An investigation of differentiation programmes governing pancreatic cell development *in vitro*

A thesis submitted for the degree of Ph D

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By

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This research work described in this thesis was performed under the supervision of Prof Martin Clynes and Dr Lorraine O Driscoll

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I hereby 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 save and to the extent that such work has been cited and acknowledged within the text of my own work.

Signed: Eadaoin Ní Chiarna ID No.: 97342599

Date: 18/09/06

This thesis is dedicated to my sister Grace, I believe she was watching over me every step of the way

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Abbreviations

AA	Activin A
ABC	Avidin-Biotin Complex
ACTH	Adrenocorticotropic Hormone
ADP	Adenosine Diphosphate
AFP	Alpha-feto Protein
ALS	Amyotrophic Lateral Sclerosis
AP	Alkaline Phosphatase
Ara-C	Cytosine Arabinoside
AS	Adult Stem
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
Basp1	Brain Acid Soluble Protein 1
Bax	Bcl2-associated x protein
BCIP	5 Bromo-4chloro-3-indolyl-phosphate, 4 toluidine salt (X-phosphate, 4 toluidine salt
bFGF	Basic Fibroblast Growth Factor
bHLH	Basic Helix-Loop-Helix
BMP	Bone Morphogenic Protein
βNGF	β nerve growth factor
BSA	Bovine Serum Albumin
BTC	Betacellulin
cAMP	Cyclic Adenosine Monophosphate
Cck	Cholecystokinin
cDNA	Complementary DNA
CEACAM	Carcinoembryonic Antigen-Related Cellular Adhesion Molecule
Chgb	Chromogranin B
Ckd4	Cyclin-Dependent Kinase 4
CNS	Central Nervous System
C-peptide	Connecting Peptide
Cpm	Counts Per Minute
CRE	cAMP Responsive Elements

CREB	CRE Binding Protein
cRNA	Complimentary RNA
DAB	Diaminobenzidine
DAPI	4', 6-Diamidino-2-phenylindole
DBD	DNA Binding Domain
DEPC	Diethyl Pyrocarbonate
Dhh	Desert Hedgehog
Dlk	Delta-Like Homologue 1
Dll-1	Delta-Like 1
DM	Diabetes Mellitus
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate (N = A, C, T, G)
EB(s)	Embryoid Body (s)
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal Growth Factor
Egr1	Early growth response 1
ELISA	Enzyme Linked Immunosorbent Assay
Epo	Erythropoietin
ES	Embryonic Stem
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
Gad1	Glutamate Decarboxylase
GAP43	Growth Associated Protein 43
GAPDH	Glyceraldehyde-6-Phosphate Dehydrogenase
GATA4	GATA Binding Protein 4
Gck	Glucokinase
GF(s)	Growth Factor (s)
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GLP1	Glucagon-like Peptide 1
Glut2	Glucose Transporter 2

GSIS	Glucose Stimulated Insulin Secretion
HDAC	Histone Deacetylase
hEpoR	Human Erythropoietin Receptor
hES	Human Embryonic Stem
HGF	Hepatocyte Growth Factor
HLXB9	Homeobox Gene HB9
HNF(6/3β)	Hepatocyte Nuclear Factor (6/3β)
IAPP	Islet Amyloid Polypeptide
ICC	Immunocytochemistry
ICCs	Islet-like Cell Clusters
ICM	Inner Cell Mass
IDDM	Insulin Dependant Diabetes Mellitus
IF	Immunofluorescence
IGRP	Islet-Specific Glucose-6-Phosphate (G6Pase) Catalytic Subunit Related Protein
IHC	Immunohistochemistry
Ihh	Indian Hedgehog
IL-3	Interluekin-3
IMS	Industrial Methylated Spirits
Isl1	Islet 1
ITSFn	Insulin-transferrin-selenium-fibronectin medium
IVF	*In Vitro* Fertilization
kDA	Kilo Daltons
k_m	Michaelis Constant
LIF	Leukaemia Inhibitory Factor
LIFR	Leukaemia Inhibitory Factor Receptor
LSCM	Laser Scanning Confocal Microscopy
MBP	Myelin Basic Protein
MEF(s)	Mouse Embryonic Fibroblasts
mES	Mouse Embryonic Stem
Mm	Minutes
ML	Metabolic Labelling
MLR	Multiple Linear Regression
MODY	Maturity Onset Diabetes of the Young

MRIA	modified RIA
mRNA	messenger RNA
NAD	Nicotinamide Adenine Dinucleotide
NCAM	Neural Cell Adhesion Molecule
ND	Not Determined
NF-L	Neurofilament-Light
NGF	Nerve Growth Factor
Ngn3	Neurogenin 3
NIC	Nicotinamide
NIDDM	Non-Insulin Dependent Diabetes Mellitus
Npy	Neuropeptide Y
OD	Optical Density
ODC	Ornithine Decarboxylase
PA	Pentanoic Acid
PBS	Phosphate Buffered Saline
PC1	Prohormone Convertase 1
PC2	Prohormone Convertase 2
PCR	Polymerase Chain Reaction
Pdx1	Pancreatic duodenal homeobox factor 1
PHEM	PIPES, HEPES, EGTA, MgCl Buffer
PKA	Protein Kinase A
PKC	Protein Kinase C
Pld1	Phospholipase D1
PLIER	Probe Logarithmic Intensity ERror Estimation
PMP(s)	Pancreatic Multipotent Precursor (s)
PP	Pancreatic Polypeptide
PPI	Preproinsulin
PVDF	Polyvinylidene Fluoride
qPCR	Real-time PCR
RA	Retinoic Acid
RCF	Rotational Speed at the Relative Centrifugal Force
RER	Rough Endoplastic Reticulum
RIA	Radio-immunoassay
RNA	Ribonucleic Acid

RNase	Ribonuclease
RNasin	Ribonuclease Inhibitor
RPM	Revolutions Per Minute
RQ	Relative Quantity
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SB	Sodium Butyrate
Scg3	Secretogranin III
Sgne 1	Secretory granule neuroendocrine protein 1 (7B2 protein),
Shh	Sonic Hedgehog
Som	Somatostatin
SSEA	Stage-Specific Embryonic Antigen
STZ	Streptozotocin
SUR1	Sulfonylurea Receptor 1
TBE	Tris-Boric Acid EDTA Buffer
TBS	Tris Buffered Saline
TGFβ1	Transforming growth factor β1
TGN	Trans-Golgi Network
TH	Tyrosine Hydroxylase
Tjp1	Tight Junction Protein
TRITC	Tetramethylrhodamine Isothiocyanate
Trp53	Transformation Related Protein 53
TSA	Trichostatin A
TV	Trypsin
UHP	Ultra High Pure Water
UV	Ultraviolet
v/v	Volume/Volume
VSMCs	Vascular Smooth Muscle Cell (s)
w/v	Weight per Volume
YY	Hormone Peptide YY

Abstract

The murine embryonic stem (ES) cell line, ES-D3 was used in this project as a model for (a) investigating controversial aspects of current protocols used for the derivation of β cell types from ES cells and (b) investigating the potential to regulate the differentiation of ES cells into endodermal derivatives i e β-like cells, using extra-cellular factors previously associated with various aspects of pancreatic development

Several groups have reported the derivation of insulin-expressing cell types from ES cells Many believe that these insulin-positive cells were a result of insulin uptake from supplements used in the culture medium Others have suggested that the ES-derived insulin-expressing cells may be of a neuronal or extra-embryonic lineage, rather than an endocrine lineage Using an adapted version of the Lumelsky protocol (Lumelsky et al, 2001), we demonstrated the induction of 3-dimensional clusters similar to those described in the original study, which expressed a range of pancreatic, extra-embryonic and neuronal transcripts Insulin protein was detected in the differentiated clusters by ELISA A novel method developed to detect insulin biosynthesis by the cells, and analysis of C-peptide expression, indicated that this insulin is probably derived from the culture medium, not from the differentiated ES cell

Culture of ES cells in suspension leads to the formation of embryoid bodies (EBs) Treatment of these aggregates with retinoic acid (RA) was previously shown to induce endodermal differentiation We exposed RA_EBs to secondary treatment with agents associated with pancreatic endocrine differentiation, e g Activin A (AA), Sodium Butyrate (SB) and Betacellulin (BTC), with the aim of developing a novel protocol for the regulated derivation of insulin-expressing cell types The resulting differentiated populations were analysed for transcript expression of a range of multi-lineage markers i e neuronal, myogenic, exocrine and endocrine pancreas, extra-embryonic and apoptotic markers Immunohistochemistry methods were established and applied for the analysis of a selection of the chosen markers, following formalin-fixing and paraffin-embedding Applying 2-stage regulated differentiation protocols developed

during the course of the study, we demonstrated the derivation of an intermediate multi-potential population (RA_EBs) from undifferentiated ES cells that preferentially gave rise to pancreatic endocrine insulin-expressing cell types in the presence of SB and neuronal-like cell types in the presence of AA or BTC

Low passages of the MIN-6 insulinoma cell line are glucose-responsive whereas at high passage they become glucose non-responsive This loss in the glucose stimulated insulin secretion (GSIS) phenotype correlates with changes in morphology, increased proliferation and increased alkaline phosphatase (AP) activity, suggesting that the loss of GSIS may be due to de-differentiation of the high passage MIN6 cells To investigate this hypothesis further, gene expression differences between low passage and high passage MIN6 cells were investigated by microarray and real-time PCR (qPCR) analysis

Section 1.0: Introduction

1 1 Stem Cell Biology

Stem Cells are defined as 'clonogemc cells capable of both self renewal and multilineage differentiation' (Berna et al, 2001) Differentiation is the process by which an unspecialized stem cell becomes equipped to perform specific functions i e hormone secretion, nerve cell communication, muscle contraction, etc The organs of the body are predominately composed of functional specialized cells that are all progeny of stem cells from the developing embryo (Figure 1 1 1) Stem cells are proving a useful tool in understanding the genes, molecules, growth factors and key events in embryonic development (Kirschtein & Skirboll, 2001) The physiological or experimental conditions that induce stem cells to give rise to functional cells are of particular importance to scientists The belief is that in the future these cells may be a potential source of cell replacement therapy for degenerative diseases such as Parkinson's disease, diabetes, multiple sclerosis, heart disease etc (http //stemcells nih gov/info/basics/basics1 asp) They may also serve as vectors to carry and express genes in target organs in the course of gene therapy (Henningson et al, 2003)

1 1 1 Differentiation

Differentiation may be symmetric or asymmetric Symmetric differentiation will give rise to two daughter cells, which have both either remained as stem cells or which have both differentiated Asymmetric differentiation will give rise to two daughter cells, one that has differentiated, while the other remains as a stem cell (Hall & Watt, 1989, Watt & Hogan, 2000) There are a number of intermediate cells between the stem cell and its terminally differentiated progeny These cells are partially differentiated precursor/progenitor cells All multi-cellular organisms are an arrangement of cell types in an organised pattern that have descended from a single stem cell - the fertilised egg (Figure 1 1 1) (Kirschstein & Skirboll, 2001, Xavier Doss et al, 2004)

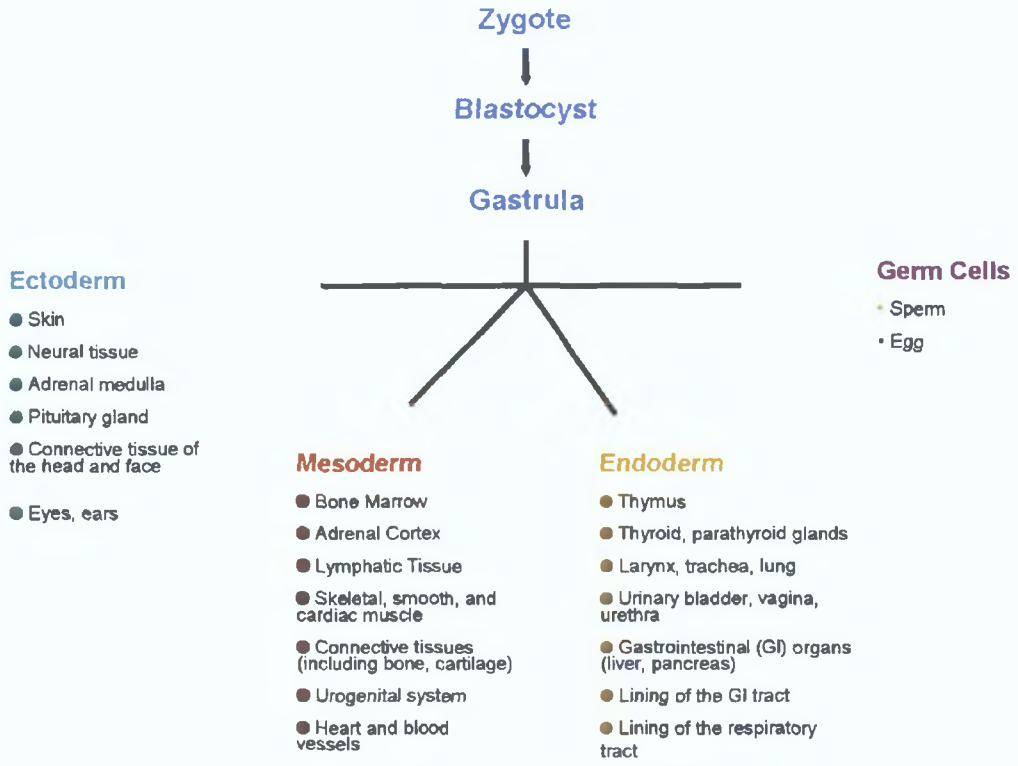


Figure 1.1.1 Embryonic Development and Differentiation

1.1.2 Classification of Stem Cells

Stem Cells can be broadly classified into (1) Adult Stem (AS) Cells (2) Embryonic Stem (ES) Cells. It is the latter, which is of primary importance in this thesis.

1.1.2.1 Adult Stem Cells

AS cells are dispersed throughout differentiated cells in tissues and organs of the body. Like all other stem cells they are capable of both self-renewal and differentiation. They can be difficult to define in that no one is entirely sure of their origin. There is only a small number of AS cells within a tissue and they remain quiescent until activated by disease or injury (Robey, 2000; Fortier, 2005). AS cells have been found in the bone marrow, brain, peripheral blood, blood vessels, skeletal muscle, skin and liver cells (http://stemcells.nih.gov/info/basics/basics4.asp). It was previously thought that the primary function of these cells was to replace dead and injured cells within

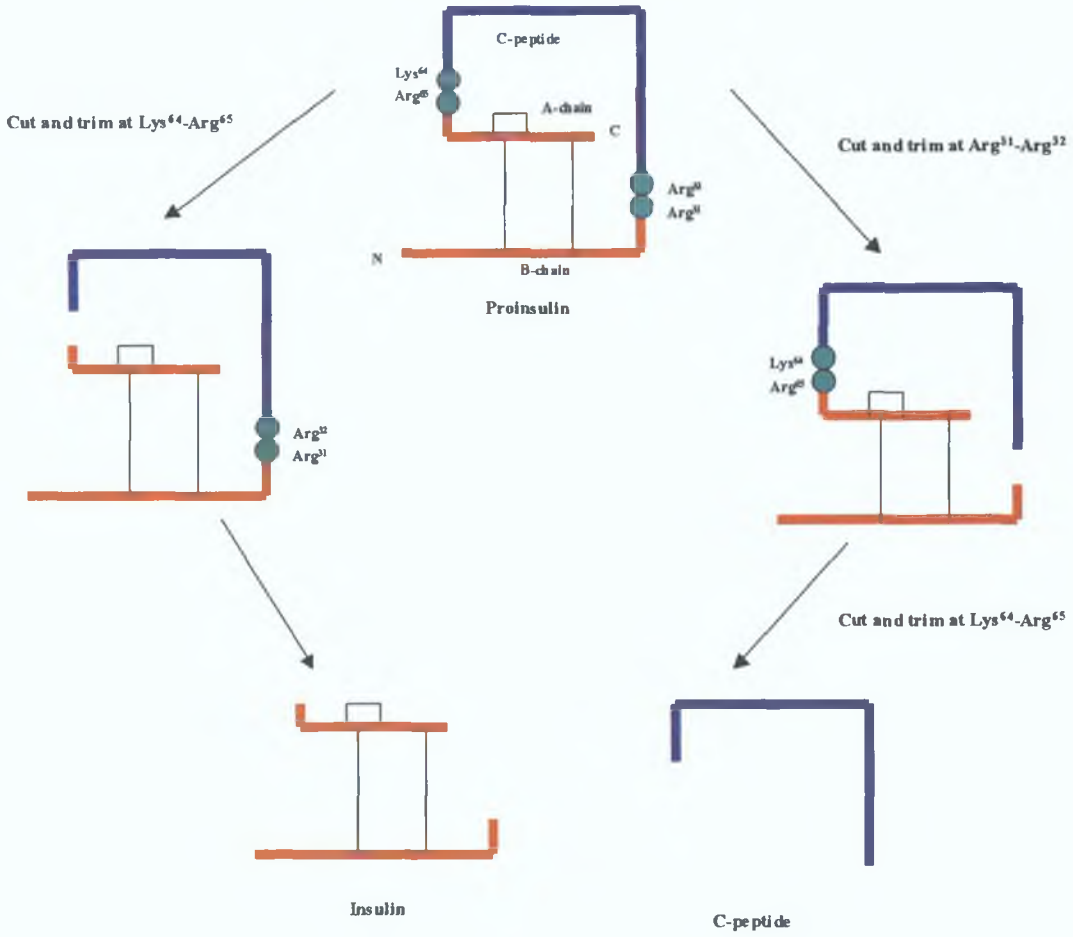


Figure 1.3.3 Processing of proinsulin to insulin. Following initial cleavage by either PC1 or PC2, trimming of the residual C-terminal residues is carried out by the action of ubiquitously expressed carboxypeptidases.

1.3.3 Glucose Sensing in the β Cell

To correctly control blood glucose levels, the β cell must be able to accurately sense/determine how much glucose is present in the blood. The glucose level is determined from the metabolism of the blood glucose and the subsequent changes in blood metabolites (Ashcroft, 1980; German, 1993). GSIS requires that the metabolism of glucose can be increased within the β cell over the entire physiological range of glucose (Marshall et al, 1993; Ishihara et al, 1994). The most important components of the glucose sensor are (I) glut2, which facilitates rapid glucose uptake regardless of the extracellular sugar concentration and (II)

the tissue of residence (Fortier, 2005) For example neural stem cells in the brain give rise to all 3 major cell types nerve cells (neurons), astrocytes and oligodendrocytes (Reynolds & Weiss, 1992, Williams et al, 1991) However, recent studies have suggested that an AS cell can give rise to differentiated cells characteristic of other tissue types, regardless whether the tissue is derived from the same germ layer or not The ability of AS cells to give rise to multiple cell types is variously referred to as plasticity, unorthodox differentiation or transdifferentiation (Filip et al, 2004, Fortier, 2005) The use of AS cells as a source of cells for replacement therapy has potential in that the expansion and reintroduction of a patient's own cell type back into the body would avoid rejection by the immune system One of the problems associated with this is the lack of successful methods for expanding AS cells in culture (Hipp & Atala, 2004)

1 1 2.2 Embryonic Stem Cells

The ES cell as defined by its origin is derived from the inner cell mass (ICM) of the blastocyst ES cells may be totipotent or pluripotent Totipotent stem cells form when a fertilised egg initially divides They are capable of generating all cells and tissues that form an embryo Pluripotent stem cells are isolated approximately 3 - 5 days after fertilisation from the blastocyst stage of the embryo (Figure 1 1 2) The blastocyst is composed of the trophoblast (outer layer of cells), the blastocoel (hollow fluid filled cavity) and ICM (cluster of cells on interior) (Kirschstein & Skirboll, 2001) Pluripotent stem cells are capable of giving rise to differentiated progeny representing all three germ layers ectoderm, mesoderm and endoderm, as well as extra embryonic tissue that support development (Reubinoff et al, 2000) Characteristics of ES cells are

- Long term self renewal in culture
- Exhibit a stable, normal karyotype
- Maintain high levels of telomerase activity (A telomere is a repeating sequence of double stranded deoxyribonucleic acid (DNA) located at the end of a chromosome Long telomeres are associated with immortalization of cells)

- Capable of giving rise to a colony of genetically identical cells (clonogenic)
- Expression of classical stem cell markers i e transcription factor Oct 4 and AP
- ES cells are pluripotent in that they can differentiate into cells from all three germ layers both *in vivo* and *in vitro* and, therefore, can give rise to a wide range of cell types

It is these unique properties that make ES cells an attractive universal source of donor cells for transplantation and regeneration therapies (Kirschstein & Skirboll, 2001, Henningson et al, 2003) ES cell lines are currently available from rodents, rabbits, pigs and primates (Xavier Doss et al, 2004)

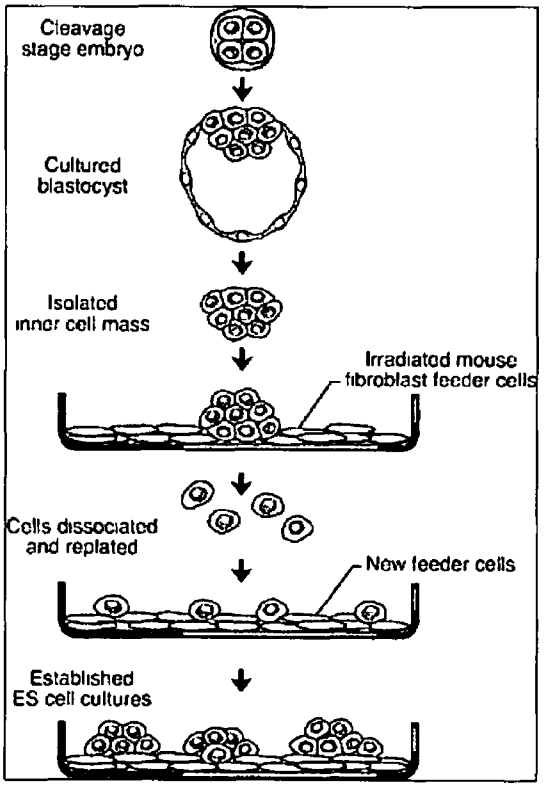


Figure 1.1 2 Derivation of ES cell lines (permission to use Figure received from Dr Jon S Odorico, M D , University of Wisconsin- Madison)

1 1 3 Mouse Embryonic Stem Cells

Prior to the isolation of human stem cells in 1998 the majority of knowledge on stem cells and embryonic development was based on mouse model systems Mouse ES cells were first isolated in 1981 from that blastocyst, which is the stage of embryonic development prior to implantation Mouse ES cells are derived from the ICM of the 3 5-day-old blastocyst (Evans and Kaufman, 1981, Martin, 1981) The multi-step process for deriving stem cells is as follows -

- Removal of the trophectoderm surrounding the ICM Cells are then grown in petri dishes containing a nutrient broth known as culture medium The medium is supplemented with fetal calf serum (FCS) The medium may contain cytokine leukaemia inhibitory factor (LIF) and if not, the petri dishes are coated in an inactivated layer of mouse embryonic fibroblasts (MEFs) (Cells inactivated using mitomycin C or gamma irradiation to prevent cell division)
- Approximately 1 week later the cells are removed and dispersed into new culture dishes coated with MEF feeder layers/in presence of LIF ES cells aggregate to form colonies
- Nondifferentiating single colonies are dissociated and replated into fresh culture dishes The replating process establishes a clonal ES cell line generated from a single ES cell

Mouse ES cells can be maintained in culture for two or more years (Kirschstein and Skirboll, 2001) In culture, mouse ES cells grow in tight rounded colonies with indistinct cell borders (Pera & Trounson, 2004)

1 1 3 1 Factors Involved in Maintaining Mouse ES Cells in an Undifferentiated State

Undifferentiated murine ES cell lines require an exogenous supply of LIF (Table 1 1 1) The mouse ES cells can be propagated on mitotically inactivated MEFs that produce LIF or the medium can be supplemented with a relatively high concentration of LIF (Berna et al, 2001, Xavier Doss et al, 2004) Pathways mediated by LIF are critical in regulating self-renewal of mouse ES cells In brief, LIF binds to the LIF receptor (LIFR) and GP130 receptor-signalling complex, activating the JAK-STAT downstream pathways Activation of the Jak kinases results in the recruitment, tyrosine phosphorylation and dimerization of

STAT3 The activation of STAT3 is essential for ES cell self-renewal (Niwa et al, 1998, Burdon et al, 2002)

Recent studies have suggested that LIF is required, but not sufficient, for maintaining pluripotent mouse ES cell expansion The presence of serum or feeder layers is necessary indicating that signals are provided by the serum or feeders that are necessary to support self-renewal (Yamane et al, 2005) Components of serum i e bone morphogenic protein (BMP) are thought to act along with LIF in maintaining the undifferentiated state of ES cells Prolonged self-renewal of ES cells in serum free media supplemented with LIF and BMP has been demonstrated (Ying et al, 2003) The effect of BMP is dependent on the presence of LIF In the absence of LIF, BMP induces mesodermal differentiation

Oct 4 is present in the mouse blastocyst and is encoded by the Pou5f1 gene Its expression is confined to pluripotential cells and has been shown to be a requirement for the establishment of pluripotent cell lineages during mouse embryonic development (Nichols et al, 1998) Precise levels of Oct 4 are required in maintaining the ES cell undifferentiated state (Niwa et al, 2000) Oct 4 is therefore a marker for undifferentiated ES cells It has been shown that Oct 4 is negatively regulated by RA signalling, little is known as to what acts as a positive regulator of Oct 4 (Rao, 2004)

The interaction of LIF signalling pathways with Oct 4 signalling pathways remains unclear but LIF itself doesn't appear to regulate Oct 4 Likewise Oct 4 doesn't appear to regulate JAK-STAT signalling The pathways may work in parallel whilst converging on common downstream targets (Rao, 2004) Although LIF is essential for *in vitro* murine ES cell maintenance, the establishment of ES cell lines from LIF receptor-null, GP130-null embryos has been demonstrated providing evidence that other factors outside of LIF may be sufficient to maintain undifferentiated ES cells (Berger & Sturm, 1997, Gendall et al, 1997)

More recently, a homeobox domain containing protein known as Nanog has been identified as a necessity in murine ES cell self-renewal It is highly expressed in ES cells and over expression of Nanog is sufficient to maintain Oct 4 levels (Chambers et al, 2003, Mitsui et al, 2003)

1 1 4 Human Embryonic Stem Cells

In 1998 human ES cells were derived from the ICM of a human blastocyst produced by *in vitro* fertilization (IVF) and donated for research purposes (Thomson et al, 1998) The derivation of human ES cell lines is similar to that of mouse ES cell lines (Figure 1 1 2) Researchers drew on animal studies i e mouse, to develop culturing methods LIF is not sufficient to prevent differentiation of human ES cells and they require continuous propagation on feeder layers (Odorico et al, 2001) Human ES cells are maintained in medium containing serum or serum replacement medium with supplemental basic fibroblast growth factor (bFGF) It's been suggested that the use of bFGF improves cloning efficiency of human ES cells growing at low density (Amit et al, 2000) The population doubling time of human ES cells is 36 hours, this is slow in comparison to their murine counterparts (population doubling time of 12 hours) Morphologically human ES cells differ from murine ES cells in that they form relatively flat loose aggregates with more distinct borders than murine cells (Table 1 1 1) (Kirschstein & Skirboll, 2001, Pera & Trounson, 2004) Differences are also noted in regard to cell surface antigen phenotype, for example human ES cells express stage specific embryonic antigens 3 and 4 (SSEA-3 and SSEA-4) where as murine ES cells do not express these antigens but do express SSEA-1 In the absence of feeder layers and in suspension culture human ES cells spontaneously form EBs and can activate the expression of genes restricted to each of the three embryonic germ layers (Odorico et al, 2001) The mouse EB initially forms a bilayered structure with the extra embryonic endoderm on the outside and primitive ectoderm on the inside, however, it is not clear if human EBs display any consistent organisation (Doetschman et al, 1985) Human ES cells fulfil the criteria of pluripotent ES cells in that they are capable of indefinite self-renewal and multilineage differentiation, both *in vivo* and *in vitro* They also express the classical markers of pluripotent ES cells i e Oct 4 and AP (Table 1 1 1) Human ES cells have been shown to differentiate *in vitro*

to neural progenitors, blood cell precursors, endothelial cells, osteogenic cells, cardiomyocytes, insulin-producing cells, hepatocytes, keratinocytes and trophoblast cells (Pera & Trounson et al, 2004) When transferred into an *in vivo* environment, human ES cells can give rise to derivatives of all three embryonic germ layers (Odorico et al, 2001)

The data on human ES cells is still somewhat limited The understanding of molecular regulation of mammalian studies may provide a framework for differentiation of human ES cells There is high hope to use human ES cells in transplantation therapy and drug testing but there are still many hurdles to overcome in regards to this area of research The ethical debate over the use of human embryos in research is ongoing Large-scale culture of human ES cells is labour intensive and time consuming The presence of feeder layers and animal based ingredients incur a risk of cross-transfer of pathogens Prolonged growth may lead to genomic instability and cause chromosomal aberrations (Pera & Trounson, 2004, Stojkovic et al, 2004) The differentiation studies in this thesis employ mouse cell models

Table 1.1 1 Comparison of mouse ES cell characteristics to human ES cell characteristics (permission to use Table received from the National Institutes of Health)

Marker Name	Mouse ES	Human ES
SSEA-1	+	-
SSEA-2	-	+
SSEA-3	-	+
Alkaline Phosphatase	+	+
Oct 3/4 (known as Oct 4)	+	+
Telomerase Activity	+	+
Feeder Cell Dependent	Yes in absence of alternative LIF	Yes
Factors aiding in stem cell self renewal	LIF and other factors acting through GP130 receptor can substitute for feeder layer	Feeder Cell + serum, feeder layer + serum-free medium+bFGF
Growth Charactenstics *in vitro*	Form compact, rounded clumps, can form EBs	Form, flat loose aggregates, can form EBs
Teratoma formation *in vivo*	+	+
Chimera Formation	+	+

1 1 5 Directing the Differentiation of ES Cells

The formation of organised tissue during embryonic development is controlled by complex inductive events and cell-cell interactions Differentiation studies using ES cells contribute to an understanding of the elements regulating both differentiation and embryonic formation (Henningson et al, 2003) If differentiation is to proceed, mouse ES cells must be removed from LIF, which promotes the division of undifferentiated ES cells Common strategies employed in directing differentiation are -

(1) Addition of growth factors to the medium For example Kawamorita et al (2002) studied the *in vitro* differentiation of mouse ES cells in the presence of RA

(2) Altering the chemical composition of the surface on which the cells are propagated Adherent substrates aid in preventing cells from interacting and differentiating In 2005, Flaim et al used a microarray platform to

study the effect of a combination of extracellular matrix molecules on murine ES cellular differentiation

(3) Introduction of a foreign gene into the genetic complement of the ES cell, which may in turn drive the cell along a specific differentiation pathway Blyszczuk et al (2003) demonstrated that expression of pax 4 in ES cells promotes differentiation of nestin-positive progenitor and insulin-producing cells

(4) Mimicking the naturally occurring cell-to-cell interaction of the *in vivo* environment by co-culturing ES cells with inducer tissues/cells (Odorico et al, 2001, Soria et al, 2001a)

Many differentiation studies employ EBs rather than monolayer culture In the absence of LIF, ES cells in suspension culture spontaneously differentiate into multi-cellular cell aggregates over a 2 - 5 day period The theory is that cell-to-cell interaction within the EB mimics the natural *in vivo* development of the embryo In general the formation of these aggregates is thought to initiate spontaneous differentiation of ES cells to the three embryonic germ layers (Henningson et al, 2003, Xavier Doss et al, 2004) *In vitro* differentiation can be disorganised and variable from one EB to another within the one culture The cultures obtained using differentiation methods are relatively heterogeneous in that they give rise to a mixture of cell types The current strategies to isolate a single population of a desired cell type include -

- genetic engineering of cells with a selection marker under the control of a tissue specific promoter
- employing selective culture conditions to promote the growth of a desired cell type
- Fluorescence activated cell sorting based on fluorescent-labelled antibodies raised against unique cell surface markers of a desired cell type

(Xavier Doss et al, 2004, Shufaro & Reubinoff, 2004)

Optimum protocols will be fundamental in the production of an unlimited source of cells for transplantation therapies Any future therapeutic application implies the current need to drive homogeneous differentiation of the ES cells to a desired cell type (Roche et al, 2003)

Murine ES cells have been differentiated *in vitro* to cardiomyocytes, haematopoietic progenitors, yolk sac, skeletal myocytes, smooth muscle cells adipocytes, hepatocytes, chondrocytes, neurons, glia, pancreatic islet cells, primitive endoderm etc The list is ever increasing but the main hurdle researchers have to face is development of a desired cell phenotype rather than a mixed population (Xavier Doss et al, 2004)

1 1 6 ES-D3 Cell Line as an Embryonic Stem cell Model

ES-D3 is a pluripotent ES cell line derived from a murine 129/Sv blastocyst (Doetschman et al, 1985) ES-D3 cells are maintained in an undifferentiated state by

(I) frequent subculture on inactivated feeder layers of MEFs or STOs (STO-SNL2) Inactivation of feeder layers is achieved by mitomycin C treatment or irradiation Inactivation prevents the cells from dividing, whilst allowing them to maintain their metabolic activity and release LIF into the medium

or

(II) frequent subculture in media supplemented with commercially available LIF (in the presence of serum)

The undifferentiated cells are routinely subcultured every 2 – 3 days In the absence of feeder layers or LIF the cells spontaneously differentiate into embryonic structures i e EBs (*American Type Culture Collection (ATCC) Data Sheet*)

1 1 6 1 Differentiation Potential of ES-D3 Cells

Identifying the factors and elements that influence ES-D3 differentiation is necessary for studies using these stem cells to achieve a desired cell type In 1991, Wiles & Keller used a semi-solid culture system (methyl cellulose media) to encourage the development of EBs These EBs contained a number of differentiated cell types including haematopoietic precursor cells The cells were exposed to both erythropoietin (Epo) and interleukin-3 (IL-3) Epo increased the frequency of EBs with erythropoietic activity to more than 60% whereas IL-3 did not increase the frequency of EBs but increased the number of erythropoietic

cells associated with them, clearly indicating that using well-defined conditions ES-D3 cells could give rise to haematopoietic precursors

ES-D3 cells can be genetically modified by gene targeting and transfection techniques In 1996 Klug *et al* used a genetic selection technique to derive pure cultures of cardiomyocytes from differentiated murine ES-D3 cells A transgene consisting of the α- cardiac myosin heavy chain promoter fused to a complementary DNA (cDNA) encoding aminoglycoside phosphotransferase was transfected into undifferentiated ES-D3 cells via electroporation Resulting cell lines underwent differentiation and drug (G418) selection Analyses showed that selected cardiomyocyte cultures were greater than 99% pure and were highly differentiated These cardiomyocytes were used to form stable intercardiac grafts in dystrophic mice Kolossov et al (1998) followed up this research with a study investigating the functional characteristics of ES-D3 cell derived cardiac precursor cells Once again genetic selection was used to identify the cardiac precursor cells A vector containing the green fluorescent protein (GFP) gene under the control of the cardiac α-actin promoter was successfully transfected into the undifferentiated ES-D3 cells by electroporation Strongly fluorescent-contracting regions distinguished cardiac precursor cells amongst the differentiated progeny Other studies use transduction techniques to introduce genes into ES-D3 cells that enhance the formation of a particular cell type upon differentiation of the ES cells In 2000, Dai et al used retrovirus mediated gene transfer to introduce human erythropoietin receptor (hEpoR) cDNA into ES-D3 cells Expression of hEpoR gene in the cells was found to enhance the primitive and definitive erythropoiesis in differentiation of EBs

In 1999a, Li et al demonstrated that transplanted ES-D3 cells could grow in the eyes of nude mice with a tendency to differentiate into neurons and retina-like structures Ge et al, 2000 demonstrated that exposure of ES-D3 cells to RA and cytosine arabinoside (Ara-C), resulted in differentiated neuronal phenotypes Both studies demonstrate ES-D3 cell potential in giving rise to neuronal phenotypes

Both Karbanova & Mokry (2002) and Toumadje et al (2003) examined the *in vitro* spontaneous differentiation potential of ES-D3 cells Karbanova & Mokry used histological and histochemical analysis to determine that the most distinct cell populations arising in spontaneously formed EBs were alpha-fetoprotein-positive (AFP) endodermal cells and myogenic cells that expressed desmin, myogenin or smooth muscle actin Toumadje at al used immunocytochemical and flow cytometric methods to monitor ES-D3 EB differentiation over a 21-day period Oct 4, SSEA-1, and EMA-1 persisted for at least 7 days, whereas the primitive endoderm marker cytokeratin endo-A was expressed at increasing levels from day 6 Localization of these antigens within the EBs indicated segregation of the embryonic ectoderm- and primitive endoderm-derived tissues Localized expression of class III beta-tubulin and sarcomeric myosin also indicated that representatives of all three embryonic germ layer were present in EBs Both studies demonstrated the usefulness of the ES-D3 cell model for studying early embryogenesis

In 2003, Kahan et al evaluated the ability of ES-D3 cells to differentiate into pancreatic and islet lineage-restricted cell types Following differentiation in nonselective medium containing serum, murine ES cells spontaneously differentiated into cells individually expressing each of the four major islet hormones insulin, glucagon, somatostatin (Som), and pancreatic polypeptide (PP) Pancreatic duodenal homeobox factor 1 (Pdx1) immuno-positive cells appeared first, before hormone-positive cells had emerged Hormone-positive cells appeared within focal clusters of cells coexpressing Pdx1 and the hormone markers peptide YY (YY) and islet amyloid polypeptide (IAPP) in combination with the definitive hormones, characteristic of endocrine cells appearing during early pancreatic neogenesis (Section 1 2) In 2005, Milne et al used ES-D3 cells to present evidence that murine ES cells, which readily differentiate into cells displaying endodermal and ß cell characteristics are likely to be extra-embryonic in origin

In 2004, Ward et al compared the differentiation properties of the ES-D3 cell line to 4 other murine ES cell lines All stem cell lines were cultured in defined medium in the absence of feeder layers The study demonstrated that all

undifferentiated ES cell lines exhibited pluripotency, similar morphology and transcript expression However upon induced differentiation, the cell lines differed in their response to RA resulting in significant variations in cell number and morphology between lines Oct 4 and Neurofilament-68k messenger RNA (mRNA) transcripts were detected in all undifferentiated cell lines

Judging by studies carried out over the past 10 – 15 years it appears that the ES-D3 cells can under certain culture conditions differentiate into a range of cell types They are also suitable for use in studies were genetic manipulation is to play a key role in (a) directing differentiation and (b) selecting desired cell types from differentiated progeny Although research has been carried out on the differentiation of ES-D3 into neuronal, cardiac, haematopoietic and erythroid precursor cells, the derivation of endoderm derivatives from this cell line is a relatively new research area and only currently being studied

1 1 7 Directing the Differentiation of ES Cells towards Neuronal Phenotypes

In the mid-1990's neuroscientists became aware that parts of the adult brain can be regenerated under certain circumstances The existence of neural stem cells/progenitors that give rise to the three major neural lineages i e neurons (main message carriers in the nervous system), oligodendrocytes and astrocytes (crucial support cells) in the foetal and adult brain (Figure 1 1 3) (Gage, 2000) gave way to hope that cell replacement therapy based on the derivation of neural cell types from ES cells may be able to repair damage caused by degenerative disorders such as Parkinson's disease, Alzheimer disease, amyotrophic lateral sclerosis (ALS also known as Lou Gehrig's disease) and others (Kirschstein & Skirboll, 2001, Henningson et al, 2003) The formation of neural precursors and neural lineages from ES cells has been extensively studied over the past 20 years (Section 1 1 7 1) However, before neural stem cells/progenitors can be used for transplantation therapy, we need to learn more about the factors that control their expansion and proliferation as well as investigating the signalling molecules that direct the differentiation of their daughter cells (Gage et al, 2000, Kirschstein & Skirboll, 2001)

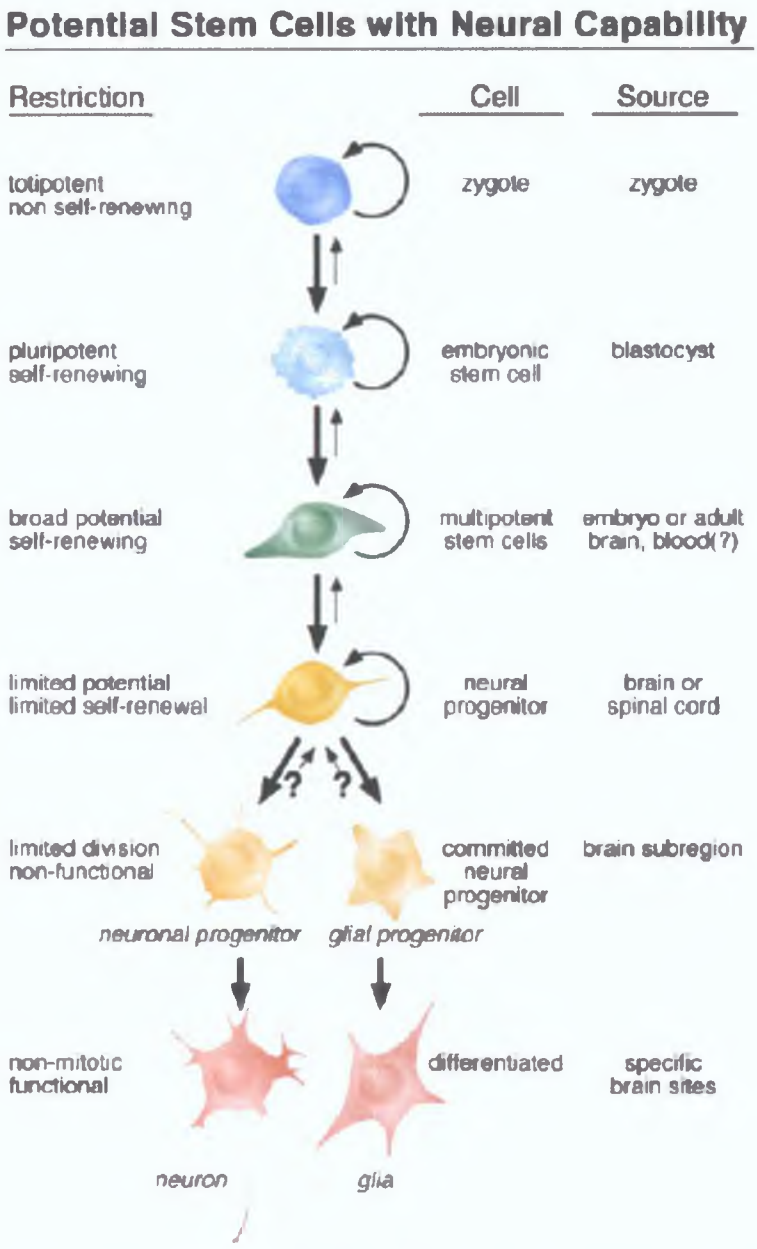


Figure 1.1.3 An illustration proposing the classes of stem cells that can give rise to neurons (permission to use Figure received from Prof. Fred H. Gage, The Salk Institute for Biological Studies)

Parkinson's disease is a good model for studying the use of stem cell therapy in curing degenerative disorders. This disease is a neurodegenerative disorder affecting motor control and causing movement disorder. Occurrence of the disease is due to loss of neurons that secrete dopamine in the brain. A therapy for this disease requires the replacement of one cell type in a particular location in the brain. Other disorders such as spinal cord injury would require the replacement of multiple cell types. Investigations into a therapy for Parkinson's

disease have found that mature dopaminergic neurons do not survive direct transplantation and transplanted cells find it extremely difficult to establish the required connections within the striatum The use of fetal dopaminergic neurons in transplant therapies has achieved some success Limited donor supply and ethical concerns are major obstacles to the widespread use of these cells (Henningson et al, 2003, Shufaro & Reubinoff, 2004) ES cells may be a potential alternative source of these transplantable dopaminergic neurons

1 1.7 1 Protocols Used in the Derivation of Neuronal Phenotypes from Mouse ES Cells

Protocols for the derivation of competent neural precursors have been developed in the mouse ES system In 1995, the chemical induction of neuronal cell types from ES cells using RA was demonstrated by three independent research groups (Bain et al, 1995, Fraichard et al, 1995, Strubing et al, 1995) The formation of EBs in the presence of RA resulted in 50 – 70% surviving cells exhibiting neural and glial characteristics after replating into serum free-medium (Fraichard et al, 1995, Strubing et al, 1995, Li et al, 1998), however, follow-on studies demonstrated that these populations were not homogeneous and contained contaminating cell populations (Li et al, 1998, Liu et al, 2000) RA-treated EBs promoted some recovery in mouse models of spinal injury suggesting a capacity for integration and function (McDonald et al, 1999, Liu et al, 2000)

Selective pressure during culture has been used to enrich neural progenitors derived from mouse ES cells An enriched population of nestin-positive neuroepithelial precursor cells was successfully derived from murine ES cells in serum free conditions These cells proliferate in the presence of bFGF and upon withdrawal of bFGF differentiate into both neurons and glia Subsequent differentiation of the cells in serum-containing medium induces expression of a wide variety of neuron-specific genes (Okabe et al, 1996)

The differentiation of mouse ES cells into dopaminergic neurons *in vitro* was initially achieved through selection and differentiation of nestin-positive cells from EBs Sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF8) (both have key roles in midbrain development) were used to direct the neural

precursors into dopaminergic neurons (Lee et al, 2000) Derivation of dopaminergic neurons from mouse ES cells has also been achieved by the direct transplantation of undifferentiated mouse ES cells into the midbrain of 6-OH-dopamine lesioned rats allowing for *in situ* differentiation into dopaminergic neurons (Bjorklund, et al, 2002) However, undifferentiated ES cell transplantation caused formation of teratoma tumours *in situ* More recently, Barberi et al (2003) demonstrated the derivation and engraftment of dopaminergic neurons from ES cells resulting in functional improvement in animal models of parkinsonism

1 1 7 2 Protocols Used in the Derivation of Neuronal Phenotypes from Human ES Cells

Most protocols for the derivation of neural lineages from human ES cells involve spontaneous differentiation, followed by selection of neural progenitors, and subsequent culture under neural stem cell promoting conditions (Shufaro & Reubinoff, 2004) Using this strategy Reubmoff et al (2001) reported the generation of an enriched population of proliferating neural progenitors isolated from human ES cells that were capable of differentiating *in vitro* into the three neural lineages--astrocytes, oligodendrocytes, and mature neurons *In vivo*, the transplanted progenitor cells migrated along established brain migratory tracks in the host brain and differentiated in a region-specific manner A subsequent study by Zhang et al (2001) reported the *in vitro* differentiation, selection and transplantation of neural precursor cells Upon transplantation into the neonatal mouse brain, these human ES cell-derived neural precursors were incorporated into a variety of brain regions, where they differentiated into both neurons and astrocytes

Perrier et al (2004) reported the differentiation of human ES cells into dopaminergic neurons A stromal feeder cell based differentiation system was used to induce neuroectodermal differentiation Regional specification was achieved by means of the sequential application of defined molecules that direct *in vivo* midbrain development i e Shh and FGF8 Progression toward a midbrain dopamine neuron fate was monitored by the sequential expression of key transcription factors e g pax 5, pax 2 and measurement of dopamine release

1 1 8 Directing the Differentiation of ES Cells towards Pancreatic Phenotypes

Researchers studying the directed differentiation of ES cells towards pancreatic phenotypes are all working towards specific goals -

- the development of a stable insulin-producing cell line with regulated hormone secretion
- the development of cells constituting normal pancreatic islets

(Roche et al, 2003, Kirschstein & Skirboll, 2001)

ß Cells, the major components of the pancreatic islets of Langerhans, secrete insulin in response to blood glucose levels The loss of insulin production by the pancreatic ß cells is central to the development of a metabolic disorder known as Diabetes Mellitus (DM) Annually DM affects more people and causes more deaths than AIDS and cancer combined Type I diabetes is characterised by auto-immune destruction of ß cells, whereas type II diabetes is characterised by the inefficient production or use of insulin resulting in glucose accumulation Treatments for diabetes such as insulin injections and controlled diets do not rigidly control glucose levels and do not solve serious long-term complications (Blyszczuk & Wobus, 2004a, Roche et al, 2005) Replacing ß cells/islets cells has been a therapeutic goal for decades and could prevent the mortality associated with DM The success of whole pancreas and islet transplantations in restoring normal glycaemic control verifies that replacing the β cells is an effective therapy for DM Isolated islets from at least two compatible donors are necessary for transplantation (Shapiro et al, 2000) and as these aren't readily available, the demand for donor tissue greatly outweighs it availability The self-replication and differentiation potential of ES cells has made them an attractive potential source of transplant islet/ß cells for future diabetes therapy Studies on the propagation and differentiation of ES cells into a pancreatic lineage are ongoing (Section 1 4) An understanding of the key factors affecting the differentiation programmes in endocrine pancreatic development will be central to the successful production of a transplantable β/islet cell line (Section 1 2) (Roche et al, 2003)

1 2 Development of the Pancreas

Gastrulation is the process by which the simple structure of the blastula is transformed into the complex, 3-dimensional structure of the basic body plan by a series of cell shape changes and rearrangements It takes place at an early stage in the development of the embryo resulting in the occurrence of the three embryonic germ layers The embryonic endoderm serves as a template for the gastrointestinal tract (duodenum) from which the embryonic pancreas is derived (Edlund, 2001, Trucco M , 2005) In mice the pancreas is derived from two buds occurring on the dorsal and ventral sides of the gut at the junction of the foregut and the midgut at the embryonic age 9 5 days (e9 5) (Watada, 2004) As the gut rotates during development the dorsal and ventral buds branch and eventually fuse to form the vertebrate pancreas (Edlund, 2002, Watada, 2004) The pancreatic buds give rise to the ducts and acinar (exocrine) components of the pancreas The exocrine acinar cells synthesize digestive enzymes, which are secreted into the intestine via branched ductal epithelium (Bishop & Polack, 1991, Madsen et al, 1996) The endocrine progenitors proliferate from the budding ducts and form the differentiated islets of Langerhans (Trucco, 2005) The endocrine pancreas represents approximately 1% of the pancreatic gland (Malaisse, 2005)

The early commitment of the region of the embryonic gut to dorsal and ventral budding is still being studied It is known that transcription factors HLXB9 (homeobox gene HB9), Pdx1, HNF6 (hepatocyte nuclear factor 6) and HNF3β (hepatocyte nuclear factor 3β) are expressed in the region of foregut endoderm committed to pancreatic fate (Soria et al, 2001b) Mice and human lacking Pdx1 do not form a pancreas (Jonsson et al, 1994, Stoffers et al, 1997) However Pdx1 must act downstream of the initial commitment signal as Pdx1 knockout mice ($Pdx^{-/-}$) still form the pancreatic buds and appearance of early pancreatic markers is unaffected (Kim et al, 1997, Edlund, 1999) HLXB9 is similar to Pdx1 in that it is expressed in regions of the gut that commit to pancreatic morphogenesis and its expression is later restricted to ß cells (Li et al, 1999b) Phenotypic comparison of HLXB9 and Pdx1 deficient mice indicate that HLXB9 acts upstream of Pdx1 (Edlund, 1999) Disruption to HLXB9 expression does not

affect Pdx1 expression in the ventral bud but does diminish Pdx1 expression in the dorsal bud as well as preventing dorsal bud morphogenesis (Chakrabarti & Raghavendra, 2003) HNF6 induces HNF3β (also known as foxa2) expression and is also required for neurogenin3 (ngn3) expression (Section 1 2 3 2) (Soria et al, 2001b) Deletion of HNF3β in mice causes disruption in the formation of the endoderm suggesting that HNF3β is necessary for endodermal patterning and subsequent pancreas development The targeted disruption of HNF3β in ES cells or β cells causes a reduction in other β cell transcription factors i e Pdx1, placing HNF3β at an early stage in pancreas development (Lee et al, 2002, Chakrabarti & Raghavendra, 2003)

The developing ventral and dorsal endoderms receive a series of inductive signals from surrounding tissues (Soria et al, 2001b) The dorsal endoderm is in direct contact with the notochord (a flexible structural organ extending over the length of the body) Removal of the notochord from embryonic mice prior to pancreatic fate specification prevents expression of Pdx1 and islet 1 (Isl1) and the dorsal bud does not form Expression of both genes is crucial in dorsal mesenchyme formation (Ahlgren et al, 1997) The ventral bud is unaffected by removal of notochord (Kim et al, 1997) Signals from the notochord such as the release of factors activin -beta B and FGF2 repress expression of Shh, a negative regulator of Pdx1, thus allowing normal morphogenesis (Hebrok et al, 2000) Mesenchymal tissue surrounding the dorsal pancreatic bud expresses Isl1 $Isl1^{-/-}$ mutants fail to form dorsal mesenchyme and do not form the dorsal pancreatic bud However the expression of Pdx1 and beta 2 in $Isl1^{-/-}$ mutants indicates that Isl1 expression and thus mesenchymal factors are not initiator signals for embryonic foregut commitment (Alhgren, 1997)

Ngn3, nkx2 2, beta 2/Neuro d, pax 4 and pax 6 are other transcription factors involved in proliferation and specification of early endocrine pancreas The individual roles of the transcription factors will be discussed in detail in Section 1 2 3

1.2 1 Source of New Islet Cells in Adult Pancreas

The adult pancreas has demonstrated the regenerative ability following tissue loss and injury (Pearson et al, 1977, Bouwens 1998, Trucco, 2005) The source of new islet cells in adulthood is somewhat controversial The theories are

- The existence of a bonafide endocrine progenitor located within or close to the pancreatic ducts Seaberg et al (2004) and Zulewski et al (2001) both isolated what appeared to be pancreatic multipotent precursors (PMPs) from both islet- and duct- derived populations
- Pancreatic ductal cells transdifferentiate into β cells efficiently activated by increased metabolic demand and tissue injury (Bonner-Weir & Sharma, 2002)
- Islet cells arise from stem cells in the blood (Kirschtein and Skirboll, 2001)

1.2 2 Development of Pancreatic Islet Cell Types

The pancreas contains exocrine, endocrine and ductal cell types (Figure 1 2 1), which are all derived from the one region of the gut epithelium in the developing embryo This study is primarily focused on the development of the endocrine pancreas The endocrine islets of Langerhans consist of four cell types organised in a topological order These are (a) glucagons producing α cells (b) insulin producing β cells (c) PP producing γ cells and (d) Som producing δ cells (Trucco, 2005) Knowledge of the development and order of appearance of each cell type is important for implementing this information in deriving replacement islet/β cells for diabetes therapy

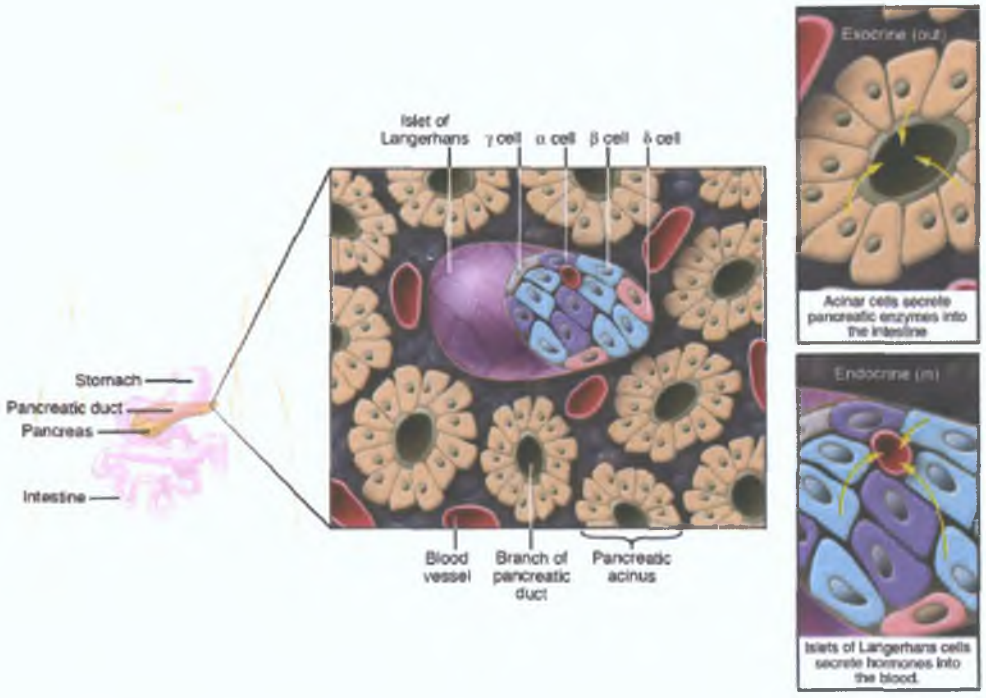


Figure 1.2.1 Cross section of the pancreas (permission to use Figure received from Dr. Massimo Trucco, M.D., University of Pittsburgh School of Medicine)

1.2.2.1 Chronological Appearance of Islet Cell Types

Gittes and Rutter (1992) analysed the transcriptional expression of pancreatic hormones in microdissected mouse embryos. The study indicated that induction of hormone gene expression occurred prior to formation of ventral and dorsal buds. Som RNA (ribonucleic acid) was initially detected followed by insulin and glucagon. PP was not detected until fusion of the dorsal and ventral buds occurs during formation of the pancreas. The appearance of immuno-reactive hormone protein differs in the order of appearance of their mRNA transcripts. Immunohistochemistry analysis indicates that co-expressing insulin and glucagon cells are initially detected (e9.5). All of the insulin cells co-express glucagon but there are also exclusive glucagon expressing cells at this stage (Teitelman et al, 1993). The pancreatic α cells are the first differentiated cells to appear followed by the appearance of β cells. Som is detected at a later stage. The first δ cells differentiate about e14 and PP cells appear shortly before birth (Watada, 2004). The initial belief that insulin-producing cells differentiated from a glucagon precursor was discredited by Herrera (2002). A Cre-loxP system based study helped in identifying cell lineages involved in pancreatic

development and demonstrated that each islet cell type has a different precursor (Kulkarni et al, 2004)

1 2 3 Endocrine Cell Determination through the Expression of Transcription Factors

The differentiation of islet cells is a complex process regulated by multiple transcription factors (Chakabarti & Raghavendra, 2003) Knowledge of the cascades and interrelationships of transcription factors can be implemented in differentiation studies to ascertain which lineage a specific cell is committed to For example the expression of Pdx1 in a differentiating cell population would indicate pancreatic fate A selection of the most significant transcription factors which are strong candidates for specifying lineage commitment are discussed below and their interrelationship is summarised in Figure 1 2 2

1 2 3 1 Pdx1 Expression in the Developing Pancreas

The homeobox transcription factor Pdx1 also known as Ipf1, Stf1 and Idx1 was initially identified in mouse β tumour cells in 1991 (Ohlsson et al, 1991) and cloned in 1993 Pdx1 is synthesised in the early rudiment that comprises the pancreatic buds as well as the surrounding gut epithelium The gut epithelium destined to dorsal pancreas commitment receives signals from the notochord to repress Shh expression, a negative regulator of Pdx1, thus facilitating Pdx1 expression (Section 1 2) (Hebrok et al, 2000) Pdx1 expression becomes restricted to β cells as the pancreas matures (Soria et al, 2001b)

A number of studies have identified Pdx1 as a crucial regulator of pancreatic development In 1994, Jonsson et al demonstrated that transgenic mice homozygous for a targeted Pdx1 gene mutation lacked a pancreas In 1997, Stoffers et al attributed pancreatic agenesis (lack of pancreas) in a patient to a homozygous point mutation in the Pdx1 coding region, which resulted in a lack of functional Pdx1 expression In 1998, Ahlgren et al employed the Cre-Lox system to disrupt Pdx1 in β cells Pups were found to be normal at birth but became diabetic within 5 months Subsequent analysis revealed that they had 60% less insulin-positive cells than normal animals and 17% of these cells co-expressed Pdx1 Further investigation revealed that mutant pups had a greater

number of α cells than normal pups Insulin/glucagon co-expressing cells were also found in mutant pups Using clonal populations of the rat INS-1 cell line, Wang et al (2001a) demonstrated that elimination of Pdx1 in the transitory INS1αβ population promoted α cell differentiation Overexpression of brain-4 (an α cell differentiation marker) did not display a similar effect The study concluded that loss of Pdx1 function is a prerequisite for α differentiation Pdx1 is proposed as an α versus β cell fate determinant

Expression of pancreatic genes in particular β cell genes are dependent on Pdx1 expression level Pancreatic glucose transporter (glut2) expression was nonexistent when Pdx1 was knocked out of β cells (Alhgren et al, 1998) The overexpression of Pdx1 in INS1β cells promoted expression of genes encoding insulin, IAPP, glut2 and nkx6 1 (Wang et al, 2001a) In 2004, Miyazaki et al used an ES cell line in which endogenous Pdx1 expression was regulated by a Tet-off system to examine the effects of Pdx1 expression during *in vitro* EB differentiation Pdx1 expression enhanced expression of insulin II, Som, kir6 2, glucokinase (gck), ngn3, p48, pax 6, prohormone convertase 2 (PC2) and HNF6 genes in resulting differentiated cell types All are associated to pancreas development

Through monitoring Pdx1 expression it has been observed that co-expression of other transcription factors is often necessary to mediate the effects of Pdx1 For example transfection experiments demonstrate that Pdx1 can stimulate insulin gene expression but in many cases the co-expression of beta 2 is necessary to do so (Serup et al, 1996, Madsen et al, 1997) Given the number of transcription factors involved in pancreatic development it is more than likely that Pdx1 mediates its effects though the co-operation of the other factors (Figure 1 2 2)

1 2 3 2 Neurogenin 3 Expression in the Developing Pancreas

Ngn3 is a member of the basic helix-loop-helix (bHLH) family of transcription factors Ngn3 is expressed in both the developing pancreas and in neural cells Ngn3 is initially expressed at the time of pancreatic budding (Watada, 2004) Expression of the pro-endocrine ngn3 is both necessary and sufficient to drive progenitor cells in the developing pancreas to an endocrine fate Gradwohl et al

(2000) demonstrated that ngn3-null mice failed to generate any pancreatic endocrine cells and died shortly after birth from neonatal diabetes The expression of Isl1, pax 4, pax 6 and Neuro D was lost in these mice placing ngn3 upstream of these targets (Chae et al, 2004) Ngn3 initiates the islet differentiation program but its expression is diminished before terminal differentiation of endocrine cells Smith et al (2005) concluded that ngn3 functions as a transcriptional activator, initiating a cascade of gene expression i e nkx2 2, pax 4 events leading to endocrine differentiation However it represses its own expression in doing so

1 2 3 3 Beta 2 Expression in Developing Islet Cells

Neuro D/beta 2 is a bHLH transcription factor implicated in insulin gene expression and islet cell differentiation (Naya et al, 1997) It is also known to play a role in terminal differentiation of neurons (Lee et al, 1995)

Beta 2 mRNA is initially expressed in the mouse at e9 5 and continues to be expressed in β cells throughout maturation (Chae et al, 2004) Beta 2 is a downstream target of ngn3 This is based on the knowledge that the neurogenin family regulates protein expression of the Neuro D family during neurogenesis and overexpression of ngn3 in stable transfected endocrine cell lines activates endogenous levels of beta 2 mRNA Ngn3 deficient mice also fail to express beta 2 (Huang et al, 2000, Gradwohl et al, 2000) Pax 6 and pax 4 are both likely downstream targets of beta 2 within pancreatic endocrine development (Chae et al, 2004) Within the β cell the insulin gene was the first identified target of beta 2 (Naya et al, 1995) Beta 2 heterodimerizes with ubiquitous bHLH proteins of the E2A family to regulate insulin gene transcription More recently a number of β cell target genes have been identified namely sulfonylurea receptor 1 (SUR1), islet-specific glucose-6-phosphate (G6Pase) catalytic subunit related protein (IGRP), and β-glucokinase (βGK) (Chae et al, 2004, Huang et al, 2000)

Newborn beta 2 null mice fail to form islets and die from hyperglycaemia within 5 days from birth There is a significant decrease in the β cell number, as well as a reduction in α cells Typically in normal islets, α cells surround β cells, but in

beta 2 null mice this is not the case i e α and β cells are clustered separately along the ductal structure (Naya et al, 1997)

1 2 3 4 Islet 1 Expression in Developing Islet Cells

Isl1 is a LIM homeodomain factor expressed in the embryonic mesenchyme (surrounding the dorsal bud) and in all endocrine islet cell types (Chakrabarti & Raghavendra, 2003) Isl1 appears in glucagon positive cells at e9 5, prior to the appearance of differentiated insulin and Som expressing cells (Gradwohl et al, 2000) Ahlgren et al (1997) used mice engineered with a disrupted Isl1 gene to demonstrate independent requirement of Isl1 in formation of pancreatic dorsal mesenchyme and islet cells The low level of Isl1 expression in β cells would suggest it doesn't have a role in the regulation of insulin gene transcription (Habener et al, 2005) The importance of mesenchymal secretion has previously been discussed in Section 1 2

1 2 3 5 Pax Gene Expression in Developing Islet Cells

Pax genes are members of the paired homeobox transcription factor family They are similar to homeobox factors with the exception that they contain an additional paired domain (DBD) This additional DBD may increase specificity for promoter binding (Chakabarti & Raghavendra, 2003) Previous studies have demonstrated the necessity for pax activity in development of the eye, brain, kidney, thyroid gland, immune system and pancreas (Dohrmann et al, 2000) Both pax 4 and pax 6 are involved in pancreatic islet formation They appear to be strong candidates for specifying endocrine lineage differentiation (Chae et al, 2004, Chakabarti & Raghavendra, 2003)

Pax 6 expression is detected during development of the dorsal and ventral buds (approx e9 0) and its expression becomes restricted to the 4 islet cell types in newborn animals *Small eye*^{Neu} mice (mice with a spontaneous mutation of the pax 6 gene) have disorganised islets and a reduced number of α, β, δ and PP cells (Sander et al, 1997) Knockout pax 6 mice die within minutes of birth and fail to form islets (St Onge et al, 1997) Vetere et al (2003) demonstrated that transfection of ngn3 into RA-derived-endoderm F9 cells induced expression of

pax 6, whilst Marsich et al (2003) proposed that Neuro D/beta 2 activates the pax 6 gene

In contrast to pax 6, pax 4 is only required in cells destined to β and δ cell fate Pax 4 is initially expressed in pancreatic precursor cells and becomes restricted to β and δ cells prior to being turned off at birth (Chae et al, 2004) Mice deficient for pax 4 fail to develop insulin-producing β cells and Som producing δ cells within their pancreas The mice have an abundance of α cells (Sosa-Pineda et al, 1997) Pax 4 expression commits endocrine precursors towards a β and δ cell fate whereas pax 6 expression promotes α cell specification (Chakrabarti & Raghavendra, 2003) Studies by Gradwohl et al (2000) and Smith et al (2003) supported the hypothesis that ngn3 functions as an upstream activator of pax 4 Gradwohl et al demonstrated that the expression of Neuro D, pax 6 and pax 4 is lost in ngn3 deficient mice, whereas Smith et al demonstrated that expression of both ngn3 and HNF1α is necessary for activation of the pax 4 gene in NIH3T3 fibroblast cells Neuro D/beta 2 has also been proposed as a candidate upstream activator of pax4 but binding of Neuro D/beta 2 to the pax 4 promoter has not been confirmed (Chae et al, 2004) In a study by Blyszczuk et al (2003), the constitutive expression of pax 4 combined with histotypic cultivation drove ES cell differentiation along a pancreatic lineage, leading to the formation of insulin secreting spheroids These islet-like spheroids normalised blood glucose levels when transplanted into streptozotocin-treated diabetic mice Wang et al (2004) suggested that the role of pax 4 in β cell differentiation is mediated via interaction with nkx2 2

1 2 3 6 Nkx Gene Expression and Islet Cell Determination

The nkx factors are homeobox transcription factors containing a conserved ten ammo acid sequence (the NK decapeptide) that interacts with transcriptional co-repressors Nkx2 2 and nkx6 1 play crucial roles in islet cell formation particularly β cell lineage differentiation (Chakrabarti & Raghavendra, 2003)

The initial expression of nkx2 2 coincides with the dorsal pancreatic bud evagination at e9 5 The majority of pancreatic epithelial cells express nkx2 2 however expression of the gene becomes more restricted to a subset of ngn3

expressing islet precursors Unlike ngn3, nkx2 2 continues to be expressed in the mature endocrine cells α, β and PP cells (Watada, et al, 2003, Habener et al, 2005) Nkx2 2 is a downstream target of ngn3 Neuro D/beta 2 may act to maintain nkx2 2 expression after the disappearance of ngn3 (Watada, 2004, Smith et al, 2005) Mice deficient for nkx2 2 have no insulin-producing β cells and have a reduced number of α and PP cells There is a population of incompletely differentiated cells within these mutants, which express some β cell markers i e Pdx1 and IAPP but do not express other definite β cell markers i e nkx6 1 (Sussel et al, 1998)

Expression of nkx6 1 is widely distributed in the pancreas until e10 5 but specifically expressed in β cells by the end of gestation (Chakrabarti & Raghavendra, 2003) Mice with a targeted disruption of the nkx6 1 gene form islets with minimal to absent β cell content, whereas all other cell types within these islets develop normally (Sander et al, 2000)

Expression of nkx2 2 immediately precedes that of nkx6 1 in the developing pancreas and it appears to be a requirement for the maintenance of nkx6 1 expression (Sussel, et al, 1998, Sander et al, 2000) Both nkx6 1 and nkx2 2 play crucial roles in the final differentiation of β cells The insulin promoter contains binding sites for both nkx genes leading to speculation that these factors may cause β cell differentiation through direct activation of insulin in precursor cells (Chakrabarti & Raghavendra, 2003)

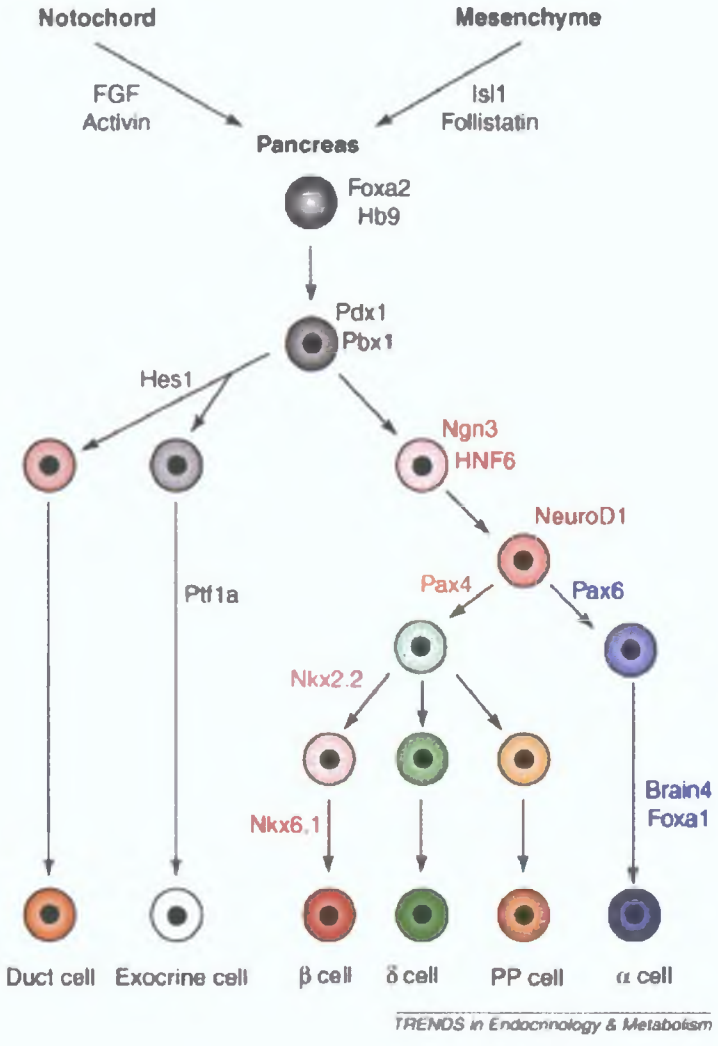


Figure 1.2.2 Model for the role of transcription factors during cellular differentiation within the developing pancreas. Data was accumulated from transgenic and knockout mice, as summarized within the previous text. The positioning of each transcription factor is based on the predominant timing of its function and not necessarily the timing of its synthesis. Although some factors act at more than one point during differentiation, for simplicity a single point is indicated for each (permission to use Figure received from Dr. Raghu G. Mirmira, MD, PhD, University of Virginia Diabetes Center).

1 3 The Islet β Cell

The primary function of β cells to produce insulin was first recognised in 1922 in studies carried out on dogs (Banting & Best, 1990) β cells are located adjacent to blood vessels These cells quantitively synthesize and secrete bioactive insulin in response to nutrients, hormones and nervous stimuli Blood glucose level is the primary modulator of insulin secretion Insulin facilitates the uptake of glucose into the tissues of the body In adjusting the insulin production in response to blood glucose concentrations β cells maintain glucose levels within a tight physiological range allowing for optimal functioning of all tissues in the body (Kirschstein & Skirboll, 2001, Kulkarni, 2004) As previously discussed in Section 1 2 the differentiation to pancreatic β cells is quite complex Furthermore, within the β cell there is a highly regulated complex network controlling cell function

1 3.1 The Insulin Gene

Insulin mRNA is initially expressed in the mouse embryo at e9 0 in the foregut from which the pancreas originates (Gittes & Rutter, 1992) In postnatal life it is widely accepted that insulin gene expression is restricted to β cells although there is some evidence of insulin expression in the central nervous system (CNS) and in the retina (Alpert et al, 1988, Devaskar et al, 1993, Budd et al, 1993) The human insulin gene is located on the short arm of chromosome 11 whereas the two mouse insulin genes are located on individual chromosomes i e Insulin I on chromosome 19 and insulin II on chromosome 7 The ancestral rodent insulin II and human insulin genes contain three exons whereas rodent insulin I lacks the second exon (Wentworth et al, 1986, Melloul et al, 2002)

1 3 1 1 Regulation of Insulin Gene Transcription

Tissue specific regulation is controlled by 5' flanking regulatory regions of the insulin gene Insulin genes share a number of conserved DNA motifs within the 5' flanking region suggesting that they may be regulated by similar factors (German et al, 1995) Deletion and mutational analysis of various insulin promoter regions in transgenic mice and transfected cell lines and have led to the identification of critical cis-acting elements in insulin gene transcription

Positive and negative regulation of insulin gene transcription relies on the interaction of sequence motifs in the promoter with specific transcription factors (Figure 1 3 1) These interactions also determine the inducibility of the insulin gene by physiological stimuli i e glucose, glucagon-like peptide 1 (GLP-1) (Melloul et al, 2002)

Systematic mutagenesis of the rat insulin I promoter and transfection studies led to the identification of two regulatory sequences within the 5' region namely E1 and E2 Disruption of these E boxes led to abolishment of transcriptional activity (Karlsson et al, 1987, Philippe, 1991) E boxes bind bHLH proteins, which function as transcriptional activators of tissue specific genes by forming heterodimers between ubiquitous and cell restricted members (Section 1 2 3 3) For example IEF1 is a heterodimer of the ubiquitous E2A gene product(s) E47 and beta 2, which binds to the insulin promoter activating transcription

AT rich A boxes (A1-A5) are additional regulatory elements within the insulin promoter These A boxes with the exception of A2 contain a core TAAT sequence, which binds homeodomain transcription factors i e Isl1 and HNF1 (Melloul et al, 2002, Chakrabarti & Raghavendra, 2003) Pdx1 is known to directly bind to the A box elements and leads to the activation of the insulin promoter in conjunction with other transcription factors (Peshavaria et al, 1997) Pdx1 is present in about 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 transactivates the insulin gene, gck, glut2 and IAPP (Mac Farlane et al, 1999, McKinnon & Docherty, 2001, Serup et al, 1996, Melloul, 2002) β cell specific inactivation of Pdx1 results in fewer insulin positive cells and reduced insulin and glucagon expression causing diabetes in mice (Ahlgren at al, 1998)

Hedgehog signalling has previously been discussed in relation to pancreatic development (Section 1 2) but it also appears to play a role 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).

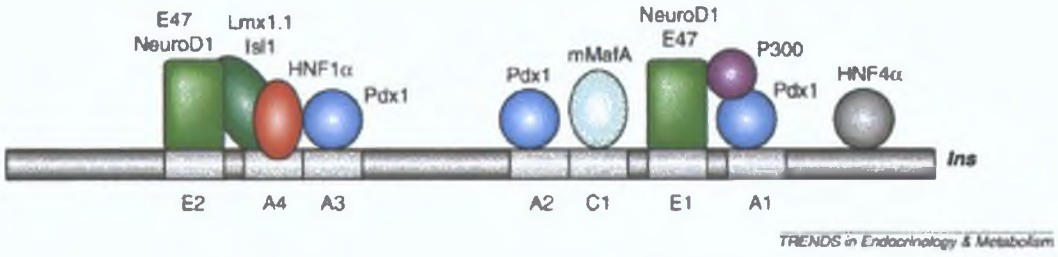


Figure 1.3.1 Major transcription factor complexes that activate the insulin promoter in β cells (permission to use Figure received from Dr. Raghu G. Mirmira, MD, PhD, University of Virginia Diabetes Center).

1.3.1.2 Glucose Regulation of Insulin Gene Expression

Glucose homeostasis requires tight regulation of insulin production and secretion. Glucose must be metabolized within the ß cell to stimulate insulin release. The first indication of a glucose effect on the insulin gene was reported over 35 years ago (Jarret et al, 1967) and since then the complexity of molecular elements regulating this effect has been studied (Melloul et al, 2002). It was initially believed that the short-term effects of glucose were predominately at a post-transcriptional level i.e. stabilisation of insulin mRNA. In 1998, Leibiger et al demonstrated that short-term exposure of HIT-T15 ß cells to stimulatory levels of glucose led to significant increases in insulin gene transcription. Direct interaction of glucose with the insulin enhancer through the Z element of the 5' flanking region of the human insulin gene has been observed (Sander et al, 1998).

Inducible transcription of the insulin gene appears to depend on the same regulatory sequences within the promoter/enhancer region that control the cell tissue specificity of the insulin gene. Thus glucose responsiveness is mediated by cis-elements within the 5' flanking region of the gene (Section 1.3.1.1). It appears that glucose regulates a number of transcription factors interacting with

the insulin promoter For example it has been shown that extracellular glucose concentrations regulate the binding of Pdx1 to the A3 element within the 5' region of the insulin gene (Melloul et al, 2002) Glucose also effects insulin gene transcription through the action of cyclic adenosine monophosphate (cAMP) response elements Glucose and other hormones such as GLP-1 increase intracellular cAMP (Melloul et al, 2002, Charles et al, 1975, Nielsen et al, 1985, Hammonds et al, 1987, Inagaki et al, 1992) There are 4 cAMP responsive elements (CRE) within the insulin gene contributing to cAMP-inducibility of insulin transcription (Inagaki et al, 1992) The interaction of cAMP with these elements is mediated by a CRE binding protein (CREB) Thus cAMP could almost be described as a form of second messenger for glucose activation of the insulin gene (Melloul et al, 2002)

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

1 3 1 3 GLP-1 Regulation of Insulin Gene Expression

GLP-1 is a physiological incretin hormone secreted from enteroendocrine $_{L}$–cells of the intestine GLP-1 is derived from post-translational processing of proglucagon and is released from the gut in response to nutrient ingestion GLP-1 is known to stimulate insulin gene transcription, expand β cell mass and augment GSIS The effect of GLP-1 on insulin gene expression is mediated via the CRE in the promoter region of the insulin gene (Section 1 3 1 2) The effects of GLP-1 on ß cell function are in part attributed to increases in cAMP concentration with concomitant activation of protein kinase A (PKA) (Melloul et al, 2002) The effects of GLP-1 on insulin transcription and cell proliferation have also been attributed to an upregulation of Pdx1 (MacDonald et al, 2002) Pdx1 translocation to the nucleus in RIN 1046-38 cells has shown to be dependent on cAMP/ PKA (Wang et al, 2001b)

1.3.2 Insulin Biosynthesis and Processing

The insulin mRNA is translated as a single chain 11.5 kDa precursor preproinsulin (PPI). The removal of PPI's signal peptide during insertion into the endoplasmic reticulum generates proinsulin. The proinsulin (9 kDa) consists of three domains: an amino terminal B chain, a carboxyl terminal A chain and a connecting peptide in the middle known as the C-peptide fragment. Disulphide bond formation occurs within the Golgi apparatus. Proinsulin is then passed through the Golgi cisterne to the trans-Golgi network (TGN) where it is packaged into immature secretory granules. The C-peptide aligns the proinsulin for cleavage to mature insulin (Howell, 1991; Docherty, 1997). The immature secretory granules can either form part of a constitutive pathway or regulated pathway of protein secretion. If constitutively secreted, the immature granules are transported immediately to the β cell membrane and a mixture of insulin and proinsulin is released. During regulated secretion the proinsulin is stored in secretory granules where it is converted to mature insulin via the action of endoproteases and carboxypeptidase (Figure 1.3.2). The endoprotease PC2 and PC1 (prohormone convertase) are enzymes that are restricted to neuroendocrine cells (Smeekens et al, 1991; Bennet et al, 1992; Docherty et al, 1997). PC1 cleaves the sequence at the B-chain C-peptide junction and PC2 cleaves at the A-chain C-peptide junction. These two intermediate molecules are converted to mature insulin and C-peptide by carboxypeptidase action (Figure 1.3.3). Insulin (6 kDa) and C-peptide (3 kDa) is released from the β cell in eqimolar amounts by exocytosis upon stimulation of insulin secretion by glucose. The large concentration of protein/hormone in the storage granules facilitates the secretion of large amounts upon stimulation by a secretagogue (Halban & Irmanger, 1994).

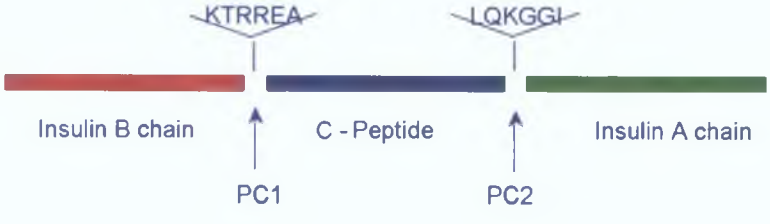


Figure 1.3.2 Cleavage Points for PC1 and PC2

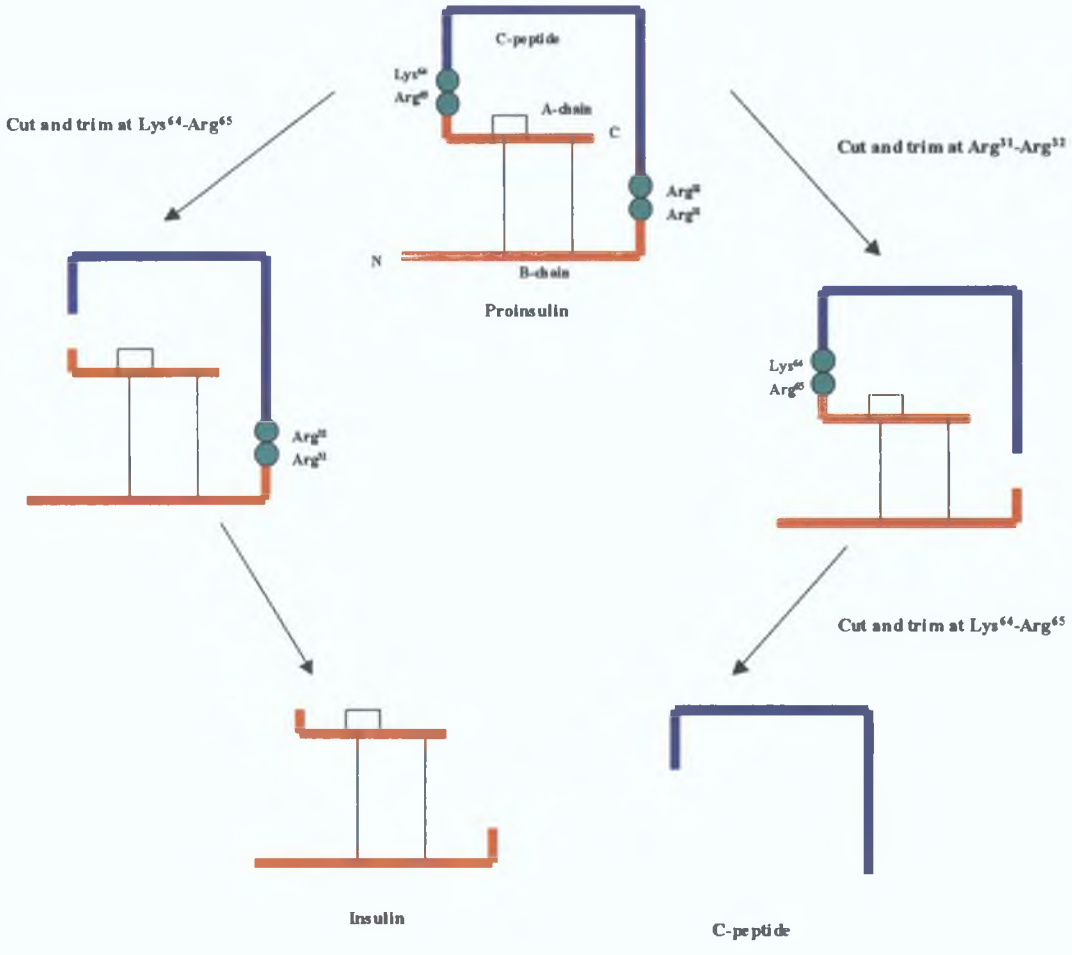


Figure 1.3.3 Processing of proinsulin to insulin. Following initial cleavage by either PC1 or PC2, trimming of the residual C-terminal residues is carried out by the action of ubiquitously expressed carboxypeptidases.

1.3.3 Glucose Sensing in the β Cell

To correctly control blood glucose levels, the β cell must be able to accurately sense/determine how much glucose is present in the blood. The glucose level is determined from the metabolism of the blood glucose and the subsequent changes in blood metabolites (Ashcroft, 1980; German, 1993). GSIS requires that the metabolism of glucose can be increased within the β cell over the entire physiological range of glucose (Marshall et al, 1993; Ishihara et al, 1994). The most important components of the glucose sensor are (I) glut2, which facilitates rapid glucose uptake regardless of the extracellular sugar concentration and (II)

glucokinase (gck), the activity of which is the rate-limiting step for glucose metabolism (Schuit et al, 2001).

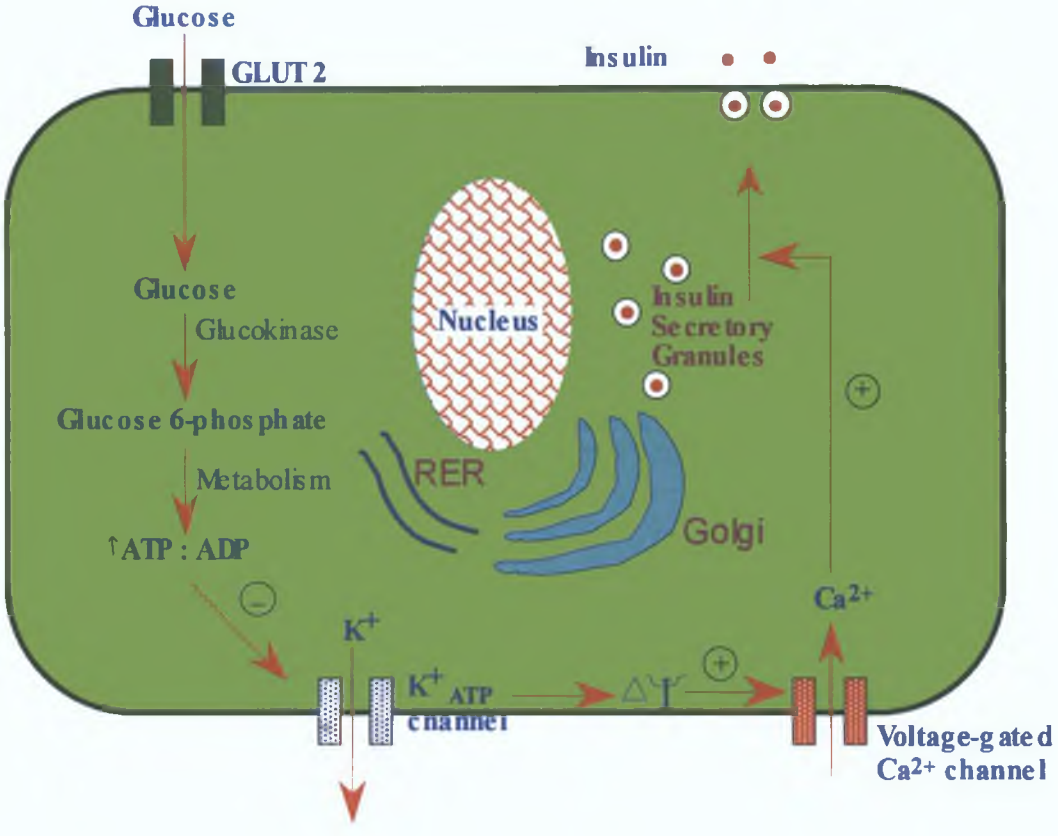


Figure 1.3.4 Glucose stimulated secretion from ß cells depends on the presence of correct glucose sensing, insulin storage and regulated secretion pathways.

The relative importance of the two components of the glucose sensor were compared by the overexpression of their low K_m counterparts i.e. glut1 and hexokinase I in the MIN6 cell line. Expression of hexokinase I increased glucose utilisation and release at concentrations below 10 mM. Glut1 expression had no effect on GSIS (Ishihara et al, 1994). Patients with maturity onset diabetes of the young (MODY) exhibit impaired GSIS due to a point mutation in the gck gene. A similar disruption of gck gene in transgenic mice causes the same phenotypic effect (Bali et al, 1995). It has also been demonstrated that mutations in the glut2 gene abolishes glucose transport in a patient with non-insulin dependent diabetes mellitus (NIDDM) while the expression of glut2 antisense has led to diabetes in transgenic mice (Mueckler et al, 1994; Valera et al, 1994). The presence of gck is

the main rate limiting glucose sensor but a combination of glut2 and gck is required for effective glucose sensing For example MIN7 insulinoma cells (isolated at same time as MIN6) were found to be glucose insensitive due to a combination of high levels of hexokinase I and Glut1 (Miyazaki et al, 1990)

1 3 4 Stimulated Insulin Secretion from β Cells

Insulin injections do not provide the minute-to-minute control of blood glucose level and are therefore not efficient at maintaining glucose homeostasis In contrast, β cells use highly regulated secretion pathways to provide a proportional release of stored insulin in response to the blood glucose level

1 3 4 1 Regulated Pathways of Protein Secretion

The majority of secretory proteins from eukaryotic cells originate in the RER and are then transferred to the Golgi apparatus The TGN plays a crucial role in directing proteins in the secretory pathway to the appropriate cellular destination The proteins may enter a constitutive pathway of secretion or a regulated secretion pathway (Kelly, 1985, Traub & Kornfeld, 1997)

Secretion via the regulated pathway allows the release of protein to be controlled by 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 earliest form of granule is clathrin coated The bulk of prohormone conversion occurs as the clathrin-coated granule matures The clathrin coat is eventually lost Secretion via 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) When cDNA encoding proinsulin was transfected into a mouse cell line secreting adrenocorticotropic hormone (ACTH) via the regulated pathway, storage, processing and secretion of the proinsulin upon stimulation occurred (Moore *et al*, 1983, Motoyoshi *et al*, 1998) Thus, it seems that regulated proteins such as proinsulin have structural determinants, which cause them to be sorted in to the regulated secretion pathway (Halban & Irminger, 1994, Traub & Kornfeld, 1997)

1 3.4 2 Glucose Stimulated Insulin Secretion

Insulin secretagogues can be divided into two groups i e initiators and potentiators Initiators can stimulate secretion on their own where as potentiators can enhance insulin secretion in the presence of an initiator Glucose is the most important initiator of insulin secretion ß cells are sensitive to changes in glucose from approx 5-8 mM to 20 mM (Knaack et al, 1994, Poitout et al, 1996, Best, 2000) i e there is no glucose stimulation below 5 mM and maximum stimulation insulin secretion occurs at 20 mM approximately

Knowledge of the mechanisms involved in insulin stimulus-secretion has increased remarkably in the last 15 years Studies have demonstrated 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 knock-on effects from glucose metabolism Increased blood glucose leads to increased glucose metabolism, determining the extent of insulin secretion Increased glucose metabolism results in an increase in the ATP/ADP (adenosine triphosphate/adenosine diphosphate) ratio within the β cell causing the closure of ATP sensitive potassium channels on the cell membrane Once these channels are closed membrane depolarisation occurs i e the cell becomes less negative a result of the build up of K^+ ions Membrane depolarisation results in the opening of the voltage gated Ca^{2+} channels in the β cell membrane, thus allowing Ca^{2+} ions to flow into the cell This increase in intracellular calcium results in translocation of the insulin storage granules to the cell membrane and facilitates release of stored insulin In resting β cells the K^+ channels are open allowing a steady flow of K^+ ions out of the cell, holding the membrane potential of the cell at a negative level of approx –70 mV(Ashcroft & Gribble, 1999, Best, 2000, Howell, 1991, Ullrich et al, 1996) See Figure 1 3 4

Evidence for an alternative Ca^{2+} independent mode of secretion has been also been reported Glucose has been shown to augment insulin secretion in Ca^{2+} - depleted rate islets in the absence of both extracellular Ca^{2+} and a rise in $[Ca^{2+}]_i$ This augmentation effect operates best when PKA and PKC are maximally activated in the ß cell (Komatsu et al, 1997, Bratanova-Tochkova et al, 2002) In MIN6 cells it was observed that adenylate cyclase activator forskolin (increases

intracellular cAMP) caused an increase in insulin secretion without an increase in intracellular calcium This and other observations involving glucose, leucine and arginine in Ca^{2+} -free systems indicate that there is another mechanism of secretion that involves cAMP and cellular alkinalization (Sakuma et al, 1995)

1 3 4 2.1 Factors affecting GSIS

The response of ß 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 ß cells is heterogeneous due to differing ß 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 islets 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 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) Homologous contact between ß cells rather than with other islet cell types is critical to effective GSIS (Bosco et al, 1989) Gap junctions are cell-to-cell channels, which allow direct transfer of ions and metabolites e g cAMP, calcium between cells Channels are formed by the interaction of hemichannels on one cell with the open channel of another (Willecke et al 1991)

1 3.4 2 2 The Effect of GLP-1 on GSIS

GLP-1 binds to a G protein-coupled receptor on ß-cells to stimulate insulin gene transcription, GSIS and ß cell growth (Section 1 3 1 3) GLP-1 is proposed to modulate GSIS by regulating the activity of the ion channels involved in depolarization of the ß cell membrane It has been observed that GLP-1 inhibits ß cells K_{ATP} channels (previously referred to as ATP sensitive potassium channels in Section 1 3 4 2) resulting in membrane depolarization triggering Ca^{2+} influx and subsequent exocytotic release of insulin The inhibitory effect of GLP-1 on K_{ATP} channels is thought to be cAMP/PKA-dependent (MacDonald et al, 2002, Melloul et al, 2002)

1 3 4 2.3 MIN6 Insulinoma Cell Line as a ß cell Model to Study GSIS

The potential use of replacement β cells/islets as a cell therapy for type 1 diabetes relies on the ability to culture such cells/islets *in vitro,* whilst maintaining their functional status The GSIS phenotype is relatively unstable in long-term culture and current research is focused on understanding the factors affecting functional GSIS The MIN6 cell line is an attractive model for studying phenotypic and genomic changes associated with the loss of GSIS in culture

MIN6 cells, originally isolated from insulinomas generated within SV40 T antigen-expressing mice and shown to share many characteristics with freshly isolated β cells (Miyazaki et al, 1990), have been described as a rare example of a transformed mouse β cell line that has retained many relevant aspects of a β cell, including GSIS (Lilla et al, 2003) MIN6 cells are also known to express glucagon, Som and IAPP (Ohgawara et al, 1995, Kanatsuka et al, 1992)

The reported stability of MIN6 in culture varies MIN6 cells have been characterised as being stable for 30-40 passages *i e* with an intact GSIS response MIN6 can exhibit an increase in insulin secretion of almost 10-fold upon stimulation with 25 mM glucose (Miyazaki et al, 1990) Other researchers, however, have noted only moderate GSIS responses (approximately 1 2 fold) from MIN6 cells by passage 16 (Kayo et al, 1996)

In a study of low/early passage (passage 17-19) MIN-6 cells, Webb et al (2000) investigated expression profile of β cells exposed to high (25 mM, continuously) and low (5 5 mM, for 24 hours) glucose to identify genes involved in insulin secretion (using the Affymetrix Mu6500 oligonucleotide array) The two largest clusters of genes differentially expressed between low and high glucose conditions were those encoding secretory pathway components and enzymes of intermediary metabolism It must be considered that optimised conditions for culturing β cells are typically supra-physiological *i e* MIN6 – 25 mM (Miyazaki et al, 1990, Lilla et al, 2003, O'Driscoll et al, 2004a) Following isolation of glucose-responsive (m9) and a non-responsive (m14) MIN6 clonal populations at passage 18, Minami et al (2000) reported 10 genes (from 216 combinations of

anchored and arbitrary primers) to be differentially expressed, using differential display techniques These included gck (involved in glucose phosphorylation, levels in m9>m14), stanniocalin/STC (involved in calcium regulation, levels in m9>m14), and delta-like homologue 1 (dlk) /Pref-1 (involved in β cell differentiation and growth, levels in m9>m14) Other gene transcripts, including glut2, were unchanged Two clonal populations of MIN6 (β1 responsive to insulin and other secretagogues, C3 non-responsive) were isolated by Lilla et al (2003), using the same approach, and differentially expressed genes were identified using suppression subtractive hybridisation techniques and oligonucleotide (Affymetrix, MG-U74) microarrays Genes clustered as being involved in metabolism, intracellular signalling, cytoarchitecture, and adhesion were identified as being of potential interest MIN6-β1 cells, however, tend to lose their GSIS beyond approximately 30 passages also (V Lilla, pers comm)

1 3 4 2 3 1 Loss of GSIS in the MIN6 maybe attributed to De-differentiation

Loss of GSIS is accompanied with a decrease in proinsulin processing enzymes PC1 and PC2 with a concommitment increase in furin that was linked to MIN6 adopting a de-differentiated state with increasing passage number (Kayo et al, 1996)

1 4 Advances in Stem Cell Therapy for Diabetes Mellitus (DM)

Stem cell therapy for DM implies the replacement of diseased or lost functional insulin-producing pancreatic cells, with pancreas or islet-cell transplants As previously stated (Section 1 1 8) there is a shortage of donor organs, which has spurred on the current research into alternative means of generating β-cells/islets Table 1 4 1 summarizes the studies to date that have reported the generation of ES-derived insulin-expressing cell types

1 4 1 Protocols Used in the Derivation of Pancreatic Phenotypes from Mouse ES Cells

A number of studies reporting the derivation of insulin-secreting structures from mouse ES cells have been published within the last 6 years Some of the more interesting findings are discussed below

In February 2000 Bernat Soria and his research team derived insulin-secreting cells from R1 mouse ES cells that normalised glycemia in streptozotocin induced diabetic mice Researchers used a cell trapping technique that involved transfecting the mouse ES cells with a construct, which coupled the regulatory region of the human insulin gene to a neomycin resistance gene i e those cells which expressed insulin would also express neomycin resistance This chimeric gene was also fused to the hygromycin resistance gene (pGk-hygn) to allow for the selection of transfected cells The hygromycin resistant mouse ES cells were grown in suspension culture in medium lacking supplemental LIF The differentiated cells underwent a final maturation step based on the use of nicotinamide (NIC) and non-stimulatory glucose concentrations Over 3 weeks the insulin content of the cells increased 20 fold and the overall yield of glucose responsive insulin-positive cells was high Clusters of the resulting clone were transplanted into the spleen of streptozotocin induced diabetic mice The mice corrected hyperglycemia within 1 week and restored body weight within 4 weeks 12 weeks after transplantation 40% of the mice developed hyperglycaemia and it was thought that this was due to the short lifespan of the transplanted cells Whilst further studies were needed to characterise the clones developed by Soria et al, the study was an encouragement to other scientists in

the pursuit of a diabetes therapy using ES cells (Soria et al, 2000, Soria et al, 2001a)

In 2001, Lumelsky et al used a 5-stage protocol based on the enrichment of ES derived nestin-positive cells to yield islet-like clusters containing 15% insulin-positive cells Glucagon, Som and PP expressing cells were also present in these clusters *In vitro* the clusters demonstrated GSIS whilst *in vivo* the clusters managed to extend the lifespan of diabetic mice but had no effect on hyperglycaemia (Lumelsky et al, 2001) In 2002, a study using a modified version of the Lumelsky protocol (addition of a P13K inhibitor i e LY294002) gave rise to structures similar in topology to the pancreatic islets of Langerhans These cells produced insulin at levels greater than previously reported and completely rescued survival in diabetic mice, whilst improving glycemic control (Hori et al, 2002) Both Miyazaki et al (2004) and Blyszczuk et al (2003) modified the Lumelsky protocol by introducing transcription factors (i e Pdx1 and pax 4) to generate pancreatic-phenotypic cells demonstrating variable insulin release

Kahan et al (2003) used a different approach to demonstrate the ability of ES cells to differentiate into pancreatic and islet lineage restricted stages (Section 1 1 6 1) ES cells grown and differentiated in non-selective medium containing serum gave rise to differentiated cell types individually expressing insulin, glucagon, Som and PP Antibodies that specifically distinguish between C-peptide cleaved from proinsulin I and proinsulin II were used to determine whether differentiated cell types were producing insulin I and insulin II

1 4 2 Protocols Used in the Derivation of Pancreatic Phenotypes from Human ES Cells

The techniques used to differentiate human ES cells towards pancreatic cell types are somewhat different than those used for murine ES cells In 2000 Schuldiner and his colleagues carried out a study to look at the effects of eight growth factors (GFs) on the differentiation of human ES cells (H9 clone 1) The growth factors tested were as follows bFGF, transforming growth factor β1 (TGF-β1),

activin A (AA), BMP-4, hepatocyte growth factor (HGF), epidermal growth factor (EGF), β nerve growth factor (βNGF) and RA 5-day old EBs (in suspension) were tested for the presence of the growth factor's respective markers to ensure that the growth factors could have an effect on the human ES cells As expected with pluripotent cells a broad range of receptors for the growth factors was detected EBs were then dissociated and plated After a 10 day exposure to the GFs, differentiated cells became more homogeneous and resembled specific cell types Reverse transcriptase polymerase chain reaction (RT-PCR) gene expression results indicate that treatment with NGF and HGF activates transcription of a variety of liver and pancreas restricted genes during differentiation of ES cells Both untreated EBs and those treated with NGF expressed Pdx-1 (gene that controls insulin transcription and is associated to the formation of ß cells) Prior to the formation of EBs Pdx-1 was not detectable These results suggested that (a) islet cells maybe one of the cell types present after spontaneous differentiation into EBs and (b) NGF may be a significant signalling factor in directing differentiation towards β cells (Schuldiner et al, 2000)

A study by Assady and colleagues (2001) complemented the above findings by further characterising insulin-producing cells in EBs The researchers allowed human ES-H9 cells to spontaneously differentiate *in vitro* in both adherent and suspension cultures Immunohistochemistry using anti-insulin antibody indicated that 1 – 3% of the cells present in EBs were insulin producing β-cells Islet specific gck and glut2 mRNAs, which play important roles in β cell function, were detected after but not before differentiation Although the investigators did not demonstrate glucose responsiveness, they did show that cells cultured in glucose containing medium secrete insulin into the culture medium Evidently EBs contained a subset of cells that have many of the characteristics of a functioning β cell

In 2004, Segev et al presented a protocol for the differentiation of human ES cells into insulin-producing clusters The protocol consisted of several steps EBs were cultured and plated in insuhn-transferrin-selenium-fibronectin medium

(ITSFn), followed by medium supplemented with N2, B27, and bFGF Next, the glucose concentration in the medium was lowered, bFGF was withdrawn, and NIC was added Dissociating the cells and growing them in suspension resulted in the formation of clusters which exhibited higher insulin secretion and had longer durability than cells grown as monolayers RT-PCR analysis revealed an enhanced expression of pancreatic genes in the differentiated cells Immunofluorescence and in situ hybridization analyses revealed a high percentage of insulin-expressing cells in the clusters In addition to insulin, most cells also coexpressed glucagon or Som, indicating a similarity to immature pancreatic cells

1 4 3 Controversy Surrounding Published Protocols

Several authors have successfully shown differentiation of ES cells into β cell types, however, subsequent studies have been unable to confirm these results (Sipione et al, 2004 Kama et al, 2004) The most common criticism of these studies is the lack of evidence to support *de novo* insulin synthesis Both Rajagopal et al and Sipione et al found that the reported detection of insulin mRNA in ES-derived insulin-positive cells was inconsistent and weak Exogenous insulin (in the form of supplements i e B27, N2 etc) was added to the culture medium in several protocols ES-derived cells had small condensed nuclei and appeared to be undergoing apoptosis Thus, uptake of insulin and release by cells undergoing apoptosis contributed to the illusion of msulin-expression and secretion It has been suggested that demonstration of C-peptide biosynthesis and secretion is necessary to confirm insulin production within ES-derived progeny (Rajagopal et al, 2003, Hansson et al, 2004, Sipione et al, 2004)

Both pancreatic and neuronal cells were generated by the majority of published protocols (Lumelsky et al, 2001, Hori et al, 2002, Rajagopal et al, 2003, Sipione et al, 2004, Miyazaki et al, 2004, Hansson et al, 2004) In the study by Sipione et al, the ES-derived insulin-positive cells rarely showed immunoreactivity for C-peptide and were mostly apoptotic The mam insulin producers within the differentiated culture were those with a neuronal phenotype Kania et al (2004) suggested that the selection for nestin-positive cells and the use of bFGF within

published protocols induced commitment to a neuronal fate rather than a pancreatic fate In 2005, Milne et al demonstrated that insulin-positive cells derived by spontaneous differentiation of ES cells appeared to be extra-embryonic in origin Whilst these cells expressed insulin, they also expressed increased levels of Oct 4, Pdx1 and AFP mRNA which is consistent with extra-embryonic lineage specification

In summary, current protocols yield a small fraction of immature and/or apoptotic insulin-expressing cell types within a heterogeneous population that may be of a neuronal, extra-embryonic or endocrine lineage Studies such as those by Sipione et al (2004), Rajagopal et al (2003), Hansson et al (2004) and Milne et al (2005) highlighted the need for stringent criteria in defining β cell identity i e the evaluation of transcript expression of both target and non-target cell types, expression of key β cell proteins and demonstration of *de novo* insulin synthesis

1 4 4 Nestin-Positive Pancreatic Progenitors

ß cells in the pancreas have a life span of approximately 50 days at which time they are replaced by neogenesis of progenitor cells Identification of these progenitors is essential in understanding islet cell regeneration A number of studies in recent years have proposed that cells expressing nestin represent multipotent progenitor cells of the pancreas (Hunziker et al, 2000, Zulewski et al, 2001, Wiese et al, 2004) Nestin is an intermediate filament protein normally found in neural precursors but is more recently emerging as a potential marker of intra-islet stem cells

Zuleweski et al (2001) isolated nestin-positive cells from adult rat and human pancreas and differentiated these cells *ex vivo* into pancreatic endocrine, exocrine and hepatic phenotypes Treatment of nestin-positive cells with GLP-1 induces differentiation to insulin secreting cells through the activation of Pdx1 (Abraham et al, 2002) During embryogenesis nestin expression has been shown to precede the appearance of ß cells (Abraham et al, 2002, Huang & Tang, 2003) In 2005, Wang et al demonstrated that a heterogeneous population of nestin progenitors could be expanded from islet-derived epithelial monolayers A subpopulation of

nestin/Pdx 1 progenitors identified within these progenitors could yield insulin-producing cells

Despite these findings, controversy still exists over the use of nestin as a pancreatic progenitor marker Studies reporting nestin immunoreactivity in ductal epithelium (a pancreatic compartment known to harbour islet progenitors) were challenged by others reporting that nestin is only a marker of mesenchymal or endothelial cell types (Klein et al, 2003, Lardon et al, 2002, Selander & Edlund, 2002) Kania et al (2004) presented a detailed comparison of protocols used to generate pancreatic phenotypes and concluded that the selection of ES-derived nestin-positive cells should be omitted as these cells are already committed to a neural fate prior to pancreatic differentiation

As discussed previously in Sections 1 4 1 and 1 4 2 the selection for nestin-positive cells (by ITSFn) has become a common step in protocols developed to generate insulin-expressing cell types

1 4 5 Endodermal/Ectodermal Multipotential Progenitors?

Research into pancreatic and neuronal development has indicated a close relationship between neural and pancreatic cell types Some of the characteristics shared between pancreatic endocrine cells and neuronal cells are electrical excitability, neuronal adhesion molecules (i e NCAM), communication between cells through specialized molecules (i e cadherins), expression of neuropeptide-processing enzymes and expression of glucose transporters Transcription factors implicated in the development and function of islet cells i e Isl1, ngn3, Neuro D, pax 6, pax 4, nkx2 2, are also implicated in the development of the CNS (Hunziker & Stein, 2000, Kania et al, 2004, Moritoh et al, 2003) Nestin-positive progenitors are also common to both neuronal and pancreatic development (Section 1 4 3) The hypothesis that similar mechanisms are involved in the development of both neuronal and β cells, has led to attempts to generate insulin-producing cells from ES cells using modifications of a protocol originally developed to generate neurons e g Lumelsky et al, 2001

Studies supporting the existence of neuroendocrine progenitors have been published Seaberg et al (2004) reported the isolation of endodermal/ectodermal multi-potential precursors from adult pancreatic tissue Induced differentiation of these cells resulted in neural and pancreatic cell types, including neuronal and glial cells, pancreatic endocrine β-, α- and δ- cells and pancreatic exocrine and stellate cells More recently Burns *et al* (2005) reported the *in vitro* differentiation of rat neural stem cells into an insulin-expressing phenotype These insulin-positive cells express functional responses typical of pancreatic β cells, including glucose-dependent increases in metabolism and rapid elevations in intracellular Ca^{2+} in response to tolbutamide or to increased glucose concentration

Table 1 4.1 A summary of studies relevant to the derivation of insulin-expressing cells from ES cells

Author	Year	Cells	Genetic Modification	Treatment	Analysis	[Insulin]	Comment
Soria	00	mES 1B/3x-99 (no feeder)	Ins Prom-cell trap	EB + Nicotinamide + low glucose	GSIS RIA/ *In Vivo*	16 5 ng/µg content 318 pg/µg release	Human insulin gene
Lumelsky	01	mES E14 1/B5		5 Stage	ICC (ins, gluc, som, PP)/GSIS ELISA	145 ng/mg content 2 87 ng/mg release	Nestin + selection
Assady	01	hES H9		Spontaneous (EB formation versus monolayer culture)	IHC (ins)/GSIS ELISA	315 µU/ml release (not in response to glucose)	
Hori	02	mES JMI/ROSA		Modified 5 Stage	IHC (ins, C-pep, glut2, gck, Pdx1)/GSIS ELISA *In vivo*	11300 ng/mg content	+ Ly294002 = PI3K inhibitor
Blyszczuk	03	mES R1	Pax 4+/Pdx1+	Modified 5 Stage	IF (ins, gluc, som, PP) /ELISA GSIS *In vivo*	98 7 ng/mg	Pax 4 important
Kahan	03	mES D3		Spontaneous->EBs plated on gelatin	IHC(ins, C-pep, gluc, som, PP)	Data not shown	Very low % spontaneous Ins+ cells
Rajagopal	03	mES E14/R1 & hES H9		5 Stage	IF (ins, C-pep)	Data not shown	Insulin absorption
Moritoh	03	mES EB3 (Oct 4 Prom-cell trap)	Ins Prom-LacZ (no feeder)	Modified 5 Stage	Bgal/IHC(ins, gluc, Pdx1)/GSIS ELISA	3 255-4 725 pg/mg release	Exo+endo w/o pdx1 induction
Blyszczuk	04	mES R1 (Pax4+)	Pax 4+	Multi-stage, no nestin selection	IF(C-pep/ins)/GSIS ELISA /*In vivo*	168 ng/mg content 27 ng/mg release	C-pep+/ins+ cells generated
Miyazaki	04	mES EB3 (no feeder)	Tet+/-Pdx1	Modified 5 stage (±dox)	IHC(C-pep/ins)/GSIS ELISA	8-10 ng/mg release (not in response to	Timing of Pdx1

						glucose)	induction important
Segev	04	hES H9		Modified 5 Stage	IF(C-pep, ins, gluc, som)/GSIS ELISA	225 uU/ml/10^5cells/hr release	
Sipione	04	mES AB1/D388/J1	Ins Prom-GFP	5 stage	ICC(ins, C-pep, gluc)/GSIS RIA	75-150 ng/10^6 cell content (Released insulin but not in response to glucose)	Nestin+ cells do not lead to beta cells
Leon-Quinto	04	mES D3 (no feeder)	Nkx6 1 Prom-cell trap	Reduced serum/Nicotinamide/ Anti-Shh/Pancreas rudiments	ICC(ins/Pdx1, nkx6 1),/GSIS RIA/*In vivo*	150-200 ng/mg content	Pancreatic rudiments most effective
Micallef	05	mES W9 5	Pdx1-GFP	RA	GFP Fluorescence/ Flow Cytometry	Data not shown	Pancreatic Endoderm->Pdx1
Yan-Shi	05	mES R1 (no feeder)	Ins Prom-GFP	AA/RA EBs->B27, NIC, N2, bFGF	IHC(C-pep/ins)/GSIS ELISA/*In vivo*	1 25 ng/mg release	Difficult treatment!
Milne	05	mES D3, CCE (no feeder)		High density growth (no EB formation)	IF(Oct 4 only)/Ca++ microfluorimetry	Data not shown	Ins+ cells are from primitive endoderm

Note ELISA, Enzyme Linked Immunosorbent Assay, RIA, radioimmunoassay, IF, immunofluorescence, ICC, immunocytochemistry, IHC, immunohistochemistry, mES, mouse embryonic stem cell, hES, human embryonic stem cell, GSIS, glucose stimulated insulin secretion, C-pep, C-peptide (connecting peptide), Ins, insulin, EB, embryoid body, Pdx1, pancreatic duodenal homeobox factor 1, PP, pancreatic polypeptide, som, somatostatin, gluc, glucagons, Shh, sonic hedgehog, RA, retinoic acid, AA, activin A, bFGF, basic fibroblast growth factor, GFP, green fluorescent protein, exo, exocrine, endo, endocrine, w/o, without

1 5 Common Agents Employed in Directing the Differentiation of ES cells

Spontaneous differentiation of ES cells can give rise to numerous cells types Current research is focused on enriching differentiated populations for a desired cell type As previously mentioned in Section 1 1 5 the addition of differentiating agents/growth factors to the culture media is one of the current methods employed in directing the differentiation of ES cells The success achieved with some of these agents is discussed below

1 5 1 Retinoic Acid

RA is a powerful morphogenic derivative of vitamin A It plays a role in embryonic development in particular neural differentiation and specification in the developing CNS (Morris-Kay & Sokolova, 1996, Maden et al, 2000, MacCaffery & Dragner, 2000) The formation of neural lineages from pluripotent ES cells in response to RA was previously discussed in Section 1 1 7 1

RA has also been identified as an inducer of endodermal and endocrine differentiation using a variety of mouse models Both Vetere et al (2003) and O'Driscoll et al (2004b) used RA to induce differentiation of endodermal derivatives from the mouse embryonic teratocarcinoma cell line, F9 These cells were further manipulated to express pancreatic transcription factors and endocrine cell markers All-trans-RA stimulated an up-regulation of Pdx1 coinciding with induced endocrine differentiation in the embryonic pancreas (Tulachan et al, 2003) Micallef et al (2005) also demonstrated that RA regulates the commitment of mouse ES cells to form Pdx1-positive endoderm

1 5 2 Activin A

AA is a member of the transforming growth factor (TGFβ) family of secreted proteins Treatment of AR42J cells with AA resulted in neuronal-like differentiation However these cells also expressed glut2 and PP and displayed what appeared to be sulphonurea receptors and calcium channels, characteristic of β cells In a subsequent study on the treatment of AR42J cells with a wider range of differentiation agents it was found that a combination of betacellulin (BTC) and AA resulted in 10% of treated cells expressing insulin (Ohnishi et al, 1995, Mashima et al, 1996) A combination of AA and BTC promoted regeneration of pancreatic β cells and

improved glucose metabolism in neonatal streptozotocin (STZ)-treated rats (Li et al, 2004) AA increases pax 4 gene transcription and ß-cell proliferation in rat islets (Brun et al, 2004) The transcription factor pax 4 plays an important role in islet cell development (see Section 1 2 3 5)

Previous studies have presented evidence of a possible neurotrophic/neurodifferentiation role for AA (Kos et al, 1997, Trudeau et al, 1997) It has been suggested that exogenous AA can rescue striatal interneurons and striatal projection neurons in an animal model of human Huntingdon's disease (Hughes et al, 1999) The effects of AA were examined on the multipotent neural stem cell line MEB5 and the astrocyte progenitor cell line AP-16 (both established from the murine CNS) Treatment with AA induced glial fibrillary acidic protein (GFAP) in AP-16 cells but not in MEB5 cells GFAP is a marker of astrocytic cells LIF-induced astroglial differentiation of MEB5 cells, however, was enhanced by treatment with AA (Satoh et al, 2000) Bao et al (2005) presented data suggesting that AA may work with bFGF in the development of tyrosine hydroxylase (TH)-positive neurons

1.5 3 Betacellulin

BTC is an epidermal growth factor, which was initially discovered in the conditioned medium of ß tumours (Shing et al, 1993) It has mitogenic activity on Balb/c 3T3 fibroblasts (Watanabe et al, 1994) and on foetal pancreatic tissue in culture (Demeterco et al, 2000) It was shown to have potent effects on the proliferation of cultured ß cells (INS 1), with no effects on insulin levels (Huotari et al, 1998) BTC is capable of differentiating small percentages (<5%)' of AR42J cells into insulin-secreting cells (Mashima et al, 1996) Pdx1-transfected epithelial cells and α cell-derived lines only expressed insulin after BTC treatment (Watada et al, 1996, Yoshida et al, 2002) Islet-like clusters, predominately composed of β cells were generated in the ducts of glucose intolerant mice after injections of BTC (Yamamoto et al, 2000) Neuro D-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice (Kojima et al, 2003) BTC exerts a similar effect to AA on rat islets i e increases pax 4 gene transcription and ß-cell proliferation in rat islets (Section 1 5 2) (Brun et al, 2004) Jiang et al (2005) demonstrated that Lammin-1 and EGF family members i e BTC etc co-stimulate fetal pancreas cell proliferation and colony formation

BTC is known to be growth factor of vascular smooth muscle cells (VSMCs) Mifune et al (2004) demonstrated that BTC promotes growth and migration of VSMCs through activation of EGFR, ErbB2, and downstream serine/threonine kinases The study also suggested a critical role for BTC in vascular remodelling

1 5 4 Sodium Butyrate

SB is a short chain aliphatic acid that occurs naturally in the colon through fermentation of dietary fibre (Otonkoski et al, 1999) SB was identified as a differentiation agent for insulinoma cells in a study relating cessation of proliferation with differentiation (Philippe et al, 1987) SB increased both insulin and glucagon levels in RIN cell lines through the recruitment of hormone negative cell to differentiate (Powers et al, 1998) A combination of SB and NIC resulted in the increased content of insulin in both INS 1 cells and porcine fetal islet-like cell clusters (ICCs) (Huotari et al, 1998, Otonkoski et al, 1999) The effects of exposure to NIC and SB both alone and in combination on β cell function were investigated in cultured rat insulin-secreting BRIN-BD11 cells NIC and/or SB caused a decrease in growth, insulin content and basal insulin secretion SB also reduced cell viability whilst NIC had no significant effect Treatment with either agent abolished β cell glucose sensitivity but increased insulin secretory responsiveness to a wide range of β cell stimulators e g protein kinase C, were enhanced (Liu et al, 2004) SB 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 SB operates

1 5 5 Nicotinamide

NIC is a metabolite of the vitamin niacin (vitamin B3) NIC 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 streptozoticin to cause breaks in the DNA strand of β cells which induces poly (ADP-ribose) synthetase, resulting in reduced nicotinamide adenine dinucleotide (NAD), thus causing inhibition of β cell function (Yamamoto & Okamoto, 1980) ICCs isolated from the pancreata of fetal pigs are of limited use as they secret low amounts of

insulin, however upon exposure to NIC containing medium these clusters synthesise and secrete 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) Inhibitors of polyamine synthesis blocked the effect of NIC (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)

NIC has been found to have a potent insulinotropic effect on fetal pancreatic tissue (resulting in β cell outgrowths) and on cultured β cells (Otonkoski et al, 1993, Ohgawara et al, 1995, Huotari et al, 1998) Lumelsky et al (2001) used NIC in the final stages of differentiation of mouse ES cells into insulin-secreting structures (Section 1 4 1)

1 6 Aims of Thesis

The aims of this study were

- to investigate whether an adapted version of the original Lumelsky protocol (Lumelsky et al, 2001), applied to a different ES cell line, could yield insulin-producing cells
- to develop novel assays for the determination of *de novo* insulin synthesis that could be applied to insulin-positive ES-derived differentiated cell types
- to further characterize cell types derived from an adapted version of the Lumelsky protocol (2001) by evaluating multi-lineage marker expression within the differentiated population
- to identify extra-cellular factors capable of inducing endocrine pancreas differentiation (without the use of exogenous insulin), in order to improve on existing protocols or to establish a novel, simplified, protocol for the derivation of pancreatic endocrine cell types
- to determine the effects of these extra-cellular factors on temporal gene expression and lineage specification using an *in vitro* 3-dimensional EB differentiation system
- to investigate models of directed differentiation, in parallel with a model of spontaneous differentiation, with particular emphasis placed on the successful derivation of pancreatic endocrine cell types within complex differentiated cultures
- to use microarray, bioinformatics and real-time PCR technologies to investigate gene expression changes associated with the loss of the GSIS phenotype, after continuous culture of MIN6 cells
- to assess the possible functional significance in GSIS of the observed changes in gene expression

Section 2.0: Materials and Methods

2 1 CELL CULTURE METHODS

2 1 1 Water

Ultra high pure water (UHP) was used in the preparation of all media and solutions This water was 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 UHP 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 22μm 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 10x media were added to sterile UHP water, buffered with HEPES (N-[2-Hydroxyethyl]-N'-[2-ethanesulphonic acid])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 500 ml sterile bottles at 4°C The basal media used during routine cell culture were prepared according to the formulations

shown in Table 2 1 1 Sterility checks were carried out on each 500 ml 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 were prepared as 100 ml aliquots and supplemented as required These were stored for up to 3 weeks at 4°C, after which time, fresh culture medium was prepared

Table 2 1 1 Preparation of basal media

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

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

ES-D3 cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS (ATCC, 30-2020), 0 1 mM β-mercaptoethanol (Sigma, M6250), 1400 units/ml LIF (Chemicon, LIF2010) and 1% penicillin streptomycin (Gibco, 15070-063) MIN6 cells were routinely grown in DMEM supplemented with 20% FCS (heat inactivated for 30 min at 56^{0}C) 4TI cells were cultured in DMEM supplemented with 5% FCS and 800 µg/µl G418 (Sigma, G5013) FVB#c cells were cultured in DMEM supplemented with 10% FCS

2 2 MAINTENANCE OF 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 15 mm 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

2.2.2 Culture of Adherent Cell Lines

The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2 2 1 Lines were generally maintained in vented 25 cm^2 (Costar, 3056) and 75 cm^2 (Costar 3376) and fed every 1-2 days

ES-D3 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 °C for at least 2 hours (usually overnight) The flasks were then rinsed 3X in sterile UHP These coated flasks were stored at room temperature until required

MIN6 cells, 4TI and FVB#c cells were maintained in vented T75 cm^2 flasks

Table 2.2 1 Cell lines used in this thesis, including sources, growth conditions and types

Cell Line	Source of Cell Line	Medium	Cell Type	CO_2 Requirement
ES-D3	[1]ATCC (CRL-1934)	DMEM	Undifferentiated Murine ES Cell Line	5% CO_2
MIN6	Dr Per Bendix Jeppesen	DMEM	Murine β Cell Line	5% CO_2
4TI	Dr Sharon Glynn NICB	DMEM	Murine Breast Cell Line	5% CO_2
FVB#c	NICB Culture Collection	DMEM	Murine Ear Fibroblast Cell Line	5% CO_2

[1]ATCC = American Type Culture Collection

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

2.2 3 1 Subculture of ES-D3/4TI/FVB#c cell line

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% ethylene diamine tetraacetic acid (Sigma, E5134) solution in phosphate buffered saline (PBS) (Oxoid, BR14a)) The purpose of this was to eliminate any naturally occurring TV inhibitor, which would be present in residual serum Fresh TV was then placed on the cells (1 ml/25cm^2 flask or 2 ml/75cm^2 flask) and the flasks were incubated at 37°C until the cells were seen to have detached (2 min) The TV was deactivated by addition of an equal volume of growth medium (containing serum) The entire solution was transferred to a 30 ml sterile universal tube (Sterilin, 128a) and centrifuged at 1,000 rpm (revolutions per minute) for 5 min 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 Cells were never re-seeded back into the same flask

2 2 3 2 Subculture of MIN6 Cell Line

MIN6 flasks were emptied of conditioned medium and the cells were rinsed in pre-warmed 1X Ca^{2+}/Mg^{2+}- free PBS (Gibco 14200-067) TV was then added (1 ml/ 25cm^2 flask and 2 ml/75cm^2 flask) and incubated at 37°C for 2 min exactly The flask was tapped sharply to dislodge the cells and then 5 ml of culture medium was added to inhibit the TV 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 min 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 ES-D3 cells in mini/petri bacteriological-grade dishes (Greiner, 627102/633183), resulted in the formation EBs These cells grew in suspension and thus did not require enzymatic detachment Subculture was performed 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 700 rpm for 10 min 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 EBs were trypsinzed for cell counting, lysis and re-plating for immunofluorescence analysis

2 2 4 1 Trypsinization of Embryoid Bodies

EBs were separated from the medium using gentle centrifugation at 700 rpm for 10 min Following centrifugation the waste medium was removed carefully using a Pasteur pipette The pellet was re-suspended in PBS and re-spun at 100 rpm for 10 min The EBs were transferred to 2 mM EDTA for 10 min at room temperature followed by TV for 10 mm at 37°C (2 ml/pellet) 2 ml media was added to inhibit TV The cell suspension was centrifuged at 700 rpm for 10 min and the resulting pellet was washed in PBS (2X) before being counted, lysed or re-plated

2 2 5 Cell Counting

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

An aliquot of trypan-blue was added to a sample from a single cell suspension at 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 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 trypan-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 MIN6 cells were also stored at -80°C

2 2.6 1 Freezing of ES-D3/4TI/FVB#c Cells

Cells 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 dimethyl sulphoxide (DMSO)/serum (1 5 v/v for ES-D3, 1 10 v/v for 4TI) freezing solution (pre-chilled on ice as were cryovials (Greiner, 122 278)) The freezing solution was slowly added (drop-wise) to the cell suspension (as DMSO is toxic to cells) The suspension was diluted to a final concentration of at least 4 x 10^6 cells/ml The suspension was then aliquoted into cryovials, which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -

80°C) After 3 to 4 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required

2 2 6 2 Freezing of MIN6 Cells

Prior to trypsinization of MIN6 cells, a freezing medium containing DMSO and culture medium was prepared (1 5) 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 trypsinized as outlined previously (Section 2 2 3 2) The pellet was re-suspended in culture medium to give a cell density of 5 x 10^6 – 1 x 10^7 cells/ml To this suspension, an equal volume of freezing medium was added, drop wise This suspension was aliquoted to cryovials The cryovials were cooled on ice for 10 min, and then transferred to a –20°C freezer in a Boehringer Mannheim enzyme box for 30 mm Following this, the cells were placed at – 80°C for at least 12 hours Reserve stocks were placed in liquid nitrogen containers

2 2 7 Cell Thawing

2.2 7 1 Thawing ES-D3 Cells

Prior to thawing ES-D3 cells, 5 ml of culture medium was placed in a gelatin coated T25 cm^2 flask (vented) and conditioned for temperature and pH in a CO_2 incubator for at least 30 min The frozen ES-D3 cryovial was then thawed and the contents were transferred directly into 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 7 2 Thawing MIN6/4TI/FVB#c Cells

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 (10 ml/25cm^2 flask and 15 ml/75cm^2 flask) and allowed to attach overnight Medium was pre-warmed and conditioned in a vented flask in the presence of 5% CO_2 for at least 30 min prior to thawing 24 hours after thawing, the cells were re-fed with fresh medium to remove any residual traces of DMSO

2 2 8 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and TV 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) 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 This analysis was preformed by Aine Adams and Michael Henry at the National Institute of Cellular Biotechnology (NICB)

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 and incubated with supernatant from test cell lines to test 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 cells 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 occasionally 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 1 ml of cell-free (cleared by centrifugation at 1,000 rpm for 5 mm) supernatant from each test cell line was then inoculated onto a NRK coverslip 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 2 ml 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 2 ml of Hoechst 33258 dye (BDH) (50 ng/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 05 M citric acid and 0 1 M disodium phosphate and examined using a fluorescence microscope with a UV (ultraviolet) filter

2 3 SPECIFIC CELL CULTURE TECHNIQUES EMPLOYED IN THIS THESIS

2 3 1 Directed Differentiation of ES-D3 Cells towards Insulin-Expressing Structures (based on the Lumelsky protocol (2001))

Stage 1 Undifferentiated ES-D3 cells were cultured in pre-coated gelatin T75cm^2 tissue culture flasks in the presence of LIF for at least 3 passages

Stage 2 Culture of ES-D3 cells in media without LIF at a cell density of 2 x 10^5 cells/ml in mini bacteriological-grade petri dishes (Greiner, 627102), resulted in the formation of aggregates called EBs These cells grew in suspension and thus did not require enzymatic detachment After 5 days the EBs were separated from the medium using gentle centrifugation at 700 rpm for 10 min Following centrifugation the universals were treated gently as the pellet was quite loose and the waste medium removed carefully using a Pasteur pipette (Section 2 2 4) The pellet was gently re-suspended in pre-warmed medium and the EBs were transferred to new tissue culture 6-well plates (Costar, 3516) Cells from one mini-dish were seeded per well and allowed to attach overnight in ES-D3 media (-LIF)

Stage 3 The attached EBs were fed with ITSFn (1X neurobasal media (Biosciences, 21103-049) supplemented with insulin @ 5 µg/ml (Sigma, I1882), transferrin @ 50 µg/ml (Sigma, T0665), sodium selenite @ 30 nM (Sigma, S9133) and fibronectin @ 5 µg/ml (Sigma, F2006)) 1 5 ml of medium is sufficient to cover the well The EBs were re-fed twice over a 7-day interval

Stage 4 Each well was fed with 1 5 ml of 1X neurobasal media supplemented with 1X N2 Supplement (Biosciences, 17502-048), 1X B27 Supplement (Biosciences, 17504-044) and 10 ng/ml bFGF Cells were re-fed twice over a 7-day interval

Stage 5 Each well was fed with 1 5 ml of 1X neurobasal medium supplemented with 1X N2 Supplement, 1X B27 Supplement and 10 mM NIC (Sigma, N0636) Cells were re-fed twice over a 6-day interval

Cells/EBs were taken down at the end of each stage for RT-PCR (Section 2 3 3 1) Stage 1 and stage 5 cells were dissociated (Section 2 2 4 1) and re-plated for analysis of protein expression by immunofluorescence (Section

2 3 3 3), assayed for protein/insulin content (Section 2 3 3 4) and GSIS capability (Section 2 3 5) and were also carried through to the radio-active labelling assay (Section 2 3 4) Western blot analysis was preformed on stage 1, stage 5 and control cells (Section 2 3 3 5)

2 3 2 Differentiation Studies

Differentiation Studies were carried out using the differentiation agents listed in Table 2 3 1

Table 2 3 1 Differentiation agents used in the course of this work

Differentiation Agent	Supplier	Stock Concentration	Working Concentration
All trans Retinoic Acid (RA)	Sigma (R2625)	1 mM	100 nM
Activin A (AA)	Sigma (A4941)	20 µg/ml	2 nM
Betacellulin (BTC)	Sigma (B3670)	2 µg/ml	1 nM
Sodium Butyrate (SB)	Sigma (B5887)	1 M	1 mM
Pentanoic Acid (PA)	Sigma (V9759)	9 2 M	1 mM
Trichostatin A (TSA)	Sigma (T8552)	3 3 mM	30 nM
Nicotinamide (NIC)	Sigma (N0636)	122 12 M	10 mM

2 3 2 1 Two-Stage Differentiation Studies

2.3 2 1 1 Reconstitution of Activin A, Betacellulin and Sodium Butyrate

RA was reconstituted in 95% ethanol to a final concentration of 1 mM RA was aliquoted and wrapped in tinfoil as it is light sensitive, it was routinely stored at –80°C The stock solutions of the recombinant proteins AA and BTC were made up in PBS-0 1% bovine serum albumin (BSA) (Sigma, A2153) to the desired concentration These were aliquoted in working volumes and stored at –20°C until required SB stocks solutions were made up in UHP The stock solution of SB was stored at 4°C immediately prior to the differentiation assay, these stock solutions were diluted in complete medium to reach working concentration and filter sterilised using a 0 22 μm filter

2 3 2 1 2 Stage 1 Differentiation

EB experiments were carried out in mini/petri dishes On day 1 ES-D3 EBs were generated as follows,

ES-D3 cells were seeded at 2 x 10^5 cells/1 5 mls/mini dish or 1 4 x 10^6 cells/10 5 mls/petri dish in media (-LIF) and incubated overnight On day 2 the EBs were treated with RA (100 nM) The cells were exposed to this compound for 72 hours at 37°C in a 5% CO_2 atmosphere The plates and dishes were wrapped in tinfoil as RA is light sensitive Untreated EBs were used as a control

2 3 2 1 3 Stage 2 Differentiation

On days 5 and 7, RA solution was removed and EBs were re-fed with one of the following compounds AA, BTC or SB as listed in Table 2 3 1 Untreated EBs were used as a control Day 9 marked the endpoint of the treatment Samples of the EBs and conditioned media were taken at day 5 and day 9 for RT-PCR, immunohistochemistry, immunofluorescence and insulin/protein content analysis (See Figure 2 3 1 and Section 2 3 3)

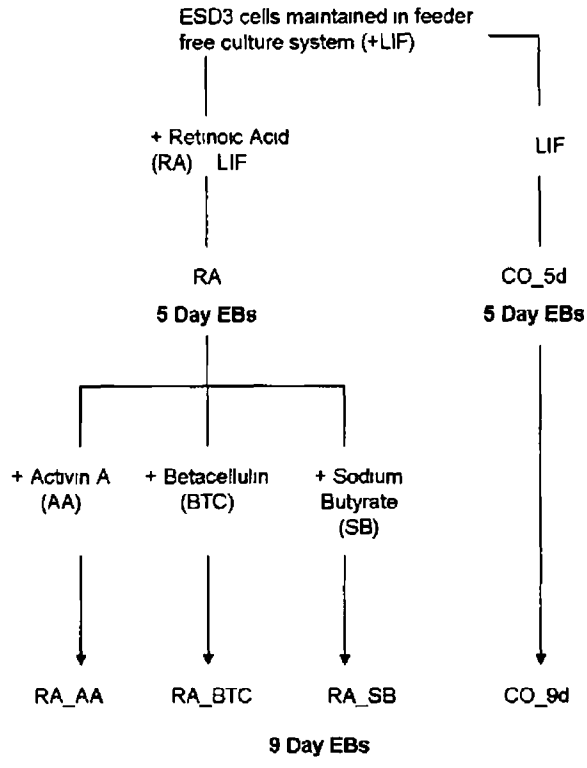


Figure 2 3 1 Two-stage differentiation protocol used in differentiation studies LIF, leukaemia inhibitory factor, CO, control, RA, retinoic acid, AA, activin A, BTC, betacellulin, SB, sodium butyrate, EBs, embryoid bodies

2 3.2 2 Time Course Assay on Transcript Expression in the RA_SB Culture

The RA_SB differentiation study was preformed as previously described in Sections 2 3 2 1 Samples were taken at 2 day intervals for RT-PCR analysis (Section 2 3 3 1) Samples were also taken for RT-PCR analysis at 2 and 4 days after removal from treatment Cell counts were preformed on day 1, day 5 and day 9 to ascertain the effects of treatment on proliferation (Section 2 2 5) The cell numbers are approximate as some cells are lost through dissociation of EBs (Section 2 2 4 1)

2 3 2 3 Investigation of Alternative RA_SB Differentiation Protocols

The RA_SB differentiation study was performed as previously described in Sections 2 3 2 1, however, the following protocol adjustments were tested as a means of improving cell yields from the RA_SB differentiation study

(a) shortening the SB part of the treatment to 48 hours instead of 96 hours

(b) removing the RA step and allowing spontaneous formation of EBs prior to secondary differentiation in the presence of SB

(c) culturing the cells in 10 mM NIC after the removal from RA_SB treatment

(d) using alternative SB treatments such as pentanoic acid (PA) and Trichostatin A (TSA) (Table 2 3 1), which are known to have similar effects as SB See Figure 2 3 2

Samples were taken for RT-PCR analysis at day 1, day 6, day 7 and day 9 (Section 2 3 3 1) Samples were taken for protein extraction and quantification on day 6, day 7 and day 9 (Section 2 3 3 4) Protein derived from cell lysates was used to determine effects of treatments on cell yields

2 3 2 3 1 Reconstitution of Trichostatin A, Pentanoic Acid and Nicotinamide. TSA was reconstituted in ethanol to a final concentration of 3 3 mM and stored at -80°C until required PA and NIC stocks were stored at room temperature and ready for use Immediately prior to assay, these stock solutions were diluted to working concentrations in complete medium (Table 2 3 1)

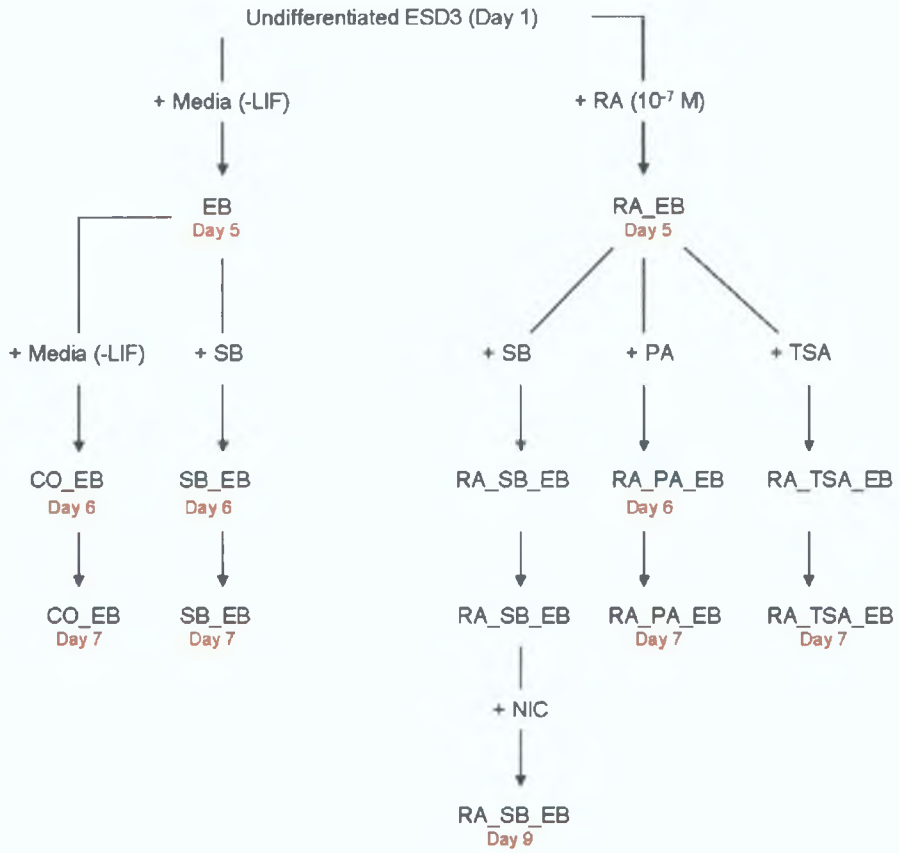


Figure 2.3.2 Alternative RA_SB differentiation protocols. LIF, leukaemia inhibitory factor; CO, control; RA, retinoic acid; SB, sodium butyrate; EBs, embryoid bodies; PA, pentanoic acid; TSA, trichostatin A; NIC, nicotinamide.

2.3.3 Sampling Differentiation Studies

2.3.3.1 Taking Samples for RT-PCR Analysis

Pellets were generated by directly centrifuging the EBs/cells from solution as during subculture (Sections 2.2.3 and 2.2.4). Pellets washed 2X in PBS. 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 2.4.1.2).

2 3 3 2 Fixing Samples for Immunohistochemistry

Pellets were generated by directly centrifuging the EBs/cells from solution as during subculture (Sections 2 2 3 and 2 2 4) Pellets washed 2X in PBS EBs/cells were resuspended in 10% buffered formalin (Serosep Ltd) Fixed samples were then stored at room temperature prior to paraffin embedding Embedding, slicing and immunohistochemistry are described in Section 2 4 2 2

2 3 3 3 Fixing Samples for Immunofluorescence

Pellets were generated by directly centrifuging the EBs/cells from solution as during subculture (Sections 2 2 3 and 2 2 4) EBs were dissociated into single cell suspension (Section 2 2 4 1) and re-plated onto poly-l-ornithine (15 µg/ml in PBS) coated chamber slides Cells were allowed to attach before being fixed in 4% paraformaldehyde (Sigma, P6148) (45 min at 4°C) Immunofluorescence (Section 2 4 2 3) was usually carried out on samples immediately, but samples could be stored at -20° C until required

2 3 3 4 Taking Samples for Insulin Content ELISA/Protein Quantification

Cells/EBs were trypsinized (Sections 2 2 3 and 2 2 4) and the resulting pellet was washed 2X in PBS 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 lysed in chilled 0 1 M NaOH The amount of lysis buffer varied depending on size of pellet Lysed samples were stored at –20°C until required for protein quantification (Section 2 4 2 1) and ELISA (Section 2 4 2 6)

2 3 3 5 Taking Samples for Western Blot

Cells/EBs were trypsinized (Section 2 2 4 1) and the resulting pellet was washed 2X in PBS 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 lysed in chilled 1X Protease Inhibitor Cocktail (Roche, 16974978) in UHP The amount of lysis buffer varied depending on size of pellet Samples were then sonicated (Labsonic U) Number of pulses varied with sample Cell debris was removed from the lysed samples by centrifugation at 2000 rpm Lysed samples

were stored at –20°C until required for protein quantification (Section 2 4 2 1) and western blot (Section 2 4 2 4)

2 3 4 Radio-Active Labelling Assay

2 3 4 1 ^{35}S-L-cysteine Labelling

Control cells were plated at $1x10^6$ cells per well overnight in a 6 well plate In the case of the ES-derived cells from the Lumelsky based protocol (Section 2 3 1), the ^{35}S-L-cysteine labelling was performed on differentiated cell cultures on the final day of the protocol Growth medium was removed from cell cultures and cells were washed with PBS 1 ml of cysteine- medium (DMEM lacking cysteine and 10% dialysed FCS (Sigma)) was added to each well and incubated at 37°C for 20mins to deplete intracellular cysteine levels This was removed and replaced with 1 ml of the same medium supplemented with 100 - 250 uCi/ml ^{35}S-L-cysteine (Specific Activity >1000 Ci/mmol) Control 'cold' cultures received complete, normal growth medium The cultures were then incubated at 37°C for 6 hours in 5% CO_2 Cells were lysed, after removing the labelling medium and washing twice with cold PBS, by addition of 400 µl 0 1 M NaOH These extracts were stored at –80°C before analysis (Section 2 4 2 5 and 2 4 2 6)

2 3 5 Insulin Secretion Assays for Monolayer Cells

2 3 5 1 Glucose Stimulated Insulin Secretion Analysis of Cultured Cells

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 2)) These cells were allowed to grow for 72 hours prior to the GSIS assay In the case of the ES-derived cells from the Lumelsky based protocol (Section 2 3 1), the GSIS assay was preformed on differentiated cell cultures (on a 6 well plate) on the final day of the protocol On the day of the assay 1X Krebs Ringer Buffer (KRB) was prepared from an 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 mm) 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 (twice) in 1X KRB The MIN6 cells were then equilibrated at 3 3 mM glucose for 30 min at 37°C After equilibration, the 3 3 mM glucose was removed, and the glucose containing stimulation medium was administered (1 ml/well) GSIS was carried out over 60 min 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 2000 rpm for 2 min and 200 µl of the supernatant was taken and stored at –20°C until analysis (Section 2 4 2 6) The attached cells were lysed in 200 - 400 µl/well 0 1 M NaOH (depending on well size) and stored at –20°C for later protein quantification (Section 2 4 2 1) and insulin content analysis (Section 2 4 2 6)

2 4 ANALYTICAL TECHNIQUES AND ASSAYS

2 4 1 RNA Analysis

2.4 1 1 Preparation for RNA Analysis

RNA is easily degraded by RNase (ribonuclease) 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 test-RNA, were prepared from sterile UHP that had been treated with 0 1% diethyl pyrocarbonate (DEPC) (Sigma, D5758) before autoclaving All eppendorfs PCR (polymerase chain reaction) tubes 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 RNA degradation) and changed frequently

2 4 1 2 RNA Isolation

2 4 1 2 1 RNA Isolation using TriReagent

RNA was extracted from cultured cells and cells following differentiation treatment In all cases the cells were initially trypsinized (Sections 2 2 3 and 2 2 4) to pellet form, lysed in TriReagent (Section 2 3 3 1) 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 min to ensure complete dissociation of nucleoprotein complexes 0 2 ml of chloroform per ml of TriReagent was added into the sample This was shaken vigorously for 15 seconds and the samples were allowed to stand for 15 min at room temperature The samples were then centrifuged at 13,000 rpm for 15 min at 4°C Following centrifugation, the sample separated into three layers, the upper aqueous layer containing the RNA, the interphase (DNA) and the red layer containing the protein The upper layer was carefully removed to a fresh eppendorf and 0 5 ml

of isopropanol (Fluka, 59304) per ml of TriReagent used initially, was added The tubes were mixed well and allowed to stand at room temperature (15 - 20 min) to let the RNA precipitate After centrifugation at 13,000 rpm for 10 min the RNA was washed (2X) in 75% ethanol and air dried for 5 - 10 min The recovered RNA pellets were then dissolved in 12-20 µl DEPC treated H_2O with repeated pipetting To aid re-suspension of the RNA, the samples were heated to 55°C for 10 min followed by cooling on ice RNA solution was aliquoted and stored at –80°C until required

2 4 1 2 2 Qiagen Kit RNA Isolation

Alternatively, high quality RNA used for microarray studies was isolated from cells using the Rneasy mini-kit (Qiagen, 74104) 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 (supplemented with 10 µl/ml of β-mercaptoethanol) and vortexed to loosen the pellets The lysed pellets were aspirated a number of times and vortexed until the samples were completely homogenised 1 volume (1 2 ml) of 70% ethanol was added to the homogenised samples 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 (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 seconds This was closely followed by 2 washes in buffer RPE (also followed by centrifuging at 8,000 rpm for 15 seconds) To completely dry the spin column, it was placed in a fresh collection tube and centrifuged at full speed for 1 min 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 min The eluted RNA was then quantified (Section 2 4 1 3)

2 4 1 3 RNA Quantification

RNA (like DNA) was quantified using an ND-1000 spectrophotometer The ND-1000 software automatically calculated the quantity of RNA in the samples using the OD_{260}

i e OD_{260} x 40 x Dilution Factor/1000 = RNA content (µg/µl)

The software simultaneously measured the OD_{280} of the samples allowing the purity of the sample to be estimated

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 the reverse transcription (RT)

2 4 1 4 Reverse-Transcription Polymerase Chain Reaction Analysis

2 4 1 4 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 at 70°C for 10 min 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 of RNA secondary structures and allows the oligo dT to bind the poly $(A)^+$ tail of the RNA

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

2 µl of a 10x buffer (Sigma, B 8559)

1 µl RNasin (ribonuclease inhibitor) (40U/µl) (Sigma R2520)

1 µl dNTPs (deoxynucleotide triphosphate) (10 mM of each dNTP) (Sigma DNTP100)

10 µl DEPC -treated water

1 µl MMLV-RT (200U/µl) (Sigma, M1302)

Once the RNA mixture had cooled (~2 min) 15 µl of the master mix was added and mixed by flicking The mixture was centrifuged 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 it was maintained at –20°C

2 4 1 4 2 Polymerase Chain Reaction

The cDNA was 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 mixture

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 (Eurogentec Ltd /MWG)

0 5 µl each of the forward and reverse primers to the housekeeping gene of interest i e β Actin (25 – 50 ng/µl) or GAPDH (glyceraldehyde-6-phosphate dehydrogenase, 10 - 25 ng/µl), (also synthesised by Eurogentec Ltd)

0 25 µl Taq Polymerase (5 U/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 4 1

95°C for 3 min	(Denaturation step)
followed by	
25-30 cycles of	
- 95°C for 30 seconds	(Denaturation)
- 52-60°C for 30 seconds	(Annealing)
- 72°C for 30 seconds	(Extension)
and	
- 72°C for 7 min	(Extension)

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

2 4 1 4 3 Gel Electrophoresis of PCR Products

Typically, 2% agarose (Sigma, A9539) gels were used for PCR gel electrophoresis, which were prepared and run in 1X TBE (10 8g Tris base, 5 5 g Boric Acid, 4 ml 0 5 M EDTA (pH 8 0) and made up to 1 L with UHP) and 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 Sample wells were formed by placing a comb into the top of the gel prior to hardening

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 the mixture was 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 have migrated to the required extent, the gel was taken to the gel analyzer (an EpiChemi II Darkroom, UVP Laboratory Products), photographed and densitometrically analysed using Labworks software (UVP)

2.4 1 5 Real-Time PCR

cDNA synthesised according to Section 2 4 1 4 1 was amplified using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) Two chemistries were used to detect PCR products

- TaqMan Chemistry (uses a fluorogemc probe to enable the detection of a specific PCR product as it accumulates during the PCR cycles)
- SYPR Green dye Chemistry (uses SYBR Green dye, a highly specific, double stranded DNA binding dye, to detect PCR product as it accumulates during the PCR cycles)

2 4 1 5 1 TaqMan PCR

Relevant probes and primer pairs were designed for this purpose (Table 2 4 1) using Primer Express 2 0 software

Standardised Taqman PCR mix

cDNA	2 5 µl
2X Master Mix	12 5 µl
Forward Primer (10 µM)	2 µl
Reverse Primer (10 µM)	2 µl
Probe (5 µM)	1 25 µl
H_2O	4 75 µl

The temperature profile of the reaction was 50°C for 2 min, 95°C for 10 mm and 40 cycles of 95°C for 15 seconds and 60°C for 1 min Expression levels were normalized using the GAPDH/β-actin signal (internal control) compared to reference sample (set to 1) Individual samples were run in triplicate

2 4 1 5 2 SYPR Green PCR

Relevant primer pairs were designed for this purpose (Table 2 4 1) using Primer Express 2 0 software

Standardised SYBR Green PCR mix

cDNA	2 5 µl
2X Master Mix	12 5 µl

Forward Primer (10 μM)	2 μl
Reverse Primer (10 μM)	2 μl
H_2O	6 μl

The temperature profile of the reaction was 95°C for 10 min, 35 cycles of denaturation at 95°C for 40 seconds, annealing (see Table 2 4 1 for specific temperatures) for 40 seconds, and extension at 72°C for 40 seconds Expression levels were normalized using the GAPDH/β-actin signal (internal control) compared to reference sample (set to 1) Individual samples were run in triplicate

Table 2 4 1 Primer sequences used during the course of this work These primers sequences were used in both semi-quantitive PCR analysis and qPCR analysis

Gene	Sequence (5' to 3')	Annealing Temp (°C)	Fragment Size (bp)	Ref Seq	Gene ID
Pdx1	ggtggagctggcagtgatgtt accgcccccactcggggtcccgc	60	125	NM_008814	18609
gck	gatgctggatgacagagccaggatg agatgcactcagagatgtagtcga	54	392	NM_010292	103988
pax 4	tggcttcctgtccttctgtgagg tccaagacacctgtgcggtagtag	52	242	NM_011038	18506
pax 6	aagagtggcgactccagaagt accatacctgtattcttgcttcagg	60	545	NM_013627	18508
Ihh	aaggcccacgtgcattgctct tgtccgcaatgaagagcaggtg	60	298	NM_010544	16147
Shh	ggaagatcacaagaaactccgaac ggatgcgagctttggattcatag	60	354	NM_009170	20423
Islet 1	acgtctgatttccctgtgtgttgg tcgatgtggtacaccttagagcgg	60	276	NM_021459	16392
nkx2 2	cggagaaaggtatggaggtgac ctgggcgttgtactgcatgtgctg	60	188	NM_010919	18088
beta2	cttggccaagaactacatctgg ggagtagggatgcaccgggaa	60	229	NM_010894	18012
GAP43	gctgctaaagctaccactga taggtttggcttcgtctaca	56	223	NM_008083	14432
npy	tgaccctcgctctatctctg aagggtcttcaagccttgtt	58	249	NM_023456	109648
cck	gatggcagtcctagctgctg ccaggctctgcaggttctta	60	167	NM_031161	12424
egr1	atggacaactaccccaaact attcagagcgatgtcagaaa	56	240	NM_007913	13653
pld1	tcttatcccttcctgctacc ccaccttgttgaacacaact	56	250	NM_008875	18805
scg3	ggatactggtgttggtgctc tttctgttattgccctcagc	58	234	NM_009130	20255
chgb	atccaagtccagtgttccaa tctcattgcctaccttcgtc	58	227	NM_007694	12653
CEACAM1	aatctgcccctggcgcttggagcc	60	246	NM_011926	26365

	aaatcgcacagtcgcctgagtacg				
sgne1	ttcagtgaggatcaaggcta ctgggtagtcatgttctgga	56	157	NM_009162	20394
trp53	agctttgaggttcgtgtttg ggaacatctcgaagcgttta	58	220	NM_011640	22059
Gad1	aaggatgcaaccagatgtgt ttggcgtagaggtaatcagc	58	205	NM_008077	14415
tjp1	acgacaaaacgctctacagg gagaatggactggcttagca	58	213	NM_009386	21872
claudin 11	caagagtgcccatgtctaag tgggcattaactgagctaga	56	234	NM_008770	18417
dll-1	agccctccatacagactctc cagaacacagagcaaccttc	56	201	NM_007865	13388
ODC	ctgccacatccttgatgaag cgttactggcagcatacttg	60	349	NM_013614	18263
nestin	ggctacatacaggattctgctgg caggaaagccaagagaagcct	60	85	NM_016701	18008
GATA4	cgccgcctgtccgcttcc ttgggcttccgttttctggtttga	60	193	NM_008092	14463
Amylase	caggcaatcctgcaggaacaa cacttgcggataactgtgcca	58	484	NM_009669	11723
AFP	cctgtgaactctggtatcag gctcacaccaaagcgtcaac	58	406	NM_007423	11576
PC1	atctgatgatgatcgtgtga tgtctgtgtgattccactcc	56	260	NM_013628	18548
PC2	cacagatgactggttcaaca tcacagttgcagtcatcgta	56	359	NM_008792	18549
PPI	agcgtggcttcttctacacacc ggtgcagcactgatccaccatg	54	158	NM_008386/ NM_008387	16333/ 16334
insulin I	tagtgaccagctataatcagag acgccaaggtctgaaggtcc	62	288	NM_008386	16333
insulin II	ccctgctggccctgctctt aggtctgaaggtcacctgct	62	212	NM_008387	16334
insulin I	gtgaagtggaggacccacaagt atgctggtgcagcactgatc Probe (5'FAM-3'TAM) acctccaacgccaaggtctgaaggt	60		NM_008386	16333

insulin II	gtgaagtggaggacccacaagt atgctggtgcagcactgatc Probe (5'FAM-3'TAM) cacctccagtgccaaggtctgaaggt	60		NM_008387	16334
glut2	Taqman	60	N K	NM_031197	20526
dlk1	Taqman	60	N K	NM_010052	13386
NF-L	cgccgaagagtggttcaagag gtaggagctgctctgcaagcc	60	472	NM_010910	18039
MBP	aagcacacagcagacccaaagaa aaggatgcccgtgtctctgt	58	309	NM_010777	17196
GFAP	ctttctctccaaatccacac ctccttgtctcgaatgactc	58	457	NM_010277	14580
β III tubulin	ctcagtcctagatgtcgtgcg gcggaagcagatgtcgtaga	58	294	NM_023279	22152
desmin	gacgctgtgaaccaggagtt acgagctagagtggctgcat	62	384	NM_010043	13346
myogenin	cctgccctgagttgagagag cggcagctttacaaacaaca	62	141	NM_031189	17928
Oct4	ggcgttctctttggaaaggtgttc ctcgaaccacatccttctct	60	293	NM_013633	18999
bax	tgcagaggatgattgctgac gatcagctcgggcactttag	60	173	NM_007527	12028
caspase3	agtctgactggaaagccgaa taacggcgagtgagaatgtgc	60	270	NM_009810	12367
glucagon	actcacagggcacattcacc ccagttgatgaagtccctgg	60	353	NM_008100	14526
somatostatin	ccgtcagtttctgcagaagt cagggtcaagttgagcatcg	60	356	NM_009215	20604
PP	aggatggccgtcgcatactg gagctgcactccaggaagtc	60	249	NM_008918	19064
β-actin	gaaatcgtgcgtgacattaaggagaagct tcaggaggagcaatgatcttga	AR	383	NM_001101	11461
β-actin	Taqman	60	N K	NM_007393	11461
β-actin	acggccaggtcatcactattg caagaaggaaggctggaaaaga Probe (5'JOE-3'TAM) caacgagcggttccgatgccc	60	70	NM_007393	11461

GAPDH	catgaccacagtccatgccatc caccctgttgctgtagccgtattc	AR	451	NM_001001303	407972
GAPDH	tgtgtccgtcgtggatctg accaccttcttgatgtcatcatactt Probe (5'JOE-3'TAM) tgccgcctggagaaacctgcc	60	68	NM_001001303	407972

Note qPCR, real-time PCR, Pdx1, Pancreatic duodenal homeobox factor 1, gck, glucokinase, Ihh, Indian hedgehog, Shh, sonic hedgehog, GAP43, growth associated protein 43, npy, neuropeptide Y, cck, cholecystokinin, egr1, early growth response 1, pld1, phospholipase D1, scg3, secretogranin III, chgb, chromogranin B, sgne 1, secretory granule neuroendocrine protein 1 (7B2 protein), trp53, transformation related protein 53, Gad1, glutamate decarboxylase, tjp1, tight junction protein, dll-1, delta-like 1, ODC, ornithine decarboxylase, GATA4, gata binding protein 4, AFP, alpha-feto protein, PC1, prohormone convertase 1, PC2, prohormone convertase 2, PPI, preproinsulin, glut2, glucose transporter 2, bax, bcl2-associated x protein, dlk, delta-like homologue 1, NF-L, neurofilament light, MBP, myelin basic protein Fam/Tam/Joe, combinational fluorescence energy transfer tags

2 4 1.6 Affymetrix GeneChips®

The microarray gene expression experiments of this study were performed using Affymetrix® Murine Genome U74A GeneChips® Affymetrix GeneChip probe microarrays are manufactured using technology that combines photolithography and combinatorial chemistry Tens to hundreds to thousands of different oligonucleotide probes are synthesised and each of these oligonucleotides is located in a specific area on the microarray slide, called a probe cell Each probe cell contains millions of copies of a given oligonucleotide and each feature size on the Affymetrix U74A GeneChip is 24 microns

The most important aspect in efficient probe design is the quality of the sequence information used Probe selection and array design are two major factors in reliability, sensitivity, specificity and versatility of expression probe arrays Probes selected for Affymetrix gene expression arrays are generated from sequence and annotation data obtained from multiple databases such as GenBank, RefSeq and dbEST Sequences from these databases are collected and clustered into groups of similar sequences Using clusters provided by UniGene database as a starting point, sequences are further subdivided into subclusters representing distinct transcripts

This categorisation process involves alignment to the mouse genome, which reveals splicing and polyadenylation variants The alignment also extends the annotation information supplied by the databases pinpointing low quality sequences These areas are usually trimmed for subsequent generation of high quality consensus sequences or alternatively Affymetrix employ quality ranking to select representative sequences, called exemplars, for probe design

In general, Affymetrix use 11 to 16 probes which are 25 bases in length for each transcript The probe selection method used by Affymetrix for their U74A GeneChips takes into account probe uniqueness and the hybridisation characteristics of the probes which allow probes to be selected based on probe behaviour Affymetrix use a multiple linear regression (MLR) model in the probe design that was derived from thermodynamic model of nucleic acid duplex formation This model predicts probe binding affinity and linearity of signal

changes in response to varying target concentrations An advantage of this type of model-based probe selection system is that it provides a physical and mathematical foundation for systematic and large-scale probe selection Also, an essential criterion of probe selection by Affymetrix for quantitative expression analysis is that hybridisation intensities of the selected probes must be linearly related to target concentrations

A core element of Affymetrix microarray design is the Perfect/Mismatch probe strategy For each probe that is designed to be perfectly complimentary to a given target sequence, a partner probe is also generated that is identical except for a single base mismatch in its center These probe pairs, called the Perfect Match probe and the Mismatch probe, allow the quantitation and subtraction of signals caused by non-specific cross-hybridisation The differences in hybridisation signals between the partners, as well as their intensity ratios, serve as indicators of specific target abundance

2.4 1 6 1 Sample and Array Processing

After RNA isolation, quantification and purification using the Qiagen Rneasy isolation method (Section 2 4 1 2 2), cDNA was synthesised using the GeneChip T7-Oligo (dT) Promoter Primer Kit (Affymetrix, 900375) from 10μg total RNA First strand cDNA synthesis was then performed using the SuperScript Choice Kit (BioSciences, 11917-010) First strand cDNA synthesis involved 'primer hybridisation' where the T7-Oligo (dT) primer was incubated with the RNA and DEPC-treated H_2O at 70^0C for 10 mins, followed by a short incubation in ice, 'temperature adjustment' where 5X first strand buffer, DTT and dNTP mix were added to the RNA mix and incubated at 42^0C for 2 mins, and 'First Strand synthesis' where SuperScript II RT was added to the mix and incubated at 42^0C for 1 hour Second strand cDNA synthesis was performed and purified using GeneChip Sample Cleanup module (Affymetrix, 900371) as recommended by the manufacturers instructions

Complimentary RNA (cRNA) was then synthesised and biotin-labelled using the Enzo BioArray HighYield RNA Transcript Labelling Kit (Affymetrix, 900182)

Biotin-labelled cRNA was purified using the GeneChip Cleanup Module Kit (Affymetrix, 900371) and quantified. The value obtained was adjusted to reflect carryover of unlabelled total RNA. A sample of biotin-labelled cRNA was taken for gel electrophoresis analysis. The labelled cRNA was then fragmented before hybridisation onto the Affymetrix GeneChip probe microarrays. The aliquot of fragmented sample RNA was stored at -20^0C until ready to perform the hybridisation step.

Hybridisation of cRNA onto the Affymetrix GeneChip probe murine microarrays (Affymetrix, U74A) was performed in the Conway Institute, University College Dublin, where the Affymetrix Hybridisation Oven and Fluidics Station is set up along with the Affymetrix GeneChip Scanner, which exported the data directly into the Affymetrix analysis software, MicroArray Suite 5.1 (MAS 5.1).

2.4.1.6.2 Microarray Data Normalisation

The purpose of data normalisation is to minimise the effects of experimental and technical variation between microarray experiments so that meaningful biological comparisons can be drawn from the data sets and that real biological changes can be identified among multiple microarray experiments.

2.4.1.6.2.1 Probe Logarithmic Intensity Error Estimation (PLIER)

All the data from the MIN6 biological triplicates assessed were normalised using the PLIER algorithm within the GREX package (Affymetrix). The PLIER algorithm (http://www.affymetrix.com) is a new tool introduced by Affymetrix for the use in data analysis of their GeneChips and has replaced the need to normalise microarray data by using the 'per chip' and 'per gene' normalisation methods. This algorithm incorporates model-based expression analysis and non-linear normalisation techniques. PLIER accounts for differences in probes by means of a parameter termed "probe affinity". Probe affinity is a measure of how likely a probe is to bind to a complimentary sequence, as all probes have different thermodynamic properties and binding efficiencies. Probe affinities determine the signal intensities produced at a specific target concentration for a given probe, and are calculated using experimental data across multiple arrays. By accounting for these observed differences, all the probes within a set can be

easily compared An example of how the PLIER algorithm works is if one probe is consistently twice as bright as other probes within a set, PLIER appropriately scales the probe intensities In the case of a probe set, this enables all set numbers to be compared and combined accurately

PLIER also employs an error model that assumes error is proportional to the probe intensity rather than that of the target concentration At high concentrations, error is approximately proportional to target concentration, since most of the intensity is due to target hybridisation signal However, at the low end, error is approximately proportional to background hybridisation intensity, which is the largest component of the observed intensity Due to this effect, it is inaccurate to treat errors as a proportion of target concentration in all circumstances The PLIER error model smoothly transitions between the low end, where error is dependent upon background, and the high end, where error is dependent on signal

The PLIER algorithm supports a multi-array approach that enables replicate sample analysis PLIER ensures consistent probe behaviour across experiments to improve the quality of results in any one given experiment and helps to discount outliers Benefits of this algorithm include an improved coefficient of variation of signals from probe sets while retaining accuracy Also higher differential sensitivity for low expressers maybe achieved using PLIER

2 4 1 6 3 Bioinformatics Criteria for Selecting Induced/Suppressed Genes and Functional Assignment

Following data normalisation, gene lists were generated using a students t-test with a P value cut-off of 0 01, to identify genes that were statistically differentially expressed between the three high passage arrays and low passage arrays (used as the reference/"control") These gene lists were segregated into lists containing genes, who's expression was either significantly higher or lower, in the high passage MIN6 cells compared to the low passage MIN6 cells A 2-fold cut-off (increase or decrease) was then applied

2 4 1 6 4 Quality Control Clustering using the Spearman Correlation

The Spearman correlation is a non-parametric test for the strength of the relationship between pairs of variables Using a GeneSpring (Affymetrix) interpretation, gene lists from 3 samples each representing the "control" (low passage MIN6) and the "test" (high passage MIN6) were used to develop a condition tree investigating their similarity Using the All Samples (log) interpretation and the Spearman (rank-based) correlation, the six samples were clustered Samples with a similar expression profile were grouped together

2 4 2 Protein Analysis

2 4 2 1 Protein Quantification

Lysed samples were removed from the freezer and placed on ice A BSA standard curve (0 – 1 mg/ml) was prepared in UHP 5 µl of standard and 5 µl of sample were placed in triplicate wells on a 96 well plate (Costar, 3596) The Biorad D_c Protein Assay was used for protein quantification 25 µl of Reagent A (containing 20 µl Reagent S (Biorad, 500-0115) per ml of Reagent A (500-0113)) followed by 200 µl of Reagent B (500-0114) were added to each test well The plate was kept at room temperature for 15 min prior to reading on the Spectra Max Plus using a softmax Lowry protein assay (750 nm) program

2 4 2 2 Immunohistochemical Analysis of Paraffin Embedded Samples

2 4 2 2 1 Plasma Clotting and Paraffin Embedding of Samples

The samples were fixed in 10% formalin (Section 2 3 3 2) and stored at room temperature Samples were spun down at 10,000 g for 15 seconds to form pellet The pellet was resuspended in 1% Fibrinogen (Sigma, F4753) prepared in DMEM Samples spun at 10,000 g for 15 seconds and resuspended in 100 µl 1% Fibrinogen solution 2 drops of thrombin solution (Sigma, T6884) was added to the sample to form a clot The clot was removed and placed in an embedding tray The clot was then exposed to 10% formalin for 30 - 60 min

Clots were soaked in 95% ethanol (2 x 10 min), 100% ethanol (2 x 10 min), xylene (2 x 10 min), paraffin/wax (2 x 10 min) The clots were embedded in paraffin (BDH, 298682F) and allowed to dry overnight

2 4 2 2 2 Sectioning of Paraffin Embedded Samples

The paraffin embedded samples were placed on ice for 30 - 60 min prior to cutting Four-micron thick slices were cut using the Reichert-Jung 2030 Biocut Slices were placed in 1% ethanol, followed by removal to warm water Slices were mounted onto poly-l-lysine coated slides (slides treated with ethanol and

coated with 10% poly-l-lysine) The mounted samples were placed at 37°C for 2 hours before storing at 4°C

2 4 2 2.3 Immunohistochemistry 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 coloured precipitate is indicative of primary antibody reactivity

The following procedure was used

The sections were dewaxed in Xylene (2 x 3 mm), 100% ethanol (2 x 3 min), 90% ethanol (2 x 3 min) and 70% ethanol (2 x 3 min) Sections were washed with tris buffered saline (TBS) (Sigma, T5912)/0 1% Tween (Sigma, T1379) and incubated with 10 mM sodium citrate buffer, pH 6, (DAKO, S2369) in a microwave (750W, 2 x 5 min) Sections were washed with TBS/0 1% Tween and endogenous peroxidase was blocked by incubation in 3% H_2O_2 (BDH, 101284N) for 7 min Sections were rinsed with UHP and placed in TBS for 5 min Sections were incubated at room temperature with the corresponding serum (depending on antibody host) diluted 1 10 in TBS to block non-specific binding After removal of serum 50-100 µl of optimally diluted primary antibody was added The sections were placed on a tray and incubated overnight at 4° C The primary antibodies used in these studies are shown in Table 2 4 2

The next day sections were washed in TBS/ 0 1% Tween, 3x for 5 min, and incubated for 30 min with a biotinylated secondary antibody diluted in TBS (Table 2 4 2) The sections were washed with TBS/0 1% Tween, incubated with ABC complex (Vector, PK-7100) for 30 min at room temperature and rinsed 3x with TBS/ 0 1% Tween The sections were incubated with a DAB solution

(Vector, SK-4100) for 7 min Excess DAB solution was rinsed off with UHP water The sections were counter stained with hematoxilin (Vector, H-3404), washed with UHP and dehydrated using 70% ethanol, (2 x 3 min), 90% ethanol (2 x 3 min), 100% ethanol (2 x 3 min) and Xylene (2 x 3 mm) Sections were mounted using a commercial mounting solution (DAKO, S3023) Controls for immunospecificity were included in all experiments by replacing the primary antibody with TBS and matching concentrations of serum

2 4 2 3 Immunofluorescence Analysis of Dissociated EBs and Monolayer Cells

Samples were fixed as previously described in Section 2 3 3 3 Fixed samples were permeabilized with 0 2% Triton X100 in PHEM buffer ((60 mmol/l PIPES, 25 mmol/l HEPES, 10 mmol/l EGTA, 2 mmol/l MgCl, pH 6 9) for 2 - 4 hours and free aldehyde groups were reduced with 0 1% (w/v) sodium borohydride in PBS Samples were blocked in relevant serum overnight (Table 2 4 2, diluted 1 10 in 5% BSA, 0 2% Triton X100 in PHEM buffer) The following morning the serum was removed and the primary antibody was applied (antibodies and dilutions are listed in Table 2 4 2) This was incubated overnight at 4°C in a moist environment The following day, the primary antibody was removed and the slides were washed three times in PHEM-0 2% Tween at 5 mm per wash The fluorescent secondary antibodies (light sensitive) were prepared in the dark room under dim conditions and were coated in foil The cells were incubated with secondary antibodies (Table 2 4 2) for 60 min and the slides were wrapped in tinfoil All work from this point onwards was carried out in the dark to prevent 'quenching' fluorescent signal After 60 min incubation the antibodies were removed and the slides were washed three times in PHEM-0 2% Tween The slides were then mounted using vectashield containing a DAPI (4', 6-diamidino-2-phenylindole) nuclear stain (Vector, H1000) and covered with coverslips (Chance Propper) Slides were examined by confocal laser scanning microscopy using Leica TCS AOBS

Table 2 4 2 Antibodies used in Immunofluorescent/Immunocytochemical studies including the labelled secondary antibodies (FITC, Fluorescein isothiocyanate, TRITC, Tetramethylrhodamine Isothiocyanate)

Antibody	Host	Supplier	Dilution	Serum for Blocking
C-peptide 1	rabbit	Dr S Heller Hagedorn Research Institute	1 1200 -1 2000	swine/goat
C-peptide 2	rabbit	Dr S Heller Hagedorn Research Institute	1 1200 -1 2000	swine/goat
glucagon	mouse	Sigma (G2654)	1 2000	rabbit
glut2	rabbit	Chemicon (AB3412)	1 300	swine/goat
gck	rabbit	Abgent (AP7901c)	1 50-100	swine/goat
AFP	rabbit	NeoMarkers (RB-315-R7)	Ready for Use	swine
insulin	rat	R&D systems (MAB1417)	1 50 - 1 200	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
Oct4	rabbit	Chemicon (AB3209)	1 300	swine/goat
nestin	mouse	Chemicon (MAB353)	1 600	rabbit
desmin	mouse	Sigma (d1033)	1 40	rabbit
myogenin	mouse	Sigma (m5815)	1 500 -1 1000	rabbit
NF-L	mouse	Abcam (AB4572)	1 500	rabbit
GFAP	mouse	Chemicon (MAB360)	1 400	rabbit
β-III tubulin	mouse	R&D systems (MAB1195)	1 100	rabbit
O4	mouse	R&D systems (MAB1326)	1 150	rabbit
Anti Rat	rabbit	Sigma	1 30	swine

(TRITC)		(T4280)		
Anti Mouse (FITC)	rabbit	DAKO (F0261)	1 30	N/A
Anti Rabbit (TRITC)	goat	DAKO (R0156)	1 40	N/A
Anti Rat (Alexa Fluor 488)	goat	Molecular Probes (A11006)	8 µg/ml	N/A
Anti Mouse (Biotin)	rabbit	DAKO (E0354)	1 300	N/A
Anti Rabbit (Biotin)	goat	DAKO (E0432)	1 300	N/A
Anti Rat (Biotin)	rabbit	DAKO (E0468)	1 300	n/a

2 4 2 4 Western Blot Analysis

An aliquot of lysed cells (Section 2 3 3 5) was mixed with an equal volume of 2X Laemmli sample buffer (Sigma, S3401) and boiled for 3 min 10 - 20 µg of protein was loaded to each well of a 12% bis-tris gel (NuPAGE, NP0341) The samples were run in parallel with multimark multi-coloured standard (Invitrogen, LC5725) The electrophoresis conditions were 200 V and 90 mA The gels were run for 40 min Following electrophoresis, gels were equilibrated in 1X tris/gly transfer buffer (Biorad, 161-0734) for 15 min with agitation Protein gels were transferred to a PVDF (polyvinylidene fluoride) membrane (Boehringer Mannheim, 1722026) 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 PVDF, 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 PVDF 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 PVDF at a current of 34 mA at 15 V for 20 mm 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 PVDF blot to all reagents

The PVDF membranes were blocked for 2 hours at room temperature with fresh, filtered, 5% non-fat dried milk (Cadburys, Marvel skimmed milk) in 1X TBS (Sigma, T5912)/ 0 1% Tween (Sigma, P1379) After blocking, the membranes were rinsed with TBS-tween and incubated with primary antibody (Table 2 4 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 min in TBS/Tween Bound antibody was detected using enhanced chemiluminescence (ECL) Following chemi-luminescent detection, blots were again washed and blocked as described and re-probed for an internal standard e g GAPDH This was carried out as before

Table 2 4 3 Antibodies used for western blotting

ANTIBODY	HOST	SUPPLIER	DILUTION *
C-peptide 1	rabbit	Dr S Heller Hagedorn Research Institute	1 5000
C-peptide 2	rabbit	Dr S Heller Hagedorn Research Institute	1 5000
GAPDH	mouse	Abcam (AB9484)	1 5000 - 1 10000
Mouse IgG	sheep	Sigma (A6782)	1 1000
Rabbit IgG	goat	Sigma (A4914)	1 5000

*All antibodies were diluted in 1X TBS

2 4 2 5 Quantification of ^{35}S Labelled Insulin and Proinsulin

Cell lysates were thawed and spun for 5 mins at 12,000 rpm at 4°C An aliquot of the supernatants was kept aside for total protein determination (Section 2 4 2 1) and quantification of total insulin content (Section 2 4 2 6) The remainder was passed through a 50 kDa cut-off spin filtration unit (Centricon, YM50) to remove high molecular weight proteins For Competitive RIA analysis of the samples, an ELISA kit (Mercodia, 10-1124-10) was used with the following modifications

Blocking 50 µl of Conjugate Buffer was added to each well and 25µl of a 1 in 10 dilution of 'cold' matched sample lysate (i e MIN6 wells blocked with 'unlabelled' MIN6 lysate), followed by 1 hour incubation at room temperature on a shaker at 100 rpm

Competitor The blocking solution was removed and the wells were washed (6X) with 1 X Washing Solution (supplied in kit) 50 µl Binding Buffer was added to each well followed by 25 µl of a dilution series of pure insulin (Sigma, I1882) ranging from 250 ng/µl down to 2 5x10^{5} ng/µl The plate was again incubated at room temperature for 1 hour on a shaker at 100 rpm to allow equilibration

Sample Binding 25 µl of a 1 in 5 dilution of the radio-labelled, ultra-filtered lysate was added to each well and incubated at room temperature for 2 hours on a shaker at 100 rpm

Measurement Unbound protein was removed by discarding the binding solution and washing the wells (6X) with Washing Buffer Bound protein was eluted from the plate by proteolytic cleavage of the capture antibody with 100 µl/well of papain-digestion buffer at 37°C overnight (200 mM sodium phosphate, pH7, 2 mM EDTA, 10 mM L-cysteine and 0 2 mg/ml papain (Sigma, P3125) The amount of radio-labelled material released from each well was measured in a Beckman-Coulter scintillation counter (100 µl digestion buffer in 5 ml Ecolite scintillation fluid, 1 min reading time on ^{35}S-channel)

2 4 2 6 ELISA Analysis for Insulin and Proinsulin

Insulin secretion studies have already been described (Section 2 3 5) Analyses on MIN6 cells and conditioned media were carried out using a proinsulin ELISA kit (Mercodia, 10-1124-10) and ES-derived cells were analysed using a mouse ultra-sensitive insulin ELISA kit (Mercodia, 10-1150-01) The kits were fully inclusive and all reagents, standards etc were supplied The generalised protocol for their use is outlined below Samples were diluted with the 0 standard supplied and mixed well by vortexing 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 into the wells) and incubated at room temperature for 2 hours on a belly dancer set to maximum speed Following this,

the conjugate/sample mixes were tapped off in to a sink and the wells were washed 6X 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 wrapped in tinfoil) for 15 - 30 min and 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 4 2 7 Alkaline Phosphatase Determination

AP determination was carried out on fresh cells cultured on 6 well plates AP activity was measured by adding the assay components (16 5µl BCIP (5 Bromo-4chloro-3-indolyl-phosphate, 4 toluidine salt (X-phosphate, 4 toluidine salt)) (Roche, 1383 221) and 33 µl NBT (nitro blue tetrazolium chloride) (Roche, 1383, 213)) to 5 ml 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 and being transformed to 5 bromo-4 chloro-3 indoxyl, which generates an insoluble purple dye Nitro blue tetrazolium chloride was used as an electron acceptor to enhance the colour reaction

The staining solution was applied directly to the test wells and the colour was allowed to develop Once the colour had developed the wells were washed 3X in UHP and the plates were photographed

Section 3.0: Results

3.1 AN INVESTIGATION INTO THE DERIVATION OF INSULIN-EXPRESSING CELL TYPES USING AN ADAPTED VERSION OF THE ORIGINAL LUMESLKY PROTOCOL

Since 2000, several important and exciting reports have been published describing the derivation of insulin-positive cells from both mouse and, to a lesser extent, human ES cell lines (Table 1 4 1) Many of these studies have been based on the derivation of insulin-producing cells using modified protocols that were originally designed to generate neurons, on the premise that the pancreas and central nervous system share developmental pathways (Section 1 4 5) A study published by Lumelsky *et al* (2001) described a 5-stage protocol that gave rise to 3-dimsional clusters which stained for insulin in addition to the other pancreatic endocrine hormones The cells secreted insulin in response to glucose but did not correct hyperglycemia when injected into diabetic mice Several further studies modified this protocol resulting in the generation of transplantable insulin-producing cells (Hori Y et al, 2002, Blyszczuk et al, 2003) The success of these protocols, and others, in generating authentic β cells has been questioned due in part to uncertainty regarding cell lineage but also due to controversy regarding *de novo* insulin synthesis (Section 1 4 3) It is apparent that stringent criteria are necessary to define β cell identity This might include (a) measuring transcript expression of β cell-associated genes as well as genes associated with other lineages, (b) measuring expression of key β cell marker proteins and (c) demonstrating functional *de novo* insulin synthesis

In the present study we attempted to address some of the more controversial issues associated with the identification of 'β-like' cells derived from mouse ES cells We devised a novel assay for detection of *de novo* insulin synthesis, in order to identify functional, insulin-producing cells *in vitro* The murine insulinoma cell line, MIN6, was used to develop the assay (This assay was developed in collaboration with Dr Niall Barron) The assay was applied to cells derived via a slightly modified version of the original Lumelsky protocol which reported insulin-producing cells containing 145 ng of insulin per milligram of protein (Figure 3 1 1) In addition, we investigated alternative means for determining C-peptide expression which does not rely on immunostaining alone We also measured expression of a range of multi-lineage markers to further

characterize the complex mixture of cell types derived from the protocol used in this and other studies.

We found that cells derived via a modified version of the Lumelsky protocol expressed a broad range of multi-lineage markers indicating the derivation of a mixed population. Insulin was detected in this ES-derived culture by both immunofluoresence and ELISA; however, we conclude that the insulin content is a result of insulin uptake from the feed medium rather than *de novo* synthesis. We present evidence suggesting that immunostaining methods for C-peptide reactivity may not be definitive proof of, and may over-estimate, β cell differentiation in ES-derived cultures and should be performed only in conjunction with other analysis methods. We also report the development of a novel assay that can identify *de novo* insulin synthesis.

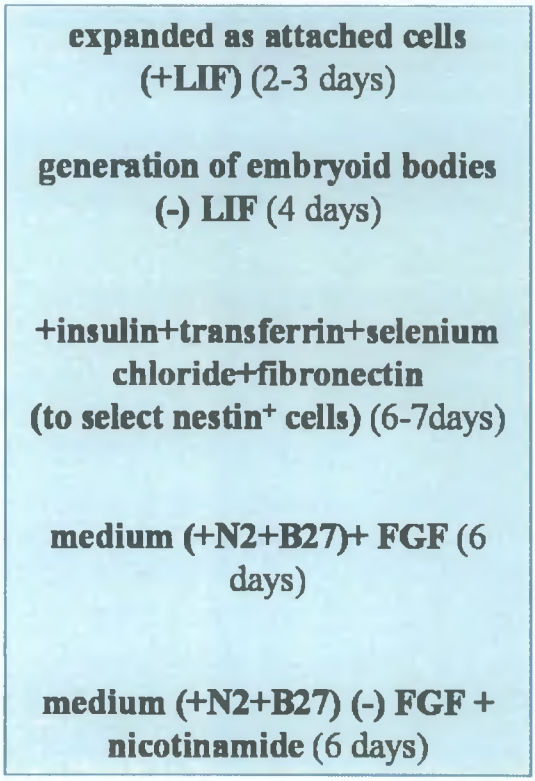


Figure 3.1.1 5-Stage Differentiation of ES Cells (Section 2.3.1). Stages 3 – 5 are serum-free.

3 1 1 Morphological Changes Occuring over the 5 Stages of the Lumelsky-Based Protocol

ES-D3 cells cultured on gelatin grew in tight multi-layered colonies (Figure 3 1 2 A & B) Embryoid body (EB) formation occurred during stage 2 of the protocol (Figure 3 1 1) EBs in suspension tended to be heterogeneous in size and appeared individually or as aggregates (Figure 3 1 2 C & D) Stage 3 ITSFn serum-free medium has been reported to enrich for nestin-positive cells (Lumelsky et al, 2001) The EBs attached to gelatin coated tissue culture plates and adopted a flattened morphology (Figure 3 1 2 E & F) Cell clusters expanded and differentiated during stage 4 and stage 5 Neuronal-like projections became more visible and dense clusters adopted a 3-dimensional topology (Figure 3 1 2 G - J) These findings were broadly in keeping with what was reported elsewhere (Lumelsky et al, 2001, Sipione et al, 2004)

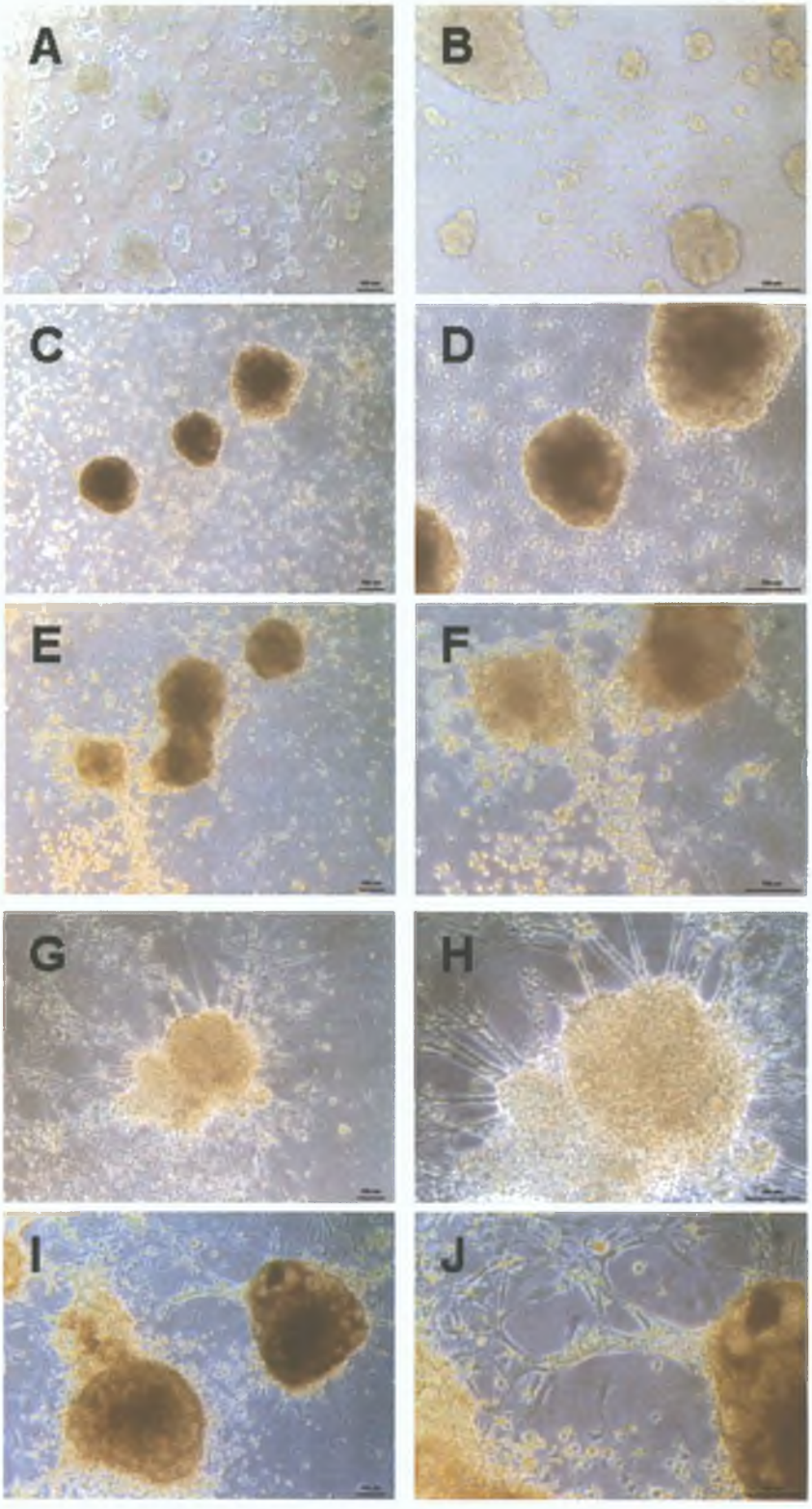


Figure 3.1.2 Light microscopy images of stage 1 ES-D3 cells (A & B), stage 2 EBs (C & D), stage 3 cultures (E & F), stage 4 cultures (G & H), stage 5 cultures (I & J). Scale bar = 100 μm. (A, C, E, G & I = 10X) (B, D, F, H & J = 20X)

3.1.2 Alkaline Phosphatase Activity in ES-D3 cells and Stage 2 EBs

AP activity is a characteristic of undifferentiated ES cells (Lumelsky et al, 2001; Berrill et al, 2004). AP activity is decreased following differentiation. Using BCIP as a substrate for alkaline phosphatase, Figure 3.1.3 A indicates that the majority of the stage 1 ES-D3 culture stained for alkaline phosphatase activity. Stage 2 EBs consisted of both stained and unstained cells. The stained cells were at the centre of the EB surrounded by a halo of unstained cells. The pattern of staining demonstrates that the cells constituting the outer layer of the EB had a decreased level of alkaline phosphatase activity indicating that these cells had differentiated (Figure 3.1.3 B).

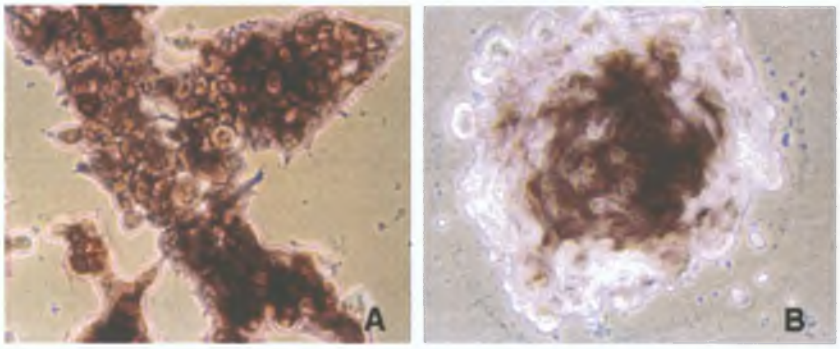


Figure 3.1.3 AP activity at stage 1 (A) and stage 2 (B) of Lumelsky based protocol. In stage 1 undifferentiated cells stained positive for AP. In stage 2 (B), embryoid bodies (EBs) consisted of both stained (undifferentiated) cells and unstained (differentiated) cells.

3.1.3 Analysis of Transcript Expression in Stages 1 – 5 of the Lumelsky Based Protocol

Cultures at stages 1 - 5 were analysed by semi-quantitive RT-PCR and qPCR analysis for expression of an extensive list of target genes representative of a number of lineages (Table 3.1.1 & Table 3.1.2).

A number of early developmental markers i.e. GATA 4 and nestin were detected at low levels in stage 1 ES-D3 cells (Figure 3.1.4 I & J). Germ-line specific transcription factor Oct 4 was down-regulated between stage 1 and stage 5. Multi-lineage marker expression was detected in stage 5 cultures (Table 3.1.1). Induced expression of pancreatic transcription factors Pdx1 (stage 2 – 5) and pax 4 (stage 5) occurred during the differentiation protocol (Figure 3.1.4 E & F). The

stage 5 culture expressed pancreatic β cell markers, type 2 glucose transporter (glut2), insulin I, insulin II (Figure 3 1 5 A - C) and alpha cell marker, somatostatin (Figure 3 1 4 C) Pancreatic polypeptide (PP) was detected at a low level in stages 1, 2 and 4 (Figure 3 1 4 B) However, this result varied from one biological repeat to the next (Table 3 1 1) Glucagon mRNA was not detected at any stage (Stage 3 1 4 D) Increased expression of the neuronal marker, myelin basic protein (MBP) and induced expression of glial fibrillary acidic protein (GFAP) was observed between stage 1 and stage 5 (Figure 3 1 4 L & M) Differentiated stage 5 cells also expressed extra-embryonic markers GATA 4, Oct 4 and alpha-feto protein (AFP) (Table 3 1 1) Apoptotic marker expression i e bcl-2 associated X protein (bax) and caspase 3 remained unchanged between stage 1 and stage 5 (Figure 3 1 4 N, O)

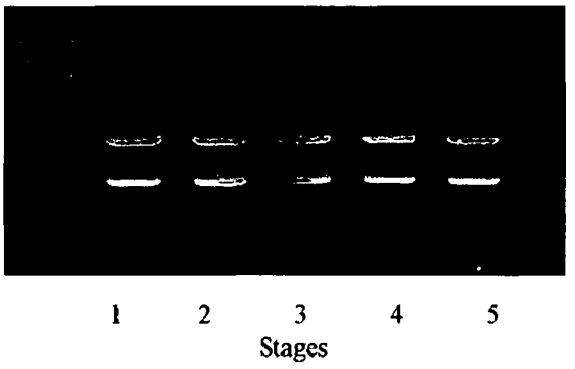
We found that ES-derived stage 5 clusters expressed markers representative of the neuronal/glial lineage (GFAP, MBP, β III tubulin), extra-embyronic lineage (GATA 4, AFP, Pdx1) and pancreatic endocrine lineage (pax 4, insulin I, insulin II, glut2) indicating the presence of multiple cell types in stage 5 clusters

Table 3.1.1 Data shown is the relative quantity (RQ) of gene expression in stage 1 – 5 cultures from 2 independent experiments (repeat 1, black; repeat 2, red). Refer to Figure 3.1.4 A – O for representative gels. RQ was determined using densitometry analysis. The RQ in the stage 1 ES-D3 cells was set to 1. In cases where there was no target detected in the ES-D3 cells, the stage at which expression was first induced was set to 1. A, Absent; ND, not determined; P, present.

Target	Stage									
	1		2		3		4		5	
Pluripotent										
Octamer 4	1	1	0.95	0.85	0.69	0.71	0.79	0.72	0.85	0.84
Endocrine Pancreas										
PP	1	1	A	0.473	A	A	0.456	A	A	A
Som	A	A	1	1	44.09	48.80	14.03	30.53	15.62	15.71
Glucagon	A		A		A		A		A	
Transcription Factors										
Pdx1	A	A	1	1	0.36	0.61	1.31	1.77	1.565	2.47
Pax 4	A	A	ND	ND	ND	ND	ND	ND	P	P
Exocrine Pancreas										
Amylase	A	A	A	A	A	A	A	1	P	0.893
Extra-embryonic										
AFP	A	A	1	1	0.27	0.11	0.51	0.73	3.83	1.42
GATA 4	1	1	0.73	0.87	1.26	1.34	1.53	1.358	1.70	2.16
Neuronal/Glial										
Nestin	1	1	1.66	1.71	1.86	2.06	1.17	2.02	1.39	1.36
B III Tubulin	1	1	0.88	0.86	1.19	1.01	1.05	1.00	0.94	0.86
GFAP	A	A	A	A	A	A	1	1	1.05	2.46
MBP	1	1	1.49	1.70	1.69	3.46	2.23	2.90	2.72	3.69
Apoptotic										
Bax	1	1	0.93	1.11	0.94	1.11	0.96	1.02	1.08	1.00
Casp3	1	1	1.08	0.94	1.49	0.99	0.82	1.02	1.01	1.07

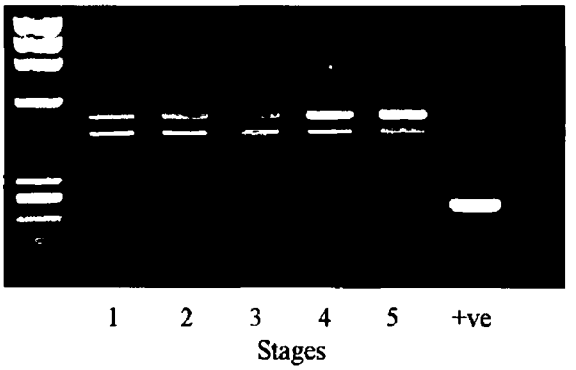
Figure 3 1 4 Semi-quantitive RT-PCR analysis of differential gene expression in Stage 1 – 5 Cultures Individual transcript analysis labelled A – O

Pluripotent Markers

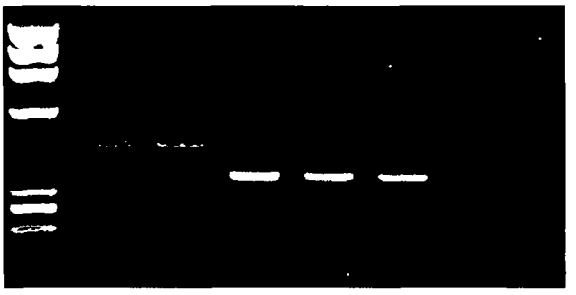


(A) Oct 4 was expressed in cultures from all stages of the differentiation protocol There was an overall decrease in expression between stage 1 and stage 5

Endocrine Pancreas Markers



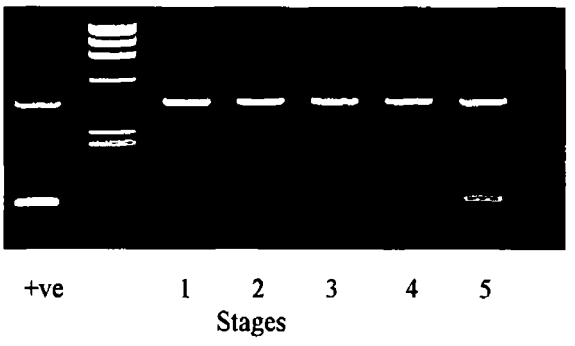
(B) Low level expression of PP was detected in the stage 1 culture PP expression was also detected at low levels in cultures from other stages of the differentiation protocol, however, this varied from one biological repeat to another The third band visible on the gel is perhaps due to a splice variant amplified by the PP primer set



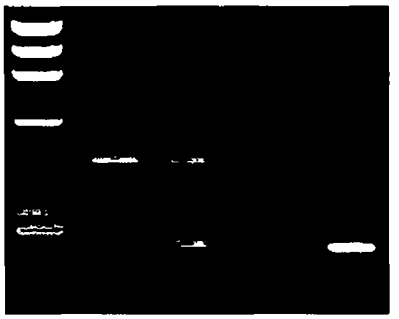
(C) Somatostatin expression was detected at a very low level in the stage 2 culture An up-regulation in expression was observed in the stage 3 culture, however, expression was subsequently down-regulated at stage 4 and 5

(D) Glucagon was not expressed in cultures at any stage of the differentiation protocol

Transcription Factors



(E) Pdx1 was expressed in cultures at stages 2 to 5 Highest Pdx1 expression was observed in the stage 5 culture

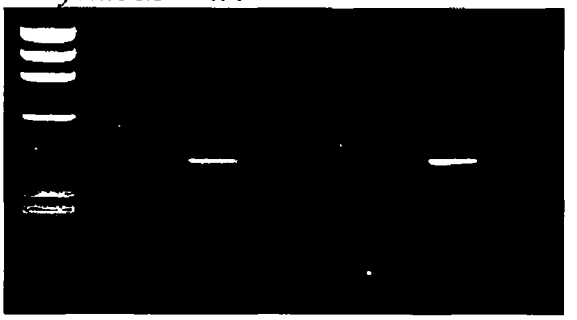


(F) Pax 4 was absent in the stage 1 culture and present in the stage 5 culture

Exocrine Pancreas Markers

(G) Amylase was expressed at a low level in the stage 5 culture

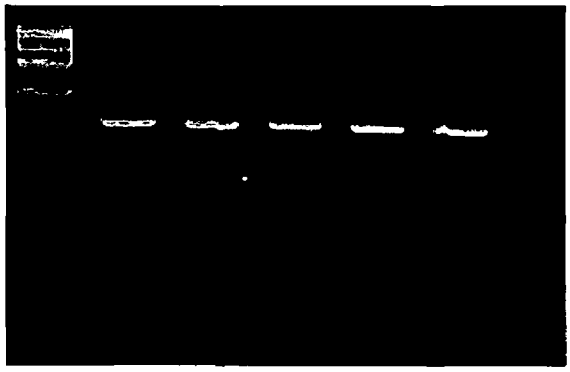
Extra-Embryonic Markers



(H) AFP was expressed in cultures at stages 2 to 4 with highest expression observed in the stage 5 culture

(I) GATA 4 was expressed in cultures at stages 1 to 5 Expression was up-regulated between stage 1 and stage 5

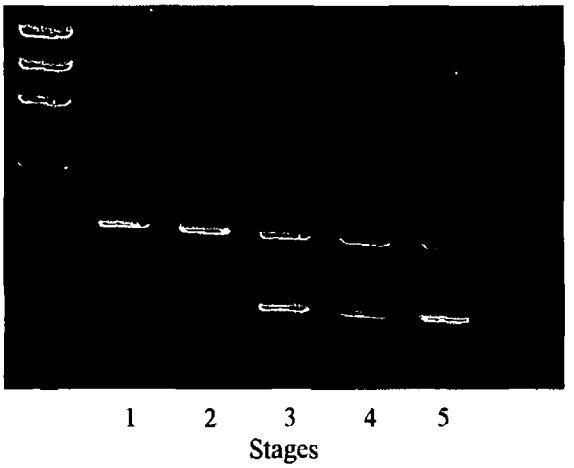
Neuronal/Glial Markers



(J) Nestin expression was detected at a low level in the stage 1 culture and up-regulated in cultures at stages 2 to 5

(K) β III Tubulin was expressed at a similar level in cultures at stages 1 to 5

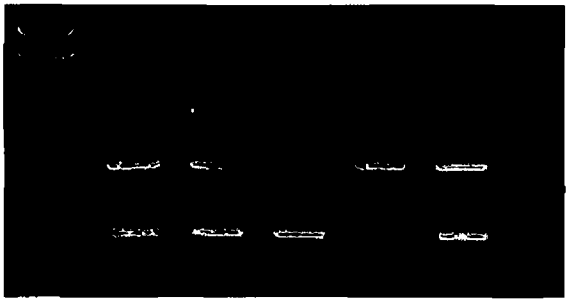
(L) GFAP was expressed in cultures at stage 4 and 5 with the highest level of expression observed in the stage 5 culture



(M) MBP was expressed in cultures at stages 1 to 5 An up-regulation in MBP expression was observed over the 5 stages of the differentiation protocol

Apoptotic Markers

(N) Bax was expressed at similar level in cultures at stages 1 to 5



(O) Caspase 3 was detected in all cultures at stages 1 to 5 There was no significant change in expression levels over the 5 stages

Table 3.1.2 Data shown is the relative quantity (RQ) of gene expression in stage 1 - 5 cultures from 2 independent repeats (repeat 1, black; repeat 2, red). Refer to Figure 3.1.5 for representative graphs. The RQ in the stage 1 ES-D3 cells was set to 1. A, absent; ND, not determined.

Target	Stage									
	1		2		3		4		5	
Insulin I	1	1	14.91	7.77	5.53	13.23	37.8	37.83	41.05	83.85
Insulin II	1	1	18.45	7.99	34.5	31.64	101.95	65.71	176.02	114.99
Glut2	1	1	ND	ND	ND	ND	ND	ND	61.29	65.67

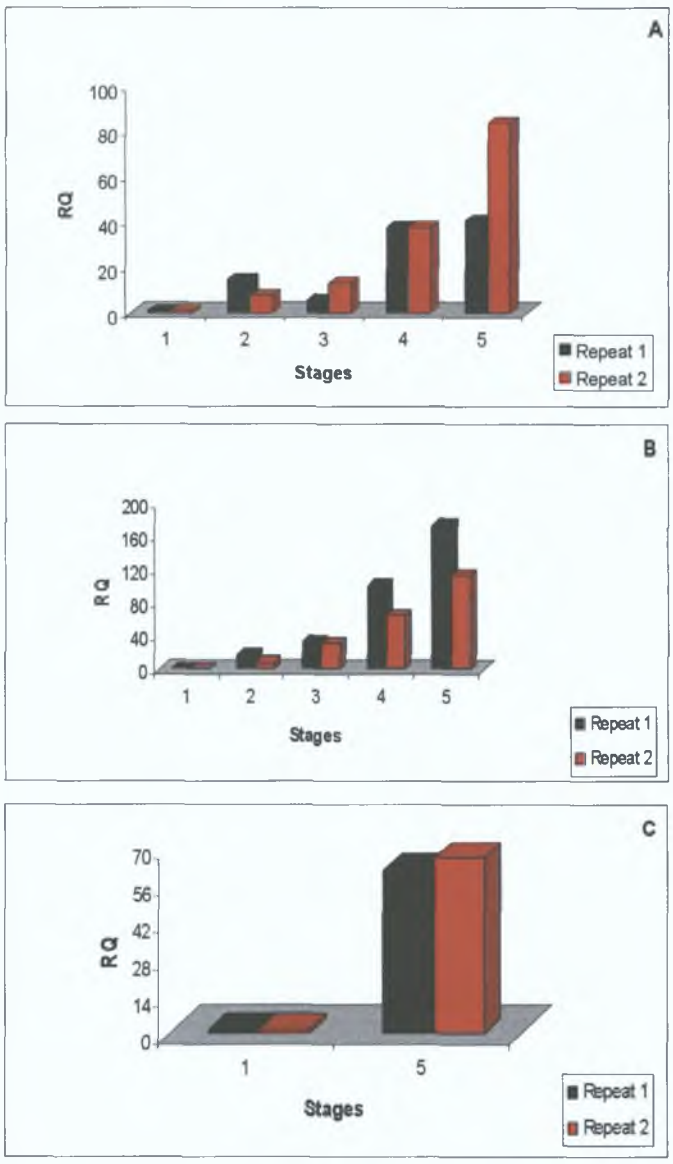


Figure 3.1.5 Graphs representative of qPCR results shown in Table 3.1.2. (A) insulin I, (B) insulin II and (C) glut2.

3 1 4 Analysis of Insulin/C-peptide Protein Expression in ES-derived Stage 5 cultures

Immunofluorescence staining was used to identify insulin, C-peptide 1 and C-peptide 2 positive cells in the differentiated stage 5 culture Stage 1 ES-D3 cells and the murine insulinoma cell line, MIN6, represented negative and positive biological controls, respectively Stage 1 ES-D3 cells were negative for insulin (Figure 3 1 6 A) but positive for C-peptide 1 (Figure 3 1 7 A) and C-peptide 2 (Figure 3 1 8 A) C-peptide 2 staining localised to the nucleus MIN6 cells stained positive for insulin (Figure 3 1 6 D), C-peptide 1 (Figure 3 1 7 C) and C-peptide 2 (Figure 3 1 8 C) The staining had a distinct punctate pattern for all proteins tested and was localised to the cytoplasm An isolated population of stage 5 differentiated clusters displayed a similar insulin-staining pattern to that of the MIN6 'β-cell' phenotype (Figure 3 1 6 B & C) Immunoreactivity for C-peptide 1 and C-peptide 2 was detected in a significant proportion of stage 5 differentiated cultures (Figure 3 1 7 B and Figure 3 1 8 B) In most cases, the anti-C-peptide antibodies stained in a cytoskeletal-like pattern Unexpectedly, immunoreactivity for C-peptide 1 and C-peptide 2 was also observed in the mouse mammary cell line, 4TI, which was thought to be a negative control (Figure 3 1 7 D and Figure 3 1 8 D)

Specificity of the C-peptide antibodies was checked by western blot (Figure 3 1 7 E and Figure 3 1 8 E & F) MIN6 cells were used as a positive control Stage 1 ES-D3, 4TI and FVB#C (fibroblast cell line derived from mouse ear) were used as negative control cell lines A band for C-peptide 1 was not detected at the appropriate size (3 kDa) by western blot in any of the cell lines including the positive control cell line, MIN6, however, a number of non-specific bands were detected at > 17 kDa (Figure 3 1 7 E) The C-peptide 2 antibody recognised a specific band for the C-peptide 2 protein at the expected size, 3 kDa, in the MIN6 cell line (Figure 3 1 8 E) Multiple non-specific higher molecular weight bands were observed in both positive and negative control cell lines A band for C-peptide 2 was not detected in stage 5 differentiated cell types (Figure 3 1 8 F 2 repeats, S5A and S5B)

These results suggest that the stage 5 differentiated clusters may contain a very small number of insulin-expressing cell types C-peptide staining may be overrepresented significantly in relation to actual levels in ES-derived cell populations Hence, C-peptide reactivity may not be definitive proof of, and may overestimate, β-cell differentiation in ES-derived cultures The C-peptide 2 western blot is a more reliable, quantitive method for detecting C-peptide 2 expression

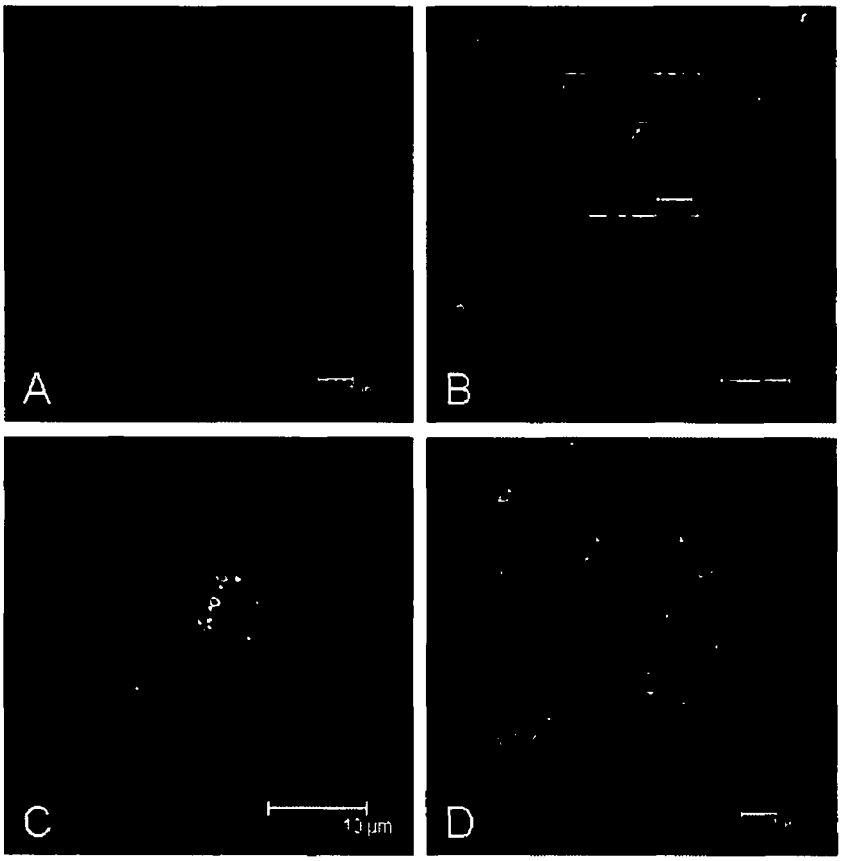


Figure 3 1.6 Insulin (A) Stage 1 ES-D3 cells were negative for insulin (B & C) Punctate insulin staining was visible in an isolated population of stage 5 differentiated cells Diffuse background staining was visible throughout culture (D) MIN6 cells, positive control, Insulin was expressed in a high proportion of cells (Insulin, green, DAPI nuclear stain, blue) Scale bar = 10 μm (A, C, D) and 20 μm (B)

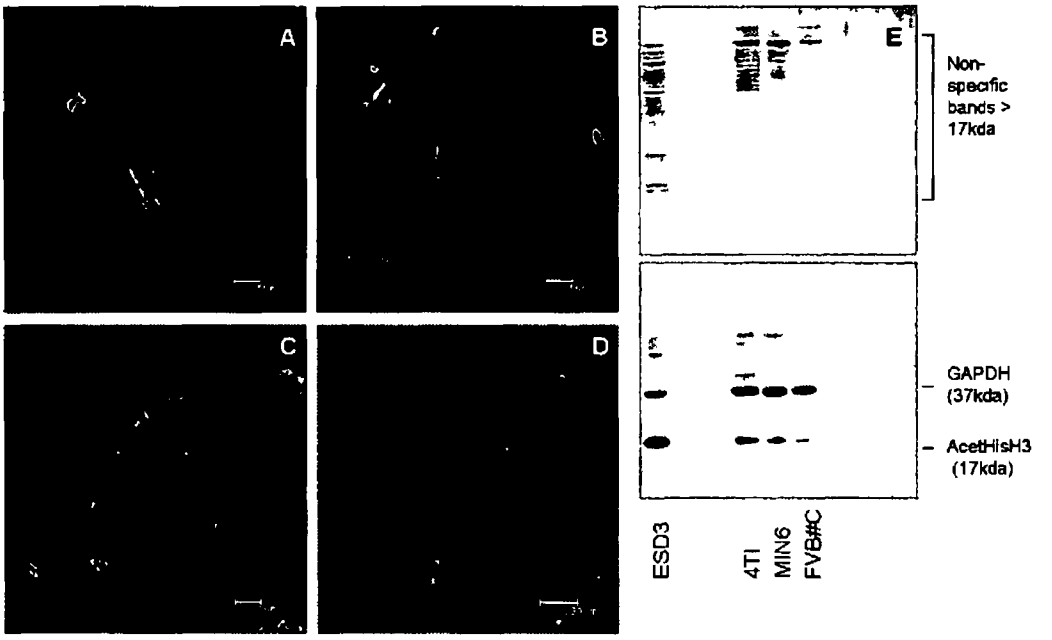


Figure 3 1 7 C-peptide 1 stage 1 ES-D3 cells (A), stage 5 differentiated cell types (B), positive control MIN6 (C) and negative control 4TI (D) were positive for C-peptide 1 The staining pattern indicative of granular staining exhibited in the MIN6 positive control (C) differed from the staining pattern exhibited by all other samples (A, B & D) Scale bar = 10 μm (A – C) and 30μm (D) Antibody specificity was subsequently checked by western blot (E) A band was not detected at the appropriate size by western blot in any of the cell lines including the positive control cell line MIN6, however, there was a number of non-specific bands detected at > 17 kDa 15μg protein loaded on western blot in all cases

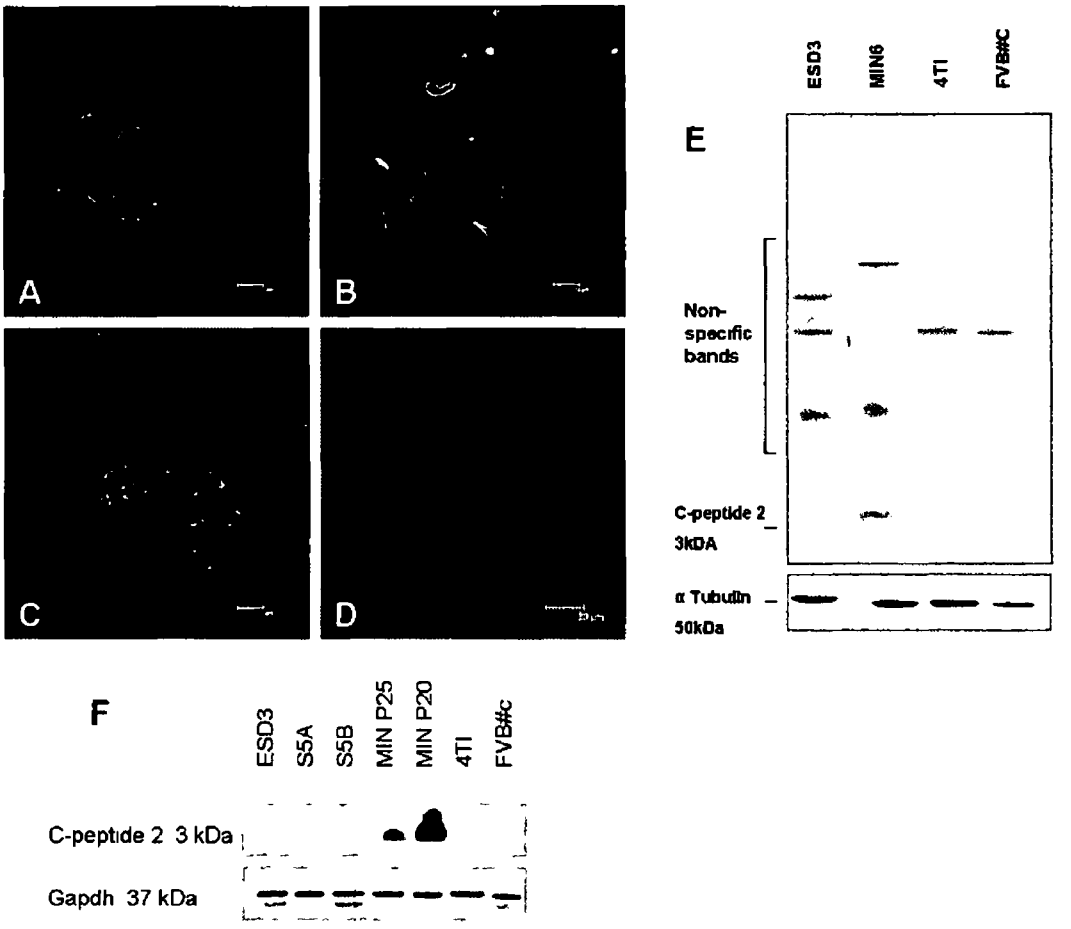


Figure 3 1 8 C-peptide 2 stage 1 ES-D3 cells (A), stage 5 differentiated cell types (B), positive control MIN6 (C) and negative control 4TI (D) were positive for C-peptide 2 The granular staining pattern indicative for positive staining exhibited in MIN6 (D) differed from the staining exhibited by all other samples Scale bar = 10 μm (A – C) and 30μm (D) Antibody specificity was subsequently checked by western blot (E) A band was detected at the appropriate size in the positive control cell line MIN6, however, a number of non-specific bands were detected by western blot in the negative control cell lines ES-D3, 4TI and FVB#c A C-peptide 2 western blot was also performed on stage 5 differentiated cell types (2 repeats, S5A and S5B) (F) Both were negative for C-peptide 2 expression 15 μg protein loaded on western blot in all cases

3 1 5 Intracellular Insulin Content and *In Vitro* Glucose-Stimulated Insulin Secretion

Stage 1 ES-D3, positive control MIN6, stage 5 differentiated cells, repeat 1 (S5A) and stage 5 differentiated cells, repeat 2 (S5B) were exposed to 3 3 mM, 16 7 mM and 26 4 mM stimulatory glucose concentrations for 1 hour Cells were lysed (one well of a 6 well plate) and analysed for insulin/protein content (Figure 3 1 9) The glucose buffers removed from the cells were also tested for insulin content to check for glucose stimulated insulin secretion (GSIS) (Figure 3 1 10) Stage 1 ES-D3 cells did not contain insulin when assayed by ELISA Stage 5 differentiated cultures contained approximately 36 ng insulin per mg protein (Figure 3 1 9) These ES-derived cultures also secreted insulin although they did not exhibit a significant increased secretory activity in response to glucose (Figure 3 1 10) The intracellular content and quantity of insulin released from ES-derived cultures varied between biological repeats of the experiment MIN6 cells demonstrated functional GSIS by increasing insulin release approx 4-fold over the glucose range tested (3 3 – 26 7 mmol/l)

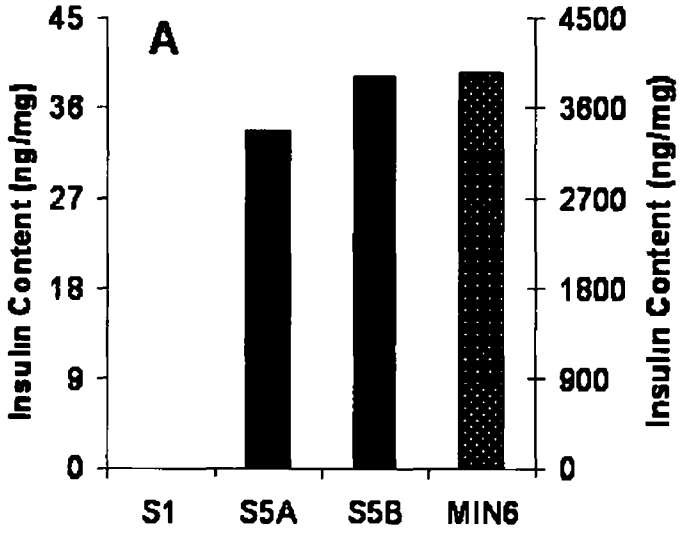


Figure 3.1 9 Intracellular insulin content Insulin content was normalised to the amount of protein in each sample Stage 1 ES-D3 (S1) and MIN6 represent negative and positive biological controls, respectively Stage 5, repeat 1 (S5A) and stage 5, repeat 2 (S5B) contained 33 7 and 39 ng insulin/mg protein, respectively

(MIN6 data plotted on secondary axis)

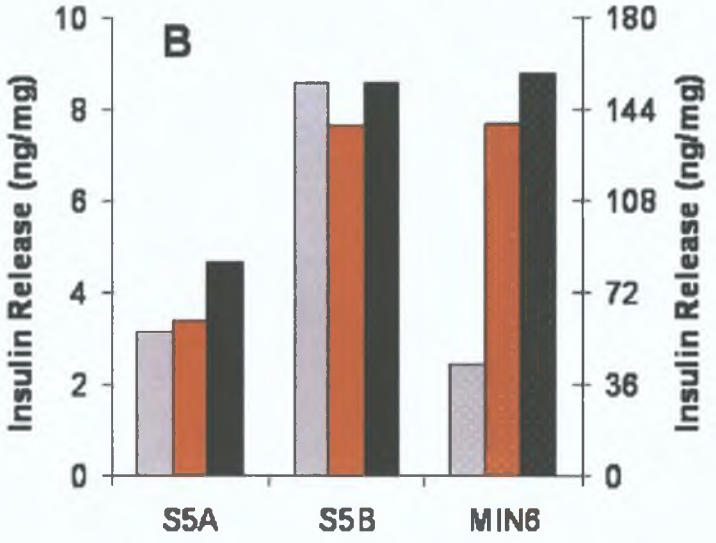


Figure 3.1.10 Insulin secretion was measured by ELISA and normalised to total cellular protein. Stage 5 differentiated clusters were pre-incubated for 1 hour with Krebs buffer containing 3.3 mM glucose. Cells were subsequently incubated with Krebs buffer containing 3.3 mM (grey), 16.7 mM (red) and 26.4 mM (black) glucose. Data is representative of two independent experiments, stage 5A (S5A) and stage 5B (S5B). MIN6 represented a positive biological control. *(MIN6 data plotted on secondary axis)*

3 1 6 Demonstration of *De Novo* Insulin Synthesis

The novel assay we designed to identify *de novo* insulin synthesis consisted of metabolic labelling (ML) of total cellular protein over a 6 hour time period followed by a modified radio-immunoassay (MRIA) We used the MIN6 cell line as a positive control to develop and prove the utility of the assay The 6 hour labelling time frame ensured that a significant percentage of newly synthesized insulin incorporated ^{35}S-L-cysteine (mature murine insulin contains 6 cysteine residues) Initial experiments investigated a 1 hour ML time frame compared to a 20 hour ML time frame Labelling for 20 hours compared to 1 hour required the use of a higher concentration of cold insulin competitor to compete with labelled insulin (Figure 3 1 11) In subsequent experiments, an ML time frame of 6 hours was chosen for technical reasons (i e ability to perform labelling step within a 1 day period contributed to the user-friendliness of the assay) It was found that labelled insulin competed with similar concentrations of 'cold' insulin competitor in both the 20 hour and 6 hour ML experiments (Figure 3 1 11 B compared to Figure 3 1 12 B & C)

Total protein was extracted and the lysates were fractionated by ultra-filtration to remove larger proteins (> 50 kDa), including a large population of radio-labelled, 'sticky' proteins We found that this step dramatically decreased the level of background signal in the subsequent MRIA and greatly improved assay performance (See Figure 3 1 12 A compared to Figure 3 1 12 B & C) It should be noted that this step also removes a small proportion (5 - 10%) of preproinsulin (11 5 kDa) and insulin (6 kDa) due to the technical performance of the ultra-filtration membrane (data estimate from Centricon source) Wells on the ELISA plate were pre-blocked with non-labelled cell lysate, which decreased the noise in sampling i e standard deviation (See Figure 3 1 12 B compared to Figure 3 1 12 C)

In the subsequent MRIA assay, radio-labelled insulin in the lysate was bound by the antibody coated on the ELISA plate, subsequently eluted by digestion with papain and the radioactive signal was measured by scintillation counting By including known, increasing concentrations of competitor substrate (unlabelled insulin) during binding, we were able to detect a drop in radio-labelled insulin

bound to the antibody At low concentration of competitor (up to $2.5x10^{-4}$ mg/ml) there was no change in the amount of labelled insulin bound to the antibody but as this increased (maximum 0 25 mg/ml) there was a sudden drop in counts from the plate (Figure 3 1 13 A) By plotting various dilutions of MIN6 protein lysate (expressed in terms of total insulin measured by conventional ELISA) against the cpm (counts per minute) detected, we were able to generate a standard curve that could be used to determine the minimum detectable level of labelled insulin in the MIN6 sample (Figure 3 1 13 B) Applying the equation of the slope of the line we determined that the lowest theoretical concentration of *de novo* synthesised insulin detectable in this assay is 0 4 ng/ml However, this would be very close to background levels therefore using a cut-off of 3 X SD above this as a minimum would be more practical (SD = ± 12 for highest dilution) This gives a minimum detectable quantity of 0 71 ng/ml

Having successfully identified *de novo* insulin synthesis in the control MIN6 cells we applied the assay to differentiated ES-D3 cells (stage 5), with untreated ES-D3 cells (stage 1) as a negative control First we measured the amount of total insulin in the lysates by conventional ELISA, including MIN6 as a positive control The control is included to account for inter-assay variations in specific activity of radioisotope, labelling efficiency, cell lysis, etc We also measured total protein content prior to ultra-filtration As can be seen in Table 3 1 3, there was a small but significant quantity of insulin detected by ELISA in the stage 5 cells (88 ng/ml), similar to levels found previously When analysed using the competitive MRIA, the lysate from undifferentiated ES cells showed a low level of radioactive counts (~ 400 cpm) and there was no detectable decrease at high concentrations of competitor, as expected (Figure 3 1 14) The background signal was similar for the stage 5 cells but, significantly, high concentration competitor did not cause any drop in the signal demonstrating absence of detectable, bound, radio-labelled insulin This would suggest that the cells do not synthesize the majority of the 88 ng/ml insulin detected by standard ELISA

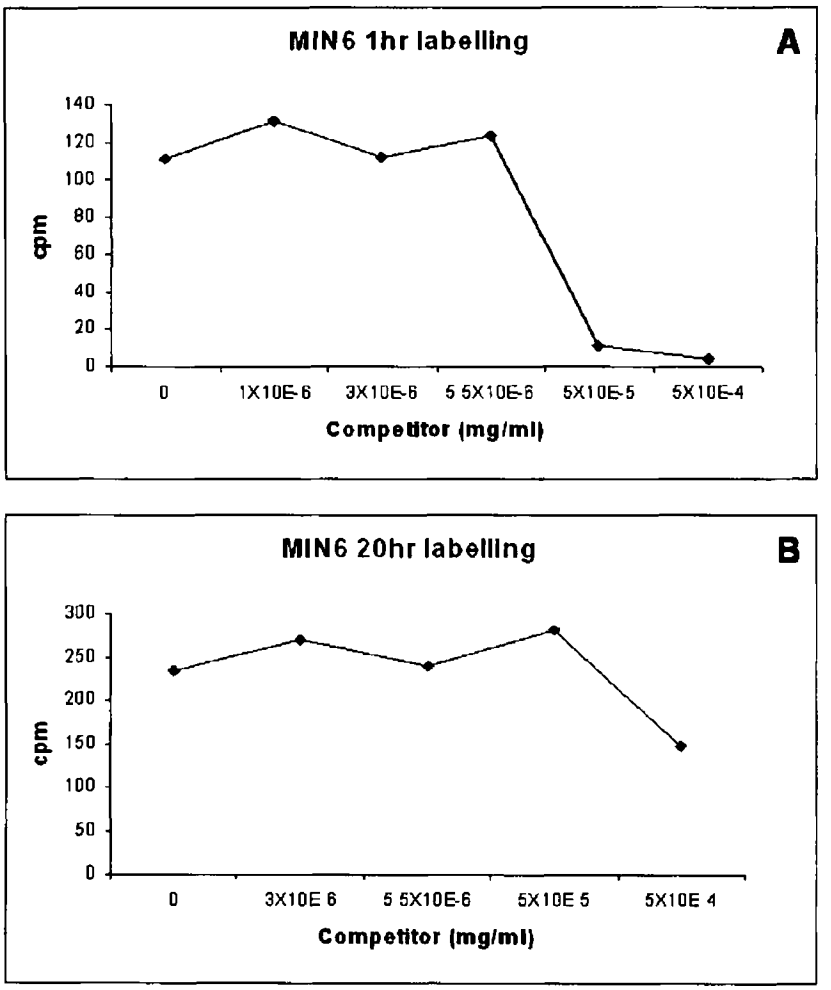


Figure 3 1 11 Metabolic Labelling Times were asessed on MIN6 cells (A) 1 hour time frame (B) 20 hour time frame Labelling for 20 hours compared to 1 hour required the use of a higher concentration of cold insulin competitor to compete out labelled insulin A 6 hour labelling time frame was chosen for subsequent experiments

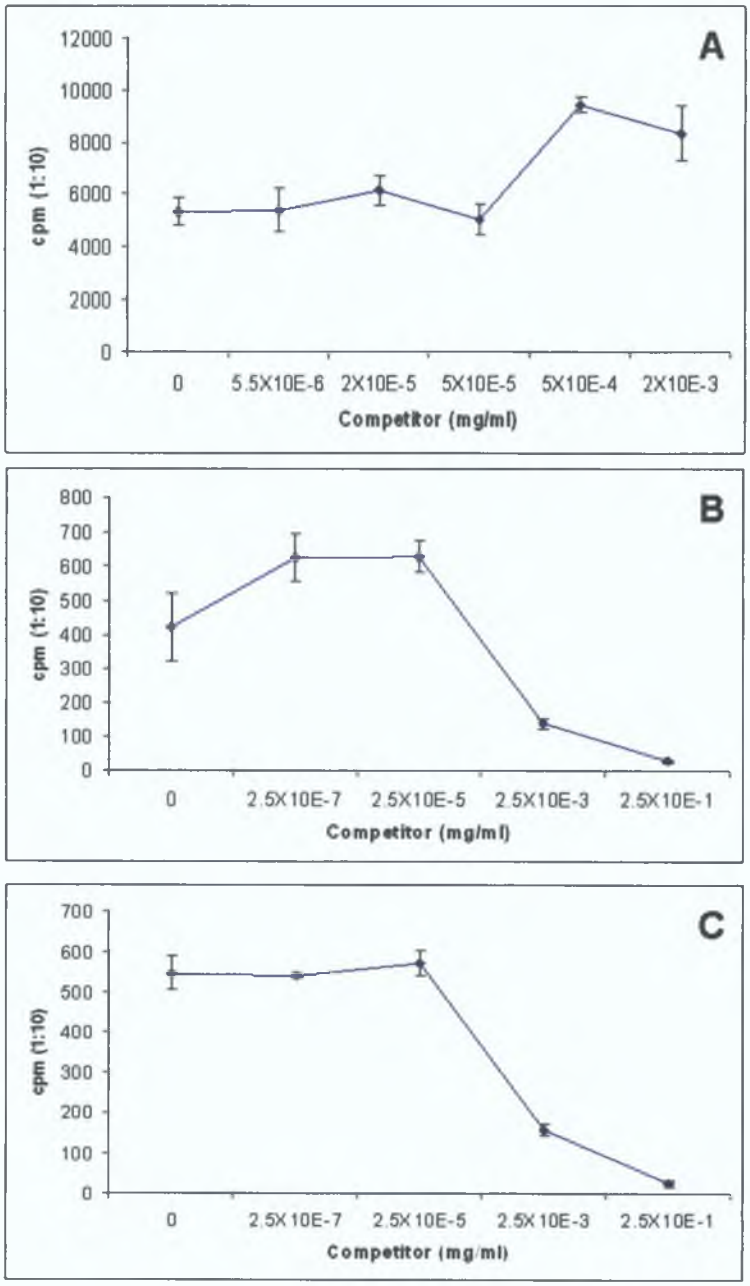


Figure 3.1.12 ^{35}S labelled MIN6 competitive MRIA optimisation. Ultra-filtration of lysates decreased background levels and improved assay performance. See Figure A (without filtration) compared to Figure B &C (with filtration). A pre-blocking step introduced to the assay reduced the noise in sampling (Standard Deviation, SD) in the MRIA step. See Figure B (no blocking) compared to Figure C (blocking included). Data represents mean ± SD from triplicate sample cpm readings.

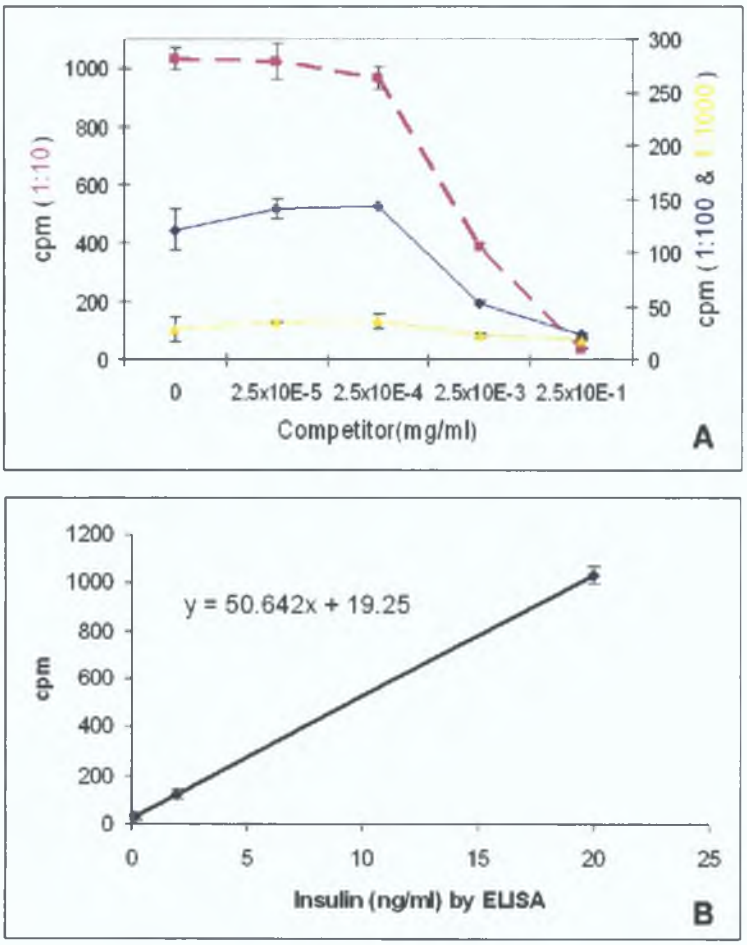


Figure 3.1.13 (A) Competitive binding of radio-labelled and non-labelled insulin to ELISA plate. Increasing the amount of 'cold' Competitor insulin from 0 to 0.25 mg/ml in the well displaced bound MIN6-derived labelled insulin. Metabolicly labelled MIN6 lysate diluted 1:10 (– – –), 1:100 (——), 1:1000 (——). (B) Radioactive counts (cpm) versus total insulin concentration. A standard curve was generated by plotting total bound cpm in non-competed, serial (10-fold) dilutions of MIN6 lysate against total insulin concentration as determined by ELISA. For very precise quantitation in any cell extract, a standard curve must be prepared as part of each experiment, due to inter-assay variations in labelling efficiency and specific activity. Data represents mean ± SD from triplicate sample cpm readings.

Table 3.1.3 Detection of *de novo* insulin in cell lysates. Protein refers to total protein concentration prior to ultra-filtration. Total insulin concentration was determined by mouse (sensitive) ELISA. Cpm are those detected in diluted samples (1:5).

	Protein (mg/ml)	Total Insulin(ng/ml)	Insulin ng/mg Protein	Cpm*	Cpm**
ES-D3	0.323	1.96	6.07	452	438 +/- 13
Stage 5	2.35	88	37.4	380	368 +/- 16
MIN6	0.341	2986	8757	2861	155 +/- 4

*cpm detected without addition of competitor **cpm detected after addition of high conc. competitor

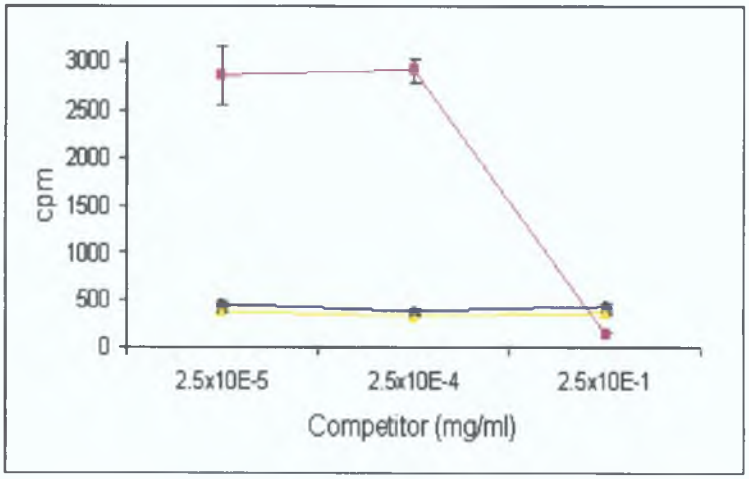


Figure 3.1.14 Competitive MRIA: (A) MIN6 cells (—), stage 1 ES-D3 cells (—), stage 5 ES-derived differentiated cells (—). All samples lysed in NaOH and diluted 1:5. MIN6 showed a decrease in cpm with increasing competitor indicating *de novo* insulin synthesis (total insulin). ES-D3 and ES-derived stage 5 differentiated cells did not show a decrease in cpm with increasing competitor despite the fact that the stage 5 ES-derived differentiated population contained approximately 45 times more total insulin (ng/ml) than the stage 1 ES-D3 population measured by ELISA. Data represents mean ± SD from triplicate sample cpm readings.

3 2 TWO-STAGE DIFFERENTIATION STUDIES

The derivation of definitive endoderm and in particular endocrine cell types from undifferentiated ES cells remains difficult to achieve Multi-step regulated differentiation protocols described for the derivation of pancreatic cell types are long, cumbersome, often involve the use of exogenous insulin and also result in low yields of insulin-producing cells The validity of these protocols has been questioned on the basis that ES-derived cells can absorb insulin from the feed media and may not produce insulin themselves (Section 3 1) In this study, we investigated the potential to regulate the differentiation of ES cells into endodermal derivatives (in particular insulin-producing cells) using extra-cellular factors previously associated with various aspects of pancreatic development

Previous studies, both in our laboratory and by others, have demonstrated that retinoic acid promotes both neuronal and endodermal differentiation (Section 1 1 7 1 & 1 5 1) It has also been shown that similar elements control the development of both the pancreas and central nervous system (CNS) (Lumelsky et al, 2001) In this study, undifferentiated ES-D3 cells were removed from LIF and allowed to form EBs in the presence of retinoic acid (RA) (stage 1 differentiation) (Section 3 2 1) The RA-treated EBs were subsequently exposed to one of the following Sodium Butyrate (SB), Betacellulin (BTC) and Activin A (AA) (stage 2 differentiation) (Figure 2 3 1) A comparative analysis was performed on these models of directed differentiation in parallel with a model of spontaneous differentiation Lineage differentiation was determined by profiling multi-lineage transcript expression (neuronal, glial, muscle, exocrine and endocrine pancreas, extra-embryonic and apoptotic) (Section 3 2 3) and subsequent protein expression within ES-derived cultures (Section 3 2 4)

Using the 2-stage differentiation protocols developed during this study, we successfully demonstrated the derivation of an intermediate multi-potential population (RA-treated EBs) from undifferentiated ES cells that preferentially gives rise to pancreatic endocrine insulin-expressing cell types in the presence of SB, and neuronal- and glial-like cell types in the presence of AA or BTC The short 2-stage RA_SB protocol developed in this study does not require

exogenous insulin for the derivation of insulin-positive cell types, indicating that any insulin expressed is *de novo* synthesized

3 2 1 Formation of Embryoid Bodies in the Presence of Retinoic Acid

Undifferentiated ES-D3 cells are seeded over-night in suspension culture prior to being treated with 100 nM RA After 3 days in suspension culture in the presence of RA the cells have formed tight rounded aggregates resembling EBs (Figure 3 2 1)

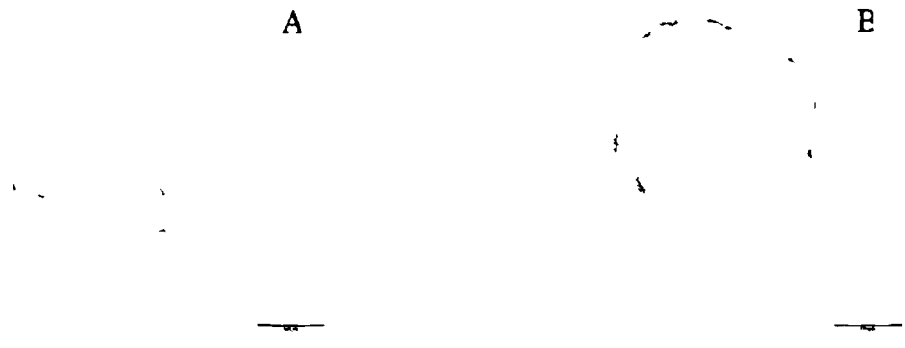


Figure 3 2 1 Formation of RA-treated EBs (A) ES-D3 grown in monolayer, scale bar = 200 μm (B) RA-treated EBs grown in suspension, scale bar = 100μm (A = 10X)(B = 20X)

3 2 2 Alkaline Phosphatase Activity in ES-D3 cells and 5 Day ES-derived Cultures

AP activity is a characteristic marker of undifferentiated pluripotent stem cells Down-regulation of AP activity is indicative of differentiation (Lumelsky et al, 2001, Prelle et al, 2002, Berrill et al, 2004) Day 1 ES-D3 cells, day 5 control EBs (CO_5d) and day 5 RA-treated EBs were analysed for AP activity to ascertain the differentiation status of the cells both before and after stage 1 differentiation EBs were plated onto a tissue culture surface prior to AP analysis Day 1 ES-D3 cells stained positive for AP activity (Figure 3 2 2 A & B) Day 5 RA-treated EBs (Figure 3 3 2 C & D) were distinctive in both morphology and staining pattern from CO_5d EBs (Figure 3 2 2 E & F) RA-treated EBs had neuronal like projections extending from the edge of the EBs that only stained weakly for AP activity and were larger in size than CO_5d EBs

These neuronal like projections were most likely a result of differentiation. CO_5d EBs were predominately stained for AP activity (Figure 3.2.2 E & F). Intensity of staining decreased towards the outer layer of the EB suggesting that some of these cells may have started to differentiate.

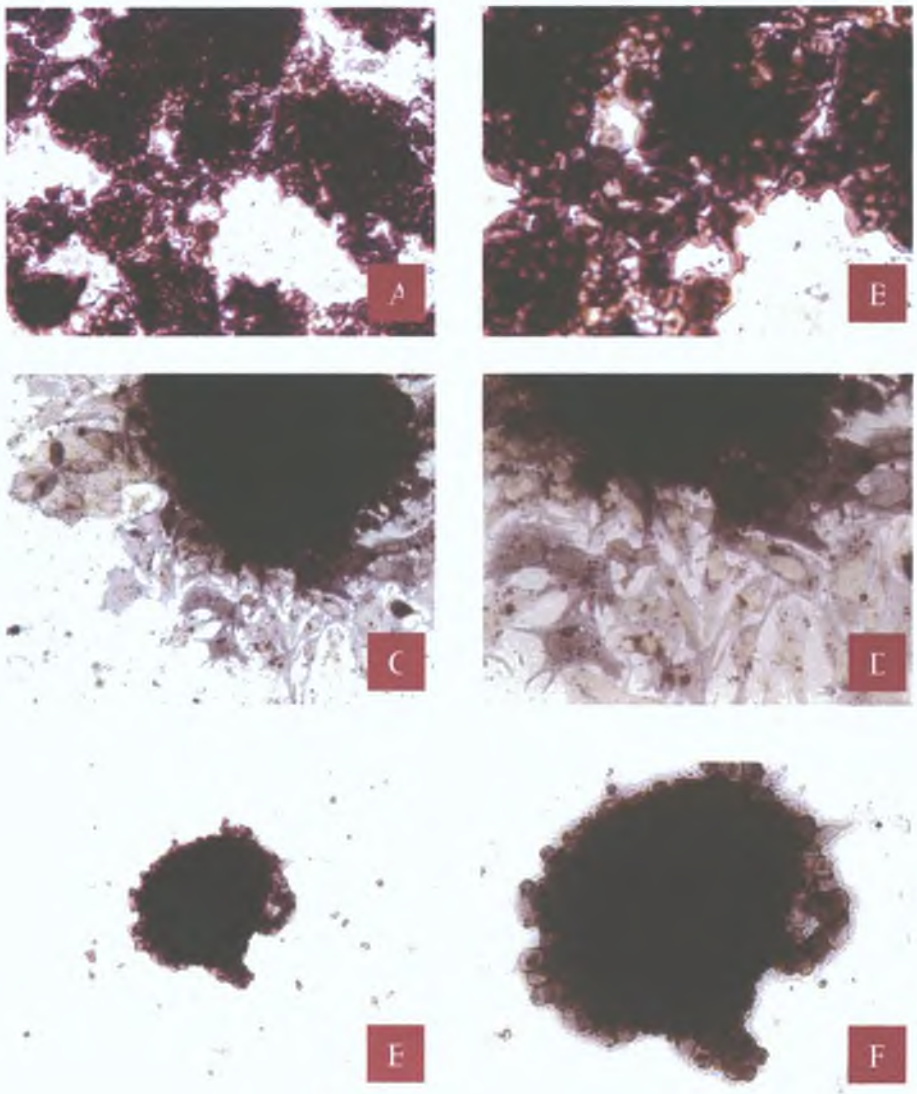


Figure 3.2.2 AP expression in ES-D3 cells and 5 day ES-derived Cultures. ES-D3 cells stained intensely for AP expression (A & B). RA-treated EBs had neuronal like projections extending from the edge of the EBs that stained weakly for AP expression (C & D). CO_5d EBs predominately stained for AP expression (E & F). Intensity of AP staining decreased towards the outer layer of the CO_5d EBs suggesting that some of the outer cells may have started to differentiate. (A, C & E = 20X) (B, D & F = 40X)

3.2 3 Analysis of Transcript Expression in ES-D3 and ES-derived Cultures

We evaluated the expression of multi-lineage marker transcripts (neuronal, glial, pancreatic exocrine & endocrine, extra-embyronic, muscle and apoptotic) in ES-derived cultures by semi-quantitive RT-PCR to characterize the cell types derived using the 2-stage differentiation protocols (Table 3 2 1 and Figure 3 2 3) Particular emphasis was placed on expression analysis of pancreatic endocrine β cell markers qPCR was used to accurately determine the relative quantity of insulin I, insulin II and glut2 transcript expression in ES-derived samples (Figure 3 2 4) Optimisation experiments were performed for the qPCR analysis as shown in Section 3 2 3 2 Two endogenous controls were tested by qPCR to ensure that the presence of extra-cellular factors had not affected the endogenous control expression thus affecting overall trends in target gene expression Both GAPDH and β-actin resulted in similar trends in target gene expression (Figure 3 2 5) PCR efficiency using insulin I, insulin II, GAPDH and β-actin primer and probe sets was checked on serial 10 fold dilutions of cDNA from a positive control cell line i e MIN6 (Figure 3 2 6) All primer and probe sets demonstrated an efficiency of > 90% (Table 3 2 3)

3 2 3.1 Summary of Results from Semi-Quantitive RT-PCR and qPCR Analysis of Multi-lineage Marker Expression in ES-D3 and ES-derived Cultures

ES-D3 cells cultured in the absence of feeder layers expressed early developmental markers (e g nestin, islet-1, ngn3) indicating that initiation of differentiation takes place early in a feeder-free culture system (Figure 3 2 3 B, J, L) Spontaneously-formed 5-day control EBs (CO_5d) expressed a broad range of multi-lineage markers (Figures 3 2 3 & Figure 3 2 4) Addition of RA to the culture media during EB formation enhanced the expression of a subset of these markers (nestin, neurofilament-light (NF-L), myelin basic protein (MBP), islet-1, pax 6, pcsk2, alpha-fetoprotein (AFP), glut2) and induced the expression of sonic hedgehog (shh) and somatostatin (Table 3 2 1 and Figure 3 2 4) RA repressed expression of the skeletal muscle regulatory gene, myogenin (Figure 3 2 3 G) Transcript expression of β cell-specific insulin I and insulin II was marginally higher in the CO_5d culture compared to the RA culture (Figure 3 2 4 A, B),

however, expression of pancreatic transcription factor Pdx1 was greatly increased in the CO_5d culture (Figure 3 2 3 K)

The addition of differentiation-inducing agents to RA-treated EBs altered gene expression (Table 3 2 1 and Figure 3 2 4) SB promoted the mRNA expression of pancreatic transcription factors (Pdx1, nkx2 2, beta2), pancreatic endocrine hormones, pancreatic polypeptide (PP) and extra-embryonic markers, AFP between day 5 (RA) and day 9 (RA_SB) of the differentiation protocol SB also greatly induced expression of β cell mRNAs (pre-proinsulin, insulin I, insulin II and glut2) (Table 3 2 1 and Figure 3 2 4) SB repressed expression of the neuronal marker, NF-L, the transcription factors, shh, the δ cell marker, somatostatin and the enzyme, pcsk2 (Table 3 2 1)

AA and BTC promoted the mRNA expression of the astrocyte marker, glial fibrillary acidic protein (GFAP) and shh, somatostatin and pcsk2 in differentiated 9 day, RA_AA and RA_BTC cultures, respectively (Table 3 2 1) The oligodendrocyte marker, MBP, and neuronal markers, NF-L and β III tubulin, were also expressed within these cultures but their level of mRNA expression remained unchanged between day 5 and day 9 of the differentiation protocol Insulin I, insulin II and glut2 mRNA expression was repressed in RA_AA and RA_BTC cultures (Figure 3 2 4) Apoptotic marker expression i e bcl2-associated protein (bax) and caspase 3, remained unchanged between ES-D3 cells and ES-derived EB cultures (Figure 3 2 3 Y, Z) Pax 4 (pancreatic transcription factor), glucagon (marker of pancreatic endocrine α cells) and amylase (marker of the exocrine pancreas) were undetected in ES-D3 and ES-derived cultures (Figure 3 2 3 O, U, W) There was no dramatic change in multi-lineage mRNA expression between spontaneously-formed EBs at day 5 (CO_5d) and day 9 (CO_9d) with exception to the muscle markers, both desmin and myogenin were expressed at higher levels in the CO_5d culture compared to the CO_9d culture (Table 3 2 1)

The range of transcript expression identified in the RA culture suggested a mixed population of multi-potential precursors Stage 2 differentiation of the RA culture in the presence of sodium butyrate encouraged pancreatic endocrine marker

expression Stage 2 differentiation in the presence of AA or BTC promoted neuronal- and glial-specific transcript expression

Table 3 2 1 Data is representative of the relative quantity (RQ) of gene expression in undifferentiated ES-D3 and ES-derived cultures Refer to Figure 3 2 3 for gels and associated densitometry RQ was determined using densitometry analysis Target expression detected in the ES-D3 cells was set to 1 In cases were there was no target detected in the ES-D3 cells, the sample expressing the lowest target level was set to 1 (A, Absent)

	ES-D3	RA	CO_5d	CO_9d	RA_AA	RA_SB	RA_BTC
Markers							
Differentiation							
Oct 4	1	0 788	0 867	0 839	0 790	0 584	0 827
Neuronal/Glial							
Nestin	1	1 242	0 583	0 356	1 085	1 060	1 049
GFAP	1	A	A	A	2 342	A	0 956
NFL	1	1 339	0 653	0 814	1 004	0 723	0 913
MBP	1	1 257	0 893	0 930	0 740	0 840	0 834
β III Tubulin	1	1 008	0 907	0 923	1 032	0 942	0 973
Muscle							
Myogenin	1	1 641	3 619	2 413	1 208	1 611	1 242
Desmin	1	1 403	1 426	0 582	1 085	1 486	1 443
Trancription Factors							
Shh	A	5 994	A	A	4 599	1	4 284
Islet 1	1	2 069	0 638	0 694	0 421	0 701	0 521
Pdx1	1	2 780	5 941	4 547	2 527	4 072	2 829
Ngn3	1	1 784	1 826	1 521	0 825	0 440	0 765
Beta2	1	0 746	0 845	0 796	0 794	1 149	0 732
Pax 6	1	1 440	0 450	0 848	0 726	0 811	0 808
Pax 4	A	A	A	A	A	A	A
Nkx2 2	1	3 992	4 378	3 571	4 233	6 447	4 992
Endopeptidases							
PC1	1	0 521	0 455	0 865	0 601	0 397	0 506
PC2	1	1 387	0 821	0 422	2 390	0 573	1 768

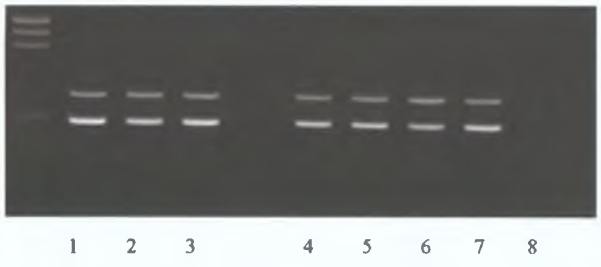
Endocrine Pancreas							
Som	1	8 460	1 364	A	5 706	1 571	10 310
PP	1	1 090	A	0 341	A	0 955	A
Glucagon	A	A	A	A	A	A	A
PPI	1	2 396	5 862	2 905	1 111	13 383	2 451
Exocrine Pancreas							
Amylase	A	A	A	A	A	A	A
Extra-embryonic							
AFP	A	2 947	1	1 394	3 499	5 279	3 749
Apoptotic							
Bax	1	1 057	1 050	1 063	0 897	0 986	0 893
Caspase 3	1	1 179	1 031	1 142	1 141	0 945	1 036

Table 3.2.2 Loading order for gels shown in Figure 3.2.3

Lane	Sample
	Marker
1	ES-D3
2	RA
3	CO_5d
4	CO_9d
5	RA_AA
6	RA_SB
7	RA_BTC
8	Negative Control: H_20
9	Positive Control: MIN6

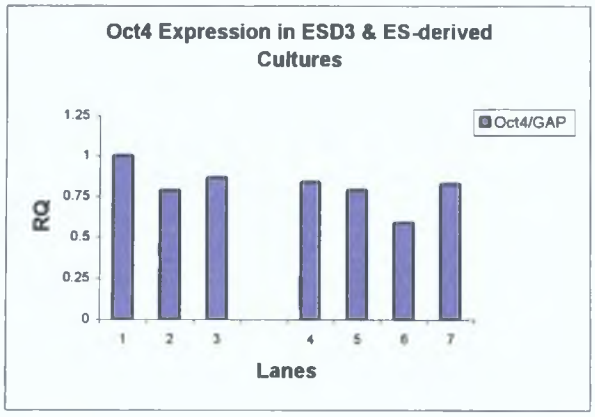
Figure 3.2.3 Semi-quantitive RT-PCR analysis of differential gene expression in undifferentiated ES-D3 and ES-derived cultures. Individual transcript analysis labelled A-Z. See Table 3.2.2 for loading order of gels. RQ, relative quantity.

Differentiation Markers



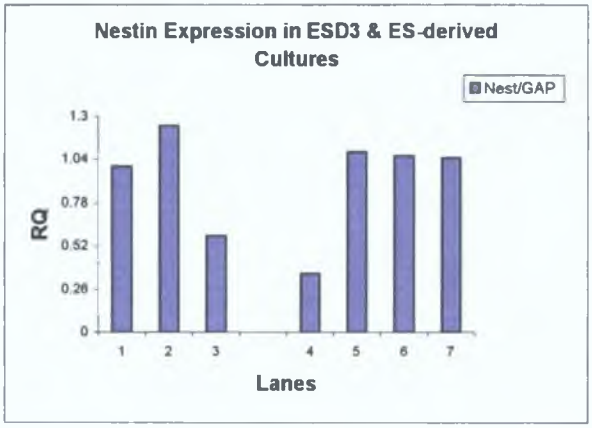
GAPDH

Oct 4

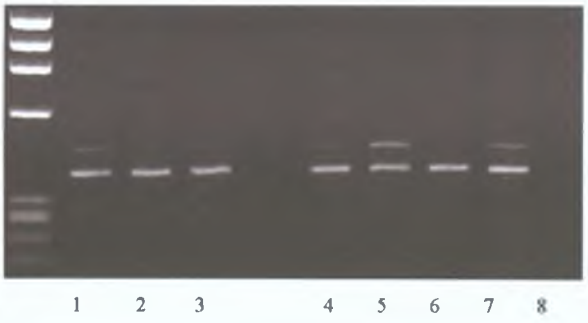


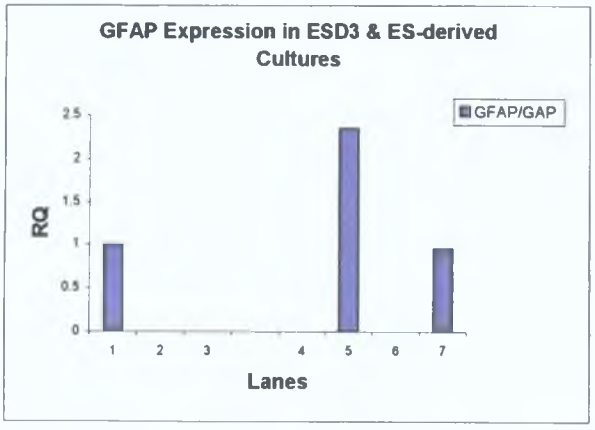
(A) Oct 4 mRNA expression was observed in ES-D3 cells and was down-regulated in all ES-derived cultures. The lowest level of Oct 4 expression was in the RA_SB culture.

Neuronal/Glial Markers

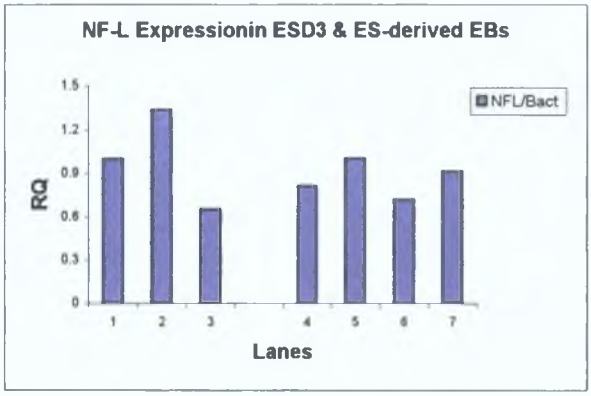


(B) Nestin mRNA expression was observed in ES-D3 cells. Nestin expression was up-regulated in the RA culture and subsequent treated EB cultures, but down-regulated in the spontaneously formed CO_5d and CO_9d cultures.

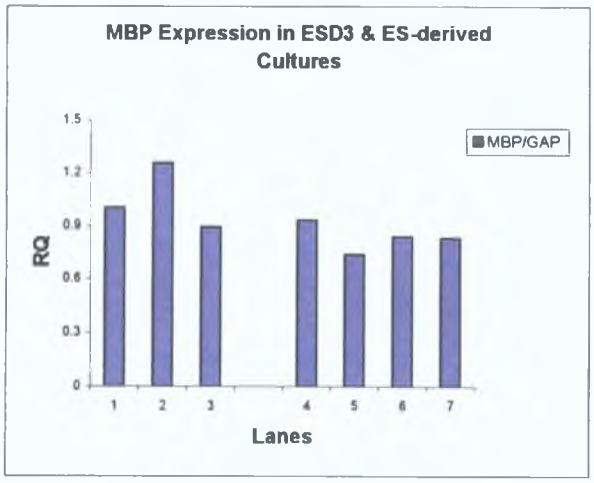




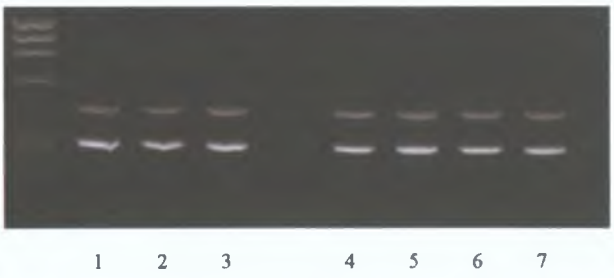
(C) GFAP mRNA expression was observed in ES-D3 cells and down-regulated in all ES-derived cultures with the exception of the RA_AA and RA_BTC cultures. GFAP expression was up-regulated in the RA_AA culture.



(D) NF-L mRNA expression was observed in ES-D3 cells and up-regulated in the RA culture. The level of NF-L expression was maintained in the presence of AA and BTC but was down-regulated in the presence of SB. NF-L expression was also down-regulated in control EBs (CO_5d and CO_9d).



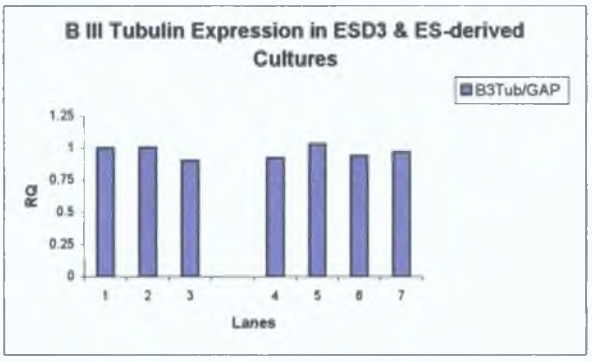
(E) MBP mRNA expression was observed in ES-D3 cells. MBP was up-regulated in the RA culture; however, there was no significant changes in expression in the other ES-derived cultures.



GAPDH

β III Tubulin

1 2 3 4 5 6 7



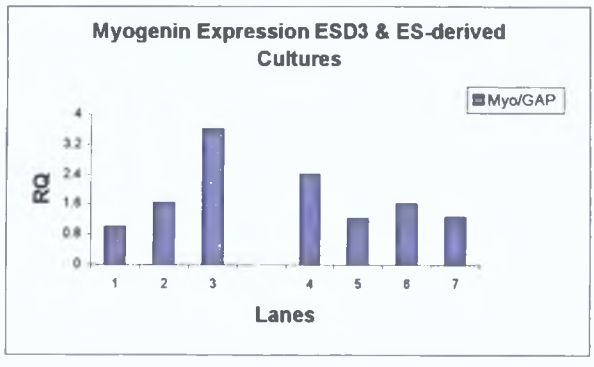
(F) β III Tubulin mRNA was expressed at similar levels in ES-D3 cells and all ES-derived cultures.

Myogenic Markers

GAPDH

Myogenin

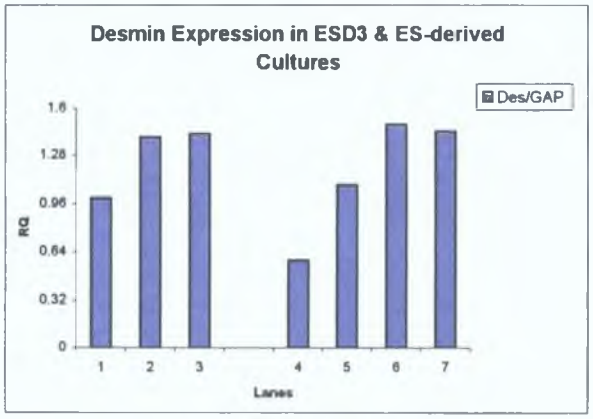
1 2 3 4 5 6 7 8



(G) Low level myogenin mRNA expression was observed in ES-D3 cells and all ES-derived cultures. Myogenin expression was up-regulated in spontaneously formed control EBs (CO_5d and CO_9d).

GAPDH
Desmin

1 2 3 4 5 6 7 8



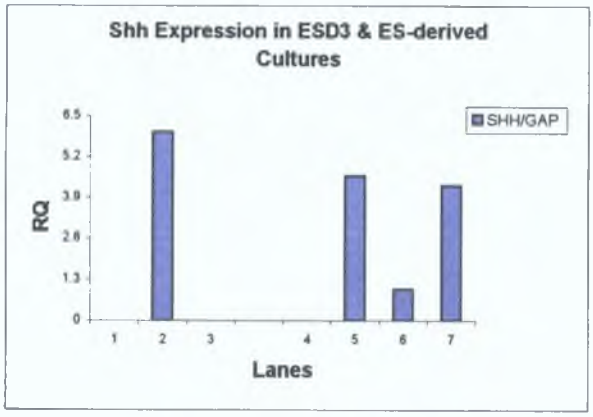
(H) Desmin mRNA expression was observed in ES-D3 cells and was up-regulated in all ES-derived cultures with the exception of the CO_9d culture.

Transcription Factor Markers

GAPDH

Shh

1 2 3 4 5 6 7 8 9

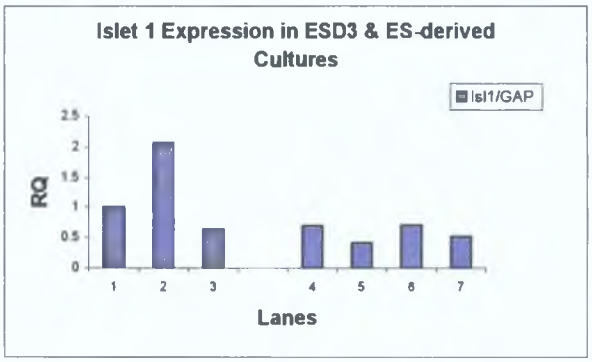


(I) Shh mRNA expression was induced in the RA culture and maintained in the RA_AA and the RA_ BTC cultures; however, shh expression was down-regulated in the RA_SB culture. Shh was not expressed in the control cultures (CO_5d and CO_9d).

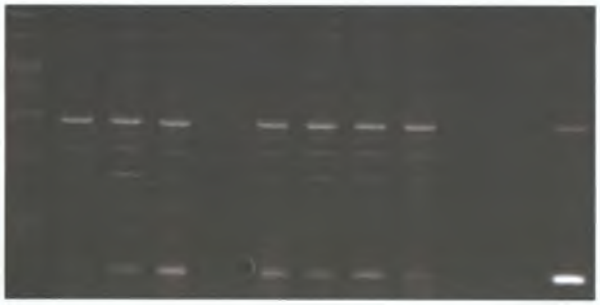
GAPDH

Islet1

1 2 3 4 5 6 7 8 9



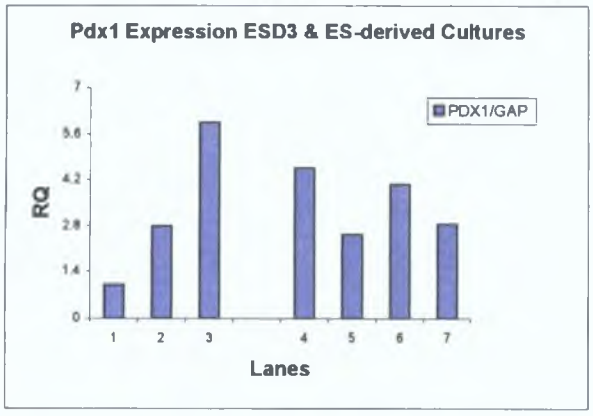
(J) Islet 1 mRNA expression was observed in ES-D3 and all ES-derived cultures. Islet 1 expression was enhanced by the presence of retinoic acid.



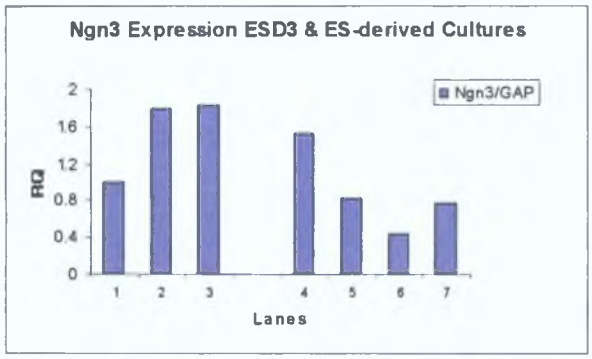
GAPDH

Pdx1

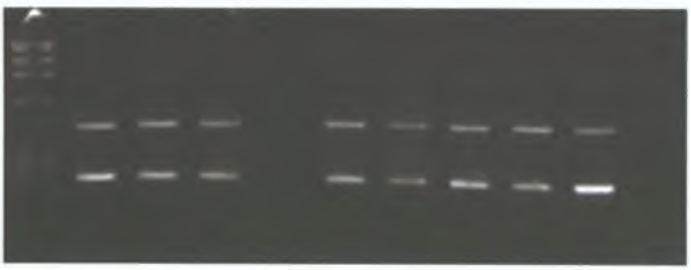
1 2 3 4 5 6 7 8 9

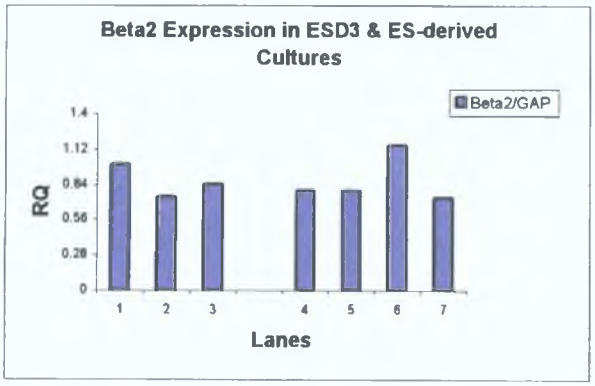


(K) Pdx1 mRNA was expressed at a low level in ES-D3 cells, but up-regulated in all ES-derived cultures with the highest level observed in sponataneously-formed control EB cultures (CO_5d and CO_9d). Pdx1 expression was up-regulated between the 5 day RA culture and the 9 day RA_SB culture.

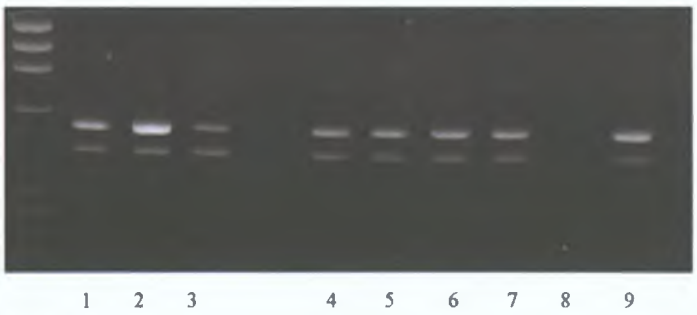


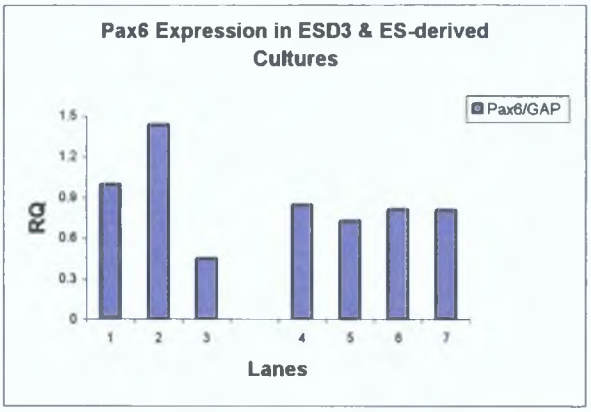
(L) Ngn3 mRNA expression was observed in ES-D3 cells and up-regulated in the 5 day RA and the CO_5d culture. Ngn3 expression was down-regulated by secondary differentiation in the presence of AA, SB or BTC. High levels of ngn3 expression were maintained in the untreated, spontaneously-formed CO_9d culture.



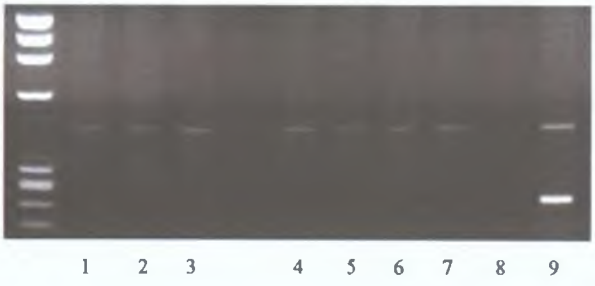


(M) Beta2 mRNA expression was observed in ES-D3 cells and all ES-derived cultures. An up-regulation in beta2 expression was induced in the RA_SB culture.

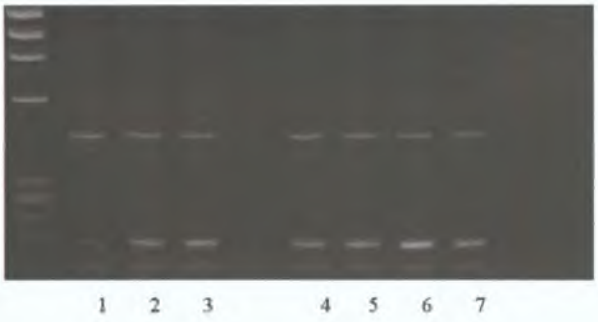


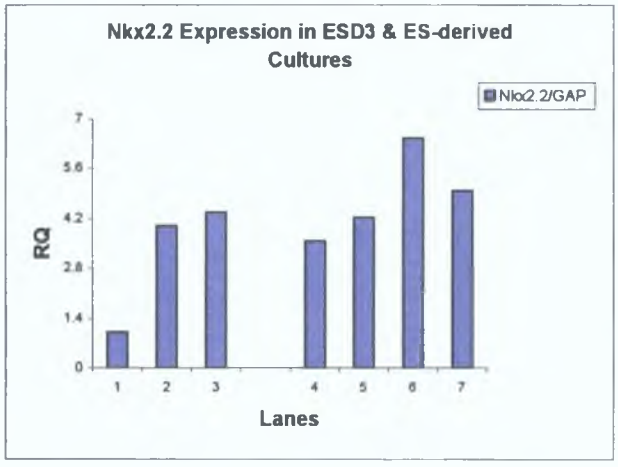


(N) Pax 6 mRNA expression was observed in ES-D3 cells and up-regulated in the RA culture. There was no change in pax 6 expression in other ES-derived cultures.



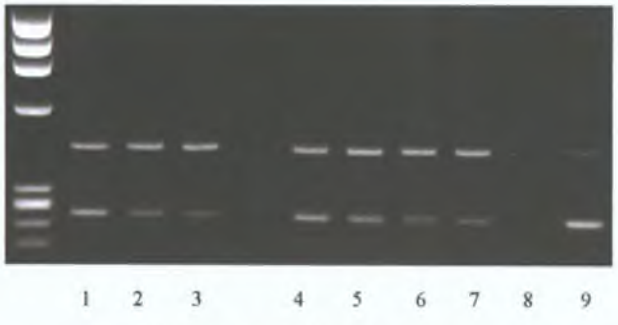
(O) Pax 4 mRNA was not detected in ES-D3 cells or ES-derived cultures.

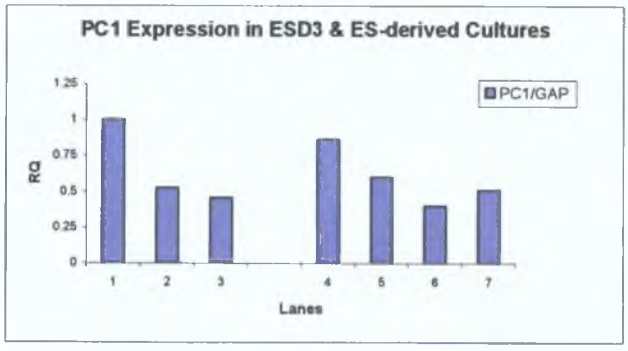




(P) Low level nkx2.2 mRNA expression was observed in ES-D3 cells and up-regulated in all ES-derived cultures. The RA_SB culture exhibited the highest level of nkx2.2 expression.

Endopeptidases

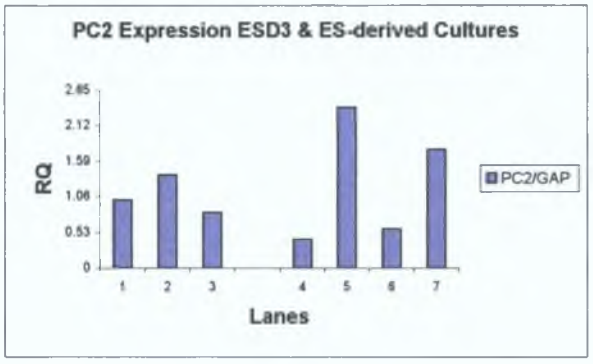




(Q) PC1 mRNA expression was observed in ES-D3 cells and down-regulated in ES-derived cultures with the exception of the CO_9d culture.

GAPDH
PC2

1 2 3 4 5 6 7 8 9

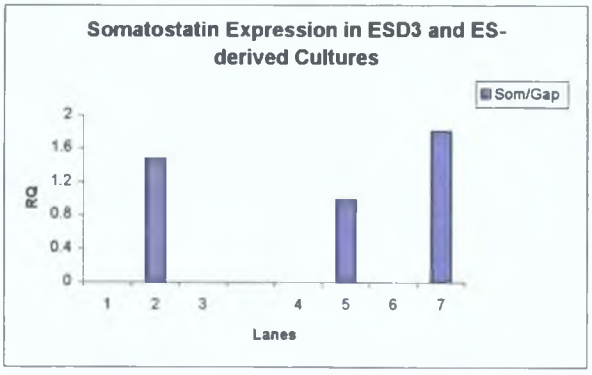


(R) PC2 mRNA expression was observed in ES-D3 cells and up-regulated in the 5 day RA culture, the 9 day RA_AA and RA_BTC cultures.

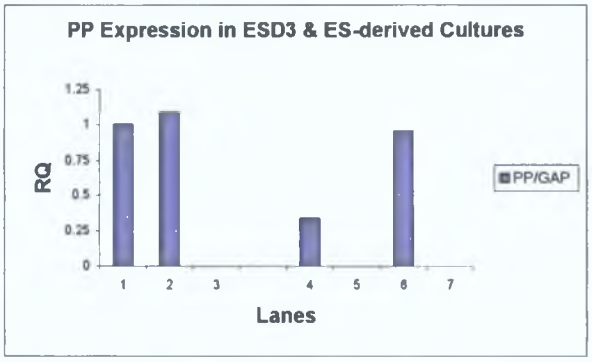
Endocrine Pancreas Markers

GAPDH
Som

1 2 3 4 5 6 7 8 9

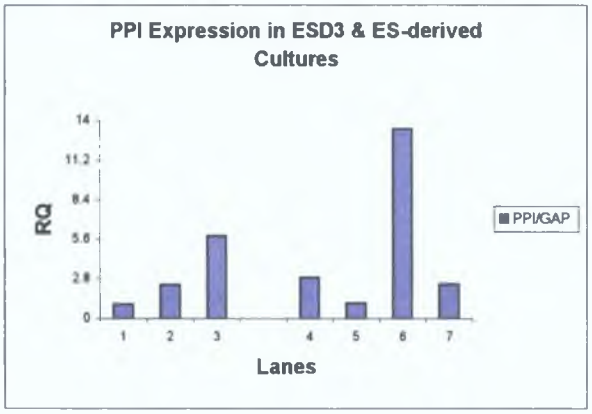


(S) Somatostatin mRNA expression was induced in the RA culture. The induced expression level was maintained by subsequent treatment with AA and BTC, however expression levels were down-regulated by subsequent treatment with SB. Expression of somatostatin was not observed in spontaneously formed CO_5d and CO_9d cultures.



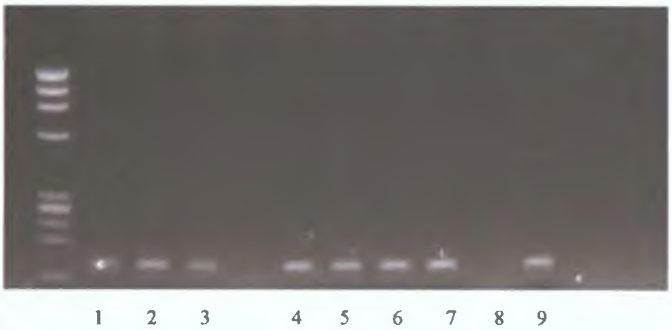
(T) Low level PP mRNA expression was detected in ES-D3 cells, the RA culture, the CO_9d culture and the RA_SB culture

(U) Glucagon mRNA was not expressed in ES-D3 cells or ES-derived cultures



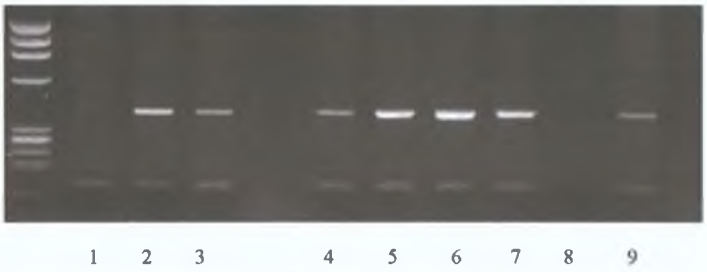
(V) PPI mRNA expression was detected at low levels in all ES-derived cultures with the exception of the RA_SB culture. PPI expression was dramatically increased by secondary differentiation in the presence of SB.

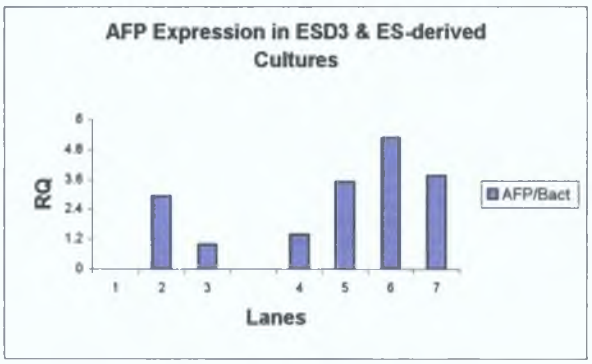
Exocrine Pancreas Markers



(W) Amylase mRNA was not detected in ES-D3 cells or ES-derived cultures

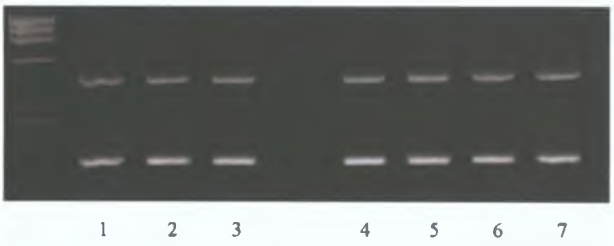
Extra-Embryonic Markers

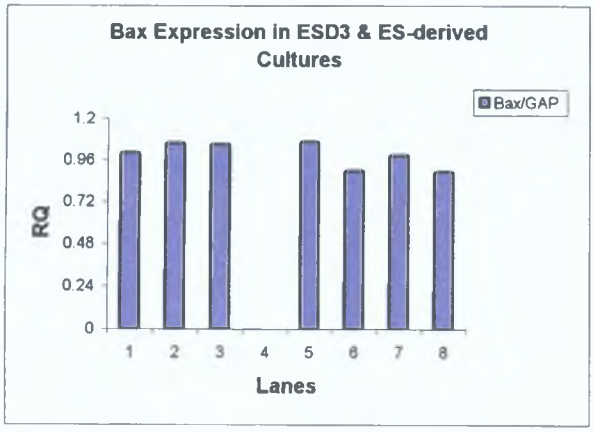




(X) AFP mRNA expression was induced in all ES-derived cultures with highest levels observed in the RA_SB culture.

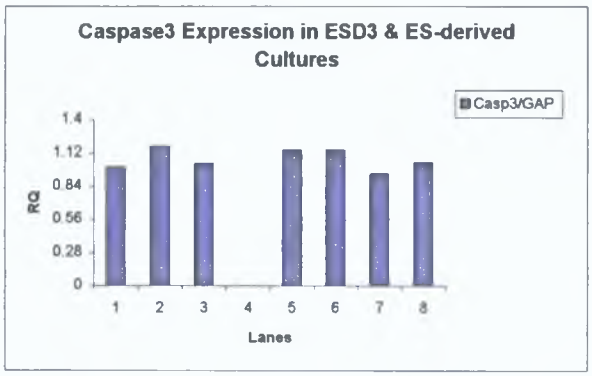
Apoptotic Markers





(Y) Bax mRNA was expressed at similar levels in ES-D3 and ES-derived cultures

1 2 3 4 5 6 7 8



(Z) Caspase3 mRNA was expressed at similar levels in ES-D3 and ES-derived cultures.

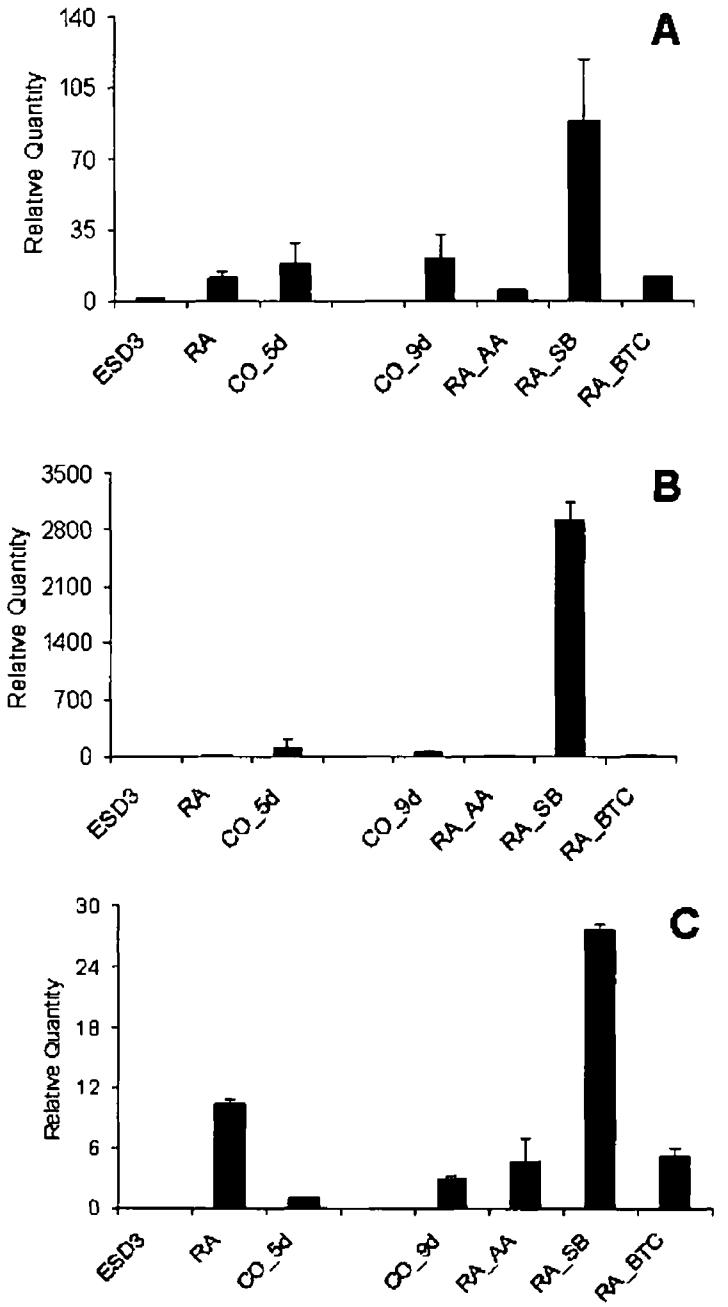


Figure 3 2 4 (A – C) qPCR analysis of (A) insulin I, (B) insulin II and (C) glut2, respectively Data presented (mean ± SD) is from two independent experiments, of which both sample sets were analysed in triplicate

3.2.3.2 qPCR Optimisation Experiments

An endogenous control comparison was performed for qPCR analysis of ES-D3 and ES-derived cell types to ensure that the presence of extra-cellular factors in the 2-stage differentiation studies hadn't affected the endogenous control expression thus affecting overall trends in target gene expression. Both controls showed similar trends in expression (Figure 3.2.5). The efficiencies of insulin I, insulin II, β-actin and GAPDH probe and primer sets were also checked prior to the qPCR experiments (Figure 3.2.6). All primer and probe sets had an efficiency of > 90% (Table 3.2.3). The glut2 primer and probe set was an 'assay on demand' designed by ABI with guaranteed efficiency.

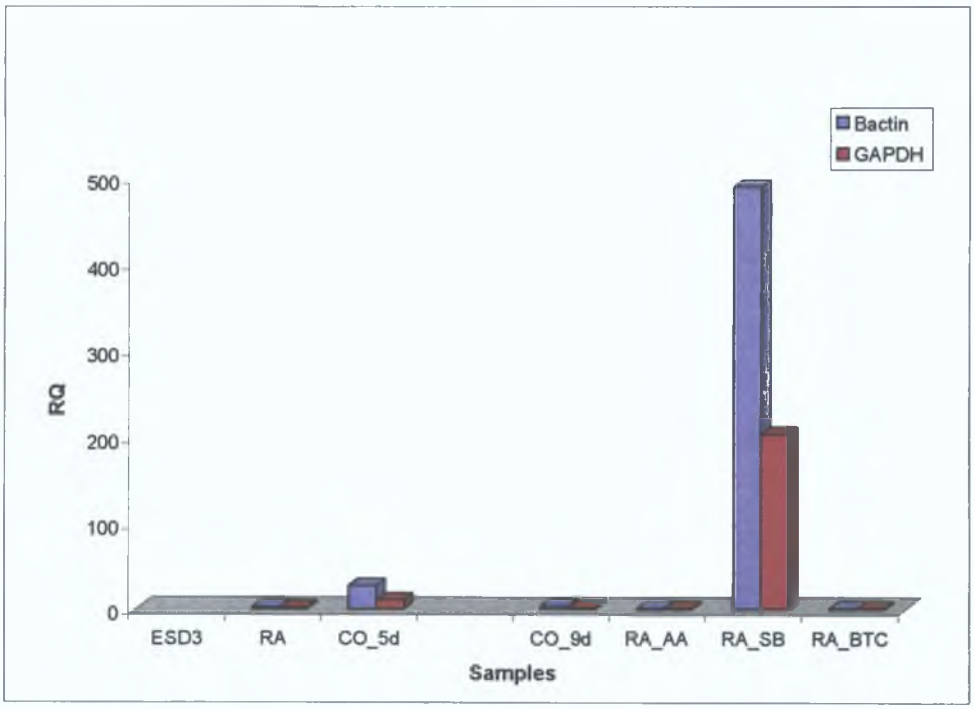


Figure 3.2.5 qPCR analysis on target expression in ESD3 & ES-derived cultures normalised by both β-actin and GAPDH. RQ, Relative Quantity. ES-D3 was set to an RQ of 1. Both GAPDH and β-actin resulted in similar trends in target gene expression.

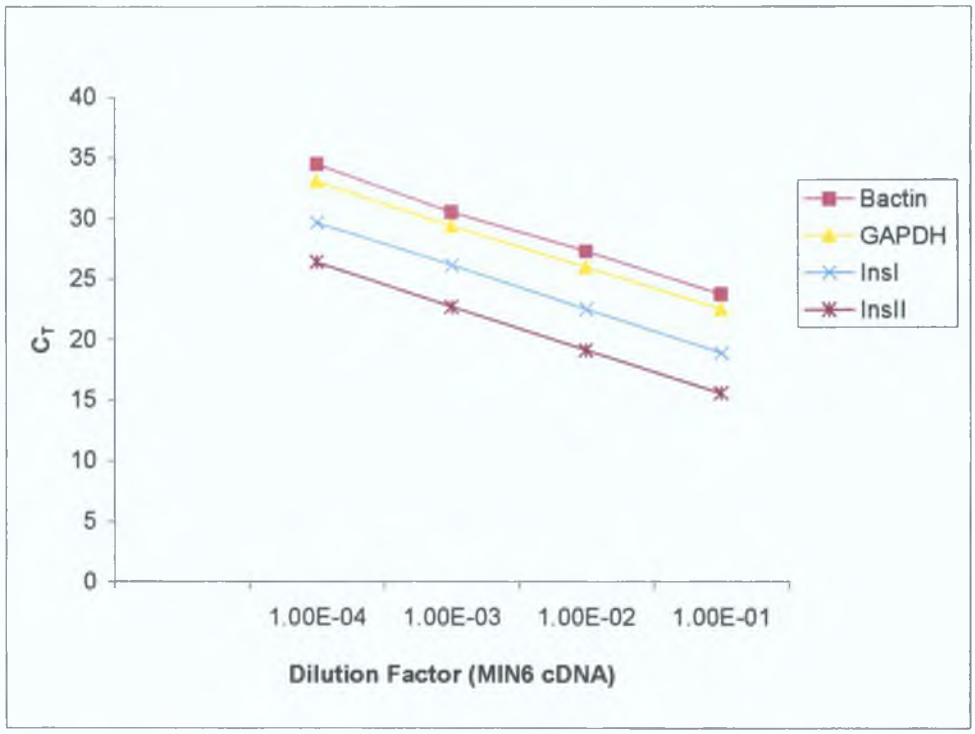


Figure 3.2.6 Plots used to determine the efficiency of insulin I (Ins I), insulin II (Ins II), β-actin and GAPDH probe and primer sets. 10-fold serial dilutions of MIN6 cDNA were prepared and used as test sample. The qPCR was performed with the gene of interest and control primers under standard conditions. The C_T was plotted against [10-fold serial dilutions of MIN6]. The efficiency was calculated using the following equation:

$$\mathbf{E} = (10^{-1/\text{slope}}-1) \times 100$$

Table 3.2.3 Data is representative of the slope of the lines shown in Figure 3.2.6 The efficiency was calculated with these slopes using the equation shown above.

Primer & Probe sets	Slope	Efficiency
β-actin	-3.5423	91.55%
GAPDH	-3.5245	92.17%
Insulin I	-3.5853	90.06%
Insulin II	-3.5862	90.02%

3 2 4 Analysis of Protein Expression in ES-D3 and ES-derived Cultures

In Section 3 2 3, qPCR and semi-quantitive RT-PCR analysis was used to profile multi-lineage marker mRNA expression in ES-D3 and ES-derived cultures In this section immunohistochemistry analysis was used to profile protein expression of a subset of these markers (i e pluripotent, neuronal, glial, pancreatic and muscle markers) in ES-D3 and ES-derived cultures The analysis was preformed on random sections of paraffin embedded samples from two independent biological experiments (Figure 3 2 7 and Figure 3 2 8) The slides were scored by 2 independent observers (Table 3 2 4) (Immunohistochemistry analysis was performed in collaboration with Prof Michael Kasper)

Omission of the primary antibody was used as a technical negative control to test for the specificity of the individual antibodies involved Staining was not visible in any of the test samples after omission of the primary antibody (Section 7 1, appendix A) The variability in protein expression within the cultures tested allowed for positive and negative biological controls within test samples

3 2 4 1 Summary of Results Obtained from Immunohistochemistry Analysis of Multi-lineage Marker Expression

Oct 4, a characteristic marker of pluripotent stem cells, was strongly expressed in the ES-D3 culture (Figure 3 2 7 A and Figure 3 2 8 A) Cells within this culture also expressed the muscle-specific transcription factor, myogenin and the pancreatic transcription factor, Pdx1 (Figure 3 2 7 F, H and Figure 3 2 8 F, H) Low level expression of NF-L (neuronal marker), C-peptide 2 (bi-product of insulin II synthesis) and PP (endocrine hormone) were also detected in ES-D3 cells (Figure 3 2 7 B, J, N and Figure 3 2 8 B, J, N)

The CO_5d culture expressed a broad range of markers i e NF_L, β III tubulin, myogenin, desmin, Pdx1, C-peptide 1, C-peptide 2, glut2, somatostatin and PP (Table 3 2 4) Formation of EBs in the presence of RA enhanced the expression of a subset of these markers (neuronal markers, NF-L and β III tubulin, β cell marker, Glut2) and induced expression of the multi-potential marker, nestin (Table 3 2 4) Stage 2 differentiation in the presence of BTC and AA further enhanced/induced nestin, GFAP (astrocyte marker), NF-L and β III tubulin

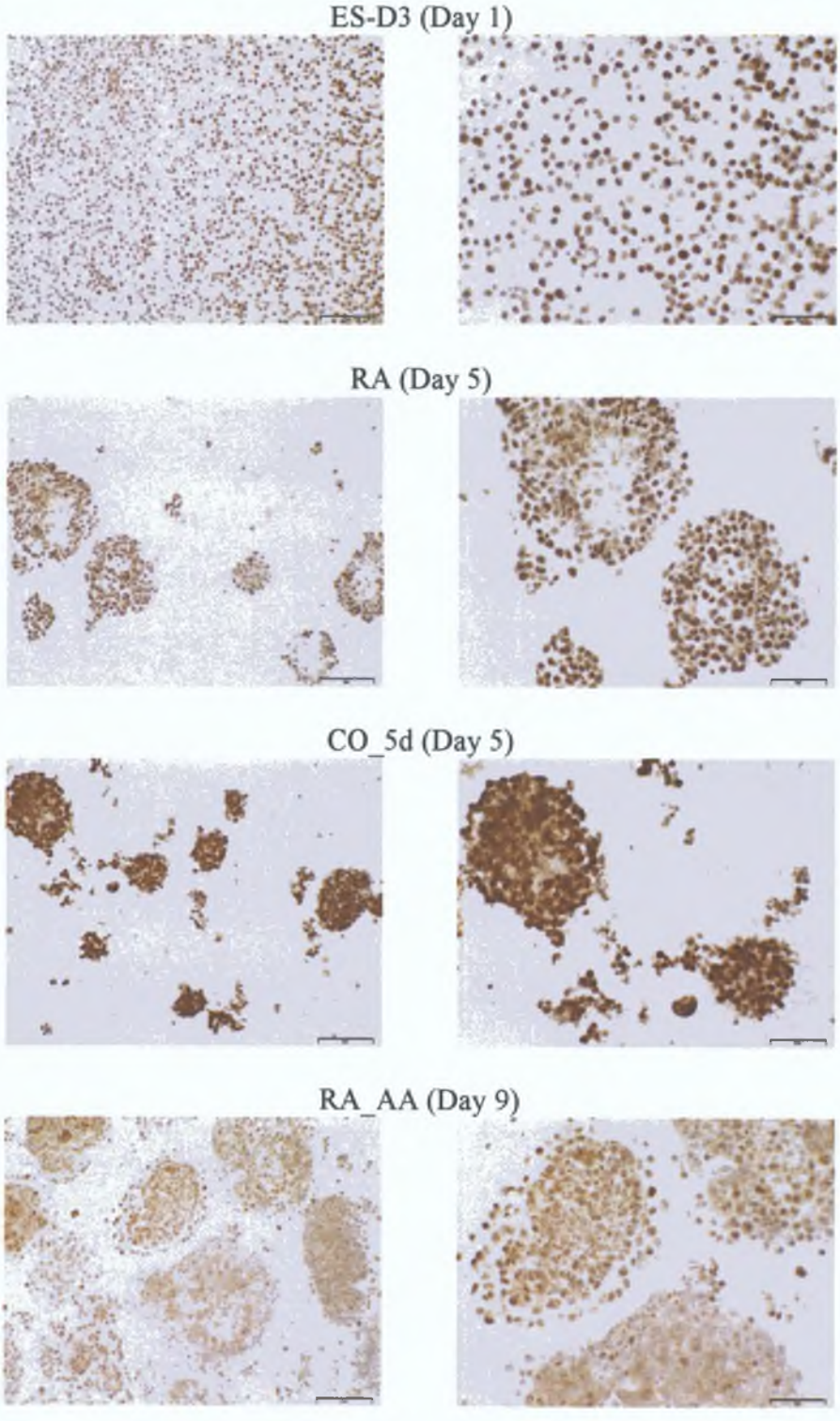
expression (Table 3 2 4) Stage 2 differentiation in the presence of SB enhanced expression of the muscle marker, desmin, the β cell marker, glut2 and induced expression of the β cell hormone, insulin (Table 3 2 4) Pdx1, C-peptide 1 and C-peptide 2 were strongly expressed in the 5 day cultures (RA and CO_5d) (Table 3 2 4) Stage 2 differentiation of the RA culture in the presence of SB did not enhance the overall expression of these markers, however, the RA_SB culture did contain subpopulations of cells that maintained a high level of Pdx1 and C-peptide 2 expression (Figure 3 2 7 H, J and Figure 3 2 8 H, J) Expression of the pancreatic endocrine hormones, PP and somatostatin, was induced in all differentiated populations at both day 5 and day 9

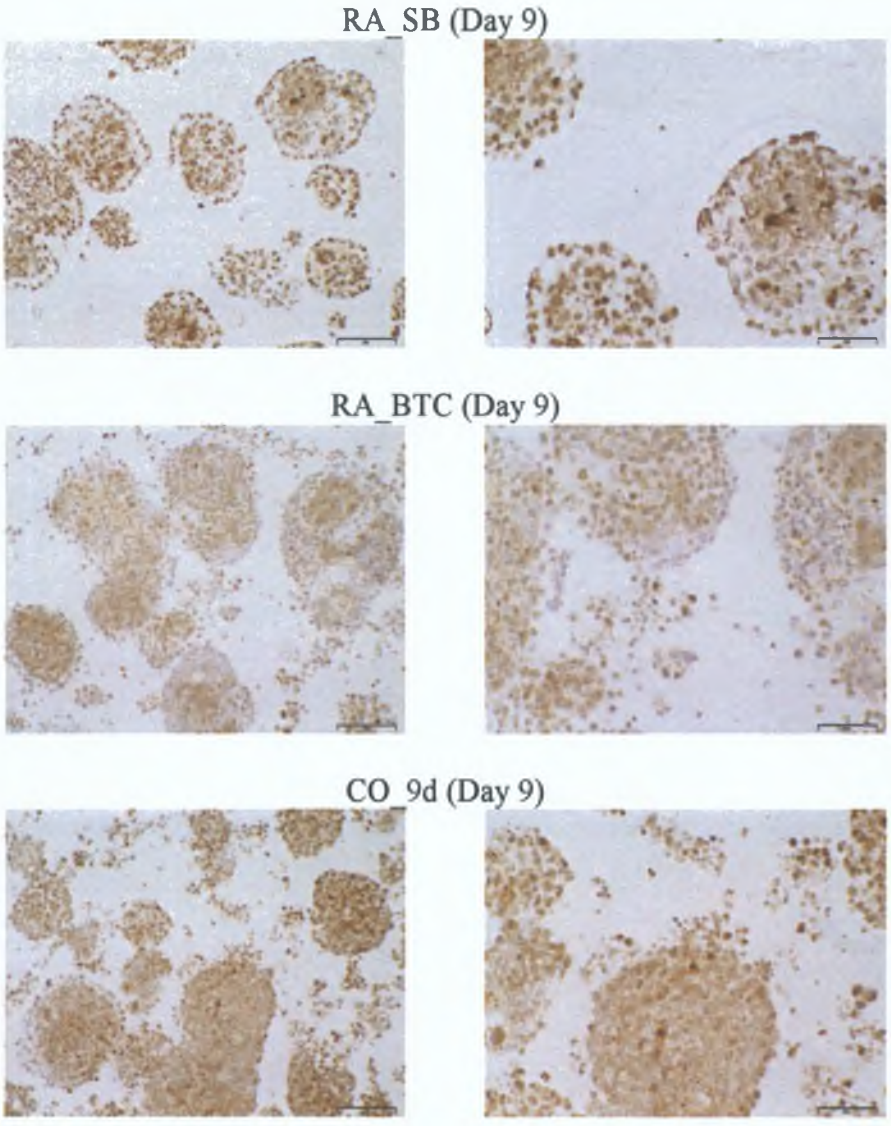
These findings in combination with the results from Section 3 2 3 suggest that the intermediate RA culture consists of a mixed population of multi-potential precursors Stage 2 differentiation of the RA culture in the presence of SB promotes pancreatic endocrine differentiation, and in the presence of AA or BTC, promotes neuronal/glial differentiation

Table 3.2.4 Summary of slide scores from immunohistochemistry analysis of protein expression in ES-D3 and ES-derived cultures. Slides were scored by two independent observers. The percentage of stained cells was graded as follows: A (<25%), B (25 – 50 %) or C (>50%). Intensity of staining was graded as follows: +1 (weak), +2 (moderate) or +3 (strong). Data is from 2 independent experiments: Repeat 1 in white column, Repeat 2 in blue column.

	ES-D3		RA		CO_5d		RA_AA		RA_SB		RA_BTC		CO_9d	
Markers														
Differentiation														
Oct 4	C+3	C+3	C+3	C+3	C+3	B+2; A+3	C+2	C+2	C+2; A+3	C+1; A+3	C+1	C+2; A+3	C+2	C+1
Neuronal														
NF-L	B+1	A+1	C+2	C+2	B+2	B+1	C+2; A+3	B+2	C+2; A+3	C+2	C+2; A+3	B+2	C+1	C+1
GFAP	Neg	Neg	Neg	Neg	Neg	Neg	A+3	A+3	Neg	Neg	A+3	A+3	Neg	Neg
nestin	Neg	Neg	A+1	Neg	Neg	Neg	A+2	A+3	A+1	Neg	A+2	A+3	Neg	Neg
β III Tubulin	Neg	Neg	A+3	A+3	A+1	A+1	B+3	A+3	A+2	A+2	B+3	B+3	A+2	A+1
Muscle														
myogenin	A+1	C+1	C+2	C+2; A+3	C+3	C+2	C+1; A+2	C+1; A+2	A+2	A+2	Neg	Neg	A+2	C+1; A+2
desmin	C+2	C+3	C+1; A+2	C+3	C+3	C+3	C+2; A+3	C+1; A+3	C+3	C+3	C+1; A+2	C+2; A+3	C+2	C+2; A+3
Pancreatic														
Pdx1	B+2	C+3	C+3	C+3	C+3	C+3	C+2	C+2	C+1; A+3	C+2; A+3	C+2; A+3	C+2	C+1	C+2
C-peptide 1	Neg	Neg	B+2	B+2	C+3	B+3	B+1	C+1	A+1	B+1; A+2	C+1	ND	A+1	B+1
C-peptide 2	A+1	Neg	C+3	C+3	C+3	C+2	C+1	C+1; A+2	B+2	C+2; A+3	B+2	C+1; A+2	C+1; A+3	B+2
insulin	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	A+3	A+1	Neg	Neg	Neg	Neg
glut2	Neg	Neg	B+1;A+2	B+1;A+2	C+1	A+1	C+1;A+2	C+2	C+2;A+3	B+2;A+3	B+1;A+2	C+1;A+3	ND	C+1
somatostatin	Neg	Neg	A+3	B+1	A+2	A+2	C+2;A+3	B+1;A+2	B+2;A+3	C+2	B+2;A+3	A+2;A+3	Neg	Neg
PP	A+1	A+2	B+1;A+3	A+2	B+2	B+3	B+1;A+3	B+1;A+3	B+2;A+3	B+2;A+3	A+3	A+3	A+2	A+2

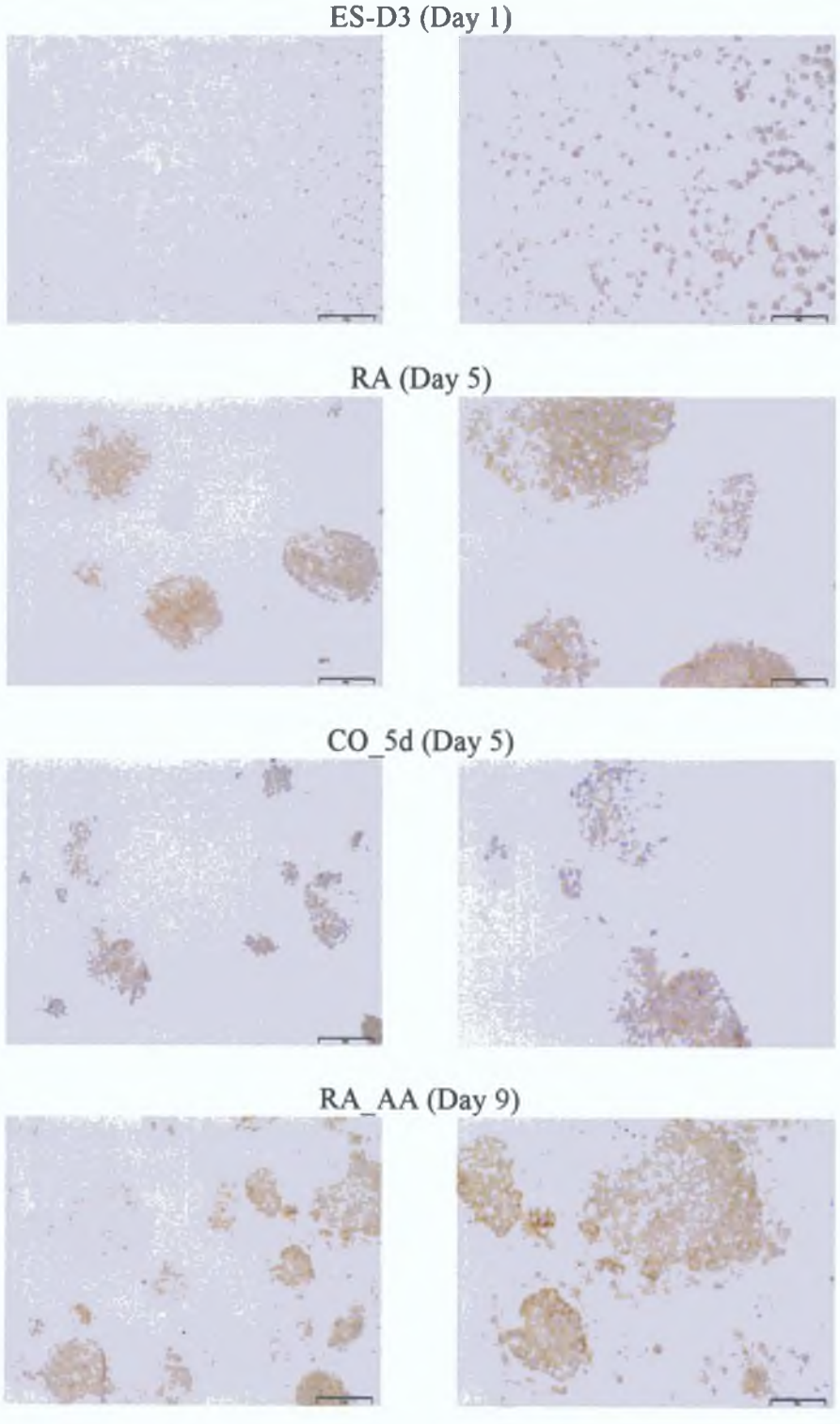
Figure 3.2.7 (A) Immunohistochemistry analysis of Oct 4 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

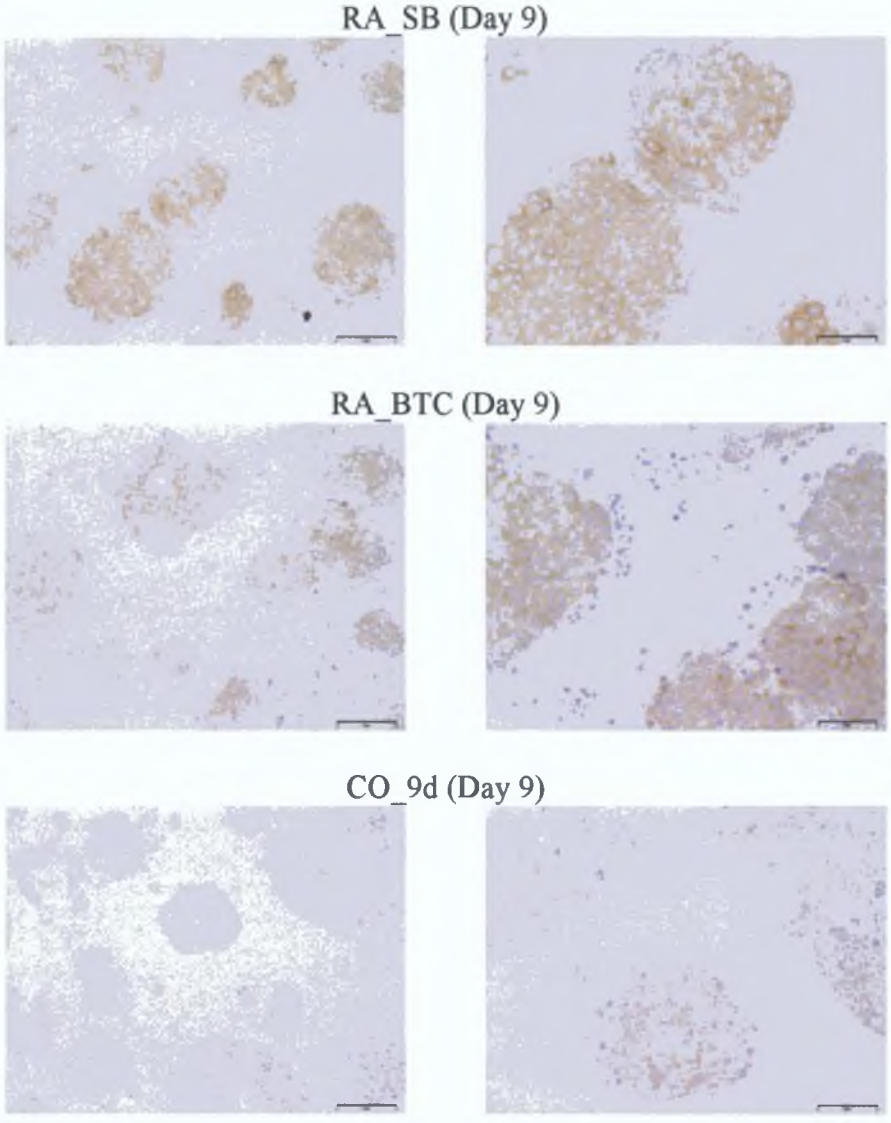




The majority of cells in the ES-D3 and the 5 day cultures (RA and CO_5d) stained intensely for Oct 4; however the majority of cells in the 9 day cultures (RA_AA, RA_BTC, RA_SB, CO_9d) stained moderately - weakly for Oct 4, indicating an overall down-regulation in Oct 4 protein expression between day 5 and day 9. The RA_BTC culture appeared to exhibit the lowest level of expression.

Figure 3.2.7 (B) Immunohistochemistry analysis of NF-L protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

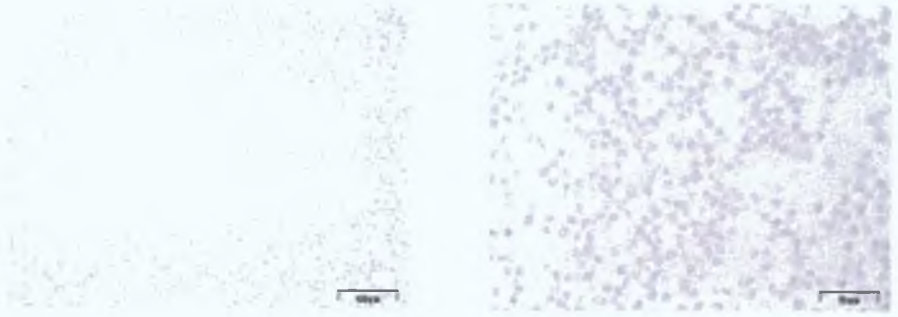




(B) A subpopulation of ES-D3 cells stained weakly for NF-L where as a subpopulation of day 5 (RA and CO_5d) cultures stained moderately for NF-L, suggesting an up-regulation in NF-L protein expression between day 1 (ES-D3) and day 5 (RA and CO_5d). The RA culture contained a higher number of moderately stained cells than the CO_5d culture. Day 9 cultures which had been exposed to AA, SB and BTC all contained both moderately and intensely stained cells indicating an up-regulation in NF-L expression between the RA culture at day 5 and the treated cultures at day 9. Cells in the CO_9d culture stained weakly for NF-L, suggesting a down-regulation in NF-L expression between the CO_5d culture and the CO_9d culture.

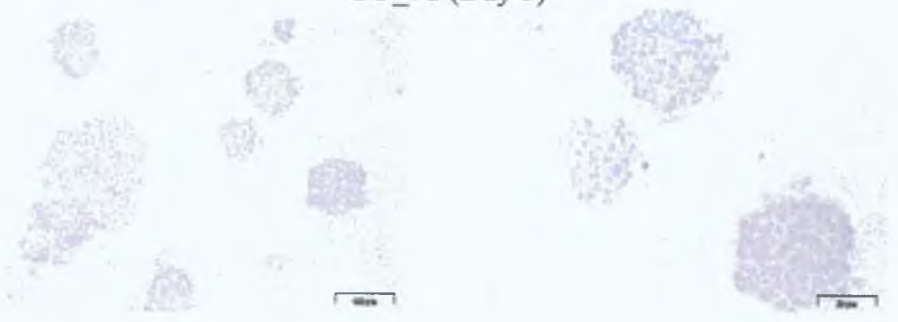
Figure 3.2.7 (C) Immunohistochemistry analysis of GFAP protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

ES-D3 (Day 1)

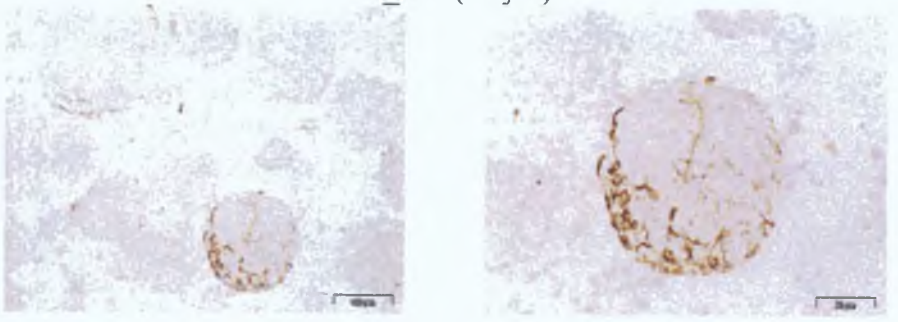


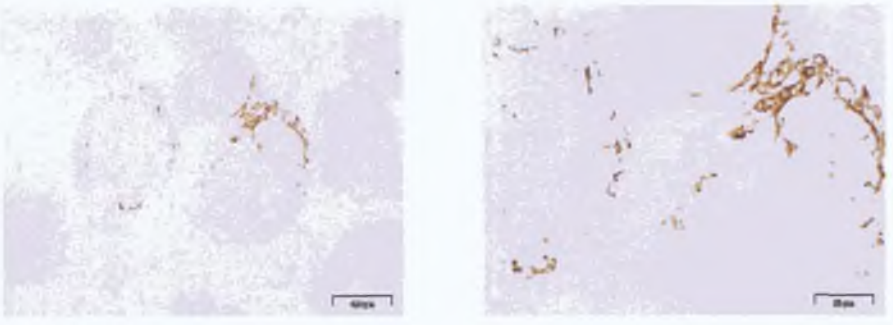
RA (Day 5)

CO_5d (Day 5)



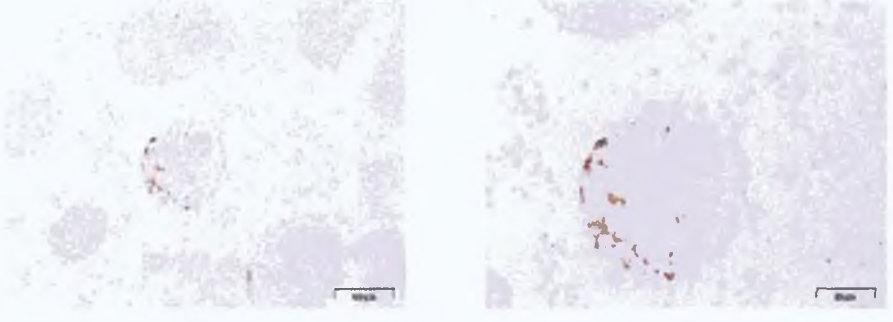
RA_AA (Day 9)





RA_SB (Day 9)

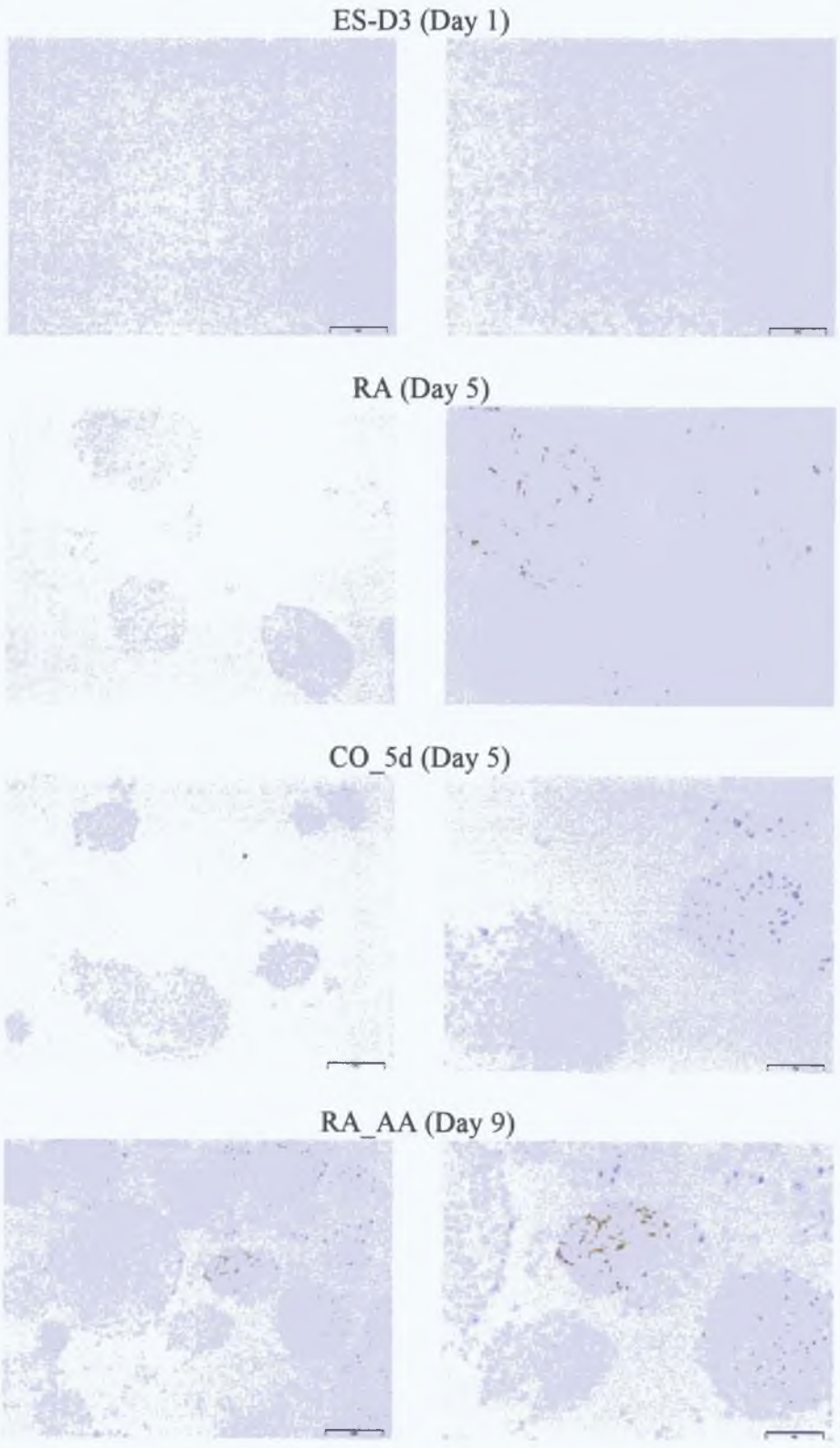
RA_BTC (Day 9)

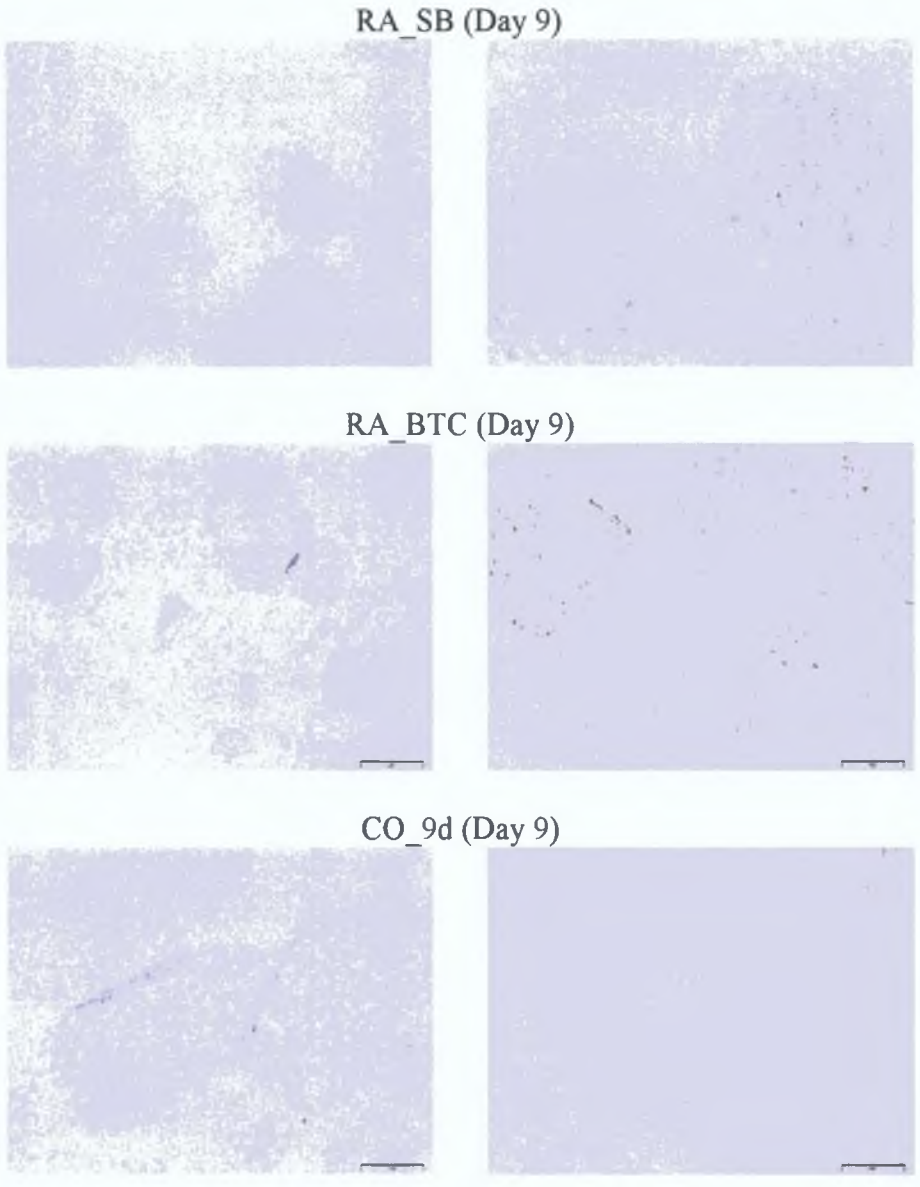


EB Control (Day 9)

GFAP protein expression was not detected in ES-D3 cells or in ES-derived 5 day cultures (RA and CO_5d). An intense distinctive staining pattern was observed in subpopulations of both the RA_AA and RA_BTC cultures at day 9, indicating that GFAP expression was induced within these cultures. Expression was not detected in the RA_SB culture or the CO_9d culture

Figure 3.2.7 (D) Immunohistochemistry analysis of nestin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

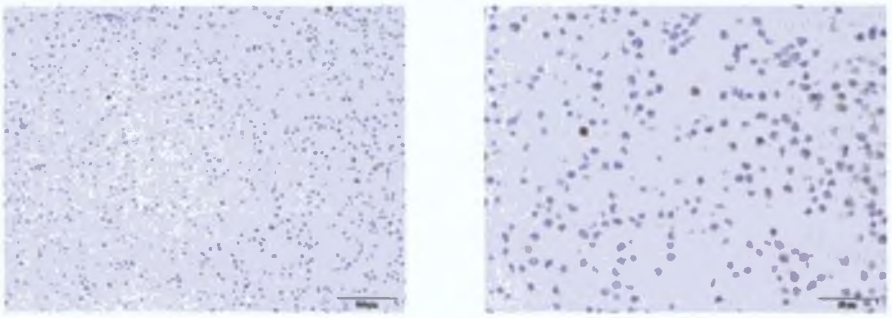




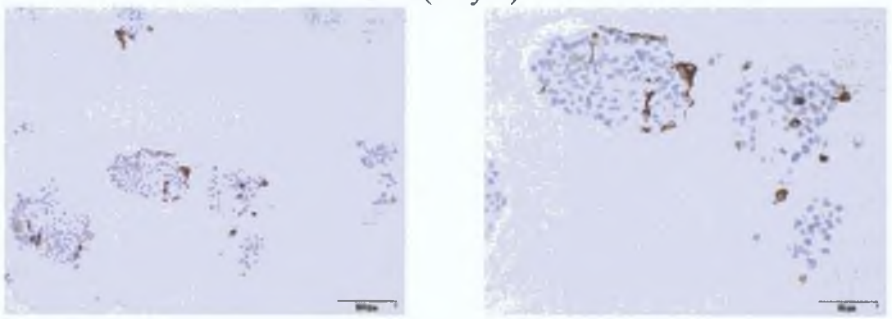
Nestin protein expression was not detected in ES-D3 cells. A filamentous staining pattern was observed in the RA culture at day 5, indicating the induced expression of nestin within a subpopulation of this culture. The intensity of staining for nestin was maintained in the RA_SB and increased in the RA_AA and RA_BTC cultures at day 9. Expression was not detected in spontaneously-formed CO_5d and CO_9d cultures.

Figure 3.2.7 (E) Immunohistochemistry analysis of β III tubulin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

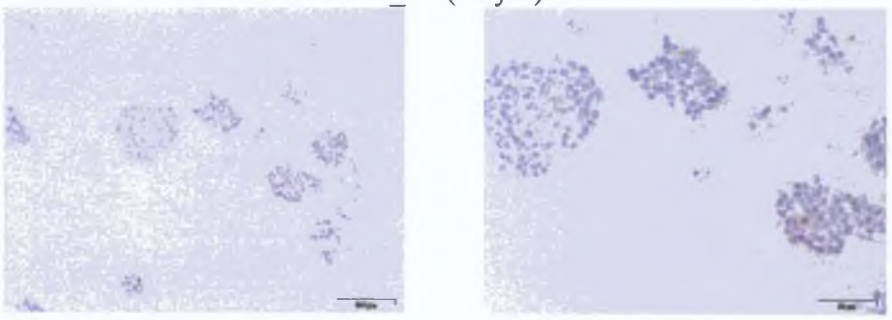
ES-D3 (Day 1)



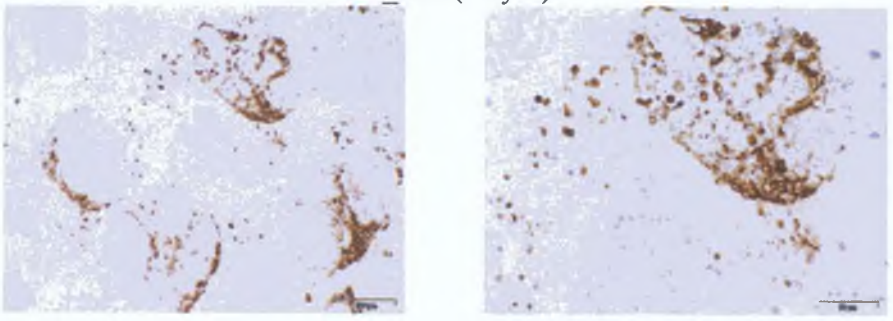
RA (Day 5)



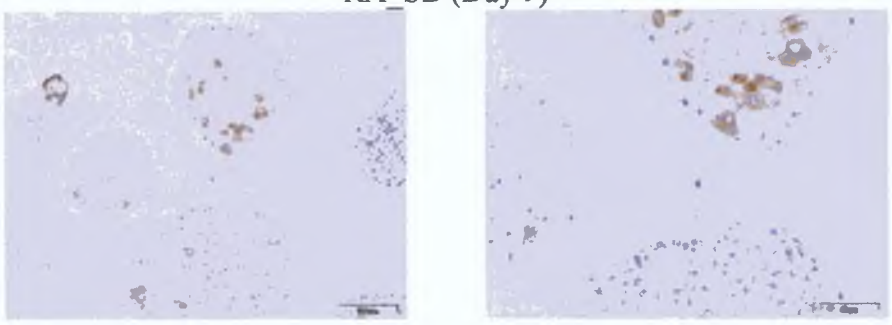
CO_5d (Day 5)



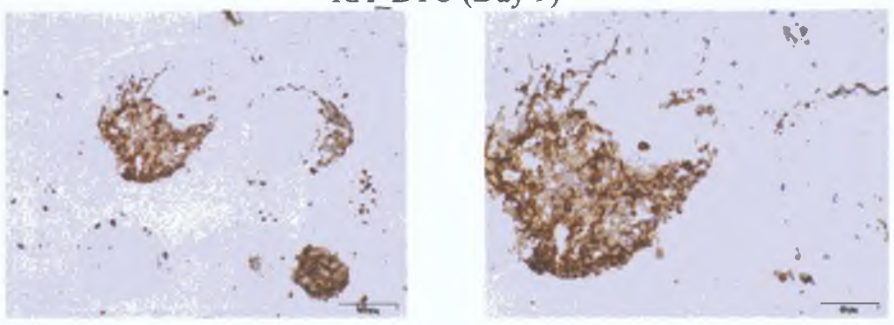
RA_AA (Day 9)



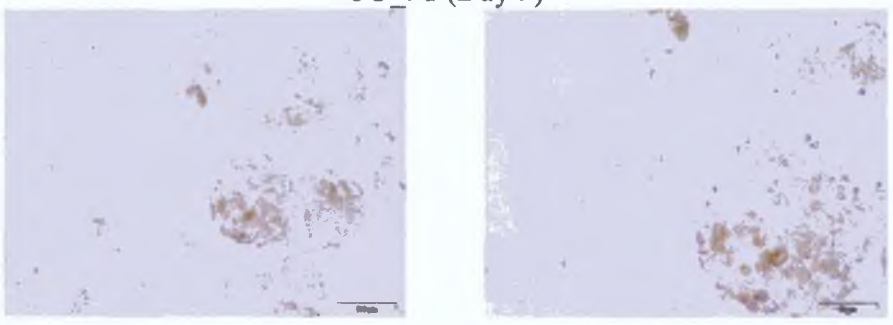
RA_SB (Day 9)



RA_BTC (Day 9)

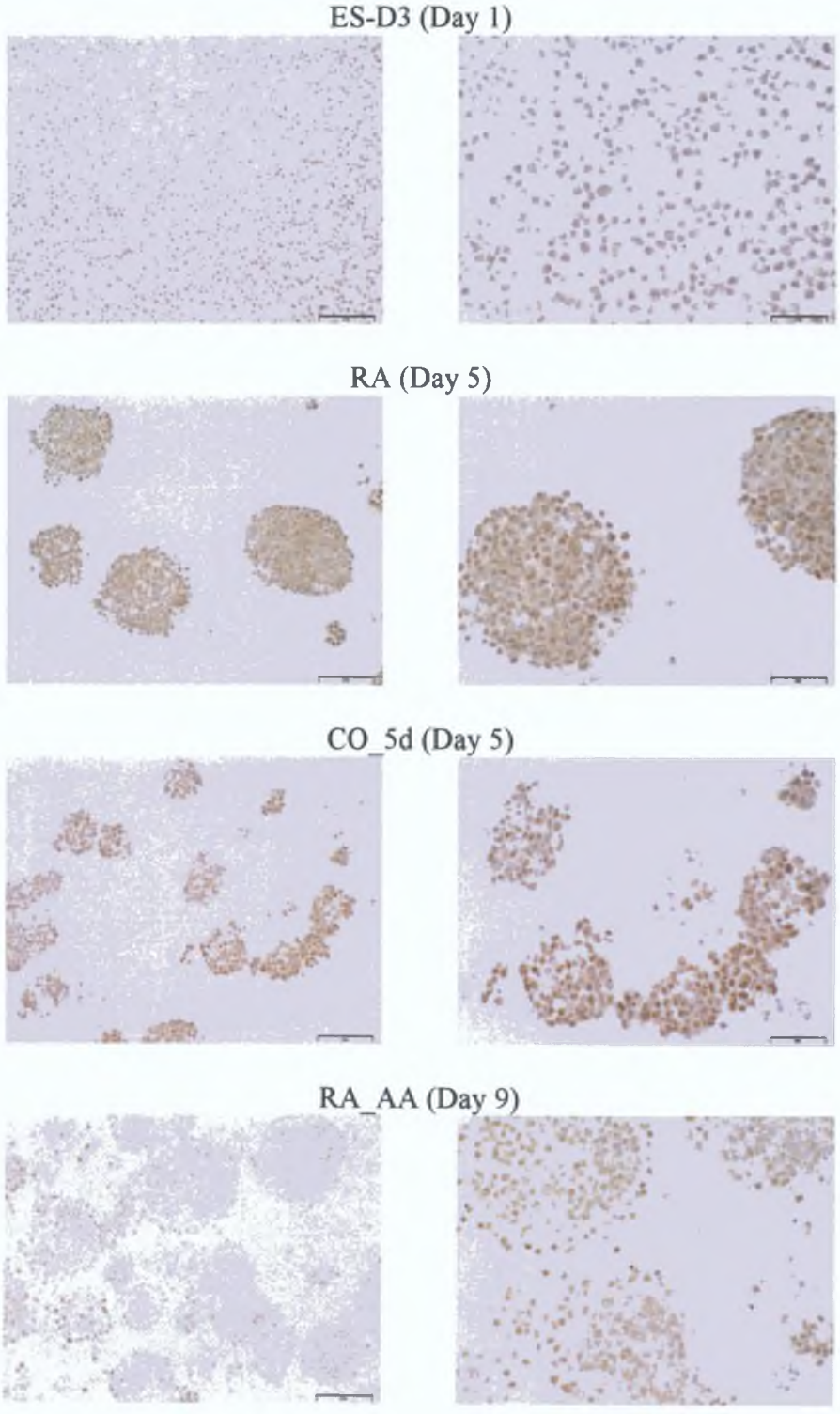


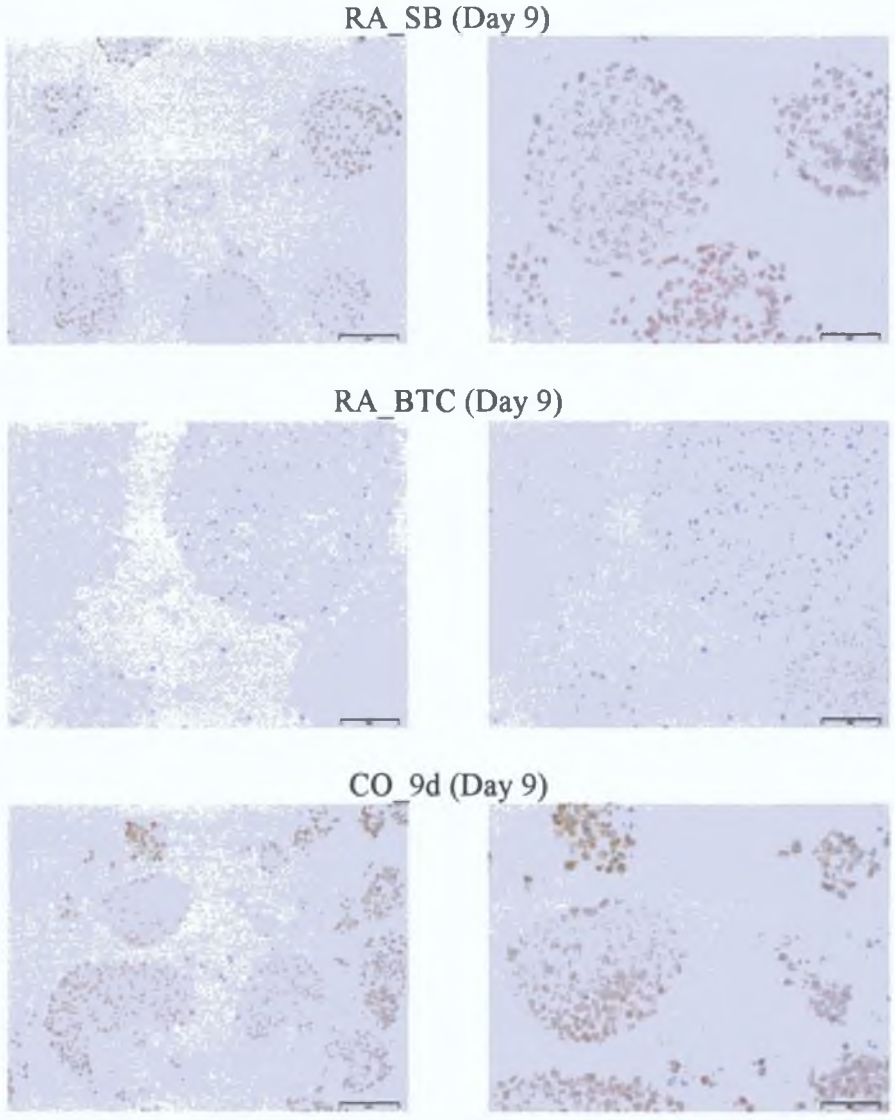
CO_9d (Day 9)



β III tubulin protein expression was not detected in ES-D3 cells. A subpopulation of the RA culture at day 5 stained positive for β III tubulin indicating that β III tubulin protein expression was induced within this culture. The percentage of staining was increased in the RA_AA and RA_BTC cultures at day 9 indicating an up-regulation in expression. β III tubulin expression was not detected in the spontaneously-formed CO_5d. A very small percentage of cells in the CO_9d culture stained weakly-moderately indicating low level expression of β III tubulin in a subpopulation of cells within this culture.

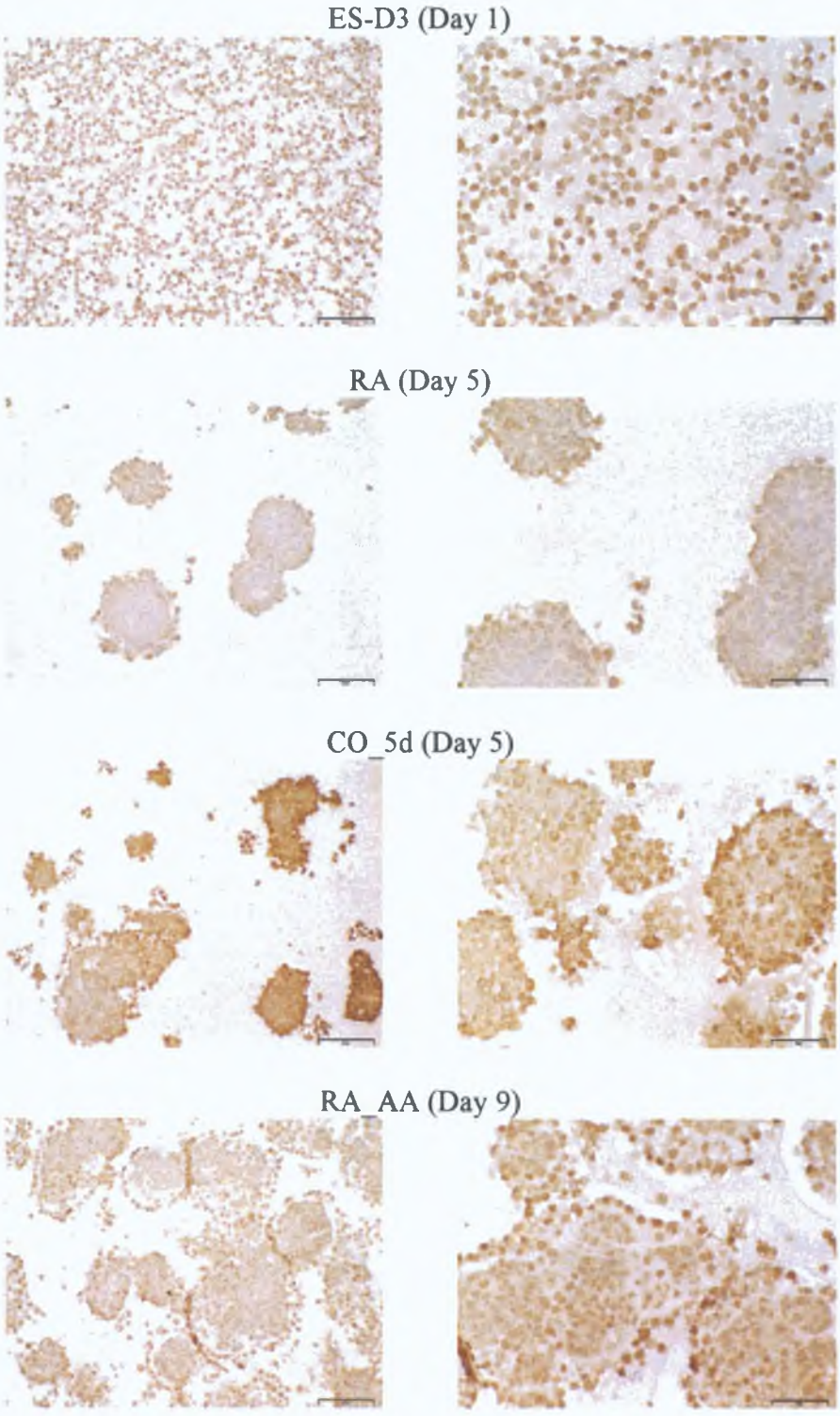
Figure 3.2.7 (F) Immunohistochemistry analysis of myogenin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

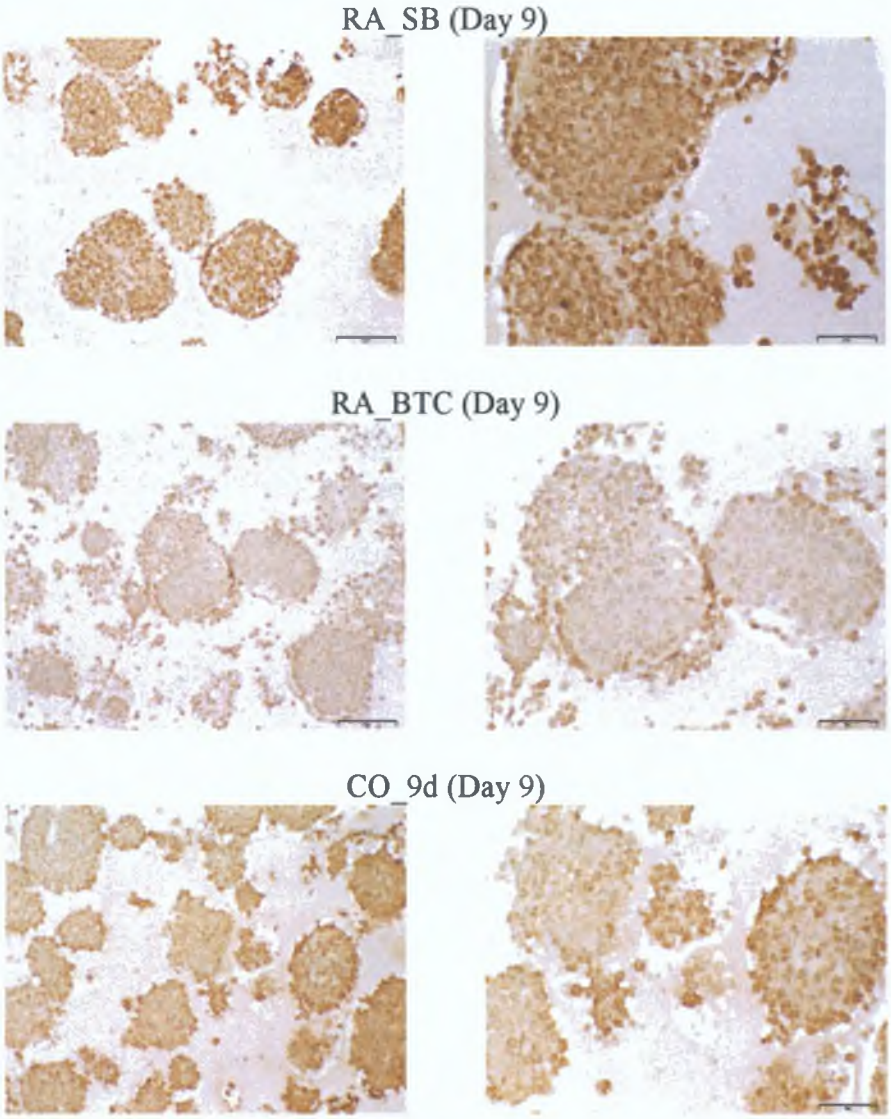




A subpopulation of ES-D3 cells stained weakly for myogenin indicating low level expression of the protein within this culture. An increase in percentage and intensity of staining was observed in day 5 cultures indicating that myogenin expression was up-regulated in both the RA culture and CO_5d culture. Subsequent differentiation resulted in a decrease in the percentage and intensity of staining indicating a down-regulation of myogenin expression in 9 day cultures, including treated (RA_AA, RA_SB, RA_BTC) and spontaneously-formed cultures (CO_9d).

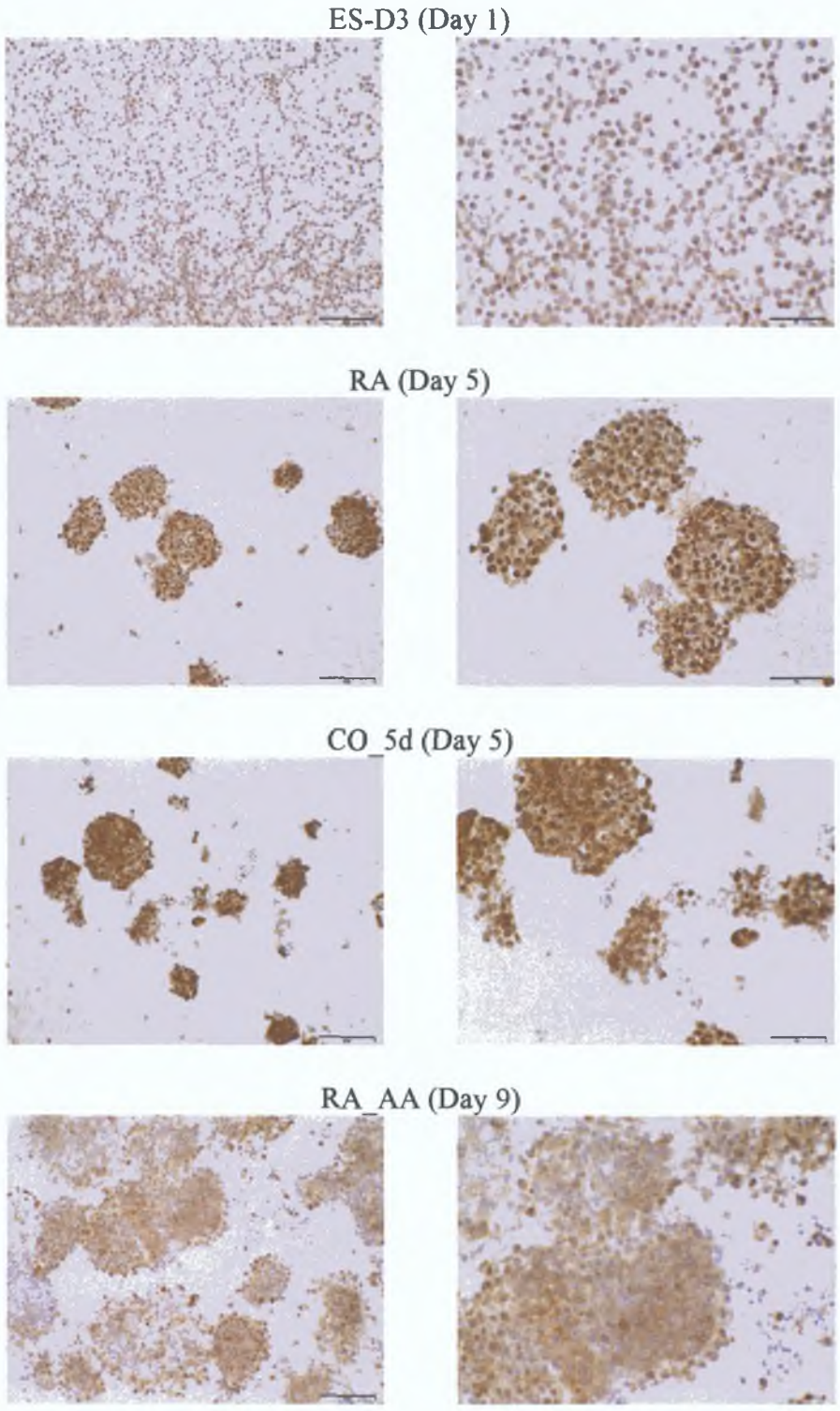
Figure 3.2.7 (G) Immunohistochemistry analysis of desmin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

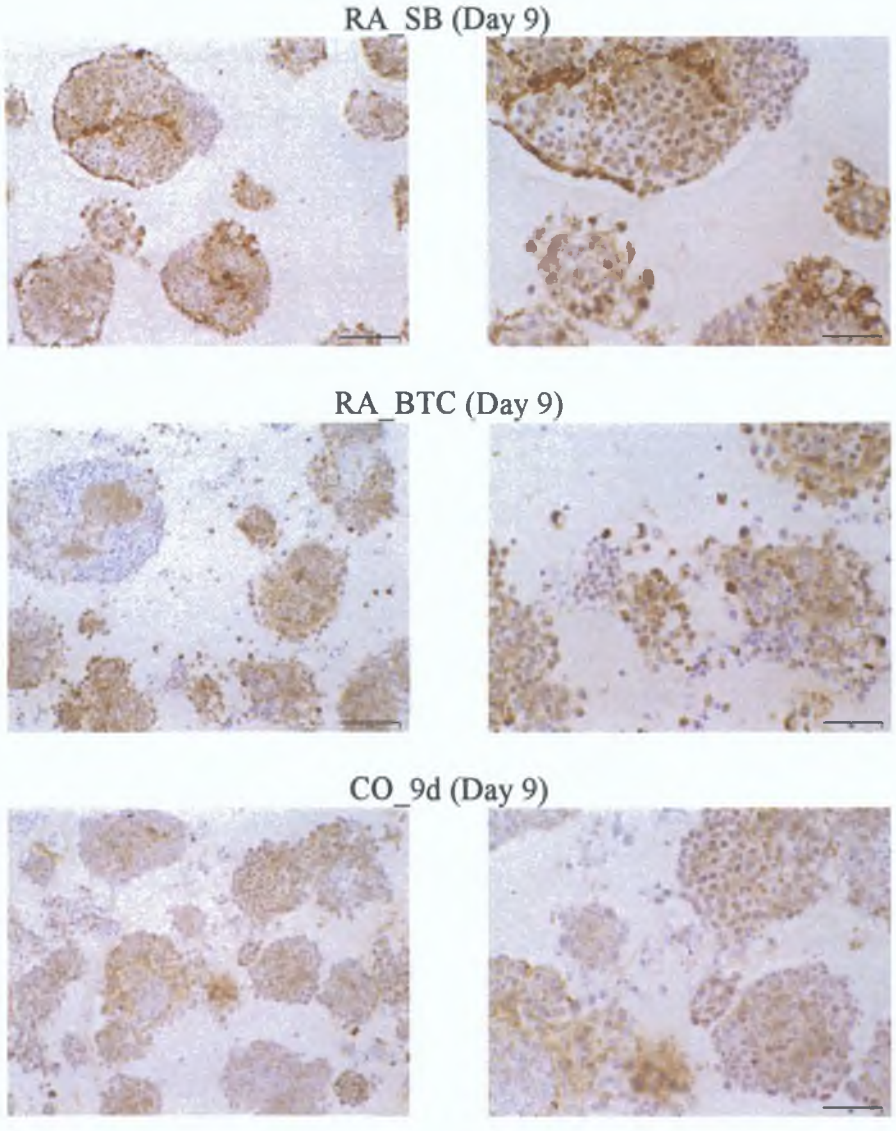




ES-D3 and all ES-derived cultures stained positive for desmin expression. Increases in staining intensity in both the spontaneously formed CO_5d and CO_9d cultures suggested that desmin protein expression was up-regulated within these cultures. Desmin also appeared to be expressed at a high level in the RA_SB culture.

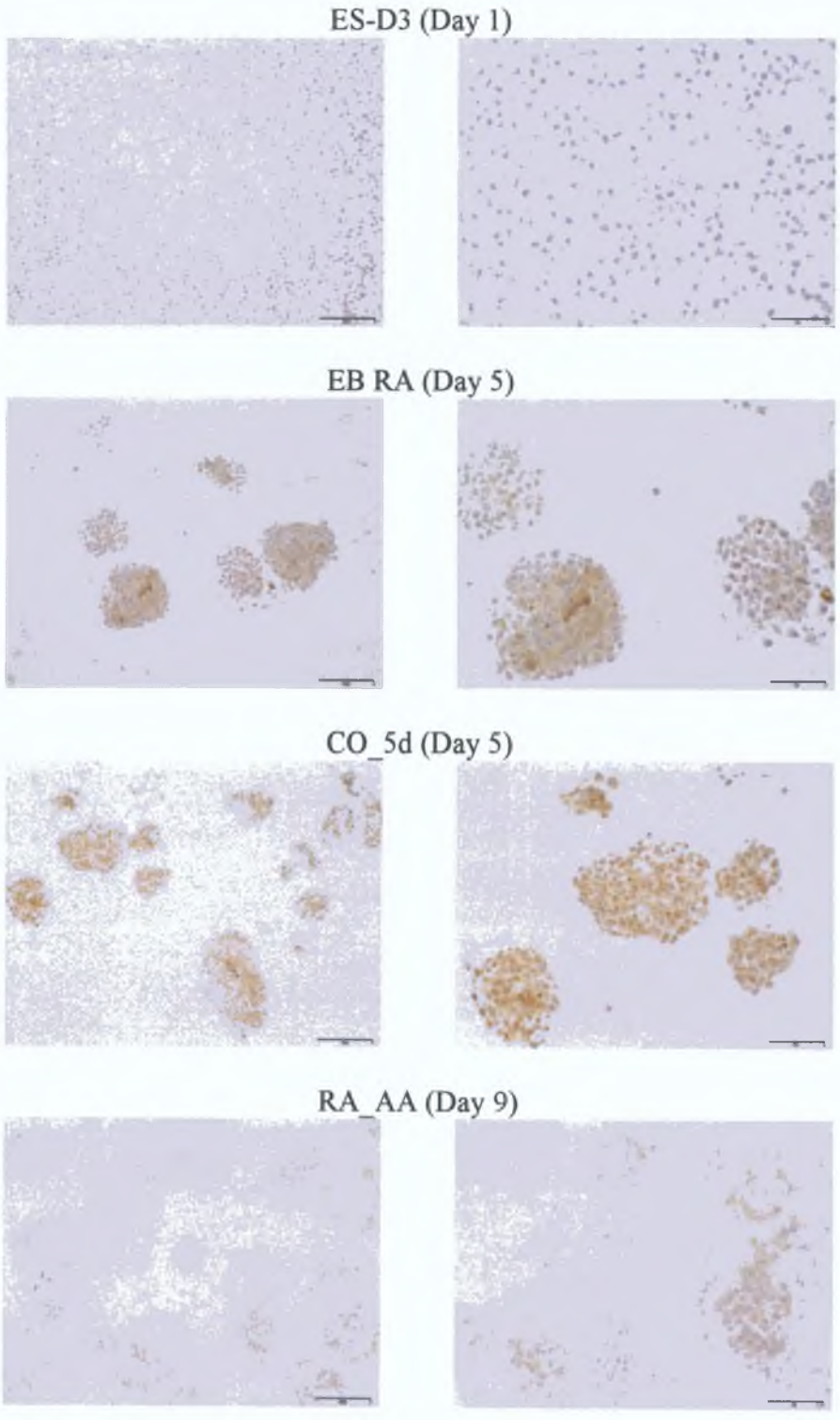
Figure 3.2.7 (H) Immunohistochemistry analysis of Pdx1 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



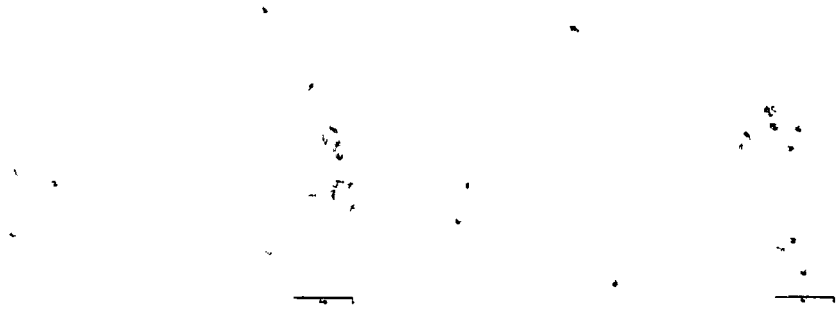


ES-D3 cells stained moderately for Pdx1 protein expression. The RA culture and CO_5d cultures both stained intensely for pdx1 expression indicating an up-regulation in expression between day 1 and day 5. A down-regulation in expression was observed between day 5 and day 9 cultures. The majority of cells in day 9 cultures (RA_AA, RA_BTC and CO_9d) exhibited moderate - weak staining for pdx1, with exception to the RA_SB culture. An intense distinct staining pattern within individual EBs was observed within the RA_SB culture.

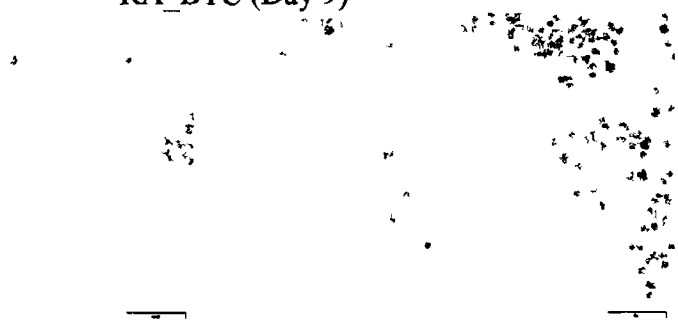
Figure 3.2.7 (I) Immunohistochemistry analysis of C-peptide 1 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



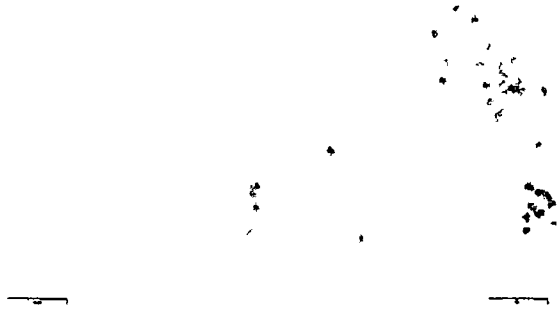
RA_SB (Day 9)



RA_BTC (Day 9)

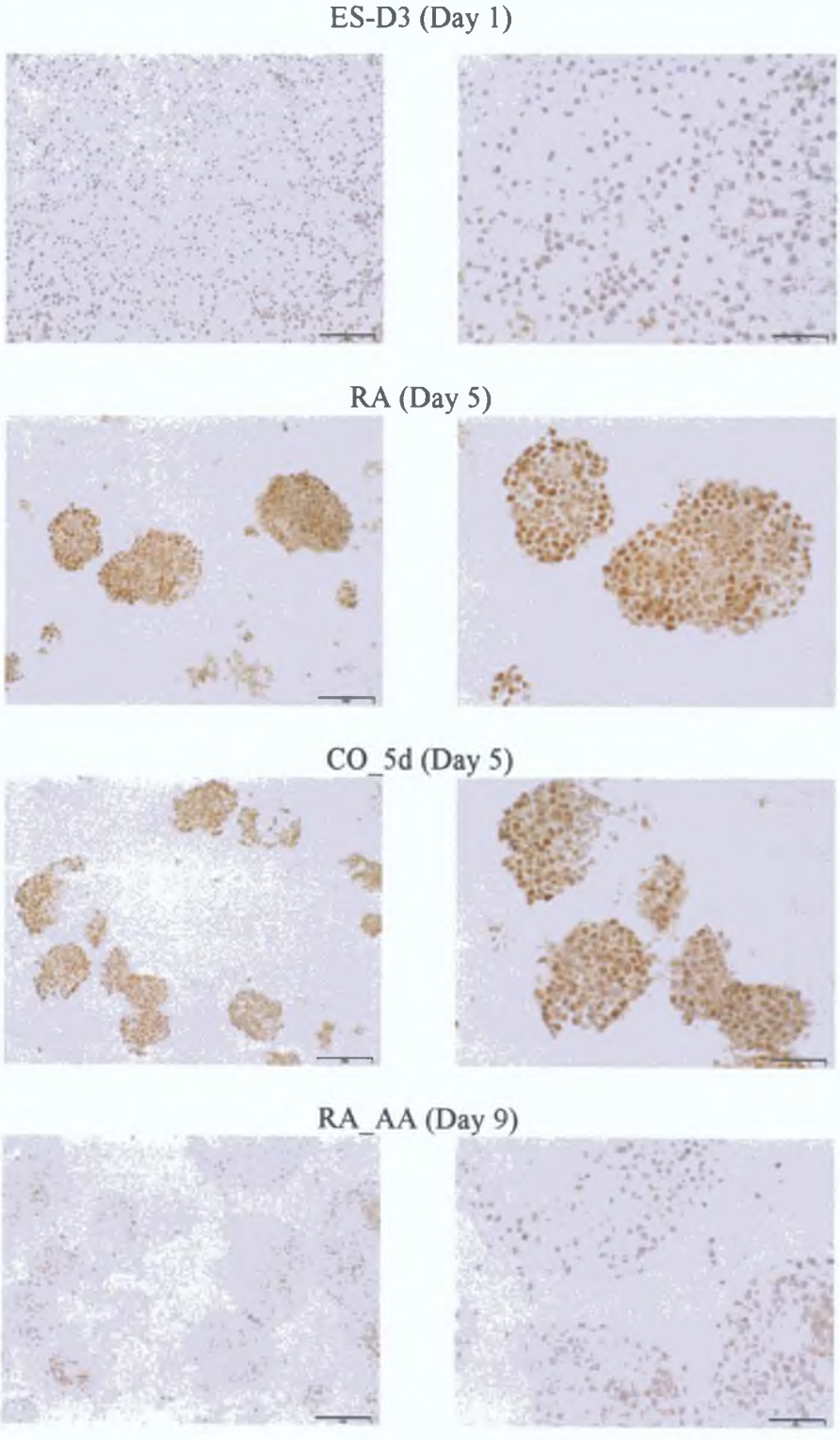


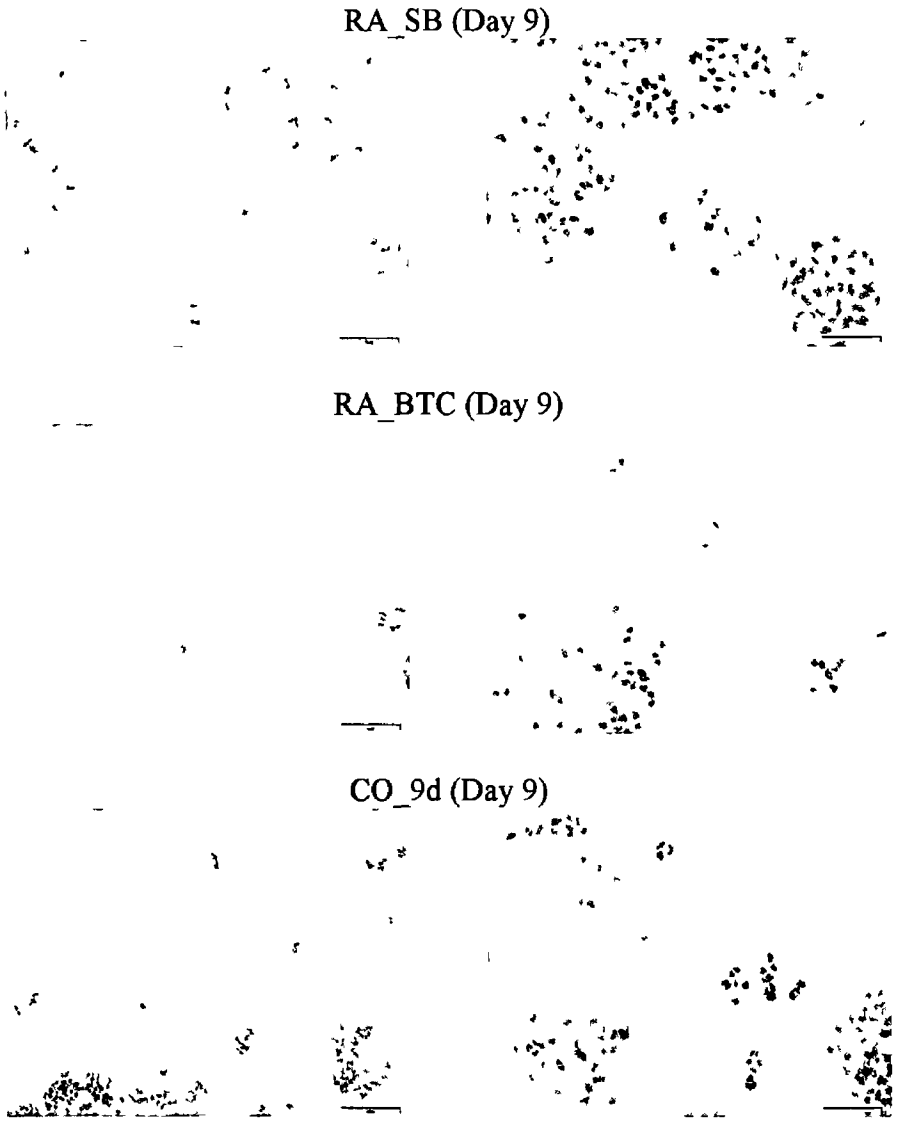
CO_9d (Day 9)



C-peptide 1 protein expression was not detected in ES-D3 cells Subpopulations of the RA and CO_5d culture stained positive for C-peptide 1 indicating induced expression of the C-peptide 1 protein within these cultures The weak staining pattern observed in day 9 cultures (RA_AA, RA_SB, RA_BTC and CO_9d) indicated that stage 2 differentiation resulted in a down-regulation of C-peptide 1 expression

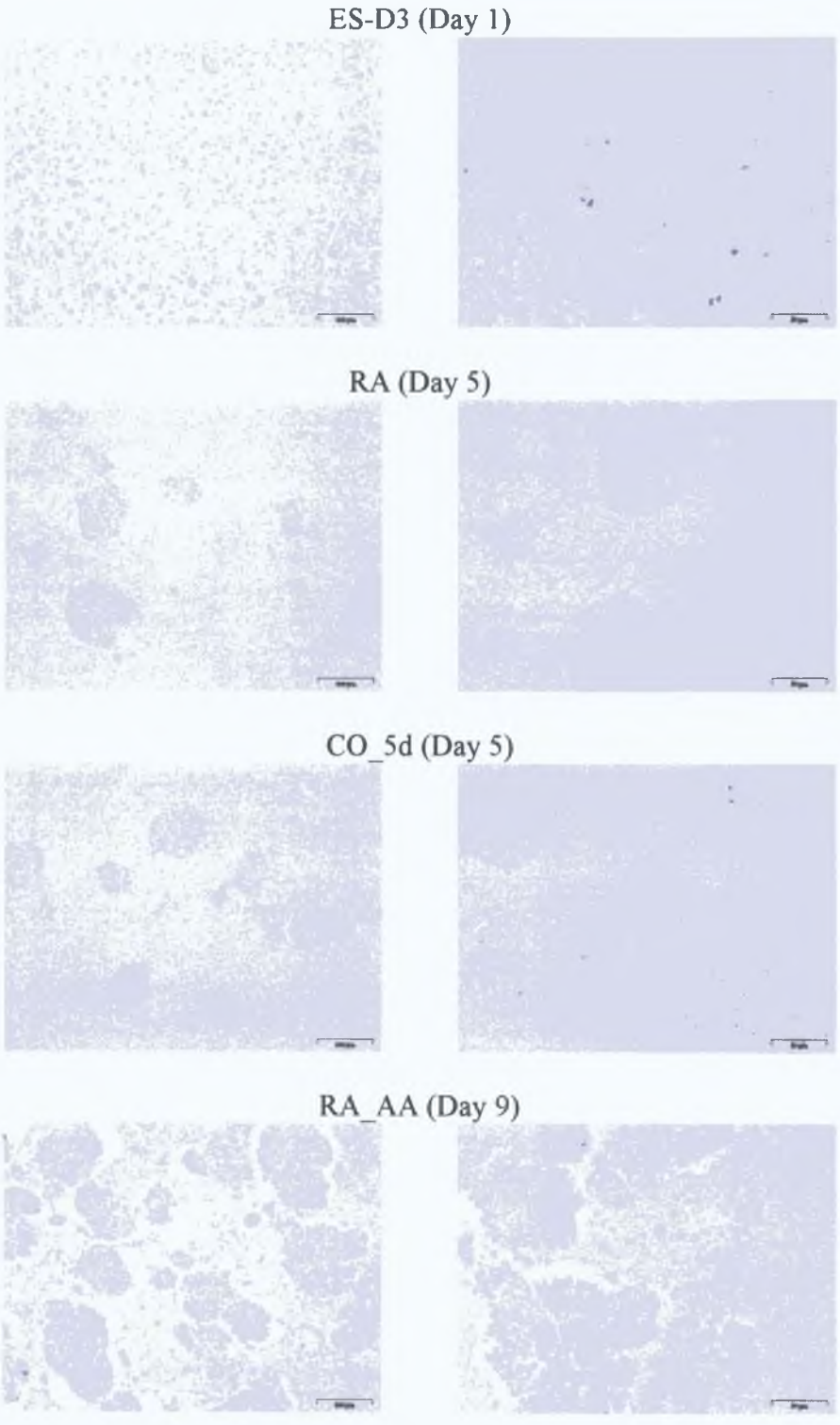
Figure 3.2.7 (J) Immunohistochemistry analysis of C-peptide 2 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



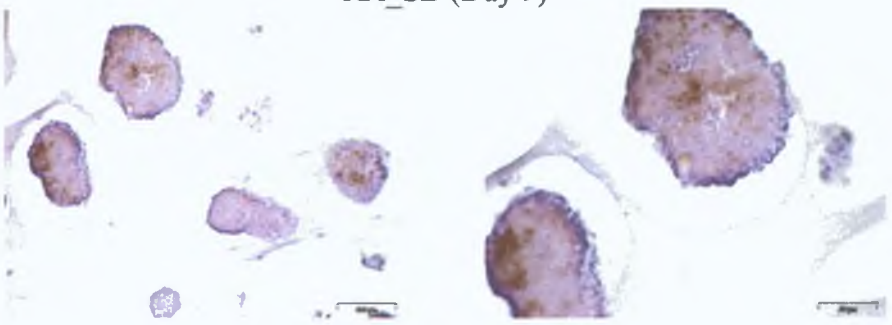


A small subpopulation of ES-D3 cells stained weakly for C-peptide 2 indicating a low level expression of the protein in these cells The increase in percentage and intensity of staining in the RA and CO_5d cultures suggested an up-regulation of C-peptide 2 expression in these cultures Stage 2 differentiation appeared to down-regulate C-peptide 2 expression However, a strong staining intensity observed in subpopulations of the RA_SB and RA_BTC cultures indicated that these isolated cells still maintained a high level of c-peptide 2 expression

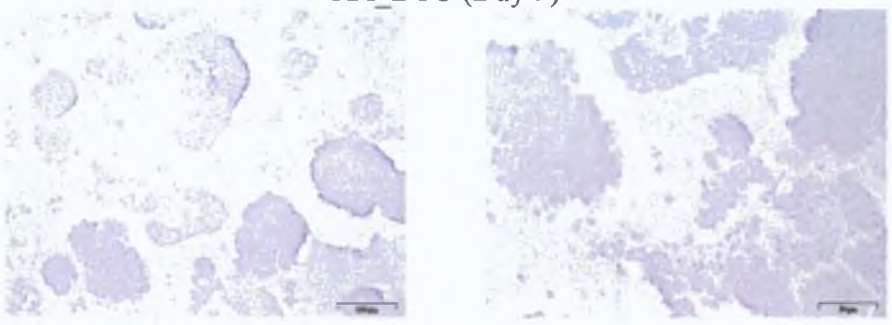
Figure 3.2.7 (K) Immunohistochemistry analysis of insulin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



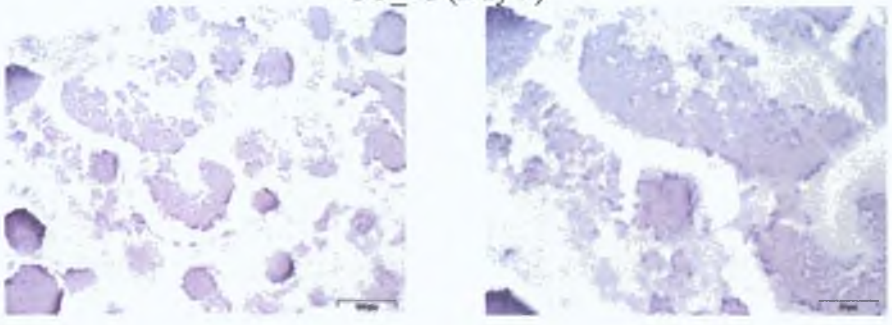
RA_SB (Day 9)



RA_BTC (Day 9)

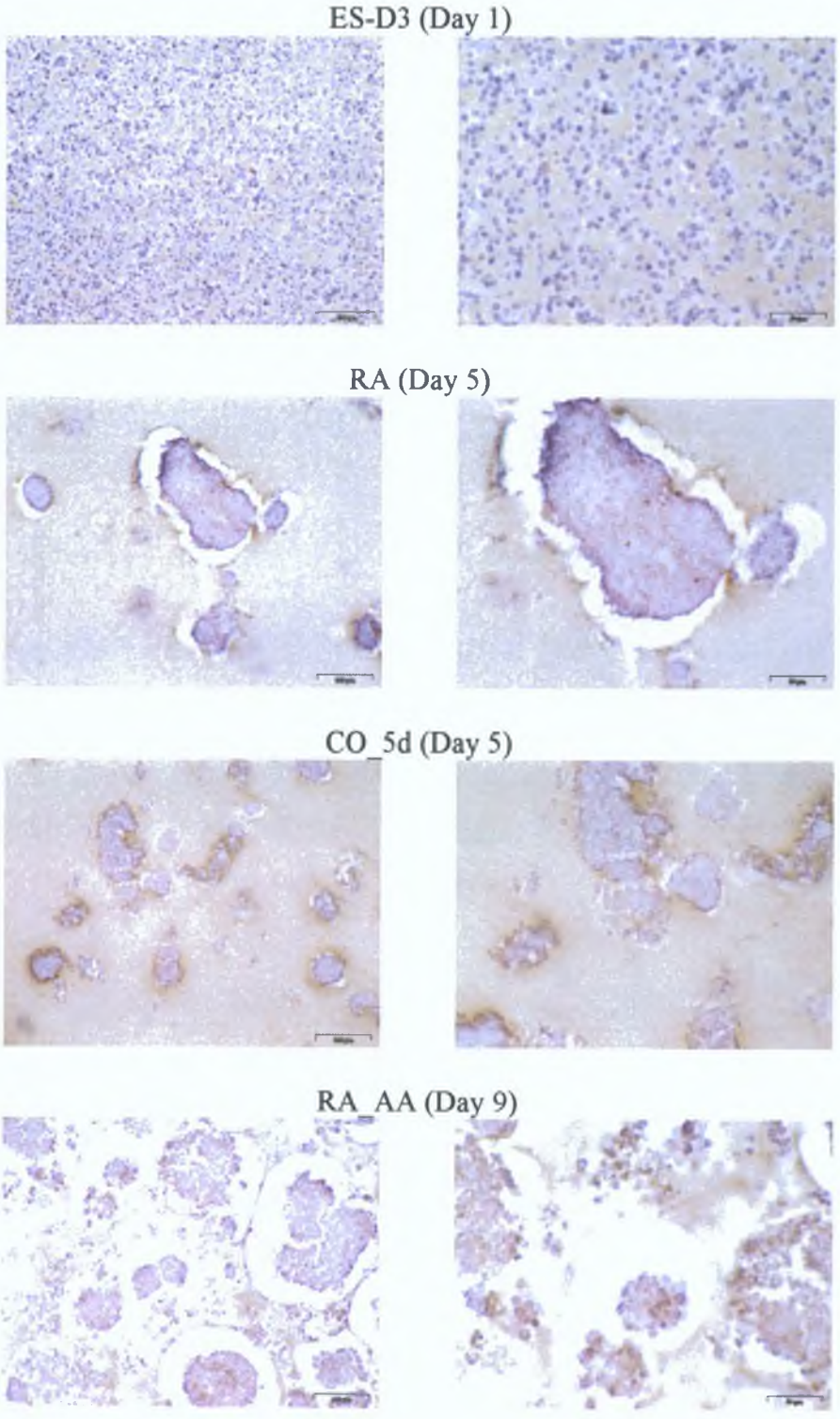


CO_9d (Day 9)

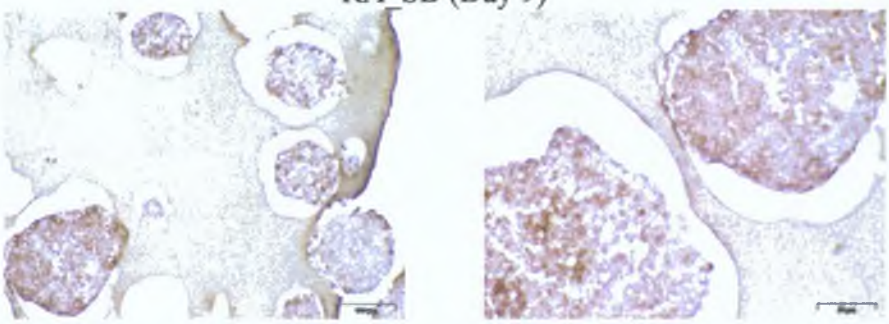


Insulin protein was not detected in the ES-D3 cells or in ES-derived cultures with exception to the RA_SB culture. A subpopulation of the RA_SB culture stained intensely for insulin indicating induced expression of the insulin protein in isolated cells within this culture.

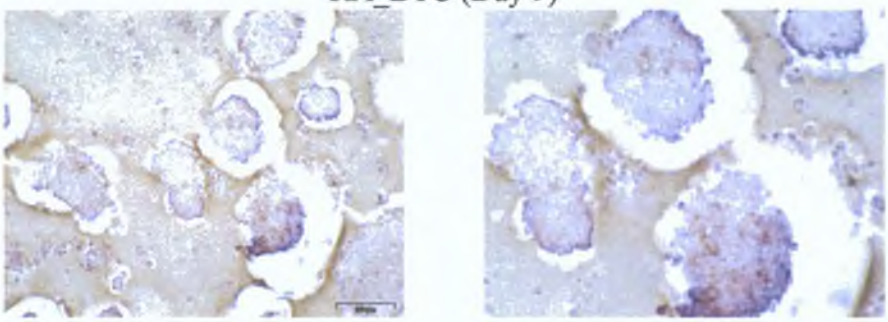
Figure 3.2.7 (L) Immunohistochemistry analysis of glut2 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



RA_SB (Day 9)



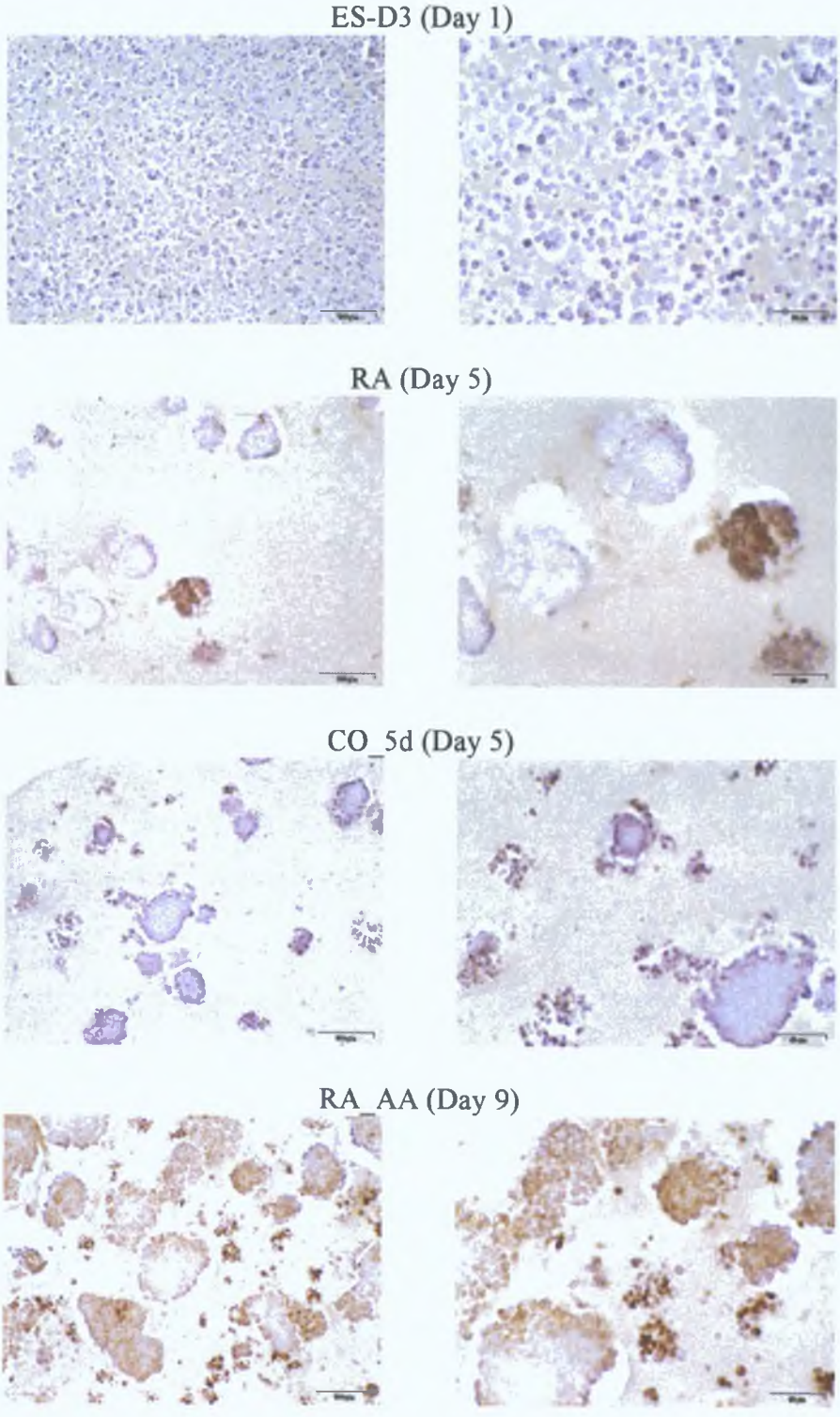
RA_BTC (Day 9)



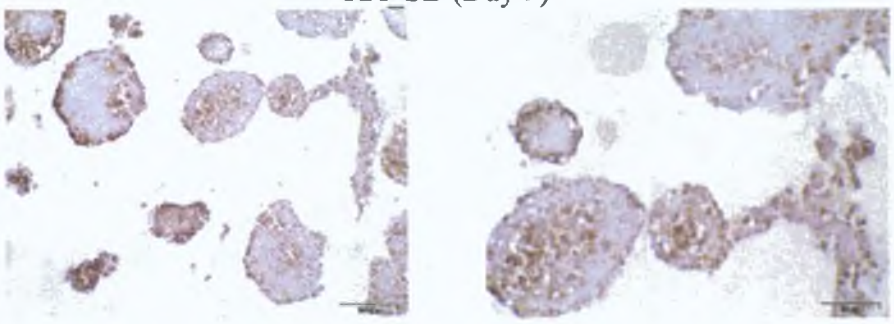
CO_9d (Day 9)
Not Determined

Glut2 protein expression was not detected in ES-D3 cells. The appearance of weak to moderately stained cells in the day 5 cultures (RA and CO_5d) indicated that glut2 protein expression was induced at a low level within these cultures. There was a noticeable increase in the percentage and intensity of staining within the RA_SB culture suggesting an up-regulation in glut2 protein expression between the RA culture at day 5 and the RA_SB culture at day 9.

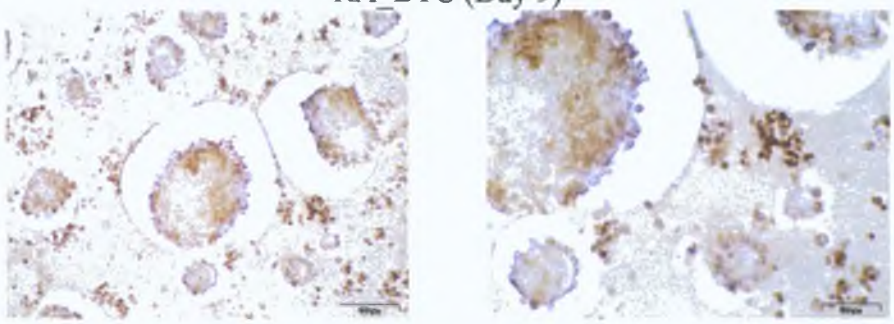
Figure 3.2.7 (M) Immunohistochemistry analysis of somatostatin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



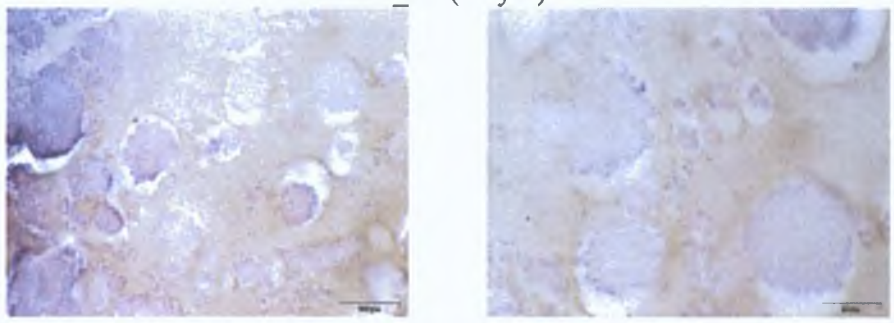
RA_SB (Day 9)



RA_BTC (Day 9)

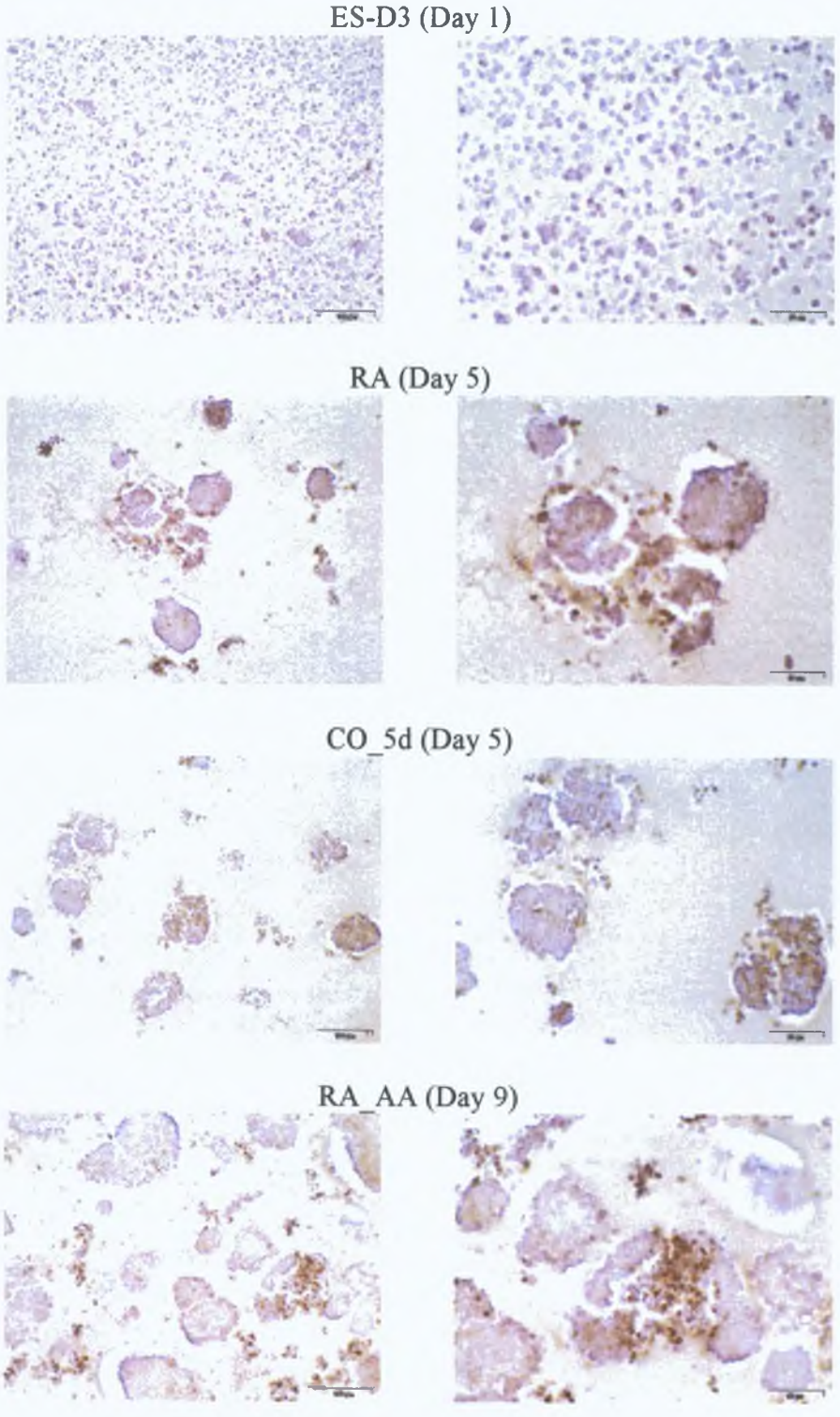


CO_9d (Day 9)

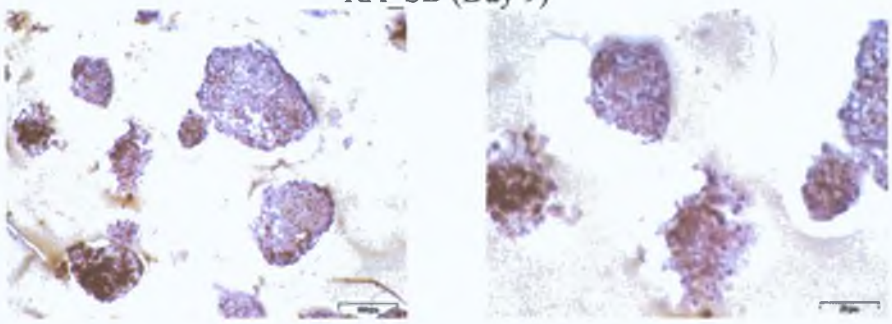


Somatostatin protein expression was not detected in ES-D3 cells. The appearance of stained cells in the 5 day RA culture and to a lesser extent in the CO_5d culture indicated that somatostatin expression had been induced within these cultures. Subsequent differentiation in the presence of AA, SB and BTC increased the percentage and intensity of staining suggesting an up-regulation in somatostatin expression between the RA culture at day 5 and the treated cultures (RA-AA, RA_SB, RA_BTC) at day 9. The spontaneously-formed CO_ 9d culture does not appear to express somatostatin.

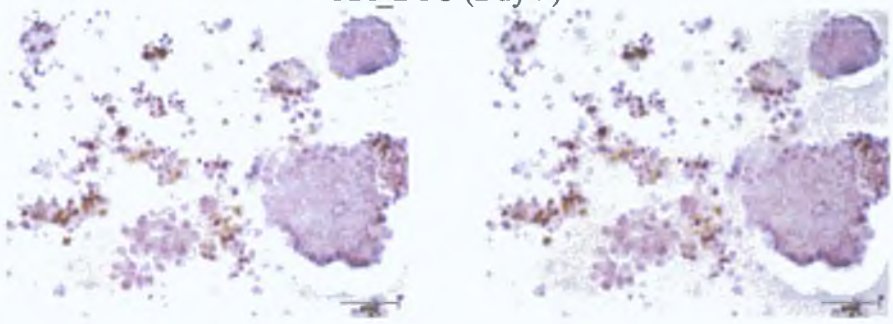
Figure 3.2.7 (N) Immunohistochemistry analysis of PP protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 1 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



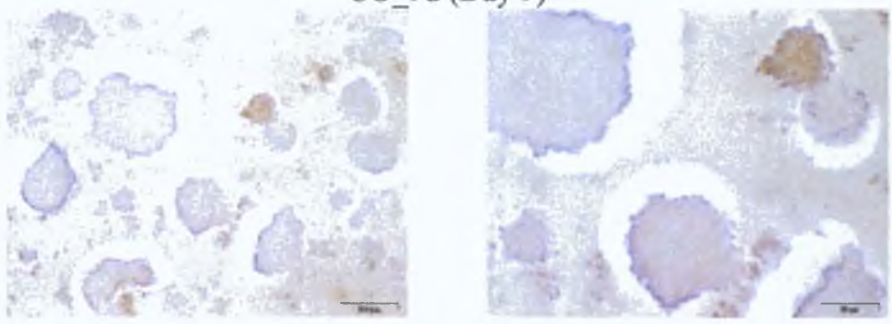
RA_SB (Day 9)



RA_BTC (Day 9)

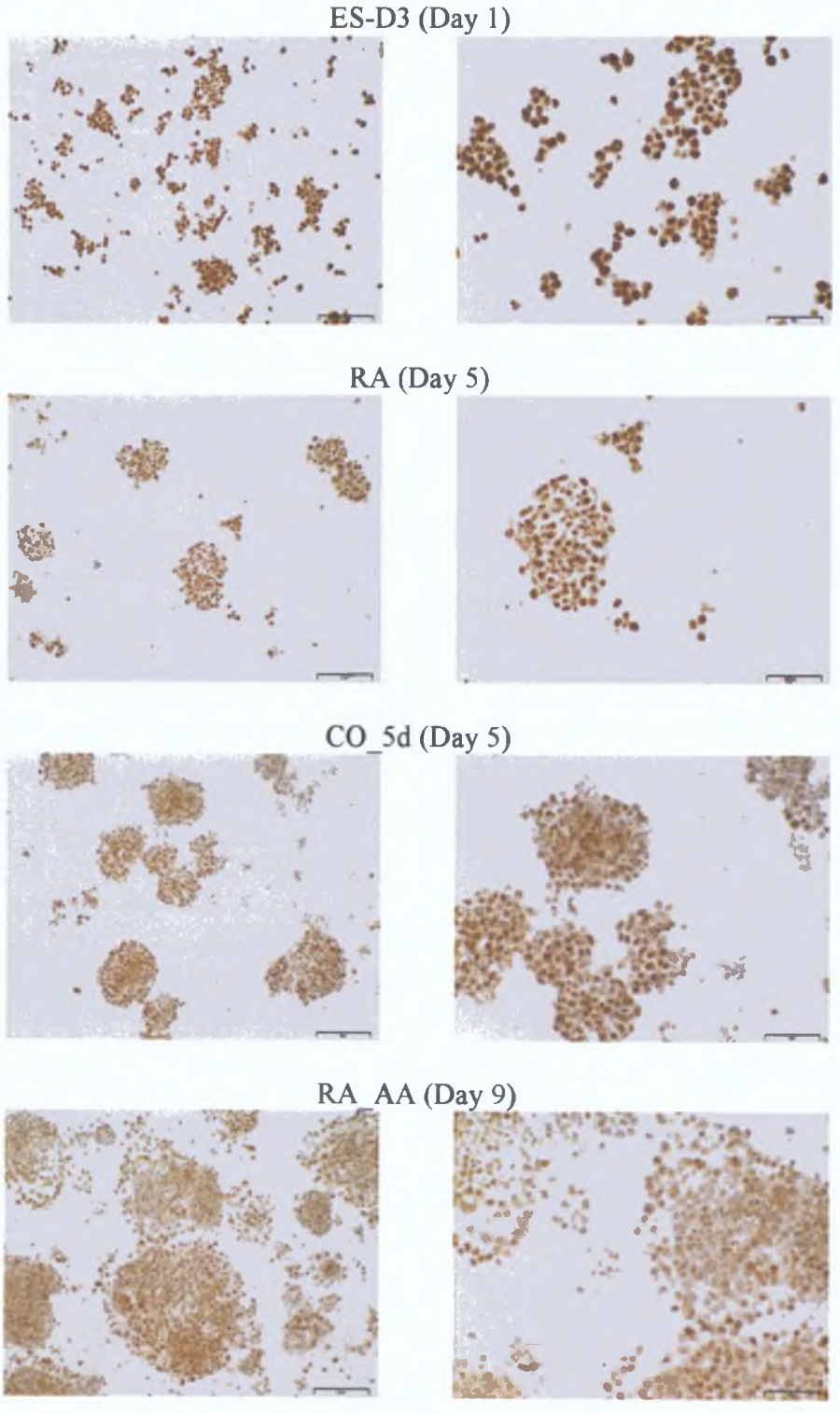


CO_9d (Day 9)

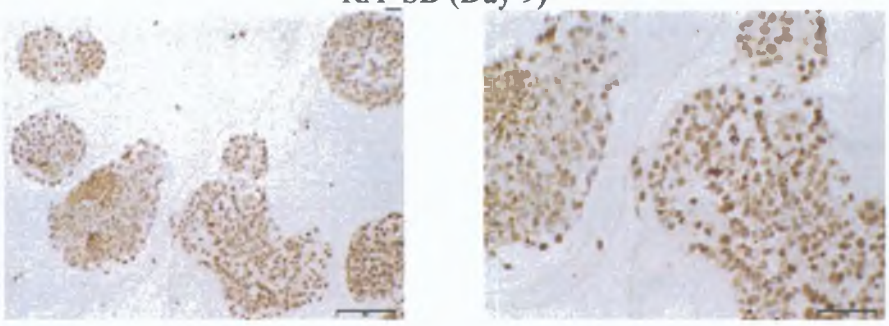


The detection of a small subpopulation of weakly stained cells in the ES-D3 culture indicated that PP protein expression was expressed at a low level within this culture. All differentiated ES-derived cultures contained subpopulations of moderately - intensely stained cells suggesting an induced up-regulation in protein expression upon differentiation.

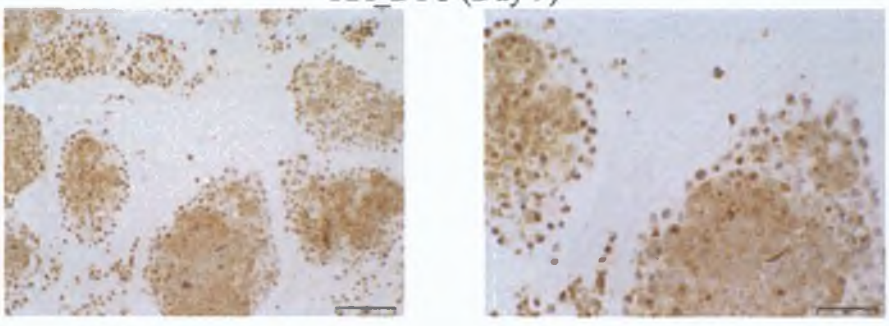
Figure 3.2.8 (A) Immunohistochemistry analysis of Oct 4 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



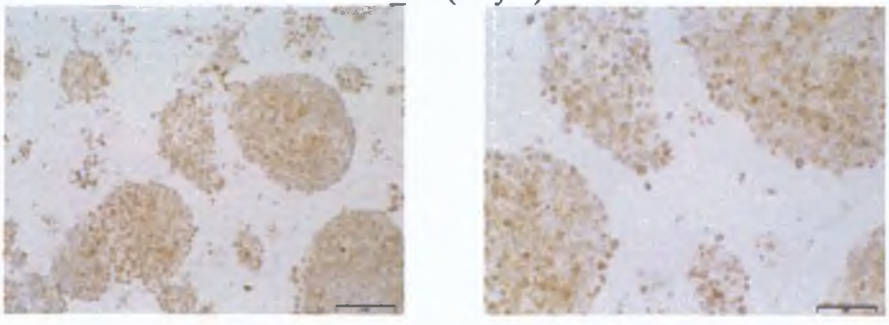
RA_SB (Day 9)



RA_BTC (Day 9)

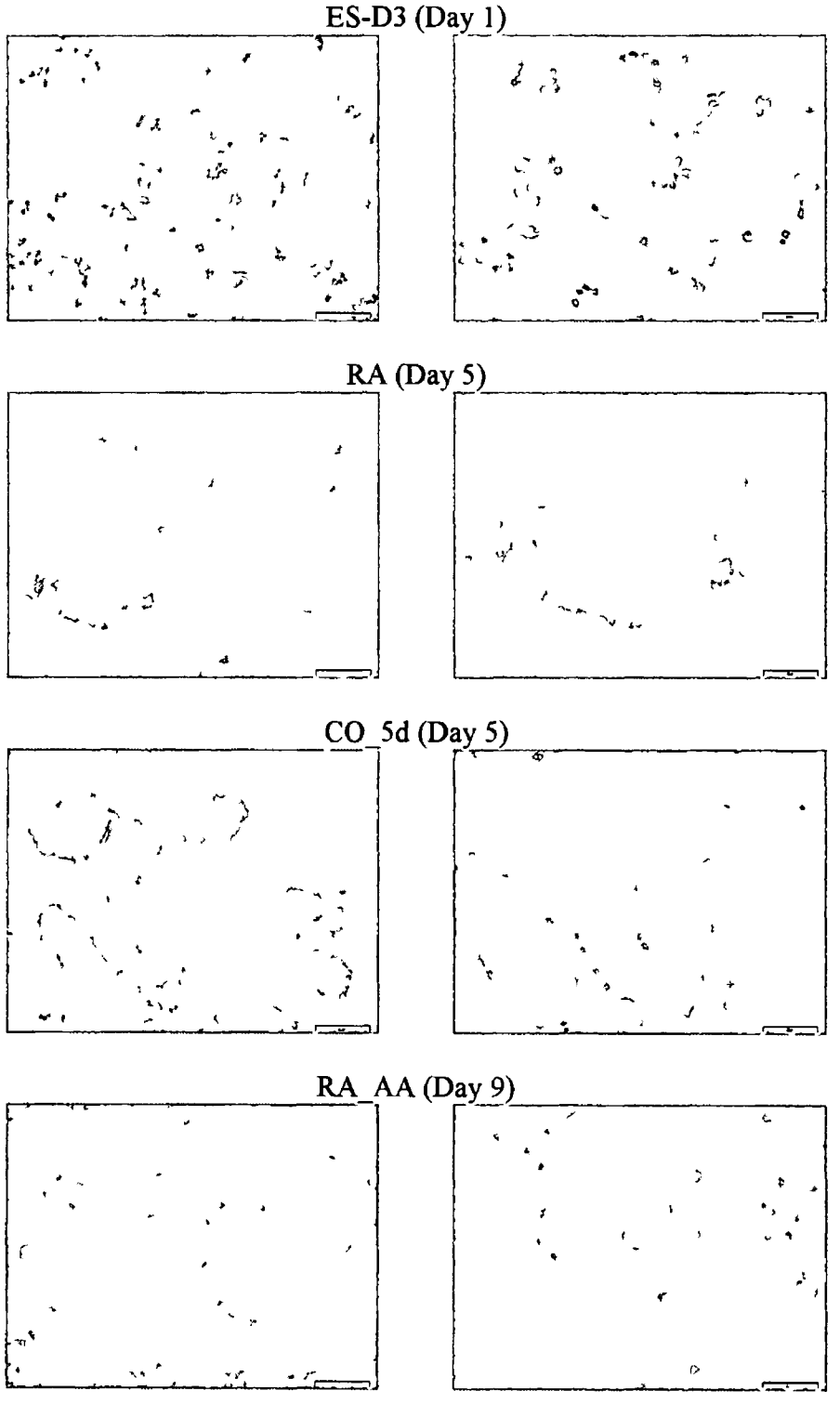


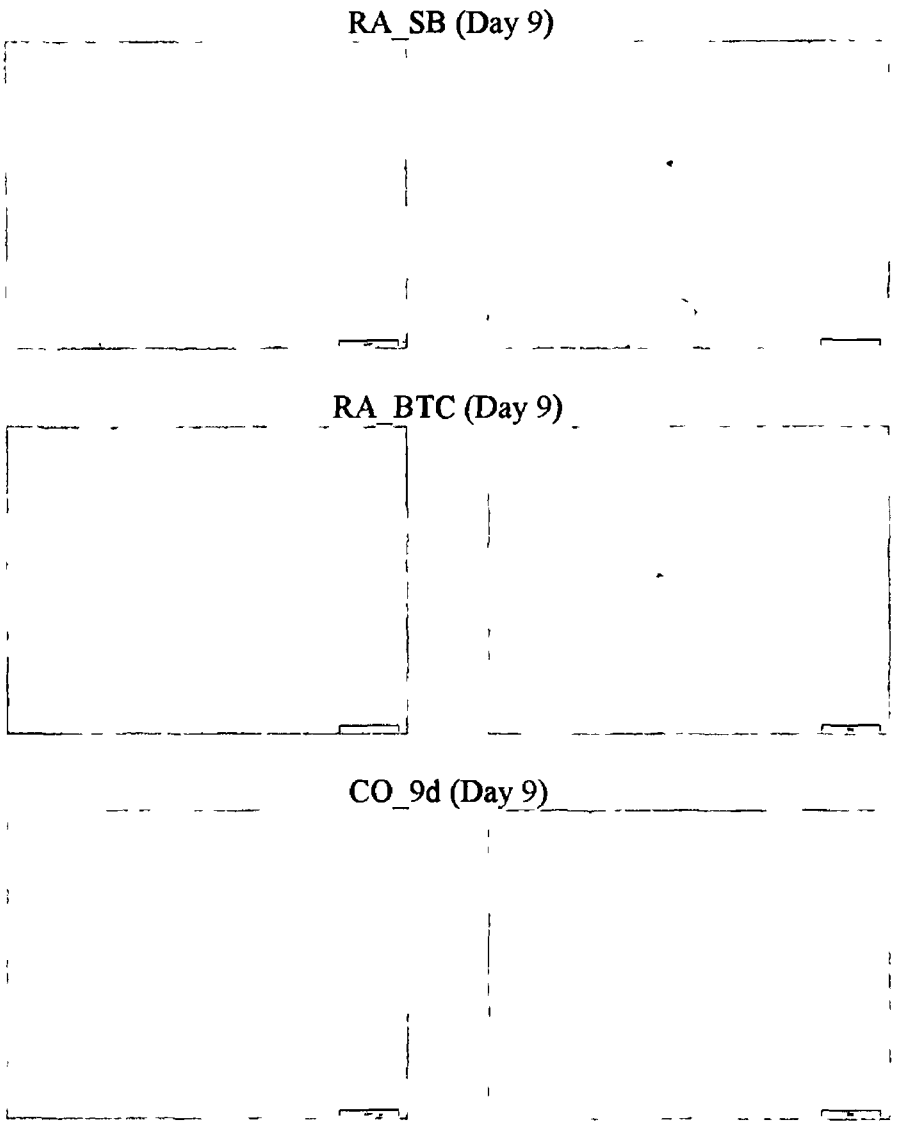
CO_9d (Day 9)



The majority of cells in the ES-D3 and the 5 day cultures (RA and CO_5d) stained intensely for Oct 4; however the majority of cells in the 9 day cultures (RA_AA, RA_BTC, RA_SB, CO_9d) stained moderately - weakly for Oct 4, indicating an overall down-regulation in Oct 4 protein expression between day 5 and day 9. The CO_9d culture appeared to exhibit the lowest level of expression.

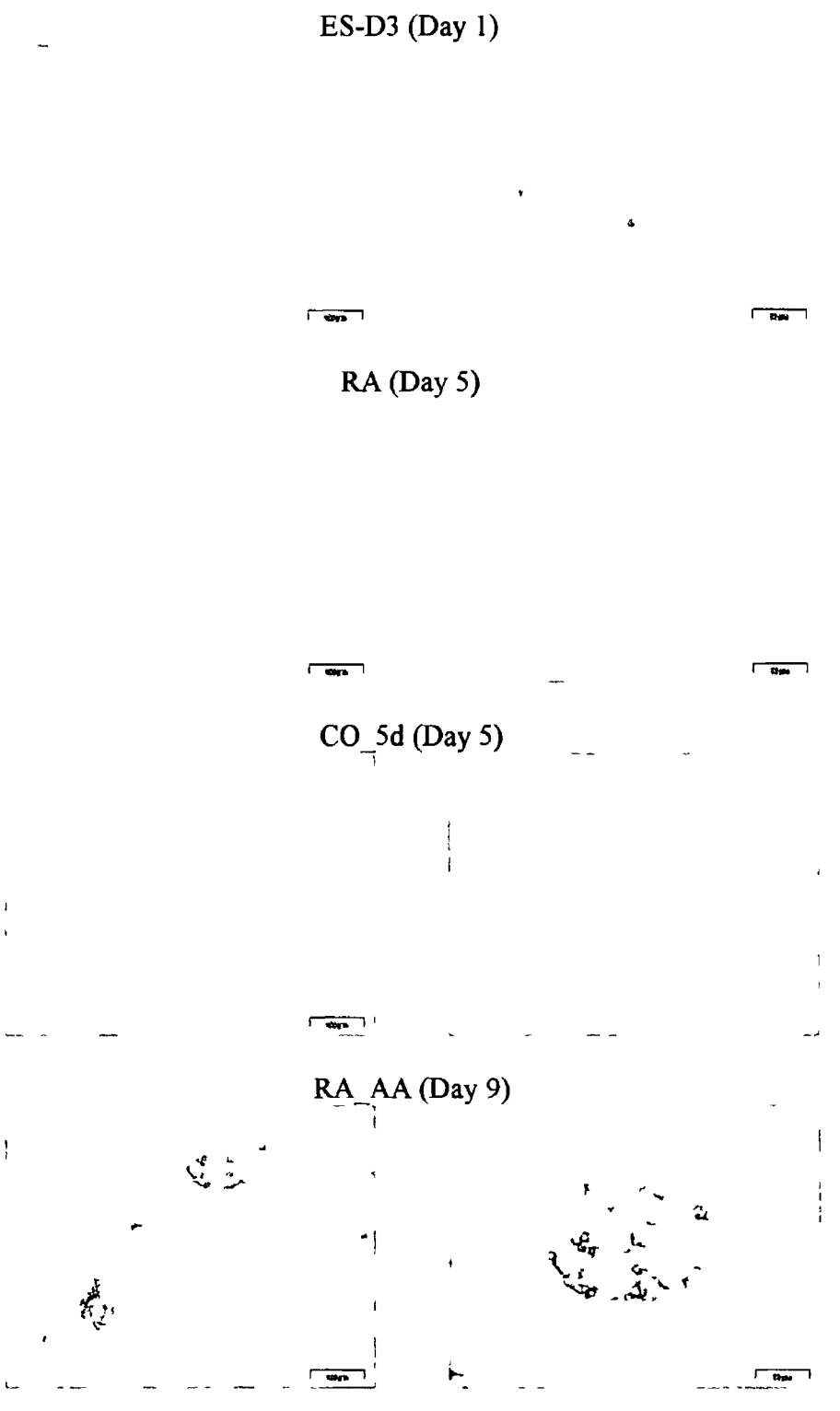
Figure 3 2 8 (B) Immunohistochemistry analysis of NF-L protein expression in ES-D3 and ES-derived cultures The images shown are representative of repeat 2 in Table 3 2 4 Images on left, scale bar = 100 μm (20X) Images on right, scale bar = 50 μm (40X)

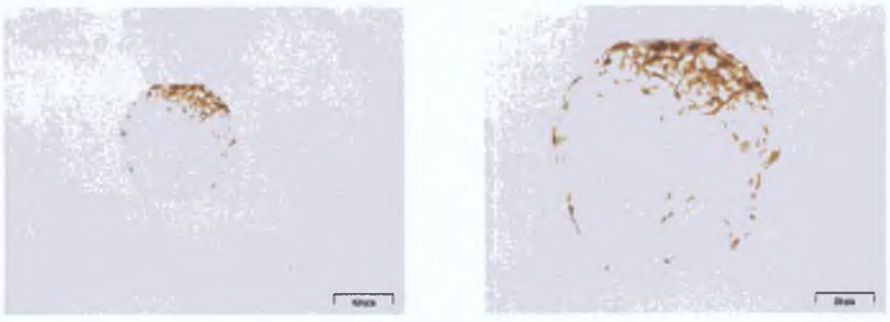




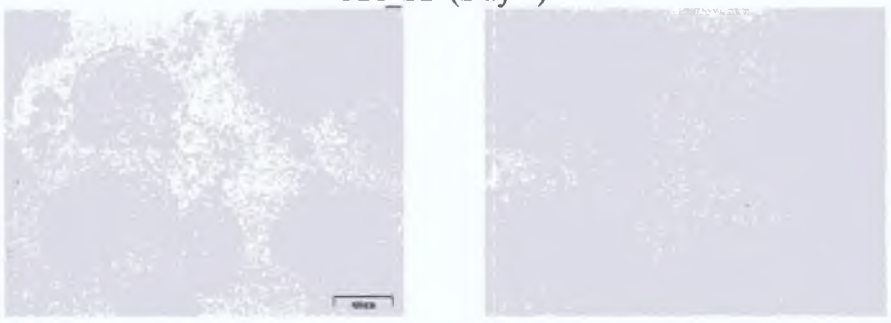
A subpopulation of ES-D3 cells stained weakly for NF-L where as a subpopulation of the day 5 RA culture stained moderately for NF-L, suggesting an up-regulation in NF-L protein expression in the RA culture Day 9 cultures which had been exposed to AA, SB and BTC all contained moderately stained cells indicating that NF-L expression levels had been maintained in these cultures The CO_5d culture and the CO_9d culture stained weakly for NF-L indicating low level of expression within these cultures

Figure 3 2 8 (C) Immunohistochemistry analysis of GFAP protein expression in ES-D3 and ES-derived cultures The images shown are representative of repeat 2 in Table 3 2 4 Images on left, scale bar = 100 μm (20X) Images on right, scale bar = 50 μm (40X)

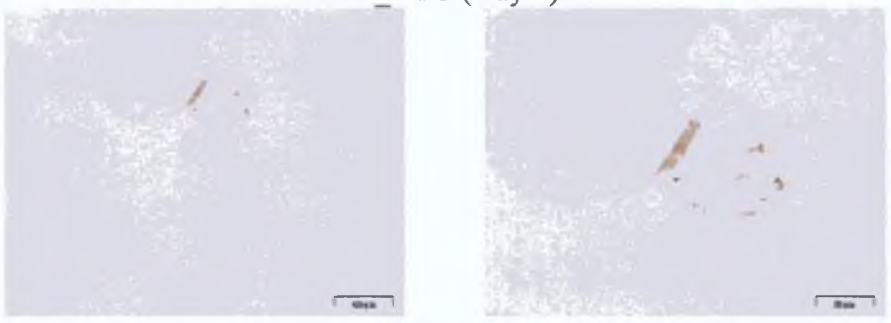




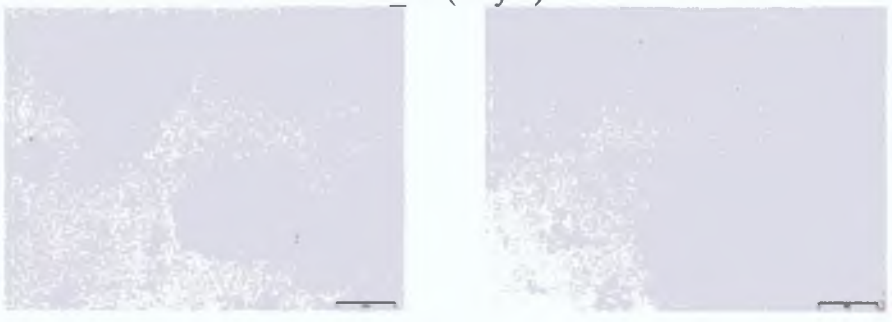
RA_SB (Day 9)



RA_BTC (Day 9)

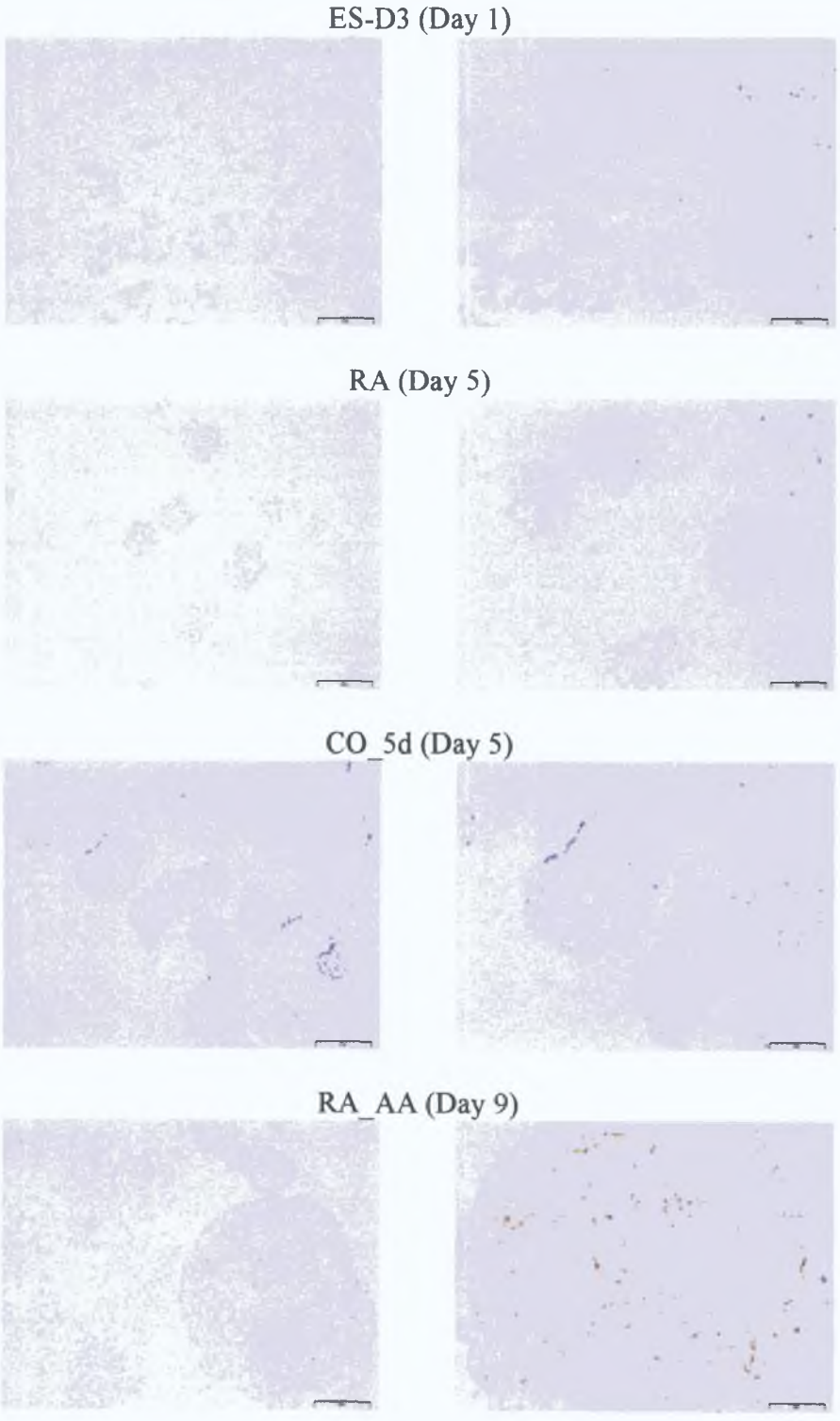


CO_9d (Day 9)



GFAP protein expression was not detected in ES-D3 cells or in ES-derived 5 day cultures (RA and CO_5d). An intense distinctive staining pattern was observed in subpopulations of both the RA_AA and RA_BTC cultures at day 9, indicating that GFAP expression was induced within these cultures. Expression was not detected in the RA_SB culture or the CO_9d culture.

Figure 3.2.8 (D) Immunohistochemistry analysis of nestin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



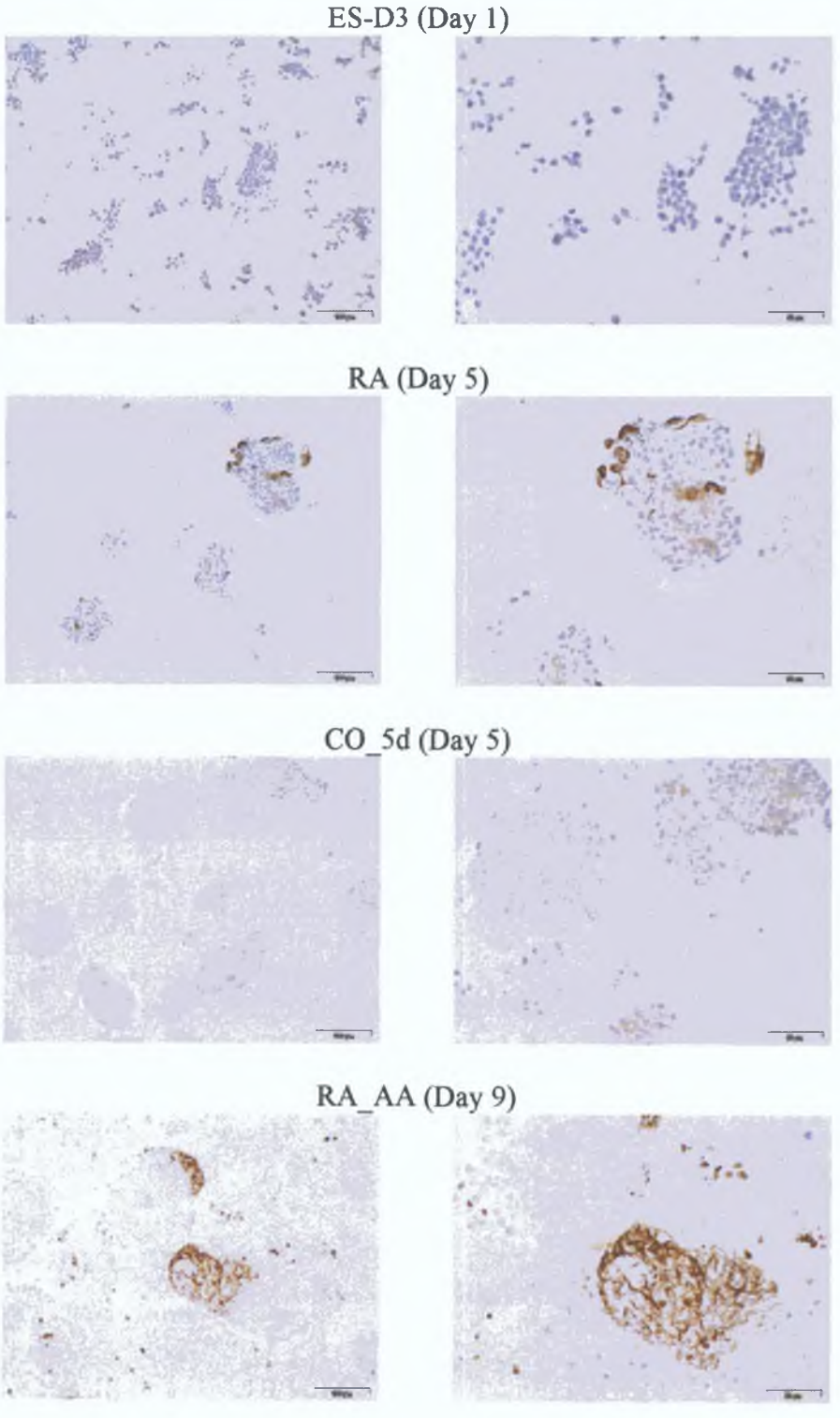
RA_SB (Day 9)

RA_BTC (Day 9)

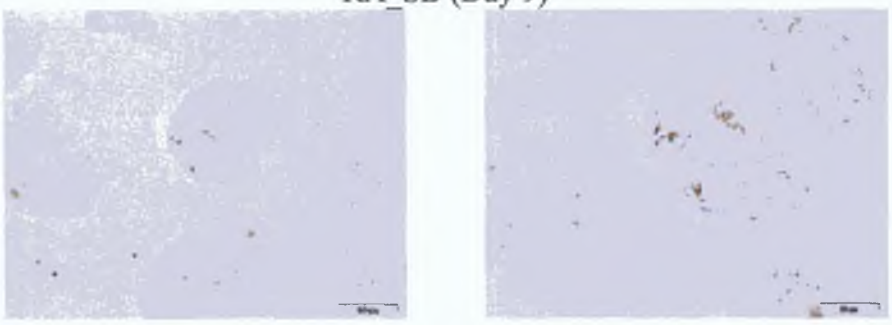
CO_9d (Day 9)

Nestin protein expression was not detected in ES-D3 cells A filamentous staining pattern was observed in the RA_AA and RA_BTC cultures, indicating the induced expression of nestin within subpopulations of this culture Expression was not detected in spontaneously-formed CO_5d, CO_9d cultures and the RA_SB culture

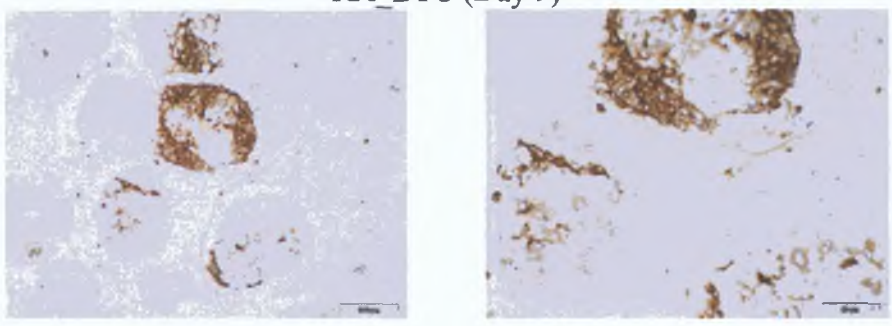
Figure 3.2.8 (E) Immunohistochemistry analysis of β III tubulin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



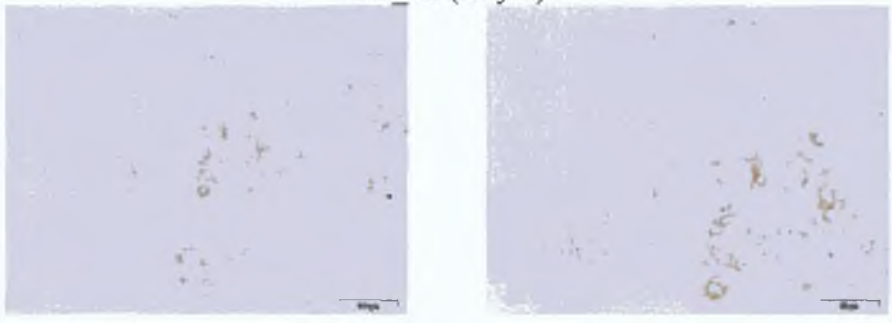
RA_SB (Day 9)



RA_BTC (Day 9)

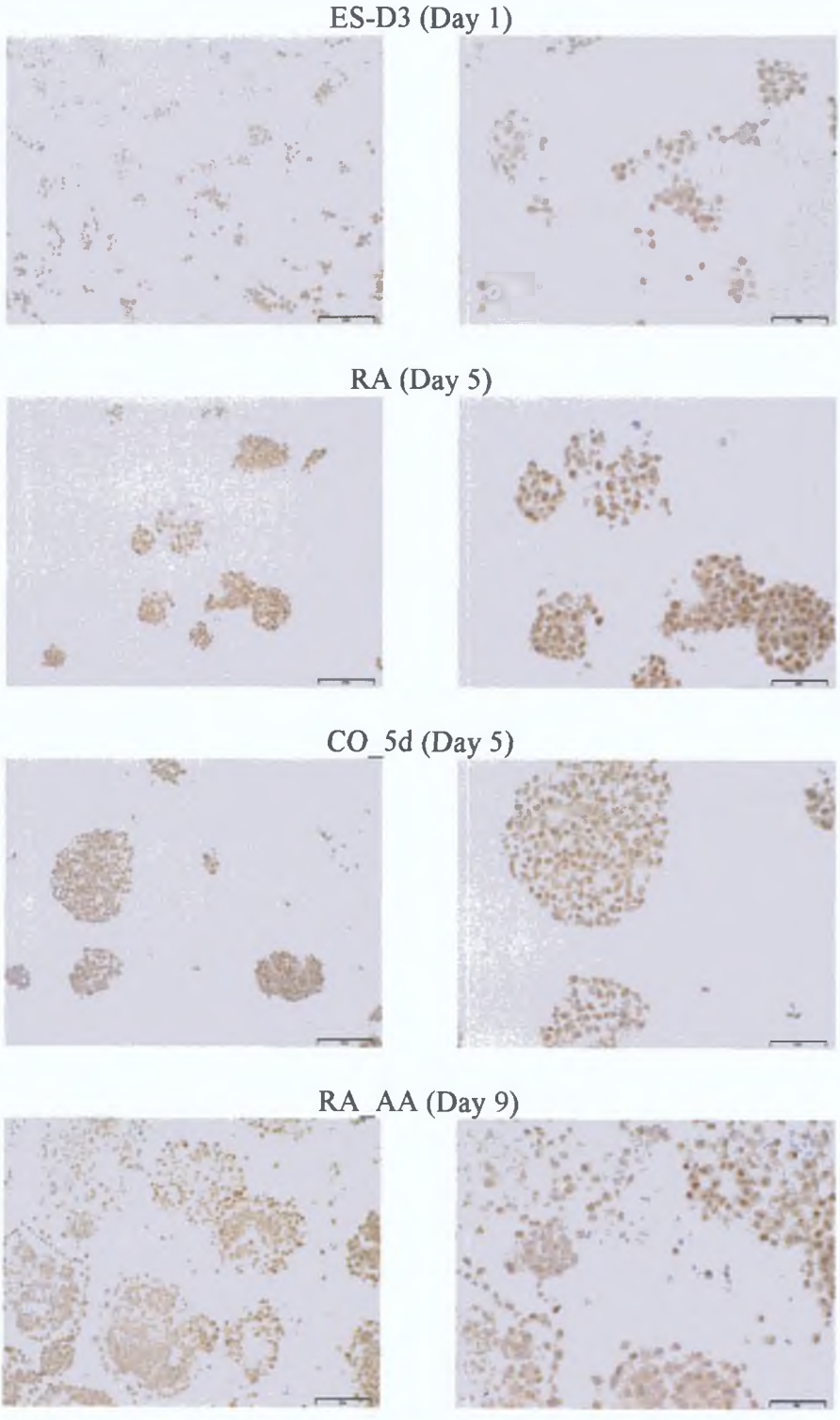


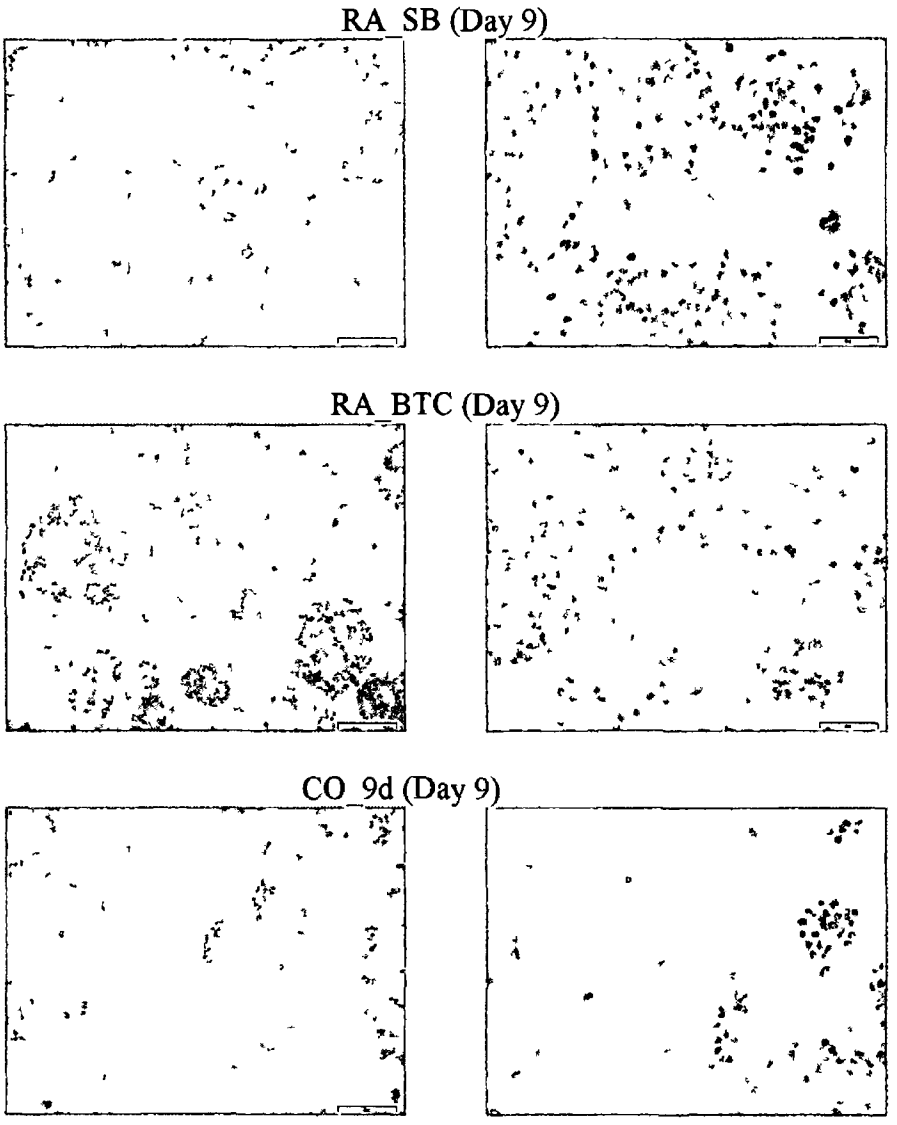
CO_9d (Day 9)



β III tubulin protein expression was not detected in ES-D3 cells. A subpopulation of the RA culture at day 5 stained positive for β III tubulin indicating that β III tubulin protein expression was induced within this culture. The percentage of staining was increased in the RA_AA and RA_BTC cultures at day 9 indicating an up-regulation in expression. β III tubulin expression was not detected in the spontaneously-formed CO_5d. A very small subpopulation of cells in the CO_9d and RA_SB culture stained weakly-moderately indicating low level expression of β III tubulin in subpopulations of cells within these cultures.

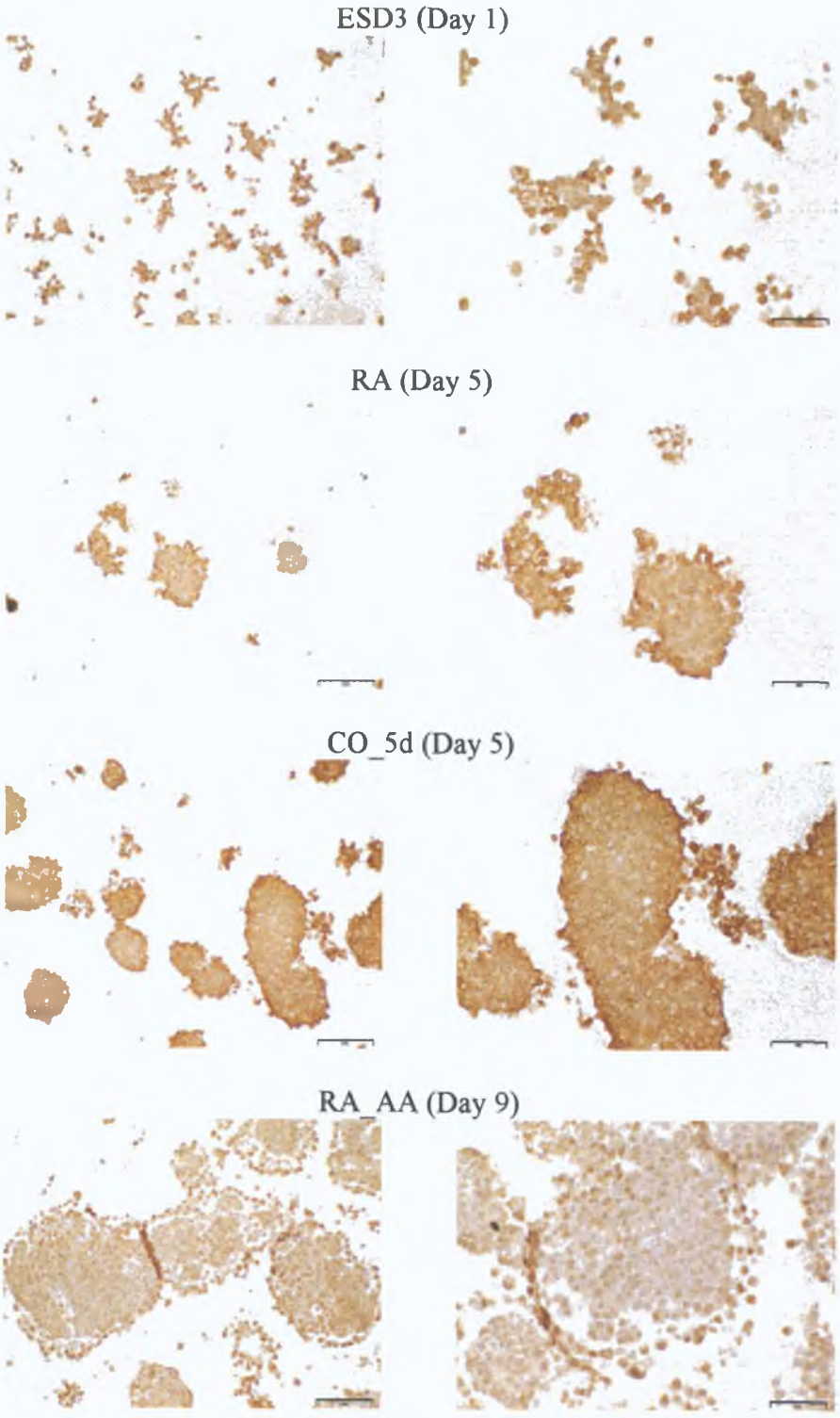
Figure 3.2.8 (F) Immunohistochemistry analysis of myogenin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

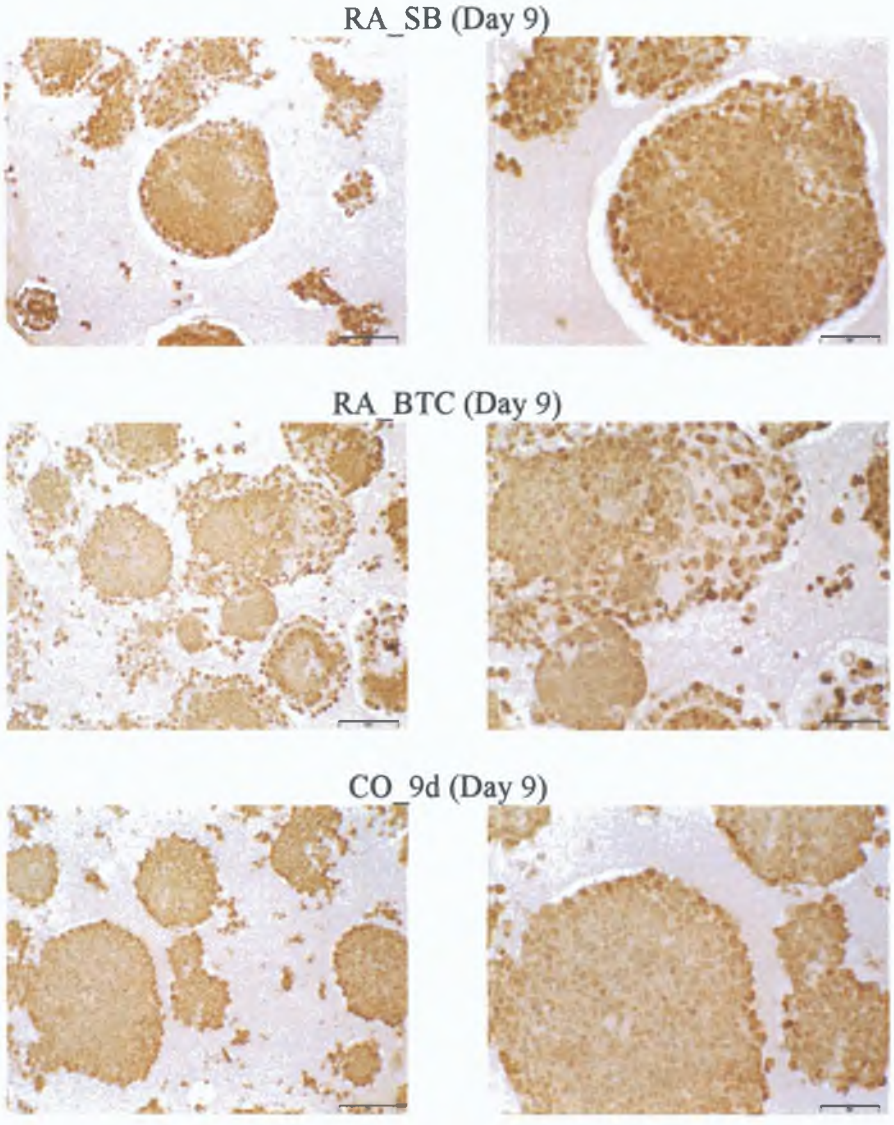




A subpopulation of ES-D3 cells stained weakly for myogenin indicating low level expression of the protein within this culture An increase in percentage and intensity of staining was observed in day 5 cultures indicating that myogenin expression was up-regulated in both the RA culture and CO_5d culture However, the RA culture contained a subset of intensely stained cells where as cells in the CO_5d culture were moderately stained Subsequent differentiation resulted in a decrease in the percentage and intensity of staining indicating a down-regulation of myogenin expression in 9 day cultures, including treated (RA_AA, RA_SB, RA_BTC) and spontaneously-formed cultures (CO_9d)

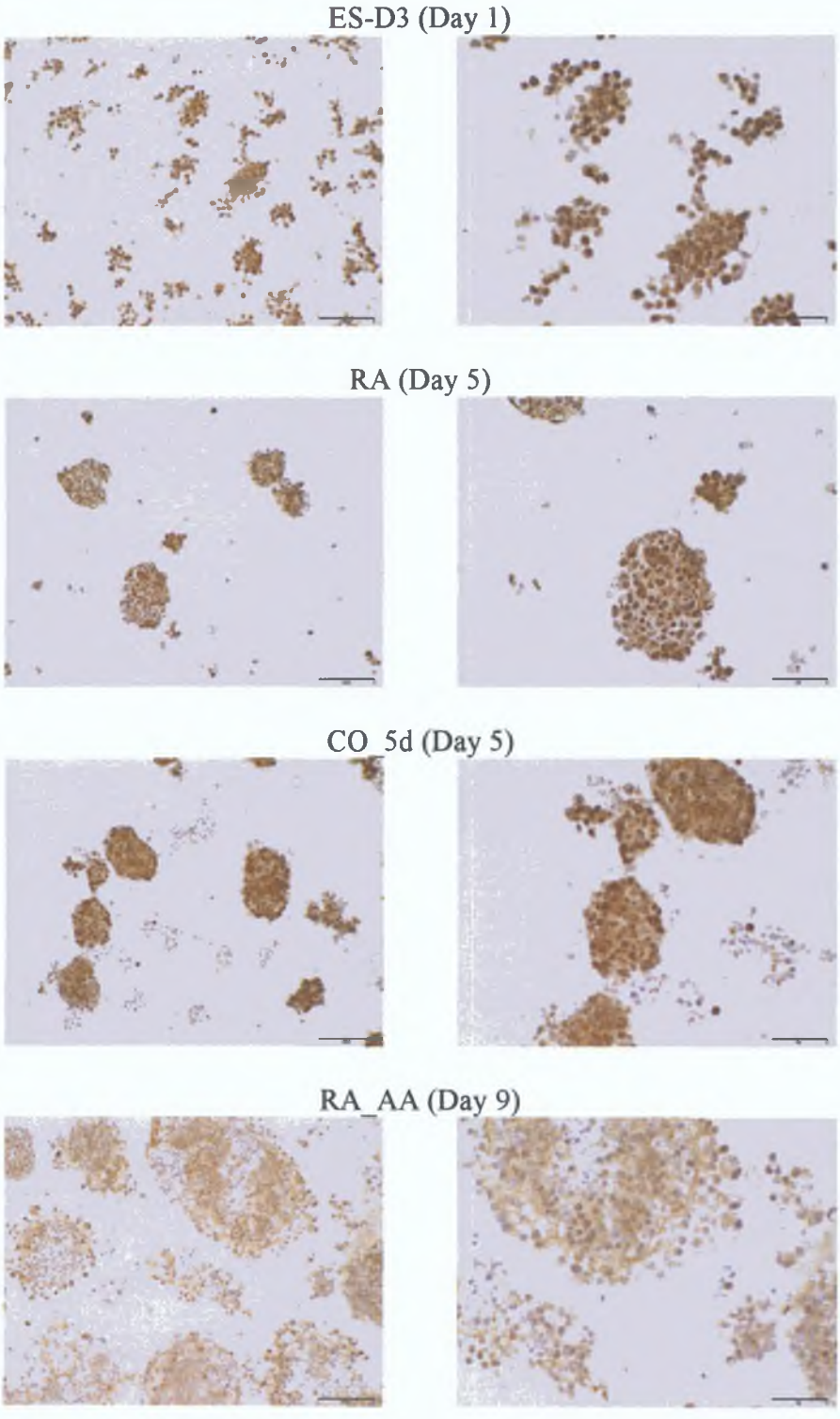
Figure 3.2.8 (G) Immunohistochemistry analysis of desmin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).

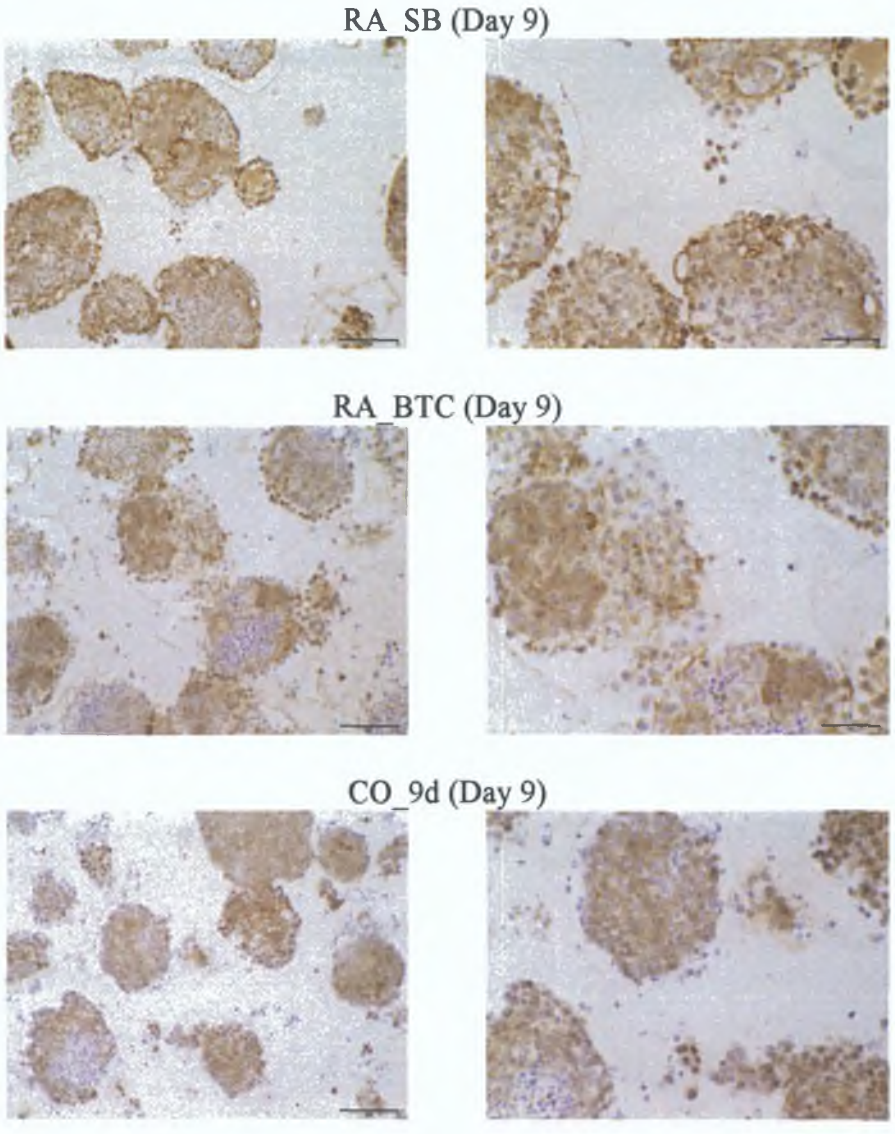




ES-D3 cells and all ES-derived cultures stained positive for desmin expression. Decreases in staining intensity in both the spontaneously formed CO_9d culture and the RA_AA and RA_BTC culture suggested that desmin protein expression was down-regulated within these cultures.

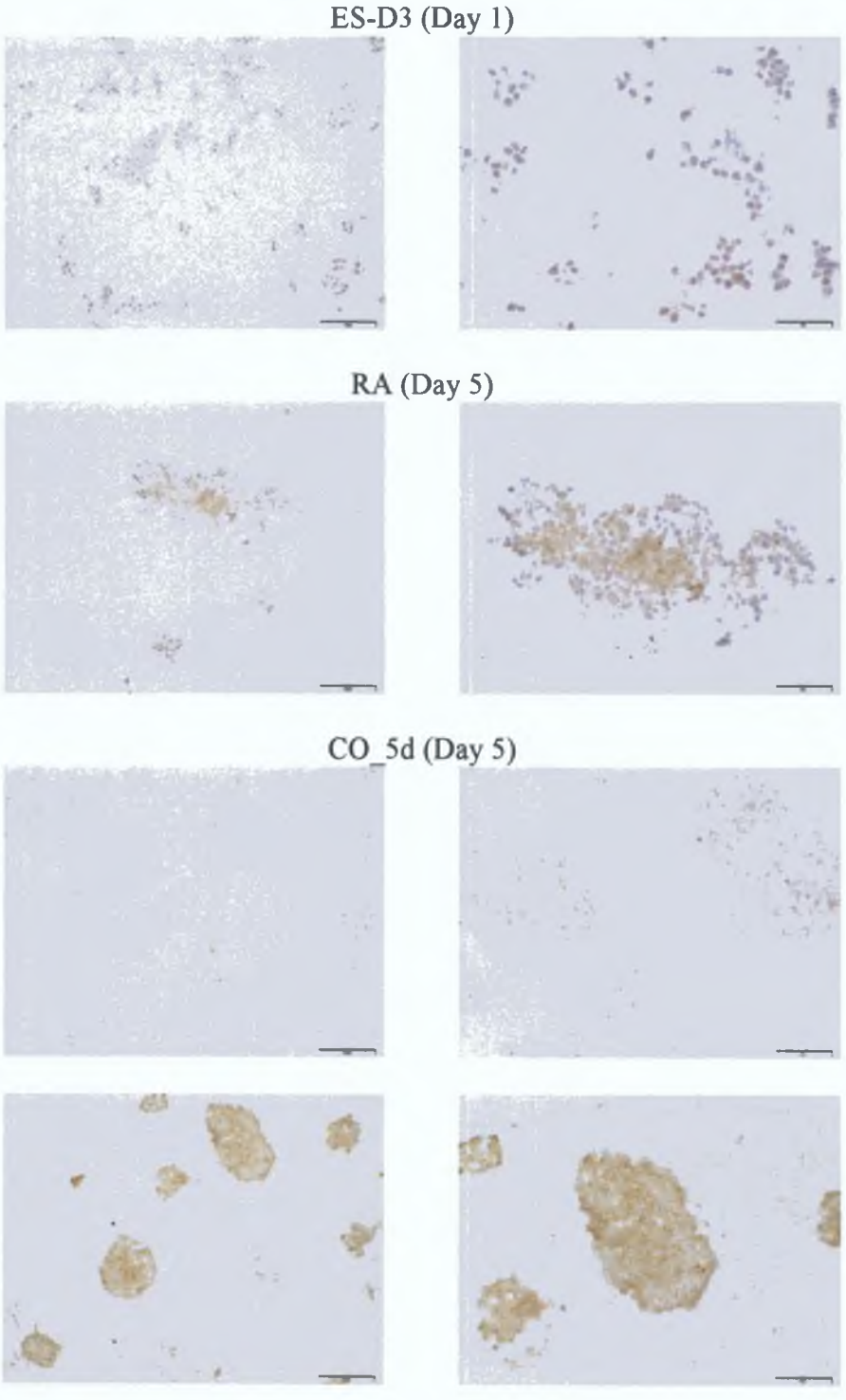
Figure 3.2.8 (H) Immunohistochemistry analysis of Pdx1 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



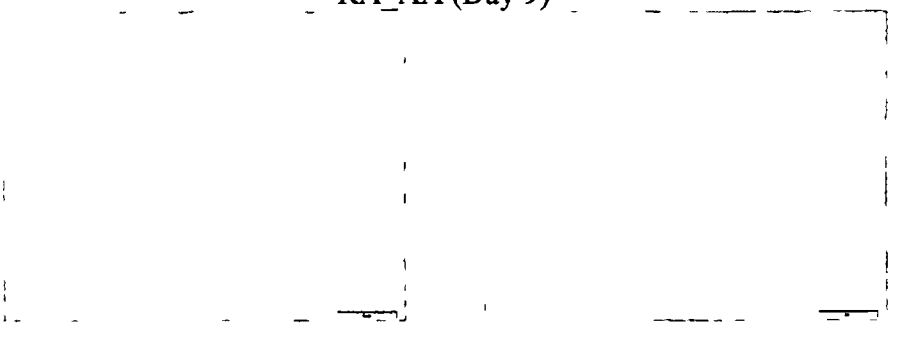


ES-D3 cells (Day 1), the RA culture and CO_5d cultures (Day 5) stained intensely for Pdx1 protein expression indicating. A down-regulation in expression was observed between day 5 and day 9 cultures. The majority of cells in day 9 cultures (RA_AA, RA_BTC and CO_9d) exhibited moderate - weak staining for Pdx1, with exception to the RA_SB culture. An intense distinct staining pattern within individual EBs was observed within the RA_SB culture.

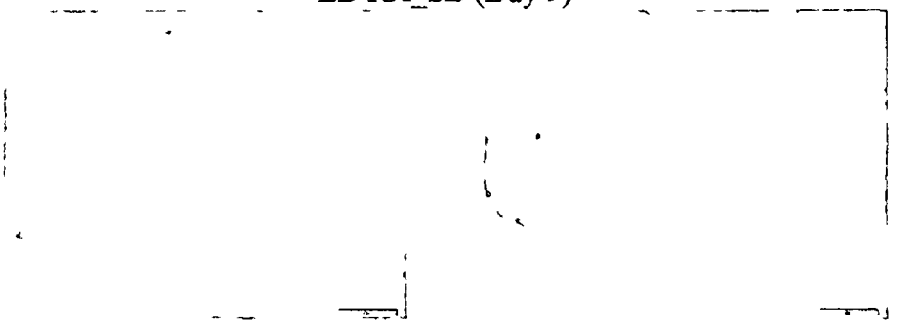
Figure 3.2.8 (I) Immunohistochemistry analysis of C-peptide 1 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



RA_AA (Day 9)

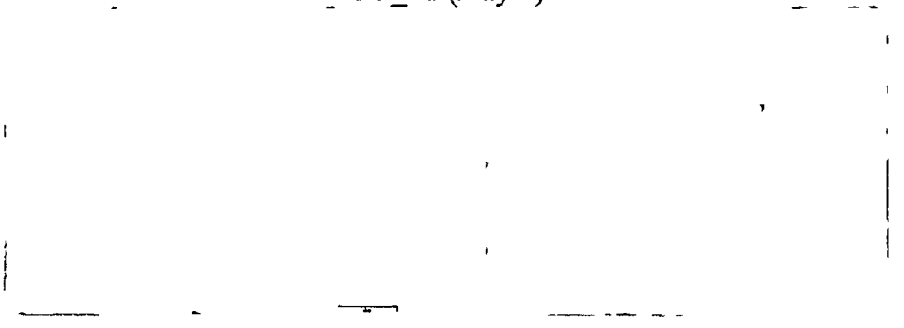


EB RA_SB (Day 9)



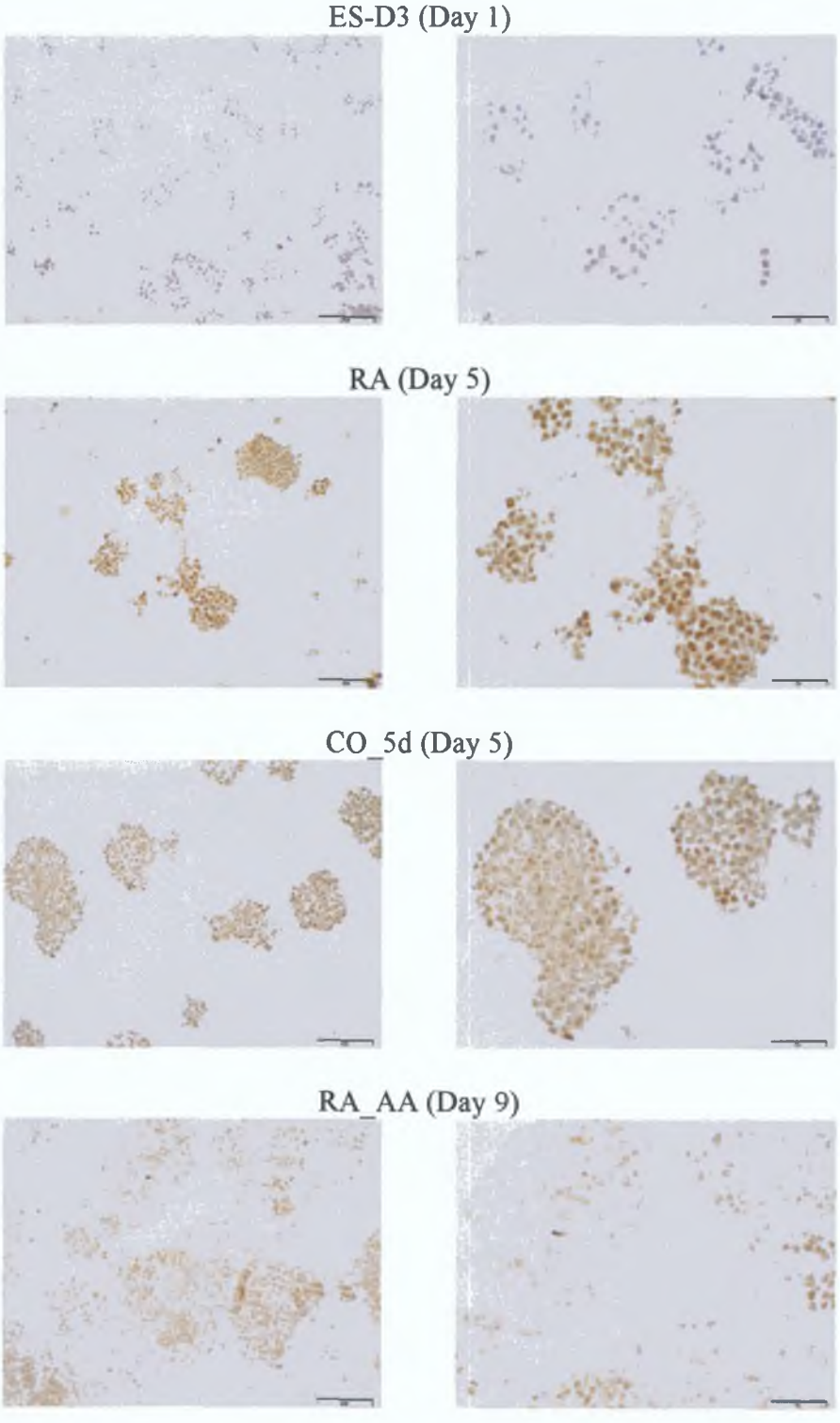
RA_BTC
Not Determined

CO_9d (Day 9)

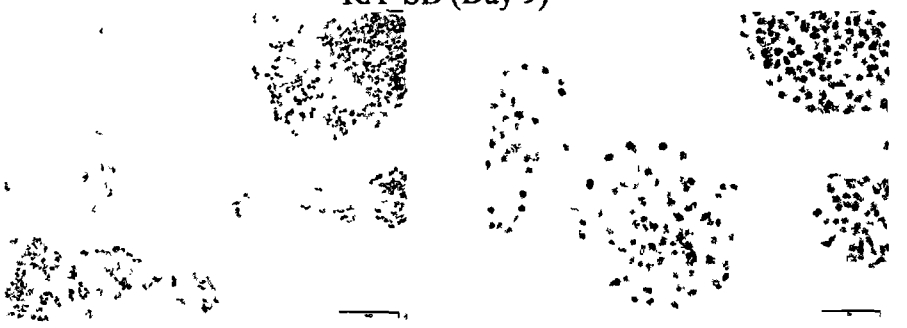


C-peptide 1 protein expression was not detected in ES-D3 cells Subpopulations of the RA and CO_5d culture stained positive for C-peptide 1 indicating induced expression of the c-peptide 1 protein within these cultures The weak staining pattern observed in day 9 cultures (RA_AA, RA_SB, RA_BTC and CO_9d) indicated that stage 2 differentiation resulted in a down-regulation of C-peptide 1 expression

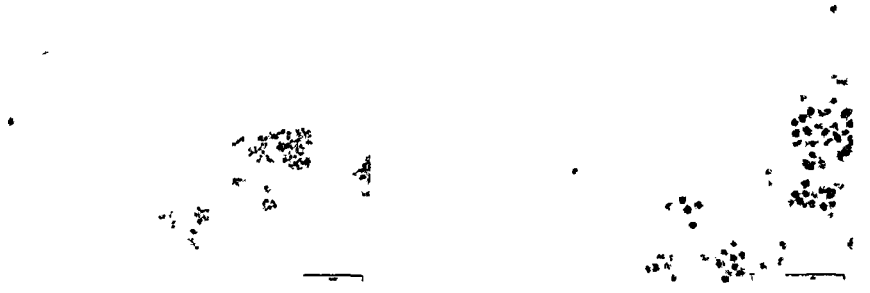
Figure 3.2.8 (J) Immunohistochemistry analysis of C-peptide 2 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 µm (20X). Images on right, scale bar = 50 µm (40X).



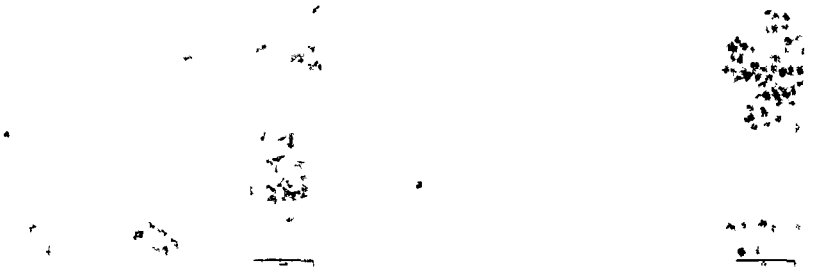
RA_SB (Day 9)



RA_BTC (Day 9)

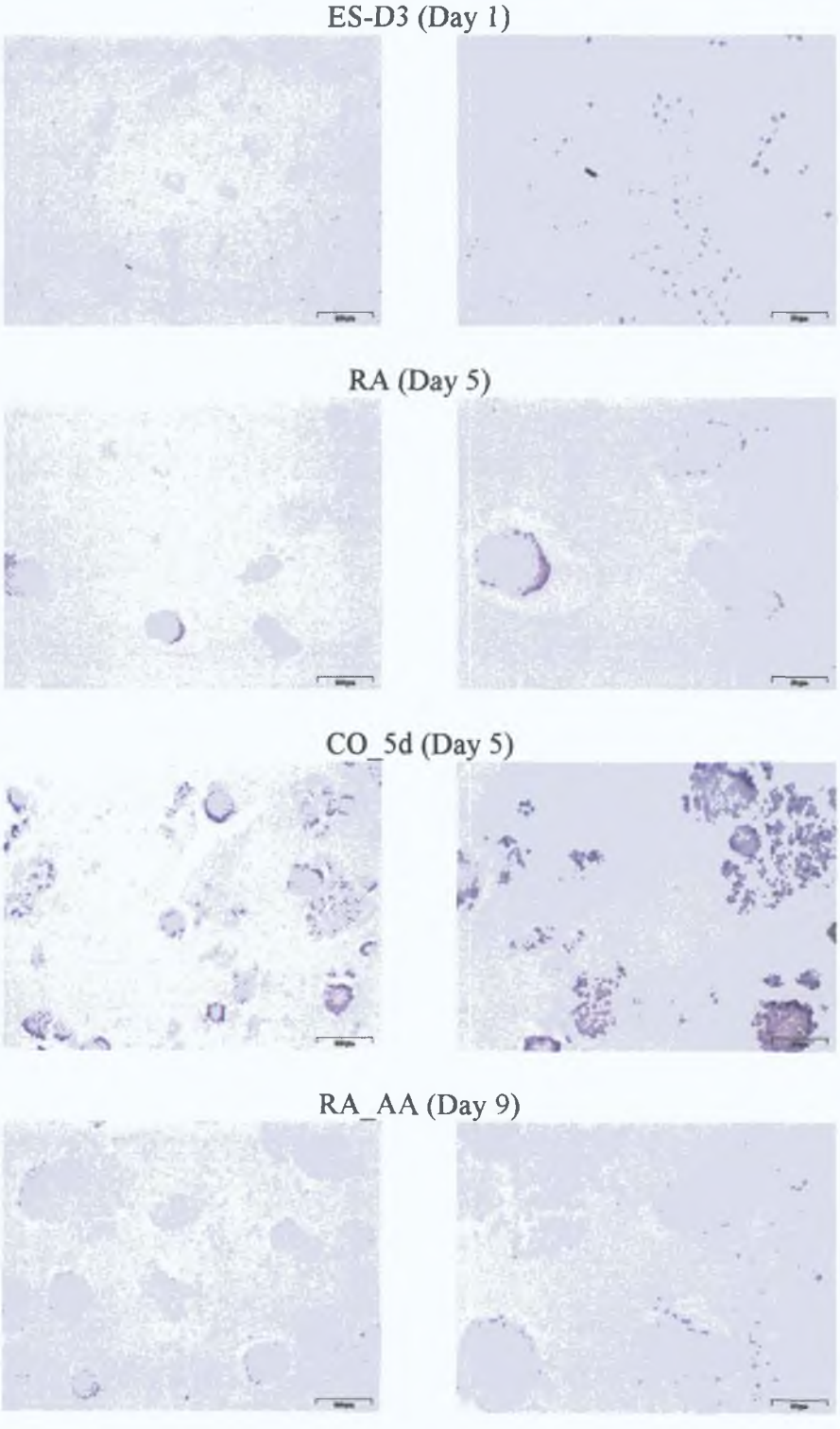


CO_9d (Day 9)

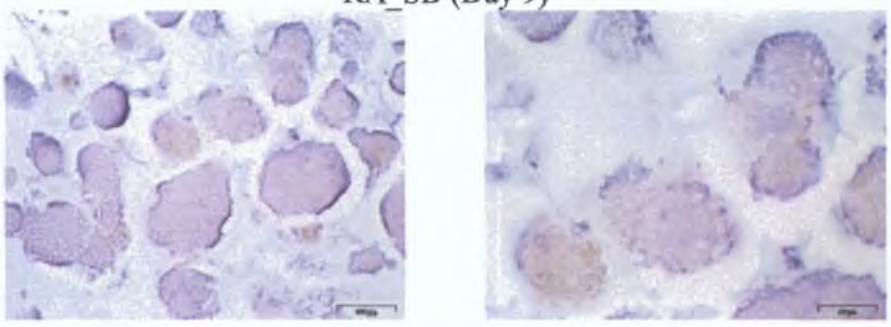


C-peptide 2 protein expression was not detected in ES-D3 cells The appearance of stained cells in the RA and CO_5d cultures suggested an up-regulation of C-peptide 2 expression in these cultures The intensity of staining in the RA culture was stronger that that of the staining in the CO_5d culture Stage 2 differentiation appeared to down-regulate C-peptide 2 expression However, a strong staining intensity observed in subpopulations of the RA_SB and RA_BTC cultures indicated that these isolated cells still maintained a high level of C-peptide 2 expression

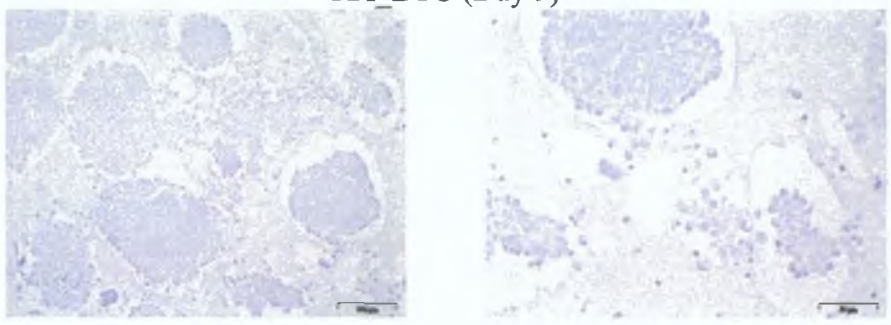
Figure 3.2.8 (K) Immunohistochemistry analysis of insulin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



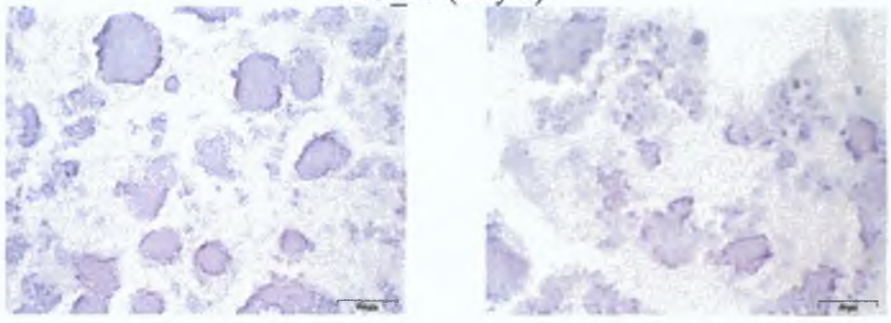
RA_SB (Day 9)



RA_BTC (Day 9)

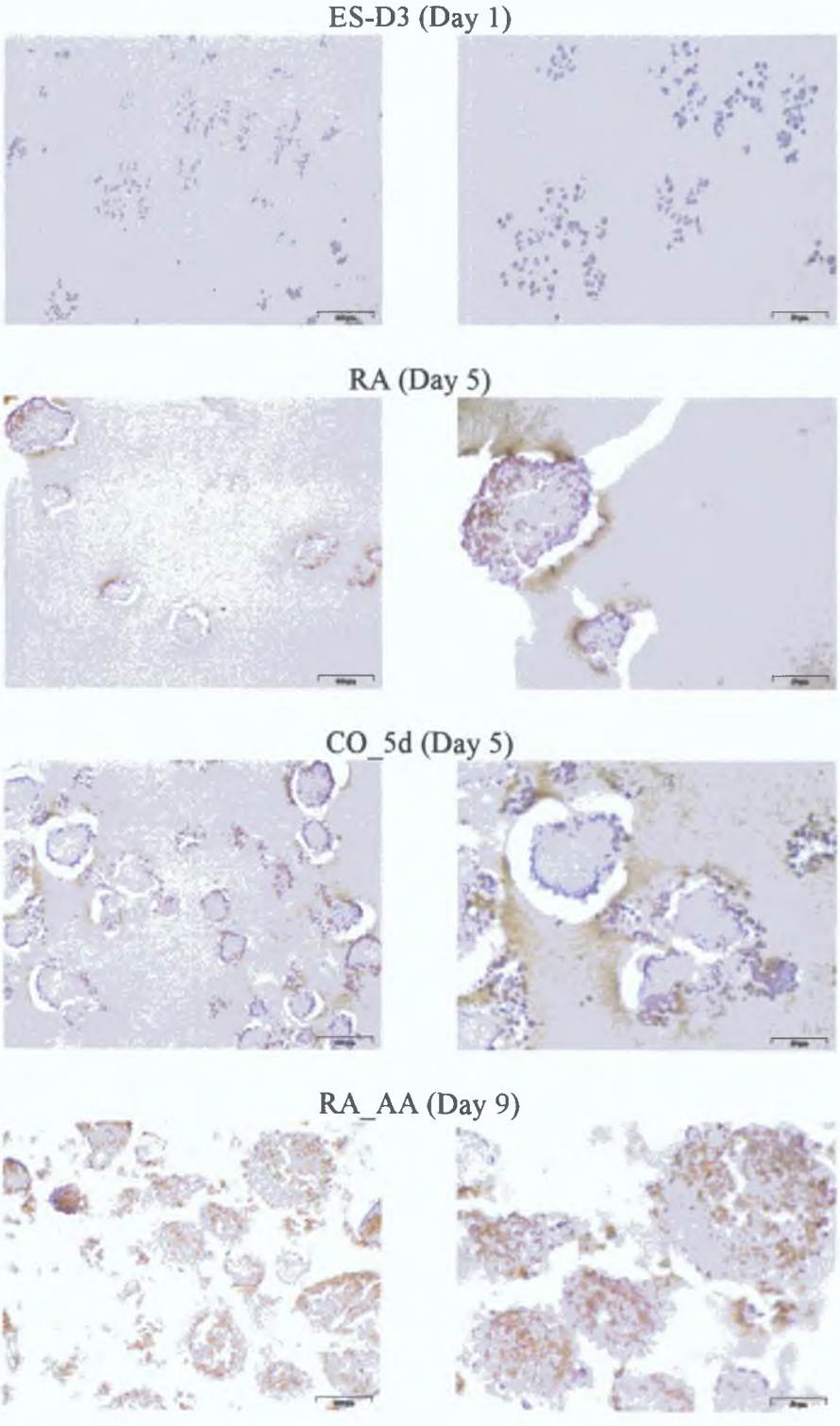


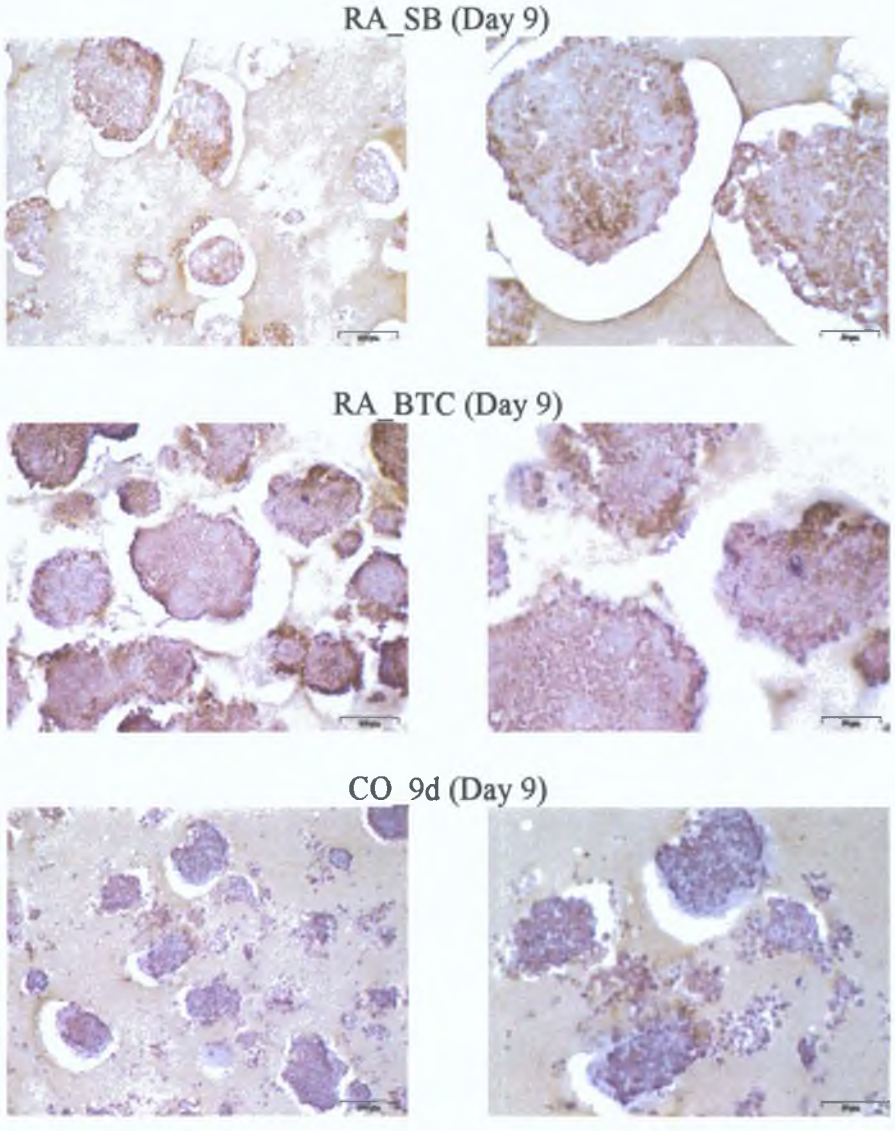
CO_5d (Day 9)



Insulin protein was not detected in the ES-D3 cells or in ES-derived cultures with exception to the RA_SB culture. A subpopulation of the RA_SB culture stained weakly for insulin indicating induced expression of the insulin protein in isolated cells within this culture.

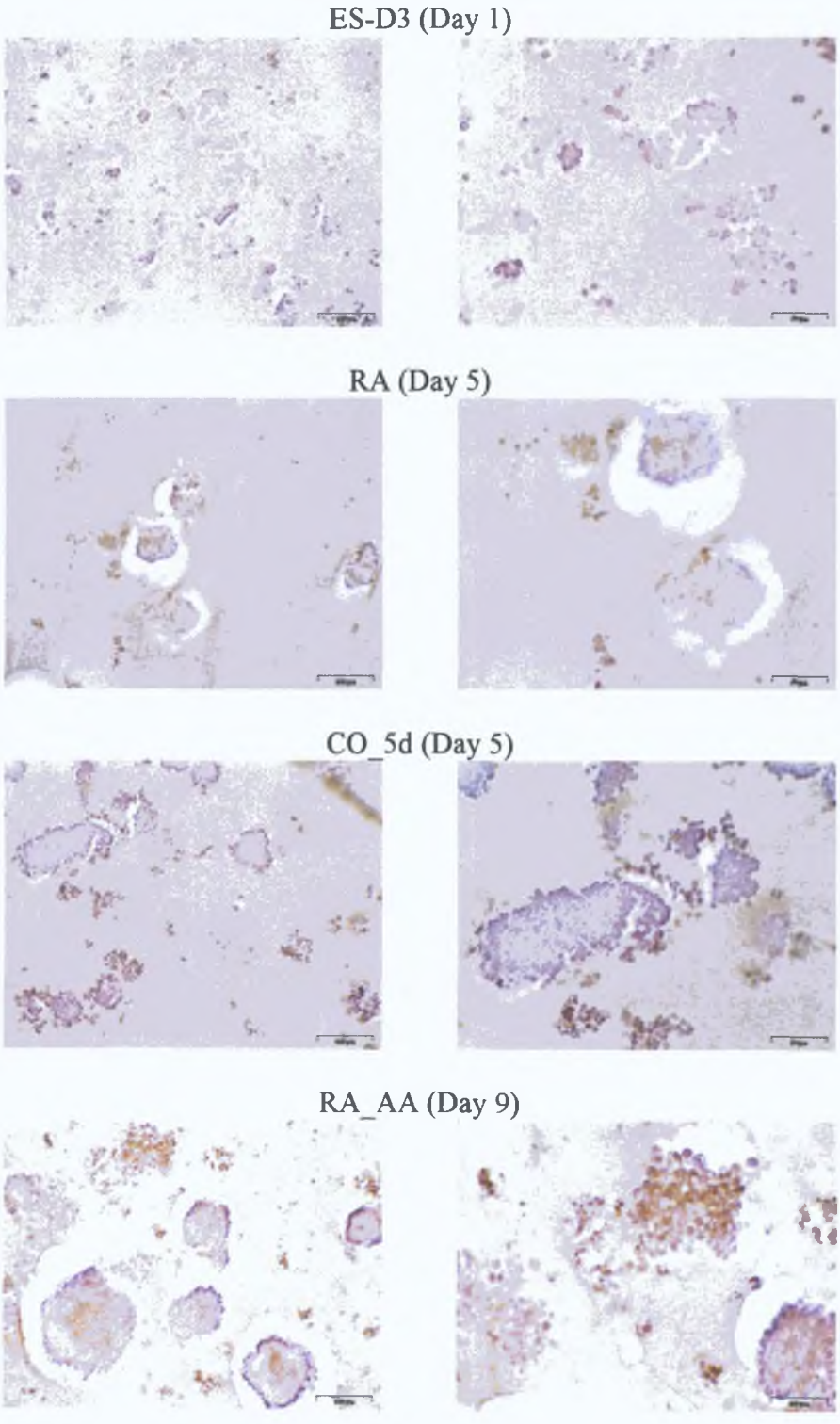
Figure 3.2.8 (L) Immunohistochemistry analysis of glut2 protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).



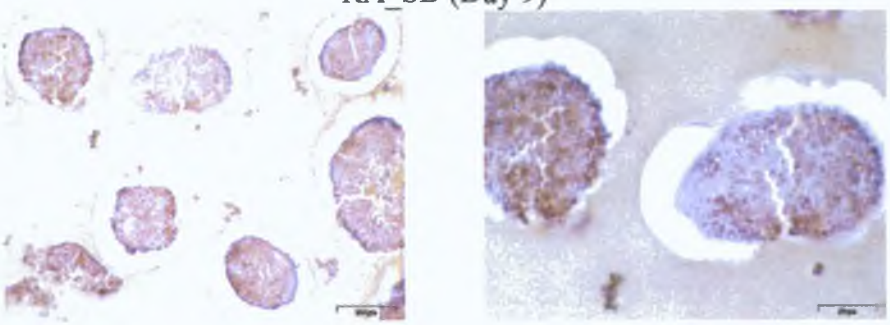


Glut2 protein expression was not detected in ES-D3 cells. The appearance of weak to moderately stained cells in the day 5 cultures (RA and CO_5d) indicated that glut2 protein expression was induced at a low level within these cultures. There was a noticeable increase in the percentage and intensity of staining within the RA_SB culture suggesting an up-regulation in glut2 protein expression between the RA culture at day 5 and the RA_SB culture at day 9.

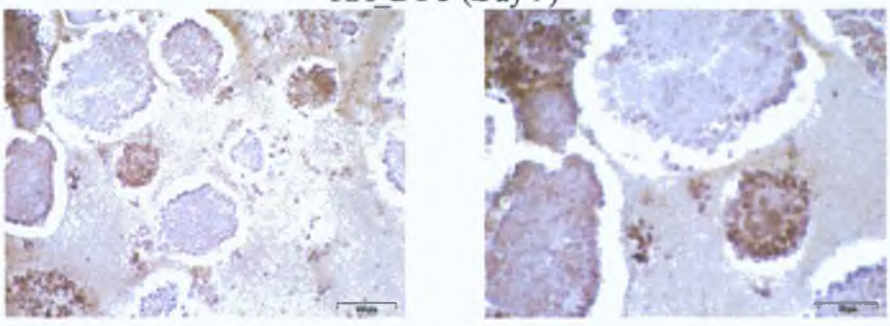
Figure 3.2.8 (M) Immunohistochemistry analysis of somatostatin protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 µm (20X). Images on right, scale bar = 50 µm (40X).



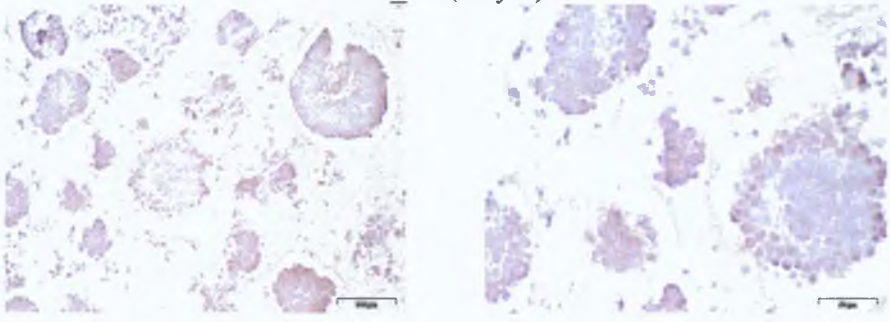
RA_SB (Day 9)



RA_BTC (Day 9)

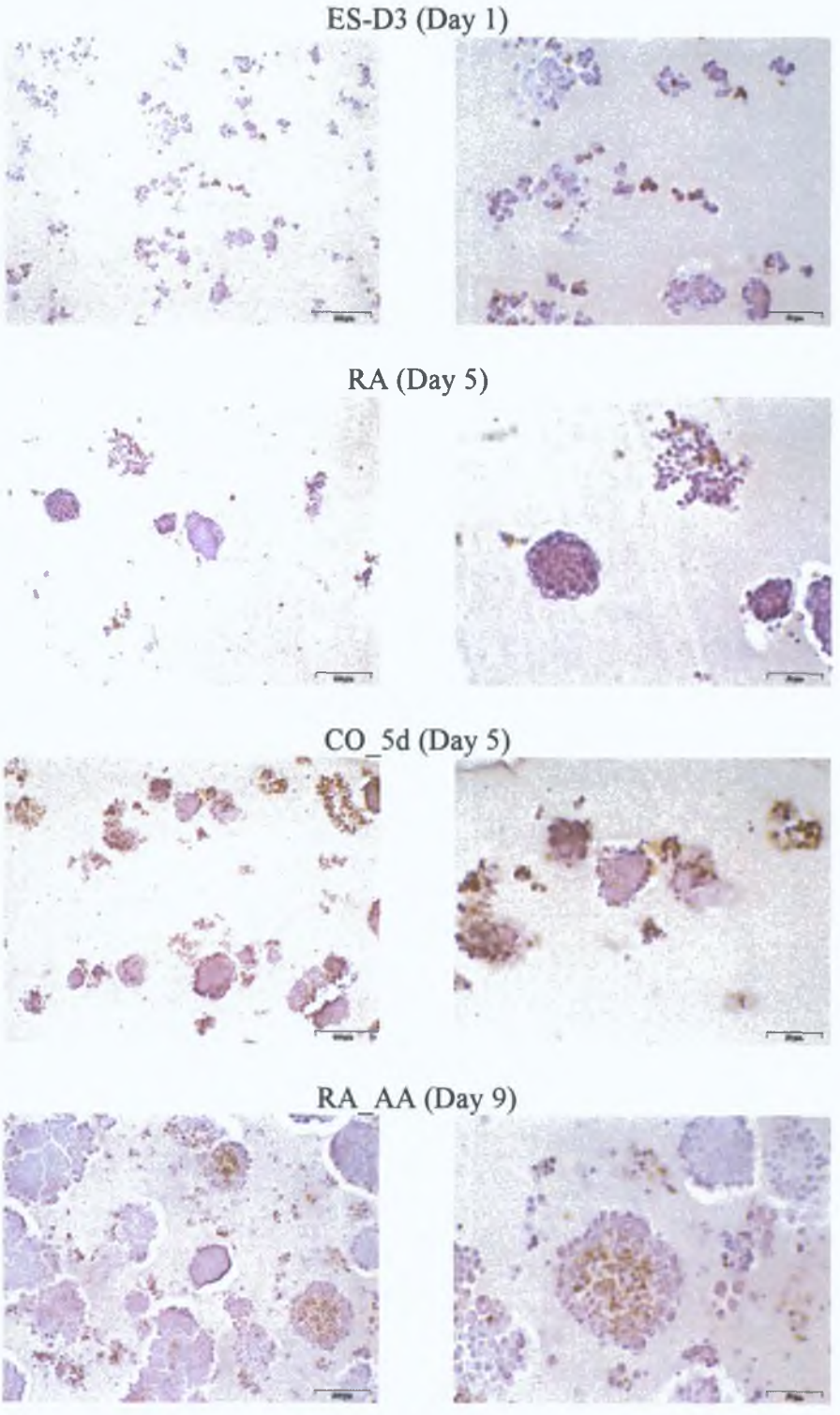


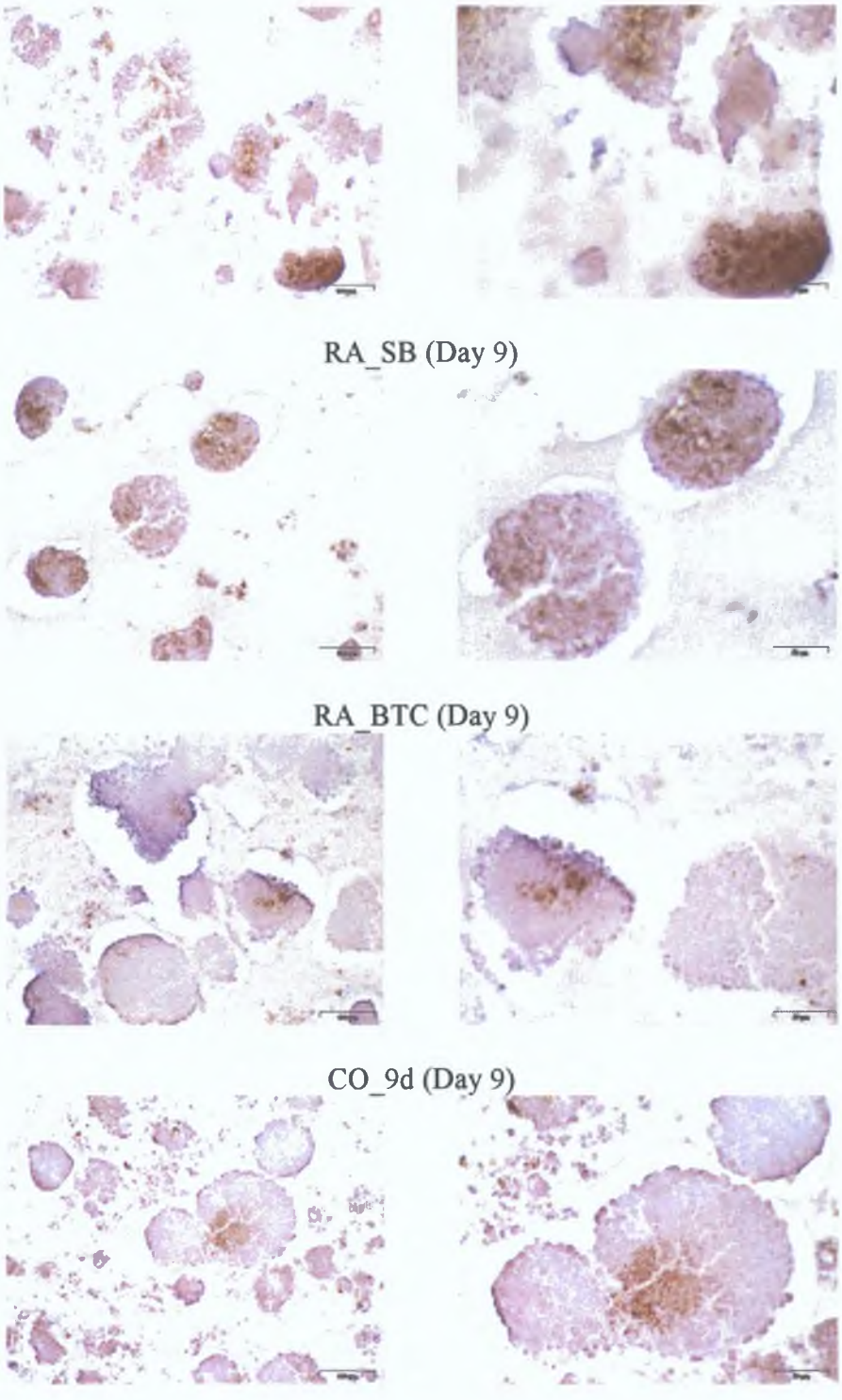
CO_9d (Day 9)



Somatostatin protein expression was not detected in ES-D3 cells. The appearance of stained cells in the 5 day RA culture and to a lesser extent in the CO_5d culture indicated that somatostatin expression had been induced within these cultures. Subsequent differentiation in the presence of AA, SB and BTC increased the percentage and intensity of staining suggesting an up-regulation in somatostatin expression between the RA culture at day 5 and the treated cultures (RA-AA, RA_SB, RA_BTC) at day 9. The spontaneously-formed CO_ 9d culture does not appear to express somatostatin.

Figure 3.2.8 (N) Immunohistochemistry analysis of PP protein expression in ES-D3 and ES-derived cultures. The images shown are representative of repeat 2 in Table 3.2.4. Images on left, scale bar = 100 μm (20X). Images on right, scale bar = 50 μm (40X).





The detection of a small subpopulation of moderately stained cells in the ES-D3 culture indicated that PP protein expression was expressed within this culture. All differentiated ES-derived cultures contained subpopulations of moderately - intensely stained cells suggesting an induced up-regulation in protein expression upon differentiation.

3 2 5 Immunofluorescence Analysis of Insulin/C-peptide Protein Expression in the RA_SB Culture

The 2-stage RA_SB differentiation protocol resulted in the derivation of insulin expressing cells (Section 3 2 4) To confirm these results we preformed immunofluorescence analysis on C-peptide and insulin expression within this culture At day 9 the entire RA_SB culture was dissociated and the resulting cell suspension was plated onto poly-L-ornithine for immunofluoresence analysis Biological controls used in this study were ES-D3 cells and dissociated RA, CO_5d and CO_9d cultures MIN6 cells were used as a positive control

Initial screening of samples was preformed using a standard fluorescent microscope (Figure 3 2 9) MIN6 cells were positive for insulin (Figure 3 2 9 A) ES-D3, CO_5d, RA and CO_9d cultures were negative for insulin (Figure 3 2 9 B – E) A small number of insulin-positive cells were detected in the RA_SB culture (Figure 3 2 9 F - I) Insulin/C-peptide co-expression in RA_SB cells versus CO_9d cells was analysed by LSCM (Figure 3 2 10) Strong insulin staining was detected in the RA_SB cells (Figure 3 2 10 A & B) but not in CO_9d cells (Figure 3 2 10 C & D), whereas C-peptide was detected in both the RA_SB cells and CO_9d cells (Figure 3 2 10 A – D) Insulin expression in the RA_SB cells did not co-localise with C-peptide 1 or C-peptide 2 expression (Figure 3 2 10 A & B), indicating a problem with C-peptide antibody specificity (previously addressed in Section 3 1)

These results verify previous findings reporting the derivation of insulin-expressing cells using the 2-stage RA_SB differentiation protocol (Section 3 2 4) These results also support previous findings questioning the specificity of the C-peptide antibodies used in *immunofluorescent* techniques (Section 3 1)

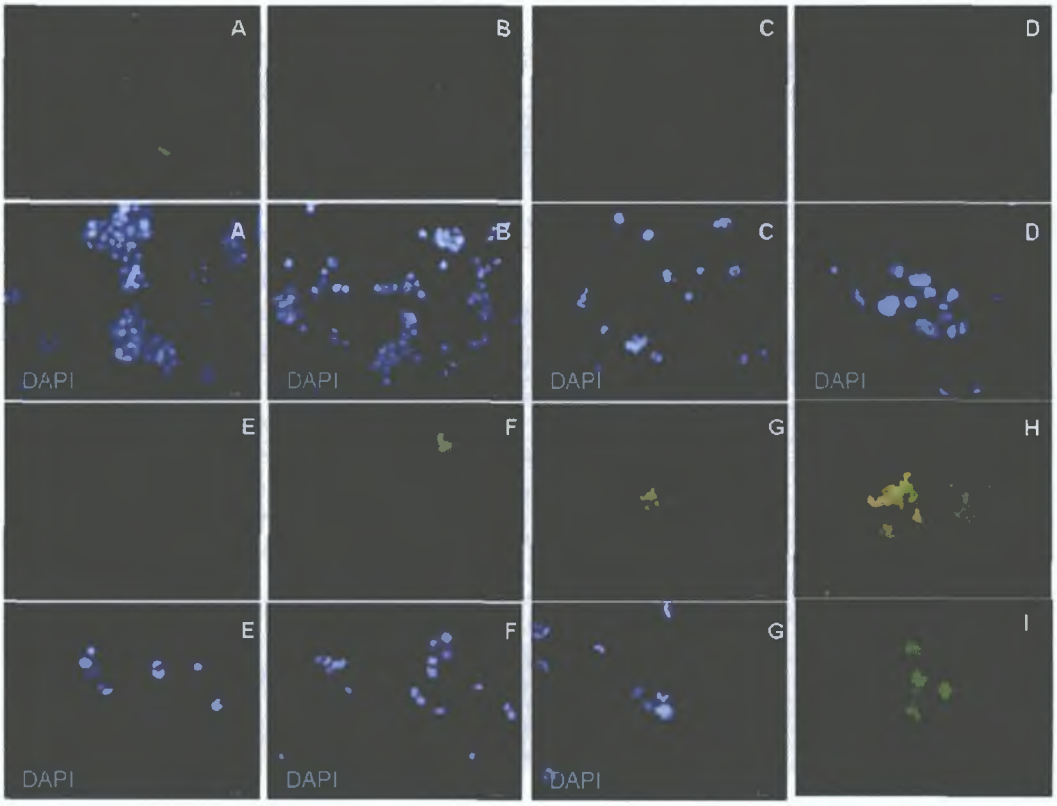
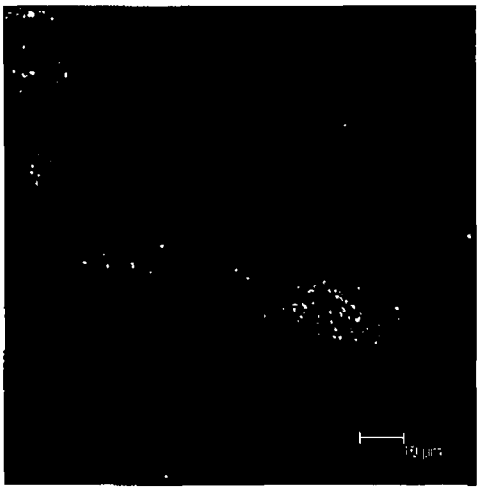
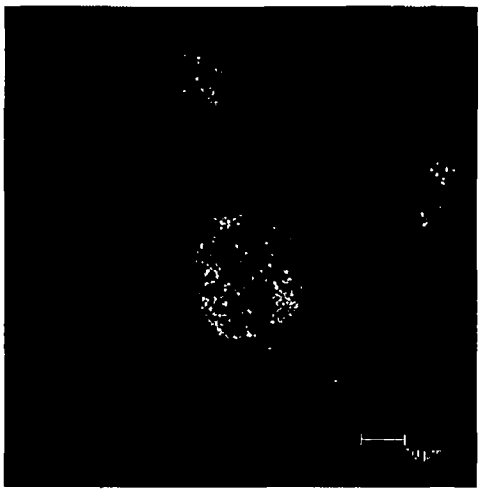


Figure 3.2.9 Insulin expression analysis using a fluorescence microscope. MIN6 (A) were positive for insulin: ES-D3 (B), CO_5d cells (C), RA-treated cells (D) and CO_9d cells (E) were negative for insulin. A small percentage of RA_SB cells (F & G) were positive for insulin. Enlarged view of the RA_SB insulin-expressing cells (H & I). Scale bar = 50 μm (40X). Insulin, green; DAPI nuclear stain, blue.

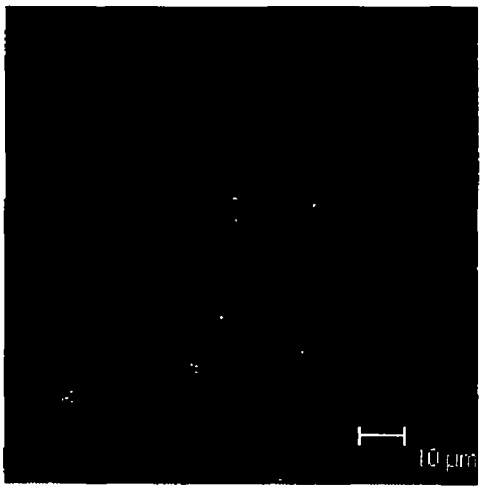
(A) RA_SB
Insulin (green), C-peptide 1(red),
DAPI nuclear stain (blue) &
C-peptide 1 overlaying DAPI (pink)



(B) RA_SB
Insulin (green), C-peptide 2 (red),
DAPI nuclear stain (blue) &
C-peptide 2 overlaying DAPI (pink)



(C) CO_9d
Insulin (green), C-peptide 1 (red),
DAPI nuclear stain (blue) &
C-peptide 1 overlaying DAPI (pink)



(D) CO_9d
Insulin (green), C-peptide 2 (red),
DAPI nuclear stain (blue) &
C-peptide 2 overlaying DAPI (pink)

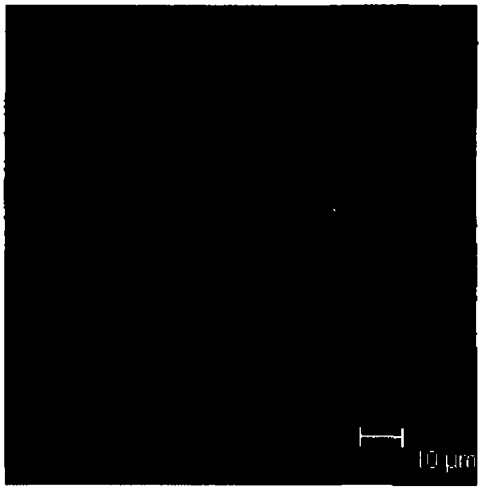


Figure 3 2 10 Insulin/C-peptide co-localisation analysis of RA_SB cells and CO_9d cells using LSCM RA_SB cells are positive for insulin (A & B), C-peptide 1 (A) and C-peptide 2 (B) Insulin expression does not co-localise with C-peptide expression CO_9d cells are negative for insulin (C & D) but positive for C-peptide 1 (C) and C-peptide 2 (D) Scale bar = 10 µm

3.2.6 Insulin ELISA on Lysed Cells

The overall aim of the 2-stage differentiation studies was to derive pancreatic endocrine cell types, in particular insulin-producing cells, from undifferentiated ES-D3 cells Insulin transcript (Section 3 2 3) and protein expression (Section 3 2 4) were previously detected in differentiated RA_SB cells Insulin ELISAs were preformed on lysates from both ES-D3 and ES-derived cell cultures to quantify insulin expression

Insulin content was assayed using a Mercodia mouse insulin ELISA All insulin readings were within the lower range of the standard curve on the ELISA plate and were considered non-specific background readings Insulin was not detected in any of the differentiated samples using the ELISA kit It was previously demonstrated by both immunohistochemistry (Section 3 2 4) and immunofluorescence (Section 3 2 5) analysis that only a small isolated number of cells within the entire RA_SB population contained insulin Therefore, the quantity of insulin per µl lysate may have been below the range of detection of the ELISA

3 2 7 Growth Assay on the RA_SB and the CO_9d Culture

The RA_SB differentiation protocol yielded interesting results in regard to the expression of pancreatic markers (Section 3 2 3, Section 3 2 4 and Section 3 2 5) However, it had been observed that the cell yield was extremely low in the RA_SB culture compared to the other ES-derived cultures A growth assay was preformed to ascertain the effects of the extra-cellular factors i e RA and SB on cell proliferation (Figure 3 2 11) The addition of RA to the culture medium resulted in a slight decrease in proliferation in the RA culture compared to the CO_5d culture The secondary treatment of RA-treated EBs with SB significantly reduced cell number (RA_SB) compared to spontaneously differentiated control EBs (CO_9d) at the same time-point The cell yield from the RA_SB culture was approx 7 fold lower than the cell yield attained from the CO_9d culture

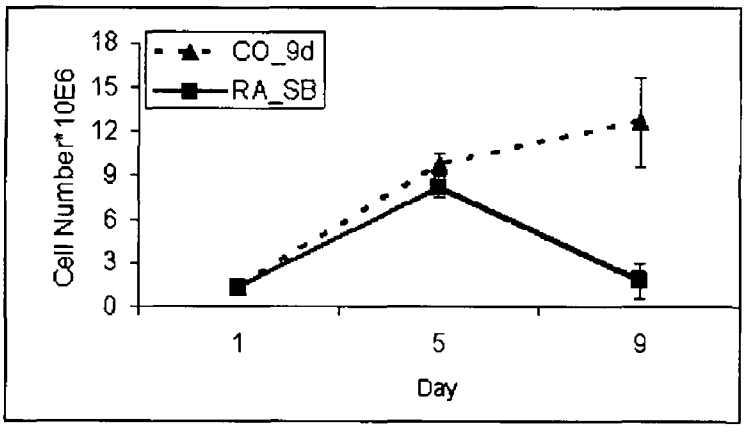


Figure 3.2.11 Growth curves comparing the cell counts from the CO_9d culture and the RA_SB culture on day 1, day 5 and day 9 of the 2-stage differentiation protocol Data presented (mean ± SD) is from two independent experiments

3 2 8 Time Course Assay on Insulin Transcript Expression in the RA_SB Culture

The derivation of insulin expressing cell types using the 2-stage RA_SB differentiation protocol was previously shown in Section 3 2 4 and Section 3 2 5 It has also been shown that very low cell yields are attained from this protocol (Section 3 2 7) An alteration in protocol was necessary to improve cell yields and in turn increase the number of ES-derived insulin-expressing cells However, before altering the protocol it was necessary to identify the time-points at which insulin transcript expression is at its highest

A time course assay (2-day intervals) of insulin I and insulin II transcript expression was preformed on the RA_SB culture to determine at which point over the course of the differentiation protocol induction/up-regulation and down-regulation of the insulin transcripts occur After the 9 day RA_SB differentiation protocol, the RA_SB culture was removed from supplemented medium and re-fed with normal ES-D3 medium (-LIF) The culture was maintained under these conditions for a further 4 days Samples were taken at 2-day intervals i e day 11 and day 13 to determine if the high transcript expression observed in RA_SB culture is maintained after removal from extra-cellular factors

The qPCR results demonstrated that both insulin I and II transcripts were up-regulated in cultures at day 7, day 9, day 11 and day 13 (Figure 3 2 12) Highest levels of expression were observed at day 9 and day 11 Shortening the 9 day RA_SB protocol or withdrawing the cells from treatment agents to recover, may allow for an improved cell yield whilst maintaining a high insulin transcript expression Further analysis was carried out on alternative RA_SB protocols with a view to improving cell yields (Section 3 2 9)

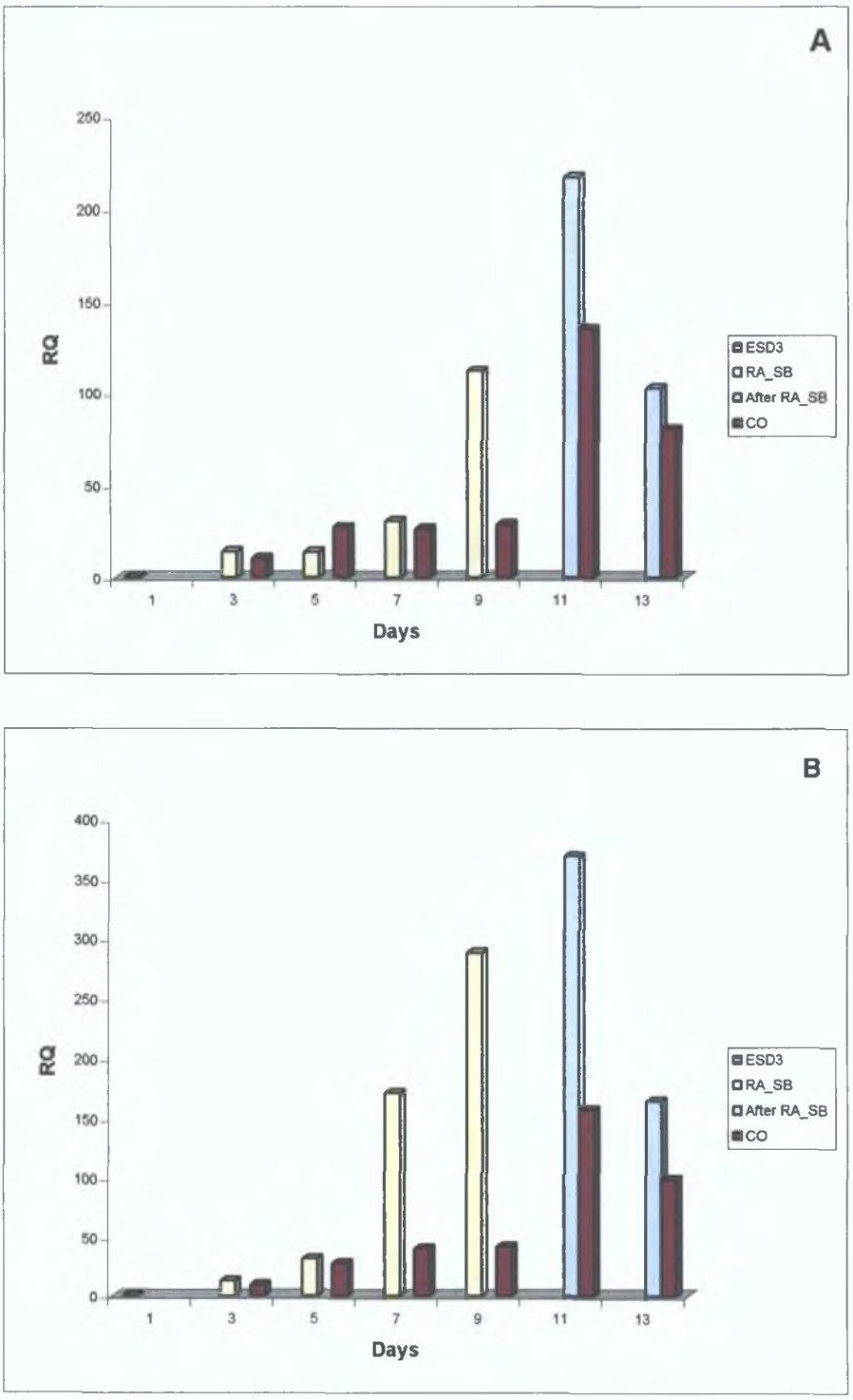


Figure 3.2.12 qPCR analysis of insulin I (A) and insulin II (B) transcript expression in ES-derived cells at 2-day intervals over the 9 day RA_SB differentiation protocol and at 2-day intervals after removal from the differentiation agents. CO, Control.

3 2 9 Investigation of Alternative RA_SB Differentiation Protocols

The 2-stage RA_SB differentiation protocol yielded interesting results in terms of pancreatic differentiation (Section 3 2 3 – 3 2 5), however the cell yield from this treatment was very low (Section 3 2 7) The following adjustments were tested on the original RA_SB protocol in order to improve the cell yield

(a) shortening stage 2 of the differentiation protocol i e reducing the exposure time to SB (Section 3 2 8)
(b) omitting RA from stage 1 of the differentiation protocol
(c) culturing RA_SB cells in nicotinamide after removal from the protocol
(d) introducing an alternative to SB in stage 2 of the differentiation protocol, PA and TSA are known to have similar effects as SB

The alternative RA_SB differentiation protocols are outlined in Figure 2 3 2 The resulting ES-derived populations were analysed for protein content (Figure 3 2 13) Protein content was representative of cell yields within cultures Insulin I and insulin II transcript expression was also analysed in the ES-derived populations (Figure 3 2 14)

Cultures exposed to SB only (SB_EB) had the highest amount of protein next to controls All other alternative treatments yielded similar amounts of protein to that of the RA_SB treatment (Figure 3 2 13) RA_SB cultures expressed high levels of insulin I transcript at day 9 whereas insulin II transcript expression was high at both day 7 and day 9 (Figure 3 2 14) RA_SB cultures which were removed from treatment at day 7 and transferred to media containing NIC exhibited the highest levels of insulin I transcript expression (Figure 3 2 14 A) The SB_EB culture expressed the highest level of insulin II transcript (Figure 3 2 14 B)

Ideally we had hoped that one of the alternative protocols would allow for improved cell yields as well as high levels of both insulin I and insulin II expression None of the protocols tested fulfilled the criteria The most promising protocol tested was the SB_EB protocol This protocol allowed for improved cell yields as well as high insulin II transcript expression

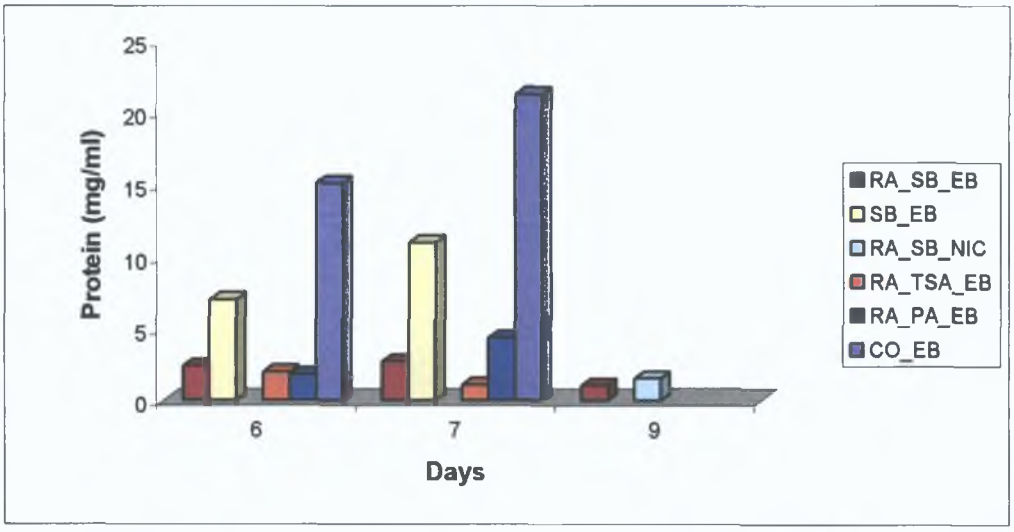


Figure 3.2.13 Data is representative of the protein derived from alternative RA_SB differentiation protocols outlined in Figure 2.3.2. Cultures were assayed for protein content at day 6, day 7 and day 9.

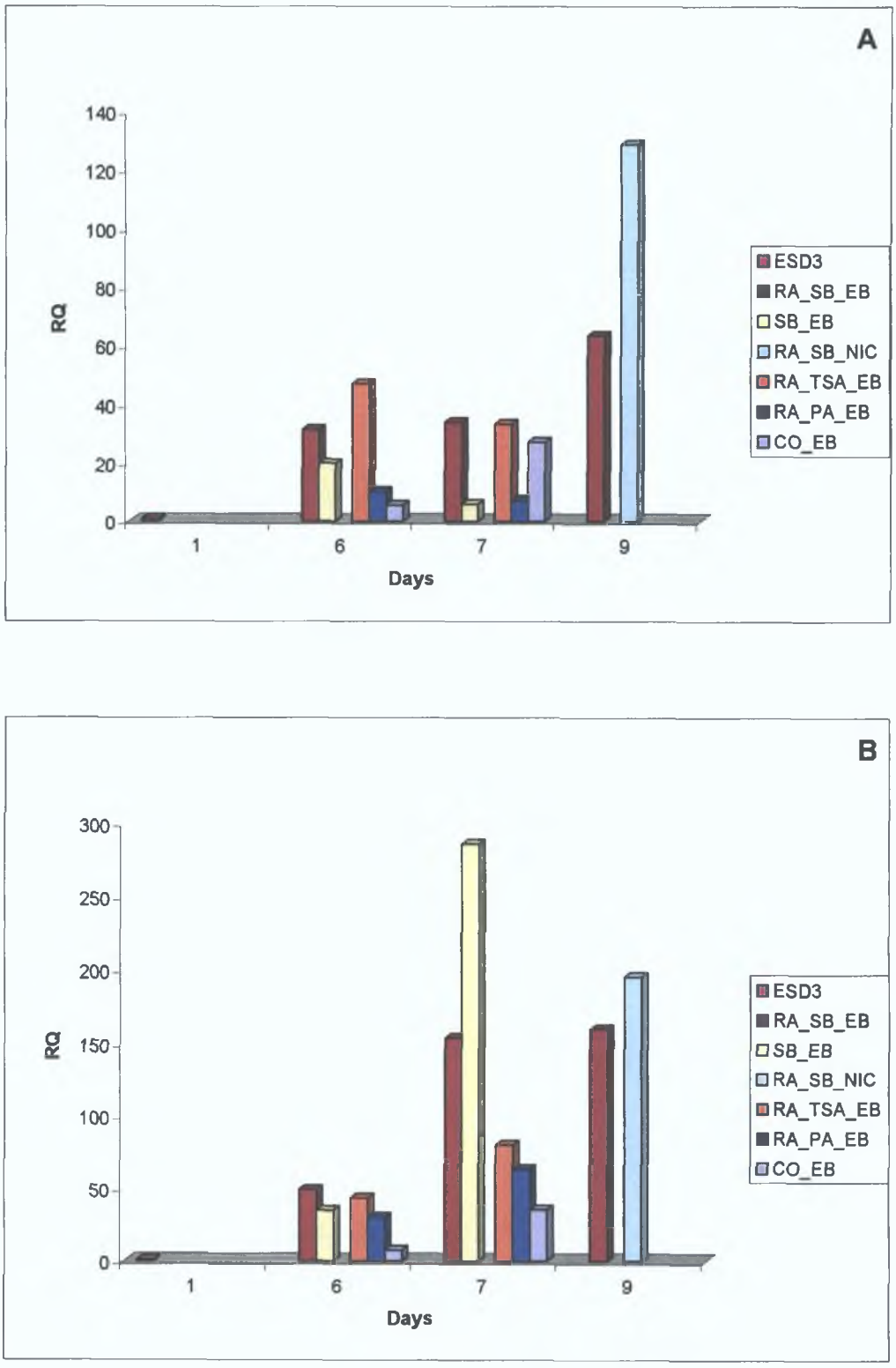


Figure 3.2.14 qPCR analysis of insulin I (A) and insulin II (B) transcript expression in cultures derived from alternative RA_SB differentiation protocols outlined in Figure 2.3.2

3 3 MICROARRAY STUDY COMPARING GENE EXPRESSION IN LOW PASSAGE MIN6 (GLUCOSE RESPONSIVE) TO HIGH PASSAGE MIN6 (GLUCOSE-NON-RESPONSIVE)

MIN6 cells have been used throughout this thesis as a positive control in the majority of ES experiments i e Competitive RIA, insulin secretion assays and RT-PCR analysis of β cell specific markers Previous studies have indicated that the glucose stimulated insulin secretion (GSIS) phenotype is relatively unstable in long-term culture (Section 1 3 4 2 3) The initial study performed in our laboratory, found that long-term culture was associated with changes in morphology, growth rate, alkaline phosphatase expression, as well as loss of GSIS (Gammell, 2002, O Driscoll et al, 2004) We subsequently preformed three separate expression microarray experiments on glucose responsive low passage MIN6 (P18) and glucose non-responsive high passage MIN6 (P40) A comparison of the differential gene expression between the two populations was used to elucidate the molecular mechanisms involved in loss of GSIS function in high passage MIN6 β cells

Loss/reduced levels in high passage cells of transcripts associated with the mature β cell coincided with increased expression of neuron-/glia- mRNAs These findings suggested that, with long-term culture, MIN6 cells may revert to a poorly differentiated precursor-like cell type This observation is supported by earlier findings demonstrating an increase in proliferation and an increase in expression of the embryonic stem cell marker, alkaline phosphatase in high passage MIN6 cells This de-differentiation model may be useful for the identification of crucial β cell markers and may also provide information relevant to the derivation of β-like cell types from ES cells It is not yet clear whether the functional de-differentiation of the MIN6 population is due to (a) de-differentiation of the population as a whole or (b) overgrowth by a faster growing poorly-differentiated sub-population

3 3 1 Morphological Changes Associated with Continuous Culture of MIN6 Cells

Continuous culture of MIN6 cells resulted in an alteration in the typical MIN6 morphology and growth pattern Low passage MIN6 cells grow in tight, multi-layered localised clumps (Figure 3 3 1 A) As the passage number was increased, the cells no longer associated into clumps and were found to form even monolayers of stretched cells, some with extended protrusions resembling neurites (Figure 3 3 1 B)

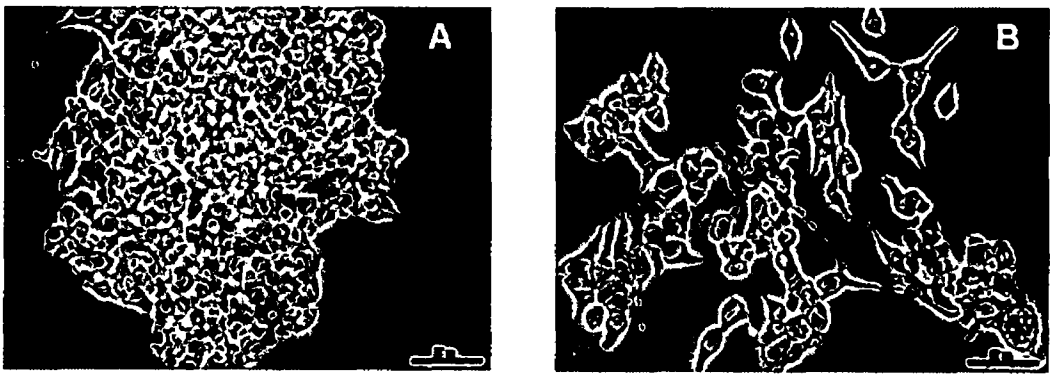


Figure 3 3 1 Low passage MIN6 cells associate into rounded multi-layered clumps (A) High passage MIN6 cells tend to form a monolayer and no longer form discrete colonies (B) In this experiment MIN6 cells were grown for 7 days with 1 re-feed Scale bar =100 μM (A & B = 40X)

3 3 2 Proliferation Assays on Low and High Passage MIN6

The proliferation rates (doubling times) of the low and high passage MIN6 cells were determined by monitoring their growth rates over consecutive 24 hour periods Growth curves indicated that the rate of proliferation in high passage MIN6 cells was approximately 2-fold greater than that of low passage MIN6 cells Continuous sub-culturing resulted in an increase in proliferation rate (Figure 3 3 2)

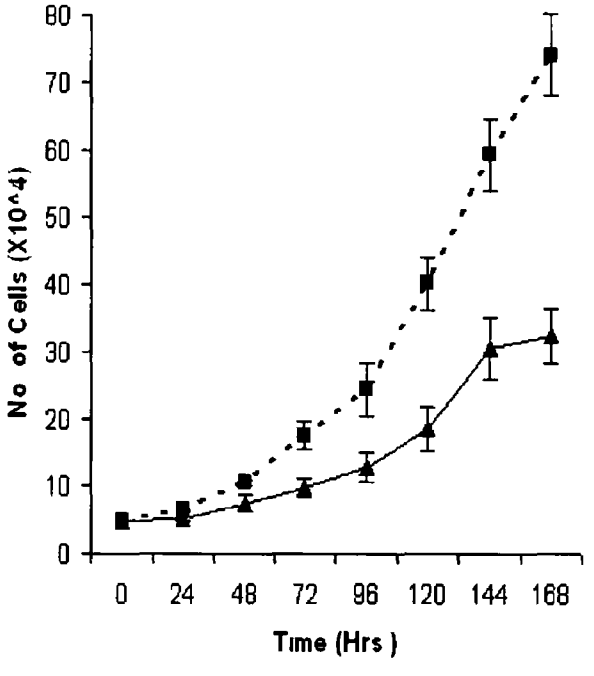


Figure 3 3 2 Proliferation data comparing MIN-6(L) (——) and (H) (-------) Cells were cultured in 24 well plates and 3 wells were counted daily The experiment was repeated on ≥ 3 independent cell populations The data presented equals mean cell number ± SD

3 3 3 Alkaline Phosphatase Expression in Low and High Passage MIN6

AP activity is a characteristic of undifferentiated stem cells and can be seen to decrease following differentiation of the ES cells (Lumelsky et al, 2001, Prelle et al, 2002, Berill et al, 2004) Using BCIP as a substrate for alkaline phosphatase, Figure 3 3 3 (A & C) indicates that the extent of AP staining is much more widespread in the high passage MIN6 population (Figure 3 3 3 C) than in the low passage MIN6 population (Figure 3 3 3 A) At higher magnifications the intensity of the staining apparently also increased in high passage MIN6 cells (Figure 3 3 3 B & D)

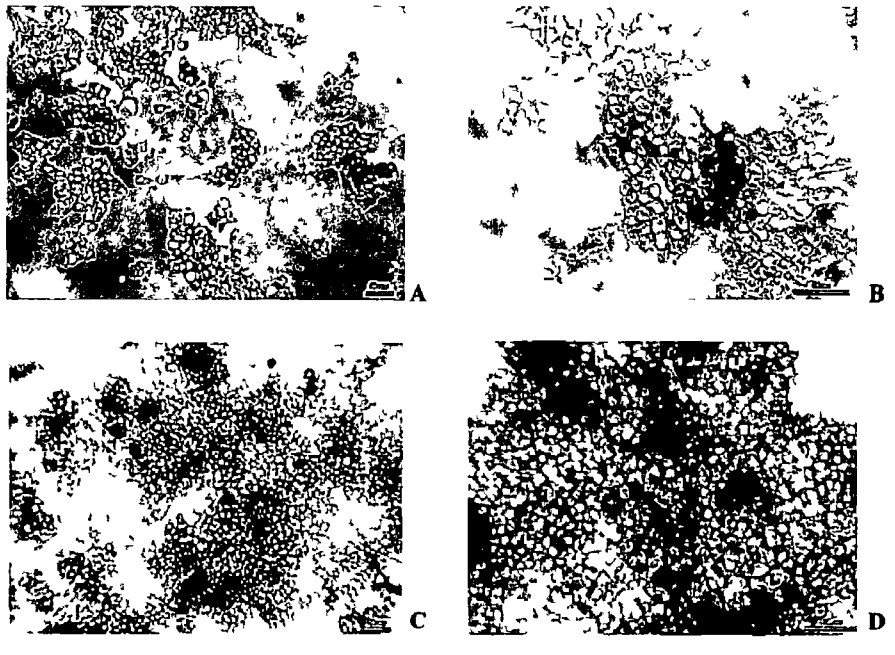


Figure 3 3 3 MIN-6 cells stained for AP expression Low passage MIN6 cells (A & B) were found to have much less staining than high passage MIN6 cells (C & D) Scale bar = 50μm (See Gammell, 2002) (A & C = 10X) (B & D = 20X)

3 3 4 GSIS ELISA on Low and High passage MIN6

Each sample set (low and high MIN6) were analysed by GSIS ELISA prior to going onto the array The ELISA confirmed that low passage MIN6 cells demonstrated GSIS whereas high passage MIN6 cells had lost the ability to do so (Figure 3 3 4)

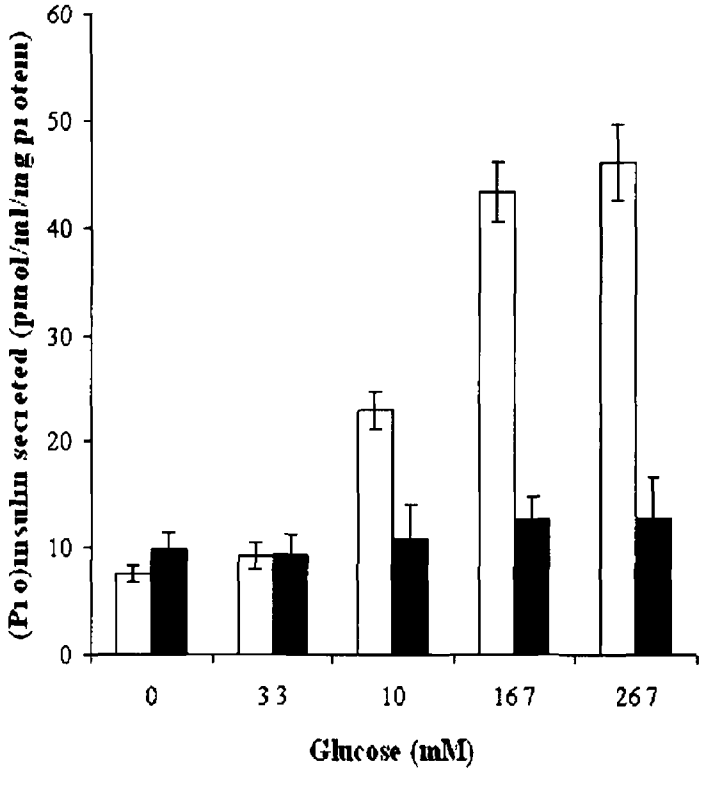


Figure 3.3 4 (Pro)insulin secretion profiles with increasing glucose concentrations in low (white bars) and high passage MIN6 cells (black bars) Results are presented as pmol/ml/mg protein to allow for the increased growth rate of high passage MIN6 cells Results represent means ± SD from three experiments

3.3 5 Analysis of Serially Passaged MIN6 cells by Microarray, Bioinformatics and qPCR

The results presented in Section 3 3 1 – Section 3 3 4 clearly indicate that there is a change in the properties of MIN6 cells after long-term culture, most notably the loss of GSIS Gene expression changes associated with the loss of GSIS were investigated using microarray, bioinformatics and qPCR technologies Three separate microarray experiments were preformed on glucose responsive low passage MIN6 (P18) and glucose non-responsive high passage MIN6 (P40)

Following data normalisation, gene lists were generated using a students t-test with a P value cut-off of 0 01, to identify genes that were statistically differentially expressed between the three high passage arrays and the low passage arrays (used as the reference/"control") (Section 2 4 1 6 3) Approximately 4 % (500) of the transcripts on the microarray were significantly increased and approximately 3 5 % (461) were significantly decreased in three biological repeat studies of high passage MIN6 compared to low passage MIN6 cells Eighty-eight genes were overexpressed 2-fold or more in high passage MIN6 cells, while 185 were down-regulated by this factor in high passage MIN6 cells compared to low passage MIN6 cells The 2-fold cut-off gene lists are available in Table 3 3 1

Passaging of MIN6 cells resulted in many changes in gene expression, including both up-regulation and down-regulation of many transcripts associated with crucial aspects of β cell biology In order to validate the microarray results, qPCR was performed on a selection of chosen genes to demonstrate that the increase/decrease observed between high and low passage MIN6 were real expression changes

Table 3 3 1 (A) Genes up-regulated 2-fold or more in high passage MIN6 cells compared to low passage MIN6 cells (B) Genes down-regulated 2-fold or more in high passage MIN6 cells compared to low passage MIN6 cells

A Up-regulated Genes			
Probe Set ID	Gene Title	Gene Symbol	Fold
100044_at	claudin 11	Cldn11	3 791
100050_at	inhibitor of DNA binding 1	Idb1	3 119
100465_i_at	Clone IMAGE 3371572, mRNA	—	2 752
100566_at	insulin-like growth factor binding protein 5	Igfbp5	9 66
100946_at	HLA-B-associated transcript 9	Bat9	2 799
101007_at	MAP kinase-interacting serine/threonine kinase 2	Mknk2	2 137
101059_at	necdin	Ndn	2 307
101108_at	nuclear autoantigenic sperm protein (histone-binding)	Nasp	2 265
101135_at	calcitonin receptor	Calcr	3 144
101178_at	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	Sema3c	8 671
101372_at	thyroid hormone receptor interactor 13	Trip13	2 805
101526_at	homeo box, msh-like 1	Msx1	2 792
101540_at	thymine DNA glycosylase	Tdg	2 108
101571_g_at	insulin-like growth factor binding protein 4	Igfbp4	2 143
101631_at	SRY-box containing gene 11	Sox11	6 347
102204_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	Mafb	12 491
102217_at	G protein-coupled receptor kinase 5	Gprk5	2 643
102859_at	DNA segment, Chr 6, ERATO Doi 253, expressed	D6Ertd253e	6 088
103061_at	glutamic acid decarboxylase 1	Gad1	8 446
103088_at	cell adhesion molecule with homology to L1CAM	Chl1	3 133
103212_at	cDNA sequence BC006933	BC006933	2 221
103234_at	neurofilament heavy polypeptide	Nefh	2 645
103238_at	wingless-related MMTV integration site 4	Wnt4	2 896
103452_at	proline dehydrogenase (oxidase) 2	Prodh2	4 674
103460_at	DNA-damage-inducible transcript 4	Ddit4	3 223
103504_at	single-stranded DNA binding protein 2	Ssbp2	2 214
103549_at	nestin	Nes	4 217
103585_at	spastic paraplegia 4 homolog (human)	Spg4	
103723_at	interleukin 13 receptor, alpha 1	Il13ra1	2 346
104154_at	transformation related protein 53	Trp53	2 074
104275_g_at	transformation related protein 53	Trp53	2 103
160068_at	sin3 associated polypeptide	Sap30	2 24
160131_at	angiomotin like 2	Amotl2	3 223
160135_at	DNA segment, Chr 16, ERATO Doi 502, expressed	D16Ertd502e	2 238
160227_s_at	bystin-like	Bysl	2 185
160246_at	tumor necrosis factor, alpha-induced protein 8	Tnfaip8	4 35
160547_s_at	thioredoxin interacting protein	Txnip	2 494
160934_s_at	—	—	3 456
160965_at	RAS p21 protein activator 4	Rasa4	4 644
161051_at	hairy and enhancer of split 5 (Drosophila)	Hes5	2 675
161077_f_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d member 2	Smarcd2	2 178
161603_r_at	erythrocyte protein band 4 1-like 4a	Epb4 1l4a	6 405
92208_at	C1q domain containing 1	C1qdc1	3 092

92231_at	erythrocyte protein band 4 1-like 4a	Epb4 1l4a	4 35
92244_at	exonuclease 1	Exo1	2 195
92266_at	interleukin 11	Il11	3 633
92697_at	forkhead box A1	Foxa1	3 809
92931_at	delta-like 1 (Drosophila)	Dll1	5 132
92932_at	cerebellin 1 precursor protein	Cbln1	3 147
92995_at	visinin-like 1	Vsnl1	2 629
93018_at	interferon induced transmembrane protein 2	Ifitm2	2 548
93550_at	cysteine and glycine-rich protein 2	Csrp2	5 067
93669_f_at	SRY-box containing gene 11	Sox11	6 216
94026_at	pro-opiomelanocortin-alpha	Pomc1	2 846
94429_at	eukaryotic translation elongation factor 1 alpha 2	Eef1a2	2 032
94439_at	oxysterol binding protein-like 11	Osbpl11	2 283
94451_at	DNA segment, Chr 13, Wayne State University 123, expressed	D13Wsu123e	2 189
94452_g_at	DNA segment Chr 13, Wayne State University 123, expressed	D13Wsu123e	2 382
94477_at	—	—	3 494
94556_at	sorting nexin 10	Snx10	2 333
95064_at	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	Acaa2	2 126
95122_g_at	polymerase (DNA-directed), epsilon 4 (p12 subunit)	Pole4	2 057
95152_g_at	RIKEN cDNA 2810052M02 gene	2810052M02Rik	3 873
95363_at	granzyme M (lymphocyte met-ase 1)	Gzmm	3 374
95471_at	cyclin-dependent kinase inhibitor 1C (P57)	Cdkn1c	6 703
95550_at	retinitis pigmentosa GTPase regulator	Rpgr	2 05
95673_s_at	brain abundant, membrane attached signal protein 1	Basp1	3 084
96244_at	ubiquitin carboxy-terminal hydrolase L1	Uchl1	2 43
96598_at	RIKEN cDNA D430039C20 gene	D430039C20Rik	2 228
96651_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	Smarce1	2 487
96663_at	surfeit gene 6	Surf6	2 246
96752_at	intercellular adhesion molecule	Icam1	
96783_at	mucin and cadherin like	Mucdhl	3 038
96804_at	nucleolar protein 1	Nol1	2 361
96849_at	translocase of inner mitochondrial membrane 8 homolog a (yeast)	Timm8a	2 506
97203_at	MARCKS-like protein	Mlp	3 675
97426_at	epithelial membrane protein 1	Emp1	2 202
97521_at	argininosuccinate synthetase 1	Ass1	2 46
97703_at	brix domain containing 1	Bxdc1	2 226
97824_at	nucleolar protein family A, member 2	Nola2	3 251
97890_at	serum/glucocorticoid regulated kinase	Sgk	4 32
98111_at	heat shock protein 105	Hsp105	2 858
98122_at	LIM domain only 4	Lmo4	2 169
98129_at	M musculus mRNA for testis-specific thymosin beta-10	—	2 469
98446_s_at	Eph receptor B4	Ephb4	2 342
98624_at	RNA-binding region (RNP1, RRM) containing 1	Rnpc1	2 374
98839_at	sine oculis-related homeobox 2 homolog (Drosophila)	Six2	3 237
99328_at	distal-less homeobox 3	Dlx3	9 246
99833_at	calpain 9 (nCL-4)	Capn9	3 025
99935_at	tight junction protein 1	Tjp1	2 203
99956_at	kit oncogene	Kit	2 595

B Down-regulated Genes			
Probe Set ID	Gene Title	Gene Symbol	Fold
100131_at	secretory granule neuroendocrine protein 1, 7B2 protein	Sgne1	-3 554
100136_at	lysosomal membrane glycoprotein 2	Lamp2	-2 593
100303_at	histocompatibility 2, M region locus 10 1	H2-M10 1	-9 487
100329_at	serine (or cysteine) proteinase inhibitor, clade A, member 1d	Serpina1d	-2 485
100505_at	Ras-like without CAAX 2	Rit2	-3 113
100513_at	development and differentiation enhancing	Ddef1	-2 439
100572_at	transmembrane 9 superfamily member 2	Tm9sf2	-2 169
100607_at	phospholipase D3	Pld3	-2 299
100633_at	RIKEN cDNA 2810484M10 gene	2810484M10Rik	-2 082
100771_at	B-cell linker	Blnk	-11 91
100772_g_at	B-cell linker	Blnk	-13 75
100931_at	arylsulfatase A	Arsa	-2 009
100956_at	klotho	Kl	-2 637
100962_at	Ngfi-A binding protein 2	Nab2	-2 899
101001_at	RIKEN cDNA 5031439A09 gene	5031439A09Rik	-2 991
101055_at	protective protein for beta-galactosidase	Ppgb	-2 356
101217_at	—	—	-3 042
101352_g_at	G protein-coupled receptor, family C, group 2, member A, related sequence 1	Gprc2a-rs1	-3 023
101384_at	RIKEN cDNA 2210008A03 gene	2210008A03Rik	-2 283
101389_at	scavenger receptor class B, member 2	Scarb2	-2 302
101426_at	ceramide kinase	Cerk	-2 259
101446_at	tumor protein D52-like 1	Tpd52l1	-2 351
101565_f_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	Serpina1a	-2 139
101574_f_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	Serpina1a	-2 975
101575_i_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	Serpina1a	-2 92
101576_f_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	Serpina1a	-2 518
101676_at	glutathione peroxidase 3	Gpx3	-6 505
101681_f_at	histocompatibility 2, blastocyst	H2-Bl	-2 051
101735_f_at	angiogenin, ribonuclease A family, member 2	Ang2	-2 686
101851_at	antigen identified by monoclonal antibody MRC OX-2	Mox2	-10 72
101887_at	angiotensinogen	Agt	-5 079
101908_s_at	CEA-related cell adhesion molecule 2	Ceacam2	-29 38
101918_at	transforming growth factor, beta 1	Tgfb1	-5 567
101975_at	delta-like 1 homolog (Drosophila)	Dlk1	-2 242
102064_at	caspase 1	Casp1	-2 099
102094_f_at	—	—	-2 377
102161_f_at	histocompatibility 2, D region locus 1	H2-D1	-2 177
102163_at	—	—	-2 622
102197_at	nucleobindin 2	Nucb2	-4 534
102296_at	proprotein convertase subtilisin/kexin type 2	Pcsk2	-2 555
102313_at	GTP cyclohydrolase 1	Gch	-2 399
102318_at	sialyltransferase 8 (alpha-2, 8-sialyltransferase) D	Siat8d	-2 409
102370_at	dehydrogenase/reductase (SDR family) member 8	Dhrs8	-6 895
102389_s_at	growth associated protein 43	Gap43	-2 028
102414_i_at	DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	-4 326
102415_r_at	DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	-4 65

102431_at	microtubule-associated protein tau	Mapt	-5 619
102727_at	brain derived neurotrophic factor	Bdnf	-4 25
102742_g_at	microtubule-associated protein tau	Mapt	-5 243
102748_at	coagulation factor V	F5	-3 092
102784_at	RIKEN cDNA 4632413K17 gene	4632413K17Rik	-2 395
102804_at	CEA-related cell adhesion molecule 2	Ceacam2	-6 734
102805_at	CEA-related cell adhesion molecule 1	Ceacam1	-25 39
102806_g_at	CEA-related cell adhesion molecule 1	Ceacam1	-17 71
102886_at	glypican 4	Gpc4	-11 09
102905_at	caspase 4, apoptosis-related cysteine protease	Casp4	-4 072
102920_at	RIKEN cDNA 9130422G05 gene	9130422G05Rik	-2 352
102926_at	glial cell line derived neurotrophic factor family receptor alpha 3	Gfra3	-2 127
102960_at	recombination activating gene 1 gene activation	Rga	-2 456
103235_at	neuropeptide Y	Npy	-23 98
103247_at	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	Mpp3	-3 227
103394_at	FXYD domain-containing ion transport regulator 5	Fxyd5	-3 917
103456_at	endothelin converting enzyme 1	Ece1	-2 322
103494_at	transmembrane 4 superfamily member 3	Tm4sf3	-6 378
103562_f_at	---	---	-3 884
103637_at	N-acetyl galactosaminidase, alpha	Naga	-2 256
103647_at	galactosidase, beta 1	Glb1	-4 016
103671_at	HIV-1 tat interactive protein 2, homolog (human)	Htatip2	-3 795
103793_at	major vault protein	Mvp	-2 245
103882_at	carboxypeptidase N, polypeptide 1	Cpn1	-12 59
103933_at	yolk sac gene 2	Ysg2	-2 579
104018_at	Similar to Lactase-phlonzin hydrolase precursor (Lactase-glycosylceramidase) (LOC226413), mRNA	---	-3 455
104065_at	ER degradation enhancer, mannosidase alpha-like 1	Edem1	-4 532
104066_at	expressed sequence AW548124	AW548124	-2 478
104143_at	coatomer protein complex, subunit zeta 2	Copz2	-3 039
104182_at	hepatocyte growth factor activator	Hgfac	-5 285
104407_at	activated leukocyte cell adhesion molecule	Alcam	-5 567
104422_at	protein tyrosine phosphatase, receptor type, N	Ptprn	-2 16
104449_at	glycine receptor, beta subunit	Glrb	-2 158
104564_at	secretogranin III	Scg3	-3 456
104680_at	receptor (calcitonin) activity modifying protein 1	Ramp1	-2 885
104686_at	glutamate receptor, ionotropic, NMDA1 (zeta 1)	Grin1	-3 303
104714_at	DNA segment, Chr 14, Wayne State University 89, expressed	D14Wsu89e	-2 139
160104_at	hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 7	Hsd3b7	-5 018
160108_at	nuclear protein 1	Nupr1	-47 94
160127_at	cyclin G1	Ccng1	-2 28
160139_at	heat shock 27kDa protein 8	Hspb8	-2 021
160181_at	synaptophysin	Syp	-2 263
160270_at	lectin, mannose-binding, 1	Lman1	-2 112
160428_at	succinate-Coenzyme A ligase, GDP-forming, beta subunit	Suclg2	-2 369
160461_f_at	RIKEN cDNA 2310057H16 gene	2310057H16Rik	-6 736
160516_at	glucosidase, alpha, acid	Gaa	-2 847
160580_at	mannosidase 1, alpha	Man1a	-8 397
160611_at	cytochrome P450 family 4, subfamily v, polypeptide 3	Cyp4v3	-4 593
160711_at	2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	-3 328
160732_at	aminopeptidase-like 1	Npepl1	-12 22

160767_at	sterol O-acyltransferase 1	Soat1	-2 157
160799_at	Transcribed sequence with moderate similarity to protein pir:A47643 (M musculus) A47643 hypothetical protein - mouse	—	-5 915
160819_at	N-myc downstream regulated gene 4	Ndrg4	-2 119
160899_at	Purkinje cell protein 4	Pcp4	-2 828
160968_at	alanine and arginine rich domain containing protein	Aard	-10 19
160971_at	expressed sequence AI842353	AI842353	-2 436
160977_at	Rho guanine nucleotide exchange factor (GEF) 5	Arhgef5	-2 781
160991_at	NFKB inhibitor interacting Ras-like protein 1	Nkiras1	-2 054
161132_at	sciellin	Scel	-4 541
161139_f_at	development and differentiation enhancing	Ddef1	-2 407
161294_f_at	clusterin	Clu	-6 402
161754_f_at	galactosidase, beta 1	Glb1	-2 298
161987_at	prodynorphin	Pdyn	-6 189
162108_f_at	salivary protein 1	Spt1	-15 25
162237_f_at	secretogranin III	Scg3	-2 615
162275_f_at	UPF3 regulator of nonsense transcripts homolog B (yeast)	Upf3b	-2 067
92271_at	paired box gene 6	Pax6	-2 171
92378_at	protein tyrosine phosphatase, receptor type Z, polypeptide 1	Ptprz1	-16 22
92403_at	sialyltransferase 7 ((alpha-N-acetylneuraminyl 2,3-betagalactosyl-1,3)-N-acetyl galactosaminide alpha-2,6-sialyltransferase) E	Siat7e	-4 12
92426_at	transmembrane 4 superfamily member 9	Tm4sf9	-2 696
92474_at	phospholipase D1	Pld1	-4 065
92475_g_at	phospholipase D1	Pld1	-2 666
92730_at	diphtheria toxin receptor	Dtr	-4 019
92793_at	tumor necrosis factor receptor superfamily, member 1a	Tnfrsf1a	-2 934
92806_at	RIKEN cDNA 1110004D19 gene	1110004D19Rik	-2 08
92841_f_at	chromogranin B	Chgb	-2 961
92880_at	milk fat globule-EGF factor 8 protein	Mfge8	-9 964
92936_at	contactin 1	Cntn1	-23 68
93009_at	glutathione S-transferase, mu 2	Gstm2	-4 464
93039_at	plasma glutamate carboxypeptidase	Pgcp	-5 807
93066_at	granulin	Grn	-2 634
93083_at	annexin A5	Anxa5	-2 277
93109_f_at	serine (or cysteine) proteinase inhibitor, clade A, member 1a	Serpina1a	-3 069
93120_f_at	histocompatibility 2, K1, K region	H2-K1	-2 383
93163_at	gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 2	Gabrg2	-4 631
93240_f_at	UPF3 regulator of nonsense transcripts homolog B (yeast)	Upf3b	-2 039
93285_at	dual specificity phosphatase 6	Dusp6	-6 373
93373_at	alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB)	Naglu	-2 013
93451_at	LIM domain only 7	Lmo7	-5 068
93568_i_at	RIKEN cDNA 2610042L04 gene	2610042L04Rik	-7 439
93569_f_at	RIKEN cDNA 2610042L04 gene	2610042L04Rik	-7 723
93575_at	gamma-glutamyl hydrolase	Ggh	-4 232
93605_r_at	immunoglobulin superfamily, member 4A	Igsf4a	-2 235
93606_s_at	immunoglobulin superfamily, member 4A	Igsf4a	-3 603
93626_at	ATP-binding cassette sub-family G (WHITE) member 2	Abcg2	-5 508
93714_f_at	histocompatibility 2, K1, K region	H2-K1	-2 136
93731_at	FK506 binding protein 9	Fkbp9	-2 799
93736_at	transcobalamin 2	Tcn2	-2 889

93842_at	death-associated protein	Dap	-2 383
94047_at	RIKEN cDNA 0610031J06 gene	0610031J06Rik	-2 346
94152_at	salivary protein 1	Spt1	-7 313
94153_g_at	salivary protein 1	Spt1	-9 954
94247_at	RIKEN cDNA 5730453H04 gene	5730453H04Rik	-2 392
94270_at	keratin complex 1, acidic, gene 18	Krt1-18	-2 004
94284_at	diaphorase 1 (NADH)	Dia1	-2 209
94350_f_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	-2 426
94351_r_at	NAD(P)H dehydrogenase, quinone 1	Nqo1	-3 413
94366_at	RIKEN cDNA 2310079N02 gene	2310079N02Rik	-2 042
94392_f_at	angiogenin, ribonuclease A family, member 1	Ang1	-3 176
94515_at	sulfide quinone reductase-like (yeast)	Sqrdl	-5 559
94545_at	reticulon 1	Rtn1	-4 232
94633_at	glucagon	Gcg	-3 041
94733_at	ATP-binding cassette, sub-family B (MDR/TAP), member 4	Abcb4	-3 318
94840_at	hexosaminidase A	Hexa	-3 057
94872_at	sphingomyelin phosphodiesterase, acid-like 3A	Smpdl3a	-3 453
94906_at	alcohol dehydrogenase 1 (class I)	Adh1	-2 073
95052_at	RIKEN cDNA 1110035L05 gene	1110035L05Rik	-2 464
95102_at	scotin gene	Scotin	-2 411
95104_at	syndecan 2	Sdc2	-3 123
95286_at	clusterin	Clu	-3 691
95290_at	corticotropin releasing hormone receptor 1	Crhr1	-3 608
95330_at	glucose-6-phosphatase, catalytic, 2	G6pc2	-3 3
95436_at	somatostatin	Sst	-16 82
95453_f_at	S100 calcium binding protein A1	S100a1	-2 07
95465_s_at	protein distantly related to to the gamma subunit family	Pr1	-2 713
95520_at	RIKEN cDNA 2310061B02 gene	2310061B02Rik	-3 274
95552_at	deiodinase, iodothyronine, type I	Dio1	-5 582
95557_at	bone morphogenetic protein 1	Bmp1	-2 232
95620_at	dehydrogenase/reductase (SDR family) member 7	Dhrs7	-2 549
95661_at	CD9 antigen	Cd9	-13 26
95709_at	vitamin K epoxide reductase complex, subunit 1	Vkorc1	-2 279
95885_at	RIKEN cDNA 1200009F10 gene	1200009F10Rik	-2 218
96047_at	retinol binding protein 4, plasma	Rbp4	-4 986
96055_at	cholecystokinin	Cck	-6 531
96114_at	protein phosphatase 1, regulatory (inhibitor) subunit 1A	Ppp1r1a	-2 279
96115_at	deleted in polyposis 1	Dp1	-2 36
96336_at	glycine amidinotransferase (L-arginine glycine amidinotransferase)	Gatm	-9 755
96346_at	cysteine dioxygenase 1 cytosolic	Cdo1	-2 492
96426_at	thymosin, beta 4, X chromosome	Tmsb4x	-3 979
96591_at	reelin	Reln	-4 396
96603_at	quiescin Q6	Qscn6	-2 477
96605_at	RIKEN cDNA 0610011I04 gene	0610011I04Rik	-2 233
96634_at	RIKEN cDNA 5730469M10 gene	5730469M10Rik	-4 054
96653_at	ribonuclease T2	Rnaset2	-2 639
96657_at	spermidine/spermine N1-acetyl transferase 1	Sat1	-2 146
96672_at	homeobox only domain	Hod	-4 655
96708_at	RIKEN cDNA 1200002G13 gene	1200002G13Rik	-2 525
96767_at	membrane bound C2 domain containing protein	Mbc2	-2 677
96948_at	quininoid dihydroptendine reductase	Qdpr	-2 18

97125_f_at	histocompatibility 2, K1, K region	H2-K1	-2 091
97197_r_at	—	—	-2 011
97206_at	serine protease inhibitor, Kunitz type 1	Spint1	-2 08
97255_at	CUG triplet repeat,RNA binding protein 2	Cugbp2	-2 467
97415_at	RAB3D, member RAS oncogene family	Rab3d	-3 024
97498_at	four and a half LIM domains 1	Fhl1	-4 38
97520_s_at	neuronatin	Nnat	-2 732
97540_f_at	histocompatibility 2, D region locus 1	H2-D1	-2 243
97811_at	ADP-ribosylation factor GTPase activating protein 3	Arfgap3	-2 026
97964_at	FK506 binding protein 11	Fkbp11	-3 584
97980_at	lymphotoxin B receptor	Ltbr	-3 598
98059_s_at	lamin A	Lmna	-2 026
98133_at	calbindin-28K	Calb1	-6 052
98316_at	cysteine-rich hydrophobic domain 1	Chic1	-3 382
98398_s_at	apolipoprotein B editing complex 1	Apobec1	-5 727
98435_at	adenylosuccinate synthetase, muscle	Adss	-2 104
98472_at	RIKEN cDNA C920025E04 gene	C920025E04Rik	-2 18
98579_at	early growth response 1	Egr1	-5 545
98588_at	fumarylacetoacetate hydrolase	Fah	-2 537
98931_at	glucosamine (N-acetyl)-6-sulfatase	Gns	-2 128
99062_at	poliovirus receptor-related 3	Pvrl3	-2 336
99065_at	casein kappa	Csnk	-7 343
99197_at	group specific component	Gc	-18 19
99390_at	wingless-related MMTV integration site 5A	Wnt5a	-2 207
99444_at	receptor (calcitonin) activity modifying protein 2	Ramp2	-3 858
99475_at	suppressor of cytokine signaling 2	Socs2	-2 577
99494_at	serine (or cysteine) proteinase inhibitor, clade I, member 1	Serpini1	-2 115
99580_s_at	UDP-glucuronosyltransferase 1 family, member 2	Ugt1a2	-19 18
99584_at	kangai 1 (suppression of tumorigenicity 6, prostate)	Kai1	-6 293
99840_at	prodynorphin	Pdyn	-8 344
99902_at	hyperpolarization-activated, cyclic nucleotide-gated K+ 1	Hcn1	-2 566
99972_at	tryptophan hydroxylase 1	Tph1	-4 225

3.3.5.1 Quality Control Clustering using the Spearman Correlation

The spearman correlation is a non-parametric test for analysis of data i.e. it makes no assumptions about data to determine the strength of the relationship between pairs of variables. Spearman correlation analysis of gene lists (normalised using PLIER algorithm, Section 2.4.1.6.2.1) from the 6 samples making up the "control" (low passage MIN6) and the "test" (high passage MIN6), were used to make a condition tree investigating their similarity. Through the careful standardisation of the culture conditions used for MIN6 cells, good concordance between biological repeats were obtained (Figure 3.3.5 A). The overall comparison of the genes flagged present in high passage MIN6 cells compared to low passage MIN6 cells indicated that these cells lines are very different (Figure 3.3.5 B).

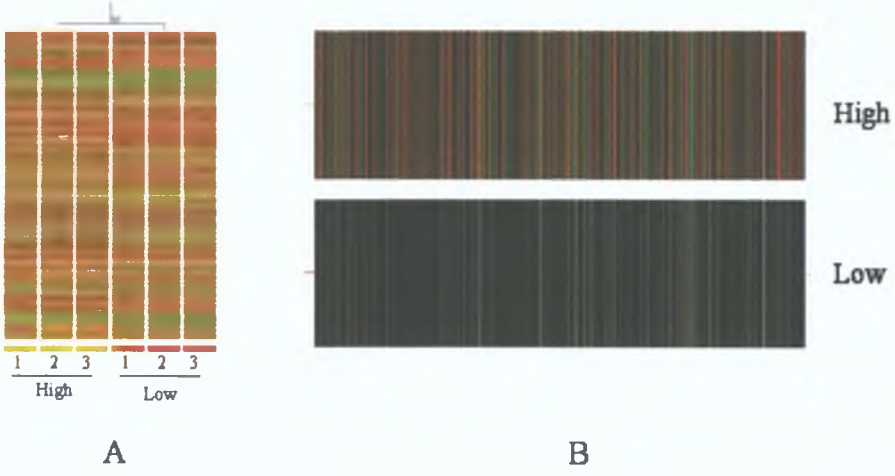


Figure 3.3.5 (A) Spearman correlation analysis of the 6 samples making up the "control" (low passage MIN6) and "test" samples (high passage MIN6) indicates the high quality and reproducibility of the biological replicates, as indicated by the close clustering of the test and control samples into two discrete groups illustrated in the condition tree above. Up- and down-regulated genes are represented by red and blue, respectively. R (Spearman correlation co-efficient) between triplicate low passage MIN6 is 0.991; between triplicate high passage MIN6 is 0.986; and between low and high passage MIN6 is 0.96. (B) Hierarchical clustering analysis of the 6,605 genes flagged 'Present' in this analysis (where each vertical line is a gene) clearly demonstrates the large differences in global gene expression upon serial passaging of MIN6 cells.

3 3 5 2 Categories of Gene Transcripts Changed after the Serial Passage of MIN6 cells

The 2-fold up-regulated/down-regulated genes (Table 3 3 1) were classified into the following categories metabolism, growth proliferation, differentiation, regulation of transcription, signal transduction, protein transport secretion, cell adhesion, apoptosis, defense/immunity, proteolysis/peptidolysis, development, hormone, other (includes cytoskeletal, stress, RNA and protein binding), unknown (includes RIKEN cDNAs and newly discovered gene products that have not yet been assigned functions)

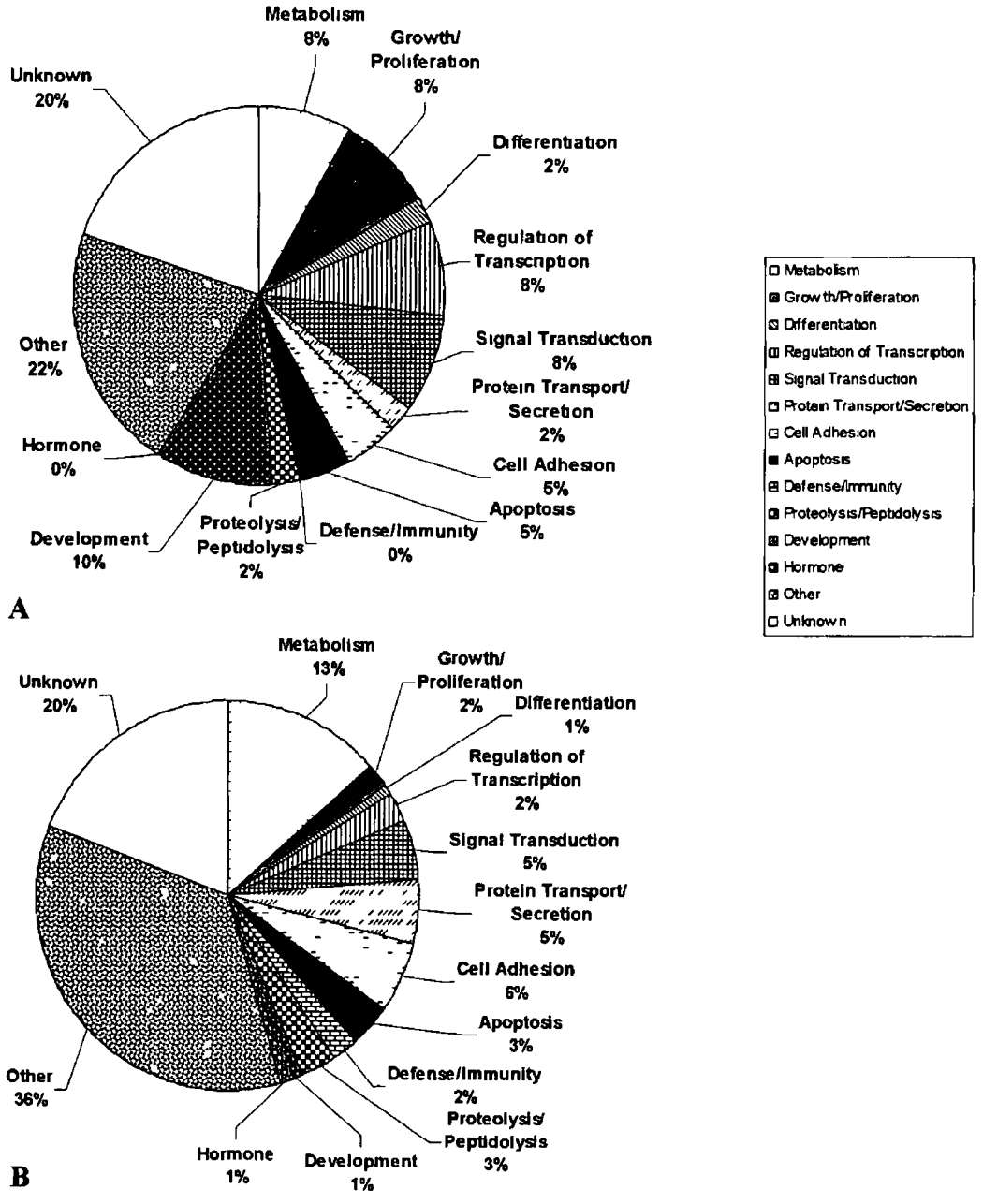


Figure 3.3 6 Representations of the overall categories of gene transcripts that were either up-regulated (A) or down-regulated (B) in high passage MIN6 cells compared to low passage MIN6 cells

3 3 5 3 qPCR Confirmation Analysis of Genes Detected on Microarray

In order to validate the gene lists and to demonstrate that the increase/decrease observed were real expression changes, a set of genes were selected for qPCR analysis from the gene lists generated (Table 3 3 1) Genes of interest included those involved in the categories listed in Figure 3 3 6 Primers were designed for these genes using Primer Express Software 2 0 (Table 3 3 3) qPCR analysis was preformed on the three MIN6 sample sets (Table 3 3 2) In the majority of cases the results confirmed the trends observed in the microarray experiment (Table 3 3 3)

Table 3 3 2 RQ of transcript expression in the three MIN6 samples sets as determined by qPCR RQ of low passage MIN6 was set to 1 MIN6-L, low passage MIN6, MIN6-H, high passage MIN6 RQ, relative quantity

Genes	MIN6-L RQ			MIN6-H RQ			SD
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3	
Up regulated							
glut2	1	1	1	2 366	1 741	7 674	3 260
dll1	1	1	1	1 613	1 387	1 144	0 235
claudin11	1	1	1	3 594	6 925	10 069	3 238
Gck	1	1	1	1 212	1 029	1 217	0 107
trp53	1	1	1	2 005	2 199	1 248	0 502
tjp	1	1	1	1 449	2 027	1 13	0 455
nestin	1	1	1	2 427	2 354	1 37	0 590
Down- regulated							
egr1	1	1	1	0 172	0 094	0 191	0 051
sgne1	1	1	1	0 094	0 163	0 166	0 041
GAP43	1	1	1	0 173	0 242	0 294	0 061
PC2	1	1	1	0 121	0 187	0 144	0 033
pld1	1	1	1	0 074	0 221	0 134	0 074
PPI	1	1	1	0 433	0 401	0 43	0 018
npy	1	1	1	0 082	0 005	0 187	0 091
scg3	1	1	1	0 176	0 222	0 15	0 036
chgb	1	1	1	0 054	0 039	0 04	0 008
Pdx1	1	1	1	0 446	0 363	0 348	0 053
Isl1	1	1	1	0 338	1 009	0 766	0 340
nkx2 2	1	1	1	0 374	0 593	0 453	0 111
beta2	1	1	1	0 546	0 496	0 457	0 045
ODC	1	1	1	0 718	0 726	0 733	0 007
CEACAM1	1	1	1	0 027	0 038	0 036	0 006
insulin I	1	1	1	0 1	0 269	0 36	0 132
insulin II	1	1	1	0 386	0 379	0 51	0 074
pax 6	1	1	1	0 3	0 3	0 402	0 059
Ihh	1	1	1	1 002	0 37	0 255	0 402
cck	P	P	P	A	A	A	0
pax 4	1	1	1	0 959	1 018	0 711	0 163
Genes not in agreement with array							
dlk1	A	A	A	P	P	P	0

Table 3 3.3 Validation of microarray data (induced/up-regulated and down-regulated) by qPCR

Induced/Up-Regulated	Function/Physiological Role	Primer Sequence (5' to 3')	Microarray[a] *(fold)*	Real-time PCR[b] *(fold)*
Secretion				
gck	phosphorylation of glucose to G-6-P	gatgctggatgacagagccaggatg agatgcactcagagatgtagtcga	NS	1 03-1 22
glut2	facilitative glucose transporter	Taqman	NS	1 74-7 67
Adhesion				
tjp	paracellular permeability barrier	acgacaaaacgctctacagg gagaatggactggcttagca	2 2	1 13-2 03
claudin 11	interacts with extracellular matrix at tight junctions	caagagtgcccatgtctaag tgggcattaactgagctaga	3 8	3 59-10 07
Differentiation				
nestin	marker of neuronal-like cells (16)	ggctacatacaggattctgctgg caggaaagccaagagaagcct	4 2	1 37-2 43

dll1	controls development of neurons
trp53	has been found to negatively impact terminal differentiation

Down-Regulated

Secretion

egr1	elicited by glucose secretagogues
pld1	regulates GSIS
scg3	associated with secretory granule membrane
chgb	islet secretory glycoprotein
sgne1 (7B2 protein)	required for PC2 activation

agccctccatacagactctc	5 13	1 14-1 61
cagaacacagagcaaccttc		
agctttgaggttcgtgtttg	2 1	1 23-2 20
ggaacatctcgaagcgttta		
atggacaactaccccaaact	5 54	5 2-10 6
attcagagcgatgtcagaaa		
tcttatcccttcctgctacc	4 07	4 5-13 5
ccaccttgttgaacacaact		
ggatactggtgttggtgctc	3 45	4 5-6 7
tttctgttattgccctcagc		
atccaagtccagtgttccaa	2 96	18 5-25 6
tctcattgcctaccttcgtc		
ttcagtgaggatcaaggcta	3 55	6-10 6
ctgggtagtcatgttctgga		

PC2	processes proinsulin to mature insulin
cck	stimulates insulin secretion from β cells
Proliferation	
dlk1	β cell autocrine/proliferation factor
Adhesion	
CEACAM1[d]	cell adhesion & proliferation suppressor
ODC	inhibition may prevent polyamine-regulated β cell replication
Differentiation	
pax 6	expression required for islet cell development, facilitates GSIS
pax 4	transcription factor involved in pancreatic development

cacagatgactggttcaaca	2 56	5 3-8 2
tcacagttgcagtcatcgta		
gatggcagtcctagctgctg	6 53	A
ccaggctctgcaggttctta		
Taqman Probe	2 2	P
aatctgcccctggcgcttggagcc	25 4	26 3-37
aaatcgcacagtcgcctgagtacg		
ctgccacatccttgatgaag	1 92	1 36-1 39
cgttactggcagcatacttg		
aagagtggcgactccagaagt	2 17	2 5-3 3
accatacctgtattcttgcttcagg		
tggcttcctgtccttctgtgagg	NS	0 9-1 4
tccaagacacctgtgcggtagtag		

Pdx1	essential for pancreatic development and insulin secretion
Isl1	development of the dorsal pancreatic bud and all endocrine islet cells
nkx2 2	pancreatic cell & neuron development
beta2/NeuroD	pancreatic, hippocampus & cerebellum development
npy	induced in insulin$^+$ cells at islet formation
Ihh	hedgehog signalling required during pancreas development
GAP43	neurite outgrowth & synaptic plasticity

Insulin

PPI

ggtggagctggcagtgatgtt	NS	2 2-2 9
accgcccccactcggggtcccgc		
acgtctgatttccctgtgtgttgg	1 47	1-2 9
tcgatgtggtacaccttagagcgg		
cggagaaaggtatggaggtgac	NS	1 7-2 7
ctgggcgttgtactgcatgtgctg		
cttggccaagaactacatctgg	NS	1 8-2 2
ggagtagggatgcaccgggaa		
tgaccctcgctctatctctg	23 98	5 3-200
aagggtcttcaagccttgtt		
aaggcccacgtgcattgctct	A	1-3 9
tgtccgcaatgaagagcaggtg		
gctgctaaagctaccactga	2 03	3 4-5 8
taggtttggcttcgtctaca		
	NA	2 3-2 5
agcgtggcttcttctacacacc		
ggtgcagcactgatccaccatg		

insulin I		tagtgaccagctataatcagag acgccaaggtctgaaggtcc	NS	2 8-10
insulin II		ccctgctggccctgctctt aggtctgaaggtcacctgct	NS	1 9-2 6
β Actin	β-actin was co-amplified with other cDNAs as endogenous control, annealing temperature (temp) used was as relevant for cDNA of interest	gaaatcgtgcgtgacattaaggagaagct tcaggaggagcaatgatcttga & Taqman		

Note qPCR = quantitative/real-time PCR, [a] = mean of 3 microarray experiments, [b] = range of fold changed detected by (n=3) qPCR, P = induced in MIN-6(H) from absent in MIN-6(L), A = present in MIN-6(L) but absent from MIN-6(H), NA = not represented on array, NS = Not Significantly Changed

Section 4.0: Discussion

4.1 General Introduction

The global incidence of insulin dependant diabetes mellitus (IDDM) in children and adolescents is increasing with an estimated overall annual increase of around 3%. (http://www.eatlas.idf.org/Incidence/) IDDM can be managed through insulin injections; however, the poor control in glucose fluctuations associated with this therapy leads to severe long-term complications such as blindness, kidney failure, heart disease, stroke and amputations. The Diabetes Control and Complications Trial (DCCT) has shown that rigid control of glucose levels can diminish longterm complications (http://diabetes.niddk.nih.gov/dm/pubs/control/). Over the past decade, several researchers have achieved varying degrees of success at using whole pancreas and islet transplantations as a therapy for IDDM; however, the demand for donor tissue greatly outweighs its availability. Investigators are now looking for alternative sources of donor tissue. One possible solution being investigated is the derivation of β/islet cells from ES cells using genetic modification and directed differentiation (Berná et al, 2001; Soria et al, 2001a).

Many of the ES differentiation studies reported to date have been carried out on mouse models. Mouse ES cells were the first to be characterised and are also more readily available to laboratories than human ES cells (Pedersen, 1999). Ethical concerns are a limiting factor when it comes to human ES cell research thus emphasising the importance of non-human primate ES cells as relevant models in studying human development (Jacobson et al, 2001). ES-D3 is a pluripotent ES cell line derived from a mouse 129/Sv blastocyst (Doetschman et al, 1985). It is used as an ES model within this thesis. Studies performed over the past 15 years have shown that the ES-D3 cell line is suitable for use in differentiation studies using both genetic and culture based techniques (Section 1.1.6.1).

Several publications in recent years have described differentiation protocols (both genetic- and culture-based) for the derivation of insulin-expressing cell types (Lumelsky et al, 2001; Hori et al, 2002; Blyszczuk et al, 2003; Moritoh et al, 2003; Miyazaki et al, 2004; Shi et al, 2005). These multi-step regulated differentiation protocols described for

the derivation of pancreatic cell types are long, cumbersome, often involve the use of exogenous insulin and also result in low yields of insulin-producing cell The success of these protocols in generating authentic β cells has been questioned due in part to uncertainty regarding cell lineage (Kania et al, 2004, Milne et al, 2005) but also due to the controversy regarding *de novo* insulin synthesis (Rajagopal et al, 2003, Hansson et al, 2004) In an attempt to address the latter, we designed a novel assay that can identify *de novo* insulin synthesis The method is based on metabolic labelling combined with a modified radio-immunoassay (MRIA) We also presented means of quantifying C-peptide expression These assays, in combination with more commonly used techniques, including Western blotting to detect and quantify C-peptide 2, were employed to characterise the ES-derived populations generated using an adapted version of the controversial protocol published by Lumelsky et al in 2001 (Section 3 1)

Limited numbers of pancreatic β-like cells have also been successfully derived from ES cells by spontaneous differentiation (Kahan et al, 2003) Whilst this is an important finding in this field, it is recognised that a more controlled differentiation is necessary to maximise the number of ES cells successfully differentiated to islet-like cell types However, the regulated multi-step protocols discussed previously appear to be suboptimal for use We investigated the potential of extra-cellular factors in inducing endocrine pancreas differentiation - without the use of exogenous insulin - in order to improve on existing protocols or to establish a novel, simplified protocol for the derivation of pancreatic endocrine cell types Using a 2-stage differentiation protocol developed during this study, we successfully demonstrated the derivation of an intermediate multi-potential population (RA_EBs) from undifferentiated ES cells that preferentially gives rise to pancreatic endocrine insulin-expressing cell types in the presence of SB, and neuronal- and glial-like cell types in the presence of AA or BTC (Section 3 2)

Previous MIN6 studies, by both ourselves and others, have indicated that the GSIS phenotype is relatively unstable, in long-term culture This loss in functional GSIS correlated with changes in morphology, increased proliferation and increased AP activity,

suggesting that the loss of GSIS may be due to de-differentiation of the high passage MIN6 cells (Kayo et al, 1996, Gammell, 2002, O' Driscoll et al, 2004a) In this study, we used microarray, bioinformatics and qPCR technologies to investigate the gene expression changes associated with this loss of GSIS Lossed/reduced levels of certain transcripts associated with the mature β cell, in high passage cells, together with increased levels of neuron-/glia- associated mRNAs, are suggesting that, with time in culture, MIN6 cells may revert to an early, poorly-differentiated, "precusor-like" cell type This observation is supported by increased expression of the stem cell marker, alkaline phosphatase The MIN6 cells (low and high passage) were therefore considered a potential model for (a) identifying crucial β cell markers and (b) studying the molecular mechanisms involved in loss of GSIS and hypothetical β cell de-differentiation (Section 3 3)

4 2 Feeder-free Culture of Mouse ES Cells

The *in vitro* differentiation process begins with a non-differentiated, pluripotent ES cell line Generally *in vitro* studies with mouse ES cells include maintenance on feeder layers (mouse embryonic fibroblasts) However, some groups have demonstrated that inclusion of LIF at high concentrations can maintain pluripotency of ES cells in the absence of feeder layers (Smith et al, 1988, Nichols et al, 1990, Burdon et al, 1999)

In this study ES-D3 cells were cultured on gelatin, in the absence of feeder layers and in the presence of 1400 units/ml (700 pM) LIF It has been shown previously that long-term culture of mouse ES cells at > 50 pM LIF, allows for a more homogenous, stable population of ES cells (Berrill et al, 2004) The ES-D3 cells exhibited characteristics of pluripotency i e growth in tight multi-layered colonies (Figure 3 1 2 A & B), expression of alkaline phosphatase activity (Figure 3 1 3 A and Figure 3 2 2 A & B) and expression of Oct 4 (Figure 3 2 7 A and Figure 3 2 8 A) (Prelle et al, 2002) However, these cells also express a broad range of mRNAs for developmental markers (nestin, islet 1, pax 6) and lineage specific markers (β III tubulin, MBP) (Figure 3 1 4 and Figure 3 2 3) The presence of pancreatic markers in undifferentiated cells has previously been reported by Lumelsky et al (2001), who demonstrated that removal of ES cells from feeder layers in the presence of LIF triggered expression of Pdx1 mRNA, and Soria et al (2001b), who demonstrated that somatostatin and glucagon mRNAs may be detected in undifferentiated ES cell cultures Ward et al (2004) also demonstrated that the neuronal marker, NF-L is expressed in a variety of undifferentiated ES cell lines Directed differentiation, therefore, requires the repression of inappropriately expressed genes, in addition to the induction of lineage specific markers

Upon removal of LIF and formation of EBs, there is a decrease observed in alkaline phosphatase activity in the cells constituting the outer layer of the EB, indicating the initiation of differentiation (Section 3 1 2 and Section 3 2 2) Previous work (Berrill et al, 2004, Sandstorm et al, 2000) showed a direct correlation between alkaline phosphatase activity and increasing LIF concentrations

4 3 Characterization of the Insulin-Containing Cells Derived using an Adapted Version of the Original Lumelsky-based Protocol

ES-derived cell types generated in recent protocols (Lumelsky et al, 2001, Hori et al, 2002, Blyszczuk et al, 2003, Moritoh et al, 2003, Miyazaki et al, 2004, Shi et al, 2005) have remained largely uncharacterised, apart from the identification of a small proportion of insulin/C-peptide positive cells within isolated sub-populations Some of these protocols have reported the derivation of neuronal phenotypes (Lumelsky et al, 2001, Hori et al, 2002, Miyazaki et al, 2004, Rajagopal et al, 2003, Sipione et al, 2004, Hansson et al, 2004) within differentiated populations whereas a more recent publication has suggested that ES-derived insulin positive cells are actually extra-embyronic in origin (Milne et al, 2005) In light of these findings we investigated the nature of the cells as well as developing another means of establishing the presence of *de novo* synthesized insulin (Section 3 1)

4 3.1 Multi-lineage Transcript Expression in Stage 5 Clusters

We found that the ES-derived stage 5 clusters expressed markers representative of neuronal/glial lineage (GFAP, MBP, β III Tubulin), extra-embyronic lineage (GATA 4, AFP, Pdx1) and pancreatic endocrine lineage (pax 4, insulin I, insulin II, glut2) indicating the presence of multiple cell types in stage 5 clusters (Section 3 1 3) The derivation of such a complex culture may be a result of the nestin selection at stage 3 of the protocol (serum-free, ITSFn-supplemented medium) Nestin has been accepted as a marker of neuronal progenitor cells, however the cumulative data from recent studies suggest that nestin expression in ES-derived progeny represents a property of multi-lineage progenitors that have the potential to develop into neuroectodermal, endodermal and mesodermal lineages (Wiese et al, 2004) The initial Lumelsky study had reported the derivation of cells expressing all endocrine hormones (Lumelsky et al, 2001), however, both the present study and others (Sipione et al, 2004) failed to detect PP or glucagon mRNA expression in the stage 5 ES-derived cultures This may be due to slight variations in the protocols such as lack of feeder cells, handling (trypsinization at certain stages), cell type

4 3 2 Stage 5 Clusters May Contain Cells of an Extra-Embryonic Lineage

Many of the published differentiation protocols rely on the expression of endodermal markers in determining pancreatic β cell differentiation However, it must be kept in mind that extra-embyronic structures such as the yolk sac are also derived from the endoderm (Milne et al, 2005) High expression levels of markers such as Pdx1 and PPI in ES-derived cells (as observed in this and other studies) are common to both extra-embryonic and pancreatic β cell differentiation In addition to these markers we observed an increase in extra-embryonic markers (AFP and GATA 4) and β cell markers (insulin I and glut2) in the stage 5 clusters indicating the presence of both lineages within differentiated cultures (Section 3 1 3) Insulin I is considered a more definitive marker of the β cell than insulin II, the reason being that insulin I expression is restricted to β cells whereas insulin II expression is more widespread (Kahan et al, 2003) If extra-embryonic differentiation was dominating over that of pancreatic differentiation we would expect to see an increase in Oct 4 expression rather than a decrease between stage 1 and stage 5 of the differentiation protocol Oct 4 is expressed at increased levels in the primitive endoderm compared to undifferentiated cells (Milne et al, 2005)

4 3 3 Stage 5 Clusters Contain cells of a Neuronal/Glial lineage

After ITSFn nestin-positive selection at stage 3 of the differentiation protocol, neuronal-like projections become visible in the stage 4 and stage 5 cultures (Section 3 1 1) These morphological changes coincided with an up-regulation in oligodendrocyte marker, MBP and astrocyte marker, GFAP transcript expression Neuron-specific β III tubulin was also expressed in these cultures (Section 3 1 3) The occurrence of neuronal-like phenotypes at this time point of the differentiation protocol was previously reported by Lumelsky et al (2001) and Sipione et al (2004) It was suggested that the introduction of ITSFn (stage 3) and FGF-2 (stage 4) induced the commitment of ES-derived cells to a neuronal fate and that any further processes to induce pancreatic differentiation after this time-point activated apoptotic pathways (Kania et al, 2004)

4 3 4 Stage 5 Clusters Lack Evidence Supporting Apoptosis

ES-derived cells did not show any sign of apoptosis RT-PCR analysis demonstrated that there was no change in bax or caspase 3 mRNA expression between stage 1 and stage 5 (Section 3 1 3) Stage 5 cell nuclei were round and robust rather than small and fragmented (as would be expected during apoptosis) (Section 3 1 4) This finding contradicted that of recent publications (Sipione et al, 2004, Hansson et al, 2004) The discrepancy may be due to a modification in protocol at the end of stage 3 In this protocol all cells present at the end of stage 3 were maintained in culture without disruption and carried through to stage 4 In other studies (Lumelsky et al, 2001, Sipione et al, 2004, Hansson et al, 2004), cell cultures were dissociated at this point and allowed to reattach before entering stage 4, perhaps putting the cells under stress and inducing apoptosis or perhaps selecting for a subpopulation which becomes apoptotic

4.3 5 Insulin/C-peptide Protein Expression in Stage 5 Cultures

The identification of a β cell phenotype within differentiated populations has proven to be a challenge to all research groups Commonly used supplements such as B27 and N2 include a high insulin content contributing to an overall insulin concentration in the culture medium exceeding that of physiological circulating levels by 1000-fold (Rajagopal et al, 2003) It is therefore difficult to ascertain whether insulin-positive cells are a direct result of insulin uptake or genuine *de novo* synthesis and secretion

Immunoreactivity to C-peptide (Rajagopal et al, 2003) as well as assaying C-peptide release (Hansson et al, 2004), in conjunction with that of insulin, have subsequently been employed as a more definitive means of demonstrating authentic β cell function However, in the present study we found that the polyclonal C-peptide antibodies (i e anti-C-peptide 1 and 2) detected C-peptide expression in all ES-derived cells (Figure 3 1 7 and Figure 3 1 8), whereas insulin protein expression was only detected in an isolated population of cells (Figure 3 1 6) Using the same antibodies, Kahan et al had found that the anti-C-peptide antibodies detected significantly more positive cells in a region than either of two monoclonal antibodies to insulin (Kahan et al, 2003)

In the present study we drew our conclusions from immunofluorescence (IF) data on the pattern and localization of staining, including positive and negative biological controls We found that C-peptide 1 and C-peptide 2 staining in stage 5 ES-derived cells was not indicative of granular insulin staining as observed in the positive control phenotype, MIN6 (Figure 3 1 7 and Figure 3 1 8) The anti-C-peptide antibodies also significantly stained 'negative' control cell lines, i e stage 1 ES-D3 and mouse mammary 4TI cells Western blot analysis confirmed that the C-peptide 2 antibody recognised a specific band for C-peptide 2 at the expected size (3 kDa) in the MIN6 cell line (Figure 3 1 8 E), however, did not detect a 3 kDa band for C-peptide 2 in negative control cell lines or stage 5 ES-derived cells (Figure 3 1 8 F) The C-peptide 2 antibody does not detect proinsulin by western blot analysis, therefore positive IF staining is not due to unprocessed proinsulin This C-peptide 2 antibody is therefore useful in western blot analysis where one can distinguish between specific and non-specific bands based on molecular weight (Figure 3 1 8 E), however more caution is required for IF/IHC analysis It is our belief, based on these findings, that stage 5 ES-derived cells did not express C-peptide 2 (at a significant level to be detected by western blot) and that apparent C-peptide 2 positivity in this IF study and perhaps in other published IF/IHC studies may be overrepresented significantly in relation to actual levels in ES-derived cell populations

4 3 6 Intracellular Insulin Content of Stage 5 Cultures and *In Vitro* GSIS

We detected approximately 36 ng insulin per mg of protein in stage 5 cultures using a mouse ultra-sensitive insulin ELISA (Figure 3 1 9) This quantity varied from one biological repeat to another Insulin was released from stage 5 cells but not in a glucose dependant manner (Figure 3 1 10) The original Lumelsky protocol reported the derivation of cells containing 145 ng insulin per mg protein These cells were also reported to have secreted insulin in response to glucose in a dose dependant manner, however, subsequent studies using both the original protocol (Sipione et al, 2004) and a modified version of the protocol (Miyazaki et al, 2004) were unable to demonstrate functional GSIS by ES-derived cells

Hansson et al (2004) demonstrated that ES-derived cells generated using the Lumelsky protocol released insulin in the presence of glucose However, the insulin release was variable and did not follow the release kinetics associated with genuine β cells It was also found that C-peptide, a bi-product of insulin synthesis was not secreted along with insulin, indicating that any insulin released was not *de novo* synthesised

4 3 7 Lack of Evidence Supporting *De Novo* Insulin Synthesis in Stage 5 Clusters

In a further effort to definitively identify *de novo* insulin biosynthesis in cultured cells, we developed a novel assay based on metabolic labelling (ML) followed by a modified radio-immunoassay (MRIA) In summary, the ML step radioactively labels all newly translated, cysteine-containing proteins (6 residues in insulin) by inclusion of ^{35}S-L-cysteine in the culture medium The subsequent MRIA specifically binds insulin present in the cell lysate - both labelled and unlabelled – the former of which is detected by scintillation counting

To develop and test the MRIA we used the mouse insulinoma cell line, MIN6, which actively synthesizes and secretes insulin. We were particularly interested in determining the sensitivity limits of the assay given its intended use on ES cell-derived cultures that would be expected to synthesize very small quantities, if any, of the hormone We first established that 6 hours was sufficient time for the ML step (Section 3 1 6 and Figure 3 1 11) It should be noted that this is based on results generated using MIN6 cells and that insulin synthesis and turnover (secretion) rates are likely to be different depending on cell type A second concern addressed was the specificity of the MRIA Although the assay uses a commercial insulin ELISA plate for insulin capture (immunosorption), it does not include binding of the secondary insulin-specific, enzyme-linked antibody To discount the possibility of non-specific protein-antibody interaction, we opted to include a titration step based on competitive antigen binding using non-labelled (cold) insulin By increasing the amount of cold competitor in the binding solution we demonstrated concentration-dependent reduction in the radioactive signal bound to the plate (Figure 3 1 12 B & C and Figure 3 1 13 A) This also served the purpose of establishing the background radioactive signal resulting from non-specific protein-plate binding events

Initial experiments revealed two further challenges in successful implementation of the MRIA Firstly, the background measurement was too high for sensitive applications This was most likely caused by non-specific binding of 'sticky' proteins, such as albumin, to the plate Secondly, there was a large standard deviation across replicate sample measurements Both these problems were overcome by incorporating an ultra-filtration step to remove proteins greater than 50 kDa from the sample lysates and by pre-blocking the wells with non-labelled cell lysate (Section 3 1 6 and Figure 3 1 12)

To investigate the sensitivity of the MRIA (and further confirm the insulm-specificity), a standard curve was generated by measuring the bound signal (cpm) detected in dilutions of labelled MIN6 lysate and plotting this against the concentration of *total* insulin in this dilution series (Figure 3 1 13 B) Having established the utility of the assay in detecting insulin biosynthesis in MIN6 cells routinely down to levels below 0 71 ng/ml, we were unable to detect *de novo* synthesis in lysate from stage 5 cells that contained **88** ng/ml insulin (as determined by standard commercial ELISA) (Figure 3 1 14) Although it could be argued that the rate of insulin translation may be very low in these cells, it is unlikely to account for the high concentrations indicated by ELISA and the failure to detect any reduction in radioactive counts at high concentrations of competitor in the MRIA This is further supported by the IF results that reveal a very small number of insulin-positive cells with a punctate staining pattern indicative of β cell granules for storage of protein (and possibly lower turnover) The IF analysis also reveals a large amount of faint, more diffuse, non-granular insulin staining, not usually associated with a β cell phenotype Taking these separate pieces of evidence together we propose that the diffuse staining seen in IF analyses is responsible for the high levels of insulin detected in stage 5 cell lysates by ELISA, but it is not cell-derived as it is not detected by MRIA. These findings are in agreement with previous reports suggesting an insulin uptake from the culture medium to be responsible for the high insulin content in cells exposed to similar 5 stage differentiation protocols

4 3 7 1 Factors Affecting the Overall Performance of the MRIA

The percentage of radio-labelled versus unlabelled insulin present in the cell lysates is unknown, therefore, the MRIA assay does not facilitate absolute quantification of labelled *de novo* synthesised insulin in cell cultures Determination of a limit of detection for the MIN6 sample required a standard curve (as shown with the MIN6 dilution series, Figure 3 1 13) Inter-assay variations necessitate the generation of a new standard curve with each independent assay These inter-assay variations, which affected assay performance were as follows

- Specific activity of the labelled amino acid substrate
- Cell line to cell line variations such as protein turnover
- Lysis efficiency
- Exposure time to the ^{35}S-L-cysteine/metabolic labelling time

Irrespective of these variables it is possible to identify *de novo* insulin synthesis using the MRIA assay based on competition between labelled insulin and cold competitor (Figure 3 1 14)

4 4 Two-Stage Differentiation Protocols

Induced differentiation of endoderm-derivatives from ES cells has proved more difficult than the induced differentiation of ecto- and meso-dermal lineages (Schuldiner et al, 2000, Mfopou et al, 2005) Thus, the application of candidate inductive signalling compounds in ES differentiation cultures to enrich for endoderm derivatives is of current interest In this study, a number of compounds previously linked to endocrine differentiation or functional enhancement of β cells were introduced to the culture medium of differentiating ES-D3-derived EBs (Section 3 2) We used an *in vitro* 3-dimensional EB differentiation system, which in many respects mimics the early stages of development (Schuldiner et al, 2001, Czyz & Wobus, 2001), to investigate the effects of these compounds on temporal gene expression and lineage specification Models of directed differentiation were developed and investigated in parallel with a model of spontaneous differentiation, with particular emphasis placed on the successful derivation of pancreatic endocrine cell types within complex differentiated cultures (see schematic, Figure 4 4 1)

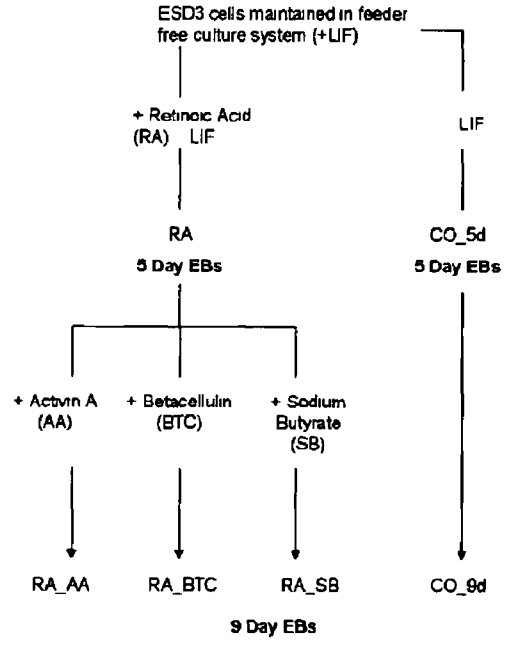


Figure 4 4 1 Two-stage differentiation protocol used in differentiation studies LIF, leukaemia inhibitory factor, CO, control, RA, retinoic acid, AA, activin A, BTC, betacellulin, SB, sodium butyrate, EBs, embryoid bodies (Repeat of Figure 2 3 1)

4 4.1 Formation of EBs in the presence of Retinoic Acid

Differentiation of mouse ES cells in the presence of retinoic acid has previously been shown to induce both neuronal/glial differentiation (Bain et al, 1995, Schuldiner et al 2001, Tang et al, 2002) and endodermal differentiation (Vetere et al, 2003, O Driscoll et al 2004, Micallef et al, 2005) The concentration of RA, the time-point of introduction and the culture type i e monolayer versus suspension, varied from one study to another In the present study, ES-D3 cells were seeded overnight in suspension culture prior to being exposed to 100 nM RA for 72 hours (stage 1 differentiation) (Section 3 2 1) Previous results within our laboratory had demonstrated that these conditions were suitable for induction of endodermal differentiation (Gammell et al, 2002)

At day 5, after 72 hours in the presence of RA, neuronal-like projections extended from the edge of RA-treated EBs when plated These outer cells appeared to have a decrease in alkaline phosphatase activity when compared to the inner cells of the RA-treated EBs The outer layer of EBs in the untreated CO_5d culture also appeared to have a decrease in alkaline phosphatase activity but not to the same extent as that observed in the RA-treated EBs The pattern of staining in both cultures indicated that the cells constituting the outer EB layer had started to differentiate (Section 3 2 2)

4 4 2 Multi-lineage Marker Expression in ES-derived cultures

In vitro cultivation of ES cells as EBs results in derivatives representative of all three primary germ layers (Doetschman et al, 1985, Rohwedel et al, 1996) Given the possibility of a broad range of lineage outcomes and the fact that many markers are expressed in multiple lineages, specific combinations of marker mRNAs expected to be expressed or not expressed, were evaluated at various time points in our 2-stage differentiation models, to determine differentiation towards a specific lineage (D'Amour et al, 2005) The choice of markers for the analysis of such regulated differentiation of cells was based on the outcome of previous studies which demonstrated that ES cells spontaneously differentiate into pancreatic endocrine (Kahan et al, 2003), extra-embryonic (Milne et al, 2005), neuronal (Wiese et al, 2004) and myogenic cell types

(Karbanova et al, 2002) Apoptotic marker expression was also assessed to determine the effect of extra-cellular compounds on cell viability (Section 3 2 3)

4 4 2 1 Multi-lineage Marker Expression in the CO_d5 Culture and the RA culture after Stage 1 Differentiation

Oct 4, a characteristic marker of pluripotent stem cells, was strongly expressed at both an mRNA level and protein level in the ES-D3 culture (Section 3 2 3 and Section 3 2 4) After stage 1 differentiation, the transcript level was down-regulated slightly in both the CO_5d culture and the RA culture, however, there was no visible sign of down-regulation at a protein level This minimal reduction in Oct 4 expression was not surprising as it had been previously shown that Oct 4 mRNA levels in ES cells are only partially diminished in LIF free conditions after 5 – 6 weeks culture (Berrill et al, 2004)

The CO_5d culture expressed a broad range of markers at both mRNA level (Nestin, NF-L, MBP, β III Tubulin, myogenin, desmin, islet 1, Pdx1, ngn3, beta2, pax 6, nkx2 2, PC1, PC2, somatostatin, PPI, AFP, bax and caspase 3) (Table 3 2 1) and protein level (NF_L, β III tubulin, myogenin, desmin, Pdx1, glut2, somatostatin and PP) (Table 3 2 4) Primary differentiation in the presence of RA enhanced expression of the multi-potential marker, nestin (Zulewski et al, 2001, Wiese et al, 2004), at both RNA and protein level, early transcription factors common to both neuronal/glial and pancreatic development (pax 6 and islet 1) at mRNA level (Hunziker et al, 2000, Kania et al, 2004, Moritoh et al, 2003), the oligodendrocyte marker, MBP, at mRNA level, the neuronal markers, NF-L, at both mRNA and protein level, and β III Tubulin, at protein level, the proinsulin processing enzyme, PC2 at an mRNA level, and the glucose transporter, glut 2, at mRNA and protein level

RA slightly repressed expression of β cell markers (insulin 1, insulin II and Pdx1) at mRNA level only (Section 3 2 3 1) The repression of Pdx1 coincided with the induced expression of Shh mRNA (a known inhibitor of pancreas induction (Soria et al, 2001b, Mfopou et al, 2005)) The difference in our findings compared to Micallef et al (who reported that RA induced Pdx1-positive endoderm expression in differentiating mouse ES

cells) (Micallef et al, 2005), is possibly due to variations in RA concentration, timing of its addition to the culture medium, and the use of a different mouse ES cell line A high level of ngn3 mRNA expression was detected in both the RA culture and the CO_5d culture Ngn3 is a pancreatic transcription factor expressed at an early stage in cells differentiating toward an endocrine fate (Habener et al, 2005) The range of transcript and protein expression identified in the RA culture would suggest a mixed population of multi-potential precursors Elevated Pdx1 and PPI mRNA expression in the spontaneously formed CO_5d culture would suggest the presence of endodermal cell types, however, unregulated secondary differentiation of the CO_5d cultures did not result in the derivation of endocrine cell types (CO_9d)

Secondary differentiation of the RA culture in the presence of other compounds altered the specification of lineage fate, resulting in neuronal- and glial-specific transcript expression prevailing in the presence of AA or BTC (Section 4 4 2 2) and pancreatic endocrine marker expression dominating in the presence of SB (Section 4 4 2 3) These findings support those reported by Seaberg et al (2004) who identified the presence of a multi-potential population in the adult pancreas, that can generate both neural and pancreatic lineages

4 4 2 2 Preferential Differentiation of the RA culture along a Neuronal/Glial Lineage in the Presence of AA or BTC

Neuronal and glial differentiation is characterized by the development of nestin-expressing precursor cells in the RA culture and subsequent down-regulation of nestin expression in the RA_AA and the RA_BTC cultures, coinciding with an up-regulation of the astrocyte-specific transcript, GFAP (Section 3 2 3) The co-expression of the nestin protein (in a small percentage of both the RA_AA and the RA_BTC cultures) with the lineage-specific proteins, GFAP, β III tubulin and NF-L, is known to occur at time points that define the onset of cell lineage specification preceding terminal differentiation *in vitro* (Section 3 2 4) (Wiese et al, 2004) The profile of somatostatin mRNA expression was similar to that of neuronal and glial markers in that it was expressed at an intermediate stage in the RA culture and maintained at a high level of expression in the

RA_AA and the RA_BTC cultures (Section 3 2 3) Somatostatin protein was detected in subpopulations of both these cultures (Section 3 2 4) Somatostatin is most commonly known as a marker of the endocrine islet δ-cell type (Soria et al, 2001b) but is also expressed in the brain (Hokfelt et al, 1975, Johansson et al, 1978, Jarvi et al, 1987)

AA and BTC have previously been identified as inducers of β cell differentiation (Mashima et al, 1996, Huotari et al, 1998, Miyagawa et al, 1999), however, given the substantial phenotypic similarity between pancreatic and neuronal/glial cell types it is understandable that the same compounds may regulate both developmental pathways (Lumelsky et al, 2004, Stoffel et al, 2004) (Section 1 4 5) In the present study, it would seem that in combination with RA, both AA and BTC independently gave rise to neuronal- and glial-like phenotypes

4 4 2 3 Preferential Differentiation of the RA culture along a Pancreatic Endocrine Lineage in the Presence of SB

SB is known to induce β cell differentiation (Otonkoski et al, 1997) and increase insulin content (Philippe et al, 1987, Bartholomeusz et al, 1989), but in turn, induce cell death (Soria et al, 2001) In this study, pancreatic differentiation in the RA_SB culture is characterized by the expression of pancreatic developmental control genes at day 5 (Pdx1, ngn3, beta 2, pax 6, nkx2 2), followed by the up-regulation of β cell specific genes (insulin I, insulin II and glut2) at day 9 (Section 3 2 3) The down-regulation of ngn3 between the RA culture at day 5 and the RA_SB culture at day 9 was indicative of islet cell differentiation Ngn3 initiates the islet differentiation program but its expression is diminished before terminal differentiation of endocrine cells (Smith et al, 2005) Semi-quantitive PCR demonstrated an induced expression of PPI mRNA in the 9 day RA_SB culture (Figure 3 2 3 V), however, qPCR was much more effective at quantifying the dramatic induction of insulin I, insulin II and glut2 mRNA expression within this culture (Figure 3 2 4) Shh expression was repressed in the RA_SB culture, which in turn promoted Pdx1 expression (Section 3 2 3) Pdx1 is known to play a role in pancreas maturation, β cell fate specification and insulin gene expression (Soria et al, 2001b) Subsequent IHC analysis of the RA_SB culture demonstrated that Pdx1, glut2 and insulin

mRNAs were translated into protein over the course of our 2-stage differentiation protocol (Section 3 2 4)

Markers characteristic of the γ and δ endocrine islet cells were also detected in the RA_SB culture mRNA expression of PP was detected in the early stages of differentiation (RA culture) and its expression was maintained in the presence of SB (RA_SB culture) (Section 3 2 3) Both **PP** and somatostatin proteins were detected in subpopulations of the RA_SB culture by IHC analysis (Section 3 2 4) The expression levels of these proteins in ES-derived cultures do not correlate with transcript expression For instance, somatostatin was detected at protein level in the RA_SB culture, however, it was not detected at mRNA level There are two possible explanations for these results (i) low level transcript expression of both genes may not have been detected in specific samples by RT-PCR or (ii) the individual antibody molecules recognised epitopes that may be shared among different proteins

The transcription factor, pax 4 and the endocrine α cell hormone, glucagon were not detected at mRNA level at any point over the 2-stage RA_SB differentiation protocol (Section 3 2 3) Although we do not know the reason for this, we speculate that the exogenous RA_SB treatment can prime pancreatic progenitors during EB formation, but may suppress some endocrine specific genes at the latter stages of differentiation Pax 4 appears in the final stages of the cascade of transcription factors thought to be involved in the endocrine pancreas differentiation programme (Figure 1 2 2)

In the present study, it would appear that in combination with RA, SB gave rise to pancreatic endocrine-like cell types, including insulin-positive cells This novel 2-stage differentiation protocol warranted further investigation (Section 4 4 3 – Section 4 4 6)

4 4 2 4 C-peptide 1 and C-peptide 2 Expression in ES-D3 and ES-derived Cultures

Immunoreactivity to C-peptide, in conjunction to that of insulin, has been used as a definitive means of proving *de novo* insulin synthesis in ES-derived cells (Kahan et al, 2003, Miyazaki et al, 2004, Segev et al, 2004) In this study, C-peptide 2 expression was

detected in ES-D3 cells C-peptide 1 and C-peptide 2 expression was detected in both the RA and CO_5d cultures after stage 1 differentiation The expression of both these proteins appeared to be down-regulated in the 9 day cultures including the RA_SB culture Despite C-peptide being a product of insulin bio-synthesis, the trend in C-peptide 1 and C-peptide 2 expression did not correlate with the trend in insulin expression (Section 3 2 4)

Insulin/C-peptide co-localization analysis was performed on cells from the RA_SB culture (Section 3 2 5) The cells were shown to express insulin, C-peptide 1 and C-peptide 2, however, the C-peptide staining did not co-localize with that of insulin C-peptide 1 and C-peptide 2 were also detected in the majority of cells in the CO_9d culture, however these cells were negative for insulin These IF results verify previous findings in Section 3 1 demonstrating that these C-peptide antibodies, when used for immuno-analysis, over-exaggerate actual protein levels

Taking both the IF (Section 3 1 4 and Section 3 2 5) and IHC (Section 3 2 4) results into account it is apparent that immuno-analysis using these particular antibodies is unreliable for determining *de novo* insulin synthesis

4 4 3 *De Novo* Insulin Synthesis in the ES-derived RA_SB Culture

Other protocols used for the derivation of insulin-expressing cell types have been long, complex (Lumelsky et al, 2004, Hori et al, 2002), often require genetic modification (Blyszczuk et al, 2003, Miyazaki et al, 2004), the addition of exogenous insulin to the culture medium (Lumelsky et al, 2004, Hori et al, 2002, Blyszczuk et al, 2003, Miyazaki et al, 2004) and result in the derivation of apoptotic bodies (Rajagopal et al, 2003) The insulin-positive cells derived from such protocols may be a direct result of insulin uptake upon apoptosis rather than *de novo* synthesis (Rajagopal et al, 2003, Hansson et al, 2004) The short 2-stage RA_SB differentiation protocol developed in this study did not require exogenous insulin for the derivation of insulin-positive cell types, indicating that any insulin expressed was *de novo* synthesized (Figure 3 2 10) LSCM analysis of RA_SB

insulin-positive cells demonstrated a punctate staining pattern indicative of β cell granules

4 4 4 Lineage Specification of Insulin-Positive cells in the RA_SB Culture

Milne et al has suggested that insulin-positive cells derived from ES cells are extra-embryonic in origin (Milne et al, 2005) Extra-embryonic endodermal cells express elevated levels of Oct 4 and AFP mRNA In our study, the RA_SB culture containing insulin-positive cells had an elevated level of AFP mRNA expression, however, a decrease in Oct 4 mRNA was observed between undifferentiated ES-D3 and the RA_SB culture coinciding with an increase in insulin I, insulin II and glut2 mRNA (Figure 3 2 4), which is more consistent with β cell differentiation than extra-embryonic lineage specification

4 4 5 Apoptotic Bodies Are Not Detected in the RA_SB Culture

As differentiation proceeded and cells within the RA_SB culture became committed to an endocrine pancreatic fate, we observed a decrease in proliferation capacity (as illustrated by cell counts, Figure 3 2 11) Previous studies have suggested that SB induces apoptosis (Soria et al, 2001), however, in the present study there was no change observed in apoptotic marker expression (Figure 3 2 3) and no sign of small fragmented nuclei (a characteristic of apoptosis) in the RA_SB culture (Figure 3 2 10), suggesting that the reduced cell number in this culture compared to the CO_9d culture was not related to apoptosis induced by extra-cellular compounds The low cell number in the RA_SB culture was most likely due to the inhibition of DNA synthesis and cell division by SB (Hagopian et al, 1977) In general, a reduction in proliferation is inversely proportional to differentiation (Soria et al, 2001b)

4.4.6 An Investigation of Alternative RA_SB Differentiation Protocols

The cell yield in the RA_SB culture was approximately 7-fold lower than the cell yield attained from the CO_9d culture. An alteration in protocol (Figure 4.4.2) was necessary to improve cell yields and in turn increase the number of ES-derived insulin-expressing cells.

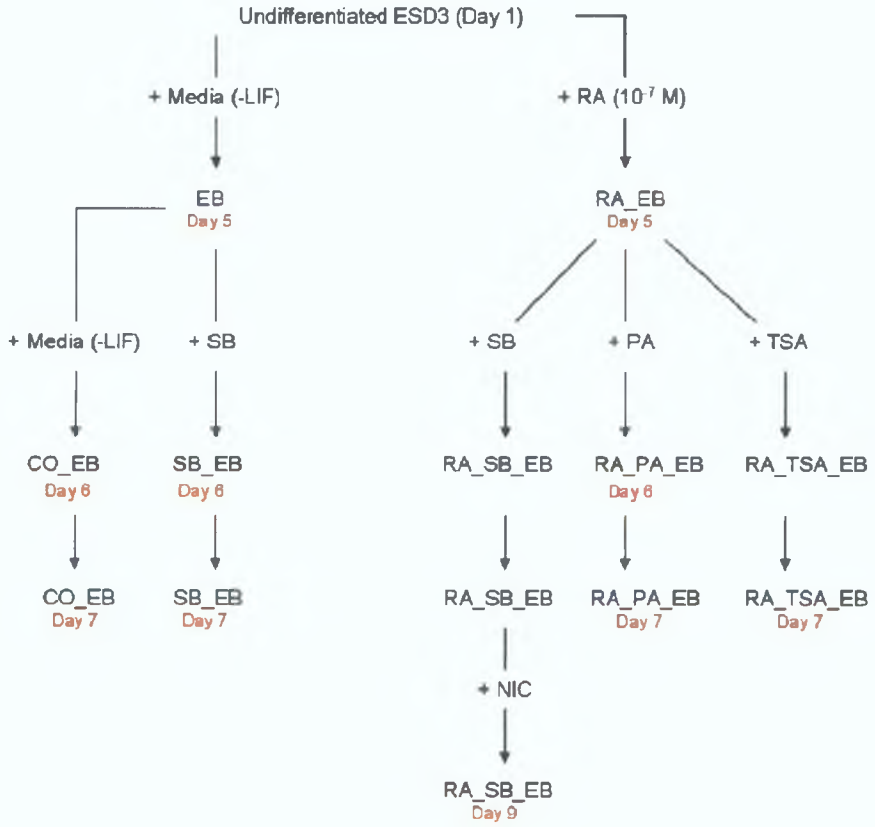


Figure 4.4.2 Alternative RA_SB differentiation protocols. LIF, leukaemia inhibitory factor; CO, control; RA, retinoic acid; SB, sodium butyrate; EBs, embryoid bodies; PA, pentanoic acid; TSA, trichostatin A. (Repeat of Figure 2.3.2)

4 4 6 1 Tertiary Treatment with Nicotinamide

It was established that insulin I and insulin II transcripts were increased > 30-fold by day 7 of the 2-stage RA_SB differentiation protocol (Figure 3 2 12) It was also demonstrated that transcript expression was maintained for at least four days after removal from the stage 2 differentiation treatment Based on these results it was hypothesized that (a) removal of the RA_SB culture from SB supplemented media by day 7 of the protocol and (b) introduction of the culture to a tertiary treatment such as nicotinamide, may allow for an overall improvement in cell yield and may also encourage translation of the insulin transcripts, allowing for higher insulin protein expression (see schematic, Figure 4 4 2)

Nicotinamide was chosen as a tertiary treatment based on a previous study demonstrating that a combination of SB and NIC increased insulin content of islet-like cell clusters by 90-fold (Otonkoski et al, 1999) However in the same study, the presence of SB caused a severe reduction in cell number Independently, NIC was found to enhance insulin content and encourage β cell differentiation without affecting cell number (Sjoholm et al, 1994, Otonkoski et al, 1999)

In the present study, there was no improvement in cell yield observed in the RA_SB_NIC culture (Figure 3 2 13) However, insulin I transcript expression was twice that observed in the original RA_SB culture and insulin II transcript expression was also noticeably higher (Figure 3 2 14)

4 4 6 2 Omission of RA from Stage 1 Differentiation

It was previously noted that (a) the CO_5d culture had a higher level of Pdx1 transcript expression than the RA culture (Figure 3 2 3) (b) the RA culture consisted of a mixed population (Section 3 2 3) (c) cell numbers were lower in the RA culture compared to the CO_5d culture (Section 3 2 7) It was therefore hypothesized that the omission of RA from stage 1 differentiation may allow for a higher percentage of endodermal cell types at

day 5 thus increasing the percentage of cells that would commit to a pancreatic endocrine fate in the presence of SB (stage 2 differentiation) See schematic, Figure 4 4 2

The omission of RA from stage 1 differentiation allowed for a dramatic increase in cell yield (Figure 3 2 13) as well as a dramatic increase in insulin II transcript expression (Figure 3 2 14 B) However, insulin I transcript expression was extremely low in these cultures (Figure 3 2 14 A) These results demonstrated that the low cell yield observed in the original RA_SB protocol (Section 3 2 7) was due to the combined effect of RA and SB, not SB alone

4 4 6 3 Pentanoic Acid or TSA as Alternatives to SB

Pentanoic acid and TSA were chosen as alternatives to replace SB in stage 2 of the differentiation protocol (Figure 4 4 2) It was thought that these two agents may induce pancreatic endocrine differentiation in a similar manner to SB without inhibiting growth rate (to the same extent as SB)

TSA is similar to SB in that it is a HDAC inhibitor This class of compounds inhibit histone deacetylase allowing histone hyperacetylation to occur Hyperacetylation leads to chromatin relaxation and modulation of gene expression resulting in high transcription of DNA and protein biosynthesis (Philippe et al, 1987, Gan et al, 2005) Pentanoic acid is similar to SB in that it is a short chain carboxylic acid Liu et al (2001) demonstrated that pentanoic acid had a similar effect to SB on protein biosynthesis whilst having less of an effect on growth suppression

TSA and PA did not improve cell yields (Figure 3 2 13) nor did they improve insulin II transcript expression (Figure 3 2 14 B) However, TSA did allow for an insulin I transcript expression level similar to that observed in the original RA_SB culture (Figure 3 2 14 A)

The alterations in the RA_SB differentiation protocol discussed in Section 4 4 6 1 – Section 4 4 6 3 were not suitable for improving the original RA_SB differentiation protocol

4 5 The MIN6 Cell Line as a Potential Model for Studying β Cell De-differentiation

Great advancements have been made in the study of β cell biology by the development of cultured β cell lines (Poitout et al, 1996) These lines are preferable over isolated islet material in that they offer a pure population with which to work One of the most commonly studied clonal β cell lines is MIN6 This cell line maintains accurate β cell function at early passages i e the cells respond synchronously and physiologically to changes in glucose concentration (Webb et al, 2000)

Previous MIN6 studies, by ourselves (O' Driscoll et al, 2004a) and others (Miyazaki et al, 1990, Ishihara et al, 1995, Kayo et al, 1996), have indicated that the GSIS phenotype is relatively unstable in long-term culture Loss of GSIS function in MIN6 cells has been accompanied by a decrease in the insulin processing enzyme pcsk2 (PC2) and an increase in furin These changes were considered indicative of a de-differentiating system i e well differentiated β cells were directed to a poorly differentiated state by furin cleaved bio-active proteins (Kayo et al, 1996) It is also possible that the loss of differentiation in MIN6 cells is attributed to the overgrowth of a rapidly proliferating, poorly differentiated population such as those identified by Minami et al, 2000

In investigating potential factors associated with the loss of the GSIS phenotype it should be considered that optimised conditions for culturing β cells are typically supra-physiological i e MIN6, 25mmol/l (Miyazaki et la, 1990, Lilla et al, 2003, O' Driscoll et al, 2004a) Continuous exposure to high glucose has wide ranging effects such as increased rates of insulin secretion at low glucose, glycogen deposition and increased proliferation These changes are correlated with changes in the expression of genes associated with differentiation, glucose regulation, insulin processing, proliferation and transcription of islet specific hormones (Kayo et al, 1996, Roche et al, 1997, Roche et al, 1998, Jonas et al, 1999, Itkin-Ansari et al, 2000)

The MIN6 system, i e glucose responsive low passage cells versus glucose non-responsive high passage cells, has the potential to highlight crucial pathways/systems in β cell development It is of utmost importance to identify a means of preserving β cell

function, i e GSIS while in culture, for possible future applications involving the use of cultured replacement tissue To investigate this, a study was performed in our laboratory on MIN6 cells with respect to their GSIS capability and their proliferation rates Upon reaching the proposed 'less differentiated state' (glucose non-responsive), microarray, bioinformatics and qPCR techniques were employed to compare gene expression profiles of fully functional MIN6 cells (glucose-responsive) and the proposed 'poorly-differentiated' MIN6

4 5 1 Loss of GSIS in MIN6 Cells After Long-term Culture

P18 MIN6 cells respond to changes in glucose concentrations, producing an approximate 6-fold GSIS in response over the glucose range tested (0 – 26 7 mmol/l) After continuous culture to passage 40 this GSIS was no longer present (Section 3 3 4) These findings were consistent with other reports such as those by Miyazaki et al (1990), Ishihara et al (1995) and Kayo et al (1996) There is an increase in mature insulin/proinsulin secretion observed at 0 mM glucose This may be attributed in part to the progressive loss of pcsk2 (PC2) with increasing passage, allowing for an increase in unprocessed proinsulin which in turn increases constitutive secretion (Simpson et al, 1995, Kayo et al, 1996) It is also possible that the high passage cells are unable to store insulin in granules as efficiently as low passage cells This theory is supported by findings by Kayo et al, who reported the down-regulation in chromogranin A expression in high passage MIN6 cells Chromogranin A is a matrix constituent of secretory granules in neuroendocrine cells (Zimmer et al, 1999) In the present study scg3, a granin family member, also associated with secretory granules, was down-regulated in high passage MIN6 cells along with other transcripts associated with insulin processing and secretion (Section 4 5 3 1) Human islets show a marked decrease in storage granules when cultured at high glucose levels for prolonged periods (Bjorklund & Grill, 1999)

4 5 2 Phenotypic Changes Correlating with the Loss in GSIS After Long-term Culture

Low passage glucose-responsive cells typically grow in multilayered clumps facilitating cell-to-cell interactions and formation of gap junctions High passage glucose non-

responsive cells do not form clumps They grow in a more conventional monolayer pattern resulting in a loss in cell-to-cell contact (Section 3 3 1) Previous studies have reported disruptions in GSIS when MIN6 cells are unable to interact and form gap junctions (Linzel et al, 1988, Meda et al, 1991, Vozzi et al, 1995)

Glucose non-responsive high passage MIN6 cells proliferate at almost double the rate of glucose responsive low passage cells The low passage cells reach a plateau after 6 days at which point the clumps have reached their maximum size However, high passage cells continue to grow until the entire tissue culture surface is covered (Section 3 3 2) Other studies have found that in general, differentiation is inversely proportional to proliferation i e differentiated cells exhibit a lower proliferate capacity than undifferentiated cells (Soria et al, 2001b) A study by Zimmer et al (1999) indicated that growth arrest of a proliferating β cell line stimulated the expression of genes associated with β cell function and resulted in a phenotype that more closely resembled that of the mature terminally differentiated, β cell

High passage glucose non-responsive MIN6 cells were found to express a considerable amount of alkaline phosphatase compared to their low passage counterparts (Section 3 3 3) The difference in alkaline phophatase activity in the two cell populations is indicative of variable differentiation status In stem cell studies, a high level of alkaline phosphatase expression is characteristic of undifferentiated pluripotent stem cells, whereas a decrease in this expression is consistent with differentiation (Lumelsky et al, 2001, Berrill et al, 2004) In the case of MIN6 cells, the increase in alkaline phosphatase activity after long-term culture suggests a de-differentiating process

4 5 3 Microarray Profiling of Gene Expression in High (glucose non-responsive) and Low (glucose responsive) MIN6

Microarray techniques allow for large scale gene expression profiling, something which is not possible with conventional PCR techniques Gene expression profiles can be compared to phenotypic changes and the resulting information can be used to identify novel pathways that warrant further investigation Microarray technology has previously

been used in the field of β cell research to demonstrate the glucose regulation of secretory and metabolic genes (Webb et al, 2000) and the effect of growth arrest on cultured β cells (Zimmer et al, 1999)

In this study almost 1000 genes were considered significantly, differentially expressed (P<0 01) in high passage MIN6 compared to low passage MIN6 Eighty-eight of these genes were over-expressed 2-fold or more in the high passage MIN6 cells whereas 185 genes were down-regulated by the same factor in the high passage compared to the low passage MIN6 Genes involved in regulated secretion, proliferation, adhesion and development were among those affected (Figure 3 3 6)

Minami et al (2000) reported 10 genes to be differentially expressed in a glucose non-responsive clonal population (m14) compared to a glucose responsive population (m9) Glucokinase (involved in glucose phosphorylation), stanniocalcin/STC (involved in calcium regulation) and dlk/Pref-1(involved in differentiation and growth) were expressed at higher levels in glucose responsive cells Lilla et al (2003) also reported the differential expression of genes involved in metabolism, intracellular signalling and adhesion in glucose responsive (B1) and non-responsive (C3) populations

4 5 3 1 Reduced Levels of Transcripts Associated with Insulin Processing and Secretion

It was hypothesized in Section 4 5 1 that a combination of both ineffective processing and depleted storage granules might be responsible for increased constitutive proinsulin secretion by high passage MIN6 cells in glucose-free conditions Kayo et al previously reported the loss of gene transcripts involved in insulin processing and regulated secretion with increased passage number (Kayo et al, 1996) Our microarray analysis indicated that gene products associated with vesicle formation/transportation and secretion were expressed at a significantly lower level in high passage MIN6 compared to low passage MIN6 (Table 3 3 3) These down-regulated genes are discussed in further detail below

Egr1 is specifically associated with insulin secretion The induction of egr1 by glucose in MIN6 cells was inhibited by EDTA suggesting a dependence on influx of extra cellular Ca2+ (Josefsen et al, 1999) Egr1 was down-regulated 5 5-fold in high passage MIN6 cells Pld1 is known to regulate secretagogue-stimulated insulin release in β cells It was also established that pld1 activity is required for both the first and the second phase of glucose-stimulated insulin release (Hughes et al, 2004) Pld1 was down-regulated 4-fold in high passage MIN6 cells Scg3 is transported to the secretory granules in neuroendocrine cells This protein and the other granin family members are important for condensing secretory proteins to secretory granules with co-aggregation (Hosaka et al, 2004) Scg3 was down-regulated 3 45-fold in high passage MIN6 cells Chgb is an acidic glycoprotein present in neuroendocrine tissue and localised to intragranular components in the RINm5F insulinoma cell line It was previously shown that chgb plays a regulatory role in IAPP and insulin secretion (Bargsten, 2004) Chgb was down-regulated 2 96-fold in high passage MIN6 cells Sgne1 is a neuroendocrine protein necessary for PC2 activity (Lee et al, 2006) PC2 processes proinsulin to mature insulin (Smeekens et al, 1992) Sgne1 was down-regulated 3 55-fold in high passage MIN6 cells Cck is a gastrointestinal hormone and a pancreatic neuropeptide It has been shown to activate cck receptors on the β cell and stimulate insulin secretion (Simonsson et al, 1998) Cck was down-regulated 6 53-fold in high passage MIN6 cells

Our microarray analysis indicated that transcript expression levels for the 'glucose sensory components' such as glut2 and gck where not significantly affected by long-term culture These findings are in agreement with Minami et al (2000) who reported that glut2 expression was similar in a glucose responsive and glucose non-responsive population

4 5 3 2 Changes in Transcripts Associated with Cell Adhesion and Proliferation

As previously discussed in Section 4 5 2, cell-cell interaction is extremely important for GSIS function A comparison of insulin levels, secretion rates and GSIS capabilities of intact islets, dispersed islets, and re-aggregated islets revealed that the dispersed cells and

re-aggregated cells had impaired response to glucose (Linzel et al, 1988) Intracellular contacts in islets are established through adherens junctions, gap and tight junctions

In the present microarray study, transcript levels of a number of cell adhesion molecules including CEACAM-1, CEACAM-2, contactin 1 and E-cadherin were reduced after long-term culture The down-regulation in these genes coincided with the up-regulation of tight junction protein (tjp) and claudin 11 (tight junction transmembrane protein (Hashizume et al, 2004)) The loss of E-cadherin (1 6-fold) in non-responsive β cells is in agreement with a similar observation by Lilla et al (2003) in a non-glucose responsive MIN6 clone (C3) and its association with undifferentiated pancreatic progenitor cells (Jensen et al, 2005) Reelin, known to be involved in cell-cell interactions in the brain, was down-regulated (4 4-fold) in high passage MIN6 cells (Lilla et al, 2003)

The absence of connexins (components of cell-cell communication in islets) from the microarray study may reflect a limitation of microarray technology where probe selection can exclude important transcripts from final gene lists, our experience has been that present calls on the microarray are generally confirmed by RT-PCR Exceptions to this are glut2 (where semi-quantitative RT-PCR, but not qPCR, confirmed microarray results) and dlk1 (where triplicate microarrays indicated > 2-fold down-regulation in high passage MIN6 compared to low, but qPCR suggested an "absent" to "present" call, in this comparison These limited conflicting results between microarrays and qPCR are likely to reflect differing locations of microarray probes and qPCR primers on dlk1 gene transcripts (Table 3 3 2 and Table 3 3 3)

CEACAM1 may have an additional role in the loss of differentiation state and GSIS in cultured MIN6 separate from its role in adhesion, as it can act as a suppressor of cellular proliferation (Singer et al, 2000), thus, its absence at high passages may in fact promote growth Other proliferation related genes were found to be altered in high passage MIN6 including increased (1 96-fold) levels of cdk4 (cyclin-dependant kinase 4) mRNA

expression and decreased levels of dlk1 mRNA, which, respectively, promote (Marzo *et al*, 2004) and attenuate (Friedrichsen et al, 2003) proliferation of β cells This loss of dlk1 in non-responsive cells is in agreement with a study by Minami et al (2000)

The increase in cdk4 (1 96-fold) in high passage MIN6 cells coincided with a decrease in TGFβ1 (5 6-fold) Growth arrest has been induced by TGFβ1 in several cell types, which perhaps explains its down-regulation in the rapidly proliferating high passage MIN6 cells It has been suggested that the inhibitory action of TGFβ1 is mediated by the deactivation of kinase complexes i e cdk4, which may also explain why cdk4 is only up-regulated in the high passage MIN6 when TGFβ1 is decreased (Herrera et al, 1996)

Ornithine decarboxylase (ODC) is a rate limiting enzyme in polyamine metabolism ODC is thought to play a role in polyamine-regulated β cell replication and growth of pancreatic tissue during early development (Morisset & Grondin, 1987, Sjoholm et al, 1994) It may also play a role in pancreatic tissue regeneration (Hayakawa, 1996) It is not surprising to find ODC expressed at a high level in low passage MIN6 cells and down-regulated (1 92-fold) in high passage cells that are thought to have de-differentiated towards a precursor-like cell type

4 5 3 3 Changes in Transcripts Associated with β cell Development and Function

qPCR analysis demonstrated that genes specifically expressed during terminal β cell differentiation and in the mature β cell are down-regulated in high passage MIN6 cells Examples of such genes are pax 4, a transcription factor that is necessary for the specification of β cell fate (Chakrabarti & Raghavendra, 2003, Chae et al, 2004) (Section 1 2 3 5), Pdx1, a transcription factor which plays a role in pancreas maturation, β cell specification and insulin gene expression (Soria et al, 2001), insulin I, the insulin transcript which is specifically expressed in β cells, insulin II, the insulin transcript which is expressed in β cells as well as other tissues (Kahan et al, 2003)

Lilla et al (2003) previously reported that Pdx1 expression is 2 5-fold higher in a non-glucose responsive population (C3) compared to a glucose responsive population (B1) These findings are contrary to what is reported in the present study i e Pdx1 expression was > 2-fold lower in the glucose non-responsive high passage MIN6 cells compared to the glucose responsive low passage MIN6 as determined by qPCR. Earlier studies in our laboratory had determined by semi-quantitive PCR that insulin transcript expression was unaffected by long-term culture (O' Driscoll et al, 2004a), however, in the present study we demonstrate by qPCR that PPI (2 3 – 2 5 fold), insulin I (2 8 – 10 fold) and insulin II (1 9-2 6 fold) are down-regulated in high passage MIN6 cells qPCR is much more effective in accurately quantifying expression levels

4 5.3 4 De-differentiation and/or Emergence of a Poorly Differentiated Sub-Population

The loss of GSIS coupled with increased proliferation, the morphological changes observed, and the increase in the level of the stem cell marker alkaline phosphatase (Section 4 5 2) are all indicative of a major transition occurring within serially passaged MIN6 cells These changes involve functional de-differentiation of the cell population, but whether this is due to de-differentiation of the population as a whole, or over-growth by a faster growing poorly-differentiated sub-population, remains unclear It must be remembered that MIN6 is a transformed cell line, not a completely normal β cell Studies by Lilla et al (pers comm) showing that clones with a high and stable level of GSIS do eventually lose this phenotype suggests a model in which fast-growing poorly-differentiated cells arise, perhaps by mutation, these cells may then over-grow and eventually dominate the differentiated populations Less differentiated β cells, such as RINm5F, have higher proliferative rates than more differentiated β cells This effect has previously been linked to the increased expression of furin in less differentiated β cells However, it is important to note that furin levels were not significantly different in low and high passage MIN6

The reduction in pax 6 levels, coupled with the appearance of nestin, correlates with the less differentiated state of the high passage cells Nestin has been used as a marker of cells that can differentiate towards a β cell-like cell type (Lumelsky et al, 2001) and has more recently been described as a marker of multi-potential precursor cells (Zulewski et al, 2001, Wiese et al, 2004) The increased mRNA levels of other neural markers tend to agree with the hypothesis that the higher passage MIN6 cells may be reverting to a more primitive neuroendocrine-like cell type i e delta-like 1 (dll1) which is essential for proper migration and differentiation of neural crest cells (De Bellard et al, 2002) and involved in controlling development of neurons/glia from neural stem cells (Grandbarbe et al, 2003), cerebellin-1, which is widely distributed in the CNS (Albertin et al, 2000), junctophilin type 3, which is specifically expressed in neurons of the brain (Nishi et al, 2002), and necdin, suggested to mediate terminal differentiation and survival of nerve growth factor-dependent dorsal root ganglion neurons In fact, most of the "development" associated genes in the 2-fold up-regulated genelist (Figure 3 3 6) are actually involved in neurogenesis and brain development

The origins of pancreatic cells and neurons have many crucial transcription factors in common including ngn3, Isl1, NeuroD1/beta 2, pax 6 and nkx2 2 (Edlund, 1999, Kania et al, 2004, Moritoh et al, 2003), many of which are differentially expressed in low and high passage MIN6 cells (Table 3 3 2 and Table 3 3 3) Furthermore, brain tumours have been identified that express insulin and proinsulin, resulting in severe hypoglycaemia for the patient (Nakamura et al, 2001) This suggests that both neuronal and β cells may have similar progenitor cells and that it is these cell types to which de-differentiating MIN6 cells are reverting This hypothesis is strongly supported by two recent reports Seaberg et al (2004) suggested a novel endodermal/ectodermal multi-potential precusor in embryonic development that persists in adult pancreas, Burns et al found that rat neural stem cells differentiated *in vitro*, into an insulin-expressing phenotype, showed functional responses typical of pancreatic β cells (Burns et al, 2005)

The increased expression of nestin and Baspl (brain acid soluble protein 1) mRNA in parallel with decreased levels (2-fold) of GAP43 (growth associated protein 43) gene transcript expression in high passage MIN6, compared to low passage MIN6, may indicate that TGFβ1 has at least a partial role in the de-differentiation towards a "neuroendocrine precusor" cell type (Figure 4.4.3). TGFβ1 levels are lower in high passage MIN6 cells, and under normal circumstances, would have a negative effect (Loo et al, 1995) on nestin levels (4.2-fold up-regulated) and a positive effect on GAP43 levels (2-fold down-regulated). While Baspl and GAP43 can functionally substitute for one another, GAP43 negatively affects Baspl expression (Frey et al, 2000) which may explain why Baspl levels are 3-fold up-regulated in high passage MIN6. As TGFβ1 positively regulates clusterin levels (Jin & Howe, 1997), the reduction in TGFβ1 (5.6-fold) may also explain the unexpected decrease in clusterin levels (6.4-fold), which would possibly have been expected to be elevated in more rapidly proliferating high passage MIN6 cells (Kim et al, 2001). The reduced levels of TGFβ1 are also in keeping with the more rapidly proliferating phenotype of high passage MIN6 as TGFβ1 is a potent growth inhibitor. Early reports by Kim et al (2001) suggested clusterin was involved in pancreatic islet formation during early development as well as islet regeneration and a recent study by the same group demonstrated that the over-expression of clusterin induced differentiation of pancreatic duct lining cells into insulin-secreting cells (Kim et al, 2006). It is therefore not surprising that in our study clusterin is down-regulated in high passage MIN6 cells that have lost β cell function.

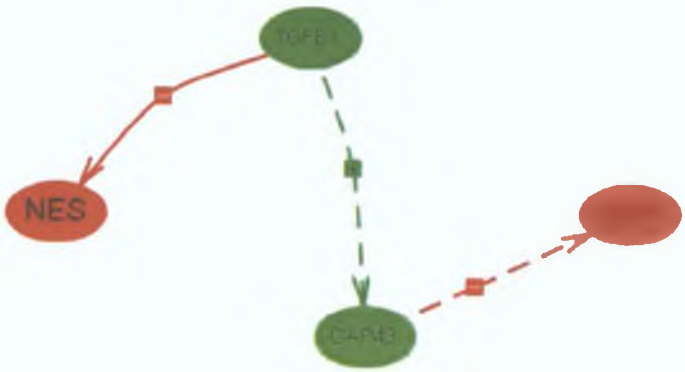


Figure 4 4 3 TGFβ1 exerts an effect on Nestin (NES), GAP43 and Basp1 expression

4 5 3 5 Summary of Changes in Gene Expression

Several genes that are involved in crucial β cell function are down-regulated in high passage glucose non-responsive cells Loss/reduced levels of these transcripts together with the increased levels of neuron-/glia- associated mRNAs may suggest, that after long-term culture, MIN6 cells revert to a poorly-differentiated, precursor-like population

Section 5.0: Conclusions and Future Work

5 1 Conclusions

5 1 1 Feeder-Free Culture of Mouse ES Cells

- Undifferentiated ES-D3 cells cultured on gelatin, in the absence of feeder layers, and in the presence of LIF exhibit characteristics associated with pluripotency However, these cells also express a range of developmental markers, which may suggest that some of the cells are in the early stages of differentiation

5 1 2 An Investigation into the Derivation of Insulin-Expressing Cell Types using an Adapted Version of the Original Lumelsky Protocol

- Stage 5 differentiated clusters derived from ES-D3 cells using a modified version of the original Lumelsky protocol expressed markers representative of the neuronal/glial lineage (GFAP, MBP, β III tubulin), extra-embyronic lineage (GATA 4, AFP, Pdx1) and pancreatic endocrine lineage (pax 4, insulin I, insulin II, glut2) indicating the presence of multiple cell types in these clusters
- The appearance of neuronal striations in cultures at stages 4 and 5 correlated with an increase in neuronal/glial transcript expression supporting a previous suggestion by Kania et al (2004) that the stage 3, nestin-selection step promotes neuronal/glial differentiation
- Endocrine pancreatic differentiation appeared to dominate over extra-embryonic differentiation during the 5 stages of the modified Lumelsky protocol An increase in β cell marker transcripts i e insulin I, insulin II, pax 4 and glut2 was observed in the stage 5 culture whereas Oct 4 expression was down-regulated An increase in Oct 4 expression is usually associated with extra-embryonic differentiation
- Stage 5, ES-derived cells did not show any sign of apoptosis There was no change in apoptotic marker transcript expression between stage 1 and stage 5 Stage 5 cell nuclei were round and robust rather than small and fragmented (as would be expected during apoptosis)
- C-peptide 1 and C-peptide 2 expression detected by IF/IHC analysis over-represented actual C-peptide protein levels in the stage 5, ES-derived culture A

C-peptide western blot optimised in our laboratory demonstrated that stage 5 clusters did not express C-peptide 2 at a significant level for detection

- IF analysis demonstrated that an isolated subpopulation of the stage 5, ES-derived culture expressed granular staining indicative of insulin staining An insulin content of 88 ng/ml was detected in lysates from this culture by ELISA Stage 5 cultures also released insulin, but not in a glucose dependent manner
- A novel MRIA assay developed during the course of this study was used to identify *de novo* insulin synthesis (down to a level of 0 71 ng/ml) in the MIN6 insulinoma cell line The same assay was applied to the stage 5, ES-derived culture (containing 88 ng/ml insulin measured by ELISA) The assay was unable to detect any *de novo* insulin synthesis in the ES-derived culture
- Using both the novel MRIA assay and the C-peptide 2 western blot developed during the course of this study, we were unable to find evidence, which would support *de novo* insulin synthesis in the stage 5, ES-derived culture These findings are in agreement with previous reports suggesting that insulin uptake from the culture medium is responsible for the high insulin content in cells exposed to similar 5 stage differentiation protocols

5 1 3 Two-Stage Differentiation Studies

- Formation of EBs in the presence of RA resulted in a mixed population expressing a range of multi-lineage transcripts (neuronal/glial, pancreatic endocrine, transcription factor, apoptotic, extra-embryonic, endoprotease and muscle) and proteins (neuronal/glial, pancreatic endocrine, muscle) suggesting the presence of a multi-potential precursor population
- Secondary differentiation of the RA culture by the presence of other compounds altered the specification of lineage fate, resulting in neuronal- and glial-specific marker expression prevailing in the presence of AA or BTC and pancreatic endocrine marker expression dominating in the presence of SB
- Neuronal/glial differentiation was characterized by the development of nestin-expressing precursor cells in the RA culture and subsequent down-regulation of nestin expression in the RA_AA and the RA_BTC cultures, coinciding with an

up-regulation in GFAP β III tubulin, GFAP and NF-L proteins were expressed in subpopulations of both the RA_AA and RA_BTC culture

- Pancreatic differentiation in the RA_SB culture was characterized by the expression of pancreatic developmental control genes at day 5 (Pdx1, ngn3, beta 2, pax 6, nkx2 2), followed by the up-regulation of β cell specific genes (insulin I, insulin II and glut2) at day 9 Pdx1, glut2 and insulin proteins were expressed in subpopulations of the RA_SB culture
- The short 2-stage RA_SB differentiation protocol developed in this study did not require exogenous insulin for the derivation of insulin-positive cell types, indicating that any insulin expressed was *de novo* synthesized LSCM analysis of RA_SB insulin-positive cells demonstrated a punctate staining pattern indicative of β cell granules containing insulin
- Insulin staining in the RA_SB cells did not co-localize with C-peptide 1 or C-peptide 2 staining C-peptide 1 and C-peptide 2 protein expression was detected in the majority of cells in the CO_9d culture, however these cells were negative for insulin These IF results verify previous findings in Section 3 1 demonstrating that these C-peptide antibodies, when used for immuno-analysis, over-exaggerate actual protein levels
- The trend in transcript expression observed over the 2-stage RA_SB differentiation protocol indicated that the RA_SB population was of a pancreatic endocrine lineage more so than an extra-embryonic lineage An increase in pancreatic endocrine marker transcripts, and a decrease in Oct 4 expression was observed between the untreated ES-D3 culture and the RA_SB culture
- The decrease in proliferation capacity observed in the 2-stage RA_SB differentiation protocol did not appear to be related to apoptosis There was no change in apoptotic marker expression between undifferentiated ES-D3 cells and RA_SB cells Small fragmented nuclei were not observed in the RA_SB culture (as would be expected with apoptosis)
- Further studies investigating alternative RA_SB protocols (Section 3 2 7 – Section 3 2 9) were not successful at improving cell yields

- The combinations of extra-cellular factors described in this study may be of value in future protocols for directed differentiation of stem cells to neuronal, glial and pancreatic endocrine cell types
- Spontaneous formation of EBs did not appear to encourage lineage specific differentiation and did not result in the derivation of insulin expressing cell types

5 1 4 MIN6 Microarray Study

- P18 MIN6 cells respond to changes in glucose concentrations, producing an approximate 6-fold GSIS in response over the glucose range tested (0 – 26 7 mmol/l) After continuous culture to passage 40, this GSIS was no longer present
- The loss in functional GSIS after continuous culture correlated with phenotypic changes including an increased proliferation rate, an increase in AP activity and a change in cell morphology
- The difference in AP activity and proliferation rates between the two cell populations (i.e P18 MIN6 and P40 MIN6) is indicative of de-differentiation.
- Microarray and qPCR analysis demonstrated that mRNAs involved in secretion, adhesion, proliferation and β cell development were affected by continuous culture
- The reduced expression of transcripts associated with the mature β cell in high passage cells (P40) compared to low passage cells (P18), together with the increased level of neuron-/glia- associated mRNAs, suggested that with time in culture, MIN6 cells reverted to a less differentiated 'precursor-like' population
- This observation was supported by (a) the elevated level of AP activity in high passage cells compared to low passage cells and (b) the suggestion by Kayo et al (1996) that well differentiated MIN6 cells were directed to a poorly differentiated state at higher passage numbers by furin cleaved bioactive proteins
- The phenotypic and gene expression changes observed between low and high passage MIN6 involve functional de-differentiation of the cell population, but whether this is due to de-differentiation of the population as a whole, or over-growth by a faster growing poorly-differentiated sub-population, remains unclear

5 2 Future Work

- Culture conditions and marker expression in undifferentiated ES cells vary from one study to another Therefore, one can assume that the differentiation status of these ES cells also varies from one study to the next This is an issue that needs to be addressed A comparative analysis of multi-lineage marker transcript expression (including pluripotent markers) by qPCR, in ES cells cultured both in the absence and presence of feeder layers, would determine if these varying culture conditions were affecting the overall undifferentiated status of the cells
- ES lines express high levels of Oct 4 and precise levels of this gene are critical in maintaining the ES cell state (Rao, 2004, Berill et al, 2004) It has been observed both in this study and in others (Berill et al, 2004) that Oct 4 mRNA expression levels are only slightly diminished after 5 – 6 weeks in culture lacking LIF Future work could investigate the effects of Oct 4 down-regulation using SiRNA technology on differentiation Down-regulation of Oct 4 in combination with some of the treatments investigated in Section 3 2 may improve on the derivation of lineage specific cell types
- Alternative RA_SB differentiation protocols investigated in Section 3 2 demonstrated that NIC and SB are both potent enhancers of insulin transcript expression It may be interesting to study the effects of these extra-cellular factors on differentiation and maturation of ductal stem cells
- Future studies on MIN6 cells could focus on determining specific passage numbers at which GSIS is lost GSIS analysis and microarray analysis of serial passage numbers may yield more information on the molecular mechanisms associated with the loss of GSIS function
- The prevention of the loss of functional GSIS with continuous culture is the main aim in MIN6 studies A study by Zimmer et al (1999) demonstrated that growth arrest correlated with increased levels of secretory granule genes including chromogranin A and insulin In the present study, genes associated with proliferation where among those significantly affected by continuous culture These genes i e dlk1, cdk4, CEACAM1 are potential targets for future functional

studies (i e siRNA knockdown or cDNA transfections) to identify whether de-differentiation can be prevented by controlling proliferation

- A study by Bouckenooghe et al (2003) demonstrated that SB induced β cell gene (re)expression after expansion and de-differentiation of human pancreatic islets It may be interesting to investigate whether sodium butyrate has a similar effect on gene expression in high passage MIN6 cells which are also thought to have de-differentiated to a precursor-like cell type
- This study by Bouckenooghe et al (2003) proposed that the expansion of human pancreatic islets represented a de-differentiating system It may be interesting to study this system in parallel to our MIN6 model (low and high passage cells) which is also thought to represent a de-differentiating system, using both micro-array and proteomics technologies

Section 6.0: Bibliography

Section 5 0 Bibliography

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