Sourcing cells for gut tissue engineering – understanding and inducing embryonic stem cell differentiation to the intestinal cell lineage

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"Minds are like parachutes –
they need to be fully open to
operate properly"
Anon.



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Abstract

Sourcing cells for gut tissue engineering – understanding and inducing embryonic stem cell differentiation to the intestinal cell lineage

Tissue engineering of any tissue type requires the combination of a supporting scaffold, a range of biological factors and a suitable source of cells. This source of cells must satisfy a number of criteria:-

- The ability to form all of the mature/specialised cell types found in the target tissue.
- Readily obtainable.
- Readily maintainable in the laboratory without requiring excessive resources or time.

For intestinal tissue engineering there are a number of issues associated with the use of tissue derived stem cells particularly quantity of normal tissue available (from the patient) and maintenance and expansion of the cells when cultured *in vitro*. Using embryonic stem cells offers a potential alternative strategy but methods must be established to efficiently differentiate the cells towards the desired fate. Many strategies for differentiating embryonic stem cells are based upon treatment with growth factors *in vitro.* There is a (logistical) limit to the degree of complexity that these systems can achieve and therefore a limit to the number of differentiation signals that occur during *in vivo* development that can be mimicked. In recent years using embryonic tissue to provide signals to undifferentiated cells has proved a successful method of directing the differentiation of naïve cells towards a particular fate (with the choice of tissue determined by the desired target cell type).

The aims of this thesis were to explore the potential of differentiating embryonic stem cells towards the intestinal progenitor fate using a combination of *in vitro* cell culture treatment with the growth factor Activin-A and *ex vivo* co-culture with embryonic chick gut tissue. Previous studies [Kubo et al 2004, Tada et al 2005, Yasunaga et al

2005, D'Amour et al 2005, MacClean et al 2007] have shown that Activin-A treatment will induce embryonic cells to more efficiently differentiate to definitive endoderm, the germ layer from which the intestines (and other visceral organs) arise. These techniques were applied to the Columnar Epithelial Epiblast murine embryonic stem cell line and cell differentiation was then evaluated at the molecular level using Reverse Transcription-Polymerase Chain Reaction, immunocytochemistry and Western blotting. Activin-A treatment produced an upregulation of definitive endoderm markers at both the mRNA and proteomic levels compared to the control conditions. However the cell population produced retained expression of pluripotent markers and showed some expression of markers of other cell lineages.

Further studies [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005, Coleman et al 2007, Krassowska et al 2006] have shown that co-culture of embryonic stem cells with early stage embryonic tissue can induce the formation of particular tissue types; the tissue must be selected based on proximity to the target cell type during development. This exposes the embryonic stem cells to the signals that prompt differentiation towards the target tissue during normal development. With gut tissue much signalling occurs between the different tissue layers that make up the whole organ both during development and in adult tissue. *Ex vivo* co-culture of murine embryonic stem cells with embryonic chick gut tissue was used to direct their differentiation to the intestinal epithelial stem cell fate. Before the co-culture was carried out various experiments were carried out to establish if the proposed protocol was viable e.g. defining how long chick gut tissue explants could survive in culture.

Once this had been established co-culture experiments were undertaken and cell differentiation was then evaluated at the molecular level using Reverse Transcription-Polymerase Chain Reaction, immunocytochemistry and Western blotting. The cells showed some expression of intestinal epithelial stem cell markers at both the mRNA and proteomic levels following co-culture. The cells were also assessed at a physiological/functional level by evaluating their ability to form a functional intestinal epithelial barrier. This was achieved using an *in vitro* co-culture model with intestinal subepithelial myofibroblasts by measuring transepithelial resistance, permeability to protein and morphology in a simple tissue model co-culture. The cells did not display

the morphological or physiological characteristics associated with intestinal epithelial cells in the model system.

Overall this work has shown that co-culturing pluripotent mES cells with embryonic chick gut tissue can induce differentiation towards the ISC fate. Pre-treating the cells with growth factors *in vitro* did not seem to enhance this differentiation but there was scope to refine these techniques. Following the differentiation protocols the cells did not display the desired physiological characteristics but again there was scope to refine the techniques particularly with regard to selecting cells positive for the expression of the chosen molecular markers. These techniques show promise but do require some further development.

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Chapter One: Introduction

List of Abbreviations

Å Angstroms

AB-AM Antibiotic & Antimycotic

Act-A Activin-A

AFP α-fetoprotein

A-G-M Aorta-Gonad-Mesonephros

Apc Adenomatous polyposis coli

APES 3-Aminopropyltriethoxysilane

ASC Adult stem cells

α-MEM α Modified Eagles Media

BCA Bicinchoninic acid

Bmi-1 BMI1 polycomb ring finger oncogene

BMPs Bone Morphogenic proteins

bp base pair

BrdU 5-bromodeoxyuridine

BSA Bovine Serum Albumin

β-MCPE β-Mercaptoethanol

CBC Crypt base columnar

CD133 Cluster of differentiation 133

CDP CCAAT displacement protein

Cdx1/2 Caudal type homeobox 1/2

CEE Columnar Epithelial Epiblast

cES cell Cynomolgus monkey embryonic stem cell

CFU colony forming unit

ChGA Chromogranin A

CM Conditioned media

COX Cyclooxygenase enzyme

CRESIP Colon-repressive element of the SI promoter

CXCR4 Chemokine C-X-C motif Receptor 4

DAB Diaminodenzidine

DABCO 1-4 Diazabicyclo-2-2-2-octane

DBA Dolichos Biflorus Agglutinin

DE Definitive Endoderm

DMEM Dulbecco's Modified Eagle's Media

DMSO Dimethylsulphoxide

DPX Distyrene, Plastisier & Xylene

DTT Dithiothreitol

EBs Embryoid bodies

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

EM Eagle's Media

Eph Ephrins

Epi Ant Epithelial Antigen

ES Cell Embryonic Stem Cell

EtBr Ethidium Bromide

Eth D-1 Ethidium D-1 Homodimer

EVOM Epithelial Voltometer

FACS Fluorescence Activated Cell Sorting

FBS/FCS Foetal Bovine Serum/Foetal Calves Serum

FGF Fibroblast Growth Factor

FITC Fluorescein isothiocyanate

FoxA2 Forkhead Box A2

FSH Follicle Stimulating Hormone

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

GATA GATA Binding protein

GFP Green Fluorescent Protein

GI Gastrointestinal

GM Genetically modified

GM-CSF Granulocyte macrophage colony stimulating factor

GPR49 G-protein receptor 49

Gsc Goosecoid

H & E Haematoxylin & Eosin

HBSS Hank's Balanced Salt Solution

HDAC Histone Deacetylase

hES cell Human Embryonic Stem Cell

HGF Hepatocyte Growth Factor

HMG High mobility group

HNF Hepatic Nuclear Factor

HRP Horse Radish Peroxidase

HSA Hepatic Specific Antigen

IBS Irritable Bowel Syndrome

ICM Inner Cell Mass

IL Interleukin

ILGF2 Insulin-like Growth Factor 2

IMS Industrial Methylated Spirit

iPS cell Induced pluripotent stem cell

ISC Intestinal (Epithelial) Stem Cell

ISEMF Intestinal sub-epithelial myofibroblast

IVF *In vitro* Fertilisation

KGF Keratinocyte Growth factor

L-Glut L-Glutamine

LDL Low Density Lipoprotein

Lgr5 Leucine-rich-repeat-containing G-protein-coupled receptor 5

LIF Leukaemic Inhibitory Factor

M-CSF Macrophage colony stimulating factor

MEF Mouse/Murine Embryonic Fibroblast

MEM Modified Eagles Media

mES cell Murine Embryonic Stem Cell

MSCs Mesenchymal Stem Cells

Msi-1 Mushashi-1

Mt2 *Metallothionein-2*

NGF Neural Growth Factor

Oct4 Octamer-4

Ω Ohms

PAA Polyallylamine

PAC Puromycin N-acetyl-tranferase

PAS Periodic Acid Schiff

PBS Phosphate Buffered Saline

PCS Portacaval shunt

PDGFRα Platelet derived growth factor receptor α

Pdx1 Pancreatic and duodenal homeobox 1

Pen/Strep Penicillin and Streptomycin

PET Polyethylene Terephthalate

PF Protein free

PFA Paraformaldehyde

PGA Polyglycolic acid

PGD Preimplantation genetic diagnosis

PGP9.5 Protein Gene Product 9.5

PI3K Phosphatidylinositol 3-Kinase

PLA Polylatic acid

PMSF Phenylmethanesulphonylfluoride

POU5F1 POU class 5 Homeobox 1

PROM1 Prominin-1

PVA Polyvinylacetate

Pyy *Peptide YY*

qPCR Quantitative/Real-Time PCR.

RA Retinoic acid

RPM Revolutions per minute

RT-PCR Reverse Transcription - Polymerase Chain Reaction

SAGE Serial Analysis of Gene Expression

SBR Small bowel resection

SBS Short Bowel Syndrome

SCs Stem cells

SCID Severely compromised immunodeficient

SDF-1 Stromal derived factor-1

SEM Standard Error of the Mean

Shh Sonic Hedgehog

SMCs Smooth Muscle Cells

SMαA Smooth Muscle α Actin

SM-MHC Smooth Muscle Myosin Heavy Chain

SNL Sto Neo Leukaemic

Sox Sex Determining Region Box

SR Serum Replacement

SSEA Stage specific embryonic antigen

TBE Tris-Borate EDTA

TBS Tris Buffered Saline

TCP Tissue Culture (treated) Plastic

TEM Transmission Electron Microscopy

TER Trans-epithelial resistance

TGF-β Transforming Growth Factor-β

Tm Melting temperature

TMCC Tissue model co-culture

TRITC Tetramethylrhodamine isothiocyanate

TSSC Tissue Specific Stem Cells

TTBS Tween-Tris Buffered Saline

TTF1 Thyroid transcription factor 1

TTR Transthyretin

VLA Very late activation

YFP Yellow fluorescent protein

Note: With gene and protein names when referred to in italics this indicates the *gene* only whilst the PROTEIN will be referred to in capitals. When in normal text this refers to the factor at both levels.

Chapter One: Introduction

Literature review

1.1 Tissue Engineering

The field of tissue engineering utilises a combination of engineering materials, known as scaffolds, cells and biochemical reagents, such as growth factors, to produce tissue structures outside of the body [Langer and Vacanti, 1993, Ikada, 2006]. Scaffolds, as the name suggests, form the framework around which the tissue structure can form. The specific architecture and composition of the scaffold can be designed to influence and aid the growth and differentiation of the tissue. The cellular component forms the foundation of the new biological material as the cells grow and divide. Biochemical factors fulfil a number of functions including directing the differentiation of the cells, stimulating growth and aiding cellular interaction with the scaffold. Biological agents, such as growth factors (e.g. Bone Morphogenic Proteins (BMPs) [Kanczler et al 2010], Transforming Growth Factor-β (TGF-β) [Miyanishi et al 2006]), and hormones (e.g. Insulin [Kellner et al 2001]) and chemical agents, such as steroids (e.g. Dexamethasone [Tanaka et al 2004]) can be used for these applications.

There are two main approaches followed in experimental tissue engineering strategies (Figure 1.1). The first uses cell free scaffolds, usually impregnated with growth factors, as an aid to *in vivo* tissue regeneration and repair and would be of use in clinical situations only. The second approach combines cells with scaffolds and growth factors to generate tissue structures outside the body, usually using a culture system known as a bioreactor (Figure 1.2).

The first strategy is useful for repairing damage to part of a tissue where the wound is too extensive for the body's natural healing process to cope by itself, for example damage to diseased joints or non-union breaks to bones (breaks that exceed the size that the bone can self-repair) [Yang et al 2004], and has more immediate potential for use in some clinical work [Braddock et al 2001]. The second strategy is still very much at the research stage of development but is advancing rapidly [Ikada 2006] with some 'products' recently reaching the clinic e.g. TE bladder [Atala et al 2006] and TE windpipe [Macchiarini et al 2008]. Some target tissues in this field include insulin

secreting islet β cells [Moritoh et al 2003], liver/hepatocytes [Dvir-Ginsberg et al 2003] and intestinal epithelium [Day et al 2006, Gupta et al 2006].

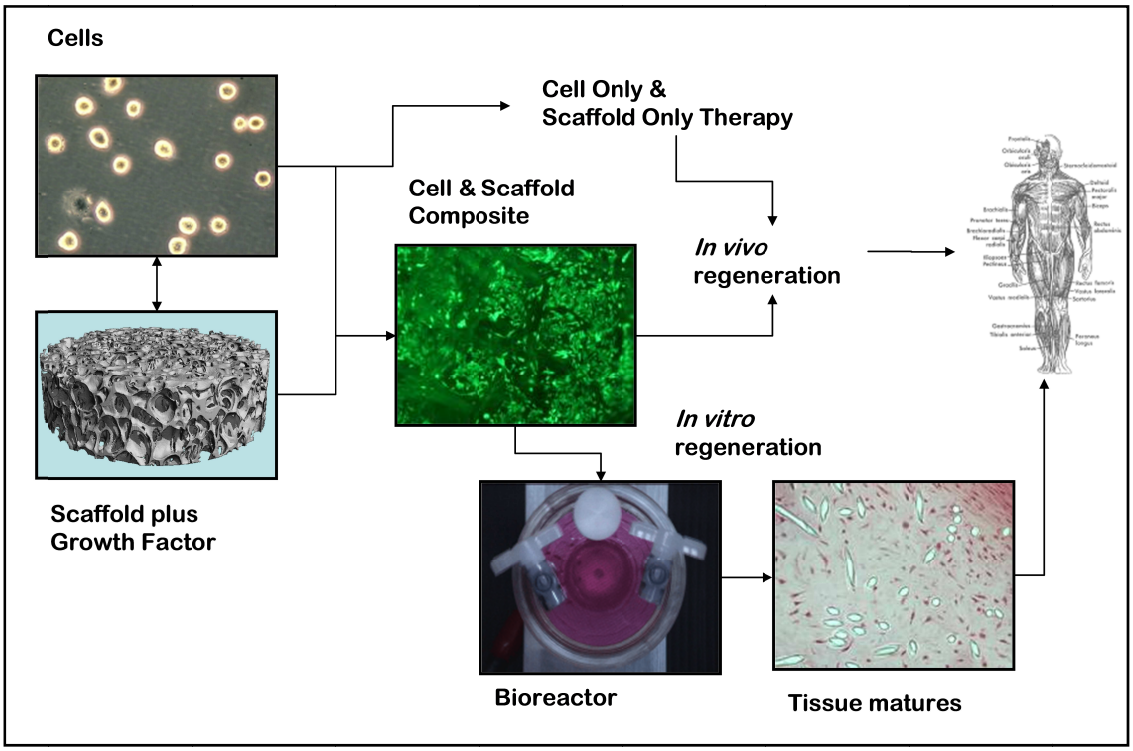


Figure 1.1: An overview of the various tissue engineering strategies employed by researchers.

These engineered tissues have a wide range of potential medical and research applications. Tissue engineered organs could provide a more readily available supply for transplant operations than donated material. In 2007 there were over 100000 people awaiting transplant operations in the UK alone and with surgical techniques steadily improving, the scope for carrying out these operations is consistently on the rise but the supply of donor organs is short. The waiting time for a suitable donor organ to become available can be many months [National Institute for Health & Clinical Excellence, NICE, www.nice.org.uk] and in many cases the patient may deteriorate considerably in that time. Some tissue engineering strategies have already been used to address this delay/shortage with clinical augmentation of skin healing using *in situ* scaffolds or engineered skin, such as MySkin® (for repairing burns for example) [MacNeil 2007, MacNeil 2008], being a common application [Kumbar et al 2008, Powell et al 2009]. Engineered tissues could also be used for pharmaceutical testing and studying mechanisms in both diseases [Jean et al 2009], including cancer

[Verbridge et al 2010], and developmental processes. For example, engineered intestine could be used to examine how readily drug candidates can cross the intestinal epithelial barrier and enter the circulation or to examine the progression of diseases such as Crohn's disease (and the effectiveness of potential treatments) [Day 2006]. This could incorporate replacing some animal experiments with tests carried out using tissue engineered constructs [www.nc3rs.org.uk, Holmes et al 2009].



Figure 1.2: Bioreactors (spinner flasks) containing scaffolds in culture media.

The supply of cells can be a rate limiting step in tissue engineering strategies as many cell types, such as intestinal epithelium (see Section 1.2.4) [Gupta et al 2006, Day 2006], are slow growing and/or difficult to culture *in vitro*. Factors such as potential rejection of tissue engineered organs produced using allogenic cells must also be considered. Therefore any techniques by which a reliable, suitable cell source can be generated would prove an extremely useful addition to the tissue engineers' armoury.

1.2.1 Gastrointestinal (GI) Biology and the maintenance of the stem cell niche

The mucosa of the GI tract consists of a variety of layers including an epithelial layer with a highly complex structure and organisation. It forms the lining of the intestinal lumen and in turn is surrounded by outer layers of submucosa and smooth muscle (Figure 1.3) that is responsible for digestive transit (peristalsis). The intestinal epithelium is a columnar epithelial layer and is responsible for digestion, nutrient and liquid absorption and immune defence. In the small intestine the epithelium has a characteristic crypt-villous architecture with numerous microvilli protruding into the lumen of the small intestine interspersed with small invaginations into the intestinal wall known as Crypts of Lieberkuhn. In the small intestine a number of different cell types (Figure 1.4) with a variety of functions arise from the small population of intestinal epithelial stem cells (ISC). Stem cells are also responsible for maintaining the cell complement in the large intestinal epithelium but the majority of research into ISC has been carried out in the small intestine. The spatial niche occupied by the stem cells is well defined with differentiating progeny becoming more specialised as they migrate away from it. The generally accepted theory of how this occurs is outlined below (Figures 1.5 and 1.6) [Cheng and LeBlond 1974, Clevers et al 2009].

The ISC reside at the base of the Crypts of Lieberkuhn; as the stem cells divide one daughter cell takes on a stem cell fate (ensuring the self-renewal of the ISC population) whilst the other begins to migrate up the wall of the Crypt of Lieberkuhn. At first this second daughter cell continues to divide, forming the transit-amplifying cells. As the cells migrate further up the crypt wall and out onto the villus surface they differentiate (and cease to proliferate) into either the absorptive enterocytes or secretory cell types (Goblet or Enteroendocrine) as shown in Figure 1.4. The exceptions to this pattern are the Paneth cells (that only occur in the small intestine); they remain in the base of the Crypts of Lieberkuhn after they have differentiated [Crosnier et al 2006].

Differentiated cells continue to migrate up the crypt/villi until they die by apoptosis and are sloughed off into the intestinal lumen. The mechanism of epithelial renewal can be observed by pulse labelling the dividing cells at a particular time (using

tritiated thymine for example, which is only taken up by dividing cells) and then following the fate and movements of their progeny [Cheng and LeBlond 1974].

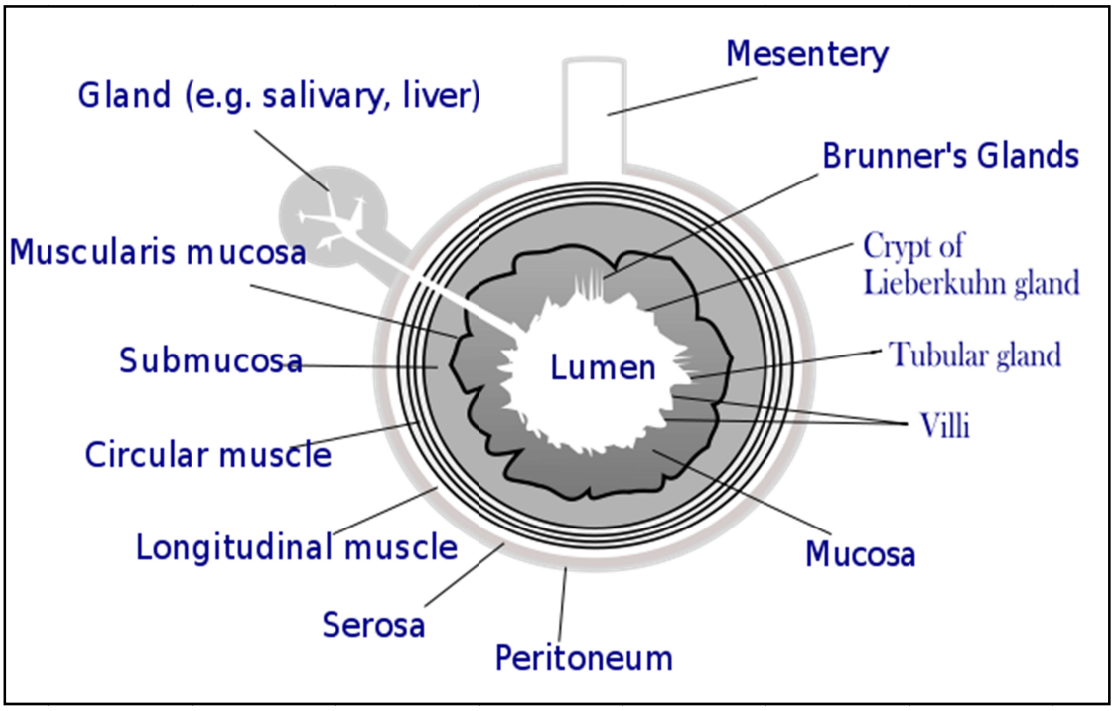


Figure 1.3: Cross section of the small intestine showing the various tissue layers that comprise the intestinal wall. The intestinal epithelium surrounds the lumen and consists of the villi-crypt architecture.

In vertebrates the correct patterning of the GI tract during development involves complex signalling interactions between the endoderm and the surrounding mesoderm that gives rise to the smooth muscle layer surrounding the GI tract [Roberts et al 1998]. Key players in this signalling include *Sonic Hedgehog (Shh), Bmp-4, Wnt* and the members of the *Hox* gene family (Figure 1.5). Overexpression of the *Wnt* pathway leads to greatly enlarged crypts whilst blocking it results in a complete absence of crypts [Roberts et al 1998]. Maintenance of the ISC niche in adult intestine also requires complex signalling interactions although this is believed to be with the surrounding connective tissue rather than the muscle layer [Roberts et al 1998].

In humans one of the initiating events for most colorectal cancers is the loss through mutation of both copies of the *Adenomatous polyposis coli (Apc)* gene resulting in the

permanent activity of the *Wnt* pathway. *Apc* homologues have been found in mice and Zebrafish where the loss of both copies also results in the formation of intestinal adenomas [Mariadson et al 2005]. The *Wnt* signalling in the crypt has a positive feedback on itself meaning it is strongly active in the stem cell niche and is essential for maintaining intestinal stem cell character (Figure 1.5) [Madison et al 2005, Gregorieff et al 2005].

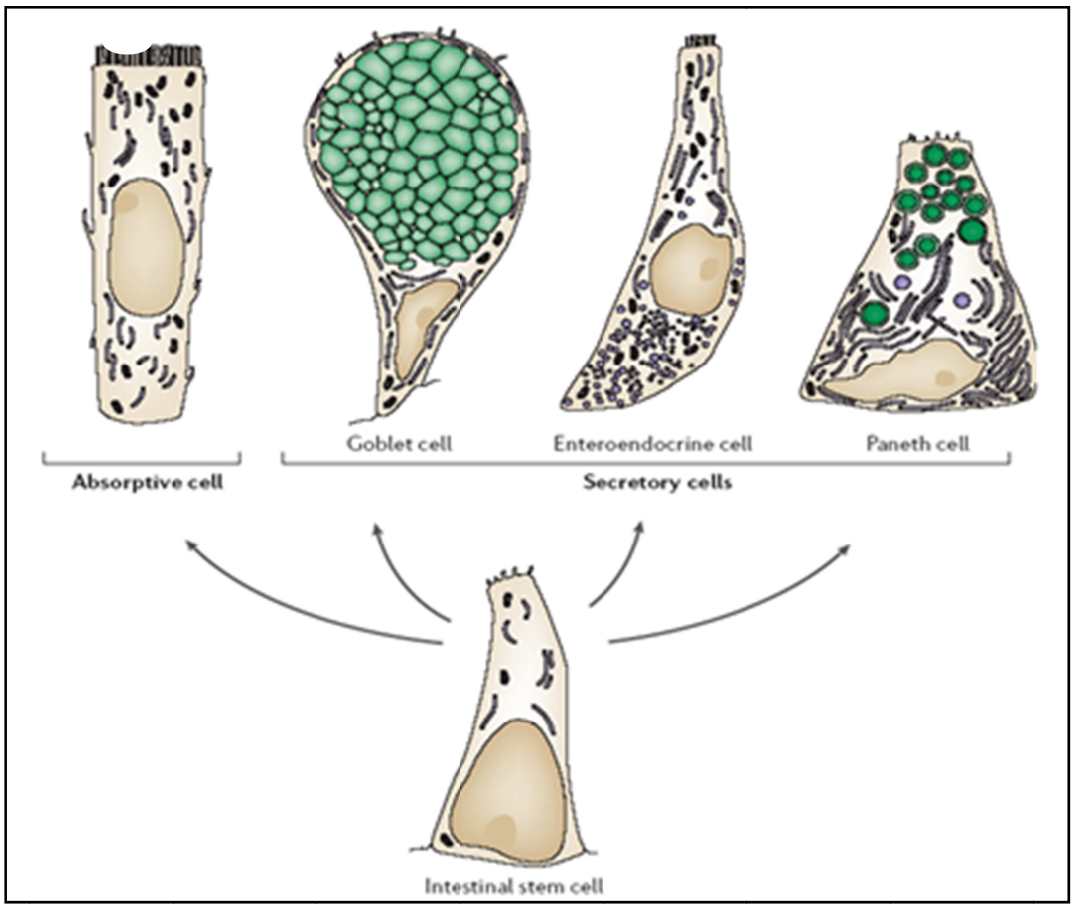


Figure 1.4: The different cell types found in the intestinal epithelium all arise from multipotent stem cells found at the base of the Crypts of Lieberkuhn [Crosnier et al 2006].

Ephrin (Eph) receptors (and their ligands, the ephrins) are membrane-associated proteins involved in communication between neighbouring cells. They play a key role in the correct migration and arrangement of all the differentiated cells along the crypt-villous axis (Figure 1.5 and Figure 1.6). Deletion of *Eph* genes results in the abnormal patterning of these cells, e.g. Paneth cells are no longer restricted to the base of the crypts and proliferating cells are scattered along the length of the crypt-villous

axis. This is also shown by the fact that the loss of particular Eph receptors (EPHB) is associated with the start of invasive behaviour in colorectal cancer [Mariadson et al 2005].

Different patterns of integrin expression (specifically the very late activation (VLA) family of integrins) and the subsequent interactions with surrounding cells along the basement membrane also play a role in controlling differentiation and migration along the crypt-villus axis [Beaulieu 1992].

The intestinal epithelium contains various cell types that are intermixed with each other throughout the crypt-villous architecture. As the variety of differentiated cell types all arise from the ISC there must be a mechanism for specifying the correct number of each type of cells is produced. *Wnt* signalling is important in specifying the secretory cell types in the gut lineage [Mori-Akiyama 2007]. If *Wnt* signalling is blocked enterocytes will differentiate normally but all classes of secretory cells are lost. Overexpression of *Wnt* leads to incomplete differentiation in all types of cells with a marked increase in the number of Paneth cell precursors [Crosnier et al 2006].

Secretory cell precursors seem to inhibit their neighbours from also adopting a secretory fate via *Notch* signalling (Figure 1.5). Partial inactivation of the *Notch* pathway results in an increase in the number of goblet and entero-endocrine cells whilst constitutive activation of *Notch* leads to a drastic reduction in all three secretory cell types. This signalling (and resulting fate commitment) occurs whilst the cells are still in the crypts and still dividing. Secretory precursors express *Delta* enabling them to activate *Notch* in their neighbours [Stanger et al 2005].

The blocking of *Notch* signalling doesn't just cause a predominance of secretory cells to form; it also results in loss of proliferation at the crypt base i.e. the stem cell population also differentiates. Overexpression of *Wnt* is not enough to reverse this so it appears stem cells are dependent on *Wnt* and *Notch* to drive proliferation (and prevent differentiation). *Wnt* signalling appears to activate *Notch* in the crypt base and it is this small region of *Notch+/Wnt+* signalling that maintains the stem cell niche [Stanger et al 2005].

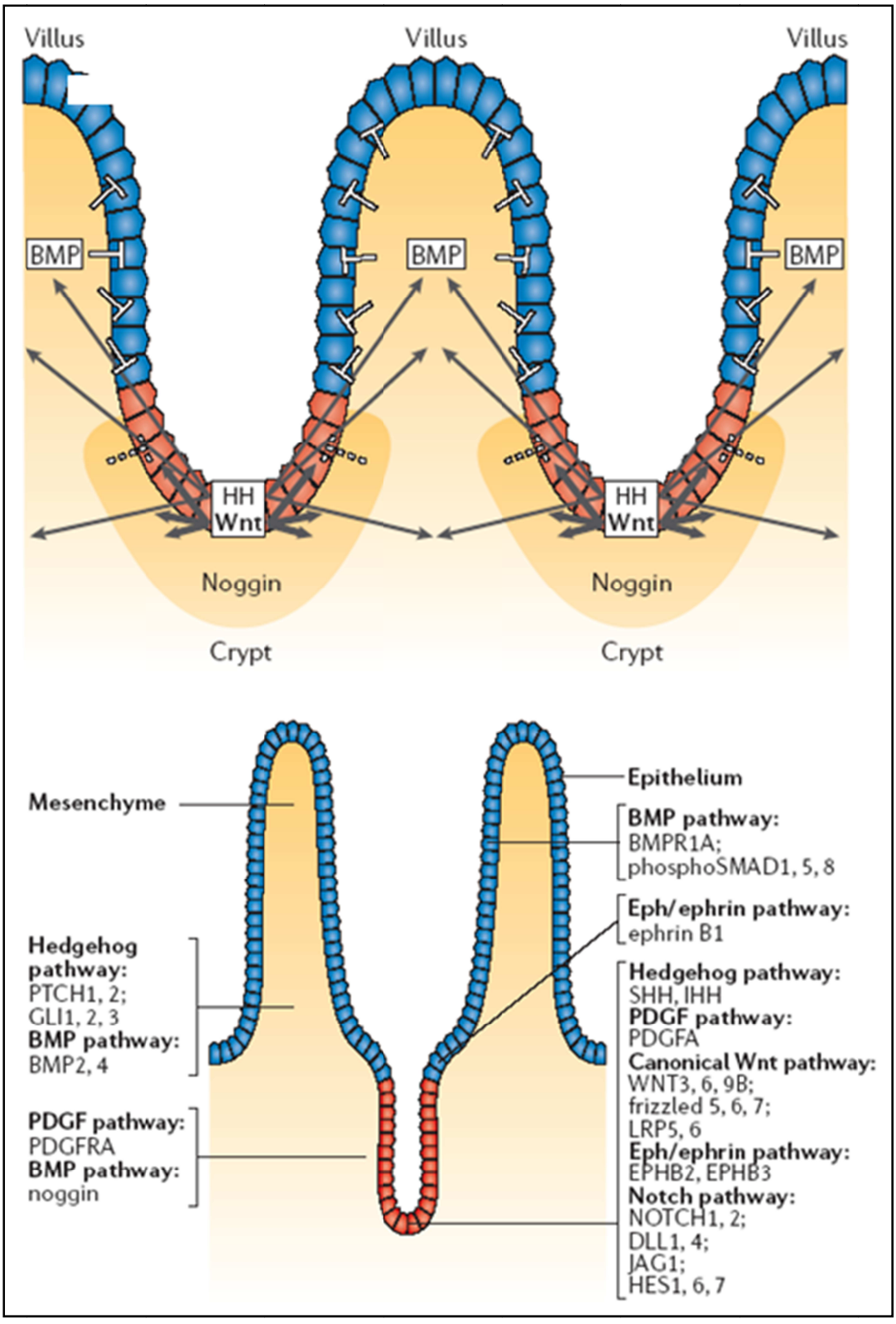


Figure 1.5: Summary of signalling in the crypt-villous structure. Hedgehog, Noggin, *Wnt, Bmp* and *Eph* specify and maintain the crypt-villous axis whilst *Wnt* and *Notch* control cell fate [Crosnier et al 2006].

The overexpression of Notch (through a gain of function mutation in mice) gives rise to a number of (reversible) defects in embryonic gut morphogenesis which suggest Notch plays a key role in regulating the ratio of secretory and absorptive cell types in the intestinal epithelium [Stanger et al 2005].

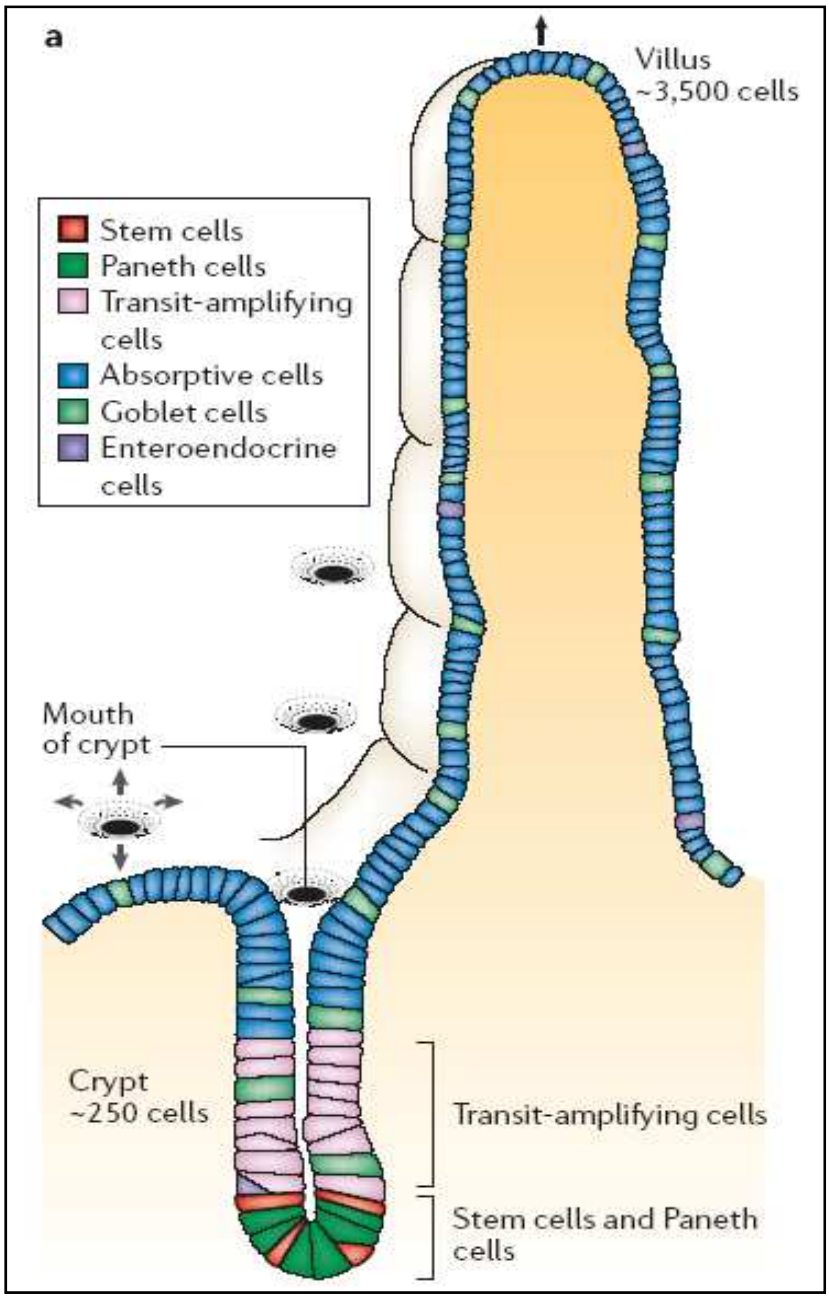


Figure 1.6: The location of the various cell types within the small intestinal crypt-villous structure [Crosnier et al 2006].

The dynamics of how the ISC maintain the intestinal epithelium have also been investigated in mice [Bjerknes and Cheng 1999]. By causing a (chemically introduced) mutation in a particular gene locus (*Dlb-1*) that produces a marker in the affected cells at random throughout the intestinal epithelium and then tracing the clones of the mutated cells, the lineages of the various cell types could be identified.

The results showed that there were 'long lived' progenitors capable of forming all the cell types in the epithelium (the ISC), 'relatively long lived' cells (a lifespan measured in months) that were either mucus cell progenitors or columnar cell progenitors and 'short lived' cells that only produced one or two daughter cells. Analysis of branching crypts showed that clones were localised to one crypt. Clones were also shown to be widely dispersed despite the fact that the intestinal epithelial contains strong cell-cell associations [Bjerknes and Cheng 1999].

1.2.2 GI Development

The GI tract, along with the rest of the viscera, arises from the (definitive) endoderm (DE) germ layer during development. The development and differentiation of the GI epithelium and other intestinal structures is controlled by a variety of regulatory mechanisms. These include some epigenetic mechanisms where genes are silenced or activated according to the state of the chromatin/DNA [Tou et al 2004]. Class I Histone Deacetylases (HDACs) play an important role in mammalian gut development illustrated by the fact that overexpression of HDACs causes misexpression of a number of intestinal epithelial developmental markers including *Apo1a* and *metallothionein-2* (*Mt2*) [Tou et al 2004].

As mentioned above *Wnt* signalling plays a key role in maintaining the ISC niche in adult intestine and is restricted to the base of the Crypts if Lieberkuhn but it is also a key component in the development of the intestinal epithelium [Kim et al 2007]. TOP-GAL and LacZ reporter constructs were used to identify the location of *Wnt* expression in developing mouse embryos. *Wnt*-reporter expression is first seen in the intestine as villi begin to form at around embryonic day 14. General patterns of expression similar to those observed in the adult intestine develop rapidly in the villi

whilst transcripts that localise to adult crypts are found in the intervillus cells (from which the Crypts of Lieberkuhn will eventually arise).

Postnatal *Wnt* expression only becomes apparent three days *post partum* in the intervillus regions whereas it is elevated in the villi in late gestation and immediately following birth. This does not correspond with what was expected and may be caused by incongruities in timing between reporter activity and real *Wnt* signalling or indicate a sensitivity threshold for detecting activity [Kim et al 2007].

The environment in the intestine continues to change *post partum* following the switch from *in utero*/parenteral nutrition to ingestion of food. In mammals these changes take on an extra stage as the change from suckling to solid food takes place. In mice there is a complex pattern of expression of key transcription factors and enzymes which shows both temporal and spatial variation [Fang et al 2006].

The (temporal and spatial) expression of Sucrase-isomaltase is partially controlled by the novel regulatory elements such as CCAAT displacement protein (CDP aka *Cux*) [Boudreau et al 2002]. This element represses the expression of Sucrase-isomaltase in the colon via the Colon-repressive element of the SI promoter (CRESIP).

1.2.3 ISC markers and isolation

The discovery of characteristic (putative) markers for GI stem cells has been a key development in this field as previously the ISC were only identified by their (supposed) location at the +4 cell position at the base of the Crypts of Lieberkuhn. MUSASHI-1 (MSI-1) is a RNA binding protein first observed in neural progenitor cells where it is involved in asymmetric cell division – the mechanism by which ISC are believed to proliferate. It should therefore only be expressed in the proliferative compartment of the intestinal epithelium i.e. the ISC and their immediate daughters the transit amplifying cells. Antibody staining against MSI-1 protein showed this to be the case in mice [Potten et al 2003].

BDF1 mice were dosed with radiation (1 – 14 Gy) and the small intestine collected up to 12 days later. Non-irradiated neonatal and adult small intestine and colon tissue samples and a range of small intestinal adenomas were also analysed. MSI-1 (at the predicted location at the base of the crypts) expression was evident in neonatal (two day old) and adult samples as well as those regenerating after radiation damage. The adenoma samples were also strongly positive for MSI-1. Some human tissue samples (from both the small and large intestine) were also examined but the antibody staining was weak and therefore clear results were not obtained [Potten et al 2003].

The antibody staining in mice was followed up with *in situ* hybridisation and qPCR investigation of *Msi-1* expression. The *in situ* hybridisations showed *Msi-1* mRNA expression in the same locations as the positive MSI-1 antibody staining. The qPCR analysis showed elevated levels of *Msi-1* expression in tumours compared with normal tissue. This suggests *Msi-1* levels are elevated in more rapidly proliferating cells within the intestinal epithelium, be they cancerous cells or the ISC and their immediate progeny [Potten et al 2003].

In recent years a number of other markers for ISCs have been discovered. *Leucine-rich-repeat-containing G-protein-coupled receptor five* (*Lgr5*), otherwise known as G-protein receptor 49 (GPR49), is a *Wnt* target whose expression is restricted to the ISC at the base of the intestinal crypts [Barker et al 2007]. *Lgr5* was initially selected from a panel of Wnt target genes because of its (spatial) expression pattern, specifically that its expression was confined to the putative ISC loci at the base of the Crypts of Lieberkuhn when analysed by *in situ* hybridisation. *Lgr5* was also shown to be expressed in intestinal adenomas in Apc^{min} mice indicating it is expressed in rapidly proliferating cancer cells. Using an *Lgr5*-LacZ reporter knock in construct (which gives a blue colouration for positive expression) the expression of Lgr5 was examined further [Barker et al 2007].

In adult mice expression was seen in scattered cells in the brain, eye, reproductive organs and mammary glands. In the small intestine expression was restricted to the base of the crypts with three - four positive cells in each crypt interspersed amongst the Paneth cells. The morphology of the *Lgr5*+ cells was distinct from that of the Paneth cells so they were referred to as Crypt Base Columnar (CBC) cells. They were

also (frequently) positive for the expression of the proliferation marker Ki67. Through 5-bromodeoxyuridine (BrdU) pulse labelling the cycling time of the cells was established as approximately 24 hours. A further Green Fluorescent Protein (GFP) reporter construct was introduced and the same expression pattern was seen as with the LacZ reporter construct. A further inducible Cre-LacZ reporter was used to permanently label *Lgr5*+ cells and their progeny to allow lineage tracing.

The initial location of the labelled cells was not the +4 position, the previously hypothesised locus for the ISC, but (as stated above) interspersed amongst the Paneth cells. The CBC cells showed much reduced sensitivity to radiation doses than those cells in the +4 position. However the labelled CBC cells were capable of repopulating the intestinal epithelium as the cell population turned over [Bjerknes and Cheng 1999]. One day after induction just a few labelled cells were observed at the bases of the crypts but after a further one, five, 35 and 60 days the percentage of the cells (in 200 crypts) that carried the permanent label were 22%, 39%, 25% and 36%. The cells at 60 days were also positive for markers of Goblet cells (Periodic Acid Schiff (PAS)), Paneth cells (PAS expressed at the base of the crypt) and Enteroendocrine cells (Synaptophysin, a membrane glycoprotein) as well as demonstrating the morphological character of enterocytes. These cell types were present in similar ratios in labelled crypts as compared with unlabelled adjacent crypts. The *Lgr5*+ CBC cells demonstrate all the properties indicative of the ISC in the small intestine. Similar analysis was carried out on the epithelium of the colon generating almost identical observations. This suggests that Lgr5+ expression is characteristic of ISC throughout the intestinal epithelium [Barker et al 2007].

BMI1 polycomb ring finger oncogene (Bmi-1), a member of the *Polycomb* gene family, plays an important role in self renewal of a number of Adult Stem Cell (ASC) populations, particularly haematopoietic [Park et al 2003] and neural stem cells [Park et al 2003 (2)] where it distinguishes stem cell maintenance from progenitor proliferation. It was also found to be expressed in the ISCs in the epithelium of the small intestine [Sangiorgi & Capecchi 2008]. This study localised the expression of *Bmi-1* in the intestine to cell position four in the base of the crypts using a genetically modified (GM) mouse carrying an inducible *Cre* reporter gene in the *Bmi-1* locus that

would generate a permanent fluorescent signal in cells that were positive for *Bmi-1* (LacZ or Yellow fluorescent protein (YFP)) [Sangiorgi & Capecchi 2008].

Some cells retained expression of BMI-1 as they differentiated and migrated up the wall of the crypt but the *Bmi-1* transcript was only present in cells at the base of the crypt. *Bmi-1* expression did not appear to be uniform along the length of the small intestine. Crypts in the 10 cm nearest the pylori showed the highest number of *Bmi-1*+ crypts with a gradual decrease further along the small intestine. This suggests that not all ISC express *Bmi-1*. One month after the reporter-*Bmi-1* constructs had been induced the labelled progeny of the *Bmi-1*+ cells were examined to identify which cell fates they had differentiated to. The *Bmi-1*+ lineage included all of the differentiated (secretory) cell types present in the intestinal epithelium (Figure 1.3), Paneth cells that expressed lysozyme, Goblet cells that expressed Dolichos Biflorus Agglutinin (DBA, a cell surface protein) and Enteroendocrine cells that expressed Chromogranin A (ChGA) [Sangiorgi & Capecchi 2008].

Crypts that were positive for *Bmi-1* remained so up to 12 months after reporter induction. This persistence of expression is an indicator of self-renewal, a key property of stem cell populations. *Bmi-1*+ cells were also shown to be present in intestinal adenomas. Disrupting *Bmi-1*+ cells had a significant impact on the animals in question. Mice where disruption was induced over several days died. On examination the intestines of these mice were full of dead cells.

Mice where a single disruption was induced survived but were slow to gain mass. The intestines of these mice initially showed several areas devoid of crypts but these areas were gradual 'repopulated' by surrounding tissue over time. This data indicates that *Bmi-1* positive cells are required for crypt maintenance which is another key feature of ISC. Although *Bmi-1* does not appear to be expressed in every crypt it does seem to be a good marker for the ISC [Sangiorgi & Capecchi 2008].

Other potential markers can be used to help identify the differentiation of ISCs from mES cells such as Epithelial Antigen (EpiAnt) and Cluster of differentiation (CD) 133. EpiAnt encodes a homophilic cell surface adhesion molecule that is expressed widely throughout all epithelial cells. CD133 is a glycoprotein, also known as

Prominin-1 (PROM1). It forms part of a transmembrane glycoprotein complex that localises to cellular protrusions [Corbeil et al 2000]. It is expressed in a wide range of adult stem cell/progenitor populations [Yin et al 1997, Corbeil et al 2000, Shmelkov et al 2005, Kordes et al 2007] including putative colonic stem cells [Samuel et al 2008] and is conserved in structure across a number of mammalian species [Corbeil 2001].

ISC potentially share some similarities from cancer initiating cells in the intestine. Cancer initiating cells in the colon have been shown to be CD133+ [Ricci-Vitiani et al 2006]. When tumours were examined c.2.5% of the cells were found to be CD133+. These cells had the capacity to initiate tumours when injected into SCID mice (eight - 10 weeks from injection until termination) whereas CD133- cells did not. In some respects these cells acted like stem/progenitor cells. A small fraction of the total tumour mass consisted of cells responsible for the maintenance and growth of the tumour in the same way that many adult tissues are maintained by a small population of ASC [Ricci-Vitiani et al 2006].

Other important intestinal markers such as *Cdx1* are also associated with intestinal cancers [Bonhomme et al 2008]. *Cdx1* (and its paralogue *Cdx2*) is a homeobox gene and is involved in anterioposterior embryo patterning. It is expressed in the intestinal epithelium throughout life and often shows altered expression in many adenocarcinomas (with loss of expression being most common with overexpression seen rarely). The effects of loss of function and overexpression of *Cdx1* in mice were investigated with particular reference to intestinal development and intestinal cancers.

Neither loss of nor overexpression of *Cdx1* had any effects on intestinal development or morphology although it did produce an inverse proportional effect on *Cdx2* activity. The incidence of tumours was also unaffected but overexpression of *Cdx1* did increase the severity of tumours in some cases. These results indicate that *Cdx1* is redundant in intestinal development; whilst it does have a significant function this is replicated by other mechanisms [Bonhomme et al 2008].

The homeobox gene *Cdx2,* which is a homologue of the Drosophila gene *Caudal,* plays a key role in the control of GI tract patterning and intestinal epithelial

development [Beck et al 1999] and is a tumour suppressor in the colon [Bonhomme et al 2003]. These properties make it a potential molecular marker for intestinal epithelium. In *Cdx2*+/- mice multiple *Cdx2*- lesions that did not contain normal intestinal epithelial structures developed [Beck et al 1999]. Examination of 98 animals showed that these polyp-like lesions formed as two distinct types of epithelium developed in the intestine; normal (*Cdx2*+) and a more gastric epithelial-like layer (*Cdx2*-). Where the two tissue types met intercalary growth occurred, in effect filling in the gaps which resulted in the formation of lesions. *Cdx2* expression is vital to the correct formation of intestinal epithelium to the extent that haploinsufficiency results in serious tissue malformation [Beck et al 1999].

It was found that Cdx2 expression is reduced in tumours of the colon [Bonhomme et al 2003]. To investigate this *Cdx2*+/- and wildtype mice were treated with the mutagen Azoxymethane and then analysed for the incidence of intestinal tumours. Three months following Azoxymethane treatment *Cdx2*+/- animals displayed increased tumour (adenocarcinomas) formation confined to the distal colon (spatially distinct from Cdx2's field of activity in correct intestinal patterning); this was not observed in wildtype animals. The haploinsufficient animals also showed reduced cell apoptosis following radiation treatment of the tumours; the tumours in *Cdx2*+/- animals did not respond to treatment as favourably as those in wildtype animals [Bonhomme et al 2003].

1.2.4 Culture and characterisation of Intestinal epithelium

It has proved to be quite challenging to culture intestinal epithelium *in vitro/ex vivo* which has hindered the development of model systems to study developmental processes and diseases such as intestinal cancers. The proliferating component of the tissue, the ISC and the transit amplifying cells, constitute a very small proportion of the total tissue mass so isolating them can be difficult. Even when they are isolated ISC prove to be slow growing and do not survive for many passages *in vitro* (around 14 days) [Dekaney et al 2005]. Much GI tissue engineering has been carried out by culturing intact crypts/small tissue masses (known as organoids that can be maintained in culture (see Section 1.5) [Evans et al 1992, Tait et al 1995, Choi and

Vacanti 1997, Choi et al 1998]. In recent years some success has been achieved in maintaining intestinal epithelium *in vitro* [Perreault & Beaulieu 1998, Dekaney et al 2005, Quinlan et al 2006].

Primary foetal intestine from between 15 - 20 weeks post fertilisation was washed and the epithelium separated by non-enzymatic dissociation [Perreault & Beaulieu 1998]. The dissociated epithelium was transferred to primary culture in Dulbecco's Modified Eagle's Media (DMEM), 5% Foetal Calf Serum (FCS), human epidermal growth factor (EGF) and insulin on tissue culture plastic (TCP) that had been Collagen I coated.

The cells initially proliferated well and reached confluence after three - four days. After seven days the culture contained both goblet and absorptive cells that demonstrated a range of the functional characteristics associated with *in vivo* intestinal epithelium. Mucins and enzymes such as lactase were expressed (evaluated by antibody staining), the cells formed tight junctions and the rate of proliferation/DNA synthesis was negligible. This showed that the isolated epithelium, and the different cell types within it, was maintaining its functional characteristics *in vitro* as well as remaining viable [Perreault & Beaulieu, 1998].

The identification of putative markers has allowed the isolation and study of murine ISC *in vitro* [Dekaney et al 2005]. A cell fraction was isolated from either whole mucosa or epithelium of the jejunum and a population of cells that was negative for CD45 and other surface markers but that exhibited *Msi-1* expression was isolated and maintained *in vitro* for up to 14 days. CD45+ and dead cells were excluded and the isolated cell population was selectively sorted by FACS against a range of haematopoietic stem cells markers.

A population of cells that exhibited Msi-1 expression was isolated from both whole intestine and epithelium only samples. These cells were maintained *in vitro* and remained viable at seven days when they still excluded Trypan blue but by 14 days most of the cells had undergone apoptosis. These results demonstrate that whilst it is possible to isolate a putative population of intestinal progenitor cells it is difficult to maintain those cells *in vitro* for any significant duration. Intestinal epithelial cells

have a limited lifespan *in vivo* as they differentiate and migrate up the villi before being sloughed off into the intestinal lumen after around four - five days. ISC also require a complex array of signalling (detailed in Section 1.2.1) to maintain the stem cell niche that is not replicated under *in vitro* culture conditions [Dekaney et al 2005].

Embryonic mouse intestinal explants were maintained on fibronectin coated coverslips for a number of days [Quinlan et al 2006]. The explants maintained the expression of the key intestinal marker, the transcription factor *cdx2* as well as columnar epithelial and smooth muscle markers. The explants also began to show later markers of intestinal epithelial development (and of the specialised cell types found therein) as the *in vitro* culture progressed [Quinlan et al 2006].

1.3 Embryonic stem (ES) cells

A stem cell is defined as a cell that is both multipotent (able to form multiple cell types) and self-renewing (each time a stem cell divides at least one of its daughter cells will also be a stem cell). Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of pre-implantation (eight cell stage/three – four days post-fertilisation) blastocysts (Figure 1.7). When generating human embryonic stem cell (hES) lines the cells are taken from embryos (following informed consent) that have not met quality control standards for use in clinical *in vitro* fertilisation (IVF) treatments and are donated for research use.

They were first generated from mouse embryos in 1981 by two separate research groups, one at the University of Cambridge and one at the University of California, San Francisco respectively [Evans and Kaufmann 1981, Martin 1981] (it was Gail Martin who coined the phrase embryonic stem cell), and from human embryos in 1998 at the University of Wisconsin, Madison [Thomson et al 1998].

Evans and Kaufmann's technique involved blocking the implantation of the embryo to allow the ICM to increase in size whilst remaining in the uterus for four – six days. This was achieved by removing the mothers' ovaries and dosing her with progesterone. Following their removal from the uterus the embryos were cultured *in vitro* on Mitomycin-C inactivated fibroblast cells (see Section 2.1.2) where they formed cylinder shaped structures. These were then dissociated into single cells that were cultured on inactivated fibroblasts to generate clonal cell lines with pluripotent characteristics [Evans and Kaufmann 1981].

Martin's technique involved removing the embryo around three days post-fertilisation and then culturing it overnight. The ICM was then removed and cultured on inactivated fibroblasts. After one week of culture colonies of cells grew out of the ICM and these were used to establish clonal cell lines with pluripotent characteristics [Martin 1981].

Generating hES cell lines requires human embryos and so there are ethical considerations as well as technical ones when it comes to the generation and use of hES cell lines. The embryos were cultured to the blastocyst stage at which point the

ICM was isolated. Following isolation the ICM were plated on inactivated (by irradiation) MEF and after (up to) 15 days in culture any outgrowths were dissociated into small clumps that were replated on irradiated MEF. Uniform colonies were identified and replated following enzymatic dissociation (with Collagenase IV). This protocol was based on a technique first used to generate ES cells from other primate species [Thomson et al 1995]. Five hES cell lines were established from five different embryos with lines H1, H13 and H14 being XY (normal) karyotype and lines H7 and H9 being XX (normal) karyotype [Thomson et al 1998].

ES cells have two defining properties. Firstly, they are pluripotent which means that they are capable of differentiating into any of the cell types found in the body including those of the germ line (Figure 1.7). Pluripotent ES cells must be distinguished from totipotent cells, that can form any cell type found within the body and those found in extraembryonic structures, and multipotent (adult/tissue specific) stem cells (see below). Secondly, ES cells have the capacity for self-renewal; they are capable of propagating themselves and therefore maintaining a population of stem cells indefinitely.

ES cells also have a number of characteristics that can be examined experimentally. They express a panel of pluripotency marker genes including *Octamer-4* (*Oct-4)* and *nanog* as well as various Stage Specific Embryonic Antigen (SSEA) sugar epitopes [Draper et al 2002, Lanctot et al 2007]. They are capable of teratoma formation when injected *in vivo* in their undifferentiated state; they will form tumours along with a population of self-renewing stem cells [Cao et al 2007]. ES cells can contribute to chimaeras [Robertson et al 1986]; chimaera formation involves injecting a population of donor ES cells into a recipient early stage embryo. The injected ES cells will contribute to the tissues of the developing organism (usually a mouse). The donor cells can sometimes be observed phenotypically, for example if the donor cells were from a brown mouse with the recipient being white you might see patches of brown and white fur, or karyotypically by using donor cells from a male mouse in a female recipient and looking for the Y chromosome carrying cells. The donor cells can also be furnished with a fluorophore label, e.g. GFP, to allow tracing using a microscope [Lakshmipathy et al 2004].

ES cells proliferate rapidly and will continue to divide indefinitely in an undifferentiated state under the appropriate *in vitro* culture conditions. They express a characteristic profile of molecular markers, as mentioned above, although some variations exist between different cell lines [Nieden et al 2001, Nagano et al 2005, Nunomura et al 2005]. During long term culture some mutations will occur but the core molecular profile (and therefore the key characteristics of the cells) should remain consistent [Berrill et al 2004, Palmqvist et al 2005]. For murine ES (mES) cell culture a protein known as Leukaemic Inhibitory Factor (LIF) is required to maintain the cells in an undifferentiated state [Ogawa et al 2006]. This can either be added to the culture media or the cells can be grown on a 'feeder' layer of cells that secrete LIF; for some mES cell lines a combination of the two approaches is used (see Section 2.1.1). Feeder layer cells also provide extracellular matrix (ECM) components that help the ES cells adhere to the culture vessel. Removal of LIF from the culture media will allow differentiation to be initiated. A number of pathways, such as Grb2-Mek signalling that represses Nanog, then become active [Hamazaki et al 2006].

For hES cells either feeder layers to provide ECM components or an ECM substitute such as MatriGel© (an ECM protein mixture produced by Engelbreth-Holm-Swarm mouse sarcoma cells marketed by BD Biosciences®) are always required. A variety of factors such as β-Fibroblast Growth Factor (FGF) and BMPs are used to maintain the cells' pluripotency. Generally hES cell lines are slower to proliferate and more difficult to maintain than mES cell lines. There are some significant differences between mES and hES cells beyond the factors required to maintain them in a pluripotent state in culture [Ginis et al 2004].

If the factors that suppress ES cell differentiation are removed from the culture media the ES cells will begin to randomly differentiate. This is enhanced if they are aggregated into structures known as embryoid bodies (EBs) [Abe et al 1996]. EBs were formed in suspension culture and following seven - eight days of formation the EBs resembled a primitive yolk sac as seen in post-implantation embryos. At this stage the expression of the endodermal markers α-Fetoprotein (AFP) and Transthyretin (TTR) were examined by Northern blotting, *in situ* hybridisation and Reverse Transcription – Polymerase Chain Reaction (RT-PCR). The expression of

the selected markers was strong in all the samples examined indicating that a significant number of cells had been directed towards the endodermal fate by aggregation into EBs [Abe et al 1996].

In the first 12 hours of differentiation as EBs a number of key factors involved in transcription, intracellular signalling were upregulated compared to controls when assessed using DNA Microarrays [Hailesellasse-Sene et al 2007]. The EBS were generated by allowing ES cells to form multicellular aggregates in suspension culture. The factors that inhibit differentiation were removed in suspension culture. The removal of this inhibition allowed the formation of patches of similarly differentiating cells; this is most easily observed when cardiomyocytes are formed as groups of beating cells [Kehat et al 2001, De Smedt et al 2008]. Some cell surface molecules are early differentiation markers in mES cells, e.g. 5T4 antigen [Ward et al 2003] and therefore are potentially useful as indicators of loss of pluripotency. Some factors seem to be common between hES and mES cells when the switch from pluripotent to differentiating occurs, e.g. Lamin A-C [Constantinescu et al 2006].

Recently it has been shown that it is possible to generate a hES cell line without destroying the embryo. In IVF treatments a single cell is extracted at the eight-cell stage to conduct genetic tests, known as preimplantation genetic diagnosis (PGD). If this cell is allowed to divide one cell can be used for the tests whilst the other can be used to generate a hES cell line [Klimanskaya et al 2006]. Multiple biopsies were taken from a number of embryos (which were not cultured further) and the single isolated blastomeres were allowed to grow overnight. In total 19 samples gave ES like outgrowths with two stable hES cell lines being established. These lines proliferated in an undifferentiated state for more than eight months and maintained the expression of a number of pluripotency markers including *Oct-4,* SSEA-3 and SSEA-4 [Klimanskaya et al 2006].

Ethical concerns led to research into characterising the pluripotent character of ES cells which in turn has led to the development of techniques to produce ES-like cells by treating skin cells with a combination of signalling factors. These cells, termed Induced Pluripotent Stem (iPS) cells, have been shown to have many of the fundamental characteristics of ES cells (detailed above) and in the future may offer an

alternative to using ES cells. They were first generated from mouse cells in 2006 [Takahashi and Yamanaka 2006] and from human cells in 2007 [Yu et al 2007, Takahashi et al 2007].

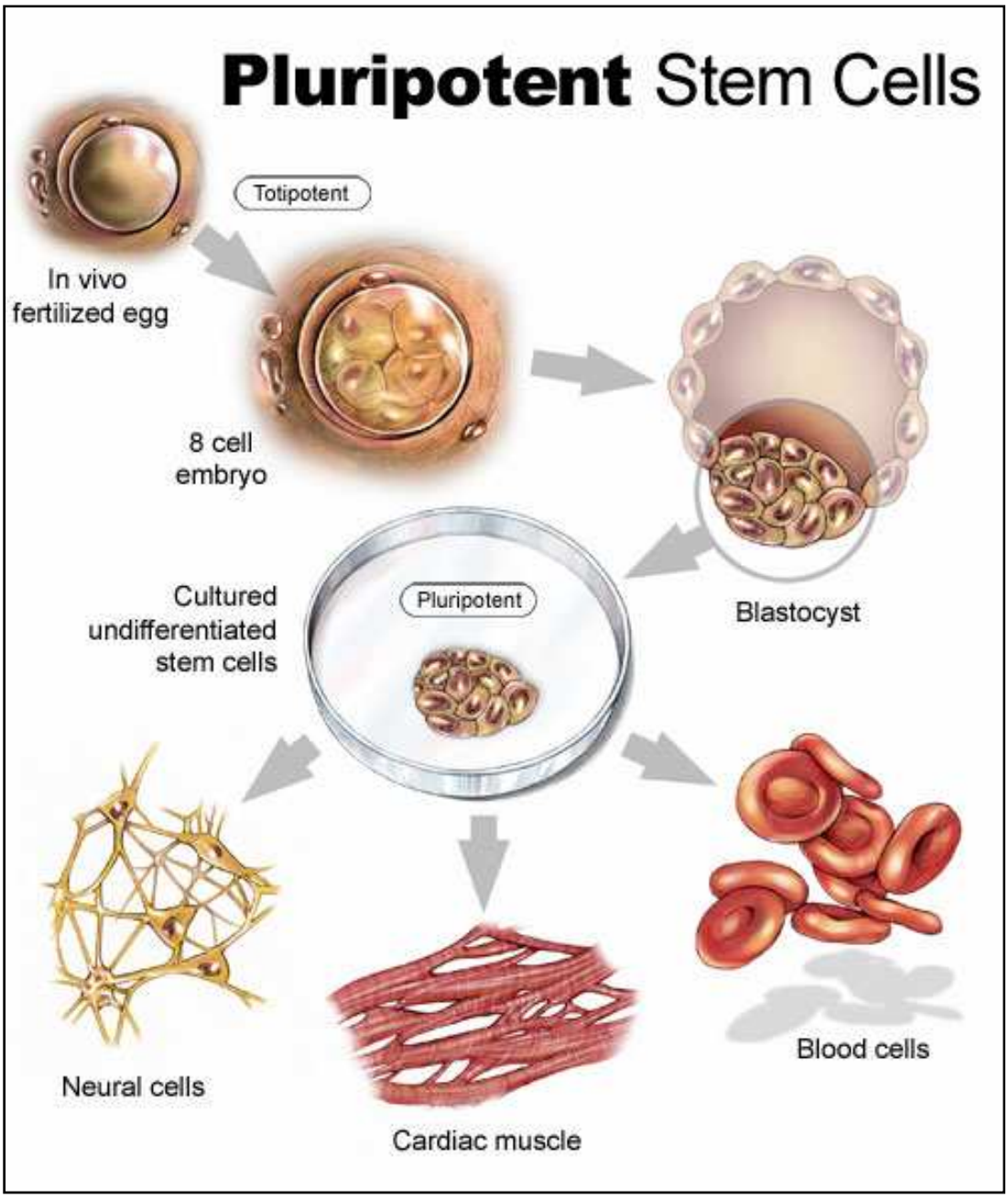


Figure 1.7: Embryonic stem cells.

Mouse skin cells were retrovirally transfected with *Oct-3/4, Sex determining region box (SOX) 2, c-Myc,* and *Klf4* and then cultured under antibiotic selection for *Fbx15* to isolate the transfected cells [Takahashi and Yamanaka 2006]. This first iPS cell line showed some aberrant characteristics, namely a failure to produce chimaeras and further changes were made to the protocols by the original research group plus a number of other researchers [Takahashi and Yamanaka 2006, Takahashi et al 2007, Wernig et al 2007, Maherali et al 2007], specifically using a different marker, *nanog*, for selection purposes.

iPS cells have the added potential bonus of offering a patient specific cell source for tissue engineering. This would reduce the risk of rejection of transplanted tissue engineered organs to an absolute minimum as the cells should be recognised by the hosts' immune system as self. iPS-like cells have also been generated from adult human testis using similar methods [Conrad et al 2008]. Recently iPS cells have been used in periodontal tissue engineering applications [Duan et al 2011].

1.4 Stem cells and their potential applications in tissue engineering

ASC, sometimes called tissue specific stem cells, have many advantages from the tissue engineering standpoint. Whilst they remain (largely) undifferentiated they are limited in the number of cell types they can form, usually to just those found in a single tissue type (although there are some exceptions such as haematopoietic stem cells). The problems with using tissue specific ASC are associated with their isolation and maintenance in culture. The ASC populations are often embedded deep in their respective tissues and only constitute a small fraction of the total cell number within a tissue. This means that extracting and isolating the stem cells can be technically difficult. Maintaining ASC in culture can also be problematic as they are often dependant on signalling from the surrounding tissues *in vivo* and once extracted from the *in vivo* tissue are programmed to undergo apoptosis. This can mean that generating sufficient numbers of cells for *in vitro* tissue engineering applications is not straightforward.

With ES cells the logistical challenges are different and there are also significant ethical concerns surrounding their use. If a small sample of ES cells was taken from an individual and stored it would be a potential source for any cell based therapies they needed in their lifetime. This can be achieved by the process known as 'Cord Banking' where a sample of pluripotent cells are taken from the umbilical cord following birth and cryogenically stored in anticipation of those cells being useful in future medical treatments [Harris et al 2007]. These cells would be immunogenically matched to the individual ('self') so there would be no risk of rejection as there is with donated organs. The cells would also have the potential to develop into any type of cell providing that techniques to drive their differentiation towards the desired fate were available; the pluripotency of ES cells is an attractive feature but ways need to be developed to reproducibly and reliably differentiate them towards the desired cell fate.

As detailed in Section 1.3 most techniques for doing this involve the generation of embryoid bodies. This technique is not always reliable and is not very efficient as the ES cells will randomly differentiate along multiple different pathways within the

aggregate. If a specific cell type is desired it must be identified and isolated from the total population.

The yield of a particular cell type may be enhanced by the addition of certain growth factors. Once the cells have differentiated the desired cell type may be selected and purified providing an appropriate panel of markers is available. The *in vitro* effects of eight different growth factors on the differentiation of hES cells (H9 clone) was examined [Schuldiner et al 2000]. hES cells were cultured on feeder layers in an undifferentiated state before being aggregated and cultured as EBs in suspension for five days. The cells were then disaggregated using trypsin and replated in monolayer on fibronectin coated TCP. The cells were then cultured with one of βFGF, TGF-β1, Activin-A (Act-A), BMP-4, Hepatocyte Growth Factor (HGF), EGF, Neural Growth Factor (NGF) and Retinoic Acid (RA) for a further 10 days (Figure 1.8).

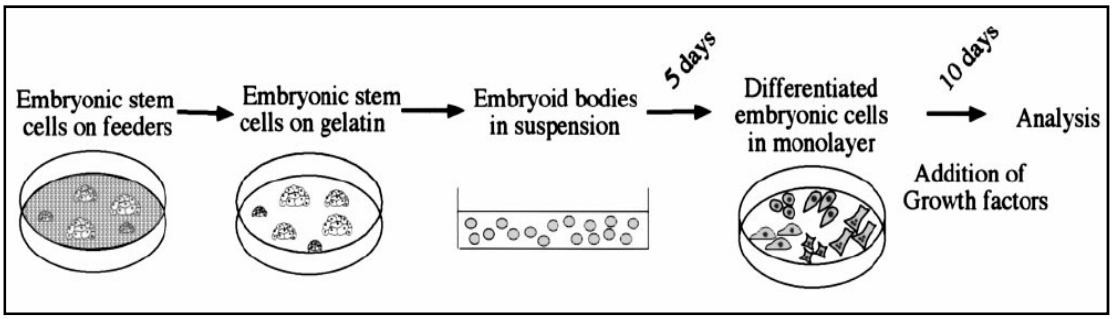


Figure 1.8: Generation of embryoid bodies to produce differentiated cells from ES precursors [Schuldiner et al 2000].

The cells were then analysed for the expression of a range of markers of differentiation to the three germ layers by RT-PCR. Firstly the expression of the receptors for each of the growth factors used was analysed. The expression of some of the receptors were negative/low in undifferentiated ES cells but were positive in five day EBs and in cells cultured in monolayer for 10 days after aggregation. This indicated that the cells were receptive to the influence of the selected growth factors in the differentiation culture. The expression of a panel of germlayer markers by the cells was then analysed. The selected ectodermal markers were *NF-H* (brain lineages), *keratin* (skin) and *DβH* (adrenal). The selected mesodermal markers were *enolase* (muscle), *CMP* (bone), *renin kallikrein* (both renal), *WT1* (uro-genital),

cActin (cardiac), *δ and β-globin* (both haematopoietic). The selected endodermal markers were *albumin, α1-AT* (both hepatic), *amylase, pdx-1, insulin* (all pancreatic) and *AFP* (definitive and visceral endoderm). Although many of these conclusions have been challenged by later studies [D'Amour et al 2005] the methodology used here has remained an excellent template for later experimental work.

Once the desired precursor cells have been isolated/generated they can be taken forward for functional analysis [Sinha et al 2006] and eventually be used in tissue engineering. Transgenic mES cells that carried a Puromycin resistance gene driven by a Smooth Muscle α-Actin (SMαA) or Smooth Muscle Myosin Heavy Chain (SM-MHC) promoter were differentiated by aggregation into EBs. The EBs were generated by the hanging droplet method with an initial formation period of three days and then a further three days in suspension culture. The cells were then cultured in selective media to isolate the Puromycin resistant Smooth Muscle Cell (SMC)-like cells. These cells were analysed for the expression of SMC markers by immunofluorescence, FACS and RT-PCR. The ES-derived cells expressed all the SMC markers tested at comparable levels with established SMC cell lines. The markers tested included SM-MHC, Myocardin and Calponin. The physiological characteristics of the ES-derived cells were also examined. They demonstrated an ability to increase Ca^{2+} levels in response to depolarisation or vasoconstrictor treatment; a function that forms part of the mechanism by which contraction is induced [Sinha et al 2006].

The cells were seeded on strips of collagen gel to form artificial muscle fibres. The contractile strength of these artificial constructs was compared to *ex vivo* muscle tissue fibres when treated with vasoactive agonists with the two sample sets proving comparable. The ES-derived cells showed a molecular profile and various physiological properties that were highly similar to established lines of SMCs. However the ES-derived cells retained significant tumuorogenic potential when injected *in vivo* into mice that were genetically compatible to the original mES cell line used (D3). Teratomas formed in a high number of injection sites in a variety of tissues where the generated SMCs had been integrated. This problem was eliminated by extending selective culture for an additional three days before the cells were

injected to remove any fraction of the cell population that retained its pluripotent character [Sinha et al 2006].

In vitro directed ES cell differentiation towards a specified cell type has been particularly successful in a number of areas including osteogenesis [Buttery et al 2001, Sottile et al 2003], pancreatic islet cells [Lumelsky et al 2001], cardiomyocytes [Kehat et al 2001], neural precursors and motor neurons [Zhang et al 2001, Wichterle et al 2002] and haematopoietic cells [Chadwick et al 2003].

ES cell differentiation towards the osteoblast fate was enhanced by treatment with ascorbic acid, β-glycerophosphate, and/or dexamethasone/RA in serum containing media or by co-culture with foetal murine osteoblasts [Buttery et al 2001]. Differentiation was assessed based on the formation of (mineralised) bone nodules, where around 50 cells are embedded in a matrix of Collagen-I and osteocalcin. Dexamethasone combined with ascorbic acid and β-glycerophosphate produced a seven-fold increase in bone nodule frequency (compared to ES cells cultured alone) when added to the cultures after 14 days. Co-culture with foetal murine osteoblasts produced a five-fold increase in bone nodule frequency [Buttery et al 2001].

hES cells have also been directed towards the osteogenic fate by *in vitro* treatments with growth factors [Sottile et al 2003]. Cell differentiation was assessed by examining the expression of osteogenic markers including osteocalcin, bone sialoprotein, osteopontin, and Collagen-I as well as observing the formation of bone nodules (that contained hydroxyapatite) in culture.

ES cells were directed towards bone and cartilage precursors via growth factor treatment [Kawaguchi et al 2005]. Initially the ES cells were aggregated into EBs and treated with RA. This suppressed mesodermal differentiation but enhanced the differentiation of neural crest which can also contribute to mesenchymal cell fates. Further treatment with BMP-4 produced osteogenic precursors whilst treatment with TGF-β produced chondrogenic precursors [Kawaguchi et al 2005].

Growth factor treatment can induce ES cell differentiation towards osteoblast, cartilage or adipogenic fates [Nieden et al 2005]. BMP-2 combined with insulin,

ascorbic acid and TGF-β produced an increase in markers of cartilage differentiation including *Sox9* (and reduced the expression of markers of other fates). Adding β-glycerophosphate and Vitamin D3 prompted these cells to differentiate towards the osteoblast fate whilst some cells expressed markers of adipogenic fates [Nieden et al 2005].

Tissue specific ECM was used to direct the differentiation of mES cells towards osteogenic fates [Evans et al 2010]. ES cells were aggregated into EBs then dissociated and cultured on (denuded) ECM prepared from the MOT3-E1 osteogenic cell line. ECM prepared from the (non-osteogenic) A549 cell line and Type I Collagen were used as controls. Mineralisation and expression of osteogenic markers were increased on the MOT3-E1 matrix compared to controls. The osteo-inductive effect was lost when the ECM was heated or treated with Trypsin suggesting that the specific protein compliment found in the ECM was responsible for the osteogenic effects [Evans et al 2010].

Other factors, including Simvastatin, have also been shown to enhance the osteogenic potential of mES cells *in vitro* [Pagkalos et al 2010]. Simvastatin concentrations of 0.1 nM were sufficient to provide an osteoinductive effect (in the absence of other factors) measured both by alizarin red staining and expression of *osteocalcin* and *osterix*. Higher (micromolar) concentrations of Simvastatin proved to be cytotoxic [Pagkalos et al 2010].

ES cell differentiation has been directed towards pancreatic cell lineages via a nestin-positive intermediate stage by culture in serum free media and treatment with basic FGF (bFGF) [Lumelsky et al 2001]. Cell differentiation was monitored by observing the expression of key molecular markers of pancreatic differentiation. Whilst most of the selected nestin positive cells developed into neurons the expression of the DE markers *Gata-4* and *HNF-3β* (*FoxA2*) and the (early) pancreatic transcription factor pdx-1 was assessed by RT-PCR in the initial stages of the differentiation protocol. In the latter stages of the differentiation protocol the expression of (murine) *Insulin I and II, Islet Amyloid Polypeptide* and (the islet α cell marker) *Glucagon* was also monitored [Lumelsky et al 2001].

The cells first expressed *Gata-4, HNF-3β* and *pdx-1* following aggregation into EBs however at this point the expression of the pluripotency markers *Oct4* and *alkaline phosphatase* was maintained. Expression of *Gata-4* and *HNF-3β* was maintained at the end of the differentiation protocol at which point all of the pancreatic markers were also expressed. Immunocytochemistry showed similar expression of the selected pancreatic markers. Around 31% of the cells stained positive for insulin. The cells released insulin when stimulated with high glucose levels and also produced other pancreatic hormones. These cells organised into islet (of Langerhans) like structures *in vitro* that were also observed following injection *in vivo* in diabetic mice where the structures also showed good vascularisation [Lumelsky et al 2001].

Treating mES cells with PI3K inhibitors produced pancreatic β-like cells [Hori et al 2002(2)]. Following treatment the cells aggregated into Islet of Langerhans-like structures *in vitro* and produced insulin in a glucose-dependant fashion. When transplanted into diabetic mice all recipients survived and exhibited reduced weight loss and improved glycaemic control compared to untreated diabetic mice. Analysis of the grafts following removal from the recipients (that subsequently 'relapsed' and died) showed no evidence of tumour formation with the ES derived cells retaining their differentiated characteristics [Hori et al 2002(2)].

Insulin producing cells have also been derived from hES cells [Segev et al 2004]. ES cells were aggregated into EBs then plated in media supplemented with fibronectin, insulin, selenium and transferrin, then media supplemented with bFGF, N2 and B27. Finally bFGF was replaced with Nicotinamide and the glucose concentration was reduced. The cells were then dissociated and placed in suspension culture. These cells formed clusters producing insulin and glucagon that exhibited elevated expression of pancreatic genes compared to controls [Segev et al 2004].

ES cells have also been differentiated towards the pancreatic lineage by co-culture with primary hepatocytes [Banerjee et al 2011]. ES cells were differentiated to definitive endoderm by co-culture with primary hepatocytes. These cells were then further differentiated by culture on Matrigel with media supplemented with Shh inhibitors and RA. >70% cells showed expression of Pdx-1, a key pancreatic transcription factor, with a number of markers of pancreatic endocrine cells, including

Ngn3 and beta2, also upregulated. These cells were then co-cultured with endothelial cells which induced upregulated expression of Insulin 1 in 60% of the population. These ES derived cells began to secrete insulin [Banerjee et al 2011].

Cardiomyocytes have been generated from hES cells by aggregation into EBs and subsequent selection of those cells that began to spontaneously contract [Kehat et al 2001]. Spontaneously contracting areas appeared in c.8% of the EBs. Cell differentiation was further assessed by antibody staining of the contracting regions and RT-PCR for various cardiac markers including cardiac myosin heavy chain, desmin and cardiac troponin I. Intracellular $Ca^{2}+$ levels showed variation that corresponded with a contraction/relaxation cycle [Kehat et al 2001].

Cardiomyocytes have also been generated from IPS cells. Human IPS cells (see Section 1.3) were differentiated towards the cardiomyocyte fate by aggregation into EBs alongside ES cells differentiated in the same fashion. Contracting cells appeared in both conditions at a similar time and both displayed upregulation of some cardiac markers. Pluripotency markers (Oct4 and nanog) were downregulated in both conditions but less so in the IPS cells (probably because both Oct4 and nanog were among the transgenes activated to induce pluripotency in the cells). Both ES and IPS-derived cardiomyocytes proliferated at a similar rate. Both sets of cells also displayed characteristics of a number of cardiac cells with evidence of sarcomere components (analysed by immunocytochemistry) and appropriate electrophysiological activity [Zhang et al 2009].

A number of studies have directed ES cells towards neuronal fates [Zhang et al 2001, Reubinoff et al 2001]. hES cells were directed towards the neural precursor fate by aggregation into EBs and treatment with FGF2. This population of cells was isolated and allowed to further differentiate (in FGF2- culture) giving rise to a number a neural cell types including neurons and astrocytes. These cells were introduced into the brains of neonatal mice where they incorporated in a number of brain regions forming neurons and astrocytes without any teratoma formation [Zhang et al 2001]. Similar results were also obtained in other studies where neural progenitors capable of forming astrocytes, neurons and oligodendrocytes when transplanted in neonatal mouse brain were generated [Reubinoff et al 2001].

ES cells differentiated towards the neuronal fate *in vitro* acquired the characteristics of particular subsets of cells following *in vivo* implantation [Peljto et al 2011]. Evidence reviewed in this study suggested that ES-derived neuronal cells contained populations of spinal (motor) neurons and cortical neurons [Peljto et al 2011].

hES cells have also been differentiated towards haematopoietic lineages by a combination of aggregation into EBs and treatment with Cytokines and BMP4 [Chadwick et al 2003]. The addition of Cytokine factors for the first 10 days of culture as EBs proved particularly effective at enhancing differentiation towards the haematopoietic progenitor fate as determined by the positive expression of the cell surface marker CD45 [Chadwick et al 2003].

WNT3a and BMP4 enrich the differentiation of haematopoietic cells from hES cells *in vitro* [Wang and Nakayama 2009]. WNT3a increased levels of both haemogenic and angiogenic activity whilst BMP4 enriched angiogenic activity in ES derived cells indicated by a population of PDGFR+/KDR+ cells. Addition of BMP inhibitors blocked the differentiating effects of WNT3a with the same effect observed with WNT inhibitors and BMP4 [Wang and Nakayama 2009].

1.5 Gastrointestinal tissue engineering

Replicating the tissue structure of the GI tract *in vitro* would be useful for drug screening, disease modelling and ultimately as an alternative to donor derived transplants. The condition Short Bowel Syndrome (SBS) arises when most of the length of the small intestine is lost, either as a result of massive resection to treat gastrointestinal cancer or as a consequence of conditions such as Crohn's disease. Radiotherapy used to treat cancer located in the pelvis or abdomen also often leads to radiation injuries to the intestine and a loss of the epithelial cell population. If less than 50 cm of small intestine is left (equating to a 75% loss) the patient will be unable to absorb sufficient nutrients to stay alive and will therefore be dependent on parenteral nutrition. Surgical intervention to slow the passage of material to the gut or to increase the surface area is possible but ultimately a transplant is required.

Aside from the usual complications associated with transplant procedures, intestinal grafts have a very low success rate. There are a large number of white blood cells found in the intestine which increases the risk of rejection whilst the large number of bacteria found in the gut increase the chances of post-operative infection. Initial graft failure occurs frequently and even when successful there is a high rate of subsequent rejection due to the complex nature of the GI immune system. Tissue ischemia and leaks from anastomosis sites are also common because of the complex nature of the surgery meaning that long term patient outlook is poor (although it is much improved compared with 10 years ago) with one year survival rates being 72% and five year survival rates being 50% (US statistics for 2010) [www.surveillance.cancer.gov/statistics/types/survival.html]. Survival rates in the UK (and W.Europe) are slightly lower than in the USA [www.cancerresearchuk.org].

Cancers of the intestine are one of the most common types of cancer in the Western world with cancers of the small intestine accounting for 0.5% of all cancers diagnosed in the USA [www.oncolink.com]. 38606 patients were diagnosed with colorectal cancer in the UK in 2007 accounting for 13% of new cancer diagnosis [www.cancerresearchuk.org]. Intestinal cancers have a very high patient mortality rate due to the frequent occurrences of primary metastasis to the liver. The far less severe but more frequent condition Irritable Bowel Syndrome (IBS) also affects many patients. There are a number of types of inflammatory bowel diseases, such as

Crohn's disease and ulcerative colitis. These conditions can be contributing factors to the development of gastrointestinal cancers [MedlinePlus, www.nlm.nih.gov] and can require severe surgical intervention themselves. Tissue engineered models could prove useful in studying the causes and progression of these diseases, for example investigation of microflora/gut interactions in IBS.

Tissue engineered intestine could also be a very useful tool in pharmaceutical testing. One of the 'gold standards' for new medicines is that they should be orally dosable and therefore any candidate drug must be shown to survive transit through the digestive system and be absorbed through the intestinal wall into the blood stream. Much of the required testing is currently carried out on animals and using Caco2 cells, which are a human cancer cell line, but tissue engineered models would provide an alternative allowing the numbers of animals used in drugs trials to be substantially reduced and providing model tissues that more closely resemble the *in vivo* environment allowing for more meaningful data to be generated. Such model systems would also be useful in studying diseases of the intestine. New EU directives on the use of animals in research are clear that pursuing alternatives to the use of live animals in experiments should be one of the top priorities for all member states. The Home Office, the government department responsible for regulating scientific research in the UK, has a series of guidelines and directives to facilitate this [http://scienceandresearch.homeoffice.gov.uk/animal-research, Animals (Scientific Procedures) Act 1986].

Most current cell based GI tissue engineering is based upon growing small intestinal epithelial crypts in (primary) culture. Tissue fragments are enzymatically digested into multicellular aggregates, known as organoids. These organoids (which contain the intact crypts), first isolated from neonatal rat intestine, consist of a layer of epithelium (including the GI stem cells which can now be identified by staining for *Msi-1,* or one of the other ISC markers detailed in Section 1.2.3) interspersed with small numbers of (mesenchymal) stromal cells [Evans et al 1992]. The presence of the stromal cells appears to be important in maintaining the stem cell niche.

Small intestinal epithelial cells were maintained *in vitro* for up to one month following isolation from suckling rats [Evans et al 1992]. The small intestines of six

day old (male and female) Wistar rats were removed following sacrifice of the animals by cervical dislocation. The tissue was cleaned and cut into sections of approximately 1 mm^3 in size. The tissue was then further broken down by a variety of enzymatic treatments including Trypsin, Collagenase, Dispase, 1 mM EDTA in Hanks Balanced Salt Solution (HBSS) and Weiser solution [Flint et al 1991].

Using either Collagenase only or a combination of Collagenase and Dispase (at 20°C with agitation) gave the best outcome with regard to further culture of the cells following disaggregation. Collagenase treatment produced small clumps of cells that adhered well to the culture vessel and demonstrated good further proliferation. Collagenase/Dispase treatment produced the organoid structures described above, where the spatial organisation of the whole tissue structure was maintained, that also demonstrated good attachment and further proliferation. Cell numbers were assessed using the crystal-violet assay and proliferation was assessed by pulse labelling with tritiated thymine. The cells were also stained with a range of antibodies to establish which cell types were present in the cultures [Flint et al 1991].

A number of media preparations were tested based on either DMEM or modified EM supplemented with FCS (between 2.5 – 10% (*v/v*)). The cells grew best in fresh media preparations rather than those prepared from concentrated stocks, where almost no growth was seen. Antibody staining showed that the vast majority (>90%) of the isolated cells were epithelial. The remaining 10% consisted of smooth muscle cells with some myofibroblast like and endothelial cells. Where higher concentrations of serum were present the smooth muscle cells tended to surround the clusters of epithelial cells [Flint et al 1991].

The epithelial cells were identified by positive staining for Cytokeratin eight with some weaker staining for Cytokeratins 18 and 19. After some time in culture following isolation as the epithelial cells spread out the Cytokeratin reaction became weaker and the cells began to express Vimentin. The non-epithelial cells showed no reactivity to the Cytokeratin antibodies. Smooth muscle cells were positive for SMαA expression; endothelial cells showed positive reactivity to OX-43 antibody and also displayed the ability to take up Low Density Lipoprotein (LDL), a functional characteristic of endothelial cells; myofibroblast cells showed positive reactivity to a

Desmin antibody. There were also some cells that displayed the morphology associated with neuronal cells such as dendrites [Flint et al 1991].

The serum concentration used in the culture media affected a number of significant experimental variables. Firstly, the serum concentration affected the growth of the isolated cells; a minimum concentration (>1%) was required to maintain the intestinal epithelial cells following isolation and the cells showed increased growth at 10% serum compared with 2.5% serum. Secondly, the serum concentration affected the growth of smooth muscle cells in the culture; at serum concentrations >2.5% the growth of smooth muscle cells was reduced and those that were present grew around the outside of the clusters of epithelial cells whilst at lower concentrations the cell types grew separately. The coating of the culture vessel had an effect on the morphology of the cultured cells but did not appear to have any influence of their proliferation; using either complete ECM or (dried) collagen coating of the TCP vessel improved the attachment of the cells [Flint et al 1991].

These organoid units have been shown to develop into small intestine like structures that can be maintained in culture and used in further tissue engineering studies [Tait et al 1995, Choi and Vacanti 1997, Choi et al 1998]. The enzymatic activity and nutrient absorption/transport capacity of engineered mucosa was examined following ISC transplantation in rats [Tait et al 1995]. ISC transplantation was used to regenerate damaged intestinal epithelium in rats and 25 days after transplantation morphologically normally tissue had formed. The functional characteristics of this tissue were evaluated by examining its nutrient transport ability and brush border (the microvillus covered intestinal epithelial surface) enzymatic activity. The activity of a panel of enzymes including lactase, sucrase, aminopeptidase N, and alkaline phosphatase activity was determined by incubation with enzyme specific substrates. Nutrient transport was assessed using labelled (C^{14}) glucose. The results were compared with those from age matched control tissue samples and the level of activity was found to be similar [Tait et al 1995].

Small intestinal organoids [Evans et al 1992 - see above] from six day old Lewis rats have been combined with biodegradable polymer scaffolds to produce tissue engineered intestine [Choi and Vacanti 1997]. These were combined with scaffolds

made of Polyglycolic acid (PGA) fibres of 15 μm in two different arrangements, either as 1 cm^2 sheets or in a tubular conformation with an internal diameter of 5 mm. The average pore size in the scaffolds was 250 μm. The organoids were seeded at a density of between 6400 – 22000 organoids per scaffold and allowed to attach for two hours prior to implantation into adult syngeneic (closely related to the donors) Lewis rats. Unseeded sheet scaffolds were implanted as controls whilst some of the seeded tubular scaffolds were cultured *in vitro* overnight or for seven days prior to implantation. The implants were recovered (following recipient sacrifice) between one and 12 weeks post implantation. Following recovery the implants were fixed, wax embedded and sectioned (5 μm sections) for histological assessment and antibody staining to identify particular cell types (anti-α-Actin for smooth muscle cells and anti-lysozyme for Paneth cells) [Choi and Vacanti 1997].

Samples from both scaffold conformations (that had been implanted shortly after seeding) at two, six and eight weeks showed cysts with neomucosal growth. The neomucosa consisted of columnar epithelium with invaginations that represented proto-crypts and villi containing both goblet and Paneth cells (positive staining for lysozyme). This epithelial layer was surrounded by vascularised tissue, fibroblasts, smooth muscle cells (indicated by positive staining for α-Actin), ECM and degrading polymer. Those scaffolds that had been cultured *in vitro* prior to implantation did not display any neomucosal formation. The controls showed evidence of some vascularisation but also stimulated a foreign body reaction/inflammation. The implants generated tissue that morphologically resembled intestinal epithelium as well containing some of the specific cell types associated with it [Choi and Vacanti 1997].

The functional properties of engineered intestinal epithelial tissue [Choi and Vacanti 1997] were examined [Choi et al 1998]. Some modifications were made to the protocol summarised above. The PGA scaffolds were sprayed with 5% polylactic acid (PLA) and some of the scaffolds were coated with Collagen I. The implants were recovered between two and 14 weeks post implantation. The morphological and histological analysis gave similar results as above but in addition the expression of the brush border enzymes sucrase and lactase was shown by fluorescent microscopy using enzyme specific substrates.

The expression pattern of Laminin was also established with the basolateral pattern suggesting that cell polarity had been established. The Transepithelial resistance (TER) of the engineered tissue was similar to native adult intestinal mucosa establishing that they had similar electrophysiological properties. The implants had generated tissue that grew and survived *in vivo* and replicated the structure, molecular and physiological properties of native intestinal tissue [Choi et al 1998].

Further studies have indicated that engineered intestinal tissue integrates well with the native tissue when transplanted following intestinal resection [Kim et al 1999, Kim et al 1999 (2), Gricksheit et al 2004, Perez et al 2002]. Tissue engineered intestine was transplanted into adult Lewis rats and the effects on tissue regeneration were examined [Kim et al 1999]. Rat intestinal epithelial organoid units [Evans et al 1992] were seeded onto biodegradable polymer tubes [Choi and Vacanti 1997] and transplanted into adult Lewis rats. The first group of animals had the seeded scaffolds implanted with no subsequent intervention. The second group of animals had the implants anastomised to the native small bowel three weeks after implantation. The third group were treated as the second but prior to implantation had had a small bowel resection.

All three groups demonstrated cysts with neomucosal growth that resembled intestinal epithelium surrounded by vascularised tissue. The anastomosis sites were all patent (the tissue was linked with an open lumen). There were increased numbers of crypt and villi that were larger in size along with increased mucosal surface area in the animals where the implants had been anastomised in comparison to the animals where the scaffolds were just implanted. The same difference was apparent between animals where the implant had been anastomised and a small bowel resection had been carried out compared with the animals where the implant had been anastomised with no resection. These results show that the scaffolds seeded with intestinal organoids can regenerate intestinal tissue and that anastomosis and small bowel resection act as stimuli for this regeneration [Kim et al 1999].

These techniques were further refined by examining which methods gave the most significant tissue regeneration [Kim et al 1999 (2)]. Some modifications were made to the protocol above with the inclusion of different experimental groups of animals.

The groups used were a control group where seeded scaffolds were implanted with no further intervention. Group two animals were implanted following a 75% small bowel resection (SBR). Group three animals were implanted following a portacaval shunt (PCS) where the hepatic portal vein is connected to the inferior vena cava. Group four animals were implanted following a partial (75%) hepatectomy – a partial resection of the liver.

The formation of neointestinal crypts was monitored *in vivo* by ultrasonography three, seven and 10 weeks after implantation. The implants were recovered 10 weeks after implantation. Cysts formed in all the animals but were larger in the SBR group at seven and 10 weeks. Histological analysis showed that the tissue was well vascularised with a neomucosal layer lining the lumen. This layer had the crypt-villous architecture as in the native intestinal epithelium. The crypts/villi were larger and more numerous and the mucosal surface area was greater in the SBR group compared with all the others. The PCS group of animals displayed a greater number of larger crypts than the control group. As with the previous study these results show that the scaffolds seeded with intestinal organoids can regenerate (well vascularised) intestinal tissue and that SBR, and to an extent PCS, act as stimuli for this regeneration [Kim et al 1999 (2)].

The functional properties of implanted engineered tissues, such as the immune cell complement, have been investigated [Perez et al 2002]. Immune defence is a key function of the intestine and therefore engineered tissue must demonstrate the same immune responses as native tissue. Engineered intestine, generated in a similar fashion to the studies above using scaffolds seeded with organoids, was implanted in rats. Some animals were implanted only whilst the other group had the implants anastomised to native tissue. Samples from the engineered tissue (cysts) and native tissue were harvested between three and 56 weeks post implantation and screened for immune cell specific antigens by immunohistochemistry.

The selected antigens were CD3 for T cells, CD32 for B cells, CD56 for NK cells and CD68 for macrophages. Cell morphology was also examined using computer based analysis. The anastomised implants showed a comparable immune cell complement to native tissue in the lamina propria and intraepithelial space 20 weeks after

implantation. In the mucosal layer the density of immune cells was less than in native tissue at both 10 and 20 weeks after implantation but the difference was less at 20 weeks. This data indicated that engineered tissue produced a similar complement of immune system cells to native tissue but these populations were restored due to tissue infiltration some time following implantation [Perez et al 2002].

The extent to which engineered tissue could replace the intestine following massive resection has also been examined [Gricksheit and Vacanti et al 2004]. In rats, the introduction of engineered small intestine significantly improved recovery after substantial resection of the small intestine. Animals that received the engineered tissue lost body mass faster than the controls at first and reached their lowest body mass first. They then showed much faster body mass gain and reached 98% of their pre-operative body mass after 40 days compared with only 76% of pre-operative body mass for animals who had not received any engineered tissue implant [Gricksheit and Vacanti et al 2004]. This data suggests that the engineered tissue integrated with the recipient at a systemic level and could contribute to recovery by significantly accelerating tissue regeneration.

Some strategies have used cell free scaffolds to provide a framework for natural regeneration of the intestine. In dogs inserting a collagen based scaffold allowed the regeneration of the epithelial layer of the small intestine [Hori et al 2001] but not the smooth muscle surrounding it. The same scaffold seeded with mesenchymal stem cells (MSCs) also allowed some regeneration of the smooth muscle responsible for peristalsis [Hori et al 2002].

A variety of types of scaffolds have been used in similar strategies. A collagen sponge scaffold seeded with SMCs was implanted in dogs where the intestine had been resected [Nakase et al 2006]. The SMCs used were autologous having been isolated from the stomach wall of the animal patient previously. After 12 weeks the seeded scaffolds showed the formation of a substantial epithelial layer with surrounding muscle tissue. The control scaffolds which were not seeded with SMCs showed some epithelial formation but only a very thin layer of surrounding muscle was present [Nakase et al 2006].

It has been shown in mice that introducing mES into a radiation damaged intestinal tract (simulating a post-radiotherapy environment) can lead to a degree of repopulation [Kudo et al 2007]. The intestines of female nude mice were irradiated with a single 30 Gy dose and then a population of male derived mES cells was transplanted into the damaged intestine. The intestines were then removed 13 or 27 days following treatment and assessed for the presence of Y-chromosome derived material by RT-PCR. The colonisation of the irradiated intestine and differentiation of the cells towards intestinal epithelial fates was assessed by histological methods and immunohistochemical analysis for SSEA-1, SMαA and cytokeratin AE1/3.

There was no improvement in the survival rate of the mice who received a cell transplant following irradiation compared to those irradiated mice that did not receive a transplant. Proliferating (donor) cells, which were positive for Y-chromosome DNA, were found to be present both 13 and 27 days after transplantation whilst in non-transplanted mice no proliferating cells were observed. Histological staining and analysis showed crypt-like structures were evident after 13 days in the transplanted mice although some undifferentiated cells were also present. There was positive reactivity to SSEA1 after 13 and 27 days which fitted with the observation that some cells remained undifferentiated.

Positive reactivity was observed for both SMαA and cytokeratin suggesting that some of the transplanted mES cells were beginning to differentiate towards either the fibroblast or epithelial fates within the environs of the irradiated intestine. This was not seen in the animals that had been irradiated but had not received a mES transplant. This study shows that undifferentiated cells may be stimulated to differentiate and become incorporated into damaged or regenerating tissue *in vivo*. This shows that mES cells are receptive to signalling from the microenvironment into which they have been transplanted [Kudo et al 2007].

Gut like structures were generated *in vitro* from mES cells [Yamada et al 2002]. EB3 mES cells were differentiated by aggregation into EBs using the hanging droplet method. Following six days of culture the EBs were plated on gelatin coated 10 cm Petri dishes to allow further growth. Some of the outgrowths formed gut like structures developing a lumen and demonstrating some mechanical activity within a

3D dome formation. These were examined to see if they displayed the electrophysiological responses associated with gut function and evaluated for smooth muscle markers by immunohistochemistry.

A number of the cellular clusters that formed demonstrated electrophysiological activity consistent with peristaltic patterns. Several of the clusters gave positive expression of the interstitial cell marker C-KIT, a cytokine receptor also known as CD117, and those that demonstrated electrophysiological activity also gave positive expression of a neuron specific Ubiquitin C-terminal Hydrolase, Protein Gene Product 9.5 (PGP9.5). The clusters also displayed spatial organisation similar to that found in the intestinal wall with an inner epithelial layer surrounded by smooth muscle cells [Yamada et al 2002].

mES cells were capable of forming gut associated structures that derive from all three germ layers [Torihashi et al 2006]. ES cells were aggregated into EBs using the hanging droplet method for six days and then replated to allow further growth. The cells were examined for markers of gut structures by immunohistochemistry and morphologically by electron microscopy between two and 28 days (EB2 – EB28) after replating. Gut like structures were transplanted under the kidney capsule of seven week old female severely compromised immunodeficient (SCID) mice. Three weeks after transplantation the animals were sacrificed and the kidney tissues examined by RT-PCR and immunohistochemistry.

Potential gut-like structures were first observed at E4/5 with weak contractions observed in these structures at E10 with the contractions noticeably stronger at E14/15. Immunohistochemical analysis showed the expression of ID2 in endoderm/epithelial formation before E10 with SMαA expressed in the surrounding smooth muscle layers from E15. Germ layer markers such as FoxA2 were also expressed. The structures began to appear more like gut structures from a morphological view with proto-villi evident.

Following transplantation the gut like structure grew in size to between three and five times their pre-transplantation size. Blood vessels and nerve fibres from the host had invaded the implanted structures. The structures retained marker expression and in

some cases where early (E6) structures were implanted had gained gut marker expression during the three week implantation period. Other gut markers such as *Cdx2* were also being expressed. In some cases the structures were maintained *in vivo* for an extended time period of 42 days. There was no sign of teratoma formation even from the early stage implants. These methods provide a way of generating small sections of intestinal tissue from mES cells and demonstrate the potential for ES cells to generate intestinal tissue given the correct stimuli [Torihashi et al 2006].

An alternative approach is to produce populations of cells that can form one part of the cellular compliment in the GI tract e.g. the epithelial layer only. For example it has been shown that human MSCs will form epithelial like cells *in vitro* [Paunescu et al 2007]. Human MSCs isolated from bone marrow were cultured with EGF, HGF, Keratinocyte Growth Factor (KGF) and Insulin-like Growth Factor 2 (ILGF2) for between seven - 14 days. When the cells exhibited a change in morphology to a more rounded shape the expression of the epithelial markers Cytokeratin 18 (by qPCR) and Cytokeratin 19 (by immunocytochemistry) was assessed. Untreated MSCs did not exhibit positive expression of either marker but following growth factor treatment both markers were expressed in the cultured cells. A number of MSC marker genes were also downregulated in the treated cells indicating that the MSCs exposed to the growth factor cocktail were differentiating towards the epithelial fate [Paunescu et al 2007].

All GI tissue engineering carried out thus far has involved *in vivo* techniques and tissue derived material. This places limits on the applications that such tissue could be used for, particularly potential clinical uses. For any tissue engineered product to be sanctioned for human clinical use (e.g. a non-donor derived transplant) all the steps and material used would have to be of non-animal origin. It further highlights the need for a readily available (laboratory generated) cell source for GI tissue engineering applications.

1.6 Activin-A (Act-A) and the selective in vitro differentiation of ES cells towards the definitive endoderm (DE) fate

The growth factor Act-A is a member of the TGF-β superfamily of signalling molecules (see Figure 3.1.1, Section 3.1). It acts by binding to Type I and II Activin receptors on the cell surface which then activate SMAD 2 & 3 molecules which in turn interact with the DNA a transcriptional response activating genes involved in the specification of DE as well as genes involved in gonadal growth and placental formation.

It has been known for some years that Activin (and Inhibin) recombinant protein can influence the differentiation of multipotent cells [Broxmeyer et al 1988]. Activin enhances the release of Follicle Stimulating Hormone (FSH) *in vitro* whilst Inhibin inhibits it. Human multipotent bone marrow cells were exposed to either Act-A or Inhibin-A *in vitro* and the effects on cell differentiation were assessed by various colony forming unit (CFU) assays. The BFU-E assay assesses the erythroid colonies (at the pro-erythroblast stage), the CFU-GEMM assay assesses the formation of myeloid multipotent progenitor cells (granulocyte, erythrocyte, monocyte, and megakaryocyte) and the CFU-GM assay assesses the formation of granulocyte or macrophage progenitors.

In bone marrow cells Act-A enhanced BFU-E colony formation two-fold over controls and CFU-GEMM colony formation seven-fold over controls. Inhibin-A showed a significant suppressing influence on BFU-E and CFU-GEMM colony formation. Both molecules seemed to act via a non-direct mechanism as the addition of certain intermediate signalling factors added to or suppressed the effects of the two growth factors. Neither molecule produced significant effects in the CFU-GM assay. Treating multipotent bone marrow cells with Act-A seems to enhance the differentiation of various haematopoietic progenitors whilst treatment with Inhibin-A has the opposite effect [Broxmeyer et al 1988].

The response of undifferentiated cells to Activin is known to be concentration dependant as in development the effects of Activin signalling are reduced over distance from signal source – as the concentration gradient falls. Act-A is dispersed by passive diffusion [Gurdon et al 1994].

More recently it has been shown that Act-A can induce ES cells (both human and murine) to differentiate towards the definitive endodermal lineage [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005, D'Amour et al 2005, MacClean et al 2007]. This work is discussed in detail in Section 3.1. The definitive endoderm (DE) is the germ layer that gives rise to the GI tract and other visceral organs during development (Figure 1.9). It is distinct from the visceral endoderm that gives rise to a number of extra-embryonic structures. The other two germ layers are the mesoderm, that gives rise to the skeleton and muscles (including the smooth muscle surrounding the intestine and cardiac muscle), and the ectoderm, that gives rise to the nervous system (including the brain) and the skin.

This differentiation can be monitored by observing the expression of key marker genes at both the mRNA and protein levels. By comparing the levels of expression of these markers expressed by cells cultured in the presence of Act-A with a number of control conditions the relative efficiencies with which the cells differentiate towards a particular fate can be determined. A variety of endodermal markers have been identified (summarised in Section 3.1.2).

Chemokine C-X-C motif Receptor 4 (CXCR4), also known as Fusin, is an alpha-chemokine cell surface receptor specific for stromal derived factor-1 (SDF-1) [Kucia et al 2004]. It plays a role in embryo implantation and is important in the mechanism of HIV infection. GATA Binding protein 4 (GATA4) is a member of a family of zinc finger transcription factors that bind a core GATA motif [Liew et al 2005]. The co-expression of the transcription factors Sex Determining Region Box 17 (Sox17) [Kania-Azuma et al 2002, Tam et al 2003] and Forkhead Box A2 (FoxA2) [Ang et al 1993], also known as Hepatic Nuclear Factor 3β (HNF-3β), indicates the production of definitive endoderm. The expression of HNF factors in the absence of Sox17 is an indication of visceral rather than definitive endoderm [Duncan et al 1994]. HNF-4 expression is found in embryonic gut and nephrons but is also expressed in a number of extra-embryonic structures. In Sox17-null mutant mice endoderm derived structures, particularly the gut, are reduced [Kania-Azuma et al 2002]. It is important to distinguish this from visceral endoderm which only contributes to extra-embryonic structures such as the amnion; visceral endoderm expresses *FoxA2* but not *Sox17*.

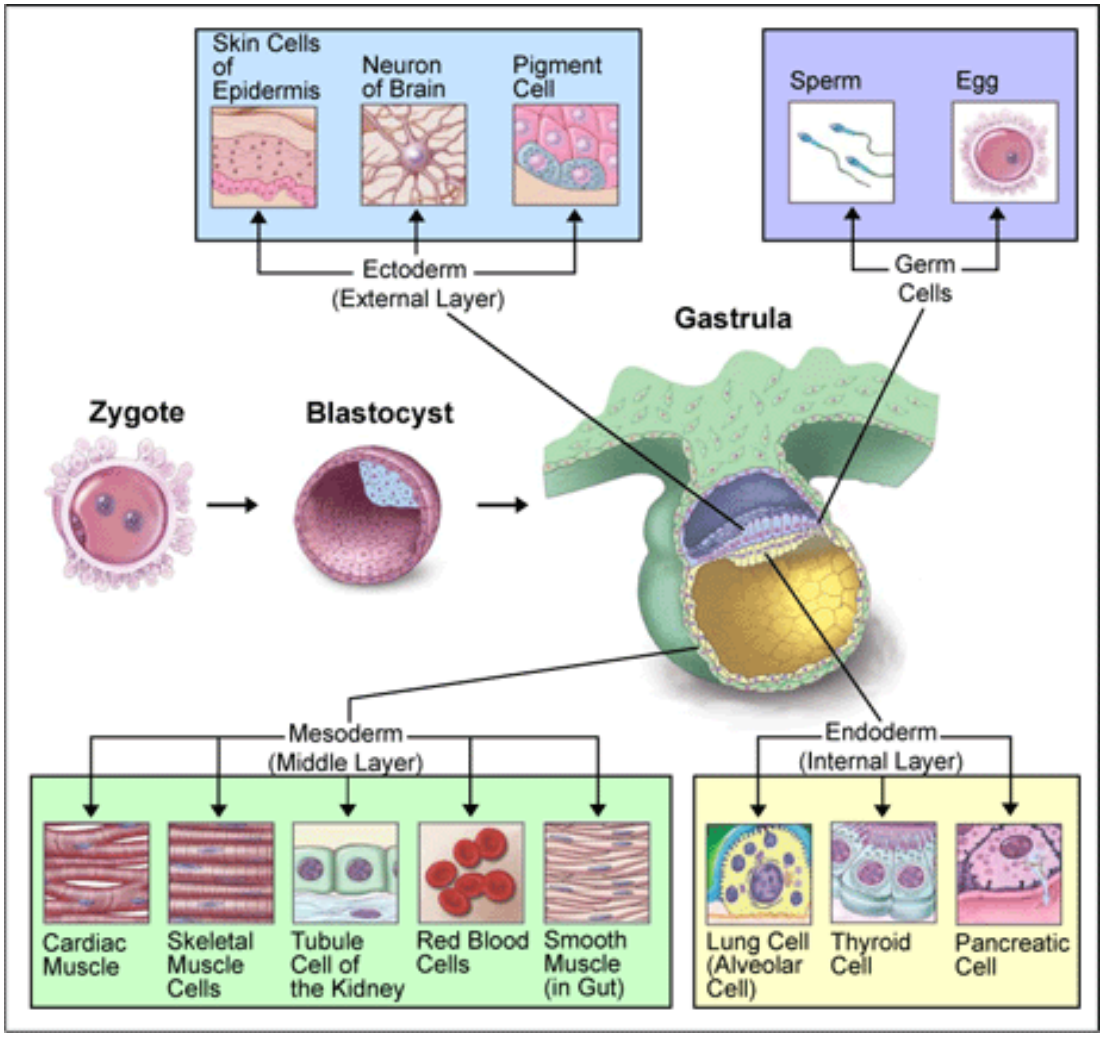


Figure 1.9: Development of the early embryo & formation of the 3 germ layers (endoderm, mesoderm & ectoderm) at gastrulation. ES cells are generated from Blastocysts.

The *Sox* gene family are transcription factors that bind to the minor groove of the DNA double helix and contain a high mobility group (HMG) box binding motif [Lefebvre et al 2007, Shivdasani 2002]. They are highly conserved across all eukaryotes. *Sox17* is a member of the *SoxF* subfamily along with *Sox7* and *Sox18*. FoxA2 is a transcription factor and contains a winged helix DNA binding domain as do the closely related *FoxA1* (HNF-3α) and *FoxA3* (HNF-3γ) [Friedman and Kaestner 2006, Kaestner 2010].

Other TGF-β family members, such as Nodal, may also play a role in DE and mesoderm differentiation of ES cells *in vitro* [Takenaga et al 2007]. Nodal signalling plays a role in the specification of mesoderm and DE during gastrulation. By

generating an ES cell line with a Tetracycline inducible promoter regulating Nodal expression, the effects of Nodal on the differentiation of the ES cells were readily observable by RT-PCR analysis of key marker gene expression. Initially upregulated Nodal expression enhanced differentiation towards the mesodermal and DE germ layers indicated by the expression of markers such as *Gsc* and *FoxA2*. Continued elevated Nodal expression resulted in teratoma formation and the maintenance of the expression of pluripotency markers such as *Oct3/4*.

If Nodal expression was downregulated following an initial period of elevated expression this lead to further differentiation towards the gut lineage indicated by the expression of markers such as *pancreatic and duodenal homeobox 1 (Pdx1)* and *Albumin 1*. Neural/ectodermal markers, such as *β-tubulin III*, were downregulated in the Nodal expressing cells. Nodal expression can be used to initiate ES cells to differentiate into mesoderm and DE but for further maturation to occur Nodal signalling must be downregulated. Sorting the cells by FACS using cell surface markers such as CXCR4 showed that the differentiation of ES towards the mesodermal and DE fates was highly efficient.

Some other TGF-β family members were upregulated in the upregulated Nodal expressing cells and therefore some of the differentiation effects may not be directly caused by Nodal activity. These Nodal expressing cells also initiated mesoderm/DE differentiation in unaltered ES cells when they were cultured together or Nodal cell CM was used. Treatment with 100 ng/ml recombinant Nodal protein did not produce this effect [Takenaga et al 2007].

Chapter One: Introduction

1.7 Aims

For tissue engineering to continue to advance from the research environment into clinical application it is crucial to have a readily available cell source for a variety of tissues. ES cells provide an attractive option to tissue engineers for a number of reasons but particularly due to their pluripotent nature. However this ability to take on multiple fates can also be something of a weakness as researchers need to generate pure populations containing one cell type, or rather the cell types found in a single tissue.

Reproducibly and reliably specifying a particular fate for ES cells is therefore one of the primary challenges facing regenerative medicine. The primary aim of this project is to generate intestinal progenitors from ES cells in sufficient numbers for intestinal epithelial tissue engineering applications (a significant proportion of the ES cells introduced into the differentiation conditions should end up specified as the desired cell type).

One way of achieving controlled ES cell differentiation is to replicate the conditions under which a particular tissue develops naturally. In the case of the GI epithelium this development involves a complex pattern of signalling with the overlying smooth muscle layer (that is derived from the mesoderm) that surrounds the epithelial layer (that is derived from the endoderm); artificially replicating the array of signals involved in this process would be highly complicated. Recent reports have demonstrated that ES cells can be induced to differentiate to tissue specific lineages by creating a microenvironment that possesses the early signalling events that drive ES cell differentiation.

Such microenvironments have been established *in vitro* using embryonic tissue from the developing mouse or chick to promote pulmonary epithelial [Van Vranken et al 2005], photoreceptor [Sugie et al 2005], haematopoietic [Krassowska et al 2006] and hepatic [Fair et al 2003] differentiation respectively. By co-culturing mES cells or

hES cells with early embryonic chick gut tissue early signalling factors would be present and may therefore provide an environment that is able to direct ES cell differentiation towards the endodermal and hence gut lineages.

A number of studies have been conducted [D'Amour et al 2005, MacClean et al 2007, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005] to show that fairly simple cell culture treatments can favour the formation of DE, the germ layer from which the gastrointestinal epithelium arises, from ES cells. By combining these *in vitro* treatments with the co-culture of ES cells with early chick gut tissue the ES cells should be directed to differentiate toward the intestinal fate.

Hypothesis: The differentiation of murine and human ES cell into intestinal progenitors can be directed specifically by signalling from the gut mesenchymal microenvironment of the developing chick.

Based on this hypothesis it is proposed to establish methodologies to derive intestinal stem cell/progenitor populations *in vitro* in a controlled fashion for future tissue engineering research projects. To test the hypothesis, gut tissue from chick embryos will be isolated at various stages of development. The tissue will be maintained in culture and ES cells (both undifferentiated and those that have been differentiated towards DE using *in vitro* stimulation) will be injected into the mesenchymal tissue surrounding the gut organoid.

Following culture, the cells will be extracted from the tissue and purified using flow cytometry and profiled using RT-PCR, immunocytochemistry and western blotting for markers of definitive endoderm and intestinal epithelial differentiation. If the cells display the molecular characteristics of intestinal precursors they will be functionally evaluated by culturing them in a model co-culture system with intestinal subepithelial myofibroblasts (ISEMFs) [Beltinger et al 1999] to examine if they organise and behave in a similar fashion to tissue derived cells.

Therefore the main aims of this project are to:-

- Establish the viability of early embryonic chick gut tissue in culture measuring any degradation of tissue quality and viability that occurs over time.

- Replicate previously described procedures for differentiating mES (& hES) cells to DE *in vitro* to determine the method is consistent for the Columnar Epithelial Epiblast (CEE) mES cell line being used in our laboratories.

- To co-culture mES cells with early chick gut tissue and show that the injected cells can be recovered and purified and that they are differentiating into gastrointestinal epithelial progenitors and/or differentiated intestinal cells.

- To evaluate the resulting cells in existing co-culture model systems.

Chapter Two: General methods

2.1 Cell culture

2.1.1 Maintenance of undifferentiated mouse embryonic stem cells in culture

CEE mES cells (between passages 15 - 30) [Buttery et al 2001, Gothard et al 2010] were maintained on (LIF producing) Sto Neo Leukaemic feeder cells (inactivated mouse fibroblasts, SNLs) [Williams et al 1988] at 37°C, 5% CO_2 in air in T25 tissue culture treated plastic (TCP) flasks (Nunc, UK) that had been gelatin (Sigma, UK) coated. The co-cultures were maintained in a monolayer in DMEM (Gibco, UK) supplemented with 10% (*v/v*) FCS (Sigma, UK), 2 mM L-Glut (Sigma, UK), 1% (*v/v*) antibiotics and antimycotics (AB-AM, Sigma, UK), 100 μM β-Mercaptoethanol (β-MCPE, Sigma, UK) and LIF (Sigma, UK) at 5 x 10^3 units/ml (referred to as mES complete media, see Section 6.4 for full media 'recipes').

When the cells reached c.80% confluence they were passaged by treatment with 0.25% (*v/v*) Trypsin (Sigma, UK) in 0.02% (*w/v*) EDTA (Sigma, UK). Please note that all Trypsin treatments were carried out using this formulation referred to as Trypsin/EDTA henceforth. The media was removed from the flask and the cells were washed with Phosphate Buffered Saline (PBS, Sigma, UK) to remove any residual proteins from the media that might inactivate the Trypsin and to remove non-viable cells. Trypsin/EDTA (3 ml) in PBS that had been pre-warmed was added to each flask for a few seconds (until cells could be seen lifting off the bottom of the flask). The cell suspension was transferred to a 15 ml centrifuge tube and aspirated up and down to generate a single cell suspension. mES complete cell media (6 ml) was added to each tube to inactivate the Trypsin. The cell suspension was centrifuged at 200 *g* for five minutes to pellet the cells. The supernatant was discarded and the cells were resuspended in 4 - 20 ml of fresh mES complete media. Cell suspension (1 ml) was seeded into 4 ml of mES complete media in a fresh flask and incubated.

2.1.2 Inactivation of SNL Fibroblasts with Mitomycin-C

Confluent T75 cell culture treated flasks (Nunc, UK) of SNL Fibroblasts were treated with 1 ml of 0.1 mg/ml Mitomycin-C (Sigma, UK, final concentration 0.01 mg/ml) This chemical causes the cell cycle to arrest by blocking DNA synthesis and nuclear division preventing further proliferation of the cells whilst maintaining cell viability [Tomasz 1995]. The cells were then incubated with Mitomycin-C for two hours at 37°C in 5% CO_2 in air after which time the flasks were washed three times with PBS then treated with Trypsin/EDTA to dissociate the cells. The cells were then transferred to a 15 ml Falcon tube containing SNL complete media (as mES complete media above but LIF-, see Section 6.4) supplemented with 10% (*v/v*) FCS to inactivate the Trypsin. The cell suspension was then centrifuged at 200 *g* for five minutes. The supernatant was then removed and the pellet resuspended in SNL complete media (2 ml). A cell count was carried out using an Improved Neubauer Haemocytometer.

SNL complete media (5 ml) was added to gelatin coated cell culture flasks (see Section 2.1.3 below) and the cells seeded at 8 x 10^4 cells/ml (4 x 10^5 cells/flask). The flasks were then gently agitated to ensure an even distribution of the cells and incubated overnight at 37°C, 5% CO_2 in air to allow the SNL cells to adhere to the cell culture surface before being seeded with mES cells. The flasks remained usable for two weeks after preparation; maintenance of fibroblast morphology and distribution was confirmed prior to seeding with mES cells.

2.1.3 Gelatin and Collagen IV coating of tissue culture plastic vessels

Gelatin was made up at 2% (*w/v*) solution and stored at 4°C. This was diluted to 0.1% (*v/v*) in PBS (5 ml gelatin in 95 ml PBS) and filtered; 3 ml was then added to a cell culture flask or 1 ml per well of a six well TCP plate (Nunc, UK). The flasks or plates were then left for at least two hours at room temperature; excess gelatin solution was removed before cell seeding.

Collagen IV (Fluka, UK; used to enhance mES cell adherence to the TCP surface [Khoshnoodi et al 2008]) was made up at 0.5% (*w/v*) solution in acidified water and stored at 4°C. This was diluted to 0.01% (*v/v*) in PBS, filtered (using a 0.2µm filter) and 1 ml added per well of a six well TCP plate. The plates were left for at least two hours at room temperature before being seeded with cells.

2.1.4 Generation of EBs

CEE mES cells that had been maintained as described in Section 2.1.1 for between 15 - 25 passages were washed with PBS (3 ml) and treated with Trypsin/EDTA (2 ml) to generate a single cell suspension; the concentration of the cell suspension was determined by counting with a Neubauer improved haemocytometer. The CEE mES cells were seeded ($5 \times 10^4 - 1 \times 10^6$ cells/ml) in non-TCP six well plates in DMEM supplemented with 1% (*v/v*) FCS, 1% (*v/v*) Penicillin/Streptomycin (Pen/Strep, Sigma, UK, 100U Penicillin and 100 ng Streptomycin per ml) and 1% (*v/v*) L-Glut (2 mM). The plates were then placed in agitated culture at 37°C, 5% CO_2 in air (on an orbital plate shaker set at 15 rpm) for a minimum of six hours. The media was supplemented further to gain a final concentration of 10% (*v/v*) FCS (by the addition of 90 µl FCS per ml of media/ 180 µl per well) and the plates were transferred to static culture at 37ºC, 5% CO_2 in air for a minimum of two days.

When media changes were required, based on the colour change of the Phenol red pH indicator in the media [Rahat and Reich 1983], standard SNL complete media (10% (*v/v*) FCS) was used. Phenol red is yellow at pH $\leq$ 6.8, red between pH 6.8 – 8.2 and pink at pH $\geq$ 8.2. If any specific growth factors were to be used the media was supplemented with them at the required concentration (details given in the methods sections of the relevant results chapters). To reseed the cells in monolayer the EBs were washed with PBS then treated with pre-warmed Trypsin/EDTA (3 ml) to disaggregate them and then reseeded in a gelatin coated TCP six well plate.

2.2 Molecular analysis

2.2.1 RNA sample collection

2.2.1i Cultured cells

At the designated timepoints for each experiment media was removed from the cells in culture which were then washed three times with PBS (3 ml). The cells were then detached/disaggregated by treatment with Trypsin/EDTA that had been pre-warmed to 37°C. The cell suspension was then transferred to a 15 ml centrifuge tube and neutralised by the addition of DMEM supplemented with 10% (*v/v*) FCS and the cell suspension was centrifuged at 200 *g* for five minutes to pellet the cells. The supernatant was removed and the cells were resuspended in lysis buffer; either Tri-reagent (Sigma, T9424) or RNeasy MiniPrep kit RLT buffer (Qiagen, 74101) were used. The samples were then stored at -80°C until required.

2.2.1ii Tissue samples

Samples were washed thoroughly with PBS. Larger tissue pieces were manually broken up using sterile forceps prior to enzymatic digestion. The tissue pieces were broken down using a dispase-collagenase digestion media (DMEM, 1% (*v/v*) FCS, 1% (*w/v*) AB/AM, 1% (*w/v*) L-Glut, 75 U/ml collagenase XI, Sigma, 20 µg/ml dispase, Sigma). The explants were transferred to fresh non-TCP six well plates containing the digestion media and incubated for two - three hours at 37°C on a shaker. The cell suspension produced from the dispase-collagenase digest was pelleted by centrifugation at 200 *g* for five minutes, then washed with PBS and repelleted before being resuspended in lysis buffer as detailed above in Section 2.2.1i. The samples were then stored at -80°C until required.

2.2.2 RNA sample preparation

2.2.2i RNA sample preparation using Tri-reagent.

Samples lysed in tri-reagent were purified by ethanol precipitation. The samples were thawed and allowed to stand at room temperature for five minutes. Chloroform (that did not contain isoamyl alcohol or other additives) was added to each sample at a

volume of 0.2 ml per 1 ml of Tri-reagent used in the lysis of the sample. The sample was covered, shaken vigorously and then allowed to stand at room temperature for between two - 15 minutes prior to centrifugation at 12000 *g* for 15 minutes at 4°C. The sample separated into three distinct phases; a (red) organic phase at the bottom containing protein, a narrow interphase band containing DNA and a (colourless) aqueous phase uppermost containing RNA.

The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added per 1 ml of tri-reagent used in the original sample preparation. The sample was well mixed and allowed to stand at room temperature for five - 10 minutes and then centrifuged at 12000 *g* for 10 minutes at 4ºC; the RNA formed a pellet at the bottom and side of the tube. The supernatant was removed and the sample was washed in (at least) an equal volume of 75% *(v/v)* ethanol in water per volume of tri-reagent used in the original sample preparation. The sample was vortexed and then centrifuged at 7500 *g* for five minutes at 4°C. Most of the supernatant was removed and then the pellet was allowed to air dry (in a flow hood). The pellet was then resuspended in an appropriate volume of nuclease free water.

2.2.2ii RNA sample preparation using RLT buffer.

Samples lysed in RLT buffer were thawed and then purified using RNeasy MiniKit/columns (all the buffers used are components in the kit, Qiagen) according to the manufacturer's instructions. The sample was homogenised by adding an equal volume of 70% *(v/v)* ethanol. The sample was mixed and then transferred to an RNeasy column in a 2 ml tube. This was centrifuged at ≥8000 *g* for 15 seconds and the flow through discarded. RW1 buffer (700 µl) was added to the column which was centrifuged at ≥8000 *g* for 15 seconds and the flow through discarded. RPE buffer (500 µl) was then added to the column which was centrifuged at ≥8000 *g* for 15 seconds and the flow through discarded. A further 500 µl of buffer RPE was added to the column which was centrifuged at ≥8000 *g* for two minutes and the flow through discarded.

The column was transferred to a fresh 2 ml tube and centrifuged at full speed (13000 *g*) for one minute. The column was then transferred to a 1.5 ml collection tube. RNase free water (50 µl) was added to elute the RNA from the column. This was then centrifuged at ≥8000 *g* for one minute. The elution step was then repeated to give a final sample volume of 100 µl. The RNA content of all the purified samples were then quantified using a NanoDrop spectrophotometer (Thermo Scientific) and stored at -80°C until required for further use.

2.2.3 Immunocytochemical sample preparation

At the designated time points for each experiment (details given in individual results chapters) media was removed from the cells in culture which were then washed with PBS. The cells were then fixed by treatment with 4% (*w/v*) Formalin (Sigma, HT501128) for 20 minutes at room temperature. The cells were then washed three times with PBS and then stored at 4°C under PBS until required. The plates were sealed with Parafilm to prevent desiccation. See Chapter Four, sections 4.2.13 and 4.2.15 for details of post co-culture immunocytochemical analysis.

2.2.4 Western blot sample collection

2.2.4i Western blot sample collection from cultured cells

At the designated timepoints for each experiment media was removed from the cells in culture which were then washed three times with PBS (3 ml). The cells were then detached/disaggregated by treatment with Trypsin/EDTA that had been pre-warmed to 37°C. The Trypsin was then neutralised by the addition of DMEM supplemented with 10% (v/v) FCS and the cell suspension was centrifuged at 200 *g* for five minutes to pellet the cells.

2.2.4ii Western blot sample collection from tissue samples

Samples were washed thoroughly with PBS. Larger tissue pieces were manually broken up. The tissue pieces were broken down using a dispase-collagenase digestion media (DMEM, 1% (*v/v*) FCS, 1% (*w/v*) AB-AM, 1% (*w/v*) L-Glut, 75 U/ml

collagenase XI, 20 µg/ml dispase). The explants were transferred to fresh non-TCP six well plates containing the digestion media and incubated for two - three hours at 37°C on a shaker. The cell suspension produced from the dispase-collagenase digest was pelleted by centrifugation at 200 *g* for five minutes.

2.2.5 Western blot sample preparation

Cells or tissue samples (as collected in sections 2.2.4i and 2.2.4ii) were suspended in freshly prepared lysis buffer (1% (*v/v)* Triton X-100, 20 mM Tris HCl, 150 mM NaCl) containing 1:1000 EDTA-free protease inhibitor cocktail (Boehringer Mannheim, Germany), 1 mM Phenylmethanesulphonylfluoride (PMSF) and 1 mM EDTA in deionised water (dH_2O). The samples were then lysed on ice for 30 minutes. The lysate was centrifuged at 14000 *g* for 10 minutes and the supernatant containing the solubilised protein was collected and then stored at -80°C until required. The protein content was determined by the Bicinchoninic acid (BCA) assay or using the NanoDrop spectrophotometer.

2.2.6 BCA assay

The BCA assay (aka the Smith assay) [Smith et al 1985] was carried out using a QuantiPro® BCA assay kit (Sigma, UK) according to the manufacturers' instructions. A series of protein (Bovine Serum Albumin (BSA)) standard solutions were prepared (0 – 10 mg/ml total protein) to produce a standard curve including blanks. A volume of each sample (40 µl) was combined with 40 µl of the reaction mixture in a flat bottomed 96 well plate and incubated for one hour at 37°C in the dark. The absorbance reading at 560 nm was then determined using a KC4 plate reader (BioTek). A standard curve was generated by plotting protein concentration (X-axis) against absorbance at 560 nm (Y-axis) using the data from the known standard solutions. The absorbance values for the unknown samples were then used to determine their protein content using the equation of the straight line generated from the standard curve.

2.2.7 Primer design

Primers were designed using Ensemble (www.ensembl.org) for sequence information and Primer 3 (http://frodo.wi.mit.edu/). Sequences were then checked using BLAST (www.ncbi.nlm.nih.gov) to ensure that they were specific for the desired gene only. *GAPDH* primers that had previously been designed within the Tissue Engineering group were used as a ubiquitously expressed control to confirm the RT stage of the reaction had worked. The primers were then ordered from MWG-Operon.

Reaction conditions for each primer pair were optimised using RNA prepared from undifferentiated mES cells, embryoid bodies, early stage mouse embryos and total mouse RNA (Applied Biosystems UK, AM7800) for the murine primers. Chick primers were optimised using RNA generated from whole chick embryos (at various stages of development), decapitated embryos and excised gut tissue. Full primer details can be found in the relevant experimental chapter methods section (Section 3.2.2i and Section 4.2.14).

2.2.8 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT and PCR reactions were carried out on a Px2 Thermal Cycler (Thermo Electron Corporation) in thin walled 200 µl PCR reaction tubes (Qiagen, 11402-8100). In the RT reaction, 1 µg of total RNA was used for each sample. This was added to the reaction mixtures detailed in Table 2.1A below. Reaction Mixture One was incubated at 65°C for 10 minutes to denature any secondary RNA structures. Reaction Mixture Two was then added and the total mixture was incubated at 55°C for two hours. The temperature was then raised to 85°C for 10 minutes to denature the reverse transcriptase enzyme, halting the reaction. The cDNA product was then stored at -20°C until required.

The product from the RT reaction was used as the template in the PCR reaction mixtures outlined in Table 2.1B. Aliquots of Master Mix One and Two (enzymes and buffers from Roche, dNTPs from Sigma, UK) were mixed and 5 µl of template was

added. These were then run on a PCR program with the general parameters of an initial denaturation incubation of 10 minutes at 94°C. This was followed by *n* cycles (see Table 3.2.4 and 4.2.1 for primer pair details) at 94°C for 30 seconds, *X*°C for 30 seconds (see Table 3.2.4 and 4.2.1 for primer pair details) and then 72°C for one minute. This was followed by a final extension incubation of 10 minutes at 72°C. The products of these reactions were then analysed by gel electrophoresis (see Section 2.2.9).

2.2.9 Gel electrophoresis

Agarose gels were prepared by melting 2% (*w/v*) high grade agarose (Sigma, UK) in Tris-Borate EDTA (TBE, Sigma, UK) electrophoresis buffer in a microwave. This was allowed to cool to approximately 60°C before Ethidium Bromide (EtBr) was added to the molten gel. The molten gel was poured into a gel tray, combs inserted in place and the gel allowed to set. Once the gel had set, it was transferred to an electrophoresis tank filled with TBE buffer. The samples were mixed with 1 µl loading buffer (Sigma, UK) per 9 µl of sample. Each sample (10 µl) was then loaded into a separate well in the gel. A ladder consisting of known fragment sizes (see Figure 2.1) was also loaded into a well. An electric current (100 mV, 100 mA) was then passed across the gel. The negatively charged DNA migrates towards the positive terminal. The fragments separate according to size as smaller fragments are able to move faster through the gel matrix. After 20 – 30 minutes the current was stopped and the gel removed from the tank and imaged using a UV transilluminator.

Table 2.1A: Reverse Transcription master reaction mixture recipe tables. U = units of enzyme

Reverse Transcription 1

Reagent	Stock conc	Volume	Final conc
Template	Variable	Variable	c.1ug total
Oligo dT	500ng/ul	1ul	25ng/ul
H2O	n/a	up to 13ul	n/a
	Total	13ul	

Reverse Transcription 2

Reagent	Stock conc	Volume	Final conc
RT buffer	5X	4ul	1X
RNase inhibitor	40u/ul	0.5ul	20u
dNTP mix	10mM each	2ul	1mM each
RTase	20u/ul	0.5ul	10u
	Total	7ul	

Table 2.1B: PCR master reaction mixture recipe tables. U = units of enzyme

PCR master mix 1

Reagent	Stock conc	Volume/reaction	Final conc
dNTP mix	10mM each	1ul	0.2mM each
Forward primer	5uM	5ul	0.5uM
Reverse primer	5uM	5ul	0.5uM
Template (RT)	Unknown	Variable	Unknown
H2O	n/a	up to 25ul	n/a
	Total	25ul	

PCR master mix 2

Reagent	Stock conc	Volume/reaction	Final conc
PCR buffer	10X	5ul	1X
Taq polymerase	20u/ul	0.25ul	5u
H2O	n/a	19.75ul	n/a
	Total	25ul	

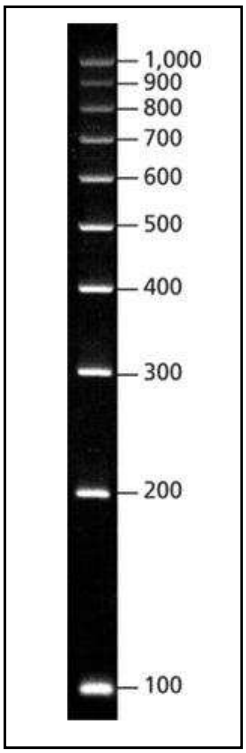


Figure 2.1: 100 base pair (bp) fragment PCR ladder.

Following imaging the bands obtained were (subjectively) scored on a scale of one – seven based upon relative size (pixel count) and brightness (pixel intensity). A guideline to these scores can be found in Section 6.4.

2.2.10 Immunocytochemistry

The expression of proteins of interest within samples was analysed using the appropriate antibodies against the products of the genes of interest at the protein level by either fluorescence or enzymatic immunocytochemistry. Details of the primary antibodies used are given in Table 2.2.

2.2.10i Fluorescence immunocytochemistry

Fixed samples (as prepared in Section 2.2.3) were washed in PBS. The samples were then permeabilised by treatment with 0.1% (*v/v*) Triton X-100 for one hour at 4°C, non-specific protein binding sites were blocked with 2% (*w/v*) BSA in PBS for one hour before being incubated overnight at 4°C in primary antibody (Santa Cruz

biotechnology, see Table 2.2) diluted 1:200 in PBS including the appropriate blocking serum (i.e. serum from the animal species in which the secondary antibody was raised). The samples were then washed in PBS before being incubated in fluorescently tagged secondary antibodies overnight at 4°C. The samples were then washed in PBS and all proteins immobilised by treatment with 4% (*w/v*) Paraformaldehyde (PFA) in PBS for five minutes at room temperature. The samples were washed with PBS and then incubated with DAPI for 60 seconds and then washed with PBS again. Finally the samples were mounted using a fluorescence preserving mounting agent such as 2.5% (*w/v*) Polyvinylacetate (PVA)/1-4 Diazabicyclo-2-2-2-octane (DABCO) or Vectashield (Vector Labs, H-1000). The samples were then visualised using a Nikon fluorescence microscope.

2.2.10ii Enzymatic immunocytochemistry

The fixed samples were washed in PBS. The samples were then permeabilised by treatment with 0.1% (*v/v*) Triton X-100 for one hour at 4ºC. The cells were then treated with 1 - 3% (*v/v*) Hydrogen Peroxide (H_2O_2) for one hour at room temperature to quench any endogenous peroxidase activity. The cells were then washed in PBS and non-specific protein binding sites blocked by treatment with 0.5% (*w/v*) Saponin in PBS for 10 minutes at 37°C. The samples were then incubated overnight at 4°C in primary antibody in 0.05% (*w/v*) Saponin in PBS plus appropriate blocking serum.

The cells were then washed with PBS and incubated overnight at 4°C in biotinylated anti-primary secondary antibody (Sigma, UK). The samples were then washed with PBS and incubated in a 1 μg/ml solution of Horse Radish Peroxidase (HRP)-Streptavidin conjugate in 1% (*w/v*) blocking reagent (Diaminodenzidine (DAB) histochemistry kit, Invitrogen, D22187) for one hour. The samples were then washed with PBS and incubated in 1 mg/ml DAB in PBS containing 0.03% (*v/v*) H_2O_2 for between 10 seconds and five minutes (until a significant colour change was observed). The samples were washed with PBS and then incubated with DAPI for 60 seconds and then washed with PBS again. The samples were then visualised using a Nikon Eclipse microscope.

Table 2.2: Table of Primary antibodies (Santa Cruz Biotechnology)

Target species	Target protein	Primary AB raised in	Cat no	Secondary AB	Raised in
Mouse (Mus musculus)	Sox17	*Goat (Capra aegagrus)*	sc-17356	FITC-Anti Goat IgG	Rabbit
Mouse (Mus musculus)	FoxA2	*Goat (Capra aegagrus)*	sc-6554	FITC-Anti Goat IgG	Rabbit
Mouse (Mus musculus)	Oct4	*Goat (Capra aegagrus)*	sc-8628	FITC-Anti Goat IgG	Rabbit
Mouse (Mus musculus)	GATA4	*Rabbit (Lagomorpa Leporidae)*	sc-9053	FITC-Anti Rabbit IgG	Goat
Mouse (Mus musculus)	Brachyury	*Goat (Capra aegagrus)*	sc-17743	FITC-Anti Goat IgG	Rabbit
Mouse (Mus musculus)	Nestin	*Rabbit (Lagomorpa Leporidae)*	sc-20978	FITC-Anti Rabbit IgG	Goat

2.2.11 Western blots

Protein samples (see sections 2.2.4, 2.2.5 and 2.2.6) were diluted to 10 µg/µl and 10 µl of sample was mixed with 10 µl loading buffer (Invitrogen, LC2676). The samples were heated at 60°C in a waterbath for five minutes to fully denature the proteins and then centrifuged at 13000 *g* for five minutes to remove any cellular debris that might interfere with the blot [Dunbar 1994]. A 12% (*w/v*) Tris-Glycine gel (Invitrogen, EC6005BOX) was loaded into the gel holder in the Invitrogen X-Cell Surelock gel tank. The central tank was filled with 1X Novex® Tris-Glycine gel Running Buffer (Invitrogen, LC2675) and left for two minutes to check for leaks. The surrounding reservoirs were then filled with 1X Running Buffer. Pre-stained molecular weight markers (10 µl, Invitrogen, LC5800) were loaded into the outer wells of the gel followed by 10 µl of the samples to the remaining wells. The gel was then run using an Invitrogen PowerEase 500 power pack set to 125 V, 35 mA and 5 W for 75 minutes. The gel was removed and soaked in 1X Novex® Tris-Glycine gel Transfer Buffer (Invitrogen, LC3675) containing 20% (*v/v*) methanol for 10 minutes. The blotting tank was then prepared by placing two pads (pre-soaked in 1X Transfer Buffer) and a filter paper in the tank. The gel was then carefully aligned and placed

on the filter paper. A nitrocellulose membrane was then placed on top of the gel with another filter paper and two more pads added on top of that. The blotting tank was then placed in the gel tank with the Nitrocellulose membrane towards the positive (red) terminal. The blotting tank and the reservoirs were filled with 1X Transfer Buffer and the blotting process run at 25 V, 125 mA and 17 W for 90 minutes.

If the transfer was successful the coloured bands from the pre-stained standards were seen on the membrane. This was blocked in Tween-Tris Buffered Saline (TTBS, 15mM Tris-HCl, 150mM NaCl, 0.01% (*v/v*) Tween 20 at pH 7.6) containing 1% (*w/v*) BSA for 30 minutes. This was then rinsed in TTBS and incubated overnight in TTBS containing 0.1% (*w/v*) BSA plus primary antibody. The membrane was then washed three times in TTBS 0.1% (*w/v*) BSA (a total duration of 10 minutes) and incubated for one hour in TTBS 0.1% (w/v) BSA plus the appropriate anti-primary secondary antibody (AntiRabbit-HRP conjugate from Invitrogen, G21234 & AntiGoat-HRP conjugate from Invitrogen, R21459). The membrane was then washed three times in TTBS 0.1% (*w/v*) BSA and once with Tris Buffered Saline (TBS). Detection solution (Sigma, T0565-5ml) was added for 15 minutes to allow the colour to develop. Positive expression was indicated by a band at the expected molecular weight determined using the molecular weight standard markers (Figure 2.2).

2.3 FACS of the samples post co-culture

Single cell suspension samples were sorted for positive expression of GFP using a Beckman-Coulter Altra Flow Sorter (http://genomics.nottingham.ac.uk). This work was carried out in the University of Nottingham's Central FACS Facility (based in the QMC, Nottingham) with expert assistance from Dr Adrian Robbins and Miss Nina Lane. The sorted cells were then either returned to culture or used to prepare RNA or immunocytochemistry samples [Robinson 1993].

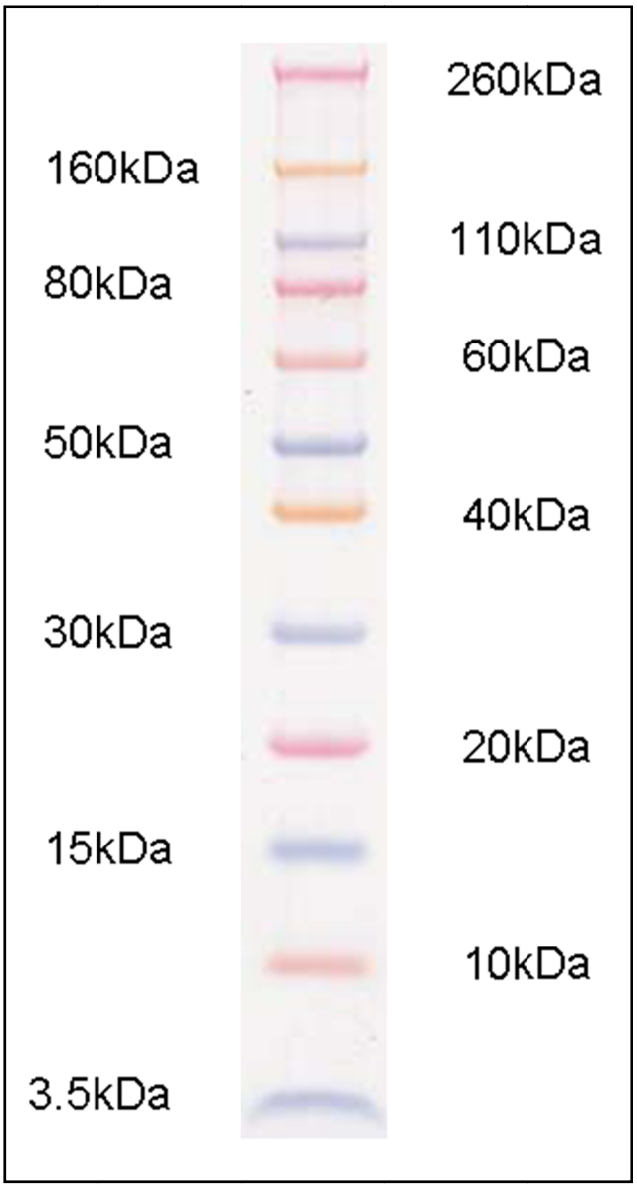


Figure 2.2: Molecular weight markers for Western blots.

Chapter Three: *In vitro* cell culture

3.1 Introduction

The differentiation of particular cell types during development and in mature tissue maintenance is a complicated and multi-staged process. Therefore directing the differentiation of pluripotent mES cells into ISC is unlikely to be simple. By deconstructing the process by which the ISC (and all the cells in the intestinal epithelium) arise during development it is possible to identify certain steps that might be replicable *in vitro*.

As discussed in Section 1.2 the intestinal epithelium and all the other visceral organs, such as the lungs, originate from the definitive endoderm germ layer that is formed, along with the ectoderm and the mesoderm, at gastrulation (Chapter One, Figure 1.9). A number of studies have shown that it is possible to direct the differentiation of both mES [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005] and hES cells [D'Amour et al 2005, MacClean et al 2007] towards the DE germ layer fate using particular *in vitro* culture conditions as discussed in detail below [Loebel and Tam, 2005].

3.1.1 Differentiation techniques

The growth factor Act-A is a member of the TGF-β superfamily of signalling molecules (Figure 3.1.1). It acts by binding to Type I and II Activin receptors on the cell surface which then activate SMAD 2 and 3 molecules which in turn interact with the DNA producing a transcriptional response activating genes involved in the specification of DE as well as placental formation and gonadal growth. As discussed in Section 1.3 there are significant differences between hES and mES cells. It is therefore encouraging that Act-A treatment seems to induce the same response in cells from both species as it suggests that the mechanism of action is highly conserved.

The further differentiation potential of the cells following the *in vitro* treatments was also investigated.

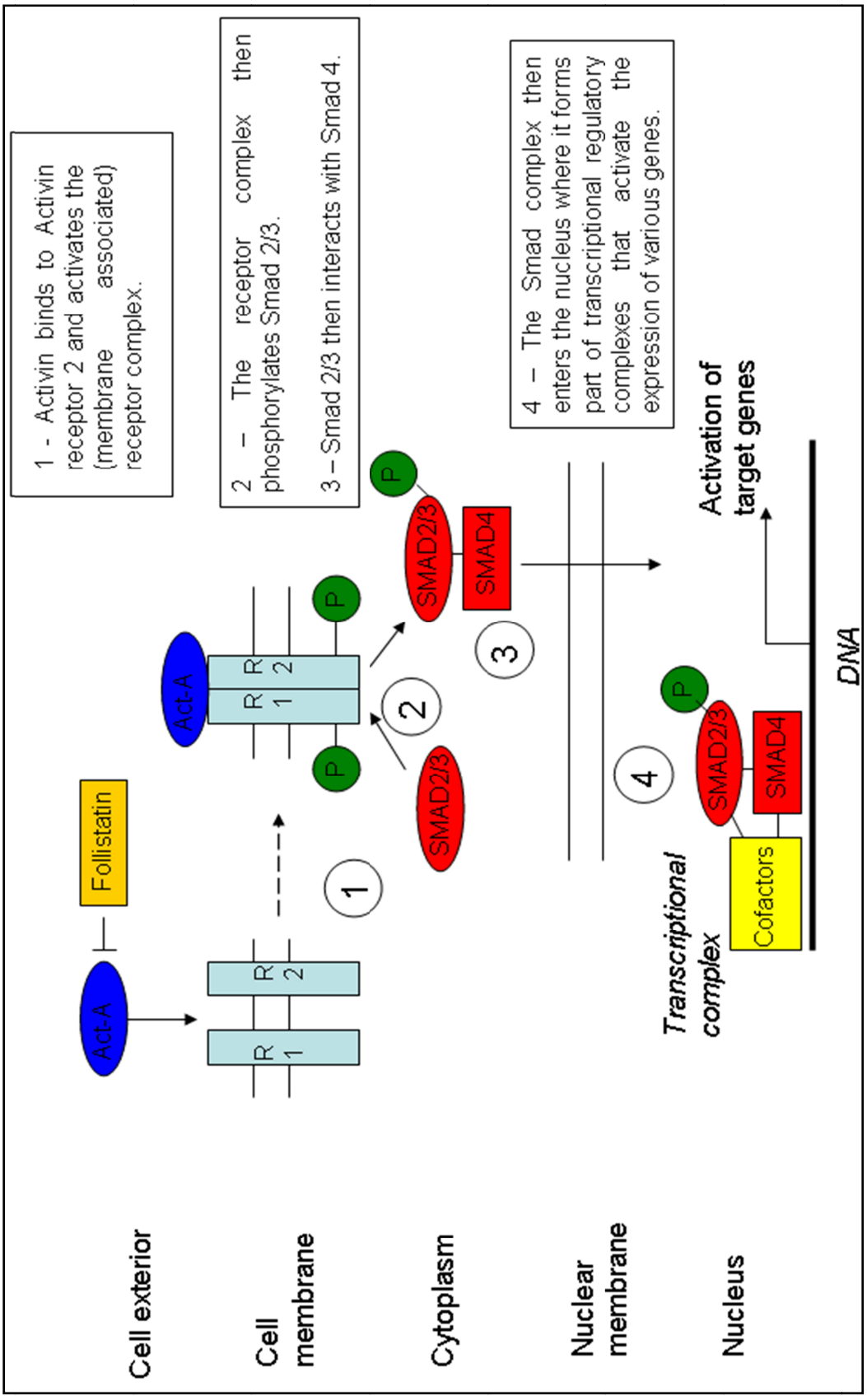


Figure 3.1.1: The Activin-A signalling pathway [Arnold and Robertson 2009].

The differentiation of mES cells to the DE fate was initiated using a combination of Act-A treatment and low serum or serum replacement (SR) media [Kubo et al 2004].

To initiate DE differentiation *GFP-Brachyury* mES cells were aggregated into EBs for 2.5 days then cultured in IMDM media with 15% SR or aggregated for two days then cultured with IMDM media with 15% SR plus Act-A (at a range of concentrations). Expression of DE markers (such as *FoxA2* and *Sox17*) was enhanced by reduced serum levels compared to controls when evaluated by RT-PCR and immunostaining.

The expression of GFP (representative of Brachyury) was reduced in low serum/SR conditions. Act-A treatment induced upregulated expression of both endodermal (*FoxA2* and *Sox17*) and mesodermal (*Brachyury*) markers (see Section 3.1.2 below). These cells were further differentiated to initiate the hepatic (endodermally derived) and haematopoietic progenitor (mesodermally derived) fates. The cells that differentiated towards the hepatic fate showed upregulated expression of a variety of hepatic markers including AFP and albumin. The cells which had been exposed to reduced serum levels showed a corresponding reduction in haematopoietic potential reflecting the observed reduction in mesodermal markers. Some of the treated cells were transplanted into the kidney capsules of SCID mice. After three to four weeks the kidneys were removed and analysed. The cells had formed some endoderm derived structures such as gut epithelial-like and bronchial epithelial-like cells. Some mesoderm derived structures had also developed such as skeletal muscle [Kubo et al 2004].

Both the DE and mesoderm arise from a common precursory germ layer known as the mesendoderm. Characterising the changes that occur during the specification of the germ layers is required if these processes are to be manipulated [Tada et al 2005]. A *GFP-Gsc* mES cell line was established to investigate this as *Gsc* expression is indicative of mesendoderm formation. Selective *in vitro* culture of these mES cells in serum free SFO3 media with the TGF-β family members Activin and Nodal initiated the formation of mesendoderm as indicated by the positive expression of Gsc-GFP along with E-cadherin and Platelet derived growth factor receptor α (PDGFRα). This population then split into two fractions, one of which remained positive for all three markers whilst the other retains only Gsc-GFP and E-cadherin expression. The latter, PDGFRα negative, population gave rise to DE whilst the former, PDGFRα positive, population gave rise to the mesoderm. These populations were also examined for the

expression of markers of endoderm (*FoxA2* and *Sox17*) or mesoderm (*Vegfr2*) formation by RT-PCR analysis. These markers showed upregulation in the selective culture conditions when compared to controls [Tada et al 2005].

Using selective *in vitro* culture conditions mES cells (carrying reporter genes) have also been differentiated towards the DE fate [Yasunaga et al 2005]. These cells were then assessed for cell surface markers that distinguished DE cells. These markers were then used to assess the differentiation of (genetically) unmanipulated mES cells that had been cultured in the selective conditions. A *GFP-Gsc* mES cell line also carrying the *CD25* reporter gene at the *Sox17* locus was generated. *Sox17* is expressed both in the DE and also in the visceral endoderm. Cells which are positive for the expression of both marker genes can therefore be identified as being of the DE lineage. Culturing cells in SFO3 serum free media with 10 ng/ml Act-A gave a high proportion of Gsc^+ cells. A further six days of culture in the continued presence of Act-A gave an cell population that was nearly 100% Gsc^+ with around a quarter of the cells also being $Sox17^+$. Although no specific conditions seemed to induce visceral endoderm a high proportion of $Gsc^-/Sox17^+$ cells were observed in cultures seeded in SFO3 media alone. These cell populations were purified by FACS and their complement of cell surface markers was determined using Microarray experiments. Of the markers expressed by DE but not visceral endoderm only CXCR4 had a suitable antibody available so this marker was selected, along with E-cadherin, to distinguish the DE fraction from the mesoderm fraction following selective culture of unmanipulated mES cells [Yasunaga et al 2005].

The specific differentiation of DE from hES cells can also be achieved using the growth factor Act-A [D'Amour et al 2005]. hES cells were cultured in RPMI media plus Glutamax with Penicillin and Streptomycin with varying concentrations of (defined) FBS with Act-A present at 100 ng/ml. The serum concentration in the culture media appeared to influence the differentiation to DE. The selected (definitive) endodermal markers (Sox17, FoxA2, Gsc) showed the highest levels of expression after five days of culture under low serum (0.5% FBS) conditions with Act-A treatment. Markers associated with visceral endoderm (Sox7) were not upregulated under these conditions. Single cell analysis of the cells for SOX17 showed that around 80% of the cells differentiated to the DE fate. These cells were

then sorted by selection for CXCR4 by FACS giving an almost pure DE cell population. The cells were examined for further differentiation potential by implantation in the kidney capsule of SCID mice where they remained *in vivo* for five weeks. The cells had organised into small structures which were analysed for the expression of intestinal (CDX2 and villin) and hepatic (Hepatic Specific Antigen, HSA) markers and compared with the expression levels seen in primary tissue. The implanted cells showed comparable expression of these markers with primary tissue when evaluated by immunohistochemistry [D'Amour et al 2005].

This study was taken further [MacClean et al 2007] where other specific conditions for improving the efficiency of differentiation to the DE fate were examined. Act-A induction of DE from hES cells (and the effects of other members of the TGF-β family of signals) was found to be more efficient when PI3K signalling was suppressed. When PI3K inhibitors including LY294002 and AKT1-II were added to hES cells in normal maintenance culture (where the cells should remain undifferentiated) changes to the cells morphology and molecular profile began to occur. E-cadherin and the pluripotency markers CD9 and Oct4 were downregulated and T and Sox17 showed upregulation. This suggests that PI3K signalling plays a role in hES cell self-renewal.

Further investigation highlighted that those genes that showed upregulated expression were those associated with the formation of mesendoderm (T and MIXL1) and then DE (*FoxA2, Sox17, Gsc*, GATA4 and GATA6) indicating that the differentiation that was occurring was towards a specific set of cell fates. To confirm this hypothesis the expression of CXCR4 and Sox17 were examined by immunostaining and flow cytometry. LY294002 treatment generated a cell population that was 70 - 80% SOX17+ and greater than 80% CXCR4+ after five days. Of the cells that were SOX17+, greater than 95% were also FOXA2+ indicating that it was DE being formed. This is supported by the data that shows that the cells differentiate to DE via the, T+, mesendoderm intermediate. AKT1-II (another PI3K inhibitor) treatment also produced an increase in the transcripts of the DE marker genes. The observations were reproducible in a number of hES cell lines (H1, BG01 and BG02).

The hES cell derived DE generated by LY294002 treatment were expression profiled using Affymetrix® GeneChips© and compared with the expression profile of hES cell derived DE generated by Act-A treatment. A number of genes (75) showed significant (10 fold) increases in expression in both conditions. These included all of the key DE markers including *Sox17, FoxA2, CXCR4, Gsc* and *GATA4*. A number of the other upregulated genes had been previously associated with murine embryonic endoderm [MacClean et al 2007]. Further investigation showed that Act-A/Nodal signalling was required in the low PI3K signalling conditions for DE differentiation to occur. A range of inhibitors to TGF-β signalling factors were added to the hES culture along with LY294002. Inhibitors of BMPs (e.g. Chordin) had no effect but inhibitors of Nodal/Activin (e.g. Follistatin) blocked the effects of LY294002. Specific blocking of the Nodal receptor, Cripto, using Lefty-A did not block the effects of PI3K suppression. This suggests that Activin in the MEF-CM stimulates differentiation to DE when PI3K signalling is suppressed. This suggests that PI3K plays a role in the self-renewal of hES cells by blocking differentiation stimuli from Activin/Nodal signalling [MacClean et al 2007].

To determine the minimum signalling requirements of the culture system the MEF-CM was substituted with unconditioned media and 20% SR with or without Activin or Nodal. In the absence of any other signalling factors in the media LY294002 suppression of PI3K was insufficient to initiate DE differentiation. The addition of Act-A or Nodal to the culture produced an upregulation in Sox17 expression comparable to that seen in the MEF-CM conditions. This could explain why in previous studies have shown more efficient DE differentiation in low serum conditions, where PI3K signalling may be reduced [MacClean et al 2007].

The hES cell derived DE cells generated by PI3K suppression with LY294002 were transplanted into the kidney capsule of SCID mice. After six weeks the grafts displayed high levels of a range of endodermal lineage markers when they were immunostained. These included hepatic markers (AFP and HSA), lung/thyroid markers (Thyroid transcription factor 1, TTF1) and intestinal (epithelial) markers (gastrin and villin). The control grafts from untreated hES cells did not show expression of any of these markers in all but one sample. The qPCR analysis showed that albumin and FABP1 were expressed 10 fold higher in the treated cells than in the

naïve hES cells. The hES cell derived DE showed considerably increased differentiation into further DE lineages than untreated hES cells [MacClean et al 2007].

Whilst these studies suggest that Act-A has a similar effect on both hES and mES cells it is not certain that the mechanism of action will be the same. As discussed in Section 1.3 there are significant differences between hES and mES cell lines so it is possible that the mechanism by which they differentiate in these studies is not the same. This is encouraging in some regards as it suggests that the effects of Act-A are fairly universal and therefore similar results should be obtainable using the CEE mES cell line.

Other TGF-β family members, such as Nodal, may also play a role in DE and mesoderm differentiation of ES cells *in vitro*. Nodal signalling plays a role in the specification of mesoderm and DE during gastrulation [Takenaga et al 2007].

3.1.2 Identification of desired cell types

As alluded to in the studies discussed above the differentiation of cells towards a particular fate can be monitored by observing the expression of key lineage marker genes at both the mRNA and protein levels. A lineage marker is a gene that has patterns of expression (both spatial and temporal) that are limited to a particular set of cells during development. In some instances using multiple markers can be useful; if co-expression of two factors is indicative of a particular lineage it reduces the chance of false positive results e.g. co-expression of *Sox17* and *FoxA2* is indicative of DE formation. By comparing the levels of expression of these markers expressed by cells cultured in the presence of Act-A with a number of control conditions the relative efficiencies with which the cells differentiate towards a particular fate can be determined. A variety of endodermal markers have been identified [Hou et al 2007]. By screening the gene expression of the early DE of mice by Serial Analysis of Gene Expression (SAGE) a number of novel candidate DE markers were identified along with a panel of previously identified markers (including *Sox17* and *Gata4*) [Hou et al 2007].

CXCR4 has been shown to be a marker of DE formation in mice [Yasunaga et al 2005] and humans [D'Amour et al 2005]. GATA4 is involved in the differentiation of a number of endoderm derived organs [Holtzinger and Evans, 2005, Watt et al 2007]. The co-expression of the transcription factors Sox17 [Kania-Azuma et al 2002, Tam et al 2003] and FoxA2 [Ang et al 1993] indicates the production of definitive rather than visceral endoderm (that only expresses FoxA2). It is important to distinguish DE from visceral endoderm which only contributes to extra-embryonic structures and therefore would be of no use in generating intestinal precursors [Duncan et al 1994].

The production of the other two germ layers also needs to be assessed to evaluate how much of the differentiation observed is occurring randomly following the removal of the factors that suppress differentiation (LIF). *Brachyury* (a T domain transcription factor) is an early marker of mesoderm [Smith et al 1991] whilst *Nestin* (a Type VI intermediate filament protein) is an early marker of ectoderm [Dahlstrand et al 1995]. The expression *Oct4* [Nichols et al 1998] and *Nanog* [Pan and Thomson 2007] (both are homeodomain transcription factors) can be used to assess if any undifferentiated cells remain in the population – they are markers of pluripotency. Downregulation of the pluripotency markers during differentiation culture should also occur and serves as an indicator of the efficiency of differentiation.

3.1.3 Aims

The aims of the experiments in this Chapter were to reproduce the results obtained in the literature reviewed above (and in Section 1.6) in the CEE mES cell line. If these results can be reproduced in this study it may prove a useful first step towards specifying ES derived cells (ISC-like cells) suitable for intestinal tissue engineering applications.

Specifically the aims are:

- To investigate the effects of serum concentration on the behaviour of CEE mES cells in culture with regard to their differentiation towards the DE fate *in vitro*.

- To investigate the effects of EB aggregation on the differentiation of CEE mES cells towards the DE fate *in vitro*.

- To investigate the effects of treatment with the growth factor Act-A on the differentiation of CEE mES cells towards the DE fate *in vitro*.

- Assess the differentiation of the CEE mES cells at the RNA level using RT-PCR analysis and at the proteomic level using immunocytochemistry and western blotting.

- To generate a population of DE-like cells for use in further (differentiation) experiments.

Chapter Three: *In vitro* cell culture

3.2 Materials and Methods

3.2.1 Cell differentiation

3.2.1i *In vitro* differentiation Experiment One - mES cell differentiation in FCS free media

A number of sources in the literature detailed methods for differentiating ES cells towards the DE fate [D'Amour et al 2005, MacClean et al 2007, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005]. These methods were used as the basis for the following experimental procedure. CEE mES cells that had been maintained in an undifferentiated state (see Section 2.1.1) for between 15 and 25 passages were transferred into culture in differentiation media for 120 hours with and without the addition of 10 ng/ml Act-A (Sigma, UK) in six well tissue culture plates (one plate per condition) that had been previously gelatin coated. For the first 24 hours the cells were cultured in differentiation media A (LIF-, FCS-, see Section 6.4) with or without Act-A. For the second 24 hours the cells were cultured in differentiation media B (LIF-, 0.2% (*w/v*) FCS, see Section 6.4) with or without Act-A. For the remainder of the experiment the cells were cultured in differentiation media C (LIF-, 2% (*w/v*) FCS, see Section 6.4) with or without Act-A. Two control plates were also run; one where the cells were cultured in (complete) embryonic stem cell media (ES media, LIF+, 10% (*w/v*) FCS) and one where the cells were cultured in LIF- ES media (SNL media, LIF-, 10% (*w/v*) FCS). The media was changed every 24 hours.

3.2.1ii *In vitro* differentiation Experiment Two - mES cell differentiation in serum replacement media

Based on the results observed from the above experiments (see Section 3.3.1) and sources in the literature [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005] some adaptations to the differentiation culture conditions were made. CEE mES cells that had been maintained in an undifferentiated state (see Section 2.1.1) for between 15 and 25 passages were transferred into culture in differentiation media (see below) for 120 hours with or without the addition of 10 ng/ml Act-A in 12 well tissue culture

plates (one plate per condition) that had been previously coated with Collagen IV (see Section 2.13). Three differentiation conditions and two control conditions were used. In conditions one and two, cells were cultured in 10% (*v/v*) SR (Sigma, UK, S0638) media for 120 hours; condition two had Act-A added. In condition three, cells were cultured in SR media for 72 hours then SNL media (10% (*w/v*) FCS) for the final 48 hours. Conditions four and five were controls (both containing 10% (*v/v*) FCS as in Section 3.2.1i), four being ES media (LIF+) and five being SNL media (LIF-) cultured for 144 hours. The media was initially changed after 48 hours and then every 24 hours thereafter.

Duplicate RNA samples for RT-PCR were prepared at 48, 96 and 144 hours using tri-reagent as described in Section 2.2.1 and analysed as described in Sections 2.2.5 and 2.2.6. Remaining wells were prepared for immunocytochemistry at 120 or 144 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7. The PCR timepoints were evenly spaced along the time course of the experiment to observe changes in the mRNA expression of the selected markers throughout. The immunocytochemical samples were prepared at the terminal timepoints only as initiation of protein expression is slower than mRNA.

3.2.1iii *In vitro* differentiation Experiment Three – Optimising seeding and culture conditions

Part A: To establish if the coating applied to the TCP prior to cell seeding influenced the attachment and proliferation of the cells in the experiment the growth of cells on Collagen IV or gelatin coated TCP was compared. CEE mES cells were seeded at 1 x 10^5 cells/ml in SNL media (10% (*w/v*) FCS) on six well TCP plates that had been previously coated with either 0.01% (*w/v*) Collagen IV or 0.1% (*v/v*) Gelatin (see Section 2.1.3). Initially the cells were cultured for 120 hours with images taken every 24 hours to assess the viability & proliferation of the cells. The experiment was then repeated with a lower initial seeding density of 5 x 10^4 cells/ml and the culture period extended to 168 hours.

Part B: The proliferation of cells in either FCS or SR was compared. CEE mES cells were seeded at 5 x 10^4 cells/ml in gelatin coated six well TCP plates. The cells were cultured in media that had been supplemented with either 10% (*v/v)* FCS or 10% (*v/v*) SR for 72 hours. Cell counts (n = 3), using an improved Neubauer haemocytometer, and images, using a Nikon light microscope, were taken every 24 hours.

Part C: To establish a suitable initial seeding density CEE mES cells were seeded at a range of densities (1 x 10^4, 5 x 10^4, 1 x 10^5, 1.5 x 10^5, 2 x 10^5 and 2.5 x 10^5 cells/ml) in SNL media on gelatin coated six well TCP plates for up to 168 hours. Cell counts (n = 3), using an improved Neubauer haemocytometer, and images, using a Nikon light microscope, were taken every 24 hours.

Table 3.2.1: Table of optimisation media compositions from Experiment Three, Part D detailing basal media used and the supplements added.

Media name	Basal media	Serum	Supplements
SNL media	DMEM	10% FCS	1% L-Glut, 1% AB/AM, 100μM β-MCPE
SR media	DMEM	10% SR	1% L-Glut, 1% AB/AM, 100μM β-MCPE
α media	α-MEM	10% FCS	1% L-Glut, 1% AB/AM, 100μM β-MCPE
Low serum	DMEM	1% FCS	1% L-Glut, 1% AB/AM, 100μM β-MCPE
α low serum	α-MEM	1% FCS	1% L-Glut, 1% AB/AM, 100μM β-MCPE
mES media	DMEM	10% FCS	1% L-Glut, 1% AB/AM, 100μM β-MCPE, 1x10^3 units/ml LIF
Act-A media	DMEM	10%SR	1% L-Glut, 1% AB/AM, 100μM β-MCPE, 10ng/ml Act-A

Part D: Cells were seeded at 5 x 10^4 cells/ml in a variety of media formulations in gelatin coated six well TCP plates for up to 168 hours. The FCS concentration, the basal media and some of the supplements used were changed in the different conditions (see Figure 3.2.1 and Section 6.4). Cell counts (n = 3), using an improved Neubauer haemocytometer, and images, using a Nikon Eclipse light microscope, were taken every 24 hours.

3.2.1iv *In vitro* differentiation Experiment Four

CEE mES cells that had been maintained in an undifferentiated state (see Section 2.1.1) for between 15 and 25 passages were cultured under a variety of conditions in the presence and absence of Act-A at 10 ng/ml with either 10% (*v/v*) FCS or 10% (*v/v*) SR. All media formulations were DMEM supplemented with L-Glut, AB-AM and β-MCPE.

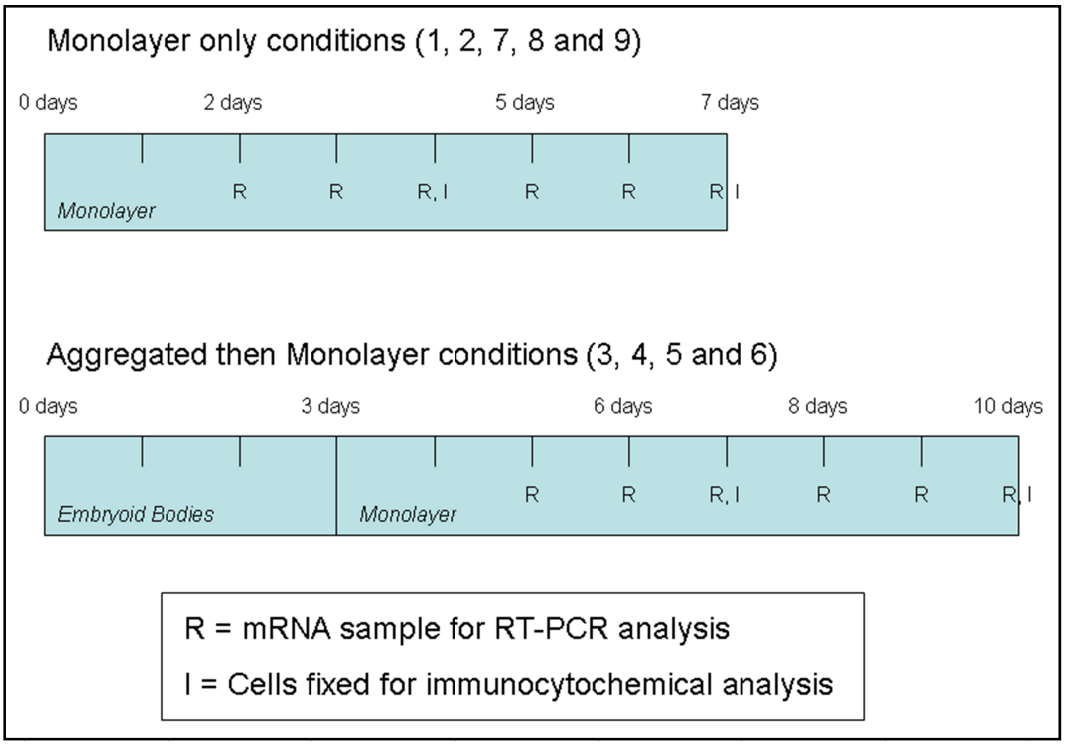


Figure 3.2.2: Schematic of *in vitro* cell culture conditions from Experiment Four showing the timepoints at which samples were taken for analysis.

In the conditions where the cells were not aggregated CEE mES cells were seeded at 1 x 10^5 cells/ml in monolayer culture for seven days on gelatin coated TCP six well plates. In those conditions where cells were aggregated CEE mES cells were seeded at 5 x 10^5 cells/ml in suspension culture for three days in non-TCP six well plates as described in Section 2.1.4. The EBs formed were then dissociated by treatment with pre-warmed Trypsin/EDTA for 30 seconds and pipetting and the cells reseeded in monolayer culture at 1 x 10^5 cells/ml on gelatin coated TCP six well plates; all culture conditions are summarised in Figure 3.2.2.

Table 3.2.2: Summary table of *in vitro* cell culture conditions from Experiment Four.

Condition	Aggregated	Serum/SR	Act-A	LIF
1	×	Serum	×	✓
2	×	Serum	×	×
3	✓	Serum	✓	×
4	✓	SR	✓	×
5	✓	Serum	×	×
6	✓	SR	×	×
7	×	Serum	✓	×
8	×	SR	×	×
9	×	SR	✓	×

RNA samples were collected after 48 hours in monolayer culture and every 24 hours thereafter (until the end of the experiment) and prepared using a Qiagen kit as described in Section 2.2.1 and analysed as described in Sections 2.2.5 and 2.2.6. Samples for immunocytochemistry were prepared after 96 and 168 hours in monolayer culture as described in Section 2.2.2 and analysed as described in Section 2.2.7. The PCR timepoints were evenly spaced along the time course of the experiment to observe changes in the mRNA expression of the selected markers throughout. The immunocytochemical samples were prepared in the latter part of the experiment and at the terminal timepoint to observe any protein expression.

3.2.1v *In vitro* differentiation Experiment Five

Following on from the results obtained from the above experiment (Chapter Three, Section 3.3.4) CEE mES cells that had been maintained in an undifferentiated state (see Section 2.1.1) for between 15 and 25 passages were cultured under a variety of conditions in the presence and absence of Act-A at 10 ng/ml with 10 % (*v/v*) FCS.

In condition one CEE mES cells were aggregated for three days in the presence of Act-A in suspension culture seeded at 5 x 10^5 cells/ml (see Section 2.1.4) in uncoated non-TCP six well plates. The EBs formed were then dissociated by treatment with pre-warmed Trypsin/EDTA and reseeded in monolayer culture at 1 x 10^5 cells/ml for nine days in the presence of Act-A in gelatin coated TCP six well plates. RNA samples were collected after 0, 72, 216 and 288 hours and prepared using a Qiagen kit as described in Section 2.2.1 and analysed as described in Sections 2.2.5 and 2.2.6. Samples for immunocytochemistry were prepared after 144 and 288 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7.

In condition two CEE mES cells were seeded in monolayer culture at 1 x 10^5 cells/ml for nine days in the presence of Act-A on gelatin coated TCP six well plates. RNA samples were collected after 0, 72 and 216 hours and prepared using a Qiagen kit as described in Section 2.2.1 and analysed as described in Section s 2.2.5 and 2.2.6. Samples for immunocytochemistry were prepared after 72 and 216 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7.

In condition three CEE mES cells were seeded in monolayer culture at 2.5 x 10^5 cells/ml for two days in the presence of Act-A on gelatin coated TCP six well plates. The cells were then aggregated in suspension culture at 5 x 10^5 cells/ml for three days (see Section 2.1.4) in the presence of Act-A in uncoated non-TCP six well plates. The EBs formed were then dissociated by treatment with pre-warmed Trypsin/EDTA and reseeded in monolayer culture at 1 x 10^5 cells/ml for nine days in the presence of Act-A on gelatin coated TCP six well plates. RNA samples were collected after 0, 48, 120, 288 and 336 hours and prepared using a Qiagen kit as described in Section 2.2.1 and analysed as described in Sections 2.2.5 and 2.2.6. Samples for immunocytochemistry were prepared after 144, 240 and 336 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7.

In condition four CEE mES cells were seeded in monolayer culture at 1 x 10^5 cells/ml for nine days on gelatin coated TCP six well plates. RNA samples were collected after 0, 72 and 216 hours and prepared using a Qiagen kit as described in Section

2.2.1 and analysed as described in Section s 2.2.5 and 2.2.6. Samples for immunocytochemistry were prepared after 72 and 216 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7.

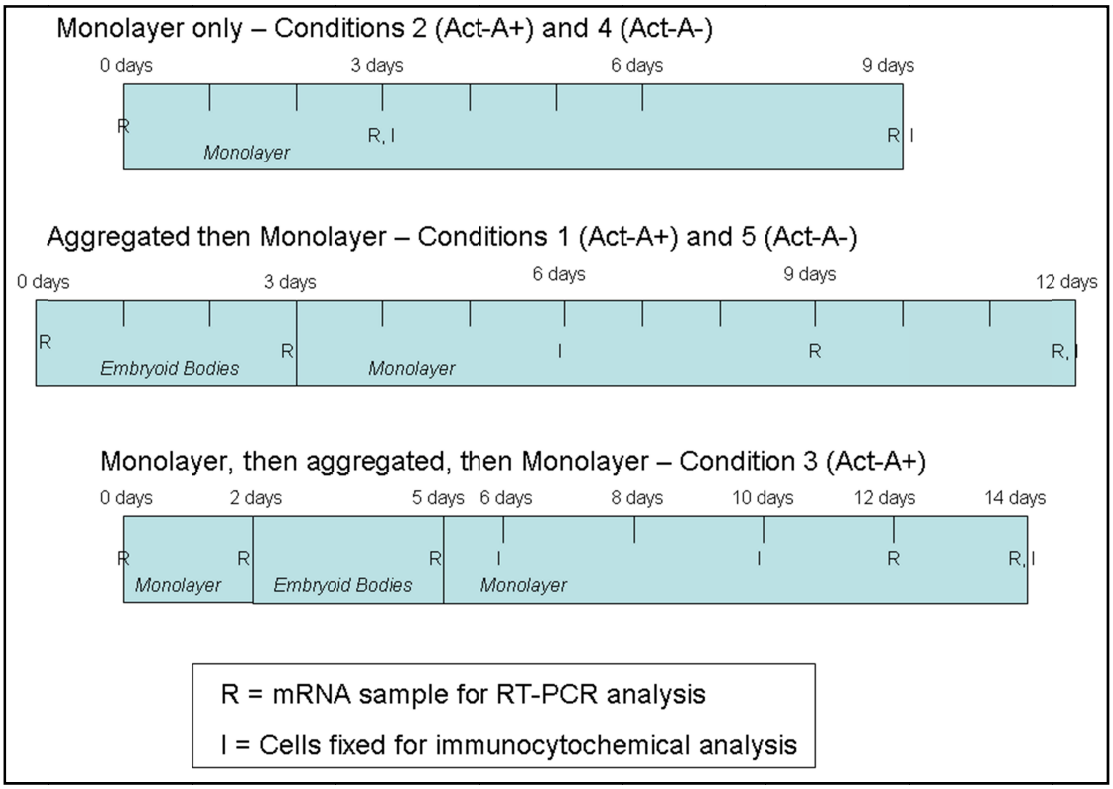


Figure 3.2.3: Schematic of *in vitro* cell culture conditions from Experiment Five showing the timepoints at which samples were taken for analysis.

In condition five CEE mES cells were aggregated for three days in suspension culture seeded at 5 x 10^5 cells/ml (see Section 2.1.4) in uncoated non-TCP six well plates. The EBs formed were then dissociated by treatment with pre-warmed Trypsin/EDTA and reseeded in monolayer culture at 1 x 10^5 cells/ml for nine days on gelatin coated TCP six well plates. RNA samples were collected after 0, 72, 216 and 288 hours and prepared using a Qiagen kit as described in Section 2.2.1 and analysed as described in Section s 2.2.5 and 2.2.6. Samples for immunocytochemistry were prepared after 144 and 288 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7. The PCR timepoints were evenly spaced along the time course of the experiment to observe changes in the mRNA expression of the selected markers throughout or at key points of each experiment such as the transition from EBs back to monolayer culture. The immunocytochemical samples were prepared in the latter

part of the experiment and at the terminal timepoint or close to key points of each experiment such as the transition from EBs back to monolayer culture to observe any protein expression.

Table 3.2.3: Table of *in vitro* cell culture conditions from Experiment Five.

Condition	Stage 1	Stage 2	Stage 3	Act-A
1	Aggregated - 3 days	Monolayer - 9 days	N/A	+
2	Monolayer - 9 days	N/A	N/A	+
3	Monolayer - 2 days	Aggregated - 3 days	Monolayer - 9 days	+
4	Monolayer - 9 days	N/A	N/A	-
5	Aggregated - 3 days	Monolayer - 9 days	N/A	-

3.2.1vi *In vitro* differentiation Experiment Six

CEE mES cells that had been maintained in an undifferentiated state (see Section 2.1.1) for 20 passages were seeded in monolayer culture at 2.5 x 10^5 cells/ml for two days in the presence of Act-A on gelatin coated TCP six well plates. The cells were then aggregated in suspension culture (see Section 2.1.4) seeded at 5 x 10^5 cells/ml for five days in the presence of Act-A in uncoated non-TCP six well plates. The EBs formed were then dissociated by treatment with pre-warmed Trypsin/EDTA and reseeded in monolayer culture at 1 x 10^5 cells/ml for nine days in the presence of Act-A on gelatin coated TCP six well plates. DMEM supplemented with 10% *(v/v)* FCS and 1% *(v/v)* L-Glut, 1% *(v/v)* AB-AM and 100μM β-MCPE was used throughout. RNA & protein samples were collected after 0, 48, 168, 264 and 384 hours and prepared as described in Sections 2.2.1 (Qiagen kit) and 2.2.3 respectively. RNA samples were analysed as described in Sections 2.2.5 and 2.2.6 whilst protein samples were analysed by western blotting as described in Section 2.2.8. Samples for immunocytochemistry were prepared after 48, 192, 264 and 384 hours as described in Section 2.2.2 and analysed as described in Section 2.2.7.

3.2.2 Post experimental sample analysis

3.2.2i Primer design

Primers were designed as described in Section 2.2.4. The selected markers for Definitive Endoderm were *Foxa2, Sox17* (transcription factors) [Kania-Azuma et al 2002, Tam et al 2003, Ang et al 1993], *CXCR4* (cell surface receptor) [D'Amour et al 2005] and *GATA4* [Holtzinger and Evans 2005, Rojas et al 2009]. The selected marker for Mesoderm was *Brachyury* [Smith et al 1991] and the marker for Ectoderm was *Nestin* [Dahlstrand et al 1995]. The selected marker for undifferentiated/pluripotent mES cells was *Oct4* [Nichols et al 1998]. New primer pairs for CXCR4, *Oct4* and *Brachyury* were designed as described in Section 2.2.4. Primers for *GATA4*, *Nestin* and *GAPDH* had been previously designed within the group. Primer sequences for *Sox17* and *FoxA2* were from published literature [Kubo et al 2004] (Table 3.2.4). The primers were then ordered from MWG-Operon.

Table 3.2.4: Details of murine germ layer marker RT-PCR primers.

Gene	Seq (5' - 3')	Annealing temp	Fragment size (bp)	Cycle no.
CXCR4→	GGCTGTAGAGCGAGTGTTGC	60°C	489	30
CXCR4←	CTTTTCAGCCAGCAGTTTCC	60°C		
Oct4→	AGCACGAGTGGAAAGCAACT	60°C	248	30
Oct4←	AGATGGTGGTCTGGCTGAAC	60°C		
GAPDH→	TGAGGCCGGTGCTGAGTATGTCG	60°C	302	30
GAPDH←	CCACAGTCTTCTGGGTGGCAGTG	60°C		
Sox17→	GCCAAAGACGAACGCAAGCGGT	60°C	211	35
Sox17←	TCATGCGCTTCACCTGCTTG	60°C		
Foxa2→	TGGTCACTGGGGACAAGGGAA	60°C	289	30
Foxa2←	GCAACAACAGCAATAGAGAAC	60°C		
GATA4→	CTGGAAGACACCCCAATCTC	55°C	130	30
GATA4←	GTAGTGTCCCGTCCCATCTC	55°C		
Brachyury→	GCTGTTGGGTAGGGAGTCAA	60°C	380	30
Brachyury←	CCCCGTTCACATATTTCCAG	60°C		
Nestin→	AGGCGCTGGAACAGAGATT	55°C	150	30
Nestin←	TTCCAGGATCTGAGCGATCT	55°C		

Chapter Three: *In vitro* cell culture

3.3 Results

3.3.1 *In vitro* differentiation experiment one - mES cell differentiation in low serum media

Evidence in the literature suggested that low serum concentrations and the presence of Act-A induced mES cells to differentiate towards the DE lineage in culture with greater efficiency than as EBs in standard cell culture media [D'Amour et al 2005, MacClean et al 2007, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005]. This experiment aimed to show that these results could be achieved using the CEE mES cell line. Complete (CEE mES) cell culture media contains around 10% FCS so the initial experiment aimed to establish if the CEE mES cell line could tolerate low serum (2% or lower) conditions and to observe any discernible effects on their differentiation under these conditions.

When cultured under low serum conditions the CEE mES cell line did not proliferate or adhere as it did when cultured in CEE complete cell culture media (10% FCS, LIF+/-) resulting in almost total cell death and therefore no samples were taken for molecular analysis (see Appendices, Section 6.3.1 for results figures).

3.3.2 *In vitro* differentiation experiment two - mES cell differentiation in SR media

The presence of Act-A in serum replacement (SR) media rather than low serum has been previously shown to promote differentiation to the DE fate without having a negative effect on cellular attachment and proliferation [D'Amour et al 2005, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005]. In this study, the ability to culture CEE mES cells in SR media was assessed. In addition, the effect of using different ECM components to coat the culture vessel was also investigated.

The cells did not appear to attach or proliferate any better in SR complete media compared with low serum conditions. There was no apparent difference between cellular attachment on Gelatin or Collagen IV coated TCP (see Appendices, Section 6.3.1 for results figures).

3.3.3 *In vitro* differentiation experiment three – Optimising seeding and differentiation culture conditions

Studies in the literature had induced mES cells to differentiate to the DE lineage in low serum/SR conditions [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005]. However in early experiments (see Section 3.2.1 and 3.2.2) the selected mES cell line did not attach or proliferate well in these conditions (see Section 6.3.1). Therefore a more extensive series of optimisation experiments were carried out to investigate the effects of culture vessel treatments, seeding density, and media composition on the attachment, proliferation and, ultimately, the differentiation of the CEE mES cells.

3.3.3i Culture vessel treatments

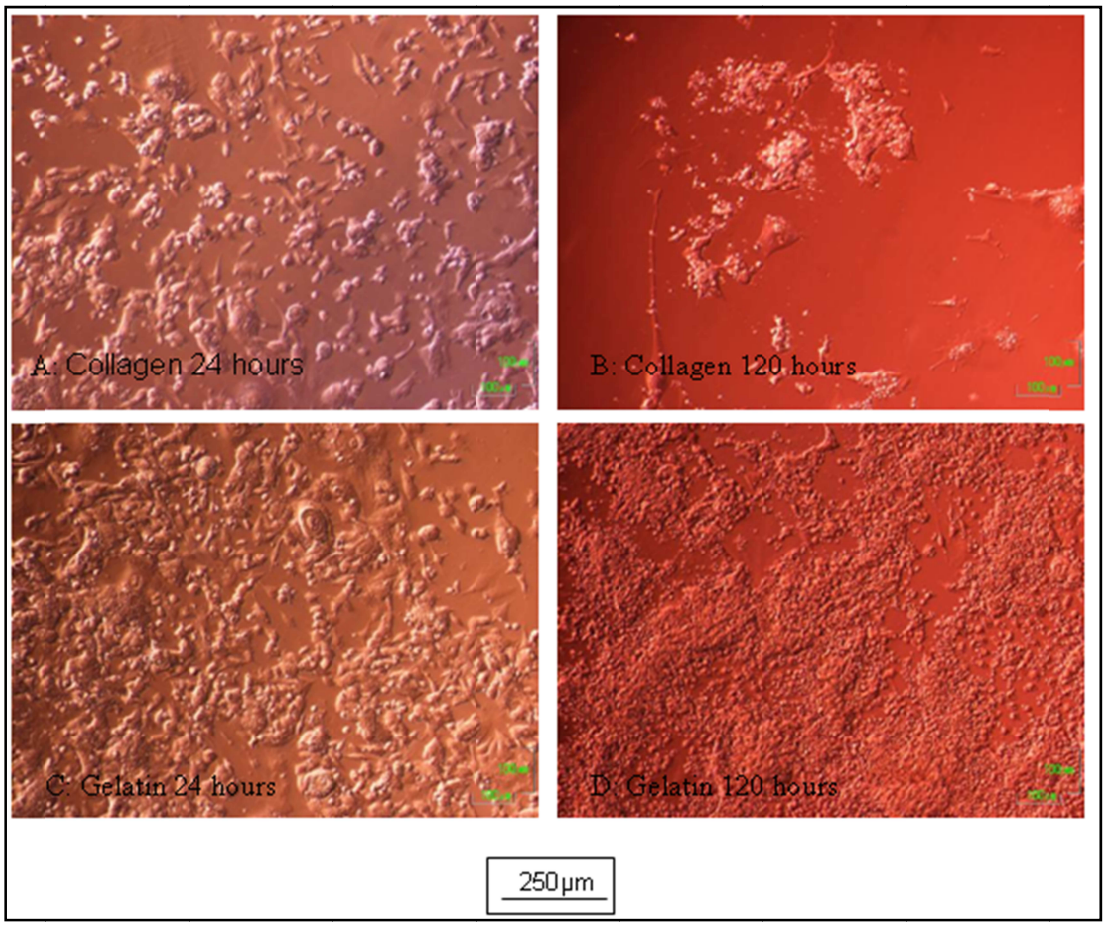


Figure 3.3.3i: Images of mES cells cultured on Gelatin or Collagen IV coated TCP for up to 168 hours (A-D = Batch A, E-H = Batch B). A – Collagen 24 hours. B – Collagen 120 hours. C – Gelatin 24 hours. D – Gelatin 120 hours.

The images in Figure 3.3.3 show that the cells adhered and proliferated no differently on Gelatin than they did on collagen coated TCP. In the first part of the experiment good initial cell attachment was seen on both coatings after 24 hours in culture (Figure 3.3.3A and C). However the cells did not proliferate well on the collagen coated TCP with a reduction in cell coverage apparent after 120 hours (Figure 3.3.3B). On the Gelatin coated TCP the cells continued to grow and had reached approximately 85% confluence after 120 hours (Figure 3.3.3D).

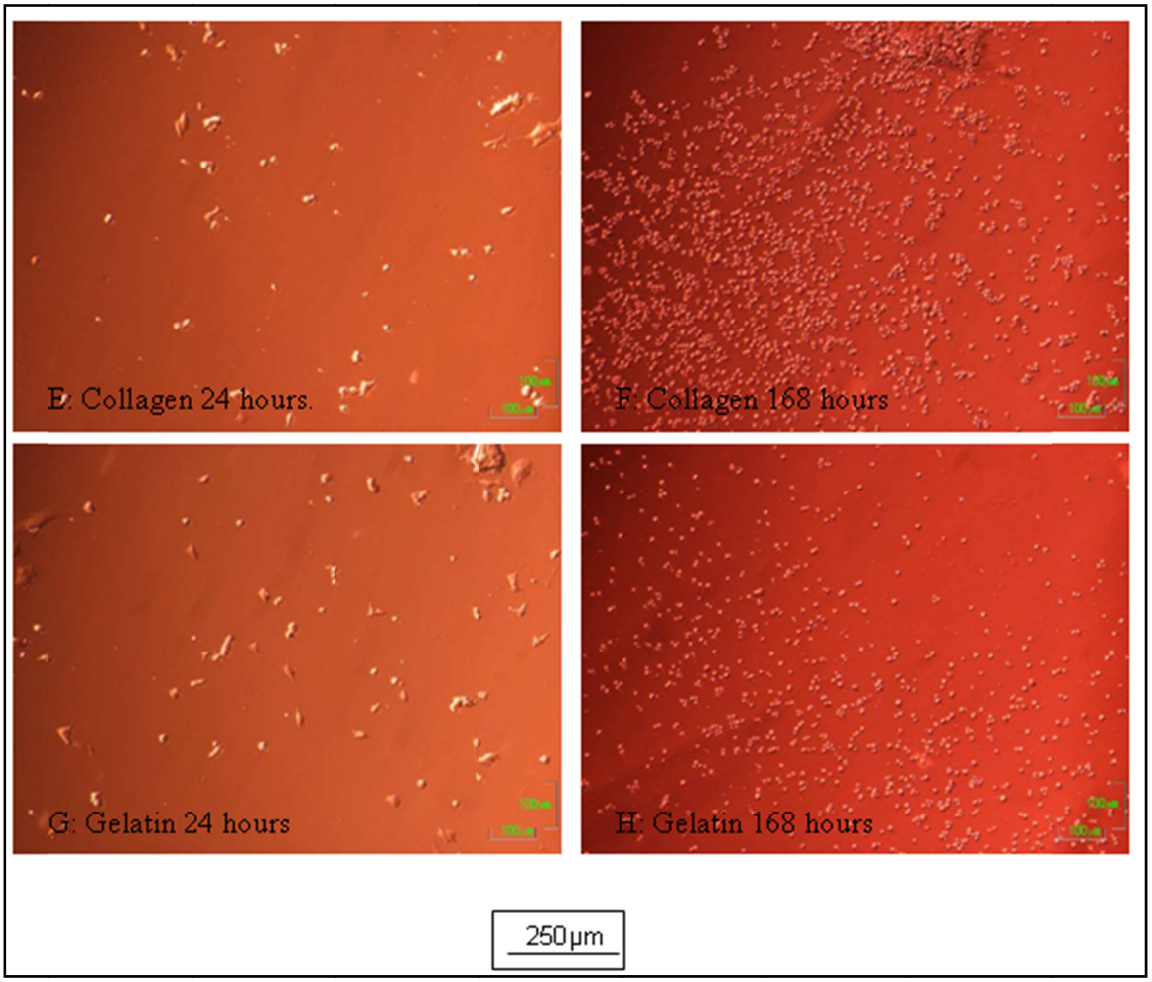


Figure 3.3.3ii: Images of mES cells cultured on Gelatin or Collagen IV coated TCP for up to 168 hours (A-D = Batch A, E-H = Batch B). E – Collagen 24 hours. F – Collagen 168 hours. G – Gelatin 24 hours. H – Gelatin 168 hours.

In the second part of the experiment where the initial seeding density of the cells was reduced the initial attachment of cells was very similar on collagen (Figure 3.3.3E) and gelatin (Figure 3.3.3G). After 168 hours the cells growing on collagen coated TCP had reached approximately 60% confluence (Figure 3.3.3F) whilst those growing

on gelatin had reached around 40% confluence (Figure 3.3.3H). Overall there appeared to be no significant differences in cellular attachment or proliferation between the gelatin or collagen coated TCP.

3.3.3ii Media Composition

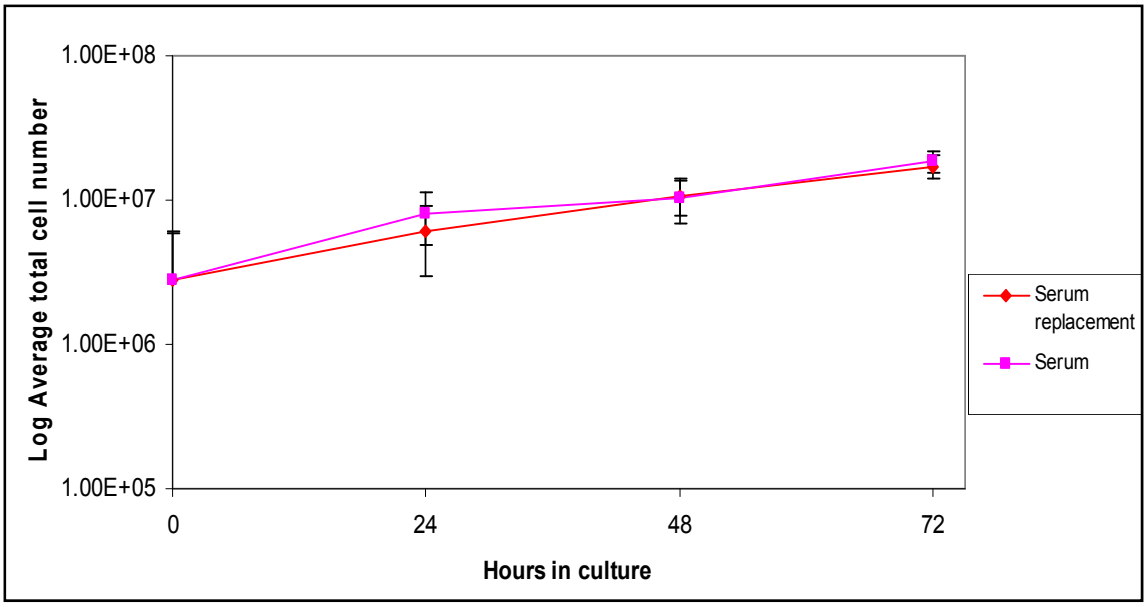


Figure 3.3.4: Graph showing the Log average total cell number of CEE mES cells seeded and cultured in 10% (*v/v*) FCS vs 10% (*v/v*) SR complete media on Gelatin coated TCP over 72 hours in culture (n = 3, error bars show SEM).

Figure 3.3.4 shows that there was no significant difference between the cells grown in 10% (*v/v*) SR when compared to 10% (*v/v*) FCS in terms of their rate of proliferation. The cultures were originally seeded at the same density and the total cell number remained similar throughout the time in culture. The data shows that there was very little variation between the replicates for either condition particularly at the 48 hour and 72 hour timepoints.

3.3.3iii Seeding density

There were considerable variations in the number of cells attaching to the culture surface (gelatin coated TCP) after 24 hours reflecting the initial cell seeding density

(Figure 3.3.5). At the lower seeding densities a scattering of cells across the field of view was observed at 24 hours (Figure 3.3.5A – D) whilst cells seeded at higher densities, particularly 2.5 x 10^5 cells/ml (Figure 3.3.5F), already demonstrated a much higher degree of confluence. After 168 hours cells seeded at higher seeding densities (Figure 3.3.5J - L) had all reached 100% confluence with all but the lowest seeding density, 1 x 10^4 cells/ml (Figure 3.3.5G), having reached > 90% confluence.

3.3.3iv Media Composition

Figure 3.3.6 illustrates that the mES cells proliferated at varying rates dependant on the type of media in which they were cultured when seeded on Gelatin coated TCP. The highest cell counts obtained after 72 hours in culture were from the controls, SNL and CEE mES complete cell culture media, with in excess of 3 x 10^6 cells per sample. These two media compositions were identical (both contained 10% (*v/v*) FCS) except that CEE mES complete cell culture media was LIF+ (at 1 x 10^3 units/ml) (LIF suppresses differentiation).

The cells grown in SR complete media (FCS-) proliferated at a slower rate with each sample containing around 2.5 x 10^6 cells after 72 hours culture in Act-A- SR complete media and approximately 2 x 10^6 cells in Act-A+ SR complete media. The cells in both the DMEM low FCS and α-MEM low FCS media proliferated very slowly; this data is not shown on the graph. The cells exhibited moderate proliferation in the 10% FCS α-MEM complete media with between 1 x 10^6 – 1.5 x 10^6 cells per sample. Adding Act-A to the SR media reduced the rate of cell growth further but the cells were clearly proliferating.

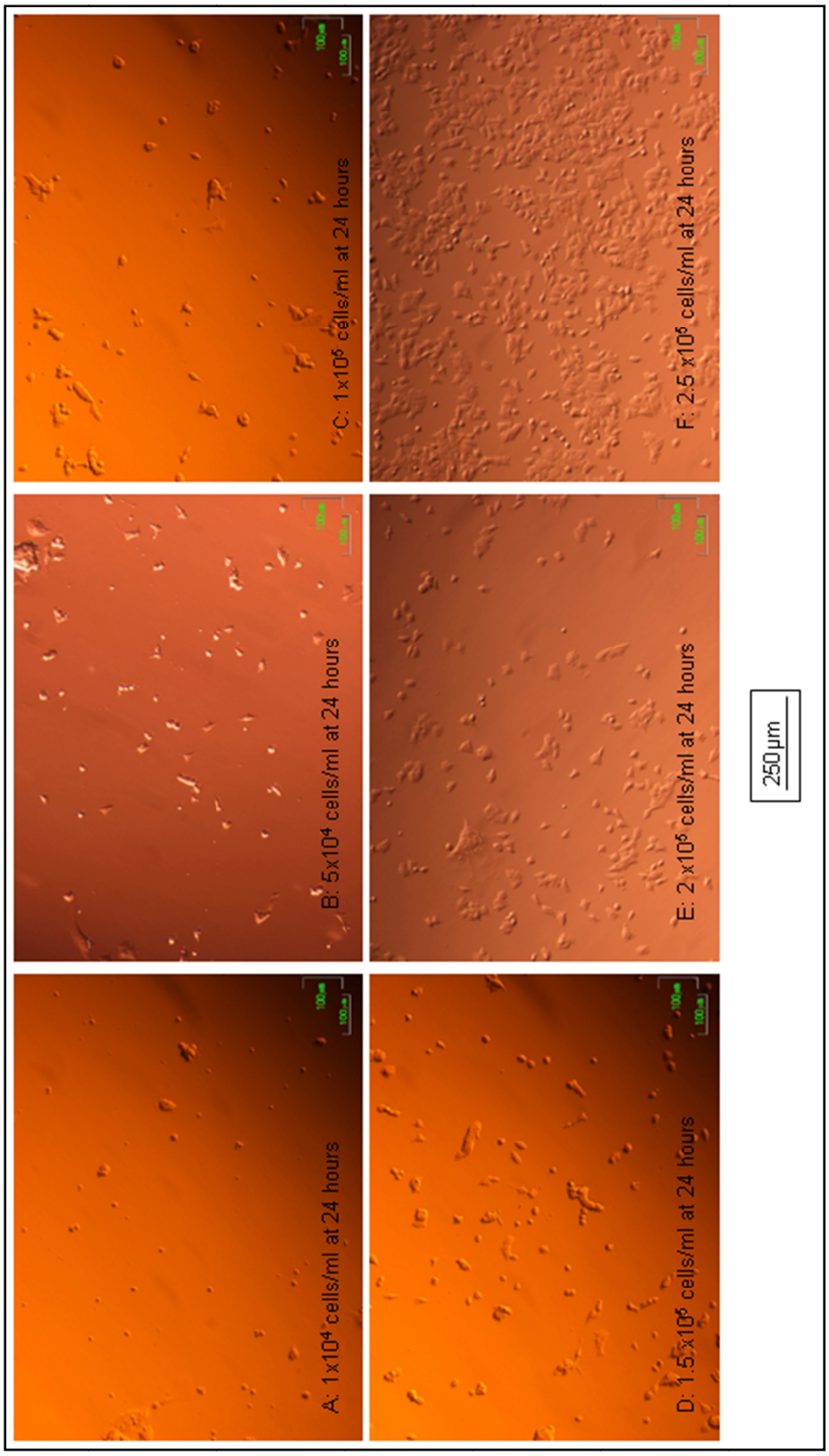


Figure 3.3.5i: Images showing the extent of initial cell attachment after 24 hours (A - F) and the extent of proliferation after 168 hours (G - L) of mES cells seeded on Gelatin coated TCP at a range of initial seeding densities between 1 x 10^4 and 2.5 x 10^5 cell/ml.

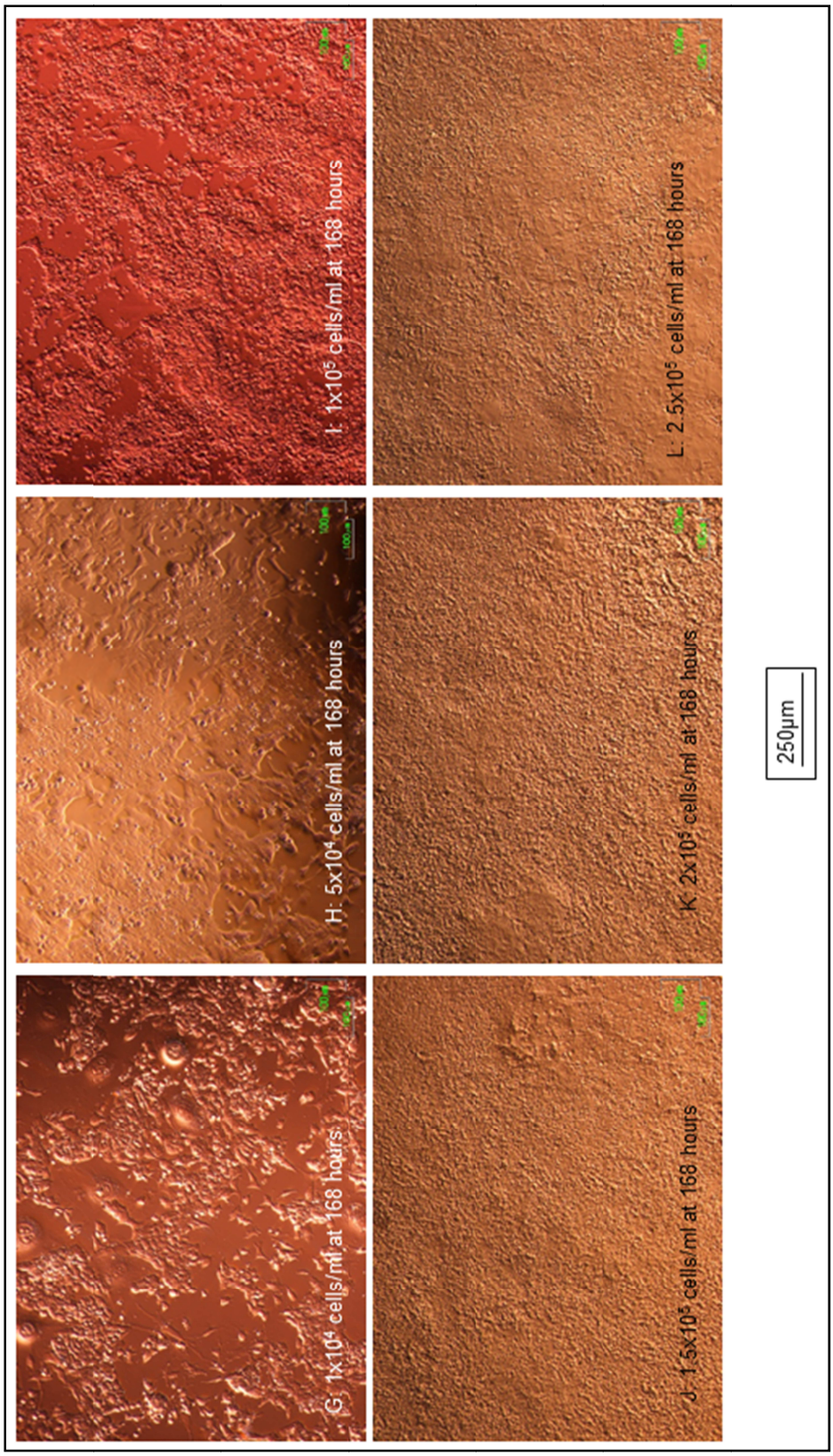


Figure 3.3.5ii: Images showing the extent of initial cell attachment after 24 hours (A - F) and the extent of proliferation after 168 hours (G - L) of mES cells seeded on Gelatin coated TCP at a range of initial seeding densities between 1 x 10^4 and 2.5 x 10^5 cell/ml.

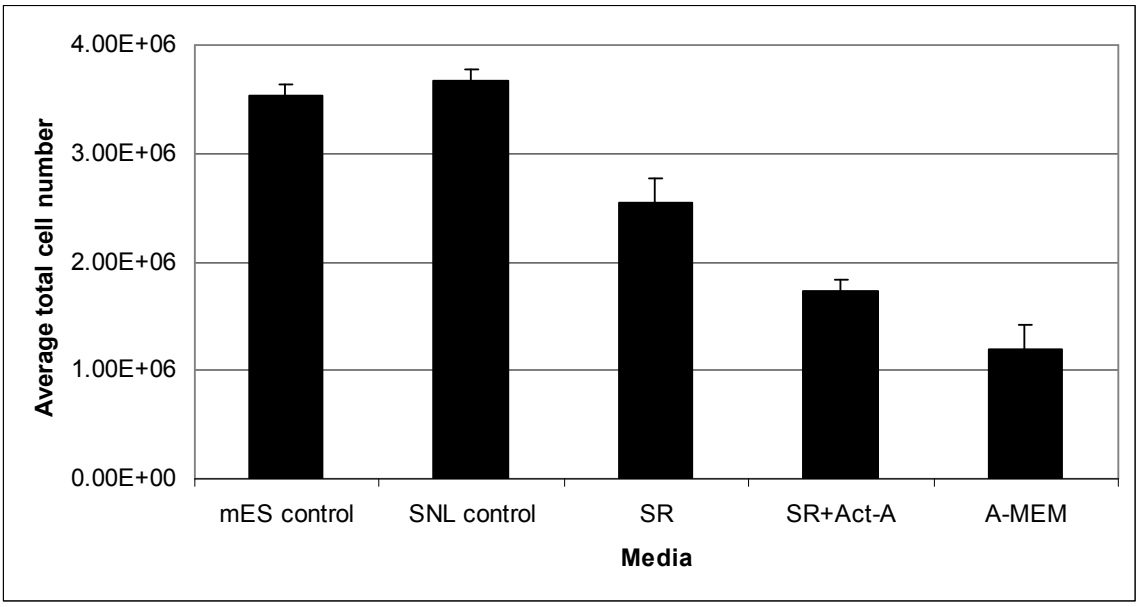


Figure 3.3.6: Graph showing the average total number of cells obtained after mES cells were cultured for 72 hours on Gelatin coated TCP in a variety of different media formulations containing 10% serum or SR (n = 3, error bars represent SD). Change labels

3.3.4 *In vitro* differentiation experiment four - Differentiation of mES cells using Act-A

CEE mES cells were removed from maintenance culture on feeder layers and seeded in a range of (feeder free) conditions designed to investigate the effects of treatment with the growth factor Act-A, aggregation into EBs and serum or SR usage on the differentiation of the cells [Kubo et al 2004, Tada et al 2005, Yasunaga et al, 2005]. In conditions where EBs were generated the cells were aggregated and cultured as EBs for 3 days prior to reseeding in monolayer culture for up to 7 days. Other conditions were immediately seeded in monolayer culture for up to 7 days. RNA samples were generated after 48 hours in monolayer culture and every 24 hours thereafter. Cells were fixed for immunocytochemical analysis after 4 and 7 days in monolayer culture.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis, was used as a ubiquitous control in the RT-PCR analysis to confirm that the RT reaction had worked; all samples should have shown positive expression of GAPDH. The strength of the GAPDH bands was then assessed to guide biased loading in the selected marker gene PCR reactions (see Sections 2.2.8 and 2.2.9). All the samples generated in condition 6 (EB in SR, Act-A-) did not contain sufficient material for analysis.

PCR expression of all of the selected marker genes was noticeably stronger in the conditions where Act-A was present (Figure 3.3.8C and F) compared to the control conditions (Figure 3.3.8A and B). Expression of *FoxA2* in cells derived from EBs (Figure 3.3.8C) and from monolayer derived cells (Figure 3.3.8F) cultured in the presence of Act-A was strongly up-regulated in the later timepoints compared to the monolayer control (Act-A-) conditions (Figure 3.3.8A and B).

In the monolayer derived cells there was some expression of *FoxA2* after 48 hours but this then disappeared at the 72 and 96 hour timepoints (Figure 3.3.8F). Weak CXCR4 expression was observed in the later timepoints in the presence of Act-A with none in the conditions without Act-A. *Sox17* expression followed a similar pattern to that of *FoxA2* but the level of expression was lower. However, *Oct4* expression was still in evidence in all conditions throughout the duration of the experiment.

This data is summarised in Figure 3.3.7. In the conditions where SR was used (Figure 3.3.8D, G and H) the cells grew slowly. Consequently the RNA samples obtained were of poor quality and yielded little or no data. The immunocytochemical images obtained were of poor quality and are not shown.

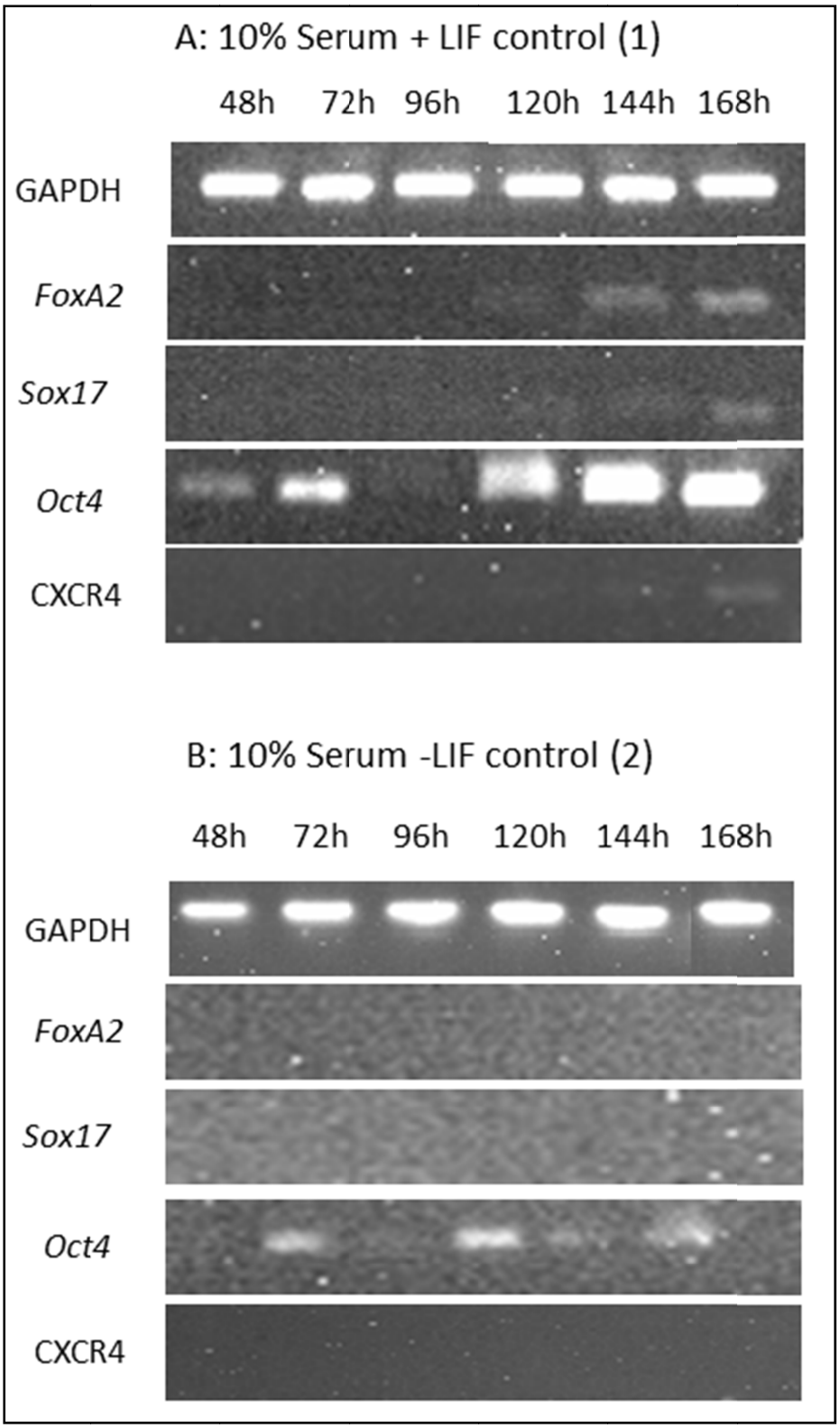


Figure 3.3.8A: Expression of marker genes (*CXCR4, FoxA2 and Sox17* for endoderm, *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis after 48, 72, 96, 120, 144 and 168 hours in culture (number in brackets refers to condition number in the table in Figure 3.2 in Section 3.2.1iv).

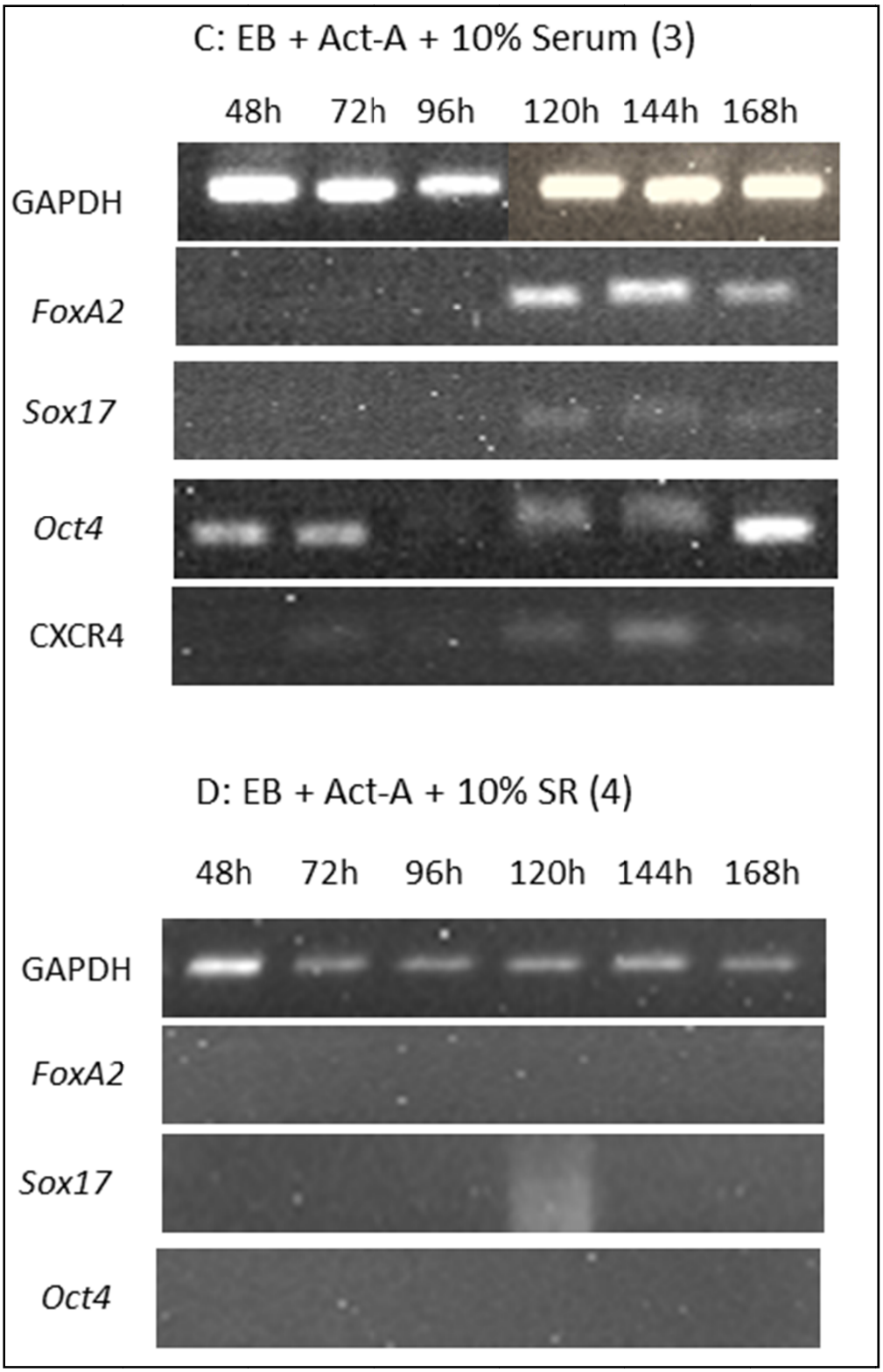


Figure 3.3.8B: Expression of marker genes (*CXCR4, FoxA2 and Sox17* for endoderm, *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis after 48, 72, 96, 120, 144 and 168 hours in culture (number in brackets refers to condition number in the table in Figure 3.2 in Section 3.2.1iv).

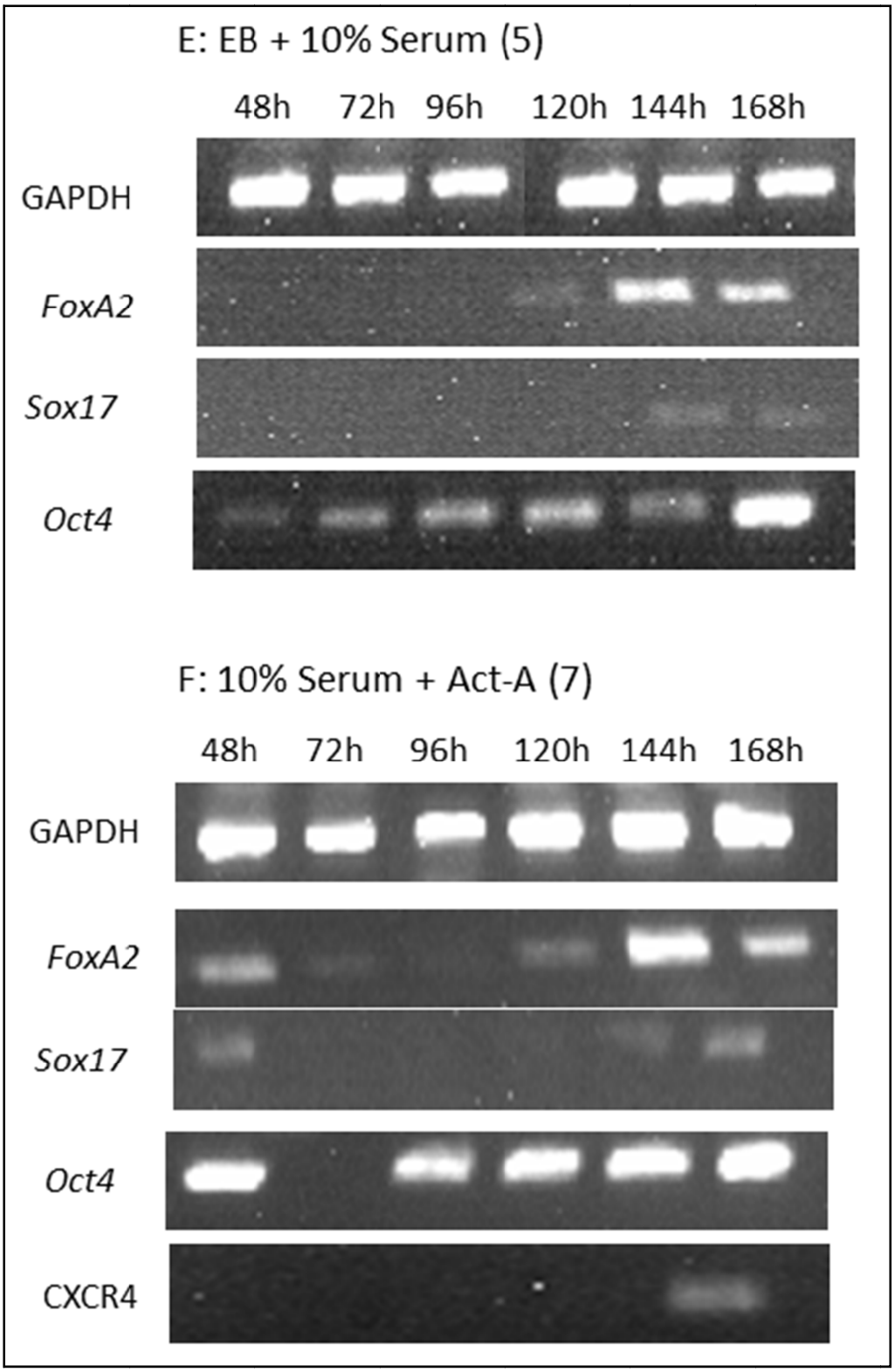


Figure 3.3.8C: Expression of marker genes (*CXCR4, FoxA2 and Sox17* for endoderm, *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis after 48, 72, 96, 120, 144 and 168 hours in culture (number in brackets refers to condition number in the table in Figure 3.2 in Section 3.2.1iv).

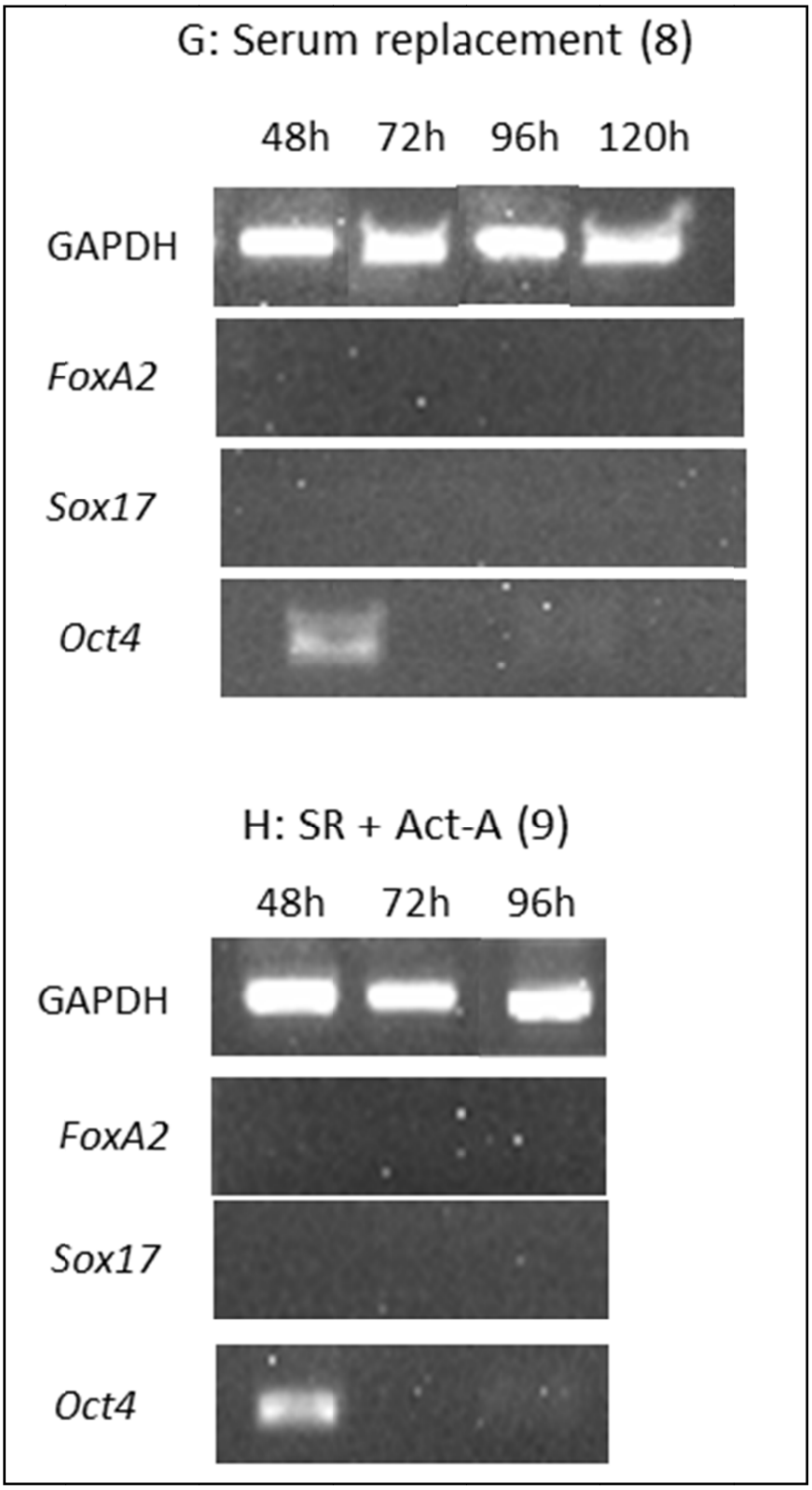


Figure 3.3.8D: Expression of marker genes (*CXCR4, FoxA2 and Sox17* for endoderm, *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis after 48, 72, 96, 120, 144 and 168 hours in culture (number in brackets refers to condition number in the table in Figure 3.2 in Section 3.2.1iv).

3.3.5 *In vitro* differentiation experiment five - Differentiation of mES cells using Act-A (part two)

CEE mES cells were removed from maintenance culture on feeder layers and transferred into five differentiation conditions designed to examine the effects of treatment with the growth factor Act-A and aggregation into EBs on the differentiation of the cells towards the DE fate. Given the extremely poor (RNA and fixed cell) samples generated in low serum and SR culture conditions in previous experiments all culture conditions contained 10% (*v/v*) FCS. RNA samples were generated (timepoints differ between conditions – see Section 3.2.1v and Figure 3.3.11) and cells fixed for immunocytochemistry (timepoints differ between conditions - see Section 3.2.1v, Figure 3.2.3).

Given the poor cell growth observed in some of the differentiation conditions used previously it was decided to evaluate and record the attachment and proliferation of the cells throughout the time course of the differentiation experiment by taking light microscopy images of the live cell cultures. The images showed good initial attachment and rapid proliferation in all conditions immediately after seeding. The rate of proliferation appeared to slow in the later stages of the experiment.

In condition one (initial three day EB phase, monolayer thereafter, Act-A+) numerous EBs were formed over the initial 72 hours. When dissociated and reseeded as a monolayer (Figure 3.3.9A and B) the cells attached well and proliferated throughout the time course of the experiment. The proliferation slowed slightly towards the end of the time course but at the time the experiment was terminated the cells had formed a confluent monolayer. In condition two (monolayer throughout, Act-A+) the cells attached well initially and proliferated rapidly throughout the time course (Figure 3.3.9C and D). Cell proliferation appeared to slow slightly towards the end of the time course but at the time the experiment was terminated the cells had formed an over-confluent monolayer.

In condition three (two days in monolayer then three day EB phase, monolayer thereafter, Act-A+), where the initial seeding density was higher than the other conditions, good attachment and rapid proliferation was seen producing nearly confluent monolayers by 48 hours (Figure 3.3.9E and F).

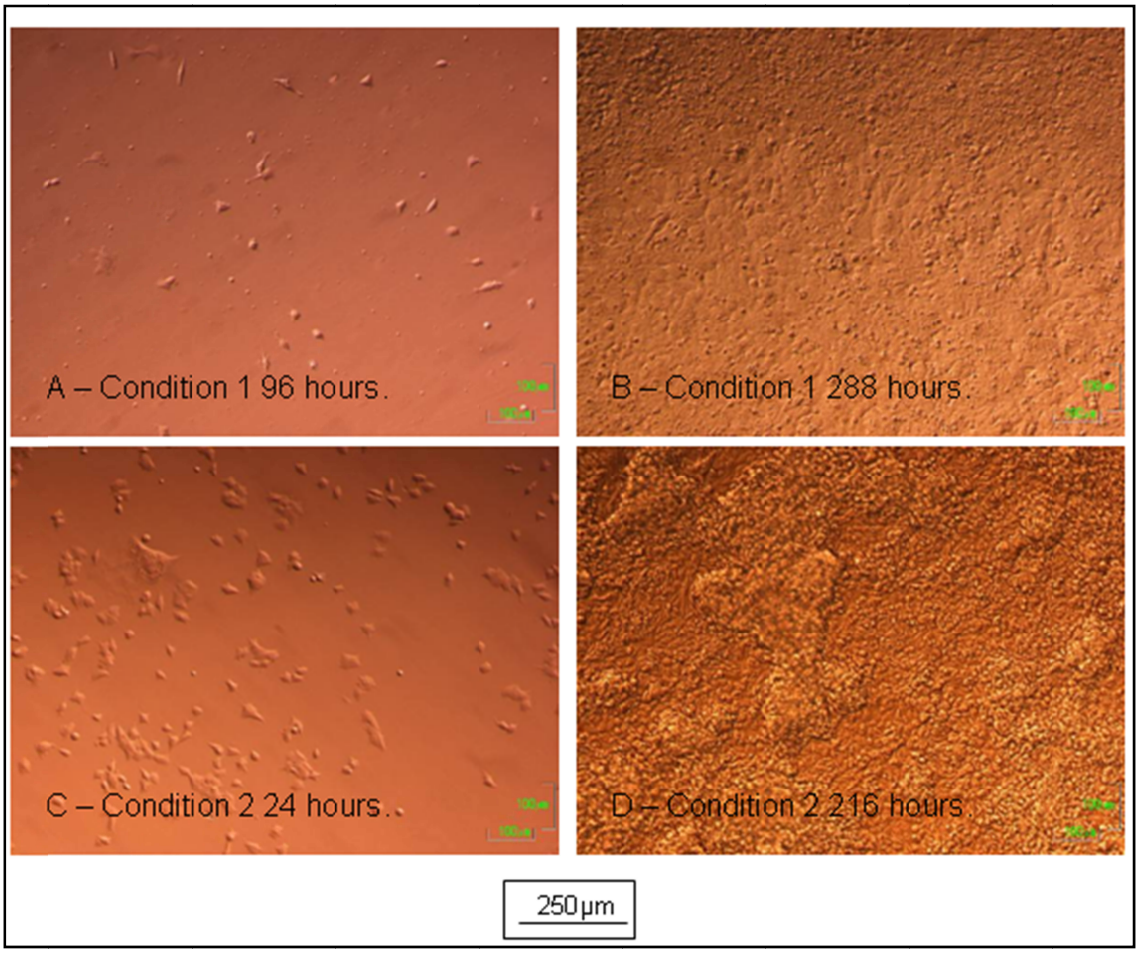


Figure 3.3.9i: Light microscopy images from Condition one (initial three day EB phase, monolayer thereafter, Act-A+) and two (Monolayer throughout, Act-A+). A – C1 24 hours, B – C1 288 hours. C – C2 24 hours, D – C2 216 hours.

The cells were then aggregated and formed numerous EBs that were then disaggregated and reseeded in monolayer where the cells attached moderately well (Figure 3.3.9G). Although the cells did not proliferate as rapidly thereafter by the time the experiment was terminated the cells had formed a confluent monolayer (Figure 3.3.9H).

In condition four (monolayer throughout, Act-A-) the cells attached well initially and proliferated rapidly throughout the time course (Figure 3.3.10A and B). The proliferation slowed slightly towards the end of the time course but at the time the experiment was terminated the cells had formed an over-confluent monolayer.

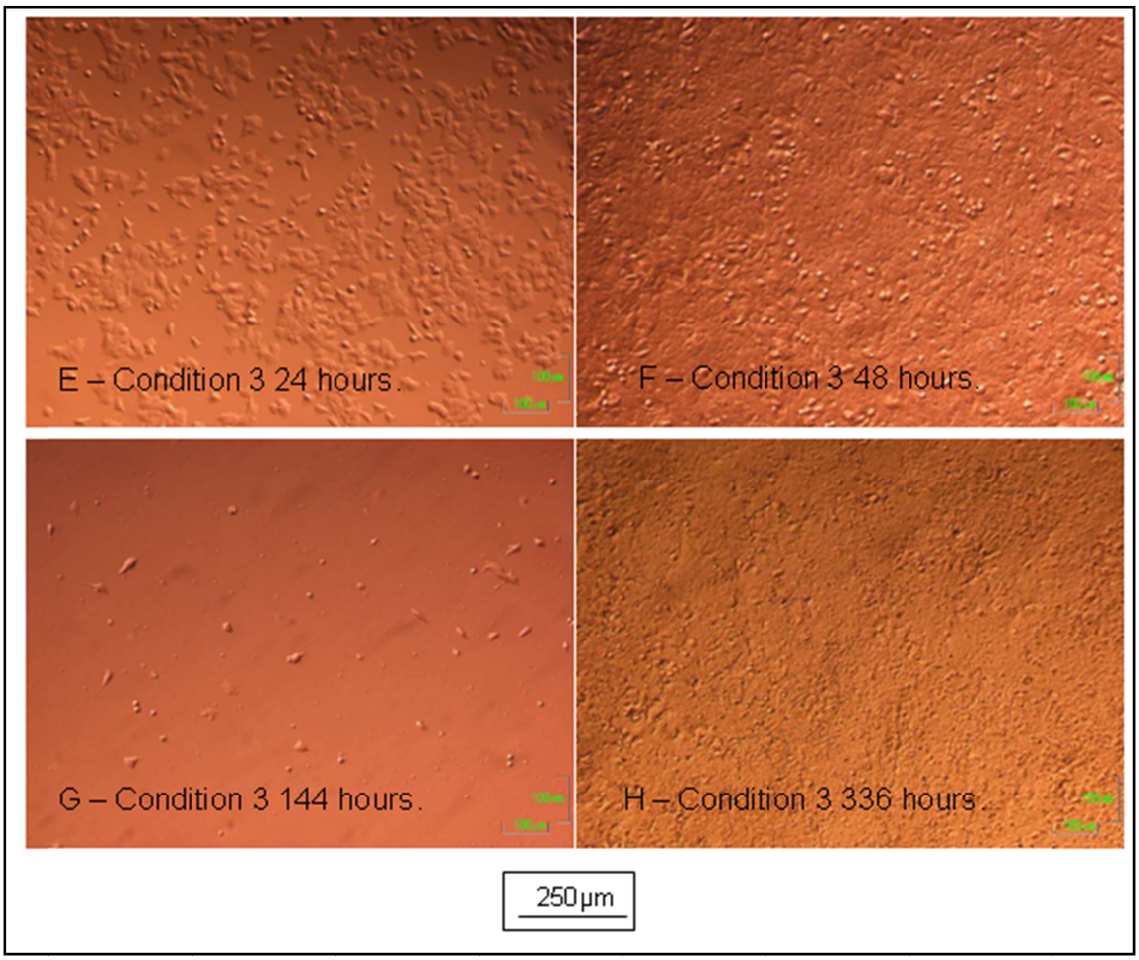


Figure 3.3.9ii: Light microscopy images from Condition three (two days in monolayer then three day EB phase, monolayer thereafter, Act-A+). E – C3 24 hours, F – C3 48 hours, G – C3 144 hours, H – C3 336 hours.

In condition five (initial three day EB phase, monolayer thereafter, Act-A-) numerous EBs were formed over the initial 72 hours. When dissociated and reseeded as a monolayer (Figure 3.3.10C and D) the cells attached well and proliferated throughout the time course of the experiment. The proliferation slowed slightly towards the end of the time course but at the time the experiment was terminated the cells had formed a confluent monolayer.

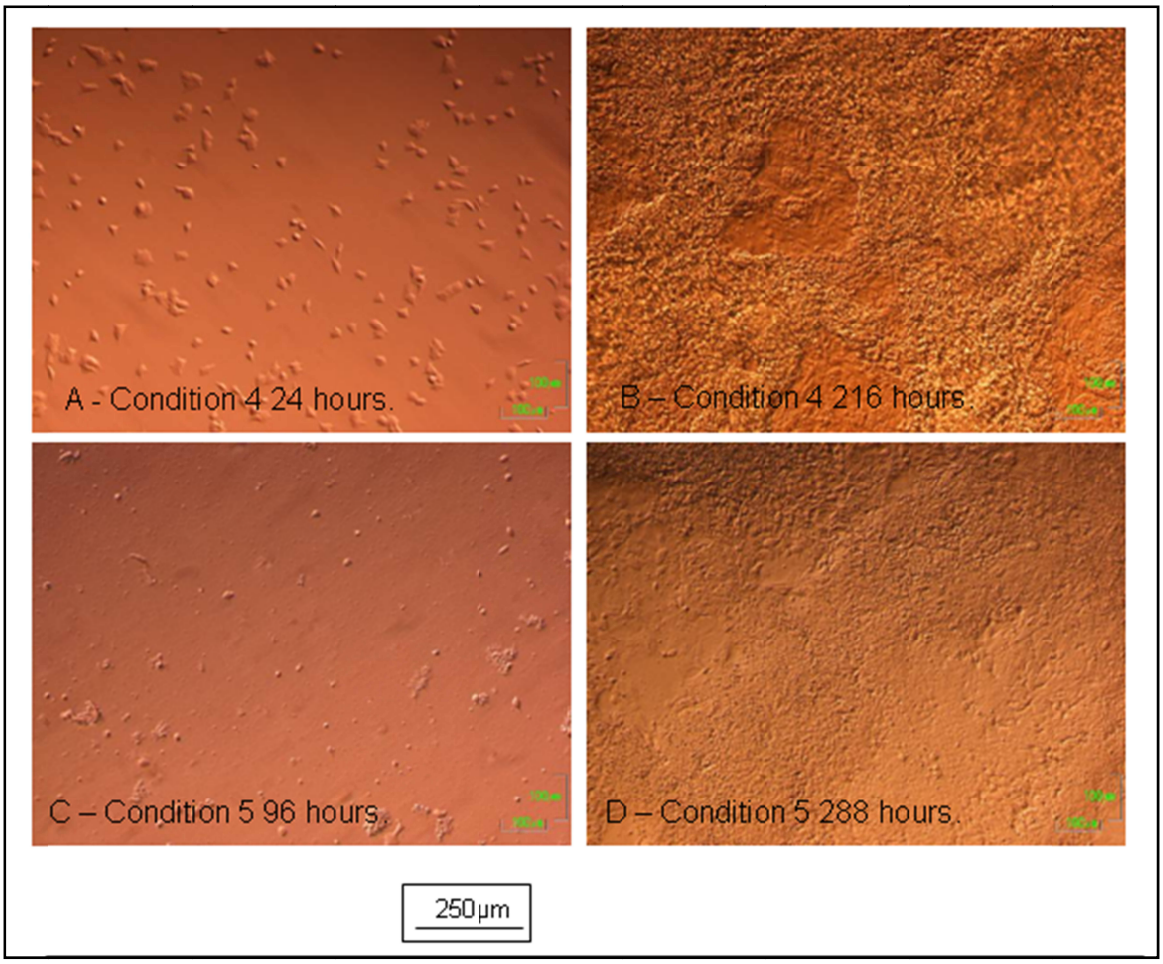


Figure 3.3.10: Images from condition four (monolayer throughout, Act-A-) and condition five (initial three day EB phase, monolayer thereafter, Act-A-). A – Condition four at 24 hours, B – Condition four at 216 hours, C – Condition five at 96 hours and D – Condition five at 288 hours.

In the RT-PCR evaluation (Figure 3.3.11) of the RNA samples from each condition strong bands were obtained for all *GAPDH* reactions indicating that the RTs had all been successful. The relative strength of the GAPDH bands was used to determine biased loading in the marker gene PCR reactions. Moderate *Oct4* expression was retained in all conditions throughout the experiment.

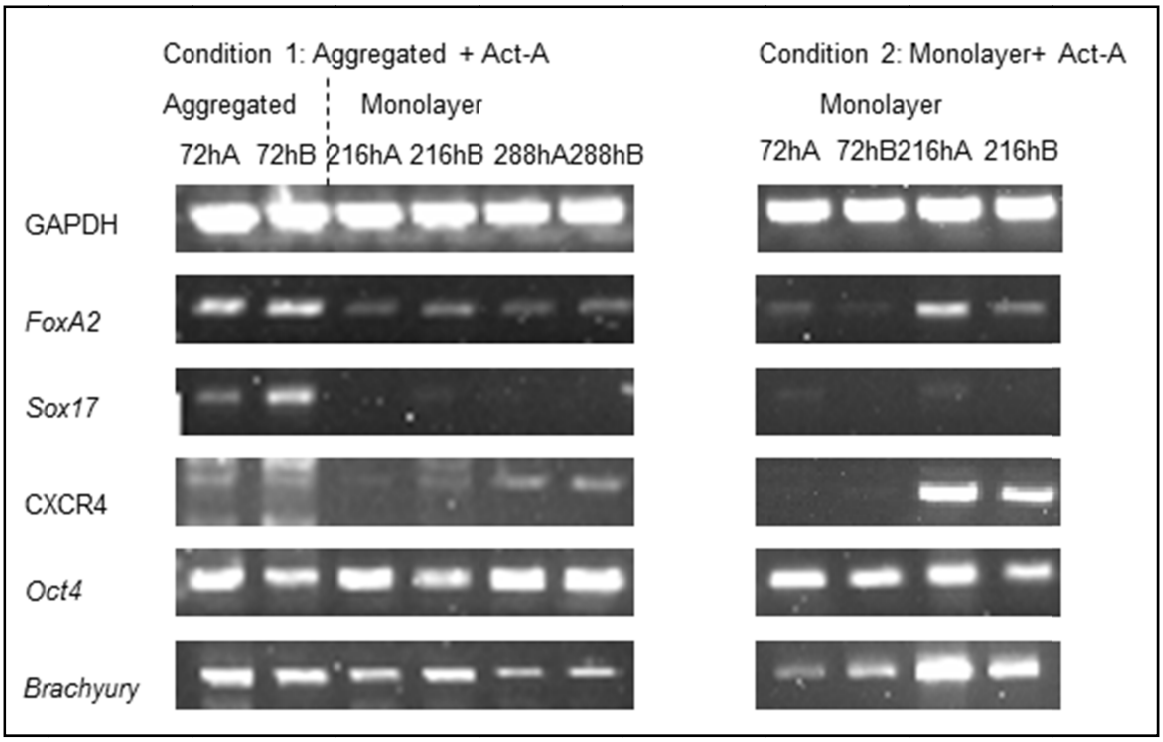


Figure 3.3.11A: Expression of markers of early differentiation (*CXCR4, FoxA2 and Sox17* for endoderm, *Brachyury* for mesoderm and *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis. Condition one (Aggregation plus Act-A) after 72, 216 and 288 hours in culture and Condition two (Monolayer plus Act-A) after 72 and 216 hours in culture.

In condition one (Figure 3.3.11A left), where the cells had been aggregated and treated with Act-A, *FoxA2* and *Sox17* were expressed after the initial 72 hours as aggregates. The same level of expression was also observed in condition five (Figure 3.3.11B left), where the cells had been aggregated but without Act-A treatment. In both conditions the levels of *FoxA2* expression had reduced by the 216 hour timepoint and were at a similar level by the final 288 hour timepoint. There were no further bands of *Sox17* expression in either condition other than a very faint band in one replicate of the 288 hour timepoint in condition five. In condition one faint bands of *CXCR4* expression were present throughout with even fainter bands present in condition five. In condition one there was also moderate expression of *Brachyury* after 72 hours but the level of expression decreased after 216 hours and again after 288 hours. Slightly stronger bands of *Brachyury* expression were present in condition

five after 72 hours but the level of expression fell significantly after 216 hours before increasing again after 288 hours.

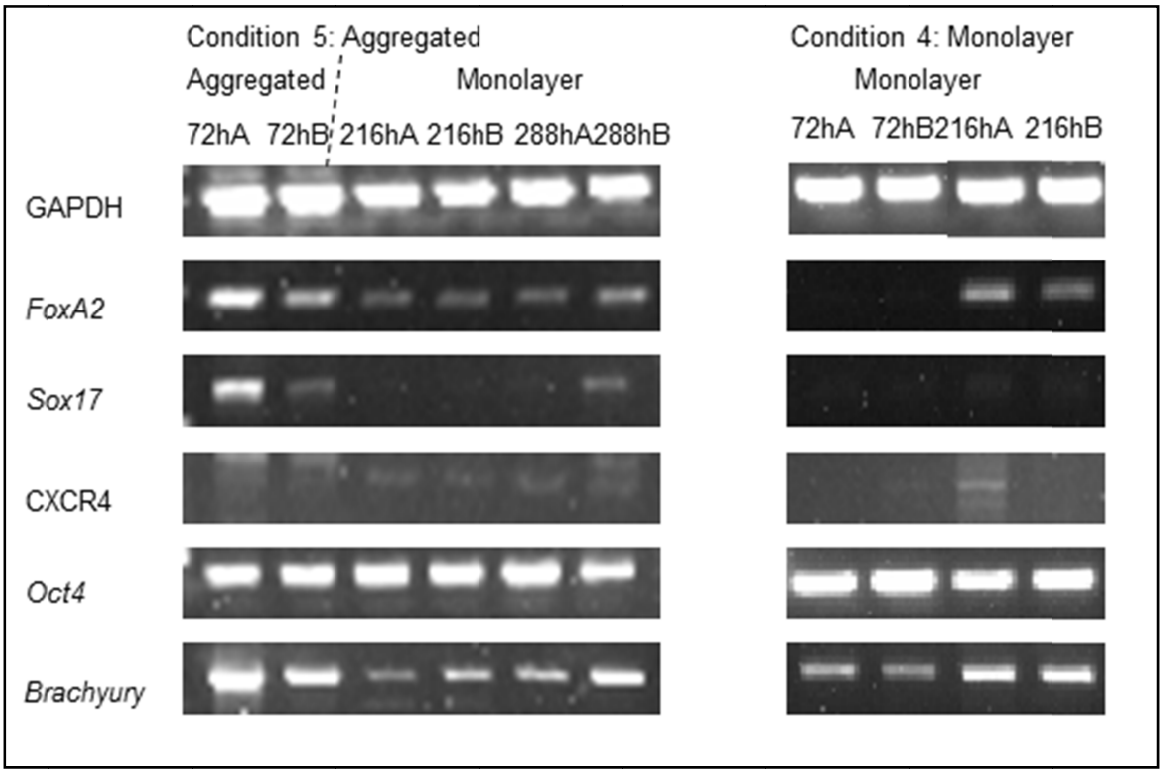


Figure 3.3.11B: Expression of markers of early differentiation (*CXCR4, FoxA2 and Sox17* for endoderm, *Brachyury* for mesoderm and *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis. Condition four (Aggregation) after 72, 216 and 288 hours in culture and Condition five (Monolayer) after 72 and 216 hours in culture.

In condition two (Figure 3.3.11A right), where the cells had been cultured in monolayer throughout and treated with Act-A, there was very weak expression of *FoxA2* after 72 hours and moderate expression at the final 216 hour timepoint. In condition four (Figure 3.3.11B right), where the cells had been cultured in monolayer throughout without any Act-A treatment, there was no expression of *FoxA2* after 72 hours and moderate expression after 216 hours. In both conditions there were no bands of *Sox17* expression. In condition two there was no expression of *CXCR4* after 72 hours but strong bands were present after 216 hours. There was one faint band of *CXCR4* expression in one replicate after 216 hours in condition four but not in the

duplicate sample. In both conditions there was moderate expression of *Brachyury* after 72 hours which had increased after 216 hours. The bands were stronger in condition two than in condition four.

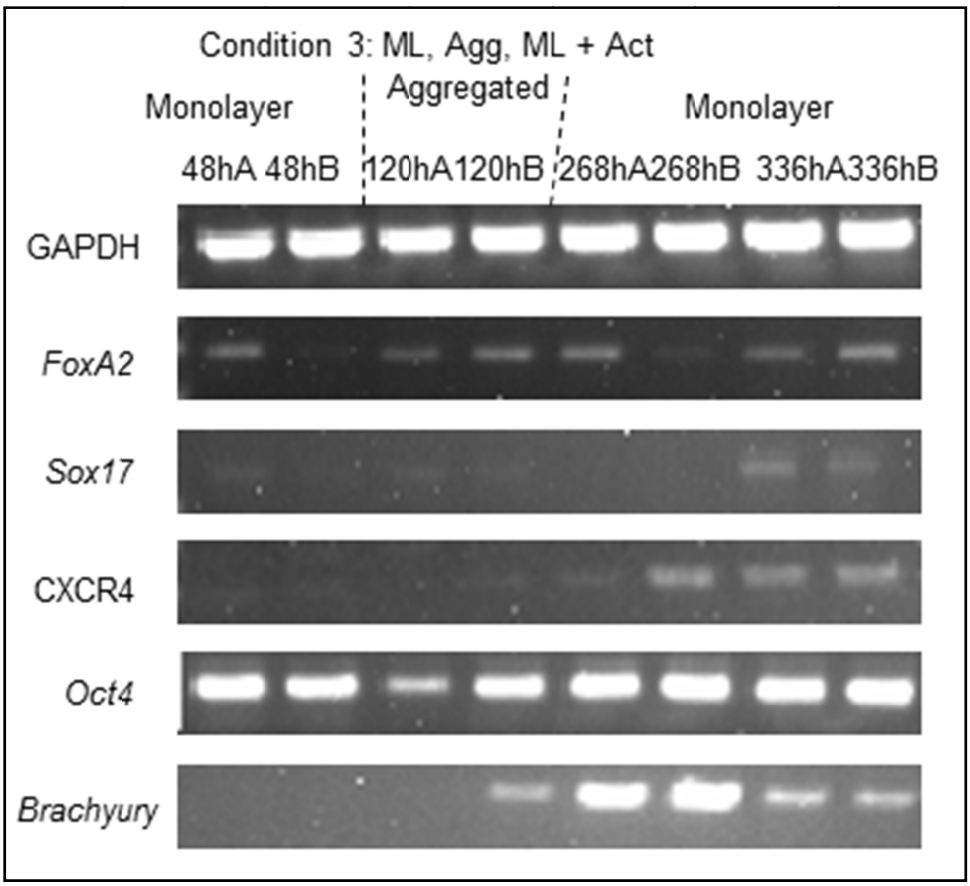


Figure 3.3.11C: Expression of markers of early differentiation (*CXCR4, FoxA2 and Sox17* for endoderm, *Brachyury* for mesoderm and *Oct4* for undifferentiated cells) under different culture conditions evaluated by RT-PCR analysis. Condition three (Monolayer then aggregation plus Act-A) after 48, 120, 264 and 336 hours in culture. There are duplicate samples (A and B) for each timepoint.

In condition three (Figure 3.3.11C), where the cells had been cultured in monolayer for two days, aggregated for three days then returned to monolayer culture for a further nine days with Act-A present throughout, weak expression of *FoxA2* was observed at all time points. There was also weak expression of *Sox17* in the 336 hour (final) samples and weak expression of *CXCR4* in the last two timepoints (268 and 336 hours). Weak expression of *Brachyury* was present in one replicate following the

aggregation stage (120 hours). There was then strong expression of *Brachyury* after 268 hours but this had become significantly weaker after 336 hours (final timepoint) in culture.

Figure 3.3.12 shows the results of the RT-PCR analysis of the RNA samples produced from the repeat of *in vitro* experiment five. All the GAPDH reactions in all the conditions produced strong bands indicating that all of the RT reactions had worked. Note that the two series of bands shown for *Sox17* represent the PCR reaction carried out with a different number of cycles. Unless specifically stated it is the 35 cycle bands that are commented on. This data is summarised in Section 6.3, Table 6.3.2.

In condition one (Figure 3.3.12A), where the cells had been aggregated and treated with Act-A throughout, *Oct4* expression was maintained throughout the time course with the exception of one of the replicates at 216 hours where the reaction appeared to have failed. *Oct4* expression was also maintained throughout the time course in condition five (Figure 3.3.12A), where the cells had been aggregated but not treated with Act-A, although the bands were slightly weaker when compared to those produced in condition one.

Nanog, another pluripotency marker, was also expressed throughout the time course in both condition one and five. In condition one there was very weak expression of *Sox17* in one replicate at each time point and strong expression of *FoxA2* throughout. In condition five there was a very weak band of *Sox17* in one of the 72 hour replicates but it was not expressed throughout. Moderate expression of *FoxA2* was observed throughout the experiment except for one of the 72 hour replicates where the reaction appeared to have failed. In condition one there was weak expression of *GATA4* throughout. *CXCR4* was expressed weakly at 72 hours but then highly expressed after 216 hours. It then appeared to fall again with one very weak band and one moderate band in the 288 hour samples.

In condition five there are weak bands of *GATA4* and *CXCR4* expression at 72 hours with a weak band present for each gene in one replicate at 216 and 288 hours. In condition one there was a strong band of *Brachyury* expression in one 216 hour replicate but no other expression. There was weak expression of *Nestin* throughout

the time course. In condition five there was weak expression of *Brachyury* in one 72 hour replicate and one 216 hour replicate with a moderate band in one 288 hour replicate. There was weak expression of *Nestin* after 72 hours with one moderate band after 216 hours.

In condition two (Figure 3.3.12B), where the cells had been cultured in monolayer and treated with Act-A, *Oct4* and *Nanog* expression were maintained throughout. In condition four (Figure 3.3.12B), where the cells were cultured in monolayer without Act-A treatment, *Oct4* expression was maintained throughout but was slightly lower than in condition two. *Nanog* expression was comparable to that in condition two.

In condition two there was no expression of *Sox17* and moderate expression of *FoxA2* whilst in condition four there was very weak expression of *Sox17* with moderate expression of *FoxA2*. In both conditions two and four there were very weak bands for both *CXCR4* and *GATA4* but they were of comparable strength to those observed in the negative controls (untreated CEE mES cells). In condition two there were very weak bands for *Brachyury* in the 216 hour sample with slightly stronger bands seen in condition four. In both conditions weak bands for *Nestin* were seen in all samples.

In condition three (Figure 3.3.12C), where the cells were cultured in monolayer for 48 hours then aggregated for 72 hours before being returned to monolayer culture with Act-A present throughout, there was strong expression of *GAPDH* in all the experimental samples. Both *Oct4* and *Nanog* were strongly expressed throughout. *Sox17* was expressed weakly at 264 hours with an increase in expression at 336 hours with *FoxA2* showing moderate expression at all timepoints although only one of the 48 hour duplicates showed expression.

CXCR4 shows expression at the 264 and 336 hour timepoints and *GATA4* showed weak expression from the 120 hour timepoint onwards. *Brachyury* showed moderate expression at 264 hours but this had reduced at the 336 hour timepoint. *Nestin* showed very weak expression at 120 hours with moderate expression at 264 hours. This was then reduced after 336 hours.

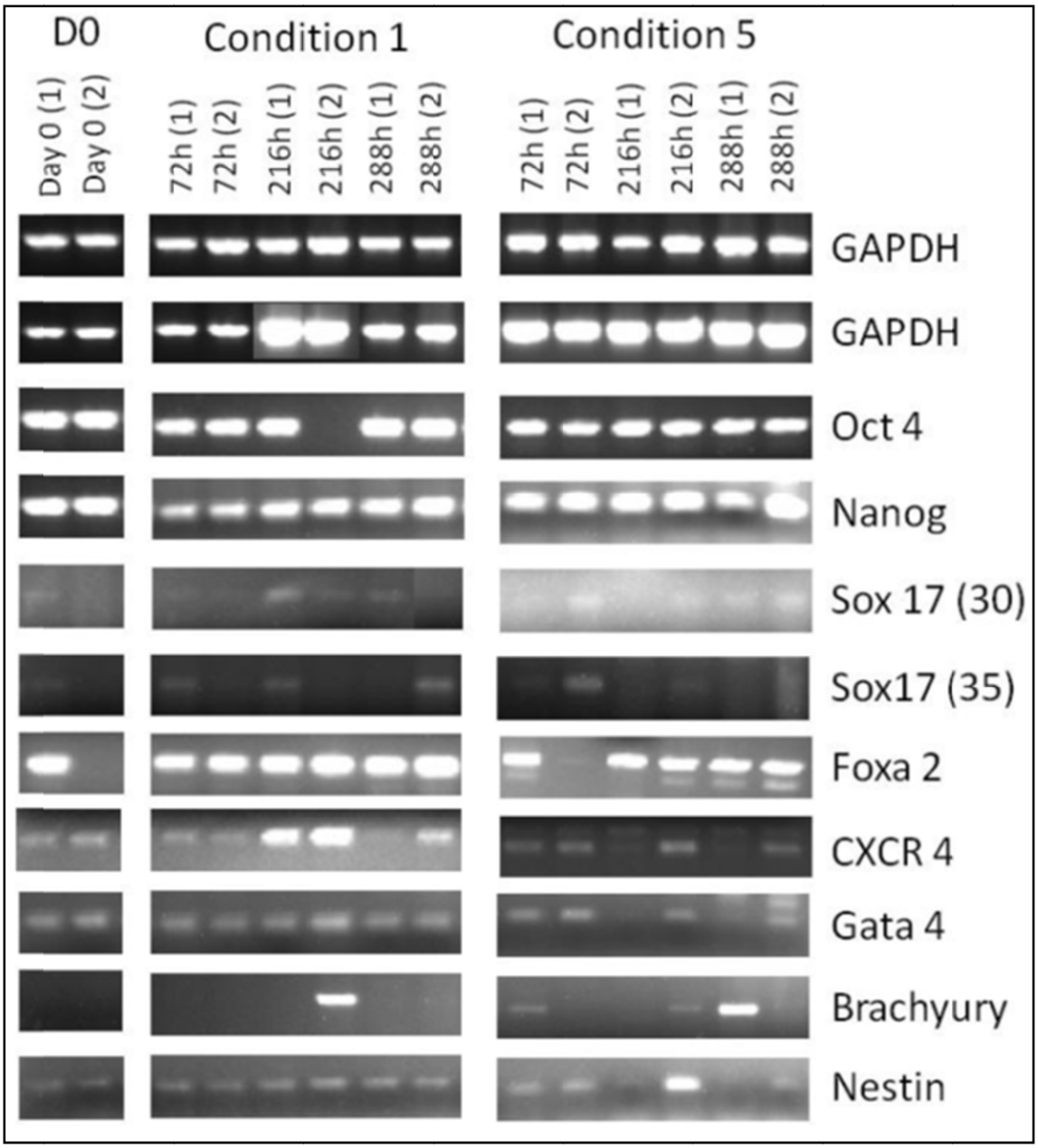


Figure 3.3.12A: Expression of marker genes (*CXCR4, GATA4, FoxA2 and Sox17* for endoderm, *Oct4* and *Nanog* for undifferentiated cells, *Brachyury* for mesoderm and *Nestin* for ectoderm) under different culture conditions (Experiment five, Part B, conditions one and five) evaluated by RT-PCR analysis.

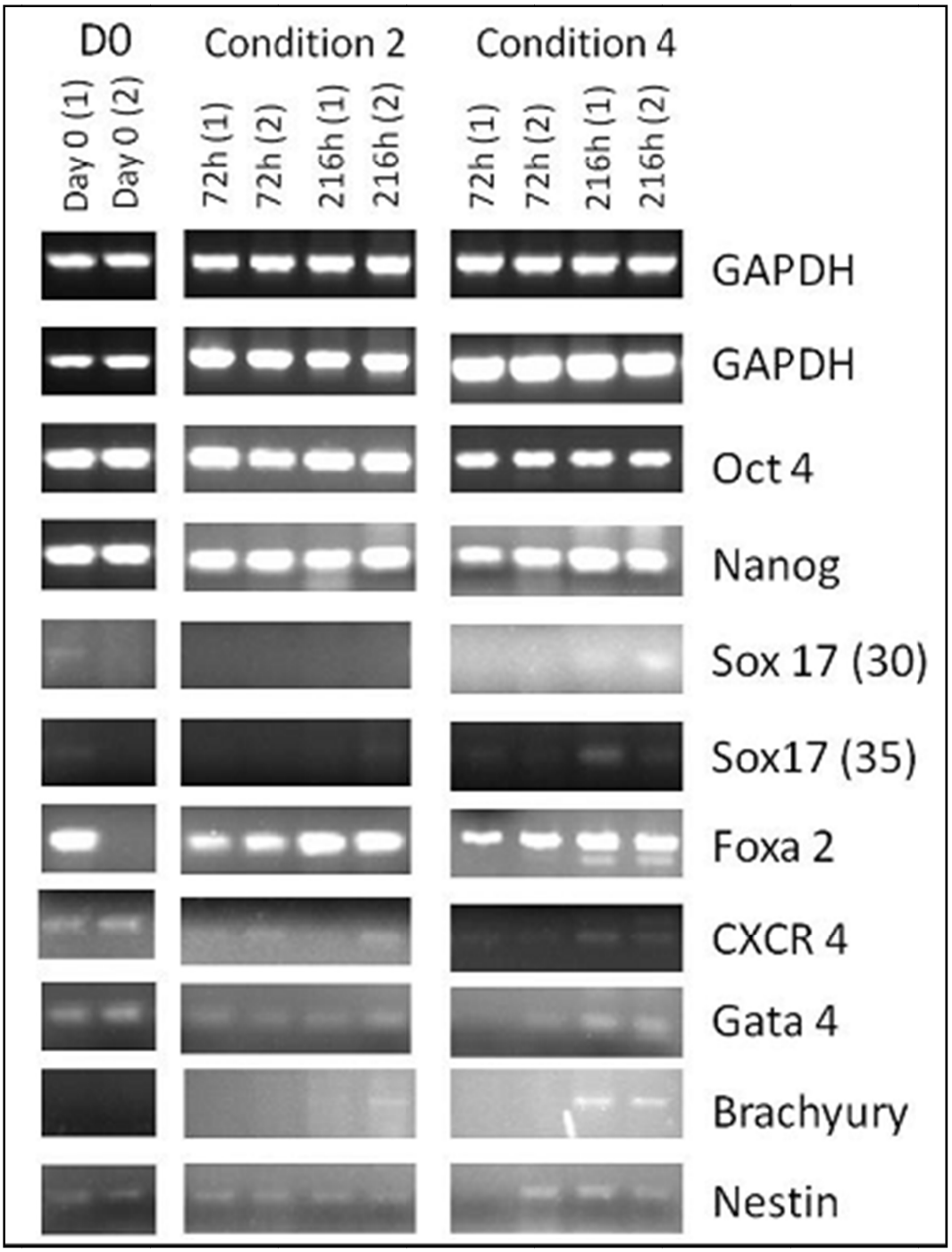


Figure 3.3.12B: Expression of marker genes (*CXCR4, GATA4, FoxA2 and Sox17* for endoderm, *Oct4* and *Nanog* for undifferentiated cells, *Brachyury* for mesoderm and *Nestin* for ectoderm) under different culture conditions (Experiment five, Part B, conditions two and four) evaluated by RT-PCR analysis.

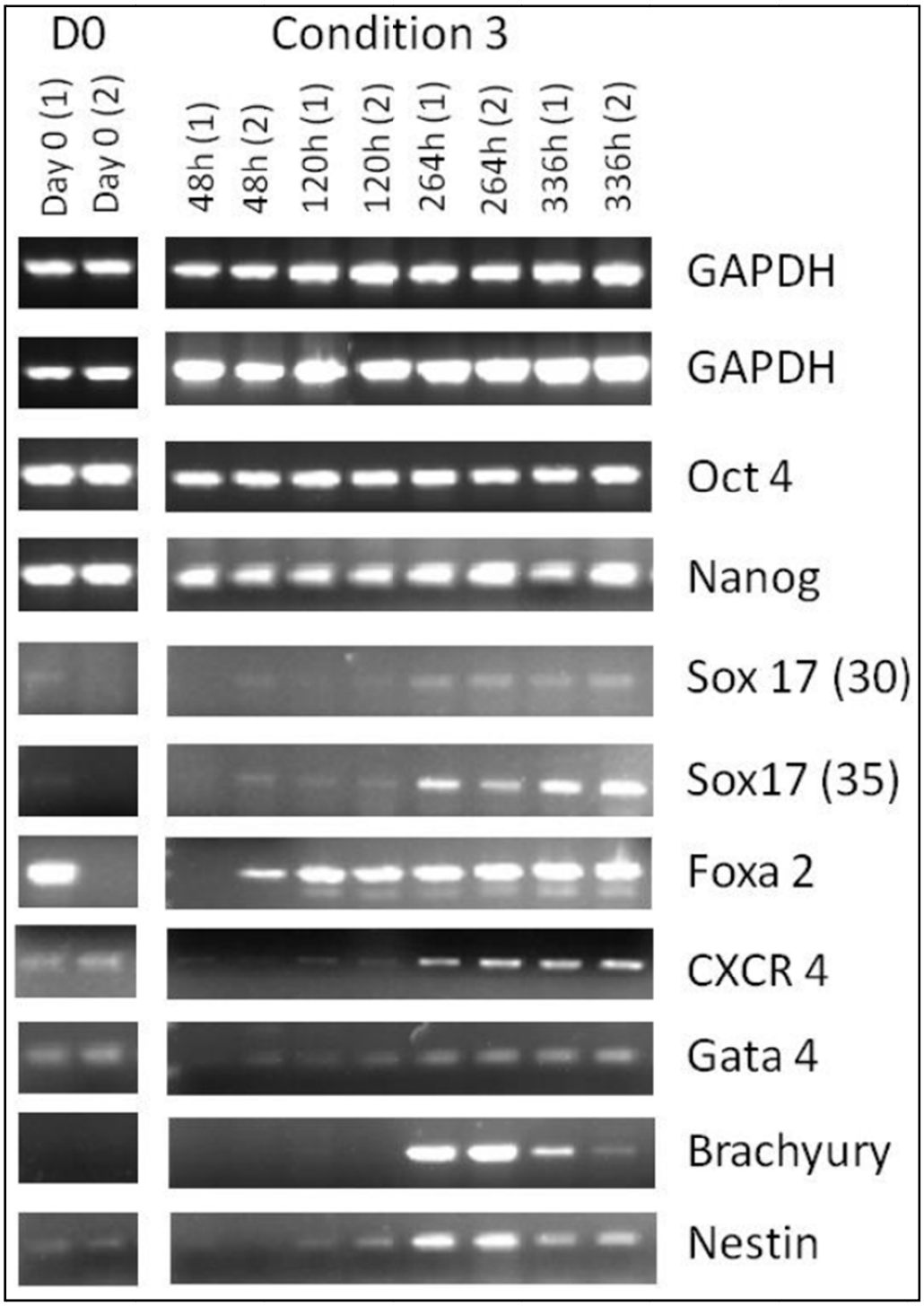


Figure 3.3.12C: Expression of marker genes (*CXCR4, GATA4, FoxA2 and Sox17* for endoderm, *Oct4* and *Nanog* for undifferentiated cells, *Brachyury* for mesoderm and *Nestin* for ectoderm) under different culture conditions (Experiment five, Part B, condition three) evaluated by RT-PCR analysis.

Figures 3.3.13 – 3.3.17 show the fluorescent immunocytochemical results obtained from the antibody staining of the fixed cell samples from *in vitro* experiment five. The markers examined are the expressed proteins of some of the marker genes examined in the RT-PCR analysis. These are OCT4 as a marker of pluripotency, GATA4 and co-expression of SOX17 and FOXA2 as markers of (definitive) endoderm and BRACHYURY as a marker of mesoderm. A secondary control is also shown to illustrate the level of background signal and to highlight any non-specific binding of the Fluorescein isothiocyanate (FITC) tagged secondary antibody.

Figure 3.3.13 shows the results from culture condition one (three day EB phase, monolayer thereafter, Act-A+). The secondary control at 72 hours showed good staining in the DAPI channel with only faint staining in the FITC channel indicating that limited non-specific binding had occurred. The DAPI stains in the marker samples all show a good number of cells in each field of view. There is little marker staining for OCT4 and only weak signal for BRACHYURY which may have been caused by background autofluorescence. FOXA2 and SOX17 both show moderate signal across the field of view with a few patches of stronger signal. The GATA4 image has strong background signal making it difficult to clearly visualise the image although there do appear to be a few patches of genuine signal present.

The secondary control at 288 hours shows a high level of background signal which is also present in a number of the marker stains (FOXA2 and GATA4). The DAPI stains also show that there are more cells compared to the earlier timepoint (the cells will have continued to proliferate throughout the time course of the experiment). There is strong marker signal for OCT4 in the 288 hour sample but much of this appears to be background interference (autofluorescence). There appear to be some patches of FOXA2 expression but the levels of background interference make these unclear. There are some clear patches of SOX17 expression. There are some faint patches of signal for BRACHYURY but these may be background signal. The GATA4 image is hard to interpret due to the high background signal but there do appear to be some patches where the signal is stronger which may indicate positive expression.

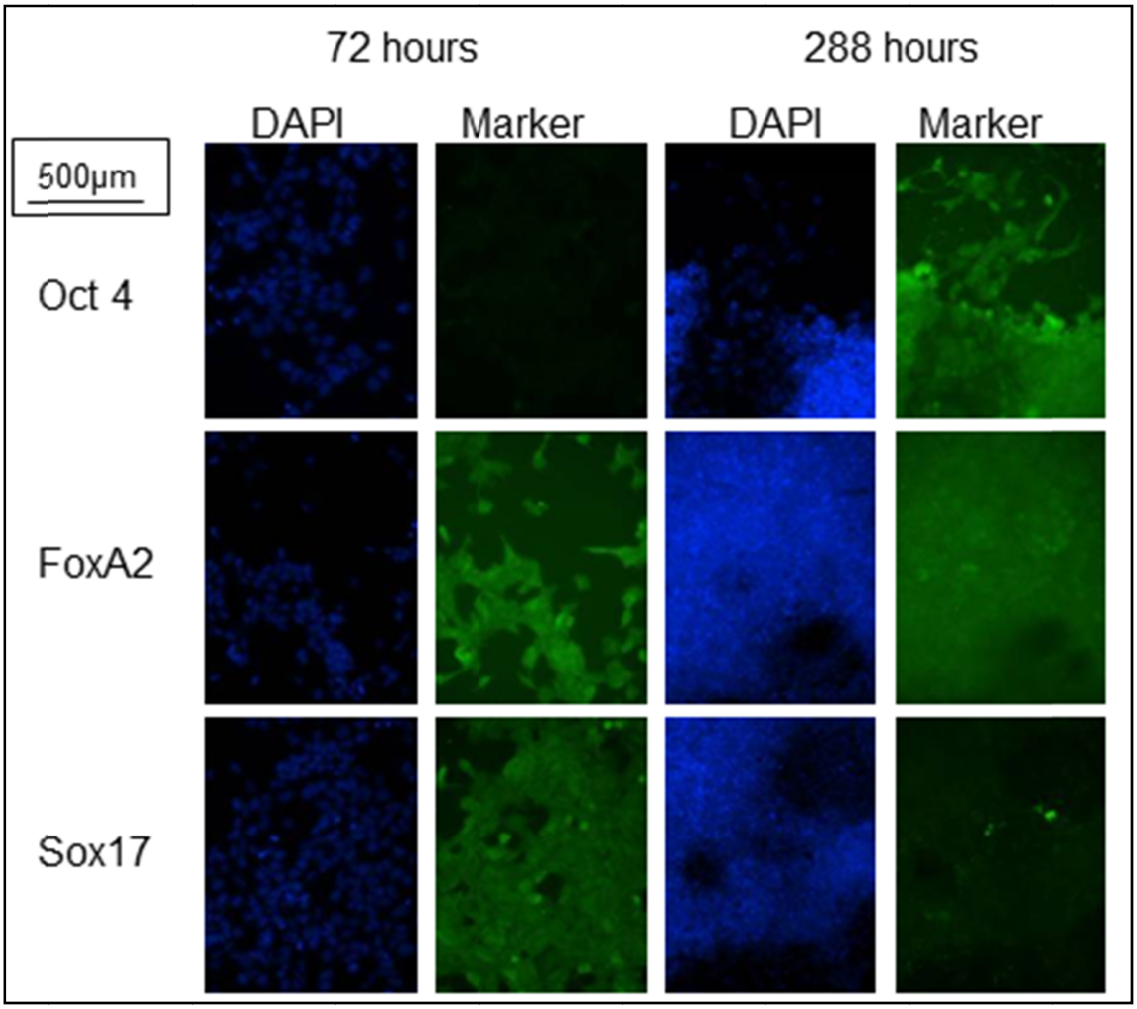


Figure 3.3.13A: Representative expression of key marker proteins from condition one (three day EB phase, monolayer thereafter, Act-A+) for expression by fluorescence immunocytochemistry after 72 and 288 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

Figure 3.3.14 shows the results from culture condition two (monolayer plus Act-A). As with condition one the secondary control image for the 72 hour timepoint shows little background interference but the image for the 216 hour timepoint shows more significant background signal. The DAPI stain images for all the 72 hour samples show a good coverage of cells growing in large clusters. The DAPI stains for the 216 hour samples show that the cells have grown to a confluent monolayer in the culture vessel. At 72 hours there is weak signal for OCT4 expression.

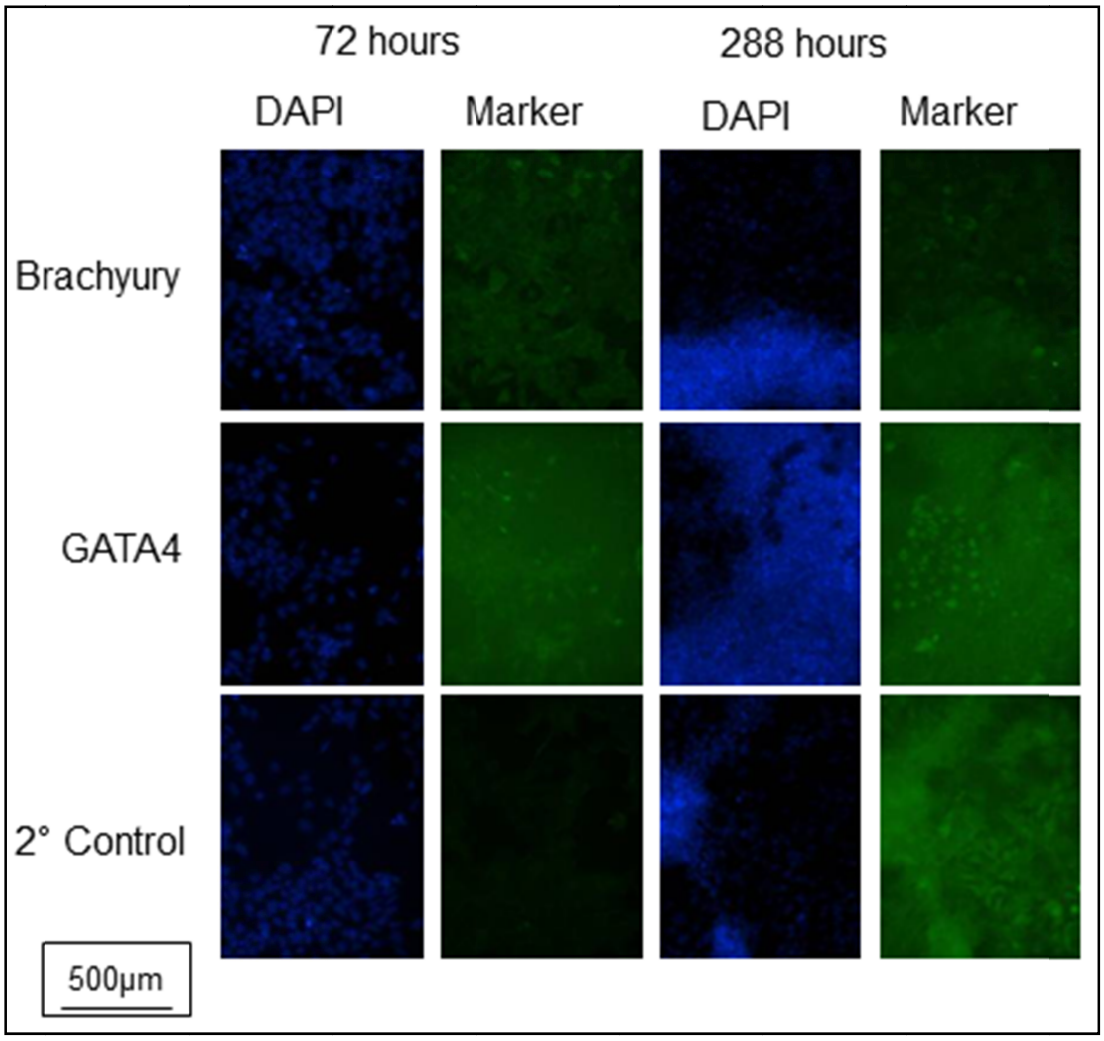


Figure 3.3.13B: Representative expression of key marker proteins from condition one (three day EB phase, monolayer thereafter, Act-A+) for expression by fluorescence immunocytochemistry after 72 and 288 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

There is clear signal for both FOXA2 and SOX17 although there are a few patches where the signal is stronger than others suggesting that the expression is not evenly distributed. There is some scattered expression of BRACHYURY but no apparent expression of GATA4 although the level of background signal makes the data unclear. At 216 hours there is still moderate expression of OCT4. FOXA2 and SOX17 appear to be expressed but FOXA2 signal is very weak whilst the levels of background signal for SOX17 make the data unclear. Both GATA4 and

BRACHYURY appear to still be expressed but as with SOX17 the levels of background interference reduce the quality of the data.

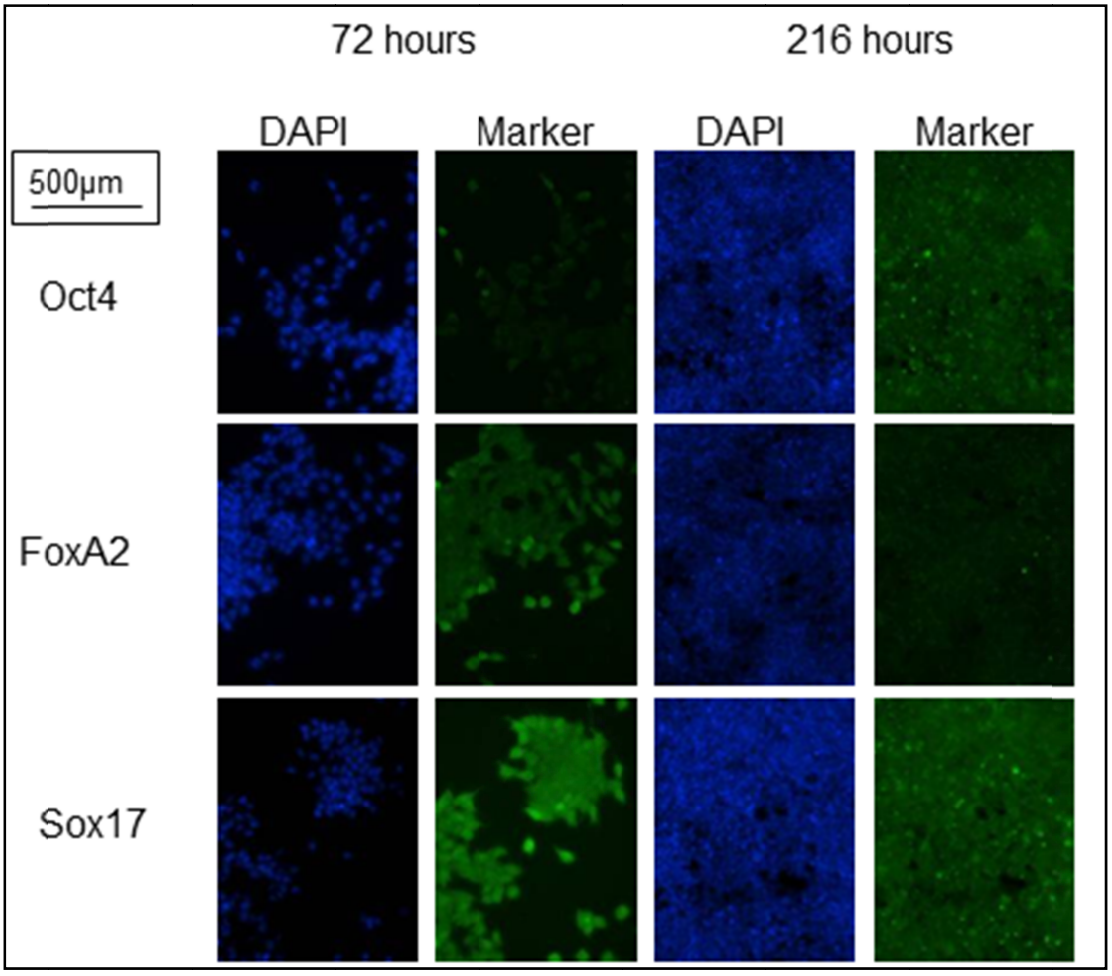


Figure 3.3.14A: Representative expression of key marker proteins from condition two (Monolayer throughout, Act-A+) for by fluorescence immunocytochemistry after 72 and 216 hours in culture. DAPI staining (of nuclei) in blue, FITC staining of the marker antibody in green.

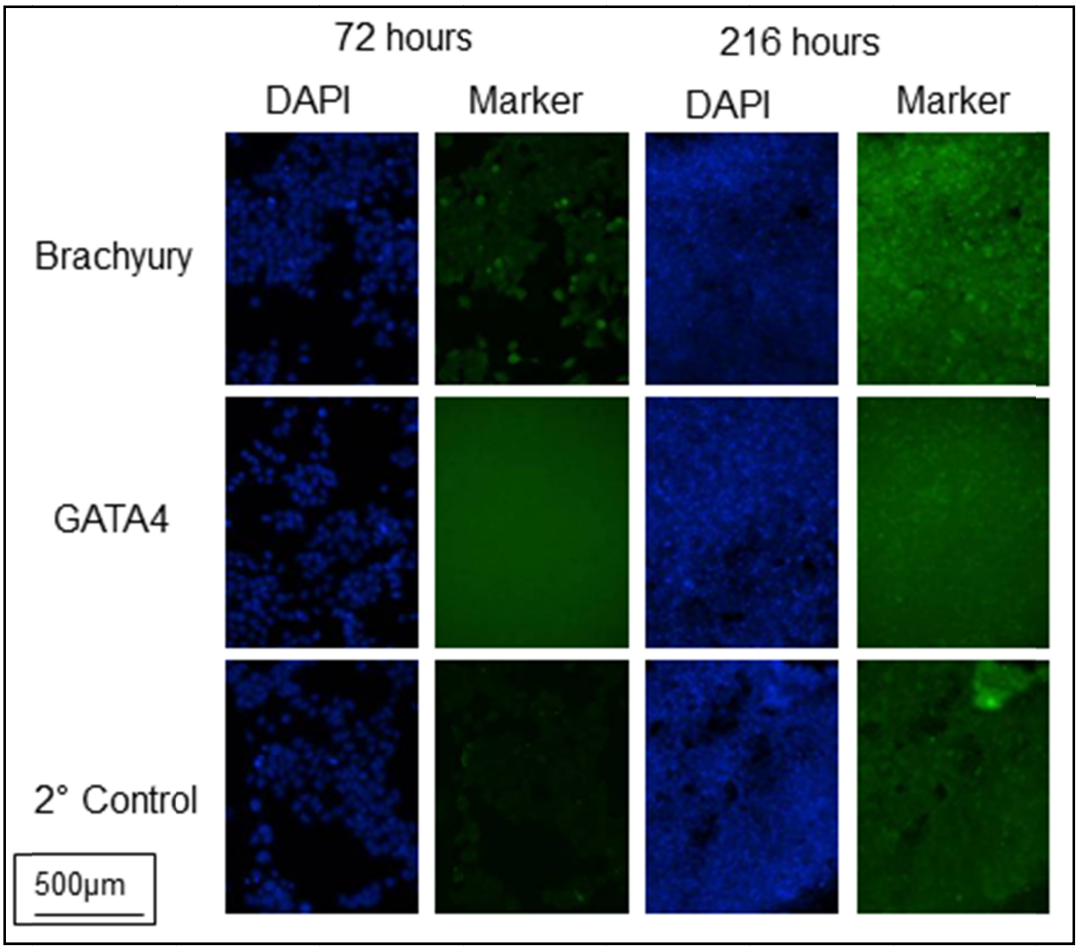


Figure 3.3.14B: Representative expression of key marker proteins from condition two (Monolayer throughout, Act-A+) for by fluorescence immunocytochemistry after 72 and 216 hours in culture. DAPI staining (of nuclei) in blue, FITC staining of the marker antibody in green.

Figure 3.3.15 shows the results from culture condition three (monolayer, then aggregation, then monolayer plus Act-A). There are three timepoints in this result set because the length of the culture period was longer than the other experimental conditions. The images of the secondary control sample at 48 hours showed high levels of background signal but this interference is not seen in the majority of the antibody stains. Background signal is low in the secondary control at 240 hours but is higher in the 336 hour (terminal) timepoint.

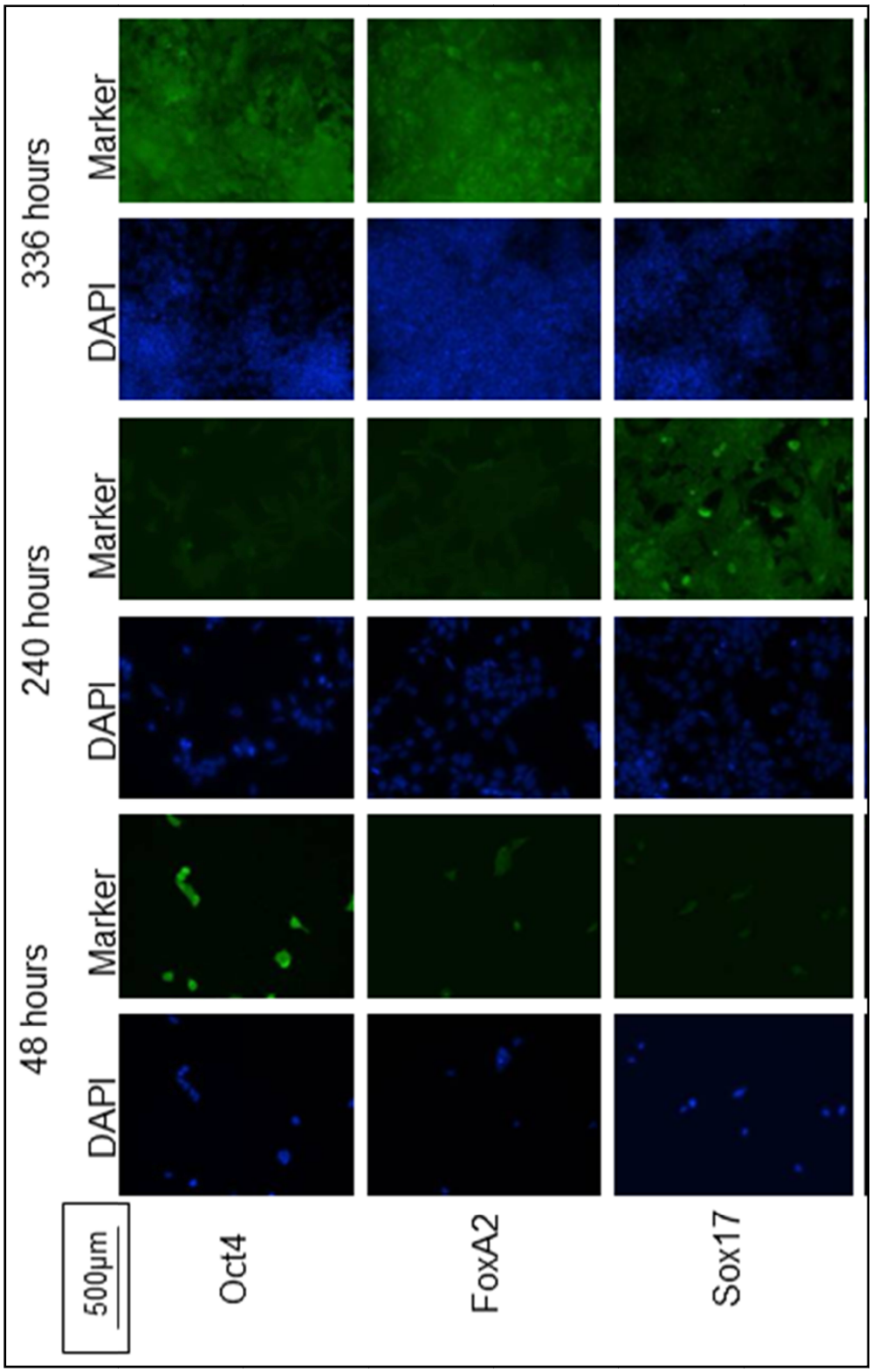


Figure 3.3.15A: Representative expression of key marker proteins from condition three (two days in monolayer then 3 days EB phase, monolayer thereafter, Act-A+) for expression of key marker proteins by fluorescence immunocytochemistry after 48, 240 and 336 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

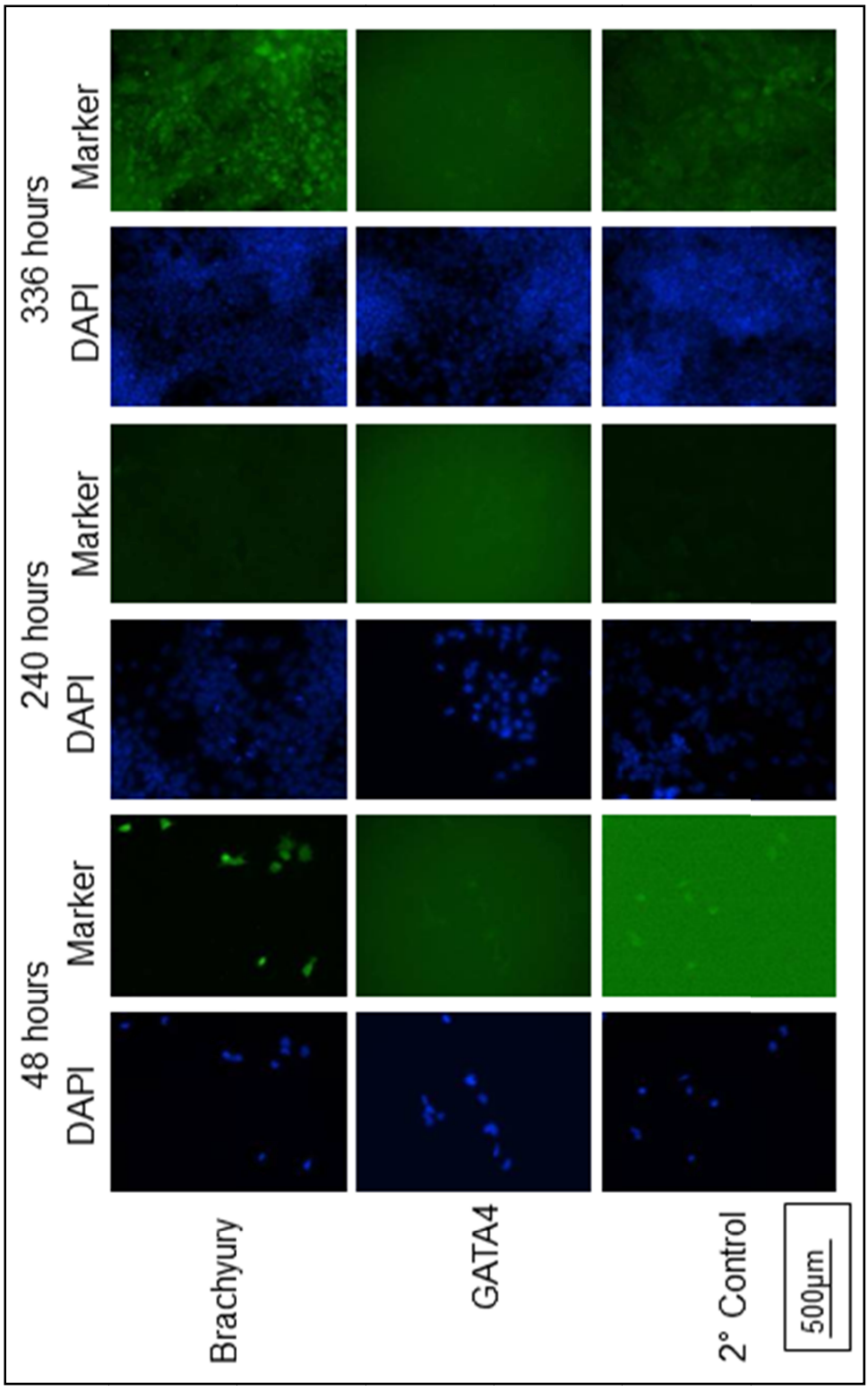


Figure 3.3.15B: Representative expression of key marker proteins from condition three (two days in monolayer then **3** days EB phase, monolayer thereafter, Act-A+) for expression of key marker proteins by fluorescence immunocytochemistry after 48, 240 and 336 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

OCT4 is clearly evident at 48 hours. There is weak signal for SOX17 and FOXA2 and moderate expression of BRACHYURY. In the GATA4 sample the level of background interference was high and the level of expression is unclear. At 240 hours OCT4 expression was present at low levels as was FOXA2. SOX17 was expressed at moderate levels but there is some background interference in the image.

There is no signal in the BRACHYURY sample and, as with the 48 hour sample, the level of background signal in the GATA4 sample made the level of actual expression unclear. In the 336 hour timepoint there is moderate signal in the OCT4 image but this may be background signal. The same is true of the FOXA2 image but there are some patches of stronger signal which may indicate genuine expression. There is weak expression of SOX17. The BRACHYURY image is similar to the FOXA2 and OCT4 with extensive signal that may be background with some patches of stronger signal that indicate real expression. As with the other timepoints the level of background signal in the GATA4 image made any genuine expression difficult to identify.

Figure 3.3.16 shows the results from culture condition four (monolayer, no Act-A). The secondary control sample for the 72 hour timepoint shows moderate levels of background signal whilst the control for the 216 hour timepoint shows quite high levels of background signal. There is widespread signal for OCT4 at 72 hours but much of this is autofluorescent interference. There are some localised patches of stronger signal that suggest some genuine expression. There is some background signal for FOXA2 and no signal for SOX17 and BRACHYURY. The background interference in the GATA4 image makes any real signal unclear. At 216 hours there is widespread expression of OCT4 and scattered but strong signal for FOXA2 and SOX17 expression. The signal for BRACHYURY is so strong that some must be caused by background interference. There was one isolated patch of GATA4 signal.

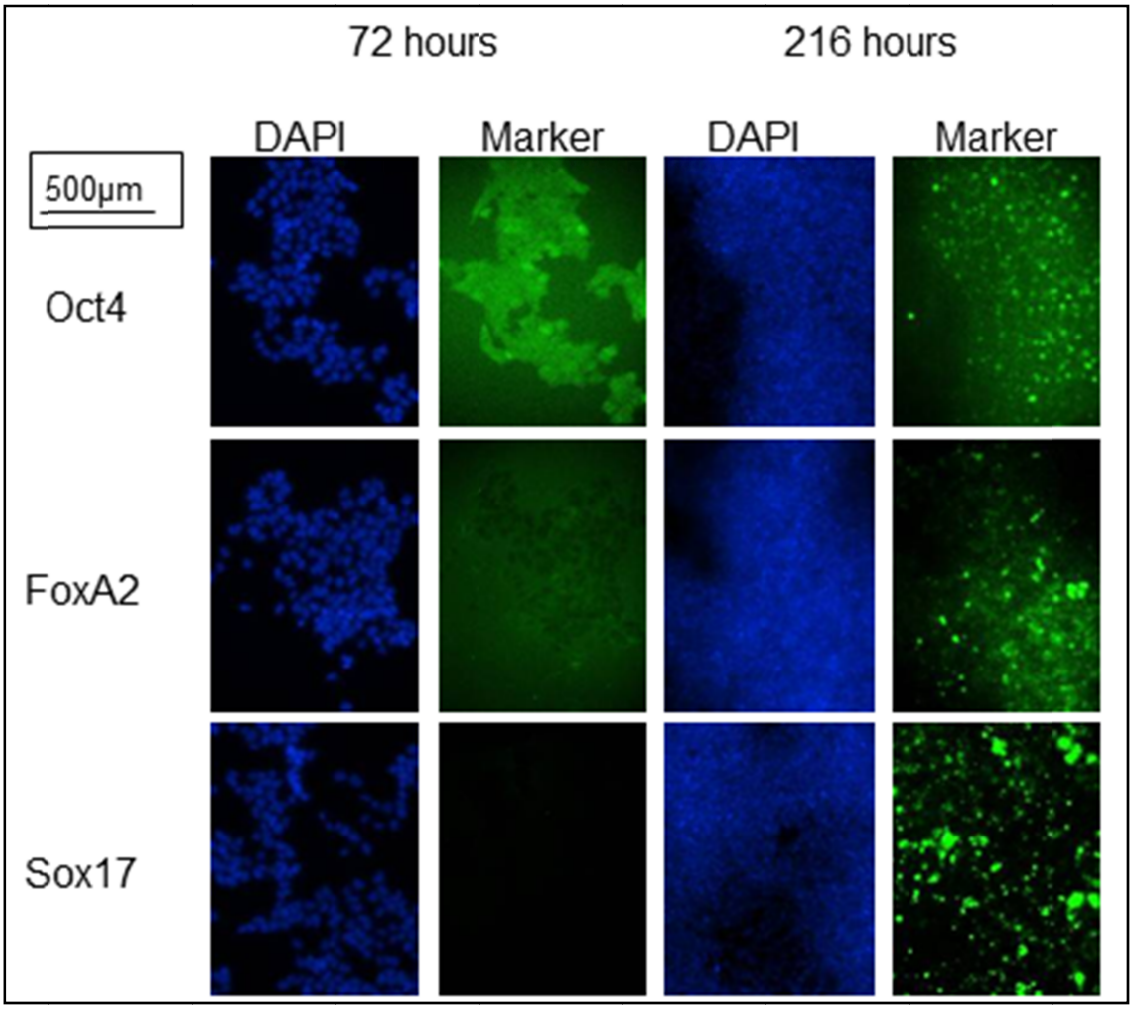


Figure 3.3.16A: Representative expression of key marker proteins from condition four (Monolayer throughout, Act-A-) for by fluorescence immunocytochemistry after 72 and 216 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

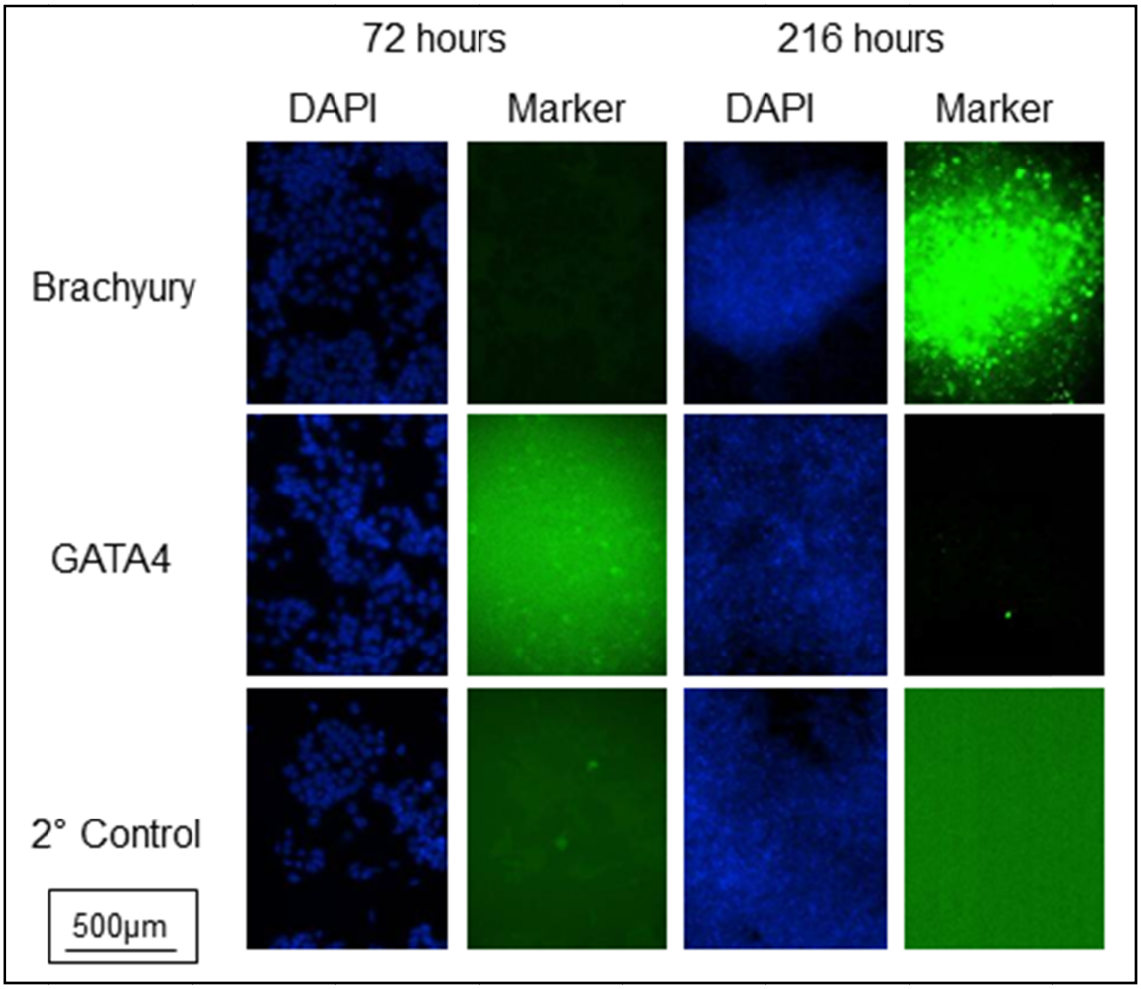


Figure 3.3.16B: Representative expression of key marker proteins from condition four (Monolayer throughout, Act-A-) for by fluorescence immunocytochemistry after 72 and 216 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

Figure 3.3.17 shows the results from culture condition five (aggregation, no Act-A). The secondary control sample for the 72 hour timepoint shows moderate background signal and cellular autofluorescence whilst the 288 hour control shows a high level of background signal. At 72 hours there is high cellular autofluorescence in the OCT4, FOXA2, SOX17 and BRACHYURY images making any genuine expression unclear. There are high levels of background signal in the GATA4 image. A similar pattern is present in the 288 hour timepoint images with some stronger patches of signal in the OCT4 and BRACHYURY images suggesting some genuine expression.

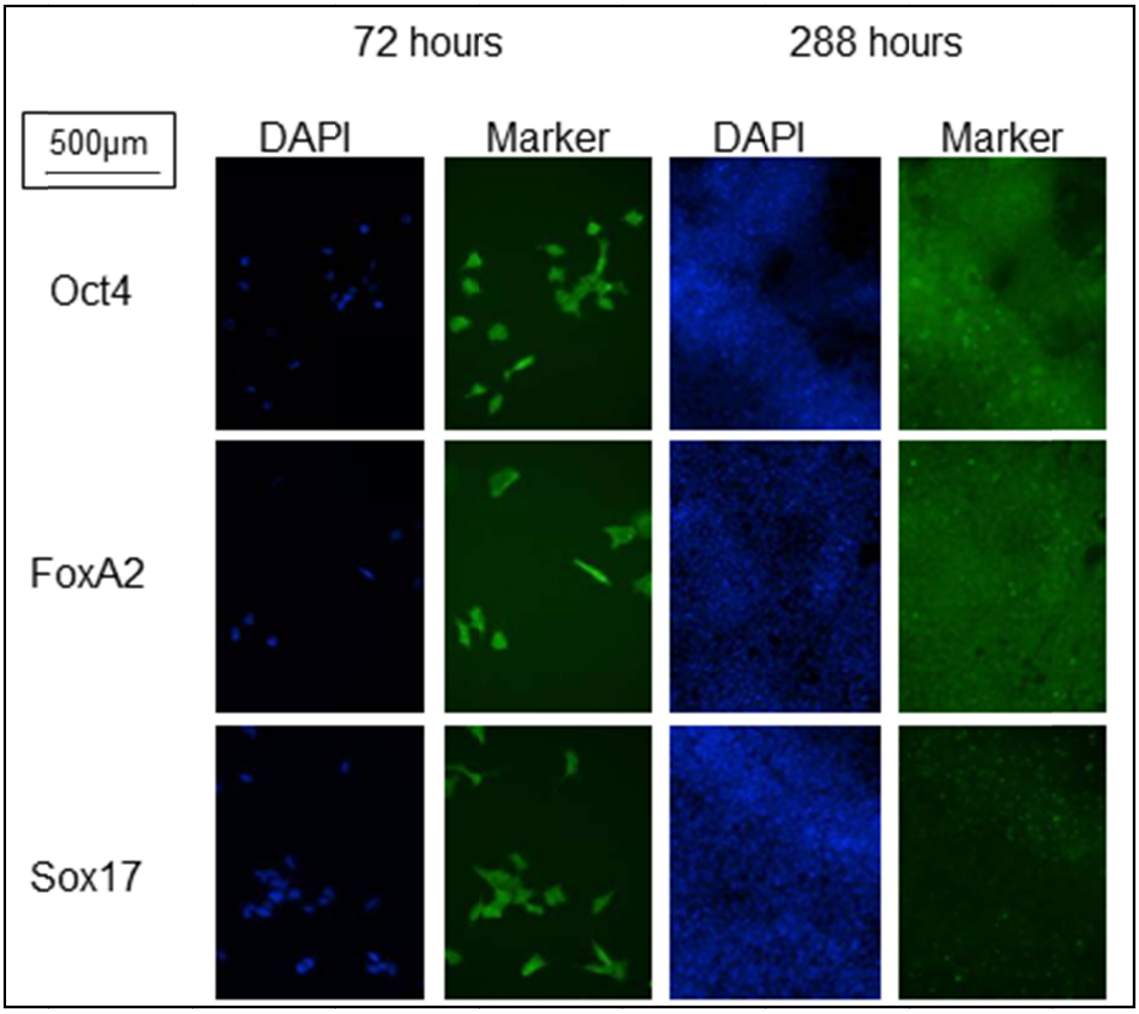


Figure 3.3.17A: Representative expression of key marker proteins from condition five (three day EB phase, monolayer thereafter, Act-A-) for by fluorescence immunocytochemistry after 72 and 288 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

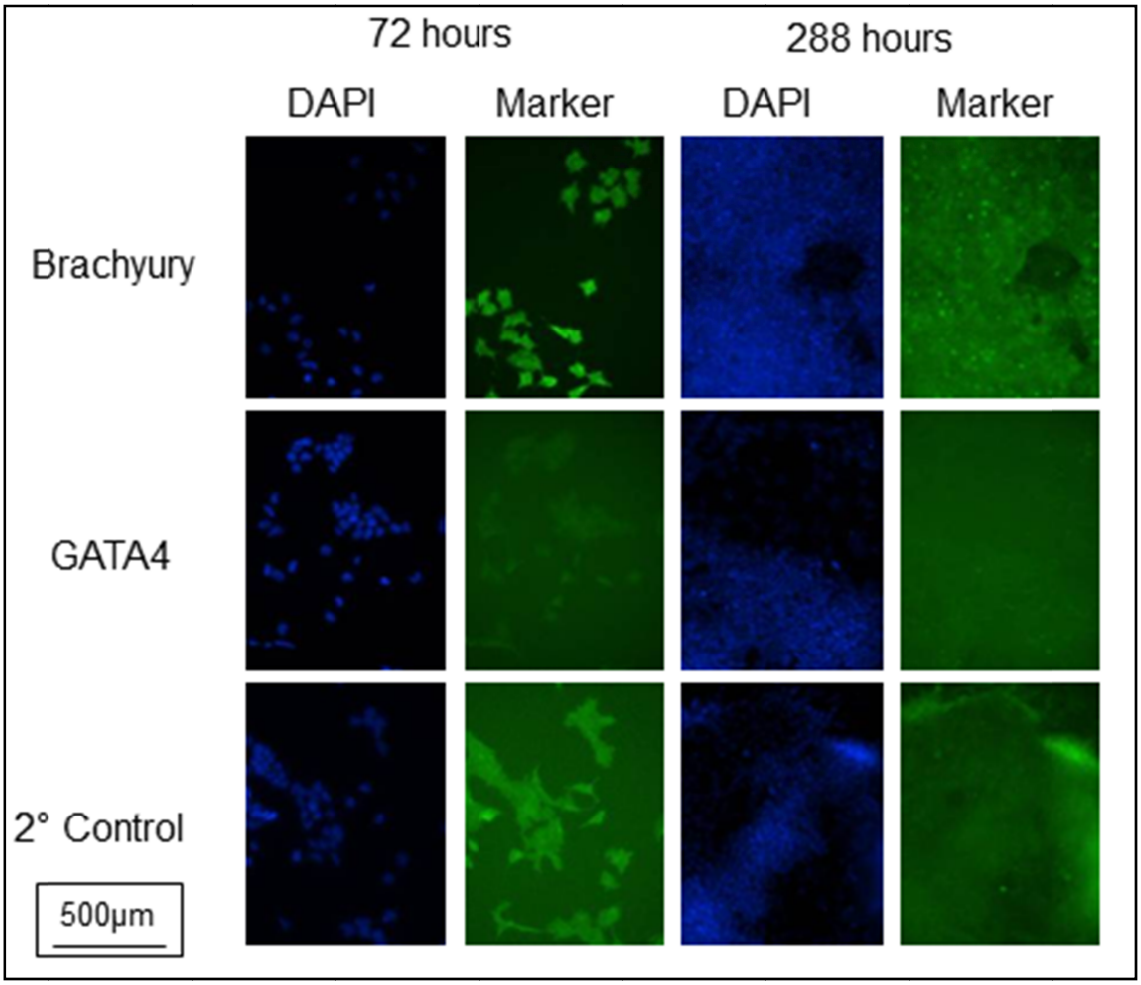


Figure 3.3.17B: Representative expression of key marker proteins from condition five (three day EB phase, monolayer thereafter, Act-A-) for by fluorescence immunocytochemistry after 72 and 288 hours in culture. DAPI staining (for nuclei) in blue, FITC staining of the marker antibody in green.

3.3.6 *In vitro* experiment six - Differentiation of mES cells using Act-A (part three)

CEE mES cells were cultured in a modified version of the most promising culture condition from experiment five (condition three – two days in monolayer, 3 days as EBs then a further nine days in monolayer, Act-A+). In this experiment the EB phase was extended from three to five days as the selected DE markers showed the strongest expression following the EB phase. Figure 3.3.18 shows the expression of key marker genes in RNA samples generated from *in vitro* experiment six evaluated by RT-PCR.

All of the experimental samples and controls gave strong bands for *GAPDH* indicating that all the RT reactions had worked well. *Oct4* expression is moderate in the 48 hour and 168 hour timepoints, being at a comparable level to the negative controls (unmanipulated CEE mES cells). In the 264 hour and 384 hour replicates expression is reduced but still present with the exception of one of the 264 hour replicates where expression is still moderate. This reduction in *Oct4* levels is not seen in the other conditions. There are weak bands of *CXCR4* expression after 168 hours (post-aggregation) and very weak bands after 384 hours but no expression in the controls. There are very weak bands of expression of *FoxA2* in the mES controls but no expression of *Sox17*. There are weak bands of *FoxA2* expression in the 48 hour, 264 hour and 384 hour timepoints but they are comparable to the levels in the mES controls. In the 168 hour samples there is moderate expression of *FoxA2*. There are a few very weak bands of *Sox17* expression with the exception of the 168 hour samples where there are stronger bands.

There are very weak bands for *Brachyury* in the mES controls. There are very weak bands in the 264 hour samples of similar strength to the controls. There are slightly stronger bands in the 48 hour and 384 hour timepoints. There is moderate expression of *Brachyury* in the 168 hour timepoint.

The levels of the markers of DE (*CXCR4* and co-expression of *FoxA2* and *Sox17*) are generally higher than those seen in previous *in vitro* differentiation experiments.

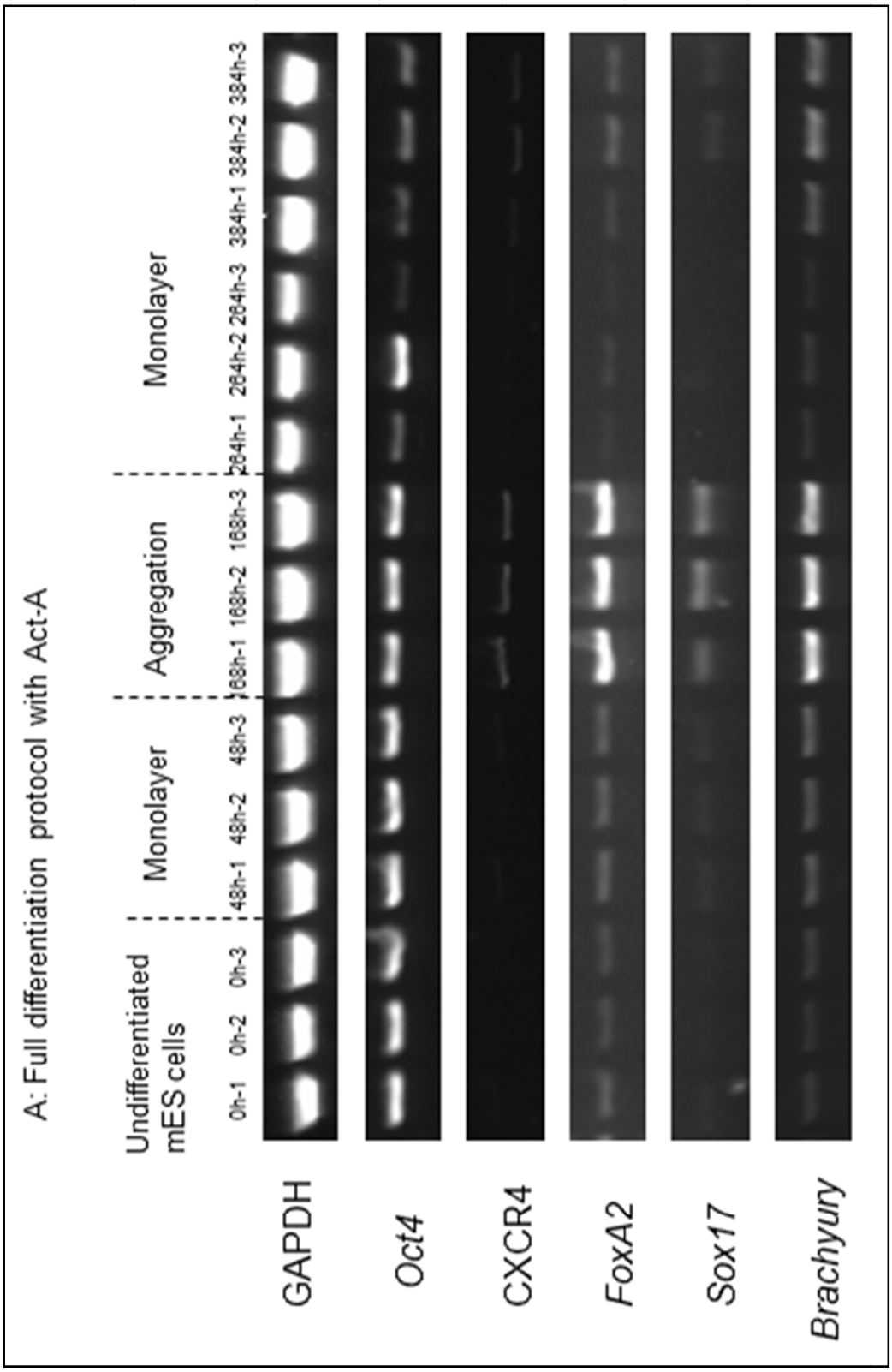


Figure 3.3.18A: Expression of key marker genes by CEE mES cells differentiated by A - two days in monolayer, five days in aggregation then a further nine days in monolayer culture with Act-A present throughout evaluated by RT-PCR.

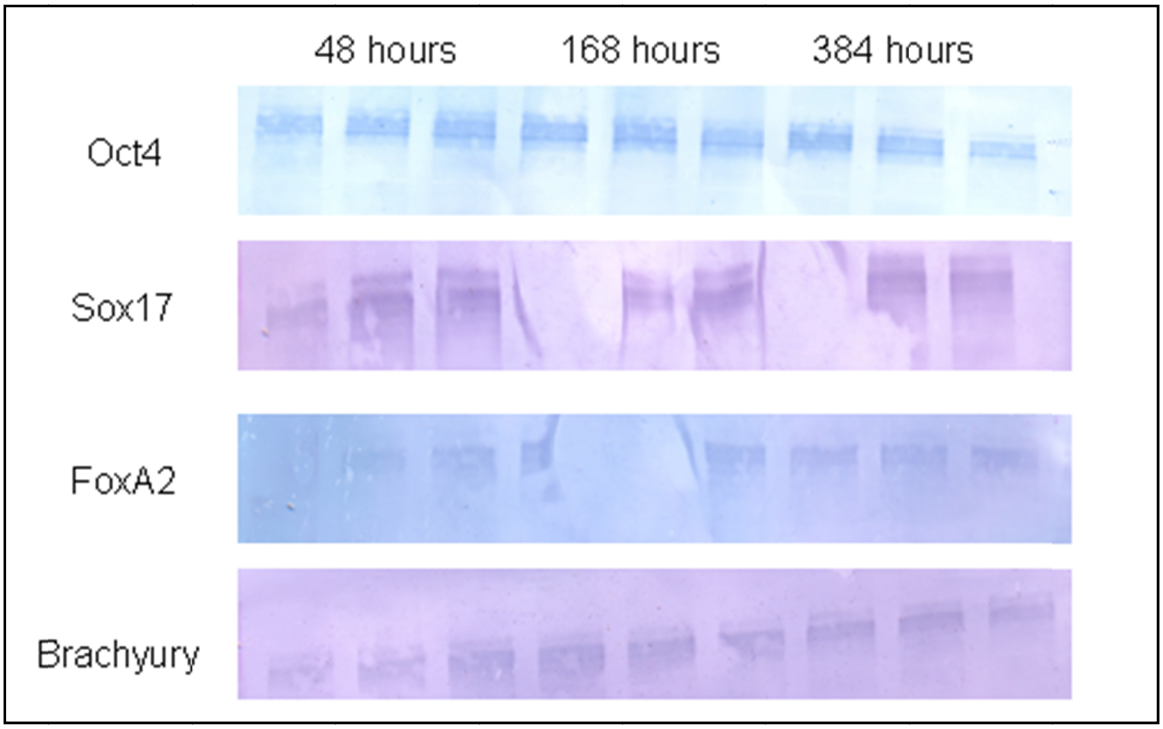


Figure 3.3.19: Expression of key marker proteins (OCT4 = 38.2 kDa, SOX17 = 44.6 kDa, FOXA2 = 48.3 kDa, BRACHYURY = 47.4 kDa) by mES cells following two days culture in monolayer, five days in aggregation and a further nine days in monolayer culture with Act-A present throughout evaluated by western blotting.

Figure 3.2.19 shows the results of the western blot using antibodies against certain key markers carried out on the protein samples generated from the treatment of mES cells with Act-A. The markers used were FOXA2 and SOX17 for DE, OCT4 for pluripotent cells and BRACHYURY for mesoderm. The three timepoints were taken after the initial two days in monolayer culture, after the five day EB phase and at the terminal timepoint of the culture period. Before the samples were tested using the differentiation markers they were blotted against the ubiquitously expressed protein α-Actin as a control (data not shown). As with the RT-PCR analysis OCT4 expression is maintained throughout the course of the experiment. BRACHYURY is also weakly expressed throughout at the protein level as it was at the RNA level. Both of the DE markers show expression but SOX17 gives stronger bands than FOXA2. Not every replicate was positive for expression of SOX17 with one replicate at 168 hours and

384 hours being negative. At 168 hours only one of the replicates shows clear FOXA2 expression.

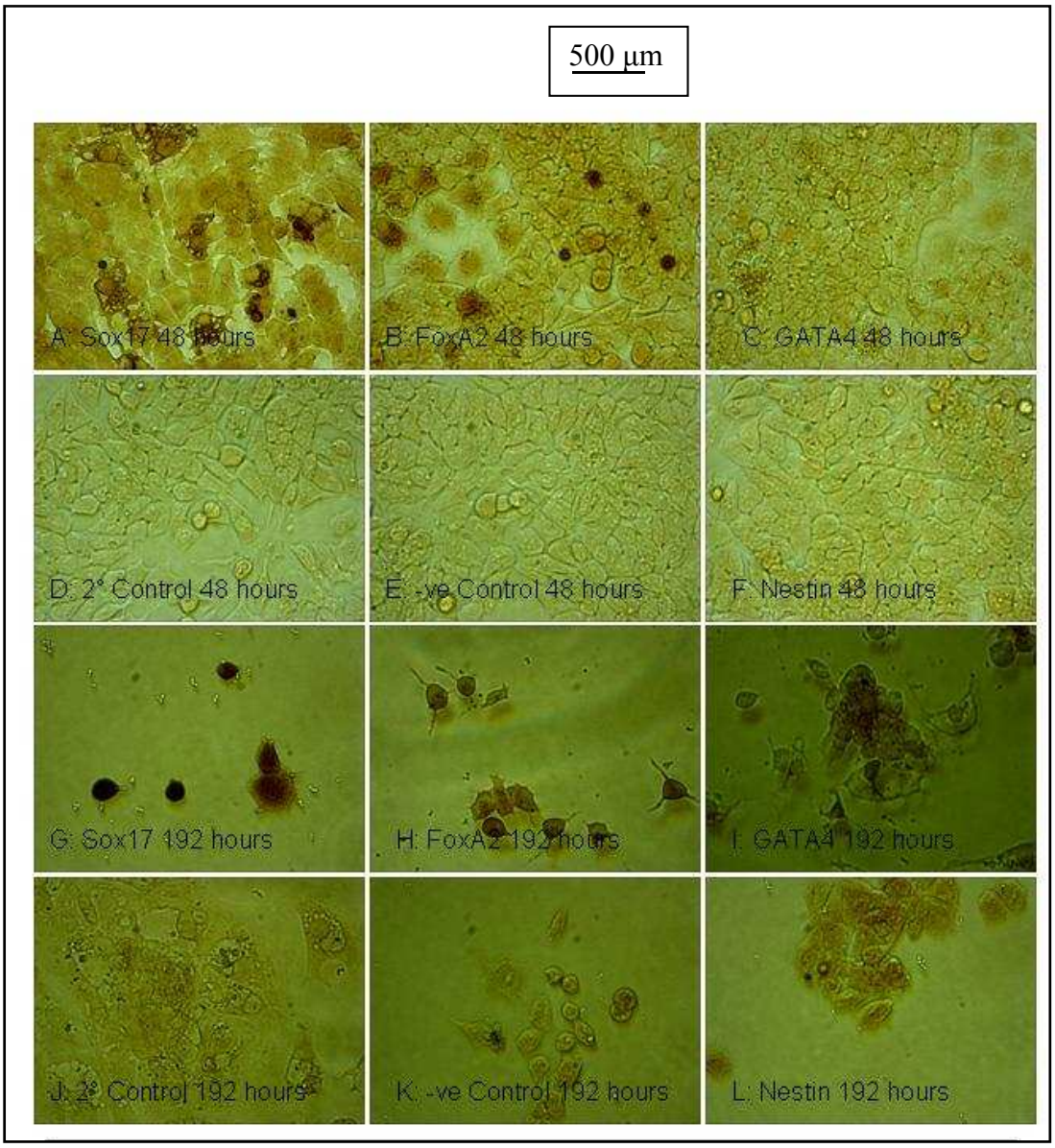


Figure 3.3.20: Expression of key markers in mES cells cultured in monolayer for two days then aggregated for five days then in monolayer for a further nine days evaluated by immunocytochemistry (enzymatic activity). All images at 400X magnification

Figure 3.3.20 shows the expression of key differentiation markers evaluated by enzymatic immunocytochemistry. The two timepoints are following the initial two days in monolayer culture and 24 hours after the cells had been reseeded in monolayer

following the five day EB phase. Secondary antibody and negative control images are included for each timepoint along with the DE markers SOX17, FOXA2 and GATA4 and the ectodermal marker NESTIN. There is no staining in any of the control conditions at either timepoint. After 48 hours some expression of SOX17 and FOXA2 is present – indicated by several areas of positive (brown) staining. There is no expression of GATA4 or NESTIN. After 192 hours there is continued positive staining for SOX17 and FOXA2 and positive expression of GATA4. There is still no evidence of NESTIN expression.

Table 3.3.1: Table summarising all the expression data for the selected differentiation markers from in vitro experiments four, five, six and controls. The selected markers were Oct4 and Nanog for undifferentiated cells, Sox17, FoxA2, CXCR4 and GATA4 for (definitive) Endoderm, Brachyury for Mesoderm and Nestin for Ectoderm. Expression of RNA expression was evaluated by RT-PCR whilst expression of protein expression was evaluated by (fluorescence or enzymatic) immunocytochemistry and Western blotting.

			Experiment					
			3.3.4	*In vitro*	experiment	four		
		Conditions	1 - Monolayer	10% FCS	2 - Monolayer	10% FCS	3 - EB	10% FCS
		Timepoints	LIF+				Act-A+	
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Positive	Strong	Weak	Weak	Weak	Positive
FoxA2	RNA	RT-PCR	Negative	Very weak	Negative	Negative	Negative	Positive
Sox17	RNA	RT-PCR	Negative	Very weak	Negative	Negative	Negative	Very weak
CXCR4	RNA	RT-PCR	Negative	Very weak	Negative	Negative	Negative	Very weak

			Experiment					
			3.3.4	*In vitro*	experiment	four		
		Conditions	4 - EB	10% SR	5 - EB	10% FCS	6 - EB	10% SR
		Timepoints	Act-A+					
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Negative	Negative	Weak	Moderate	N/A	N/A
FoxA2	RNA	RT-PCR	Negative	Negative	Negative	Positive	N/A	N/A
Sox17	RNA	RT-PCR	Negative	Negative	Negative	Very weak	N/A	N/A
CXCR4	RNA	RT-PCR	N/A	N/A	N/A	N/A	N/A	N/A

			Experiment					
			3.3.4	*In vitro*	experiment	four		
		Conditions	7 - Monolayer	10% FCS	8 - Monolayer	10% SR	9 - Monolayer	10% SR
		Timepoints	Act-A+				Act-A+	
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Strong	Strong	Positive	Negative	Positive	Negative
FoxA2	RNA	RT-PCR	Weak	Moderate	Negative	Negative	Negative	Negative
Sox17	RNA	RT-PCR	Very weak	Very weak	Negative	Negative	Negative	Negative
CXCR4	RNA	RT-PCR	Negative	Very weak	N/A	N/A	N/A	N/A

			Experiment					
			3.3.5	*In vitro*	experiment	five	part A	
		Conditions	1 - EB	10% FCS	2 - Monolayer	10% FCS	3 - Monolayer	EB
		Timepoints	Act-A+		Act-A+		10% FCS	Act-A+
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Strong	Strong	Strong	Moderate	Strong	Strong
	Protein	Immunocytochemistry	Negative	Positive	Weak	Weak	Positive	Weak
FoxA2	RNA	RT-PCR	Moderate	Weak	Very weak	Positive	Weak	Weak
	Protein	Immunocytochemistry	Positive	Weak	Positive	Weak	Weak	Weak
Sox17	RNA	RT-PCR	Positive	Negative	Very weak	Very weak	Negative	Very weak
	Protein	Immunocytochemistry	Positive	Weak	Positive	Positive	Weak	Weak
CXCR4	RNA	RT-PCR	Very weak	Weak	Negative	Strong	Negative	Weak
Brachyury	RNA	RT-PCR	Moderate	Positive	Positive	Strong	Negative	Moderate
	Protein	Immunocytochemistry	Weak	Weak	Weak	Positive	Weak	Positive
GATA4	Protein	Immunocytochemistry	Weak	Weak	Negative	Weak	Negative	Weak

			Experiment			
			3.3.5 *In vitro*	experiment	five	part A
		Conditions	4 - Monolayer	10% FCS	5 - EB	10% FCS
		Timepoints				
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Strong	Strong	Strong	Strong
	Protein	Immunocytochemistry	Positive	Positive	Positive	Weak
FoxA2	RNA	RT-PCR	Negative	Positive	Moderate	Weak
	Protein	Immunocytochemistry	Weak	Positive	Positive	Weak
Sox17	RNA	RT-PCR	Negative	Negative	Moderate	Very weak
	Protein	Immunocytochemistry	Negative	Positive	Positive	Weak
CXCR4	RNA	RT-PCR	Negative	Very weak	Very weak	Very weak
Brachyury	RNA	RT-PCR	Positive	Moderate	Strong	Moderate
	Protein	Immunocytochemistry	Negative	Positive	Positive	Positive
GATA4	Protein	Immunocytochemistry	Weak	Weak	Weak	Weak

			Experiment					
			3.3.5	*In vitro*	experiment	five	part B	
		Conditions	1 - EB	10% FCS	2 - Monolayer	10% FCS	3 - Monolayer	EB
		Timepoints	Act-A+		Act-A+		10% FCS	Act-A+
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Strong	Strong	Strong	Strong	Strong	Strong
FoxA2	RNA	RT-PCR	Strong	Strong	Moderate	Strong	Positive	Strong
Sox17	RNA	RT-PCR	Very weak	Very weak	Negative	Negative	Positive	Strong
CXCR4	RNA	RT-PCR	Very weak	Positive	Very weak	Very weak	Negative	Positive
Brachyury	RNA	RT-PCR	Negative	Positive	Negative	Very weak	Negative	Positive
Nestin	RNA	RT-PCR	Weak	Weak	Very weak	Very weak	Negative	Positive
Nanog	RNA	RT-PCR	Moderate	Strong	Strong	Strong	Moderate	Strong
GATA4	RNA	RT-PCR	Weak	Weak	Very weak	Very weak	Very weak	Weak

			Experiment			
			3.3.5 *In vitro*	experiment	five	part B
		Conditions	4 - Monolayer	10% FCS	5 - EB	10% FCS
		Timepoints				
Marker	**Molecule**	**Method**	Initial	Terminal	Initial	Terminal
Oct4	RNA	RT-PCR	Strong	Strong	Strong	Strong
FoxA2	RNA	RT-PCR	Moderate	Strong	Positive	Strong
Sox17	RNA	RT-PCR	Negative	Very weak	Very weak	Very weak
CXCR4	RNA	RT-PCR	Very weak	Very weak	Weak	Weak
Brachyury	RNA	RT-PCR	Negative	Weak	Weak	Positive
Nestin	RNA	RT-PCR	Very weak	Very weak	Weak	Very weak
Nanog	RNA	RT-PCR	Strong	Strong	Strong	Strong
GATA4	RNA	RT-PCR	Very weak	Very weak	Weak	Very weak

			Experiment			
			3.3.6 *In vitro*	experiment	six	Controls
		Conditions	Monolayer	EB		CEE mES
		Timepoints	10% FCS	Act-A+		cells
Marker	**Molecule**	**Method**	Initial	Post EB	Terminal	
Oct4	RNA	RT-PCR	Moderate	Moderate	Positive	Strong
	Protein	Immunocytochemistry	N/A	N/A	N/A	Positive
	Protein	Western Blotting	Positive	Positive	Positive	Positive
FoxA2	RNA	RT-PCR	Weak	Moderate	Weak	Very weak
	Protein	Immunocytochemistry	Weak	Positive	N/A	Negative
	Protein	Western Blotting	Weak	Weak	Weak	Negative
Sox17	RNA	RT-PCR	Negative	Positive	Very weak	Negative
	Protein	Immunocytochemistry	Weak	Positive	N/A	Negative
	Protein	Western Blotting	Positive	Positive	Positive	Negative
CXCR4	RNA	RT-PCR	Negative	Weak	Very weak	Negative
Brachyury	RNA	RT-PCR	Weak	Moderate	Positive	Very weak
	Protein	Immunocytochemistry	N/A	N/A	N/A	Negative
	Protein	Western Blotting	Weak	Weak	Weak	Negative
Nestin	RNA	RT-PCR	N/A	N/A	N/A	Negative
Nanog	RNA	RT-PCR	N/A	N/A	N/A	Strong
GATA4	RNA	RT-PCR	N/A	N/A	N/A	Negative
	Protein	Immunocytochemistry	Negative	Positive	N/A	Negative

Table 3.3.1 shows a summary of the expression of the selected markers from *in vitro* experiments four, five and six along with selected controls. Where the expression of a particular marker was not assessed it will read N/A. RNA levels are given as negative, where no expression was observed in any samples, then very weak where some very faint bands were seen, then weak where faint bands appeared in most samples. With these samples expression was equivocal to a degree. Values of positive, where bands were present in (almost) all samples, moderate, where stronger bands were seen and strong, were very bright bands were seen all indicate that the selected marker was definitely expressed. Protein levels are given as negative, weak or positive.

3.4 Discussion & Conclusions

The experiments carried out in this section were aimed at replicating the results reported from a number of published studies [D'Amour et al 2005, MacClean et al 2007, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005] where DE had been generated from both human and murine ES cells *in vitro.* This study aimed to do this for the first time using the CEE mES cell line available in our laboratory [Buttery et al 2001, Gothard et al 2010].

In the above studies low serum and SR culture conditions were used. In experiment one (Section 3.2.1i and 3.3.1) the attachment and proliferation of the CEE mES cell line in these conditions was investigated. There were some problems regarding the proliferation of the cells in some of the experimental conditions. Using CEE mES complete media (used for maintaining the mES cells in proliferation culture), that contained 10% FCS, cells attached to the plates and then expanded suggesting the cells could proliferate in the absence of a feeder layer. In the differentiation media(s) where the serum concentration was lower (≤ 2%) this did not occur. The cells could be cultured in a feeder free environment for a sufficient time period intended for the differentiation experiments so it was not the absence of a feeder layer or the ECM components it provides that caused the cell attachment problems.

The lack of proliferation, due to cell death, in the differentiation conditions indicated that the mES cells would not grow in serum free/low serum conditions (it is known that other researches within the Tissue Engineering group at Nottingham University have encountered similar results when conducting similar experiments) and as a consequence it was not possible to evaluate the extent to which the cells had differentiated. It was decided to try using SR media in future and to reduce the seeding density of mES cells (to avoid them reaching confluency prior to the end of the experiment).

Following the issues with cell growth in low serum media observed in experiment one culture conditions using SR supplemented media were investigated in experiment two

(Section 3.2.1ii and 3.3.2). The two control conditions (CEE mES complete media, LIF+/-) that contained 10 % serum (FCS) showed greater proliferation in cell number over the course of the experiment than the experimental conditions, where 10% SR was used. This was largely due to very low cell attachment following cell seeding in the differentiation conditions. There was also some loss of cells throughout the experiment and the combination of these two factors left very few surviving cells in the differentiation conditions. The seeding density may have been reduced too much from the previous experiment but the major problem seemed to be in the initial attachment of the mES cells to the culture surface.

It was concluded that in further experiments the initial conditions when the mES cells are transferred to the culture plates must favour their adherence. The differentiation media would then be introduced once the cells had adhered. The key conclusions from these experiments were that:

- CEE mES cells will not readily attach and proliferate in the absence of serum.

- It is crucial to ensure that the CEE mES cells to adhere to the culture plate at the start of the differentiation experiments.

- CEE mES cells will proliferate in feeder free culture on gelatine or collagen IV coated plates for at least six days.

- Plates should be seeded with cells in maintenance media (containing serum) to allow initial adherence before culturing in low serum conditions (differentiation media).

These conclusions were investigated further in a series of optimisation experiments, termed experiment three, (Section 3.2.1iii and 3.3.3) before any future differentiation experiments were conducted.

Culture vessel treatment: mES cells were seeded on either gelatin or Collagen IV coated TCP in 10% (v/v) FCS containing media to see if using a different matrix component to aid cell attachment made any difference to the poor cell attachment

observed in earlier experiments. Gelatin is a mix of a number of irreversibly hydrolysed Collagens and is therefore a non-specific aid for cell attachment whilst Collagen IV consists of six polypeptide chains and is a specific (unaltered) ECM component that is found exclusively in basement membranes where it plays an important role in cell adhesion [Khoshnoodi et al 2008]. No significant differences were observed between the mES cells seeded on gelatin coated TCP or those seeded on collagen IV coated TCP. This suggested that the poor attachment and proliferation observed in the initial experiments above was not due to the cell culture vessel and its (gelatin) coating.

Media composition: The attachment and rate of proliferation of mES cells cultured (feeder free) in 10% FCS was compared to that in 10% SR media on gelatin coated TCP. No significant differences were observed between the rate of proliferation and total cell number when the mES cells were cultured in either 10% (*v/v*) FCS or 10% (*v/v*) SR (a chemically defined alternative to serum that contains heated treated serum components required for cell maintenance but does not contain any bioactive proteins such as growth factors and hormones, etc). Having shown that the mES cells would not attach and proliferate in low serum conditions 10% (*v/v*) SR was used in some of the culture conditions in further experiments as an alternative to both high and low serum conditions.

Seeding density: mES cells were seeded in CEE mES complete media (10% FCS, LIF-) at various cell densities between 1 x 10^4 and 2.5 x 10^5 cells/ml on gelatin coated TCP. The initial (total) number of cells that attached increased with increasing seeding density. The optimum seeding density for these experiments was between 5 x 10^4 and 1 x 10^5 cells/ml based on an experiment of between nine – 16 days duration. The actual seeding density chosen for future experiments was decided depending on the planned duration of the experiment.

Media composition: The proliferation of mES cells was investigated in a range of different media formulations including low (1%) and normal (10%) FCS levels, serum and SR, and DMEM and α-MEM. The CEE mES cells proliferated at different rates in the different media compositions tested although growth was observed in all conditions with the exception of the low serum conditions. Greater cell numbers were

observed when cultured in 10% (*v/v*) FCS (CEE mES complete media, LIF+/-) after 72 hours compared with the 10% (*w/v*) SR media and the α-MEM media. α-MEM was used as it contains the antioxidant ascorbic acid (Vitamin C) which is thought to aid attachment by limiting the peroxidation of a range of lipid membrane components including α-Tocopherol (Vitamin E) facilitating greater interactions between cell surface molecules and the tissue culture vessel surface [Smith et al 2002].

However, the number of cells attaching was comparable in all conditions. Cell numbers in all conditions were sufficient for the generation of RNA samples and preparation of samples for immunocytochemical analysis. These optimisation experiments showed that the CEE mES cell line would proliferate on gelatin coated TCP in a range of media formulations but that if low serum conditions were used the cells would not proliferate. Gelatin coated TCP along with DMEM based media formulations containing 10% (v/v) FCS or SR were taken forward for use in future experiments.

In experiment four (Section 3.2.1iv and 3.3.4) CEE mES cells were seeded in nine different conditions designed to investigate the effects of aggregation into EBs, treatment with the growth factor Act-A and serum levels on the specification of DE. The levels of key marker expression in the RT-PCR analysis show some marked differences between the different culture conditions. Although cell attachment and proliferation occurred in all the conditions the quality of RNA samples generated varied considerably which may have been an indicator that some conditions more favourable for cell growth and differentiation than others. The cells seemed to show the sensitivity to low serum levels that had been previously observed in experiments one and two in the culture conditions where SR was used but this conflicted with what has been reported in the literature where mES cells (although not CEE mES cells) had been successfully differentiated in media containing SR [Kubo et al, 2004].

The RT-PCR analysis showed upregulation of DE markers when Act-A was added to the culture media. The co-expression of *FoxA2* and *Sox17* along with the expression of *CXCR4* indicates that DE formation was favoured in these conditions compared to conditions where Act-A was not present. This corresponds with the upregulation of DE markers seen following Act-A treatment of mES cells in the literature [Kubo et al

2004, Tada et al 2005, Yasunaga et al 2005]. As detailed in Sections 1.6 and 3.1.1 Act-A is a member of the TGF-β superfamily of growth factors. It triggers a signal pathway that prompts SMADs 3 and 4 to enter the nucleus where they join transcriptional complexes. These complexes then mediate the expression of various genes including those responsible for DE specification during development [Abe et al 1996].

Oct4 was expressed throughout the time course of the experiment suggesting that either an undifferentiated population of cells remained or that the differentiating cells retained some undifferentiated characteristics [Nichols et al 1998]. Some expression of Oct4 has been reported in adult stem cell populations suggesting that Oct4 expression is retained as cells differentiate further [Lee et al 2010]. The cells that had been aggregated into EBs showed slightly elevated levels of differentiation markers expressed, in some instances, at earlier timepoints in comparison with those that had been cultured in monolayer.

Whilst none of the differentiation conditions in this experiment directed the cells to differentiate towards the DE fate with great efficiency, the variations in marker expression observed suggest that the culture conditions investigated, particularly addition of Act-A and aggregation into EBs, did influence the differentiation of the mES cells. Further experiments incorporated extended culture times to increase the time that cells were exposed to the factors hypothesised to induce differentiation (see sections 3.2.1v). These conclusions agreed with those from the literature [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005] where the addition of growth factors and the subsequent activation of specific signalling pathways promoted the differentiation of mES cells towards the DE fate shown by the co-expression of *FoxA2* and *Sox17* and the expression of *CXCR4*. Some of the techniques employed in the literature involved selective stages based on positive expression of CXCR4 and E-Cadherin [Yasunaga et al 2005] or CXCR4 alone (using hES cells) [D'Amour et al 2005] to achieve a purer population of DE cells.

In experiment five (Section 3.2.1v and 3.3.5) CEE mES cells were seeded in five different conditions designed to investigate further the effects of aggregation into EBs and treatment with the growth factor Act-A on the specification of DE. Following the

poor outcome of low serum and SR conditions in the earlier experiments all conditions were supplemented with 10% (v/v) FCS. The proliferation of the cells was monitored by eye through the course of the experiment to ensure sufficient cells were present for the generation of samples for analysis (by RT-PCR and immunocytochemistry). The cells in all the culture conditions attached and then proliferated throughout the time course of the experiment.

The cells that had been aggregated (3 days as EBs) prior to monolayer culture began to express the selected DE markers, *FoxA2, Sox17* and *CXCR4,* but the fact that there was an apparent reduction in the level of expression later in the experiment (when in monolayer culture for 9 days) suggested that extending the aggregation time might have aided DE differentiation [Abe et al 1996]. In the literature aggregation/culture times of 2.5 days followed by up to 7.5 days in monolayer in the presence of Act-A induced expression of hepatic markers [Kubo et al, 2004]. In this study there was little difference between the Act-A treated aggregates and those with no Act-A. This could mean that the concentration of Act-A was insufficient to exert an effect or that the culture duration needed to be extended to increase the exposure time of cells to the growth factor or that the effectiveness of the growth factor is reduced in a 3D environment.

Act-A disperses by passive diffusion in culture [Gurdon et al, 1994] so may have been unable to penetrate to the core of larger aggregates. Greater differences were apparent between the cells treated with Act-A and those that had not been treated in the monolayer only conditions. This could mean that the differentiation initiated by aggregating the cells was not supported further with growth factor treatment. It also suggested that the differentiation effects of the growth factor treatment and of aggregation were not combinatorial. In condition three, where the cells went from monolayer to aggregation then back to monolayer culture, the DE markers were expressed at a fairly constant level until the terminal samples which showed a slight increase in expression. Again this suggested that an extension to the duration of the experiment might prove worthwhile.

The expression of *Brachyury* in a number of culture conditions suggested that some mesodermal differentiation was taking place alongside any differentiation towards

DE. The same observations have been reported in the literature [Kubo et al 2004, Tada et al 2005]. The two germ layers have been shown to arise from a common precursor known as the mesendoderm [Tada et al 2005] and are therefore closely related. The ectodermal marker *Nestin* was expressed at low levels in some of the conditions. There was no real pattern to this expression but the strongest bands were observed in conditions were Act-A was absent indicating that the ectodermal lineage was not favoured by Act-A treatment.

The immunocytochemical analysis provided few clear conclusions although expression of the selected DE markers was present in a number of the samples. In the later timepoints (216, 288 or 336 hours dependant on which culture condition) high background interference, shown in the secondary antibody controls, made analysis difficult. However in the earlier timepoints co-expression of FOXA2 and SOX17 was seen in conditions one and two, where Act-A was present, and to a lesser extent in condition five, where the cells had been aggregated without Act-A treatment. Act-A treatment and aggregation appeared to support the expression of the DE markers at the protein level. This corresponds with the results in the literature [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005].

OCT4 expression was observed in all conditions although it was strongest in condition four, where the cells were in monolayer culture and had no differentiation stimuli. The cells had retained the greatest pluripotent character in the culture condition where factors to induce differentiation were absent but some pluripotent marker expression remained in all conditions (as with the RT-PCR analysis). The mesodermal/mesenodermal marker BRACHYURY was also expressed at the protein level. Its expression was strongest in the conditions were Act-A was absent (conditions four and five). This conflicts with some observations in the literature that suggested that Act-A can induce upregulation of mesodermal markers [Kubo et al 2004, Tada et al 2005].

The immunocytochemical results and the RT-PCR analysis point towards the same general conclusions, namely that both Act-A treatment and aggregation favour DE specification when compared with the monolayer controls under 10% FCS conditions with the CEE mES cell line. However the differentiation is not limited exclusively to

DE lineages as mesodermal markers are also in evidence. As previously stated most of the studies in the literature involved a selection stage to purify the population of DE cells [Yasunaga et al 2005, D'Amour et al 2005, MacClean et al 2007] but such selection proved difficult in these experiments because of the lack of robustness of the cells when subjected to FACS.

In experiment six (Section 3.2.1vi and 3.3.6) a modified version of the most promising culture condition from experiment five (condition three – two days in monolayer, 3 days as EBs then a further nine days in monolayer with Act-a present throughout) was run (Section 3.2.1vi and 3.3.6). Extending the aggregation phase by 48 hours (2 days in monolayer then 5 days as EBs followed by a further 9 days in monolayer with Act-A present throughout) resulted in clear RT-PCR co-expression of all three selected DE markers, *FoxA2, Sox17* and *CXCR4*, at the 168 hour (post aggregation phase) timepoint.

As previously observed the marker expression then decreased when the cells were reseeded in monolayer (despite continued exposure to Act-A) before gradually increasing by the end of the culture period at the 384 hour timepoint. The same pattern was also seen for the mesodermal/mesendodermal marker *Brachyury*. Expression of the pluripotency marker *Oct4* was maintained at a consistent level through to the 168 hour timepoint after which it began to fall. *Oct4* was still expressed in the terminal 384 hour timepoint but at a noticeably reduced level.

Western blot analysis showed that the DE markers were being expressed at the protein level. However the resolution of the data produced meant it was difficult to say whether a marker was present at greater levels in one sample than another, only whether it was present or absent. The selected DE markers, FOXA2 and SOX17, were expressed but the cells also expressed OCT4, suggesting a retention of some pluripotent character or that some of the cell population had not differentiated, and BRACHYURY, indicating some mesodermal differentiation.

The enzymatic immunocytochemical analysis also showed the expression of FOXA2, SOX17 and GATA4 indicating that specification of DE was occurring. There was no

evidence of NESTIN expression suggesting that no ectodermal differentiation was taking place.

3.4.7 Summary

A number of the variables examined through the course of these experiments seem to induce cells to differentiate towards the DE fate. However the process appeared to be far from efficient. The sensitivity of the CEE mES cell line to serum levels even when SR was used proved an obstacle to fully investigating the range of culture conditions that favour DE specification. The ultimate conclusions from this chapter of work are:

Aggregating the cells into EBs promoted differentiation when compared to cells cultured in monolayer but did not specifically favour DE specification. In many cases when cells were reseeded in monolayer culture after an aggregation phase the strength of differentiation marker expression reduced. This phenomenon was still observed when the aggregation phase was extended from three to five days in duration.

Act-A treatment seems to favour the specification of DE when compared to control conditions but the induction effect was not comprehensive or particularly strong. DE markers were generally expressed at elevated levels after Act-A treatment but markers of other fates, particularly mesoderm (see below) were sometimes present [Kubo et al 2004, Tada et al 2005]. The pluripotent marker Oct4 [Nichols et al 1998] was also still expressed although occasionally at reduced levels.

Some differentiation towards mesoderm occurred in the conditions that favour DE specification. This was perhaps unsurprising as the two germ layers arise from a common precursor [Tada et al 2005]. There was little evidence of any ectodermal differentiation in the conditions that most favoured DE formation.

Whilst germ layer specification is an important stage in establishing cell lineages during development the cells are still some way from being fully differentiated into specific cell types and a level of maturation is still required. These *in vitro* techniques (specifically a five day EB phase in Act-A+, 10% (v/v) FCS media) will be used further in Chapter 4 to investigate the ability to enhance DE differentiation from the

CEE mES cell line with subsequent co-culture (following *in vitro* DE differentiation) of the CEE mES cells with early stage embryonic chick gut tissue with the ultimate aim of further differentiating the CEE mES cells towards the intestinal progenitor/ISC fate. Co-culture with specific embryonic tissue types has been shown to induce differentiation towards specific cell lineages [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005] with the target cell lineage dictating the choice of tissue e.g. to produce pulmonary epithelium mES cells were co-cultured with pulmonary mesenchymal tissue [Van Vranken et al 2005].

Chapter Four: *Ex vivo* co-culture

4.1 Introduction

4.1.1 Differentiating ES cells by co-culture with embryonic tissue ex vivo

As cell differentiation moves further down the developmental pathway the signals that control its differentiation become more complex both in terms of the number of factors involved as well as spatial and temporal levels of control [Chadwick and Marsh 1992, Kaufmann 2005, Tickle 2003]. As a consequence this pattern of signalling becomes increasingly difficult to replicate *in vitro* making it a challenge to expose cells to further differentiation stimuli. A number of studies have shown that co-culturing pluripotent cells with *ex vivo* early embryonic tissue explants or cells (from a variety of different species) can direct undifferentiated cells towards a particular fate as discussed below.

To further differentiate cells towards a particular tissue/cell population or to maintain a particular cell type a complex network of signalling interactions is usually involved. These can be hard to reproduce *in vitro* for a variety of reasons including the number of signalling factors involved to the spatial nature of the signals. As previously stated the intestinal progenitor niche is maintained in the base of the Crypts of Lieberkuhn by a complex range of signals (Section 1.2.1, Figures 1.5 and 1.6) that act in a spatially dependant manner. A number of signalling factors are involved that do not originate from the same source. The strengths of the different signals therefore vary depending on the physical location of where they overlap (dependant on the methods by which the signalling factors diffuse) and these variations can produce different responses in the receptor cells.

To prompt ES cells towards a particular developmental fate it stands to reason that they must be exposed to the signals from the location in the embryo where they arise and this can direct the choice of tissue for co-culture. This has been demonstrated with a number of target cell types. A number of the studies detailed below also included an initial *in vitro* differentiation step (involving growth factor treatments and/or aggregation into EBs) before the co-culture stage, usually targeting a specific germ layer. This mirrors the process that would occur in normal development as

differentiation into specific cell and tissue types would occur following the specification of the three germ layers (endoderm, mesoderm and ectoderm) at gastrulation [Kaufmann 2005, Chadwick and Marsh 1992].

Photoreceptor cells have been generated by co-culturing mES cells with embryonic chick retina [Sugie et al 2005]. In this study mES cells were initially aggregated into EBs for four days, by the hanging drop method, and treated with retinoic acid *in vitro* for a further four days to initiate differentiation towards the neural fate. The cells were then sorted by selection for markers of neural differentiation and the positive cells were co-cultured with (day six) embryonic chick retinal tissue for 10 days. At the end of the co-culture period the mES derived cells were assessed for the expression of Rhodopsin, a key functional protein found in photoreceptor cells, by immunohistochemistry with approximately one fifth staining positive. The expression of the photoreceptor specific transcription factor *crx* and the rod photoreceptor markers *IRBP* and *recoverin* was assessed by RT-PCR. These all showed increased expression in the co-cultured cells versus controls [Sugie et al 2005].

Culturing mES cells with cardiac mesoderm has been shown to promote differentiation towards the hepatic fate [Fair et al 2003]. Cardiac mesoderm would be found proximal to hepatic endoderm during embryonic development and it is therefore likely that the two areas share signalling interactions. GFP-labelled mES cells were co-cultured with embryonic chick cardiac mesoderm for up to four days. The cells were then analysed for the expression of markers of the hepatocyte fate. It was shown that the cells in co-culture were positive for the expression of the early endodermal/hepatic markers *Sox17α*, *GATA4* and *HNF3β* compared to control cells when assessed by RT-PCR after one day. After two and four days the hepatic markers α-fetoprotein and albumin also showed upregulated mRNA expression [Fair et al 2003].

Co-culturing mES cells with pulmonary mesenchyme was found to promote differentiation towards the pulmonary epithelial fate [Van Vranken et al 2005]. mES cells that had been aggregated into EBs were co-cultured with pulmonary mesenchymal tissue from day 11.5 or 13.5 embryonic mice for five or 12 days. The cells were then analysed for the expression of markers of pulmonary epithelium. The

co-cultured cells showed up-regulation of the epithelial markers *cytokeratin* and *TTF1*. Expression of Surfactant protein C, a marker of type II pneumocytes, was detected in some of the co-cultured cells. These markers were not expressed in the experimental controls [Van Vranken et al 2005].

Co-culturing mES cells with five day post-natal rat auditory hair cells or auditory neurons promoted differentiation into bipolar neurons following an initial *in vitro* treatment with RA to induce ectodermal/neural differentiation [Coleman et al 2007]. EBs and dissociated cells were co-cultured with auditory neurons for seven days and with auditory hair cells for three days. The most successful technique involved the co-culture of intact EBs with hair cell explants which gave the highest number of neuron-like cells (by morphological assessment) that expressed neurofilament protein when evaluated by immunolabelling [Coleman et al 2007].

Introduction into the Aorta-Gonad-Mesonephros (A-G-M) microenvironment induced haematopoietic activity in (labelled) mES cells [Krassowska et al 2006]. EBs that had been generated by the hanging droplet method were co-cultured with a number of stromal cell lines and samples were collected at defined timepoints. The haematopoietic activity of the sampled cells was assessed using the CFU-A and the HPP-CFC (colony forming) assays [Krassowska et al 2006].

Tissue derived cells also seem able to promote stem cell differentiation. Cynomolgus monkey ES (cES) cells cultured with (day 14) embryonic mouse liver derived cells differentiated into hepatocyte like cells [Saito et al 2006]. The expression of *AFP, albumin, α-1-antitrypsin* and *HNF-4α* were upregulated in the co-cultured cells compared to controls when evaluated by RT-PCR. The co-cultured cells also showed expression of Cytochrome P450 7A1, a putative marker for endoderm derived hepatocytes, whilst the controls did not. Immunocytochemical analysis showed an increased number of ALBUMIN positive cells in the co-cultured cells compared to controls. The co-cultured cells also showed some functional activities associated with hepatocytes, namely urea synthesis and glycogen storage [Saito et al 2006].

Some studies have used intact embryos from a variety of species rather than specific tissue explants or cells to induce the differentiation of SCs towards a target cell fate.

Culturing hMSCs within whole rodent or murine embryos has been shown to be able to initiate differentiation towards kidney cell types [Yokoo et al 2005]. The embryos were excised at embryonic day 9.5 for the mice and embryonic day 11.5 for the rats. Bone derived hMSCs were injected into the embryos which were then cultured *ex vivo* for four days.

At this point the metenephroi (the third stage of kidney development) were dissected and cultured *in vitro* for a further six days. The hMSCs were then extracted and analysed for the expression of a number of markers of early kidney development including *aquaporin-1*, *parathyroid hormone receptor 1* and *glomerular epithelial protein 1* by RT-PCR. Based on increased levels of marker expression compared to controls the hMSCs cultured with rodent embryos appeared to take the initial differentiation steps towards the kidney fate during the whole embryo culture with further differentiation occurring during the organ culture phase [Yokoo et al 2005].

ES also differentiated according to the microenvironment in which they were transplanted in vivo [Kudo et al 2007]. mES cells seeded into the intestinal environment following radiation damage contributed to the repair and repopulation of the intestinal epithelium (see Section 1.4) [Kudo et al 2007].

The chicken is a model organism for genetic and developmental studies and therefore much is known about its molecular biology and development including a fully sequenced genome. These closely resemble mammalian processes so it is hypothesised that inducing signals in the chick will have the desired effect on the murine (and human) cells [Bellairs and Osmond 2005, Chadwick and Marsh 1992]. Embryonic chick tissue was used in conjunction with mES in a number of the studies discussed above [Sugie et al 2005, Fair et al 2003]. However, there are sufficient differences to allow for molecular analysis of the murine cells following co-culture without any chick nucleic acid or protein giving false positive results (i.e. it is possible to design species specific RT-PCR primers and species specific antibodies are available).

4.1.2 Transfection of CEE mES cells with a GFP expressing plasmid

To allow for easier identification of the CEE mES cells when in co-culture with the chick tissue explants the cells were transfected with a GFP (Figure 4.1.1) expressing plasmid (VS01 plasmid – Figure 4.1.2). This enabled the cells to be visualised whilst in co-culture and permitted easier separation of the cells, by methods such as FACS, at the end of the co-culture period. As its name suggests GFP fluoresces green (emission wavelength 509 nm) when exposed to the correct excitation stimuli (blue light at wavelength 395 nm) [Inouye and Tsuji 1994]. GFP is a naturally occurring protein consisting of 238 amino acids (Mw 26.9 kDa) originally found in the jellyfish *Aequorea victoria* in the 1960's [Prendergast and Mann 1978, Tsien 1998, Shimomura et al 1962]. Another version of GFP is found in the Sea Pansy (*Renilla reniformis*) that has a different excitation peak (wavelength 498 nm) [Tsien 1998].

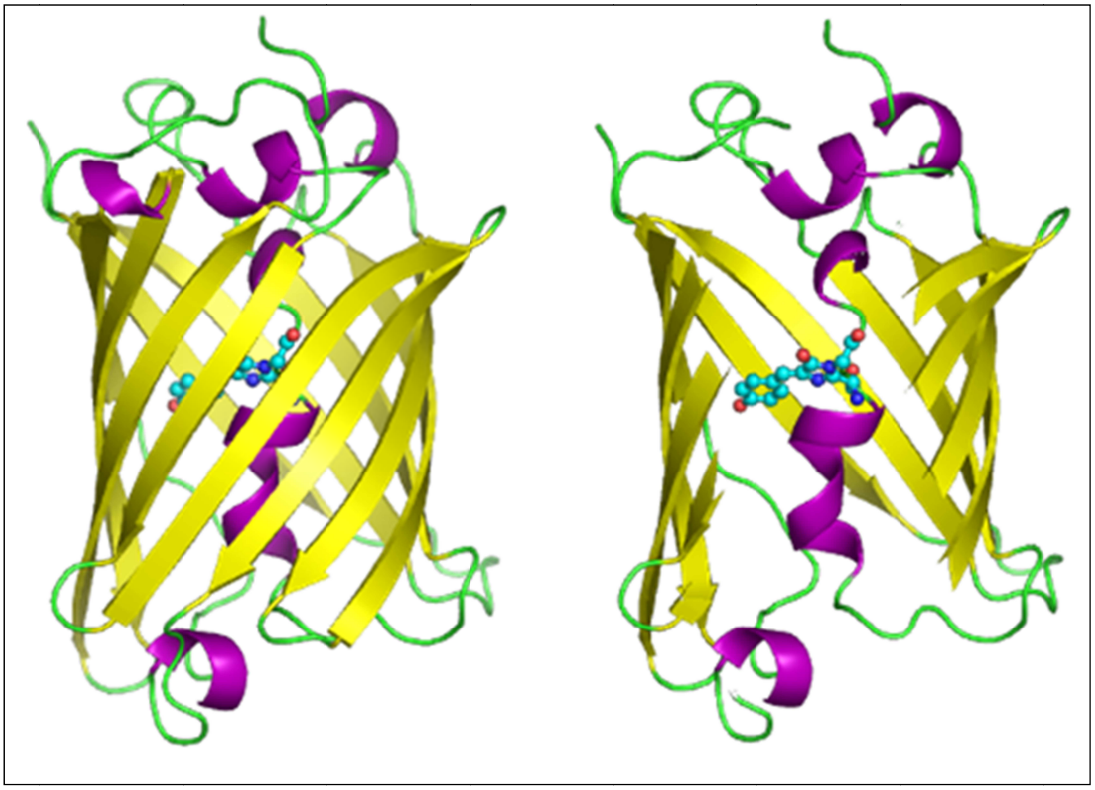


Figure 4.1.1: Structure of GFP shown fully (left) and with the side of the β barrel cut away to reveal the chromophore (right) [Tsien 1998].

Most GFP variants used in the laboratory today are modified recombinant versions of the wild-type protein that have been engineered to have more favourable fluorescence characteristics for particular uses. Martin Chalfie, Osamu Shimomura and Roger

Tsien were awarded the 2008 Nobel Prize in Chemistry for their discovery and development of GFP [http://nobelprize.org/nobel_prizes/ chemistry/laureates/2008/]. GFP consists of a β-sheet with α-helixes forming a β barrel structure (Figure 4.1.1). The chromophore is contained within the α-helixes running through the centre of the molecule. The structure of the protein excludes solvent molecules from the interior and thus protects against its fluorescence being quenched (by water) [Ormö et al 1996, Yang et al 1996, Prasher et al 1992].

The VS01 plasmid (Figure 4.1.2) also conferred resistance to a specific antibiotic, Puromycin (Figure 4.1.3), on the cells. This allowed selection for the cells that had been successfully transfected by supplementing the media they were grown in with a concentration of Puromycin known to kill non-resistant cells. Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger* that specifically inhibits peptidyl transfer on both prokaryotic and eukaryotic ribosomes and thus disrupts normal protein synthesis [Blobel and Sabatini 1971, Nathans 1964, Yarmolinsky and De La Haba 1959]. The antibiotic inhibits the growth of Gram positive bacteria and various animal [Blobel and Sabatini 1971] and insect cells.

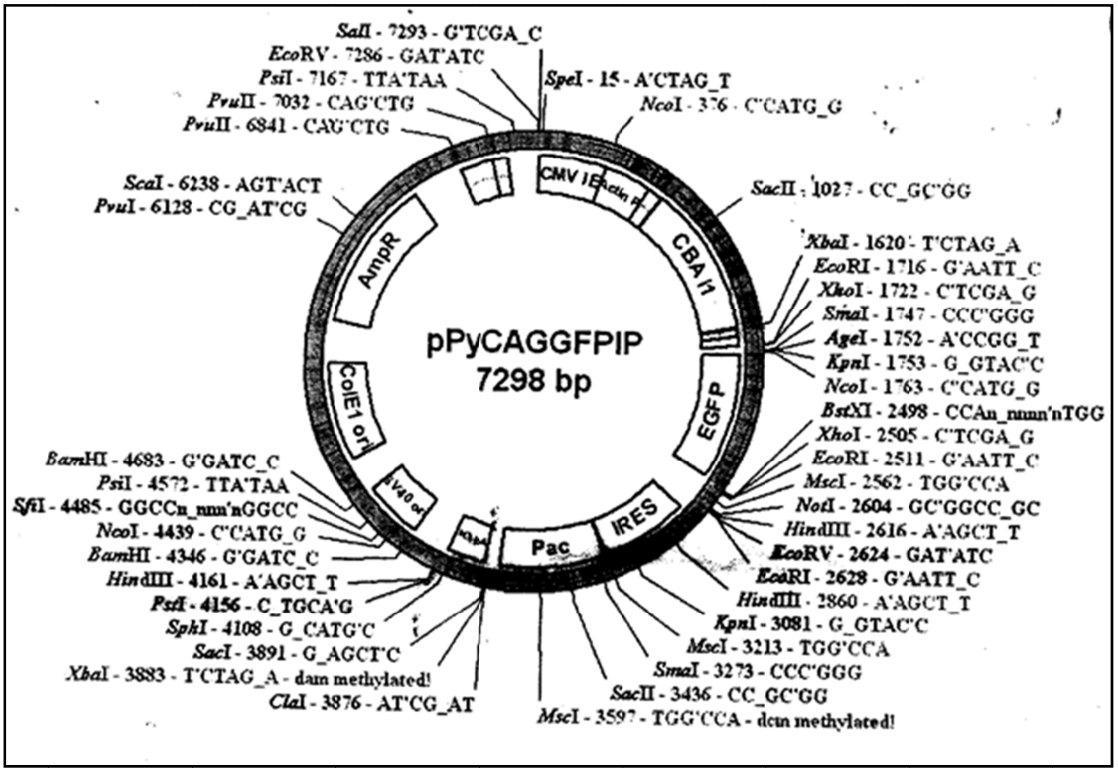


Figure 4.1.2: VS01 plasmid.

Puromycin is usually toxic at a range of 10 – 100 μg/ml but can be toxic for some eukaryotic cells at concentrations ≤1 μg/ml. Puromycin selection is rapid; it can kill up to 99% of (non-resistant) cells within 48 hours. The *pac* gene encoding a Puromycin N-acetyl-tranferase (PAC) has been isolated from a *Streptomyces* producing strain. It is located in a region of the *Pur* cluster linked to the other genes determining the Puromycin biosynthetic pathway. Transfecting mammalian cells with the *pac* gene confers Puromycin resistance on them [Vara et al 1986].

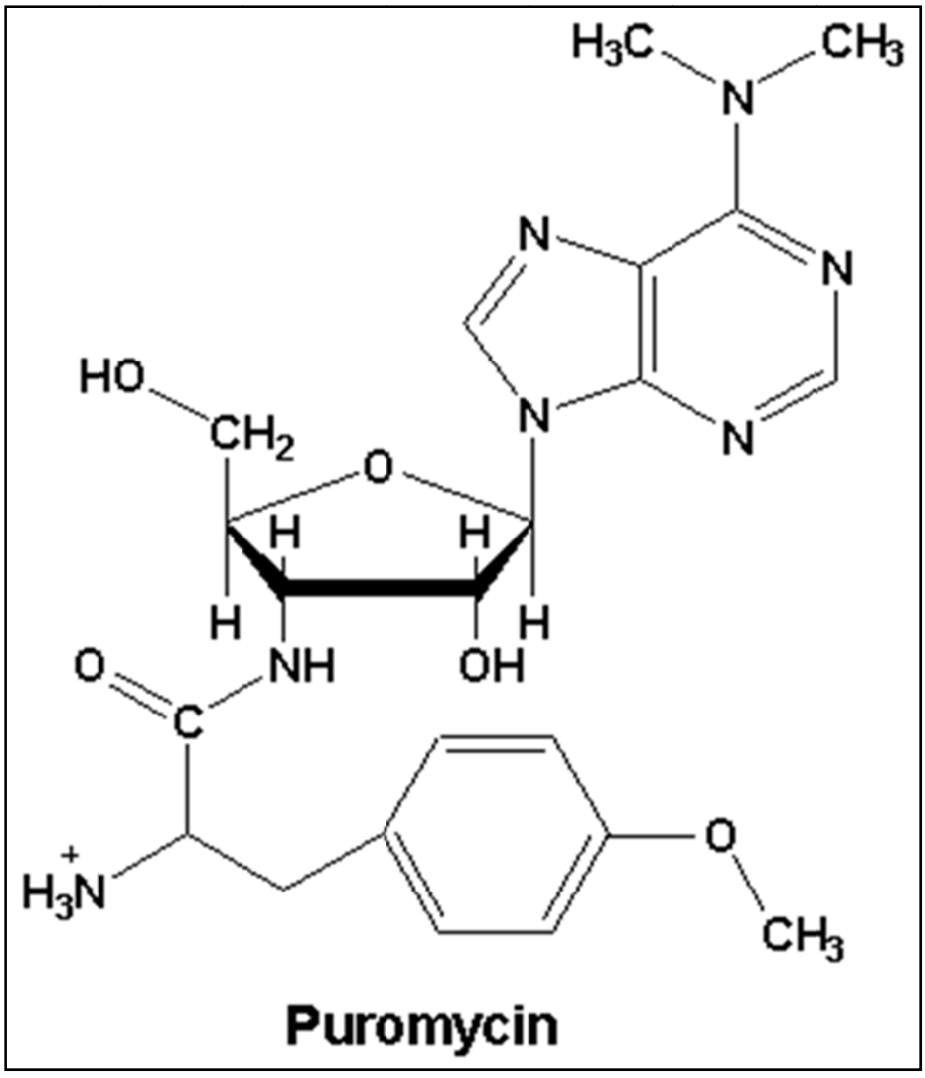


Figure 4.1.3: The structure of the antibiotic Puromycin [Yarmolinsky and De La Haba 1959].

Aims

The experiments in this chapter sought:

- To ascertain if it was feasible to excise and maintain *ex vivo* embryonic chick gut tissue for sufficient time to carry out the proposed co-culture protocols.

- To transfect CEE mES cells with a GFP expressing plasmid.

- To establish if mES cells could be introduced to, cultured within and then extracted from *ex vivo* chick gut tissue explants.

To co-culture CEE mES cells, some of which had been through the *in vitro* differentiation protocols detailed in Chapter Three, with embryonic chick gut tissue and to evaluate the effects of this co-culture on stem cell differentiation to the definitive endoderm and intestinal precursor lineages. Cell differentiation was assessed at the RNA level by RT-PCR and the proteomic level by immunocytochemistry and western blotting.

Chapter Four: *Ex vivo* co-culture

4.2 Materials and Methods

4.2.1 Egg dissection to excise embryonic chick gut tissue

Fertilised chicken eggs were obtained at one day post-fertilisation (Henry Stewart & Company Ltd, Lincolnshire, UK) and stored at 12°C until required. The eggs were then incubated at 37ºC for six days. The top of the egg was then gently cracked open and a sufficient part of the shell removed to allow access around the embryo (Figure 4.2.1A). A small volume of PBS was pipetted in and the membrane was then pierced to allow the PBS to drain through to aid in detaching the embryo from the membrane (Figure 4.2.1B). The membrane was then cut around the embryo which was then lifted out of the shell and placed in a Petri dish. Any membrane remaining attached to the embryo was then trimmed away and the embryo was cleaned with PBS (Figure 4.2.1C). The head and the heart were then removed, the embryo pinned down and an incision made along the ventral side. The endodermal tissues, including the early intestine were then excised [Bellairs and Osmond 2005].

Embryonic chick tissue was chosen for a number of reasons. Embryonic chicks are far easier to obtain & store than (mammalian) species that give birth to live young. Fertilised eggs can be obtained from a specialist supplier in large numbers and can be kept at 8 - 12°C in a chilled unit for a few days (where development is arrested) before being transferred to an incubator at 37°C until they reach the desired stage of development. In addition, during the first half of development (up to 10 days as the eggs would take approximately 21 days to hatch) work can be carried out without the need for a home office licence.

4.2.2 Processing tissue samples & wax embedding

Tissue explants were washed in PBS and then fixed by 4% (*w/v*) formalin treatment for 20 - 30 minutes at room temperature. The tissue samples were then processed overnight using a Leica TP1020 tissue processor that dehydrates the tissues through a series of solvent and alcohol treatments. The samples pass through a Xylene

treatment and then a series of alcohol washes (initially 100% decreasing through four steps to 50%) before finally being permeated with molten wax. The processed samples were then embedded in wax blocks using a Leica EG1160 wax embedder. The blocks were chilled to allow the paraffin wax to harden and then were stored at 4°C [Bancroft and Stevens 1982, Young, Lowe, Stevens and Heath 2006].

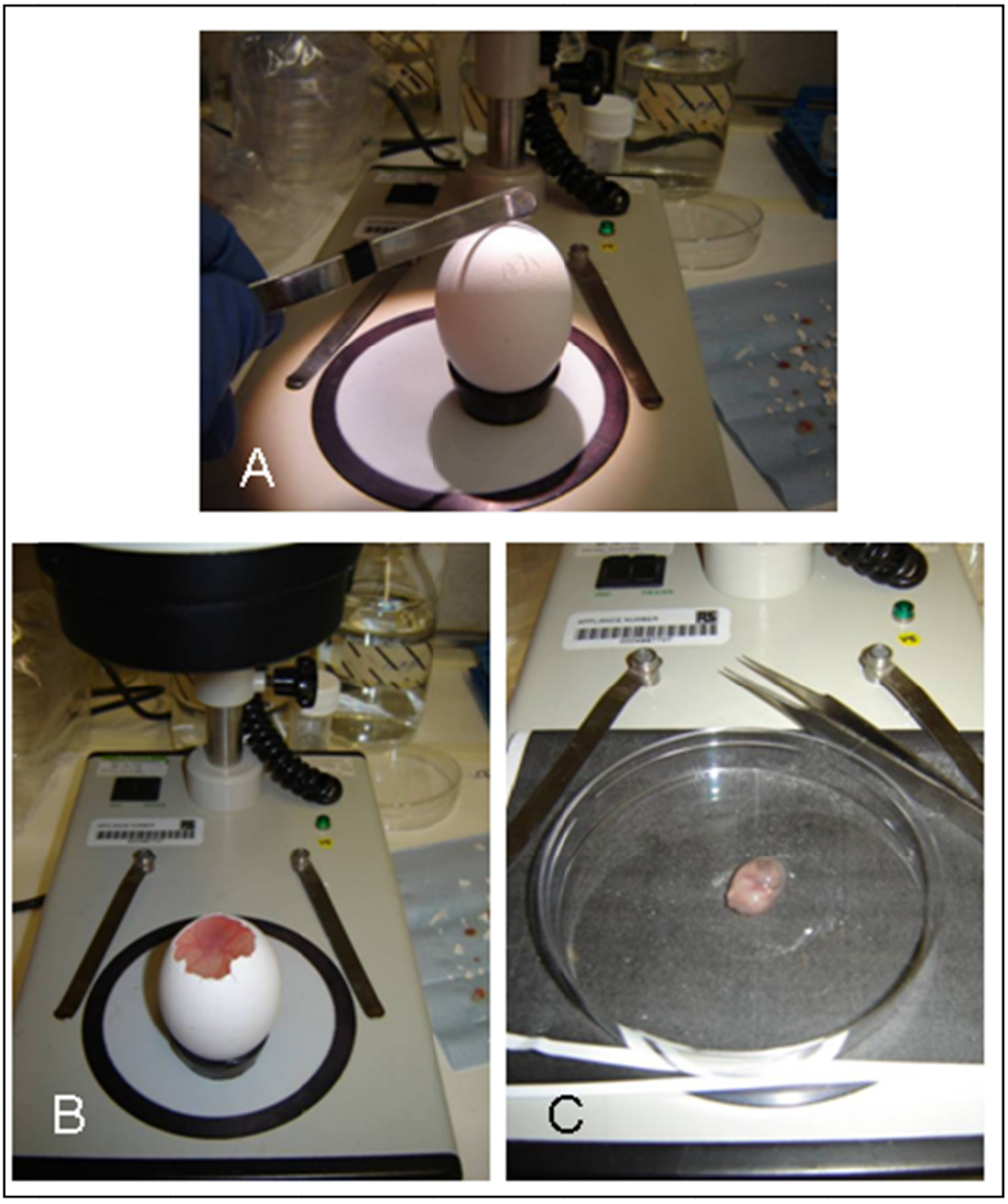


Figure 4.2.1: Removal of the chick embryo from the egg. A - The top of the eggshell was gently cracked. B – A small amount of PBS was added and the membrane was pierced. C – The embryo was lifted out of the shell, placed in a Petri dish and then cleaned with PBS.

4.2.3 Processing tissue samples for cryo-sectioning

Tissue explants were washed in PBS and then fixed with 4% (*w/v*) formalin treatment for 20 minutes. The samples were mounted in OCT embedding matrix (Cellpath, UK). The blocks were set by immersion in isopentane cooled by immersion in liquid N_2 and stored at -80°C.

4.2.4 3-Aminopropyltriethoxysilane (APES) coating of slides for mounting tissue samples

Poly-lysine slides (VWR, 631-0107) were soaked in acetone (or methanol) for two minutes and then air dried to ensure that the slides were clean. A 2% (*v/v*) solution of 3-Aminopropyltriethoxysilane (APES) in acetone was made by adding 2 ml APES (Sigma-Aldrich, A3648) to 98 ml Acetone. This solution was used on the day it was made and was subsequently discarded. The (dry) slides were soaked in the 2% (*v/v*) APES solution for two minutes. The slides were then washed twice in distilled water and then dried completely in a 37ºC oven and allowed to cool. The slides were then autoclaved and then stored at room temperature until required.

4.2.5 Sectioning of tissue samples

Wax embedded samples were sectioned (5 - 10 μm thickness) using a Leica RM2165 Microtome. The sections were transferred to a 50°C water bath to stretch the wax and then mounted onto an APES coated slide. The slides were placed on a hot plate set at 65°C to melt the wax and remove any air bubbles. The slides were allowed to cool and then the mounted samples were stored at room temperature.

Cryo-embedded samples were sectioned (5 - 10 μm thickness) using a Bright Instrument Company 5030 Cryostat. The interior of the instrument was chilled to -30°C to keep the samples frozen whilst the sample blocks are stored in dry ice when removed from the -80°C freezer to keep them as cold as possible. Sections were mounted on an APES coated slide and placed at room temperature to allow the embedding matrix to melt and dry to the slide. The mounted samples were then stored at -20°C until required.

4.2.6 Characterisation of the excised tissue

The tissue explants were tested for markers to ensure that intestinal tissue was being excised. Mounted tissue sections were stained by fluorescence immunohistochemistry (see Section 2.2.7) using an antibody raised against the expressed protein for CDX2, a marker of early intestinal lineages (Cell Signalling Technology, USA, 3977S) [Traber and Silberg 1996].

RNA samples were prepared from whole chicks, freshly excised tissue and from tissue explants that had been cultured *ex vivo* for up to 10 days (three and seven days in batch A then three, seven and 10 days in batch B - see Section 2.2.1) by digestion with dispase-collagenase (see Section 4.2.11).

These samples were evaluated for the expression of *GAPDH*, a ubiquitously expressed 'house-keeping' gene, and *cdx2* by RT-PCR analysis (as described in the Sections 2.2.5 and 2.2.6). Details of the primer pairs can be found in the table below (Table 4.2.1). Please note these primers were designed against chicken (*Gallus gallus*) sequences and not murine sequences.

4.2.7 Maintenance of the tissue explants in *ex vivo* culture

Tissue explants were washed with PBS and then transferred to non-tissue culture treated six well plates in DMEM + 10% (*v/v*) FCS that had been pre-warmed to 37°C. The tissue was then maintained in culture for up to 25 days with fresh media added as required, usually every day. Samples were prepared for RT-PCR analysis of key marker expression after three, seven and 10 days (see Sections 2.2.5, 2.2.6 and Section 4.2.6). Samples were fixed and prepared for sectioning and histological staining after one, four, six and eight days in culture (see Section 4.2.8). Live-dead analysis was also carried out after three, five, six, eight, 10, 20 and 25 days in culture (see Section 4.2.9).

4.2.8 Haematoxylin and Eosin (H & E) staining

Wax embedded sections or cryo-sections mounted on slides of explanted tissue that had been maintained in *ex vivo* culture for between zero (freshly explanted tissue) and

eight days were stained with H & E [Bancroft and Stevens 1982, Young, Lowe, Stevens and Heath 2006] to allow examination of the tissue architecture. Reagent recipes can be found in Section 6.4.

The wax sections were rehydrated by immersion through the following sequence of solvents: Xylene for three x one minute, 100%, 90%, 70% and 50% (*v/v*) Industrial Methylated Spirit (IMS) for one minute each and then H_2O for one minute. The slides were immersed in Haematoxylin for five - 10 minutes then rinsed with H_2O to remove any excess stain. The slides were then immersed in Scott's Tap Water substitute for two minutes. The slides were then washed with H_2O and then dehydrated through the following sequence of solvents: 50%, 70% and 90% (*v/v)* IMS for one minute each. The slides were then dipped in 1% (*w/v*) Alcoholic Eosin for three seconds. The dehydration sequence was then completed by further solvent immersions: 100% IMS two x two seconds and Xylene two x five seconds. The slides were then air dried and the sections mounted using Distyrene, Plastisier and Xylene (DPX) (Sigma) under a coverslip and dried overnight. The sections were then imaged using a Nikon Eclipse light microscope.

4.2.9 Live-Dead staining of tissue explants

The required amount of Live-dead working solution was made up from a Live-dead® cell viability/cytotoxicity kit (Molecular Probes, L-3224). Ethidium D-1 homodimer (Eth D-1, 20 µl of 2 mM) was added to 9.975 ml PBS (final concentration 4 µM) and vortexed thoroughly. Calcein AM (5 µl of 4 mM) was then added to the working solution (final concentration 2 µM) and vortexed thoroughly.

The media was aspirated from the tissue in culture and the tissue washed three times with PBS. Enough live/dead working solution to cover the tissue was applied and incubated for 30 - 40 minutes at room temperature. The live-dead working solution was then removed and the tissue was washed three times with PBS. The tissue was then covered with UV mountant (DABCO) and visualised using a Nikon Eclipse fluorescence microscope (excitation/emission 495/515 nm Calcein AM (green = live cells): excitation/emission 495/635 nm Eth D-1 (red = dead cells)).

4.2.10 Transfection of CEE mES cells with a GFP expressing plasmid

LB broth was supplemented with Ampicillin to a final concentration of 30 μg/ml (150 ul of 100 mg/ml stock in 500 ml). 10 ml of this media was aliquoted into a universal and inoculated with a scraping from a glycerol stock of the bacteria carrying the GFP/Puromycin resistance expressing plasmid (VS01 – see Figure 4.1.2). This culture was incubated in a shaking incubator at 37ºC for two - three hours. This allowed the bacteria to start growing in a small and concentrated environment. The contents of the universal were transferred into a large conical flask containing the remainder of the media. This was incubated overnight in the shaking incubator at 37°C.

The medium was cloudy the following day indicating the bacteria had grown. The culture was aliquoted into 50 ml centrifuge tubes and spun at 2000 *g* for 10 - 15 minutes. The supernatant was removed. The pellets were stored at -20°C until required.

The minimum concentration of Puromycin required to kill mES cells not carrying the plasmid was determined by carrying out a kill curve – untransfected cells were cultured in media (DMEM + 10% FCS + 1% AB/AM + 1% L-Glut + 5 x 10^3 units/ml LIF) containing 0.05 – 3 μg/ml Puromycin in a gelatin coated 24 well TCP plate (Figure 4.2.2) for several days and observed throughout. A control was cultured in Puromycin free media. The kill curve was carried out in triplicate.

The plasmid was prepared and purified using an RNeasy® MaxiKit (Qiagen, Cat no 12162). The plasmid yield was then quantified using a Nanodrop Spectrophotometer. The plasmid was linearised by digestion with PstI restriction enzyme overnight at 37°C. The linearised plasmid was checked by electrophoresis on an agarose gel (Figure 4.2.2).

CEE mES cells at passage number 20 in suspension at 1 x 10^7 cells/ml (400 μl) and plasmid suspension at 20 μg/ml (250 μl) were mixed with PBS (150 μl, Gibco, Cat no 10010) to give a total reaction volume of 800 μl. This was transferred to a cuvette and placed in a Multiporator. A charge of 450 V was then passed across the cuvette

for 500 μs [Bushman F, 2002, Lateral DNA Transfer - Mechanisms and Consequences: Cold Spring Harbor Laboratory Press, Lakshmipathy et al, 2004].

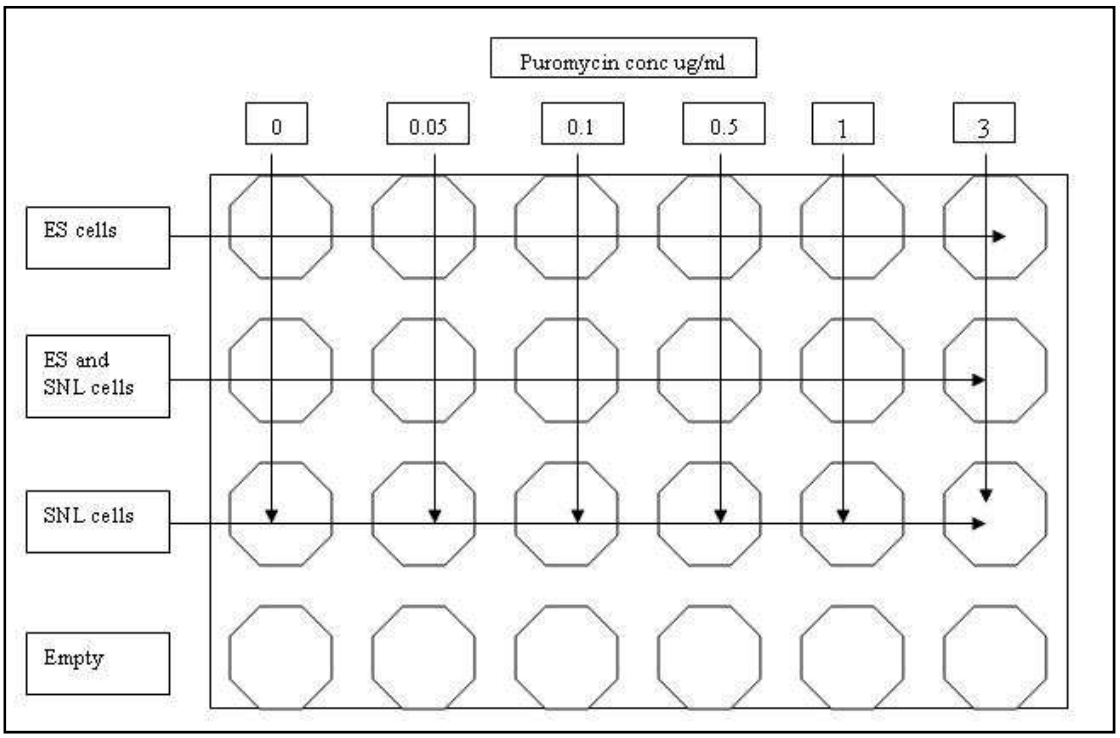


Figure 4.2.2: Kill curve plate layout

The reaction mix was then diluted out in cell culture media and transferred to six well tissue culture treated plates. The media contained Puromycin at 1 μg/ml (as determined by the kill curve – Figure 4.3.5 - 7). The electroporated cells were cultured under Puromycin selection for 14 days on gelatin coated TCP six well plates to ensure only cells that had been transfected would survive. The surviving cells were then transferred into ordinary mES cell proliferative culture conditions. The cells' GFP expression was monitored throughout using a Nikon Eclipse fluorescence microscope.

4.2.11 Co-culture of mES cells with tissue explants

Tissue explants were washed with PBS and then transferred to non-TCP treated six well plates containing DMEM + 10% (*w/v*) FCS. The explants were then injected

with 100 µl of mES or GFP-mES cells in suspension (some of which had been pre-treated *in vitro* with Act-A), at 1 x 10^6 cells/ml, using a fine hypodermic needle and syringe. The tissue-cell co-cultures were then incubated at 37ºC, 5% (*v/v*) CO_2 in air for up to seven days. In the optimisation experiments the live co-cultures were examined under a fluorescent microscope to establish if the injected GFP-mES cells remained associated with the tissue mass; some of the (intact) cell-tissue co-cultures were embedded and sectioned as above and examined using a fluorescence microscope. The sections were examined using a Nikon Eclipse Fluorescence microscope before H and E staining as it was found that H and E staining produced high levels of background fluorescence in the GFP emission wavelength. Therefore the fields of view for the two images produced (GFP vs H and E staining) were slightly offset (see Section 4.3, Figure 4.3.7).

At the end of the co-culture period the tissue explants were broken down using a dispase-collagenase digestion media (DMEM, 1% (*v/v*) FCS, 1% (*w/v*) AB/AM, 1% (*w/v*) L-Glut, 75 U/ml collagenase XI, 20 µg/ml dispase). The explants were transferred to fresh non-TCP six well plates containing the digestion media and incubated for two - three hours at 37°C on a shaker. RNA samples were prepared from the cell suspension as described in Section 2.2.1 and analysed by RT-PCR as described in Section 2.2.5 and 2.2.6. Protein samples were prepared as described in Section 2.2.3 and analysed by western blotting as described in Section 2.2.8. Samples were prepared for immunocytochemistry as described in Section 4.2.14 and analysed as described in Section 2.2.7.

These experiments were conducted a number of times as there was a limit to the number of samples that could be run at any one time. These experiments were termed batch A – F. In Batch A naïve GFP-mES cells were injected into six intact embryonic chick gut tissue explants. In Batch B naïve GFP-mES cells were injected into six intact embryonic chick gut tissue explants whilst GFP-mES cells that had been aggregated into EBs and Act-A treated *in vitro* (treatment based on condition three in Chapter Three, Section 3.2.1v and 3.3.5 consisting of two days in monolayer followed by a five day EB phase with a further nine days in monolayer in Act-A+, 10% (*v/v*) FCS media throughout) were injected into a further six intact embryonic chick gut tissue explants. In Batch C naïve GFP-mES cells were injected into six intact

embryonic chick gut tissue explants. In Batch D naïve GFP-mES cells were injected into five intact embryonic chick gut tissue explants whilst GFP-mES cells that had been aggregated into EBs and Act-A treated *in vitro* were injected into a further four intact embryonic chick gut tissue explants. In Batch E mES cells were injected into nine intact embryonic chick gut tissue explants. In Batch F mES cells were injected into nine intact embryonic chick gut tissue explants and co-cultured for six days.

4.2.12 Co-culture of mES cells with dissociated embryonic chick gut tissue cells

Tissue explants were washed with PBS and then broken down using a dispase-collagenase digestion media as described above (Section 4.2.10). The dissociated cells were then resuspended and seeded in non-TCP six well plates in DMEM + 10% (*v/v*) FCS. GFP-mES cells in suspension (100 µl at 1 x 10^6 cells/ml), some of which had been pre-treated *in vitro* with Act-A, were intermixed with the dissociated gut cells using a fine hypodermic needle and syringe. The cell-cell co-cultures were then incubated at 37ºC & 5% (*v/v*) CO_2 in air for up to seven days. RNA samples were prepared from the cell suspension as described in Section 2.2.1 and analysed by RT-PCR as described in Section 2.2.5 and 2.2.6. Protein samples were prepared as described in Section 2.2.3 and analysed by western blotting as described in Section 2.2.8. Samples were prepared for immunocytochemistry as described in Section 4.2.14 and analysed as described in Section 2.2.7.

This experiment was run in parallel to intact tissue co-cultures as part of the experiment termed batch D. In Batch D naïve GFP-mES cells were used to set up five co-cultures with cells derived from embryonic chick gut tissue explants and co-cultured for seven days whilst GFP-mES cells that had been aggregated into EBs and Act-A treated *in vitro* (treatment based on condition three in Chapter Three, Section 3.2.1v and 3.3.5 consisting of two days in monolayer followed by a five day EB phase with a further nine days in monolayer in Act-A+, 10% (v/v) FCS media throughout) were used to set up four co-cultures with cells derived from embryonic chick gut tissue explants and co-cultured for seven days.

4.2.13 FACS of the samples post co-culture

The mES cells used in the co-culture experiments (Batches A – D inclusive) had been stably transfected with a GFP expressing plasmid (see Chapter 3A). The cell suspension (from batches A - C) produced by the dispase-collagenase digest was sorted for positive expression of GFP using Beckman-Coulter Altra Flow Sorter instrument prior to sample preparation (see Section 2.3). The sorting parameters were optimised using a positive and negative control sample to ensure all negative cells were excluded. The sorted cells were then either returned to monolayer culture or used to prepare RNA or protein samples (see Section 2.2.1 or Section 2.2.3).

4.2.14 Preparation of samples for immunocytochemical analysis from co-culture experiments

The cell suspension produced from the dispase-collagenase digest was pelleted by centrifugation at 200 *g* for five minutes and washed three times with PBS. The cells were then fixed by treatment with 4% (*w/v*) formalin for 20 minutes at room temperature. The cells were then washed three times with PBS and resuspended at high density in a small volume of PBS. Between 10 - 20 µl of this cell suspension was placed on an APES coated poly-lysine microscope slide and spread to cover an area approximately 5 mm in diameter. The slides were then allowed to air dry in fumehood. Between five and eight slides were prepared from each co-culture incubation.

4.2.15 Primer design

Primers were designed as described in the general methods section. The selected markers were *Lgr5/GPR49* [Barker et al 2007], *Msi-1* [Potten et al 2003], both of which are markers of ISC, *CD133* [Kania et al 2005], a marker commonly associated with ASC populations known to be expressed in colonic stem cells [Samuel et al 2008] & *Epi Ant* [Engelhardt et al 1993]. As described previously the selected marker for undifferentiated cells was *Oct4 & GAPDH* primers were used as a ubiquitously expressed control to confirm the RT stage of the reaction had worked (Table 4.2.1A). Primers were also designed for chick *cdx2* and chick *GAPDH* (Table 4.2.1B).

Table 4.2.1A: RT-PCR primer pair details for chicken markers of interest.

Primers	Seq (5' - 3')	Annealing temp C	Fragment size bp	Cycle no.
Cdx2-F	TCGGTAGCCAAGTCAAAACC	60	647	30
Cdx2-R	AACAGCATCGCTCAGACCTT			
GAPDH-F	CGTCCTCTCTGGCAAAATG	60	507	30
GAPDH-R	CATCCACCGTCTTCTGTGTG			

Table 4.2.1B: RT-PCR primer pair details for (murine) ISC markers.

Primers	Seq (5' - 3')	Annealing temp C	Fragment size bp	Cycle no.
CD133-F	CAGGAGGCAGAAGAGTCCAC	55	186	30
CD133-R	TAGCCCCAGGAGTGTTATGG			
EpiAnt-F	TGTTGGGTAGCGGAAAGAAC	55	163	30
EpiAnt-R	TCAACAGCATCCTTTGCATC			
LGR5mm-F	AGCATACCCGTTTCTGGATG	55	173	30
LGR5mm-R	AGTCATGGGGTAAGCTGGTG			
MSI1-F	ATGGTGGAATGCAAGAAAGC	55	191	30
MSI1-R	TAGGTGTAACCAGGGGCAAG			

4.2.16 Immunocytochemical analysis of post co-culture cells

Where co-culture batches A - D were analysed only the enzymatic method (see Section 2.2.9) was used as the samples contained GFP labelled cells. For batches E - H unlabelled cells were used so both fluorescent and enzymatic techniques were employed (see Section 2.2.9). The expression of the ISC marker LGR5, the DE markers FOXA2 and SOX17 and the pluripotency marker OCT4 were examined.

Chapter Four: *Ex vivo* co-culture

4.3 Results

4.3.1 Characterisation of the explanted tissue

Before the explanted tissue could be used in the co-culture experiments it was necessary to confirm that the tissue being excised was the developing intestinal tissue. Therefore the tissue was examined at the molecular level for the expression of key marker genes and corresponding proteins.

Day six/stage 20 tissue was used because work carried out and general information [Bellairs and Osmond 2005] showed that it was at this stage that the early intestine had formed a tissue mass of appropriate size and morphology. This was confirmed by histological and immunohistochemical staining of whole embryo sections. Day six chick tissue was also used in one of the studies in the literature [Sugie et al 2005].

4.3.1i Antibody staining of sections of the excised tissue

Sections of wax embedded (day six) embryonic chick gut tissue displayed strong expression of the intestinal marker CDX2 when evaluated by fluorescence immunohistochemistry (Figure 4.3.1). Figure 4.3.1A shows H & E staining of a whole (day six) embryonic chick section with the area from which tissue was excised circled. Figures 4.3.1B and C show the secondary antibody control (i.e. sections incubated with PBS in place of the primary antibody but with secondary antibody to determine if any positive signal was given by non-specific binding). The DAPI staining clearly shows that material was present but there was no strong signal in the FITC channel. There was moderate background interference in the FITC channel but no areas of strong positive staining. This indicated that there was minimal non-specific binding of the tagged secondary antibody. Figures 4.3.1D – G show the images of the primary antibody staining from two representative sections. The DAPI images show all the cells present in this area of the section whilst the FITC channel illustrate expression of the target protein, CDX2, which was expressed in both samples (Figure 4.3.1E and G).

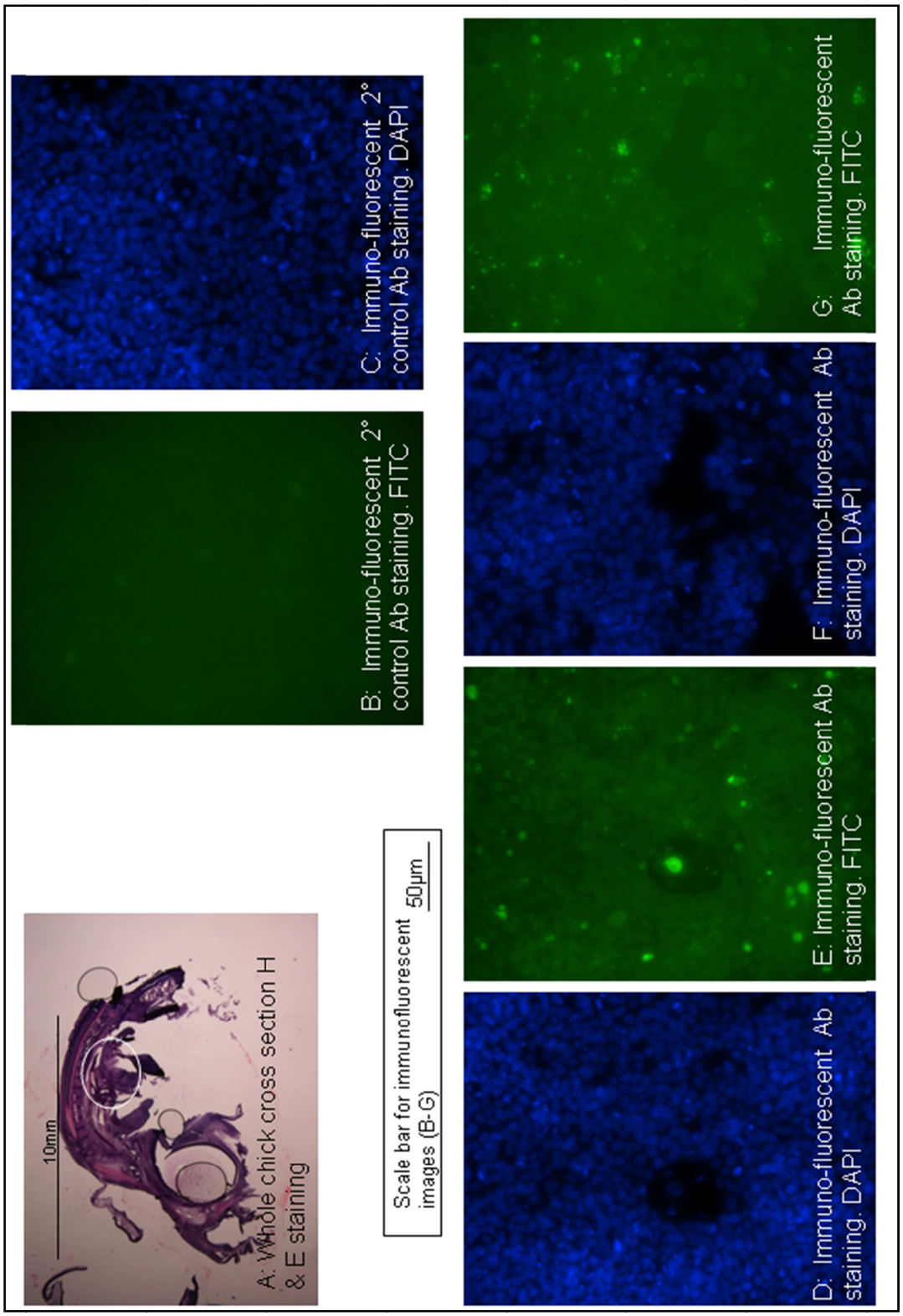


Figure 4.3.1: Representative images of wax embedded sections illustrating that the target tissue was excised. A: H & E staining of whole (day six) chick. White circle shows the region in which the intestine is found. B & C: Secondary antibody control staining of excised gut tissue sections. D – G: Fluorescent antibody staining for gut marker CDX2 (green) and DAPI (blue) of excised gut tissue sections.

4.3.2 Characterisation of chick gut tissue explants in *ex vivo* culture

Before the cells could be co-cultured with the tissue explants the viability of the tissue in *ex vivo* culture was investigated. The tissue explants were placed in culture for up to 25 days and analysed for signs of necrosis and degradation. By eye observations were made and samples were collected for histology, immunohistochemistry and RT-PCR analysis. Live-dead staining was also carried out.

4.3.2i Expression of marker genes by excised chick gut tissue that had been maintained in *ex vivo* culture

Figure 4.3.2 shows the levels of RNA expression of the ubiquitous marker, *GAPDH*, and the intestinal marker, *cdx2* by chick gut tissue explants cultured *ex vivo* for 0, 3, 7 and 10 days. This data supported the immunohistochemical results shown in Figure 4.3.1 and also demonstrated that the explanted tissue not only survived in culture but also maintained RNA expression. The top two rows in Figure 4.3.2 show the results from the first batch of this experiment. Stronger *GAPDH* bands were achieved from whole chick explants than the other samples due to the varied levels of *GAPDH* expression in different tissues. *GAPDH* expression was maintained throughout the time course without any significant difference in the strength of the bands from the samples prepared from tissue samples immediately after excision or those from tissue that had been in culture for seven days.

This was also true for the intestinal marker *cdx2* although one of the three replicates at the seven day timepoint showed no expression. In the second batch of the experiment (the lower two rows in Figure 4.3.2) there was some reduction in the strength of the *GAPDH* bands over the (longer) time course but expression was still maintained although more variation between replicates was apparent than in batch A. *Cdx2* expression also appeared reduced in the later timepoints, again with more variation between replicate samples. Some of this corresponded to the variation in the *GAPDH* bands (day 10 samples) and therefore may have been caused by reduced efficiency of the RT reactions but some did not (day seven samples). The variation could also have been caused by the tissue beginning to break down leading to a reduction in RNA expression.

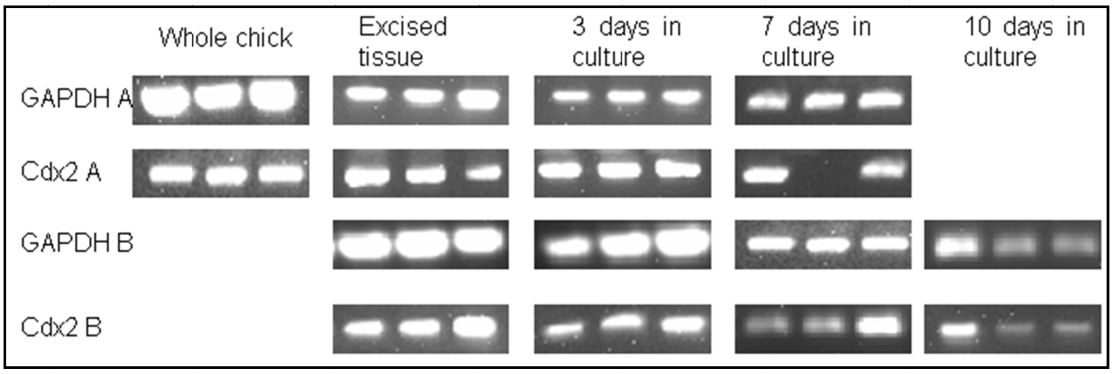


Figure 4.3.2: Expression of the ubiquitous control gene, *GAPDH*, and a key gut marker, *Cdx2*, by RNA samples prepared from excised gut tissue immediately after excision and after three, seven or 10 days in culture evaluated by RT-PCR. In batch A the tissue explants were cultured for up to seven days, in batch B for up to 10 days.

4.3.2ii Histology

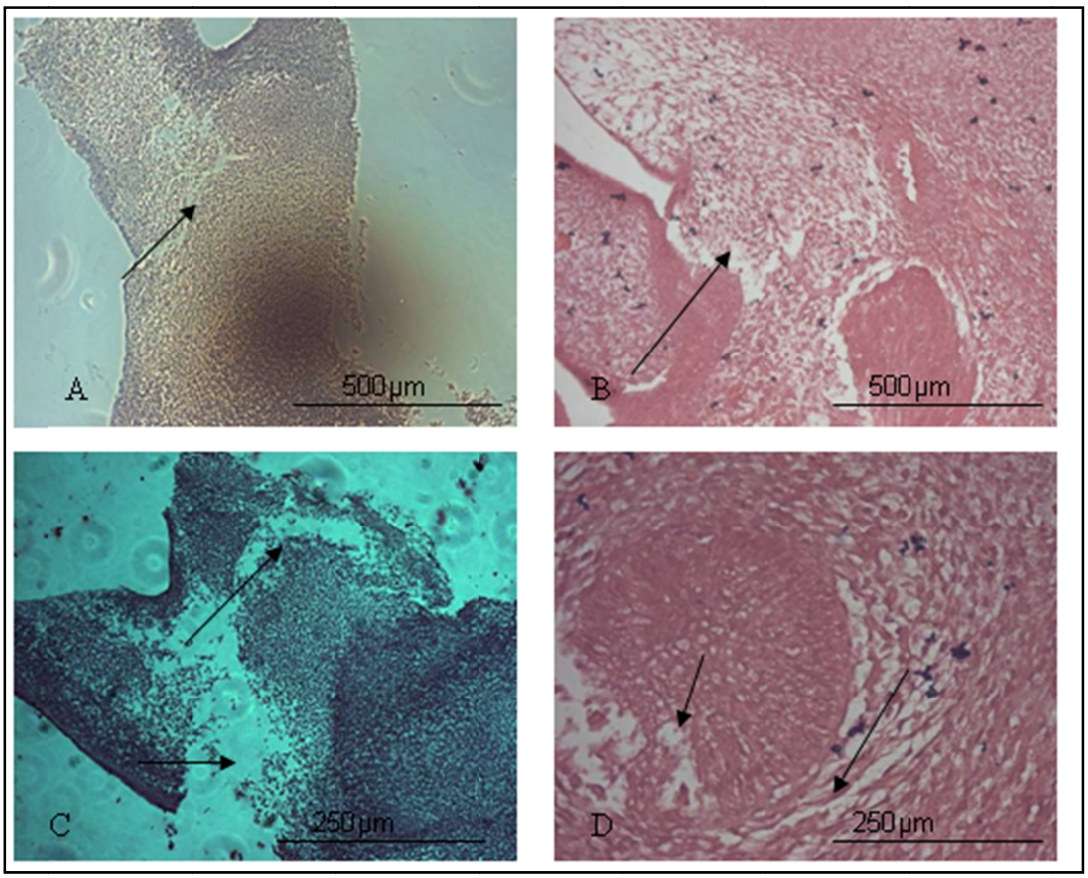


Figure 4.3.3: Representative images of H & E staining of (day six) embryonic chick gut tissue sections following up to eight days in *ex vivo* culture. A: One day in culture (100X magnification). B: Four days in culture (100X magnification). C: Six days in culture (200X magnification). D: Eight days in culture (200X magnification).

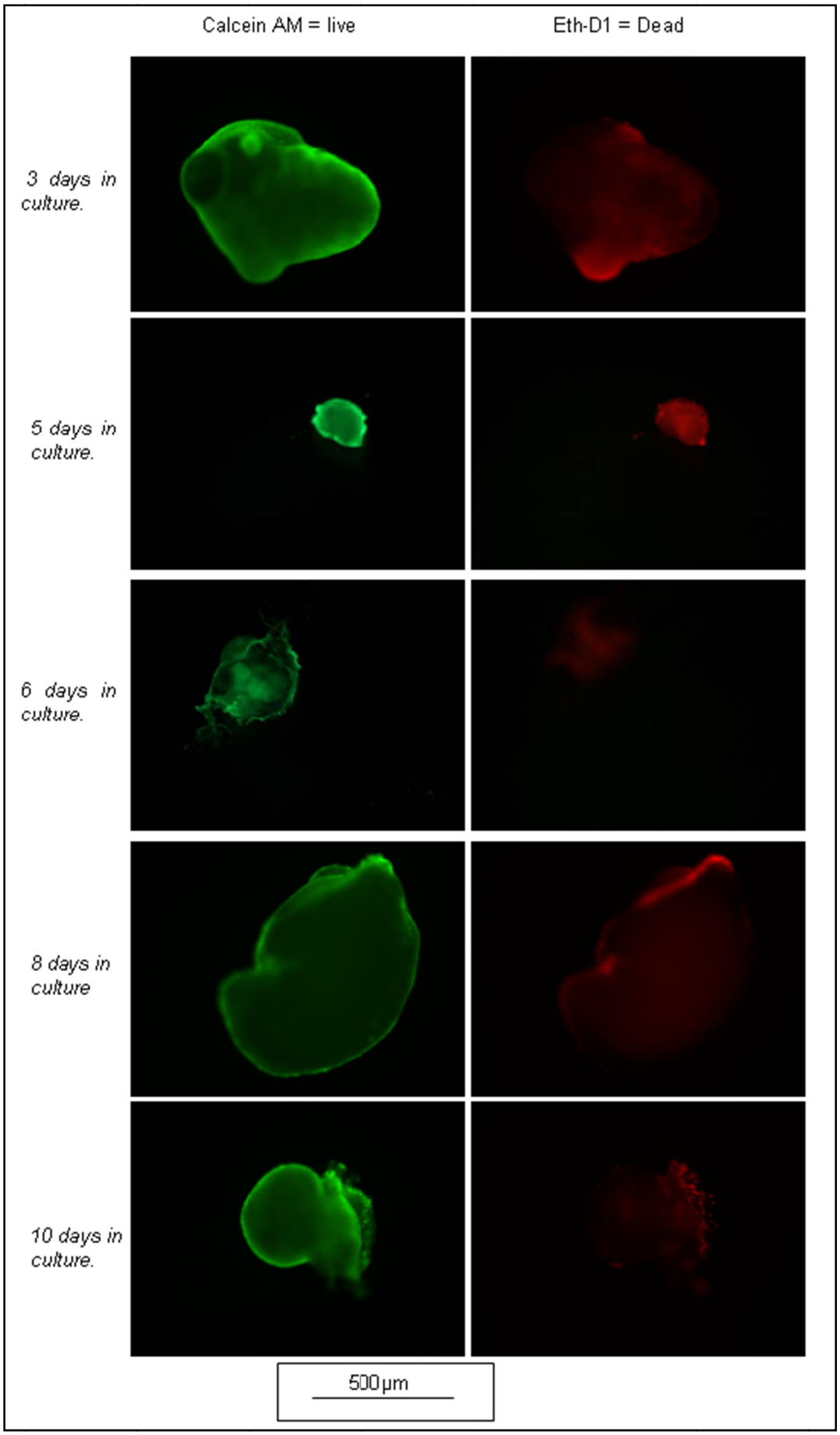
In the earlier timepoints (Figure 4.3.3A and B) there was little sign of any tissue degradation although some mechanical damage from the sectioning process was visible. At the later timepoints (Figure 4.3.3C and D) some lesions and signs of degradation were visible. This corresponded with the observations, made using a dissection microscope, of changes in the appearance of the tissue and culture media, summarised in Table 4.3.1, and the data shown in Figure 4.3.2 (i.e. the continued expression of RNA), that the tissue appeared to remain intact throughout the culture period.

Table 4.3.1: Summary table of observations of chick gut tissue in *ex vivo* culture (changes in comparison with the previous observation, i.e. day six with day four, not the initial day zero observation).

Days in culture	Observations
0	Tissue pieces pale in colour. Surfaces generally smooth apart from areas where mechanical damage occurred during extraction.
1	Tissue loosely attached to the culture plate in places. Colour and texture unchanged.
4	Tissue firmly attached to the culture plate in some places. Colour appears slightly darkened but texture unchanged. Media change required.
6	Tissue firmly attached to the culture plate. Colour slightly darkened. Surface texture slightly roughened.
7	Tissue detached from culture plate in some places. Surface texture considerably roughened. Some tissue pieces spreading out on culture plate. Media change required.
8	No change from day 7.

4.3.2iii Live-dead staining

Although there was some evidence of dead cells after between three and 10 days in culture the signal from the live staining was much stronger (Figure 4.3.4A - E). This indicates that the majority of the cells on the surface of the tissue explant were still alive. The strength of the live signal was less after six days onwards than it had been up to five days.


Calcein AM = live
Eth-D1 = Dead
3 days in culture.
5 days in culture.
6 days in culture.
8 days in culture
10 days in culture.
500µm

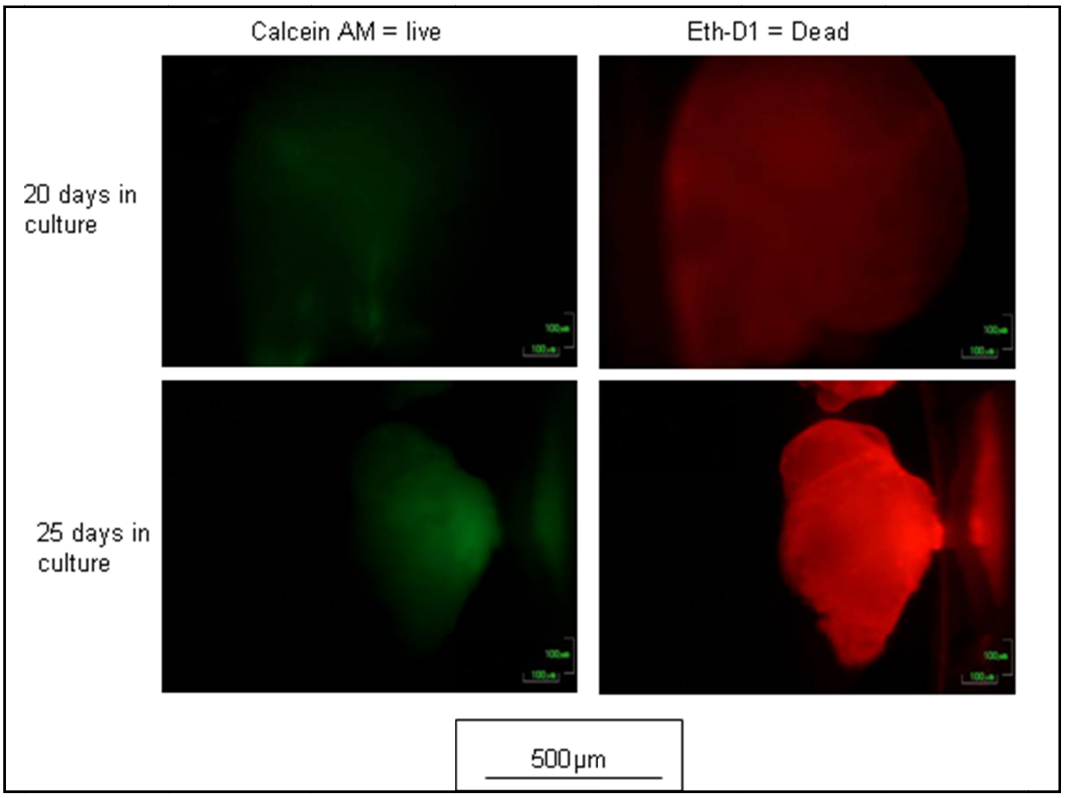


Figure 4.3.4: Representative Live-Dead® staining of day six embryonic chick gut explants after up to 25 days in *ex vivo* culture. Green stain (Calcein AM) = live, red stain (Eth-D1) = dead.

Some morphological changes were observed in the tissue explants in that they lost their shape but not by a significant degree. The explants that were cultured for 20 or 25 days (Figure 4.3.4F and G) showed a much higher proportion of dead cells. The explants had also begun to disintegrate and lose their original morphology.

4.3.2iv Transfection of CEE mES cells with a GFP expressing plasmid - Kill curve

After 24 hours under Puromycin selection there were a noticeably higher number of dead cells at the highest concentration (Figure 4.3.5D, E and F) than in the controls (Figure 4.3.5A, B and C). There were still large numbers of live cells at all concentrations of Puromycin.

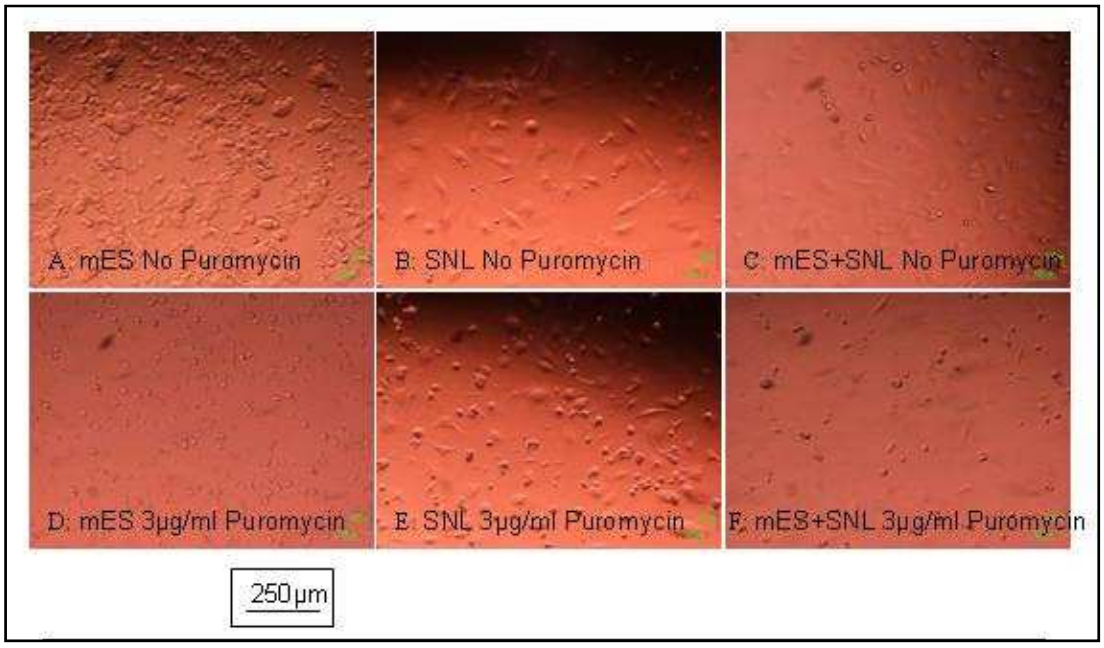


Figure 4.3.5: Kill curve. Light microscopy images showing mES and SNL cell growth at *x* µg/ml Puromycin after 24 hours. mES and inactivated SNL feeder layer cells were cultured alone and mES cells on a feeder layer of inactivated SNL cells. A: mES No Puromycin. B: SNL No Puromycin. C: mES+SNL No Puromycin. D: mES 3 µg/ml Puromycin. E: SNL 3 µg/ml Puromycin. F: mES+SNL 3 µg/ml Puromycin.

After 96 hours little difference was observable between mES cells in the control (Figure 4.3.6A) and in 0.05 µg/ml Puromycin (Figure 4.3.6B). Far fewer cells remained viable in 0.1 µg/ml Puromycin and 0.5 µg/ml (Figure 4.3.6C and D) but live cells were still present. At the highest concentrations of Puromycin used, 1 µg/ml and 3 µg/ml, >95% of the cells were dead (Figure 4.3.6E and F). The inactivated SNLs showed a reduced sensitivity to the Puromycin.

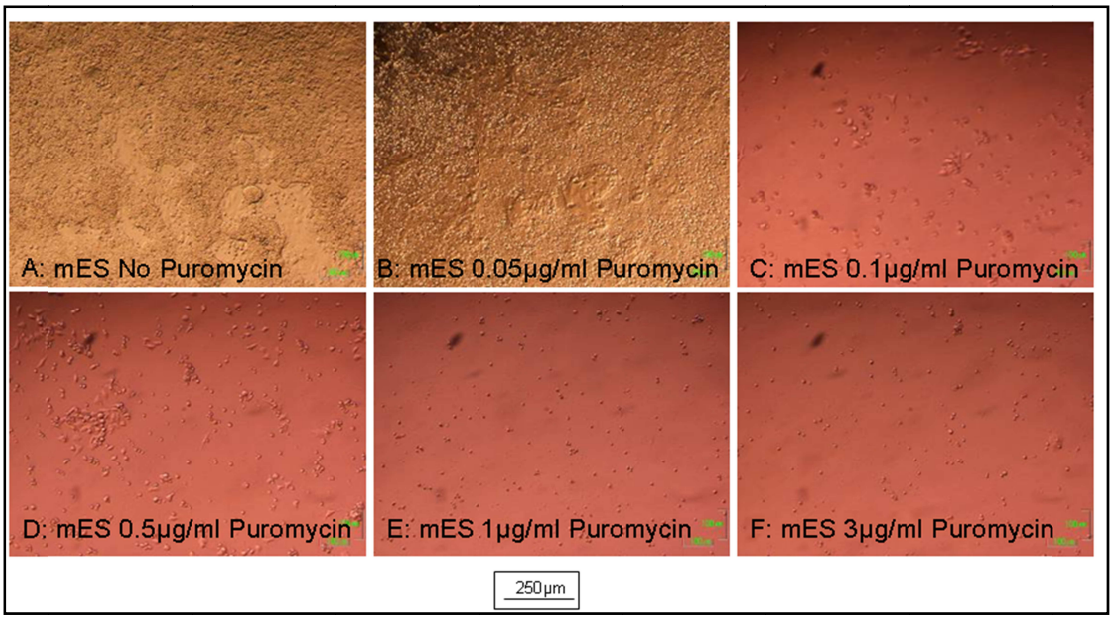


Figure 4.3.6i: Kill curve. Light microscopy images showing mES cell growth at *x* µg/ml Puromycin after 96 hours.

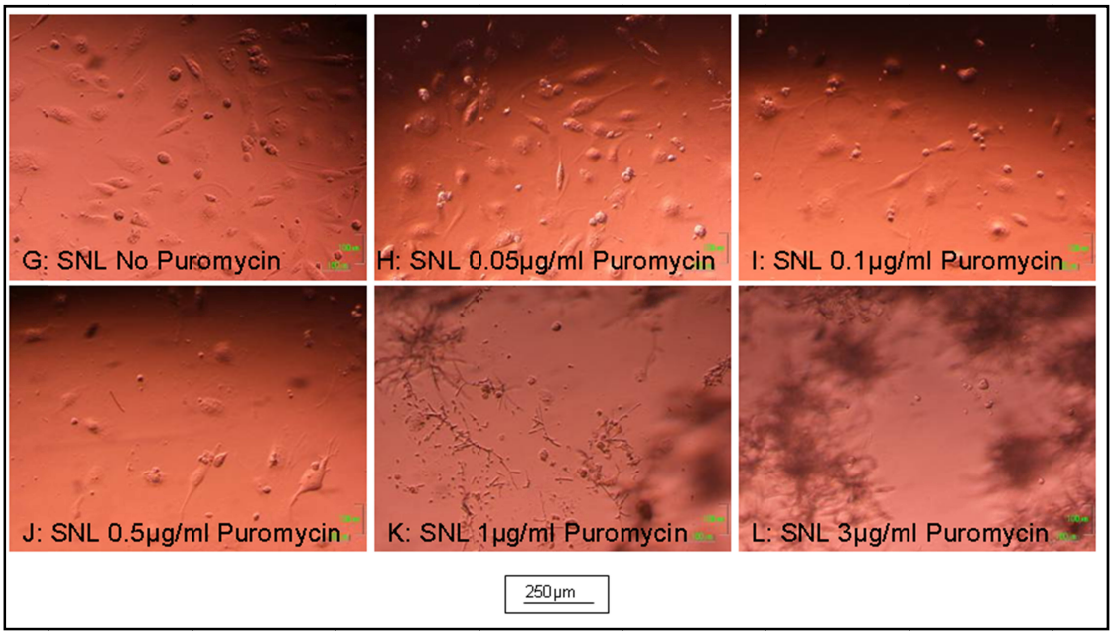


Figure 4.3.6ii: Kill curve. Light microscopy images showing SNL cell growth at *x* µg/ml Puromycin after 96 hours.

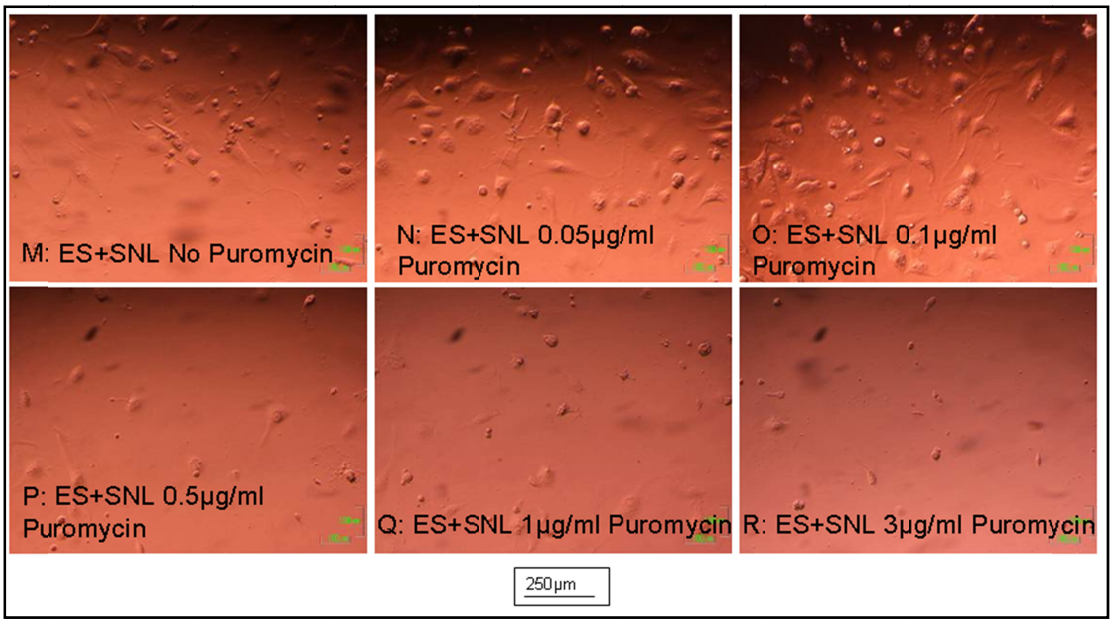


Figure 4.3.6iii: Kill curve. Light microscopy images showing mES cells on a feeder layer of inactivated SNL cells growth at *x* µg/ml Puromycin after 96 hours.

Figure 4.3.6: Kill curve. Light microscopy images showing mES and SNL cell growth at *x* µg/ml Puromycin after 96 hours. mES and inactivated SNL feeder layer cells were cultured alone and mES cells on a feeder layer of inactivated SNL cells.

After 120 hours mES cells grown on gelatin coated TCP had proliferated at comparable rates in the control conditions (Figure 4.3.7A) and 0.05 µg/ml Puromycin (Figure 4.3.7B). Some cell death was observed at 0.1 µg/ml Puromycin (Figure 4.3.7C) and 0.5 µg/ml Puromycin (Figure 4.3.7D) whilst almost all the cells (>95%) were dead at 1 µg/ml Puromycin (Figure 4.3.7E) and 3 µg/ml Puromycin (Figure 4.3.7F). The Mitomycin C treated SNL cells showed no significant changes from the control (Figure 4.3.7G) other than at 3 µg/ml Puromycin (Figure 4.3.7L) where >80% of the cells were dead. Where mES cells were grown on inactivated SNL feeder layers the SNL cells showed a similar response to when they were cultured on their own.

The kill curve (Figure 4.3.5 - 7) showed that a concentration of 1 µg/ml Puromycin was sufficient to kill non-resistant cells after 120 hours in culture.

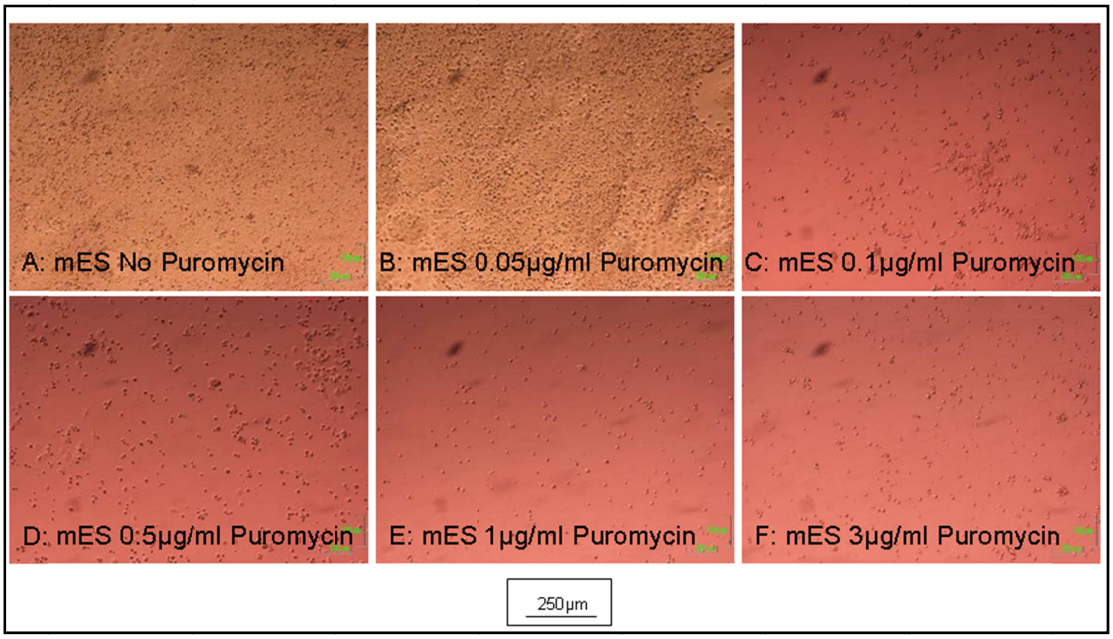


Figure 4.3.7i: Kill curve. Light microscopy images showing mES cell growth at *x* µg/ml Puromycin after 120 hours.

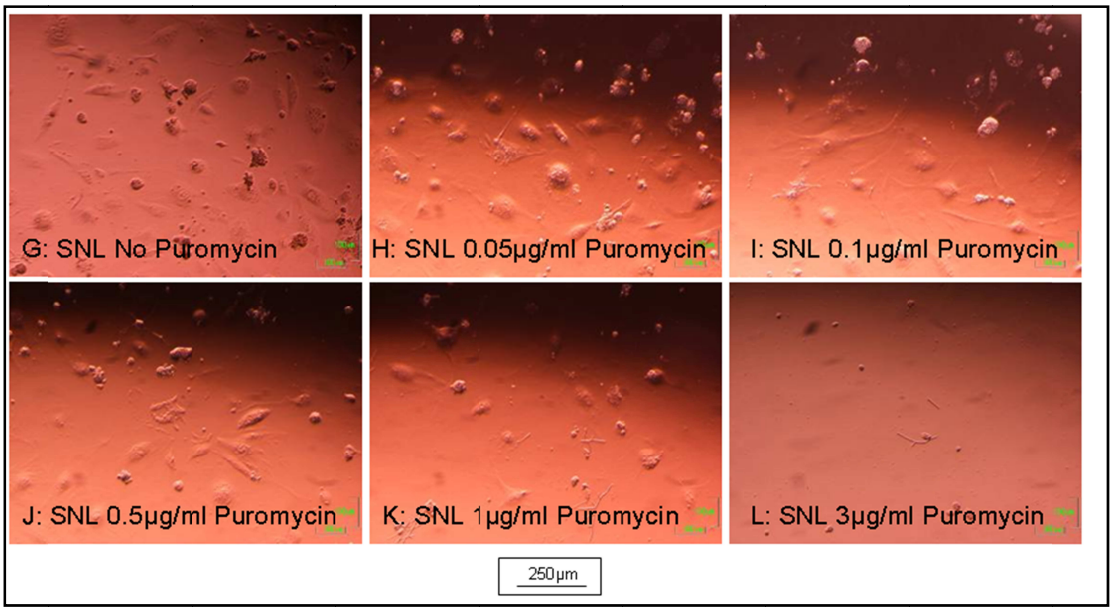


Figure 4.3.7ii: Kill curve. Light microscopy images showing SNL cell growth at *x* µg/ml Puromycin after 120 hours.

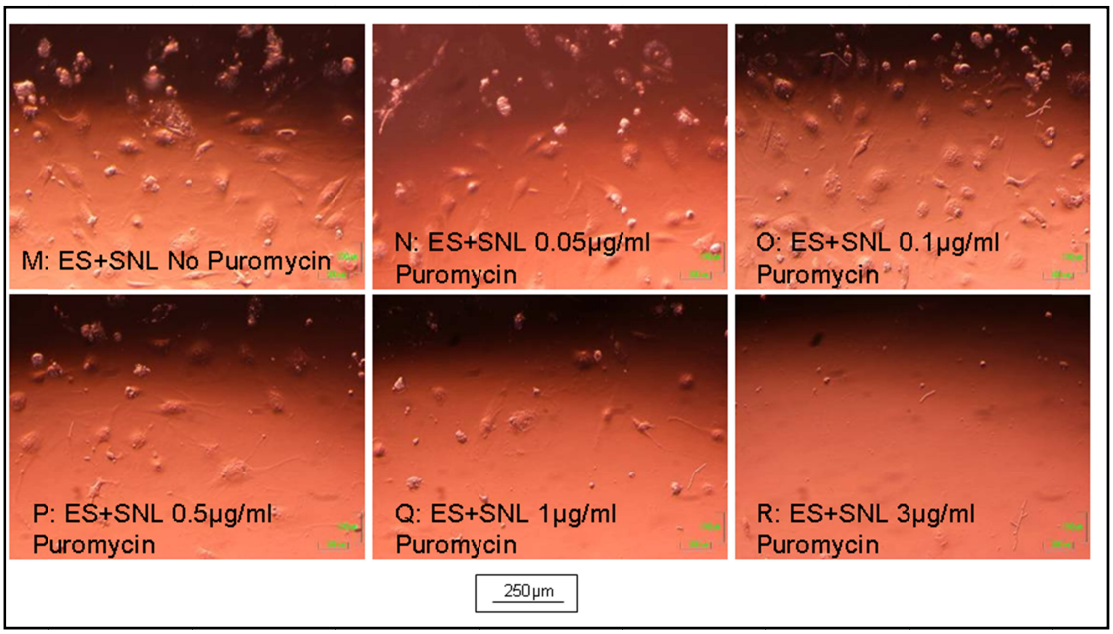


Figure 4.3.7iii: Kill curve. Light microscopy images showing mES cells on a feeder layer of inactivated SNL cell growth at *x* µg/ml Puromycin after 120 hours.

Figure 4.3.7: Kill curve. Light microscopy images showing mES and SNL cell growth at *x* µg/ml Puromycin after 120 hours. mES and inactivated SNL feeder layer cells were cultured alone and mES cells on a feeder layer of inactivated SNL cells.

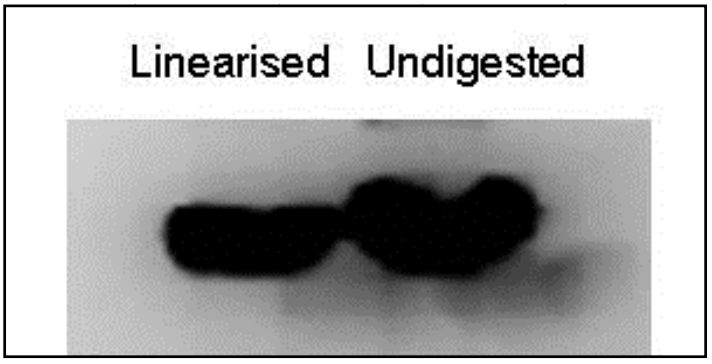


Figure 4.3.8: Linearised plasmid run next to the undigested plasmid on an agarose gel.

4.3.2v Transfection of CEE mES cells with a GFP expressing plasmid - GFP expression following transfection

The transfected cells began to express GFP after around six days following electroporation. After 14 days all the surviving cells showed GFP expression. The cells continued to show GFP expression in normal proliferation culture (Figure 4.3.9A - B) and also after aggregation into embryoid bodies (Figure 4.3.9C - E).

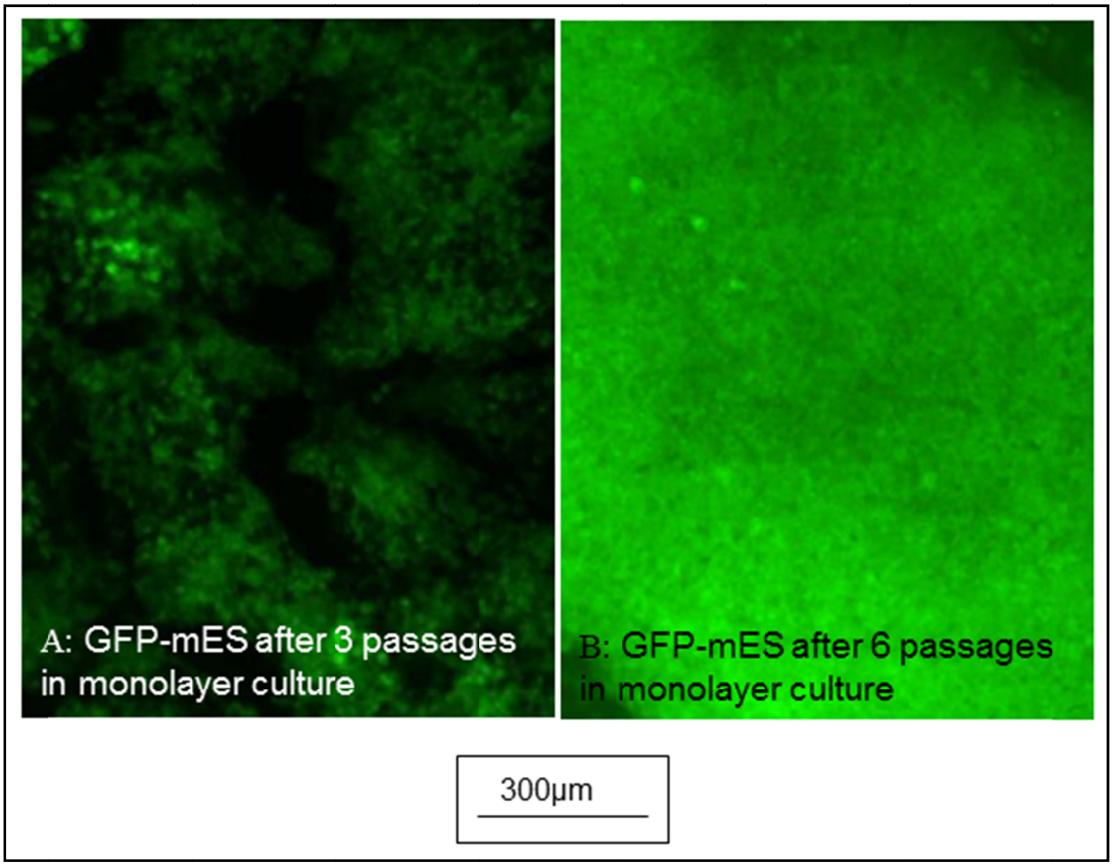


Figure 4.3.9i: Transfected mES cells expressing GFP in monolayer culture after three or six passages. A: GFP-mES after three passages in monolayer culture. B: GFP-mES after six passages in monolayer culture. The cells in panel A were approaching confluence whilst those in panel B had formed a confluent monolayer.

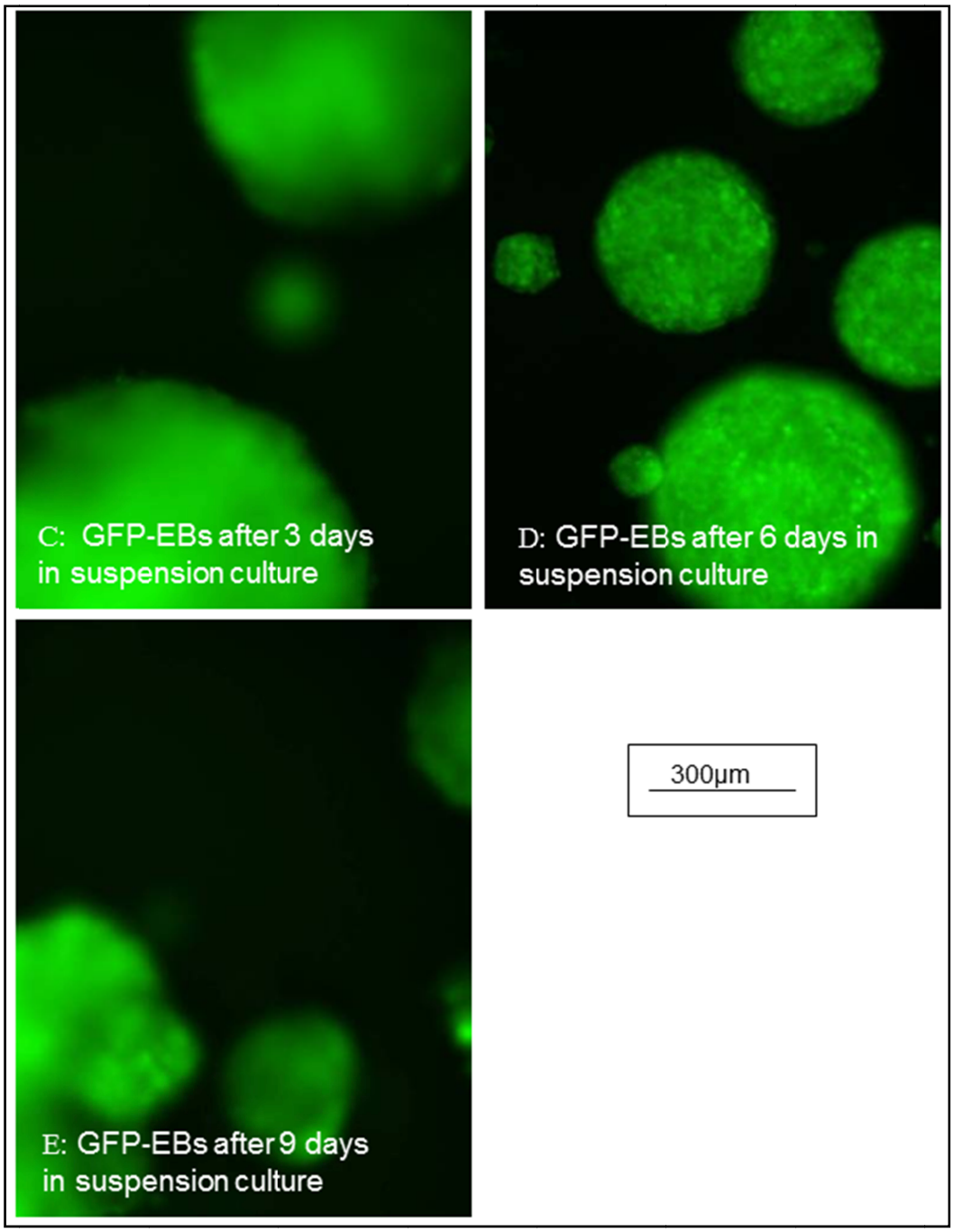


Figure 4.3.9ii: Transfected mES cells expressing GFP following 3, 6 or 9 days in suspension culture after aggregation into EBs. C: GFP-EBs after three days in suspension culture. D: GFP-EBs after six days in suspension culture. E: GFP-EBs after nine days in suspension culture.

4.3.2vi Retention of injected cells in *ex vivo* co-culture

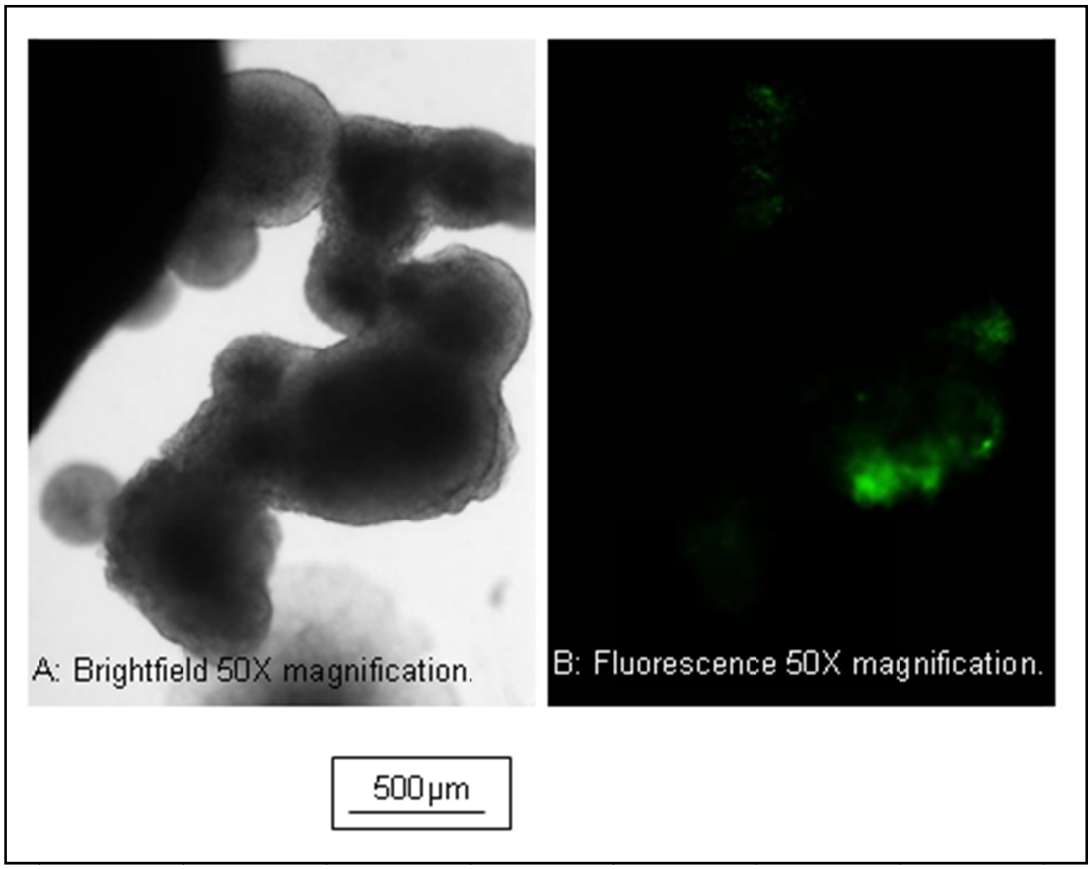


Figure 4.3.10: GFP cells retained in live co-culture with day six embryonic chick gut tissue. Tissue explants were injected with GFP-mES cells and co-cultured. The co-cultures were then examined under a fluorescent microscope. A – Live tissue under Brightfield view at 50X magnification after seven days in *ex vivo* co-culture. B – GFP cells retained in the live tissue under fluorescent view at 50X magnification.

Figure 4.3.10 shows a live gut tissue explant that had been injected with GFP-mES cells and co-cultured for seven days. The brightfield image (Figure 4.3.10A) showed that the tissue had remained intact without significant signs of any (morphological) degradation other than a roughening of the explant surface supporting observations in Figure 4.3.3. Media changes were required with increased frequency when compared to the tissue only cultures, possibly because of the extra requirements of the additional injected cell population. The fluorescent image (Figure 4.3.10B) showed the positive

GFP signal from the GFP-mES cells. The positive signal coming from the tissue explant demonstrated that (at least some of) the GFP expressing cells that were injected had been retained throughout the time course of the co-culture. Although there was one area of strong positive signal in the centre of the explant there were also other weaker areas of GFP signal at the 'top' and 'bottom' of the explant as it appears in the image. This suggested that portions of the injected population of cells had migrated away from the initial site of injection. In the live imaging of the co-culture it was impossible to visualise the interior of the tissue explant so only GFP positive cells near the surface were visible in this image.

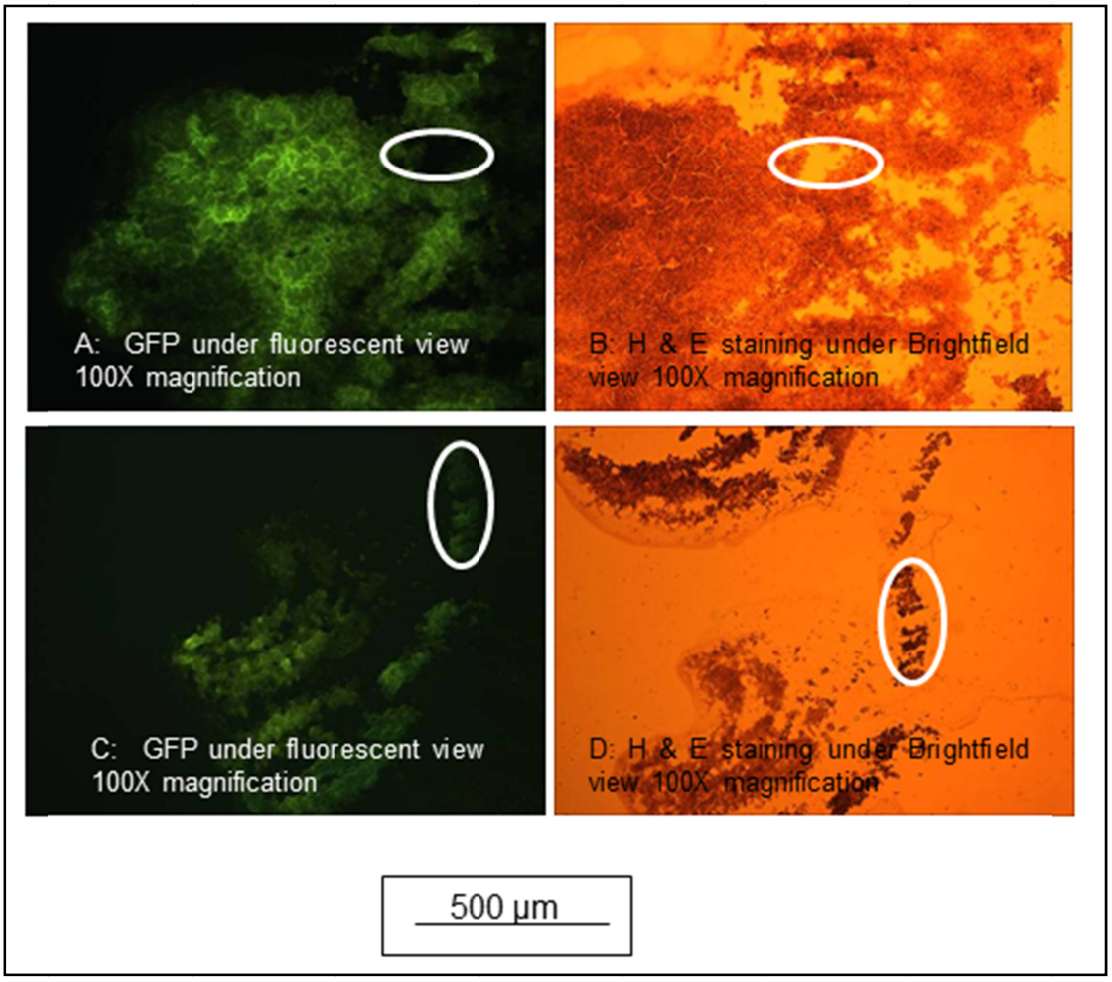


Figure 4.3.11: Representative images from 5 µm cryo-sections of GFP-mES/day six embryonic chick gut tissue co-cultured for six days. A and C - GFP cells under fluorescence at 100X magnification. B and D – H & E staining to show tissue present in sections under Brightfield at 100X magnification

Figure 4.3.11 shows cross sections of co-cultures that had been fixed, cryo-preserved/embedded and sectioned following seven days in culture. For each sample two images are displayed; the left hand images (Figure 4.3.11A and C) are fluorescent images scanning for GFP, the right hand images (Figure 4.3.11B and D) are H & E stains to show the extent of the tissue on the sample slide. Whilst in Figure 4.3.10 above only GFP signal from cells on or near the surface of the explants is detected these sections show the extent to which the GFP positive mES cells were distributed within the chick tissue. The fluorescent and H and E images are slightly offset but the main features can be seen in both images (circled).

4.3.3 Co-culture of naïve and Act-A treated GFP-mES cells with intact chick gut tissue explants (Batches A, B and C)

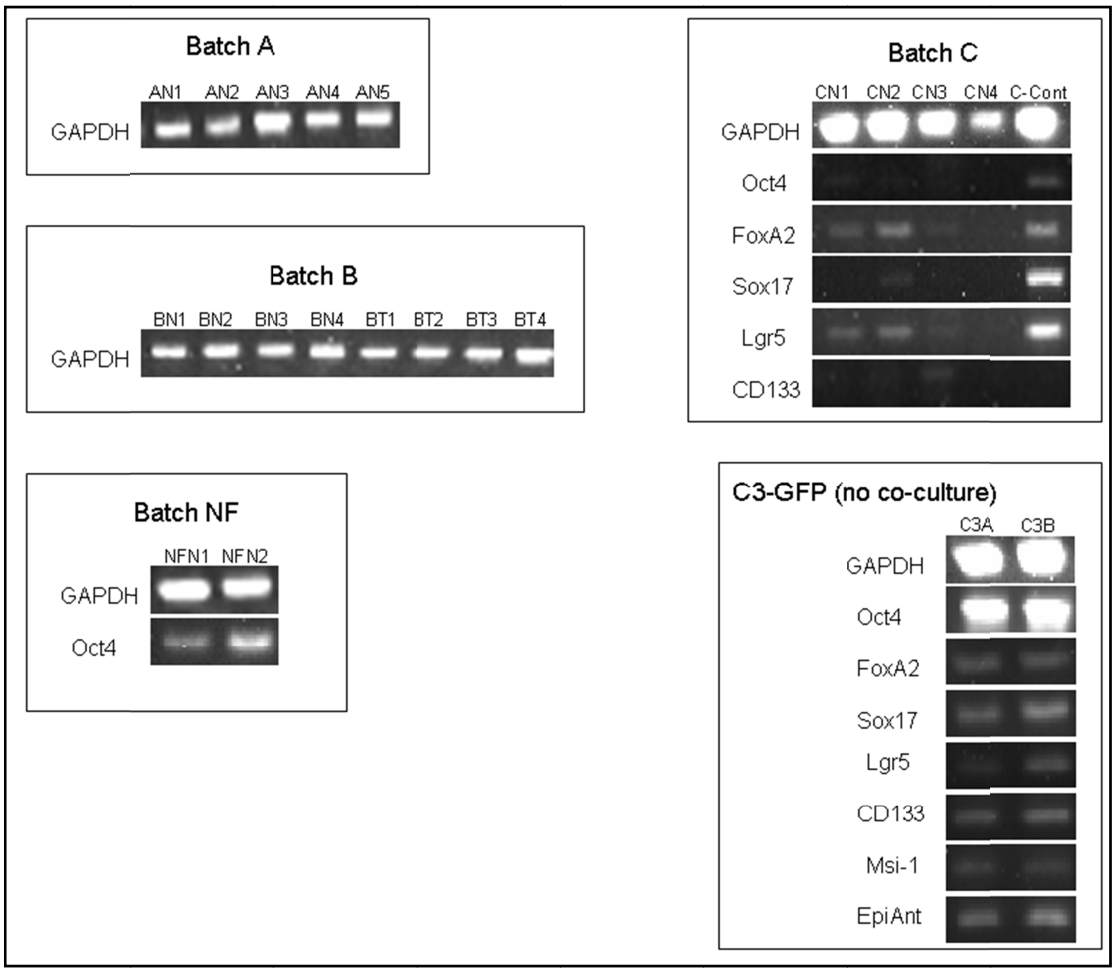


Figure 4.3.12: RT-PCR analysis of the recovered cells (following FACS sorting) for markers of early intestinal fate. Expression in naive mES cells, following *in vitro* differentiation culture & following co-culture (of naïve & pre-treated cells) with day six embryonic chick gut tissue. Only markers where expression was observed are included

Figure 4.3.12 shows the results obtained from the first three co-culture experiments (batches A - C) plus control conditions. These batches were carried out using either naïve GFP-mES cells or GFP-mES cells that had been pre-treated with Act-A (treatment based on condition three in Section 3.2.1v and 3.3.5 consisting of two days in monolayer followed by a five day EB phase with a further nine days in monolayer in Act-A+, 10% (*v/v*) FCS media throughout). In

Batch A naïve GFP-mES cells were injected into six intact embryonic chick gut tissue explants and co-cultured for seven days. In Batch B naïve GFP-mES cells were injected into six intact embryonic chick gut tissue explants and co-cultured for seven days whilst GFP-mES cells that had been aggregated into EBs and Act-A treated *in vitro* were injected into a further six intact embryonic chick gut tissue explants and co-cultured for seven days. In Batch C naïve GFP-mES cells were injected into six intact embryonic chick gut tissue explants and co-cultured for seven days. In both batches A and B expression of *GAPDH* was observed but the bands produced were weaker than were produced in most reactions. There were no bands produced with any other primer pair which may have been a reflection of the poor quality of the RNA samples generated post-FACS or may simply have been because none of the selected markers were expressed.

4.3.4 Co-culture of naïve and Act-A treated GFP-mES cells with intact chick gut tissue explants or dissociated cells derived from the same (Batch D)

Figure 4.3.13 shows the results of the RT-PCR analysis of the RNA samples generated from co-culture experiment (batch) D. Naïve GFP-mES cells were injected into five intact embryonic chick gut tissue explants whilst GFP-mES cells that had been aggregated into EBs and Act-A treated *in vitro* (treatment based on condition three in Section 3.2.1v and 3.3.5 consisting of two days in monolayer followed by a five day EB phase with a further nine days in monolayer in Act-A+, 10% (v/v) FCS media throughout) were injected into a further four intact embryonic chick gut tissue explants and co-cultured for seven days. Naïve GFP-mES cells were also used to set up five co-cultures with cells derived from embryonic chick gut tissue explants and co-cultured for seven days whilst GFP-mES cells that had been aggregated into EBs and Act-A treated *in vitro* were used to set up four co-cultures with cells derived from embryonic chick gut tissue explants and co-cultured for seven days.

Figure 4.3.13A shows the results from GFP-mES cells co-cultured with intact tissue explants, Figure 4.3.13B shows the results of GFP-mES cells cultured with

dissociated embryonic chick gut cells whilst Figure 4.3.13C shows the control mES cells that had not been co-cultured. In both the intact explants and dissociated cell experiments samples one and two were with Act-A treated cells whilst samples three - five were with naïve GFP-mES cells. The 'scores' assigned to these bands are summarised in tabular form in Figure 6.3.6 in Section 6.3.

No significant expression was observed for any of the markers in the chick tissue controls with the exception of *GAPDH*. The sequence homology between species is higher for *GAPDH* than for the marker genes used so some expression was not unexpected. The expression of *GAPDH* in the chick tissue controls was noticeably weaker than in other samples. The exception is control sample B1 where the *GAPDH* expression was stronger (& weak expression is observed in a number of the marker genes). This was probably due to contamination with murine cells as the other two controls showed no expression of the markers. *Oct4* expression was observed in all the samples but at relatively low levels. The levels of *Oct4* expression were reduced in the experimental samples when compared to the controls. Moderate expression of *FoxA2* was seen in all the samples but expression of *Sox17* was less consistent. *FoxA2* expression was comparable between the intact explant and dissociated cell co-cultures but *Sox17* expression was stronger in the dissociated cell samples. Moderate expression of *Brachyury* was seen in all the samples but was stronger in the dissociated cell samples than the intact explant samples. There was no expression of *CXCR4* or *Nestin* in either condition.

Moderate expression of *Lgr5* was seen in both conditions. There was weak expression of *CD133* and *Epi Ant* in all the samples with the dissociated cell samples showing stronger bands than the intact explants. There was no expression of *Msi-1* in either condition. None of the ISC markers selected showed any expression in the controls.

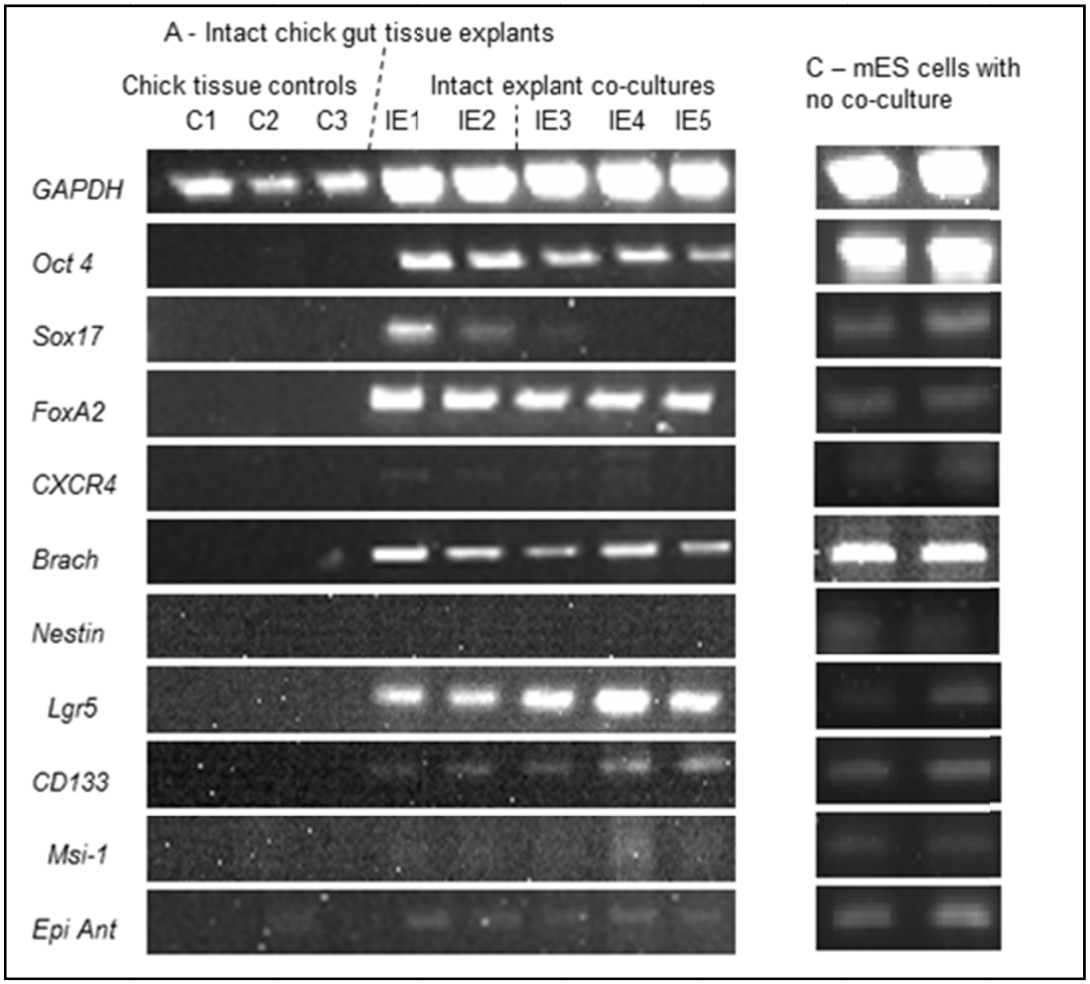


Figure 4.3.13A: Expression of germ layer and intestinal stem cell markers in A - GFP-mES cells co-cultured with intact (day six) embryonic chick gut explants for seven days. C – GFP-mES cells following *in vitro* Act-A treatment but no co-culture with embryonic chick tissue.

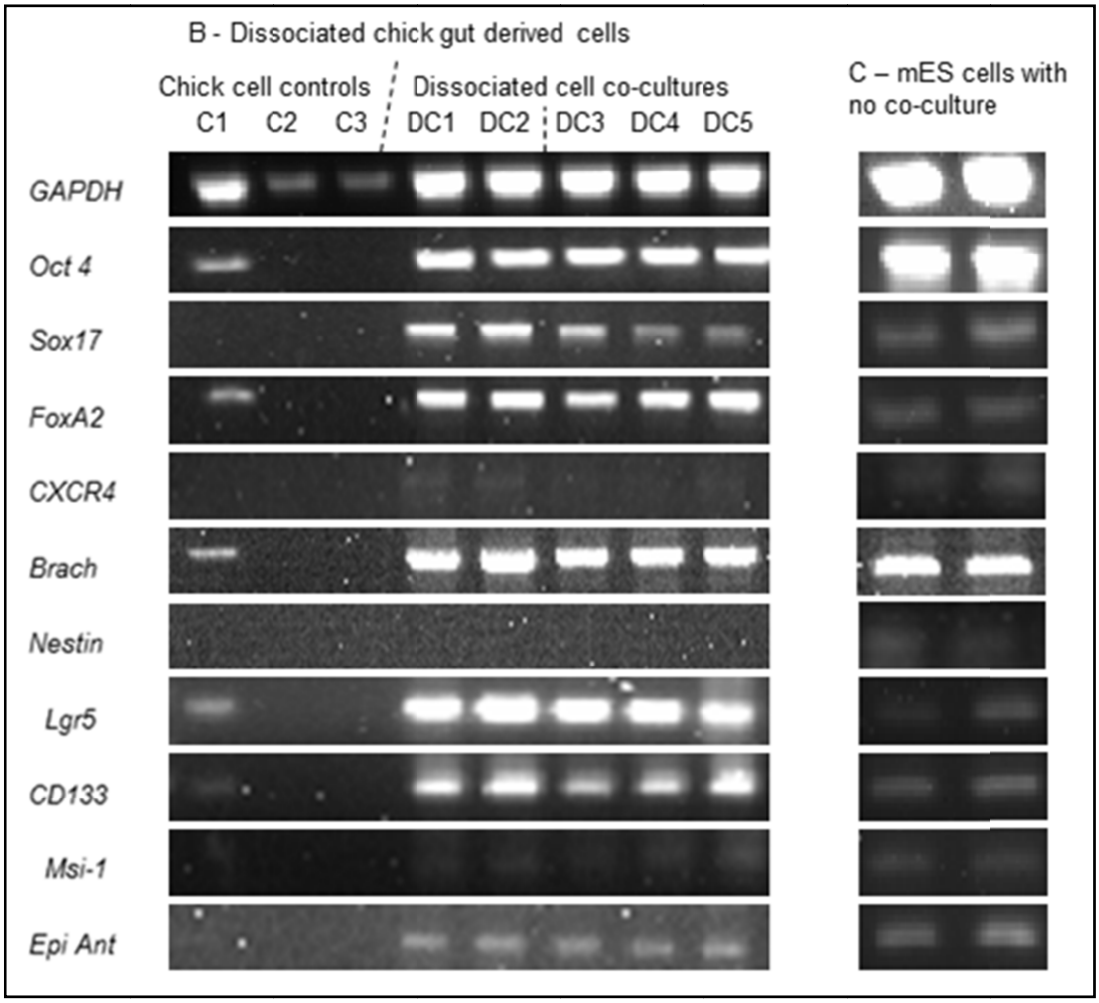


Figure 4.3.13B: Expression of germ layer and intestinal stem cell markers in B - GFP-mES cells co-cultured with dissociated (day six) embryonic chick gut tissue cells for seven days. C – GFP-mES cells following *in vitro* Act-A treatment but no co-culture with embryonic chick tissue.

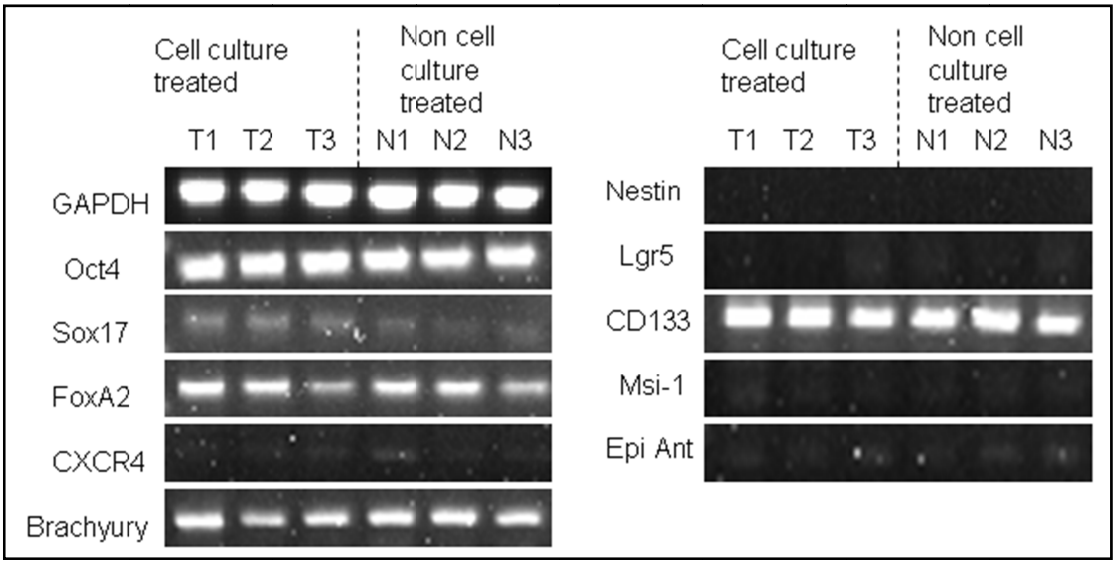


Figure 4.3.14: Expression of germ layer and intestinal stem cell markers in GFP-mES cells cultured without any embryonic chick gut material present. Cultured on cell culture treated (T) and non-cell culture treated (N) plastic six well plates in CEE mES complete media (LIF-).

Figure 4.3.14 shows the expression of key marker genes in mES cells cultured for seven days in six well culture plates in the absence of any chick gut material. Both TCP (samples T1 - 3) and non TCP (samples N1 - 3) plates were used to ensure that switching from TCP vessels to non-TCP vessels had not caused any changes in the expression profile of the cells.

All the samples produced strong *GAPDH* bands indicating that all of the RT reactions had worked well. Moderate *Oct4* expression was observed in all the samples. *FoxA2* and *Brachyury* were expressed in all samples as was *Sox17* at very low levels. There was no expression of *CXCR4* or *Nestin*. *CD133* was strongly expressed in all the samples. *Lgr5* was not expressed in either of the control samples. Neither *Msi-1* nor *Epithelial Antigen* were expressed to any significant degree (although very faint bands may be present in some of the samples). This data is summarised in table 6.3.6B in Section 6.3.

4.3.5 Co-culture of naïve GFP-mES cells with intact chick gut tissue explants (Batches E and F)

4.3.5i Marker gene expression of GFP-mES cells following co-culture with intact chick gut tissue explants evaluated by RT-PCR

Figure 4.3.15 shows the results from the RT-PCR analysis of the RNA samples generated in co-culture experiments (batch) E (Figure 4.3.15A) and F (Figure 4.3.15B). In Batch E naïve (unlabelled) mES cells were injected into nine intact embryonic chick gut tissue explants and co-cultured for 7 days. In Batch F naïve (unlabelled) mES cells were injected into nine intact embryonic chick gut tissue explants and co-cultured for six days. Control samples from mES cells that had not been co-cultured are also shown (Figure 4.3.15C).

GAPDH expression was seen in the two chick tissue controls but at a lower level than in the samples. This was believed to be due to the especially high degree of inter-species sequence homology for this gene (as detailed above). No other markers were expressed in the controls.

Oct4 expression was seen in every sample however it was reduced in the experimental samples when compared to the controls. Also *Oct4* expression was higher in batch E than in batch F. Strong expression of *FoxA2* but no expression of *Sox17* was apparent in batch E whilst in batch F *FoxA2* expression was weaker but *Sox17* was also weakly expressed. Very weak bands for *Sox17* and *FoxA2* were just visible in the controls. Moderate expression of *Brachyury* was present in both conditions. Weak expression of *Nestin* was observed batch E but was not present in batch F. There was no expression of *CXCR4* in either condition.

Moderate expression of *Lgr5* was present in both batches as was strong expression of *CD133*. There was no expression of *Msi-1* in batch E but weak expression was observed in batch F. There was no expression of *Epi Ant* in either condition. The ISC markers were not expressed in the controls.

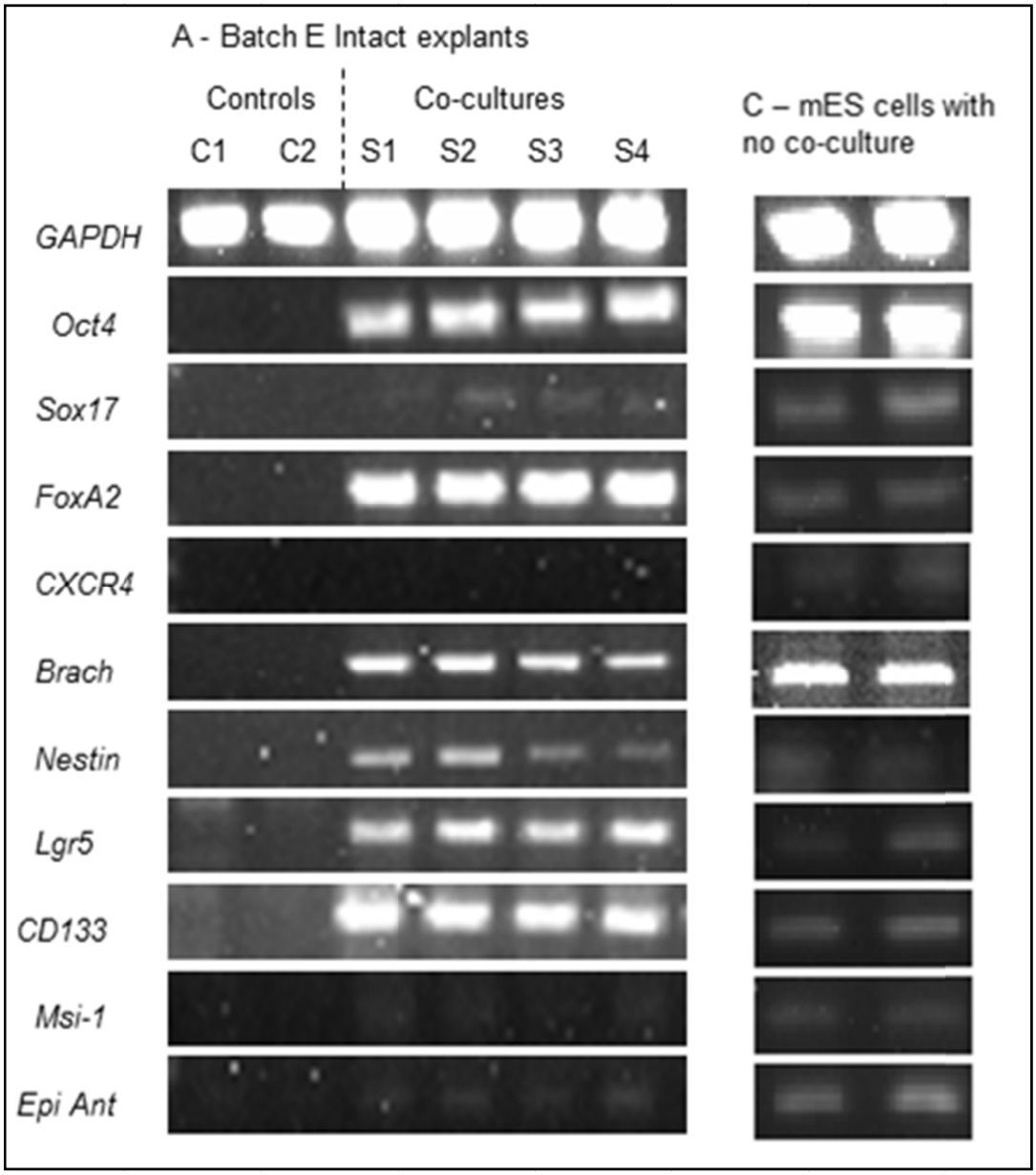


Figure 4.3.15A: The expression of germ layer and intestinal stem cell markers in A - mES cells co-cultured (batch E) with intact (day six) embryonic chick gut tissue explants for seven days evaluated by RT-PCR. C –mES cells that had been aggregated and Act-A treated *in vitro* but had not been co-cultured with embryonic chick tissue evaluated by RT-PCR.

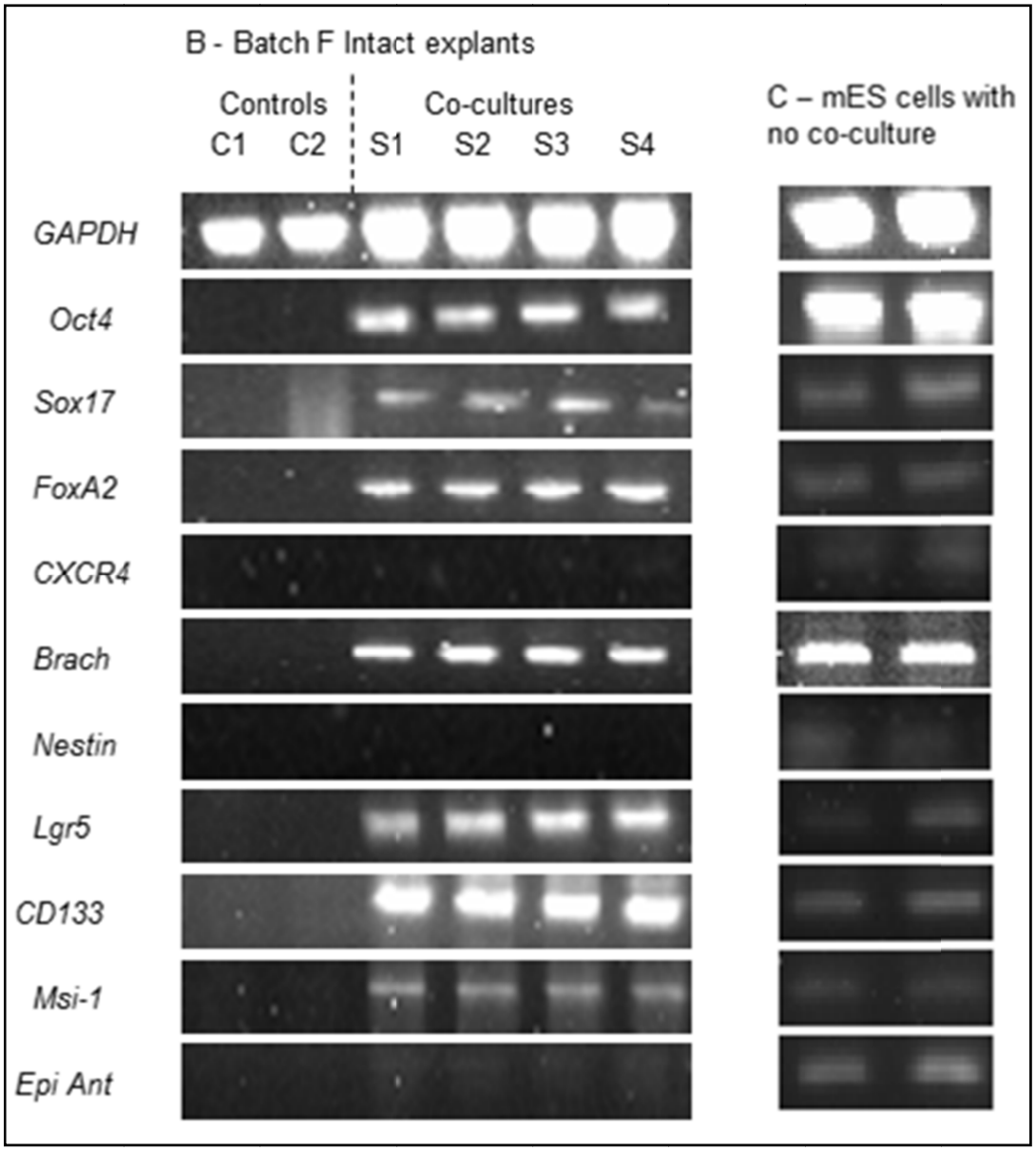


Figure 4.3.15B: The expression of germ layer and intestinal stem cell markers in B - mES cells co-cultured (batch F) with intact (day six) embryonic chick gut tissue explants for six days evaluated by RT-PCR. C –mES cells that had been aggregated and Act-A treated in vitro but had not been co-cultured with embryonic chick tissue evaluated by RT-PCR.

4.3.5ii Marker protein expression of GFP-mES cells following co-culture with intact chick gut tissue explants evaluated by immunocytochemistry

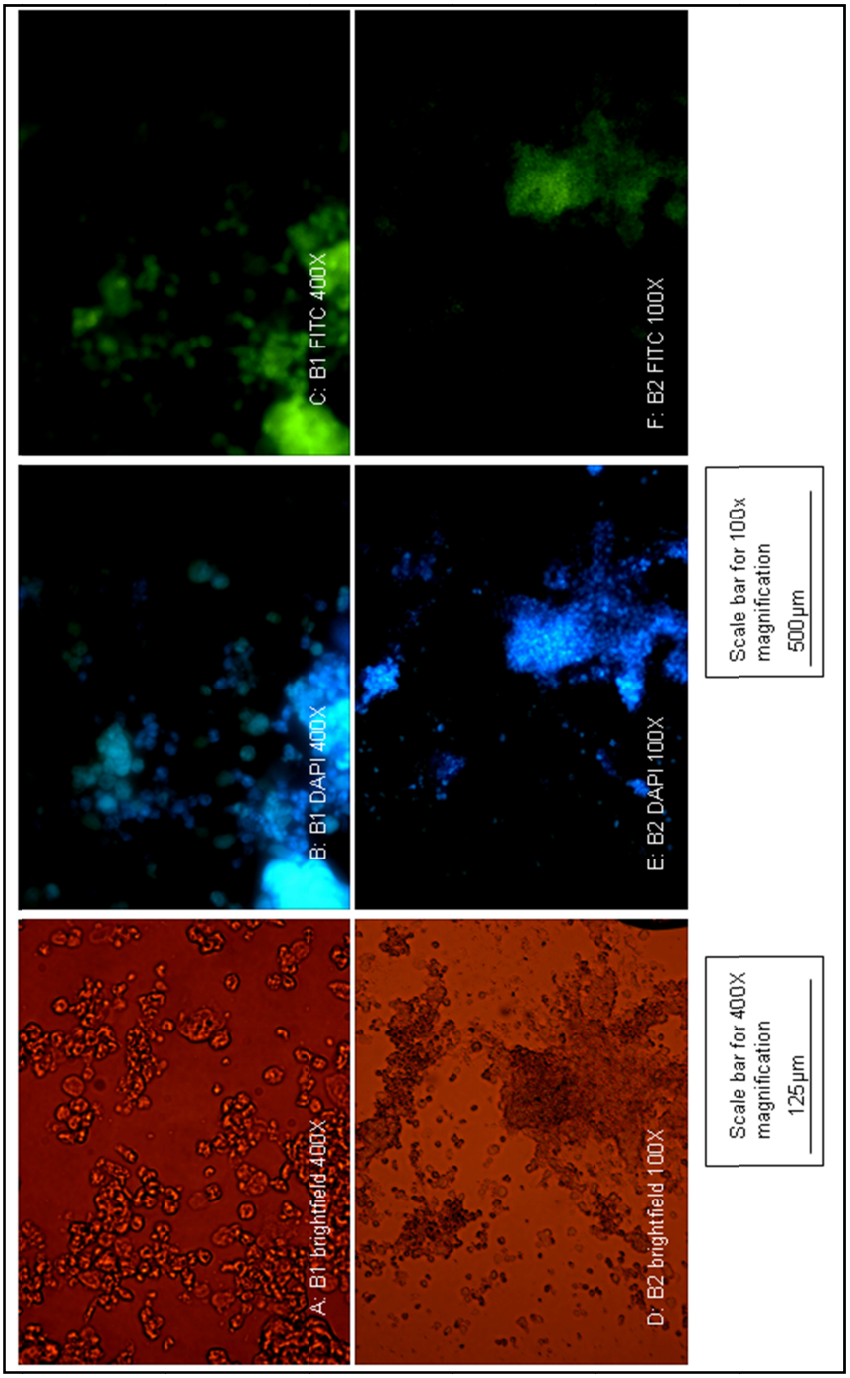


Figure 4.3.16i: Representative LGR5 expression in Batch E samples determined by fluorescence immunohistochemistry. A – Sample B1, Brightfield 400X magnification. B – Sample B1, DAPI 400X. C – Sample B1, FITC 400X. D – Sample B2, Brightfield 100X. E – Sample B2, DAPI 100X. F – Sample B2, FITC 100X.

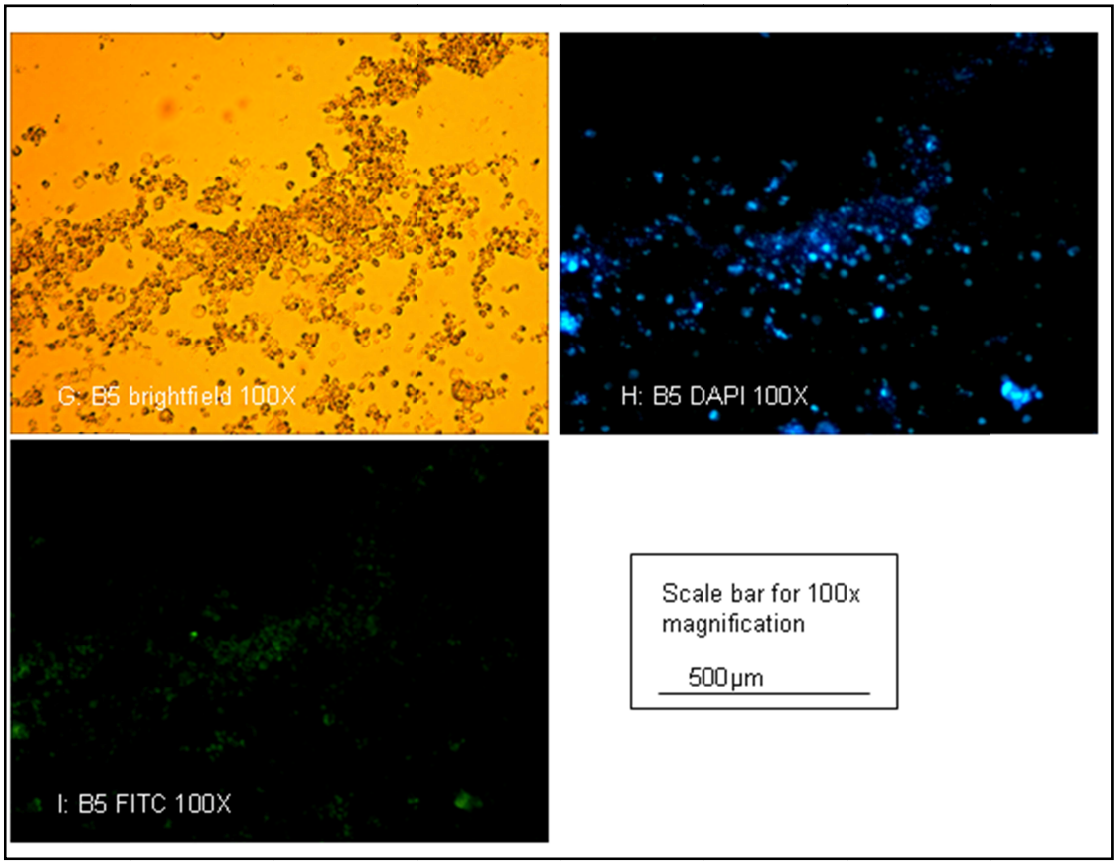


Figure 4.3.16ii: Representative LGR5 expression in batch E samples determined by fluorescence immunohistochemistry. G – Sample B5, Brightfield 100X. H – Sample B5, DAPI 100X. I – Sample B5, FITC 100X.

Figure 4.3.16i and 4.3.16ii show the results of the immunofluorescent staining of sample slides prepared from co-culture experiment (batch) E (unlabelled, naive mES cells injected into chick gut tissue explants and cultured for seven days) with an anti-LGR5 antibody. For each sample there is a brightfield image to show the extent of cell coverage on the slide, a nuclear DAPI stain and a FITC image showing the extent of LGR5 protein expression. Figures 4.3.16i and 4.3.16ii (panels A – I) show experimental samples, Figure 4.3.16iii (panels J – L) shows positive control samples with Figure 4.3.16iii (panels M – O) showing secondary antibody controls.

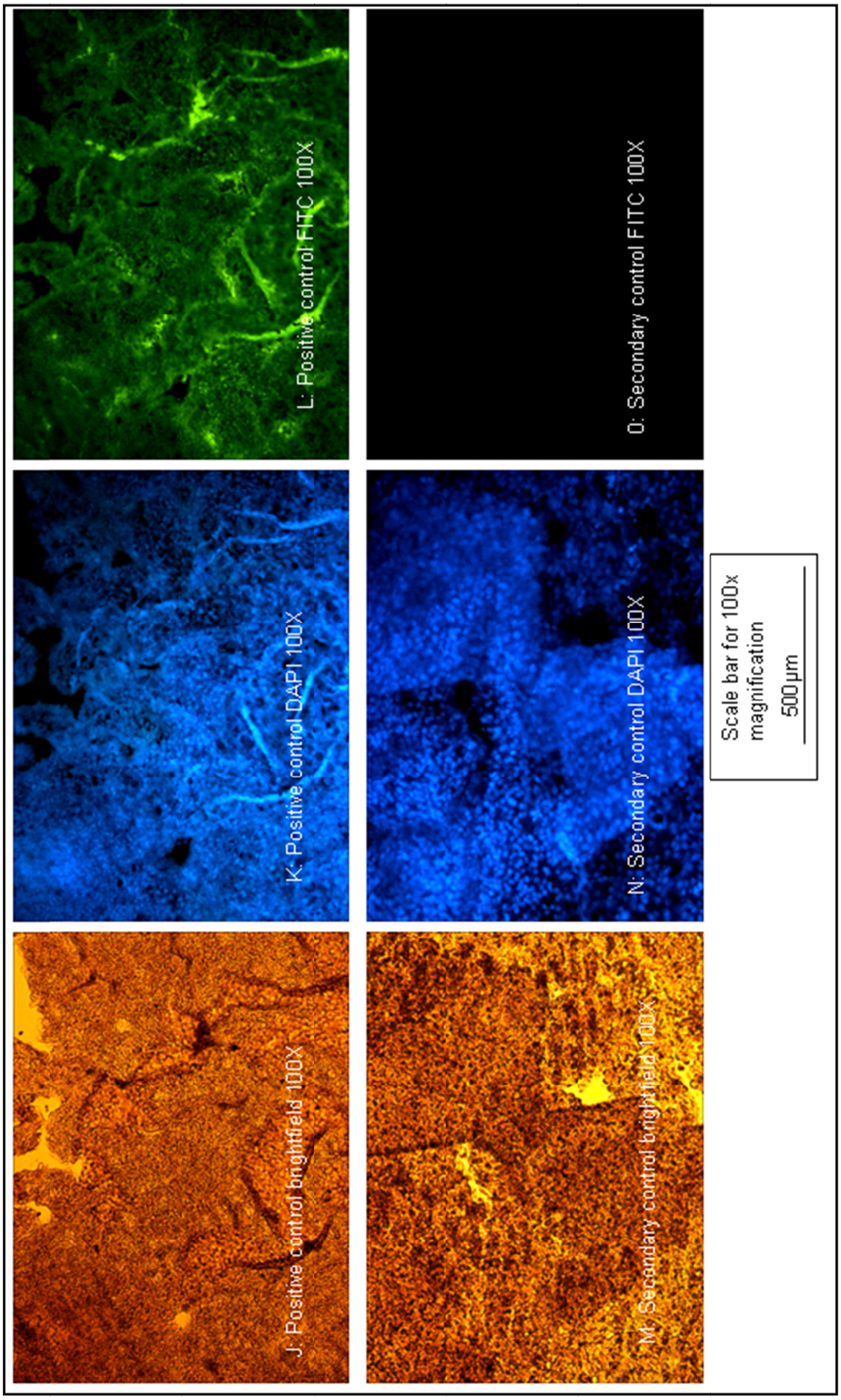


Figure 4.3.16iii: Representative positive control LGR5 staining of wax section from a (day 9.5) mouse embryo. J – Brightfield 100X, K – DAPI 100X, L – FITC 100X. Secondary antibody control staining. M – Brightfield 100X, N – DAPI 100X, O – FITC 100X.

In sample B1, where naïve mES cells had been co-cultured with an intact embryonic chick gut tissue explant for seven days, (Figure 4.3.16i, panels A - C)

the brightfield image showed a good number of cells in the field of view with these cells mostly being found in clusters. The clusters of cells in the bottom left hand quadrant gave strong FITC signal and those in the top left hand quadrant gave moderate signal indicating the presence of LGR5 protein. Although LGR5 expression was extensive it was not expressed in every cell.

In sample B2, where naïve mES cells had been co-cultured with an intact embryonic chick gut tissue explant for seven days, (Figure 4.3.16i, panels D - F) the brightfield image (at lower magnification than B1) showed large clusters of cells. The large cluster of cells in the bottom right hand quadrant gave a moderate FITC signal indicating the presence of LGR5 protein but the other clusters of cells did not appear to express LGR5 protein (at a detectable level).

In sample B5, where naïve mES cells had been co-cultured with an intact embryonic chick gut tissue explant for seven days, (Figure 4.3.16ii, panels G - I) the brightfield image showed that most of the cells in this sample were towards the centre of the field of view. The FITC signal was weaker than the previous samples. This could have reflected lower levels of LGR5 protein expression or may have been an experimental or imaging artefact.

The positive control samples (Figure 4.3.16iii, panels J - L) were sections of embryonic mouse intestinal tissue mounted on a slide and stained with anti-LGR5 antibody. The brightfield and DAPI images showed that almost all the field of view contained tissue. Strong FITC signal was obtained across the sample with some very strong signal in some areas. In the secondary control sample there were large numbers of cells present as illustrated by the brightfield image (Figure 4.3.16iii, panel M) and the DAPI stain (Figure 4.3.16iii, panel N) but no FITC signal was present indicating that there was no non-specific binding of the antibodies and therefore no false positive signal in the experimental samples.

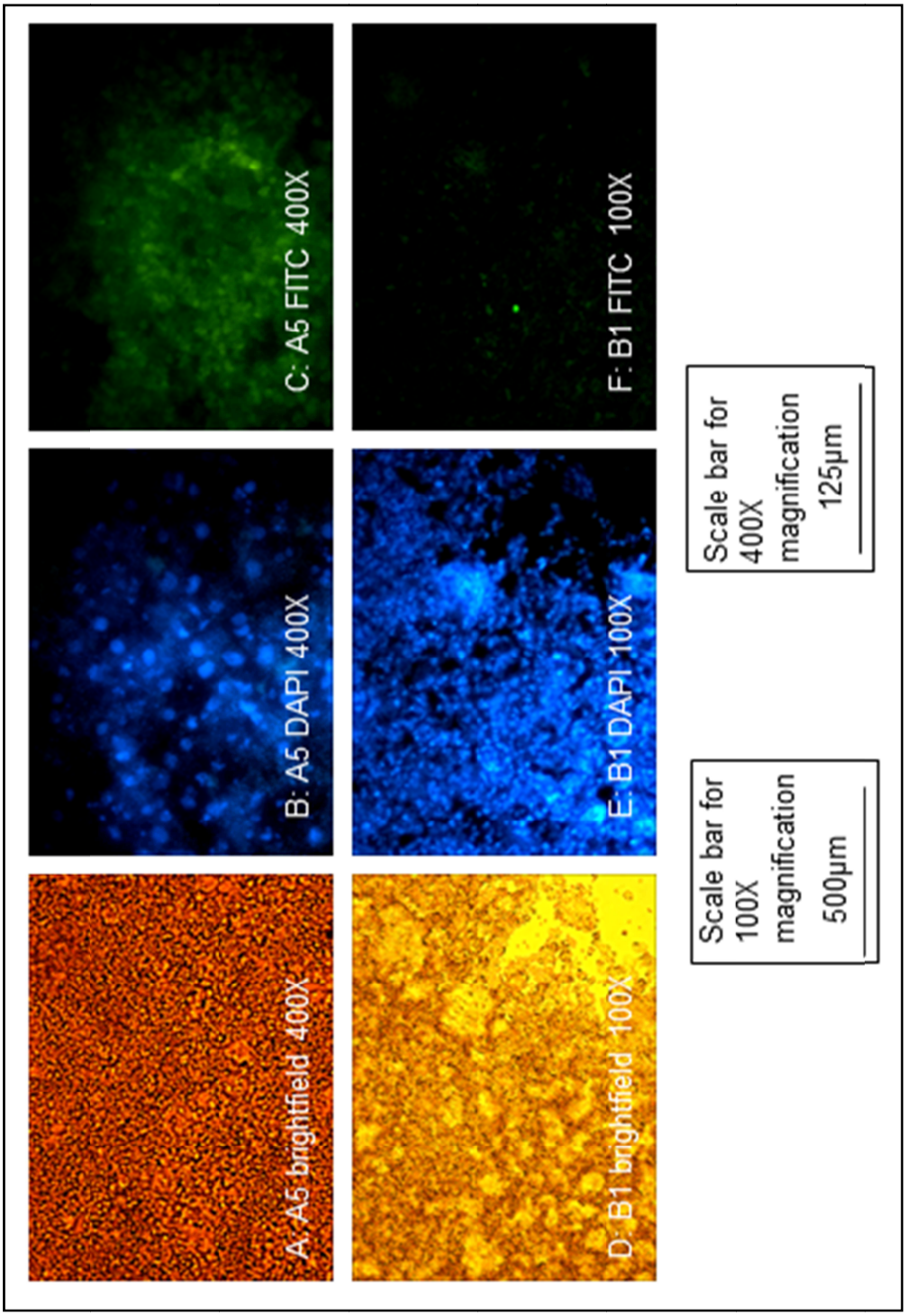


Figure 4.3.17i: Representative Lgr5 expression from batch F samples determined by fluorescence immunohistochemistry. A – Sample A5, Brightfield 400X magnification. B – Sample A5, DAPI 400X. C – Sample A5, FITC 400X. D – Sample B1, Brightfield 100X magnification. E – Sample B1, DAPI 100X. F – Sample B1, FITC 100X.

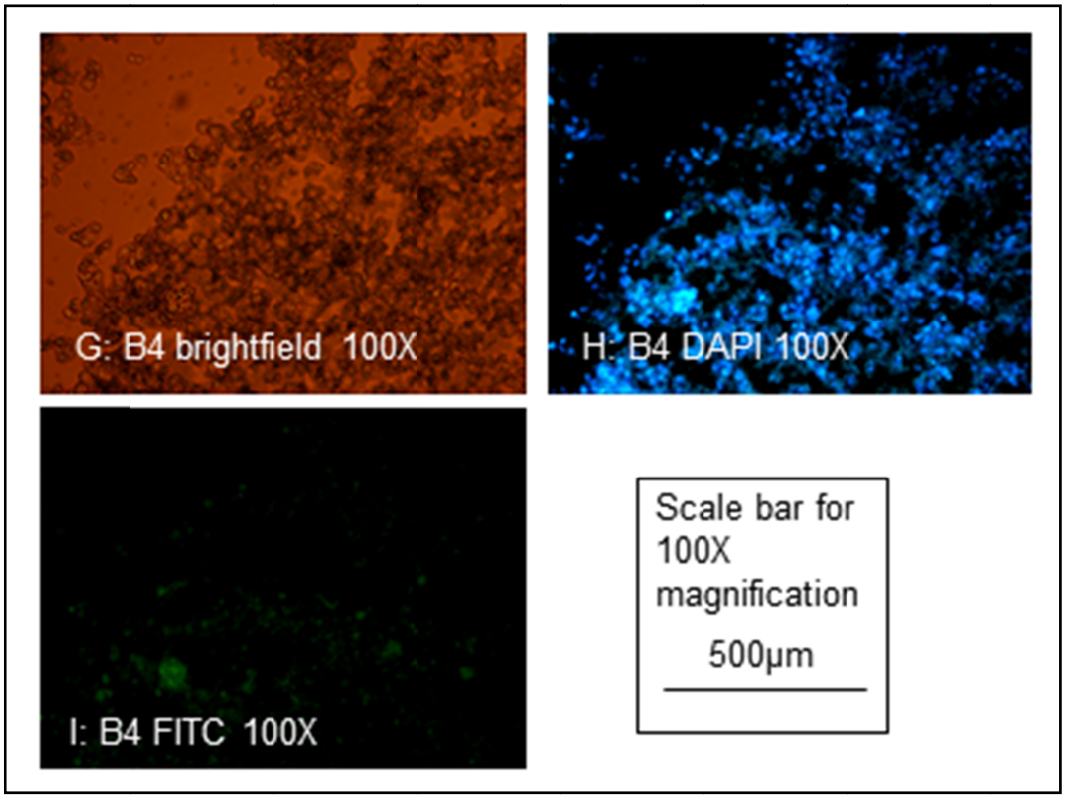


Figure 4.3.17i: Representative Lgr5 expression from batch F samples determined by fluorescence immunohistochemistry. G – Sample B4, Brightfield 100X magnification. H – Sample B4, DAPI 100X. I – Sample B4, FITC 100X.

Figure 4.3.17 shows the results of the immunofluorescent staining of sample slides prepared from co-culture experiment (batch) F (unlabelled mES cells injected into chick gut tissue explants and cultured for seven days) with an anti-LGR5 antibody. For each sample there is a brightfield image to show the extent of cell coverage on the slide, a nuclear DAPI stain and a FITC image showing the extent of LGR5 protein expression. Figures 4.3.17A - I show experimental samples, Figures 4.3.17J - L show positive control samples with Figures 4.3.17M - O showing secondary antibody controls.

In sample A5, where naïve mES cells had been co-cultured with an intact embryonic chick gut tissue explant for six days, (Figure 4.3.17A - C) the field of view was entirely filled with cells. A large number of the cells showed moderate FITC signal indicating LGR5 expression.

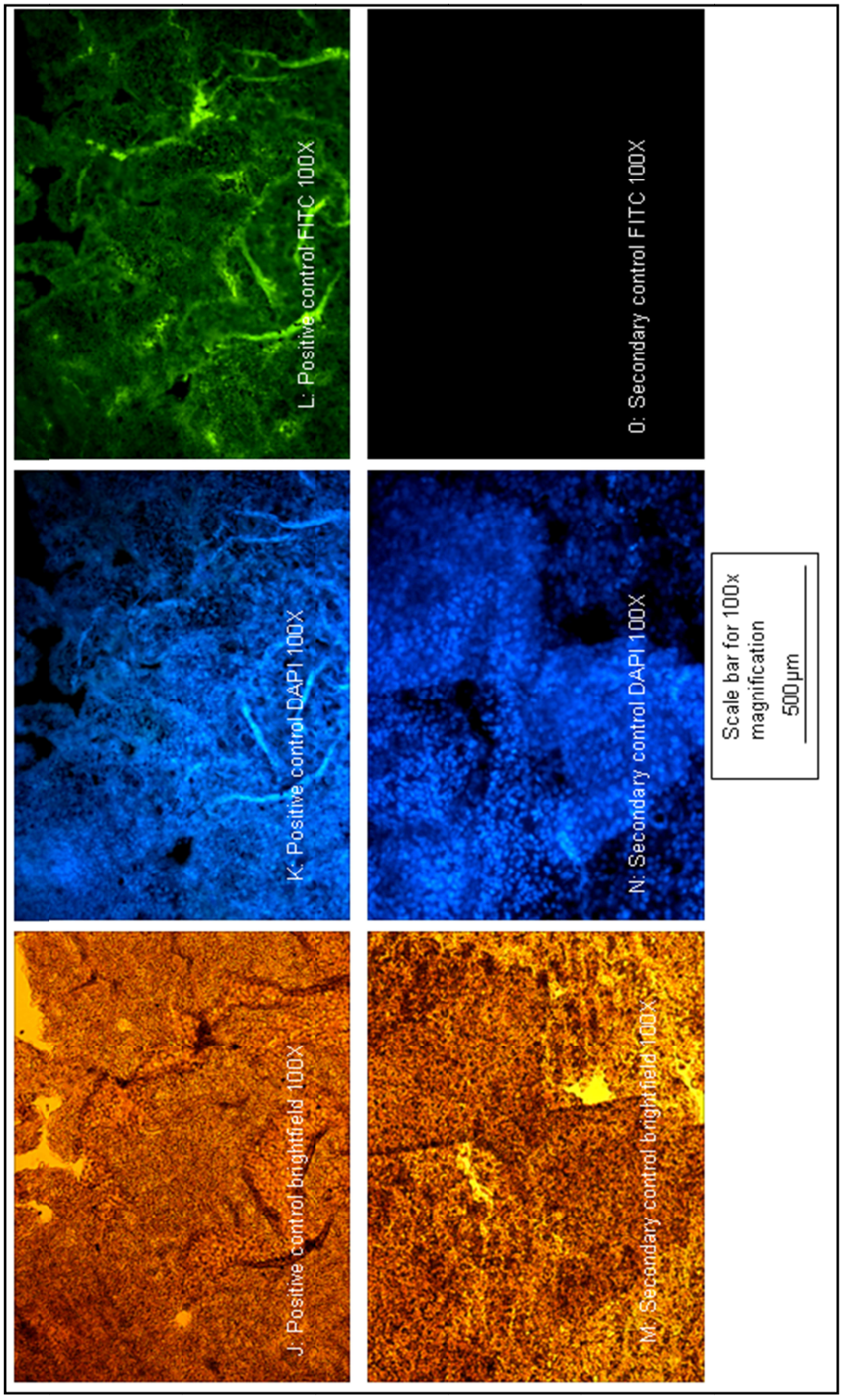


Figure 4.3.17iii: Representative positive control LGR5 staining of wax section from a (day 9.5) mouse embryo. J – Brightfield 100X, K – DAPI 100X, L – FITC 100X. Secondary antibody control staining. M – Brightfield 100X, N – DAPI 100X, O – FITC 100X.

In sample B1, where naïve mES cells had been co-cultured with an intact embryonic chick gut tissue explant for six days, (Figure 4.3.17D - F) the field of

view was also entirely filled with cells. There were a number of small patches of low FITC signal generally to the left hand side of the image (Figure 4.3.17F) indicating areas where LGR5 protein was expressed.

In sample B4, where naïve mES cells had been co-cultured with an intact embryonic chick gut tissue explant for six days, (Figure 4.3.17G - I) the field of view was largely full of cells, although there were none in the top left hand corner. There were a number of patches of low FITC signal in the bottom half of the image (Figure 4.3.17I) indicating areas of LGR5 protein expression.

The three experimental samples shown all exhibited some expression of LGR5 protein however there was considerable variation in the apparent distribution and strength of this expression with sample A5 showing stronger expression than the other two samples.

The positive control sample (Figure 4.3.17J - L) was a section of embryonic mouse intestinal tissue mounted on a slide and stained with anti-LGR5 antibody. The brightfield image shows that almost all the field of view contained tissue. Strong FITC signal was obtained across the sample with some very strong signal in some areas. In the secondary control sample there were large numbers of cells present as illustrated by the brightfield image (Figure 4.3.17M) and the DAPI stain (Figure 4.3.17N) but no FITC signal was present indicating that there was no non-specific binding of the antibodies. Please note that the controls are common to Figures 4.3.16 and 4.3.17.

4.3.5iii Expression of OCT4 and ISC marker LGR5 in mES derived cells following co-culture with embryonic chick gut tissue for six days evaluated by Western blotting

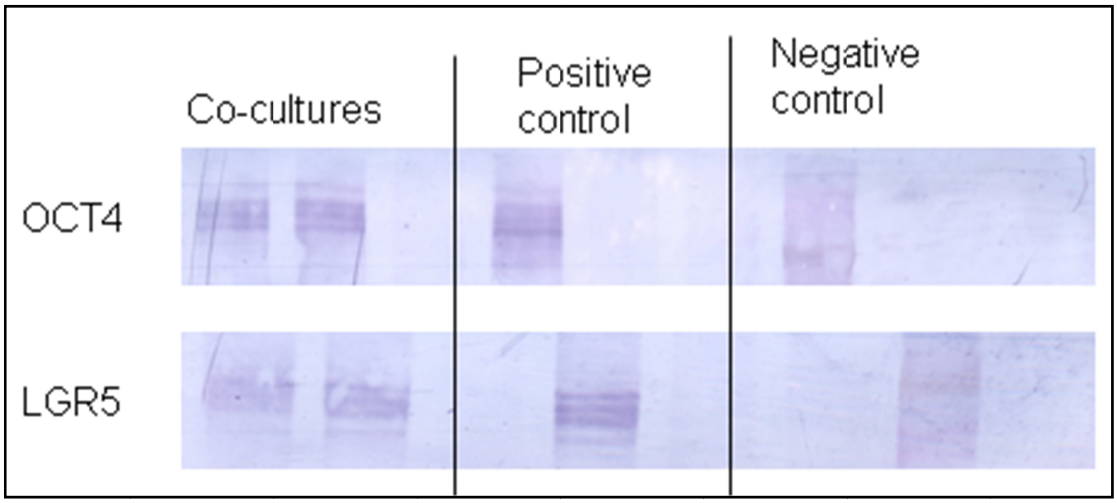


Figure 4.3.18: Expression of OCT4 (38.2 kDa) and ISC marker LGR5 (99.7 kDa) in mES derived cells following co-culture with (day six) embryonic chick gut tissue for six days evaluated by Western blotting. For OCT4 the positive control was protein prepared from undifferentiated mES cells, for LGR5 the positive control was protein prepared from mouse intestine. The same control samples were used in reverse for the negative controls.

Figure 4.3.18 shows the results of western blots using antibodies against the pluripotency marker OCT4 and the ISC marker LGR5 in protein samples prepared from mES derived cells co-cultured with embryonic chick gut tissue explants for seven days (batch F). The negative controls showed no bands for either marker whilst the positive controls gave strong bands for both markers. In the experimental samples OCT4 expression was still present as in the RT-PCR analysis. LGR5 expression was evident in both experimental samples although the bands were not as strong as in the positive control sample.

Figure 4.3.19 (below) shows the expression of DE germ layer markers in samples from batch F evaluated by fluorescence immunohistochemistry. The cells were positive for the expression of FOXA2 and SOX17 but they also retained

expression of the pluripotency marker OCT4. This corresponded with the RNA expression observed in the RT-PCR analysis.

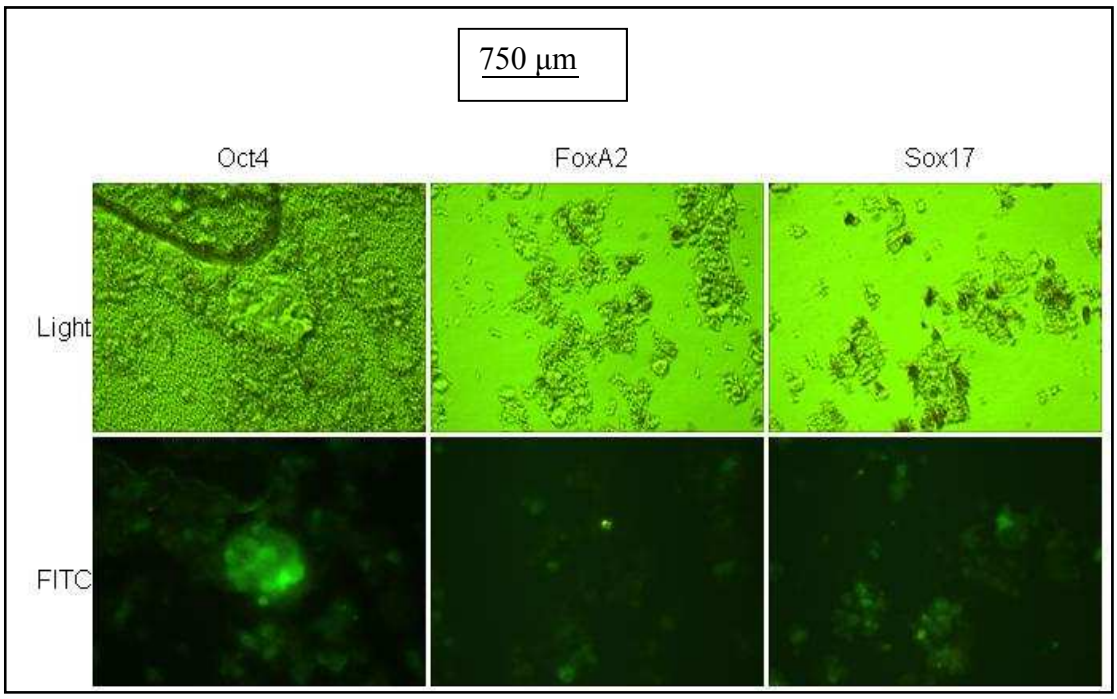


Figure 4.3.19: Representative images illustrating the expression of key (germ layer) markers in mES derived cells following co-culture with (day six) embryonic chick gut tissue for seven days. Cells were fixed and dried on glass slides before being stained by immunofluorescence. Light images (top) show the location of the cells on the slide, fluorescent images showing positive primary FITC antibody staining.

Table 4.3.2: Table summarising all the expression data for the selected differentiation markers from all the co-culture experiments (and controls) detailed above. The selected markers were Oct4 for undifferentiated cells, Lgr5, Msi-1, CD133 and EpiAnt for Intestinal progenitor cells/ISC, Sox17, FoxA2 and CXCR4 for (definitive) Endoderm, Brachyury for Mesoderm and Nestin for Ectoderm. Expression of RNA expression was evaluated by RT-PCR whilst expression of protein expression was evaluated by fluorescence immunocytochemistry (immuno-fluor) and Western blotting.

Marker	Molecule	Method	Experiment					
			4.3.4 Batch D	4.3.4 Batch D	4.3.5 Batch E	4.3.5 Batch F	Control	
			Intact Explants	Dissociated cells	Intact Explants	Intact Explants	mES cells	
Oct4	RNA	RT-PCR	Moderate	Moderate	Moderate	Positive	Moderate	
	Protein	Immuno - fluor	N/A	N/A	N/A	Moderate	N/A	
	Protein	Western	N/A	N/A	N/A	Weak	Weak	
Lgr5	RNA	RT-PCR	Moderate	Moderate	Moderate	Moderate	Negative	
	Protein	Immuno - fluor	N/A	N/A	Positive	Positive	N/A	
	Protein	Western	N/A	N/A	N/A	Weak	N/A	
CD133	RNA	RT-PCR	Very weak	Weak	Strong	Strong	Moderate	
Msi-1	RNA	RT-PCR	Negative	Negative	Negative	Very weak	Negative	
EpiAnt	RNA	RT-PCR	Very weak	Weak	Negative	Negative	Negative	
Sox17	RNA	RT-PCR	Weak	Positive	Negative	Weak	Very weak	
	Protein	Immuno - fluor	N/A	N/A	N/A	Positive	N/A	
FoxA2	RNA	RT-PCR	Moderate	Moderate	Strong	Positive	Positive	
	Protein	Immuno - fluor	N/A	N/A	N/A	Positive	N/A	
CXCR4	RNA	RT-PCR	Negative	Negative	Negative	Negative	Negative	
Brachyury	RNA	RT-PCR	Positive	Moderate	Positive	Positive	Positive	
Nestin	RNA	RT-PCR	Negative	Negative	Weak	Negative	Negative	

Table 4.3.2 shows a summary of the expression of the selected markers from all of the co-culture experiments and selected controls. Where the expression of a particular marker was not assessed it will read N/A. RNA levels are given as negative, where no expression was observed in any samples, then very weak where some very faint bands were seen, then weak where faint bands appeared in most samples. With these samples expression was equivocal to a degree. Values

of positive, where bands were present in (almost) all samples, moderate, where stronger bands were seen and strong, were very bright bands were seen all indicate that the selected marker was definitely expressed. Protein levels are given as negative, weak or positive.

Chapter Four: *Ex vivo* co-culture

4.4 Discussion & Conclusions

4.4.1 Transfection of CEE mES cells with a GFP expressing plasmid

The mES cells were successfully stably transfected with GFP. GFP expression was retained after several passages in monolayer culture and after the cells were aggregated into EBs indicating that GFP expression was likely to be retained if the cells were subjected to the *in vitro* differentiation protocols detailed in Chapter 3 and the *ex vivo* co-culture detailed in this Chapter.

4.4.2 Investigation and optimisation of co-culture conditions

It was established embryonic day six was the optimal point in development at which to extract tissue based upon observations of the size and development of the target tissue. This was supported by previous observations of chick development [Bellairs and Osmond 2005]. Embryonic day six tissue was also used in co-culture experiments in the literature [Sugie et al 2005]. At this point of development the endodermal organs were distinguishable and could be excised. Any earlier in development and the embryo was too small to reliably excise the correct tissue, any later and the early developmental signals might have been lost.

Antibody staining of the excised tissue was positive for the selected intestinal marker, CDX2, indicating that the gut tissue was being excised during the embryo dissections [Traber and Silberg 1996]. The tissue extracted at embryonic day six appeared to remain viable in culture for between six and eight days but some signs of degradation, such as loss of shape and colour changes, were visible at the latter stages of culture. H & E staining revealed the formation of small lesions in the tissue at days six and eight. Live-dead staining showed the tissue remained viable, in part, for up to 25 days. However significant increases in the amount of dead tissue occurred beyond 10 days of culture. Studies in the literature generally used a co-culture period of between four and 12 days [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005]. The tissue explants continued to express the *cdx2*

transcript [Traber and Silberg 1996] after 10 days in *ex vivo* culture although there was some reduction when compared to freshly excised tissue.

When mES cells were injected into embryonic chick gut tissue explants in *ex vivo* culture they were retained in sufficient numbers within the tissue mass for at least seven days to then be analysed for markers of differentiation. Studies in the literature generally used a co-culture period of between four and 12 days [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005] and this combined with the survival time of the tissue explants in *ex vivo* culture a co-culture period of seven days was targeted. It was decided therefore to co-culture CEE mES cells with the excised chick gut tissue for up to seven days.

4.4.3 *Ex vivo* chick tissue and mES cell co-cultures

Although the data generated from batches A - C (naïve and Act-A pre-treated GFP-mES cells co-cultured *ex vivo* with (day six) embryonic chick gut tissue for 7 days) was limited, because of the poor quality RNA samples not producing any bands in the RT-PCR analysis, it was still possible to glean some useful information with regard to planning further experiments. FACS recovered a good number of cells, sufficient to generate RNA samples for analysis. However the process of sorting had a detrimental effect on the viability of the cells; this resulted in very poor samples being generated for analysis and prevented further culture of the cells and as a consequence no meaningful data was generated. This was also true for the control batch of cells that had not been co-cultured prior to FACS.

It was shown that the PCR primers being used in the RNA sample analysis were species specific, with the exception of the highly conserved *GAPDH*, which meant that it would be possible to analyse the mES cells following co-culture without separating them from the chick tissue derived cells; this approach was used for subsequent experiments. Using the existing *GAPDH* primers made it harder to equalise loading in the PCR reactions (because the chick RNA present contributed to the strength of the *GAPDH* bands). However as the samples were used for an endpoint comparison rather than multiple timepoints through the course of the

experiment the data collected still clearly indicated where expression of the selected ISC markers had been initiated.

In co-culture experiment batch D (naïve and Act-A pre-treated GFP-mES cells co-cultured *ex vivo* with either intact embryonic (day six) chick gut tissue explants or dissociated cells derived from the same for 7 days) the lack of expression in the chick only controls confirmed that the primers were species specific and therefore all the expression in the experimental samples was of murine origin eliminating the possibility of false positives. The control cells that were cultured on either TCP plates or non-cell culture treated plates showed no significant differences in marker gene expression suggesting that the non-cell culture treated plates used in the co-culture experiments had no effect on the differentiation of the cells.

In the RT-PCR analysis the germ layer marker expression indicated that DE cell fates were present (*FoxA2* and *Sox17* positive) although mesendoderm/mesoderm (*Brachyury* positive) were also present in all samples (expression was stronger in dissociated cell co-cultures compared with intact explants) [Kubo et al 2004, Tada et al 2005]. This was true for both intact explant and dissociated cell co-cultures. The expression of the ISC marker *Lgr5* [Barker et al 2007] suggested that differentiation towards the intestinal epithelial progenitor had occurred. Expression of *CD133* [Kania et al 2005] and weak expression of *EpiAnt* [Engelhardt et al 1993] also suggested that differentiation towards the desired cell fate was occurring in some cell populations although *Msi-1* expression was not present.

In comparison only *CD133* (of the selected ISC markers) was expressed in the control samples. CD133 is expressed in a range of ASC populations (as detailed in Section 1.2.3) [Yin et al 1997, Corbeil et al 2000, Shmelkov et al 2005, Kordes et al 2007, Samuel et al 2008] and this expression could be accounted for by the random differentiation of the mES cells in the control samples. *Oct4* expression was maintained indicating that some undifferentiated cells may remain or that the differentiating cells continue to express *Oct4*. The level of *Oct4* expression was reduced when compared to the (no co-culture) controls which supports the conclusion that the cells are differentiating. Some expression of Oct4 has been

reported in adult stem cell populations suggesting that Oct4 expression is retained as cells differentiate further [Lee et al 2010].

When the Act-A pre-treated and naïve GFP-mES cells are compared following co-culture it can be seen that the germ layer markers e.g. *FoxA2* and *Brachyury* are expressed at slightly elevated levels in the Act-A treated samples than the naïve cells. This pattern is reversed when examining the ISC marker *Lgr5* [Barker et al 2007] in the intact explants although in the dissociated cells the *Lgr5* and *CD133* bands are slightly stronger in the pre-treated cells. Overall there is no significant difference between the cells treated with Act-A *in vitro* prior to co-culture and naïve mES cells following seven days co-culture with intact chick gut explants or dissociated cells derived from the same.

The RT-PCR analysis of the RNA samples generated in co-culture batches E and F (naïve mES cells co-cultured *ex vivo* with intact embryonic (day six) chick gut tissue explants for six days (Batch F) or seven days (Batch E)) showed a high degree of similarity between the results from both experiments (and with the previous data) indicating that the experiments were giving reproducible results. The germ layer marker expression showed that DE specification had occurred (*FoxA2* and *Sox17* positive) and some expression of the mesendodermal/mesodermal marker *Brachyury* was present (see Section 1.6 and Section 3.1) [Tada et al 2005, Kubo et al 2004].

The selected ISC markers (see Section 1.2.3) were expressed although there was slight variation between the cells co-cultured for seven days (Batch E) and the cells co-cultured for six days (Batch F). In both batches the strong expression of *Lgr5* and *CD133* was a good indicator that ISC like cells were being specified [Barker et al 2007, Kania et al 2005]. *Msi-1* was also expressed in batch F which further supported this conclusion [Potten et al 2003]. No *EpiAnt* expression was seen but this may be because the cells had not yet differentiated to the point of expressing this specialised tissue marker [Engelhardt et al 1993].

The immunocytochemical analysis showed that the cells were expressing the ISC marker LGR5 at the protein level [Barker et al 2007], as did the western blot

analysis. In the western blot the co-culture sample produced a weaker band than the positive control sample. This supported the RT-PCR data in indicating that (some of) the cells were differentiating towards the ISC fate.

4.4.4 Summary

Whilst the protocol proved to be a viable method for initiating the expression of ISC markers not all of the mES cells differentiated towards the ISC fate. The panel of differentiation markers were expressed in most of the co-cultured cell samples but there were signs that some cells remained undifferentiated and that some other fates may have been specified (see Table 4.3.2).

- The CEE mES cells were successfully transfected with the GFP encoding VS01 plasmid.

- The desired embryonic chick tissue could be successfully explanted and maintained in *ex vivo* culture for six – seven days. Sufficient injected cells are retained within the tissue explant during co-culture.

- There were no apparent differences in DE/ISC gene/protein expression between the cells treated *in vitro* with Act-A and naïve mES cells following seven days co-culture with intact chick gut explants or dissociated cells derived from the same.

- There were no differences in DE/ISC gene/protein expression between the GFP-mES cells and the unlabelled mES cells when differentiated using the co-culture protocol.

- The co-culture seemed to induce the expression of the key ISC marker *Lgr5* [Barker et al 2007] at the RNA level in a reproducible manner. This is supported by consistent LGR5 expression at the protein level.

- The co-culture methodology used supported the differentiation of naïve mES cells to differentiate towards an ISC-like fate at a molecular level.

Similar results have been reported in the literature with different tissue types and target cell lineages [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005]. However not all the cells were differentiated or fully differentiated towards the intestinal progenitor fate.

When the studies in the literature (see Section 4.1.1) are examined it can be seen that whilst they all share the central concept of culturing undifferentiated cells with tissue from early in development to produce specific cell lineages the assessment of cell differentiation is generally limited to the molecular level. For example, to produce photoreceptor cells [Sugie et al 2005] a two stage differentiation protocol was used with *in vitro* treatment of mES cells with RA followed by selection of cells positive for neural (ectodermal) markers which were then co-cultured with embryonic chick retinal tissue. This resulted in about 20% of the co-cultured cells expressing retinal markers, such as Rhodopsin, but no functional evaluation was conducted.

The production of hepatocytes from mES cells by co-culture with cardiac mesoderm alone [Fair et al 2003] also assessed cells differentiation at the molecular level only with co-cultured cells showing upregulation of hepatic markers, such as *AFP*, when compared to controls. Generating pulmonary epithelium by co-culturing of mES cells with pulmonary mesenchyme [Van Vranken et al 2005] produced an upregulation of pulmonary epithelial markers, such as *cytokeratin,* when compared to controls but no functional tests were conducted.

Whilst the cells that had been co-cultured with embryonic chick gut tissue expressed ISC markers at the molecular level for the cells to be useful for tissue engineering applications they must also have the functional characteristics of the ISC. The cells generated by the techniques outlined in Chapters Three and Four were tested at the physiological level in Chapter Five.

Chapter Five: Tissue model co-culture

5.1 Introduction

Although it is possible to generate cells that exhibit the molecular profile of particular differentiated cell types from mES cells, the true test of how successful the process has been is to assess their ability to mature such that they display physiological and functional characteristics of the desired cell type/lineages. Therefore the aim of this Chapter was to investigate if the cells generated in Chapters Three and Four could differentiate and mature further to give rise to fully differentiated intestinal epithelial cells. This could be assessed by using a model system that had been described for investigation of epithelial barrier formation [Beltinger et al 1999]. For the cells to have potential in tissue engineering applications, such as generating *in vitro* models of disease or for drug absorption studies, this model system needs to demonstrate the fundamental barrier functional properties expected of the intestinal epithelium.

Other GI epithelial functions include digestion (enzymes and suchlike are secreted by the intestinal epithelium) and digestive transit (waves of contraction in the smooth muscle surrounding the intestine known as peristalsis move material along the GI tract), (selective) nutrient absorption (into the capillaries that permeate the intestinal wall, see Figure 5.1.1A) and immune defence (there is a large population of micro-organisms present in the gut). A number of these functions including nutrient absorption and immune defence are dependent on the integrity of the epithelial cell layer and could therefore be investigated in the proposed model. An intact epithelial cell layer acts as a barrier between the intestinal lumen and the underlying tissue and blood capillaries. Assessing the extent to which the ES derived cells can form an intact epithelial layer is a good initial test of their suitability for GI tissue engineering applications.

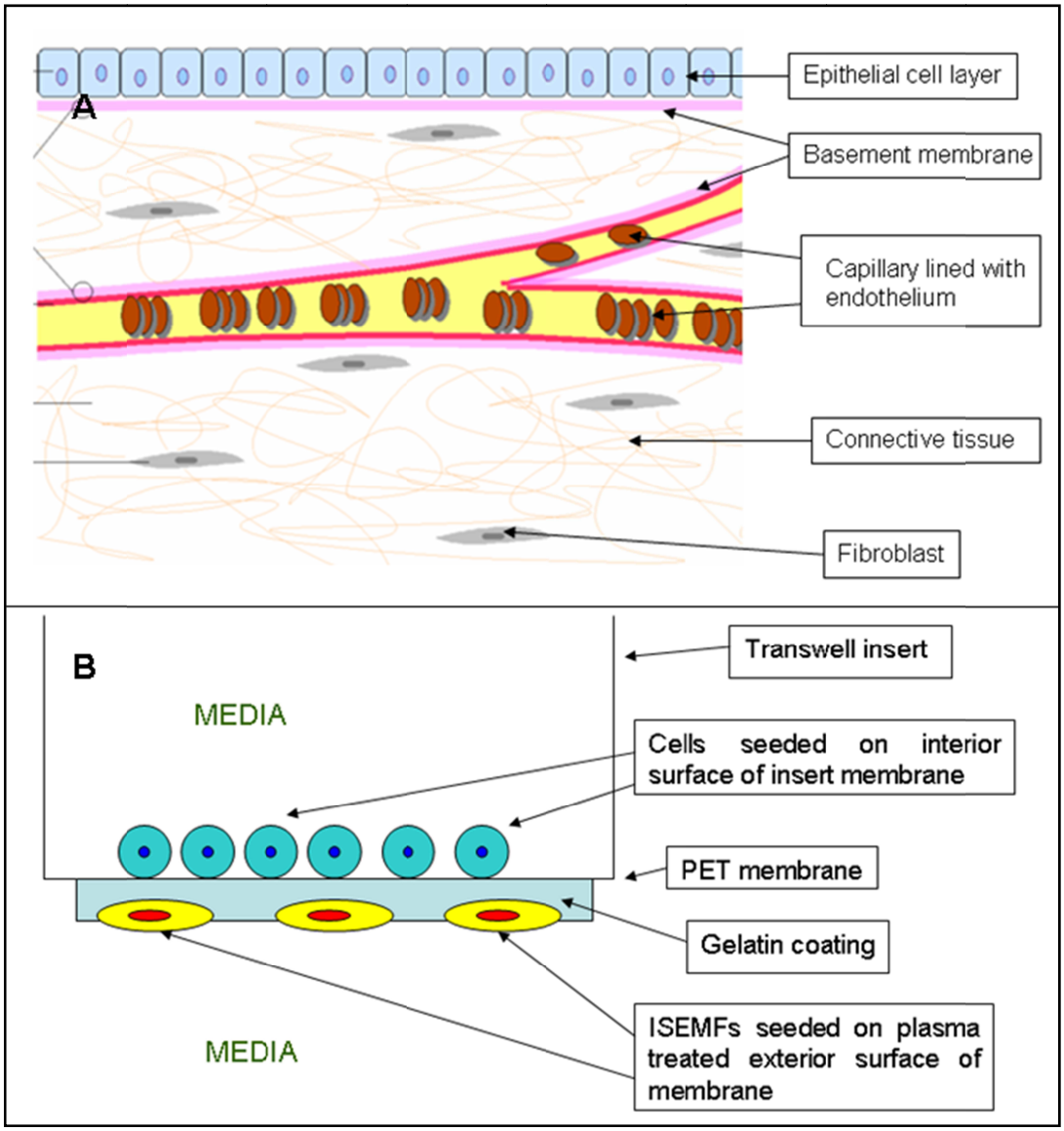


Figure 5.1.1: Schematic showing epithelial layer overlying a basement membrane and connective tissue and how this is represented in the Tissue model. A: Diagram showing an epithelial cell layer, the underlying basement membrane and connective tissue [Paulsson et al 1992]. B: Schematic of how the TMCC model system represents the structures shown in Figure 5.1.1A. The cells on the interior of the membrane represent the epithelial layer, the PET membrane represents the basement membrane and the ISEMFs and Gelatin represent the underlying connective tissue and fibroblasts.

The integrity of an epithelial layer can be assessed by examining the Transepithelial resistance (TER) across the membrane. This is a measure of the

electrical resistance of the epithelial layer; an intact epithelial layer will provide more resistance to the passage of an electrical current as charged molecules will not be able to pass across it as readily.

A simple co-culture model using ISEMF cells with either HCA-7 or T84 colonic epithelial cell lines was established (see Section 5.2, Figure 5.2.2) and certain physiological characteristics were examined [Beltinger et al 1999]. Co-cultures were established on Transwell cell culture inserts (as detailed in Section 5.2.1) with ISEMF cells seeded on the exterior surface of the membrane with either HCA-7 or T84 cells seeded on the interior surface. The TER across the membrane was then measured using an Epithelial Voltometer (EVOM) that measured in Ohms (Ω) per cm^2.

TER provides a measure of the electrical resistance across a membrane (see Figure 5.1.2). An intact epithelial layer will present a significant barrier to the passage of an electrical current and will therefore have a higher TER than an incomplete epithelial layer or negative control samples [Madara et al 1992]. TER values have also been used to assess tissue engineered GI tissue [Choi et al 1998]. The TER of control inserts that had no cellular component and that had been seeded with ISEMF cells only was also evaluated. The inserts (membrane) gave TER readings of 12 (± 0.5) Ω/cm^2 whilst the inserts seeded with ISEMF cells only gave TER values of 10.5 (± 0.4) Ω/cm^2. This indicates that the ISEMF cell layer alone did not contribute to the TER of the system.

Membranes with a confluent layer of HCA-7 cells only gave TER values of 140 (± 12.4) Ω/cm^2 whilst those with both ISEMF and HCA-7 cell layers gave TER values of 246.7 (± 20.4) Ω/cm^2. To determine if this increase in TER was caused by factors secreted by the ISEMF cells the TER of HCA-7 cells incubated in ISEMF-CM or unconditioned media were compared. HCA-7 cells in unconditioned media (0.1% FCS in DMEM) gave similar TER values as previously of 138 (± 11.4) Ω/cm^2 whereas those incubated in ISEMF-CM gave TER values of 180.7 (± 7.1) Ω/cm^2. This supports the idea that it is factors secreted by the ISEMF cells that contribute to an increase in the TER rather than the presence of the cells themselves.

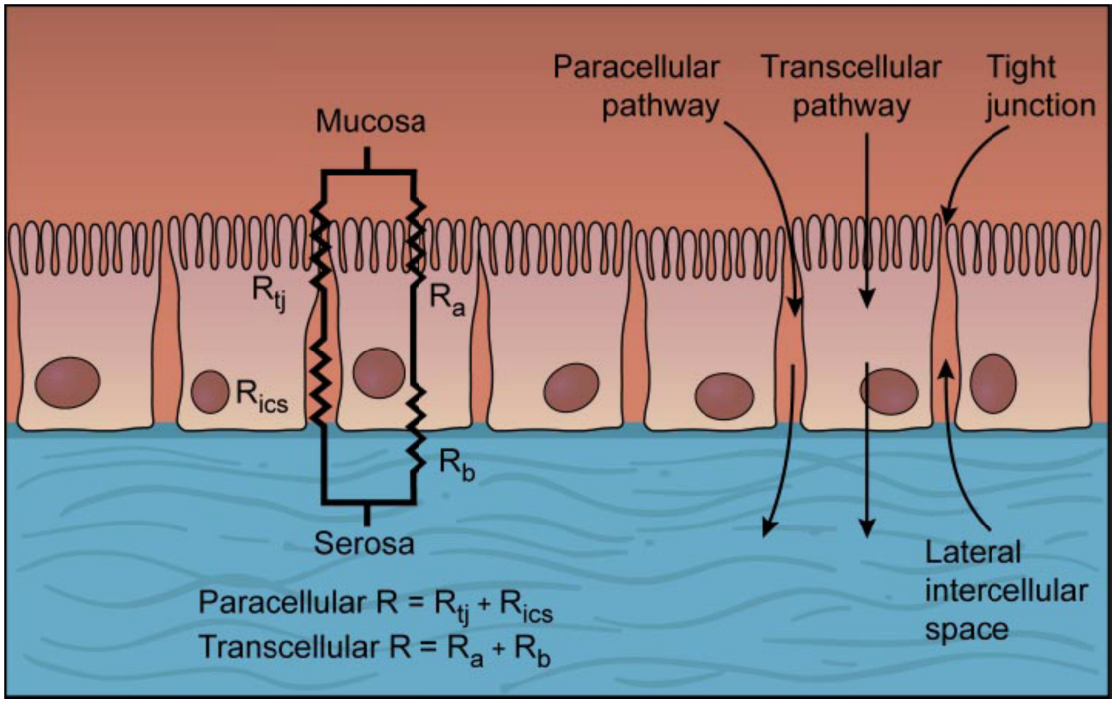


Figure 5.1.2: Diagram of the intestinal epithelium depicting components that contribute to the electrical measurement of TER. Note the critical components of this monolayer that make up the components of TER, including the epithelium itself and the paracellular space. *R*tj, tight junction resistance; *R*ics, intercellular space resistance; *R*a, apical membrane resistance; *R*b, basolateral membrane resistance. [Blikslager et al 2007].

It was also shown that TGF-β was one of the factors involved in this mechanism. The blocking of TGF-β activity using a neutralising antibody cancelled the effects of ISEMF-CM on HCA-7 cells whilst the addition of recombinant TGF-β to monolayers of HCA-7 cells induced a similar increase in TER as the CM had done. The passage of a number of different molecules across the cell-membrane barrier was also investigated [Beltinger et al 1999]. The inert marker ^{3}H-Mannitol was shown to equilibrate across the membrane in one hour in acellular and HCA-7 cell only controls. The rate of equilibration was reduced in the ISEMF cell and HCA-7 cell alone samples. Agonist stimulated ion transport was also reduced in the co-culture samples compared to controls (samples displayed between 60 – 70% the levels of ion transport seen in the controls).

The same pattern was not seen in T84 cells where the dual cell samples showed 10-fold upregulated transport activity compared with the controls. This activity was mediated by the cyclooxygenase enzymes COX-1 and COX-2. Suppression of the COX enzymes reversed the downregulation of transport activity in HCA-7 cells. The differences between HCA-7 and T84 cells were explained by the different expression of the *Cox* enzyme transcripts in each cell line; HCA-7 cells expressed the *Cox* transcripts whilst T84 cells did not. This explains why in the presence of COX positive ISEMF cells transport activity increases in T84 cells [Beltinger et al 1999]. The use of an ISEMF layer along with the epithelial cell line in the TMCC system gave a more accurate model of the properties of the intestinal epithelium.

Monolayers of T84 (intestinal epithelial) cells were cultured in monolayer on collagen coated permeable supports [Nash et al 1987]. These monolayers were then used to study the transmigration of polymorphonuclear leukocytes in the presence of a chemotactic gradient (of formyl methionyl leucyl phenylalanine) and the effects that this had on the ability of the epithelial monolayer to maintain its barrier function. The chemical treatment reduced the TER of the epithelial layer which permitted greater transmigration of leukocytes. Normal TER levels were restored following removal of the chemical agents [Nash et al 1987].

It is important to represent all of the tissue/cell components of the intestinal wall in any model as it is not just the epithelial layer that is important in maintaining the function of the intestinal lining [Powell et al 1999(1)]. Myofibroblasts play an important role in the intestinal wall where they secrete a number of growth factors, ECM components and inflammation response mediators [Powell et al 1999(1)]. The role of ISEMFs in Crohn's disease was examined [Powell et al 1999(2)]. ISEMFs play an important role in wound repair in the intestine with factors they secrete stimulating epithelial proliferation and hence restitution (where healthy tissue grows into the space left by an injury). They also help facilitate some of the active transport mechanisms by which nutrients are absorbed from the gut lumen into the bloodstream [Powell et al 1999 (2)]. For a model system to accurately mimic these functions it must therefore have an ISEMF component.

A similar model was used to examine the effect of the immune response to microbial agents on the intestinal mucosa (particularly the role of sub-epithelial myofibroblasts) with reference to Crohn's disease [Willemsen et al 2002]. Sub-epithelial myofibroblasts and T84 intestinal epithelial cells were seeded on 12mm Transwell inserts in a similar fashion as shown in Figure 5.1.1B (with controls where myofibroblasts were not present). This model was then used to investigate if excessive exposure to microbial factors could induce early symptoms of Crohn's disease by initiating an 'excessive' immune response. Leukocytes or cytokines were then introduced into the models to simulate an immune response to a microbial agent. This reduced the integrity of the epithelial barrier (assessed by monitoring TER and molecule movement (flux) across the membrane) but the effects were less marked in the model where myofibroblasts were present suggesting that they play a role in maintaining epithelial integrity. It appeared this role was facilitated by the secretion of growth factors by the myofibroblasts [Willemsen et al 2002].

A potential use for ES derived intestinal epithelial progenitors would be as a substitute for the T84 cell line in a model such as the one used in this study. IPS derived cells might also be useful in such a model as it would allow for the generation of disease models where cells from patients suffering from e.g. Crohn's disease could be compared with unaffected cells. This might allow the identification of initiating environmental factors as well as the study of the disease's progression.

Passage of labelled molecules has also been used to assess tissue engineered GI tissue in rats [Tait et al 1995]. Nutrient transport was assessed using labelled (C^{14}) glucose. The results were compared with those from age matched control tissue samples and the level of activity was found to be similar [Tait et al 1995]. Transport of D-Glucose is a sodium dependant process mediate by the neomucosa and will not occur in a normal fashion unless a functional epithelial barrier is present. By comparing the uptake of D-[U-^{14}C] glucose in animals that had received engineered intestinal tissue with a control group of animals that had only native tissue the nutrient uptake properties of the engineered tissue could be

assessed. The rate of glucose uptake was comparable in both groups of animals [Tait et al 1995].

Some of the techniques used have also been used to assess mucosal repair *in vivo* following damage to the intestinal epithelium [Blikslager et al 2007]. TER measurements were used to assess the rate and extent to which the barrier function of the intestinal epithelium was restored following injury. This occurs via a mechanism called restitution where healthy tissue surrounding a wounded area grows into the space restoring the epithelial cell population. Measuring the TER at various timepoints following intestinal injury indicate show quickly the epithelial barrier is re-established [Blikslager et al 2007].

Aims

The aims in this chapter were to establish if the cells that had been through the differentiation protocols in Chapter Four could mature further into functional intestinal epithelial cells (and generate an epithelial barrier) in a simple model system.

The experiments in this chapter evaluated if the cells produced by the methodologies described in Chapters Three and Four displayed some of the physical and functional characteristics of an (intestinal) epithelial layer (and ultimately would be able to generate a functional epithelial barrier). Transwell cell culture inserts with Polyethylene Terephthalate (PET) membranes were used as the basis for the model. The membranes represented the basement membrane, in this instance of the intestinal epithelium (see Figure 5.1.1 and Section 1.2.1, Figure 1.3) [Paulsson et al 1992, Stanley et al 1982]. These were seeded with ISEMFs on the gelatin coated exterior surface that represented the surrounding connective tissue that supports the intestinal epithelium (see Figure 5.1.1). The interior of the membrane was seeded with the mES derived cells to see if they could form an epithelial layer [Beltinger et al 1999]. The following properties were then examined (see Section 5.2 for full experimental details.

- To investigate the integrity of the putative epithelial cell layer (cultured in monolayer) the TER across the membrane was measured.

- To investigate the passage of nutrients across the putative epithelial cell layer the permeability of the membrane to (fluorescently labelled) protein was measured.

- These characteristics were used to infer whether the cells were organising into a structure that would perform the functions expected of an epithelial layer such as controlling/blocking the passage of material across the layer. Other components in the model were designed to replicate the other tissue structures that interact with the epithelial layer in the intestine (see Figure 5.1.1).

- The intestinal epithelium contains a number if distinct structures and cell types with characteristic morphologies. To observe if any of these structures, such as tight junctions, or cell types were present the cells were examined in high resolution using TEM as well as light microscopy to produce images of the cells in the tissue model environment.

Chapter Five: Tissue model co-culture

5.2 Materials and Methods

5.2.1 Experimental set up

5.2.1i Polyallylamine (PAA) plasma coating of Transwell cell culture inserts

Cell culture Transwell inserts for 12 well plates with 1.5 cm^2 PET membranes with 0.45 μm pores (BD, 353180) were coated by plasma deposition of PAA using a plasma chamber [Zelzer et al 2008] (Figure 5.2.1) to aid cell attachment to the membrane exterior surface [Harsch et al 2000]. Plasma is a fourth state in which matter can exist, where gaseous particles are highly ionised, first described by Sir William Crookes in 1879 (although the name 'Plasma' was first used by Irving Langmuir in 1928). Whilst plasmas share some properties with matter in a gaseous state (such as no defined physical shape) they also have some very distinctive properties (such as the ability to form 'structures' under the influence of a magnetic field) [Crookes 1897, Langmuir 1928].

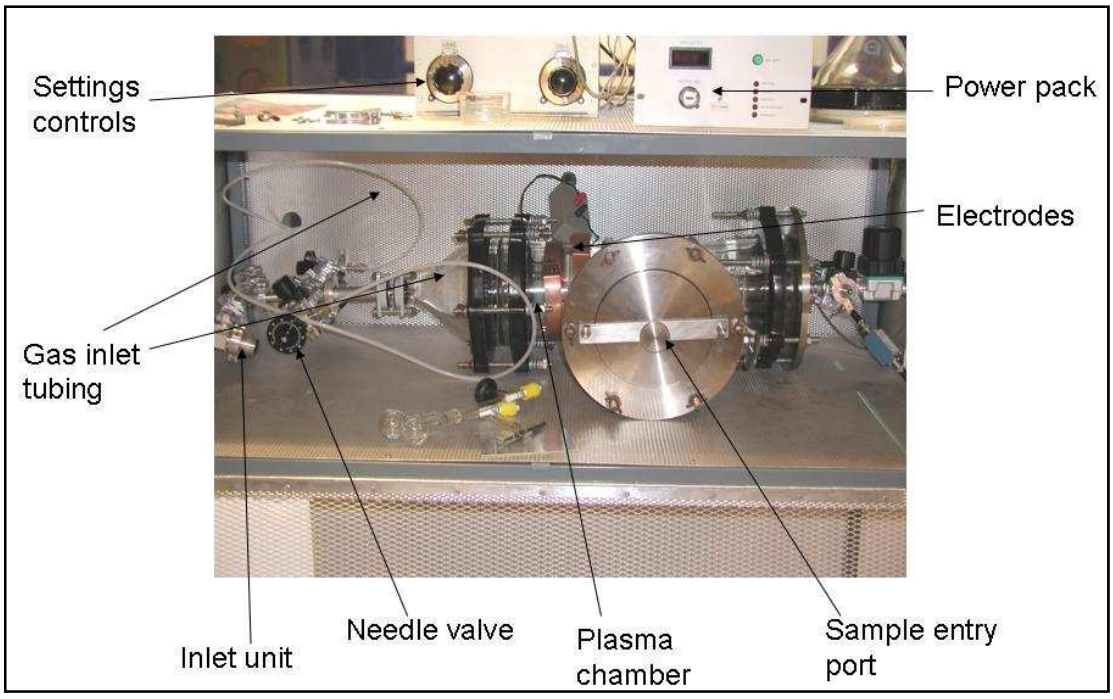


Figure 5.2.1: Plasma chamber equipment set up for plasma coating

To coat the Transwell inserts with the PAA plasma the inserts were placed into the plasma chamber which was then sealed. The Transwell inserts were first

'Oxygen etched' by the addition of oxygen at 300 mTorr with an input power of 20W for three minutes. PAA (in its plasma form) was then added to the chamber at 250 mTorr. PAA was deposited at 3.8 Angstroms (Å)/second to a thickness of 1.008 kÅ for Batch A and 2.9 Å/second to a thickness of 1.009 kÅ for Batch B. Each deposition treatment took between five and eight minutes.

5.2.1ii ISEMF cell culture

A population of ISEMF cells was provided by Michelle Jackson of the Wolfson Digestive Diseases Centre, Queens Medical Centre, Nottingham. These cells had been isolated from adult human intestine as detailed below [Mahida et al 1997]. Samples of human adult colonic mucosa were obtained from resection specimens from patients undergoing partial colectomy for carcinoma. These samples were taken at least 5 cm away from the tumour site and histologically abnormal samples were excluded. Ethical approval was obtained in 2003 – R&D GM050303 and ethics Q2050309, Nottingham City Hospitals Trust.

Mucosal samples were separated from the underlying sub-mucosa by dissection and cut into 1 cm long strips. The mucosal samples were incubated with 1 mM dithiothreitol (DTT) for 15 minutes at room temperature. After washing with calcium and magnesium free HBSS the mucosal strips were incubated on a plate shaker at 37˚C in 1 mM EDTA for a 90 minute period; the 1 mM EDTA was replaced every 30 minutes. After treatment with EDTA, the mucosal strips (that were now denuded of epithelium) were thoroughly washed with HBSS with Ca^{2+} and Mg^{2+}. Mucosal strips were cut into approximately 1 cm lengths and were subsequently cultured at 37˚C, 5% CO_2 in air, in RPMI 1640 media (100 ml total volume) supplemented with 10% (*v/v*) FCS and 1% (*w/v*) Penicillin-Streptomycin-Amphotericin, (100 µg Streptomycin, 250 ng Amphotericin B and 1000 units Penicillin B, Sigma, UK).

During culture, lamina propria lymphocytes, macrophages and eosinophils migrated via basement membrane pores and appeared both in the suspension and adherent to the culture dish. The cells in suspension were removed every 24 to 72 hour culture period for the first week and twice weekly subsequently. The

denuded mucosal tissue was maintained in culture for up to four weeks, at which point ISEMFs migrated from the tissue and populated the culture dish. Lamina propria was transferred into fresh culture dishes until no more ISEMFs migrated from the tissue.

The ISEMF cells that migrated out of the tissue were transferred to fresh T75 TCP culture flasks (Nunc, UK) and were maintained in ISEMF media (DMEM supplemented with 10% (*v/v*) FCS, 2 mM L-Glut and 1% (*w/v*) Penicillin-Streptomycin-Amphotericin, (100 µg Streptomycin, 250 ng Amphotericin B and 1000 units Penicillin B) in a humidified atmosphere at 37°C and 5% CO_2 in air.

5.2.1iii Seeding Transwell inserts with ISEMF cells

Once c.80% confluent, ISEMF cells were washed with 5 ml PBS and detached from the cell culture flask by treatment with pre-warmed 0.25% (*v/v*) Trypsin/0.02% (*w/v*) EDTA (3 ml). The cell suspension was transferred to a centrifuge tube where the Trypsin was inactivated by serum following the addition of ISEMF media. The cells were pelleted by centrifugation at 200 *g* for five minutes. The supernatant was removed and the cells were resuspended in ISEMF media and counted using an improved Neubauer haemocytometer. The concentration was adjusted to 1 x 10^5 cells/ml by adding an appropriate amount of ISEMF media.

Transwell cell culture inserts that had been plasma coated (see section 5.2.1i) were inverted in a large Petri dish so that the exterior membrane surface faced upwards. This surface had been coated with 0.1% (*w/v*) Gelatin solution for two hours, then washed thoroughly with PBS and allowed to dry. ISEMF cell suspension (150 µl) was aliquoted onto the membrane and the Transwell inserts were incubated in a humidified atmosphere at 37°C, 5% (*v/v*) CO_2 in air overnight to allow the cells to adhere (Figure 5.2.2). PBS (2 ml) was added to each Petri dish to ensure the atmosphere remained humidified. Following overnight incubation it was established that the cells had adhered to the exterior of the membrane by observation under a light microscope. The Transwell inserts were then turned and placed in 12 well (non-tissue culture treated) plates. ISEMF

media (1.5 - 2 ml) was added to the outer/lower chamber with 500 µl - 1 ml media being added to the inner/upper chamber. The Transwell inserts were cultured for a further six - seven days to allow the ISEMF cells to proliferate on the membrane exterior [Beltinger et al 1999].

5.2.1iv Seeding Transwell inserts with mES derived intestinal progenitor like cells

Both mES cells and GFP-mES cells were differentiated towards the intestinal precursor fate by *ex vivo* co-culture as described in Chapter Four, Section 4.2.10. In brief the cells were injected into day six embryonic chick gut tissue explants in *ex vivo* culture for 7 days in mES complete media (LIF-). Following co-culture the cells were sorted to isolate the mES derived cells from the chick tissue. Some of these cells were sorted by FACS as described in the Section 2.2.10 whilst some of the cells were sorted by centrifugation.

Remaining tissue was removed by sucrose density centrifugation. Following enzymatic digestion the co-culture samples were placed in a 5% (*w/v*) sucrose solution and centrifuged at 150 *g* for two minutes in a 15 ml centrifuge tube. Whilst the enzymatic digest of the tissue explants following co-culture reduced the tissue to smaller pieces the tissue was not broken down to single cells. The remaining multi-cell clusters are much greater in mass than the single murine cells. This mass differential means that when centrifuged in a viscous matrix the cells separated according to density. The upper portion of the supernatant (and the single CEE mES cells therein) was removed and the single cells washed thoroughly with PBS. These cells were then resuspended in ISEMF media and seeded onto the interior membrane surface of Transwell cell culture inserts that had been seeded with ISEMF cells (as described above) at 5 x 10^4 cells/ml in 12 well tissue culture plates (Figure 5.2.2). The plates were incubated in a humidified atmosphere at 37°C, 5% CO_2 in air.

Controls were also set up using undifferentiated mES and GFP-mES cells on ISEMF seeded inserts and Transwell inserts with ISEMF cells alone. Plasma coated Transwell inserts with no cells seeded on either side were used as negative

controls. The seeded Transwell inserts were maintained for up to 21 days in culture (Figure 5.2.2).

This model was designed to imitate the tissue structure shown in Figure 5.1.2. The PET membrane represents the basement membrane, the gelatin coating and ISEMF cells represent the underlying connective tissue and fibroblasts whilst the cells seeded on the interior of the Transwell insert represent the epithelial cell layer.

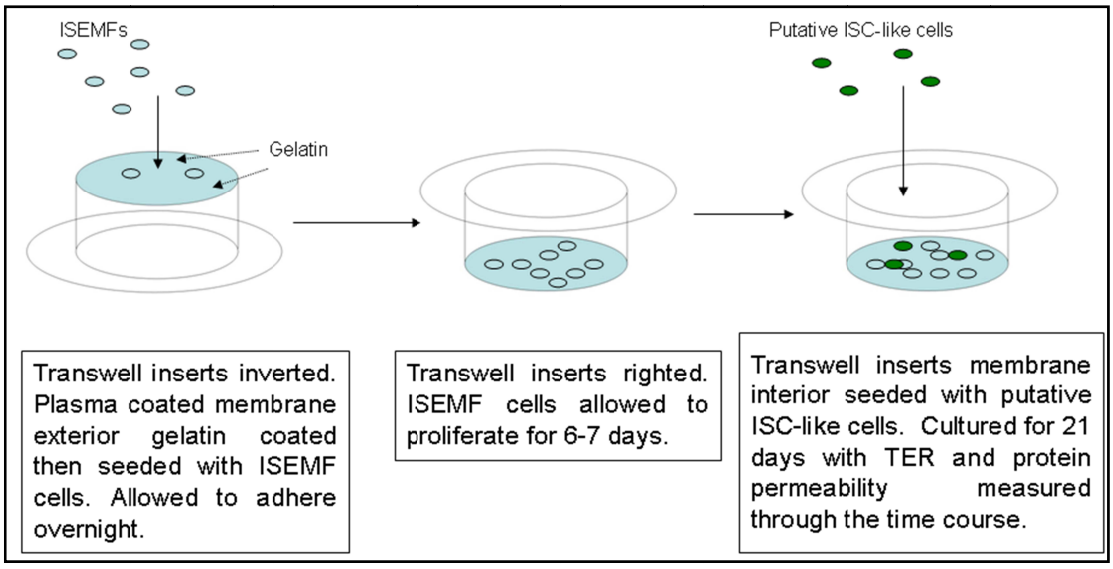


Figure 5.2.2: Schematic representation of the TMCC experimental set up.

5.2.2 Analysis

5.2.2i Measuring TER

The TER is a measure of the electrical resistance across a membrane barrier and gives an indication as to whether the cells are forming strong interactions, such as tight junctions, leading to the formation of an epithelial barrier layer (see Section 5.1, Figure 5.1.2). It can be measured using an EVOM where an electrode is placed on either side of the membrane barrier (Figure 5.2.3) and the resistance across the membrane measured in Ohms (Ω). This was measured for the samples and controls every two to three days through the course of the experiment (21

days) and the data gathered was used to determine if significant differences existed between the controls and the experimental samples.

5.2.2ii Diffusion of proteins across the *in vitro* epithelial membrane barrier

FITC-BSA (5 mg, Autogen Bioclear, ABXK14) conjugate was reconstituted in 1 ml PBS. This was then added to protein free-DMEM (PF-DMEM) at 0.1% (*v/v*). A 1% (*w/v*) solution of unlabelled BSA was also made up in PF-DMEM. The media was removed from the seeded Transwell cell culture inserts and the upper and lower chambers washed twice with PF-DMEM. BSA (1% (*w/v*)) in PF-DMEM (800 µl) was added to the lower chamber with 300 µl being added to the upper chamber. The Transwell inserts were returned to the incubator for 30 minutes. This allowed the protein levels on either side of the membrane to equilibrate and ensured there was no osmotic gradient across the membrane/cell layers.

After equilibration, an additional 700 µl 1% (*w/v*) BSA in PF-DMEM was added to the lower chamber (total volume 1.5 ml) whilst 200 µl of 0.1% (*w/v*) FITC-BSA in PF-DMEM was added to the upper chamber (total volume 500 µl) giving a final concentration of 0.04% (*w/v*) in the upper chamber and 0.01% (*w/v*) in the total volume of media. The Transwell inserts were then returned to the incubator. Every 30 minutes for the following two hours 200 µl of media was removed from the lower chamber following gentle agitation by pipetting. This was immediately replaced with 200 µl of 1% (*w/v*) BSA in PF-DMEM. After the two hour time point was complete the Transwell inserts were rewashed twice in PF-DMEM and returned to the incubator in ISEMF media.

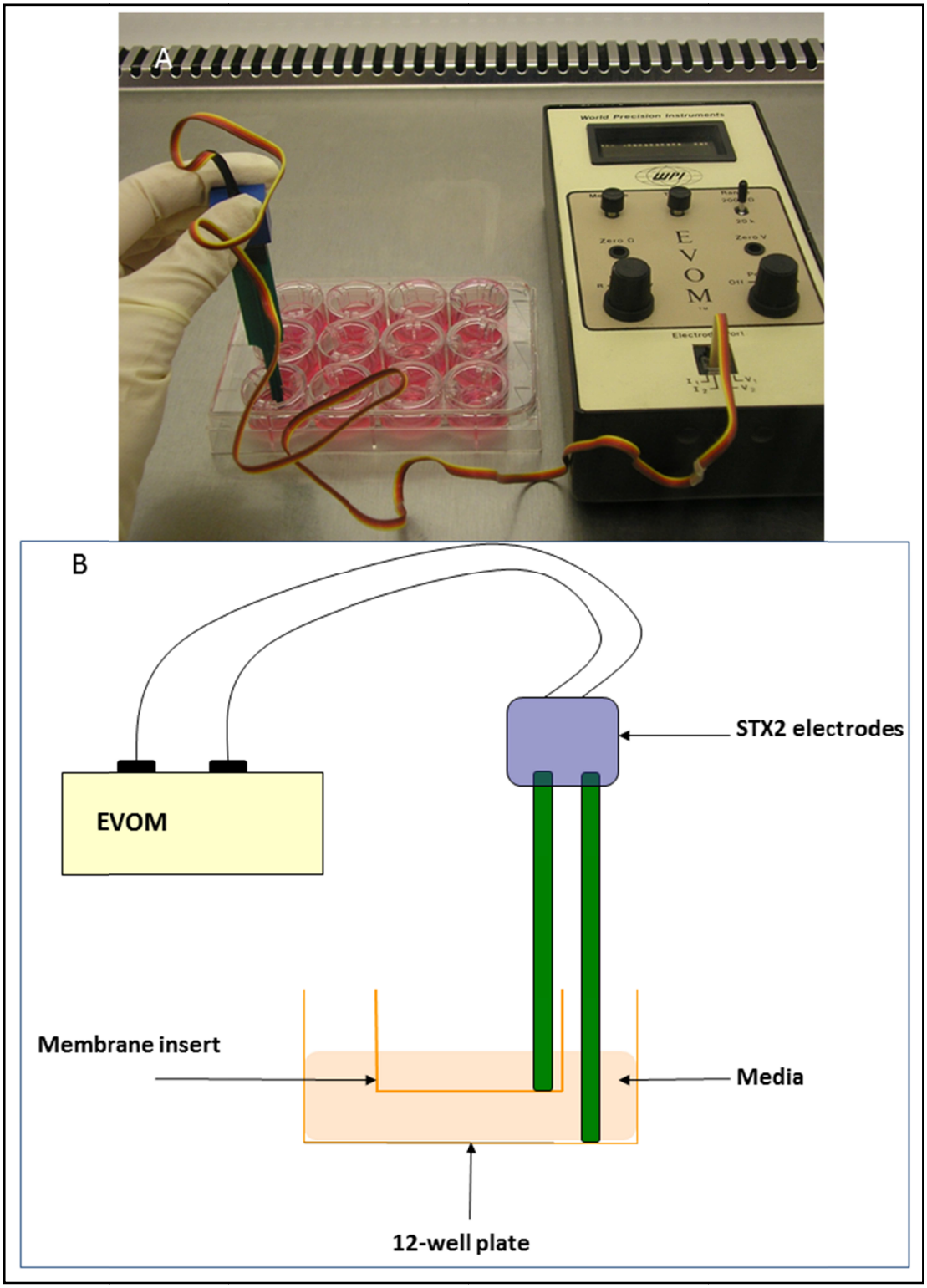


Figure 5.2.3: A – EVOM and STX2 electrodes used to measure TER of seeded inserts. B – Diagrammatic representation of electrodes on either side of seeded insert membrane.

The concentration of FITC-BSA that had permeated across the membrane was determined by measuring the fluorescence of the samples following transfer to a

flat bottomed black 96 well plate and comparing this to a standard curve of FITC-BSA in PF-DMEM using a KC4 plate reader. The fluorescence excitation and emission maxima for FITC are Excitation 495 nm and Emission 525 nm.

The standard curve was generated by making up nine standard solutions of FITC-BSA in PF-DMEM between 0 - 1% (*w/v*). The standard solutions of FITC-BSA in PF-DMEM used were 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.015625%, 0.0078125% and 0%. The fluorescence of these solutions was then measured and plotted against the concentration of FITC-BSA on a graph to produce a standard curve. The concentrations of the 'unknown' samples were then calculated using the equation from the straight line determined from standard curve.

5.2.2iii Imaging the seeded inserts

Some images of the seeded Transwell inserts were obtained through the course of the experiment by light microscopy using a Nikon Eclipse microscope and camera.

Following 21 days of culture the samples were fixed in 3% (*v/v*) Glutaraldehyde in 0.1 M Cacodylate buffer for 24 hours. Sample processing, embedding, sectioning and TEM imaging was carried out in the Advanced Microscopy Unit, Medical School, QMC, Nottingham. The inserts were washed in 0.1 M Cacodylate buffer then treated with 1% (*v/v*) aqueous Osmium Textroxide for one hour. The inserts were washed for five x one minute in H_2O and then dehydrated with two x 15 minute washes in 50% (*v/v*) Ethanol. The inserts were then stored in 70% (*v/v*) Ethanol before being cut in half. The cut inserts were then washed two x 10 minutes in 70% (*v/v*) Ethanol. The samples were then further dehydrated through the following stages: two x 15 minutes 90% (*v/v*) Ethanol, three x 20 minutes 100% Ethanol then two x 15 minutes 100% Propylene Oxide (carried out in a glass vessel).

Resin was prepared by mixing 25 ml Araldite CY212 resin, 15 ml Agar 100 resin and 55 ml Dodecenyl Succinic Anhydride (DDSA) then adding 2 ml Dibutyl

phthalate and 1.5 ml Dimethyl phthalate (DMP) 30. The mixture was stirred and then polymerised by incubation at 60°C for 48 hours. The samples were then infiltrated with resin firstly using a 1:3 mix of resin and Propylene Oxide for four hours then with 1:1 resin/Propylene Oxide for 24 hours and finally 100% resin for 2.5 hours. The samples were then placed in a mould and incubated at 60°C for (at least) 48 hours. Sections were cut using a Microtome and mounted on APES coated slides on copper grids.

The sections were then stained with 50% (*v/v*) Methanolic Uranyl Acetate (Saturated Uranyl Acetate (UA) in distilled water mixed 1:1 with 100% Methanol). A few drops of Methanolic UA were placed on each section and left for 10 minutes in the dark. The samples were then washed once in 50% (*v/v*) Methanol then three times in UltraPure™ water; for each wash the sample was briefly immersed in the liquid at least ten times. Excess liquid was removed from the sample by blotting with filter paper. The samples were then placed in 0.1 mg Lead Citrate dissolved in NaOH for 30 seconds then washed three times in UltraPure™ water as before. The samples were blotted again and allowed to dry and then stored in the dark in a sealed container until they were imaged.

Detailed images were obtained via TEM using a FEI Tecnai 12 BioTWIN electron microscope with a Soft Imaging System, Megaview III camera [Bancroft and Stevens 1982].

Chapter Five: Tissue model co-culture

5.3 Results

5.3.1 Trans-epithelial resistance (TER)

Table 5.3.1 and Figure 5.3.1 (see Section 6.3 for supplementary graphs 6.3.8 and 6.3.9) show the TER readings obtained from experimental and control seeded cell culture inserts. The negative control samples, where the inserts had not been seeded with any cells, showed a slight decrease in TER, from 175 (±7) to 143 (±4) Ω, through the course of the experiment but generally were very consistent. The ISEMF controls, where only the exterior surface of the insert membrane had been seeded, showed very little change in TER through the experiment from 185 (±0) Ω at the start to 173 (±18) Ω at day 21 with the reading never lower than 165 (±7) Ω. TER measured in the mES controls, the undifferentiated controls for batch A, were fairly constant up to day 12, having been 190 Ω initially and 185 Ω at day 12, but the TER then fell at day 14 (165 Ω) and 16 (150 Ω) before rising back to a similar level (200 Ω) for the final two timepoints. The GFP-mES controls, the undifferentiated controls for batch B, showed a decrease in TER (190 to 150 Ω) over the whole time course but this was not a consistent trend throughout.

Experimental batch A, where the inserts had been seeded with mES cells that had been through the differentiation protocol where naïve mES cells were injected into intact day six embryonic chick gut tissue explants and co-cultured for seven days (detailed in Chapter Four) but that had not been sorted using FACS, gave consistent TER readings throughout the time course (175 (±13) Ω initially, 176 (±11) Ω at day 21). However there was some variation between individual samples as shown in Supplementary Figure 6.3.3. Experimental batch B, where the inserts had been seeded with GFP-mES cells that had been through the differentiation protocols detailed in Chapter Four that had been sorted by FACS, also gave consistent readings throughout with a slight increase in TER at the end of the time course (157 (±14) Ω initially, 173 (±13) Ω at day 21). As with batch A there was some variation between the individual samples as shown in Supplementary Figure 6.3.4 but the SD values show the variation was less in batch B than it was in batch A.

The absence of any discernible patterns or trends in the TER data is shown in graphical format in Figure 5.3.1. In all cases the ratio between the experimental samples oscillates around 1:1 without showing a particular trend in either direction (see Section 6.3, Table 6.3.8).

The significance of this data can be evaluated based upon the null hypothesis that the ratio between the experimental samples and controls will be 1:1 initially and will not change significantly through the course of the experiment. This null hypothesis is proved to be true as no significant changes occurred through the course of the experiment.

Table 5.3.1A: Summary table of average TER readings in Ohms (Ω) from the cell culture inserts seeded with differentiated cells and controls over the course of 21 days. In negative and ISEMF control conditions n = 2, in batch A n = 12 and in batch B n = 8. Numbers are given to nearest whole number. Standard deviations are given in brackets where applicable.

Sample	Day 1	Day 5	Day 7	Day 9	Day 12
Negative control	175 (7)	160 (28)	165 (0)	155 (7)	143 (4)
ISEMF control	185 (0)	165 (21)	173 (4)	168 (4)	165 (7)
mES control	190	190	200	190	185
GFP-mES control	190	160	190	170	175
Seeded inserts A	175.42 (13)	169.58 (20)	177 (19)	189 (27)	175 (7)
Seeded inserts B	157.50 (14)	169.38 (14)	174 (9)	168 (8)	163 (12)

Table 5.3.1B: Summary table of average TER readings in Ohms (Ω) from the cell culture inserts seeded with differentiated cells and controls over the course of 21 days. In negative and ISEMF control conditions n = 2, in batch A n = 12 and in batch B n = 8. Numbers are given to nearest whole number. Standard deviations are given in brackets where applicable.

Sample	Day 14	Day 16	Day 19	Day 21
Negative control	150 (0)	145 (0)	153 (11)	143 (4)
ISEMF control	168 (11)	175 (7)	183 (25)	173 (18)
mES control	165	150	200	190
GFP-mES control	160	185	160	150
Seeded inserts A	175 (7)	167 (11)	168 (17)	176 (11)
Seeded inserts B	178 (15)	168 (12)	162 (10)	173 (13)

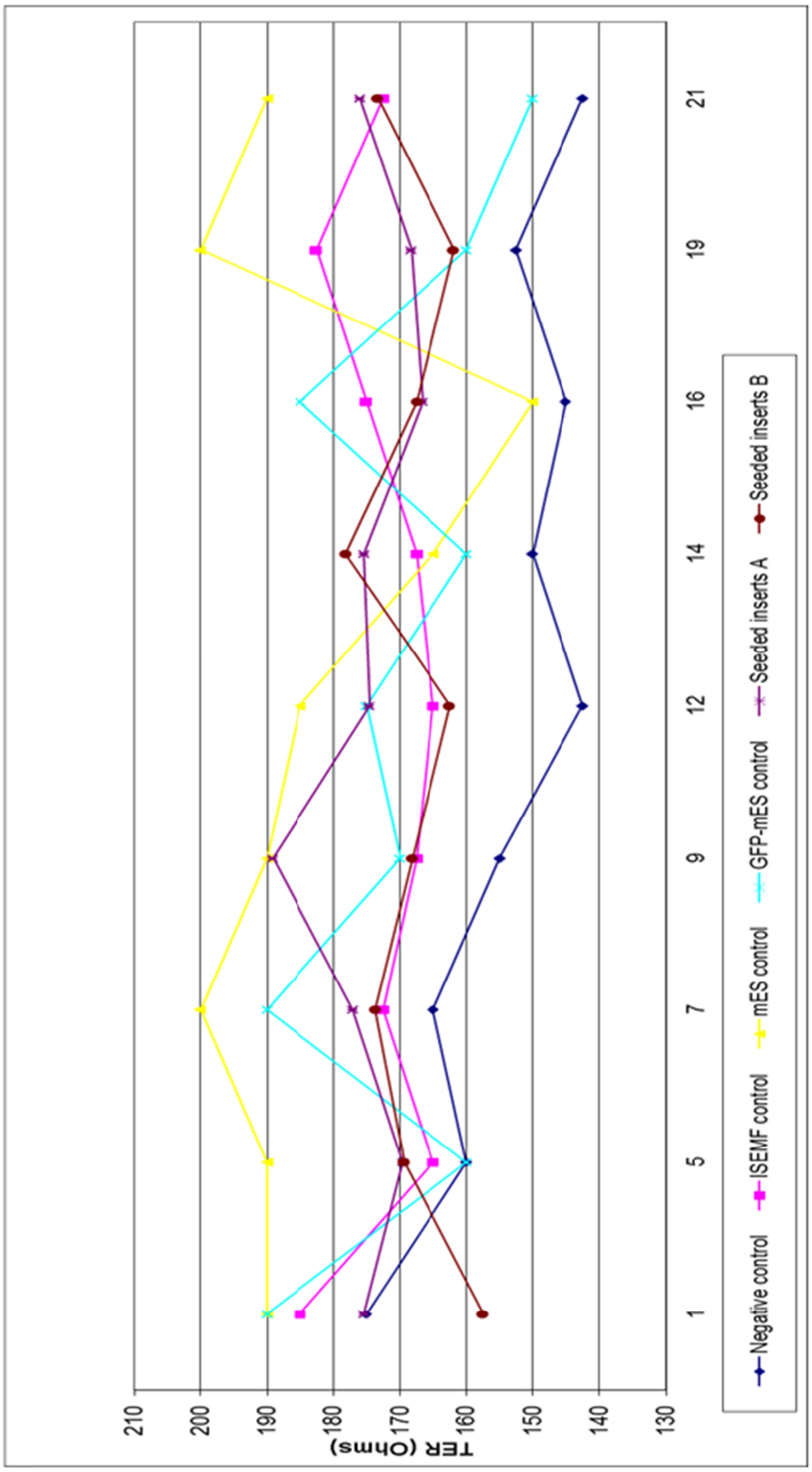


Figure 5.3.1: Graph showing mean TER values in seeded inserts and controls over the course of 21 days in culture.

5.3.2 Membrane permeability

Figure 5.3.2A shows the standard curve generated for the fluorescence readings from known concentrations of FITC-BSA and a summary table for the data. This was then used to calculate the concentration of FITC-BSA in unknown samples based on the fluorescence reading obtained. The standard curve did not pass through zero as the PF-DMEM autofluoresced at low levels at 520 nm. This was not corrected as the unknown samples were also in PF-DMEM.

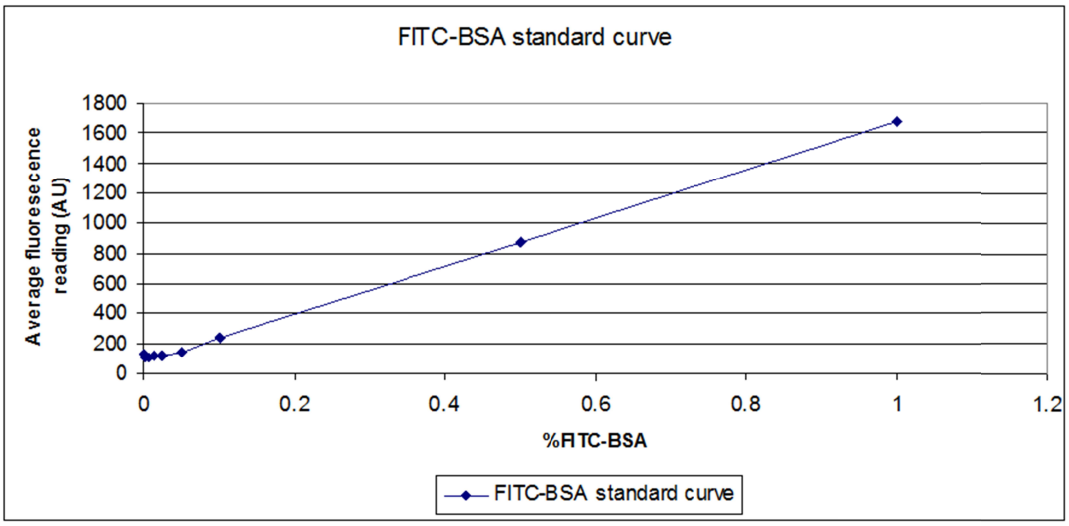


Figure 5.3.2A: FITC-BSA standard curve (n = four). Readings taken on a KC4 plate reader. The media in which the standard curve and unknown samples were carried autofluoresced at low levels in FITC emission wavelength hence the standard curve does not pass through zero.

Figures 5.3.2B and C shows a summary of the concentration of FITC-BSA based on the fluorescence readings obtained for the membrane permeability study. Some readings are given as zero values because they fell outside the lower limits of sensitivity of the standard curve. This data is also summarised in tabular format in Section 6.3 Table 6.3.9. At the 13 day timepoint the two co-cultured mES cell samples showed markedly different results. Although the total amount of FITC-BSA that permeated the membrane was similar in A1 and A2 in A1 the majority was in the first 60 minutes of the incubation whilst in A2 the vast majority crossed in the final 30 minute interval. The two samples from batch B also show similar disparities. Again the total amount of protein to have permeated across the membrane is similar in B1 and B2 the entire total for B1 was

in the latter half of the incubation whilst for B2 the entire total was in the initial 60 minutes. The control samples (mES and GFP) both showed greater amounts of protein permeating the membrane than the experimental samples. The naive mES control showed a roughly even split of protein crossover between the initial and final 60 minutes of the incubation.

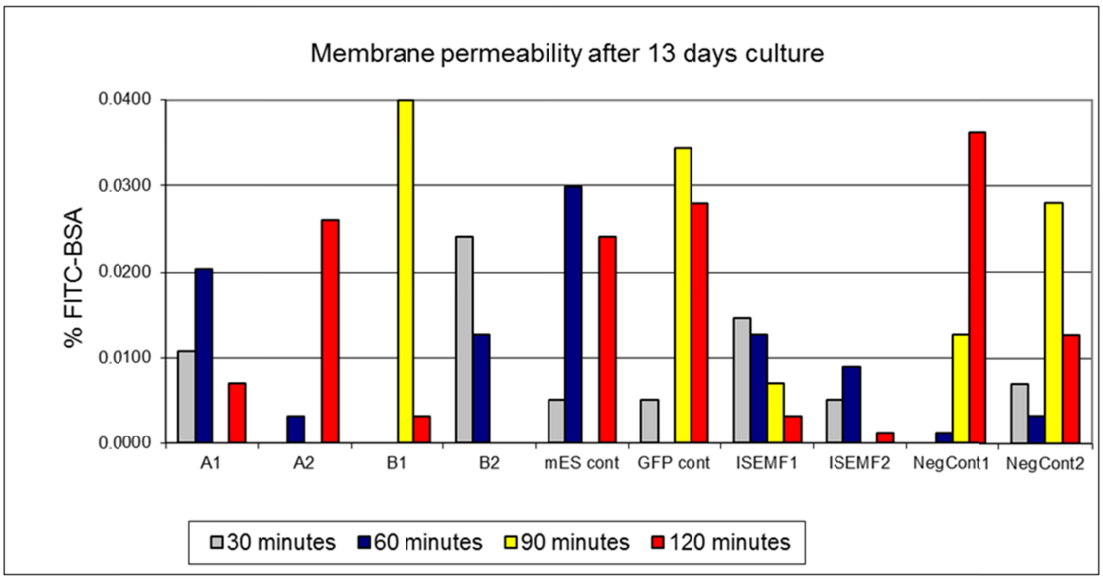


Figure 5.3.2B: Summary graph for membrane permeability data 13 days after the membranes were seeded with cells. The bar chart displays the percentage of FITC-BSA that had permeated across the cell seeded membrane in four consecutive 30 minute periods.

In the naïve GFP-mES control the vast majority of the protein crossed in the final 60 minutes of the incubation. The two ISEMF controls differed in the total amount of protein that permeated the membrane. In ISEMF1 the total was similar to that seen in the experimental samples but in ISEMF2 it was considerably lower. In both samples the majority of the total was from the initial 60 minutes of the incubation. The negative control samples both produced very similar results. The total amount of protein was higher than the experimental samples but less than the mES and GFP controls. In both negative controls the majority of the total protein crossover was from the final 60 minutes of the incubation.

At the 20 day timepoint the two co-cultured mES cell samples gave similar results. The total amount of protein was slightly higher than the day 13 samples. In both samples the majority of the total was from the initial 60 minutes of the incubation. The two co-cultured GFP-mES cell samples gave dissimilar results. Sample B1 had a total amount of protein almost double that of B2 and three times that of B1 at 13 days. Sample B2 had a total of twice the amount at 13 days. In both samples the majority of the total was from the final 60 minutes of the incubation although the distribution was more even in B1 than B2. The total amount of protein for the mES control was halved at 20 days compared with 13 days and was less than the experimental samples. The distribution of protein crossover between the first and second half of the incubation was even.

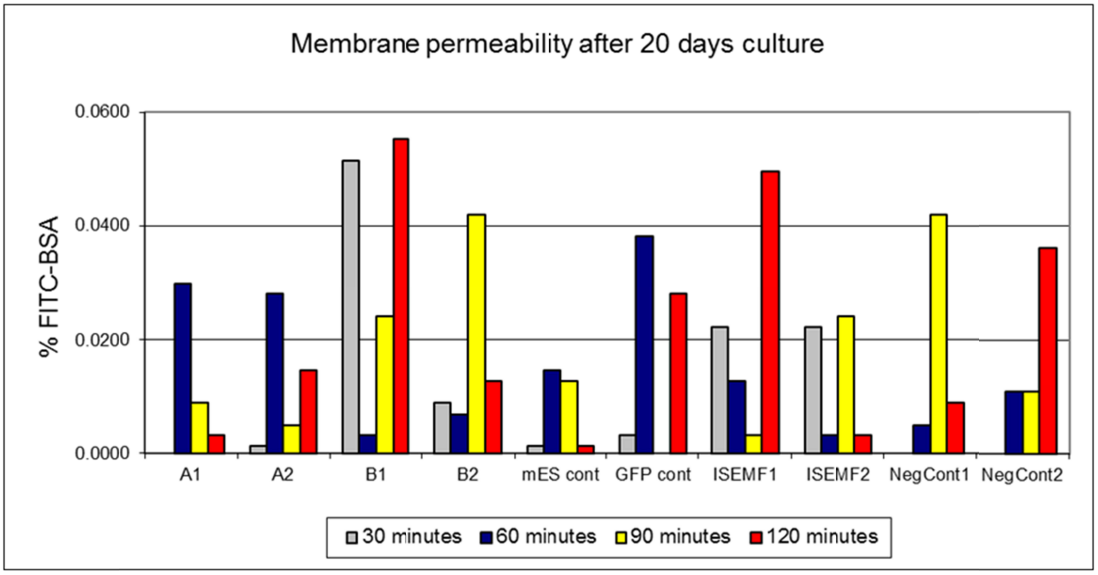


Figure 5.3.2C: Summary graph for membrane permeability data 20 days after the membranes were seeded with cells. The bar chart the percentage of FITC-BSA that had permeated across the cell seeded membrane in four consecutive 30 minute periods.

The naïve GFP-mES control at 20 days showed a similar total amount of protein crossover as it had at 13 days. The total was similar to sample B2 but much less than sample B1.The distribution of protein crossover was fairly even although the majority was from the first 60 minutes. The ISEMF samples showed much higher totals of protein crossover at 20 days than at 13 days with both being doubled. ISEMF1 was still considerably higher than ISEMF2 although the

disparity was less than at 13 days. The distribution of protein crossover was fairly even through the incubation with ISEMF1 having a slight majority in the initial 60 minutes. The negative controls showed almost identical results to the day 13 readings.

5.3.3 TEM

Figures 5.3.3i - iv (panels A - P) show the TEM images generated from the embedded membrane samples following 21 days of culture. Panels A - H show images from the mES samples, panels I - K show the images from the mES controls, panels L - O show the images from the GFP-mES samples and panel P shows the GFP-mES controls. The images generally show the membrane with any cells associated with it although some of the images focus on specific cell structures and the membrane is not shown.

Figure 5.3.3i panel A shows a single rounded cell that has detached from the membrane. Morphologically this cell resembles an undifferentiated ES cell and was around 1 μm across. Figure 5.3.3i B shows a number of cells (and surrounding cellular detritus) that have detached from the membrane. These cells also displayed the rounded morphology characteristic of undifferentiated cells. Figure 5.3.3i C and D show an ISEMF cell attached to the membrane, C shows the whole cell whilst D is at higher magnification. The cell had the expected elongated myofibroblast morphology. These images were of an insert that had been seeded with mES cells that had been co-cultured with day six embryonic chick gut tissue for seven days prior to seeding.

Figure 5.3.3ii E, F, G and H showed a single cell attached to the membrane, E showed the whole cell whilst F, G and H were at higher magnification. The cell had a rectangular morphology and a variety of cellular structures could be seen including some dark granules and lighter spaces. The cell was also much larger than the one observed in Figure 5.3.3i (around 4 μm across) and had a more rectangular morphology. These images were of an insert that had been seeded with mES cells that had been co-cultured with day six embryonic chick gut tissue

for seven days prior to seeding. Figure 5.3.3iii I, J and K showed another ISEMF cell associated with the membrane, I showed the whole cell whilst J and K were at higher magnification. The cell had the expected elongated myofibroblast morphology. These images were of an insert seeded with undifferentiated mES cells (mES control).

Figure 5.3.3iii L and 5.3.3iv M showed a single cell associated with the membrane, L showed the whole cell whilst M was at higher magnification. The cell had a square morphology although the corners are rounded and was very large (around 10 μm across). As with the cell shown in panel E there were a number of dark granules present in the cell. Figure 5.3.3iv panel N shows an ISEMF cell attached to the membrane that had the expected elongated myofibroblast morphology. These images were of an insert that had been seeded with GFP-mES cells that had been co-cultured with day six embryonic chick gut tissue for seven days prior to seeding.

Figure 5.3.3iv O showed the cellular detritus of a lysed cell that appeared to have been associated with the membrane. This showed that some damage may have occurred to the cells during sample processing for TEM. This image was of an insert that had been seeded with GFP-mES cells that had been co-cultured with day six embryonic chick gut tissue for seven days prior to seeding. Figure 5.3.3iv P showed part of an ISEMF cell attached to the membrane although the cell is partly obscured by part of the sample mounting (the dark black area). This image was of an insert seeded with undifferentiated GFP-mES cells (GFP control).

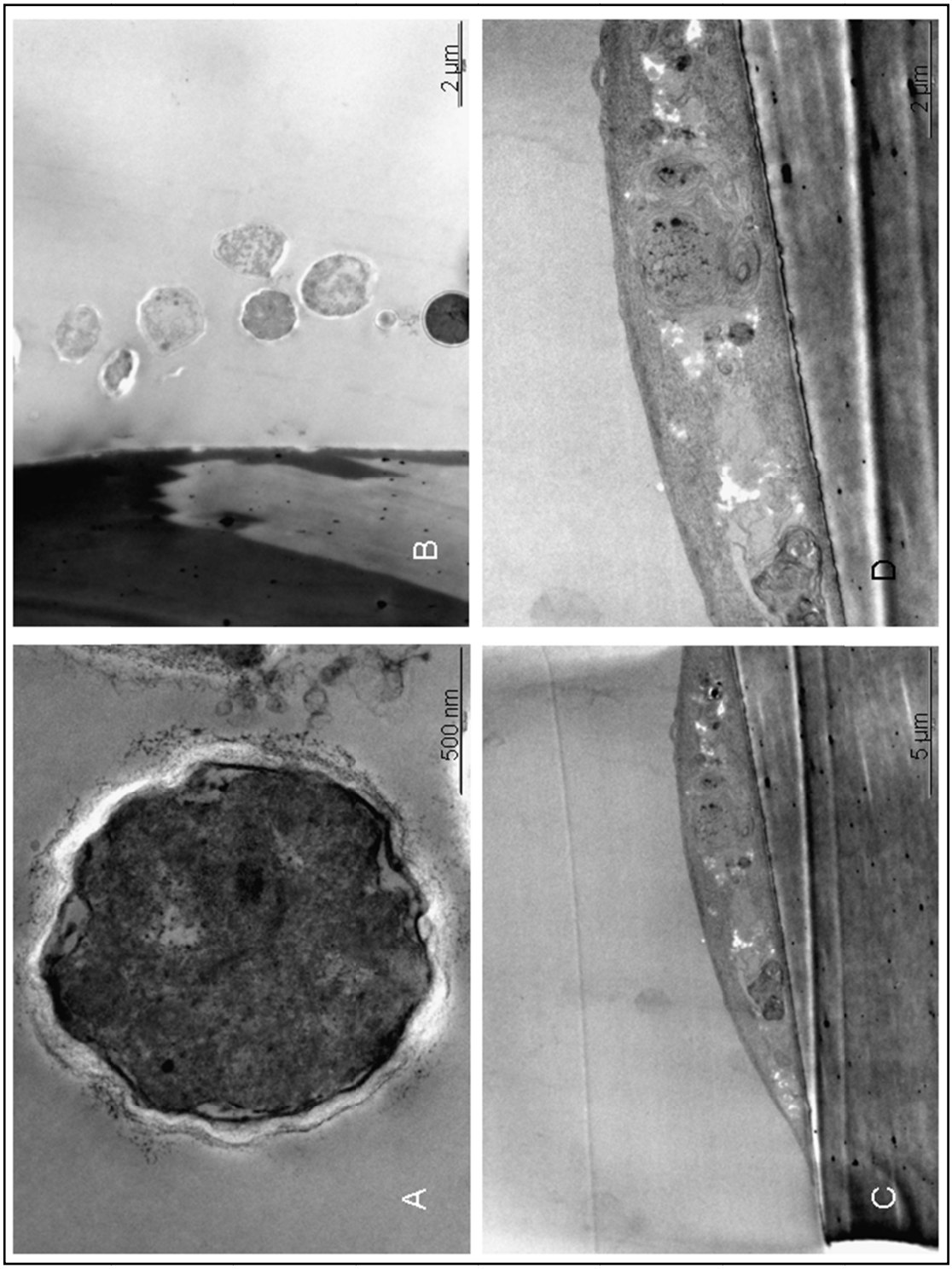


Figure 5.3.3i: TEM images from embedded seeded inserts after 21 days in culture. Panels A - D show images from the mES samples. Scale bars in bottom right corner of images.

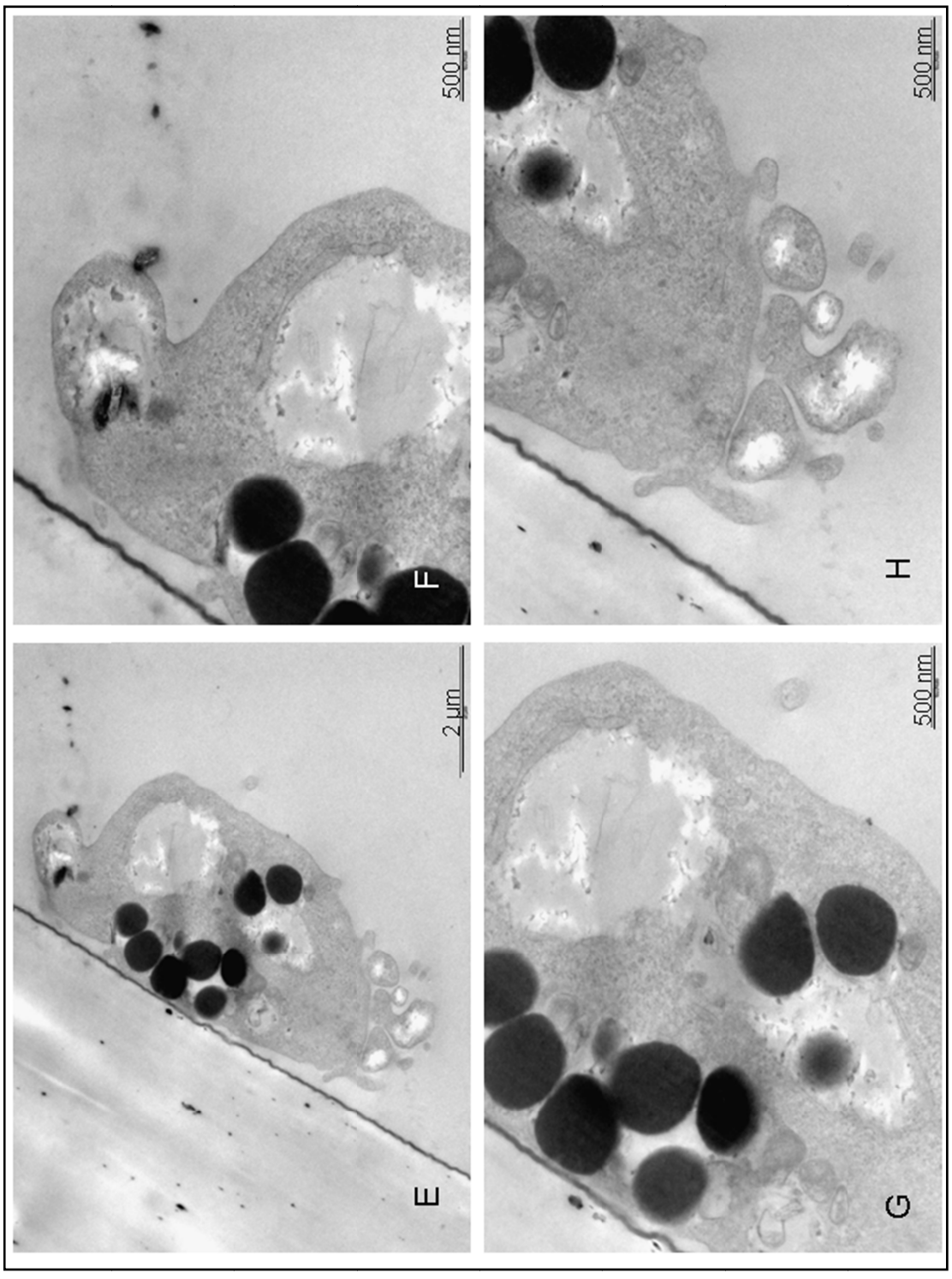


Figure 5.3.3ii: TEM images of embedded seeded inserts after 21 days in culture. Panels E - H show images from the mES samples. Scale bars in bottom right corner of images.

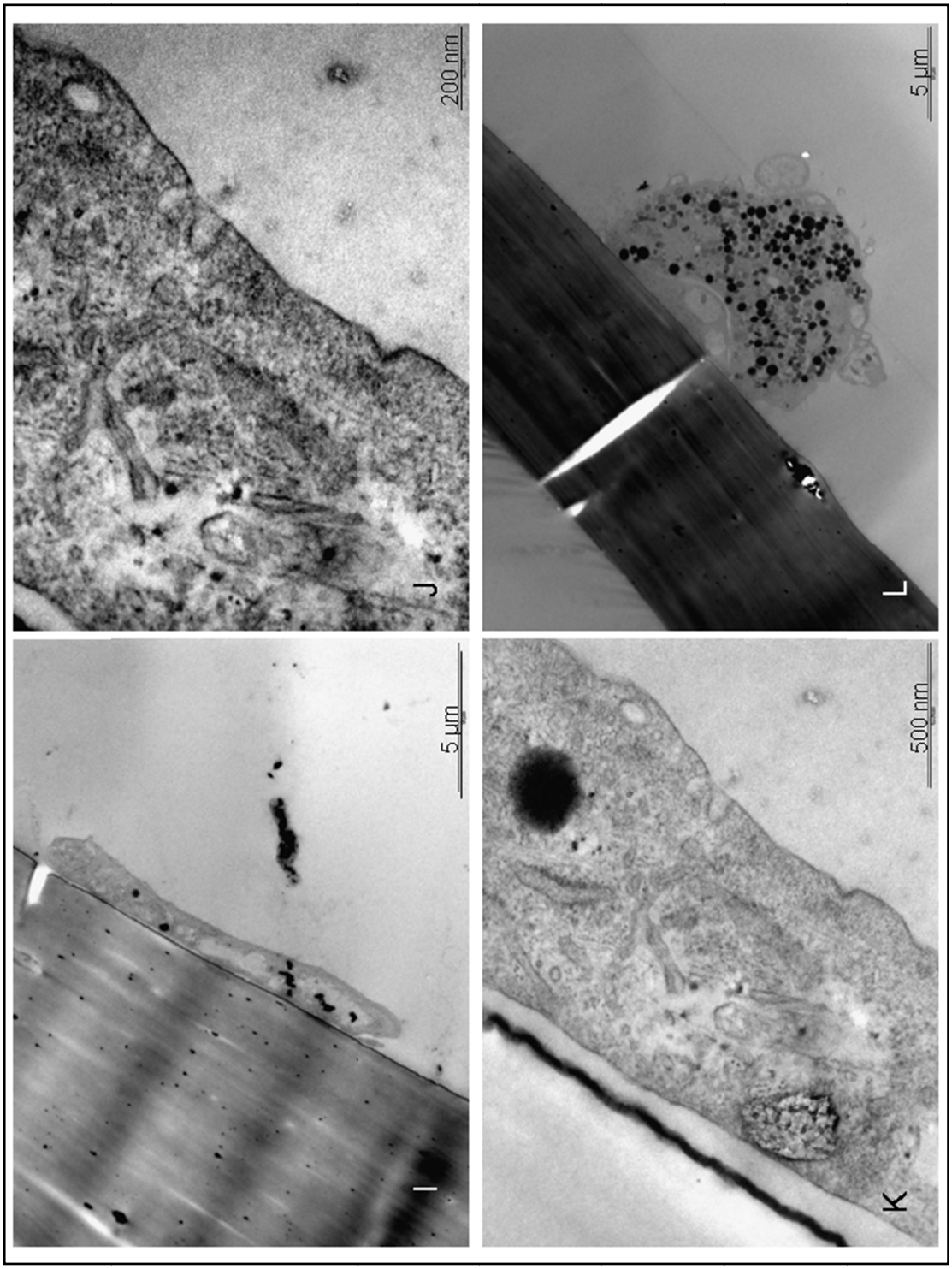


Figure 5.3.3iii: TEM images of embedded seeded inserts after 21 days in culture. Panels I - K show the images from the mES controls whilst panel L shows the first image from the GFP-mES samples Scale bars in bottom right corner of images.

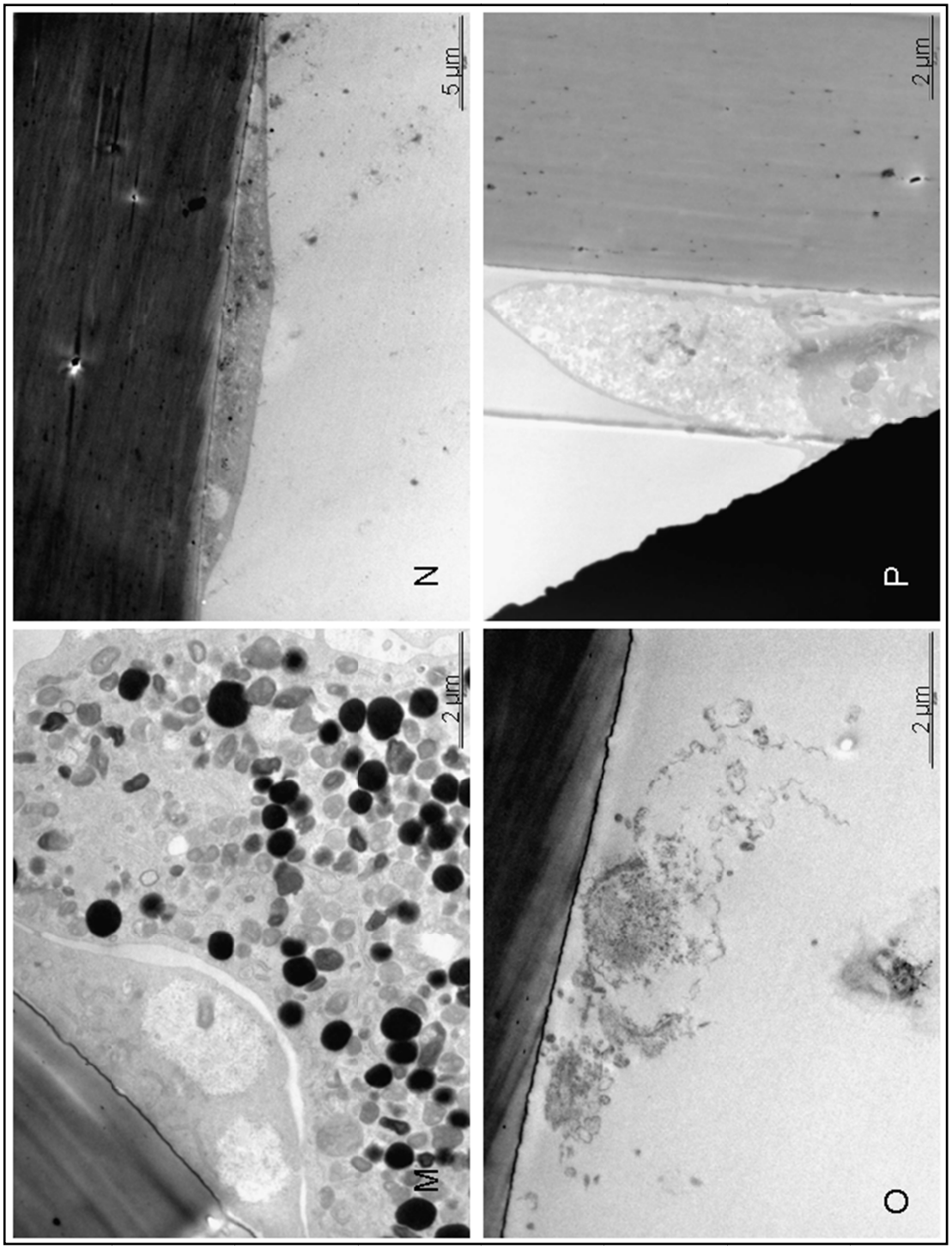


Figure 5.3.3iv: TEM images of embedded seeded inserts after 21 days in culture. Panels M - O show the images from the GFP-mES samples and panel P shows the GFP-mES control. Scale bars in bottom right corner of images.

5.3.4 Light microscopy

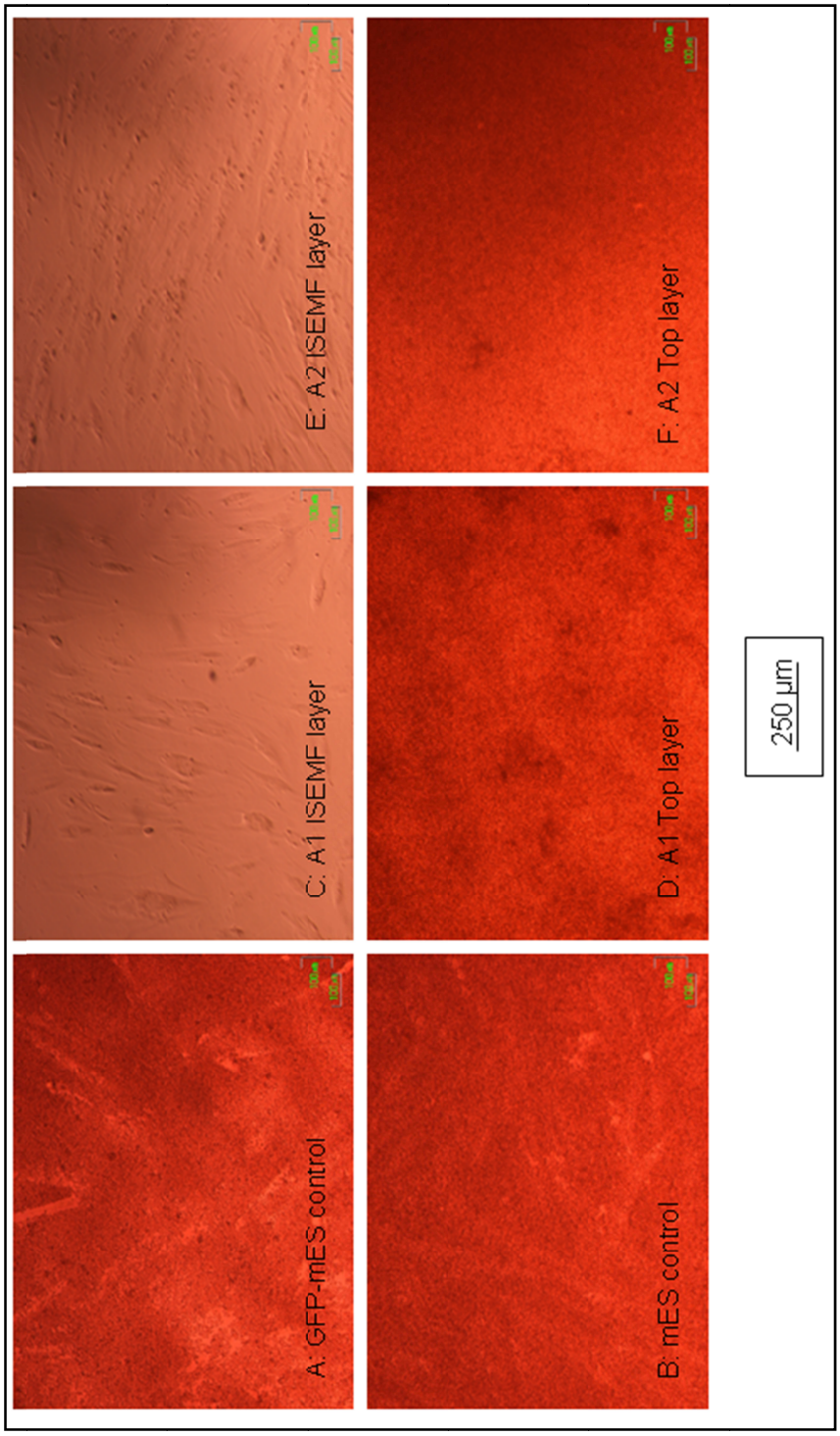


Figure 5.3.4i: Light microscopy images of seeded inserts after 15 days in culture. Control samples show ES derived cell layer only. Experimental samples show underlying ISEMF layer and top layer of mES derived cells. A = mES cells, B = GFP-mES cells.

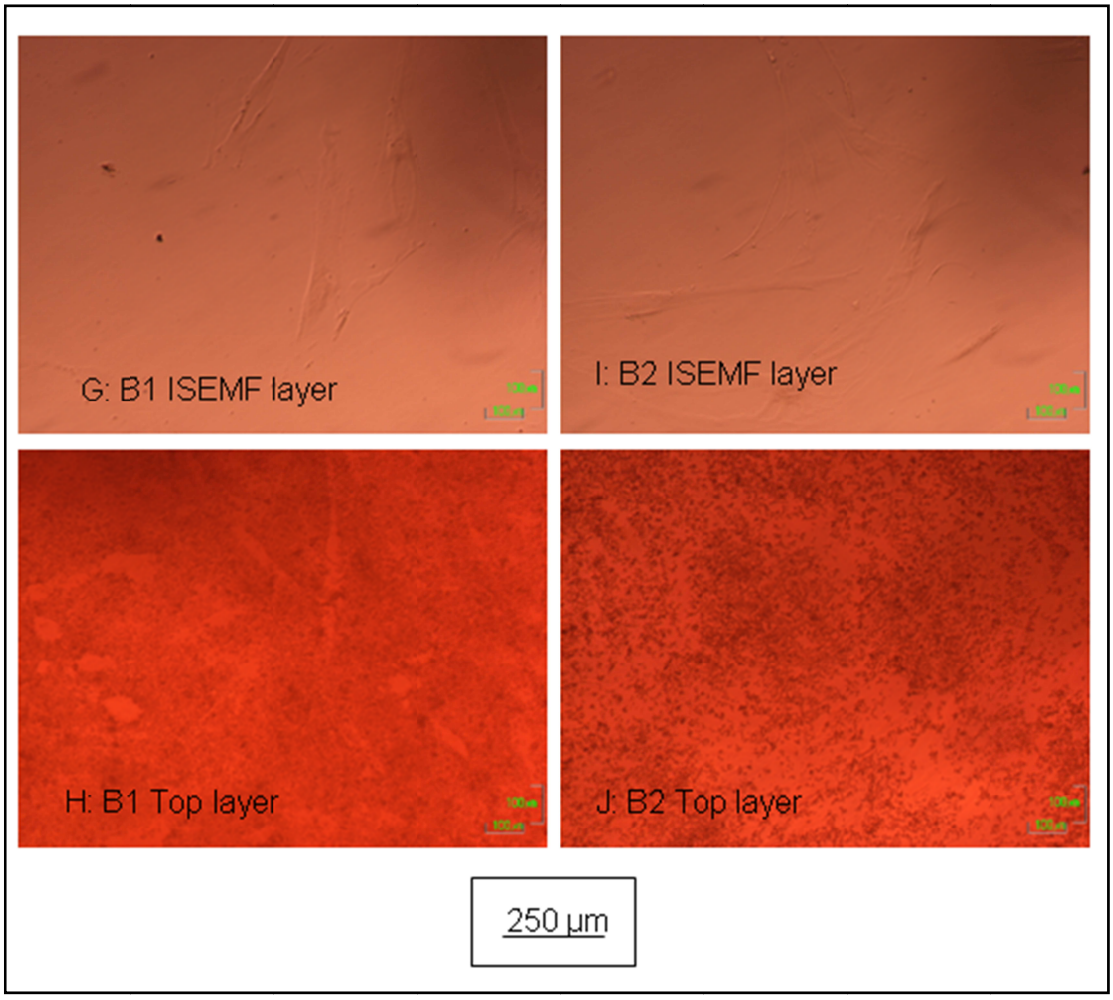


Figure 5.3.4ii: Light microscopy images of seeded inserts after 15 days in culture. Control samples show ES derived cell layer only. Experimental samples show underlying ISEMF layer and top layer of mES derived cells. A = mES cells, B = GFP-mES cells.

Figure 5.3.4 shows light microscopy images of the inserts taken 15 days after seeding, six days before the samples were fixed and processed for TEM imaging. Figure 5.3.4i panels A and B show the near confluent monolayer of cells in the GFP-mES and mES cell control conditions (no pre-seeding co-culture). The underlying ISEMF layers are not shown for the controls as they showed no difference from the experimental samples. Figure 5.3.4i C - F shows the images from the co-cultured mES cell seeded inserts (batch A). Panels C and E show the

underlying ISEMF cell layer. These images show that the underlying ISEMF cell layer had remained attached but had not achieved confluence.

Panels D and F show the top layer of mES cells (that had been co-cultured with embryonic day six chick gut tissue for seven days prior to seeding) where the seeded cells had formed a near confluent monolayer. Figure 5.3.4ii panels G - J show the same images from the co-cultured GFP-mES cell (that had also been co-cultured prior to seeding) seeded inserts (batch B). The ISEMF cells (panels G and I) in these inserts were sparse and had not reached the same degree of membrane coverage as in batch A. The co-cultured cells (Figure 5.3.4ii panels H and J) had formed near confluent monolayers.

5.4.1 Discussions and Conclusions

As detailed in Section 5.1 the Transepithelial resistance (TER) across a cell layer provides an indication of the integrity of an epithelial barrier. If solution/media (and the salts/charged molecules within) were able to freely permeate across the barrier the TER would be low (<200 Ω) and would not change significantly through the course of the experiment. In this instance an epithelial barrier was not being formed as the cells were not interacting with their neighbours or forming tight junctions If the TER rises through the initial part of the experiment (the first seven - 10 days) until reaching a steady level (>1000 Ω) this would indicate that as the cells seeded on the membrane grew to confluence they also began to interact cell to cell e.g. by forming tight junctions [Beltinger et al 1999, Blikslager et al 2007]. A more integrated (epithelial) barrier was formed and this prevented the easy passage of charged molecules (and hence electrical current) from one side of the barrier to another; this caused an increase in resistance.

Tight junctions are strong interactions between the membranes of adjacent cells [Denker and Nigam 1998] and when formed create an (almost) impermeable barrier. They link the Actin cytoskeletons of neighbouring cells via a number of membrane protein complexes with the most common proteins being Claudins [Furuse et al 1999] and Occludins [McCarthy et al 1996]. This barrier is critical to the functions of the intestinal epithelium as active transport mechanisms are required to cross it allowing the exclusion of micro-organisms (immune defence) and the selective absorption of nutrients.

The TER values for the co-cultured cells showed no significant differences from the TER readings for the control cells and negative controls with the ratio between them oscillating around 1:1 over the course of the experiment. If the samples were behaving in a significantly different manner from the controls you would expect to see this ratio change with a specific trend as the experiment progressed. If a genuine epithelial barrier had been formed the TER in the experimental

samples (and hence the ratio to the controls where the TER remained constant) would have risen steadily through the early part of the experiment as the epithelial layer formed before levelling off when an intact barrier was present. Tissue engineered intestine was compared with native tissue (see Section 1.5) following transplantation into rats. Initially (prior to transplantation) the engineered constructs had a lower TER than intact tissue but by end of the experiment the engineered tissue displayed comparable TER readings with the native tissue [Choi et al 1998].

The controls, particularly the negative control where there was no cellular component, were expected to remain at fairly steady levels, as they did. The ISEMF cell layer on the exterior membrane surface contributed little to the TER as the ISEMF controls gave similar readings to the negative controls. This corresponds with what was reported in the literature [Beltinger et al 1999]. This supports the hypothesis that it was only the formation of an intact epithelial layer along with its associated interactions, such as tight junctions [Denker and Nigam 1998] and associated structural proteins [Furuse et al 1999, McCarthy et al 1996] that would have produced a significant change in the TER. In retrospect the inclusion of a positive control where inserts were seeded with an epithelial cell type, such as HCA-7 or T84 [Beltinger et al 1999, Nash et al 1987, Willemsen et al 2002], would have provided an additional useful comparison.

One of the key functions of the intestinal epithelium is the absorption of nutrients from the intestinal lumen into the blood stream. This absorptive function is based upon concentration gradients but the epithelial layer restricts the loss of 'useful' molecules out of the circulation. This is reliant on a number of mechanisms including being a simple barrier to molecule diffusion as well as active transport mechanisms.

It would have been expected to see diffusion of the FITC-BSA across the cell-membrane barrier in the TMCC system in both the experimental and control samples. If an epithelial layer was formed in the experimental samples this would have regulated the passage of the labelled protein between the two liquid volumes on either side of the membrane. Therefore in the experimental samples the

passage of the labelled FITC-BSA should have occurred at a slower rate compared with the controls assuming that an epithelial layer was formed. The level of labelled protein should have equilibrated in all samples eventually but it should have reached equilibrium in the controls before the experimental samples.

The membrane permeability of the negative controls, which were identical at the two timepoints as there was no cellular component in these samples, gave very similar results throughout the experiment. This indicates that the results from both incubations could be compared with confidence. In a few samples the amount of FITC-BSA was at a higher percentage than the initial concentration but this could be explained by errors/variations from the standard curve and the plate reader (the limits of sensitivity of the plate reader and the standard curve must be considered when analysing these results). There was no significant change in the membrane permeability between the two timepoints which, whilst expected for the control samples where passage of the labelled protein was uninhibited [Beltinger et al 1999], indicates that there was no formation of an epithelial barrier layer in the experimental samples.

An established epithelial barrier should have slowed the passage of the labelled protein across the membrane in comparison with the controls. This is supported by the poor correlation between some of the experimental replicates. Once again a positive control using an epithelial cell type would have been useful; it has been shown that intestinal epithelial cell lines co-cultured with ISEMF cells prove a significantly greater barrier to the passage of (labelled) molecules such as ^{3}H-Mannitol than membrane only or ISEMF only controls [Beltinger et al 1999].

An identifying feature of intestinal epithelium is the distinct cell types that are found within the tissue. These cell types (see Section 1.2.1, Figure 1.4) have unique morphologies and structures that can be identified visually. The examination of the samples under the microscope (by light microscopy and by Transmission Electron Microscopy, TEM) was carried out to assess the architecture of the cells within the samples and to determine if any of them display the characteristic morphology of intestinal epithelial cell types as well as

looking for characteristic cell-cell interactions found in epithelial cell layers, particularly tight junctions [Denker and Nigam 1998].

The sample processing for the TEM imaging seems to have had a deleterious effect on the samples. Whilst some of the ISEMF cells seemed to have remained associated with the membrane the majority cells seeded on the interior side of the membrane seemed to have detached during processing (suggesting that they were strongly associated with the membrane). A few single cells were observed some of which had dissociated from the membrane. These cells were small and had a rounded morphology characteristic of unattached, dead cells. It is possible that a population of undifferentiated cells were still present following the co-culture with embryonic chick gut tissue and that these cells more readily dissociated from the membrane during sample processing for TEM.

Some cells were observed still associated with the membrane and these cells were often larger with a more rectangular morphology and noticeable internal architecture. Absorptive enterocytes are the most common cell type in the intestinal epithelium and have a rectangular morphology whilst all the cell types in the intestinal epithelium, particularly the Paneth cells and Goblet cells, have large internal structures (see Section 1.2.1) [Crosnier et al 2006].

No tight junctions were observed in any of the samples as those cells that were observed were always solitary and therefore could not interact with any neighbouring cells. There was also no evidence of the tissue morphology of the intestinal epithelium such as microvillous structures. The density of cells would suggest that an epithelial layer and its associated architecture were unlikely to have been formed (although this may have been a consequence of the cells being lost during the TEM processing). These cells had been observed (by light microscopy) throughout the experiment (as seen in Figure 5.3.5) including at the termination of the experiment when the samples were fixed. This suggests that (some of) the cells might have been lost during sample processing (see below) but if the cells association with the insert membrane had been strong (as would be expected if an epithelial layer had been formed) then it is unlikely that the cells would have detached so easily.

The TEM facility reported that during the processing and embedding phase 'something' detached from the interior of the inserts and this may account for the loss of these cells. If the cells had not attached well to the interior of the membrane this would explain the ease with which they were lost during processing in comparison to the ISEMF cells that remained associated with the exterior membrane surface. The two sets of experimental samples using mES or GFP-mES that had been through the co-culture differentiation protocol (batch A = mES cells, batch B = GFP-mES cells) were seeded with ISEMF cells from different flasks that originated from different tissue sources which may therefore have been proliferating at different rates. This could explain the differences observed (by light microscopy) in the number of ISEMF cells in different samples.

5.4.2 Summary

The data collected indicates that the putative ISC-like cells did not form into an epithelial layer in the model system. The cells did not display the morphological or functional characteristics of intestinal epithelium.

- There was no significant change in the TER through the course of the experiment. This indicates that an epithelial layer and its associated tight junctions were not being formed.

- The membrane permeability assay showed that the cells grown on the Transwell inserts (in all conditions) did not form a barrier to FITC-BSA. There were no significant changes through the course of the experiment which supports the conclusion that FITC-BSA could pass across the membrane as readily at the end of the experiment as at the start indicating that an epithelial layer had not been formed.

- The TEM images provide little information due to the loss of the cells during sample processing. Those cells that were present displayed some interesting cellular architecture but were so infrequent that making a

positive identification of the cells found in the intestinal epithelium [Crosnier et al 2006] was impossible. Nothing resembling the characteristic cellular architecture, such as microvilli, of the intestinal epithelial cell types was apparent.

Chapter Six: Summary and Appendices

6.1 Summary of conclusions

6.1.1 In vitro cell differentiation culture

A number of the variables examined through the course of these experiments seem to induce cells to differentiate towards the DE fate. However the process appeared to be far from efficient. The sensitivity of the CEE mES cell line to serum levels even when SR was used proved an obstacle to fully investigating the range of culture conditions that favour DE specification. The ultimate conclusions from this chapter of work are:

- Aggregating the cells into EBs promoted differentiation when compared to cells cultured in monolayer but did not specifically favour DE specification. In many cases when cells were reseeded in monolayer culture after an aggregation phase the strength of differentiation marker expression reduced. This phenomenon was still observed when the aggregation phase was extended from three to five days in duration.

- Act-A treatment seems to favour the specification of DE when compared to control conditions but the induction effect was not comprehensive or particularly strong. DE markers were generally expressed at elevated levels after Act-A treatment but markers of other fates, particularly mesoderm (see below) were sometimes present [Kubo et al 2004, Tada et al 2005]. The pluripotent marker Oct4 [Nichols et al 1998] was also still expressed although occasionally at reduced levels.

- Some differentiation towards mesoderm occurred in the conditions that favour DE specification. This was perhaps unsurprising as the two germ layers arise from a common precursor [Tada et al 2005]. There was little evidence of any ectodermal differentiation in the conditions that most favoured DE formation.

6.1.2 *Ex vivo* co-culture

Whilst the protocol proved to be a viable method for initiating the expression of ISC markers not all of the mES cells differentiated towards the ISC fate. The panel of differentiation markers were expressed in most of the co-cultured cell samples but there were signs that some cells remained undifferentiated and that some other fates may have been specified (see Table 4.3.2).

- The CEE mES cells were successfully transfected with the GFP encoding VS01 plasmid.

- The desired embryonic chick tissue could be successfully explanted and maintained in *ex vivo* culture for six – seven days. Sufficient injected cells are retained within the tissue explant during co-culture.

- There were no apparent differences in DE/ISC gene/protein expression between the cells treated *in vitro* with Act-A and naïve mES cells following seven days co-culture with intact chick gut explants or dissociated cells derived from the same.

- There were no differences in DE/ISC gene/protein expression between the GFP-mES cells and the unlabelled mES cells when differentiated using the co-culture protocol.

- The co-culture seemed to induce the expression of the key ISC marker *Lgr5* [Barker et al 2007] at the RNA level in a reproducible manner. This is supported by consistent LGR5 expression at the protein level.

- The co-culture methodology used supported the differentiation of naïve mES cells to differentiate towards an ISC-like fate at a molecular level. Similar results have been reported in the literature with different tissue types and target cell lineages [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005]. However not all the cells were differentiated or fully differentiated towards the intestinal progenitor fate.

6.1.3 Tissue model co-culture

The data collected indicates that the putative ISC-like cells did not form into an epithelial layer in the model system. The cells did not display the morphological or functional characteristics of intestinal epithelium.

- There was no significant change in the TER through the course of the experiment. This indicates that an epithelial layer and its associated tight junctions were not being formed.

- The membrane permeability assay showed that the cells grown on the Transwell inserts (in all conditions) did not form a barrier to FITC-BSA. There were no significant changes through the course of the experiment which supports the conclusion that FITC-BSA could pass across the membrane as readily at the end of the experiment as at the start indicating that an epithelial layer had not been formed.

- The TEM images provide little information due to the loss of the cells during sample processing. Those cells that were present displayed some interesting cellular architecture but were so infrequent that making a positive identification of the cells found in the intestinal epithelium [Crosnier et al 2006] was impossible. Nothing resembling the characteristic cellular architecture, such as microvilli, of the intestinal epithelial cell types was apparent.

Summary

Overall this work has shown that co-culturing pluripotent mES cells with embryonic chick gut tissue can induce limited differentiation towards the ISC fate with increased expression of the selected ISC markers observed when compared to controls. Pre-treating the cells with growth factors *in vitro* did not seem to enhance this differentiation but there was scope for these techniques to be refined. Following the differentiation protocols the cells did not display the desired

physiological characteristics but again there was scope to refine these techniques, particularly with regard to selecting cells positive for the expression of the chosen molecular markers. These techniques show promise but do require some further development. Ideas for how this work might be refined, expanded upon and taken further are discussed in the following section.

Chapter Six: Summary and Appendices

6.2 Future work

The sensitivity of the CEE mES cell line to FACS proved a major technical hurdle in a number of experiments. Whilst a number of factors that might have caused this such as temperature, duration of time out of 'normal' culture conditions, the stress of passing through the FACS machine and comparison with other mES cell types have been investigated the cost of using the FACS facility and time limitations meant that no adequate explanation for this sensitivity could be found. Establishing why the cells display this sensitivity, and hopefully finding a solution to the problem, would allow considerable refinement of the differentiation protocols at a number of stages.

Following the *in vitro* differentiation stage detailed in Chapter Three cells could be selected by positive expression of DE cell surface markers, such as CXCR4 [D'Amour et al 2005, Yasunaga et al 2005]. Secondly, labelled murine cells could be easily separated from chick derived cells following *ex vivo* co-culture detailed in Chapter Four. The cells could then be selected by positive expression of ISC cell surface markers, such as LGR5/GPR49 [Barker et al 2007], before being seeded in the TMCC experiments carried out in Chapter Five.

The co-culture protocols were technically difficult to carry out and there were some parts of the process that proved 'rate limiting' particularly the egg/embryo dissection stage. If embryonic gut tissue cells could be isolated and grown in continuous culture this would provide an easier way of supplying cells for co-culture. It would need to be established if the cells continue to produce the same molecular signals in this form as they do as intact explants/freshly isolated cells.

Extending the concept above further it might be possible to use cells/tissue explants to condition media that could then be used in the further differentiation of ES cells (or other multipotent cells) towards the intestinal epithelial progenitor fate [Maxson and Burg 2008, Kang et al 2009]. This would remove some of the

more time consuming stages of the co-culture protocols. Using gut cells from continuous cell culture or conditioned media rather than tissue explants would also permit the extension of the time the ES cells could be cultured under differentiation conditions as the degradation of the tissue explants would no longer be a limiting factor. It would also reduce the number of animals required for the experiments. Media could be conditioned using embryonic murine gut tissue; this would give species specific signalling factors (a consideration that would also be important in transferring the techniques to hES cells). Using conditioned media might also permit an expansion in the number of cells used in the differentiation protocols as this would not be limited by the number of cells that could be injected into a tissue explant.

These protocols could be transferred to produce different types of cells. Injecting ES cells into other embryonic tissue explants, such as the liver or the kidney, could be combined with *in vitro* DE specification to produce a range of target cell types. This could be expanded to incorporate mesoderm (e.g. muscle) and ectoderm (e.g. skin) derived tissues. A number of studies have already been conducted exploring the potential of co-culture of ES cells with early embryonic tissues [Sugie et al 2005, Fair et al 2003, Van Vranken et al 2005, Coleman et al 2007] and some have employed *in vitro* growth factor treatments [Sugie et al 2005, Coleman et al 2007] or aggregation into EBs [Van Vranken et al 2005] prior to the co-culture stage as discussed in Section 4.1.

The different parameters within the differentiation protocols could be varied or in some cases combined. For example intact EBs could be used in co-culture with embryonic chick gut tissue. EBs could be generated *in vitro* and cultured for around five days (to initiate differentiation) before being injected into embryonic chick gut tissue explants in *ex vivo* culture [Van Vranken et al 2005]. The length of the culture periods in the *in vitro* differentiation stage could be varied e.g. would extending the length of the EB phase of culture to from five to seven days increase the expression of DE markers. Resources permitting, the concentration of Act-A growth factor could be increased (from 10 ng/ml) to concentrations of up to 100 ng/ml. Other factors such as LY294002 (an inhibitor of the enzyme PI3K)

[MacClean et al 2007] could also be used to replace/compliment the effects of Act-A.

There are a number of different methods by which EBs can be generated in addition to the suspension culture method detailed in Section 2.1.4, such as the hanging droplet technique [Kurosawa 2007]. These methodologies could be compared to the existing protocol to see if they are more suited for generating DE and its derivatives. Ideally this would involve an expanded version of the experiments detailed in Section 3.2.1iv – 3.2.1vi and 3.3.4 – 3.3.6, where the different variables are all explored in parallel.

If the results in Chapter Five had been more significant it would have been necessary to repeat the experiments with the inclusion of a greater range of controls. A positive control using an epithelial cell line, such as HCA-7, seeded on the interior surface of the cell culture inserts would definitely have been included. These cells would have been expected to produce a functional epithelial barrier and would therefore give an indication of what changes in TER and membrane permeability occur as the epithelial barrier is formed [Beltinger et al 1999, Blikslager et al 2007].

Investigating the expression of a range of markers, such as tight junction associated proteins [McCarthy et al 1996, Furuse et al 1999] and markers of various intestinal epithelial cell lineage markers (see Section 1.2.3), and comparison with positive controls would also provide a further assessment of whether the cells were functioning as ISC. This study could be further expanded by examining the TER and membrane permeability (of FITC-BSA) of excised gut tissue explants in *ex vivo* culture [Tait et al 1995, Choi et al 1998]. This might also provide interesting observations as to how the values change as the tissue degrades with time and the epithelial barrier loses integrity – the reverse scenario of what you would hope to observe in the experimental samples.

Further imaging of the seeded Transwell inserts could be carried out. The light microscopy imaging carried out in Chapter Five allowed observation of the cells during the culture period but only in 2D whilst the TEM imaging gave high

magnification images of individual cells. Sectioning of the inserts at the conclusion of the experiment and staining with e.g. H & E would show if any cellular organisation/tissue structures were evident (see Section 1.2.1) [Crosnier et al 2006].

Such sections would also be suitable for antibody staining for markers of intestinal epithelial cell types (see Section 1.2.3) such as LGR5 (Intestinal Stem Cells) [Barker et al 2007], CDX2 (a key transcription factor for correct intestinal patterning during development) [Beck et al 1999], Epithelial Antigen (a cell surface marker found throughout epithelial layers) [Engelhardt et al 1993] and specific markers for Goblet Cells (Periodic Acid Schiff or Dolichos Biflorus Agglutinin), Enteroendocrine Cells (Synaptophysin or Chromogranin A) and Paneth Cells (Lysozyme or Periodic Acid Schiff expressed at the base of the Crypts of Lieberkuhn).

More extensive molecular analysis of the cells could be carried out throughout the differentiation protocols. Techniques such as Microarray analysis could quantitatively assess the expression of many thousands of genes. Differentiating cells could be compared to undifferentiated ES cells to highlight the changes in gene expression induced by the differentiation protocols and to normal intestinal epithelium to assess the full extent to which the cells are differentiating towards the target cell type. Microarray analysis would also allow comparisons of the gene expression of cells that had been through different differentiation protocols e.g. cells that had been Act-A treated and co-cultured versus cells that had only been co-cultured. This would facilitate the identification of the most 'efficient' way to differentiate the cells towards the desired target type.

If the sensitivity to FACS was resolved it would allow quantification of what percentage of the cells were differentiating towards a particular fate using a labelled antibody against an endodermal surface marker such as CXCR4 [D'Amour et al 2005, Yasunaga et al 2005]. As mentioned above, this would also allow selection of the differentiated fraction of the cell population for use in further experiments. The same process could be repeated using intestinal stem

cell surface markers, such as LGR5 [Barker et al 2007], to select the desired fraction of the cell population following the co-culture differentiation protocol.

The molecular/viability analysis carried out on the chick tissue explants could also be expanded. Further stains, such as Alamar blue (Resazurin) [Anoopkumar-Dukie et al 2005, O'Brien et al 2000] are available as alternatives to Live-Dead solution for assessing the viability of tissue in culture. A more extensive analysis of the RNA and protein expression of the tissue through *ex vivo* co-culture could also have been carried out.

The CEE mES cell line used in these studies has been successfully differentiated towards osteogenic fates [Gothard et al 2010] but did not readily differentiate towards DE under the selected conditions. Other mES cell lines that have previously been differentiated towards endodermal fates could be substituted in these experiments [Tada et al 2004, Kubo et al 2004, Yasunaga et al 2005]. Using feeder-independent mES cell lines rather than the feeder dependant CEE mES cell line might eliminate the attachment issues encountered in the early experiments in Chapter Three.

This might also provide an indication as to whether transferring the techniques to hES cells (please see below) is likely to prove successful; if results cannot be replicated in different mES cell lines it would suggest a reduced likelihood that the results would be replicated in ES from a different species. Other mES cell lines might not display the same sensitivity to serum levels shown by the CEE mES cell line which would allow the full effects of serum concentration on the differentiation of the cells to be investigated [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005, D'Amour et al 2005].

If the differentiation protocols could be refined then the research could be developed further to encompass further objectives. Collaborating with researches producing scaffolds for GI tissue engineering would provide the opportunity to show that the derived cells would work in a complete tissue engineering strategy. The cells would be seeded on a suitable scaffold and placed in 3D/*in vivo* culture and allowed to proliferate (see Section 1.4) [Choi and Vacanti 1997]. The

scaffold would be designed to reproduce the morphology of intestinal tissue. Potentially such constructs could be used for disease modelling, drug absorption studies and ultimately clinical applications.

All current strategies for intestinal epithelial engineering involve the use of isolated adult crypts (see Section 1.4) [Flint et al 1991, Evans et al 1992]. The supply of such material is limited and this would limit the potential scope for clinical use of tissue engineered intestine (much in the same way that organ replacement therapies are current limited by the number of suitable donors). Using an ES (or an IPS) derived cell source would address this issue as ES cells can be readily cultured in large numbers *in vitro.* Using ES derived cells would also allow the observation of disease progression in conditions such as Crohn's disease. Intestinal models could be established with both 'normal' and Crohn's patient derived cells and the effects of microbial and dietary factors [Nash et al 1987, Willemsen et al 2002] in terms of inducing Crohn's disease could be investigated by examining the normal cells compared to the model with cells known to have a tendency towards the disease.

Once proof of principle had been established using mES cells the next step would be to transfer the techniques to hES cells. For any tissue engineered material to have clinical potential it would need to be human tissue. Studies in the literature have shown that the DE inducing effects of Act-A on mES cells [Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005] are comparable to those on hES cells [D'Amour et al 2005]. Given the potential that IPS cells [Takahashi and Yamanaka 2006, Yu et al 2007, Takahashi and Yamanaka 2007, Duan et al 2011] (see Section 1.3) have to provide a patient specific cell source it would also be useful to see if the differentiation protocols had the same effects in them as in 'conventional' ES cells. This would be a critical step in developing tissue engineered intestine that was of clinical use.

Whilst they were only produced as a technical step to facilitate the co-culture experiments, further characterisation of the GFP-mES cells could be carried out. Although they continued to form EBs and express *Oct4* following transfection with the VS01 plasmid a more extensive molecular analysis would be required to

establish that the transfection had not caused any significant alterations to the cells molecular character (at both the nucleic acid and protein level). This could include Microarray experiments to compare the full gene expression profile of the transfected cells with the unaltered ES cell line. The ability of the cells to contribute to chimeras and form teratomas could also be investigated.

Chapter Six: Summary and Appendices

6.3 Appendix A - Supplementary data

This section contains summary tables and supplementary information for data displayed in the results sections.

6.3.1 Chapter Three

6.3.1 *In vitro* differentiation experiment one - mES cell differentiation in low serum media

Evidence in the literature suggests that low serum concentrations and the presence of Act-A induces mES cells to differentiate towards the DE lineage in culture with greater efficiency than as EBs in standard cell culture media [D'Amour et al 2005, MacClean et al 2007, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005]. This experiment aimed to show that these results could be achieved using the CEE mES cell line. Complete (CEE mES) cell culture media contains around 10% FCS so the initial experiment aimed to establish if the CEE mES cell line could tolerate low serum (2% or lower) conditions and to observe any discernible effects on their differentiation under these conditions.

Figure 3.3.1A shows that the cells rapidly reached a confluent state in the CEE complete cell culture media (10% FCS, LIF+, see Section 3.2.1i) with large single colonies formed by 24 hours. The cells continued to proliferate rapidly and by the end of the experiment a confluent monolayer had formed. The cells maintained a rounded morphology and did not appear to change in size, remaining approximately 60 - 80 μm in diameter.

Figure 3.3.1B shows that the cells grew fairly rapidly in the SNL media (10% FCS, LIF-, see Section 3.2.1i) over the first 24 hours, but not at the same rate as in CEE complete cell culture media, and then appeared to proliferate faster for the duration of the experiment. By the end of the experiment a confluent monolayer had formed. Although it was unobservable at 120 hours, at 48, 72 and 96 hours some of the cells appeared to have grown larger and altered their morphology. Some elongated cells were visible in the photos from these timepoints.

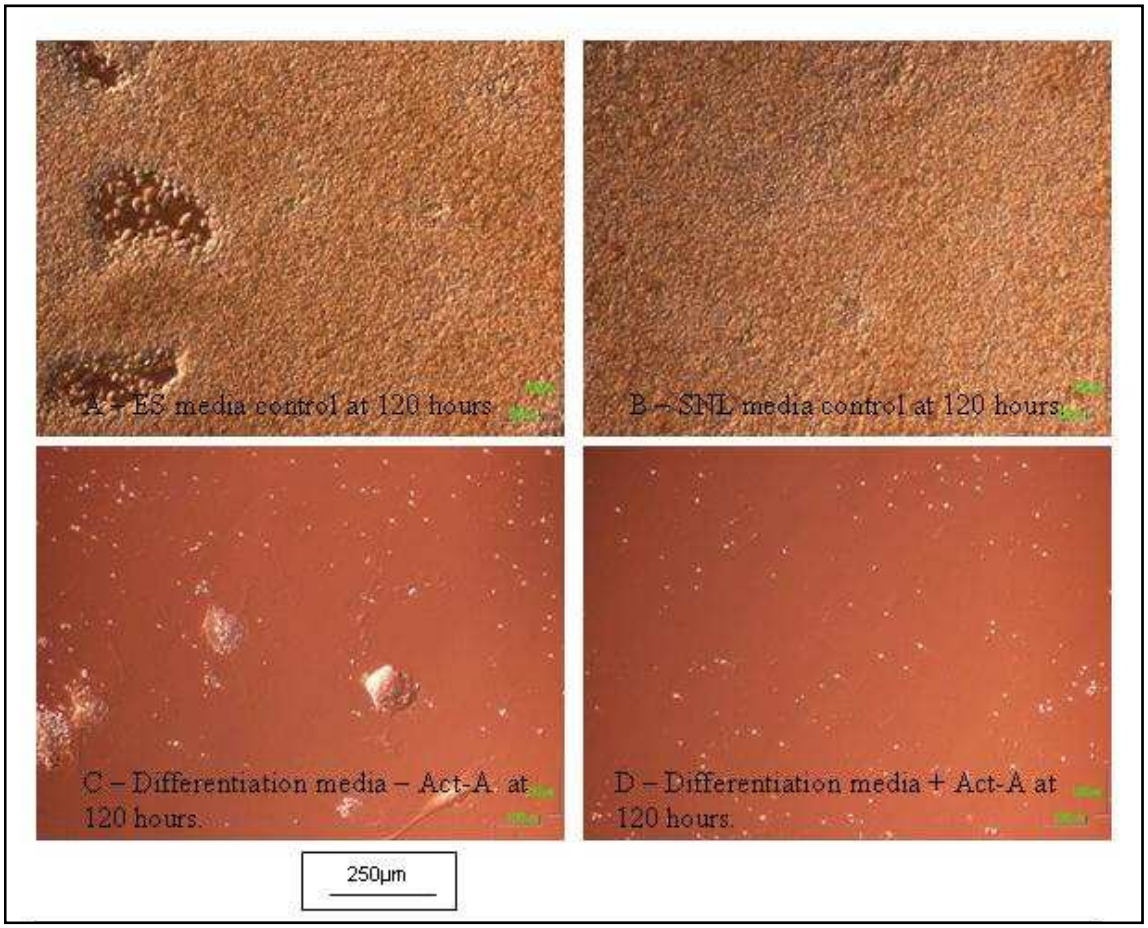


Figure 3.3.1: Representative Light microscopy images of CEE mES cells cultured in A - CEE complete cell culture media (10% FCS, LIF+) control for 120 hours, B - SNL media control for 120 hours (10% FCS, LIF-), C – Differentiation media without Act-A for 120 hours and D - Differentiation media with 10 ng/ml Act-A for 120 hours.

In Figure 3.3.1C some cells appeared to adhere to the surface of the wells but many did not resulting in early loss of cell numbers in the differentiation media without Act-A. Proliferation was also minimal. Although some cell clusters were apparent by 120 hours they appeared to be unattached to the culture plate. Figure 3.3.1D illustrates that cell growth was minimal and that only a small fraction of the cells adhered to the well surface in the differentiation media plus Act-A. Some cells appeared enlarged and spread out at 48 - 72 hours but by the end of the experiment these cells were no longer observable.

When cultured under low serum conditions the CEE mES cell line did not proliferate or adhere as it did when cultured in CEE complete cell culture media

(10% FCS, LIF+/-) resulting in almost total cell death and therefore no samples were taken for molecular analysis.

6.3.2 *In vitro* differentiation experiment two - mES cell differentiation in SR media

The presence of Act-A in serum replacement (SR) media rather than low serum has been previously shown to promote differentiation to the DE fate without having a negative effect on cellular attachment and proliferation [D'Amour et al 2005, Kubo et al 2004, Tada et al 2005, Yasunaga et al 2005]. In this study, the ability to culture CEE mES cells in SR media was assessed. In addition, the effect of using different ECM components to coat the culture vessel was also investigated.

Figure 3.3.2A shows that the cells attached to the well surface but did not reach a confluent monolayer (it should be noted that a large number of cells had not attached at 48 hours and were removed with the first media change hence the apparent reduction in cell number from 48 - 72 hours) in CEE complete cell culture media on gelatin coated TCP. Surviving cells remained small and rounded in morphology and formed multicellular aggregates. Similarly, Figure 3.3.2B shows that the cells attached to the plate surface in CEE complete cell culture media on Collagen IV coated TCP. There was a significant loss of cells between 72 and 96 hours but those that remained were able to become established in culture.

Figure 3.3.2C shows the cells attached to the culture plate and proliferated until 72 hours after seeding SNL media on Gelatin coated TCP. After this point there was a large reduction in cell number. The cells that survived showed altered (usually elongated) morphologies and were generally larger in size. Figure 3.3.2D shows the cells proliferated slowly for the first 72 hours but a significant number showed changes in morphology at 72 hours in SNL media on Collagen IV coated TCP. From 72 - 96 hours the cells proliferated rapidly but from 96 - 120 hours there was a significant loss of cells. The cells that proliferated at a faster rate (and then died) were generally small and round (unaltered ES cell morphology).

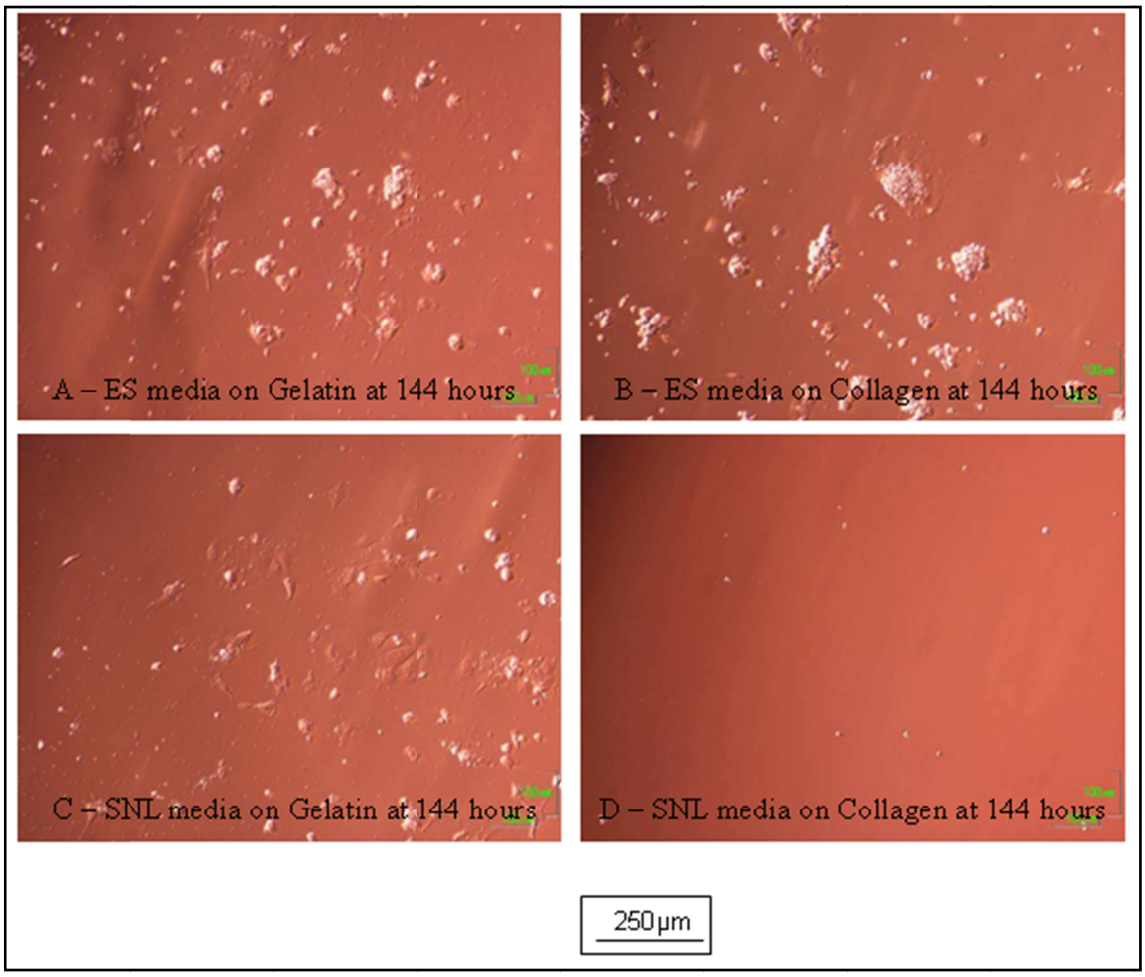


Figure 3.3.2i: Light microscopy images of mES cells after 144 hours culture under A - ES media control on Gelatin coated TCP, B - ES media control on Collagen IV coated TCP, C - SNL media control on Gelatin coated TCP, D - SNL media control on Collagen IV coated TCP.

In Figure 3.3.2E the cells showed little or no proliferation or changes in morphology during the course of the experiment in SR media on Collagen IV coated TCP. Very few cells appeared to attach to the culture plate. Similar results were observed in Figure 3.3.2F where the cells showed no observable proliferation or changes in morphology in SR media plus Act-A on Collagen IV coated TCP. Figure 3.3.2G showed that there was little initial cell adherence to the plate in SR media. Switching to SNL media after 72 hours, on Collagen IV coated TCP, the cells showed little or no proliferation or changes in morphology. The cells appeared to proliferate more quickly after the switch to serum containing culture media at 72 hours. The cells did not appear to attach or

proliferate any better in SR complete media compared with low serum conditions. There was no apparent difference between cellular attachment on Gelatin or Collagen IV coated TCP.



Figure 3.3.2ii: Light microscopy images of mES cells after 144 hours culture under E - SR media on Collagen IV coated TCP, F - SR media with Activin-A on Collagen IV coated TCP and G - SR media with Act-A for 72 hours then SNL media thereafter on Collagen IV coated TCP.

Table 6.3.1: Summary table for RT-PCR band strength scores from *in vitro* experiment 4.

Sample	GAPDH	GAPDH2	FoxA2	Sox17	Oct4	CXCR4
A1-48	5	5	0	0	2	0
A1-72	5	5	0	0	3	0
A1-96	5	5	0	0	0	0
A1-120	6	5	0	0	5	0
A1-144	5	6	1	0	6	0
A1-168	7	6	1	1	6	1

Sample	GAPDH	GAPDH2	FoxA2	Sox17	Oct4	CXCR4
B2-48	3	3	0	0	0	0
B2-72	4	4	0	0	2	0
B2-96	1	5	0	0	0	0
B2-120	3	5	0	0	3	0
B2-144	4	5	0	0	0	0
B2-168	2	6	0	0	1	0

Sample	GAPDH	FoxA2	Sox17	Oct4
D4-48	3	0	0	0
D4-72	2	0	0	0
D4-96	2	0	0	0
D4-120	2	0	0	0
D4-144	2	0	0	0
D4-168	2	0	0	0

Sample	GAPDH	FoxA2	Sox17	Oct4
G8-48	6	0	0	2
G8-72	4	0	0	0
G8-96	3	0	0	0
G8-120	4	0	0	0

Sample	GAPDH	GAPDH2	FoxA2	Sox17	Oct4	CXCR4
C3-48	5	6	0	0	3	0
C3-72	4	6	0	0	3	0
C3-96	3	6	0	0	0	0
C3-120	6	6	3	1	2	1
C3-144	6	6	3	1	2	1
C3-168	6	6	2	1	4	0

Sample	GAPDH	GAPDH2	FoxA2	Sox17	Oct4	CXCR4
F7-48	5	5	2	1	5	0
F7-72	4	5	0	0	0	0
F7-96	3	5	0	0	5	0
F7-120	6	5	1	0	5	0
F7-144	6	5	4	0	5	0
F7-168	6	5	3	1	5	2

Sample	GAPDH	FoxA2	Sox17	Oct4
E5-48	5	0	0	1
E5-72	4	0	0	2
E5-96	4	0	0	3
E5-120	7	0	0	3
E5-144	7	3	1	3
E5-168	7	2	1	5

Sample	GAPDH	FoxA2	Sox17	Oct4
H9-48	5	0	0	3
H9-72	4	0	0	0
H9-96	3	0	0	0

Table 6.3.2: Summary table showing the PCR band strength scores from *in vitro* experiment number five, Part A.

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
1-72A	7	5	1	3	2	3
1-72B	7	4	1	3	3	3
1-216A	7	5	0	1	0	3
1-216B	7	3	1	2	0	3
1-288A	6	5	1	1	0	2
1-288B	6	5	1	1	0	2

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
2-72A	6	4	0	1	0	2
2-72B	6	4	0	0	0	3
2-216A	5	4	1	3	0	5
2-216B	5	3	0	2	0	4

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
3-48A	5	4	0	1	0	0
3-48B	5	4	0	0	0	0
3-120A	5	2	0	1	0	0
3-120B	5	4	0	1	0	2
3-268A	5	4	0	1	0	4
3-268B	5	4	1	0	0	4
3-336A	5	4	1	1	1	2
3-336B	5	4	1	1	1	2

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
5-72A	7	4	0	3	3	4
5-72B	7	4	0	2	1	3
5-216A	6	4	0	1	0	1
5-216B	6	4	0	1	0	2
5-288A	6	4	0	1	0	2
5-288B	6	4	0	2	1	3

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
4-72A	7	4	0	0	0	2
4-72B	6	4	0	0	0	2
4-216A	7	4	4	2	0	3
4-216B	7	4	4	2	0	3

Table 6.3.3: Summary table of the RT-PCR band strength data from experiment five part B, experimental conditions one, two and three.

Sample	GAPDH	GAPDH2	Oct4	Nanog	CXCR4	FoxA2	Sox17 (30)	Sox17 (35)	GATA4	Brachyury	Nestin
1-72A	5	5	5	3	1	5	0	1	1	0	1
1-72B	5	5	6	3	1	5	0	0	1	0	1
1-216A	6	7	6	4	4	6	1	1	1	0	1
1-216B	6	7	0	4	5	6	0	0	2	4	1
1-288A	5	6	6	4	1	6	0	0	1	0	1
1-288B	5	6	6	5	2	6	0	1	1	0	1

Sample	GAPDH	GAPDH2	Oct4	Nanog	CXCR4	FoxA2	Sox17 (30)	Sox17 (35)	GATA4	Brachyury	Nestin
2-72A	5	6	6	6	1	4	0	0	1	0	1
2-72B	5	6	6	6	1	5	0	0	1	0	1
2-216A	5	6	6	6	1	6	0	0	1	0	1
2-216B	5	6	6	6	1	6	0	0	1	1	1

Sample	GAPDH	GAPDH2	Oct4	Nanog	CXCR4	FoxA2	Sox17 (30)	Sox17 (35)	GATA4	Brachyury	Nestin
3-48A	4	6	5	5	0	0	0	0	0	0	0
3-48B	4	5	5	5	0	3	0	0	1	0	0
3-120A	6	6	5	5	1	5	0	0	1	0	1
3-120B	6	6	5	5	1	5	0	0	1	0	1
3-268A	5	7	5	6	3	5	1	3	2	5	4
3-268B	5	7	5	6	3	5	1	3	2	5	4
3-336A	5	7	5	5	3	6	1	4	2	3	3
3-336B	5	7	5	5	3	6	1	5	2	1	3

Table 6.3.4: Summary table for RT-PCR band strength data from experiment five part B, experimental conditions four and five plus zero hour controls.

Sample	GAPDH	GAPDH2	Oct4	Nanog	CXCR4	FoxA2	Sox17 (30)	Sox17 (35)	GATA4	Brachyury	Nestin
C-0hrA	5	5	6	6	1	5	0	0	1	0	1
C-0hrB	5	5	6	6	1	0	0	0	1	0	1

Sample	GAPDH	GAPDH2	Oct4	Nanog	CXCR4	FoxA2	Sox17 (30)	Sox17 (35)	GATA4	Brachyury	Nestin
4-72A	6	7	4	5	0	3	0	0	0	0	0
4-72B	6	7	4	6	0	4	0	0	1	0	1
4-216A	6	7	4	6	1	5	0	1	1	1	1
4-216B	6	7	4	6	0	5	0	0	1	1	1

Sample	GAPDH	GAPDH2	Oct4	Nanog	CXCR4	FoxA2	Sox17 (30)	Sox17 (35)	GATA4	Brachyury	Nestin
5-72A	6	7	4	5	1	5	0	0	1	1	1
5-72B	6	7	4	5	1	0	1	1	2	0	1
5-216A	4	7	4	5	0	5	0	0	0	0	0
5-216B	6	7	4	5	1	5	0	0	1	1	4
5-288A	6	7	4	4	0	5	0	0	0	4	0
5-288B	6	7	4	6	1	5	0	0	1	0	1

Table 6.3.5: Summary table for experiment six RT-PCR data. Summary tables for control conditions from experiment five also displayed.

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
0-A	6	3	0	1	0	1
0-B	6	3	0	1	0	1
0-C	6	3	0	1	0	1
48-A	6	3	0	2	0	2
48-B	6	3	0	2	0	2
48-C	6	3	0	2	0	2
168-A	6	3	2	4	1	4
168-B	7	3	2	4	2	4
168-C	7	3	2	4	2	4
268-A	6	2	0	0	0	1
268-B	6	3	0	0	0	1
268-C	6	1	0	0	0	1
384-A	7	2	0	1	0	2
384-B	7	2	1	1	0	2
384-C	7	2	1	1	0	2

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
4-72A	7	4	0	0	0	2
4-72B	6	4	0	0	0	2
4-216A	7	4	4	2	0	3
4-216B	7	4	4	2	0	3

Sample	GAPDH	Oct4	CXCR4	FoxA2	Sox17	Brachyury
5-72A	7	4	0	3	3	4
5-72B	7	4	0	2	1	3
5-216A	6	4	0	1	0	1
5-216B	6	4	0	1	0	2
5-288A	6	4	0	1	0	2
5-288B	6	4	0	2	1	3

6.3.2 Chapter Four

Table 6.3.6: Summary table showing PCR band strength ‘scores’ for co-culture experiment (batch) D.

Sample	GAPDH	Oct4	Sox17	FoxA2	CXCR4	Brachyury	Nestin	Lgr5	CD133	Msi1	EpiAnt
IE Cont1	4	0	0	0	0	0	0	0	0	0	0
IE Cont2	3	0	0	0	0	0	0	0	0	0	0
IE Cont3	4	0	0	0	0	0	0	0	0	0	0
IE1	7	4	2	5	0	4	0	3	1	0	1
IE2	7	4	1	4	0	3	0	3	1	0	1
IE3	7	3	0	4	0	2	0	4	1	0	1
IE4	7	3	0	4	0	3	0	5	1	0	1
IE5	7	2	0	4	0	2	0	4	1	0	1
DC Cont1	5	2	0	1	0	2	0	1	1	0	0
DC Cont2	2	0	0	0	0	0	0	0	0	0	0
DC Cont3	2	0	0	0	0	0	0	0	0	0	0
DC1	7	5	3	4	0	5	0	7	4	0	1
DC2	7	4	3	5	0	5	0	7	4	0	1
DC3	7	4	2	4	0	4	0	6	2	0	1
DC4	7	4	1	4	0	4	0	6	3	0	1
DC5	7	4	1	5	0	4	0	5	3	0	1

Table 6.3.6B: Summary table showing PCR band strength scores for co-culture experiment culture vessel controls.

Sample	GAPDH	Oct4	Sox17	FoxA2	CXCR4	Brachyury	Nestin	Lgr5	CD133	Msi1	EpiAnt
Cont T1	7	6	1	4	0	4	0	0	5	0	0
Cont T2	7	6	1	3	0	3	0	0	5	0	0
Cont T3	7	6	1	2	0	3	0	0	5	0	0
Cont N1	7	6	1	3	0	4	0	0	5	0	0
Cont N2	7	5	0	3	0	4	0	0	5	0	0
Cont N3	7	5	0	2	0	3	0	0	5	0	0

Table 6.3.7: Summary table of average PCR band strength ‘scores’ for co-culture experiments (batches) E and F.

Sample	GAPDH	Oct4	Sox17	FoxA2	CXCR4	Brachyury	Nestin	Lgr5	CD133	Msi1	EpiAnt
Cont1	6	0	0	0	0	0	0	0	0	0	0
Cont2	6	0	0	0	0	0	0	0	0	0	0
E1	7	4	0	5	0	3	2	4	5	0	0
E2	7	4	1	5	0	3	2	4	5	0	0
E3	7	4	0	5	0	3	1	4	5	0	0
E4	7	4	0	5	0	3	1	4	5	0	0

Sample	GAPDH	Oct4	Sox17	FoxA2	CXCR4	Brachyury	Nestin	Lgr5	CD133	Msi1	EpiAnt
Cont1	6	0	0	0	0	0	0	0	0	0	0
Cont2	6	0	0	0	0	0	0	0	0	0	0
F1	7	4	1	2	0	4	0	4	5	1	0
F2	7	3	1	2	0	4	0	4	5	1	0
F3	7	4	2	2	0	4	0	4	5	1	0
F4	7	3	1	3	0	4	0	4	5	1	0

6.3.3 Chapter Five

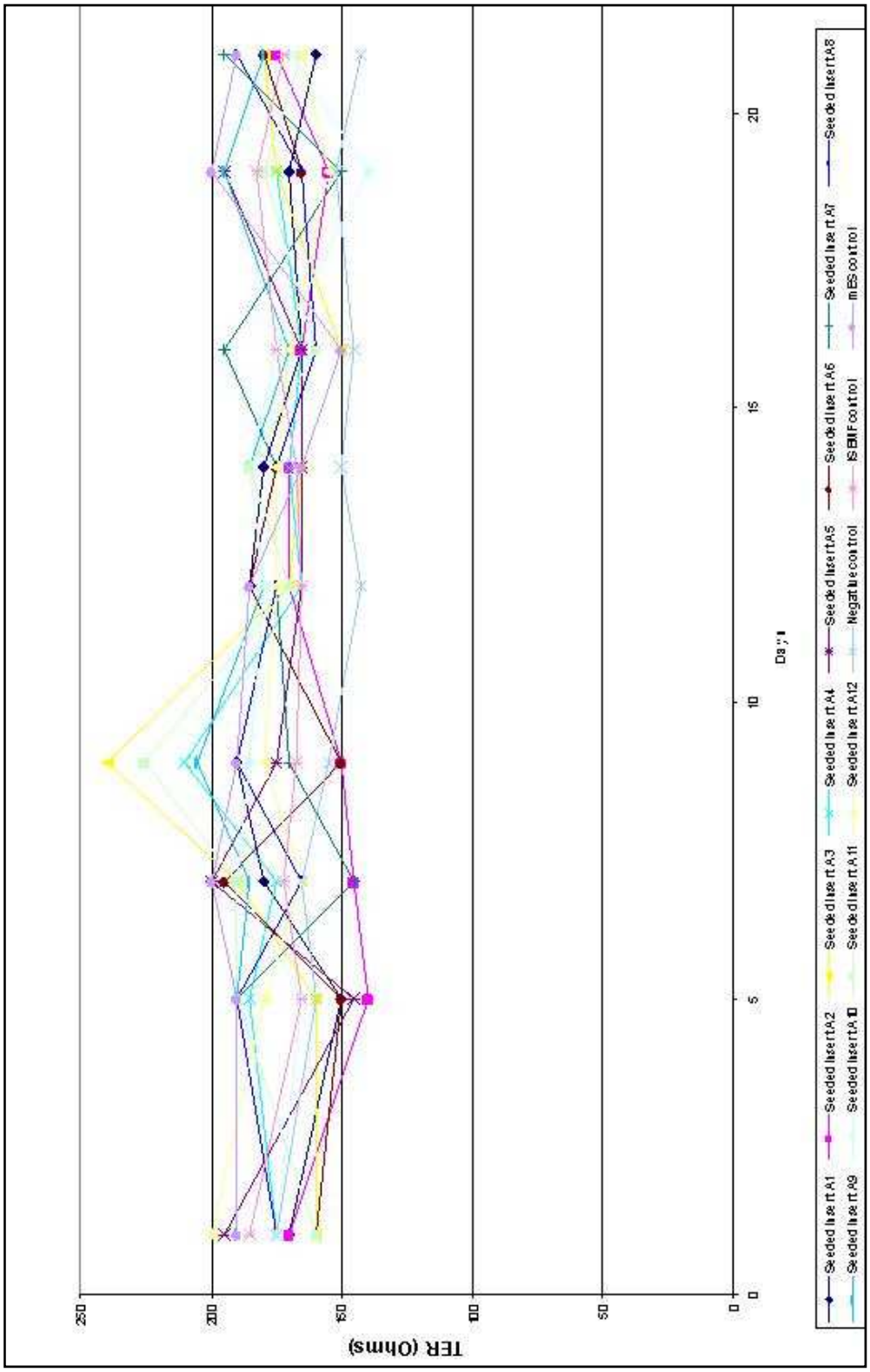


Figure 6.3.3: Graph showing TER values in mES derived cell seeded inserts and controls over 21 days in culture.

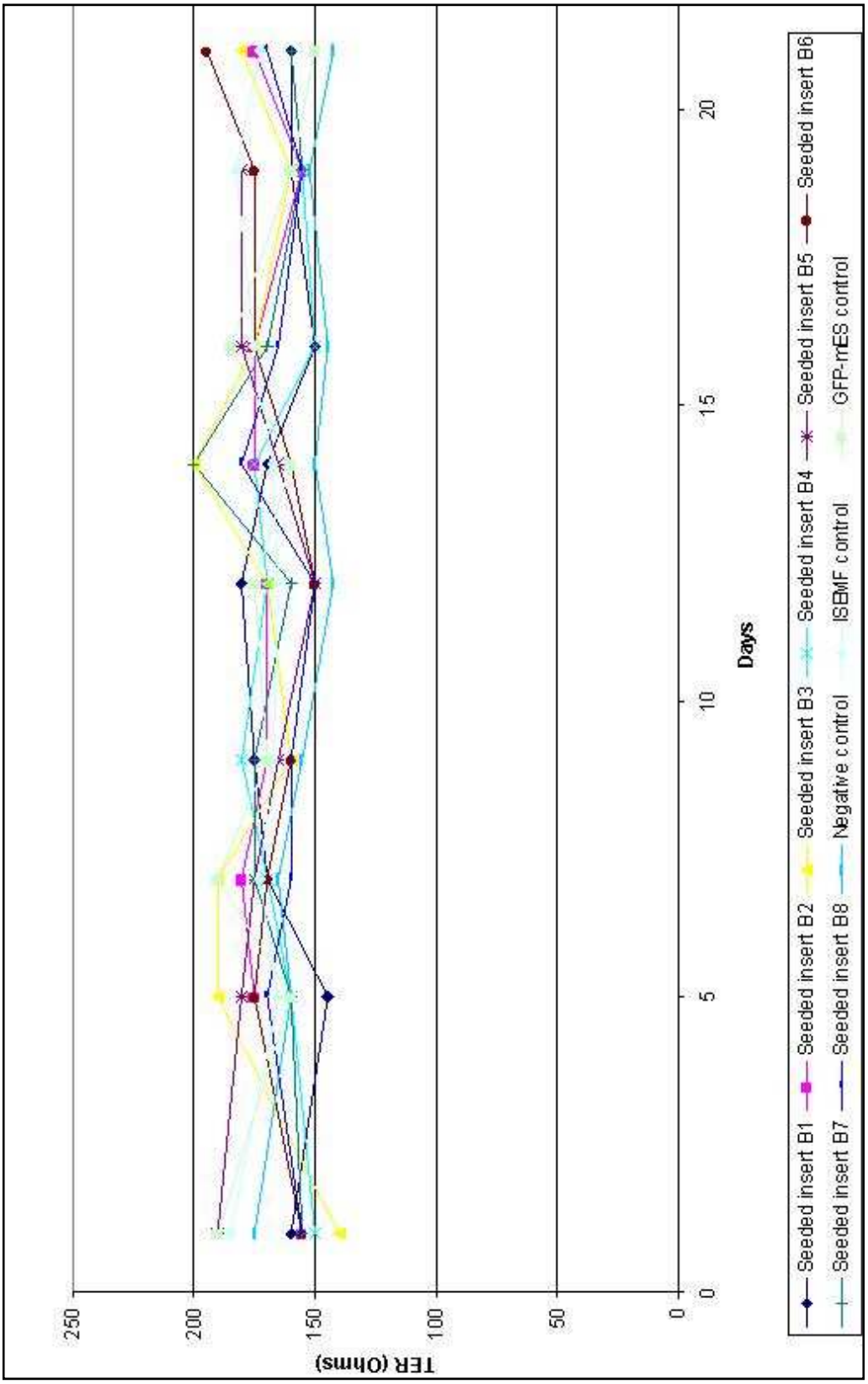


Figure 6.3.4: Graph showing TER values in GFP-mES derived cell seeded inserts and controls over 21 days in culture.

Table 6.3.8: Summary table of the ratios of the average TER readings from the experimental samples (batch A or B) and the various control conditions throughout the time course of the culture.

Day	1	5	7	9	12
A : negative control	1.00	1.06	1.07	1.22	1.23
A : ISEMF control	0.95	1.03	1.03	1.13	1.06
A : mES control	0.92	0.89	0.89	1.00	0.94
B : negative control	0.90	1.06	1.05	1.08	1.14
B : ISEMF control	0.85	1.03	1.01	1.00	0.98
B : GFP-mES control	0.83	1.06	0.91	0.99	0.93

Day	14	16	19	21
A : negative control	1.17	1.15	1.10	1.24
A : ISEMF control	1.05	0.95	0.92	1.02
A : mES control	1.06	1.11	0.84	0.93
B : negative control	1.19	1.16	1.06	1.22
B : ISEMF control	1.06	0.96	0.89	1.00
B : GFP-mES control	1.11	0.91	1.01	1.16

Table 6.3.9: Summary tables for membrane permeability study. Values given are % FITC-BSA based upon the standard curve (Figure 5.3.3A). Where 0 values are given the reading obtained fell below the lower detectable limits of the standard curve.

Day 13

Minutes	A1	A2	B1	B2	mES cont	GFP cont	ISEMF1	ISEMF2	NegCont1	NegCont2
30	0.0108	0.0000	0.0000	0.0241	0.0051	0.0051	0.0146	0.0051	0.0000	0.0070
60	0.0203	0.0032	0.0000	0.0127	0.0298	0.0000	0.0127	0.0089	0.0013	0.0032
90	0.0000	0.0000	0.0400	0.0000	0.0000	0.0343	0.0070	0.0000	0.0127	0.0279
120	0.0070	0.0260	0.0032	0.0000	0.0241	0.0279	0.0032	0.0013	0.0362	0.0127

Day 20

Minutes	A1	A2	B1	B2	mES cont	GFP cont	ISEMF1	ISEMF2	NegCont1	NegCont2
30	0.0000	0.0013	0.0514	0.0089	0.0013	0.0032	0.0222	0.0222	0.0000	0.0000
60	0.0298	0.0279	0.0032	0.0070	0.0146	0.0381	0.0127	0.0032	0.0051	0.0108
90	0.0089	0.0051	0.0241	0.0419	0.0127	0.0000	0.0032	0.0241	0.0419	0.0108
120	0.0032	0.0146	0.0552	0.0127	0.0013	0.0279	0.0495	0.0032	0.0089	0.0362

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6.4 Appendix B - Media, buffer & reagent recipes and additional experimental information

Cell culture media

500 ml SNL complete media:

439.5 ml DMEM (Gibco, UK)

50 ml FCS (10% (*v/v*), Sigma, UK)

5 ml Antibiotics & Antimycotics (1% (*v/v*), Sigma, UK)

5 ml (200 mM) L-Glutamine (1% (*v/v*), final conc 2mM) (Sigma, UK)

500 µl 10 mM β-Mercaptoethanol (0.1% (*v/v*), final conc 10 µM, Sigma, UK)

100 ml mES complete media:

99.95 ml SNL complete media (see above)

50 µl LIF (stock concentration 1 x 10^6 units/ml, working concentration 500 units/ml, Sigma, UK)

100 ml Cryopreservation media:

70 ml DMEM (Gibco, UK)

20 ml FCS (20% (*v/v*), Sigma, UK)

10 ml DMSO (10% (*v/v*) Sigma, UK)

100 ml Differentiation media A:

97.9 ml DMEM (Gibco, UK)

1 ml Antibiotics & Antimycotics (1% (*v/v*), Sigma, UK)

1 ml (200 mM) L-Glutamine (1% (*v/v*), final conc 2mM) (Sigma, UK)

100 µl (10 mM) β-Mercaptoethanol (0.1% (*v/v*), final conc 10 µM, Sigma, UK)

100 ml Differentiation media B:

97.7 ml DMEM (Gibco, UK)

1 ml Antibiotics & Antimycotics (1% (*v/v*), Sigma, UK)

1 ml (200 mM) L-Glutamine (1% (*v/v*), final conc 2mM) (Sigma, UK)

100 µl (10 mM) β-Mercaptoethanol (0.1% (*v/v*), final conc 10 µM, Sigma, UK)

200 µl FCS (0.2% (*v/v*), Sigma, UK)

100 ml Differentiation media C:

95.9 ml DMEM (Gibco, UK)

1 ml Antibiotics & Antimycotics (1% (*v/v*), Sigma, UK)

1 ml (200 mM) L-Glutamine (1% (*v/v*), final conc 2mM) (Sigma, UK)

100 µl (10 mM) β-Mercaptoethanol (0.1% (*v/v*), final conc 10 µM, Sigma, UK)

2 ml FCS (2% (*v/v*), Sigma, UK)

100 ml Serum replacement complete media:

87.9 ml DMEM (Gibco, UK)

1 ml Antibiotics & Antimycotics (1% (*v/v*), Sigma, UK)

1 ml (200 mM) L-Glutamine (1% (*v/v*), final conc 2mM) (Sigma, UK)

100 µl (10 mM) β-Mercaptoethanol (0.1% (*v/v*), final conc 10 µM, Sigma, UK)

10 ml Serum replacement (10% (*v/v*), Sigma, UK)

100 ml Digestion media:

96.75 ml DMEM (Gibco, UK)

1 ml FCS (1% (*v/v*), Sigma, UK)

1 ml Penicillin & Streptomycin (1% (*v/v*), final concentrations: 100 U/ml Penicillin; 100 µg/ml Streptomycin, Sigma, UK)

0.25 ml Gentamycin solution (0.25% (*v/v*), final concentration 25 µg/ml Gentamycin, Sigma, UK)

1 ml (200 mM) L-glutamine (1% (*v/v*) final concentration 2 mM, Sigma, UK)

6 mg collagenase XI (6% (*w/v*) final concentration 75 U/ml, Sigma, UK)

2 mg dispase I (2% (*w/v*), final concentration 20 µg/ml, 0.12 U/ml, Sigma, UK)

Note: dissolve the enzymes in an aliquot of the 100 ml DMEM and filter sterilise thorough a 0.2 µm filter back into the 100 ml.

500 ml ISEMF media:

445 ml DMEM (Gibco, UK)

50 ml FCS (10% (*v/v*), Sigma, UK)

5 ml (200 mM) L-Glutamine (1% (*v/v*), final conc 2 mM, Sigma, UK)

100 µg Streptomycin (0.002% (*w/v*), final conc 200 ng/ml, Sigma, UK)

250 ng Amphotericin B (0.005% (*w/v*), final conc 500 ng/ml, Sigma, UK)

1000 units/ml Penicillin B (Sigma, UK)

100 ml 0.1% FITC-BSA (*v/v*) in PF-DMEM:

99.9 ml DMEM (Gibco, UK)

100 µl FITC-BSA solution (Sigma, UK)

100 ml 1% BSA (*w/v*) in PF-DMEM:

100 ml DMEM (Gibco, UK)

1 g BSA (Sigma, UK)

Histology Solutions

100 ml Alcoholic Eosin

1 g Eosin (Nustain, UK).

100 ml 25% IMS.

1 litre Scotts modified tap water

20 g $NaHCO_3$ (Sigma, UK).

3.5 g $MgSO_4$ (Sigma, UK).

1 litre H_2O.

Immunohistochemical reagents

200 ml Permeabilisation solution (ph7.2):

199 ml H_2O.

20.6 g Sucrose (Sigma, UK).

584 mg NaCl (Sigma, UK).

120 mg $MgCl_2$ (Sigma, UK).

952 mg HEPES (Sigma, UK).

1 ml Triton X-100 (Sigma, UK).

Note: once the solution had been made up the pH was adjusted to 7.2 by the addition of small amounts of HCl or NaOH. The pH was measured using a Mettler Toledo pH meter. All solutions where a specific pH is given were adjusted in this fashion.

DABCO UV mountant:

Stock solution (pH 8.6) store at 4°C:

20 mg DABCO.

20 ml PBS.

Working solution (store at 4°C):

2 ml DABCO stock solution.

18 ml Glycerol (Sigma, UK).

Western blot reagents

1 litre TTBS (pH 7.6):

999.9 ml H_2O.

2.42 g Tris-HCl (Sigma, UK).

8.76 g NaCl (Sigma, UK).

100 µl Tween 20 (Sigma, UK).

Live-Dead reagents

Live-Dead working solution:

9.975 ml PBS.

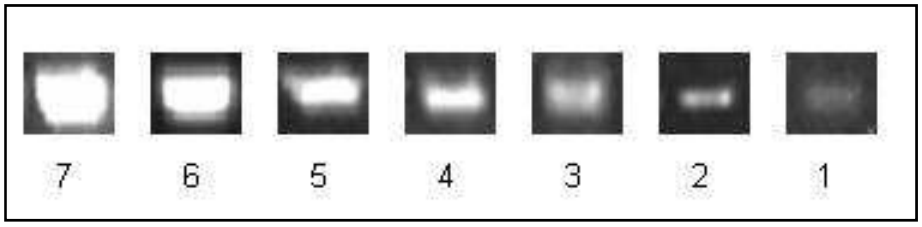
25 µl Eth D-1.

5 µl Calcein AM.

PCR band 'scoring'

Following imaging the bands obtained were (subjectively) scored on a scale of one – seven based upon relative size (pixel count) and brightness (pixel intensity).

A guideline to these scores is shown below. If no band was visible then a score of zero was assigned.



RT-PCR band scoring chart guide

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6.5 References

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