed that it was not 'switched-on' as a result of the transient transfections.

In order to increase transfection efficiency, the F9 cells were partially differentiated with RA and db cAMP prior to transfection, thus pushing the cells towards a relatively homogenous parietal fate. This may limit the differentiation potential of the cells and thus the effects that the transcription factors may have if the islets were found to be more associated with VE as indicated in some recent ES cell work (Schuldiner *et al.*, 2000; Lumelsky *et al.*, 2001).

Cell Line	Gene Transfected	Gene Expressed/Induced	Reference
αTC1.6	PDX1	IAPP	Watada *et al.* (1996)
Trm6	PDX1	Somatostatin	Itkin-Ansari *et al.* (2000)
IEC-1	PDX1	IAPP, Glucokinase & Nkx6.1	Yoshida *et al.* (2002)
Capan 1	PDX1	Insulin	Zhou *et al.* (2002)
AR42J	Ngn3	PP	Zhang *et al.* (2001)
AR42J	Nkx2.2	PP	Palgi *et al.* (2000)

Table 4.4.1 Transcription Factor Expression Studies Resulting in 'Knock-on' Gene Induction.

4.4.5.1 Previous Studies Where Transient Overexpression Studies did not Result in Knock-On Effects on Endogenous Genes

There have been a number of reported cases whereby transcription factor expression did not result in the desired knock-on effects, e.g., nuclear expression of PDX1 had no effect on insulin expression in αTC1.9 cells (Serup *et al.*, 1995), while expression of PDX-1, isl-1 and Nkx6.1 (separately, or in combination) had no effects on gene expression in AR42J-B13 cells (Palgi *et al.*, 2000). It is not always apparent why the overexpression of transcription factors (especially PDX1) can elicit such large changes in one cell line but not in another. In the case of PDX1, it is known that it can associate with PBX1b and MRG1 homeodomain proteins to enhance transcription of the pancreatic Elastase I gene in acinar cells (Swift *et al.*, 1998). This may explain its

failure to induce PPI gene transcription in AR42J-B13 cells, i.e. if PDX1 has already been sequestered by other homeodomain-binding proteins (Palgi *et al.*, 2000). Thus it is possible that complex formation between transfected homeodomain proteins and native proteins could act as transcriptional repressors in certain systems.

4.5 Continuous Culture of Min6 Cells Results in Loss of Specialised Function

The continuous culture of a number of β cell lines leads to loss of specialised function i.e. insulin synthesis, storage and secretion in response to glucose. Cell lines such as HIT-T15, RIN-38 and βTC gradually lose their β cell phenotype through negative effects involving insulin gene transcription factors, such as PDX1, and unfavourable hexokinase/glucokinase ratios (Harmon *et al.*, 1998; Clark *et al.*, 1990; Knaack *et al.*, 1994). The Min6 cell line is considered an excellent model for functional β cells in culture (due to its preserved GSIS), but these cells also lose insulin processing ability and functional GSIS over time. Loss of function in Min6 has been seen to be accompanied by a decrease in the insulin processing enzyme PC2, and an increase in furin; these changes were considered indicative of a de-differentiating system (Kayo *et al.*, 1996). It is also possible that the observed loss of differentiation in Min6 cells may be due to the overgrowth of a more rapidly proliferating, less differentiated, population of cells. Such poorly differentiated subpopulations have been previously identified in Min6 (Minami *et al.*, 2000) and the overgrowth of differentiated F9 populations with less differentiated 'differentiation-resistant' cells has also been described (Alonso *et al.*, 1991).

Min6 cells in culture are also continuously growing in the presence of supraphysiological levels of glucose (physiological glucose levels are around 5-6 mM, Min6 cells grow in 25 mM glucose). Continual exposure to high glucose has wide ranging effects on β cells, including high rates of insulin secretion at low glucose, glycogen deposition and increased proliferation (Roche *et al.*, 1997; Roche *et al.*, 1998). These changes are correlated with a number of changes in the expression of genes involved with differentiation, glucose regulation, insulin processing, proliferation and the transcription of islet specific hormones (Roche *et al.*, 1997; Kayo *et al.*, 1996; Jonas *et al.*, 1999; Itkin-Ansari *et al.*, 2000).

This system has the potential to highlight crucial pathways/systems in β cell development. It is, however, also crucial to identify methods of preserving β cell function while in culture for possible future applications involving cultured replacement tissue. To investigate this, Min6 cells were studied with respect to their

GSIS capability, and their proliferation rates. Upon arrival at the proposed 'less differentiated state' (judged by the loss of GSIS), DNA array analysis and RT-PCR were carried out to compare the gene expression profiles of fully functional Min6 cells and 'de-differentiated' Min6 cells.

4.5.1 Continuous Culture Results in Morphological and Growth Pattern Changes in Min6 Cells

Low passage Min6 cells are seen to grow in three dimensional clumps; i.e. they do not form the monolayers typical of many cultured cell types (this growth pattern facilitates cell-to-cell contact and gap junction formation). GSIS has been shown to be severely disrupted when Min6 cells are unable to contact one another and form gap junctions (Linzel *et al.*, 1988; Meda *et al.*, 1991; Vozzi *et al.*, 1995). At higher passages, however, there is a noticeable change in Min6 morphology, the cells are seen to be smaller, and exhibit a lot of pointed processes spreading outwards in all directions (Figure 3.4.1). Most significantly, however, the cells do not form clumps and instead adopt the more conventional monolayer growth pattern. Both of these changes are significant for β cell function *in vitro* i.e., the loss of cell contact (as discussed) and smaller β cells show reduced levels of insulin secretion and GSIS (Giordano *et al.*, 1993). Even though the clustered nature of low passage Min6 cells often conceals the number of cells present in a flask, it is clear that there are more cells in the higher passage flask, thus suggesting changes in the proliferation rate.

In Section 3.4.2 it can be clearly seen that the high passage Min6 cells are proliferating at almost double the rate of the lower passage cells. Towards the end of the growth curve of low passage Min6 there is a plateau that is representative of the 3-D clumps having reached their maximal size; however, the higher passage cells can continue to grow until the entire tissue culture surface is coated. This is the opposite of what occurs upon differentiation of F9 cells, i.e. lengthening of the G1 phase and a reduction in the proliferative capacity of the cells (Linder *et al.* 1981; Kurki *et al.*, 1989), and following the BrdU mediated differentiation of DLKP (McBride *et al.*, 1999). Thus based on morphology and proliferation, Min6 cells are seen to undergo

changes that are the opposite of what occurs in two differentiating species previously studied in this laboratory.

High passage Min6 cells were also found to express considerable amounts of alkaline phosphatase compared to their low passage counterparts. Again this is the opposite of what occurs upon differentiation of F9 cells where the reduction in alkaline phosphatase activity has been correlated directly to increasing differentiation (Moore *et al.*, 1986). These observations tend to confirm that high passage Min6 cells are less differentiated than their lower passage counterparts.

4.5.2 Min6 Cells Lose Functional GSIS After 40 Passages in Culture

Consistent with other reports, Min6 cells were found to have lost their functional GSIS after 40 passages in culture (Miyazaki *et al.*, 1990; Ishihara *et al.*, 1995; Kayo *et al.*, 1996). It is also interesting to note the increased secretion of insulin at low glucose levels, this is particularly noticeable at 0 mM glucose. In cells with regulated storage and processing pathways such as heptocytes, mature insulin is stored until required while some proinsulin is constitutively secreted (Simpson *et al.*, 1995). Thus with increasing passage number and progressive loss of PC2 expression (Kayo *et al.*, 1996), increased amounts of unprocessed proinsulin would accumulate for constitutive release. As the EIA kit employed in determination of immunoreactive insulin (IRI) in these experiments detects both proinsulin and mature insulin (Section 2.5.2.3), the increase in secretion at low glucose may be attributable at least in part to increased proinsulin levels. It is also possible that the high passage Min6 cells were not able to store insulin as efficiently in the granules. In the de-differentiating Min6 cells identified by Kayo *et al.* (1996), chromogranin A was also reduced (this protein is associated with the secretory granules of neuroendocrine cells). The most likely explanation for the increase in constitutive secretion is a combination of both ineffective processing and depleted storage granules (processing and storage are inextricably linked). Human islets have also been seen to preferentially secrete proinsulin and show a marked decrease in storage granules when cultured at high glucose levels for lengthy periods (Bjorklund & Grill, 1999). This ties in with observations discussed later regarding the down-regulation of crucial granule-related genes in high passage Min6 cells (Section 4.5.3.2.4).

4.5.3 Comparative DNA Array Analysis of Low and High Passage Min6 Cells

To more closely examine the differences between the GSIS-capable low passage Min6 cells and the higher passage cells, and to endeavour to explain the phenotypic changes occurring, DNA array analysis was carried out. The array chosen was the Atlas™ Mouse 1.2 array (Clontech), which is a general array with 1,173 genes covering a number of categories and functions.

4.5.3.1 An Introduction to DNA Arrays

Microarray experiments can be used to compare the levels of gene expression on a large scale that is not possible with conventional RT-PCR or RNase protection assays. The rate at which genomes are being sequenced is increasing all the time and modern drug and therapeutic-target discovery is operated on a high-throughput basis.

Microarrays consist of cDNA or oligodeoxynucleotides (oligos) immobilised on to a support such as nylon, plastic or glass. These oligos represent specific regions of particular genes. Atlas™ arrays (Clontech) have cDNAs of 200-600 bp to minimise non-specific hybridisation in their array experiments. The arrays themselves can be of low complexity (100-1000 probes), to the very high complexity arrays with 100,000 probes (Cuzin, 2001). To carry out this analysis, RNA (preferably pure poly $(A)^+$ RNA) must be purified from the cells or tissues to be examined. This poly $(A)^+$ RNA is then converted into a labelled cDNA probe via the incorporation of a labelled nucleotide during the reverse transcriptase (RT) reaction. In the array experiment carried out in Section 3.4, the probes were labelled via the incorporation of P^{33} labelled dATP (Amarsham). P^{33} was preferentially chosen due to the specificity of its signal. The probes are then hybridised to the arrays and the expression levels examined by the appropriate detection method e.g. X-ray film, phosphoimager or fluorescence reader. Analysis of the arrays can be carried out using a software package such as AtlasImage™ (Clontech), and these can be used to compare a number of experiments to highlight the differentially expressed genes.

There are numerous literature examples of research involving array analysis of a number of related samples e.g. a series of tumors. This is done to determine common

features and to compare gene expression profiles with clinical outcomes. This information can be used to identify a related series of changes e.g. novel pathways which can then be investigated via more specific means of analysis (RT-PCR, protein etc). This type of research has numerous applications for diagnosis of diseases.

This technology is also finding a number of applications in the field of β cell research e.g. to demonstrate the glucose regulation of secretory and metabolic genes (Webb *et al.*, 2000) or the effect of growth arrest on cultured β cells (Zimmer *et al.*, 1999). This has led to the manufacture of a specific diabetes microarray, the PancChip (Scearce *et al.*, 2002).

4.5.3.2 High Passage Min6 Cells Show Large Differences in Their Gene Expression Profile

The results obtained in Section 3.4.5 indicate that analysis using the Atlas™ 1.2 array is a reproducible and robust system. Of the 80 shared gene changes (i.e. upregulated and downregulated) detected in two separate experiments using different membranes and RNA samples, 85% of these were direct repeats. The results that were found to be contradictory (e.g. a gene that was found to be upregulated in high passage Min6 in the first experiment was found to be downregulated in the second experiment) between the repeat experiments, were all stress/apoptosis related. This may reflect a variation in harvesting conditions, which could effect rapid changes in the levels of those genes. 1.5 or greater fold changes in expression levels were chosen as a cut-off point for tolerance. The genes obtained were classified under a number of headings (Section 3.4.5.2), however most genes may be listed under several headings e.g., PDX1 is a transcription factor, it is part of the gluco-regulatory machinery of the β cell and it is a developmental determinant. The gene designations used here follow those outlined in the Atlas™ array gene list, and this convention is used to maintain consistency throughout.

From Figure 3.4.7 it can be seen that the main differences between the low and high passage Min6 cells are that a large number of cell cycle genes are up-regulated while genes for hormones, specialised peptides and the genes involved in processing them

are down regulated. In fact upon closer examination of all the up-regulated genes, 23% of them are associated with proliferation or proliferating cells while 29% of the down regulated genes are involved in protein secretion and processing. These observations tie-in directly with the observations already made and tend to confirm that the loss of β cell phenotype is as a result of de-differentiation. The possible mechanisms of de-differentiation are discussed in Section 4.5.4.

Certain clusters of genes affected illustrate that high passage Min6 cells are less differentiated than low passage Min6 cells and are summarised below.

4.5.3.2.1 Min6 Cells Show Increased Expression of Primitive-Cell Markers at High Passage

The polycomb group gene EED is found to be upregulated in high passage Min6 cells. This gene is required in early mouse organogenesis (Morin-Kensicki *et al.*, 2001). The EED gene product operates through the formation of multimeric protein complexes e.g., EED interacts with YY1 to induce neurogenesis in *Xenopus* (Satijn *et al.*, 2001). The groucho gene related protein (Grg) is also upregulated in high passage Min6 cells. This gene is implicated in mouse development, especially that of the mesenchyme and the developing nervous system (Mallo *et al.*, 1993; Leon & Lobe, 1997). p55CDC is not an embryonic marker, as such, but this protein has been identified in all exponentially growing mammalian cell lines. Levels of p55CDC are seen to disappear upon induction of differentiation as cells fall out of the cell cycle (Weinstein *et al.*, 1994), and significantly, it is only found in high passage Min6 cells. These findings all indicate that the high passage Min6 cells have more in common with primitive developing cells than fully differentiated cells.

4.5.3.2.2 Continuous Passage in Glucose Induces GAPDH Expression

The β cell is a uniquely fuel-sensitive cell type due to its GSIS function, thus it is not surprising that there would be changes in the genes involved in the glucose metabolism following sustained culture in high glucose. Increased expression of these genes is associated with amplified glucose metabolism, which has been linked to

increased β cell proliferation and insulin release at low glucose concentrations (Roche *et al.*, 1997). Glucose consumption and metabolism were significantly increased in a non-glucose responsive sub-clone of Min6 (m14) compared to the glucose responsive clone m9 (Minami *et al.*, 2000). The observed increase in GAPDH in the high passage Min6 cells is not surprising, as it ties in perfectly with the other observed changes i.e., the increased insulin secretion at low glucose and the rapid proliferation. That is, rapid proliferation requires increased metabolism and proliferation is negatively associated with the expression of proteins in the insulin secretory pathway (Zimmer *et al.*, 1999).

4.5.3.2.2.1 Implications for Changes in Housekeeping Genes in Normalization of Expression

The increase in GAPDH had implications for later analysis as it is considered a 'housekeeping gene' and is therefore used as an internal control in RT-PCR to assess RNA quality and quantity. As a result of the observed increase in GAPDH with high passage, β actin was used in RT-PCR as the internal control when comparing low and high passage Min6 cells (its levels were found to be unchanged at high passage). Normalization of the arrays was not based on housekeeping genes as even these genes are susceptible to change given the correct stimulus. This method of normalisation is only recommended when a very large number of differentially expressed genes are expected e.g., when comparing divergent tissues. Global normalisation is the method of choice when examining similar tissues. The 'sum method' was automatically applied in the software used for these studies (AtlasImage^{TM}) and involved the calculation of all the signals in excess of background on both arrays to calculate a normalization coefficient based on :

$$Normalization \quad Coefficient = \frac{\sum_{i=1}^{n}(Intensity - Background)i}{\sum_{j=1}^{n}(Intensity - Background)j}$$

i = Genes on Array 1

j = Genes on Array 2

n = Number of genes on array.

4.5.3.2.3 Continuous Culture Results in the Down Regulation of Differentiation Related Genes

A number of the genes downregulated in the high passage Min6 cells are markers of differentiated islet cells (e.g. preproglucagon and PC2) and their down regulation is indicative of de-differentiation. These genes are discussed separately below.

Pax6 and Delta-like homologue 1 (DLK1) were both found to be downregulated in the high passage Min6 cells. DLK is a transmembrane protein and belongs to the EGF-like homeotic protein family which also includes the Notch receptor and Notch ligands, such as Delta (Laborda, 2000). As discussed already, Notch signalling is very important in specifying β cell fate in the developing pancreas (Apelqvist *et al.*,1999; Section 1.1.3.5.2), and acts through Ngn3. Ngn3 causes increased Delta expression on the cell surfaces which can inhibit neighbouring cells from adopting an endocrine fate (Section 1.1.3.5.2). $Ngn3^{-/-}$ mutants fail to form any endocrine cells and also do not express many important transcription factors, including Pax6 (Gradwohl *et al.*, 2000). DLK appears to have a more positive role than Delta in endocrine differentiation and is preferentially localised to insulin secreting cells in adults (Carlsson, *et al.*, 1997). It is also noteworthy that, upon comparison of two clones of Min6 with differing GSIS capabilities, DLK was more highly expressed in the cell line with the functional GSIS (Minami *et al.*, 2000). That result and the observations made here tend to implicate this gene product in regulated insulin secretion also. The importance of Pax6 in the developing islet has been covered in Section 1.1.3.5.5 and outlines the role of Pax6 in islet structure and in α cell determination. However Pax6 has also been implicated in regulating PDX1 expression in β cells through interaction with HNF 3β (Foxa 2) (Samaras *et al.*, 2002). Thus, this may be at least part of the reason why PDX1 levels were reduced in the high passage Min6 cells (Figure 3.4.11(A)). (An oligo specific for PDX1 was represented on the array but it was not detected despite being shown to be present in large quantities by RT-PCR, this is discussed later (Section 4.5.3.2.6)). Pax6 can directly bind the regulatory regions of the glucagon, insulin and somatostatin genes and is known to transactivate both insulin and glucagon (Sander *et al.*, 1997), which is interesting as levels glucagon and somatostatin are found to be reduced in high passage Min6 cells (Sections 3.4.5.4, 3.4.5.5 & 3.4.6.6). It should be

mentioned, however, that Pax6 levels were not found to be downregulated using RT-PCR analysis (Figure 3.4.9). This and other observations serve to highlight the differences between RT-PCR and arrays (Section 4.5.3.2.6).

4.5.3.2.3.1 Ornithine Decarboxylase is Downregulated in High Passage Min6, Impaired Polyamine Synthesis?

Nicotinamide has been shown to have inductive effects on insulin expression in cultured islets. It aids in maintaining β cell function *in vitro* and causes differentiation of precursor cells to insulin secreting cells (Sjoholm *et al.*, 1994; Otonkoski *et al.*, 1999). This agreed with previous findings linking polyamines to β cell function (Sjoholm *et al.*, 1990; Welsh & Sjoholm, 1988). Ornithine decarboxylase (ODC) is a critical rate-limiting enzyme in polyamine synthesis (Shantz & Pegg, 1999). Inhibition of ODC can prevent polyamine-mediated β cell replication (Sjoholm *et al.*, 1994). High passage Min6 cells show reduced ODC levels (Section 3.4.5.4), therefore, if ODC is so important for maintaining β cell function, then it is not surprising to find that it is downregulated in β cells which have lost a number of their specialised functions. This could indicate a target for studies to prevent loss of function of β cells in culture i.e., to devise methods to maintain ODC levels in cultured Min6 cells.

4.5.3.2.4 High Passage Min6 Cells Exhibit Differential Gene Expression in the Protein Secretion and Processing Pathway

One of the first indicators that the Min6 population is approaching a de-differentiated state is that the cells demonstrate impaired regulated insulin secretion. This observation is confirmed by a number of changes in genes involved in the storage and processing of insulin. The regulated pathway of secretion is outlined in Section 1.2.4.1 however in summary: reduced unfolded preproinsulin is synthesised in the rough endoplasmic reticulum (RER) where it is converted to proinsulin and packaged in microvesicles. These are transported to the Golgi apparatus where the insulin is processed while the secretory granule matures. The secretory granules are then stored

until glucose signals their translocation to the membrane to release their insulin, which is facilitated by microtubules and requires Ca^{2+} signalling (Howell, 1991).

A number of the secretion/processing-related genes found to be downregulated in high passage Min6 cells are discussed below in their approximate chronological order as they occur in the secretion and processing pathway.

Nucleobindin is a resident protein within the Golgi apparatus where it is involved in maintaining Ca^{2+} homeostasis (Lin *et al.*, 1998). Ca^{2+} levels are critical in endocrine cells and Ca^{2+} 'spikes' or oscillations facilitate secretory responses upon stimulation. These 'spikes' can be propagated to neighbouring cells to allow a co-ordinated response to stimuli (Kanno, 1998), however in periods of non-stimulation, the Ca^{2+} levels need to be maintained in a steady state. Calcium signalling in response to stimuli is unique in β cells as nutrients increase Ca^{2+} levels. One of the methods through which this increase is mediated is through the proteins involved in Ca^{2+} homeostasis (Soria *et al.*, 2000). Therefore the down regulation of nucleobindin can be linked to the disruptions in regulated secretion in Min6 cells.

RAB2 is also downregulated and its role in cells involves the trafficking of vesicles from the RER to the Golgi apparatus (Tisdale & Bach, 1996; Cheung *et al.*, 2002).

A number of proteins associated with the secretory granules are downregulated in high passage Min6 cells including secretogranin II, 7B2 neuroendocrine protein (secretogranin V), chromogranin B and PC2. Of these, PC2 is possibly the most important as it is one of the pair of enzymes that process proinsulin to mature insulin within the granule (Bennett *et al.*, 1992). PC2 requires secretogranin V in the granule as a chaperone protein (Sarac *et al.*, 2002). This is similar to the observation by Kayo *et al.*, (1996), i.e., where PC2 was reported to be sensitive to passage number. Kayo *et al.* (1996) also reported that PC1 decreased only slightly, which was also found in this study using the Atlas™ 1.2 array (it was down regulated by a factor of –1.1, which was considered unchanged).

Other secretion-associated genes found to be downregulated using the Atlas™ array include β-neoendorphin-dynorphin which has been implicated in the regulation of insulin secretion (Josefsen *et al.*, 1998), cholecystokinin which in involved in enhancing post prandial insulin secretion (Hardikar *et al.*, 2002), and CAB45 which localises to the secretory pathways of mammalian cells (Honore & Vorum, 2000).

The increased proliferation in high passage Min6 cells can also be linked to the negative effects on the secretion and processing of insulin. In a situation where cultured β cells could be held in a quiescent phase through a 'tet-off'-system of controlling proliferation (the β cell line was conditionally transformed by the SV40 T antigen (Tag) under the control of the regulatory elements of the bacterial tetracycline (tet) operon), administration of tetracycline to arrest growth caused induction of the secretory granule genes, chromogranin A and synaptogyrin 3, along with insulin (Zimmer *et al.*, 1999). This is probably due to the fact that a non-proliferating situation is similar to the *in vivo* β cell condition.

4.5.3.2.5 Hormone Expression is Decreased in Serially Passaged Min6 Cells

The preproglucagon gene expression was reduced in both array and RT-PCR analysis whereas somatostatin expression was found to be eradicated based on RT-PCR alone. PPI or PP mRNA expression levels were not substantially altered in the high passage Min6 cells. PDX1 is implicated in the regulation of PPI and somatostatin, however it has been shown to continue to transactivate the PPI gene even following an 86 % reduction through antisense expression (Kajimoto *et al.*, 1997). Therefore, even though the reduction in PDX1 is substantial in high passage Min6 cells (Figure 3.4.11(A)), there is still PDX1 present and thus the PPI gene can be transcribed as normal. The somatostatin gene has been shown to be induced in non-hormone expressing islet cells via overexpression of PDX1 (Itkin-Ansari *et al.*, 2000), and it is possible that the endogenous somatostatin expression noted in Min6 cells is due to the high PDX1 levels present, therefore reducing PDX1 could adversely affect somatostatin transcription in Min6. As noted earlier, Pax6 is implicated in the expression of insulin, somatostatin and glucagon (Sander *et al.*, 1997), and this was also shown to be downregulated on the Atlas™ array. The downregulation of

specialised hormones in Min6 cells is symptomatic of a de-differentiating system and, as such, fit in with the other observations leading to this conclusion.

4.5.3.2.6 Confirmation of Array Results by RT-PCR Highlights Differences Between the Two Techniques

To determine if the array results could be considered real and to validate the results obtained, confirmatory RT-PCR was carried out on the same RNA samples as had been used in the arrays (where primers were available). On comparing the RT-PCR results with the array it can be seen that, in the examples chosen (Section 3.4.5.5), semi-quantitative RT-PCR does not appear to be as sensitive as the array, and the likelihood of a similar result in the RT-PCR is enhanced by a large change in gene expression (as indicated by the array). This leads to other somewhat contradictory observations regarding RT-PCR and arrays however, in that certain genes that were represented on the array, were not detected at all, even when found to be abundantly present within the cells when amplified by RT-PCR e.g., PDX1. When using this array a P^{33} label was employed; this is not as 'hot' as a P^{32} label and therefore is not as sensitive. P^{33} however is far less prone to signal-bleed or non-specific binding, i.e. the signal from P^{32} can give a powerful signal which can spread out and cover other loci and mask the expression of other genes. The reasons for these differences between the array and RT-PCR are unknown. Kudoh *et al.* (2000) reported only 58% success in validation of array results by RT-PCR and attributed this to various factors such as sequence content and to unknown PCR related factors.

4.5.3.2.7 RT-PCR Analysis of Other β Cell Genes Reveals the Capacity for Increased Glucose Transport in High Passage Min6 Cells

RT-PCR analysis of other Min6 genes was carried out to examine the expression of genes that were not represented on the array or that were present but were not detected. As mentioned, PDX1 levels were found to be > 2 fold lower in the high passage cells than in the lower passage Min6 cells. This has previously been observed in cultured insulinoma cells before e.g. HIT-T15 cells continuously cultured in high glucose show reduced PDX1 levels (Olson *et al.*, 1995). As PDX1 is considered to be

one of the β cells most defining features, it is clear that the loss of this gene marks the transition of high passage Min6 cells towards another less defined cell-type.

The lack of glucose sensing ability in certain cell lines is associated with the absence of GLUT2 or glucokinase expression, however glucokinase mRNA levels were similar in low and high passage Min6 cells while GLUT2 mRNA was appreciably increased in high passage cells. GLUT2 has been characterised as a glucose sensitive gene (Waeber *et al.*, 1994), and chronic elevation of glucose has been shown to increase GLUT2 transcript levels in INS1 cells while glucokinase mRNA levels were unaffected (Roche *et al.*, 1997). As already discussed, the metabolism of the high passage cells appears to be increased when compared to the low passage Min6 cells (as evidenced by the increase in GAPDH), thus the increase in GLUT2 should facilitate the increased requirement for glucose which fuels the increased metabolic and proliferation rates. Other important β cell transcription factors, i.e. Beta2 and Isl1, were unaffected by the continuous culture. The effects on insulin and the other hormone levels has already been discussed. A number of other genes critical for pancreatic function and differentiation state were not included on this array e.g. Indian hedgehog (Ihh), however this array was chosen as it was general enough to afford an overall view of all the changes taking place in the cell with increasing passage number.

4.5.4 Continuous Culture of Min6 Cells Leads to a Number of Phenotypic and Genotypic Changes Consistent With De-Differentiation

In summary, the serial passage of Min6 cells led to initial observable changes in morphology, growth and insulin secretion. These were accompanied by the increased activity of markers typical of undifferentiated cells. The increases in growth were correlated with increases in genes required for glucose transport and metabolism. The altered secretion was shown to be associated with the down regulation of a number of critical features of the regulated secretion pathway. The reduction of terminal differentiation markers such as PDX1 and the hormones glucagon and somatostatin all additively confirm that these cells are de-differentiating while continuously cultured in the presence of supraphysiological levels of glucose. The data presented here does

not indicate whether the de-differentiated population resulted from the overgrowth of the Min6 culture with rapidly proliferating, undifferentiated cells or if the de-differentiation was as a result of gradual changes in the gene expression profile of the cells leading to a less specialised cell type. Previous studies in the literature show many of the changes observed in high passage Min6 cells can occur upon culture of islets or β cells in high levels of glucose and that these changes can occur within 2 days - 1 week (Roche *et al.*, 1997; Roche *et al.*, 1998; Jonas *et al*, 1999). This is not enough time for a population of cells to be completely overgrown by a subpopulation of undifferentiated cells, therefore there is a definite possibility that the de-differentiation observed in Min6 cells could be due to a number of gradual changes in the levels of critical genes in the population as a whole. These possible mechanisms are illustrated in Figure 4.4.1.

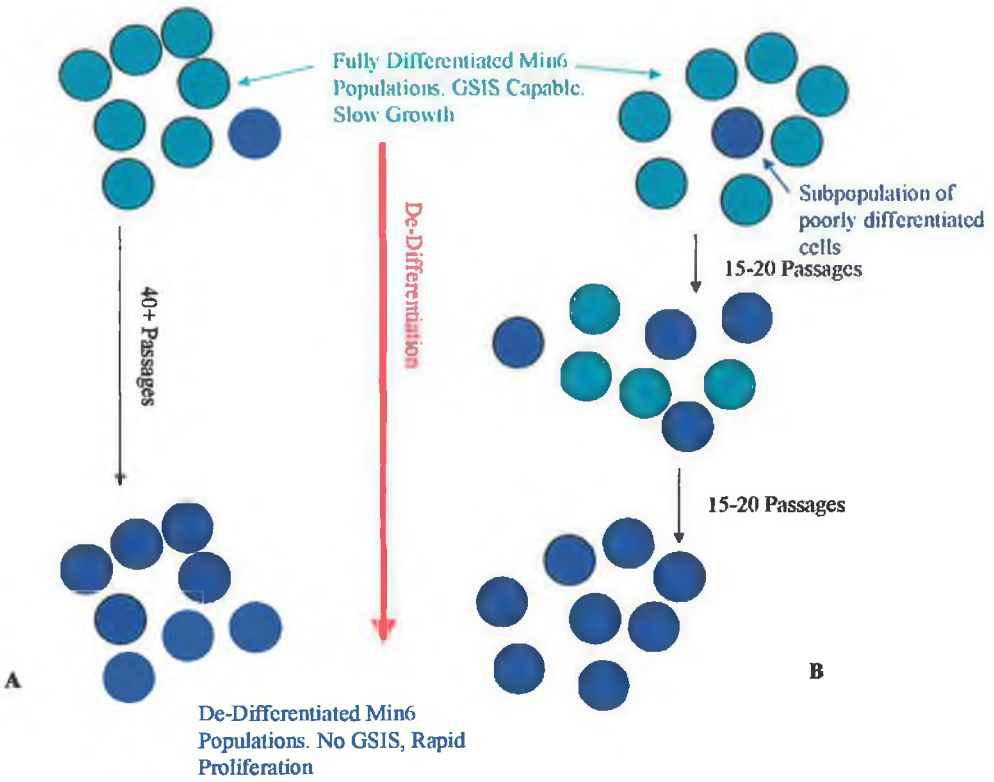


Figure 4.4.1 Possible mechanisms of Min6 'de-differentation'. (A) Continuous passaging in conditions of elevated glucose result in gradual alterations in the gene expression profile such that the cells regress to a less specialised phenotype. (B) Subpopulations of poorly differentiated cells within the Min6 cell population gradually take over the population, with time in culture, until a homogenous population of these cells results.

4.6 *In Vitro* Generation of Insulin Secreting Cells via Direct Gene Transfer

Cultured cell lines expressing human insulin have been considered for somatic cell therapy for diabetes. The techniques to express proteins of choice in cells are standardised and generally very efficient. The choice of cell line however is a critical factor. Ideally the cell line should be capable of storing and secreting protein in a regulated fashion and should also be capable of sensing glucose over the entire physiological range. The only cell line that carries out all of these functions however is the β cell; thus the goal of cell engineering for β cell replacement is to generate cells genetically modified so as to resemble a functional β cell, e.g. through the co-expression of insulin and the glucose sensing machinery. Aside from all the considerations regarding the mechanics of GSIS, the most important point regarding a modified cell for therapeutic use is that it must be considered safe to implant in to humans (in a suitably encapsulated form). Some of the more β cell-like lines secrete other proteins which might cause problems if implanted to humans e.g. the release of ACTH from AtT20 cells. BHK-21 cells have been used in a human clinical trial and, while the effect of the released protein did not significantly alter the disease state, the trial was of interest because the cells were able to grow and survive while secreting their product, without endangering the patient (Aebischer *et al.*, 1996). Thus on the basis of clinical usefulness, BHK-21 cells were considered to be an appropriate choice for an insulin delivery vehicle.

4.6.1 BHK-PPI-C16 Constitutively Release Proinsulin in an Unregulated Fashion

Following transfection of the human insulin gene into BHK-21 cells and characterisation of the 20 derived cell lines, BHK-PPI-C16 was isolated for further work. Expression of the gene was confirmed using RT-PCR, immunocytochemistry and EIA. Immunocytochemical analysis indicated that the proinsulin was present throughout the cytoplasm and there did not appear to be any storage granules present. Fibroblasts are not characterised as having these granules (Kelly, 1985), however upon transfection of insulin in to the HEP G2 cell line it was found that the new HEP G2ins cells were able to store insulin in membrane bound vesicles that were not present in the parental population (Simpson *et al.*, 1995). Characterisation of the

proinsulin synthesised by BHK-PPI-C16 revealed that it was entirely proinsulin and that it was being released constitutively, i.e. that no processing was taking place. Previous studies with fibroblasts have also identified this phenomenon (Selden *et al.*, 1987; Kawakami *et al.*, 1992; Taniguchi *et al.*, 1997; O' Driscoll *et al.*, 2002). Constitutive secretion is not typically associated with proprotein processing; however, rat hepatoma cells (FAO cells) were shown to process insulin (albeit inefficiently), while constitutively secreting it (Vollenweider *et al.*, 1992). The steady release of the proinsulin per hour accounted for approximately 34% of the cellular insulin within BHK-PPI-C16, which is similar to findings by O' Driscoll *et al.* (2002). This release rate is due to the continuous transport of proinsulin from the trans golgi network (TGN) to the plasma membrane, a process which takes approximately 10 minutes (Kelly, 1985; Halban & Irmanger, 1994). The cellular proinsulin content presumably represents the proinsulin that is 'in-transit' to the plasma membrane and the steady-state level of intracellular proinsulin over 24 hours signifies the cells 'holding capacity' rather than storage.

BHK-PPI-C16 cells were unable to regulate the secretion of proinsulin to environmental glucose levels despite expressing both components of the glucose sensor, i.e., GLUT2 and glucokinase. The observation that the cells did not modulate their proinsulin secretion in the subphysiological range of glucose concentrations (Figure 3.5.6), indicated that the lack of GSIS is not as a result of the unfavourable GLUT1/GLUT2 or hexokinase/glucokinase ratios seen in Min7 and AT20ins (Ishihara, 1993; Davies *et al.*, 1998). However, confirmation of this was sought through the overexpression of both GLUT2 and glucokinase in BHK-PPI-C16.

4.6.2 Overexpression of the Glucose Sensing Genes Does Not Confer Glucose Sensitivity Over the Physiological Range in BHK-PPI-C16

Glucokinase and a combination of glucokinase and GLUT2 were transfected into BHK-PPI-C16 and the overexpression of both at the mRNA level was confirmed by RT-PCR. Immunocytochemical analysis revealed the existence of intensely stained cells within the transfected populations, but these were relatively sparsely distributed within the population. Immunocytochemical analysis can only be considered semi-quantitative however, thus western blotting analysis was carried out to determine the

levels of the transfected proteins. Western analysis did not indicate substantial increases in the levels of glucokinase and GLUT2 in the transfected BHK-PPI-C16_GCK and BHK-PPI-C16_GCK_GLUT populations. Despite this, however, it was found that the BHK-PPI-C16_GCK population exhibited a moderate sub-physiological secretory response, whereas the BHK-PPI-C16_GCK_GLUT population was no longer found to secrete proinsulin. Transfection of glucokinase into RIN-38 cells resulted in a large increase in the capacity for GSIS which was also in the subphysiological range (i.e. < 5-6 mM glucose) (Hohmeier *et al.*, 1997). Actively regulated secretion through secretagogues, however, requires a pool of stored material which was not found in BHK-PPI-C16. Regulated secretion has been achieved previously with fibroblasts through the action of an inducible promoter (Kawakami *et al.*, 1992), however this is not regulated at the level of secretion, rather, increasing transcription increases the biosynthesis of proinsulin. Cells have a consistent 'holding-capacity', thus the extra proinsulin must be secreted at a faster rate through the constitutive pathway. This is regulation of secretion at the 'level of availability' (Halban & Irmanger, 1994). This implies that there is more insulin available for secretion in BHK-PPI-C16_GCK in the range 2-5 mM glucose, which is not surprising as it is within the range at which the cells normally grow. Assuming that there is an increase in functional glucokinase within the BHK-PPI-C16_GCK population, this could allow for increased metabolism and therefore an increase in biosynthetic activity within the optimal glucose range for BHK-21 cell culture.

The loss of insulin secretion despite unaltered mRNA expression in the co-transfected population BHK-PPI-C16_GCK_GLUT may be due to an increase in metabolic load due to the expression of too many constitutively expressed genes. Concomitant with the loss of insulin expression is the finding that the levels of the two housekeeping proteins β actin and α tubulin are also reduced. This reduction could be correlated with increased number of transfected genes. A Bradford assay (Section 2.5.2.1.2.1) of cell lysates also indicated a similar reduction in cellular protein levels following double and triple transfection. Recombinant bacteria have been shown to exhibit lower growth rates than their wild type counterparts due to the increased metabolic load/burden resulting from the expression of the exogenous protein (Glick, 1995). Similar observations have been made in mammalian cells by Kim *et al.* (1998).

Yallop & Svendsen (2001) found that aggressive selection using G418 increased the copy number of the neo^r (G418 resistance gene) gene and this was responsible for increases in the metabolic load in BHK-21 cells. In the BHK-PPI-C16_GCK_GLUT system there are 6 constitutively expressed genes i.e. PPI, glucokinase, GLUT2 and the resistance genes for G418, zeocin and hygromycin. Thus, if aggressive selection can increase the expression of the resistance genes then it is probable that the negative effects noted in BHK-PPI-C16_GCK_GLUT are due to increased metabolic load and cellular exhaustion. Although the difference in cellular protein levels between BHK-PPI-C16_GCK and BHK-PPI-C16_GCK_GLUT is minor, the protein levels in the BHK-PPI-C16_GCK_GLUT are lower. It is possible that there are other effects of increasing the metabolic load that are not visible through direct analysis of cellular protein levels, which may play a more direct role in the loss of secretion ability.

4.6.3 Agents Causing an Increase in Intracellular cAMP Increase Proinsulin Secretion From BHK-PPI-C16

cAMP has been shown to increase insulin secretion in a number of cell lines (Nielsen *et al.*, 1985; Hammonds *et al.*, 1987; Ullrich *et al.*, 1996; Simpson *et al.*, 1997; Lu *et al.*, 1997). This effect is at least partly mediated through membrane depolarisation (Ullrich *et al.*, 1996). cAMP can act as a second messenger for stimulated insulin release by insulinotropic hormones such as GLP-1 (Drucker *et al.*, 1987). Upon stimulation of BHK-PPI-C16 with forskolin and theophylline there was a heterogeneous, but consistent, increase in the proinsulin levels secreted. Forskolin acts through activation of adenyl cyclase while theophylline is a cAMP phosphodiesterase inhibitor. Using two similar compounds, glucagon (an adenyl cyclase activator) and IBMX (a cAMP phosphodiesterase inhibitor), also resulted in increased insulin secretion. As discussed already (Section 4.6.2), it seems unlikely that BHK-PPI-C16 could be stimulated to secrete proinsulin due to its inherent constitutive protein secretion pathway and that increased secretion could only be effected by an increase in intracellular insulin, i.e. to create a positive pressure to encourage increased secretion. cAMP can also directly stimulate transcription of the human insulin gene through interactions with four cAMP responsive elements (CREs) (Inagaki *et al.*, 1992), however, cAMP was not found to have any enhancing effects on PPI transcription in BHK-PPI-C16. Roesler *et al.* (1988) described cAMP as the most

important regulatory molecule with diverse functions ranging from co-ordinating metabolic processes to specific enzyme synthesis and that the effects of cAMP are varied in nature. It is therefore possible that cAMP is influencing proinsulin biosynthesis in BHK-PPI-C16, resulting in an increased rate of secretion.

4.6.4 Implications for Use of BHK Cells Secreting Human Insulin

BHK-PPI-C16 cells produce proinsulin, which can function in an insulin-like manner although the efficiency is only 8-20% of mature insulin (Selden *et al.*, 1987; Taniguchi *et al.*, 1997; Falqui *et al.*, 1999). Previous studies have shown that the continuous release of proinsulin from genetically engineered fibroblasts ($Ltk^{+}Ins$) could aid in maintaining normoglycemia in mice for up to 50 days (Taniguchi *et al.*, 1997). The Diabetes Control and Complications Trial (DCCT) (1987) stated that the residual release of insulin significantly improves metabolic control in IDDM, therefore BHK-PPI-C16 could provide such a role. This function could possibly help prevent the long-term damage associated with diabetes, e.g. renal failure and peripheral neuropathy, which are all associated with long term additive effects of transient hyperglycemic episodes, that occur during loss of diabetic control.

5.0 CONCLUSIONS AND FUTURE WORK.

5.1 Conclusions

A major focus of this thesis was the development of protocols and conditions whereby the murine EC cell line, F9, could be differentiated along an endocrine differentiation pathway. The F9 cells were differentiated along characterised endodermal differentiation pathways using retinoic acid and db cAMP (di butyryl cyclic adenosine monophosphate). These endodermal cells were then treated with secondary differentiation agents associated with β cell differentiation. Lineage determination was carried out by RT-PCR analysis of critical differentiation markers and developmentally regulated genes. To investigate the importance of the transcription factor PDX1 in endocrine differentiation, PDX1 expressing cells were isolated from differentiating F9 cells, using cell trapping techniques and were studied. Experiments were also carried out on continuously cultured β cell lines, to investigate if these cells were de-differentiating, with the aim of identifying important features for the maintenance of a β cell phenotype in culture. Finally, the use of the cultured cell line BHK-21 to express the human insulin gene was investigated.

The following conclusions were made as a result of this research:

- Endodermal differentiation of F9 cells can lead to the expression of the full complement of cytokeratins associated with the pancreas i.e., cytokeratins 7, 19, 8 & 18 with a concomitant loss in expression of the stem cell marker alkaline phosphatase.

- By shortening the duration of the RA (10^{-7}M retinoic acid) and RAC (10^{-7} M retinoic acid with 10^{-3}M di butyryl cyclic adenosine monophosphate) mediated differentiation, the potential of F9 cells to differentiate in to a wide variety of tissue types is retained.

- Examination of the morphological evidence and the expression levels of differentiation markers , such as cytokeratins and alpha fetoprotein, lead to the conclusion that RA and RAC mediated differentiation resulted in the formation of

three types of endoderm i.e. parietal endoderm (PE), visceral endoderm (VE) and primitive endoderm (PrE).

- Treatment of the three endodermal cell types with a selection of differentiation agents (characterised for their ability to direct differentiation towards an endocrine lineage), leads to a number of changes in gene expression. The developmental genes Pax6 and Ihh showed the greatest amount of variation in expression levels between the different treatments.

- RT-PCR analysis detected the expression of a number of pancreatic genes in differentiating F9 cells including preproinsulin (PPI), somatostatin, pancreatic polypeptide (PP), pancreatic duodenal homeodomain protein (PDX1), Pax6 and indian hedgehog (Ihh). This again indicates that F9 cells are capable of differentiating in multiple directions.

- The absence of typical pancreatic transcription factor cascades/pathways may indicate that the treatments are facilitating other cell types to develop e.g. mesodermal cells as was found in the treatment of ES cells with activin A (Schuldiner *et al.,* 2000). The wide range of responses of the three F9-derived endodermal types reveals that the cells differ in their responses to external stimuli.

- Sodium butyrate can lead to the expression of pancreatic polypeptide and somatostatin genes in F9 derived endoderm. Its effects can be enhanced by activin A in specific circumstances. The fact that sodium butyrate did not have any similar effects on any of the other genes examined suggests that is likely that sodium butyrate is having a direct effect on the expression of these genes.

- Sodium butyrate may be exerting its effects on the somatostatin and pancreatic polypeptide genes through direct interaction with the structure of the chromatin, as evidenced by sodium butyrate-mediated increases in acetylated histone H3.

- Overall analysis of the results obtained here tend to suggest that visceral endoderm (VE) is the lineage from which to differentiate pancreatic cells. The

gene expression profiles associated with VE derived from embryoid bodies (EBs) show more similarities to the pancreatic cell types, i.e. EBs have elevated levels of PDX1 and PPI, and show wider expression of the endocrine markers somatostatin and pancreatic polypeptide than homogenous PE derived from RAC treated cell lines. The latter do not show the same level of pancreatic differentiation.

- Cell trapping has been shown to be a powerful method for isolating populations of specific cells from a differentiating stem cell population. The cells isolated through PDX1-based cell trapping all expressed higher PDX1 mRNA levels than the parental population. Although it was not possible to visualise an increase in PDX1 protein levels, evidence for an increase in functional PDX1 protein was provided by associated increases in PPI gene and somatostatin gene expression, both of which are known to be transactivated by PDX1.

- Somatostatin gene expression appears to be transactivated by increased PDX1 levels in differentiated F9 cells. In cell trapped cells where the levels of PDX1 are continuously elevated, somatostatin expression is constitutive. Untreated EBs also show elevated PDX1 levels compared to F9 cells and constitutively express somatostatin.

- F9 cells show low transfection efficiencies while undifferentiated. Previous studies have shown that this can be overcome through endodermal differentiation (Darrow *et al.,* 1990). In this study it was shown that the transfection efficiency was substantially increased upon partial differentiation with RAC medium for only 72 hours. The morphologically differentiated PE-like cells clearly show preferential expression of the transfected cDNA.

- Overexpression of pancreatic differentiation factors in partially differentiated F9 cells does not lead to initiation of pancreatic transcription factor cascades. It is possible that the PE nature of the transfected population may be the incorrect lineage from which to initiate pancreatic development.

- Min6 cells lose the ability to regulate insulin secretion in response to glucose when passaged continuously in culture. This is associated with other changes such as increased insulin secretion at low (i.e. subphysiological) glucose levels, increased proliferation and morphological changes. RT-PCR and DNA array analysis identified that these phenotypic changes are as a result of changes in the expression of critical genes. DNA array analysis specifically indicated increases in gene transcripts such as p55CDC, which is a marker for proliferating cell lines. Critical insulin processing and secretion proteins such as PC2, secretogranin V, secretogranin II and chromograinin B were all decreased at higher passages. The concomitant reduction in the expression of genes such as Pax6, DLK, ODC, somatostatin and glucagon all contribute to the evidence that the continuous culture of Min6 cells results in de-differentiation which may be at least partly due to impaired polyamine synthesis resulting from reduced ODC expression levels. The results obtained here do not reveal the mechanism of de-differentiation, however, and it is unclear if Min6 cells undergo gradual changes in gene expression over time or if the de-differentiation is as a result of the overgrowth of the Min6 population by a subpopulation of undifferentiated cells.

- Expression of the human PPI gene in BHK-21 cells leads to a high level of gene expression, and to translation of the gene product, proinsulin. However, possibly due to the constitutive nature of fibroblast protein secretion pathways, these cells were not able to regulate the secretion with external glucose levels. This inability to regulate secretion was at least partly caused by the lack of efficient storage, which was also linked to the inability to process the proinsulin to mature insulin.

- BHK-PPI-C16 cells were found to express low levels of the glucose sensing genes, GLUT2 and glucokinase. Overexpression of glucokinase did result in a moderate subphysiological glucose-related increase in secretion. This effect is thought to be due to 'regulation through availability' which was as a result of improved metabolism. Similar work with fibroblasts has achieved this regulation through the action of inducible promoters.

- Co-expression of GLUT2 and glucokinase in BHK-PPI-C16 resulted in impaired insulin secretion. This was accompanied by the reduction in the levels of two housekeeping proteins (α tubulin and β actin), and a decrease in cellular protein as measured by the Bradford assay. These changes were linked to a deleterious increase in the metabolic load of the cells due to the constitutive expression of six exogenous genes.

- CAMP-inducing agents were found to increase proinsulin secretion from BHK-PPI-C16. It is believed that the increase in cAMP levels results in an increase in proinsulin biosynthesis, leading to increased proinsulin secretion.

5.2 Future Work

The work carried out here identified a number of points regarding endocrine differentiation thus there are a series of experiments which could be carried out to further the work outlined here.

5.2.1 Elucidation of Possible Lineages Emerging From Secondary Treated F9 Cells

To fully monitor the effects that the secondary differentiation agents may be having on F9 cells, a wider range of differentiation markers should be examined, i.e. markers outside of those involved in the developing pancreas. Examples of the type of markers that could be examined are listed below:

1. Ectodermal Markers including: Neurofilament Heavy Chain (Brain), and dopamine β hydroxylase (adrenal ectoderm).

2. Mesodermal: Enolase (Muscle), cartilage matrix protein (bone) and cardiac actin (heart).

3. Endoderm: Albumin (liver) and α1AT (liver).

5.2.2 Treatment of Differentiated F9 Cells With Bromodeoxyuridine (BrdU)

It has been shown in this laboratory that BrdU can overcome the translational repression of cytokeratin expression in DLKP cells through its induction of eIF4E expression (allowing for more efficient translation initiation). It would be interesting to determine if BrdU could elicit the same response in differentiated F9 cells which have been shown to have increased levels of PPI and PDX1 mRNA, but not their respective proteins.

5.2.3 Cell Trapping at Low Glucose

It is unknown if the seemingly poor expression of PDX1 and insulin in the cell trapped cell lines was due to differentiation in the presence of high levels (25 mM) of glucose, therefore it would be advisable to carry out the procedure at low glucose and monitor the effects on PDX1 and insulin proteins.

5.2.4 Generation of Stable F9 Cell Lines Expressing PDX1

Increased PDX1 levels were linked to the transactivation of the somatostatin gene in F9-CT (i.e. cell trapped (CT) F9 cells) cell lines. Direct transfection of undifferentiated F9 cells is inefficient and would not be suitable for transient experiments (as these experiments typically require transfection efficiencies of ≥15-20% to have any noticeable effect on endogenous gene expression), however stable cell lines could be generated. Thus an experiment to generate PDX1 overexpressing cell lines would be interesting to determine if this alone could activate the somatostatin gene. Other markers should also be investigated to determine if PDX1 could have an effect when expressed in totally undifferentiated F9 cells and without the pre-commitment to parietal endoderm (PE), as in the RAC treated F9 population.

5.2.5 Generation F9 Cells Stably Transfected with Nuclear Transcription Factors

As with the PDX1 overexpression in undifferentiated F9 cells, it would be worthwhile to generate F9 cell lines stably transfected with Beta2, Ngn3, Nkx2.2 and combinations of these. It would be interesting to determine if the expression of these proteins alone could lead to the differentiation of F9 cells. This would also serve to indicate if the PE lineage of RAC treated F9 cells, was responsible for the lack of knock-on effects (as noted in Section 3.3.2).

5.2.6 Cell Trapping of Other Embryonic Stem (ES) Cell Lines

The results obtained during the course of this thesis indicate that cell trapping is a excellent tool for cell isolation in differentiating F9 cells. For comparative purposes it

would be interesting to carry out the cell trapping protocol on differentiating ES cells and to purify the PDX1 expressing cells, that have been previously observed to be generated, following spontaneous differentiation of EBs (embryoid bodies) (Schuldiner *et al.,* 2000; Gerrish *et al.,* 2000).

5.2.7 Identification of the Mechanism of Action of Sodium Butyrate in Pancreatic Polypeptide (PP) and Somatostatin Induction

Treatment of all three types of endoderm from stage 1 differentiation with sodium butyrate resulted in PP and somatostatin expression (activin A was required for induction of somatostatin in EBs). Increases in acetylated histone H3 levels indicated that the sodium butyrate was having an effect through acetylation of chromatin. To confirm the mechanism of sodium butyrate activation of PP and somatostatin in F9 cells the following experiments should be carried out:

1. Treatment of F9 endoderm samples with another histone deacetylase (HDAC) inhibitor e.g. trichostatin A or oxamflatin (Marks *et al.,* 2000), to determine if HDAC inhibition is the mechanism by which the genes are induced.

2. Transcriptionally active chromatin is more soluble due to partial unwinding and a more open conformation, which is linked to histone acetylation. These modifications can make the chromatin more susceptible to DNase I digestion. Thus, nuclei from F9 EC and sodium butyrate treated F9 cells could be treated with DNase I, restriction digested to give fragments containing the relevant promoter region, southern blotted and probed for DNase I digestion.

3. ChIPs (chromatin immunoprecipitation protocol) analysis is another method whereby one can discover if a gene is undergoing modifications due to histone acetylation, by immunoprecipitation of all acetyl histone of interest (previously crosslinked to DNA), and then checking the recovered DNA for the gene of interest, via PCR.

5.2.8 Identification of the Mechanism of Loss of Min6 Phenotype

As already outlined in Section 4.5, the actual mechanism of de-differentiation in Min6 is unknown, i.e. whether the cells are de-differentiating as a population or if the differentiated functional cells are being overgrown by less differentiated cells with faster proliferation rates. To elucidate this, clonal populations of Min6 cells should be generated and characterised for function. This could aid identification of rapidly proliferating sub-populations within the Min6 population as a whole. By identifying fully functional clones and passaging them over time one could discover if these cells also lose their ability to secrete insulin in response to glucose and if they show similar differential gene expression to that seen in the high passage cells examined in this thesis. If these clones were not to lose their GSIS ability then it would indicate that the 'overgrowth theory' was valid. If these cells, however, were to lose their GSIS in a similar manner to ordinary Min6 cells, then a mechanism involving the gradual de-differentiation of cultured Min6 cells through changes in expression in critical genes would be confirmed.

5.2.9 Protein Analysis in High Passage Min6 Cells

The DNA array studies indicated some very interesting changes in high passage Min6 cells. These should be investigated by protein analysis, including studies of glucagon and somatostatin expression levels. Some proliferation proteins should also be investigated e.g. Ki67, as should the levels of the secretogranin and chromogranin proteins i.e. the proteins involved in regulated secretion. The polyamine levels should also be compared between high and low passage Min6 cells. These experiments would confirm the array data used to explain why Min6 cells lose their ability to regulate insulin secretion in response to glucose.

5.2.10 Maintenance of Min6 Cells in Long Term Culture

During DNA array analysis of high passage Min6 cells it was noticed that the levels of ODC were reduced. If polyamine levels are shown to be reduced in high passage Min6 cells then a repeat passaging experiment should be carried out, except that one set of flasks should be grown in nicotinamide (to induce high polyamine levels), to investigate if β cell function can be maintained longer. This should be followed by comparative DNA array analysis to determine the effects of nicotinamide treatment.

6.0 BIBLIOGRAPHY.

6.0 Bibliography

Abraham, E. J., Leech, C. A., Lin, J. C., Zulewski, H. and J. F. Habener. (2002). Insulinotropic hormone glucagon-like peptide 1 differentiation of human pancreatic islet derived progenitor cells into insulin producing cells. *Endocrinology* **143** : 3152-3161

Aebischer, P., Pochon, N. A,. Heyd, B., Deglon, N., Joseph, J. M., Zurn A. D., Baetge, E. E., Hammang, J. P., Goddard, M., Lysaght, M., Kaplan, F., Kato, A. C., Schluep, M., Hirt, L., Regli, F., Porchet, F. and N. De Tribolet. (1996). Gene therapy for amyotropic lateral sclerosis (ALS) using a polymer encapsulated xenogenic cell line engineered to secrete hCNTF. *Hum Gen Ther* **7** : 851-860

Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K. and H. Edlund. (1998). β Cell-specific Inactivation of the Mouse Ipf1/Pdx1 Gene Results in Loss of the β Cell Phenotype and Maturity Onset Diabetes. *Genes and Development.* **12** : 1763-1768

Ahlgren, U., Pfaff, S. L., Jessel, T. M., Edlund, T., and H. Edlund. (1997). Independent requirement for Isl1 in the formation of Pancreatic Mesenchyme and Islet Cells. *Nature.* **385** : 257-260.

Albano, R. M., Groome, N. and J. C. Smith. (1993). Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. *Development.* **117** : 711-723

Alonso, A., Breuer, B., Steuer, B. and J. Fischer. (1991). The F9-EC cell line as a model for the analysis of differentiation. *Int J. Dev. Biol.* **35** :389-397

Alpert, S., Hanahan, D., and G. Teitelman. (1988). Hybrid Insulin Genes Reveal a Development Lineage for Pancreatic Endocrine Cells and Imply a Relationship with Neurons. *Cell.* **53** :295-308.

Alzuherri, H. M. and R. J. White. (1999). Regulation of RNA polymerase I transcription in response to F9 embryonal carcinoma stem cell differentiation. *The Journal of Biological Chemistry.* **274** : 4328-4334

Anastasi, E., Ponte, E., Gradini, R., Bulotta, A., Sale, P., Tiberti, C., Okamoto, H., Dotta, F. and U. D. Mario. (1999). Expression of Reg and cytokeratin 20 during ductal cell differentiation and proliferation in a mouse model of autoimmune diabetes. *European Journal of Endocrinology.* **141** : 644-652

Andreoli, J. M. and K. T. Trevor. (1995). Structural and biological consequences of increased vimentin in simple epithelial cell types. *Cell Motil Cytoskeleton.* **32**: 10-25

Andrews, G. K. and E. D. Adamson. (1987). Butyrate selectively activates the metallothionein gene in teratocarcinoma cells and induces hypersensitivity to metal induction. *Nucleic Acid Res.* **15** : 5461-5475

Ang, S. L. and J. Rossant. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78** : 561-574

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendhal, U. and H. Edlund. (1999). Notch signalling controls pancreatic cell differentiation. *Nature.* **400** : 877-881

Artavanis-Tsakonas, S., Rand, M. and R. J. Lake. (1999). Notch signalling: Cell fate control and signal integration in development. *Science* **284**: 770-776

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P. A. and C. B. Wollheim. (1992). Establishment of 2-mercaptoethanol-dependent differentiated insulin secreting cell lines. *Endocrinology* **130** : 167-178

Ashcroft S.J.H. (1980). Glucoreceptor Mechanisms and the control of Insulin Release and Biosynthesis. *Diabetalogia.* **18** : 5-15

Ashcroft, F. M. and F. M. Gribble. (1999). ATP-sensitive K^+ Channels and Insulin Secretion: their Role in Health and Disease. *Diabetologia.* **42** : 903-919

Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K. L. and M. Tzukerman. (2001). Insulin production by human embryonic stem cells. *Diabetes* **50** : 1691-1697

Auricchio, A., Gao, G. P., Yu, Q. C., Raper, S., Rivera, V. M., Clackson, T. and J. M. Wilson. (2002). Constitutive and regulated expression of processed insulin following in vivo hepatic gene transfer. *Gene Therapy* **9** : 963-971

Bach, S. P., Renehan, A. G. and C. S. Potten. (2000). Stem Cells: the intestinal stem cells as a paradigm. *Carcinogenesis* **21** : 469-476

Bailey, C. J., Davies, E. L. and K. Docherty. (1999). Prospects for insulin delivery by ex-vivo somatic cell gene therapy. *J. Mol Med.* **77** : 244-249

Bali, D. Svetlanov, A., Lee, H-W., Fusco-DeMane, D., Leiser, M., Li, B., Barzilai, N., Surana, M., Hou, H., Fleischer, N., DePinho, R., Rossetti, L. and S. Efrat. (1995). Animal model for maturity onset diabetes of the young generated by disruption of the mouse glucokinase gene. *The Journal of Biological Chemistry.* **270** : 21464-21467

Banting, F. G. and C. H. Best. (1922). The internal secretion of the pancreas. *J Lab and Clin Med* **7** : 465-480

Becker, S., Wang, Z. J., Massey, H., Arauz, A., Labosky, P., Hammerschmidt, M., St-Jacues, B., Bumcrot, D., McMahon, A. L. Grabel. (1997). A Role for Indian Hedgehog in extraembryonic endoderm differentiation in F9 cells and the early mouse embryo. *Developmental Biology*. **187**.: 298-310

Bennett, D. L., Bailyes, E. M., Nielsen, E., Guest, P, C., Rutherford, N. G., Arden, S. D. and J. C. Hutton. (1992). Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryote subtilisin family. *The Journal of Biological Chemistry*. **267** : 15229-15236

Bernstine, E. G., Hooper, M. L., Grandchamp, S., and B. Ephrussi. (1973). Alkaline Phosphatase activity in Mouse Teratoma. *Proc. Natl. Acad. Sci.* **70** : 3899-3093

Best, L. (2000). Glucose-sensitive conductances in rat pancreatic β cells: contribution to electrical activity. *Biochemica et Biophysica Acta* **1468** : 311-319

Bishop, A. E., and J. M. Polak. (1991). The anatomy, organisation and ultrastructure of the islets of Langerhans. *The Textbook of Diabetes, Volume I.* Eds. Pickup, J. and G. Williams. Blackwell Scientific Publications, Oxford p57

Bjorklund, A. and V. Grill. (1999). Enhancing effects of long-term elevated glucose and palmitate on stored and secreted proinsulin-to-insulin ratios in human pancreatic islets. *Diabetes* **48** : 1409-1414

Bjornson, C. R. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. and A. L. Vescovi. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science*. **283** : 534-537

Boam, D. S. and K. Docherty. (1989). A tissue specific nuclear factor binds to multiple sites in the human insulin-gene enhancer. *Biochem J.* **264** : 233-239

Boam, D.S.W., Clarke, A.R. and K. Docherty. (1990). Positive and Negative Regulation of the Insulin Gene by Multiple Trans-Acting Factors. *J. Biol. Chem*. **265** : 8285-8296

Bockman, D.E., (1993). Anatomy of the Pancreas. *The Pancreas: Biology and Pathobiology and Disease.* 2nd ed. Eds. Vay Liang, W,. Go. *et al.* Raven Press Ltd. New York. p1

Bogler, O., Wren, D., Barnett, S. C., Land, H. and M. Noble. (1990). Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. *Proc. Natl. Acad. Sci.* **87** : 6368-6372.

Bonner-Weir, S., Taneja, M., Weir, G. C., Tatarkiewicz, K., Song, K-H., Sharma, A. and J. J. O' Neil. (2000). In Vitro cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci* **97** : 7999-8004

Bosco, D., Orci, L. and P. Meda. (1989). Homologous but not heterologous contact increases the insulin secretion of individual pancreatic β cells. *Experimental Cell Research*. **184** : 72-80

Bosco, D., Meda, P., Halban, P. A. and D. G. Rouiller. (2000). Importance of cell-matrix interactions in rat islet β cell secretion in vitor, Role of α6β1 integrin. *Diabetes* **49** : 233-243

Bouwens, L,. Wang, R-N., De Blay, E., Pipeleers, D. G. and G. Kloppel. (1994). Cytokeratins as markers of ductal cell differentiation and islet neogenesis in the neonatal rat pancreas. *Diabetes*. **43**: 1279-1283

Bouwens, L. and De Blay, E. (1996). Islet Morphogenesis and Stem Cell Markers in Rat Pancreas. *The Journal of Histochemistry and Cytochemistry*. **44** : 947-951

Bouwens, L., Lu, W. G. and De Krijger, R. (1997). Proliferation and Differentiation in the Human Fetal Endocrine Pancreas. *Diabetologia*. **40** : 398-404

Bouwens, L. (1998a). Cytokeratins and Cell Differentiation in the Pancreas. *Journal of Pathology*. **184** : 234-239.

Bouwens, L. (1998b). Transdifferentiation versus stem cell hypothesis for the regeneration of islet beta-cells in the pancreas. *Microsc Res Tech*. **15** : 332-336

Bouwens, L. and D. G. Pipeleers. (1998). Extra-insular beta cells associated with ductules are frequent in adult human pancreas. *Diabetologia*. **41** : 629-633

Bronner-Fraser, M. (1986). An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Developmental Biology* **117** : 528-536

Brüstle, O., Jones, K. N., Learish, R. D., Karram, K., Choudhary, K., Wiestler, O. D., Duncan, I. D. and R. D. G. McKay. (1999). Embryoinc stem cell-derived glial precursors: a source of myelinating transplants. *Science* **285** : 754-756

Budd, C. G., Pansky, B. and L. Glatzer. (1993). Preproinsulin mRNA in the Rat Eye. *Investigative Opthamology & Visual Science*. **34** : 463-469

Burdsal, C. A., Lotz, M. M., Miller, J. and D. R. McClay. (1994). Quantitative switch in integrin expression accompanies differentiation of F9 cells treated with retinoic acid. *Developmental Dynamics*. **201**: 344-353

Burdsal, C. A., Pedersen, R. A., Hyun, W. C. and J. J. Latimer. (1995). Novel flow cytometric method for seperating cell types in differentiated F9 embryoid bodies.*Cytometry* **21** : 145-152

Calabrese, A., Guldenagel, M., Charollais, A., Mas, C., Caton, D., Baquis, J., Serre-Beinier, V., Caille, D., Sohl, G., Teubner, B., Le Gurun, S., Trovato-Salinaro, A., Condorelli, D. F., Haefliger, J. A., Willecke, K. and P. Meda. (2001). Cx36 and the function of the endocrine pancreas. *Cell Adhes Commun.* **8** : 387-391

Carlsson, C., Tornehave, D., Lindberg, K., Galante, P., Billestrup, N., Michelsen, B., Larsson, L-I. and J. H. Nielsen. (1997). Growth hormone and prolactin stimulate the expression of rat preadipocyte factor1/Δ-like protein in pancreatic islets: Molecular cloning and expression pattern during development and growth of the endocrine pancreas. *Endocrinology* **138** : 3940-3948

Carty, M.D., Lillquist, J. S., Peshavaria, M., Stein, R. and W. Soeller. (1997). Identification of cis and trans-active factors regulating human Islet Amyloid Polypeptide gene expression in pancreatic β-cells. *The Journal of Biological Chemistry.* **272** : 11986-11993

Charles, M.A., Laweki, J., Pictet, R. and G.M. Grodsky. (1975). Insulin Secretion. Interrelationships of Glucose, Cyclic 3,5 –Monophosphate, and Calcium. *J Biol. Chem.* **250** : 6134-6140

Chertow, B.S., Blaner, W.S., Barnetsky, N.G., Sivitz, W.I., Cordle, M.B., Thompson, D. and P. Meda. (1987). Effects of Vitamin A Deficiency and Repletion on Rat Insulin Secretion In Vivo and In Vitro from Isolated Islets. *J. Clin. Invest.* **79** : 163-169

Cheung, A. Y., Chen, C. Y., Galven, R. H., de Graaf, B. H., Vidali, L., Hepler, P. K. and H. M. Wu. (2002). Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the golgi bodies and is important for pollen tube growth. *Plant Cell* **14** : 945-962

Christophe, J. (1994). Pancreatic tumoral cell line AR42J: an amphicrine model. *Am. J. Physiol.* **266** : G963-G971

Cirulli, V., Crisa, L., Beattie, G. M., Mally, M. I., Lopez, A. D., Fannon, A., Ptasznik, A., Inverardi, L., Ricordi, C., Deerinck, T., Ellisman, M., Reisfeld, R. A., and A. Hayek. (1998). KSA Antigen Ep-CAM Mediates Cell-Cell Adhesion of Pancreatic Epithelial Cells: Morphoregulatory Roles in Pancreatic Islet Development. *Journal of Cell Biology* **140** : 1519-1534.

Clairmont A., Tessmmann, D. and H. Sies. (1996). Analysis of *connexin43* expression induced by retinoic acid in F9 teratocarcinoma cells. *FEBS Letters.* **397**: 22-24

Clark, S. A., Burnham, B. L. and W. L. Chick. (1990). Modulation of glucose-induced insulin secretion from a rat clonal beta-cell line. *Endocrinology.* **127** : 2779-2788

Clarke, A. R., Wilson, M. E., Leibiger, I., Scott, V. and K. Docherty. (1995). A Silencer and an Adjacent Positive Element Interact to Modulate the Activity of the Human Insulin Promoter. *Eur. J. Biochem.* **232** : 627-632

Cockell, M., Stolarczyk, D., Frutiger, S., Hughes, G. J., Hagenbuchle, O. and P. K. Wellauer. (1995). Binding sites for hepatocyte nuclear factor 3β or 3γ and pancreas transcription factor 1 are required for efficient expression of the gene encoding pancreatic α amylase. *Mol Cell Biol* **15** : 1933-1941

Coll, J. L., Ben-Zev, A., Ezzell, R. M., Rodriguez Fernandez, J. L., Baribault, H., Oshima, R. G. and E. D. Adamson. (1995). Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion and locomotion. *Proc. Natl. Acad. Sci.* **92**: 9161-9165

Cornelius, J. G., Tchernev, V., Kao, K-J. and A. B. Peck. (1997). In vitro generation of islets in long-term cultures of pluripotent stem cells from adult mouse pancreas. *Hormone and Metabolic Research*. **29** : 271-277

Cuzin, M. (2001). DNA Chips: a new tool for genetic analysis and diagnostics. *Transfus Clin Biol* **8** : 291-296

Dabeva, M. D., Hwang, S-G, Vasa, S. R. G., Hurston, E., Novikoff, P. M., Hixson, D. C., Gupta, S. and D. A. Shafritz. (1997). Differentiation of pancreatic progenitor cells into hepatocytes following transplantation into rat liver. *Proc. Natl. Acad. Sci.* **94** : 7356-7361

Darrow, A. L., Rickles, R. J. and S. Strickland. (1990). Maintenance and use of F9 teratocarcinoma cells. *Methods Enzymol* **190** : 110-117

Davies, E. L., Shennan, K. I. J., Docherty, K., and C. J. Bailey. (1998). Expression of GLUT2 in insulin-secreting AtT20 Pituitary Cells. *Journal of Molecular Endocrinology.* **20** : 75-82

DCCT Research group. (1987). Effects of age, duration and treatment of insulin-dependent diabetes mellitus on residual beta-cell functions: observations during eligibility testing of the Diabetes Control and Complications Trial (DCCT). *J Clin Endocrinol Metab* **65** : 30-36

Demeterco, C., Beattie, G. M., Dib, S. A., Lopez, A. D. and A. Hayek. (2000). A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. *J. Clin Endocrinol Metab.* **85** : 3892-3897

Deutsch, G., Jung, J., Zhang, M., Lora, J. and K. S. Zaret. (2001). A bipotential precursor population for pancreas and liver within the embryonic ectoderm. *Development* **128** : 871-881

Devaskar, S. U., Singh, B. S., Carnaghi, L. R., Rajakumar, P. A. and S. J. Giddings. (1993). Insulin II gene expression in rat central nervous system. *Regulatory Peptides* **48** : 55-63

Dinsmore, J., Ratliff, J., Jacoby, D., Wunderlich, M. and C. Lindberg. (1998). Embryonic stem cells as a model for studying regulation of cellular differentiation. *Theriogenology.* **49**: 145-151

Docherty, K., Macfarlane, W. M., Read, M. L., Smith, S. B., Wilson, M. E., Bujalska, I. and M. Gilligan. (1996). Regulation of insulin gene transcription by nutrients. *Biochemical Society Transactions*. **24** : 368-372.

Docherty, K. (1997). Gene therapy for diabetes mellitus. *Clinical Science*. **92** : 321-330

Doetsch, F. Caille, I., Lim, D. A., Garcia-Verdugo, J. M. and A. Alvarez-Buylla. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell.* **97** : 703-716

Dohrmann, C., Gruss, P. and L. Lemaire. (2000). Pax genes and the development of hormone-producing endocrine cells in the pancreas. *Mechanisms of Development.* **92** : 47-54

Draper, J. S., Pigott, C., Thomson, J. A. and P. W. Andrews. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J. Anat* **200**: 249-258

Drucker, D. J., Philippe, J., Mojsov, S., Chick, W. L. and J. L. Habener. (1987). Glucagon-like peptide 1 stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc. Natl Acad Sci.* **84** : 3434-3438

Dudek, R. W. and I. E. Lawerence. (1988). Morphologic evidence of interactions between adult ductal epithelium of pancreas and fetal foregut mesenchyme. *Diabetes*. **37** : 891-900

Dudek, R.W., Lawerence, I. E., Hill, R. S. and R. C. Johnson. (1991). Induction of islet cytodifferentiation by fetal mesenchyme in adult pancreatic ductal epithelium. *Diabetes* **40** : 1041-1048

Dumonteil, E., Laner, B., Constant, I. and J. Philippe. (1998). Differential Regulation of the Glucagon and Insulin I gene promoters by the Basic Helix-Loop-Helix Protein Factors E47 and BETA2. *J. Biol. Chem* **273** : 19945-19954

Edlund, H. (1998). Transcribing Pancreas. *Diabetes* **47** : 1817-1823

Edlund, H., (1999). Pancreas: how to get here from the gut. *Curr. Opin Cell Biol.* **11** : 663-668

Edlund, H. (2001). Developmental Biology of the Pancreas. *Diabetes*. **50**: S5-S9

Edlund, H. Pancratic Organogenesis-Developmental Mechanisms and Implications for Therapy (2002). *Nature Reviews*. **3**: 524-532

Edlund, T., Walker, M.D., Burr, P.J. and W.J. Rutter. (1985). Cell Specific Expression of the Rat Insulin Gene: Evidence for the Role of Two Distinct 5'- Flanking Elements. *Science*. **230** : 912-916

Edwards, S. A., Rundrell, A.Y.K. and Adamson, E. A. (1988). Expression of c-fos antisense mRNA inhibits differentiation of F9 cells to parietal endoderm. *Dev Biol.* **129**: 91-102

Eglitis, M. A. and E. Mezey. (1997). Hematopoietic cells differentiate into both macroglia and macroglia in the brains of adult mice.*Proc. Natl Acad Sci.* **94** : 4080-4085

Emens, L. A., Landers D. W. and L. G. Moss. (1992). Hepatocyte nuclear factor 1 alpha is expressed in a hamster insulinoma line and transactivates the rat insulin 1 gene. *Proc Natl Acad. Sci.* **89** : 7300-7304

Esni, F., Taljedal, I-B., Perl, A-K., Cremer, H., Christofori, G. and H. Semb. (1999). Neural cell adhesion molecule (N-CAM) is required for type segregation and normal ultrastructure in pancreatic islets. *Journal of Cell Biology*. **144** : 325-337

Fairbairn, L. J., Cowling, G. J., Reipert, B. M., and T. M. Dexter. (1993). Supression of apoptosis allows differentiation and development of a multipotent hematopoitic cell line in the absence of added growth factors. *Cell*. **74** : 823-832

Falqui, L., Martinenghi, S., Severini, G. M., Corbella, P., Taglietti, M. V., Arcelloni, C., Sarugeri, E., Monti, L. D., Paroni, R., Dozio, N., Pozza, G. and C. Bordignon. (1999). Reversal of diabetes in mice by implantation of human fibroblasts genetically engineered to release mature human insulin. *Human Gene Therapy* **10** : 1753-1762

Faria, T. N., LaRosa, G. J., Wilen, E., Liao, J. and L. J. Gudas. (1998). Characterisation of genes which exhibit reduced expression during retinoic acid-induced differentiation of F9 teratocarcinoma cells: involvement of cyclin D3 in RA-mediated growth arrest. *Mol. Cell.Endocrinol.* **25**: 155-166

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and F. Mavilio. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. *Science*. **279** : 1528-1530

Furukawa, M., Eto, Y. and I. Kojima. (1995). Expression of immunoreactive activin A in fetal rat pancreas. *Endocr J.* **42** : 63-68

Gandarillas, A. and F. M. Watt. (1997). C-Myc promotes differentiation of human epidermal stem cells. *Genes and Development* **11** 2869-2882

Gat, U., DasGupta, R., Degenstein, L. and E. Fuchs. (1998). De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin *Cell.* **95**: 605-614

Gatignol, A., Durand, H. and G. Tiraby. (1988). Bleomycin resistance conferred by a drug-binding protein. *FEBS Lett.* **230** : 171-175

Gazdar, A. F., Chick, W. L., Oie, H. K., Sims, H. L., King, D. L., Weir, G. C. and V. Lauris. (1980). Continuous, clonal, insulin- and somatostatin secreting cell lines established from a transplantable rat islet cell tumor. *Proc.Natl Acad Sci.***77** : 3519-3523

German, M.S. (1993). Glucose Sensing in Pancreatic Islet Beta Cells : The Key Role of Glucokinase and the Glycolytic Intermediates. *Proc. Natl. Acad. Sci.* **90** : 1781-1785.

Gerrish, K., Gannon, M., Shih, D., Henderson, E., Stoffel, M., Wright, C. V. E. and R. Stein. (2000). Pancreatic β cell specific transcription of the PDX-1 gene. The role of conserved upstream control regions and their hepatic nuclear factor 3β sites. *The Journal of Biological Chemistry*. **275** : 3485-3492

Giordano, E, Cirulli, V., Bosco, D., Rouiller, D., Halban, P. and P. Meda. (1993). β-Cell Size Influences Glucose Stimulated Insulin Secretion. *Am. J. Physiol.* **265** : C358-C364

Githens, S.G. (1993). Differentiation and Development of the Pancreas in Animals. *The Pancreas: Biology and Pathobiology and Disease.* 2nd ed. Eds. Vay Liang, W,. Go. *et al.* Raven Press Ltd. New York. P21

Gittes, G. K. and W. J. Rutter. (1992). Onset of Cell-specific Gene Expression in the Developing Mouse Pancreas. *Proc. Natl. Acad, Sci.,* **89** : 128-1132

Glick, B. R. (1995). Metabolic load and heterologous gene expression. *Biotech Advances* **13** : 247-261

Glick, E., Leshkowitz, D. and M. D. Walker. (2000). Transcription factor BETA2 acts cooperatively with E2A and PDX1 to activate the insulin gene promoter. *The Journal of Biological Chemistry*. **275** : 2199-2204

Goslow, N. and C. Grobstein. (1962). Epitheliomesenchymal interaction in pancreatic morphogenesis. *Dev Biol.* **4** : 242-255

Grabel, L., Becker, S., Lock, L., Maye, P. and T. Zanders. (1998). Using EC and ES cell culture to study early development: recent observations on Indian hedgehog and Bmps. *Int J Dev Biol.* **42** : 917-925

Gradwohl, G., Dierich, A., LeMeur, M. and F. Guillemot. (2000). Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc. Natl. Acad. Sci.* **97**: 1607-1611

Griep, A. E. and H. F. De Luca. (1986a). Studies on the relation of DNA synthesis to retinoic acid-induced differentiation of F9 teratocarcinoma cells. *Exp Cell Res.* **164 :** 223-231

Griep, A. E. and H. F. De Luca. (1986b).Decreased c-myc expression is an early event in retinoic acid-induced differentiation of F9 teratocarcinoma cells. *Proc. Natl Acad. Sci* **83** : 5539-5543

Griep, A. E. and H. Westphal. (1988). Antisense Myc sequences induce differentiation of F9 cells. *Proc. Natl. Acad. Sci.* **85**.: 6806-6810

Gros, L., Montoliu, L., Riu, E., Lebrigand, L. and F. Bosch. (1997). Regulated production of mature insulin by non-β-cells. *Human Gene Therapy* **8** : 2249-2259

Gros, L., Virsolvy, G., Salazar, D., Bataille, D. and P. Blache. (1999). Characterization of low affinity binding sites for glibenclamide on the Kir6.2 subunit of the β cell K_{ATP} channel. *Biochem Biophys Res Commun.* **257** : 766-770

Groskerutz, D. J., Sliwkowski, M. X. and C. M. Gorman. (1994). Genetically engineered proinsulin constitutively processed and secreted as mature, active insulin. *The Journal of Biological Chemistry* **269** : 6241-6245

Guthrie, J., Williams, J. A. and C. D. Logsdon. (1991). Growth and differentiation of pancreatic acinar cells: independent effects of glucocortocoids on AR42J cells. *Pancreas* **6** : 506-513

Halban, P. A. and J-C. Irminger. (1994). Sorting and Processing of Secretory Proteins. *Biochemical Journal.* **299** : 1-18

Hardie, D. G. and P. Cohen. (1991). Insulin action and responses: the biochemistry of post receptor events.*The Textbook of Diabetes, Volume I.* Eds. Pickup, J. and G. Williams. Blackwell Scientific Publications, Oxford p99

Hammonds, P., Schofield, P.N., and S.J.H. Ashcfoft. (1987). Glucose Regulates Preproinsulin Messanger RNA Levels in a Clonal Cell Line of Simian Virus 40 Transformed β Cells. *FEBS Letters.* **213** : 149-154

Hardikar, A. A., Wang, X. Y., Williams, L. J., Kwok, J., Wong, R., Yao, M. and B. E. Tuch. (2002). Functional Maturation of fetal porcine beta cells by glucagon-like peptide 1 and cholecystokinin. *Endocrinology* **143** : 3505-3514

Harndahl, L., Jing, Z-J., Ivarsson, R., Degerman, E., Ahren, B., Manganiello, V. C., Renstrom, E. and L. S. Holst. (2002). Important role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic β cell exocytosis and release of insulin. *The Journal of Biological Chemistry.* [e-pub ahead of print 6 August]

Harmon, J. S., Tanaka, Y., Olson, L. K. and R. P. Robertson. (1998). Reconstitution of glucotoxic HIT-T15 cells with somatostatin transcription factor 1 partially restores insulin promoter activity. **47** : 900-904

Harris, T. M. and G. Childs. (2002). Global gene expression patterns during differentiation of F9 embryonal carcinoma cells into parietal endoderm. *Funct Inter Genomics* **2** : 105-119.

Hatzfeld, M. and W. W. Franke. (1985). Pair formation and promiscuity of cytokeratins: Formation in vitro of heterotypic complexes and intermediate-sized filaments by homolgous and heterologous recombinations of purified proteins. *J. Cell Biol* **101** : 1826-1841

Hauge-Evans, A. C., Squires, P. E., Persaud, S. J. and P. M. Jones. (1999). Pancreatic β-cell-to-β-cell interactions are required for integrated responses to nutrient stimuli. *Diabetes* **48** : 1402-1408

Hebrok, M., Kim., S.K. and D. A. Melton. (1998). Notochord repression of endodermal Sonic Hedgehog permits pancreas development. *Genes and Development* **12** : 1705-1713

Herrera, P. L., Huarte, J., Sanvito, F., Meda P., Orci, L., and J-D. Vassalli. (1991). Embryogenesis of the Murine Endocrine Pancreas; Early expression of the Pancreatic Polypeptide Gene. *Development.* **118** : 1257-1265

Herrera, P. L. (2002). Defining the cell lineages of the islets of Langerhans using transgenic mice. *Int J Dev Biol.* **46** : 97-103

Hogan, B. L. M., Taylor, M and E. Adamson. (1981). Cell Interaction modulates embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* **291**: 235-237

Hohmeier, H. E., BeltrandelRio, H., Clark, S. A., Henkel-Rieger, R., Normington, K. and C. B., Newgard. (1997). Regulation of Insulin Secretion From Novel Engineered Insulinoma Cell Lines. *Diabetes.* **46** : 968-977.

Hohmeier, H. E., Milder, H., Chen, G., Henkel-Rieger, R., Prentki, M. and C. B. Newguard. (2000) Isolation of INS-1 derived cell lines with robust K^+ channel-dependent and independent glucose stimulated insulin secretion. *Diabetes* **49** : 424-430

Honore, B. and H. Vorum. (2000). The CREC family, a novel family of EF-hand, low-affinity Ca(2+)-binding proteins localised to the secretory pathway of mammallian cells. *FEBS Letters* **466** 11-18

Howell, S. L. (1991). Insulin Biosynthesis and Secretion. *The Textbook of Diabetes, Volume I.* Eds. Pickup, J. and G. Williams. Blackwell Scientific Publications, Oxford p72
Huang, H-P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M. and M-J Tsai. (2000). *Molecular and Cellular Biology.* **20** : 3292-3307

Huang, H-P, Liu, M., EL-Hodiri, H. M., Chu, K., Jamrich, M. and M-J. Tsai. (2000). Regulation of the pancreatic islet specific gene BETA2 (neuroD) by Neurogenin. *Mol Cell Biol* **20** : 3292-3307

Hughes, S. D., Quaade, C., Milburn, J. L., Cassidy L. and C. B. Newguard. (1991). Expression of normal and novel glucokinase mRNAs in anterior pituitary and islet cells. *The Journal of Biological Chemistry* **266** : 4521-4530

Hughes, S.D., Johnson, J.H., Quaade, C., and C.B. Newgard. (1992). Engineering of Glucose Stimulated Insulin Secretion and Biosynthesis in non-Islet Cells. *Proc. Natl. Acad. Sci.* **89** : 688-692

Huotari, M-A, Palgi, J. and T. Otonkoski. (1998). Growth factor mediated proliferation and differentiation of insulin producing INS1 and RINm5F cells: Identification of betacellulin as a novel β cell mitogen. *Endocrinology.* **139** : 1494-1499

Inada, S., Kaneko, S., Suzuki, K., Miyazaki, J-I., Asakura, H. and M. Fujiwara. (1996). Rectification of diabetic state in C57BL/KsJ-db/db mice by the implantation of pancreatic beta cell line Min6. *Diab Res. And Clin. Pract* **32** : 125-133.

Inagaki, N., Maekawa, T., Sudo, T., Ishii, S., Seino, Y. and H. Imura. (1992). c-Jun Represses the Human Insulin Promoter Activity that Depends on Multiple cAMP Responsive Elements. *Proc. Natl. Acad. Sci.* **89** : 1045-1049

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J., and Y. Oka. (1993). Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similat to those of normal islets. *Diabetologia* **36** : 1139-1145

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J., and Y. Oka. (1994). Overexpression of Hexokinase I but not GLUT1 Glucose Transporter Alters Concentration Dependence of Glucose-stimulated Insulin Secretion in Pancreatic β-Cell line MIN6. *Journal of Biological Chemistry.* **269** : 3081-3087

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Yazaki, Y., Miyazaki, J., Kikuchi, M., and Y. Oka. (1995). Human GLUT2 overexpression does not

affect glucose-stimulated insulin secretion in Min6 cells. *Am. J Physiol.* **269** : E897-E902.

Ishiyama, N., Kanzaki, M., Seno, M., Yamada, H., Kobayashi, I. and I. Kojima. (1998). Studies on the betacellulin receptor in pancreatic AR42J cells. *Diabetologia*. **41**: 623-628

Itkin-Ansari, P., Demeterco, C., Bossie, S., Dufayet de la Tour, D., Beattie, G. M., Movassat, J., Mally, M. I,m Hayek, A. and F. Levine. (2000). PDX-1 and cell-cell contact act in synergy to promote δ cell development in a human pancreatic endocrine precursor. *Molecular Endocrinology* **14** : 814-822

Jackerott, M., Oster, A. and L-I Larsson. (1996). PYY in Developing Murine Islet Cells : Comparisons to Development of Islet Hormones, NPY, and BrdU incorporation. *J Histochem and Cytochem.* **44** : 809-817

Jan, Y. N. and L. Y. Jan. (1998). Asymmetric cell division. *Nature*. **392** : 775-778

Jensen, J., Serup, P., Karlsen, C., Nielsen, T. F. and O. D. Madsen. (1996). MRNA profiling of rat islet tumors reveals Nkx6.1 as a β cell specific homeodomain transcription factor. *The Journal of Biological Chemistry.* **271** : 18749-18758

Jensen, J., Scott Heller, R., Funder-Neilsen, T., Pedersen, E. E., Lindsell, C., Weinmaster, G., Madsen, O. D. and P. Serup. (2000). Independent requirement for pancreatic α- and β- cells from Neurogenin3-expressing precursors. A Role for the Notch pathway in repression of premature differentiation. *Diabetes* **49**: 163-176

Jonas, J-C., Sharma, A., Hasenkamp, W., Ilkova, H., Patane, G., Laybutt, R., Bonner-Weir, S. and G. C. Weir. (1999). Chronic hypoglycemia triggers loss of pancreatic β cells differentiation in an animal model of diabetes. *The Journal of Biological Chemistry.* **274** : 14112-14121

Jonsson, J., Carlsson, L., Edlund, T. and H. Edlund. (1994). Insulin Promoter Factor 1 is Required for Pancreas Development in Mice. *Nature*. **371** : 606-609

Josefsen, K., Buschard, K., Reinholdt Sorensen, L., Wollike, M., Ekman, R. and M. Birkenbach. (1998). Glucose stimulation of pancreatic β cell lines induces expression and secretion of dynorphin. *Endocrinology* **139** : 4329-4336

Kakita, K., Giddings, S. and A. Permutt. (1982). Biosynthesis of rat insulins I and II: Evidence for differential expression of the two genes. *Proc. Natl Acad Sci.* **79**: 2803-2807

Kajimoto, Y., Watada, H., Matsuoka, T-A., Kaneto, H., Fujitani, Y. and J-I. Miyazaki. (1997). Supression of Transcription Factor PDX-1/IPF-1/STF-1/IDX-1 Causes no Decrease in Insulin mRNA in MIN6 Cells. *J Clin. Invest.* **100** : 1840-1846

Kanatsuka, A., Makino, H., Yamaguchi, T., Ohsawa, H., Tokuyama, Y., Saitoh, T., Yamamura, K-I., Miyazaki, J-I. and S. Yoshida. (1992). Islet amyloid polypeptide/amylin in pancreatic β-cell line derived from transgenic mouse insulinoma. *Diabetes* **41** : 1409-1414.

Kanno, T. (1998). Intra- and intercellular Ca2+ signalling in paraneurons and other secretory cells. *Jpn J Physiol* **48** : 219-227

Karlsson, O., Edlund, T., Moss, J. B., Rutter, W.J. and M.D. Walker. (1987). A Mutational Analysis of the Insulin Gene Control Region : Expression in Beta Cells is Dependent on Two Related Sequences Within the Enhancer. *Proc Natl. Acad. Sci.* **84** : 8819-8823

Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and T. Edlund. (1990). Insulin Gene Enhancer Binding Protein Isl-1 is a Member of a Novel Class of Proteins Containing both a Homeo- and a Cys-His- Domain. *Nature.* **344** : 879-882

Kasper, M., Hahn von Dorsche, H. and P. Stoseik. (1991). Changes in the Distribution of Intermediate Filament Proteins and Collagen IV in Fetal and Adult Human Pancreas. *Histochemistry.* **96** : 271-277.

Kawakami, Y., Yamaoka, T., Yamashita, K., Itakura, M. and Nakcuchi H. (1992). Somatic Gene Therapy for Diabetes with an Immunological Safety System for Complete Removal of Transplanted Cells. *Diabetes.* **41** : 956-960

Kayo, T., Sawada, Y., Suzuki, Y., Suda, M., Tanaka, S., Konda, Y., Miyazaki, J-I. and T. Takeuchi. (1996). Proprotein processing endoprotease furin decreases regulated secretory pathway-sepcific proteins in the pancreatic β cell line Min6. *The Journal of Biological Chemistry.* **271** : 10731-10737

Kelly, F. and Condamine, H. (1982). Tumor viruses and early mouse embryos. *Biocim, Biophys. Acta.* **651**: 105-141

Kelly, R.B. (1985). Pathways of Protein Secretion in Eukaryotes. *Science.* **230** : 25-32

Kim, K. S., Kim, Y. K. and A. L. Lee. (1990). Expression of the glucose-regulated proteins (GRP94 and GRP78) in differentiated and undifferentiated mouse embryonic cells and the use of the GRP78 promoter as an expression system in embryonic cells. *Differentiation.* **42**: 153-159.

Kim, S.K & D. A. Melton (1998). Pancreas development is promoted by cyclopamine, a hedgehog signalling inhibitor. *Proc. Natl. Acad. Sci.* **95**: 13036-13041

Kim, S. J., Kim, N. S., Ryu, C. J., Hong, H. J. and G. M. Lee. (1998). Characterisation of chimeric antibody producing CHO cells in the course of dihydrofolate reductase mediated

gene amplification and their stability in the absence of selective pressure. *Biotech Bioeng* **58**: 73-84

Kim, S. K., Hebrok, M. and D. A. Melton. (1997). Notochord to endoderm signalling is required for pancreas development. *Development.* **124** : 4243-4252

Klug, M. G., Soonpaa, M. H., Koh, G. Y. and L. J. Field. (1996). Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J. clin Invest.* **98** : 216-224

Knaack, D., Fiore, D. M., Surana, M., Leiser, M., Laurance, M., Fusco-DeMane, D., Hegre, O. D., Fleischer, N. and S. Efrat. (1994). Clonal insulinoma cell line that stably maintains correct glucose responsiveness. *Diabetes* **43** : 1413-1417

Kondo, S., Hashimoto, M., Etoh, Y., Murata, M, M, Shibai, H. and M. Muramatsu. (1989). Identification of the two types of specific receptor for activin/EDF expressed on Friend leukemia and embryonal carcinoma cells. *Biochem Biophys Res Commun* **161** : 1267-1272

Koopman, P. and R. G. H. Cotton. (1987). Pluripotent differentiation of single F9 embryonal carcinoma cells. *Exp Cell Res.* **168**: 567-571

Kosaka, M., Nishina, Y., Takeda, M., Matsumoto, K. and Y. Nishimune. (1991). Reversible effects of sodium butyrate on the differentiation of F9 embryonal carcinoma cells. *Exp. Cell Res.* **192**: 46-51

Kreymann, B. and S. R. Bloom. (1991). Glucagon and gut hormones in diabetes mellitus. *The Textbook of Diabetes, Volume I.* Eds. Pickup, J. and G. Williams. Blackwell Scientific Publications, Oxford p313

Kudoh, K., Ramanna, M., Ravatn, R., Elkahloun, A. G., Bittner, M. L., Meltzer, P. S., Trent, J. M., Dalton, W. S. and K-V Chin. (2000). Monitoring the expression profiles of doxorubicin-induced and doxorubicin-resistant cancer cells by cDNA microarray. *Cancer Research* **60** : 4161-4166

Kurki, P., Laasonen, A., Tan, E. M. and E. Lehtonen. (1989). Cell proliferation and expression of cytokeratin filaments in F9 embryonal carcinoma cells. *Development.* **106**: 635-640

Laborda, J. (2000). The role of epidermal growth factor-like protein dlk in cell differentiation. *Histol Histopathol.* **15** : 119-129

Larsson, L-I. (1998). On the Development of the Islets of Langerhans. *Microscopy Research and Technique.* **43** : 284-291

Lammert, E., Cleaver, O. and D. Melton. (2001). Induction of pancreatic differentiation by signals from blood vessels. *Science*. **294** : 564-567

Lee, C. S., Sund, N. J., Vatamaniuk, M. Z., Matsch, F. M., Stoffers, D. A. and K. H. Kaestner. (2002). Foxa2 controll PDX1 gene expression in pancreatic β cells in vivo. *Diabetes* **51** : 2546-2551

Lee, S-H., Lumelsky, N., Studer, L., Auerbach, J. M. and R. D. McKay. (2000). Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nature Biotechnology*. **18** : 675-679

Lee, J. C., Smith, S. B., Watada, H., Lin, J., Scheel, D., Wang, J., Mirmira, R. G. and M. S. German. (2001). Regulation of the pro-endocrine neurogenin3. *Diabetes*. **50** : 928-936.

Lehv, M. and P.J. Fitzgerald. (1968). Pancreatic Acinar cell Regeneration. IV. Regeneration after surgical resection. *AM. J. Pathol*. **53** : 513-535

Leibiger, B., Moede, T., Scharwz, T., Brown, G.A., Kohler, M., Leibiger I.B. and P.O. Berggren. (1998a) Short Term Regulation of Insulin Gene Transcription by Glucose. *Proc. Natl Acad. Sci*. **95** : 9307-9312

Leibiger, I. B., Leibiger, B., Moede, T., Berggren, P. O. (1998b). Exocytosis of human isulin promotes insulin gene transcription via the insulin receptor/ PI-3 kinase kinase/p70 s6 kinase and CaM kinase pathways. *Mol Cell*. **1** : 933-938

Leiter, A. B., Montminy, M. R., Jamieson, E. and R. H. Goodman. (1985). Exons of the human pancreatic polypeptide gene define functional domains of the precursor. *Journal of Biological Chemistry*. **260** : 13013-13017

Lendahl, U., Zimmerman, L. B. and R. D. McKay. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **23**: 585-595

Leon, C. and C. G. Lobe. (1997). Grg3, a murine groucho-related gene is expressed in the developing nervous system and in mysenchyme-induced epithelial structures. *Dev Dyn*. **208** : 11-24

Leonard, J., Peers, B., Johnson, T., Fefferi, K., Lee, S. and M. R. Mountminy. (1993). Characterisation of Somatostatin Transactivator 1, a Novel Homebox Factor that Stimulates Somatostatin Expression in Pancreatic Islet Cells. *Mol Endocrinol*. **7** : 1275-1283.

Lethonen, E., Laasonen, A. and Jukka Tienari. (1989). Teratocarcinoma stem cells as a model for differentiation in the mouse embryo. *Int J. Dev Biol* **33** : 105-115

Levine, R. A., Campisi, J., Wang, S. Y. and L. J. Gudas. (1984). Butyrate inhibits the retinoic acid-induced differentiation of F9 teratocarcinoma stem cells. *Dev Biol* **105** : 443-450.

Li, H., Arber, S., Jessel, T. M. and H. Edlund. (1999). Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. *Nature Genetics*. **23** : 67-70.

Liesi, P., Rechardt, L. and J. Wartiovaara. (1983). Nerve growth factor induces adrenergic neuronal differentiation in F9 teratocarcinoma cells. *Nature* **306**: 265-267.

Lin, P., Le-Niculescu, H., Hofmeister, R., McCaffery, J. M., Jin, M., Hennemann, H., McQuistan, T., De Vries, L. and M. G. Farquhar. (1998). The mammalian calcium binding protein nucleobindin (CALNUC), is a Glogi resident protein. **29** : 1515-1527

Linder, S., Krondahl, U., Sennerstam, R., and N. R. Gingertz. (1981). Retinoic acid induced differentiation of F9 embryonal carcinoma cells. *Exp Cell Res* **132** : 453-460

Linzel, M., Ciccarelli, A., and R.C. Merrell. (1988). Beta Cell Contact and Insulin Release. *Biochemical and Biophysical Research Communications.* **153** : 999-1005

Logsdon, C.D., Moessner, J., Williams, J. A. and I. D. Goldfine. (1985). Glucocortocoid incresae amylase mRNA level, secretory organelles, and secretion in pancreatic acinar AR42J cells. *J. Cell Biology*. **100** : 1200-1208.

Lu, D., Tamemoto, H., Saito, I. And T. Takeuchi. (1998). Regulatable Production of Insulin from Pirmary-Cultures Hepatocytes: Insulin Production is Up-regulated by Glucagon and cAMP and Down Regulated by Insulin. *Gene Therapy*. **5** : 888-895.

Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R and R. McKay. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science*. **292**: 1389-94

Luzi l. and G. Pozza. (1997). Glibenclamide: An Old Drug with a Novel Mechanism of Action ?. *Acta Diabetol.* **34** : 239-244

Macfarlane, W. M., Smith, S. B., James, R. F. L., Clifton, A. D., Doza, Y. N., Cohen, P. and K. Docherty. (1997). The p38/Reactivating protein kinase mitogen activated protein kinase cascade mediates the activation of the transcription factor insulin upstream factor 1 and insulin gene transcription by high glucose in pancreatic β cells. *The Journal of Biological Chemistry*. **272** : 20936-20944

Macfarlane, W.M., McKinnon, C.M., Felton-Edkins, Z.A., Cragg, H., James, R.F.L., and K.Docherty. (1999). Glucose Stimulates Translocation of the Homoedomain Transcription Factor PDX1 from the Ctyoplasam to the Nucleus in Pancreatic β-Cells. *J Biol. Chem*. **274** : 1011-1016

Madsen, O. D., Jensen, J., Blume, N., Petersen, H. V., Lund, K., Karlsen, C., Andersen, F. G., Jensen, P. B., Larsson, L-I. and P. Serup. (1996). Pancreatic development and maturation of the islet β cell. Studies of pluripotent islet cultures. *Eur. J. Biochem.* **242**: 435-445.

Madsen, O. D., Jensen, J., Petersen, H. V., Pedersen, E. E., Oster, A., Andersen, F. G., Jorgensen, M. C., Jensen, P. B., Larsson, L. I. and P. Serup. (1997). Transcription factors contributing to the beta-cell phenotype. *Hormone Metabolic Research.* **29** : 265-270

Mallo, M., del Amo, F. and T. Gridley. (1993). Cloning and developmental expression of Grg, a mouse gene related to the groucho transcrit of the drosophila enhancer of split complex. *Mech Dev.* **42** : 67-76

Manglesdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S. and R. M. Evans. (1991). A direct repeat in the cellular retinol binding protein type II gene confers differential regulation by RXR and RAR. *Cell.* **66** : 555-561

Marks, P. A., Richton, V. M. and R. A. Rifkind. (2000). Histone deacetylase inhibitors: Inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst* **92** : 1210-1216

Marshall, M. O., Thomas, H. M., Seatter, M. J., Greer, K. R., Wood, P. J. and G. W. Gould. (1993). Pancreatic β cells express a low affinity glucose transporter: functional consequences in normal and diabetic states. **21** : 164-168

Martin, C. A., Ziegler, L. M. and J. L. Napoli. (1990). Retinoic Aci, di-butyryl-cAMP, and differentiation affect the expression of retinoic acid receptors in F9 cells. (1990). *Proc Natl Acad Sci.* **87** : 4804-4808

Martin, G. R. and M. J. Evans. (1975). Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies *in vitro*. *Proc. Natl. Acad. Sci.* **72** : 1441-1445

Mashima, H., Ohnishi, H., Wakabayashi, K., Mine, T., Miyagawa, J., Hanahusa, T., Seno, M., Yamada, H., and I. Kojima. (1996a). Betacellulin and Activin A Co-ordinately Convert Amylase Secreting AR42J Cells into Insulin-Secreting Cells. *J Clin Invest.* **97** : 1647-1654

Mashima, H., Shibata, H., Mine, T., and I. Kojima. (1996b). Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. *Endocrinology.* **137** : 3969-3976

Mashima, H., Yamada, S., Tajima, T., Seno, M., Yamada, H., Takeda, J. and I. Kojima. (1999). Genes expressed during the differentiation of pancreatic AR42J cells into insulin secreting cell lines. *Diabetes.* **48** : 304-309

McBride, S., Walsh, D., Meleady, P., Daly, N. and M. Clynes. (1999). Bromodeoxyuridine induces keratin protein synthesis at a post transcriptional level in human lung tumor cell lines. *Differentiation* **64** : 185-193

McCarthy, S. A. and R. Bicknell. (1994). Activin A binds to a heteromeric receptor complex on the vascular endothelial cell surface. *The Journal of Biological Chemistry.* **269** : 3909-3912

McKay, R. (1997). Stem cells in the central nervous system. *Science.* **276**: 66-71

McKinnon, C. M. and K. Docherty. (2001). Pancreatic duodenal homeobox-1, PDX-1, a major regulator of beta cell identity and function. *Diabetologia* **44** : 1203-1214.

Means, A. L., Thompson, J. R. and L. J. Gudas. (2000). Transcriptional regulation of the cellular retinoic acid binding protein I gene in F9 teratocarcinoma cells. *Cell Growth and Diff.* **11**: 71-82

Meda, P., Chanson, M., Pepper, M., Giordano, E., Bosco, D., Traub, O., Willecke, K., el Aoumari, A., Gros, D., Beyer, E. C. *et al.,* (1991). In vivo modulation of connexin43 gene expression and junctional coupling of pancreatic β cells. *Experimental Cell Research.* **192** : 469-480

Mering, J. Von and O. Minkowski. (1890). Diabetes Mellitus, nach pankreasextirpation. *Arch Exp Pathol Pharmacol* **26** : 371-387

Merten, O. W., Kallel, H., Manuguerra, J. C., Tardy-Panit, M., Crainic, R., Delpeyroux, F., Van der Werf, S. and P. Perrin. (1999). The new medium MDSS2N, free from any animal protein supports cell growth and production of various viruses. *Cytotechnology* **30** : 191-201

Michalopoulos, G. K. and M. C. DeFrances. (1997). Liver Regeneration. *Science.* **276** : 60-66

Miki, K.(1999). Volume of liquid below the epithelium of an F9 cell as a signal for differentiation into visceral endoderm. *J. Cell Sci.***112**: 3071-3080.

Miller, C. P., McGehee, R. E. and J. F. Habener. (1994). IDX1: A New Homeodomain Transcription Factor Expressed in Rat Pancreatic Islets and Duodenum that Transactivates the Somatostatin Gene. *EMBO J.* **13** : 1145-1156

Minami, K., Yano, H., Miki, T., Nagashima, K., Wang, C-Z., Tanaka, H., Miyazaki, J-I. and S. Seino. (2000). Insulin secretion and differential gene expression in glucose-responsive and –unresponsive Min6 sublines. *Am J Physiol Endocrinol Metab* **279** : E773-E781

Miralles, F., Czernichow P. and R. Scharfmann. (1998). Follistatin regulates the relative proportions of endocrine versus exocrine tissue during pancreatic development. *Development.* **125** : 1017-1024

Miralles, F., Serup, P., Cluzeaud, F., Vandewalle, A., Czernichow, P. and R. Scharfmann. (1999). Characterisation of β cells developed in vitro from rat embryonic pancreatic epithelium. *Developmental Dynamics.* **214** : 116-126

Mitanchez, D., Doiron, B., Chen, Ruihuan, C., and A., Kahn. (1997). Glucose-Stimulated Genes and Prospects of Gene Therapy for Type I Diabetes. *Endocrine Reviews.* **18** : 520-540

Mitanchez, D., Chen, R., Massias, J-F., Porteau, A., Mignon, A., Bertagna, X. and A. Kahn. (1998). Regulated expression of mature human insulin in the liver of transgenic mice. *FEBS Letters* **421** : 285-289

Miyagawa, J., Hanafusa, O., Sasada, R., Yamamoto, K., Igarashi, K., Yamamori, K., Seno, M., Tada, H., Nammo, T., Li, M., Yamagata, K., Nakajima, H., Namba, M., Kawajima, M. and Y. Matsuzawa. (1999). Immunohistochemical localization of betacellulin, a new member of the EGF family, in normal human pancreas and islet tumor cells. *Endocr. J.* **46** : 755-764

Miyashita, T., Yamamoto, H., Nishimune, Y., Nozaki, M., Morita, T. and A. Matsushiro. (1994). Activation of the mouse cytokeratin A (endo A) gene in teratocarcinoma F9 cells by the histone deacetylase inhibitor Trichostatin A. *FEBS Letters* **353** : 225-229

Miyazaki, J-I., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y. and K-I Yamamura. (1990). Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* **127** : 126-132

Moitoso de Vargas, L., Sobolwski J., Siegel, R., and L. G. Moss. (1997). Individual β Cells within the Intact Islet Differentially Respond to Glucose. *Journal of Biological Chemistry.* **272** : 26573-26577

Moll, R., Frenke, W. W. and D. L. Schiller. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31** : 11-24

Momma, S., Johansson, C. B. and J. Frisen. (2000). Get to know your stem cells. *Curr Opin Neuro* **10** : 45-49

Monaghan, A. P., Kaestner, K. H., Grau, E. and G. Schutz. (1993). Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 α, β and γ genes in determination of the difmitive endoderm, chordamesoderm and neuroectoderm. *Development.* **119** : 567-578

Montminy, M., Brindle, P., Arias, J., Ferreri, K. and R. Armstrong. (1996). Regulation of somatostatin gene transcription by cyclic adenosine monophosphate. *Metabolism* **45** : Suppl 1 4-7

Moore, H.P., Walker, M.D., Lee, F. and R.B. Kelly. (1983). Expressing a Mouse Proinsulin cDNA in a Mouse ACTH-Secreting Cell. Intracellular Storage, Proteolytic Processing and Secretion on Stimulation. *Cell.* **35** : 531-538

Moore, E. E., Mitra, N. S. and E. A. Moritz. (1986). Differentiation of F9 embryonal carcinoma cells. *Differentiation* **31** : 183-190

Moran, A., Zhang, H-J., Olson, L.K., Harmon, J.S., Poitout, V. and R.P. Robertson. (1997). Differentiation of Glucose Toxicity from Beta-Cell Exhaustion During the Evolution of Defective Insulin Gene Expression in the Pancreatic Islet Cell Line, HIT-T15. *J. Clin. Invest.* **99** : 534-539

Morin-Kensicki, E. M., Faust, C., LaMantia, C. and T. Magnuson. (2001). Cell and tissue requirements for the gene eed during mouse gastrulation and organogenesis. *Genesis* **31** : 142-146

Morrison, S. J., Shah, N. M. and D. J. Anderson. (1997). Regulatory mechanisms in stem cell biology. *Cell.* **88** : 287-298

Morrison, S. J., White, P. M., Zock, C. and D. J. Anderson. (1999). Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. *Cell.* **96** : 737-749

Motoyoshi, S., Shirotani, T., Araki, E., Sakai, K., Kaneko, H., Motoshima, H., Yoshizato, A., Shirakami, A., Kishikawa, H. and M. Shichiri. (1998). Cellular characterization of pituitary adenoma cell line (AtT20 cell) transfected with insulin, glucose transporter type 2 (GLUT2) and glucokinase genes: Insulin secretion in response to physiological concentrations of glucose. *Diabetologia.* **41** : 1492-1501

Mueckler, M., Kruse, M., Strube, M., Riggs, A. C., Chiu, K. C. and M. A. Permutt. (1994). A mutation in the Glut2 glucose transporter gene of a diabetic patient abolishes transport activity. *The Jounal of Biological Chemistry.* **269** : 17765-17767

Muller, R. and E. F. Wagner. (1984). Differentiation of F9 teratocarcinoma stem cells after transfer of c-fos proto-oncogenes *Nature* **311** : 438-442

Mummery, C. L., Feyen, A., Freund, E. and S. Shen. (1990). Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. *Cell Differentiation and Development.* **30** : 195-206

Murtomaki, S., Virtanen, I. and Liesi, P. (1999). Neurofilament proteins are constitutively expressed in F9 teratocarcinoma cells. *Int. J. Dev. Neurosci.* **17**: 829-838

Naya, F. J., Huang, H-P., Qui, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B., and Tsai, M-J. (1997) Diabetes, Defective Pancreatic Morphogenesis, and Abnormal Enteroendocrine Differentiation in BETA2/NeuroD-deficient Mice. *Genes and Development.* **11** : 2323-2334.

Naya, F.J., Stellrecht, C.M.M. and M-J. Tsai. (1995). Tissue Specific Regulation of the Insulin Gene by a Novel Basic Helix-Loop-Helix Transcription Factor. *Genes and Development.* **9** : 1009:1019

Newguard, C. B., Ckark, S., BeltrandelRio, H., Hohmeier, H. E., Quaade, C. and K. Normington. (1997). Engineered cell lines for insulin replacement in diabetes: current status and future prospects. *Diabetologia* **40** : S42-S47

Nielsen, D.A., Welsh M., Casadaban, M.J. and D.F. Steiner (1985). Control of Insulin Gene Expression in Pancreatic β-Cells and in an Insulin Producing Cell Line, RIN-5F Cells. *J Biol. Chem.* **260** : 13585-13589

Nir, U., Walker M.D. and W.J. Rutter. (1986). Regulation of Rat Insulin I Gene Expression : Evidence for Negative Regulation in Non-Pancreatic Cells. *Proc. Natl Acad. Sci.* **83** : 3180-3184.

Nishikura, K., Kim, U. and J. M. Murray. (1990). Differentiation of F9 cells is independent of c-myc expression. *Oncogene* **5**: 981-988

Nishimune, Y., Kume, A., Ogiso, Y. and A. Matsushiro. (1983). Induction of teratocarcinoma cell differentiation. Effect of inhibitors of DNA synthesis. *Exp Cell Res* **146** : 439-444

Nowling, T., Desler, M., Kuszynski, C. and A. Rizzino. (2002). Transfection of embryonal carcinoma cells at high efficiency using liposome mediated transfection. *Mol Reprod Dev* **63** : 309-317

O' Driscoll, L., Daly, C., Saleh, M. and M. Clynes. (1993). The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate gene expression in multidrug-resistant cells. *Cytotechnology* **12** : 289-314

O' Driscoll, L., Gammell, P., and M. Clynes. (2002). Engineering Vero cells to secrete human insulin. *In Vitro Cell Dev Biol.* **38** : 146-153

Ohgawara, H., Miyazaki, J-I., Karibe, S., Tashiro, F., Akaike, T. and Y. Hashimoto. (1995). Embedded culture of pancreatic β cells derived from transgenic mouse insulinoma as a potential source for xenotransplantation using a diffusion chamber. *Cell Transplantation.* **4** : 307-313

Ohlsson, H., Thor, S. and T. Edlund. (1991). Novel Insulin Promoter- and Enhancer-binding Proteins that Discriminate Between Pancreatic α and β Cells. *Molecular Endocrinology*. **5** : 897-904

Ohlsson, H., Karlsson, K. and T. Edlund. (1993). IPF1, a Homeodomain-Containing Transactivator of the Insulin Gene. *The EMBO Journal*. **12** : 4251-4259.

Ohneda, K., Mirmira, R. G., Wang, J., Johnson, J. D. and M. S. German. (2000). The homeodomain of PDX-1 mediates multiple protein-protein interactions in the formation of a transcriptional activation complex on the insulin promoter. *Molecular and Cellular Biology*. **20** : 900-911

Ohnishi, H., Ohgushi, N., Tanaka, S., Mogami, H., Nobusawa, R., Mashima, H., Furukawa, M., Mine, T., Shimada, O., Ishikawa, H. and I. Kojima. (1995). Conversion of amylase-secreting rat pancreatic AR42J cells to neuronlike cells by activin A. *J. Clin Invest.* **95** : 2304-2314.

Okabe, S., Forsberg-Nilsson, K., Cyril Spiro, A., Segal, M. and R. D. G. McKay. (1996). Development of neuronal precursor cells and functional post mitotic neurons from embryonic stem cells in vitro. *Mechanisms of Development* **59**: 89-102.

Olson, L.K., Sharma, A., Peshavaria, M., Wright, C.V.E., Towle, H.C., Robertson, R.P. and R. Stein. (1995) Reduction of Insulin Gene Transcription in HIT-T15 β-Cells Chronically Exposed to Supraphysiologic Glucose Concentration is Associated with Loss of STF-1 Transcription Factor Expression. *Proc. Natl. Acad. Sci.* **92** : 9127-9131

Øster A., Jensen J., Serup, , P., Galante, P., Madsen, O.D. and L-I Larsson. (1998b). Rat Endocrine Pancreatic Development in Relation to Two Homebox Gene Products (PDX1 and Nkx6.1). *J. Histochem Cytochem.* **46** : 707-715.

Otonkoski, T., Beattie, G. M., Mally, M. I., Ricordi, C. and A. Hayek. (1993). Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J. Clin Invest.* **92** : 1459-1466

Otonkoski, T., Beattie, G. M., Rubin, J. S., Lopez, A. D., Baird, A., Hayek, A. (1994). Hepatocyte growth factor/scatter factor has insulintropic activity in human fetal pancreatic cells. *Diabetes* **43** : 947-953

Otonkoski, T., Cirulli, V., Beattie, M., Mally, M. I., Soto, G., Rubin, J. S. and A. Hayek. (1996). A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. *Endocrinology*. **137 :** 3131-3139

Otonkoski, T., Ustinov, J., Rasilainen, S., Kallio, E., Korsgren, O. and P. Hayry. (1999). Differentiaion and maturation of porcine islet cells in vitro after transplantation. *Transplantation* **68**: 1674-1683

Palgi, J., Stumpf, E. and T. Otonkoski. (2000). Transcription factor expression and hormone production in pancreatic AR42J cells. *Molecular and Cellular Endocrinology.* **165** : 41-49

Pang, K., Mukonoweshuro, C. and G. G. Wong. (1994). Beta Cells arise from glucose transporter type2 (Glut2)-expressing epithelial cells of the developing rat pancreas. *Proc. Natl. Acad. Sci.* **91** : 9559-9563

Paulin, D., Jakob, H., Jacob, F., Weber, K. and M. Osborn. (1982). In vitro differentiation of mouse teratocarcinoma cells monitored by intermediate filament expression. *Differentiation.* **22**: 90-99

Pearson, K. W., Scott, D. and B. Torrance. (1977). Effects of partial surgical pancreatectomy in rats. I. Pancreatic Regeneration. *Gastroenterology.* **72** : 469-473

Pedersen, A. A., Petersen, H. V., Videbaek, N., Skak, K. and B. K. Michelsen, (2002). PDX-1 mediates glucose responsiveness of GAD(67), but not GAD(65), gene transcription in islets of Langerhans. *Biochem Biophys Res Commun.* **295** : 243-248

Permutt, M. A., Koranyi, L., Keller, K., Lacy, P. E., Scharp, D. W. and M. Mueckler. (1989). Cloning and functional expression of a human pancreatic islet glucose-transporter cDNA. *Proc Natl Acad Sci* **86** : 8688-8692

Peshavaria, M., Cissell, M. A., Henderson, E., Petersen, H. V. and R. Stein. (2000). The PDX1 actication domain provides specific functions necessary for transcriptional stimulation in pancreatic β cells. *Molecular Endocrinology.* **14** : 1907-1917

Peshavaria, M., Henderson, E., Sharma, A., Wright, C.V. E. and R. Stein. (1997). Functional characterisation of the transactivation properties of the PDX-1 homeodomain protein. *Molecular and Cellular Biology.* **17** : 3987-3996

Petersen, B. E., Bowen, W. C., Patrene, K D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. and J. P. Goff. (1999). Bone marrow as a potential source of hepatic oval cells. *Science.* **284** : 1168-1170

Petersen H. V., Peshavaria, M., Pedersen A. A., Philippe, J., Stein, R., Madsen, O. D. and P. Serup. (1998). Glucose stimulates the activation domain potential of the PDX-1 homeodomain transcription factor. *FEBS Letters* **431** : 362-366

Philippe J., Drucker, D. J., Chick, W. and J. F. Habener. (1987). Transcriptional regulation of genes encoding insulin, glucagon and angiotensinogen by sodium butyrate in a rat islet cell line. *Molecular and Cellular Biology* **7** : 560-563

Philippe, J. and M. Missotten. (1990). Functional Characterisation of a cAMP-Responsive Element of the Rat Insulin I Gene. *J. Biol. Chem.* **265** : 1465-1469

Philippe, J. (1991). Structure and Pancreatic Expression of the Insulin and Glucagon Genes. *Endocrinol. Rev.* **12** : 252-271

Poitout, V., Olson, L. K. and R. P. Robertson. (1996). Insulin-secreting cell lines: classification, characteristics and potential applications. *Diabetes and Metabolism* **22** : 7-14

Polak, M., Bouchareb-Banaei, L., Scharfmann, R. and P. Czernichow. (2000). Early pattern of differentiation in the human pancreas. *Diabetes* **49** : 225-232

Powers, A. C., Philippe, J., Hermann, H. and J. F. Habener. (1988). Sodium butyrate increases glucagon and insulin gene expression by recruiting immunocytochemically negative cells to produce hormone *Diabetes* **37** : 1405-1410

Ramaekers, F., Schaap, H., Mulder, M., Huysmans, A. and P. Vooijs. (1984). Cytokeratin filament expression during in vitro teratocarcinoma cell differentiation as detected by a monoclonal antibody. *Cell Biology Int. Reports.***8**: 721-730

Ramirez, L. Z. and P. Raskin. (1991). Insulin Biosynthesis and Secretion. *The Textbook of Diabetes, Volume I.* Eds. Pickup, J. and G. Williams. Blackwell Scientific Publications, Oxford p198

Ramiya, V. K., Maraist, M., Arfors, K. E., Schatz, D. A., Peck, A. B. and J. G. Cornelius. (2000). Reversal of Insulin-Dependent Diabetes Using Islets Generated from Pancreatic Stem Cells. *Nature Medicine.* **6** : 278-282

Redmon, J.B., Towle, H.C. and Robertson, R.P. (1994). Regulation of the Human Insulin Gene by Glucose, Epinephrine and Somatostatin. *Diabetes.* **43** : 546-551

Riese, D.J., Bermingham, Y., van Raaj, T.M., Buckley, S., Plowman, G.D. and D.F. Stern. (1996). Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta *Oncogene*, **12** : 345-353

Ritz-Laser, B., Estreicher, A., Gauthier, B. R., Mamin, A., Edlund, H. and J. Philippe. (2002). The pancreatic beta-cell-specific transcription factor Pax-4 inhibits glucagon gene expression through Pax-6. *Diabetologia* **45** : 97-107

Roche, E., Assimacopoulos-Jeannet, F., Witters, L. A., Perruchoud, B., Yaney, G., Corkey, B., Asfari, M. and M. Prentki. (1997). Induction by glucose of genes coding for glycolytic enzymes in a pancreatic β cell line. *The Journal of Biological Chemistry.* **272** : 3091-3098

Roche, E., Farfari, S., Witters, L. A., Assimacopoulos-Jeannet, F., Thumelin, S., Brun, T., Corkey, B. E., Saha, A. K. and M. Prentki. (1998). Long term exposure of β Ins cells to

high glucose concentrations increases anaplerosis, lipogenesis, and lipogenic gene expression. *Diabetes* **47** : 1086-1094

Roesler, W. J., Vandenbark, G. R. and R. W. Hanson. (1988). Cyclis AMP and the induction of eukaryotic gene transcription. *The Journal of Biological Chemistry.* **263** : 9063-9066

Rosenberg, L. (1995). In vivo cell transformation: neogenesis of beta cells from pancreatic ductal cells. *Cell Transplantation*. **4** : 371-383.

Ross, S. A., Ahrens, R. A. and De Luca, L. M. (1994). Retinoic acid enhances adhesiveness, laminin and integrin β1 synthesis, and retinoic acid receptor expression in F9 teratocarcinoma cells. *J. Cell. Physiol.*. **159**: 263-273.

Sakuma, N. Ishikawa, S-E., Okada, K., Miyazaki, J-I. and T. Saito. (1995). Glucose Induces Calcium Dependent and Calcium Independent Insulin Secretion from the Pancreatic Beta Cell Line MIN6. Eur. J. Endocrinol. **133** : 227-234

Samaras, S. E., Cissell, M. A., Gerrish, K., Wright, C. V., Gannon, M. and R. Stein. (2002). Conserved sequences in a tissue specific regulatory region of the pdx-1 gene mediate transcription in Pancreatic beat cells: role for hepatocyte nuclear factor 3 beta and Pax6. *Mol Cell Biol* **22** : 4702-4713

Sander, M., Neubüser, A., Kalamaras, J., Le, H.C., Martin, G. R., and M.S. German. (1997). Genetic Analysis Reveals that Pax6 is Required for Normal Transcription of Pancreatic hormone Genes and Islet Development. *Genes and Development.* **11** : 1662-1673.

Sander, M., Griffen, S.C., Huang, J. and M.S. German. (1998). A Novel Glucose-Responsive Element in the Human Insulin Gene Functions Uniquely in Primary Cultured Islets. *Proc. Natl. Acad. Sci.* **95** : 11572-11577

Santos-Rosa, H., Schneider, R., Bannister, A. J., Sheriff, J., Bernstein, B. E., Tolga Emres, N. C., Schreiber, S. L., Mellor, J. and T. Kouzarides. (2002). Active genes are tri-methylated at K4 of histone H3. *Nature* **419** : 407-411.

Sarac, M. S., Windcatt, S., Castro, M. G. and I. Lindberg. (2002). Intrapituitary adenoviral administration of 7B2 can extend life span and reverse endocrinological deficiencies in 7B2 null mice. *Endocrinology* **143**: 2314-2323

Sartorelli, V. and P. L Puri. (2001) The link between chromatin structure, protein acetylation and cellular differentiation. *Fronteirs in Bioscience* **6** : 1024-1047

Sato, Y., Nenquin, M. and J-C Henquin. (1998). Relative contribution of Ca^{2+}-dependent and Ca^{2+}-independent mechanisms to the regulation of insulin secretion by glucose. *FEBS Letters*. **421** : 115-119

Satijn, D. P., Hamer, K. M., den Blaauwen, J. and A. P. Otte. (2001). The po;ycomb group protein EED interacts with YY1, and both proteins induce neural tissue in Xenopus embryos. *Mol Cell Biol* **21** : 1360-1369

Scarpelli, D. G., and M. S. Rao. (1981). Differentiation of regenerating pancreatic cells into hepatocyte-like cells. *Proc. Natl Acad Sci.* **78** : 2577-2581

Scearce, L. M., Brestelli, J. E., McWeeney, S. K., Lee, C. S., Mazzarelli, J., Pinney, D. F., Pizzaro, A., Stoeckert, C. J., Clifton, S. W., Permutt, M. A., Brown, J., Melton, D. A. and K. H. Kaestener. (2002). Functional genomics of the endocrine pancreas. The pancreas clone set and PancChip, new resources for diabetes research. *Diabetes* **51** : 1997-2004

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A. and N. Benvenisty. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. Natl Acad. Sci.* **97** : 11307-11312

Schwartz, P. T., Perez-Villamil, B., Rivera, A., Moratella, R. and M. Vallejo. (2000). Pancreatic homeodomain transcription factor IDX1/IPF1 expressed in developing brain regulates somatostatin gene transcription in embryonic neural cells. *The Journal of Biological Chemistry* **275** : 19106-19114

Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J., Lee, J. E., Anderson, D. J., Sussel, L., Johnson, J. D. and M. S. German. (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* **127** : 3533-3542.

Seijffers, R., Ben-Davis, O., Cohen, Y., Karasik, A., Berezin, M., Newguard, C. B. and Sarah Ferber. (1999). Increase in PDX1 levels supresses insulin gene expression in RIN-1046-38 cells. *Endocrinology* **140** : 3311-3317.

Selden, R.F., Skoskiewicz, M.J., Russel, P.S. and H.M. Goodman. (1987). Regulation of Insulin Gene Expression. Implications for Gene Therapy. *New Engl. J. Med.* **317** : 1067-1076

Selle-Beinier, V., Le Gurun, S., Belluardo, N., Trovato-Salinaro, A., Charollais, A., Haefliger, J-A., Condorelli, D. F. and P. Meda. (2000). Cx36 Preferentially Connects β-Cells Within Pancreatic Islets. *Diabetes* **49** : 727-734

Serup, P., Petersen, H. V., Pedersen, E. E., Edlund, H., Leonard, J,. Petersen, J. S., Larssons, L-I. and O. D Madsen. (1995). The homeodomain protein IPF-1/STF-1 is expressed in a subset of islet cells and promotes insulin 1 gene expression dependent on intact E1 helix-loop-helix factor binding site. *Biochem J.* **310** : 997-1003

Serup, P., Jensen, J., Andersen, F. G., Jorgensen, M. C., Blume, N., Holst, J. J. and O. D. Madsen. (1996). Induction of Insulin and Islet Amyloid Polypeptide Production in

Pancreatic Islet Glucagonoma Cells by Insulin Promoter Factor 1. *Proc. Natl. Acad. Sci.* **93** : 9015-9020

Shah, N. M., Groves, A. K. and D. J. Anderson. (1996). Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. *Cell.* **85** : 331-343

Shamblott, M. J., Axelman, J., Littlefield, J. W., Blumenthal, P. D., Huggins, G. R., Cui, Y., Cheng, L. and J. D. Gearhart. (2001). Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. *Proc Natl Acad Sci* **98** : 113-118

Shantz, L. M. and A. E. Pegg (1999). Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. *Int J Biochen & Cell Biol* **31** : 107-122

Sharma, S., Jhala, U. S., Johnson, T., Ferreri, K., Leonard, J. and M. Montminy. (1997). Hormonal regulation of an islet specific enhancer in the pancreatic homeobox gene STF-1. *Mol Cell Biol* **17** : 2598-2604

Shima, K., Zhu, M. and M. Kuwajima. (1998). A role of nicotinamide-induced increase in pancreatic β cell mass on blood glucose control after discontinuation of the treatment in partially pancreatectomized OLTEF rats. *Diabetes Research and Clinical Practice.* **41** : 1-8

Shimada, N., Yamada, K., Tanaka, T., Kawata, H,. Mizutani, T., Miyamoto, K. and T. Matsuzawa. (2001). Alterations in gene expression in endoderm differentiation of F9 teratocarcinoma cells. *Mol Reprod Dev.* **60** : 165-171

Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K. and J. Folkman. (1993). Betacellulin: a mitogen from pancreatic beta cell tumors. *Science.* **259** : 1604-1607

Shiroi, A., Yoshikawa, M., Yokata, H., Fukui, H., Ishizaka, S., Tatsumi, K. and Y. Takahashi. (2002). Identification of insulin producing cells derived from embryonic stem cells by zinc chelating dithizone. *Stem Cells.* **20** : 284-292

Simpson, A.M., Tuch, B.E., Swan, M.A., Tu, J. and G.M. Marshall. (1995). Functional Expression of the Human Insulin Gene in a Human Hepatoma Cell Line (HEP G2). *Gene Therapy.* **2** : 223-231

Simpson, A.M., Marshall, G.M., Tuch, B.E., Maxwell, L., Szymanska, B., Tu, J., Beynon, S., Swan, M.A. and M. Camacho. (1997). Gene Therapy of Diabetes : Glucose Stimulated Insulin Secretion in a Human Hepatoma Cell Line. *Gene Therapy.* **4** : 1202-1215

Sjoholm, A. Welsh, N., Sandler, S. and C. Hellerstrom. (1990). Role of polyamines in mitogenic and secretory responses of pancreatic beta-cells to growth factors. *Amer J. Physiol* **259** : C828-C833

Sjoholm, A., Korsgren, O. and A. Andersen. (1994). Polyamine requirements in nicotinamide stimulated β cell differentiation in fetal porcine islet like cell clusters. *Endocrinology*. **135** : 1559-1565

Skelly, R. H., Schuppin, G., Ishihara, H., Oka, Y. and C. J. Rhodes. (1996). Glucose regulated translational control of proinsulin biosynthesis with that of the proinsulin endopeptidases PC2 and PC3 in the insulin producing Min6 cell line. *Diabetes*. **45** : 37-43

Slack, J.M.W. (1995). Developmental Biology of the Pancreas. *Development.* **121** : 1569-1580.

Smeekens, S.P., Avruch, A. S., LaMendola, J., Chan, S. J. and D. F. Steiner. (1991). Identification of a cDNA encoding a second putative prohormone convertase related to PC2 and AtT20 cells and islets of Langerhans.*Proc. Natl. Acad. Sci.* **88** : 340-344

Smith, S. B., EE, H. C., Conners, J. C. and M. S. German. (1999). Paired homeodomain transcription factor PAX4 acts as a transcriptional represor in early pancreatic development. *Mol Cell Biol* **19** : 8272-8280

Soria, B., Roche, E., Berna, G., Leon-Quinto, T., Reig, J. A. and Franz Martin. (2000a). Insulin secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes*. **49** : 157-162

Soria, B., Andreu, E., Berna, G., Fuentes, E., Gil, A., Leon-Quinto, T., Martin, F., Montanya, E., Nadal, A., Reig, J. A., Ripoll, C., Roche, E., Sanches-Andres, J. V. and J. Segura. (2000b).Engineering Pancreatic Islets. *Eur J Physiol* **440** : 1-18

Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G. and P. Gruss. (1997). The Pax4 gene is essential for differentiation of insulin-producing β cells in the mammalian pancreas. *Nature*. **386**: 399-402

Stephens, L. E., Sonne, J. E., Fitzgerald, M. L. and C. H. Damsky. (1993). Targeted deletion of β1 integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol.* **123**: 1607-1620

Stewart C, Taylor, N. A., Green, I. C., Docherty, K., and C. J., Bailey. (1994). Insulin-releasing Pituitary Cells as a Model for Somatic Cell Gene Therapy in Diabetes Mellitus. *Journal of Endocrinology.* **142** : 339-343.

Stoffers, D. A., Zinkin, N. T., Stanojevic., V., Clarke W. L. and J. F Habener. (1997). Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nature Genetics*. **15** : 106-110.

St-Onge, L., Sosa-Pineda, B., Chowdbury, K., Torres, M., Mansouri, A., and P. Gruss. (1997). Pax 6 is Required for Differentiation of Glucagon-Producing α Cells in Mouse Pancreas. *Nature*. **387** : 406-409

Strickland, S., smith, K. K. and K. R. Marotti. (1980). Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* **21** : 347-355

Sussel, L., Kalamaras, J., Hartigan-O' Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L. R. and M. S. German. Mice lacking the homodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic β cells. (1998). *Development*. **125**: 2213-2221.

Swift, G.H., Liu, Y., Rose, S. D., Bischof, L. J., Steelman, S., Buchberg, A. M., Wright, C. V. E. and R. J. MacDonald. (1998). An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through formation of a trimeric complex with PBX1b and MRG1 (MEIS2) *Mol Cell Biol* **18** : 5109-5120

Takeda, M., Kosaka, M., Nishina, Y., Sawada, K., Matsumoto, K. and Y. Nishimune. (1992). Teratocarcinoma F9 cells induced to differentiate with sodium butyrate produce bith tissue-type and urokinase-type plasminogen activators. *J. Cell Biochem* **49** : 284-289

Tanaka, T., Satoh, T., Onozawa, Y., Kohroki, J., Itoh, N., Ishidate M., Muto, N. and K. Tanaka. (1999). Apoptosis during iron chelator-induced differentiation in F9 embryonal carcinoma cells. *Cell Biol Int* **23** : 541-550

Taniguchi, H., Fukao, K. and Nakcuchi H. (1997). Constant Delivery of Proinsulin by Encapsulation of Transfected Cells. *J. Surgical. Res*. **70** : 41-45

Teitelman, G., Alpert, S., Polak, J. M., Martinez, A. and D. Hanahan. (1993). Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. *Development*. **118** : 1031-1039.

Thomas, M. K., Rastalsky, N. Lee, J. H. and J. F. Habener. (2000). Hedgehog signalling regulation of insulin production by pancreatic β cells. *Diabetes*. **49** : 2039-2047

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. and J. M. Jones. (1998). Embryonic stem cells deriverd from human blastocysts. *Nature*. **282**: 1145-1147.

Tiedge, M., Hohne, M. and S. Lenzen. (1993). Insulin secretion, insulin content and glucose phosphorylation in RINm5F insulinoma cells after transfection with human GLUT2 glucose-transporter cDNA. *Biochem J.* **296** : 113-118

Tisdale, E. J. and W. E. Balch (1996). Rab2 is essential for the maturation of pre-Golgi intermediates. *J Biol Chem* **15** : 29372-29379

Tsao, M-S. and W. P. Duguid. (1987). Establishment of propagable eipthelial cell lines from normal adult rat pancreas. *Experimental Cell Research.* **168** : 365-375

Ullrich, S., Abel, K-B., Lehr, S. and R. Greger. (1996). Effects of Glucose, Forskolin and Tolbutamide on Membrane Potential and Insulin Secretion in the Insulin-Secreting Cell Line INS-1. *European Journal of Physiology.* **432** : 630-636

Upchurch, B. R., Aponte, G. W. and A. B. Leiter. (1994). Expression of Peptide YY in all Four Islet Cell Types in the Developing Mouse Pancreas Suggests a Common Peptide YY-producing Progenitor. *Development.* 120 : 245-252

Valera, A., Solanes, G., Fernandez-Alvarez, J., Pujol, A., Ferrer, J., Asins, G., Gomis, R. and F. Bosch. (1994). Expression of GLUT-2 antisense RNA in β cells of transgenic mice leads to diabetes. *The Journal of Biological Chemistry.* **269**: 28543-28546

Van der Heyden, M. A. G., Veltmaat, J. M., Hendriks, J. A. A., Destree, O. H. J. and L. H. K. Defize. (2000). Dynamic connexin43 expression and gap junctional communication during endoderm differentiation of F9 embryonal carcinoma cells. *European Journal of Cell biology.* **79**: 272-282.

Van der Velden, A. W. and A. A. Thomas (1999). The role of the 5' untranslated region of an mRNA in translation regulation during development. *Int J. Biochem. Cell Biol.* **31** : 87-106

Vasios, G., Mader, S., Gold J. D., Leid, M., Lutz, Y., Gaub, M. P., Chambon, P. and L. Gudas. (1991). The late Retinoic acid induction of laminin B1 gene transcription involves RAR binding to the responsive element. *EMBO J.* **10**: 1149-1158

Vogel, G. (1999). Harnessing the power of stem cells. *Science.* **283**. : 1432-1434

Vollenweider, F., Inrimger, J-C., Gross, D. J., Villa-Komaroff, L. and P. A. Halban. (1992). Processing of Proinsulin by transfected Hepatoma (FAO) Cells. *Journal of Biological Chemistry.* **267** : 14629-14636

Mering, J. V. and O. Minkowski. (1890). Diabetes Mellitus nach pankreasextirpation. *Arch Exp Pathol Pharmacol.* **26** : 371-387

Vozzi, C., Ullrich, S., Charollais, A., Philippe, J., Orci, L. and P. Meda. (1995). Adequate connexin-mediated coupling is required for proper insulin production. *J. Cell Biol.* **131**: 1561-1572.

Wada, Y. (2001). Estimating the number of protein-coding genes of mouse genome. *J. Animal Genetics* **29** : 17-19

Wade, P. A., Pruss, D. and A. P. Wolffe. (1997). Histone acetylation: chromatin in action. *TIBS* **22** : 128-132

Waeber, G., Thompson, N., Haefliger, J-A. and P. Nicod. (1994). Characterisation of the murine high K_m glucose transporter GLUT2 gene and its transcriptional regulation by glucose in a differentiated insulin secreting cell line. *The Journal of Biological Chemistry* **269** : 26912-26919

Wagers A. J., Christensen, J. L. and I. L. Weissman. (2002). Cell fate determination from stem cells. *Gene Therapy* **9** : 606-612

Walker, M. D., Edlund, T., Boulet, A. M. and W. J. Rutter. (1983). Cell-specific expression controlled by the 5' flanking region of insulin and chymotrypsin genes. *Nature* **306** : 557-560

Walker, M.D., Park, C.W., Rosen, A. and A. Aronheim. (1990). A cDNA from a Mouse Pancreatic Beta-Cell Encoding a Putative Transcription Factor of the Insulin Gene. *Nuclaeic. Acid. Res.* **18** : 1159-1166

Walsh, D. (1999). Differentiation of lung cancer cell lines: A role for translational regulation of gene expression. PhD Thesis. Dublin City University, Glasnevin, Dublin 11. Ireland.

Wan, Y. J., Wang, L. and T. C. Wu. (1995). Retinoic acid induces activin receptor IIB mRNA in F9 embryonal carcinoma cells. *J. Mol Endocrinol.* **14** : 247-254

Wang, H., Maechler, P., Ritz-Laser, B., Hagenfeldt, K. A., Ishihara, H., Philippe, J. and C. B. Wollheim. (2001). Pdx-1 level defines pancreatic gene expression pattern and cell lineage differentiation. *The Journal of Biological Chemistry.* **276** : 25279-25286

Wang, R. N. Kloppel, G. and L. Bouwens. (1995). Duct to Islet Cell Differentiation and Islet Growth in the Pancreas of Duct Ligated Adult Rats. *Diabetologia.* **38** : 1405-1411

Wang, R. N. and L. Rosenberg. (1999). Maintainence of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *Journal of Endocrinology* **163** : 181-190

Watada, H., Kajimoto, Y., Miyagawa, J-I, Hanafusa, T., Hamaguchi, K., Matsuoka, T-A., Yamamoto, Y., Kawamori, R. and Y. Yamasaki. (1996). PDX1 Induces Insulin and

Glucokinase Gene Expression in αTC Clone 6 Cells in the Presence of Betacellulin.. *Diabetes*. **45** : 1826-1831

Watanabe, T., Shintani, A., Nakata, M., Shing, Y., Folkman, J., Igarashi, K. and R. Sasada. (1994) Recombinant hunam betacellulin. *The Journal of Biological Chemistry* **269** : 9966-9973

Watt, F. M., and B. L. M. Hogan. (2000). Out of Eden: Stem Cells and their Niches. *Science*. **287**. : 1427-1430

Webb, G. C., Akbar, M. S., Zhao, C. and D. F. Steiner. (2000). Expression profiling of pancreatic β cells: glucose regulation of secretory and metabolic pathway genes. *PNAS* **97** : 5773-5778

Weinstein, D. C., Ruiz I Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R. L, Jessel, T. M., and J. E. Darnell. (1994). The winged helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78** : 575-588

Weinstein, J., Jacobsen, F. W., Hsu-Chen, J., Wu, T. and L. G. Baum. (1994). A novel mammalian proteiin p55CDC, present in dividing cells is associated with protein kinase activity and has homology to the saccharomyces cerevisiae cell division cycle proteins Cdc20 and Cdc4. *Mol Cell Biol.* **14** : 3350-3363

Welsh, M., Nielsen, D. A., MacKrell, A. J. and D. F. Steiner. (1985). Control of insulin gene expression in pancreatic beta-cells and in an insulin-producing cell line, RIN-5F cells. II. Regulation of insulin mRNA stability. *Journal of Biological Chemistry*. **260** 13590-13594

Welsh, N. and A Sjoholm. (1988). Polyamines and insulin production in isolated mouse pancreatic islets. *Biochem J* **252** : 701-707

Welsh, N., Welsh, M., Steiner, D.M. and C. Hellerström. (1987). Mechanisms of Leucine- and Theophylline Stimulated Insulin Biosynthesis in Isolated Rat Pancreatic Islets. *Biochem. J.* **246** : 245-248

Wentworth, B. M., Schafer, I. M., Villa-Komaroff, L. and J. M. Chirgwin. (1986). Characterisation of the two nonallelic genes encoding mouse preproinsulin. *J. Mol Evol.* **23**: 305-312

Willecke, K., Hennemann, H., Dahl, E., Jungbluth, S. R. Heynkes. (1991). The diversity of connexin genes encoding gap junctional proteins. *Eur. J. Cell Biol.* **56**: 1-7

Willis, A. E. (1999). Translational control of growth factor and protooncogene expression. *The Int. J. Biochem and Cell Biol.* **31** : 73-86

Wu, H., Macfarlane, W. M., Tadayyon, M., Arch, J. R., James, R. F. and K. Docherty. (1999). Insulin stimulates pancreatic duodenal homobox factor-1 (PDX1) DNA-binding activity and insulin promoter activity in pancreatic beta cells. *Biochem J.* **344** : 813-818

Wu, H., Liu, X., Jaenisch, R. and H. F. Lodish. (1995). Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*. **83** : 59-67

Wu, K-L., Gannon, M., Pehsavaria, M., Offield, M. F., Henderson, E., Ray, M., Marks, A., Gamer, L. W., Wright, C. V. E. and R. Stein. (1997). Hepatocyte nuclear factor 3β is involved in pancreatic β-cell-specific transcription of the Pdx1 gene. *Mol Cell Biol.* **17** :L 6002-6013.

Xu, W., Chen, H., Du, K., Ashahara, H., Tini, M., Emerson, B. M., Montminy, M. and R. Evans. (2001). A transcriptional switch mediated by cofactor methylation. *Science* **294** : 2507-2511

Yallop, C. A. and I. Svendsen. (2001). The effects of G418 on the growth and metabolism of recombinant mammalian cell lines. *Cytotechnology* **35** : 101-114

Yamamoto, H. and K. Okamoto. (1980). Protection by picolinamide, a novel inhibitor of poly(ADP-ribose) synthetase, against both streptozotocin-induced depression of proinsulin synthesis and reduction of NAD content in pancreatic islets. *Biochem Biophys Res Commun*. **95** : 474-481

Yamamoto, K., Miyagawa, J-I, Waguri, M., Sasada, R., Igarashi, K., Li, M., Nammo, T, Moriwaki, M., Imagawa, A., Yamagata, K., Nakajima, H., Namba, M., Tochino, Y., Hanafusa, T. and Y. Matsuzawa. (2000). Recombinant human betacellulin promotes the neogenesis of β cells and ameliorates glucose intolerance in mice with diabetes induced by selective alloxan perfusion. *Diabetes*. **49** : 2021-2027

Yang, L., Li, S., Hatch, H., Ahrens, K., Corneliun, J. G., Petersen, B. E. and A. B. Peck. (2002). In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. *Proc Natl Acad Aci.* [E-pub ahead of print, 4-June].

Yasuda, H., Tanaka, S., Ohnishi, H., Mashima, H., Ogushi, N., Mine, T. and I. Kojima. (1994). Activin A: negative regulator of amylase secretion and cell proliferation in rat pancreatic acinar AR42J cells. *Am. J. Physiol.* **30** : 220-226

Yonemura, Y., Takashima, T., Miwa, K., Miyazaki, I., Yamamoto, H. and H. Okamoto. (1984). Amelioration of Diabetes Mellitus in Partially Depancreatised Rats by Poly(ADP-ribose) Synthetase Inhibitors. *Diabetes* **33**: 401-404

Yoshida, S., Kajimoto, Y., Yasuda, T., Watada, H., Fujitani, Y., Kosaka, H., Gotow, T., Miyatsuka, T., Umayahara, Y., Yamasaki, Y. and M. Hori. (2002). PDX-1 Induces

differentiation of Intestinal Epitheloid IEC-6 into Insulin Producing Cells. *Diabetes* **51** : 2505-2513

Zakany, J., Burg, K. and I Rasko. (1984). Spontaneous differentiation in the colonies of a nullipotent embryonal carcinoma cell line F9. *Differentiation.* **27**: 146-151

Zhang, Y-Q, Mashima, H. and I. Kojima. (2001). Changes in the expression of transcription factors in pancreatic AR42J cells during differentiation into insulin-producing cells. *Diabetes*. **50**: Suppliment S10-S14

Zhou, J., Wang, X., Pineyro, M. A., and J. M. Egan. (1999). Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. *Diabetes*. **48** : 2358-2366.

Zhou, J., Pineyro, M. A., Wang, X., Doyle, M. E.. and J. M. Egan. (2002). *J. Cell Physiol.* **192** : 304-314

Zhu, A. J., Haase, I. and F. M. Watt. (1999). Signalling via β1 integrins and mitogen activated protein kinase determines human epidermal stem cell fate *in vitro*. *Proc. Natl. Acad. Sci.* **96** : 6728-6733

Zimmer, Y., Milo-Landesman, D., Svetlanov, A. and S. Efrat. (1999). Genes induced by growth arrest in a pancreatic β cell line: identification by analysis of cDNA arrays. *FEBS Letters* **457** : 65-70

Zulewski, H., Abraham, E.J., Gerlach, M.J., Daniel, P.B., Moritz, W., Muller, B., Vallejo, M., Thomas, M.K. and J.F Habener. (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