The influence of the extracellular matrix on cell behaviour

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The Influence of the Extracellular Matrix on Cell Behaviour

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ABSTRACT

Stem cell research has raised great interest in the scientific community as it has the potential to form multiple cell types and it is believed they hold the key to curing many diseases. However, there is a need for better understanding of how to control these cells and further research investigating methods for controlling and directing these cells is required.

Pluripotent stem cells transplanted into immune-deficient mice 'spontaneously' differentiate and proliferate to form a complex mass of differentiated and undifferentiated cells, teratomas – teratoma assay. Such tumours are generally haphazard in their organisation however; they do contain some structures similar to those observed in the embryo. Teratoma formation is a useful model to explore the developmental potential of stem cells and study aspects of tissue development. Examination of how the anatomical location into which human pluripotent stem cells are grafted influences their growth *in situ* allows investigation of how these cells are affected by different areas within the body: cells grafted into the liver rapidly produce large tumours containing predominantly immature cells whereas, subcutaneous implants were significantly slower growing and formed tumours composed of differentiated tissues. These different growth patterns indicate how environmental cues within the niche affect stem cell behaviour. One factor which contributes to the maintenance of a niche is the extracellular matrix (ECM). To investigate how endogenous ECM affects teratoma behaviour, co-transplantation is carried out with stem cells and ECM components. The ECM extract Matrigel™ dramatically increased the success rate of teratoma formation and size with no detectable affect on teratoma composition when compared to controls and removal of the growth factors from the co-transplanted ECM extract had no effect on teratoma success rate, growth rate, or composition.

To study the effects of the ECM *in vitro*, components of the ECM are often used to coat glass or plastic surfaces to enhance cell attachment *in vitro*. Fragments of ECM molecules can be immobilised on surfaces in order to mimic the effects seen by whole molecules. In this study a novel technology developed by Orla Protein Technologies for the immobilisation of peptide sequences from ECM proteins is evaluated. By examining: the adherence of cultured PC12; neurite outgrowth from PC12 cells; and neuronal differentiation of neural stem/progenitor cells (NSPCs) it is shown that peptides from collagen I, collagen IV, fibronectin and laminin can mimic surfaces coated with ECM proteins. Collectively, this data demonstrates that peptides from ECM proteins can be immobilised in a functional fashion to control cell behaviour.

Surfaces with adsorbed proteins and biomimetic surfaces presenting peptide motifs from ECM proteins are used to investigate and explain observations from *in vivo* teratoma experiments. *In vivo,* Matrigel™ increases the gene expression of the pluripotent stem cell marker Oct4, increasing the pluripotent cell percentage and thus increases the likelihood of teratoma formation. *In vitro*, Matrigel™ also increases the gene expression of the proliferative marker Ki67, indicating that large teratomas from by the co-transplantation of stem cells with Matrigel™ could be due to increased cell proliferation.

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DECLARATION

This thesis is entirely the result of my own work. It has not been submitted for any other degree

ABBREVIATIONS

3D	Three dimensional
Aph	Aphidiolin
BDNF	Brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BM	Basememnt membrane
BME	Basement membrane extract
BrdUrd	Bromodeoxyruidine
BSA	Bovine serium albimum
CSPGs	Chondroitin sulphate proteoglycans
DBH	dopamine-β-hydroxylase
DMEM	Dulbeccos Modified Essential Medium
DPPE	Thiolipid
E.coli	Esherichia coli
ECM	Extracellular matrix
FCS	Foetal Calf Serum
FSK	Forskolin
FSK	Forskolin
GAP-43	Growth cone associated protein-43
GFAP	Gilal fibril associated protein
GFR	Growth factor reduced
H&E	Haeamtoxylin and eosin
HDT	Hexadecane thiol
ICM	Inner cell mass
IGF	Insulin growth factor
I-type	Intermediate type
MAP-2	Microtubule associated protein
MEF	Murine embryonic fibroblast

MUDA ..... Mercaptoundecanoic acid

NF ..... Neurofilament

NGF ..... Nerve growth factor

NGS ..... Normal goat serum

OmpA ..... Outer membrane protein A

RA ..... Retinoic acid

SAM ..... Self assembled monolayer

SCID ..... Severe combined immunodeficiency

SDS ..... Sodium dodecyl sulphate

SEM ..... Standard error of the mean

SSEA-3 ..... Stage specific antigen-3

S-Type ..... Substrate adherent

TH ..... tryosine hydroxylase

TPA 12-O-tetradaconoyl-phorbol-13-acetate

Trk ..... tropomyosin receptor kinase

PUBLICATIONS ARISING FROM THIS THESIS

MJ Cooke, T Zahir, SR Phillips, DSH Shah, D Athey, JH Lakey, MS Shoichet, SA Przyborski. Regulation of neurogenesis by biomimetic surfaces presenting motifs of extracellular matrix proteins. Accepted 2009 - Journal of Biomedical Material Research Part A

Xin Zhang, Irina Neganova, Stefan Przyborski, Chunbo Yang, **Michael Cooke**, Stuart Atkins, George Anyfantis, Stefan Fenyk, W. Nicol Keith, Stacey F. Hoare, Owen Hughes, Tom Strachan, Miodrag Stojkovic, Lyle Armstrong, and Majlinda Lako. The role of NANOG in human ESC. The Journal of Cell Biology. 2009 184 (1) 67-82

Chunbo Yang, Stefan Pryzborski, **Michael J Cooke**, Xin Zhang, Rebecca Stewart, Stuart Atkinson, Gabriele Saretzki, Lyle Armstrong and Majlinda Lako. A key role for telomerase reverse transcriptase unit (TERT) in modulating human ESC proliferation, cell cycle dynamics and *in vitro* differentiation. Stem Cells. 2008 Apr;26(4):850-63

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Zhang X, Stojkovic P, Przyborski S, **Cooke M**, Armstrong L, Lako M, Stojkovic M. Derivation of human embryonic stem cells from developing and arrested embryos. Stem Cells 24(12):2669-76

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CHAPTER 1 – USING THE EXTRACELLULAR MATRIX AND PEPTIDE MOTIFS TO CONTROL CELL BEHAVIOUR

1.1 Introduction

The field of stem cell research has raised much media interest, with numerous scientific groups claiming that stem cells hold the solution to a myriad of problems. Proposed uses include: investigating cellular events accompanying embryonic development [1,2]; producing model systems to explain biological observations or hypotheses [3]; cell replacement therapy [4]; tissue engineering [5,6]; and to cure various diseases [7-11]. Despite these claims, to date there are only a few examples where clinical success has been achieved. The main obstacle that must be overcome is how to control stem cells and until this is fully understood the full potential of stem cells will not be ascertained. *In vivo* experimentation can be used to investigate potential factors that can influence cell behaviour. For example, stem cells can be transplanted into animal hosts and the development of the transplanted cells investigated, allowing potential factors that control stem cell behaviour to be identified. However, the *in vivo* environment is highly complex and makes the assessment of how each individual factor is affecting the cells difficult. Factors identified from *in vivo* experiments can be used *in vitro* and presented to cells in a controlled fashion to investigate how individual components of the *in vivo* environment affect cell behaviour.

Experimentation carried out in an *in vivo* environment can provide information of how multiple factors, working collectively, affect development. However, given the vast range of factors present *in vivo* that can contribute to the control of cellular development, when using such a complex system observations are based upon all of the possible factors working in combination. This makes the evaluation of the effect of each individual component difficult. *In vitro* systems can be used to examine individual components in an environment where all the variables are controllable by the investigator and the effects of one component of interest can be observed without the effects of other interacting components. The *in vitro* system

is a simplified version of the *in vivo* environment however, care is needed and efforts should be made to mimic the *in vivo* environment to ensure it is a representative environment and not an artificial environment. Many approaches have been used including: recreation of a 3D shape [12,13]; co-culture [14]; addition of exogenous molecules to the culture media [15]; cells secreting factors [16]; and the coating of surfaces with molecules [17].

The focus of this thesis is how the extracellular matrix (ECM) can be used to control cell behaviour. The effect on stem cell behaviour in alternative anatomical locations is investigated and from this, potential factors are proposed that control cell behaviour. Focus is given to how the ECM controls cell behaviour *in vivo* and *in vitro*. The study of how both individual and combined peptide motifs affect cell behaviour is investigated to identify specific motifs that control cell behaviour.

1.2 The potential application of stem cells and their sources

1.2.1 The potential therapeutic applications of stem cells

It is proposed that stem cells can be derived from an embryo, propagated and differentiated in a controlled manner to form specific cell types for transplantation into a patient (figure 1.1, 1) [18]. An alternative strategy would be the isolation and selective differentiation of selected stem cell populations within the body and transplantation of these cells into the patient. For example, neural cells can be isolated from the hippocampus [19,20] and have been proposed for use in cell therapy to repair handicaps incurred post stroke [21] (figure 1.1, 2). An alternative source of stem cells is the bone marrow. Bone marrow can be harvested from the pelvis or the sternum and mesenchymal stem cells can be isolated and have been proposed for tissue engineering to making bone [10,22] (figure 1.1, 3). These applications depend upon the controlled growth and differentiation of the stem cell populations (figure 1.1, "?" red arrows). There is a need to gain a greater understanding of the processes that control stem cell behaviour before these cells can be controlled

efficiently. One proposed method is the creation of biomaterials, both synthetic and natural to direct stem cell growth and differentiation [23].

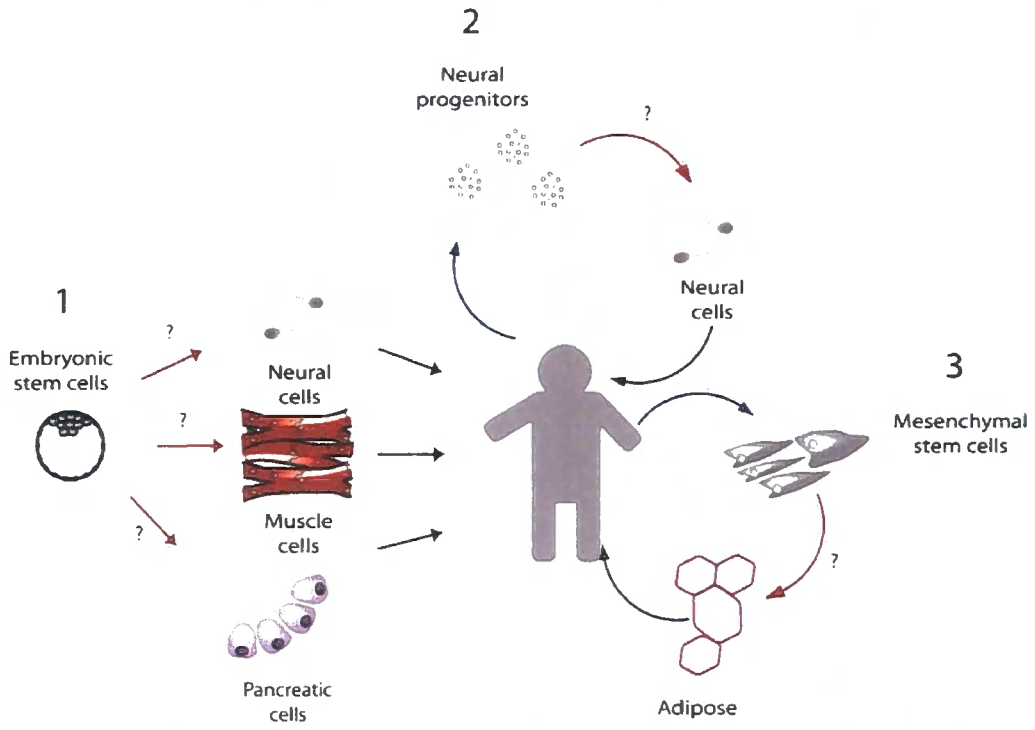

Figure 1.1 The sources and potential therapeutic uses of stem cells. Diagram shows some potential sources of stem cells and their proposed differentiated derivatives. Blue arrows indicate the isolation of stem cells. Red arrows indicate the proliferation and selective differentiation. "?" highlight the stages where knowledge is lacking on how to control stem cell behaviour. Black arrows indicate transplantation.

1.2.2 Stem cells and development

Development of animals starts with egg fertilisation, from this zygote all of the estimated >200 different cell types found within the body must be made. Every cell within the body is divided into one of two classes, germ cells (the spermatozoa and ova) and somatic cells (every other cell type within the body). Further cell type classification of somatic cells is carried out by grouping into one of three germ layers: ectoderm; endoderm; and mesoderm (Figure 1.2). The zygote undergoes numerous developmental stages initially giving rise to the foetus and finally resulting in the adult. As development progresses cells become more

specialised and their proliferation and capacity to form other cell types decreases. In the adult it is clear that the capacity of cells to form other cell types does not completely diminish as tissues with a high turnover, such as the skin, need to be maintained. Many tissues are envisaged as being composed of two cell populations: post-mitotic specialised cells responsible for the physiological activity of the tissue; and the other population retains its capacity to proliferate, differentiate and maintain the tissue. The populations of cells responsible for the maintenance of tissues are termed stem cells and are defined as cells that both retain capacity to self renew and give rise to more developmentally restricted progeny which in turn produces distinct differentiated cell types. There are numerous sources of stem cells, including embryonic and somatic.

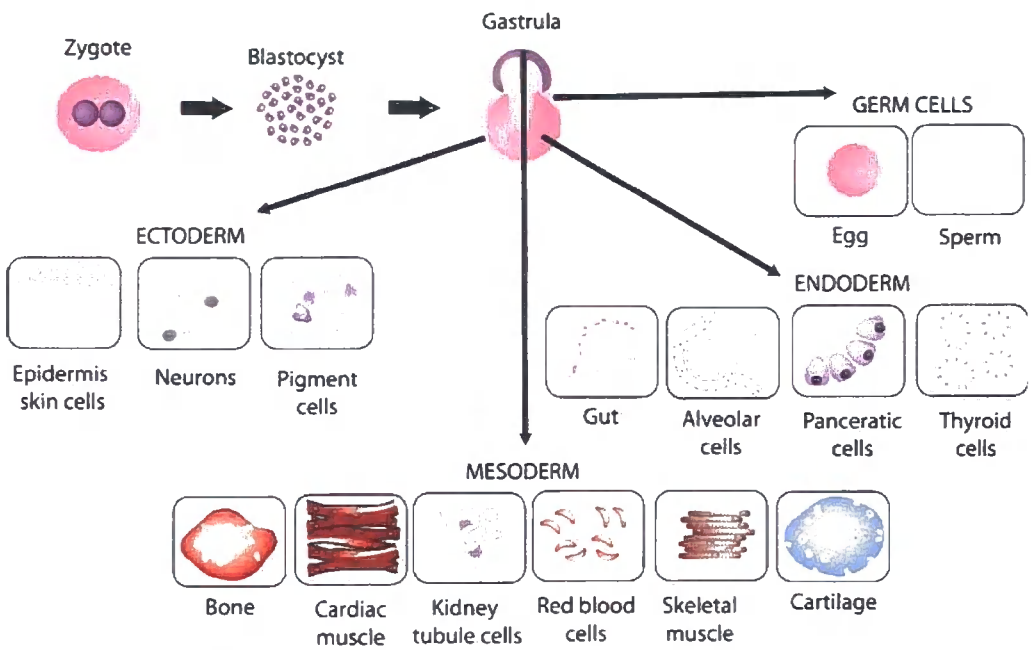

Figure 1.2 The origin of the three germ layers. Diagram shows examples of the different cell types that form within the body. All cell types within the body are grouped into one of the three germ layers, ectoderm, mesoderm and endoderm. The diagram shows examples of cell types from each of the germ layers.

1.2.3 Stem cell plasticity

The differentiation capacity of a stem cell, termed potency, is a measure of the number of different cell types that can be formed from the single cell [24]. All the cell types within the body can be classified as one of the three germ layers: endoderm (gut epithelium); mesoderm (cartilage, bone, smooth muscle and striated muscle); and ectoderm (neural epithelium, embryonic ganglia and stratified squamous epithelium) (Figure 1.1). Totipotent (from the Latin *totus*, meaning entire) cells can form cells from all three germ layers and also the extraembryonic tissues that support development [25 26]. An example of a totipotent cell is that of the zygote, it is from this single cell that all cell types within the body originate. A more restricted stem cell is pluripotent cell (from the Latin *pluris,* meaning many) which can form many of the cell types from the three germ layers [27]. Embryonic stem cells, derived from the blastocyst, are an example of pluripotent stem cells and their developmental potential has been demonstrated both *in vitro,* with embryoid bodies expressing RNA for markers of the three germ layers (α-fetoprotein, muscle and neurofilament 68) [28] and *in vivo* by the formation of a teratoma with gut, muscle and neural tissues [25]. The examples discussed so far are from embryonic cells. Stem cells have also been isolated from somatic tissues which are more restricted in developmental potential and are usually multipotent (from the Latin *multus,* meaning several), *in situ* they are responsible for maintaining tissues. An example of multipotent stem cells are neural stem cells, derived from specific regions of the brain [29].

1.2.4 Embryonal carcinoma stem cells

One source of pluripotent stem cells is teratocarcinomas, these can arise spontaneously in either the ovary or testis. The earliest reference to a teratocarcinoma was 600 – 900 B.C. and can be found in writings at the Chaldean Royal Library of Nineveh where a prophecy for the future states: *"When a woman gives birth to an infant that has three feet, two in their normal position (attached to the body), and the third between them, there will be great prosperity in the land"*

[30]. In the ovary, an oocyte can undergo spontaneous parthenogenesis (from the greek parthenos "virgin" and genesis "creation") *in situ* and behave as if it had been fertilised. For a brief period these develop as a normal embryo but subsequently they become disorganised and form a teratocarcinoma. In the testis, primordial germ cells undergo abnormal proliferation and form an embryonic ectoderm-like structure that subsequently becomes disorganised. The majority of these spontaneous tumours are benign teratomas, although occasionally they contain undifferentiated cells – embryonal carcinoma (EC) stem cells (Figure 1.3) [27].

Investigations into deriving stem cells from teratomas began in 1954 when Stevens and Little discovered that in ~1% of male mice (129 strain) teratomas spontaneously formed in the testes [31]. Later, in 1964, Stevens and Little showed that by transplanting embryos of the genital ridge from strain 129 mice to the testes of adult mice it was possible to induce the formation of a teratoma [32]. It was proved in 1964, by Kleinsmith and Pierce that a teratoma contained undifferentiated EC stem cells. Kleinsmith and Pierce showed a single EC stem cell transplanted into a host could give rise to the formation of a heterogeneous structure: a teratoma [33].

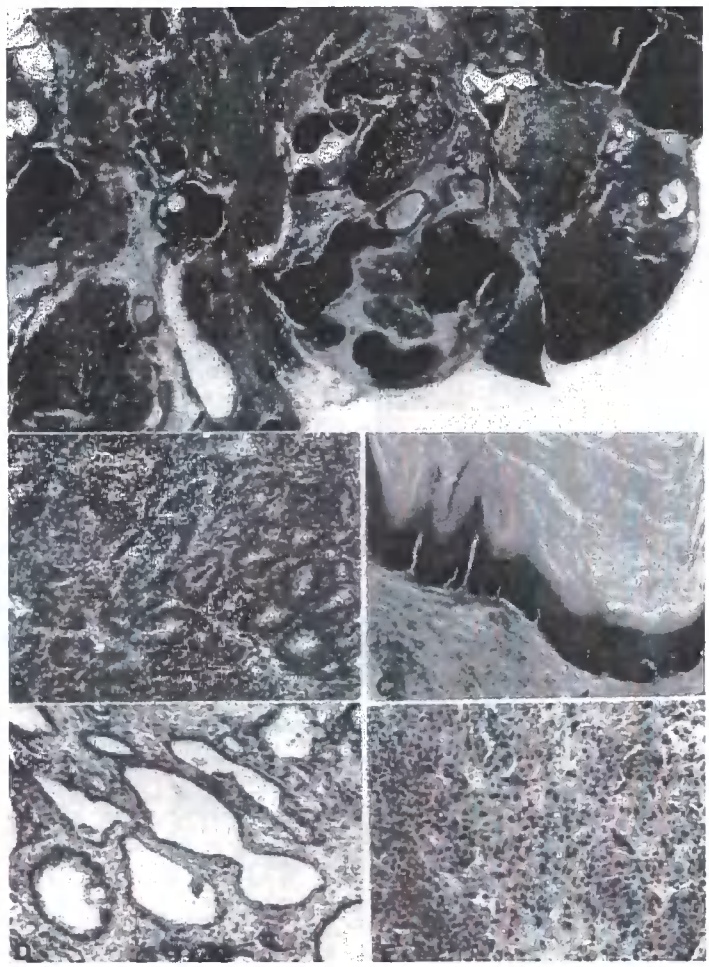

Figure 1.3 Teratocarcinomas are highly heterogeneous in composition. A) Low power examination of tumour demonstrating heterogeneity, collagen can be seen clearly in black. B) Primitive neural cells. C) Skin. D) Endodermal cysts. E) Trophoblasts with embryonal carcinoma cells to the left. From: Teratocarcinomas as a Model System for the Study of Embryogenesis and Neoplasisa Martin GR, Cell (5) 229-243 (1975).

1.2.5 Embryonic stem cells

Teratomas are not the only source of stem cells and in 1981 Evans and Kaufman were able to derive pluripotent cells from a mouse embryo [34]. Later, in 1998 the Thomson group derived stem cells from human embryos and the cells were shown to be pluripotent, these cells became known as embryonic stem (ES) cells [25]. ES cells are derived from 'spare' embryos that have been donated from *in vitro* fertilisation (IVF). This is ethically controversial and ES cells have also been

isolated from arrested embryos produced during *in vitro* culture for IVF [35]. During either process, the cells are derived from embryos, which are made up of an inner cell mass (ICM) surrounded by a layer of protective specialised tissue called the trophectoderm, a region of ~100 cells in total, 30-34 of which are present in the ICM, immunosurgery is used to separate the ICM from the trophectoderm. The cells of the ICM are isolated and plated onto a mitotically inactivated murine embryonic fibroblast (MEF) feeder layer and propagated. The colonies that arise are then selected on the basis of their undifferentiated morphology and are expanded. ES cells can be cultured in a pluripotent undifferentiated state [34,36].

ES cells are usually derived via a two step *in vitro* method using day 5-7 cultured embryos. More recently an 8 day culture process with three steps to the *in vitro* culture has been used to increase the success rate of ES cell derivitisation [37]. By growing the blastocyst to the 8 day stage the ICM is larger and contains more cells than the 5-7 day alternatives and hence the derivitisation of stem cells is greater. These cells were then fully characterised *in vitro* and *in vivo* in a similar fashion to that used by Thomson et al. (1998) and the cells were shown to be true ES cells.

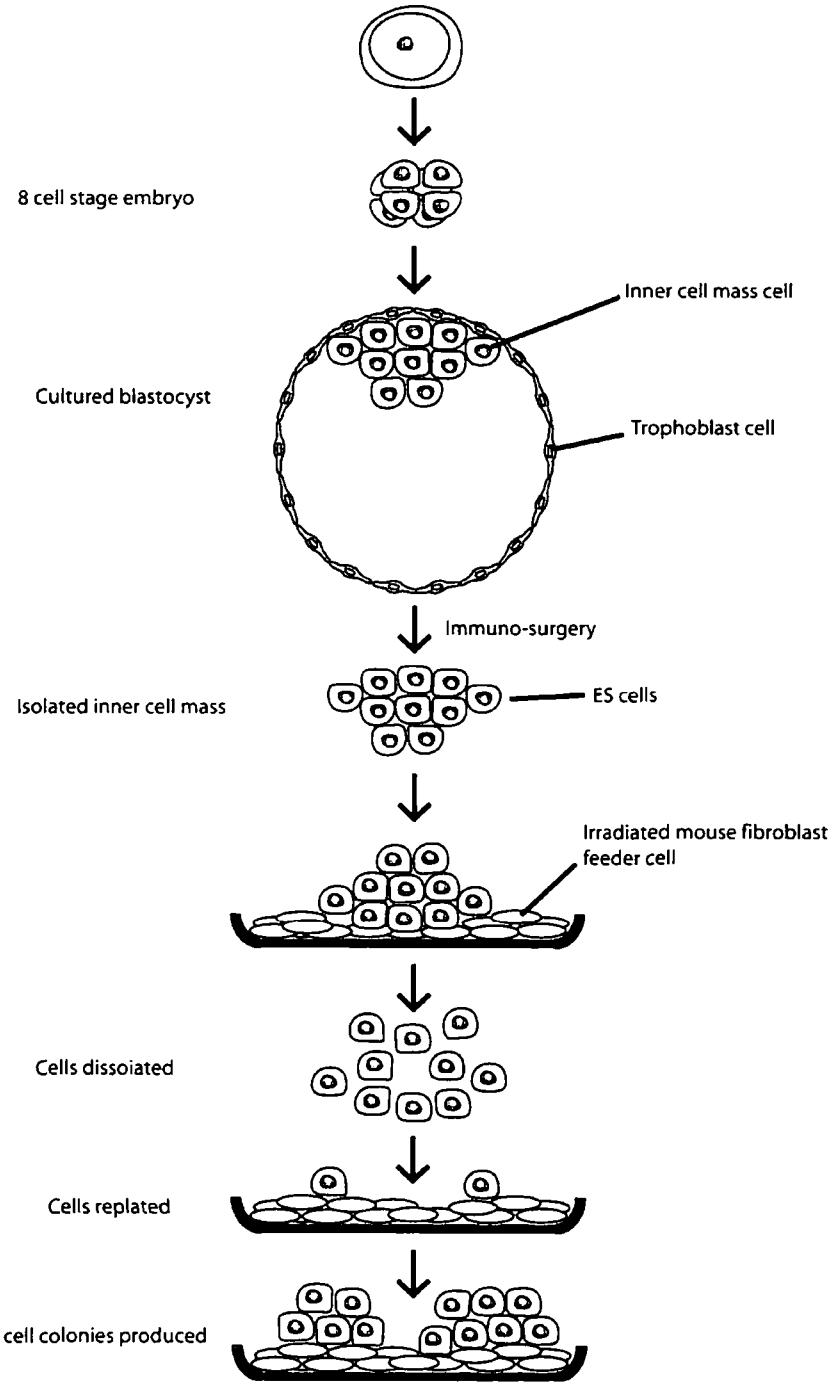

Figure 1.4 The isolation of embryonic stem cells from the blastocyst. Schematic diagram showing how the inner cell mass is removed from an embryo and cultured *in vitro* to form embryonic stem cells.

1.2.6 Maintaining embryonic stem cell pluripotency

Pluripotent stem cells spontaneously differentiate, this key behaviour needs to be minimised so that they can be expanded in an undifferentiated state without the risk of undesired differentiation occurring. Work carried out by Austin Smith demonstrated that ES cell pluripotency could be maintained when the cells were cultured in the presence of medium conditioned by buffalo rat liver (BRL) cells. The conditioned media from BRL cells was found to contain a polypeptide factor that had ES cell differentiation inhibitory activity (DIA) [38]. The polypeptide factor within BRL conditioned media was found to be related in structure and function to the leukaemia inhibitory factor (LIF) that induces differentiation in M1 myeloid leukaemic cells. ES cells were found to present receptors for LIF. Culture of cells in the presence of LIF could maintain pluripotency with 90% of cells displaying a cell surface stem cell specific antigen, whereas the control maintained in the absence of LIF could only maintain pluripotency of 1% [39]. LIF expression is known to be high in murine embryonic fibroblasts (MEFs) so by culturing cells on MEFs this provides a source of LIF [40]. Increasing the concentration of LIF added to ES culture media increases the percentage of undifferentiated cells for example, when 0, 5, 10 or 20ng/ml are added the percentage of undifferentiated cells increases as the concentration increases (34, 43, 67, 83% undifferentiated) [41]. Maintenance of stem cell pluripotency occurs by LIF binding to its heterodimer receptor LIF receptor (LIFR) and gp130. Stabilisation of this receptor complex activates Janus-associated (JAK) tyrosine kinases which cause the recruitment and dimerisation of signal transducer and activator of transcription 3 (STAT3). STAT3 is able to translocate into the nucleus where it can control genes required for regulating self-renewal [42].

1.2.7 The relationship of embryonal carcinoma stem cells to the inner cell mass and embryonic stem cells

During embryogenesis, at the pluripotent cleavage stage, cells split to give rise to two cell types: the pluripotent inner cell mass (which forms the foetus); and the

differentiated extraembryonic trophectoderm (which protects the ICM, aids implantation and the establishment of the foetal relationship with the mother). The pluripotent cells of the ICM go on to give rise to two cell types: the pluripotent foetus-forming primary ectoderm; and the differentiated extraembryonic primary endoderm. In an analogous fashion, some EC stem cell lines give rise to primary endoderm-like cells as their first differentiated derivatives *in vitro*. EC stem cells are the malignant counterpart of ES cells and considered to mimic development in a similar fashion. Direct evidence that EC stem cells are related to the ICM comes from studies where antibodies which have been raised against primitive teratocarcinoma cells were not reactive against differentiated teratoma cells or mouse cells. However, they were reactive to the 4- to 8- cell morulae [43]. Further evidence that EC stem cells are similar to ES cells comes from their antigen expression, undifferentiated EC positive for TRA-1-60 [44] SSEA3 [45] as too are ES cells [25].

Conclusive proof that EC stem cells are related to the ICM is demonstrated when murine EC stem cells are transferred into the mouse blastocyst and participate in normal development. Animals produced by the transfer of EC stem cells into the blastocyst showed patches of agouti hairs in their normal albino coloured fur. EC stem cells were derived from animals and when grafted onto chimeras were maintained significantly longer than on control animals [46,47]. Furthermore, a single EC stem cell transplanted into an embryo gives rise to an adult with tissues such as: brain; heart; kidneys; and liver that expresses glucose phosphate isomerase (GPI) and isocitrate dehydrogenase (IDH) allelic variants which are the same as the transferred EC cells [26]. Using GFP EC stem cells transferred into the blastocyst it has since been confirmed that the EC stem cells do contribute to formation of organs such as the liver. The stage in development during which the cells are transferred has been shown to affect their behaviour. The rate of tumour development in animals produced from blastocysts with EC stem cells increases as the developmental stage prior to EC cell transfer into the blastocyst progresses further. When EC stem cells are transferred into E8 blastocysts (the active stage of organogenesis) only 8% develop tumours, whereas transplantation during organ

growth at E12 results in 69% developing tumours [48]. This suggests that EC stem cells can respond to factors present in the blastocyst during development however as development progresses these factors are lost and can no longer control these cells. This gives strength to the argument that EC stem cells represent the ICM as they can respond to factors that control the ICM.

Although EC stem cells are similar to ES and hence the ICM there are some drawbacks to using these cells: as they are derived from tumours they could potentially be tumourigenic *in vivo*; they have chromosomal abnormalities [49]; and some cell lines are nullipotent (from the Latin *nullus*, meaning none) and have a reduced differentiation capacity [50,51]. It is for these reasons that many consider them as a "caricature" [52] of ES cells. It is important to study both ES and EC stem cells alongside each other to gain a true picture of the mechanisms that control cell behaviour.

1.2.8 EC stem cells as a model system to study development

EC stem cells are frequently used as a model system to study development. EC stem cells have been used to extensively study neuronal development as strong similarities exist between cells *in vivo* and EC stem cells. For example, the transcription factor neuroD is known to be involved in neural development [53], during differentiation of NTERA2 EC cells, neuroD1 expression is found to increase to a maximum following 7 days retinoic acid treatment, after which, its expression declines [54]. In agreement with this neuroD1 expression has been shown to be transiently expressed in the EC stem cell line P19, aggregates have a maximal expression at day 6 retinoic acid treatment [55]. A similar trend for neuroD RNA expression is observed *in vivo*, in the mouse embryo, it is first detected at e9.0-9.5 and a strong expression is seen at e11.5, by e14.5 neuroD expression then ceases to be seen [56].

Another aspect of neuronal development that EC stem cells have been used to study is their response to retinoic acid. Retinoic acid is involved in controlling

early development and regulation of the levels affect development. In *Xenopus* embryos, endogenous levels of all-trans-retinoic acid are similar in the doral and ventricle halves of the early neurla, whereas in the late neurla, levels are much higher in the ventricle half than the doral [57]. All-trans-retinoic acid is the biologically active derivative of vitamin A and is known to have a wide range of biological effects. The effects of retinoic acid and other retinoids are mediated by two families of receptors: retinoic acid receptors (RARs); and retinoid X receptors (RXRs). Activation of the receptors results in gene transcription through a retinoic acid response element (RARE). There are three RARs (RARα, β, γ) and three RXRs (RXRα, β, γ). Using an RA resistant subclone (NTERA2/D1-R1), over expression of RARγ results in a retinoic acid induced neural phenotype [58].

1.2.9 Neural stem cells

The study of pluripotent stem cells is important, however, other stem cell types should not be over looked. In addition to pluripotent stem cells more developmentally restricted stem cells can be isolated from the body, including neural stem cells. It was believed for a long time that the adult neural system had no scope for self repair and that in adulthood new neural cells were not generated. However, in 1992 Reynolds and Weiss demonstrated that cells could be isolated from the striatum of the adult brain and differentiate into neurons and astrocytes [59]. Stem cells were isolated from the hippocampus of Fischer 344 rats [embryonic day 16(E16)]. The hippocampi were removed, mechanically dissociated then plated onto laminin coated tissue culture plates. The cultures were analysed using immunocytochemistry and found to be almost purely neural due to the absence of astrocyte, oligodendrocyte and fibroblast markers (GFAP, galactocerebroside, vimentin, and fibronectin) [20]. These cells were shown to be multipotent *in vivo.* When labelled, cells were transplanted into the hippocampus of adult Fisher 344 rats. The cells were found to be multipotent, forming both glial and neuronal cell types. Cells that survived within the granule cell layer of the hippocampus displayed neuronal characteristics whereas cells outside of this region displayed markers and the morphology of glial cells. This showed that the cells were

multipotent in nature and that cell fate could be controlled by endogenous signals experienced by the cell.

An alternative source of neural stem/progenitor cells is the forebrain subependyma [60]. Tissue can be isolated from the subependyma of 8-12 week old rats and enzymatically dissociated, the resultant cell suspension can be grown *in vitro* as undifferentiated neurospheres. The neurospheres consist of mainly (~96%) undifferentiated cells and upon, foetal bovine serum induced, differentiation, the following is observed: βIII positive neurons (~11%); GFAP positive astrocytes (~13%); RIP positive oligodendrocytes (~41%); and a fraction continue to express nestin (~35%) [61].

1.3 Understanding how to control cell behaviour

1.3.1 Using stem cells as model systems to study cell behaviour

Stem cells are widely accepted as a model system to study the cellular events accompanying development [1,2]. Their study *in vitro* and *in vivo* provides a method of studying how their behaviour is affected when the cells are subjected to certain factors. *In vivo* experimentation enables potential factors to be identified and these can then be used *in vitro* to control cell behaviour.

1.3.2 Cell behaviour is similar in vitro and in vivo

There are similarities between cell behaviour *in vitro* and *in vivo*. It is possible to mimic how cells behave *in vivo* by changing the *in vitro* environment, for example, aggregates of cells can be made *in vitro* to mimic how cells interact with each other *in vivo*. Aggregates are clumps of cells that have cell packing densities similar to cells *in vivo* and viable cells toward the outer with necrotic regions in the centre where there is deprivation of oxygen, glucose and nutrients. These spheroids mimic tumours *in vivo* better than standard 2D cell cultures [62,63]. EC stem cells can be grown as spheroids and their behaviour becomes more

reminiscent of the *in vivo* environment with the formation of neural rosettes and pseudostratified layers. This model system has been used to demonstrate that the ECM proteins collagen IV and laminin become upregulated during development of the spheres and become polarised, confined to the basal membrane and absent from the opposite or lateral membranes of neural rosettes [64]. These results mimic the *in vivo* observation of laminin and collagen IV involvement with neural tube formation [65].

1.3.3 ES cells behave similar in vivo as they do in vitro

When cells are transplanted *in vivo* their behaviour mimics the effects seen *in vitro*. Telomerase is an enzyme that protects the ends of chromosomes from degradation during replication, it consists of two units, a telomerase RNA component (TR) containing a template for telomere elongation and a telomerase reverse transcriptase unit (TERT) that possess catalytic activity. Modulation of TERT is associated with regulation of cell growth. TERT over expressing ES cells grow faster in culture than wild type ES cells. Apoptosis is similar in both TERT over expressing ES cells and wild type ES cells so the faster growth is not due to reduced cell death in the over expressing cells. However, upon examination of DNA synthesis by bromodeoxyuridine (BrdU) incorporation, over expressing TERT cells have increased levels when compared to control cells. It would be expected that if the *in vivo* environment mimics *in vitro* cell culture upon transplantation of over expressing TERT cells into immunocompromised animals, large teratomas would be produced. Indeed, transplantation of over expressing TERT cells produces large heterogeneous teratomas (figure 1.5, A) whereas, transplantation of wild type cells results in heterogeneous teratomas that are ~50% of the size (figure 1.5, B) [66].

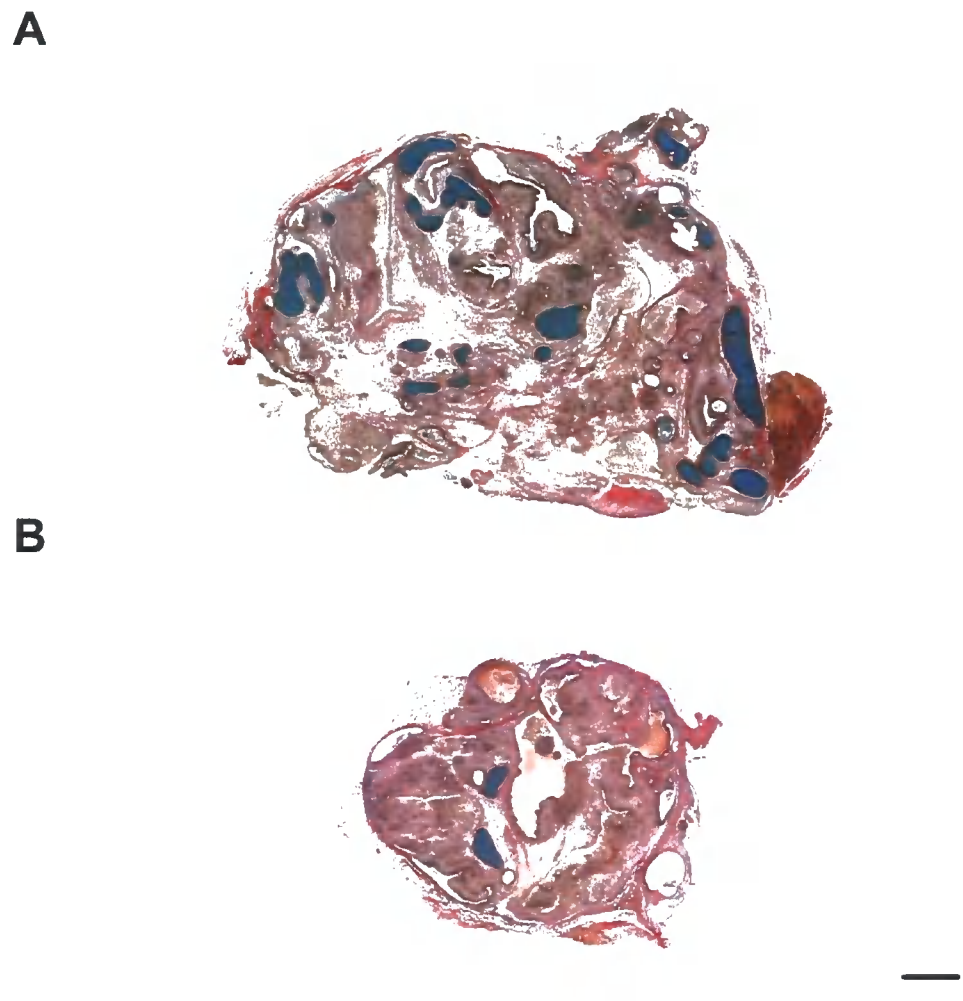

Figure 1.5 Over expression of TERT results in large teratomas. A) Teratoma produced by over expressing TERT cell line hES-NCL1 TERT. B) Teratoma produced by hES-NCL1. Histological staining: Weigerts Haematoxylin, scale bar = 1mm

1.3.4 In vivo transplantation of stem cells to form teratomas

The *in vitro* environment is artificial and not entirely representative of the natural *in vivo* environment. The mouse model is routinely used to investigate development however analysis of results is difficult as many species specific differences exist. It is not always possible to use study cells *in vivo*, for example, it is not ethical to use invasive methods to study human cell behaviour during development. One model system that can be used is transplantation of human stem cells into immunocompromised animals, which can be used to investigate their

behaviour [67]. Upon transplantation of pluripotent stem cells into immunocompromised animals 'spontaneous' differentiation occurs and the cells form a teratoma. Analysis of teratoma composition provides information on how cell behaviour has been affected. This model system can be used to study how different factors affect teratoma formation. There are limitations of this system that should be considered including: the teratoma is a haphazard arrangement; *in situ* cells are arranged in a specific fashion; and their proximity to each other is controlled; this is not the case in a teratoma. The length of time of development is also a limitation as due to regulatory limits teratomas cannot be grown indefinitely. A further drawback of this system is that the human cells are transplanted into a non-human animal and non-human factors and cells can therefore interact with the human cells. With these limitations in mind, the teratoma model system provides a good insight into human development. Study of this system will allow identification of factors controlling cell behaviour.

The rate at which a teratoma grows and develops depends upon a number of factors: anatomical location; cell types; any addition factors included in the transplantation; and any treatment to the cells prior to transplantation. In the case of EC stem cells, the teratoma produced is similar to the teratocarcinoma from which the cells are derived although due to regulations these cannot be developed as much as a teratocarcinoma and hence are far less mature. Teratomas are thought to encapsulate early embryogenesis and are a model system to study development and the control of cell behaviour. Following transplantation of ES cells, teratomas arise that are highly heterogeneous (figure 1.6, A) and composed of cell types from all three germ layers, including: gut (endoderm, figure 1.6, B); kidney (mesoderm, figure 1.6, C); neural (ectoderm, figure 1.6, D); cartilage (mesoderm, figure 1.6, E); and bone (mesoderm, figure 1.5, 6) [35].

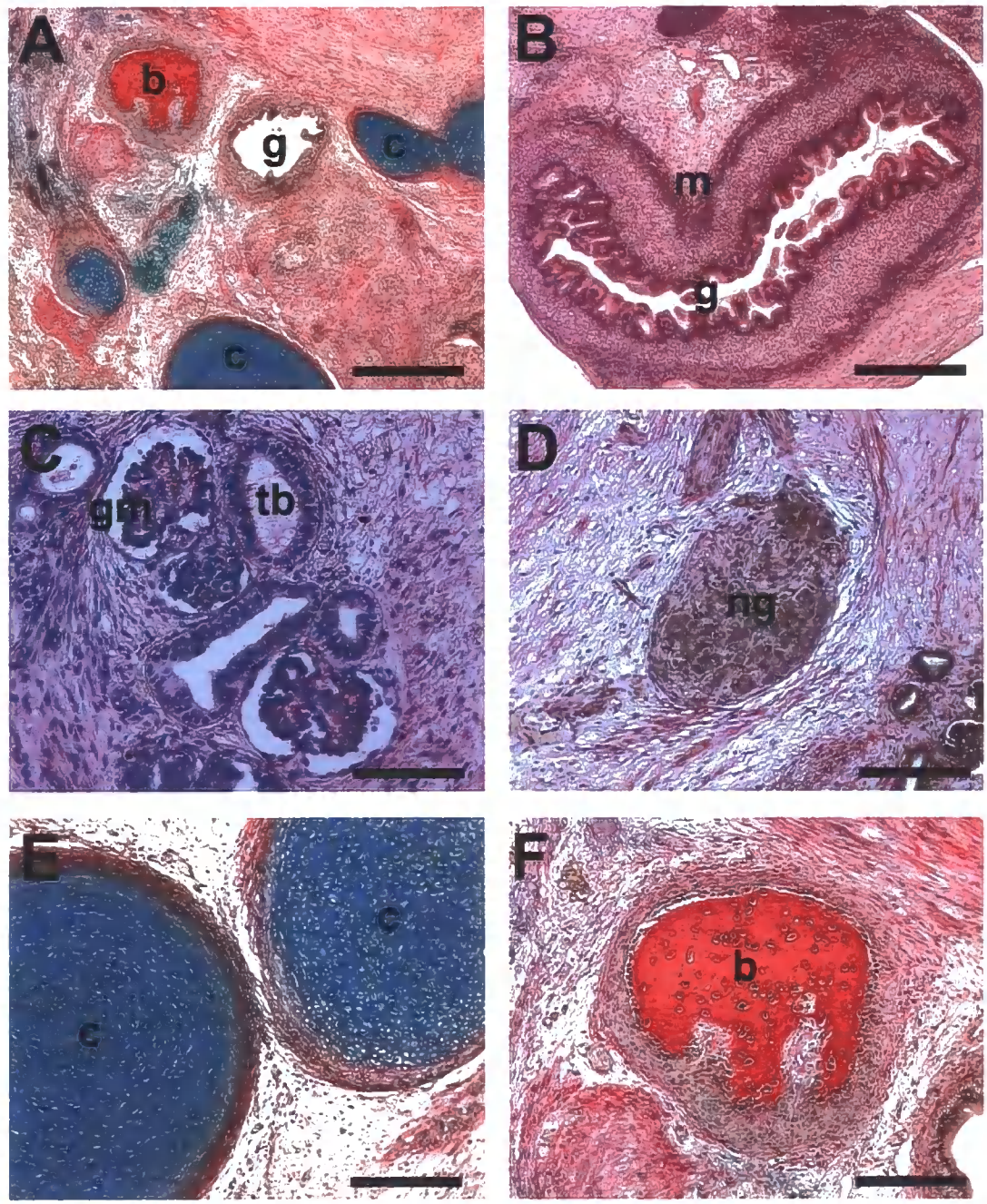

Figure 1.6 Embryonic stem cells produce teratomas following transplantation. Teratomas are composed of: bone (b); cartilage (c); gut (g); muscle (m); glomerular (gm); tubules (tb); and neural ganglion (ng). Histological staining: Weigerts Haematoxylin (A, D–F), Haematoxylin and Eosin (B, C). Scale bars: (A) 500μm; (C) 100μm; (D-F) 200μm.

Studies have used histological analysis to define the cell composition of teratomas [50,68]. However, to gain conclusive proof of the identity of the cell types present, immunohistochemical analysis is required to demonstrate the antigen expression of cells. Using antibodies against all three germ layers, true pluripotency can be demonstrated. Teratomas produce from hES cells are positive for: endoderm antibodies such as α-Fetoprotein and CDX2-positive cells (figure 1.7, A, D); ectoderm antibodies such as nestin, neurofilament 200 (NF200), glial fibrillary acidic protein (GFAP) and microtubule associated protein 2 (MAP2) (figure 1.7, B, F, G, H); and mesoderm antibodies such as smooth muscle actin (SMA) (figure 1.7, C) [66].

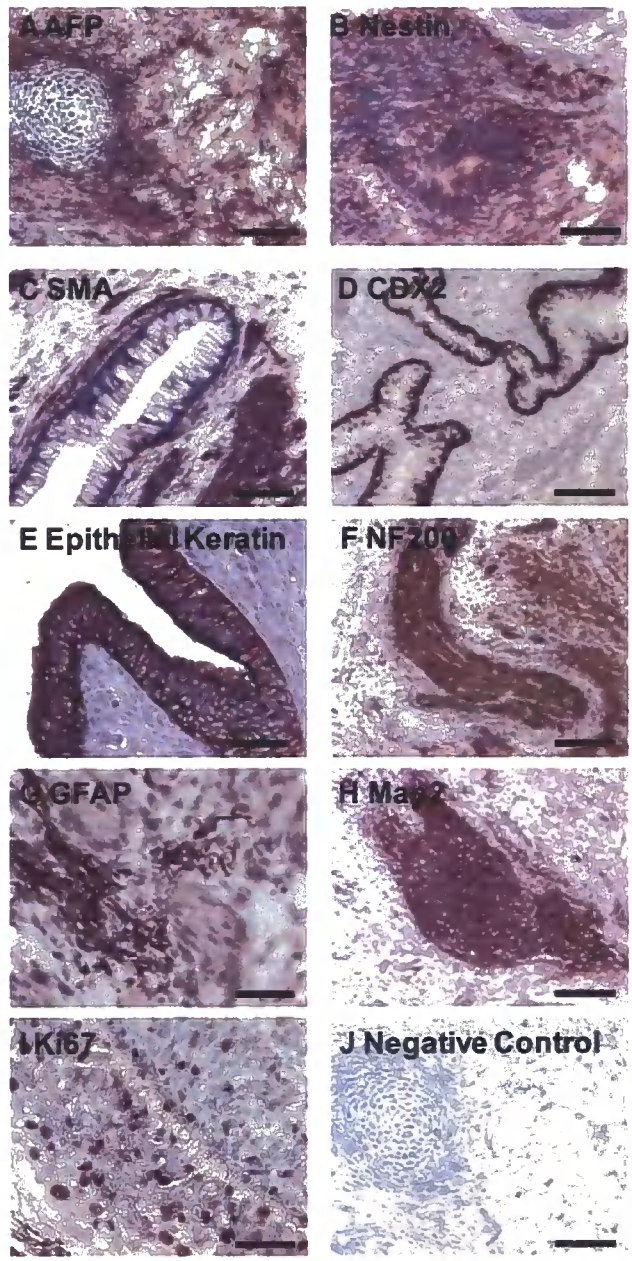

Figure 1.7 Immunohistochemical analysis of teratomas formed from grafted colonies of hES cells. hES-NCL1 TERT cells were found to be pluripotent and teratomas formed were composed of cell types from all three germ layers. A) α-Fetoprotein-positive cells (endoderm). B): Nestin-positive cells (ectoderm). C): Smooth muscle actin-positive cells (mesoderm). D) CDX2-positive cells (endoderm). E) Pan Epithelial keratin-positive cells. F) NF200. G) GFAP. H) MAP2. I): Proliferative populations of cells were shown by expression of a marker for cell proliferation Ki67. Antibody-positive regions are shown in red-purple. All negative controls showed minimal levels of background staining. J) Image corresponding to A. Scale bars: (A-C, J) 80μm; (D, F, H) 160μm; (E, G, I) 40μm.

1.3.5 Identification of factors within the in vivo environment that affect cell behaviour

One method of identifying factors controlling cell behaviour is to study transplantation of cells into alternative anatomical locations and investigate the effects on cells. It would be logical that when stem cells are transplanted into a location the factors within that particular niche are correct for directing cells to produce cell types required within the location and hence will direct the stem cells towards cell types similar to those in the location.

Within the body, there are niches, which are sets of factors acting together to maintain the correct environment to control cells so that their development is correct for that location. These factors include the ECM, growth factors, soluble molecules, cell-cell interactions, mechanical strength and 3D shape. It would be expected that when cells are transplanted into different locations, the factors within the niche will act together and direct the differentiation of the transplanted cells in a similar fashion to that of the endogenous cells. Transplantation of ES cells into different anatomical locations within the body, such as subcutaneously, intramuscular, under the kidney capsule and intratesticular result in different teratoma formation efficiencies, with the kidney capsule proving to be ideal for teratoma formation. Furthermore, following ES cell transplantation, immunohistochemical analysis demonstrates that different cell types form in different transplantation locations [69].

1.3.6 Transplantation of cells into different locations within the brain

When EC NTERA2.cl.D1 (NT2) cells are transplanted into peripheral sites such as the muscle or liver, tumour formation is observed, the cells have a high proliferation capacity and the tumour becomes large and finally, lethal. In a similar fashion, transplantation of cells into subarachnoid space, superficial neocortex or the lateral ventricles results in tumour formation. Unlike the other locations tested, transplantation in to the caudoputamen does not result in tumour

formation and the animals can live up to 15 months post transplantation. The NT2 cells ceased proliferating, ceased undergoing apoptosis and spontaneously differentiated into post mitotic neuron-like cells. The investigators concluded that there were factor(s) in the CP that modulated the behaviour of the NT2 cells but did not suggest what these factors were or how this modulation occurred [70 71].

When comparing different sites within the CNS, transplantation into different sites has been found to have little effect on maturity but a large effect on the network of processes. Cells transplanted into the caudoputamen extend processes similar to those of the host caudoputamen extending through the caudate. Whereas, cells confined to the neocortex produced processes confined almost exclusively to the immediate vicinity of the cell body [72]. In support of this, an additional study of how the transplantation site within the brain effects cell behaviour has shown that NT2N cells transplanted into white matter extend long processes whereas when transplanted into the gray matter the processes are shorter [73].

1.3.7 Transplantation into the Knee Joint

Transplantation of stem cells into locations which are rich in one particular cell type could direct the transplanted cells toward the predominant cell type. The knee joint, for example, is rich in cartilage and therefore this environment should have the correct signals and structure to promote the production of cartilage. When comparing ES cells transplanted into the knee joints of mice to the same cells transplanted subcutaneously, teratomas formed in both sites with no difference in the amount of cartilage formed. The ratio of cartilage to teratoma was however found to be greater for the knee transplantation compared to that of the subcutaneous site. In some cases the teratomas that grew completely destroyed the joint. The knee transplantation therefore showed enhanced cartilage formation but was not ideal as other tissues were also formed. The investigators also found evidence of what they believed to be tissue derived from all three germ layers [74].

The investigators did not indicate what they believe to be the reason why the knee was not ideal or explained the formation of other cell types as well as the expected cartilage. The physical transplantation of the ES cells may have disrupted the structure of the site and led to cells gaining access to areas not normally possible or it may be due to the nature of the knee environment which could only express the correct signalling molecules for cartilage differentiation at specific times during development. A possible theory for the growth of the teratoma cells is that, perhaps, the local environment did not provide a strict enough pressure to selectively differentiate the cells.

A later study by the same group demonstrated that the mechanical forces experienced by ES cells influences their behaviour. ES cells transplanted into the knee joint of rats that had their limb immobilised developed teratomas. In contrast, when the limbs were allowed to move freely, no teratoma development was observed [75].

1.3.8 The ECM affects teratoma behaviour

Studies where stem cells are transplanted into different anatomical locations indicate that the niche in which cells are transplanted affects their behaviour. One of the factors within the niche is the ECM, which could be directing and controlling the transplanted cells. The ECM occupies the intracellular space where it provides physical support to tissues. The ECM not only acts as a scaffold but it is highly dynamic and plays an important role in controlling cell behaviour. The ECM provides the base of the control of cells and tissues.

1.4 The extracellular matrix and its regulation

1.4.1 The extracellular matrix

The ECM is a meshwork of proteins and carbohydrates that occupies the intercellular space. The ECM is laid down by cells and its composition and

relative concentration of constituents is different depending on the location within the body. In humans the ECM has a key role in the control of development. One process that the ECM controls is branching, there are numerous organs that have resulted from branching of epithelial cells and include: salivary glands; airways of the lungs; and the urine-collecting ducts of the kidneys. Branched epithelia arise from unbranched precursors and control over this process comes from a multitude of different factors, including soluble factors: HGF; FGFs; TGFβ1; GDNF; BMPs and extracellular components such as: entactin; collagens; laminins; and fibronectin [76,77]. The study of branching provides evidence that the ECM has a key role in controlling cell attachment. In particular, down regulation of fibronectin inhibits branching and addition of exogenous fibronectin rescues this effect [77]. The effects of fibronectin are mediated through the integrin receptor $\alpha_5\beta_1$ and fibronectin translocation into the bud increases cell-matrix interactions and promotes cleft formation [78].

Further evidence that the ECM is essential for normal development comes from the observation that defective collagen IV production and secretion [79] results in a mutant phenotype of F9 teratocarcinoma cells [80]. Some of the main components of the ECM that are frequently investigated include: collagens; laminins; and fibronectins.

Two classes of molecules make up the ECM – Polysaccharides (specifically glycosaminoglycans (GAGs)) and fibrous proteins (such as collagens and laminins). The GAGs provide a gel-like structure and the fibrous proteins give the resulting gel tensile strength and further functionality. The interactions which occur between these molecules are important in the correct assembly of the ECM, presentation of the molecules within the ECM and the function of the ECM. The proteins that make up the ECM are known to interact with each other both directly and in interactions mediated by other cells, they are also known to interact with additional cell receptors for example, cell surface heparan sulphate proteoglycan can bind laminin, collagen IV, fibronectin and nidogen [81].

1.4.2 Collagens

Collagens are the major constituent of all ECMs and are fibrous proteins composed of three α subunits wound in a triple helical structure forming a rigid collagen superhelix. Collagens are secreted into the extracellular space and form polymers termed collagen fibrils. There are at least 19 proteins which are now formally defined as collagens. The most frequent studies of collagens are carried out using collagen I and collagen IV. Collagen I is the major structural component of connective tissue and bone and is most prevalent in the dermis, tendons and bone. Collagen I is a 300 kDa triple helical structure composed of two α1(I) chains and one α2(I) chain. Collagen I is known to undergo spontaneous assembly [82]. Collagen IV is the major collagen found in the basement membrane. Collagen IV assembles into a meshwork that constitutes a major part of the basal laminae.

Collagen matrix formation *in vitro* is easily achieved as collagen can be extracted from tissues in an acidic solution, which can then be neutralised and heated to produce a gel [83]. Although collagen gel formation is straightforward in the *in vitro* environment, the *in vivo* assembly of collagen into the ECM is a more complex process. Fibronectin is a key component in the assembly of collagen into the ECM *in vivo* as seen when blocking the 60k fragment from fibronectin inhibits the correct assembly of collagen into the ECM [84].

One potential mechanism for collagen assembly is seen by the mechanism where integrin attachment of fibronectin to the cell's surface mediates the formation of the collagen network. Following the binding of integrin and fibronectin cryptic domains are exposed and collagen I, procollagen and collagen V bind to the exposed domains. The integrin α2β1 becomes active and binds to collagen inducing a conformational change that facilitates fibril formation [85].

Collagen has the ability to form a mesh and it is the 3D effect of collagen on cell behaviour that has been most extensively studied. However, there is evidence to suggest that peptide motifs within these molecules can control cell attachment.

Synthetic mimics for collagen can be produced from peptides containing RGD that self assemble to form a structure mimicing natural collagen [86 87]. Although the 3D structure of collagen is important in controlling cell behaviour, the peptide motifs found in collagens can be used to control cell behaviour independently of the natural 3D structure of collagen [88-92]. From the non collagenous domain the peptide CNYYSNSYSFWLASLNPER inhibits cell attachment when cells are grown in media containing the soluble peptide whereas surfaces with adsorbed collagen peptide increase cell attachment [89].

1.4.3 Laminins

Laminins are a group of glycoproteins found in the basement membrane [93 94,95] and are present in a large range of tissue types [96]. Laminins are cruciform-shaped and are composed of three subunits: α-chain (~400kD); β-chain (~200kD); and a γ-chain (~200kD). Assembly of the whole molecule is mediated by specific sites in the α-helical region of the chains at the carboxyl end. Different variants of the α, β, γ chains are known and complexes of the chains result in at least 10 different possible laminin forms, for instance laminin-1 (α1β1γ1) or laminin-2 (α2β1γ1). Laminin assembles at cell surfaces via a receptor mediated mechanism. Binding of laminin to dystroglycan [97] and the integrin α7β1, increases the concentration of laminin above its critical concentration thereby inducing laminin to form a gel at the cell surface.

The effects elicited by laminin are mainly due to interactions between peptide motifs in the molecule and membrane receptors on the cell surface. To date, three laminin receptors have been identified: 67kDa; 110kDa; and 180kDa [98]. Laminin was first shown to bind the 67kDa receptor [99] and this was later identified to be the binding site for the YIGSR motif [100]. Many laminin derived peptides have been identified as having biological activity, for example, 16 peptides have been found in the laminin α1 chain [101]. The most frequently investigated peptides include YIGSR [102], IKVAV [103 104] and RGD [105 106].

1.4.4 Fibronectins

Fibronectin is a large glycoprotein composed of two subunits; dimerisation is achieved through inter-chain disulfide bonds at the carboxyl terminus. Each subunit is ~2500 amino acids and folded into five or six domains connected by flexible polypeptide linkers. Each subunit consists of type I, type II and type III repeating modules. Interactions between cells, matrix components and fibronectin are mediated through sets of repeats, which make up domains.

When cells are grown in culture, fibronectin can be detected both in the culture media and at the cell surface. Studies have predicted that 25% [107] or 50% [108] (depending on the cell type) of the secreted fibronectin synthesised is retained at the cell surface. The addition of exogenous fibronectin to culture media of cells grown *in vitro* results in the incorporation of fibronectin into the ECM [109]. Intravenous injection of fibronectin into the mouse results in co-localisation of endogenous and exogenous fibronectin [110]. These findings indicate that cells could synthesise soluble fibronectin, secrete this fibronectin and then incorporate it into the ECM.

Integrin binding has been proposed as a possible mechanism for the assembly of fibronectin into the ECM [111]. Fibronectin is secreted by cells as an inactive disulphide bonded dimer. Binding of the integrin $\alpha5\beta1$ to the RGD motif within fibronectin molecules immobilises the fibronectin molecule to the cell surface. Binding of fibronectin to the integrin molecule forms the initiation step which allows fibril elongation to occur and activates the fibronectin resulting in a conformational change that exposes cryptic domains that can bind to additional fibronectin molecules. Fibril elongation occurs as more fibronectin molecules become bound together and also to integrin [112]. Within the fibronectin molecule, there are binding sites for collagen, GAGs, fibrin, integrins and fibronectin.

Interactions of fibronectin with cells are known to occur through two key motifs, RGDS and PHSRN [113]. Cell attachment can be increased when cells are cultured

on surfaces presenting either RGDS or PHSRN. Attachment to surfaces presenting RGDS could be inhibited by preincubation with either RGDS or PHSRN soluble peptides, thus indicating that these two peptides can be competitively inhibited and they act via the same receptor [114].

1.4.5 Physical support by the extracellular matrix

One function of the ECM is to support cells, the ECM is a network of fibers which resist stress and maintain correct cell shape. Collagen forms the main structural protein within the ECM, collagen I is the most abundant. By examining the structure of collagen it is possible to identify how collagen provides support for the cell.

Collagens are known for their capacity to self assemble, for example when an initially acidic *in vitro* environment is neutralised and the temperature is increased collagen is able to self assemble to from fibril gels [115]. Collagen I forms the primary structural element within the ECM and provides tensile stiffness. Collagen I is a triple helix composed of two α1(I)-chains and one α2(I)-chain [116]. Each peptide forms a left-handed helix, with the peptides combining to form one single right-handed super helix. Collagen triple helices form a distorted hexagonal shape [117].

Collagen I molecules are approximately 300 nm, interacting with each other at the tail to form overlapping regions with gaps between these overlaps. Collagen has a high tensile strength and upon application of a force of 20 MPa per unit area a collagen fibril will extend by 4%. It is proposed that collagen fibrils resist extension by three mechanisms: collagen molecules themselves are known to allow for molecular elongation; the gaps between the overlapping regions where tail domains interact form kinked regions that can straighten to compensate for an applied force [118], increases in the gap regions provide further increases in collagen fibril extension; and slippage between laterally adjoining collagen molecules allows for further extension [119].

1.4.6 Interactions between the extracellular matrix, growth factor and cytokines

Growth factors are peptides and polypeptides secreted by cells to regulate a variety of cellular processes. Growth factors are factors produced by the body that can regulate cell proliferation and differentiation which produce their effects by binding to a corresponding receptor on a cell and then activating signalling cascades. Growth factor receptors are made up of extracellular domains that bind to the growth factor, a transmembrane domain and an intracellular domain that possesses catalytic activity. Upon growth factor binding the active receptor complex recruits a myriad of intracellular signalling molecules to bring about changes in gene transcription and cytoskeletal changes. This system allows for the control of growth factor signalling at both the extracellular and intracellular levels.

The extracellular matrix interacts directly with other cells and has additional indirect effects on cell function including influence on cell behaviour by mediating growth factor signalling. Part of the control of growth factor signalling at the extracellular level is given by the binding and interaction between growth factors and the ECM which can facilitate storage and decrease loss via decreasing diffusion.

Research has demonstrated that the ECM can increase the effects elicited by growth factors. Glomerular epithelial cells cultured in the presence of EGF on collagen I coated surfaces results in increased proliferation when compared to the same cells cultured in the presence of EGF on uncoated surfaces and also enchanced the level of the activated EGF receptor [120].

Tumour necrosis factor alpha (TFN-α) is a cytokine that mediates the immune and inflammatory response. TFN-α has two receptors, a 55 and a 75kDa, binding to these receptors activates numerous signalling cascades, including: NF-κB (a transcription factor that controls proteins involved in cell survival, proliferation, anti-apoptosis and inflammatory); MAPK/JNK (involved in cell proliferation, differentiation and pro-apoptosis); and caspase 3. Many of the processes are

counter effective and there is significant cross talk between the processes to control the overall effect. TFN-α binds to fibronectin and also to a lesser extent collagen I, collagen II, collagen IV and laminin [121]. Bone morphogenic protein 7 (BMP 7) is involved in bone homeostasis and osteoblast differentiation and is capable of binding to various ECM proteins, including collagen IV [122].

Hepatocyte growth factor (HGF) is secreted by stromal fibroblasts and becomes bound by the ECM. Release of HGF from the ECM is facilitated by urokinase ECM degradation [123]. In a similar scenario, transforming growth factor β (TGF-β) is held within the ECM and is released by plasmin or thrombin [124]. Basic fibroblast growth factor (bFGF) is secreted by cells and stored in the ECM [125]. Thrombin is known to degrade the ECM and release bFGF from the matrix. This provides a mechanism for the control of cell proliferation [126].

1.4.7 Extracellular matrix receptors

Cell interaction with the ECM is mediated by a family of cell surface proteins - integrins. Integrins can be isolated from PC12 cells and reconstituted in lipid vesicles, when these vesicles are incubated on collagen IV or laminin coated surfaces adhesion is increased when compared to vesicles without integrins present [127]. Furthermore, synthetic peptides known to bind to surface glycoproteins can be used to inhibit cell attachment of PC12 cells to collagen IV, laminin or fibronectin [127]. Integrin binding initiates dimerisation of α and β subunits, resulting in a conformational change and phosphorylation of the intracellular domain subsequently leading to the activation of intracellular signalling pathways to control cell behavior [128,129]. In a process called integrin switching a cell can regulate its integrin expression to control its behaviour. By up or down regulating specific integrins a cell can switch from proliferative to differentiated [130].

The integrin β1 is known to be involved in migration and neurite length; however with the loss of this β1 integrin, there is not a complete abolishment of activity. In addition, depending upon the culture surface, of either laminin or fibronectin, the

reduction is different, this demonstrates the existence of multiple mechanisms for controlling these processes and also indicates that different proteins act through different mechanisms [131].

1.4.8 Cell response to their environment

During cell development it is important that cells respond to their surroundings. Guidance signals from the exterior of a cell must be translated across the cell membrane into the cell where they can control cellular behaviour. Typically, extracellular signals initiate gene transcription, leading to remodeling of the cytoskeleton and specialised responses such as neurite outgrowth extracellular signals can include ECM proteins on the exterior of cells. The first evidence that the ECM and subsequent control of the cytoskeleton is important for cell behaviour came from Ali et al (1977). Transformed cells lacking fibronectin (LETS protein) did not adhere and lacked a defined actin cytoskeleton, addition of fibronectin resulted in a defined actin cytoskeleton and restored adhesion [132]. Further evidence that the ECM controls the cytoskeleton is found in co-labeling studies where fibronectin and actin have similar distributions during cell spreading [133]. It was later demonstrated that adhesion to a surface could control gene expression, when monocytes adhered to a surface, their expression of mRNA for TFN and c-fms increased [134]. In fact, the ECM has been shown to control the gene expression of many cell types, from mammary epithelial cells [135] to hepatocytes [136].

The first stage in cells sensing and responding to extracellular signals is receptors that can specifically bind to the ligands. Cell adhesion molecules (CAMs) are the group of molecules responsible for mediating signals from the exterior of cells. There are four subgroups of CAMs: integrins; selectins; immunoglobulins (Ig); and cadherins. Although, CAMs were originally identified for their role in cell adhesion, they also have a role in neurite direction. Two CAMs involved in neurite direction are: neural cell adhesion molecule (NCAM) (from the Ig group); and N-cadherin (from the cadherins group).

1.4.9 Integrins

Integrins integrate signals from cell exteriors to cell interiors. Integrins have an extracellular domain that can interact with signalling molecules such as ECM proteins and an intercellular domain that can interact with signalling molecules inside of the cell. Sixteen different α subunits are known to interact with 8 β subunits to form 22 integrin heterodimers. Most of the α-subunits (excluding α4,6,v) only associate with one β-subunit (figure 1.8).

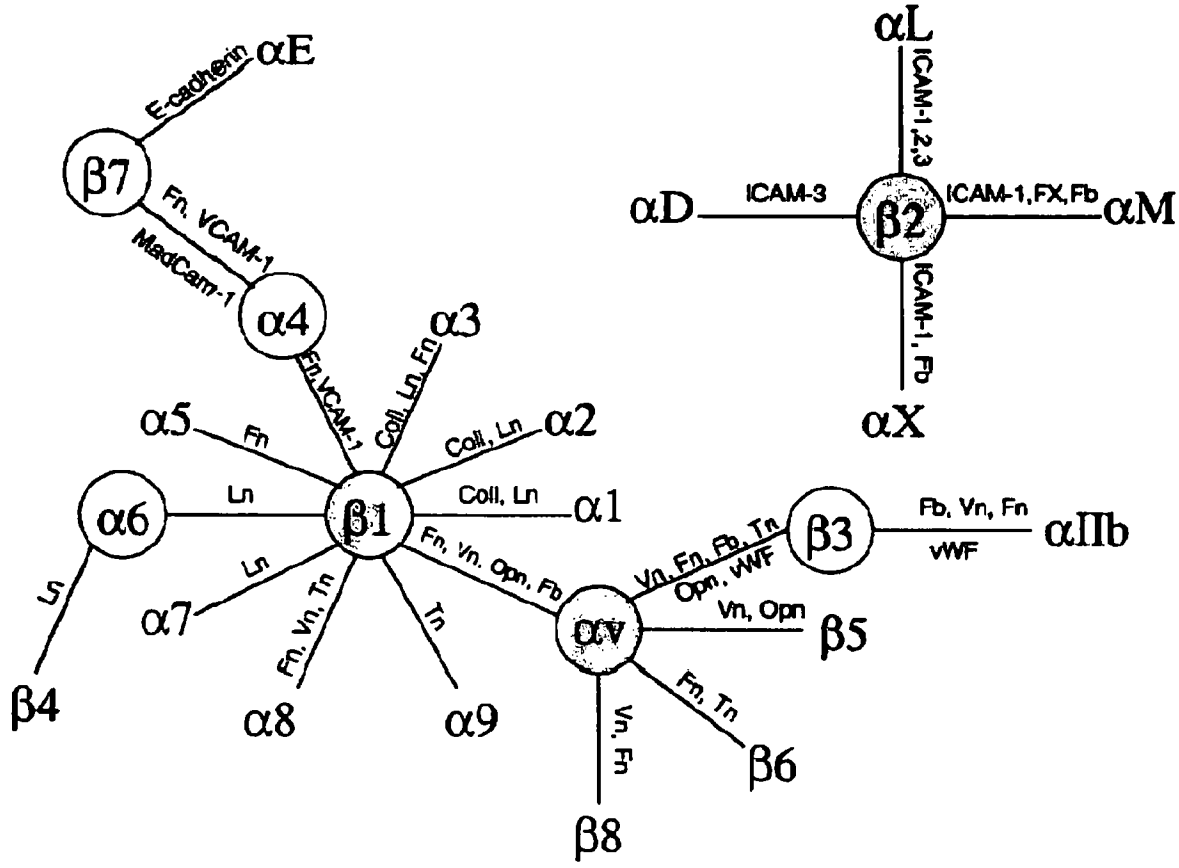

Figure 1.8 Schematic showing integrin heterodimers and their ligands. Coll, collagen, Fb, fibrinogen, Fn, fibronectin, FX, factor X, ICAM intercellular adhesion molecule, Ln, Laminin, MadCAM mucosal addressin cell adhesion molecule, Opn osteopontin, Tn, tenascin, VCAM, vascular cell adhesion molecule, Vn, vitronectin, vWF von Williebrand's factor. From: Milner Campbell 2002 J. Neurosci Res 69:286-291[137].

An example of integrin binding is the binding of integrins to the laminin RGD motif. There are numerous integrin combinations that can bind to the RGD sequence including: α5β1; α8β1; αvβ1; αvβ3; αvβ5; αvβ6; αvβ8; IIbβ3; and to a lesser extent α2β1; α3β1; α4β1; and α7β1 [138].

Integrin distribution throughout cells is not uniform. Upon solubilisation of PC12 cells grown on surfaces coated with either collagen I or laminin, not all of the integrins are solubilised and a fraction of the α1 and β1 integrins remain bound to the ECM protein. Confocal analysis of cells cultured on laminin reveals that the

distribution of integrins is polarized and the main localisation was at the periphery of the cell surface in contact with the culture surface. Immunocytochemical analysis demonstrates a high distribution of the integrin β1 uniformly throughout the growth cone of dorsal root ganglion neurons [139].

1.4.10 Control of integrin binding

Integrins typically have a low affinity to their ligands with dissociation constants (K_d) of 10^{-6} and 10^{-8} mol/litre. Although individual integrins show weak binding, the interaction of thousands of integrins allows an effective strong bond between a cell and the ECM. As individual integrin binding is weak the cell is still able to break contacts with the ECM and migrate efficiently. The binding efficiency of a cell is controlled by modulating the binding activity of individual integrin molecules [140]. Experimentally, increasing the activation of integrins by the addition of Mn^{2+} or by constitutively activating R-ras increases the percentage of cells presenting neurites when grown on laminin coated surfaces [140]. An additional level of integrin control is achieved by a variety of molecules binding to the cytoplasmic or extracellular domains of the integrin molecules. For example, binding of CD98 to the integrin β1 increases integrin activation [141].

An alternative mechanism proposed for the control of integrins is the mechanism by which cells can regulate their integrin expression by controlling the number of integrin receptors present at their surface. It was hypothesised that culturing DRGs on surfaces coated with low levels of laminin (1μg/ml) would result in increased levels of the laminin specific integrin α6 RNA and higher total protein concentrations in comparison to cells grown on surfaces coated with high levels (20μg/ml). However, the results observed showed a decreased in the level of α6 RNA expression and an increase, of four-five folds, in the level of α6 integrin expression under low laminin conditions. By culturing these cells on low laminin and high laminin coated surfaces over night the integrin expression at the cell surface could be modified, transfer of these cells to new laminin coated surfaces resulted in cells previously cultured on low laminin (high levels of cell surface α6

integrin expression) having a greater attachment and neurite outgrowth when compared to cells previously cultured on high levels of laminin. This observation was seen for both cells cultured on new surfaces with high and low levels of laminin expression. From these results it was proposed that when cells are cultured on surfaces with low levels of ligands the receptor level at the surface is maintained by constitutive levels of integrin synthesis, secretion and uptake. Whereas, when cells are cultured on high levels of ligands, targeted endocytosis and sequestration or degradation is used to remove the receptors from the surfaces [142]. Integrin function has been studied using genes which have been ablated. For example, neural progenitors derived from the forebrain of mice with the β1 integrin allele removed show a reduction in cell adhesion to laminin and fibronectin coated surfaces, increased levels of apoptotic cells in neurospheres and reduced migration on laminin and fibronectin coated surfaces [143].

1.4.11 Linking integrins to the cytoskeleton

Integrins have two mechanisms by which their effects are mediated: they provide a physical link between the ECM and the cytoskeleton; and they activate signaling pathways. With the exception of β4, the cytoplasmic domains of integrins are short (≈30-40 amino acids) and contain no enzymatic activity hence in order for integrins to mediate their effects they must recruit other signaling molecules. Binding of cells to an ECM initiates integrin clustering and stimulates reorganisation of the cytoskeleton, which in turn reorganises integrins and related signaling molecules [144]. Mutations in the cytoplasmic domain do not alter heterodimer assembly [145,146]. Whereas, deletion of the cytoplasmic tail domain [145] or mutation greatly reduces cell adhesion [146]. How contact with the ECM modulates cell attachment was first documented when it was noted that the integrin mediated binding of platelets to a surface resulted in phosphorylation of a protein tyrosine kinase, pp125FAK (FAK, focal adhesion kinase) [147].

There are numerous mediators of integrin-actin interactions including integrin-linked kinase (ILK) which is localised to cell adhesion sites by PINCH [148]. ILK

interacts with the cytoplasmic domain of β1 integrin via ILKs COOH-terminal domain [149]. In turn, affixin and CH-ILKBP bind to both ILK and the actin cytoskeleton, linking the transmembrane integrins to the actin cytoskeleton.

Aggregation of integrins results in the accumulation of at least 20 signal transduction molecules, including: c-Src; c-Fyn; RhoA; Rac1; Ras; GAP; MEK1; ERK1; ERK2; and JNK1. Integrin aggregation in combination with integrin occupancy, tyrosine phosphorylation and actin cytoskeletal integrity results in the redistribution of the following cytoskeletal molecules: F-actin; paxillin; filamin; talin; a-actinin; vinculin; and tensin [150].

1.4.12 Integrin binding modulates growth factor signalling

Integrin signalling is linked to growth factor signalling. The attachment of fibroblasts to surfaces coated in collagen I and fibronectin induces a transient tyrosine phosphorylation of platelet derived growth factor (PDGF) beta receptor, activating the receptor. This phosphorylation effect is lost with the disruption of the actin cytoskeleton using cyclochalasin D. This directly links integrin binding to activation of growth factor receptors [151]. In combination with integrins, the growth factors EGF, FGF and PDGF activate ERK but only when the integrins are occupied and clustered [152].

1.4.13 Integrin signalling cascade

Following activation of integrins, cells need to activate specific signalling cascades to respond. Integrins are known to control neurite outgrowth and studies have inhibited neurite outgrowth using both soluble peptide sequences [153] and antibodies to competitively block integrins.

The culture of PC12 cells with NGF or on laminin results in increased neurite outgrowth. In the presence of NGF or laminin, the levels of phosphorylated ERK are elevated and a synergistic effect is observed when cells are cultured on laminin

coated surfaces with the addition of NGF to the culture media [154]. Transfection of PC12 cells with Ras increases the phosphorylation ERK, this in turn results in an increase in neurite outgrowth. The culture of PC12 cells in the presence of NGF results in the down regulation of TrkA expression and the culture of PC12 cells on laminin results in the down regulation of integrin β1 receptor when compared to poly d lysine. Over expression of p75NTR results in a decrease in neurite outgrowth as p75NTR is known to increase Rho GTPase activity which is a known inhibitor of neurite outgrowth. The culture of cells on laminin results in a decrease of P75NTR expression and decreased levels of Rho activity when compared to PDL. The down regulation in p75NTR was found to be linked to an increase in PTEN, knocking out PTEN increased p57NTR expression. The primary role of PTEN is to negatively regulate the phosphatidylinositol-3-kinase (PI3K) signalling cascade. PTEN hydrolyzes phosphoinositide PI(3,4,5)P3 to produce the second messenger PI(4,5)P2, which binds to profilin to release G-actin, increasing the availability of free monomers for the extension of neurite actin filaments, allowing neurite extension [154].

Investigating cell spreading has provided some insight into how signals from the membrane bound integrins are transduced into an effect in the cell. Integrin binding to a surface results in the activation of phospholipase A2 (PLA2), this converts phospholipids into arachidonic acid (AA), which is oxidized by lipoxygenase to a metabolite that activates protein kinase C (PKC). PKC can then induce cell spreading [155].

1.4.14 Mechanotransduction

Cells within tissues and organs are in contact with each other via CAMs and other signalling molecules. Such signalling molecules influence cell behaviour directly by interacting with the corresponding receptor on the target cell. If the cell type of interest does not have the correct receptor, no effect will be observed. Thus interactions between cells using these direct signalling molecules are highly cell specific. In addition to these signalling molecules, physical cues from the

surrounding tissues can modulate their behaviour. Tissues are elastic which means that when a deforming force is removed they will recoil to assume their original conformation, providing the elastic limit is not exceeded. For cells to function correctly, they must respond to the elasticity of tissues, this is done by the reorganization of their cytoskeleton. Although, at first it would appear that cells must resist external forces applied to them to remain viable, it is becoming apparent that the application of external mechanical forces to a cell can influence the cell behaviour. The process by which cells sense their mechanical environment and transduce these extracellular signals into a cell response is termed mechanotransduction.

The resistance to stress is measured by the Young's elastic modulus (E). The stiffness or elastic modulus is dependent upon the tissue type. For example, brain tissue is softer than muscle tissue and muscle tissue is softer than skin [156]. The liver has a Young's elastic modulus of ~2 kPa whereas plastic and glass used for tissue culture have a Young's elastic modulus in the giga pascal range. In cells and therefore tissues, the Young's elastic modulus is dependent upon their actin, myosin cytoskeleton and their ECM. Posttranslational modifications of collagen, including modification by lysyl oxidase, can change the stiffness of a cell. It has been found that lysyl oxidases are up-regulated following liver injury [157] and lysyl oxidases have been found to increase the E of collagen gels *in vitro* [158].

For a cell to respond to mechanical stress, it must have two components: something that is changed due to the applied force; and a mechanism to transmit this effect from the sensory element to elicit a change. The structure that is implicated in mechanotransduction is the ECM/integrin/cytoskeleton network. The ECM is linked to the cytoskeleton via integrins and when a force is applied to a cell, any of these elements can sense the change from the resting state. It has been suggested that upon the application of force, ECM components could potentially unfold, thus providing a sensory mechanism. The integrins linking the ECM proteins to the cytoskeleton could also provide a mechanism for the sensing

of mechanical load [159]. Finally, the cytoskeleton could mediate the sensing of mechanical load [160].

The strength of a material which cells come into contact with can affect their behaviour. Increasing the stiffness of the collagen gel increases the proliferation of rat smooth muscle cells [161]. NSC cultured on surfaces with an elastic modulus of 10-10,000 Pa, show a maximal growth rate at 1,000 Pa, furthermore, the stiffness of the substrate also affects the differentiation of the NSCs, with stiffer gels favouring astrocyte differentiation and weaker gels favouring neural differentiation [162]. The response to substrate stiffness is cell type dependant. The culture of cells on a flexible surface results in decreased proliferation and increased apoptosis. In comparison, transformed 3T3 cells do not respond to these changes in surface flexibility [163].

1.4.15 Neurite outgrowth

For the nervous system to develop, neurons must project their axons over large distances to reach their targets. Axon migration is mediated by a specialised structure at the leading edge, the growth cone which has the capacity to sense the environment and respond to attractive and repulsive cues thereby guiding the axon. Growth cones determine the rate and direction of neurite outgrowth, in order for growth cones to act in this way they must be able to sense signals from the environment and transduce these into cytoskeletal rearrangements. At the periphery of the growth cone there is a layer of flat web shaped sheets of cytoplasm called lamellipodia, with finger like filopodia extending out. For growth cones to mediate the exterior signals into a response they must be able to elicit rearrangements in the cytoskeleton. The two main cytoskeletal components found in growth cones are actin filaments and microtubules. Actin filaments are helical polymers composed of globular actin (G actin) monomers. ATP-actin is added to the barbed end of the filament and hydrolysed to ADP-Pi-actin, the inorganic phosphate is lost, leaving ADP-actin. ADP-actin can be dissociated from the filament from both the pointed and barbed ends however, loss from the

pointed end is kinetically favoured. Microtubules are hollow cylindrical fibers composed of α and β-tubulin subunits, similar to actin filaments, microtubule subunits are added at one end and lost at the other.

The rate of neurite advance is linked to the shape of the growth cone, with the initial extension rate correlating with the length of the filopodia [164]. Neurite outgrowth of PC12 cells has been studied and neurite outgrowth has clear stages. Rapid extension of the filopodia allows protrusion of the growth cone. If the newly extended lamellipodium does not retract or lift off the substrate, the consolidation phase causes the lamellipodium to thicken as it fills with cytoplasm and organelles [165].

In a steady state, actin filaments undergo assembly where monomeric actin (G-actin) is polymerized to form F-actin. At the barbed end G-actin is assembled and at the pointed end it is disassembled. Monomeric ATP-actin units are added to the plus end where they are hydrolysed to ADP-Pi-actin, the inorganic phosphate is lost, leaving ADP-actin. This addition of monomeric actin to the plus end and loss at the minus end is termed treadmilling. Most of the actin filaments within the filopodia have their barbed ends facing towards the cell membrane. The actin filaments within the filopodia and lamellipodia are continually undergoing cycles of expansion and contraction [166].

In the resting state, the barbed ends of actin filaments are capped, following stimulation, free barbed ends are generated. There are a number of proposed mechanisms for the generation of barbed ends including elevation of levels of PtdIns(4,5)P2 which results in dissociation of the capping proteins, and ADF/cofilin severs filaments with Arp2/3 promoting actin nucleation as shown by Condeelis et al. 2001. Growth of actin filaments is rapid as there is a lag in Pi disassociation

During neurite extension and retraction, the retrograde of G-actin remains relatively constant and the main differences are in the assembly of actin. When

filopodia are retracting, actin assembly is low and retrograde flow is high, whereas, when filopodia are extending, although retrograde flow is still high the rate of assembly is greater and exceeds the retrograde flow [167].

1.5 Investigating how the extracellular matrix and peptide motifs control cell behaviour

1.5.1 The extracellular matrix can control cell cycle progression and cell death

Cell binding to adhesive molecules can mediate cell survival by both increasing cell cycle progression and decreasing cell death. Integrin binding can influence apoptosis. Melanoma cells cultured in gels composed of collagen I and III are viable, in contrast, melanoma cells lacking the integrin αv undergo apoptosis. Similary, antibody inhibition of integrin αvβ3 induces apoptosis [168].

When endothelial cells are seeded onto vitronectin coated surfaces they are able to bind and attach to the surface. This binding is mediated via the integrins αvβ3, αvβ5 and α5β1. When cells are seeded onto vitronectin coated surfaces in the presence of RGD peptides, these peptides are able to bind to the integrins presented by the cells and inhibit binding. This inhibition results in the failure of cell attachment and subsequently the induction of apoptosis [169].

Cell adhesion to the ECM can control cell cycle progression. Cell cycle progression through G1 and G1-S transition is controlled by transcriptional repressors. In the presence of the mitogens cyclin D, cyclin E, and cyclin A associated cyclin dependent kinase (cdk) activites increase. These act to phosphorylate retinoblastoma protein (Rb), which results in the de-repression of the genes encoding for E2F, cyclin E and cyclin A. This positive feedback continues until phosphorylation of Rb is complete and cyclin A associated cdk activity starts to inhibit E2F. Once this has taken place, cells have passed the restriction point and are committed to the cell cycle [170]. When cells adhere to a surface this results in the increase of the expression of cyclin A and cyclin D1.

Furthermore, if cells were transferred from an adhesive condition to a non-adhesive condition a decrease in the phosphorylation of Rb is seen [171].

As well as increasing cell cycle progression, cell binding can also inhibit cell death. Anoikis is a type of apoptosis that is induced when anchorage dependent cells lose their anchorage [172,173], this process is known to be controlled by integrin signalling. As well as integrin binding, growth factors are important in the control of cell behaviour. Anoikis in schwann cells can be inhibited with addition of BDNF to the culture media, although a similar trend is not observed for NGF, NT-3 or GDNF. Pretreatment of cells with BDNF prior to transplantation protects them and results in an increased level of survival [174].

Integrin signalling is important in the resistance of cells to stress induced apoptosis. Following cell stress such as serum withdrawal, the proapoptotic protein Bad becomes localised at the mitochondria, this facilitates the leakage of cytochrome C out of the mitochondria into the cytoplast. Apaf-1 mediates the formation of the apoptosome with caspase 9 and cytrochrome C. Under adhesive conditions, integrin ligation results in the activation of the Ras-Raf-MEK-ERK cascade. Raf and MEK phosphorylate the proapoptotic protein Bad resulting in its displacement from the mitochondria and 14-3-3 proteins result in its sequestration. This process prevents the loss of cytochrome C from the mitochondria and prevents the formation of the apoptosome with caspase 9 [175].

Integrin binding can regulate cell survival upon binding of β1 this activates p130Cas, paxillin, Akt and Src. Both p130Cas and paxillin inhibit JNK, this inhibits apoptosis, whereas Akt and MAPK increase cell survival [176].

One key pathway in the control of apoptosis is the Rho/ROCK pathway. Rho kinase (ROCK) is a downstream effector of Rho which directly phosphorylates myosin light chain (MLC) and also inactivates myosin phosphatise thereby increasing MLC phosphorylation. MLC mediates cell contraction which is one of the triggers to evoke apoptotic cell death. Loss of cell attachment can be

mimicked by the dissociation of cells cultured as spheres, this dissociation increases MLC phosphorylation. This increase in MLC phosphorylation can be reversed with the addition of ROCK inhibitor resulting in a decrease in cell death. ROCK is known to modulate the cytoskeleton, the interaction between ROCK and the cytoskeleton poses a potential mechanism for controlling apoptosis. No increase in cell death is observed where the cytoskeleton is disrupted prior to the initiation of cell attachment loss. This demonstrates that rearrangement of the cytoskeleton by ROCK is necessary for induction of aopotosis [177].

1.5.2 The extracellular matrix can be used in vitro to control cell behaviour

The ECM has been demonstrated to be a key factor in controlling cell behaviour *in vivo*, culture of cells on surfaces coated with alternative ECM components allows the direct study of how the ECM regulates cell behaviour. It is possible to mimic the ECM *in vitro* by adsorbing ECM components onto culture surfaces [17]. When a solution of ECM molecules is applied to a surface the proteins come out of the solution and adsorb onto the surface. This adsorption is rapid, within two hours 80% of fibronectin in the solution will become adsorbed onto the surface [178].

There are contradicting reports regarding the regulation of a cells ECM. Some groups have demonstrated that, differentiation *in vitro* is accompanied by marked changes in the cells ECM, suggesting that ECM synthesis plays an important role in differentiation [179 180]. Whereas, others have shown that the culture of ES cells on surfaces with adsorbed collagen I does not suppress the cells tendency to produce collagen I, collagen IV, fibronectin or laminin [181]. However, suppression of collagen production by ES cells using *cis*-hydroxyproline results in detachment of cells from the culture surfaces and this is not rescued when they are grown on collagen I coated surfaces, demonstrating that collagen production in these cells is necessary for cell attachment [181]. Furthermore, the presentation of peptides from ECM proteins can modulate ECM secretion and increasing the surface density of the presented RGDS sequences decreases extracellular matrix production in a number of different cell types [182 183].

Surfaces coated with ECM proteins are known to influence cell attachment, neurite outgrowth and differentiation. Laminin has been demonstrated to increase both the attachment and the number of cell bearing neurites of primary neurons from new born rat brains [184]. Using an alternative cell type of dorsal root ganglion (DRG) cells, when cultured on laminin, greater neurite outgrowth is observed than on collagen IV or fibronectin [185]. Laminin is also known to affect differentiation and accelerates neuronal differentiation of EC cells when compared to collagen I, collagen IV or fibronectin [186]. In addition to increasing the rate of differentiation, the ECM has been found to increase the range of cell types formed. When ES cells are cultured on collagen IV they can be differentiated into trophoectodermal cells, when cultured on collagen I, laminin or fibronectin their differentiation is more restricted [187]. As well as investigating how the ECM affects differentiation, how the ECM affects the inhibition of differentiation has also been investigated. Murine ES cells differentiate when cultured on surfaces presenting laminin or fibronectin whereas when cultured on collagen I or collagen IV they maintain their undifferentiated phenotype. The response of the cells is attributed to their integrin expression; they express integrins to laminin and fibronectin but not collagen I or collagen IV. [188].

1.5.3 Basement membrane extracts

Investigating cell behaviour in response to single ECM proteins provides a simplified model system. As the ECM *in vivo* is made up of multiple ECM proteins this could be considered as too simplified and to more faithfully mimic the *in vivo* environment, interactions with multiple ECM proteins is required.

ECM secretions can be used to control cell differentiation, ES cells cultured under keratinocyte inductive conditions on glass do not form keratinocytes and those cultured on gelatin rarely differentiate into keratinocytes. Keratinocyte differentiation can be increased when ES cells are cultured on surfaces with ECM

secretions from primary cell cultures. ECM secretions from different cell types result in different levels of ES cell differentiation [189].

ECM extracts can be produced consisting of multiple ECM proteins, for example, Matrigel™ is an ECM extract that is derived from Engelbreth-Holm-Swarm (ESH) murine sarcomas [190]. Matrigel™ can be used *in vitro* to mimic the *in vivo* environment. When ES cells are grown on Matrigel™ *in vitro*, they form polarised columnar epithelial-like structures, similar to those formed by ES cells *in vivo*, whereas, without Matrigel™ these structures are not observed [191].

Matrigel™ is made up of a combination of both ECM proteins and growth factors (Table 1.1). An alternative to Matrigel™ is Matrigel™ reduced growth factors (RGF), this has a similar ECM protein composition to Matrigel™ but has a reduced concentration of growth factors (Table 1.1). Matrigel™ is liquid at 4°C and forms a gel as the temperature is increased to 37°C [192] and can subsequently be denatured at higher temperatures [192]. This provides a 3D matrix that will present ECM molecules to cells along with encapsulated growth factors. An alternative basement membrane extract to Matrigel™ is Cultrex basement membrane extract (BME)® (produced by Trevigen), this is derived from the same source and is claimed to contain laminin, collagen IV, entactin and heparan sulphate proteoglycan. Although Cultrex BME® is acknowledged as being similar to Matrigel™ [193] there is little published information on Cultrex BME® (http://www.trevigen.com/) as at 5 November 2008.

	Parameter	Matrigel™ Percentage/concentration	Matrigel™ RGF Percentage/concentration
ECM PROTEINS	Laminin	56%	61%
	Collagen IV	31%	30%
	Entactin	8%	7%
GROWTH FACTORS	bFGF	0-0.1 pg/ml	0-0.1 pg/ml
	EGF	0.5-1.3 ng/ml	<0.5 ng/ml
	IGF-1	15.6 ng/ml	5 ng/ml
	PDGF	12 pg/ml	<5 pg/ml
	NGF	<0.2 ng/ml	<0.2 ng/ml
	TGF-β	2.3 ng/ml	1.7 ng/ml

Table 1.1 The main constituents of Matrigel™ and Matrigel™ RGF. Matrigel™ is made up of a variety of ECM proteins and growth factors. Table shows approximate mean quantities for each component. Matrigel™ contains the following growth factors: basic fibroblast growth factor (bFGF); epidermal growth factor (EGF); insulin-like growth factor-1 (IGF-1); platelet-derived growth factor (PDGF); nerve growth factor (NGF); and transforming growth factor beta (TGF-β). Note: The total percentage of ECM proteins does not total 100% due to some unknown constituents and batch to batch variability. Data correct as of 26.2.08, from: http://www.bdbiosciences.com/discovery_labware/products/display_product.php?keyID=230

It is known that the culture of cells on surfaces coated with individual ECM proteins is not as effective as their culture on surfaces coated with multiple ECM components. MSCs induced to differentiate, when cultured on collagen I, laminin or fibronectin behave similarly. In contrast, when MSCs are induced to differentiate on surfaces presenting multiple ECM components (Matrigel™) their differentiation is enhanced, with a greater percentage of cells differentiating and a greater neurite branching [194].

It is possible to obtain basement membrane extracts from cells. PYS-2 cells can be cultured *in vitro* and subsequently lysed, leaving their ECM attached to a surface. The ECM produced by PYS-2 cells consists of laminin, collagen IV and heparan

sulphate proteoglycan [195]. When investigating the proliferation of MSCs, the ECM prepared from PYS-2 cells performed better than, laminin or collagen IV when adsorbed to a surface. This is not surprising as the ECM from PYS-2 cells will contain a mixture of molecules and not just one single molecule. However, when compared to Matrigel™, which does contain multiple molecules the ECM prepared form PYS-2 cells performed better at increasing proliferation, the authors suggested that either some activity was lost from Matrigel™ during isolation or that an intact structure is needed [196]. However, as the ECMs were prepared from different sources it is also possible that there were some differences in the composition of the two ECMs.

When attempting to mimic cell behaviour *in vitro*, it is important to consider the *in situ* environment in which cells are normally found. When cells are grown *in vitro* with factors such as ECM proteins that they do not normally come into contact with, their development is abnormal for example, when keratinocytes are grown with collagen IV, found in the epidermal basement membrane (BM) their development is more representative of normal keratinocyte development. In contrast, when cells are grown with ECM proteins not found in the BM such as fibronectin or collagen I their behaviour is abnormal [197].

1.5.4 The identification of peptides motifs within ECM proteins

The idea that cell attachment to proteins does not require the whole molecule to elicit an effect started with the observation that proteolytic fragments of fibronectin are active in promoting cell attachment [198]. From the primary sequence of fibronectin [199] there have been small peptides identified that competitively inhibit cell attachment to fibronectin of which, the most studied are RGDS [200] and PHSRN [113]. The cell receptor for RGD was identified by coupling RGD from fibronectin Sepharose and passing detergent extracts from huma placenta or cultured cells over the column, bound molecules were then eluted using soluble RGD [201]. The identification of the fibronectin receptor was first published in 1983 [202]. The receptor identified was later fully defined as the $\alpha 5\beta 1$ integrin.

Clarification that the effects of ECM proteins are mediated by peptides is gained from inhibition experiments. Surfaces coated with laminin increase the attachment of small cell lung cancer cells in a dose dependant manner. Preincubation of the cells with cyclic YIGSR peptide decreases the percentage of cells that attach to laminin coated surfaces, this too is in a dose dependant manner [203]. This indicates that the peptide YIGSR can inhibit the attachment of cells to laminin and therefore the effects of laminin must be mediated thorough the peptide YIGSR.

Peptide motifs are frequently studied individually, however proteins have numerous peptide motifs. Direct evidence that proteins have multiple motifs comes from experiments where PC12 cells cultured on laminin are only partly inhibited for neurite outgrowth by anti-PA22-2 (a peptide containing the laminin sequence IKVAV) whereas cells cultured on PA22-2 immobilised surfaces are completely inhibited by anti-PA22-2 thus there must be other binding sites in laminin [104]. Laminin has three motifs that have been greatly investigated, YIGSR, IKVAV and RGD. However even these are an over simplification, for example, a series of peptides of laminin-1 have been synthesised 23 of which have been found to be bioactive [204].

RGD and PHSRN motifs, found in fibronectin, are two peptides which have been thoroughly investigated. When presented together these peptides are known to have a synergistic effect. When in their natural presentation in fibronectin there is a spacer between the two motifs which means they are not presented together, this spacer has been found to be important in the efficacy of the motifs. The presence of a spacer unit increases the adhesion of HUVEC cells, when the spacer is removed or the length of this spacer is increased these changes result in a decrease in cell attachment. The hydrophilic/hydrophobic properties of the spacer unit are also important, a neutral spacer unit has been shown to result in increased cell attachment when compared to hydrophilic or hydrophobic spacers [205].

The most fully investigated motifs are those from laminin and fibronectin, fewer studies have investigated the effects of collagen motifs. Collagen is frequently

investigated for its ability to increase cell attachment. In the α-chain of collagen I there is a domain that induces cell attachment which is made up of 15 amino acids and is designated P-15, the GTPGPQGIAGQRGVV motif is the motif in this domain which confers the observed effects. The immobilisation of P-15 has been found to increase cell attachment and viability of a variety of different cell types. The immobilisation of P-15 increases cell attachment of fibroblasts [206]. Fibroblasts grown on an anorganic bone mineral material without the adsorption of P-15 show a greater reduction in cell viability following serum withdrawal than fibroblasts grown on an anorganic bone material with adsorption of P-15 [207]. Particles coated with P-15 and embedded in a hydrogel have been shown to increase cell attachment and the viability of osteoblastic cells [90]. The collagen IV peptide MNYYSNS is rarely investigated. This peptide is found in the NC1 domain of the α3chain, one study has shown that when added as a soluble peptide it significantly inhibits both the attachment and migration of melanoma cells [88].

1.5.5 The advantages of using peptides from extracellular matrix proteins to control cell behaviour

Although proteins will adsorb spontaneously onto a surface there are many limitations: the surface coating process is difficult to control; adsorption may lead to the protein becoming denatured resulting in reduced functionality; the orientation; and density of the protein are difficult to control leading to batch inconsistency and reduced reproducibility. The method of incorporation; such as adsorption, covalently binding or blending at the time of surface manufacture, can affect the amount of protein presented by the surface and in turn, these result in different responses of cells cultured on the surfaces [208]. Covalent attachment of protein by chemical coupling also results in substantial loss of protein activity owing to chemical modification of critical residues, denaturation and random orientation and inaccessibility [209]. Current methods result in surfaces that are highly defective in their presentation of molecules. There are patches where no molecules have attached and the surface is exposed to interactions with cells. Molecular deformation is observed [210,211], resulting in molecules becoming

attached to the surface. However, they exhibit decreased effects due to a conformational change reducing the availability of the binding sites [212]. In addition, aggregation leads to multiple layers of molecules, having two effects: it reduces the numbers of molecules available to attach to the surface hence reducing the activity of the surface; and it can result in lower levels not being accessible [213].

There is evidence to suggest the underlying material affects cellular behaviour, for instance CNS neurons grown on laminin coated platinum do not form glial cell types following 3 days culture, whereas cells grown on laminin coated tantalum do form glial cell types [214]. It was not demonstrated whether the effects observed were due to differences in adsorption of laminin or interactions of cells with the underlying surface however it is likely that both of these factors will contribute to the observed effects.

The conformation and exposure of active motifs by adhesive proteins can be controlled by other non-adhesive proteins on a surface. Fibronectin at a low concentration alone produces a low level of breast carcinoma cell attachment but when surfaces are coated with fibronectin and a protein that does not increase adhesion, osteonectin, adhesion to the surface is significantly increased. The presence of osteonectin on a surface in combination exposes more of the fibronectin RGD motifs thus increasing cell attachment [215].

Methodologies have been developed in an attempt try and produce surfaces in a more defined manner, for example, the immobilisation of whole proteins such as fibronectin to functionalised gold surfaces has been studied to produce a monolayer without molecular aggregation taking place [216]. An ideal system would produce surfaces that are fully intact with no areas of unreacted molecules to ensure that all reactions observed are due to the molecules attached to the surface and not interaction between cells and the uncoated surface. All the molecules should retain their active conformation and be presented in the correct orientation for their active domains to be accessible by cells. Molecular aggregation should be avoided on the surface so that all the attached molecules are active.

1.5.6 Presentation of peptides affects their effectiveness

Although presentation of peptide motifs does offer advantages over whole protein molecules, there are many considerations that must be made to ensure optimal activity of the motif. Concentration has been shown to be important when investigating cell attachment; as the concentration of immobilised YISGR peptide is increased an increase in cell attachment is seen [100]. Motifs have certain residues that are essential for their activity, for example, replacement of the R residue in PHSRN results in a significant decrease its cell attachment activity [113]. Replacing I from IKVAV results in decreased cell attachment and neurite outgrowth of PC12 cells [103]. Furthermore, modification of the laminin motif YIGSR has been shown to affect its ability to increase PC12 cell adhesion, if Y is removed (or modified) or R changed this decreases the cell attachment of PC12 cells however, this had no detractive effect on the peptide's ability to promote neurite outgrowth [100]. Some motifs, such as RGD, are found in many different molecules, for example RGDS is found in fibronectin whereas RGD is found in laminin, it has been shown that the S residue is not essential for the motifs function [217,218]. Although, one group showed that adding S to RGD increases its effect in controlling cell attachment [219]. The conformation of the motif has been shown to have no effect on its efficacy. The all-$_L$- and all- $_D$- conformations of the laminin peptide motif IKVAV have similar effects on PC12 cells attachment [220] and neurite outgrowth [103]. The charge of the motif can affects its activity as addition of a NH_2 group to the end of the YIGSR [100] or RGD [219] peptides increases the activity of the peptide through removal of the peptides negative charge. The flanking sequences of a peptide are not vital to the activity of the peptide as when the motif IKVAV is inserted into equivalent regions of mouse α2 (ANSIKVSVGGGG) or drosophila a-chain (ANSIKVGVNFKP) (note there is a slight residue change but this has not effect as I is the critical residue) a similar response of PC12 cells to cell attachment and neurite outgrowth is noted [103].

The concentration of the peptide has an effect on the surface behaviour, increasing the concentration of the RGD peptide facilitates adhesion of cells [221]. It is possible

to synthesise peptides with repeating units, for example YIGSR can be synthesised in a single peptide or with 16 repeating units. Surfaces presenting repeating units of YIGSR have greater cell attachment than surfaces presenting single YIGSR units [222].

The primary structure of the motif is important in maintaining its activity, for example, a reverse sequence would be AASIKVAVSADR vs. RDASVAVKISAA. When compared the reverse sequence does not increase cell attachment whereas the forward sequence does [103]. In agreement with this Graf et al. (1987) showed that reversing the RGD sequence results in reduced activity [100]. Conflicting reports have been made for whether peptides show greater functionality presented in a linear or cyclic form. Studies have shown that different cell types prefer different presentation methods, for example smooth muscle cells have a greater cell attachment when RGDS is presented in a cyclic fashion conversely 3T3 fibroblast adhesion is greater when RGDS is presented linearly. Additionally, it was found that spreading of 3T3 fibroblast was similar for both linear and cyclic motifs [223]. When RGDS is presented as a soluble linear peptide, it inhibits cell attachment to fibronectin and vitronectin. In comparison, when presented in a cyclic peptide, RGDS has an increased ability to inhibit cell attachment to vitronectin whereas it has a decreased ability to inhibit cell attachment to fibronectin [219].

There exist many contradicting reports regarding which conformation of peptide results in the greatest effect, demonstrating that peptide presentation is cell type specific and also functionally specific. Furthermore, it emphasises the need for the presentation of peptides in a defined and controllable manner.

1.5.7 Current methods for peptide presentation

There are numerous methods by which peptide motifs can be immobilised onto a surface [224] including: direct adsorption of protein from solution [225]; modifying the molecular characteristics of the surface using complex chemistry [226]; and active site directed immobilisation [227,228]. However, these approaches have their

limitations: the surface coating process is difficult to control; adsorption may lead to the protein becoming denatured resulting in reduced functionality; the orientation and density of the protein are difficult to control leading to batch inconsistency and reduced reproducibility. Covalent attachment of protein by chemical coupling also results in substantial loss of protein activity owing to chemical modification of critical residues, denaturation and random orientation and inaccessibility [209]. Current methods result in surfaces that are highly defective in their presentation of molecules. There are patches where no molecules have attached and the surface is exposed to interactions with cells. Molecular deformation is observed [210,211], resulting in molecules becoming attached to the surface however they exhibit decreased effects due to a conformational change reducing the availability of the binding sites [212]. In addition, aggregation leads to multiple layers of molecules with the lower levels not being accessible by the cultured cells [213]. Molecules can also become attached to the surface in the incorrect orientation resulting in the active peptide not being accessible at the extremity of the surface. Molecular aggregation can result in two or more molecules joining together, this has two effects, firstly it reduces the numbers of molecules available to attach to the surface hence reducing the activity of the surface, and secondly it can result in the active domain of the molecules becoming hidden and therefore inactivating the molecule. An ideal system would produce surfaces that are fully intact with no areas of unreacted molecules so that all reactions observed are due to the molecules attached to the surface and not interaction between cells and the uncoated surface. All the molecules should retain their active conformation and be presented in the correct orientation for their active domains to be accessible by cells. Molecular aggregation should be avoided on the surface so that all the attached molecules are active (Figure 1.9).

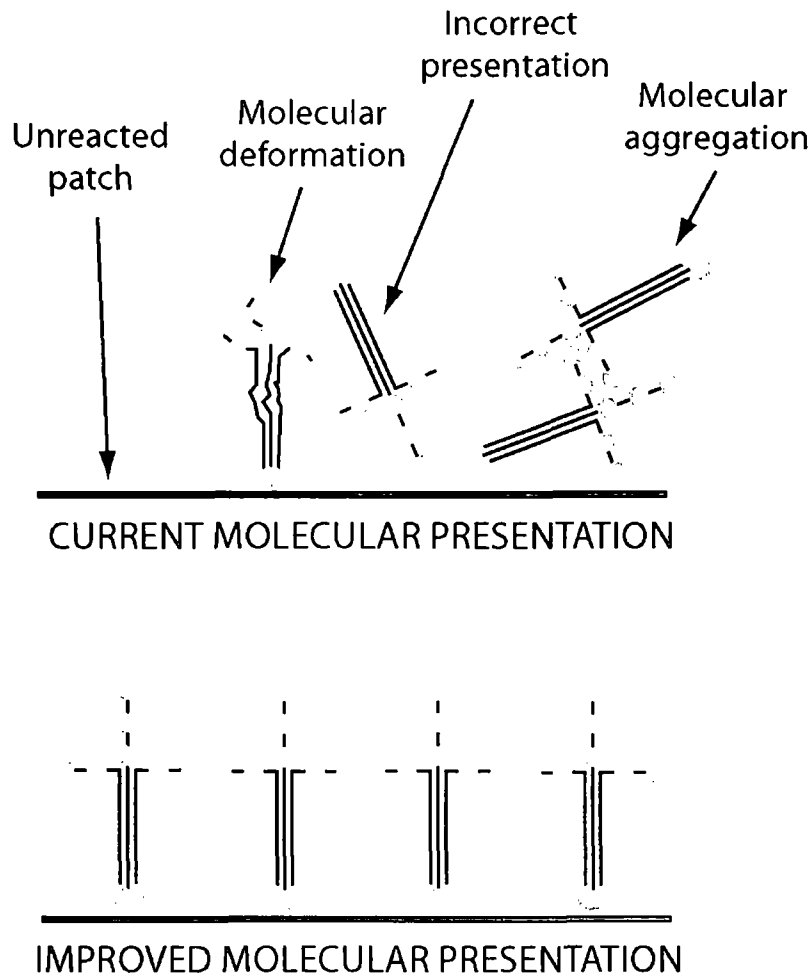

Figure 1.9 The defects in surfaces prepared using current technologies. There are a number of possible shortcomings of current methods for the attachment of molecules to surfaces (upper diagram). Unreacted patches, molecular deformation, incorrect presentation and molecular aggregation are all possible reasons for surfaces with low activity being produced. An improved molecular presentation (lower diagram) would be where the layer is intact and all of the molecules presenting in a defined orientation with their activity being maintained.

1.5.8 Self assembled monolayers – a more defined process

Self assembled monolayers (SAMs) are organic surfaces that form spontaneously from the adsorption of molecules either from gas or liquid phase to a metallic surface. SAMs are similar to biological membranes and present molecules as a

membrane would to other cells. They can be formed from molecules, such as alkanethiols. The reaction is spontaneous as the absorption of the molecules lowers the free energy of the metal substrate. SAMs have the general structure of a spacer linked to the metallic surface via a ligand with a molecule of interest attached to the surface. The spacer prevents non specific reactions of the cells with the base surface. SAMs can be formed on a number of different metals, including copper, silver, mercury and palladium. However as gold is by far the most extensively studied and most existing technologies are compatible with gold this is at present the preferred metal substrate for the formation of SAMs [224].

1.5.9 The Orla approach

In this study, we have adapted an existing technology that enables the stable self-assembly of proteins on surfaces [229] to develop a novel tool for applications in cell culture (Figure 1.10). The technology employs engineered variants of the N-terminal transmembrane (TM) domain from the Escherichia coli outer membrane protein, OmpA [230]. The motifs from ECM proteins are engineered into the flexible outer loops of OmpA. The protein attaches to the cell culture surface via a single cysteine residue thereby forming an oriented monolayer. Gaps between protein molecules are filled in using thiolipids or thioalkanes so that only the surface loops and the motifs of interest are exposed. This method of protein immobilisation results in stable, oriented monolayers that display only the motifs of interest in a functional conformation [230] and alleviates many of the problems associated with adsorption and chemical methods [231].

β-barrel proteins are very stable structures and here the bacteria Escherichia coli (E.coli) outer membrane protein A (OmpA) forms the base of this technology. OmpA is a highly stable β-barrel protein that has a 16-stranded conformation that is resistant to: protease; urea; guanidine hydrochloride; sodium dodecyl sulphate (SDS); and heat denaturation. The high stability of this protein makes it ideal to act as a scaffold. OmpA has long variable extracellular loops, ideal for engineering and short periplasmic turns that are suitable for interaction with a gold

surface. OmpA can be modified and purified with the insertion of a cysteine residue (E183C) at the fifth periplasmic turn, in a similar manner to other mutants [232,233]. It is this introduced cysteine residue that allows attachment to the gold surface via sulphur gold bond [234]. Correct insertion is judged by the ability to form trimers and its insolubility at low detergent concentrations. Self assembly onto the gold surface is achieved by samples of freshly reduced detergent-solubilised trimeric OmpA-E183C. Synthetic thiolipids containing a sulphur bearing head group and a phospholipid are then added sequentially to fill in the gaps between OmpA molecules and build up the bilayer. The infill molecule properties influence the final property of the SAM, with hydrophobic molecules preventing cell attachment and hydrophilic ones promoting attachment [235,236]. It is possible to present two peptide motifs within one OmpA molecule. Fibronectin has two well studied domains, PHSRN and RGDS, which are known to act synergistically. The spacing between these two molecules is known to be a key factor in their efficacy, this can be mimicked by the addition of a spacer [237]. This distance is also mimicked by the presentation of these two motifs in the OmpA molecule Orla 35 (Table 1.2).

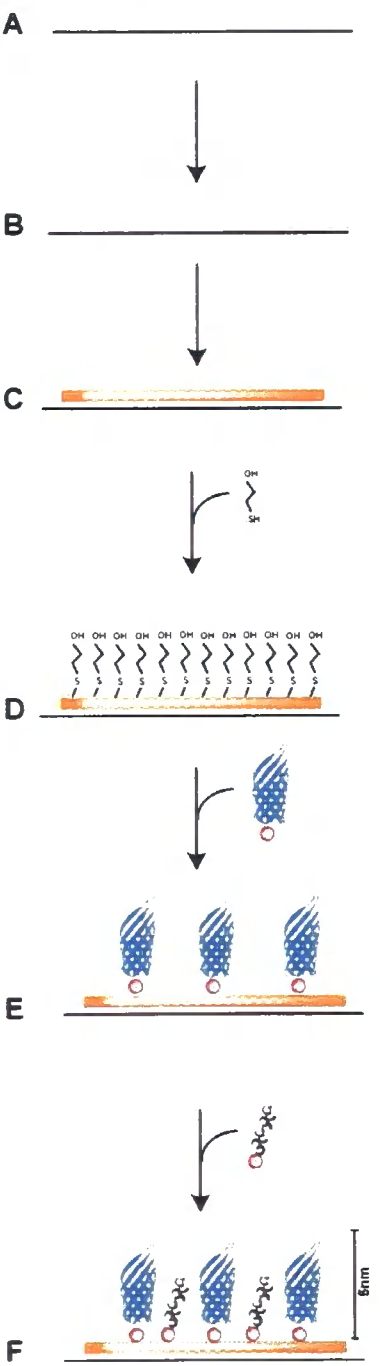

Figure 1.10 Schematic showing assembly of Orla surfaces. Surfaces are assembled in the following manner A) Glass coverslips are first cleaned to remove any residues. B) Coverslips are coated with titanium to allow the attachment of gold. C) Gold is spluttered onto the surface. D) The surface is passivated by β-mercaptoethanol to facilitate the attachment of OmpA. E) OmpA is allowed to self assemble on the surface in a controlled fashion due to the modified cysteine residue (red). F) Infill molecules are allowed to self assemble on the surface and fill the gaps between OmpA molecules to form an intact monolayer.

1.6 Stem cell therapy using the ECM

ES cells provide an unlimited source of cells and hence could potentially be used to produce an unlimited supply of skin. Experiments using murine ES cells demonstrate that the combination of surface coating, soluble molecules and culture conditions are key in the differentiation of ES cells into skin. Using surfaces coated with ECM secreted by primary human normal fibroblasts, with the addition of ascorbic acid, ES cells can be induced to differentiate into keratinoctyes. With the correct culture conditions of a liquid air interface, these ES cell derived keratinocytes can from a highly organised structure resembling skin. Although there would remain some undifferentiated, unorganised regions and hence, more knowledge is needed if this is to be used to produce skin for engraftment [189].

It is possible for the cornea to become damaged due to diseases such as Stevens-Johnson syndrome or severe microbial infection furthermore it is possible to damage the cornea by chemical or thermal burns resulting in the loss of corneal and limbal epithelial cells. This could cause loss of visual acuity and as such a method of removing damaged cells and replacing with new ones would be favourable.

The corneal epithelium is maintained by a population of stem cells, termed limbal stem cells (LSC), located in the basal layer of the limbal epithelium. It follows that if the factors contained within this niche were recreated *in vitro* this would form a favourable environment for the production of corneal epithelial cells. The basement membrane of the limbal epithelium is composed of collagen, laminin and fibronectin [238] furthermore it is known that during wounding the content of fibronectin within the ECM increases [239,240]. It has been demonstrated that murine ESC can be cultured on collagen IV coated surfaces to direct them towards corneal epithelial-like cells. Upon transplantation onto a damaged cornea the transplanted cells assume a corneal epithelial cell-like morphology.

In addition it is known that cells from the ocular surface produce a myriad of cytokines that control cell behaviour [241]. By combining factors produced by limbal fibroblasts and coating surfaces with collagen IV it has been demonstrated that human ESC can be differentiated to form corneal epithelial-like cells [242].

1.7 Current understanding shown within the literature

Currently there are a large number of proposed uses of stem cells for the cure of diseases. However, in the literature there is a significant lack of understanding of how stem cell behaviour is affected and controlled. Transplantation of stem cells into immunocompromised animals is widely used as a model system to study cell potency. In addition to the study of cell potency, the teratoma model provides a method to study stem cell behaviour with multiple factors acting together to direct and control stem cell fate.

In the literature there have been numerous different locations investigated for the transplantation of stem cells to study stem cell behaviour, however one site that has not been fully investigated is the liver. This site has a known regenerative capacity that would be predicted to provide an ideal environment for stem cell growth. This would provide an alternative environment for the control of stem cell behaviour. From *in vivo* studies, factors controlling stem cell behaviour are predicted, such as the ECM.

Expanding upon *in vivo* studies it is possible co-inject cells with ECM components to study how this affects their behaviour. The most extensively studied ECM component for transplantation is Matrigel™. Matrigel™ has been studied for a number of different applications. However, few studies have applied it to the teratoma model. Matrigel™ is a complex mixture of ECM components made up of both ECM proteins and growth factors. To date, no studies have investigated how the individual ECM proteins that make up Matrigel™ affect stem cell behaviour in the teratoma model. By studying these individual components this will allow a better understanding of how the ECM affects stem cell behaviour *in*

vivo. Having demonstrated that the ECM affects cell behaviour it is possible to investigate the effects of peptide motifs on cells.

In the literature, there are many examples of methods to present peptides to control cells. What is lacking is the study of how peptide motifs when presented in a defined and reproducible manner affect cell behaviour. By studying cell behaviour in the response to the ECM this will allow a greater understanding of how to control stem cell behaviour.

Surface name	Motif	Source
OmpA	-	-
Orla 1	RGDS	Fibronectin
Orla 11	IKVAV	Laminin
Orla 30	VFDNFVKL	Tenascin C
Orla 31	GTPGPQGIAGQRGVV	Collagen I
Orla 32	MNYYSNS	Collagen IV
Orla 34	PHSRN	Fibronectin
Orla 35	PHSRN+RGD	Fibronectin
Orla 36	YIGSR	Laminin
Orla 37	YIGSR+IKVAV	Laminin
MOS1	Collagen I Collagen IV	GTPGPQGIAGQRGVV MNYYSNS
MOS2	Fibronectin Laminin	RGDS YIGSR
MOS3	Fibronectin Collagen I Collagen IV	RGDS GTPGPQGIAGQRGVV MNYYSNS
MOS4	Fibronectin Collagen I Laminin	RGDS GTPGPQGIAGQRGVV YIGSR
MOS5	Fibronectin Collagen I Collagen IV Fibronectin Laminin	RGDS GTPGPQGIAGQRGVV MNYYSNS PHSRN YIGSR

Table 1.2 Biomimetic surfaces tested. Table shows nomenclature of biomimetic surface, motifs presented and source of motifs. Where + is shown this denotes two motifs are presented in a single OmpA molecule. Mixed Orla Surfaces (MOS) are composed of multiple OmpA molecules each presenting one single motif.

CHAPTER 2 - MODIFYING THE *IN VIVO* ENVIRONMENT TO MODULATE TERATOMA DEVELOPMENT

2.1 Introduction

The study of stem cell behaviour is important as stem cells are believed to hold the answer to both curing multiple diseases and gaining a greater understanding of the mechanisms that control development. Stem cell behaviour can be studied both *in vitro* and *in vivo*. *In vitro* study provides a simplified system where all of the environmental factors affecting cell behaviour can be controlled. In comparison, the *in vivo* environment is highly complex and less controllable where multiple factors such as: the ECM; physical forces; soluble molecules; 3D shape; and cell-cell interactions all act simultaneously to control stem cell behaviour. Although *in vivo* study is less controllable than *in vitro* studies it provides a method to investigate stem cell behaviour in an environment that is not artificial and representative of their natural environment.

To investigate stem cell behaviour, undifferentiated EC stem cells and ES cells can be transplanted into immunocompromised animals. Following transplantation, spontaneous differentiation occurs and a mass of unorganised differentiated cell types are formed – a teratoma. The main application of teratoma formation is to demonstrate the potential of the transplanted cells. This is achieved by transplanting stem cells into an immunocompromised animal (usually a mouse), allowing a teratoma to form then subsequently studying the composition of the teratoma and cell types contained within [25,35,66,243-246]. Given that this method is widely used to validate stem cell potency relatively little is known about how these unique structures develop and what controls their formation.

Although teratoma formation and analysis is seen as the gold standard to assess potency, inconsistencies exist in the method by which this assay is conducted. Previous studies have demonstrated that the anatomical location in which cells are transplanted affects their behaviour [67,69]. The anatomical location in which stem cells have been transplanted varies and can include: muscle [25 246 247]; testis [35,243];

kidney capsule [37,243,248]; or subcutaneous [67,69 249]. One factor that could influence the transplanted cells and affect their behaviour is the physical environment, for example, cells transplanted into the muscle will be subjected to continual stretching and contractions whereas cells transplanted into other sites such as the testis will not be subjected to such forces. *In vitro*, stretching of cells is used to produce muscle tissue from stem cells [250] and it is likely that the physical condition of the muscle will affect the developmental potential and differentiation of the transplanted stem cells.

Routinely, all of the above mentioned anatomical sites are used for the transplantation of stem cells. An alternative transplantation site for teratoma formation that has not been investigated is the liver. This provides an environment that is: not subject to physical strain unlike the muscle; has a high blood supply unlike the skin; and is rich in nutrients. The liver is known to be a highly regenerative organ and therefore is likely to contain the correct factors for the expansion of the transplanted cell population. Studying this location will allow factors to be proposed that can control cell behaviour.

In vitro, extracellular matrix proteins have been shown to affect stem cell behaviour, laminin, collagen IV and fibronectin have all been shown to be important in EC stem cell differentiation and the levels of secretion have been found to change during differentiation [179]. Within different anatomical locations a major component of the environment is the ECM, which contains: collagens; fibronectins; and laminins. Collagen is a major part of the ECM within the liver making up 80%[251 252]. The liver is also known to be rich in the glycoproteins, fibronectin and laminin [253]. Collagen I is also important in the skin as, 70-80% of the dry weight of the dermis is collagen I [254]. Fibronectin is also found in the dermis of the skin [255]. Hence, i*n vivo*, interactions of cells with the ECM could be key in the control of cellular behaviour.

To investigate how the ECM affects cell behaviour and teratoma development *in vivo,* stem cells can be co-injected with ECM components into immunocompromised animals to modulate teratoma formation. To date, few studies have been conducted using co-transplantation of ECM molecules and stem

cells; however, this technique has been used for tumour cells. One studied factor that has been used with tumour cells is an ECM extract - Matrigel™. Matrigel™ is a mixture of collagen IV, laminin, heparan sulphate, proteoglycan, entactin, nidogen and various growth factors (Table 1.2) that form a solid gel at 37°C and therefore when injected into a body a gel will form [192]. Co-transplantation of prostatic, breast and ovarian carcinoma cells with Matrigel™ has been shown to significantly increase the rate of tumour formation of these cells which do not readily form tumours when Matrigel™ is not present [256 257]. Furthermore the effects of Matrigel can in part be reduced with the co-injection of laminin peptide YIGSR thus implementing laminin as a key component [203].

In addition to Matrigel™, collagen I has also been co-injected to aid tumour growth [203,258], the effects observed are not as pronounced as with Matrigel™ and the effects are not observed in all cells. Matrigel™ is a complex mixture of multiple molecules and it would be expected that individual ECM proteins such as collagen I will not be as effective as multiple ECM proteins. When Matrigel™ is co-injected an increase in tumour size is observed. Potential factors contributing to the increased tumour size could be due to the observation of a decreased necrosis and increased vascularisation of tumours. Matrigel™ contains a wide number of growth factors, with the removal of these growth factors beneficial effects are still observed on tumour formation, thus indicating that the ECM components do effect tumour development [258].

In a similar manner to that implemented with tumour cells, stem cells could be co-transplanted with various ECM molecules to influence cell fate and modulate teratoma development. It would be expected that Matrigel™ would be a potent factor in increasing the success rate of teratoma formation. As Matrigel™ is a mixture of different ECM proteins it provides a method for studying how complex interactions between multiple different factors influence teratoma growth *in vivo*. This experimental paradigm is highly complex and simplification of this system will allow for a greater interpretation of the interactions between stem cells and ECM components. Matrigel™ consists of both growth factors and ECM proteins; there are alternative versions of Matrigel™ available, such as a version with reduced levels of growth factors (EGF, IGF-1, PDGF and TGF-beta). Co-

transplantation of Matrigel™ with reduced growth factors with stem cells will allow specific analysis of how the ECM affects stem cells in the absence of any effects caused by the growth factors present. Matrigel™ with reduced growth factors is a mixture of collagen, laminin and entactin and is presenting multiple different ECM proteins to the transplanted cells. To further simplify this system, stem cells can be co-transplanted with individual ECM proteins. In addition to collagen IV and laminin found in Matrigel™, other proteins that have been shown to influence cell behaviour *in vitro* include collagen I and fibronectin.

To further simplify this system, ECM mimics can be used. Extracel™-X is an ECM mimic composed of three components, thiol-modified hyaluronan (a constituent of the ECM), thiol-modified gelatin (hydrolysed collagen) and a thiol-reactive cross linker (polyethylene glycol diacrylate, PEGDA). Unlike Matrigel™, gelation is not achieved by a change in temperature but by time, dependent upon the relative quantities of cross linker added. The gel that is produced has a high water content of 97-98% giving a high permeability for oxygen and water soluble metabolites. *In vitro* cells are able to attach and gels are biocompatible *in vivo*. The biodegradability of the hydrogel can be controlled with the relative amounts of its constituents. Cells can be seeded into the hydrogels and injected subcutaneously for *in vivo* growth [259]. Extracel™-X has been used to investigate the growth of pancreatic adenocarcinoma cells transplanted into mice. Compared to cells transplanted without Extracel™-X, an increase in the time in which tumours are formed is observed. Extracel™-X increases the rate of tumour progression and subsequently the final tumour mass [260]. Extracel™-X has also been demonstrated to increase the rate of tumour formation and size of cancer cells transplanted into mice [261].

In addition to natural ECM mimics, synthetic materials have been developed to mimic the *in vivo* environment. PuraMatrix™ is a synthetic fiber made of 8-16 RAD peptide units that mimic RGD, the mutation of G to A has previously been demonstrated to not affect its activity [262]. Due to the repeating hydrophilic and hydrophobic units the peptides self-assemble into β-sheets and then into fibers with a diameter of 7-10nm. PuraMatrix™ forms a gel at 37°C, the resultant

hydrogel has a low peptide concentration (0.5-1%) and mimics the gels formed by collagen.

It has been noted that the success rate of teratoma formation can vary. Some studies have shown that failure of graft formation can be attributed to compromised viability of the transplanted cells [72], however the age of the host does not influence the teratoma [72 73]. Other studies have found that the efficiency of teratoma formation can be influenced by the site into which the cells are transplanted. Intramuscular transplantation had a success rate of 12.5% and testicular transplantation had a success rate of 60%. Also, modulating the local environment with the co-transplantation of the extracellular matrix component Matrigel™ has a dramatic increase in the rate of teratoma formation [69].

HYPOTHESIS

The anatomical site into which stem cells are transplanted are known to influence teratoma behaviour. Due to the known regenerative capacity of the liver, it is predicted that transplantation of EC stem cells and ES cells intrahepatically will produce large teratomas. As the ECM is known to be a key component of the *in vivo* environment, co-transplantation with different ECM components can be used to modulate teratoma development. Previously, Matrigel™ has been shown to increase the success rate of tumour formation, it is predicted that a similar trend will be observed for EC stem cells. Furthermore, co-transplantation with individual ECM proteins found in Matrigel™, such as collagen IV and laminin should influence teratoma success rate and behaviour similar to Matrigel™, although, the effects may be less pronounced. As well as ECM proteins found in Matrigel™, collagen I and fibronectin are tested for their abilities to influence teratoma behaviour. It would be expected that collagen I would influence the success rate of teratoma formation similar to collagen IV, however the effects on their development would be varied due to the different signaling mechanisms. Matrigel™ contains several growth factors using an alternative version with reduced growth factors, it would be expected that a reduction in teratoma growth rate would be observed. Two ECM mimics are used, Extracel™-X and PuraMatrix™. Extracel™-X contains collagen and it would be expected that its

effects on teratoma behaviour would be similar to collagen I or collagen IV. PuraMatrix™ contains RAD peptides that mimic the RGD motif found in collagen, fibronectin and laminin and would be expected to behave similar to these proteins, however as it is only presenting the peptide and not the whole protein, a reduction in the effects seen should be observed. It is predicted that the success rate of teratoma formation will be modulated depending upon the ECM component with which EC stem cells are co-transplanted. It would be expected that factors such as Matrigel™, collagen I and collagen IV, that form gels would increase teratoma success rate by encapsulating the cells and maintaining their viability.

AIM

Gain a greater understanding of teratoma development by presenting stem cells with a novel microenvironment to modulate the growth and development of the transplanted cells. In addition, co-transplantation of stem cells with ECM molecules will provide information on how the ECM regulates teratoma development.

OBJECTIVES

1. Transplant stem cells into the liver and subcutaneously to modulate cell growth and development using different microenvironments.

2. Co-transplant stem cells with ECM components to increase the success rate of teratoma formation and influence the cell types within.

2.2 Materials and Methods

Cell culture

The human EC stem line TERA2.cl.SP12 was maintained in DMEM (Lonza), 10% Foetal bovine serum (Invitrogen), 2 mM L-glutamine (Lonza), 20 units/mL of penicillin and 20 μg/mL streptomycin[263]. Cultures were passaged as required using acid cleaned glass beads. The hESC line, hES-NCL1 were grown and maintained in medium containing knockout-DMEM (Invitrogen), 100 μM β-mercaptoethanol (Sigma), 1 mM L-glutamine (Invitrogen), 100 mM nonessential amino acids; 10% serum replacement (Invitrogen), 1% penicillin-streptomycin (Invitrogen); and 4 ng/ml basic fibroblast growth factor (Invitrogen) [37].

Intrahepatic and subcutaneous cell transplantation

All procedures involving mice were conducted in accordance with guidelines and permission granted by the Institution and the Home Office (UK). Approximately $5x10^5$ cells were resuspended to a final volume of 100 μl. Using a 25G x 5/8" needle, cells were injected either beneath the skin (subcutaneous) or into the liver (intrahepatic) of adult male nude (nu/nu) mice. Each animal received one graft of either hESC or EC stem cells into one of the two locations. To validate the findings, this procedure was repeated 3-5 times in each site for each stem cell type. Mice were sacrificed after 3-12 weeks and a post-mortem examination was performed during which teratoma tissues were surgically removed.

Co-transplantation of ECM components with EC cells

The ECM components were prepared as follows: collagen I (Sigma) was dissolved in 0.1M acetic acid, chloroform sterilised and diluted to a concentration of 1mg/ml (double the final concentration required) then balanced to pH7.0 using sodium hydroxide; collagen IV (Sigma) was dissolved in 0.25% acetic acid, chloroform sterilised and diluted to a concentration of 1mg/ml (double the final concentration required) then balanced to pH7.0 using sodium hydroxide; fibronectin was used as

supplied (1mg/ml); laminin was used as supplied (1mg/ml); and Matrigel™ was used as supplied (lot concentration, ~9.8mg/ml). EC stem cells were trypsinised, resuspended in media, centrifuged and washed twice with PBS to remove any residual media. The cell number was determined and the required number were resuspended in half the final injection volume. The cell suspension was mixed with an equal volume of ECM component and loaded into a syringe with a 25G x 5/8" needle. Approximately $5x10^5$ cells were injected subcutaneously into adult male Nude (nu/nu) mice. Each animal received bilateral grafts of EC stem cells suspended in PBS and EC stem cells suspended in an ECM component. To validate the findings, this procedure was repeated 6 times for each ECM component. Injection of ECM components without any EC stem cells was carried out twice for each ECM component. Mice were sacrificed after 6-13 weeks and a post-mortem examination performed during which teratoma tissues were surgically removed.

Co-transplantation of ECM components and synthetic matrices with EC cells

Cultrex BME (AMS Biotechnology #3432-005-02, RGF #3433-005-02) was thawed from storage (-80°C) on ice at 4°C and a cell solution in PBS was added to dilute the stock concentrations of 17.3mg/ml and 14.2mg/ml to 10mg/ml. Cells were added to this solution and transplanted as previously described.

Extracel™-X (Glycosan biosystems) was prepared as described by the manufacturer. Glycosil™, Gelin-S™ and Extralink™ vials were removed from storage (-20°C) and thawed at 37°C for 30 minutes. DG water was removed from storage (-20°C) and thawed at 37°C for 15 minutes. Under aseptic conditions 2.0ml of DG water was added to both Glycosil™ and Gelin-S™ vials, these were mixed at 37°C for 30 minutes. 1.0ml of DG water was then added to the Extralink™ vial and inverted to dissolve. Equal volumes of Glycosil™ and Gelin-S™ were mixed, 10million cells were added to this solution (25µl cells:250µl Glycosil™, Gelin-S™ solution). To form the hydrogel, 250µl of cells suspended in Glycosil™ and Gelin-S™ was added to 1.0ml of Extralink™. From this

solution, 100µl of cells suspended in hydrogel were taken and loaded into a syringe and transplantation was carried out as previously described.

PuraMatrix™ (BD biosciences) was removed from storage (4°C) and sonicated to decrease its viscosity for 30 minutes in a water bath. Cells were harvested, centrifugated at 800 RPM for 2 minutes and resuspended in 100µl of filter sterilised 10% sucrose solution. Cells were then centrifugated at 800 RPM for 2 minutes, cell number was ascertained using a haemocytometer and this step repeated to produce a cell suspension of 2million cells in 100µl of 10% sucrose solution. 100µl of PuraMatrix™ was added to the cell solution, gentle pipetting was used to mix the two solutions. To initiate gelation, 200µl of PBS 1x was added to the cells/PuraMatrix™ suspension. Syringes were loaded with 100µl of this solution using a 20G needle, switched to a 30G needle and transplantation was carried out as previously described.

Tissue processing

Specimens were preserved in Bouin's fixative (70% (v/v) saturated picric acid (Sigma); 25% (v/v) formaldehyde (37%/40%, Sigma); and 5% (v/v) glacial acetic acid (Sigma)) at room temperature overnight or in 4% paraformaldehyde (4% PFA (Sigma) in phosphate buffered saline (PBS, Sigma)) overnight at 4°C. Fixed tissues were dehydrated through a series of progressively concentrated alcohol washes of 70%, 80%, 90% and 95% for two hours each and 100% dry alcohol for 24 hours. Samples were cleared in Histoclear for 24 hours, incubated at 60°C in Histoclear with wax (1:1); samples were incubated at 60°C in wax for 48 hours and embedded. Samples were sectioned to 6µm and mounted onto electrostatically charged slides. All samples were de-waxed and rehydrated for histology and immunocytochemical analysis.

Histology

Tissues fixed in Bouin's fixative were stained with either Haematoxylin and Eosin (H&E) or Weigert's Haematoxylin (Weigert's) using routine methods.

Haematoxylin and Eosin

Samples were de-waxed in Histoclear for 6 minutes and rehydrated using, 100%, 95% and 70% alcohol for 2 minutes each and washed in dH_2O for 2 minutes. Nuclear staining was achieved using Mayer's Haemalum (RALamb) for 6 minutes, which was washed in dH_2O and blued using alkaline alcohol (3ml ammonia in 100ml 70% alcohol) for 15 seconds. Samples were washed in dH_2O, dehydrated in 70% and 95% alcohols for 1 minute each and cellular staining was achieved using Eosin (RALamb) for 10 seconds, sections were finally wash in 95% alcohol and 100% alcohol for 25 seconds each, Histoclear for 4 minutes and mount in DPX.

Weigert's Haematoxylin

Samples were de-waxed in Histoclear for 6 minutes and rehydrated using, 100%, 95% and 70% alcohol for 2 minutes each and wash for 2 minutes in dH_2O. Cartilage was stained with alcian blue (1g alcian blue, 3ml glacial acetic acid, 100ml dH2O, stirred overnight) for 6 minutes and washed in dH_2O. Cellular staining was achieved using Weigert's Haematoxylin (1g Haematoxylin in 100ml 100% alcohol mixed 1:1 with 4ml 30% $FeCl_3$ + 1ml concentrated HCl + 95ml dH_2O) for 20 minutes, sections were washed in 50% alcohol and dipped in 1% acid alcohol (1ml concentrated HCl + 99ml 70% alcohol) and then sections were taken to water using 70% alcohol for 1 minute and dH2O, bone was stained using Ponceau S (10ml 1% Ponceau S (1g Ponceau S in 100ml dH_2O) + 1.2ml glacial acetic acid + 90ml saturated aqueous picric acid) for 10 seconds, sections were washed in 95% alcohol for 10 seconds and 100% alcohol for 1 minute, Histoclear 4 minutes, mounted in DPX.

Immunohistochemistry (fluorescence)

Tissues fixed in PFA, de-waxed and taken to water as previously described for haematoxylin and eosin staining and then processed for antigen retrieval by microwaving (800W) tissues in 10mM citrate buffer (stock solution: 19.2g anhydrous citric acid in 1000ml dH_2O (0.1M) pH6, Sigma) for 3x 2 minutes.

Sections were left standing in warm citrate buffer for 20 minutes and subsequently washed 3x 10 minutes in PBS. Sections were permeablilsed for 15 minutes (1% Triton X-100 (Fisher) PBS solution) and blocked for 15 minutes (5% normal goat serum (NGS, Invitrogen), 0.1% Triton X-100, PBS). Sections were incubated with the following primary antibodies: Stage-specific embryonic antigen-3 (SSEA3), 1:4, (gift from Peter Andrews); pan epithelial keratin, 1:200, (Chemicon International; http://www.chemicon.com); Nestin, 1:200, (Chemicon); Neurofilament 200kD (NF200), 1:200 (Sigma); and TRA-1-60, 1:4, (gift from Peter Andrews) in PBS containing 1% bovine serum albumin (Sigma), 0.2% Triton-X-100 (Sigma) and 5% normal goat serum (NGS, Sigma) over night at 4°C prior to washing 3x 2 minutes in PBS and incubation with fluorescence-conjugated secondary antibodies (For SSEA3 and TRA-1-60: mouse IgM, 1:200 (Sigma); for Nestin and Pan Epithelial keratin: mouse IgG, 1:200 (Sigma); For NF200: rabbit IgG, 1:400 (Sigma)) for 1 hour at room temperature. Immuno-stained tissue sections were subsequently washed 3x 10 minutes in PBS, mounted using Vectashield® (Vector Labs), examined by fluorescence microscopy and digital images were taken.

Immunohistochemistry (peroxidase)

Sections were cleared, rehydrated and antigen retrieval carried out as previously described for fluorescence immunohistochemistry. Endogenous Avidin/Biotin activity was blocked using a blocking kit (Vector Labs) and permeabilisation and blocking carried out as described for fluorescence immunohistochemistry. Sections were incubated with the following primary antibodies; Ki67 (1:200 (Abcam)), human nuclear antigen (1:25 (Chemicon)) and CDX2 (1:50 (Novocastra)). Negative controls were carried out with the omission of the primary antibody. To detect the primary antibodies, a universal ABC detection kit was used (Vector labs). Sections were incubated for 30 minutes with a biotinylated secondary antibody and washed for 5 minutes in PBS. To detect the secondary antibody, sections were incubated for 30 minutes with Vectastain® Elite ABC reagent (Vector Labs) and then washed for 5 minutes in PBS. To visualise the location of the primary antibody, sections were incubated in a purple coloured Vector VIP peroxidase substrate (Vector Labs). Sections were briefly counter

stained using Mayer's Haemalum for 10 seconds and blued for 10 seconds using 4% alkaline alcohol (4% ammonia (Fisher) in 70% alcohol). Sections were dehydrated through a series of alcohols, cleared using Histoclear and mounted using DPX (RA Lamb).

Imaging

All images were taken on a Nikon Diaphoto 300 with a digital camera using Nikon ACT1 image capture software.

Measurements of images

Measurements were determined using ImageJ software (National Institute for Health; http://rsb.info.nih.gov/ij/).

Low magnification images

Low magnification images were produced by capturing multiple images at 4x magnification and subsequently stitching these images together by hand in Adobe Photoshop. Cross sections images are compared showing the greatest cross sectional diameter of teratomas, and adjusted to account for teratoma length.

2.3 Results

Comparison of tumours formed from different anatomical locations

2.3.1 Embryonal carcinoma intrahepatic transplantation produces large teratomas in comparison to subcutaneous transplantation

Transplantation of EC cells into the liver resulted in teratomas dramatically different in structure to those seen following transplantation beneath the skin. Transplantation into the liver resulted in rapid growing teratoma masses that were terminated 3-4 weeks post transplantation. In comparison, subcutaneous transplantation resulted in much slower growing teratomas, which could be maintained for 10-12 weeks. Subcutaneous teratomas were solid in composition with no cystic regions. Intrahepatic teratomas were cystic with areas of solid material similar to subcutaneous teratomas. Cystic regions were surrounded by simple epithelial cells. Subcutaneous teratomas had an estimated volume of ~40-50mm^3 and intrahepatic teratomas were estimated to be five to eight times larger with an estimated volume of ~225-360mm^3. The mass of solid material for intrahepatic teratomas was estimated to be ~70-80% thus indicating that the cystic regions are not solely accountable for the increase in tumour size (Figure 2.1).

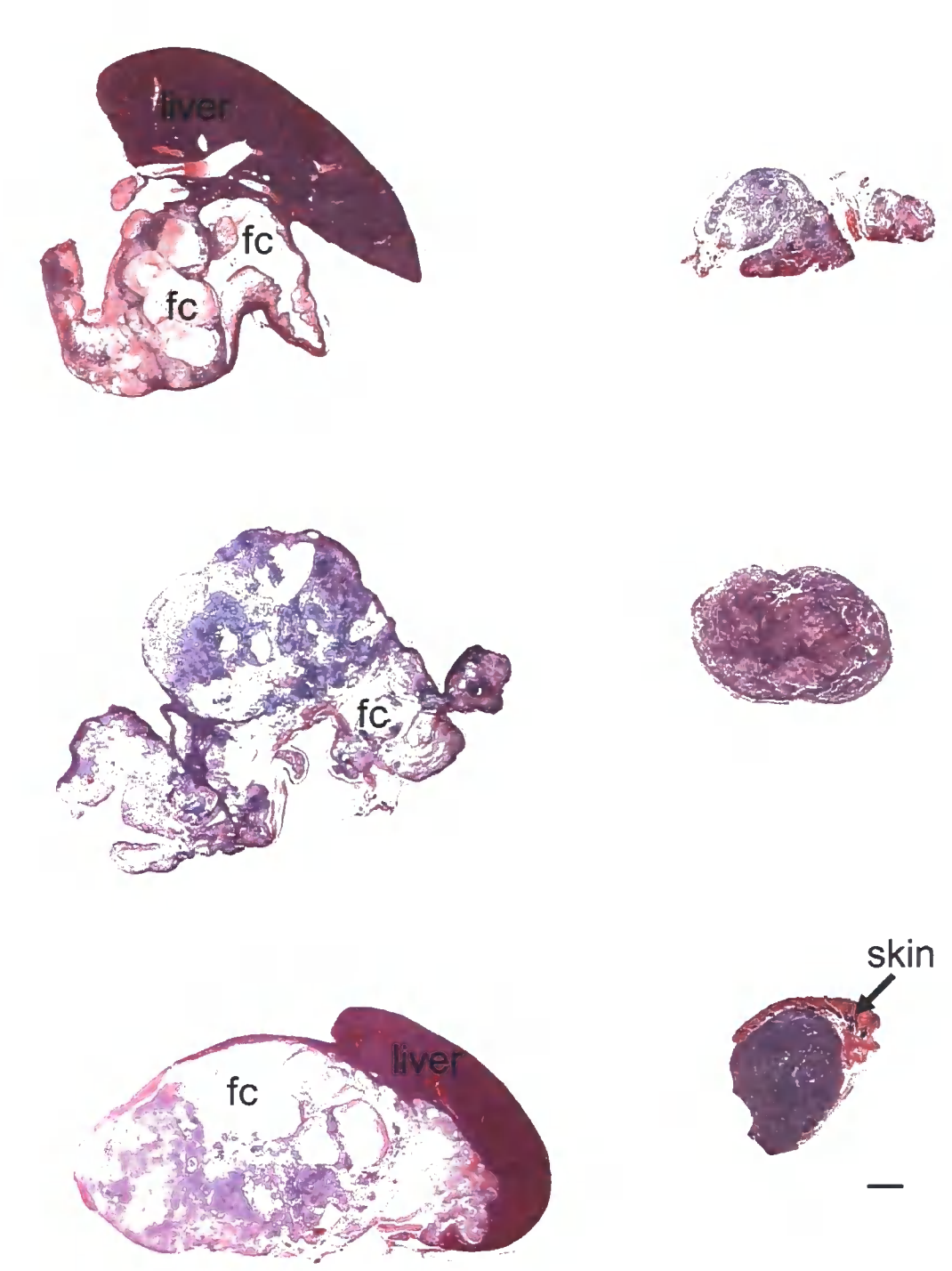

Figure 2.1 Morphological comparison of hEC stem cell subcutaneous and intrahepatic teratomas. Low magnification images show middle sections of teratomas. Larger intrahepatic teratomas are shown on the left, whereas, smaller subcutaneous teratomas are shown on the right. Fluid filled cavities (fc) were observed in intrahepatic teratomas. Histological staining: Haematoxylin and eosin, all images are shown at the same magnification. Scale bar: 1mm.

2.3.2 Embryonal carcinoma transplanted intrahepatically produces immature teratomas

To identify the cell types present within the intrahepatic teratoma, immunohistochemical analysis was carried out. Teratomas produced by hEC stem cells are known to consist of undifferentiated elements and differentiated components. The undifferentiated elements within a teratoma produced by hEC stem cells are known to be reactive to SSEA-3 [264], for this reason this antibody was used to assess the undifferentiated elements of the teratoma. Teratomas from hEC stem cells are also known to contain neural progenitor cells, the class VI intermediate filament antibody nestin, that recognises neural stem/progenitor cells was used to assess the neural progenitor cell population. Epithelial cells are abundant in teratomas produced by hEC cells, to assess this, an antibody to pan keratin that recognises a broad range of human keratins was used. To assess the presence of more differentiated neural cells, the neurofilament antibody NF200 was use to detect these.

Intrahepatic teratomas consisted of both undifferentiated and differentiated elements. Intrahepatic teratomas were found to have expression of the pluripotent stem cell marker SSEA-3 and the differentiated cell markers, nestin and epithelial keratin, indicating that both immature and mature differentiated tissues are present. Subcutaneous teratomas were found to be more differentiated with fewer undifferentiated cell types and were found to have no expression of SSEA-3 and the expression of nestin, keratin and NF200 were all greater than for intrahepatic teratomas, indicating a more differentiated teratoma. The location of the nestin expression was different in each of the two teratomas, with nuclear localisation in the subcutaneous teratoma and cytoplasmic expression in the intrahepatic teratoma. The cytoplasmic staining was reminiscent of undifferentiated pluripotent stem cells and multilineage progenitor cells *in vitro* [265] (Figure 2.2).

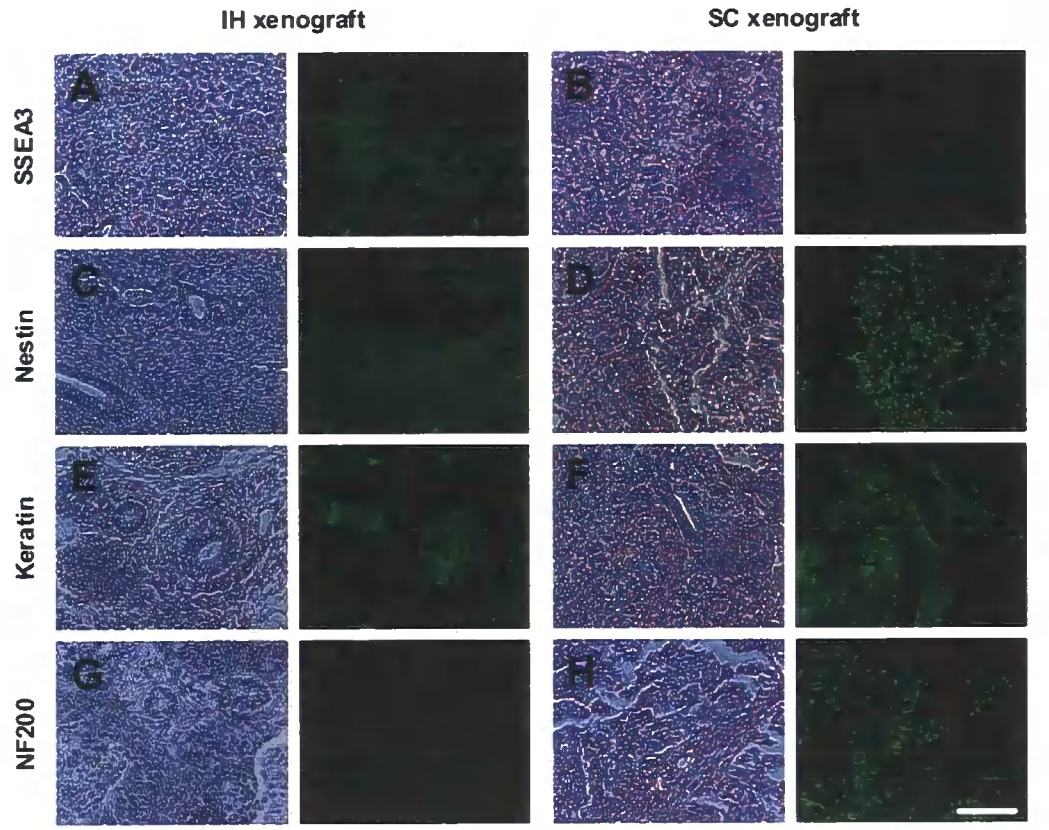

Figure 2.2 Immunohistochemical analysis of the composition of teratomas grown in intrahepatic or subcutaneous locations. Embryonal carcinoma stem cells were grafted and maintained intrahepatically (IH) for 3-4 weeks (A, C, E, G) or subcutaneously (SC) for 10-12 weeks (B, D, F, H). Samples were immunologically stained for: SSEA-3 (A, B); Nestin (C, D); Epithelial keratin (E, F); or NF200 (G, H). Brightfield haematoxylin and eosin stained images are shown with their corresponding fluorescence counterpart. All images are shown at the same magnification. Scale bar: (A-F) 100µm.

2.3.3 Human embryonic stem cells transplanted intrahepatic produces large cystic tumours

Transplantation of ES cells into the liver of SCID mice, produces large tumours within 3-4 weeks, several of these tumours grew so rapidly that they resulted in dramatically reduced health of the animal and termination of the experiment. Histological examination showed that the teratoma contained no solid material and was solely composed of fluid filled cavities, further examination through the whole of the tumour showed this to be the case throughout (Figure 2.3). Cystic regions

were not surrounded by any identifiable epithelial. The estimated volume of the tumour was ~510mm^3.

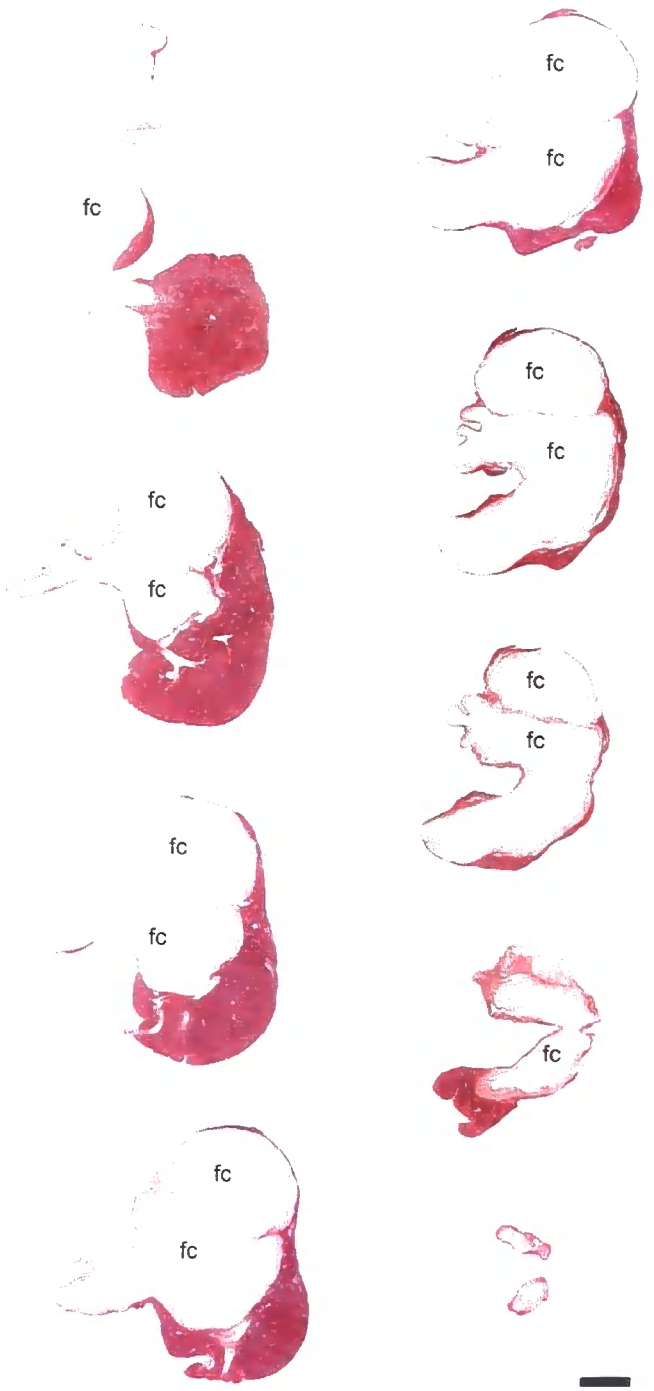

Figure 2.3 Intrahepatic transplantation of ES cells produces large cystic teratomas. Teratomas were found to be composed of fluid filled cavities (fc) with no evidence of any solid or differentiated material. Images show serial sections 600μm apart. Histological staining; haematoxylin and eosin. Scale Bar: 2mm.

2.3.4 Transplantation of embryonic stem cells subcutaneously produces teratomas composed of both solid and fluid filled cavities

Upon examination of teratomas produced by hES cells, it was found that these teratomas consisted mainly of solid material made up of: neural (n); cartilage (c); epithelial (EpK); and bone (arrows) cells (Figure 2.4). In addition there were numerous fluid filled cavities (Figure 2.4, fc). The fluid filled cavities were surrounded by both simple epithelial cells and epithelial cells reminiscent of the gut. The size of the teratoma was dramatically smaller than the tumour produced following intrahepatic transplantation; the estimated teratoma volume was ~100-125mm^3.

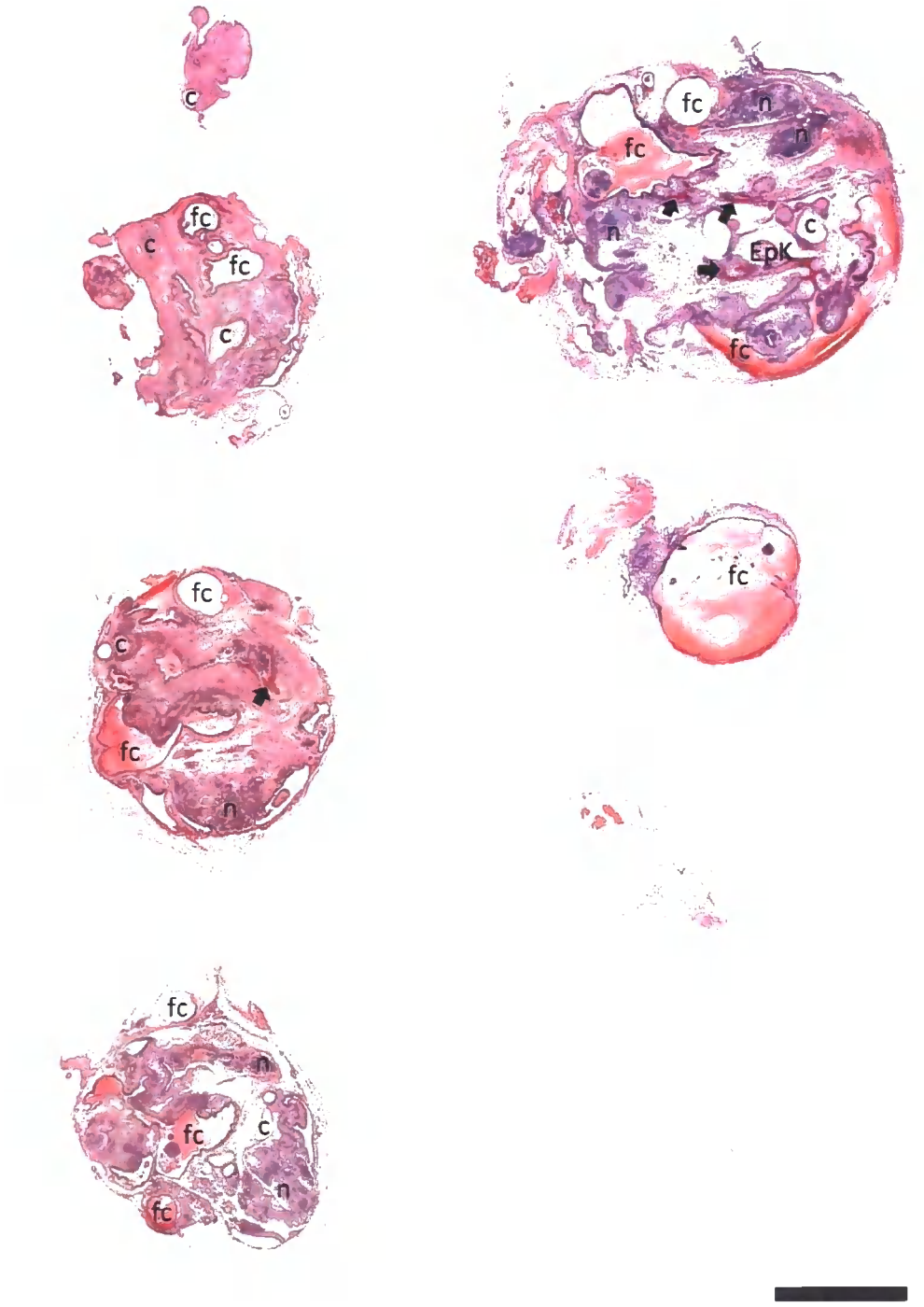

Figure 2.4 Transplantation of hES cells subcutaneously produced solid teratomas with small cystic regions. Histological examination throughout the whole teratoma demonstrated that the majority of the teratoma is solid material. Evidence of both differentiated elements including neural tissue (n), cartilage (c) and epithelial keratin (EpK) as well as fluid filled cavities (fc) was observed. Histological staining; Haematoxylin and Eosin. Images show serial sections 600μm apart. Scale Bar: 2mm.

2.3.5 Subcutaneous transplantation of embryonic stem cells produces teratomas composed of cell types from all three germ layers

In order to have a comparison for intrahepatic transplantation of hES, these cells were transplanted subcutaneously and their differentiation potential was investigated. In agreement with previous work, hES cells transplanted subcutaneously resulted in teratomas composed of cell types from all three germ layers. The teratomas produced were heterogeneous and histological analysis showed that they were pluripotent and composed of cell types from all three germ layers: bone, cartilage (mesoderm); neural epithelial (ectoderm); gut (endoderm); and primitive kidney (mesoderm) (Figure 2.5).

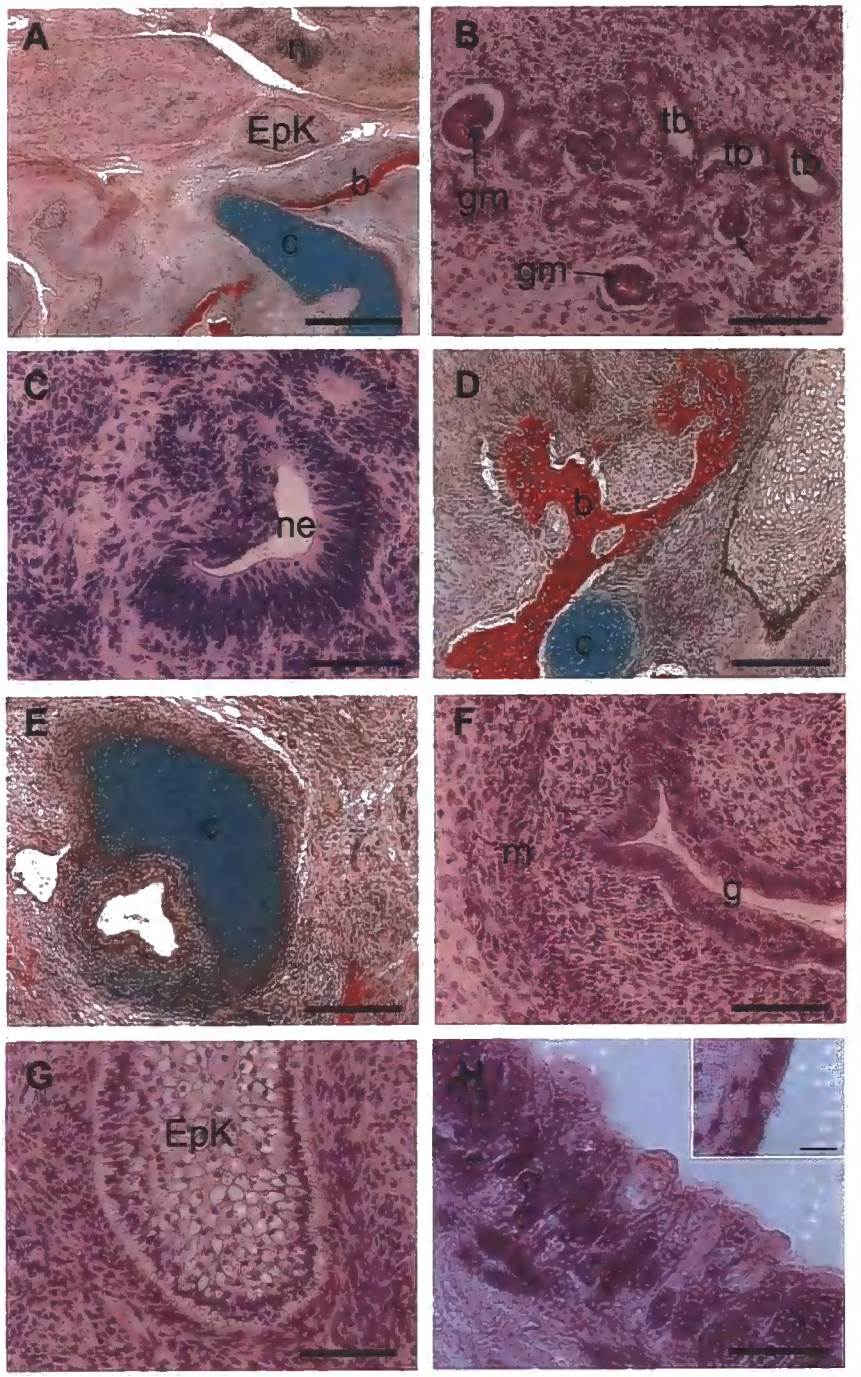

Figure 2.5 Subcutaneous transplantation of ES cells produces fully differentiated teratomas. A) Low power examination demonstrated the heterogeneous nature of the teratoma with cartilage (c), bone (b) neural tissue (n) and epithelial keratin (EpK). B) Primitive kidney glomeruli (gm) and associated tubules (t). C) Neural epithelium was observed forming neural tube-like structures. D) Bone (b) and cartilage (c). E) Cartilage (c). F) A gut like structure was also observed with a folded epithelia and lumen (g) and associated muscle layer (m). G) Epithelial keratin. H) Epithelium with goblet cell, insert shows a goblet cell secreting mucus. Histological staining; A, B, E Weigert's Haematoxylin, C, D, F, G Haematoxylin and Eosin. Scale Bars: A=400μm; B, C, F, G=100μm; D, E=200μm; H=20μm insert 20μm

2.3.6 Immunohistochemical analysis confirms that subcutaneous transplantation of embryonic stem cells produces teratomas composed of cell types from all three germ layers

Immunohistochemical analysis was performed to verify that teratomas produced by the transplantation of ES cells are composed of cell types from all three germ layers. Positive staining for the caudal-related homeobox transcription factor antibody (CDX2) confirmed the presence of cells derived from endoderm (Figure 2.6, A). Cell types positive for smooth muscle actin confirms presence of mesoderm derived cells (Figure 2.6, B). Nestin expression demonstrates that cells of an ectodermal origin are also present (Figure 2.6, C). Taken together, these results demonstrate that teratomas derived from hES cells are pluripotent and composed of cell types from all three germ layers.

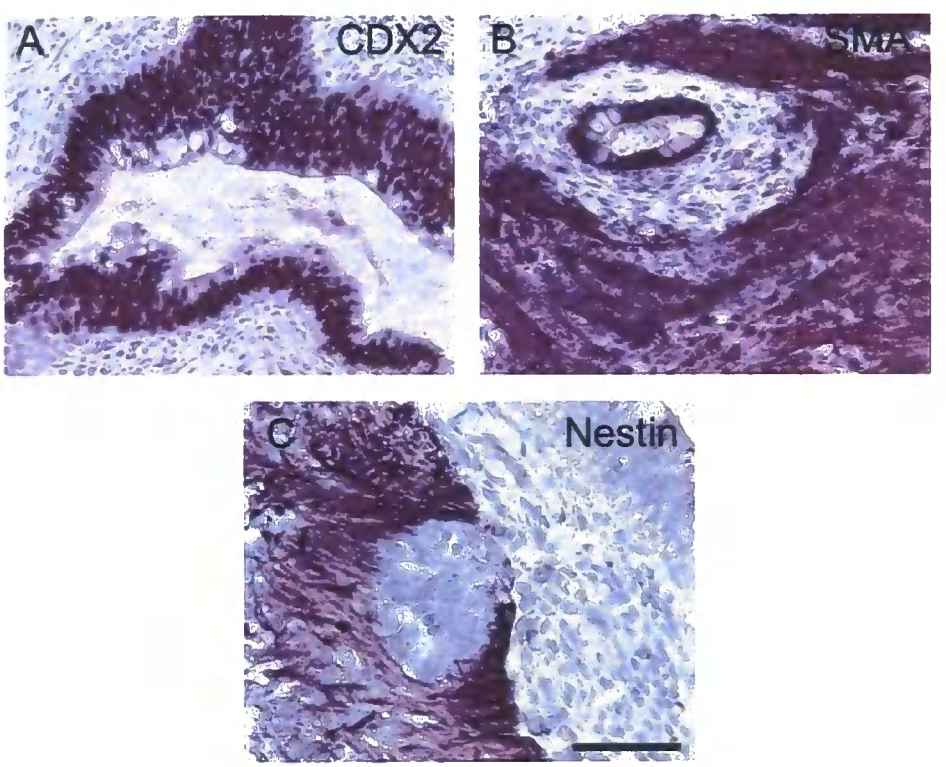

Figure 2.6 Immunohistochemical confirmation of the presence of cell types from all three germ layers. Teratomas were positive from antibodies to cell types from all three germ layers. A) Positive expression for the endodermal marker CDX2. B) Positive expression for the mesoderm marker smooth muscle actin (SMA). C) Positive expression for the ectoderm marker nestin. Positive staining is shown in purple with negative regions shown in blue. All images were taken at the same magnification scale bar; 100μm.

One factor that could contribute to the different results obtained following transplantation into alternative environments is the composition of the ECM. Co-transplantation of stem cells into a single environment with different ECM components could provide evidence of whether the ECM plays an important role in teratoma formation.

2.3.7 Co-transplantation of embryonal carcinoma cells with different extracellular matrix components increases the success rate of teratoma formation

Subcutaneous transplantation of hEC stem cells with the following solutions was carried out: PBS (control); collagen I; collagen IV; fibronectin; laminin; and Matrigel™. The success rate of teratoma formation was different depending upon the ECM component used. No teratoma formation was observed when cells were transplanted with either collagen IV or fibronectin. Teratoma formation was observed when cells were transplanted with PBS (control), collagen I, laminin and Matrigel™ (Table 2.1). No tumours formed for any ECM component transplanted without cells.

ECM component	Tumour formation rate
Control (PBS)	4% (1/24)
Collagen I	33% (2/6)
Collagen IV	0% (0/6)
Fibronectin	0% (0/6)
Laminin	33% (2/6)
Matrigel	100% (6/6)

Table 2.1 The success rate of teratoma formation of hEC stem cells co-transplanted with various ECM components. Numbers in brackets show the number of successful teratomas and the total number of animal in which transplantations were carried out.

2.3.8 Co-transplantation of embryonal carcinoma cells with different extracellular matrix components affects the growth rate of teratomas

Teratoma progression was followed by weekly measurements of the mean teratoma diameter using calipers. Cells transplanted with Matrigel™ readily formed teratomas within one week post transplantation and their growth was rapid with the experiment being terminated six weeks post transplantation. Cells transplanted with collagen I and PBS (control) formed teratomas more slowly (first palpable five weeks post transplantation) than with Matrigel™ (first palpable one week post transplantation). Collagen I teratomas grew at a slower rate than the control. Cells co-transplanted with laminin first showed signs of teratoma growth six weeks post transplantation, whilst their progression was quicker than collagen I teratomas it was not as rapid as the control teratoma (Figure 2.7 A). Following termination of the experiments, histological analysis was carried out. Teratomas produced with the co-injection of Matrigel™ appeared to have a similar cross sectional diameter similar to cells transplanted when suspended in PBS, however, the teratoma produced from co-transplantation with Matrigel™ had only been allowed to develop for six weeks, whereas, the control teratoma had been allowed to develop for thirteen weeks (over twice the time allowed for Matrigel™ co-transplantation development). Teratomas produced with the co-injection of laminin, showed a reduction in cross sectional diameter, when compared to control teratomas. Those transplanted with collagen I showed a further reduction (Figure 2.7, B).

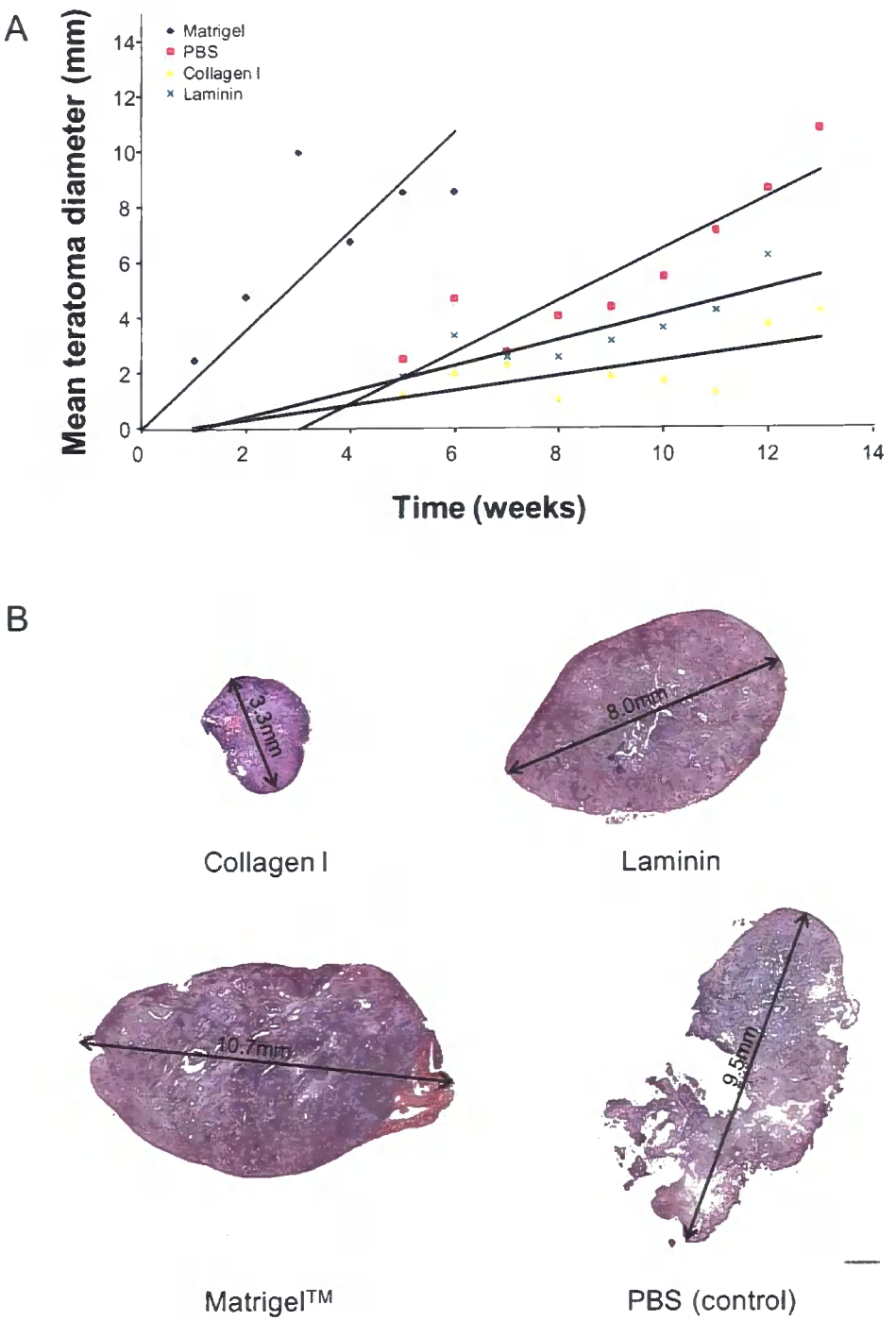

Figure 2.7 The growth of teratomas depends upon the co-transplanted ECM component. A) Graphical representation of teratoma growth rate for EC stem cells co-transplanted with PBS (control), collagen I, laminin and Matrigel™. Points represent average values for all successful teratomas in each group. B) Low power images showing cross sections of teratomas at their widest cross sectional length. Note, cross sectional areas were adjusted to account for teratoma length and areas were reduced for teratomas of shorter length to give a true representation of teratoma size. All samples are shown at the same magnification. Scale = 1mm, histological staining, haematoxylin and eosin.

2.3.9 Teratomas produced by EC stem cells co-transplanted with extracellular matrix components are solid in composition

It was previously demonstrated that teratomas can consist of both solid and cystic cavities filled with fluid (Sections 2.3.3 and 2.3.4). Upon examination of serial sections throughout the teratomas formed, no cystic areas were observed for any of the teratomas and all tumours appeared as a solid mass throughout (Figure 2.8, shows a teratoma formed by co-transplantation of EC stem cells with Matrigel™ and is representative of all teratomas formed).

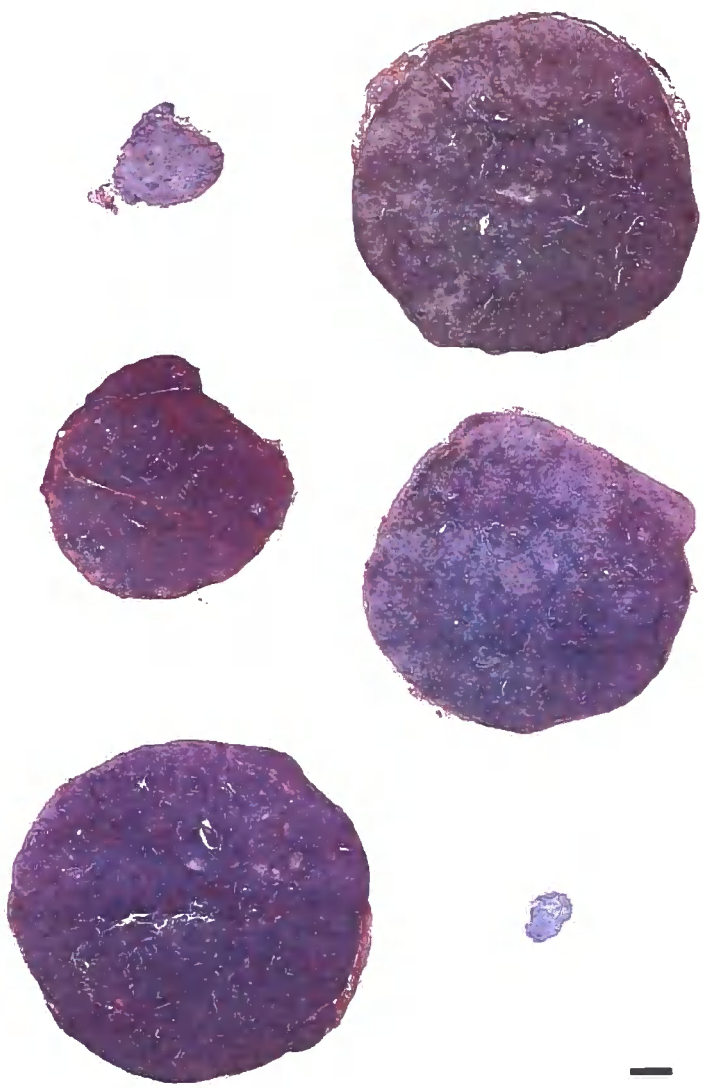

Figure 2.8 Examination of serial sections throughout a teratoma formed by co-transplantation of EC cells with Matrigel™. Sections demonstrate that the teratoma is solid throughout the whole of the material. Histological staining; Haematoxylin and Eosin. Sections are 2mm apart; all images are shown at the same magnification, Scale Bar: 1mm.

2.3.10 The extracellular matrix affects the cell types present within the teratoma

When EC stem cells were suspended in PBS without any additional ECM and transplanted subcutaneously, teratoma development was consistent with that previously reported [67] and as described herein (Section 2.3.1). Both differentiated cell types of glandular structures composed of cavities surrounded by epithelial-like cells (Figure 2.9, A) and neural rosettes (Figure 2.9, B) were observed. In addition, cell types with an undifferentiated morphology similar to that of pluripotent hEC cells were seen (Figure 2.9, C). Immunohistochemical analysis revealed that within the teratoma there existed populations of Ki67 positive cells, thus demonstrating cells within the teratomas were proliferative (Figure 2.9, D).

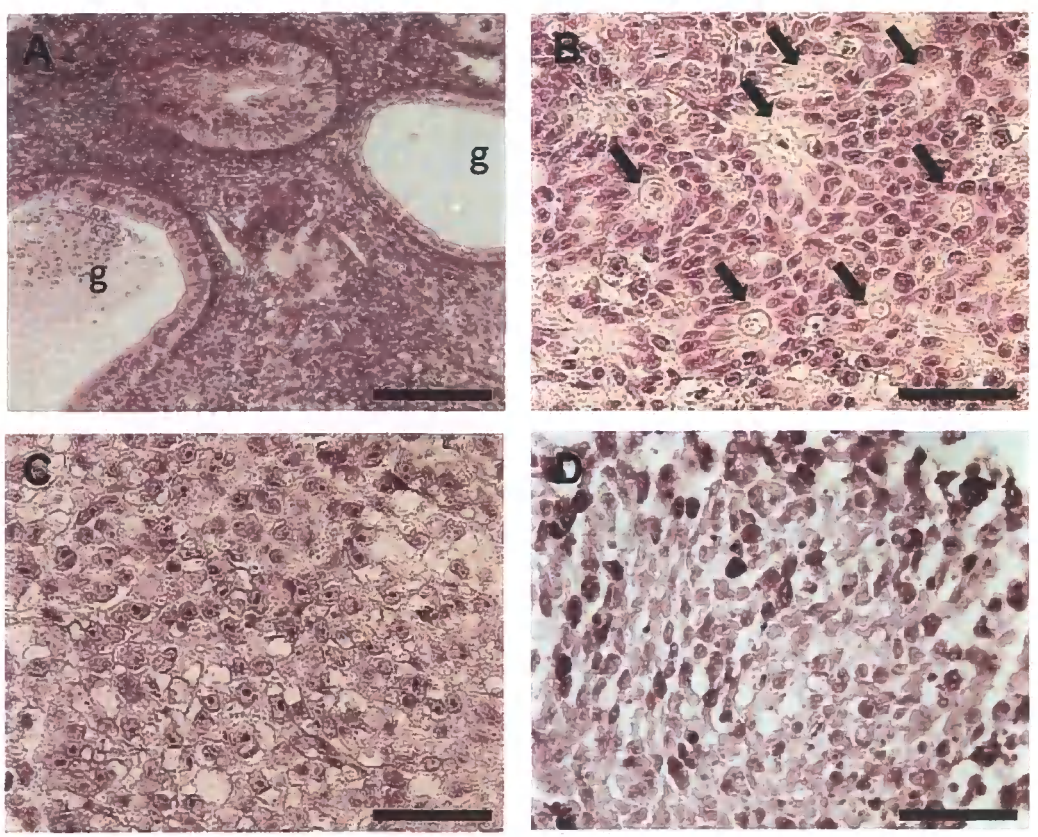

Figure 2.9 Histological examination of teratomas produced by EC cells co-transplanted with no additional ECM. Cells types indicative of EC teratomas were observed. A) Glandular structures (g). B) Neural rosettes (arrows). C) Embryonal carcinoma cells. D) Proliferative Ki67 positive cells (dark red). Histological staining: A-C Haematoxylin and Eosin. Immuno-staining: D Ki67. Scale Bars: A 100μm, B-D 50μm.

2.3.11 Co-transplantation with collagen I decreases the differentiation potential of embryonal carcinoma stem cells

Similar to control teratomas, co-transplantation of EC stem cells with collagen I produced a solid teratoma with both embryonal carcinoma cells and proliferative elements. The collagen I teratoma was the smallest of all the teratomas produced. However, differentiation appeared to be suppressed and no glandular structures or neural elements were observed (Figure 2.10, A, B). Similar to control teratomas, cells with morphology similar to undifferentiated hEC stem cells were seen (Figure 2.10, C). Also similar to control teratomas, Ki67 positive cells were seen, demonstrating that cells within the teratoma were proliferating (Figure 2.10, D).

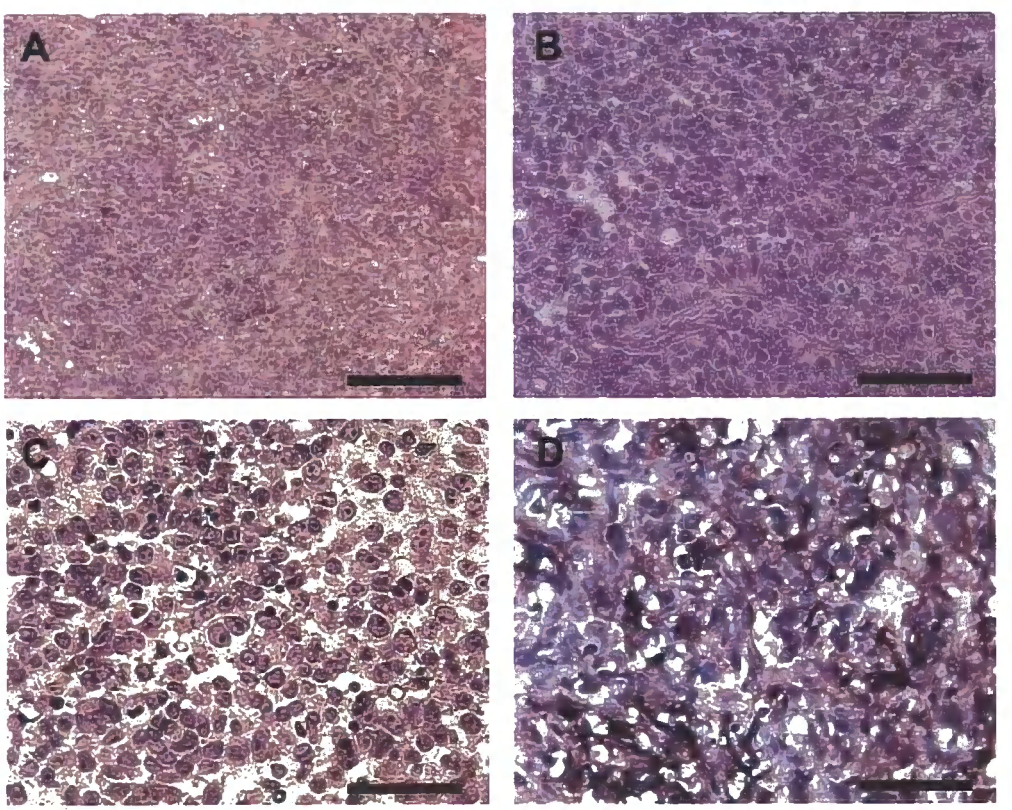

Figure 2.10 Histological examination of teratomas produced by EC cells co-transplanted with collagen I. Teratomas were solid in composition with few alternative cell types. A, B) The tumour appeared solid throughout. C) Embryonal carcinoma cells. D) Proliferative Ki67 positive cells (dark red). Histological staining: A-C Haematoxylin and Eosin. Immuno-staining: D Ki67. Scale bars; A 200μm, B 100μm, C, D 50μm.

2.3.12 Co-transplantation with laminin increases the differentiation potential of embryonal carcinoma stem cells

Co-transplantation of EC stem cells with laminin appeared to increase the developmental potential of EC stem cells. As observed in control teratomas, glandular structures (Figure 2.11, A), neural rosettes (Figure 2.11, B), pluripotent EC stem cells (Figure 2.11, C) and Ki67 positive proliferating cells (Figure 2.11, D) were all present. In addition, in one of the two teratomas produced, several nodules of cartilage were found, suggesting that mesodermal differentiation of TERA2.cl-SP12 cells is possible (Figure 2.11).

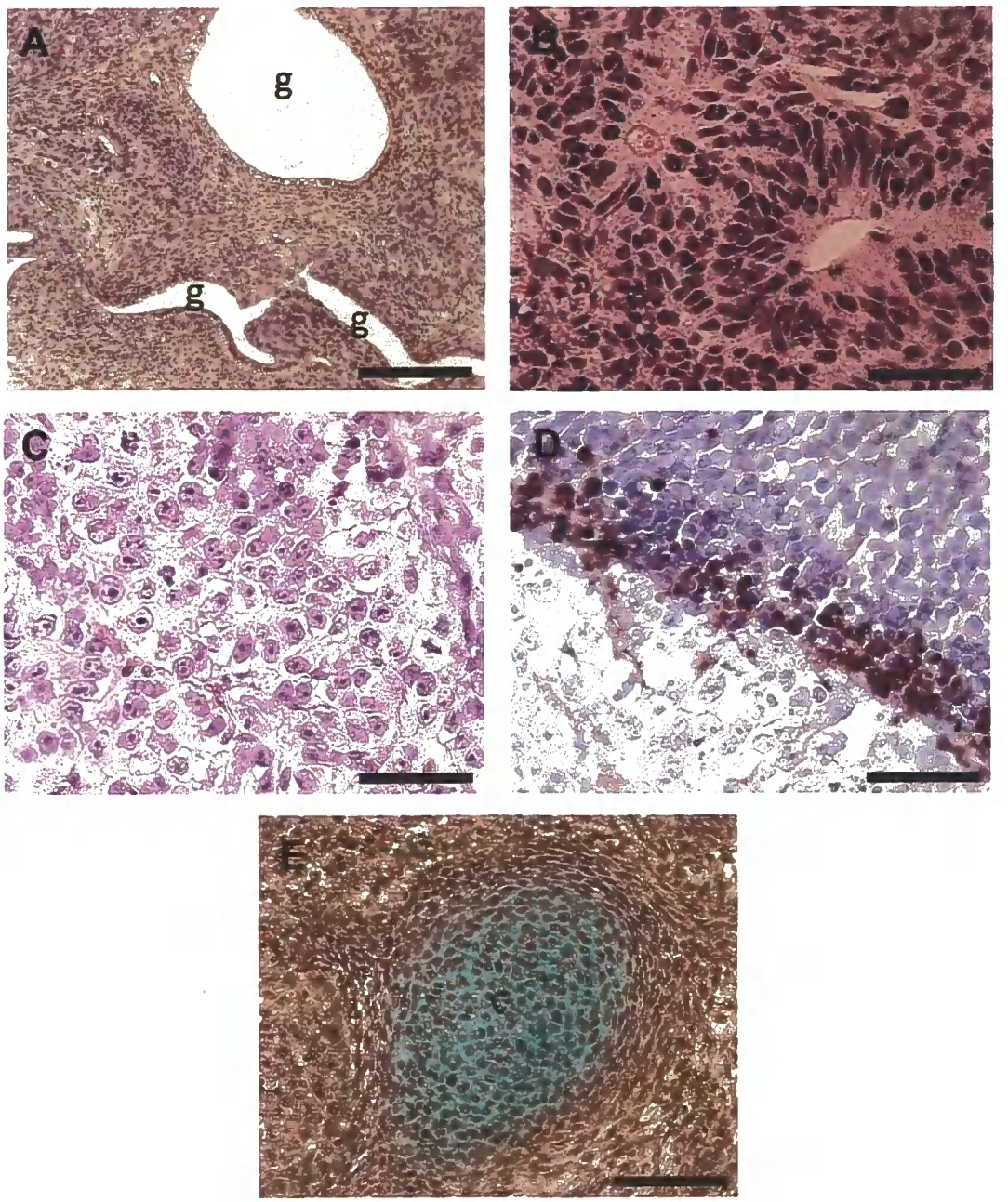

Figure 2.11 Histological examination of teratomas produced by EC cells co-transplanted with laminin. Teratomas were solid in composition with numerous cell types. A) Glandular structures (g). B) Neural tissue. C) Embryonal carcinoma cells. D) Proliferative Ki67 positive cells (dark red). E) Cartilage nodule (c). Histological staining: A-C Haematoxylin and Eosin, E Weigert's Haematoxylin. Immuno-staining: D Ki67. Scale Bars: A 200μm, B-E 50μm.

2.3.13 Co-transplantation with Matrigel™ had no apparent effect on the differentiation potential of embryonal carcinoma stem cells

Co-transplantation of EC cells with Matrigel™ produced teratomas similar in cell composition to control teratomas. Differentiated elements of glandular structures (Figure 2.12, A) and neural rosettes (Figure 2.12, B) were present. Also undifferentiated cell types with morphology similar to pluripotent EC stem cells were present (Figure 2.12, C). Immunohistochemical analysis further confirmed that teratomas produced with the co-transplantation of hEC stem cells and Matrigel™ were similar to control teratomas by demonstrating that populations of proliferative cells existed (Figure 2.12, D).

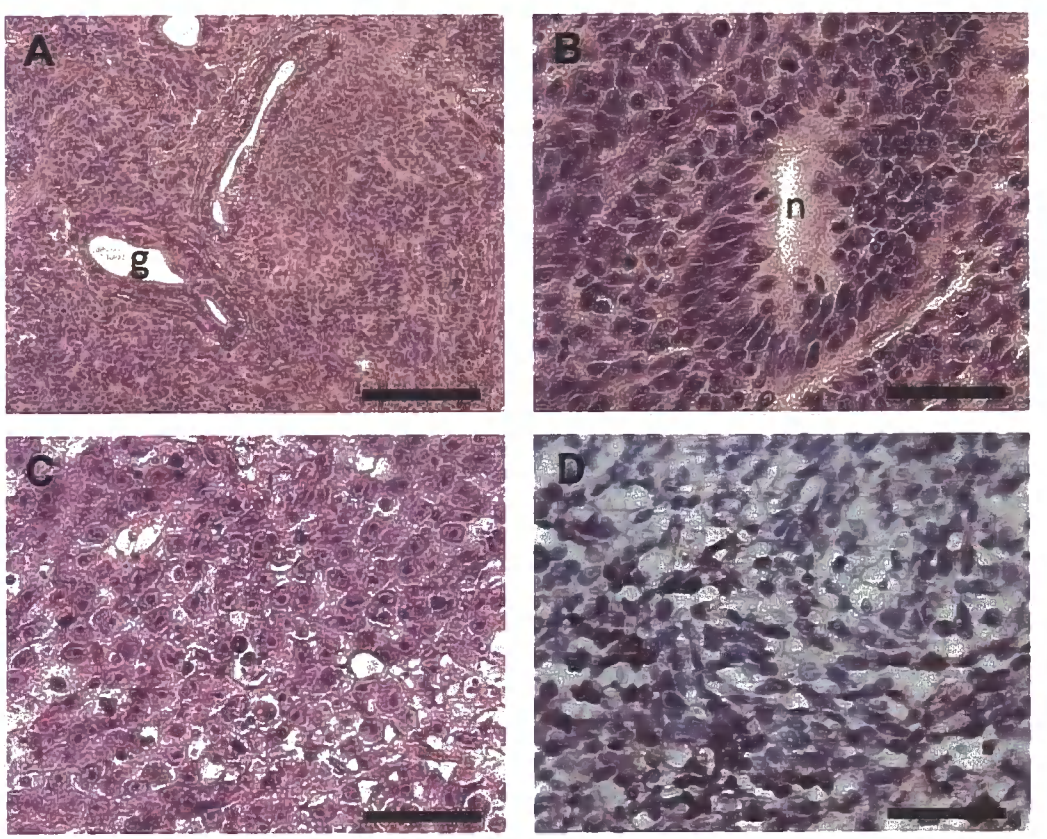

Figure 2.12 Histological examination of teratomas produced by EC cells co-transplanted with Matrigel™. Teratomas were solid in composition with cell types similar to control teratomas. A) Glandular structures (g). B) Neural rosette (n). C) Embryonal carcinoma cells. D) Proliferative Ki67 positive cells (dark red). Histological staining: A-C Haematoxylin and Eosin. Immuno-staining: D Ki67. Scale bars; A 200μm, B-D 50μm.

2.3.14 Immunohistochemical analysis of teratomas produced by co-transplantation of EC stem cells and extracellular matrix proteins

To gain a specific identification of the teratoma constituents, immunohistochemical analysis was performed. An antibody to pan keratin that recognises a broad range of human keratins demonstrated that keratinised epithelial is present in all teratomas. The class VI intermediate filament antibody nestin that recognises neural stem/progenitor cells demonstrated that neural progenitor cells were present in all teratomas. The neurofilament antibody NF200 was detected in both control teratomas and those produced from co-injection with Matrigel™, however, no expression was observed in teratomas produced from the co-transplantation of EC stem cells with collagen I or laminin. The undifferentiated EC stem cell marker TRA-1-60 was present in all teratomas; however, the levels of expression were highest in control and Matrigel™ teratomas (Figure 2.13).

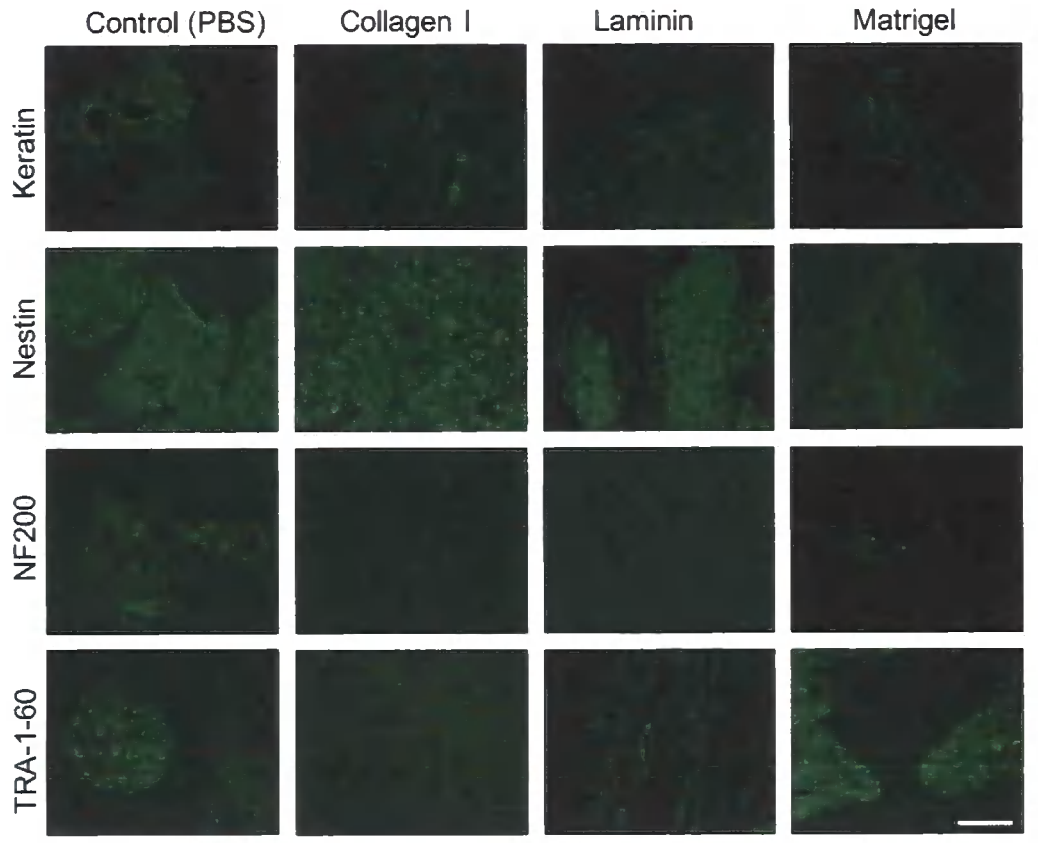

Figure 2.13 Immunohistochemical analysis of teratomas produced by co-transplantation with ECM components. Teratomas were assessed for their reactivity to antibodies for differentiated elements (keratin and NF200), neural progenitor cells (nestin) and undifferentiated EC stem cells (TRA-1-60). All images as shown at the same magnification, scale = 100μm.

2.3.15 Co-transplantation of embryonal carcinoma cells with different extracellular matrix components affects the success rate of teratoma formation

Subcutaneous transplantation of EC stem cells with the following solutions was carried out: PuraMatrix™, Extracel™, Matrigel™, Cultrex BME and Cultrex BME RGF. The success rate of teratoma formation was different depending upon the component used. No teratoma formation was observed when cells were transplanted with either PuraMatrix™ or Extracel™. Teratoma formation was observed when cells were transplanted with Matrigel™, Cultrex BME and Cultrex BME RGF (Table 2.2).

ECM component	Success rate
Extracel™	0% (0/3)
PuraMatrix™	0% (0/3)
Matrigel™	100% (3/3)
Cultrex BME	100% (3/3)
Cultrex BME RGF	100% (3/3)

Table 2.2 The success rate of teratoma formation for various ECM components. Numbers in brackets show the number of successful teratomas and the total number of animals in which transplantations were carried out.

2.3.16 Co-transplantation of embryonal carcinoma cells with different extracellular matrix components affects the growth rate of teratomas

Teratoma progression was followed by weekly measurements of the mean teratoma diameter. Cells transplanted with Matrigel™, Cultrex BME and Cultrex BME RGF all readily formed teratomas within three weeks post transplantation and their growth was rapid with the experiment being terminated eight weeks post transplantation. Growth rates were similar for Matrigel™, Cultrex BME and Cultrex BME RGF (Figure 2.14, A). Histological analysis demonstrates that cross sectional diameters of all teratomas produced were similar (Figure 2.14, B).

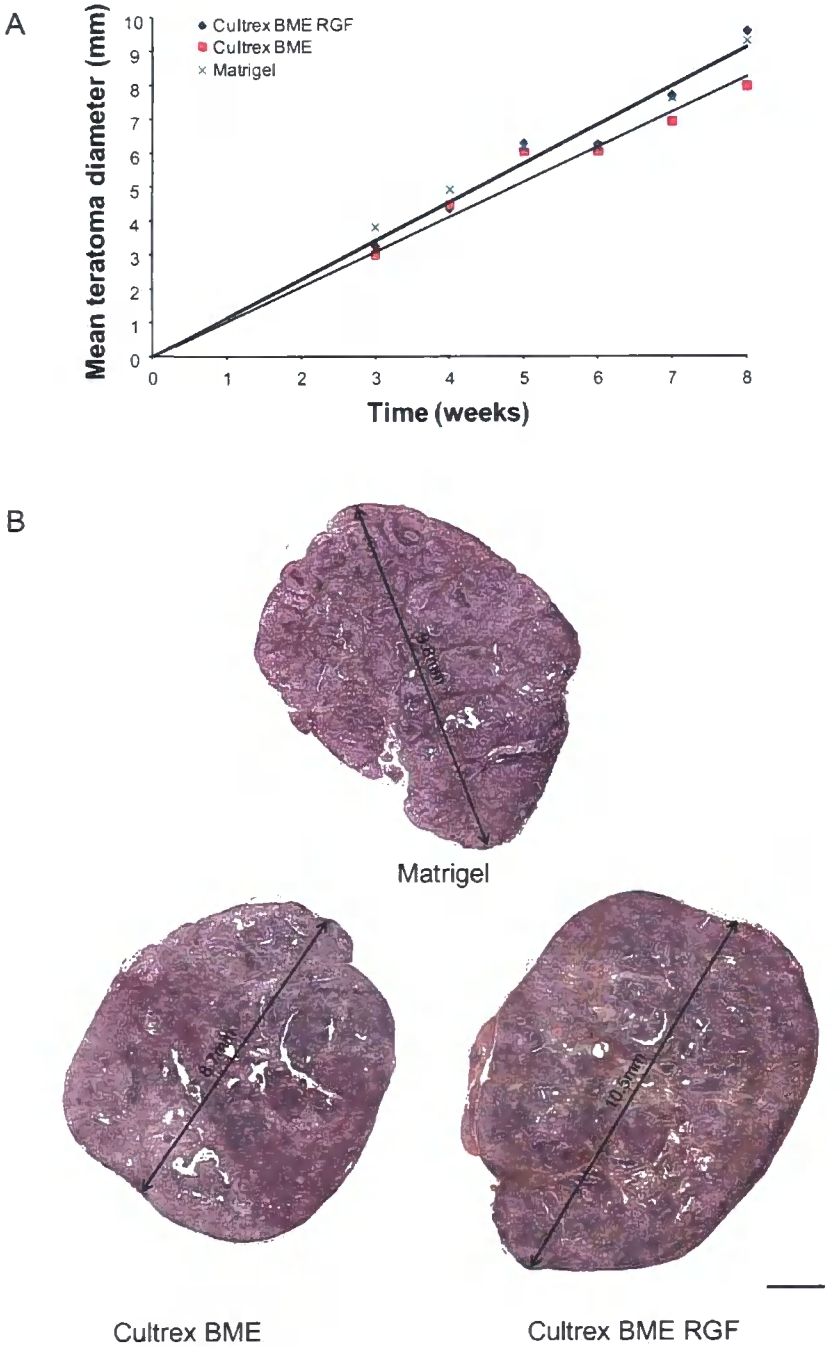

Figure 2.14 The growth of teratomas is not dependent upon growth factors. A) Graphical representation of teratoma growth rate. Points represent average values for 3 teratomas in each group. B) Low power images showing sections of teratomas at their widest cross sectional length. Note, all samples are shown at the same magnification. Scale = 1mm, histological staining, haematoxylin and eosin.

2.3.17 Teratomas produced by EC stem cells co-transplanted with extracellular matrix components are solid in composition

It was demonstrated previously that teratomas can consist of both solid and cystic cavities filled with fluid (Sections 2.3.3 and 2.3.4). It has also been shown that EC stem cells co-transplanted subcutaneously with collagen I, laminin or Matrigel™ all resulted in teratomas, which were solid in composition (Sections 2.3.8 and 2.3.9). Upon examination of serial cross sections throughout the teratomas formed by co-transplantation with Cultrex BME and Cultrex BME RGF, no cystic areas were observed and all tumours appeared as a solid mass throughout (Figure 2.15, shows a teratoma formed by co-transplantation of EC stem cells with Cultrex BME RGF and is representative of all teratomas formed).

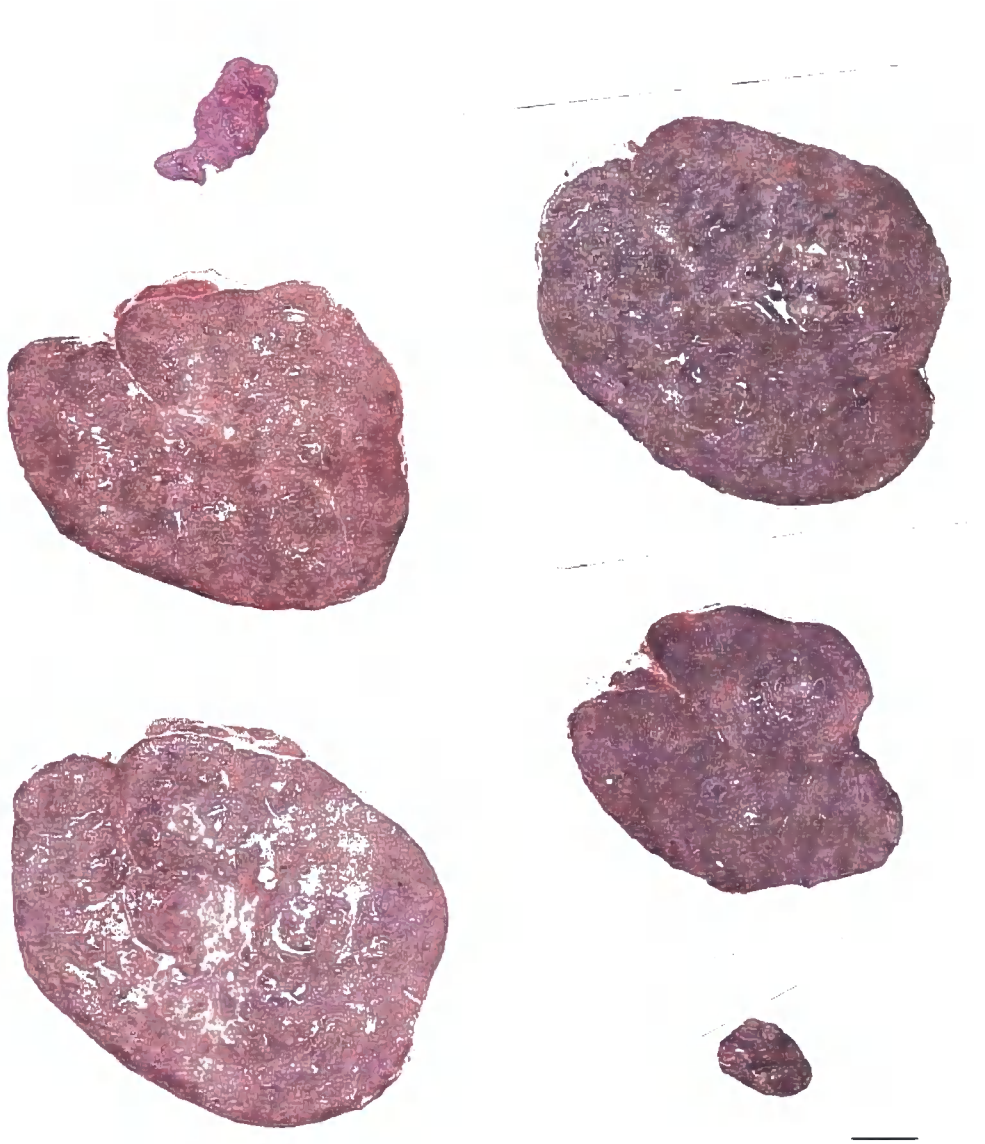

Figure 2.15 Examination of sections throughout a teratoma formed by co-transplantation of EC cells with Cultrex BME RGF. Serial sections demonstrate that the teratoma is solid throughout the whole of the material. Histological staining; Haematoxylin and Eosin. Scale Bar: 1mm.

2.3.18 Co-transplantation of EC stem cells with Cultrex BME has no effect on the differentiation potential of embryonal carcinoma stem cells when compared to controls

Co-transplantation of EC stem cells with Cultrex BME produced teratomas similar to control teratomas and those produced by the co-transplantation of Matrigel™ (Section 2.3.13). Differentiated cell types typical of teratomas produced by EC

stem cells were present. Both neural (Figure 2.16, A) and epithelial (Figure 2.16, B) differentiated cells were present, in addition, undifferentiated cell types were also present (Figure 2.16, C).

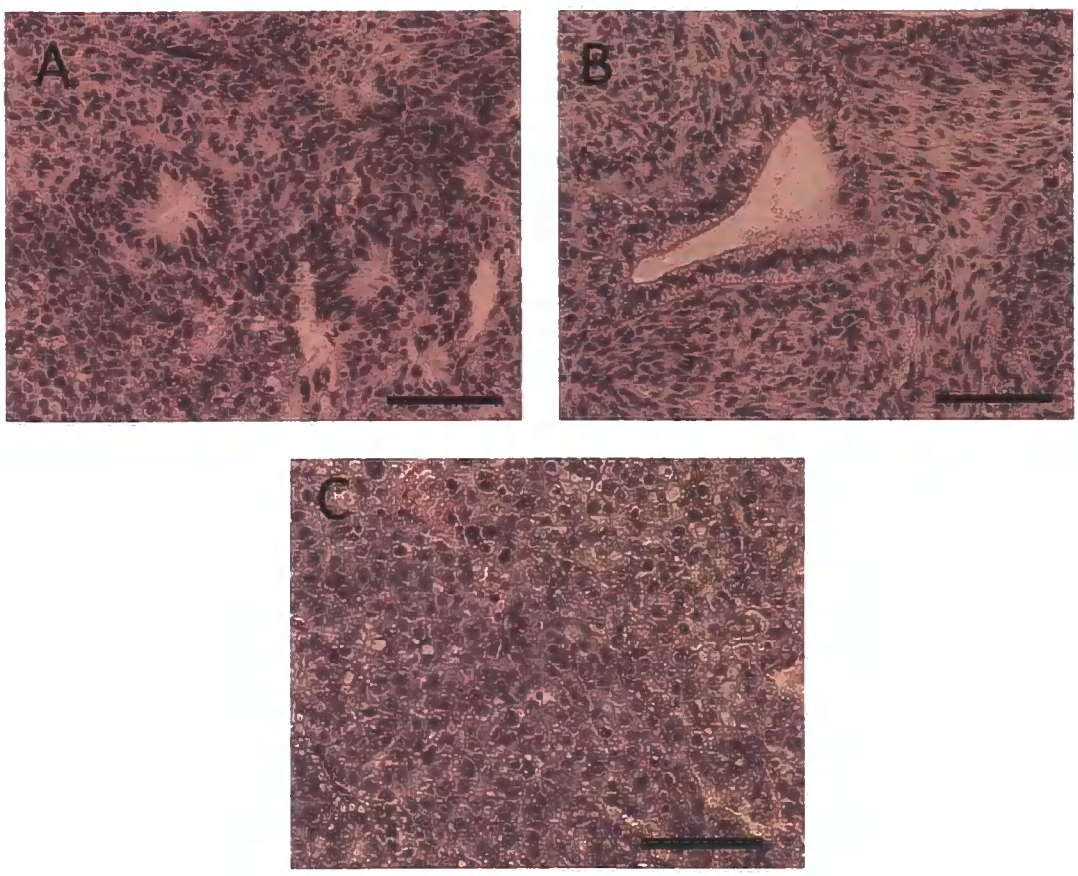

Figure 2.16 Histological examination of teratomas produced by EC stem cells co-transplanted with Cultrex BME. Teratomas were solid in composition with cell types similar to control teratomas. A) Neural cells. B) Epithelial cells. C) Embryonal carcinoma cells. Histological staining: A-C Haematoxylin and Eosin. Scale bars; A-C 100μm

2.3.19 Co-transplantation of EC stem cells with Cultrex BME RGF has no effect on the differentiation potential of embryonal carcinoma stem cells

Co-transplantation of EC cells with Cultrex BME produced teratomas similar to control teratomas and those produced by the co-transplantation of Matrigel™ (Section 2.3.13). Differentiated cell types typical of teratomas produced by EC stem cells were present. Both neural cells (Figure 2.17, A) and epithelial cells with

mucus secreting goblet cells (Figure 2.17, B) were present, and, in addition, undifferentiated cell types were also present (Figure 2.17, C).

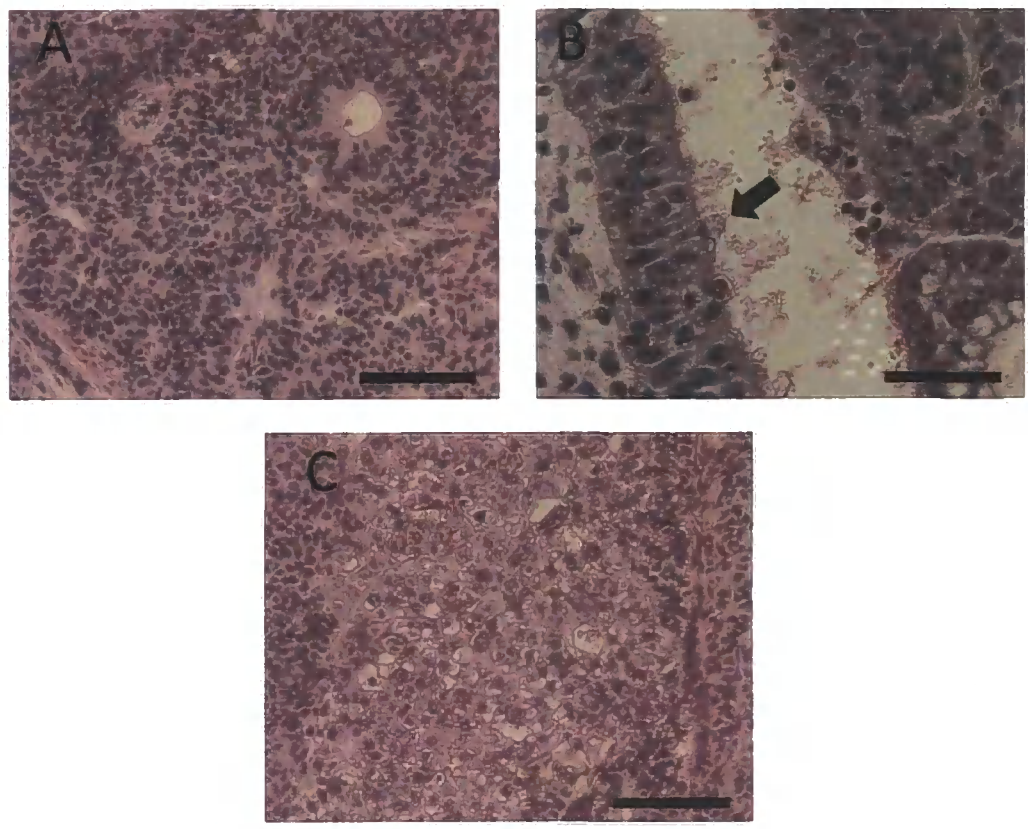

Figure 2.17 Histological examination of teratomas produced by EC stem cells co-transplanted with Cultrex BME RGF. Teratomas were solid in composition with cell types similar to control teratomas. A) Neural cells. B) Epithelial cells with goblets cells (arrow). C) Embryonal carcinoma cells. Histological staining: A-C Haematoxylin and Eosin. Scale bars; A 100μm, B 50μm, C 100μm.

2.3.20 Immunohistochemical analysis of teratomas produced by co-transplantation of EC stem cells with Cultrex BME or Cultrex BME RGF

To gain specific identification of the cell types present within the teratomas, immunohistochemical analysis was performed. Similar to teratomas produced by the co-transplantation of EC stem cells with Matrigel™ (Section 2.3.13), teratomas produced with Cultrex BME and Cultrex BME RGF were reactive to pan keratin, nestin, NF200 and TRA-1-60 (Figure 2.18).

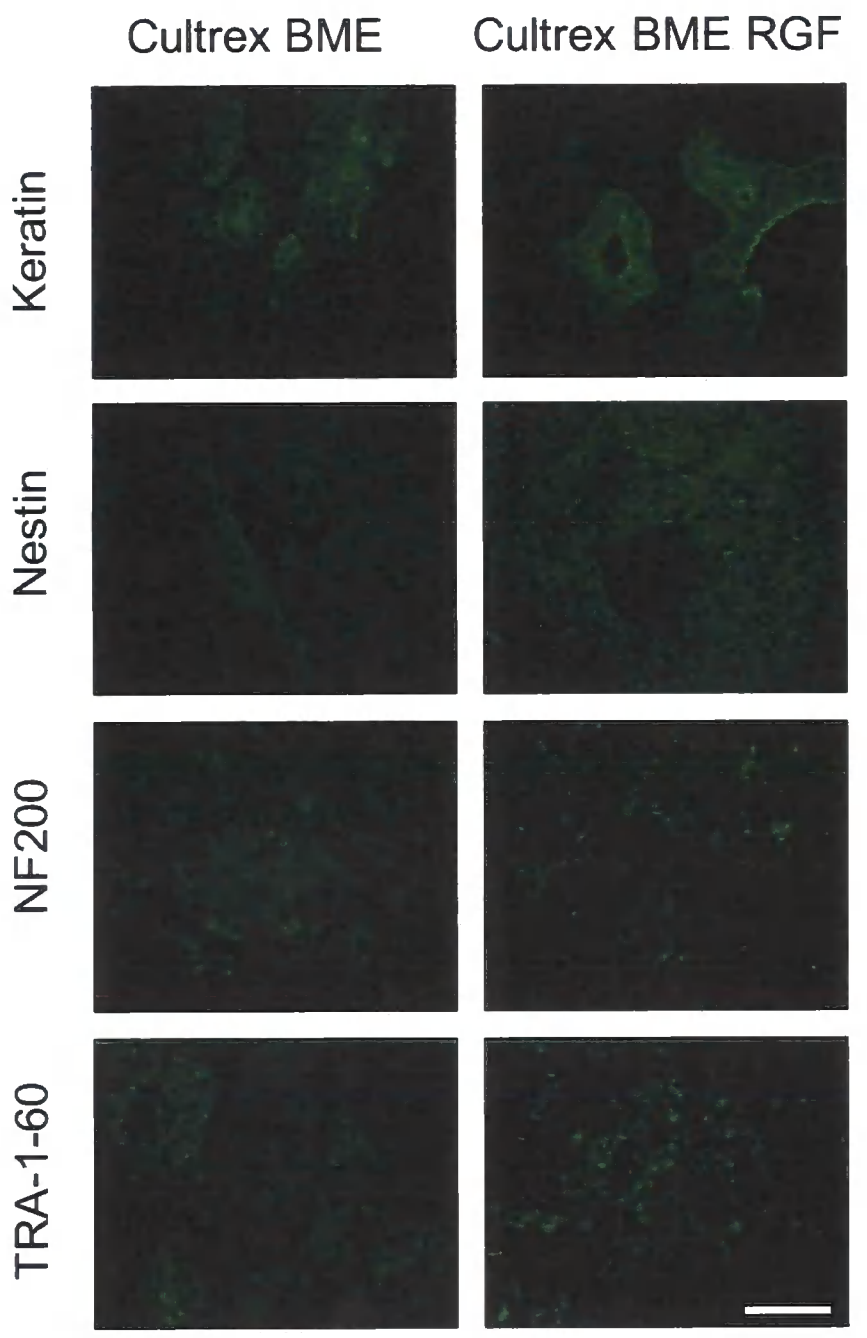

Figure 2.18 Immunohistochemical analysis of teratomas produced by co-transplantation with Cultrex BME and Cultrex BME RGF. Teratomas were assessed for their reactivity to antibodies for differentiated elements (keratin and NF200), neural progenitor cells (nestin) and undifferentiated cells (TRA-1-60). All images as shown at the same magnification, scale = 100um.

2.3.21 Teratomas are composed of human cell types

It is important to demonstrate that the structures observed originate from the transplanted cells, this can be achieved using an antibody to human nuclear antigen [243]. To demonstrate teratoma formation was due to the transplanted cells an antibody reactive to human cell types was employed. Staining revealed that all cell types within the graft were positive for human nuclear antigen thus demonstrating that the origin of the teratoma was from the human transplanted cells and not the host mouse cells. Furthermore staining with anti epithelial keratin (a cytoskeletal marker) demonstrates the specificity of the anti human nuclear antigen for the nucleus of cells. No significant levels of background staining were observed (Figure 2.19). To demonstrate antibody speficifity staining was conducted on a section where the edge of the teratoma was present. The edge of the teratoma is surrounded by host skin tissue, negative staining of this host skin demonstrated minimal cross reactivity with the host tissue.

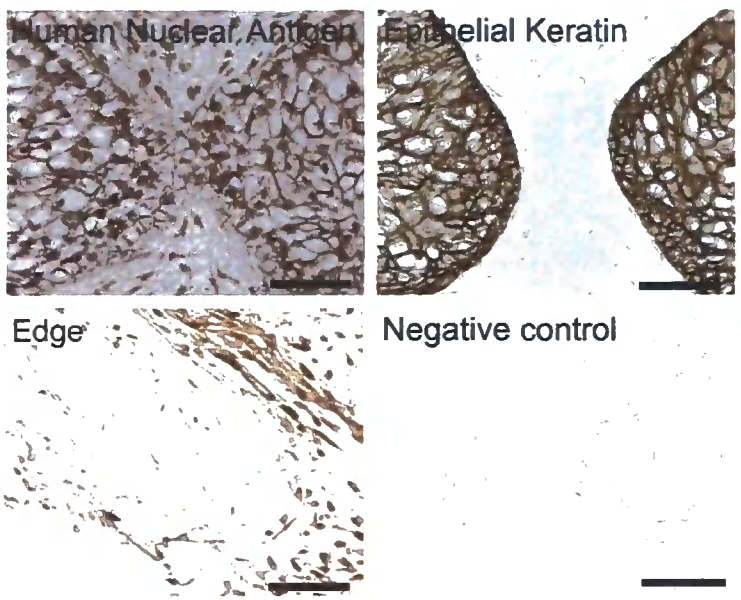

Figure 2.19 Confirmation that teratoma origin is human. Staining for human nuclear antigen produced positive staining located at the nucleus of the cells. Images show antibody nuclear localisation of human nuclear antigen and cytoplasmic localisation of epithelial keratin. All images were taken at the same magnification. Positive areas of staining are shown in brown. Scale Bar: 50μm.

Summary of results

The effects of anatomical site on teratoma development

Subcutaneous	Liver
Decreased size	Increased size
Increased differentiation	Decreased differentiation

The effects of ECM components on teratoma development

ECM component	Effect on proliferation	Effect on differentiation
Collagen I	Decrease	Decrease
Collagen IV	No teratoma formed	
Fibronectin	No teratoma formed	
Laminin	Decrease	Increase
Matrigel™	Increase	No change
Cultrex BME	Increase	No change
Cultrex BME RGF	Increase	No change

Table 2.3 Summary tables. Tables summarise the findings of this section.

2.4 Discussion

In this study it is demonstrated that the environment into which human pluripotent stem cells are grafted radically affects their ability to proliferate and differentiate. When transplanted intrahepatically cells proliferate rapidly and result in immature teratomas. When transplanted subcutaneously cells grow slower and result in more differentiated teratomas. The observed result that stem cells transplanted into the liver produce larger teratomas has since been recognised and validated by an independent group [266]. Transplantation of hES cells intrahepatically did not result in a teratoma but resulted in a large rapid growing tumour. In comparison, solid teratomas composed of all three germ layers were produced when cells were transplanted subcutaneously. Co-transplantation of hEC stem cells with Matrigel™, collagen I and laminin increased the success rate of teratoma formation. It was shown that the growth rate of teratomas can be modulated by the ECM component with which hEC stem cells are co-transplanted: Matrigel™ increased the growth rate whereas; collagen I and laminin decreased the growth rate. Using histological and immunohistological analysis, it was found that Matrigel™ has no effect on the composition of the teratoma, whereas, it appeared that collagen I decreased the different cell types present and laminin increased the cell types present. To validate the hypothesis that the ECM is the key in controlling cell behaviour, an alternative version of Matrigel™ was used with reduced levels of growth factor. Teratomas produced with the reduced growth factor ECM extract behaved similarly to those produced with growth factors, demonstrating that the ECM is the main component controlling cell behaviour.

The highly complex *in vivo* environment complicates the clarification of the precise factors eliciting the different teratoma production in the two transplantation sites, although some explanations for the observed differences can be proposed.

The type of location differs between the two transplantation sites. The liver is a highly active metabolic organ with a large supply of blood, which may be more conducive for cell growth than the subcutaneous environment. The locations also differ in their potential for cell expansion, the liver is a large malleable organ

enclosed within the body providing room to allow transplanted cells to expand whereas, cells transplanted beneath the skin are restricted in the dermal layer where the connective tissues will limit cell expansion.

The mechanical differences between the liver and skin may account for the differences in differentiation and proliferation that were observed. Mechanical load upon the cells plays a role in controlling their differentiation. When a 10% cyclic strain is applied to human ES cells grown on a deformable elastic substrate an increase in the undifferentiated stem cell marker SSEA-4 and the pluripotency marker Oct4 is observed [267]. It is possible that when transplanted into alternative locations the cells are subject to variations in the mechanical environment. One of the potential mechanisms for the transduction of mechanical signals in EC cells is the process whereby cell adhesion increases protein kinase C (PKC) and mediates tyrosine phosphorylation of pp^{125FAK} [268], a cytoplasmic tyrosine kinase. Studies have localised this at the sites of focal adhesions. When cells are treated with a tyrosine kinase inhibitor there is a decrease in cell adhesion with a corresponding decrease in the formation of stress fibers suggesting that pp^{125FAK} control cell attachment through involvement in the formation of stress fibers [269].

It is difficult to conclusively determine whether the mechanical load experienced between these two alternative anatomical locations accounts for the differences seen as no study has investigated the mechanical properties of murine skin and liver *in situ*. One study where these two were measured under the same conditions was conducted on porcine skin and liver. Although the liver would appear to be more malleable than skin under the conditions investigated by Constantinides et al the opposite was observed and the skin demonstrated a Youngs modulas of 222kPa whereas liver demonstrated a Young's modulas of 760kPa [270]. The stiffness of the liver is known to change in response to health [271], likewise the elastic recoil of skin is known to decrease following dehydration [272] and hence the amount of hydration can affect the stiffness of the skin. Hence the health of the animals used in investigations herein could greatly vary from those used by other investigators. The lack of information of the youngs modulas of murine skin and liver makes it hard to conclusively determine whether the mechanical differences between these two locations can account for the differences observed.

The experiments were conducted using immunocompromised animals. It is possible that a low level of immunity still remained within the animals which could result in the recognition and destruction of the foreign injected cells. The liver is a known immune privileged site [273,274] and is unlikely to have any level of immunity, therefore, cells transplanted intrahepatically are not likely to be destroyed by the immune system. The skin is not an immune privileged site and cells transplanted subcutaneously could be recognised and destroyed by any residual immunity. Therefore, it may be that fewer cells are successfully transplanted subcutaneously compared to intrahepatically, which would result the formation of a smaller teratoma.

The liver is an organ with substantial regenerative capacities and the capability to fully regenerate following the removal of up to 65-70% of the liver [275,276]. Upon injury of the liver several molecules are upregulated, including stem cell factor (SCF) [277]. SCF is located in the liver where it is bound to the membrane in its inactive form, upon liver damage it becomes cleaved by cell surface enzymes and released from the membrane upon which it becomes active. In the active form SCF acts as a mitogen to repair cellular damage and acts upon hepatocytes to increase their proliferation and repair the liver. SCF has been immobilised to surfaces to enhance proliferation [278]. Upon injection of stem cells intrahepatically this damage could cause SCF to become active and act upon the transplanted stem cells, this would account for the larger teratoma produced. When cells are transplanted subcutaneously there is no such increase in molecules that will influence cell proliferation. If the transplanted cells in the liver are being promoted to proliferate this would account for the higher level of immature cells when compared to those produced following subcutaneous transplantation.

Following tumour metastasis different organs will form tumours more readily than others, there are several factors which account for these differing tumour formation rates including: tumour cell adhesion to organ cells; survival; and proliferation [279,280]. Following the tail vein injection of the murine EC stem cell line F9 the majority of tumours are formed in the liver with relatively few in the lungs [281]. It has been proposed that the ECM of the liver provides favourable interactions with

the transplanted cells which promote tumour formation. Extraction of ECM from liver, lungs and kidney and subsequent adsorption onto culture surfaces provide a method for investigating how EC stem cells interact with the ECM from these organs. It was demonstrated that compared to ECM extracted from lung and kidneys the ECM from liver provides a more favourable surface for cell adhesion and increased proliferation of EC stem cells. Further investigations isolated the component that increased EC stem cell proliferation within the ECM [282]. These studies were conducted either *in vitro* or by injecting into the tail vein, which is dissimilar to the investigations conducted herein. However, they demonstrate the liver ECM has the potential to both increase cell attachment and stimulate proliferation. If the liver is capable of increasing cell attachment, it follows that transplantation into the liver will allow greater cell attachment of cells and also stimulate proliferation in comparison to transplantation into the skin. These observations are inline with those observed here and validate the observation made that the ECM is a key factor in teratoma formation.

Consistent with previous studies, Matrigel™ increased the success rate of teratoma formation [69,257]. Collagen I also increased the rate of teratoma formation but to a lesser extent than the increase seen with Matrigel™ [203,258]. The success rate of tumour formation could be due to: increased cell survival prior to and during transplantation; increased stimulation of transplanted cells; or a combination of both. Cell survival could be increased by gel formation of the ECM component reducing shear forces experienced by the cells upon exit from the needle during injection. However, gel formation was not a common factor in teratoma formation as teratomas were also produced when injected with laminin, which does not form a gel, and teratomas were not produced when cells were co-transplanted with collagen IV, which forms a gel readily. Matrigel™ is a mixture of ECM proteins and growth factors, when the growth factors are removed Matrigel™ continues to be effective [258]. No group has conclusively demonstrated the method by which Matrigel™ increases the rate of tumour formation and comparison of Matrigel™ to synthetic 3D gels could provide some way of solving the complex mechanisms of this system.

In order for cells to function correctly they must attach and interact with surrounding cells. One method by which cells interact with surrounding cells is the formation of focal contacts. One of the first observations that showed the existence of focal contacts was by Ambrose in 1961 [283]. Surface contact microscopy can be used to visualise parts of cells that are attached to surfaces. Using this technique, it was apparent that cells do not attach to a surface using the whole of their surface and discrete focal contacts are made. Interference reflection microscopy is similar to surface contact microscopy in that it shows the areas closer to the surface. Interference reflection microscopy was used to confirm these results and similar to the observations of Ambrose, cells were found to adhere to surfaces at discrete focal contacts [284]. Integrins binding is one mechanism of cell-cell interactions. Integrins mediate their effects via a whole host of adaptor proteins. The first protein to bind is tensin, tyrosine kinase FAK also binds early following integrin clustering. Ligand binding to the clustered integrins results in the recruitment of cytoskeletal components such as vinculin, talkin and α-actinin. Ligand binding and tension induce phosphorylation mediated by members of the Src protein kinase family (c-Src, Csk and Fyn) leading to activation of multiple molecules including: Rho-like GTPase; phospholipase-Cγ (PLCγ); and the ERK and JNK signaling cascades. The ability of cells to survive and proliferate in the absence of anchorage has strong correlations with tumourgenicity. Anchorage independence growth is a key step in becoming tumourgenic [285]. When cells that normally grow under adhesive conditions lose their contacts they can undergo anoikis. As shown herein, removal of EC stem cells from their culture surface can induce cell death, EC stem cells removed from their culture surface and suspended in a gel provides necessary contacts to promote cell survival. Therefore suspension of EC stem cells in a gel such as Matrigel™ could promote cell survival and therefore increase the number of viable cells being delivered to the transplantation site, thus accounting for the increase in teratoma size.

This data suggests that Matrigel™ increases hEC stem cell proliferation whereas collagen I and laminin decrease hEC stem cell proliferation. Matrigel™ is known to contain a large array of growth factors, including: bFGF; EGF; IGF-1; PDGF; NGF; and TGF-beta [192]. It has been demonstrated that bFGF [286] and EGF [287] increase proliferation of EC cells whereas, IGF-1 has no effect on EC proliferation

[288]. However, co-transplantation of hEC stem cells with a Matrigel™ equivalent Cultrex BME and Cultrex BME RGF shows that growth factors are not essential in increasing the success rate of teratoma formation and also does not affect the composition of the teratomas produced.

Differentiation was also affected: laminin increased the developmental potential; and collagen I decreased the developmental potential. It has been demonstrated that the secretion of collagen IV, laminin and fibronectin change as EC cells differentiate [179]. Embryonal carcinoma cells lines are not alike but vary in their capacity for differentiation and growth [33]. Cartilage formation *in vivo* has been observed from the EC cells GCT27X-1 [68] and NSR Cl 35 [33], prior to this study TERA2.cl.SP12 cells have not been demonstrated to form cartilage *in vivo*. This study presents the first evidence that TERA2.cl.SP12 can differentiate into cartilage. However, only one experiment has been conducted to investigate the effects of ECM components when co-transplanted with stem cells, Matrigel™ was found to increase the success rate of teratoma formation, no effects on differentiation were noted [69].

The observation of the formation of collagen nodules from EC cells co-transplanted with laminin is of particular interest. The formation of collagen nodules from NTERA2 (the parent line of Tera2.cl-SP12 cells) *in vivo* has not been reported. There has been one reported example of collagen nodule formation *in vivo* from a sub-line of NTERA2 [289]. It has been reported that the local environment can influence collagen formation by ES cells. Transplantation of ES cells into the knee increases the relative percentage of collagen formed by the cells [74].

Teratoma formation can be used to classify the potency of stem cells however, this approach is limited. Care should be taken when interpreting cell potency using the teratoma model as the factors required to guide development of a cell may not be present in a certain location. Transplantation into an alternative site could provide the necessary factors and show a more complex differentiation capacity. Detailed examination of stem cell behaviour in response to these ECM components is required to fully understand the observations made *in vivo*.

COMPARISON OF RESULTS GAINED TO HYPOTHESIS UNDER INVESTIGATION

It was hypothesised that the anatomical site into which stem cells are transplanted would influence teratoma behaviour and that due to the known regenerative capacity of the liver, it was predicted that transplantation of EC stem cells and ES cells intrahepatically will produce large teratomas. Large teratomas were indeed produced; however, there was no identifiable teratoma material when hES cells were transplanted.

Matrigel™ has been shown to increase the success rate of tumour formation, a similar trend was observed for hEC stem cells. It was hypothesised that individual components, from this cocktail of ECM components, when co-transplanted with hEC stem cells, would influence teratoma success rate and behaviour similar to Matrigel™. Collagen IV and laminin are components of Matrigel™, however, only laminin was found to increase the success rate of teratoma formation. It was hypothesised that with the removal of growth factors from Matrigel™ would affect the behaviour of transplanted cells; however no difference was observed, demonstrating that the ECM is a major factor controlling the cells. Neither of the two ECM mimics used: Extracel-X™ and PuraMatrix™ had an effect on teratoma success rate and teratoma formation was not observed. Previously, using condrocytes, when PuraMatrix™ is compared to two natural scaffolds, a telopeptide collagen I and alginate (from seaweed), it does not increase proliferation as well as the natural scaffold, furthermore, histological analysis showed that the gels formed by PuraMatrix™ were more sparsely laden with cells. The Young's modulus of the PuraMatrix™ gel was lower than those formed by the natural molecules demonstrating that the gel is weaker [290]. A weak gel formed and no increase in the rate of proliferation could explain why there is no effect on teratoma formation.

CHAPTER 3 – EVALUATING BIOMIMETIC SURFACES FOR CELL CULTURE AND THE CONTROL OF CELLULAR BEHAVIOUR

3.1 Introduction

Laboratory cultured cells are widely used in the field of biomedical science and provide scientists with model systems to investigate cell activity. The process of cell culture involves the removal of cells from their natural (*in vivo*) location and their subsequent growth in an artificial (*in vitro*) environment. Optimisation of the culture environment is critical to enable cells to grow, function and survive in a realistic and representative manner. There are many parameters that must be considered in this process including the constituents of the media, growth supplements, and the surface upon which cells grow. Often, it is necessary to tailor the culture environment for optimal growth survival and differentiation of a particular cell type. Some cell types do not attach to standard tissue culture plastic or glass unless it is coated with an appropriate growth promoting molecule that more accurately represents the environment their native counterparts' experience.

Within tissues, cells are in contact with other cells and the ECM, both of which play an important role in controlling cell behaviour. Interactions between cells and ECM components are important in many biological processes such as cell growth and development [291-293]. These interactions can be recreated *in vitro* by coating surfaces with ECM molecules [17]. In general, cells utilise integrins, a family of transmembrane glycoproteins, to attach to specific cell binding sites that are displayed by ECM proteins such as collagen, fibronectin and laminin. The integrins are able to link the cell cytoskeleton to a material surface via these proteins which are present on the surface. The adsorption or immobilisation of specific ECM proteins can therefore be used to enhance cell attachment. In order to mimic the *in vivo* environment, purified components of the ECM are frequently used in the laboratory to coat cell culture plastic or glass to enhance cell adhesion [17].

Previous work has demonstrated that immobilisation of whole ECM proteins, such as fibronectin, results in conformational changes in the molecule [210,211]. Alteration of the molecular conformation can result in decreased ECM activity by reducing the availability of functional domains to the cell [212]. In some instances of coating surfaces with whole ECM proteins, multiple layers of molecules are produced instead of a single monolayer [213]. This can result in some molecules being hidden by those above and it can become difficult to determine the actual amount of ECM protein available to interact with the cultured cells. The biological effects elicited by key components of the ECM can be attributed to certain short peptide motifs within the whole molecule [294-299]. It is possible to present these motifs to imitate the behaviour elicited by the whole molecule. Peptides can be immobilised to surfaces in a controllable manner with a defined conformation and hence it is sometimes advantageous to present the functional peptide motif(s) as opposed to the whole ECM molecule. Many factors contribute to the effective presentation of peptide motifs within biomimetic surfaces: including density of the molecules [300]; the nature of the substrate supporting the biomimetic surface [214]; the distance presented motifs are from the surface [105,301,302]; and the conformation of the presented molecules on the surface [223,303]. Accordingly, the method by which ECM peptide motifs are presented by the biomimetic surface is extremely important.

There are numerous methods by which such peptide motifs can be immobilised onto a surface [224] including, direct adsorption of protein from solution [225] or by modifying the molecular characteristics of the surface using complex chemistry [226]. However, these approaches have their limitations: the surface coating process is difficult to control; adsorption may lead to denaturation of the protein resulting in reduced functionality; the orientation and density of the protein are difficult to control leading to batch inconsistency; and reduced reproducibility. Covalent attachment of protein by chemical coupling also results in substantial loss of protein activity owing to chemical modification of critical residues; denaturation, random orientation and inaccessibility [209]. These limitations are problematic in cell culture and consequently, there is demand for improved surfaces for *in vitro* cell growth that possess known composition and more accurately represent a biologically relevant environment. Such surfaces must be manufactured in a

controlled fashion to reduce batch variability and increase the consistency and robustness of the cells cultured on such surfaces.

A proposed improvement upon current technologies for presenting peptides to cells is that developed by Orla Protein Technologies [229]. The technology employs engineered variants of the N-terminal transmembrane (TM) domain from the *Escherichia coli* outer membrane protein, OmpA [230], motifs from ECM proteins are engineered into the flexible outer loops OmpA and the protein is attached to the surface via a single cysteine residue thereby forming an oriented monolayer. Gaps between protein molecules are filled in using thiolipids or thioalkanes so that only the surface loops and the motifs of interest are exposed (figure 1.10). This method of protein immobilisation results in stable and oriented monolayers that display only the motifs of interest in a functional conformation [230] which alleviates many of the problems associated with adsorption and chemical methods [231].

To evaluate the effectiveness of this technology the adherence of the neuron-like rat pheocytochroma cell line, PC12, on OmpA surfaces presenting peptide motifs from ECM proteins is investigated. PC12 cells are known to attach poorly to uncoated glass and in order for the cells to attach and grow as an adherent monolayer the culture surface must be coated with ECM proteins [293,304]. To demonstrate that motifs are presented in a functional manner, the ability of ECM protein motifs derived from, collagen, fibronectin and laminin are presented by OmpA molecules and the rescue of PC12 cell attachment is determined. Comparisons to control surfaces presenting no motifs are made and surfaces which increase cell attachment demonstrate that the motifs are presented in a manner which cells can recognise.

In addition to cell attachment, PC12 cells are a widely used model system to study neurite behaviour as they are known to produce neurites in response to NGF [305]. PC12 cells have been used to investigate effects on: neurite length; neurites per cell; and branches per neurite [306]. It is known that the culture of PC12 cells on surfaces coated with different ECM proteins affect their neurite behaviour. Preparations of collagen I gels can be made for the culture of PC12 cells. These gels can be modified with the addition of other proteins, such as laminin or

fibronectin. In comparison to the unmodified collagen I gel, there is little effect on PC12 neurite length when laminin is added, however upon the addition of fibronectin a reduction occurs [307], thus demonstrating that ECM proteins can influence PC12 neurite behaviour. PC12 neurite outgrowth is investigated in response to biomimetic surfaces presenting motifs from collagen I, collagen IV, laminin and fibronectin. This allows investigation of how these motifs affects neurite behaviour and also provides further validation that the motifs investigated are presented in a functional manner which cells can recognise.

The differentiation of cells in tissues is affected by a complex combination of different factors within their local environment including: soluble molecules; local geometry; and interactions with the ECM. In combination these factors form a niche that allows correct cellular development and tissue function[308]. Direct evidence that local micro-environments determine the development of certain cell types has been demonstrated when neural progenitor cells transplanted into the subventricular zone, rostral migratory stream, hippocampus or striatum undergo neurogenesis in a similar fashion to endogenous cells [309], for example, cells in the subventricular zone of the brain grow in contact with ECM proteins, collagen I and laminin [310]. Combined with the correct culture media, immobilisation of the ECM proteins, entactin, collagen, and laminin, onto the culture surface partially recreates *in vivo* growth conditions and allows the differentiation of murine embryonic stem cells into cells characteristic of the subventricular zone [311].

NSPCs are of great interest as they are considered a potential source of cells for clinical applications, notwithstanding, little work has been conducted to investigate these cells fully. Several studies have investigated the effect of ECM components on neural cells [214]. However, fewer examples have been demonstrated of how peptides can be used to control these cells. Multiple motifs from laminin (RGD, YIGSR) and bone marrow homing proteins have been used to control neuronal cells [312]. Using rat neural stem cells it has been demonstrated that the peptide RGD produces similar levels of neuronal and glial differentiation to PLO/laminin, conversely the laminin peptide IKVAV was found to have no effect. Upon mixing the active RGD and the inactive IKVAV motif the greater the proportion of

inactive IKVAV the lesser the effect the surfaces have on glial and neuronal differentiation [300].

Although the presentation of individual motifs provides a simplified system for the study of cell behaviour, this could be considered far too simplified and the presentation of multiple motifs on a single surface more faithfully represents the *in vivo* environment. Several groups have studied the effects of presenting multiple motifs on a single surface and both intuitive and counterintuitive effects have been noted [313,314].

Through presenting specific ECM peptides either individually or in combination, we can identify those domains that play an important role in the differentiation of cultured neural tissues. This approach can be used to assist in the development of defined culture conditions for the generation of specific cell types for use in research and tissue engineering.

HYPOTHESIS UNDER INVESTIGATION

The main hypothesis that is under investigation is that the presentation of motifs from ECM proteins via biomimetic surfaces will influence cell behaviour. It is hypothesised that not all motifs will be effective in influencing all cell types and some motifs may not be presented in an orientation to which cells can recognise and bind. As it is known that surfaces coated with the ECM proteins collagen I, collagen IV, fibronectin and laminin all increase cell attachment of PC12 cells it is hypothesised that the presentation of these motifs will increase cell attachment. Furthermore, as the ECM protein collagen I is more effective than the ECM protein fibronectin, it is expected that attachment to the corresponding peptide motifs will have a similar effect. It is hypothesised that presentation of motifs via biomimetic surfaces will also influence neurite outgrowth, however it is predicted that the most effective motifs will be those that will be able to influence PC12 cell attachment. The most effective motifs will then be assayed for their potential to control neural stem/progenitor cells and surfaces composed of mixed motifs will be used to increase their effect. It is hypothesised that presentation of multiple

motifs from different ECM proteins will have an increased effect when compared to individual motifs.

AIM

To evaluate the presentation of motifs by OmpA biomimetic surfaces to identify motifs that are presented in a functional manner to cells and demonstrate that these surfaces can be used to control neuronal attachment and differentiation.

OBJECTIVES

1. Evaluate whether biomimetic surfaces can control the attachment of cultured cells

2. Evaluate whether this technology can control differentiation of neuronal cells

3.2 Materials and methods

Cell culture

PC12 cells

Cultures of PC12 cells were purchased from American Type Culture Collection (ATCC; http://www.lgcpromochem-atcc.com) and were maintained according to a previously described method [315]. Cell stocks were grown on cell culture plasticware (VWR; http://uk.vwr.com) pre-coated for 24 hours at 37°C with a 0.1% solution of collagen IV from human placenta (Sigma-Aldrich; http://www.sigmaaldrich.com) in 0.1M glacial acetic acid (Sigma-Aldrich). PC12 cells were grown in RPMI 1640 media (Cambrex; http://www.cambrex.com) supplemented with: 10% heat-inactivated foetal bovine serum (Invitrogen; http://www.invitrogen.com); 2mM L-glutamine (Cambrex); 20 units/mL of penicillin and 20 µg/mL streptomycin (Cambrex). Cultures were maintained at 37°C in a humidified 5% CO_2 incubator, the medium was changed three times a week. The cells were passaged as required using trypsin-EDTA solution as standard (Cambrex).

Neuroblastic cells

SH-SY5Y cells (ATCC) were maintained using RPMI 1640 (Cambrex) supplemented with: 15% heat-inactivated foetal bovine serum (Invitrogen); 2mM L-glutamine (Cambrex); 20 units/mL of penicillin and 20 µg/mL streptomycin (Cambrex). Cultures were maintained at 37°C in a humidified 5% CO_2 incubator, the medium was changed three times a week. The cells were passaged as required using trypsin-EDTA solution as standard (Cambrex).

Murine fibroblast cells

Murine fibroblast cells (ATCC) were maintained using DMEM (Sigma) supplemented with: 10% heat-inactivated foetal bovine serum (Invitrogen); 2mM L-glutamine (Cambrex); 20 units/mL of penicillin and 20 µg/mL streptomycin

(Cambrex). Cultures were maintained at 37°C in a humidified 5% CO_2 incubator, the medium was changed three times a week. The cells were passaged as required using trypsin-EDTA solution as standard (Cambrex).

Osteoblast-like cells

Cultures of MG63 (ATCC) were maintained using DMEM (Sigma) supplemented with: 10% heat-inactivated foetal bovine serum (Invitrogen); 2mM L-glutamine (Cambrex); 20 units/mL of penicillin and 20 μg/mL streptomycin (Cambrex). Cultures were maintained at 37°C in a humidified 5% CO_2 incubator, the medium was changed three times a week. The cells were passaged as required using trypsin-EDTA solution as standard (Cambrex).

Coating glass coverslips with purified ECM proteins

Borosilicate glass coverslips (VWR) were first sterilised by immersion in 70% ethanol for 15 minutes and then washed twice with sterile PBS (Cambrex). Coverslips were coated with solutions of the following at concentrations ranging from 1ng/ml to 1mg/ml: collagen I (Sigma-Aldrich) diluted in 0.1M glacial acetic acid; collagen IV (as previously described above); fi
bronectin (Sigma-Aldrich) diluted in sterile ddH_2O; or laminin (Sigma-Aldrich) diluted in sterile PBS (Cambrex). Coverslips were immersed in the coating solution for 24 hours, collagen and laminin coatings at 37°C and fibronectin coating at room temperature. Coverslips were immersed in the coating solution for 24 hours. Surfaces were then washed twice with PBS to remove any traces of un-adsorbed molecules and placed in fresh multi-well culture plates.

Protein engineering

The peptide motifs in ECM components most likely to enhance cell attachment were identified from literature (see Table 1). Oligonucleotides designed to encode the relevant motifs were synthesised (Sigma Genosys; http://www.sigmaaldrich.com/Brands/Sigma_Genosys.html). Annealed oligonucleotides were ligated into the gene encoding the modified OmpA TM

domain scaffold such that the motif was within an outer loop of OmpA. Four different plasmids were constructed with motifs as shown in Table 1. The parental modified OmpA was used as a negative control. The DNA sequence of each construct was verified before use. All constructs thus possess a 6xHis tag and a cysteine residue at the N-terminus.

Expression, purification and refolding

Exponential phase cultures of *E. coli* BL21 (Novagen; http://www.merckbiosciences.co.uk) containing expression plasmid were induced with isopropyl-β−D-thiogalactopyranoside and shaken for six hours at 37°C. After harvest and lysis, the insoluble fraction was enriched and solubilised in the binding buffer (8M Urea, 20 mM imidazole, 0.5 M NaCl, 20 mM sodium phosphate pH7.4). The OmpA based proteins were purified by Ni-affinity using an AKTA Prime system (GE Healthcare; http://www.gehealthcare.com/worldwide.html) with a step elution at 250 mM imidazole. The fractions containing the desired proteins were further purified on a QFF anion exchange column (GE Healthcare) in 30 mM ethanolamine buffer at pH9.5 with elution in the same buffer plus 80mM NaCl. The proteins were shown to be >98% homogenous as judged by gel electrophoresis (data not shown). The proteins were refolded by a very slow dilution (1/40) into the refold buffer (50 mM Tris HCl, 0.1 mM EDTA, 1% n-octyl glucopyranoside, 1 mM dithiothreitol pH8.0). Correct refolding was indicated by band shift on SDS polyacrylamide gel electrophoresis [230] and correct formation of the β-barrel structure confirmed by circular dichroism spectroscopy (data not shown).

Preparation of culture surfaces

The protein-thioalkane monolayer was manufactured essentially as described [229] Figure 1. Borosilicate glass coverslips (VWR) were cleaned, sputter coated with a 10nm layer of titanium followed by a 25nm layer of gold and cleaned in 1% Hellmanex® (Hellma; http://www.hellma.co.uk). The surfaces were passivated by incubation with 1% (v/v in ddH_2O) 2-mercaptoethanol (Fisher; http://www.fisher.co.uk) and washed with ddH_2O. OmpA proteins were diluted to

2 μg mL^{-1} in refold buffer. Hexadecanethiol (HDT, Sigma) was diluted to 0.15 mg mL^{-1} in ethanol. Protein was applied to the surface in three 1 hour incubations with 1% SDS and ddH_2O washes between each protein application. After the last protein application the gaps between the immobilised protein molecules were in-filled by overnight incubation with HDT solution followed by washes with ethanol followed by 1% SDS (Sigma) and finally ddH_2O. The coverslips with fully assembled surfaces were dried under nitrogen gas and stored at 4°C with desiccant until required. Control surfaces with only HDT or untreated gold were also prepared and stored. Prior to use, the coverslips were sterilised by immersion in 3ml 70% ethanol for 15 minutes followed by two washes with 5ml sterile phosphate buffered saline (PBS, Cambrex).

Cell attachment assay

PC12 cells growing on cell culture plastic were removed by trypsination to produce a single cell suspension and the number of cells was determined using a haemocytometer. Cells were subsequently seeded at a density of 300,000 cells/well in a 6-well culture plate (VWR) containing alternative growth surfaces. Cells were maintained at 37°C in a humidified, 5% CO_2 incubator for 24 hours. Cultures were washed once with 2ml PBS and fixed using 4% paraformaldehyde (Sigma-Aldrich) in PBS for 30 minutes, washed twice with PBS and mounted using 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) in Vectashield® (Vector Laboratories; http://www.vectorlabs.com). Cells were visualised using a Nikon Diaphoto 300 fluorescence microscope and digital images recorded using a Nikon Digital Camera DXM1200. Nine random fields of view were sampled using the 10x objective lens for each surface tested. Experiments were repeated in triplicate.

PC12 cell differentiation

In preparation for differentiation, a single cell suspension of PC12 cells was achieved by trypsinisation followed by two media washes to remove any trypsin and the number of cells determined using a haemocytometer. Cells were seeded at a density of 300,000 cells/well in a 6-well culture plate (VWR) containing alternative growth surfaces. Cells were maintained at 37°C in a humidified, 5%

CO_2 incubator for 24 hours. The medium was replaced with 50ng/ml nerve growth factor (NGF, Sigma-Aldrich) containing media and the cells were differentiated for ten days with medium changes every three days. Immunocytochemical analysis was carried out as detailed below. Experiments were repeated at least three times. Images were captured of 20 randomly selected single differentiated PC12 cells per condition. Cells were examined and the number of neurites per cell body; the length of the longest neurite; and the number of neurite branches were determined and recorded. Neurite branching data was normalised to neurite length to take into account that longer neurites are more likely to have an increased number of branches [306].

Culture and differentiation of neural stem/precursor cells

NSPCs were isolated from the subventricular zone of the lateral ventricles of adult eGFP transgenic male Wistar rat forebrains, as described previously [61]. Subependymal tissue was harvested from 8-12 week old rats and subjected to papain dissociation (Papain Dissociation System; Worthington Biochemical Corporation, Lakewood, NJ, USA; http://www.worthington-biochem.com). The resultant cell suspension was centrifuged and the pelleted cells were subjected to a discontinuous density gradient to remove cell debris. Dissociated cells were re-suspended in complete medium (CM) containing Neurobasal™ media (Invitrogen); B27 neural supplement (Invitrogen); 2 mM L-glutamine (Sigma-Aldrich); 100 µg/ml penicillin-streptomycin (PenStrep; Sigma-Aldrich); 20 ng/ml epidermal growth factor (recombinant human EGF; Gibco-Invitrogen); 20 ng/ml basic fibroblast growth factor (recombinant human bFGF; Invitrogen); and 2 ng/ml heparin (Sigma-Aldrich) and incubated at 37°C in a humidified incubator with 5% CO_2. GFP-positive neurospheres appeared in 2-3 weeks, after which cells were passaged every week.

Neurospheres were mechanically dissociated to produce a single cell suspension and the number of cells was determined using a haemocytometer. Cells were subsequently seeded at a density of 400,000 cells/well in a 6-well culture plate containing alternative growth surfaces. Cells were maintained at 37°C in a humidified 5% CO_2 incubator for 24 hours in CM. The medium was replaced with

differentiation medium containing: Neurobasal™ media; B27 neural supplement; 2 mM L-glutamine; 100 μg/ml penicillin-streptomycin; and 1% foetal bovine serum (FBS, Invitrogen); cells were differentiated for 4 days. Immunocytochemical analyses were carried out (see below), cells were visualised using a fluorescence microscope and digital images recorded. All experiments were repeated in triplicate. Images were captured of nine random fields of view per condition. Neuronal differentiation was assessed by the number of βIII positive cells observed per field of view.

Statistical analysis

The distribution of data was tested using the Kolmogorov-Smirfnov test. For data that were not normally distributed, subsequent post-hoc analysis was carried out as described by Zar [316]. Data that was normally distributed was analysed using ANOVA. $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were used to indicate levels of statistical significance.

3.3 Results

Controlling attachment using biomimetic surfaces

3.3.1 Attachment of multiple cell types on infill biomimetic surfaces

Different cell types require different culture conditions therefore it is important to use numerous cell types to gain a correct understanding of how these biomimetic surfaces control cell behaviour. Biomimetic surfaces are composed of two aspects that can control cell attachment: peptide motifs and infill molecules. To gain a greater understanding of cell responses to these biomimetic surfaces, cell behaviour of different cell lines was evaluated on biomimetic surfaces presenting infill molecules without any peptide motifs present. Surfaces were prepared presenting the following molecules: mercaptoundecanoic acid (MUDA) which is hydrophilic (due to the terminal carboxylic acid group); hexadecane thiol (HDT) which is hydrophobic (due to the terminal methyl group); and a thiolipid (DPPE) which is a lipid membrane mimic. In all tested cell types, surfaces presenting HDT were found to inhibit cell attachment when compared to gold coated surfaces. In some cell types MUDA was found to increase cell attachment when compared to uncoated surfaces however, in two cases the level of cell attachment to MUDA presenting surfaces was equal to or lower than the attachment to uncoated surfaces. In three of the four cell types examined DPPE was found to have an intermediate effect on cell attachment resulting in a level between the negative effect produced by HDT and the positive effect produced by MUDA (Figure 3.1).

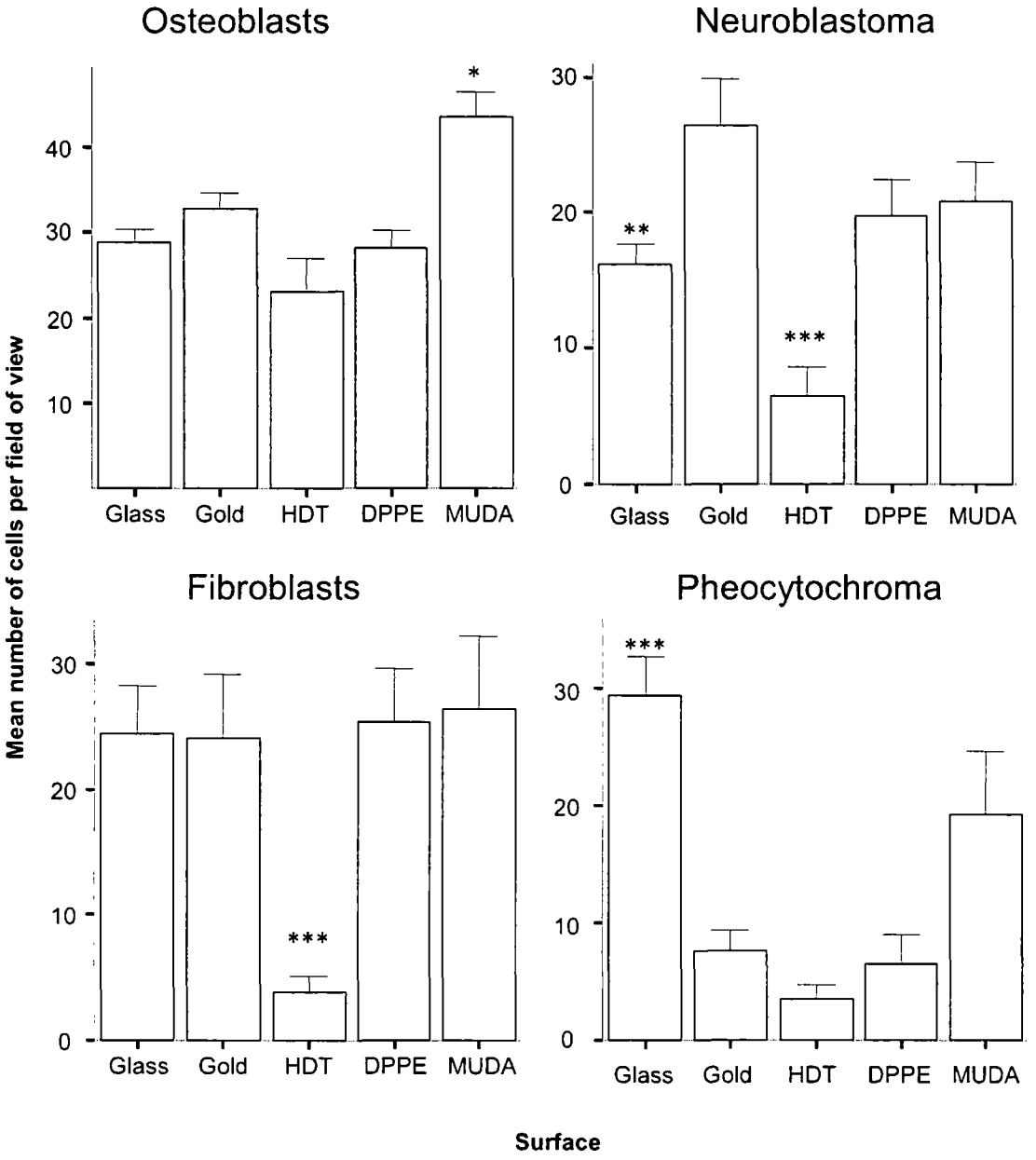

Figure 3.1 Different cell types respond differently to similar surfaces. Attachment was compared following 24 hours culture of various cell lines. The surfaces tested were: glass coverslips (glass); gold plated coverslips (gold); HDT infill coverslips (HDT); DPPE infill coverslips (DPPE); and MUDA infill coverslips (MUDA). For each of three independent experiments, nine random fields of view were examined (a total of 27 fields of view), bars represent mean values +SEM. All data was compared to gold $*p<0.05$, $**p<0.01$, $***p<0.001$

Following seven days cell culture, coverage appeared similar on MUDA, DPPE and uncoated surfaces for all cell types tested. Osteoblastic cells were shown to overcome the inhibitory effect of HDT and their attachment to these surfaces increased (Figure 3.2). Neuroblastic cells were partially able to attach to HDT

surfaces however PC12 cells and murine fibroblast cells were not able to attach to HDT regions and only able to attach to uncoated regions (Figure 3.3).

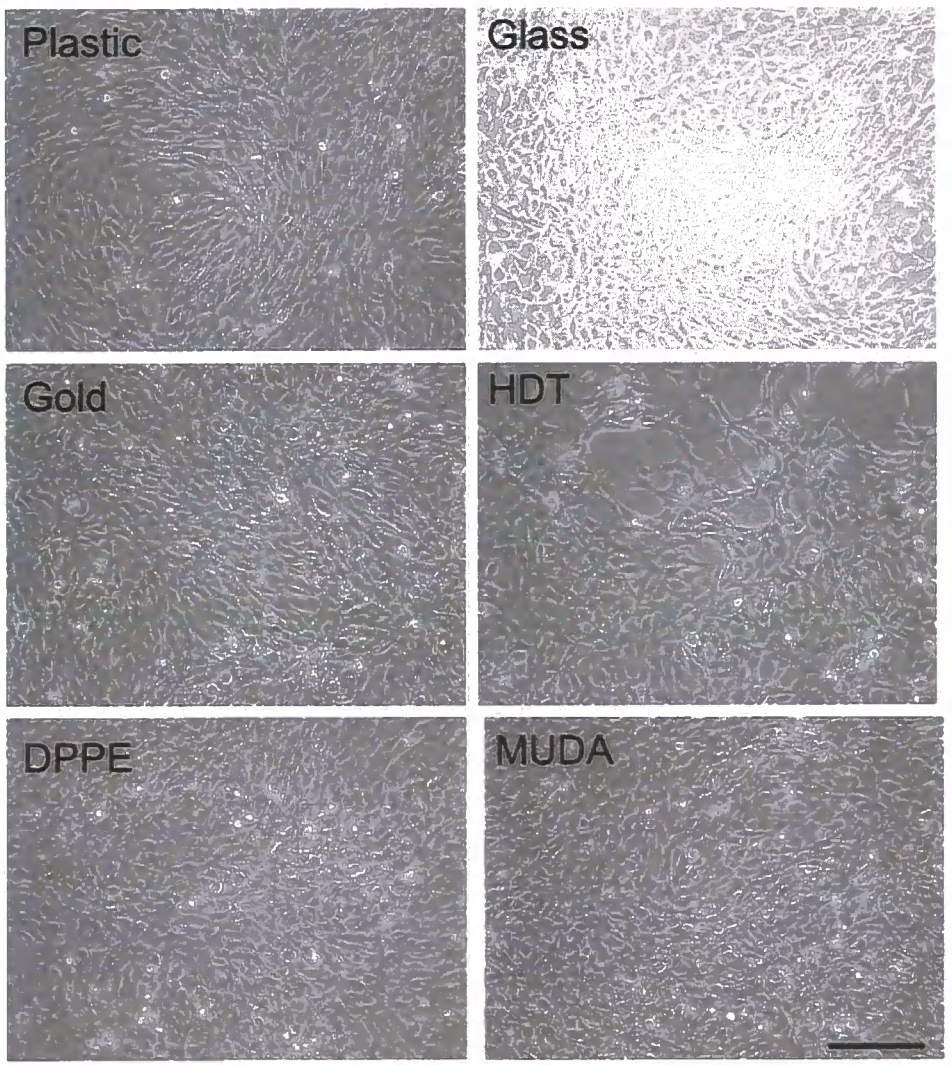

Figure 3.2 Cell attachment, growth and proliferation of human osteoblasts following seven days culture. Phase images show representative fields of view of human osteoblasts following seven days culture in proliferative medium. Scale bar 200μm.

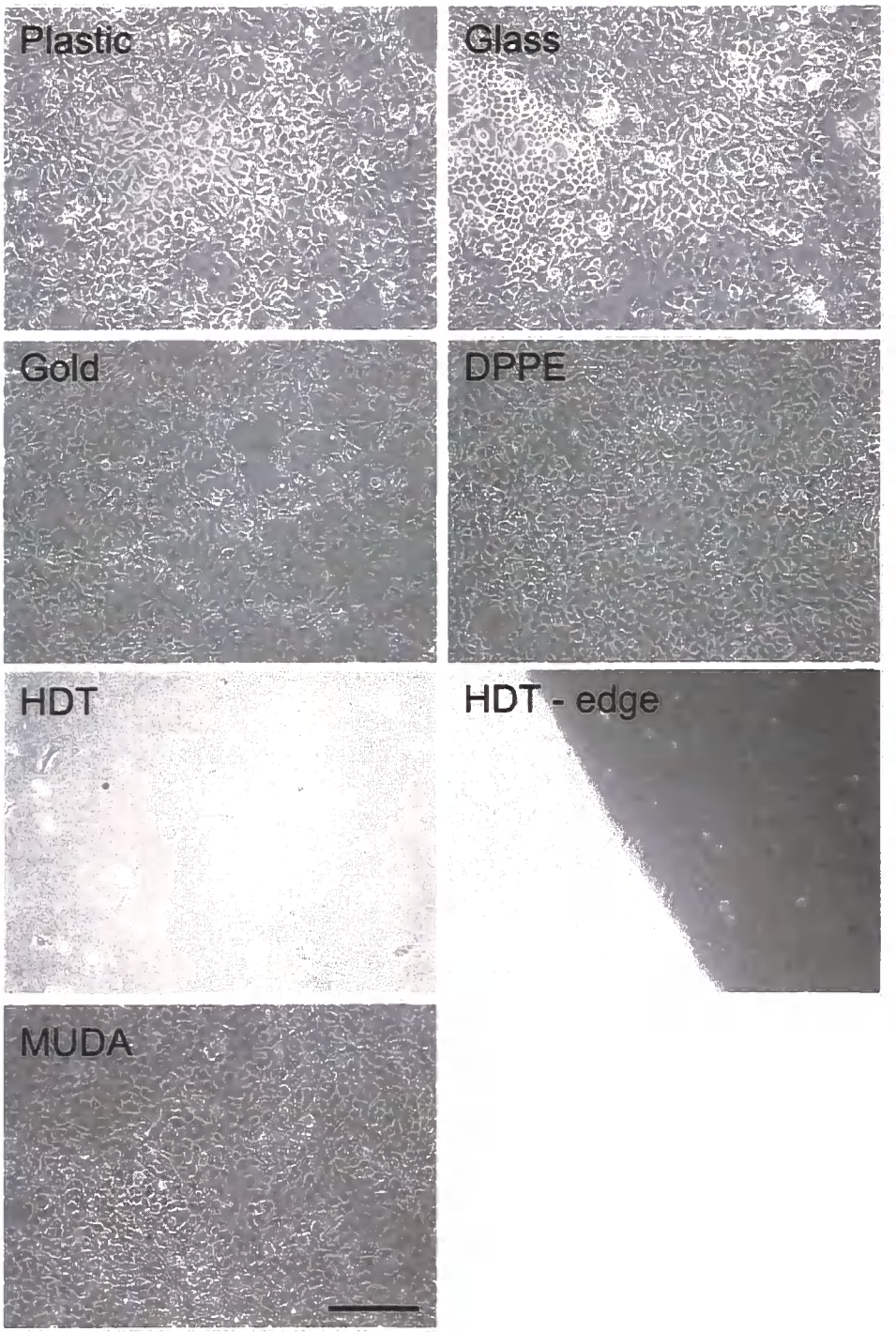

Figure 3.3 Cell attachment, growth and proliferation of murine fibroblasts following seven days culture. Phase images show representative fields of view of murine fibroblasts following seven days culture in proliferative medium. HDT-edge shows attachment to uncoated regions (left) and little attachment to HDT coated regions (right). Scale bar 200μm.

3.3.2 Comparison of PC12 cell attachment to biomimetic and control surfaces

From previously published work PC12 cells are documented as a model system for studying cell attachment [293,304]. The attachment of PC12 cells to infill surfaces was compared to a routine culture of adsorbed collagen IV. When comparing cell attachment to control levels it is clear that any differences which do arise are minimal (Figure 3.4).

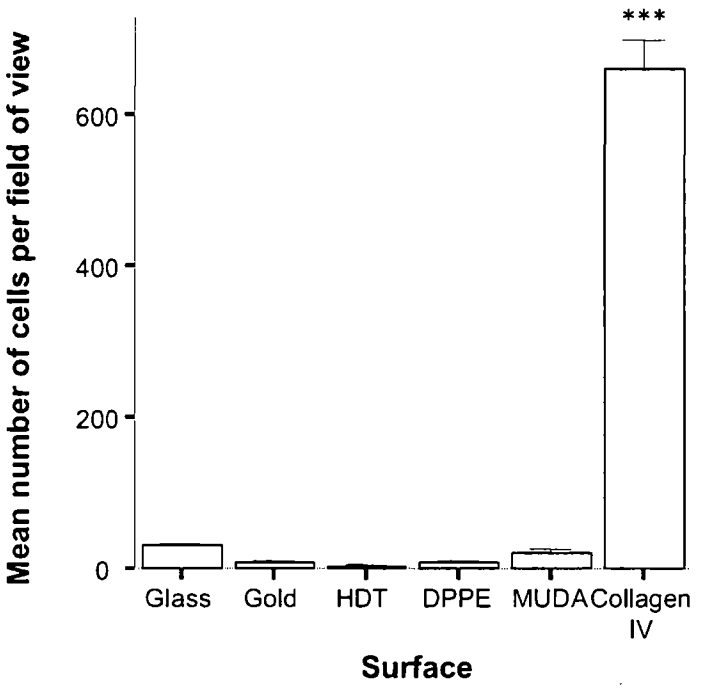

Figure 3.4 Adsorption of collagen IV to glass surfaces greatly enhances cell attachment of PC12 cells. After 24 hours cell attachment of PC12 cells to the following surfaces was compared; glass, gold, HDT, DPPE, MUDA and adsorbed collagen IV. Nine fields of view were examined. Data represents mean values +SEM of three independent experiments. All data was compared to glass*** $p<0.001$

3.3.3 Alternative ECM protein surfaces for PC12 cell attachment

Although PC12 cells are routinely cultured on collagen IV coated surfaces, alternative ECM proteins such as collagen I, fibronectin and laminin can be adsorbed onto surfaces to support the attachment of PC12 cells. However, the optimal concentration can only be determined by experimentation. Surfaces were prepared with differing protein concentrations; cell attachment was proportional to

rising concentrations of adsorbed ECM protein up to an optimal level after which no further increase in cell attachment was observed. Effective concentrations for optimal cell attachment were determined for collagen I (250μg/ml), collagen IV (8μg/ml), fibronectin (50μg/ml) and laminin (10μg/ml). In all cases (except collagen I) once the optimal concentration for cell attachment was reached a slight decrease in attachment was then observed with any further increase in concentration. This could be due to molecular aggregation taking place at these high concentrations thus reducing the effective concentration of protein (Figure 3.5).

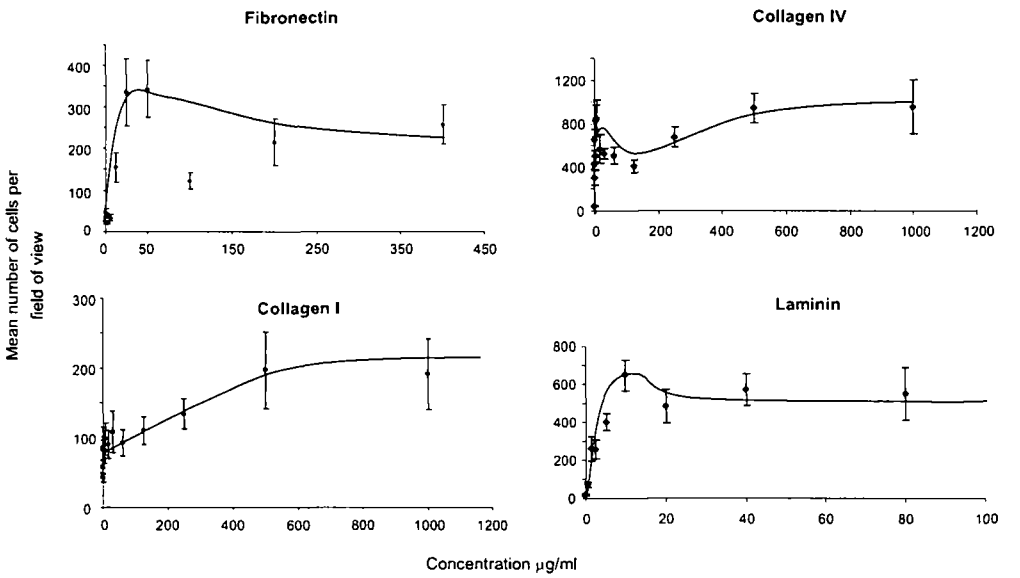

Figure 3.5 Increasing initial concentration of protein used to adsorb to surfaces increases PC12 cell attachment. As the concentration of protein is increased a corresponding increase in cell attachment following 24 hours culture is observed. However increases in protein concentrations above the optimal results in a detrimental effect on cell attachment. Data represents mean values of nine fields of view per surface ±SEM. Note different scales of *x*-axes demonstrate the effectiveness of the ECM protein tested.

3.3.4 Attachment of PC12 cells to alternative control surfaces at optimal concentration

The greatest level of PC12 cell attachment was observed on collagen IV coated surfaces. Laminin coated surfaces supported cell attachment in a similar manner to

collagen IV, with a high level of cell attachment and an even distribution of cells. On the contrary, surfaces coated with collagen I and fibronectin supported the attachment of fewer cells and their distribution was noted to be more clumped, demonstrating that cell-cell interactions are more favourable than cell-surface interactions (Figure 3.6).

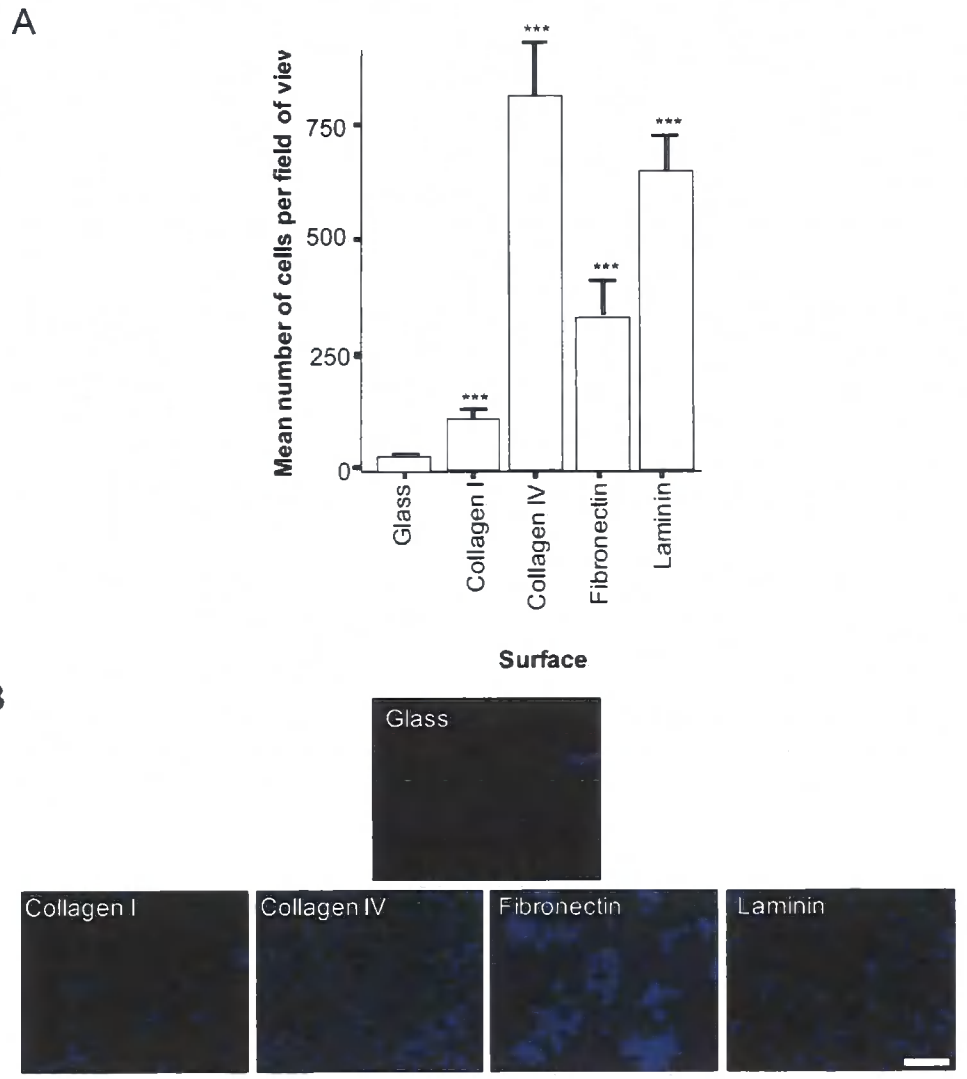

Figure 3.6 PC12 cell attachment to surfaces coated with adsorbed protein. (A) Bar chart showing the differential adhesion of PC12 cells following 24 hours culture to alternative surfaces at optimal coating concentrations including: uncoated glass; glass coated with adsorbed ECM proteins: collagen I (250μg/ml); collagen IV (8μg/ml); fibronectin (50μg/ml); and laminin (10μg/ml). Data represents mean number of cells per field of view (nine randomly selected fields were examined per surface) for three independent experiments (+SEM). (B) Fluorescence micrographs of cultures grown on either glass surfaces coated with adsorbed protein. DAPI staining revealed large numbers of cells attached to surfaces coated with adsorbed proteins;

collagen I, collagen IV, fibronectin and laminin whilst there was little if any evidence of cells adhering to glass. Scale bar: 100μm. *** $p<0.001$

3.3.5 Presentation of ECM motifs expressed in OmpA enhance cell attachment of PC12 cells

Surfaces were prepared presenting single motifs from the ECM proteins collagen I, collagen IV, fibronectin and laminin (Orla 1, 11, 31, 32, 34, 36, RGD, IKVAV, GTPGPQGIAGQRGVV, MNYYSNS, PHSRN, YIGSR respectively, Table 1.2) and the tenascin C motif (Orla 30, Table 1.2). As ECM proteins have multiple active domains, two surfaces were prepared, presenting two motifs simultaneously to more faithfully recapitulate the *in vivo* environment (Orla 35 and 37, fibronectin and laminin respectively, Table 1.2). Surfaces were infilled with HDT to minimise non-specific cell attachment and therefore any increase in attachment level would be of a direct consequence of the presented motifs.

The level of cell attachment to culture surfaces consisting of the OmpA scaffold with or without the insertion of various ECM molecule motifs was examined (Figure 1.10). Only a low level of attachment (17 cells) per field of view was observed on surfaces containing only OmpA infilled with HDT on gold (OmpA-control). Cell attachment was increased by the presence of OmpA with no motif(s) presented when compared to HDT alone. This is due to two factors: assembly of OmpA prior to HDT assembly resulted in a lower concentration of HDT present on the surface; and the outer loops of OmpA provide a roughened surface providing a topographic advantage over the flat surface presented by HDT. The inclusion of sequences from the functional domains of the ECM proteins, collagen I, collagen IV, fibronectin or laminin into OmpA significantly enhanced the attachment of PC12 cells compared to OmpA-control surfaces. When PC12 cells were cultured on adsorbed ECM proteins differences in cell spreading was observed, in agreement with this, cell spreading is greater on some biomimetic surfaces than on others. However, the trend was not analogous for each molecule/motif: cell spreading on surfaces coated with laminin or collagen IV proteins produced an even distribution of cells. In agreement the collagen IV biomimetic surface showed the least amount of clumping, conversely the laminin biomimetic surface

was not observed to produce an even cell distribution. Not all surfaces had a positive effect on cell attachment; no increase was seen from Orla 1, 30 or 11. When motifs were combined in a single OmpA molecule the level of attachment observed was similar to that of the least effect motif. Orla 36 presented singularly increased cell attachment and Orla 11 did not increase cell attachment, when the two motifs were combined in a single OmpA molecule (Orla 37). Furthermore, when presented to PC12 cells the attachment of the combined surface was similar to the least effective motif IKVAV (Orla 11). A similar trend was observed for Orla 1 combined with Orla 34 – Orla 35 (Figure 3.7).

PC12 cells are able to bind to; Orla 31, 32, 34, 36. This demonstrates that these motifs are presented in a functional manner.

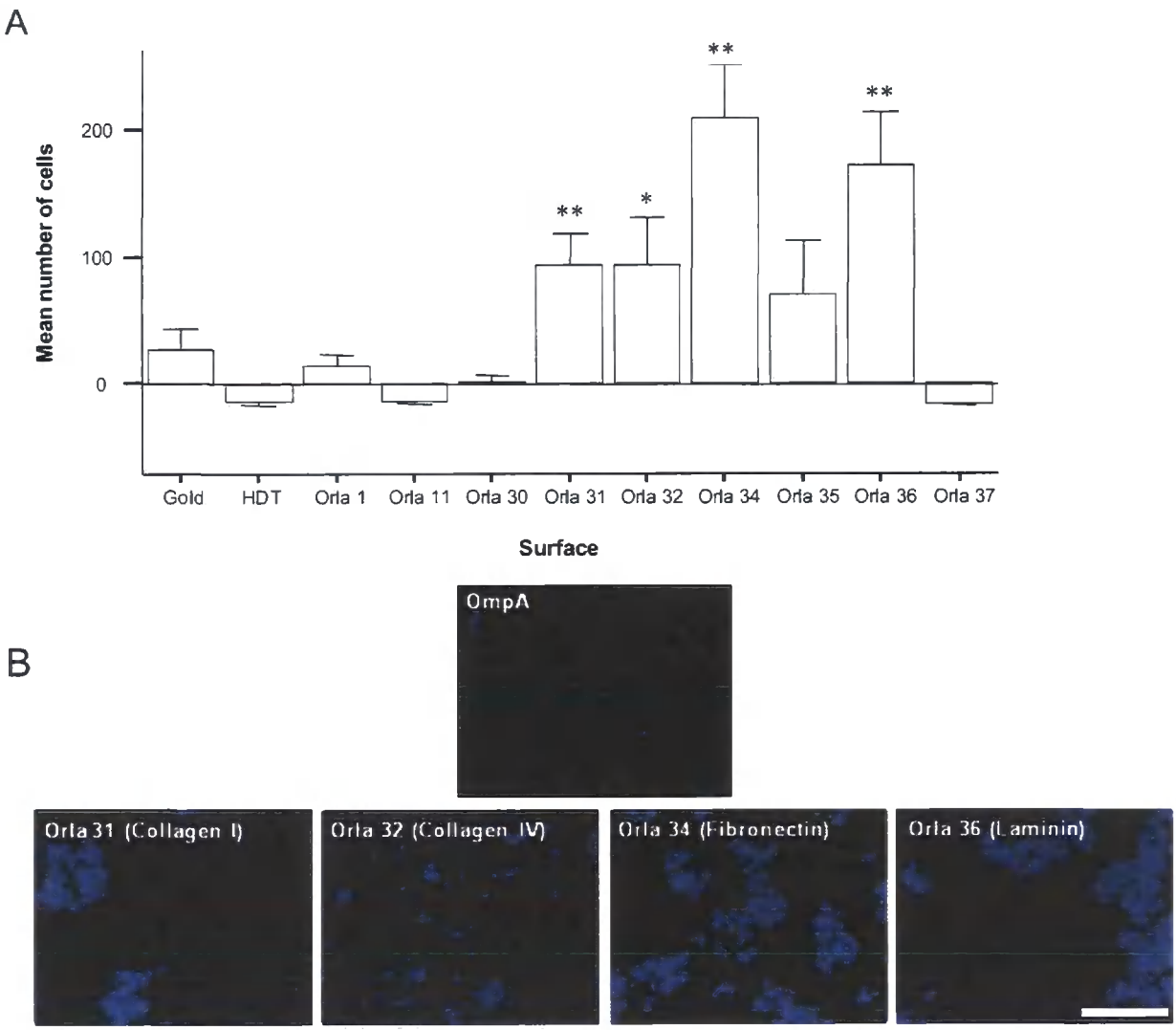

Figure 3.7 Presentation of motifs from ECM molecules increases PC12 cell attachment following 24 hours culture. Surfaces displaying ECM motifs presented by the OmpA molecule enhance the attachment of cultured cells. A) Bar chart showing mean number of cells per field of view of PC12 cells to surfaces coated with self assembled OmpA or OmpA-ECM motif protein. The protein surfaces were all completed by infilling with HDT. Data represents mean number of cells per field of view (nine randomly selected fields were examined per surface) for three independent experiments (+SEM). Values for each motif condition were normalised to the number of cells attached to surfaces coated with OmpA without a motif insertion. B) Fluorescence micrographs of cultures grown on surfaces consisting of either OmpA without any ECM motif insertion or OmpA carrying a motif from the ECM protein, collagen I, collagen IV, fibronectin or laminin. Scale bar: 100μm. *$p<0.05$, **$p<0.005$

3.3.6 Comparison of biomimetic surfaces to protein adsorbed surfaces

To compare biomimetic surfaces to protein adsorbed surfaces, the level of cell attachment on surfaces of known amounts of adsorbed ECM proteins can be compare to the number of cells attached to biomimetic surfaces. This allows an equivalent concentration of biomimetic surfaces to be deduced. Each ECM domain tested had levels of cell attachment equivalent to the following concentrations of their corresponding adsorbed protein: collagen I 129μg/ml; collagen IV 0.1μg/ml; fibronectin 18μg/ml; and laminin 2.0μg/ml (Figure 3.8).

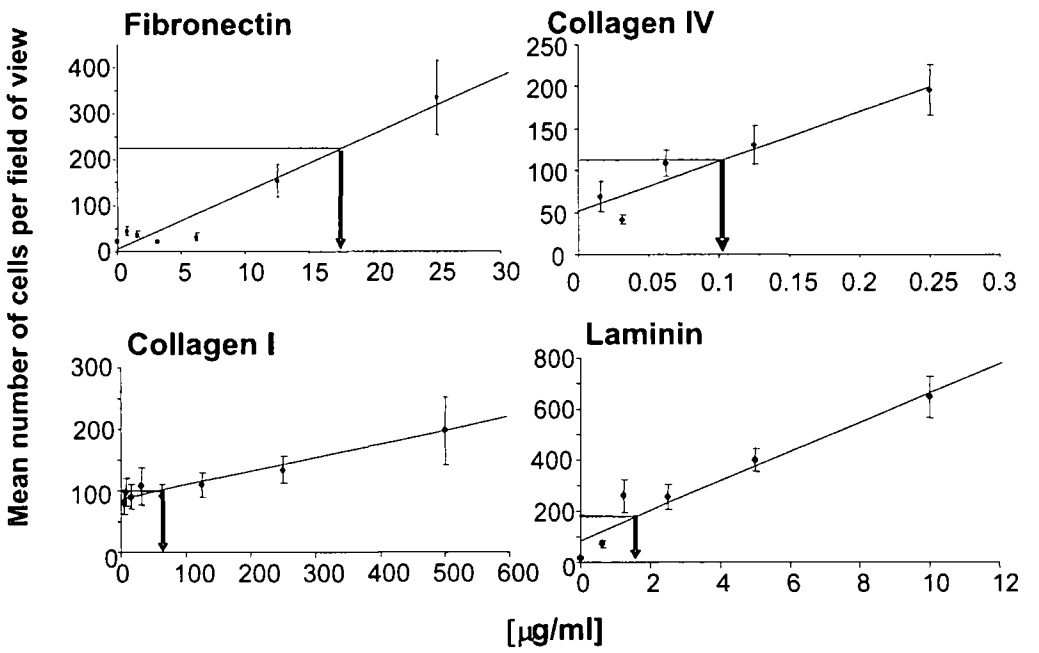

Figure 3.8 Comparison of the mean number of cell attachment following 24 hours culture between Orla peptide surfaces to that of protein coated surfaces. Points represent protein coated surfaces at various concentrations (±SEM), arrowed line illustrates mean cell attachment level of biomimetic surfaces and approximate protein concentration equivalent.

Upon calculation of the relative attachment to biomimetic surfaces as a percentage of protein coated surfaces (Table 3.1) the order of effectiveness of biomimetic surfaces is Orla 34 (fibronectin) > Orla 31 (collagen I) > Orla 36 (laminin) > Orla 32 (collagen IV). It is important to consider the level of attachment relative to adsorbed protein as the biomimetic surface Orla 31 results in one of the lowest significant results however when taking the attachment of PC12 cells to adsorbed

collagen I protein, this biomimetic surface produces the second greatest level of cell attachment.

Surface	Attachment to biomimetic surface (μg/ml)	Attachment to protein adsorbed surface (μg/ml)	Percentage (%)
Collagen I	60	250	24
Collagen IV	0.1	8	1.3
Fibronectin	18	50	36
Laminin	1.5	10	15

Table 3.1 Table gives information of the level of cell attachment to biomimetic surfaces and the maximal cell attachment level to surfaces with adsorbed protein. Final column shows the attachment of cells on biomimetic surfaces as a percentage of the maximal level of attachment of cells to protein adsorbed surfaces.

Controlling neurite behaviour using biomimetic surfaces

3.3.7 Neurite outgrowth of PC12 cells cultured on control surfaces

ECM proteins have previously been shown to affect PC12 cell growth and neural differentiation [104,225,293,304]. Previous studies have investigated multiple aspects of neurite behaviour from PC12 cells and these include: neurite length; number of neurites per cell; and the number of branches per neurite.

Using a similar paradigm, neurite behaviour of PC12 cells cultured on various adsorbed ECM proteins was investigated. Based on previous observations (Section 3.3.6) surfaces were prepared of the ECM proteins collagen I, collagen IV, fibronectin and laminin of varying concentrations from 1600μg/ml to 0.01μg/ml. The following concentrations resulted in optimal cell coverage following ten days NGF induced differentiation: collagen I (800μg/ml); collagen IV (0.5μg/ml); fibronectin (35μg/ml); and laminin (5μg/ml). A similar trend to that previously observed was seen, collagen and laminin provided the best

substrates for cell coverage, whereas collagen I and fibronectin supported fewer cells and their coverage appeared clumpy (Figure 3.9).

Surfaces of decreasing concentrations were studied and surfaces where sufficient single cells were visible were analysed for neurite outgrowth. The following concentrations of ECM proteins were used: collagen I (800μg/ml); collagen IV (0.25μg/ml); fibronectin (17.5μg/ml); and laminin (2.5μg/ml). Comparisons between cells grown on coated and uncoated surfaces gave the changes described below in comparison to control surfaces. Cells grown on uncoated surfaces had a mean neurite length of 129.4μm ±7.4μm. When normalised to cells cultured on uncoated surfaces it was found that cells grown on collagen I decreased the mean neurite length by -25.2μm ±9.8μm whereas for all the other tested substrates the following increases in mean neurite length were observed: collagen IV 35.1μm ±9.5μm; fibronectin 51.9μm ±11.5μm; and laminin 15.1μm ±8.3μm. Cells grown on uncoated surfaces had a mean number of 2.50 ±0.07 neurites per cell. Culture of cells on surfaces coated with ECM proteins increased the number of neurites per cell: collagen I 0.25 ±0.10; collagen IV 0.95 ±0.09; fibronectin 0.55 ± 0.08; and laminin 0.17 ±0.09. When analysing branching, cells grown on uncoated surfaces had 1.70 (±0.19) branches per mm of neurite. Culture of cells on fibronectin decreased the branching on neurites by -0.60 (±0.20) branches per mm. Culture of cells on all other ECM proteins tested resulted in an increase in neurite branching per mm: collagen I 4.4 (±0.58); collagen IV 3.8 (±0.45); and laminin 2.1 (±0.47).

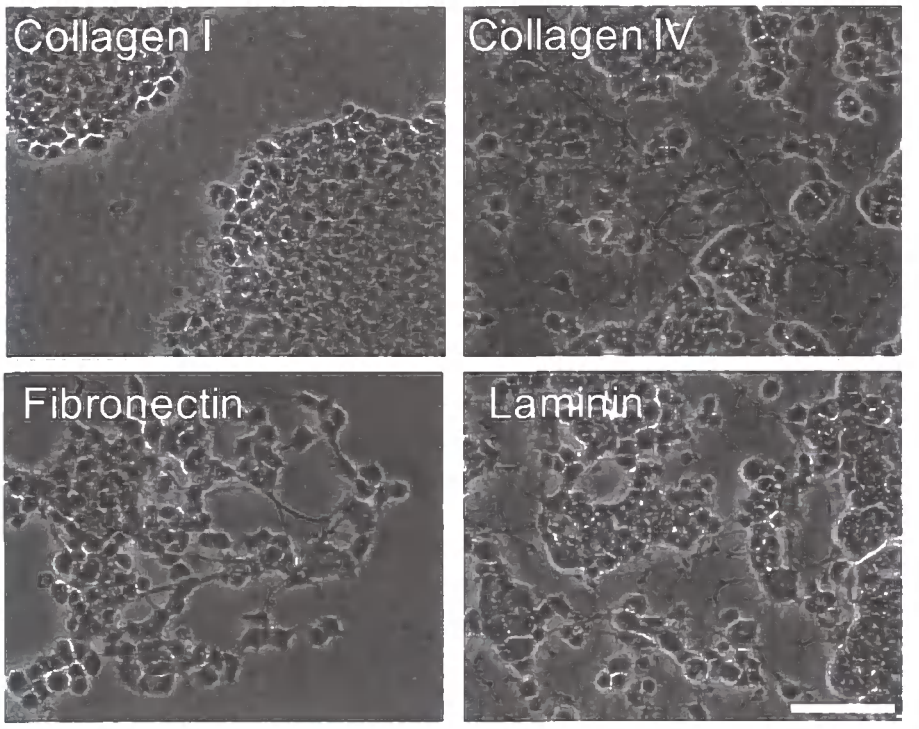

Figure 3.9 ECM proteins provide adequate surfaces for the differentiation of PC12 cells. Images show representative fields of view of PC12 cells cultured for 10 days under NGF induced differentiation conditions. All images were taken at the same magnification, scale bar = 100μm.

3.3.8 Neurite outgrowth of PC12 cells cultured on biomimetic surfaces

Having demonstrated that PC12 cells respond differently to different adsorbed ECM proteins, biomimetic surfaces presenting motifs from these ECM proteins was investigated. Motifs from collagen I, collagen IV, fibronectin and laminin were all found to increase mean neurite length. Both of the fibronectin motifs and the laminin YIGSR motif produced a significant increase in neurite length, however the laminin motif IKVAV had no effect on neurite length (Figure 3.10 A). The most effective motif in promoting neurite growth was Orla 31 (Figure 3.10 D, Orla 31). When investigating the influence on the number of neurites per cell of various biomimetic surfaces, many of the surfaces appeared to increase the number of neurites per cell, only one surface produced a significant increase (Orla 32) (Figure 3.10 B, D Orla 32). Two surfaces produced a significant increase in neurite branching: Orla 31 and Orla 36 (Figure 3.10 C). PC12 cells cultured on Orla 36 biomimetic surfaces appear highly branched (Figure 3.10 D, Orla 36).

Consistent with previous studies, the tenascin C motif had no significant effect on any of the aspects tested for neuronal behaviour.

In agreement with PC12 adhesion data (Section 3.3.7), when two motifs were presented by a single OmpA molecule these surfaces had an effect similar to that of the motif, which when presented alone gave the lesser response. Orla 36 was found to significantly increase neurite branching whereas, Orla 11 did not significantly increase neurite branching. When these two motifs were presented in a single protein (Orla 37) the level of neurite branching was similar to that of Orla 11 alone and the significant increase from Orla 36 was no longer observed. Both Orla 1 and Orla 34 had positive effects on the mean neurite length, however, when combined in a single OmpA molecule no significant increase in mean neurite length was observed suggesting that the presentation of both motifs simultaneously has a detrimental effect on their correct presentation.

PC12 cells responded to: Orla 1; Orla 31; Orla 32; Orla 34; and Orla 36. This demonstrates that these motifs are presented in a functional manner.

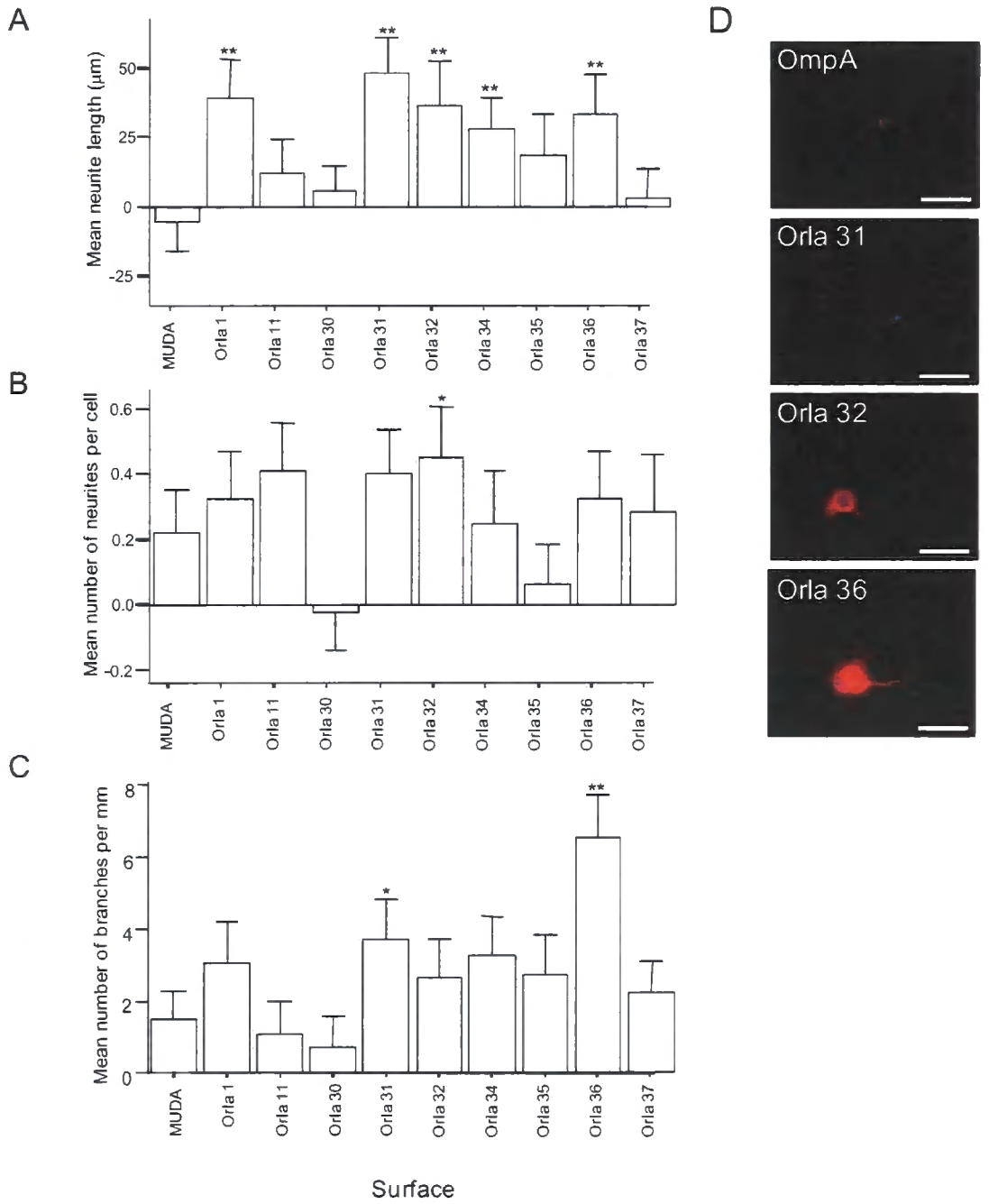

Figure 3.10 Regulation of neurite formation in response to alternative ECM motifs. Results were recorded from cultures of PC12 cells grown for 10 days under NGF differentiation conditions on different biomimetic surfaces. A) The mean neurite length. B) The mean number of neurites per cell. C) The mean number of branches/mm of neurite. Values were normalised to those of cells grown on control (OmpA) surfaces. Negative values represent a decrease when compared to control levels. Data represents mean values of 20 randomly selected single cells per surface for three repeats (+SEM). D) Fluorescence micrographs showing prime examples of cultures grown on alternative biomimetic substrates: OmpA-control; Orla 31; Orla 32; and Orla 36. Cells were

stained with the nuclear marker DAPI (blue) and the neuronal marker βIII-tubulin (red). Scale bars: OmpA-control and Orla 31: 100μm; Orla 32 and Orla 36: 25μm. *$p<0.05$, **$p<0.005$

Controlling neuronal differentiation using biomimetic surfaces

3.3.9 Coating surfaces with alternative ECM proteins modulates cell attachment

Surfaces were prepared by adsorbing laminin (following poly-d-lysine, PDL adsorption), fibronectin, collagen I and collagen IV. NSPCs neurospheres were dissociated and allowed to adhere. Following 24 hours proliferation, the media was changed to differentiation media and cells were cultured for a further four days. The confluency of the cultures appeared different, cells grown on PDL-laminin or fibronectin appeared clumpy favouring cell-cell interactions instead of cell-surface interaction, whereas cells on collagen I or collagen IV were more homogeneously spread and a greater number of single cells were apparent. The number of neuronal cells present was investigated by positive staining with βIII tubulin NSPCs grown on PDL-laminin or fibronectin appeared less differentiated whereas those grown on collagen I or collagen IV appeared more differentiated with a more extensive network of neurites (Figure 3.11 A). To quantitatively compare cell number on the different surfaces the mean cell number for each field of view was calculated, this revealed that there were no differences in cell number between PDL-laminin and fibronectin. In contrast collagen I and collagen IV resulted in significantly fewer cells than PDL-laminin and fibronectin although there was no difference between collagen I and collagen IV (Figure 3.11 B). Further analysis of neural cell types proved that cultures of cells on PDL-laminin or fibronectin had significantly fewer neuronal cells when compared to cultures grown on collagen I or collagen IV (Figure 3.11 C).

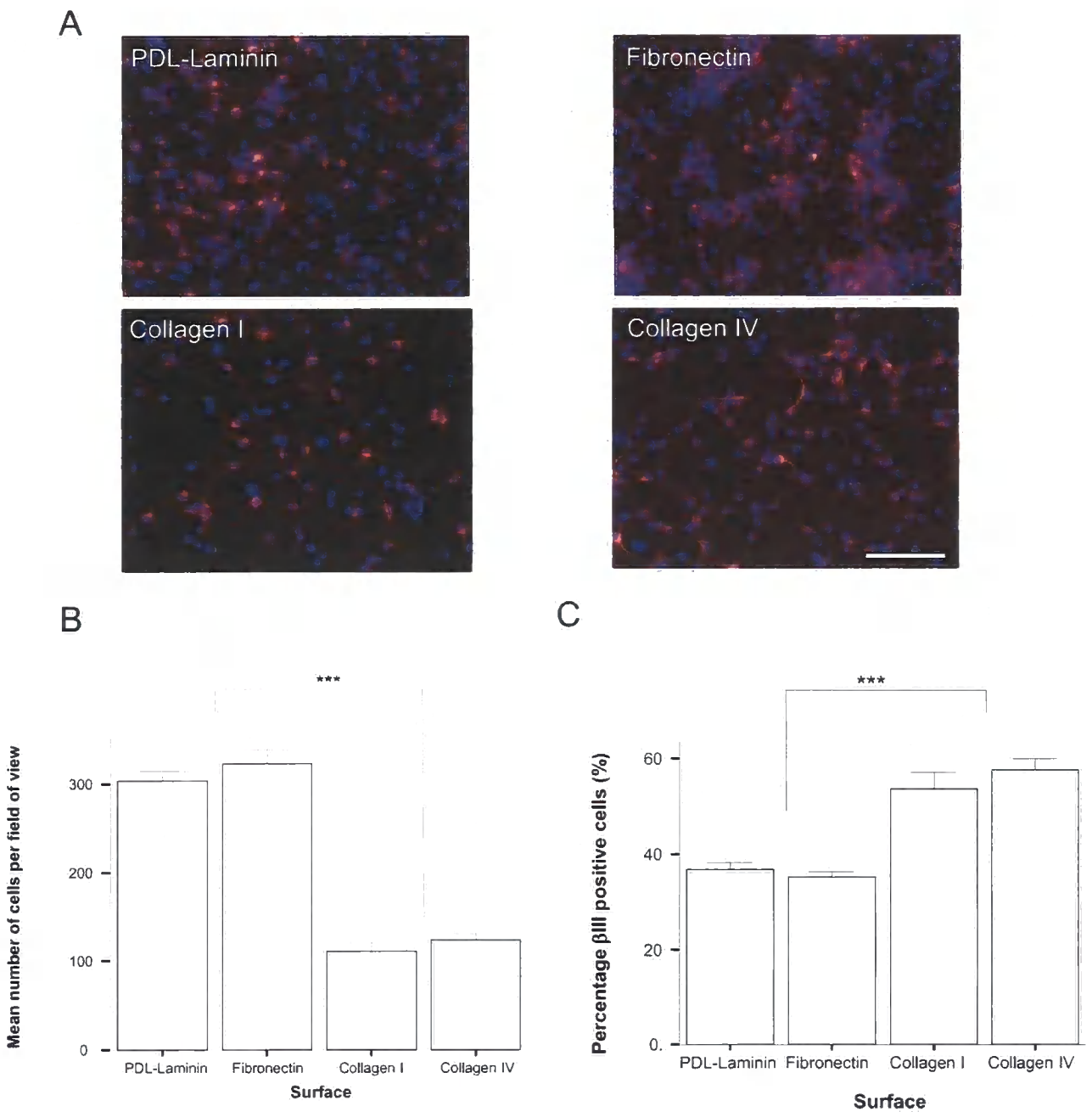

Figure 3.11 Analysis of NSPCs grown for 4 days under differentiation conditions on alternative surfaces with adsorbed ECM proteins. A) Fluorescence images show βIII positive cells (red) and nuclei (blue). Representative images are shown. Scale bar: 100μm. B) Bar chart shows the quantitative analysis of cell number on alternative growth surfaces of nine randomly selected fields. Bars show mean values +SEM, n=3. C) Quantitative analysis of βIII positive cells on alternative protein adsorbed surfaces. Data represents the mean percentage of βIII positive cells per field of view for nine randomly selected fields as a percentage of the total population per field of view for each repeat. Bars show mean values +SEM, n=3. ***$p<0.001$

3.3.10 Presentation of motifs from ECM proteins by biomimetic surfaces modulates NSPC attachment and the number of neuronal cells

Previous studies using PC12 cells have demonstrated that Orla 31, Orla 32, Orla 34 and Orla 36 are all effective when presented in our system, in a correct and accessible orientation. Therefore, these motifs were selected for further investigation. The motif RGDS (Orla 1) when presented by our system was only found to result in a positive effect in one of the assays. However, previous studies have shown RGDS has a positive effect on neural cells [298,317] and hence was included in further investigations. NSPCs were allowed to adhere for 24 hours and subsequently differentiated for a further four days. Cultures of NSPCs on biomimetic surfaces all appeared similar in cell attachment, except for cells cultured on Orla 32 where fewer cells attached (Figure 3.12 A). Quantitative analysis clearly demonstrates the differences in cell number: Orla 32 significantly reduces cell number when compared to OmpA without any inserted motif (Figure 3.12 B). Analysis of the percentage of βIII positive cells revealed that only Orla 1 significantly enhanced the percentage of neuronal cells (Figure 3.12 C). One possible explanation for this difference is that the higher cell confluency results in reduced differentiation; a 3-fold difference in cell attachment was noted whereas a 1.5-fold difference in differentiation was observed. Surfaces composed solely of MUDA produced a 5-fold increase in cell number however no increase in differentiation was observed.

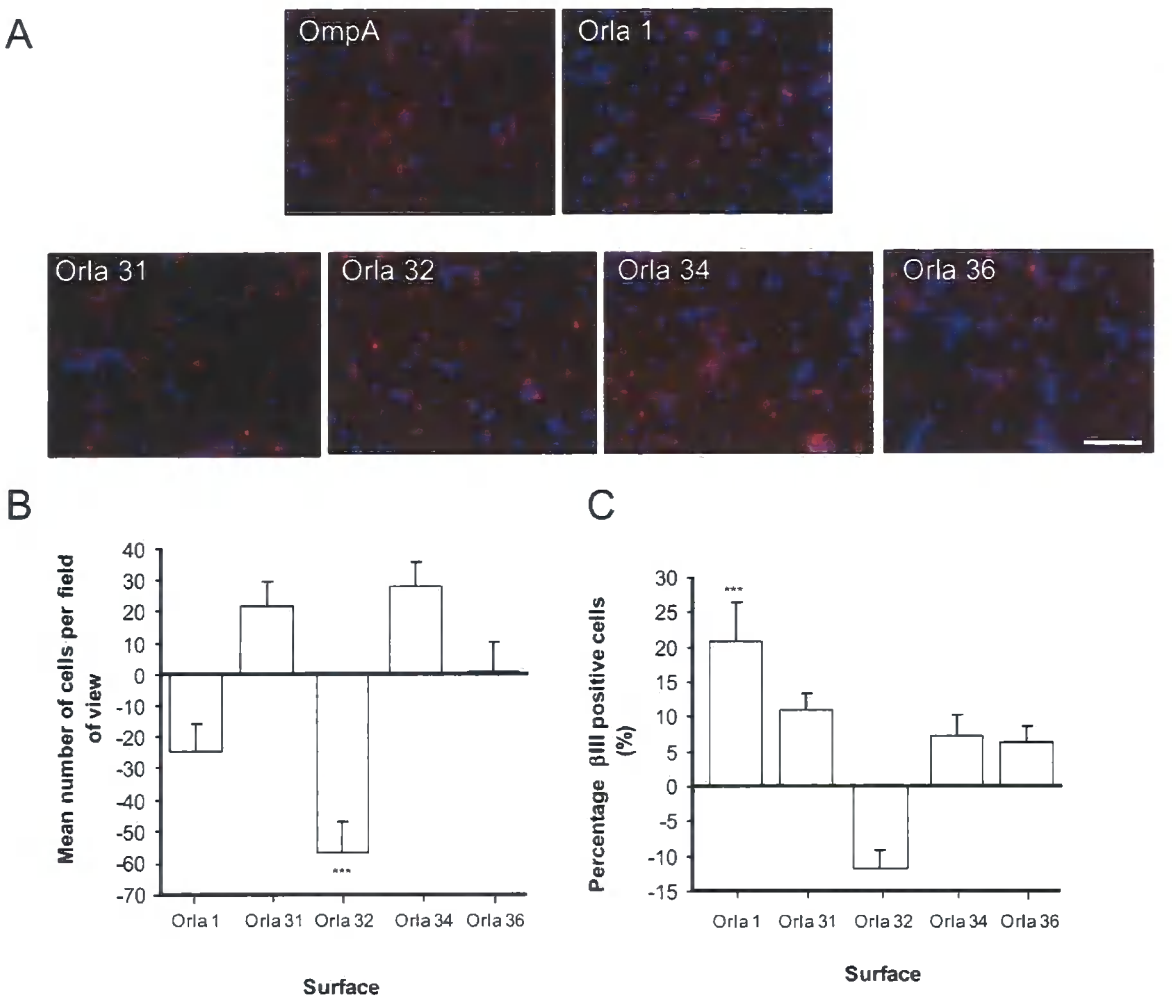

Figure 3.12 Influence of alternative ECM motifs on cell number and NSPC differentiation. A) Fluorescence images of NSPC cultures grown for 4 days under differentiation conditions on biomimetic substrates presenting multiple motifs, cells were stained with the nuclear marker, DAPI (blue), whilst neurons were labeled with βIII-tubulin (red). Scale bar: 100μm. B) The mean number of cells per field of view was compared to the number on biomimetic substrates. C) The number of βIII-tubulin positive cells were recorded on substrates presenting multiple ECM motifs compared to control (OmpA) surfaces. Values represent the number of cells different to control (OmpA) levels (zero line) and were compared statistically to the control (OmpA) surfaces. The data represents mean values of 20 randomly selected single cells per surface for three repeats (+SEM). *$***p<0.001$*

3.3.11 Presentation of mixed peptides to more faithfully recapitulate the in vivo environment

The ECM comprises of multiple different molecules, each presenting different peptide motifs. In order to mimic this, surfaces were produced which consisted of

multiple peptides - Mixed Orla Surfaces (MOS) (for information regarding the composition of MOS see Table 1.2). Observation of the cultures showed there were no clear differences in cell number between the different surfaces (Figure 3.13 A). Quantitative analysis confirmed that there were no significant differences in cell number between the different surfaces examined (Figure 3.13 B). Further analysis was conducted and the effect on neuronal differentiation assessed. MOS2 was the only surface to produce significant increases in neuronal differentiation when compared to OmpA (control). MOS2 contains Orla 1, and this result is consistent with previous studies, which showed Orla 1 to be the best motif for increasing neuronal differentiation.

The surface that had the smallest effect on neuronal differentiation was MOS1, the only surface that does not contain Orla 1 (Figure 3.13 C). As additional motifs are added to a surface, the relative coverage of each motif is decreased, for instance, MOS2 has two motifs and the coverage of RGDS is 50%, MOS3 has three motifs and the coverage of RGDS is 33%, MOS5 has five motifs thereby reducing the coverage of RGDS to just 20%. There is no difference between the presentation of RGDS alone (100% coverage) and MOS2 (50% RGDS coverage).

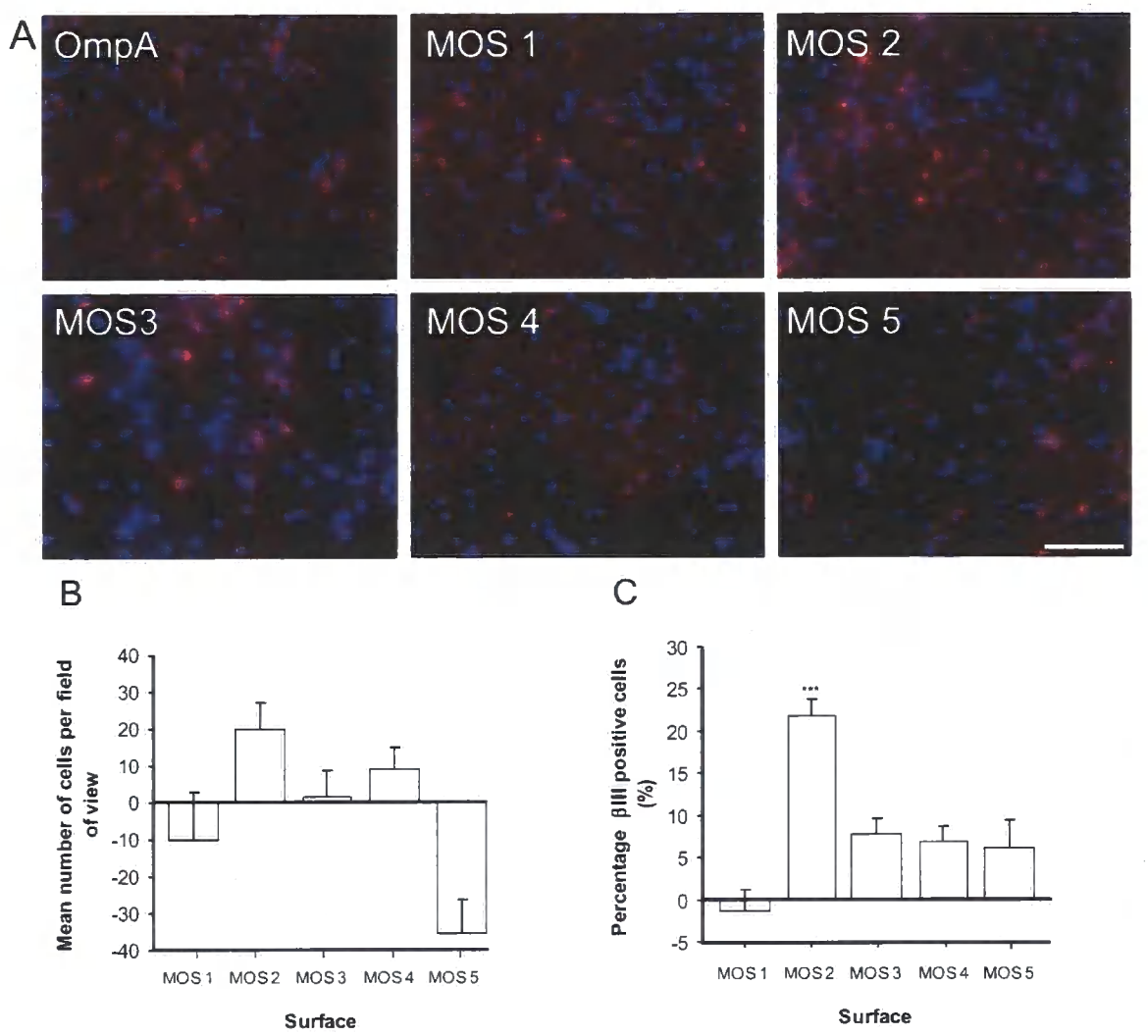

Figure 3.13 Influence of surfaces presenting multiple ECM motifs on cell number and neuroprogenitor differentiation. A) Fluorescence images of NSPC cultures grown for 4 days under differentiation conditions on biomimetic substrates presenting multiple motifs, cells were stained with the nuclear marker, DAPI (blue), whilst neurons were labelled with βIII-tubulin (red). Scale bar: 100μm. B) The mean number of cells per field of view was compared to the number on biomimetic substrates. C) The mean percentage of βIII-tubulin positive cells was recorded on substrates presenting multiple ECM motifs and compared to control (OmpA) surfaces. Values represent the number of cells different to control (OmpA) levels (zero line) and were compared statistically to the control (OmpA) surfaces. Data represent mean values of 20 randomly selected single cells per surface for three repeats (+SEM). ***$p<0.001$

Certain motifs were found to be inactive in the assays tested. The laminin motif IKVAV and the tenascin C motif did not show any significant increase in cell attachment or neurite behaviour. Surfaces where two motifs were combined within one molecule proved inactive. Of all the motifs tested for influences on neuronal differentiation, RGDS was the only motif that produced a significant increase when compared to control levels. When mixed motif surfaces were produced RGDS was found to be a key component and without this motif present in sufficient quantities little increase above control (OmpA) levels were observed (Table 3.2).

Surface name	Motif	Source	Adhesion	Neurite behaviour	Neuronal differentiation
Orla 1	RGDS	Fibronectin	-	Y	Y
Orla 11	IKVAV	Laminin	-	-	NT
Orla 30	VFDNFVKL	Tenascin C	-	-	NT
Orla 31	GTPGPQGIAGQRGVV	Collagen I	Y	Y	-
Orla 32	MNYYSNS	Collagen IV	Y	Y	-
Orla 34	PHSRN	Fibronectin	Y	Y	-
Orla 35	PHSRN+RGD	Fibronectin	-	-	NT
Orla 36	YIGSR	Laminin	Y	Y	-
Orla 37	YIGSR+IKVAV	Laminin	-	-	NT
MOS1	Collagen I Collagen IV	GTPGPQGIAGQRGVV MNYYSNS	NT	NT	-
MOS2	Fibronectin Laminin	RGDS YIGSR	NT	NT	Y
MOS3	Fibronectin Collagen I Collagen IV	RGDS GTPGPQGIAGQRGVV MNYYSNS	NT	NT	-
MOS4	Fibronectin Collagen I Laminin	RGDS GTPGPQGIAGQRGVV YIGSR	NT	NT	-
MOS5	Fibronectin Collagen I Collagen IV Fibronectin Laminin	RGDS GTPGPQGIAGQRGVV MNYYSNS PHSRN YIGSR	NT	NT	-

Table 3.2 Summary of results. Table shows, motif/s presented by surfaces, origin of motif/s and whether they showed an increase in the test variable when compared to control (OmpA) surfaces for the three independent assays. (Y) indicates that a positive effect was observed, (-) that no positive effect was observed and (NT) indicates that these motifs were not tested in that particular assay.

3.4 Discussion

The technology discussed here, developed by Orla Protein Technologies, has been assessed as a platform for the control of cell attachment, neurite outgrowth and neuronal differentiation. Surfaces composed of infill molecules without any peptide motifs could be used to control the attachment of multiple cell types, although the effects were cell type dependant. It was shown that motifs from collagen I, collagen IV, fibronectin and laminin were able to enhance the cell attachment of PC12 cells to a surface. These motifs were then demonstrated to control various aspects of neurite outgrowth from PC12 cells. The motifs that were found to have a positive effect on PC12 attachment and neurite outgrowth were then assayed for their effects on NSPCs. When investigating attachment; a peptide from collagen IV was found to significantly reduce the level of attachment, whereas the fibronectin peptide RGDS significantly increased the percentage of βIII positive cells. Surfaces were prepared where multiple motifs were presented on a single surface and this resulted in some expected and in some unexpected results.

Previous studies, supported herein, have provided evidence that topographic features of a surface can influence cell attachment and growth [318]. Cell attachment is enhanced when OmpA molecules presenting no motifs are added to a surface, however, when compared to HDT alone it is seen that for significant increases in cell attachment the presentation of ECM motifs is essential.

Cell spreading is also affected by the surface upon which cells are cultured. Surfaces where cell coverage is clumpy show that the cells prefer cell-cell interactions in comparison to cell-surface interactions. PC12 cells attached to glass surfaces coated with adsorbed fibronectin protein develop as aggregations of cells whereas cells grown on collagen IV protein coated surfaces were more uniformly distributed. Cells cultured on surfaces using OmpA to present the fibronectin domain formed clumps whereas collagen motifs resulted in a more even cell distribution.

In contrast to the data from experiments coating glass with solubilised whole proteins, surfaces that displayed the fibronectin motif, PHSRN, enabled the greatest level of cell attachment. Each surface was manufactured in such a way that the concentration of OmpA molecules presenting the different ECM domains was approximately equivalent. When considering the level of attachment to adsorbed proteins, the collagen I motif (Orla 31) resulted in the greatest level of cell attachment compared to the whole molecule. However, when compared to the level of attachment to adsorbed proteins, the collagen I motif provides the highest level of attachment.

Studies have demonstrated that the laminin motif RGD [319 223 320 295] and the fibronectin motif RGDS [321 200] are highly effective in promoting cell adhesion. In contrast to previous work, the RGDS motif was not effective in our system when presented to PC12 cells using a HDT infill background. There may be some reluctance for groups to publish negative results however, one group has shown that, in their system, RGDS does not increase cell attachment whereas YIGSR [182] and KQAGDV do increase cell attachment [183]. Many factors affect the efficacy of immobilised peptides on surfaces, including: the distance between the peptide and the substratum [105,322]; the immediate environment surrounding the functional domain [322]; and the orientation and configuration of the motif and its secondary structure [303]. These factors can affect the accessibility of the active part of the peptide to the cell, the activity of the peptide and the topography of the surface.

Surfaces were produced by which multiple motifs were presented by a single OmpA molecule, these were found to be ineffective in the assays tested. Several surfaces presenting a single motif were similarly found to be ineffective in enhancing cell attachment. Surfaces that were not effective in enhancing PC12 attachment were excluded from further analysis.

Cell differentiation is influenced by cues within their environment, notably the interaction with adjacent cells, soluble signals and the ECM. Within developing tissue, cells differentiate as a combined response to multiple signalling events. This is especially relevant to the ECM which is often composed of mixtures of different molecules that surround cells and tissues and are important in

determining cell growth, state of differentiation and cell migration [323]. It is difficult to elucidate and investigate the exact role of individual ECM molecules in a standardised and reproducible manner. The presentation of peptide motifs helps to determine the exact factor influencing cell behaviour.

PC12 cells are also known for their ability to produce neurites in response to NGF and are used as a model system to study neuritogenesis [305]. Previous studies have investigated the effects of whole proteins on PC12 neurogenesis and, upon comparison; similarities can be drawn with the data presented herein. Care should be taken when interpreting this data as, the whole protein will contain numerous motifs whereas only single motifs are presented herein.

Presentation of motifs from laminin, fibronectin, collagen I and collagen IV by OmpA molecules increased neurite length in comparison to control surfaces. This is consistent with previous reports whereby the exposure of PC12 cells to ECM molecules resulted in enhanced neurite outgrowth [324-327]. Similarly, as previously noted, no significant differences were detected between the lengths of neurites grown on collagen I or laminin [324]. However, the data for fibronectin is not completely consistent with those of Paralkar et al. [328] who showed that collagen IV and laminin produced similar levels of neurite outgrowth more effectively than fibronectin.

Laminin has been demonstrated to increase the percentage of PC12 cells producing neurites [329]. When comparing laminin and fibronectin, laminin increased the percentage of cells with neurites whereas fibronectin had no effect over that of the control [304]. Collagen I has previously been shown to significantly increase the numbers of neurites formed by PC12 cells compared to numbers induced in cultures grown on laminin [324]. Whilst the collagen I motif did increase the mean number of neurites originating from the cell body, this was not a significant change. On the other hand exposure to collagen IV significantly increases the number of processes formed per cell. There are inconsistencies between published studies and the data reported herein, potentially due to the variation in the methods used to evaluate the effect of ECM proteins on cell growth. The Nakajima group were unable to obtain attachment of their cells to collagen I or collagen IV coated

surfaces, whereas, cell attachment of NSPCs was obtained on these surfaces. These contradictory findings could be due to the different cell types however, their procedure for collagen coating differed from that recommended by the manufacturer. When coating was carried out as described by Nakajima no attachment of NSPCs was observed whereas with the recommended coating procedure attachment of NSPCs was possible. There is a need to standardise this process and the presentation of functional ECM peptide domains in a controlled and consistent manner, using well engineered synthetic surfaces, will significantly improve reproducibility.

The local influence of substrate molecules in determining distinctive growth patterns has been recognised [330-332]. To further investigate neuritogenesis, the number of branches per neurite formed was recorded. Peptide motifs for both collagen I and laminin induced significant levels of branching per neurite, inparticular, neurites grown on the laminin YIGSR motif appeared highly branched. Investigation of neurite branching has previously shown that laminin increases branching in comparison to fibronectin [333], which is consistent with the data reported herein whereby neurites growing on surfaces presenting the laminin motif YIGSR produced significantly more branches compared to cells cultured on surfaces expressing motifs RGDS or PHSRN associated with fibronectin.

The behaviour of different cells on a surface have been attributed to the expression of integrins. PC12 cells are known to attach poorly to fibronectin coated surfaces, upon comparison to cell lines that show a good level of attachment to fibronectin the differences were attributed to different expression levels of the α-integrin [127]. PC12 cells are known to express α1, 6, 3, v and weakly α5 [153].

The integrin heterodimers α1β1 and α3β1 are known to be the PC12 receptors of laminin. Their binding has been localized to two different regions on the laminin molecule, with α1β1 binding at the E1-4 region and the α3β1 binding at the E1 domain. In contrast, α1β1 is the main integrin of PC12 cells that binds to collagen I or collagen IV [326].

These results demonstrate that surfaces presenting peptide motifs can influence neuritogenesis. Interactions between the peptide motifs investigated herein and the cell are known to be mediated by integrins and these results show integrins to be important in neuritogenesis. A process related to neuritogenesis is the formation of new synapses (synaptogenesis). Integrin signalling is also important in the process of controlling synaptogenesis and is controlled by cell-cell interaction between astrocytes and hippocampal neurons. One proposed mechanism for synaptogenesis is that phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) leads to actin rearrangement, spinogenesis and finally synaptogenesis. This is mediated via up regulation of phospholipase A2 (PLA2) leading to an increase in the release of arachidonic acid. Arachidonic acid is required for the translocation of PKC(ε) from the cytosol to the plasmamembrane, where it then induces the phosphorylation of MARCKS. Using the integrin blocker echistatin a reduction is phosphorylation of MARCKS is observed, implementing integrin binding as a mechanism to control synaptogenesis [334].

The work herein demonstrates that peptide motifs from collagen, fibronectin and laminin can be used to promote neuritogenesis. These results have implications in the wider literature for the application of this technology in increasing neuritogenesis *in vivo*. Peptides can be immobilised into scaffolds for transplantation *in vivo* to aid cell transplantation. Incorporation of scaffolds with immobilised RGD has been shown to increase the number of cells within a scaffold *in vivo* [335] and RGD has also been immobilised onto scaffolds to promote repair following spinal cord injury [336]. There are many studies using IKVAV [337] and YIGSR [338] to promote neuritogenesis on scaffolds for transplantation into areas of damage. However, what has not been investigated is how the alternative peptides tested herein affect neuritogenesis *in vivo* for example, it has not been investigated how the collagen I peptide GTPGPQGIAGQRGVV could promote neuritogenesis *in vivo*. These results suggest that alternative motifs should be tested other than the currently investigated motifs.

Cell fate and the direction of cell differentiation are regulated by multiple factors including the interaction between cells and their surrounding ECM [339]. It has been investigated whether certain active ECM peptide domains influenced the

differentiation of adult NSPCs to induce the formation of βIII tubulin-positive cells, indicative of neuronal differentiation.

The effect of ECM proteins specifically on neuronal differentiation by neural stem cells (derived from the striatum of foetal rats) has been previously reported [317]. An array approach was used whereby either whole ECM molecules or artificial ECM proteins were tethered to a synthetic substrate in the presence and absence of known growth factors. It was demonstrated that growth factors were more predominant determinants for the specification of neural stem cells than matrix components, although the effects of growth factors were often influenced by the type of co-immobilised ECM. In the absence of growth factors, ECM proteins for laminin and fibronectin induced neuronal differentiation [317]. Even though a different type of neuroprogenitor cell was used in this study, the motif for fibronectin (RGDS) had a significant effect on the formation of neurons. Collagen I has been previously recognised as a key component to enhance neuronal differentiation when used as a gel for three dimensional cell growth [340]. When presented by OmpA molecules the collagen I motif did not have a significant influence on neuronal differentiation.

Of the motifs tested, only collagen IV resulted in a decreased number of βIII tubulin-positive cells formed by adult NSPCs. This is in contrast to the behaviour of foetal cortical neurons whose differentiation is promoted in the presence of collagen IV [341]. Such results draw attention to the differences between the model systems where the response of developing neurons to ECM proteins may differ as a consequence of their tissue origin. There are inconsistencies in the manner in which ECM proteins are represented in different studies, notably the use of either the whole ECM molecule adsorbed onto a solid substrate (with different studies using alternative concentrations) or the presentation of the functional ECM peptide domain alone. The conformation of adsorbed whole molecules is likely to be variable and different to that where the active peptide motif is presented in a uniform and controlled manner as reported here. This in turn is likely to influence the interaction of the cells with the substrate and consequently the differentiation response.

Proteins of the ECM can possess several different functional domains; therefore, it can be difficult to determine which domain is important for a certain function within a particular cell growth system. This is complicated further when several domains are functioning simultaneously and presentation is in such a way that the molecular conformation of such molecules is varied. *In vivo*, cells interact with the ECM composed of multiple co-localised proteins. Given the complexity of these interactions, there are significant advantages to studying the function of ECM proteins and their active domains both individually and in various combinations. To evaluate the combined function of different ECM peptide domains, biomimetic surfaces were produced which were composed of a mixture of different motifs presented in a uniform fashion (Figure 1.10). When presented as a single peptide motif, collagen I and collagen IV produced opposite differentiation results however these responses were approximately equal in magnitude. In combination these functional domain effects appeared to cancel out resulting in no change compared to control levels.

Earlier work studying rat neural stem cells demonstrated that it is possible to produce surfaces expressing the RGD peptide that appear to induce similar levels of neuronal differentiation to PLO/laminin surfaces [300]. Moreover, the laminin peptide IKVAV was found to have no effect on neuronal development. Upon bringing the two conditions together and creating a surface comprised of the active RGD and the inactive IKVAV motifs, the effect on neuronal differentiation was decreased in proportion to the dilution of the combined positive and negative effects [300]. The work described here is consistent with the observation that when ECM motifs that elicit approximately equal positive and negative effects on cell differentiation are combined, the cumulative effect amounts to no change relative to the control.

When presented singularly, laminin (YIGSR) and fibronectin (RGDS) both induced neuronal differentiation. When the domains were co-presented this resulted in a surface that was similar to fibronectin (RGDS) alone. Contrary to previous results where PC12 cells and motifs were presented in a single OmpA molecule, presentation of two motifs on a single surface did not have a detrimental effect compared to the more effective of the two motifs.

An increase in neuronal differentiation is observed when the RGDS motif is presented at 100% (Orla 1) and 50% (MOS2); however, when the motif concentration is decreased below this level no effect is seen. Previous work has shown that the concentration of peptides immobilised to surfaces is critical in controlling cell fate as; insufficient peptide concentrations result in no elicited effects [300,342].

The addition of the fibronectin RGDS motif to produce a surface in combination with the collagen I and collagen IV domains, resulted in an increase in neuronal differentiation, as would be expected. However, counterintuitive interactions were also observed. When three peptide motifs (Orla 1, 31 and 36), that singularly all produced positive effects on cell differentiation, were combined on a single surface, the resulting effect, although positive, was not significantly different from the control surface. Furthermore, when five peptide motifs (Orla 1, 31, 32, 34 and 36) were expressed in combination, it appeared that the positive effects of fibronectin (RGDS), laminin and collagen I did not significantly counteract the negative influence of collagen IV.

Studies of the differentiation of murine embryonic stem cells have shown that the summative effects of combined ECM molecules did not consistently correlate with their activities when presented individually. Cultures of these cells on surfaces presenting collagen I, fibronectin or laminin resulted in an increase in differentiation However, when fibronectin was presented on a surface in combination with collagen I, a significant decrease in differentiation is observed [313,314].

When Fittkau et al. studied cell adhesion to the peptides YIGSR, RGD and PHSRN, the greatest level of cell attachment was on RGD, then PHSRN, then YIGSR. When YIGSR was presented by a combined surface with RGD this increased cell attachment level whereas, co-presentation of RGD and PHSRN reduced cell attachment compared to levels on RGD alone, these results are surprising as, when presented alone PHSRN preformed better than YIGSR. This was consistent for two different cell types, however when investigating migration

with these cell types the results for mixed surfaces was not consistent with cell attachment data [343]. This highlights that not only are the responses of cells to combinations of peptides complex but that the responses are also subject to the behaviour being investigated.

It would appear that in certain situations it is possible to create substrates composed of ECM proteins that influence cell differentiation in predictable ways however, there are other situations where the combination of alternative ECM molecules introduces additional complexity.

As well as surfaces directing cell fate there are a number of possible explanations for the differences in the number of βIII positive cells: surfaces could affect the selective attachment of one cell type; surfaces could favour selective death; or surfaces could favour selective propagation. These factors cannot be discounted as potential reasons for the observed effects. The percentage of βIII positive cells was used to account for surfaces where reduced cell attachment was observed.

The growth of cells outside the body requires the development of technologies to recreate the natural environment that cells experience in living tissues for optimal cell survival, growth and function. Biomimetic substrates presenting the active peptide motifs of various ECM proteins can be used to regulate cell attachment, neurite behaviour and neural differentiation *in vitro*. The presentation of individual ECM peptide sequences enables direct assessment of their function whereas the production of surfaces containing more than one motif allows assessment of the combinatorial effects of different ECM peptides. The development of this technology will lead to the creation of synthetic ECM substrates designed to interact with differentiating cells and will be of direct relevance to tissue engineering and regenerative medicine.

COMPARISON OF RESULTS GAINED TO HYPOTHESIS UNDER INVESTIGATION

The main hypothesis that was under investigation is that the presentation of motifs from ECM proteins via biomimetic surfaces will influence cell behaviour. It was hypothesised that cell behaviour would have been similar to the ECM protein from which they were derived. However, other groups have found this is not the case. Cells that show similar attachment levels to the whole protein laminin do not necessarily show similar levels of attachment to the laminin peptide YIGSR. Chinese hamster ovary (CHO) cells and neuroblastoma x glioma cells (NG108-15) both show similar attachment levels to the laminin protein, however, when cultured on surfaces presenting the laminin peptide YIGSR, in comparison to CHO cells, NG108-15 cells show a reduced level of attachment of 59%. This level of attachment could not be increased by increasing the concentration of peptide used to coat the surfaces [100]. It was hypothesised that not all motifs will be effective in influencing all cell types. This was observed herein, only four of the nine motifs tested had positive effects on PC12 cell attachment. It was hypothesised that the most effective motifs for controlling PC12 neurite behaviour would be those that were able to influence PC12 cell attachment, this was not completely true as one motif that had no effect on cell attachment was able to influence neurite behaviour, this highlights the fact that cell adhesion and neurite behaviour are not controlled by the same mechanism and that one orientation/conformation of motif although it may not be correct for one function, it can still activate other pathways. It was hypothesised that presentation of multiple motifs from different ECM proteins will have an increased effect when compared to individual motifs. This hypothesis was not supported as the presentation of two motifs that had a positive effect on a single surface did not result in an increase above the level from one of the motifs and furthermore, the presentation of three motifs that each resulted in positive effects resulted in a surface that did not greatly enhance the effect.

CHAPTER 4 – HOW THE ECM AFFECTS EMBRYONAL CARCINOMA STEM CELLS IN VITRO AND THE IMPLICATIONS IN TERATOMA DEVELOPMENT

4.1 Introduction

EC stem cells have been well studied as a model system for cellular behaviour. *In vitro*, EC stem cells can be induced to differentiate towards a neuronal phenotype with the addition of retinoic acid [344]. Within seven days a marked effect on their morphology is observed, this is accompanied with a decrease in proliferation [345] [346]. This decrease in proliferation is confirmed as, in cultures of undifferentiated NTERA2 cells, ~90% of the total population is positive for the proliferation marker Ki67, following seven days retinoic acid treatment this expression is reduced to ~50% of the total population [54].

There is an increasing amount of information suggesting that stem cell differentiation can be directed by interactions with the ECM. For example, the rate at which EC stem cells secrete ECM proteins changes during differentiation, which indicates the key role of the ECM in controlling development [179]. The culture of ES cells on surfaces coated with different ECM proteins demonstrates that their developmental potential can be modulated. Culture of ES cells on collagen I, fibronectin or laminin inhibits differentiation toward trophoectoderm. Whereas, when ES cells are cultured on collagen IV coated surfaces, differentiation toward the trophoectoderm is induced [187]. In addition to individual proteins, mixtures of proteins have been found to control development. For example, it has been reported that collagen I and laminin have no effect on differentiation whereas Matrigel™ markedly increases differentiation [191]. Further data suggesting that the ECM controls stem cell differentiation comes from experiments where an increase in the rate of neuronal differentiation has been observed when EC stem cells are cultured on laminin as opposed to fibronectin, collagen I or collagen IV coated surfaces [186]. ECM proteins have also been demonstrated to affect the induction of neural differentiation, with laminin increasing the levels of tyrosine hydroxylase [347] and collagen IV inducing neuronal differentiation [348]. Taken together, this data suggests that the ECM can be used to control stem cell differentiation.

There is also evidence to suggest that under certain conditions, the ECM can inhibit differentiation and maintain a pluripotent phenotype. ES cells are routinely cultured on murine embryonic fibroblasts (MEFs) in order to maintain their pluripotency; the mechanism(s) by which this maintenance occurs is unknown. Studies suggest that the ECM has a key role in maintaining stem cell pluripotency. The ECM can be extracted from MEFs and used to support stem cell pluripotency [349]. This ECM extract consists of procollagen and fibronectin [350]. Matrigel™, an alternative ECM extract has also been demonstrated to maintain ES cell pluripotency [351]. The ECM proteins laminin [351] and fibronectin [352] when presented singularly have also been shown to maintain ES cell pluripotency. Cells cultured in the absence of fibronectin on a gelatin do not remain as undifferentiated cells, demonstrating that under these conditions the interactions with ECM proteins are having a greater effect than the 3D environment for maintaining pluripotency [352].

Proliferation is also affected by the ECM upon which the cells are cultured. Laminin coated surfaces increase the confluence of EC stem cells and hence proliferation [186]. Collagen IV, fibronectin and laminin coated surfaces have all been shown to increase the proliferation of EC stem cells when in the presence of mitogens [286]. Laminin [353] and Matrigel™ [312] have also been shown to increase proliferation.

In Chapter 2 the anatomical site in which cells were transplanted was found to influence cell behaviour [354] and the ECM was proposed as a potential factor influencing the observed effects. Further experimentation demonstrated that co-transplantation of stem cells with ECM components influences cell behaviour and confirmed the ECM as a key factor in controlling stem cell behaviour. Although the co-transplantation of stem cells with ECM molecules provided evidence that the ECM modulates teratoma behaviour, *in vivo* experimentation is complex which makes analysis of the control mechanisms difficult. Co-transplantation of ECM components appeared to have three clear effects on teratoma development: the success rate of tumour development; the size of the teratoma (proliferation of cells); and the differentiation of the transplanted cells.

The *in vivo* environment is highly complex and the culture of cells *in vitro* on surfaces coated with ECM components helps go part way to simplify this. Coating surfaces provides a method for studying the effects of whole ECM proteins on stem cell behaviour. Some ECM components, such as Matrigel™, are complex mixtures of multiple proteins and growth factors. Individual components, such as laminin can be taken from this mixture and presented alone, however, this is still complex as ECM proteins are composed of multiple motifs. To simplify this further, cells can be cultured on surfaces presenting individual ECM protein motifs to identify the exact motif having the observed effects. As previously discussed, coating surfaces with proteins can be limiting and it is important to consider how the active motif of the ECM protein is affecting cell behaviour. In Chapter 3 it was demonstrated that motifs can be presented to cells in a functional manner to control their behaviour [355], by expanding on this it is possible to investigate how motifs influence stem cell behaviour.

Transplantation of cells into the liver produced large teratomas composed of immature cells, whereas subcutaneous transplantation produced small highly differentiated teratomas. The following observations were made when EC cells were transplanted with ECM components: Matrigel™ increased teratoma success rate, teratoma size and had no effect on differentiation; collagen I decreased teratoma size and also decreased differentiation; and laminin decreased teratoma size and increased differentiation. When the cells were co-transplanted with collagen IV or fibronectin, teratoma formation did not occur.

In vitro culture provides a model system by which it is possible to elucidate how the ECM proteins are affecting the cells. In this Chapter, the control of cell behaviour is monitored by analysis of gene expression assessed by the relative quantities of mRNA. Although the presence of mRNA does not demonstrate that a functional protein will be produced this does indicate how a cell is behaving. The cell line Tera2.cl-SP12 is a model system for the investigation of differentiation induced by the addition of retinoic acid.

The differentiation of EC stem cells following retinoic acid treatment is a complex process, microarrays have demonstrated that a large number of neural transcripts are upregulated [356]. Upon differentiation, EC stem cells are known to increase the expression of βIII-tubulin [45] and POU5F1 expression decreases [357], with almost complete reduction, following 7 days retinoic acid treatment [356]. Proliferation is known to decrease as the EC stem cells undergo differentiation [54]. In this section, the induction of differentiation in Tera2.cl-SP12 cells is investigated using the following markers: neuronal markers βIII tubulin and tyrosine hydroxylase; a marker of pluripotency, POU5F1 (Oct4); and a marker of proliferation, Ki67.

HYPOTHESIS UNDER INVESTIGATION

Large teratomas are produced when EC stem cells are co-transplanted with Matrigel™ whereas transplantation with collagen I or laminin results in a reduction in teratoma size compared to controls. When cells are cultured on surfaces coated with different ECM components *in vitro*, it is predicted that the level of the proliferative marker Ki67 should be increased on surfaces coated with Matrigel™ whereas a reduction in the expression of Ki67 should be observed on surfaces coated with collagen I or laminin. EC stem cells co-transplanted with collagen I appeared to show a reduction in their differentiation potential. This reduction in differentiation could be due to maintenance of the EC stem cell pluripotency. To test this, EC stem cells are cultured on surfaces with adsorbed ECM components and the expression of Oct4 is investigated. If interactions of EC stem cells with collagen I prevents differentiation and maintains pluripotency, it would be expected that the relative levels of Oct4 will be greater than cells cultured on other surfaces. To further investigate the effects on differentiation, the gene expression levels of two neuronal markers are investigated, if differentiation is decreased when cells are cultured on collagen I, there should be a reduction in the expression of these markers. Although no teratoma formation was observed when cells were co-transplanted with collagen IV or fibronectin, these are included in this assay for comparison. The peptide motifs from collagen I, collagen IV, fibronectin and laminin are investigated to demonstrate whether these are the principle effectors of controlling EC stem cell differentiation. It would be

predicted that the motifs will have a similar effect to the whole proteins however the response should be reduced as only single peptides are presented, as opposed to multiple motifs in the whole protein. All of the motifs have previously been demonstrated to be presented in the correct fashion for the adhesion of PC12 cells. However, it is likely that Tera2.cl.SP12 cells will not have the same compliment of integrins and their response to the presented motifs will not follow the same pattern as for PC12 cells.

AIM

To culture EC cells on surfaces presenting various ECM proteins in order to elucidate how these control EC behaviour and hence provide possible explanations for the observations seen during teratoma experiments.

OBJECTIVES

1. Culture EC cells on various ECM proteins and assess their behaviour.

2. Culture EC cells on surfaces presenting peptide motifs from ECM molecules and assess their behaviour.

4.2 Materials and methods

Surface preparation

Biomimetic surfaces were prepared as previously described (Section 3.2). Surfaces with adsorbed proteins were produced as previously described (Section 3.2). Coating concentrations were used as recommended by the manufacturer and published data to ensure maximal coverage: collagen I (2mg/ml); collagen IV (2mg/ml); fibronectin (50mg/ml) [353]; laminin (20mg/ml) [353]; and Matrigel™ (1:3, stock:media without FCS, Penicillin/streptomycin or l-glutamine, stock concentration ~9.8mg/ml). Adsorption of ECM components was carried out as previously described (Section 3.2). Biomimetic surfaces presenting motifs from collagen I (Orla 31), collagen IV (Orla 32), fibronectin (Orla 34) and laminin (Orla 36) were manufactured and prepared for cell culture as previously described (Section 3.2). Experiments were repeated in triplicate.

Cell culture and induction of differentiation

Tera2.cl-SP12 cells were seeded at a density of 500,000 cells per T25 flask or 200,000 cells per well of a 6-well plate and cultured in media as previously described (Section 3.2). Induction of differentiation was carried out by addition of 10mM all-trans retinoic acid (Sigma) to the culture medium. Cultures were either maintained in proliferative media for four days or in differentiation medium for seven days.

mRNA extraction

Media was removed from cells, washed once with PBS and mRNA was extracted.

To lyse the cells, 1ml TRI® reagent (Sigma) per 10cm^2 samples were incubated for 10 minutes at room temperature and the cell lysate passed several times through a pipette. The lysate was transferred to a 1.5ml eppendorf tube and centrifugated at 12,000g for 10 minutes at 4°C. The mRNA contained within the clear supernatant, was transferred to a fresh 1.5ml tube and 0.2ml of chloroform per 1ml of TRI ®

reagent was added, tubes were vortexed for 15 seconds and incubated at room temperature for 15 minutes. Centrifugation at 12,000g for 15 minutes was carried out and the aqueous supernatant was transferred to a fresh tube.

To precipitate the mRNA, 0.5ml of isopropyl alcohol per 1ml of TRI ® reagent was added, the tubes were inverted 3 times, incubated for 10 minutes and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed once with 1ml of 75% ethanol per 1ml of TRI ® reagent. The RNA was centrifuged at 10,000g for 10 minutes at 4°C and all of the supernatant removed. The total RNA pellet was dried for 5-10 minutes, dissolved in 15-50µl of RNase free water and incubated for 5 minutes at 55°C.

The extracted total RNA was then stored at -80°C until required.

DNase treatment

DNase treatment was carried out using a TURBO DNase Treatment Kit (Ambion www.ambion.com) and was conducted as follows: 10µl of the extracted RNA was aliquoted into a fresh tube; 1µl of 10x TURBO DNase Buffer and 1µl of TURBO DNase were added and mixed gently. The samples were incubated for 20 minutes, at 37°C, inactivated, by addition of 1µl DNase inactivation reagent, mixed and incubated for 2 minutes at room temperature with occasional mixing. The samples were then centrifuged at 10,000g for 90 seconds and the RNA transferred to fresh tubes.

Reverse transcription

The RNA sample was quantified using a NanoDrop ® spectrophotometer ND-1000 and the volume to obtain 4µg calculated. Using a Taqman reverse transcription kit (Applied Biosystems) the following reverse transcription master mix was prepared (quantities are given per reaction): 2µl RNase-free water; 4.4µl 10x TaqMan RT buffer; 4.0µl dNTPs (2.5mM); 1.0µl random hexamers; 0.4µl RNase inhibitor (20U/L); and 0.5µl Reverse Transcription enzyme (50U/µl). The

master mix was aliquoted, 4μg of RNA added and RNase free water was used to make the total volume up to 20μl. These were then vortexed and centrifuged at 15,000 RPM for 5 minutes. Thermocycling was carried out in an Eppendorf Thermocycler as follow: 25°C for 10 minutes; 48°C for 30 minutes; and 95°C for 5 minutes.

qRT-PCR

A master mix was prepared containing: 40μl (200nm) forward primer; 40μl (200nm) reverse primer (VHBio, Gateshead); 5μl SYBR green PCR master mix (Applied Biosystems); and 3.2μl Molecular biology grade water (Sigma). This master mix was then aliquoted to 9μl per well of a qRT-PCR plate (Applied Biosystems). Primers were used as listed (Table 4.1). A 'no template control' was carried out by loading 1μl of dH_2O instead of any cDNA sample to confirm there was no contamination. cDNA samples were diluted 1:4 and standards prepared of the following dilutions: 1:0, 1:4, 1:9, 1:19, 1:49, 1:99; and 1:999. 1μl of cDNA sample or standard was loaded per well of a MicroAmp Fast 96 well Reaction plate (0.1ml, Applied Biosystems) and loading was repeated in triplicate. Plates were sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) and centrifuged at 15000 RPM for 5 minutes. PCR was carried out using an Applied Biosystems 7500 Fast Real-Time PCR System. A dissociation plot was produced to demonstrate a single product. Standard curves were produced with an R^2 value of ≥ 0.98. All data was normalised to the reference gene GAPDH and subsequently to that of the control surface (either uncoated plastic or OmpA presenting no motif).

Confirmation of PCR specificity

A 2% (w/v) agarose gel was prepared by adding 0.8g of agarose (162-0133, Bio-Rad) to 35ml dH_2O, boiling until all the agarose had dissolved and then adding 5ml of 5x Tris-borate (TBE (pH 8.3), 54g Tris base, 27.5g boric acid, 20ml 0.5M EDTA (pH 8.0)). Once cool, 1μl/ml of ethidium bromide was added and the solution poured into a gel mould with comb in place. Once set 0.5x TBE was

poured onto the gel and the comb removed. The running tank was then filled with 0.5x TBE and the samples loaded. Samples were pooled from multiple reactions, 10µl was taken and 0.5µl of Blue Juice gel loading buffer (Invitrogen) was added. Samples were loaded onto the gel along with a Hyper Ladder IV 100bp standard (Bioline), a fixed voltage of 120V was applied and samples run until a good separation was achieved.

Primer sequences

Primer	Sequences	Annealing temperature (°C)	Product size (bp)	Ref
GAPDH	TCG CCC CAC TTG ATT TTG GAG G ATG GGG AAG GTG AAG GTG GGA G	60	266	#
Ki67	TCC TTT GGT GGG CAC CTA AGA CCT G TGA TGG TTG AGG TCG TTC CTT GAT G	56	155	358
POU51 (Oct4)	GAG AAC CGA GAG AGA GGC AAC C CAT AGA CGC TGC TTG ATC GCT TG	60	145	359
TUJ1	GGC CAA GTT CTG GGA AGT C CGT TGT AGT AGA CGC TGA TCC	60	108	360
TH	Sequence not available	60	74	

Table 4.1 Primer and sequences used for real time RT-PCR. Table gives information of primers used, annealing temperature and expected product size. # http://medgen.ugent.be/rtprimerdb/

Statistical analysis

The distribution of data was tested using the Kruskal-Wallis test. Where there were too few samples for distribution evaluation, to increase the number of data points the mean value of each group was calculated and subtracted from each value, thus increasing the number of data points by two. The additional values have a mean of 0 and do not change the variance. For normally distributed data an ANOVA test was performed with Bonferroni post-hoc analysis. $p<0.05$ (*), $p<0.005$ (**) and $p<0.001$ (***) were used to indicate levels of statistical significance.

4.3 Results

Presentation of extracellular matrix components to control embryonal carcinoma stem cell behaviour in vitro

Surfaces were prepared presenting the same ECM components as previously investigated (Chapter 3). Tera2.cl-SP12 cells were seeded onto the surfaces under both uninduced (proliferative) and retinoic acid induced (differentiation) conditions. All of the surfaces tested proved adequate for cell attachment and cell culture.

4.3.1 βIII gene expression of embryonal carcinoma cells cultured on surfaces with adsorbed extracellular matrix components

Upon induction of differentiation using retinoic acid, Tera2.cl-SP12 cells are known to differentiate towards a neuronal lineage. To investigate neuronal differentiation the gene expression of βIII tubulin was assessed. When Tera2.cl-SP12 cells were grown under proliferative conditions, the observed expression of βIII tubulin was low. Addition of retinoic acid to the culture media resulted in differentiation of Tera2.cl-SP12 cells and the gene expression of βIII tubulin increased on all surfaces (Figure 4.1, A). Under proliferative conditions, in comparison to uncoated surfaces, collagen I, fibronectin and laminin all resulted in an increase in βIII tubulin expression, where as Matrigel™ had a negative effect and collagen IV had no effect (Figure 4.1, B). Although laminin had the greatest effect on βIII expression, a 0.7-fold increase, statistical analysis revealed that none of the surfaces differed significantly from control levels. Following induction of differentiation an increase in βIII tubulin gene expression was observed on all surfaces except Matrigel™ (Figure 4.1, C). Similar to cells grown under proliferative conditions, there was no significant difference between the tested ECM surfaces and the uncoated control.

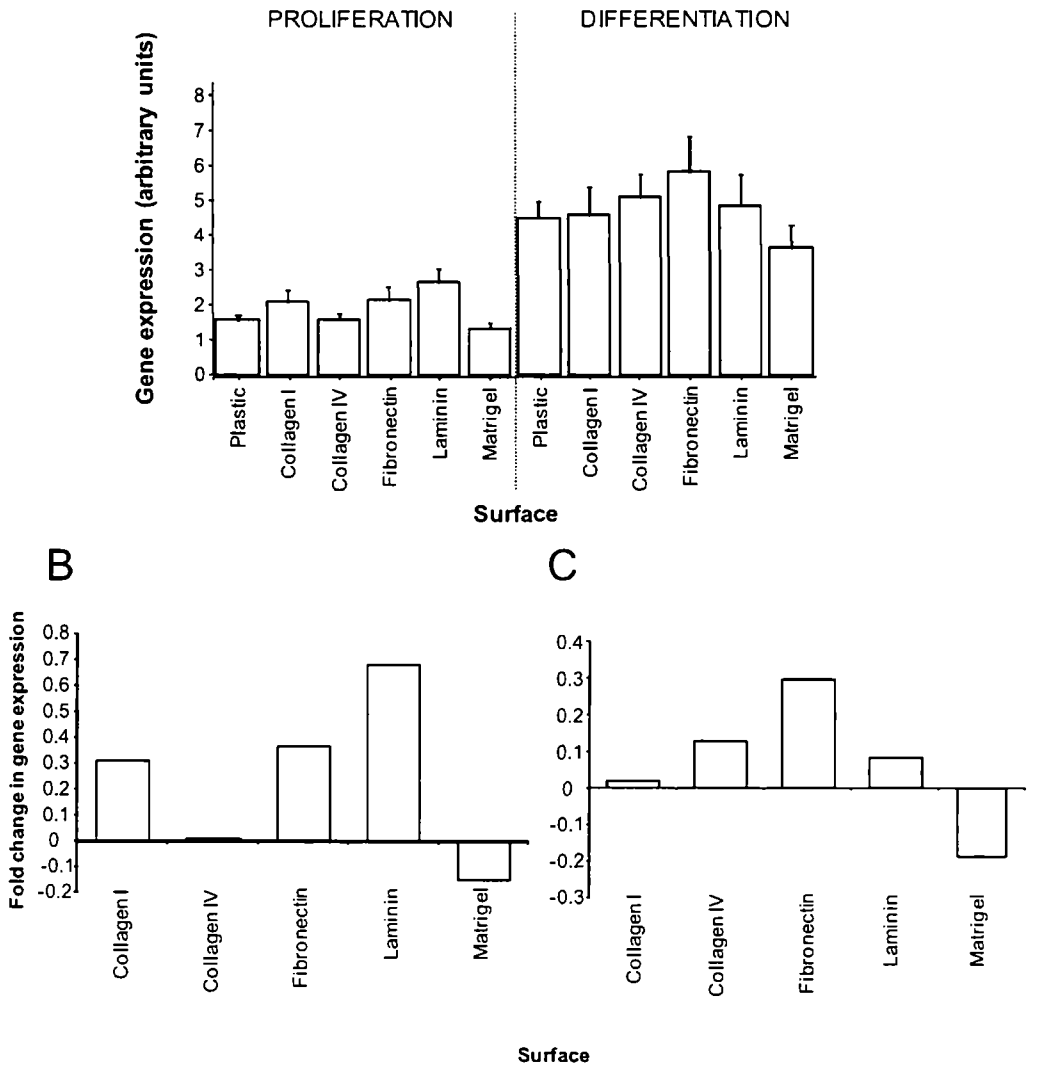

Figure 4.1 Neuronal differentiation of TERA2.cl-SP12 cells cultured on ECM protein adsorbed surfaces as assessed by βIII tubulin gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions for four days is compared to cells cultured for seven days under retinoic acid induced differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD.

4.3.2 Tyrosine hydroxylase gene expression of embryonal carcinoma cells cultured on surfaces with adsorbed extracellular matrix components

An alternative marker of neuronal differentiation is tyrosine hydroxylase. Upon retinoic acid induced differentiation, tyrosine hydroxylase gene expression increased. The difference between gene expression of cells cultured under proliferative and differentiation conditions was the smallest when cells were cultured on fibronectin or Matrigel™ (Figure 4.2, A). Under proliferative conditions, when compared to control levels, all ECM components tested resulted in an increase in tyrosine hydroxylase gene expression. Collagen IV resulted in a 2-fold increase and Matrigel™ resulted in a 4-fold increase. Although these are large increases they were not found to be statistically different from the control level. Fibronectin resulted in a dramatic 10-fold increase in tyrosine hydroxylase gene expression, this increase was found to be significantly *($p<0.05$)* different from the control levels (Figure 4.2, B). Compared to proliferative cultures, under differentiation conditions, the changes in tyrosine hydroxylase gene expression were far less. Similar to proliferative cultures, cells grown on fibronectin produced the greatest increase in tyrosine hydroxylase gene expression however, there was no significant differences between any of the ECM surfaces tested (Figure 4.2, C).

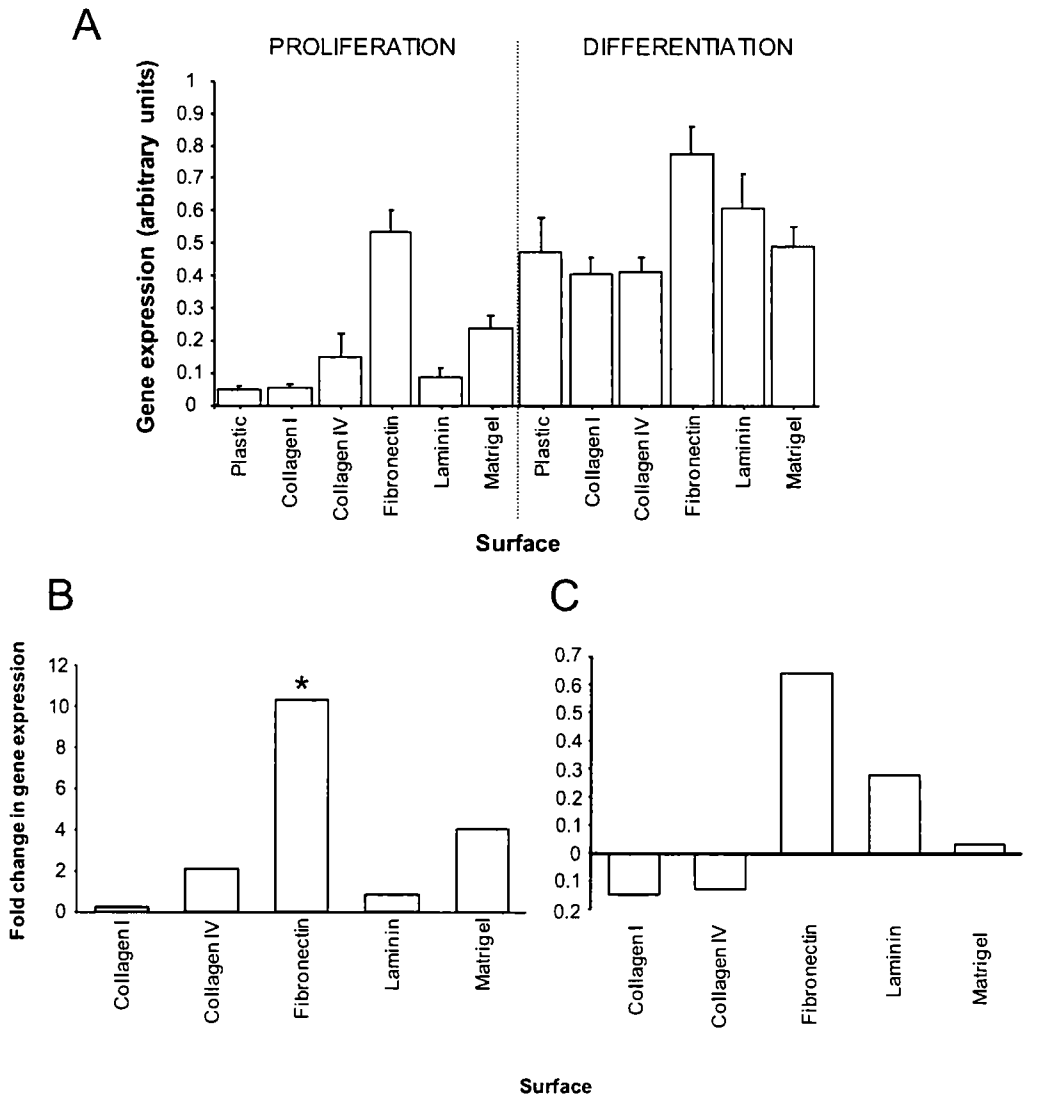

Figure 4.2 Neuronal differentiation of TERA2.cl-SP12 cells cultured on ECM protein adsorbed surfaces as assessed by tyrosine hydroxylase gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid induced differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD, $*p<0.05$.

4.3.3 Oct4 gene expression of embryonal carcinoma cells cultured on surfaces with adsorbed extracellular matrix components

Upon differentiation, the gene expression of the pluripotent stem cell marker Oct4 (*POU5F1*) was seen to decrease (Figure 4.3, A). Under proliferative conditions, laminin and Matrigel™ both decreased Oct4 gene expression, suggesting that these molecules induced differentiation. Cells cultured on collagen I had increased Oct4 gene expression, indicating that this surface maintains pluripotency and decreased differentiation. However, none of these differences were found to be significantly different from control levels (Figure 4.3, B). Upon differentiation, all surfaces except Matrigel™ were observed to have similar gene expression to the control. Expression of Oct4 was not significantly increased by culture upon Matrigel™ however it was increased 7-fold above the level of the control, suggesting Matrigel™ maintains the cells in an undifferentiated state (Figure 4.3, C).

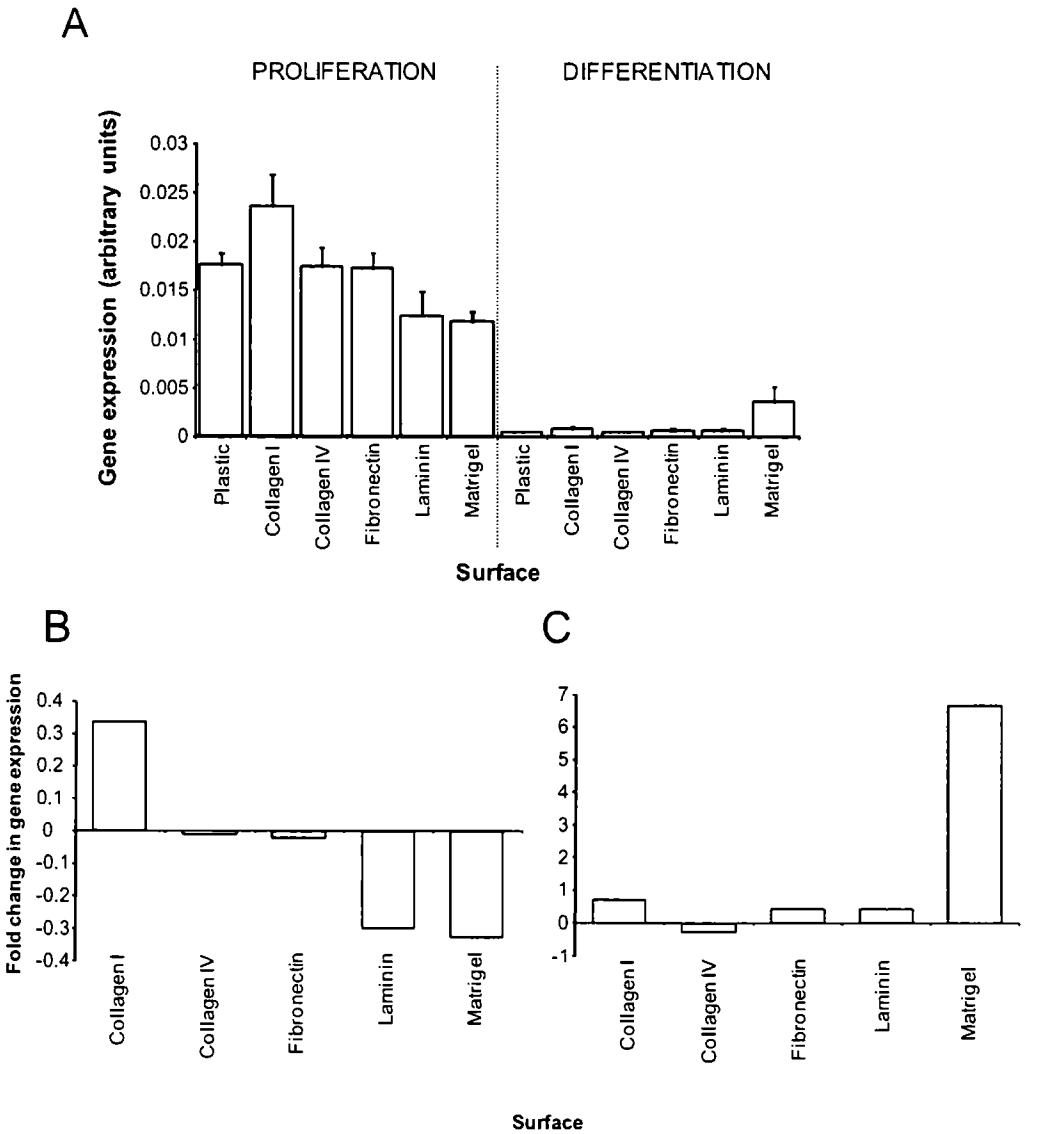

Figure 4.3 Pluripotency of TERA2.cl-SP12 cells cultured on ECM protein adsorbed surfaces as assessed by POU5F1 gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD.

4.3.4 Ki67 gene expression of embryonal carcinoma cells cultured on surfaces with adsorbed extracellular matrix components

Proliferation of cells can be evaluated by the gene expression of the cell proliferation marker Ki67. Upon differentiation, there was a slight reduction in Ki67 gene expression, it is expected that as cells start to differentiate their

proliferation rate will be reduced (Figure 4.4, A). Under proliferative conditions, all of the surfaces tested produced an increase in proliferation when compared to control levels. Fibronectin and Matrigel™ both resulted in a significant *($p<0.05$)* 2.5-fold increase in Ki67 gene expression (Figure 4.4, B). Upon differentiation, all surfaces tested produced an increase in proliferation; fibronectin and Matrigel™ both produced significant increases in proliferation (3.5- and 3-fold *($p<0.005$)*, respectively, Figure 4.4, C), similar to the results seen under proliferative conditions.

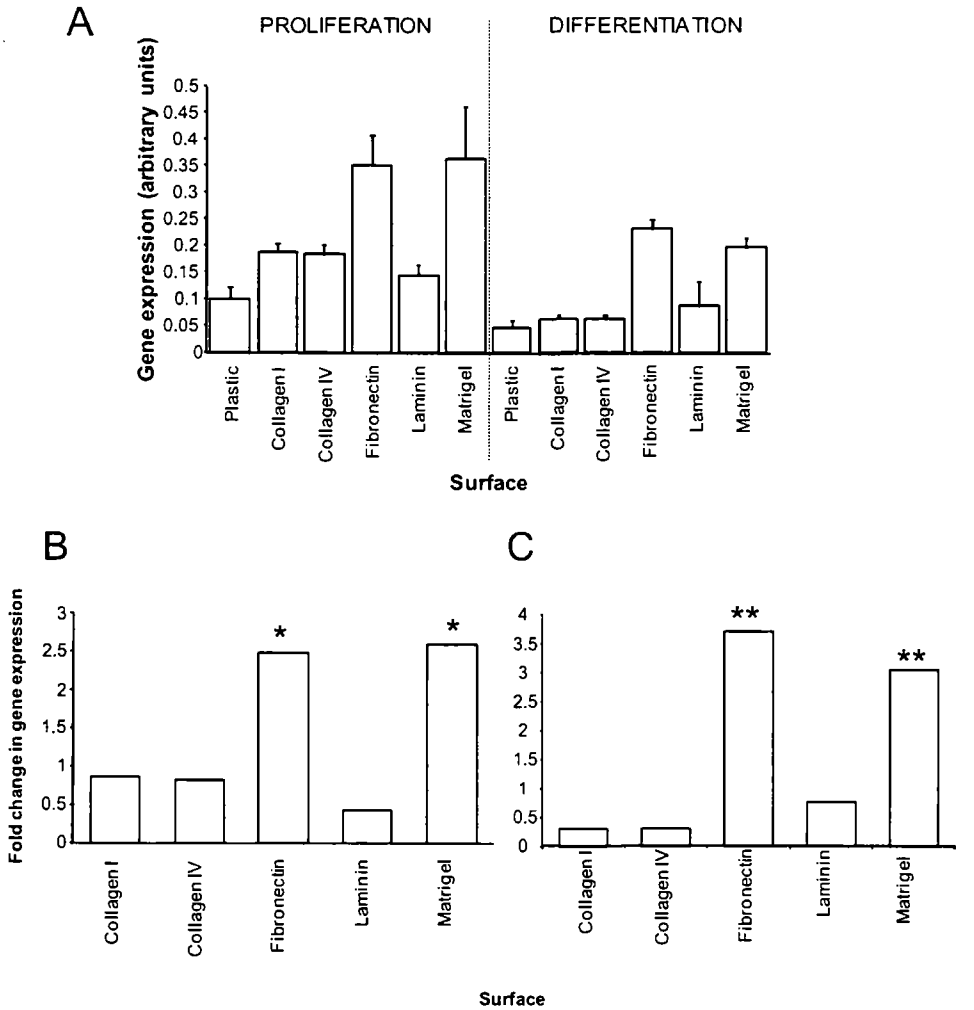

Figure 4.4 Proliferation of TERA2.cl-SP12 cells cultured on ECM protein adsorbed surfaces as assessed by Ki67 gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD, *$p<0.05$, **$p<0.005$.

Biomimetic surfaces presenting motifs from extracellular matrix proteins to control embryonal carcinoma stem cell behaviour in vitro

In Chapter 2, biomimetic surfaces presenting motifs from the ECM proteins: collagen I (Orla 31); collagen IV (Orla 32); fibronectin (Orla 34); and laminin (Orla 36) were demonstrated to be presented in an orientation to which cells could interact. Embryonal carcinoma stem cells, Tera2.cl-SP12, were cultured on each surface under proliferative and retinoic acid induced differentiation conditions. All surfaces proved adequate for cell attachment and culture. An analysis was carried out in a similar fashion to that used for surfaces coated with adsorbed proteins.

4.3.5 βIII gene expression of embryonal carcinoma cells cultured on surfaces presenting motifs from extracellular matrix proteins

Similar to surfaces presenting ECM components, retinoic acid induced differentiation increased βIII tubulin gene expression on all surfaces (Figure 4.5, A). For proliferative cultures, comparison of βIII gene expression relative to the control surface demonstrated that only minor changes are observed (Figure 4.5, B). Under differentiation conditions, all surfaces behaved similar to the control surface, except Orla 32. Orla 32 induced a 2-fold increase in βIII gene expression when compared to control levels, although this proved not to be a significant increase (Figure 4.5, C).

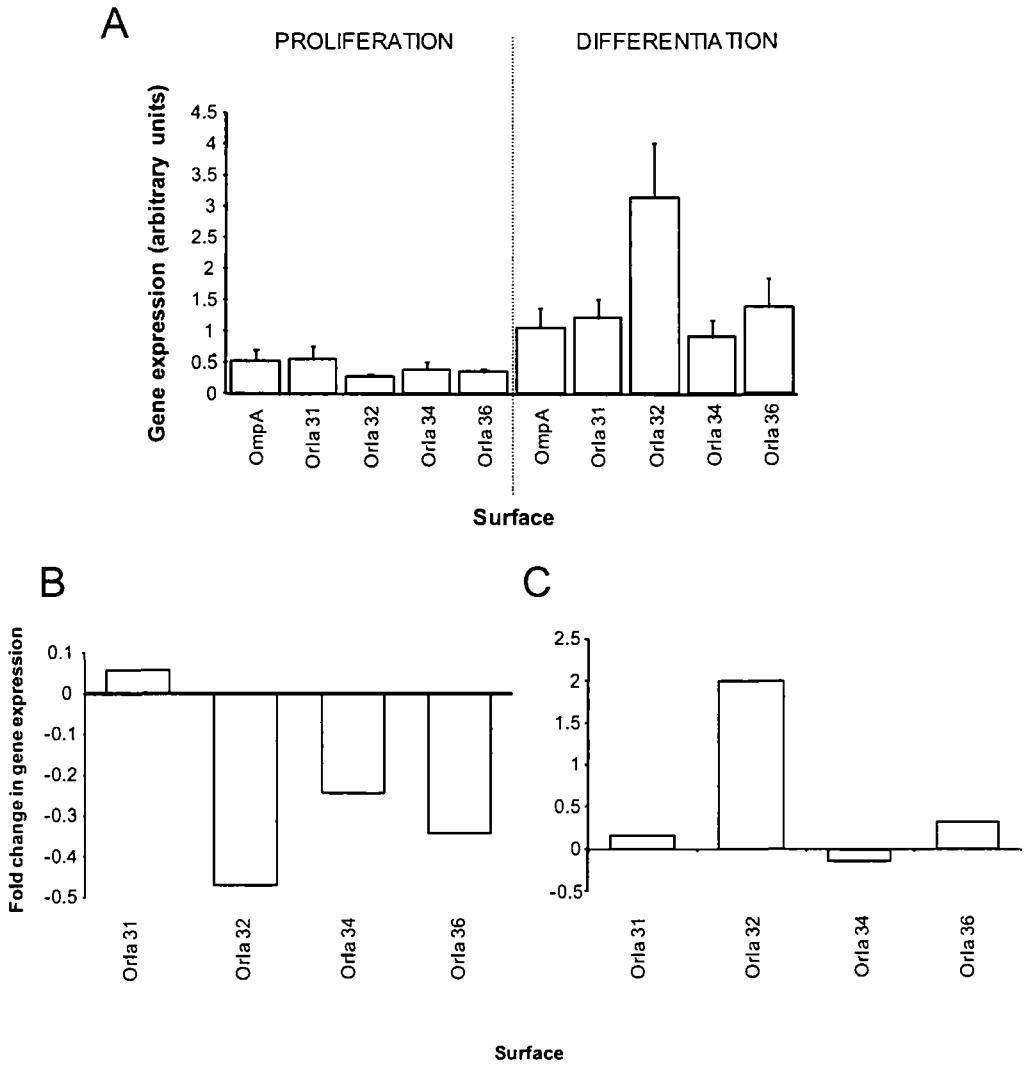

Figure 4.5 Neuronal differentiation of TERA2.cl-SP12 cells cultured on biomimetic surfaces presenting peptide motifs from ECM protein as assessed by βIII tubulin gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD.

4.3.6 Tyrosine hydroxylase gene expression of embryonal carcinoma cells cultured on surfaces presenting motifs from extracellular matrix proteins

Upon retinoic acid induced differentiation, tyrosine hydroxylase gene expression increased on all surfaces, Orla 32 and 36 only resulted in modest increases (Figure 4.6, A). For proliferative cultures, comparison of tyrosine hydroxylase gene expression relative to the control surface demonstrated that the surfaces were having an effect on neuronal differentiation: Orla 31 and 32 increased gene expression 1-fold; Orla 36 increased gene expression 3-fold; and Orla 34 increased gene expression 7-fold (Figure 4.6, B). Under differentiation conditions, none of the effects observed for proliferative cultures were seen and all surfaces behaved similar to the control surface (Figure 4.6, C). Statistical analysis proved none of the differences observed were significant.

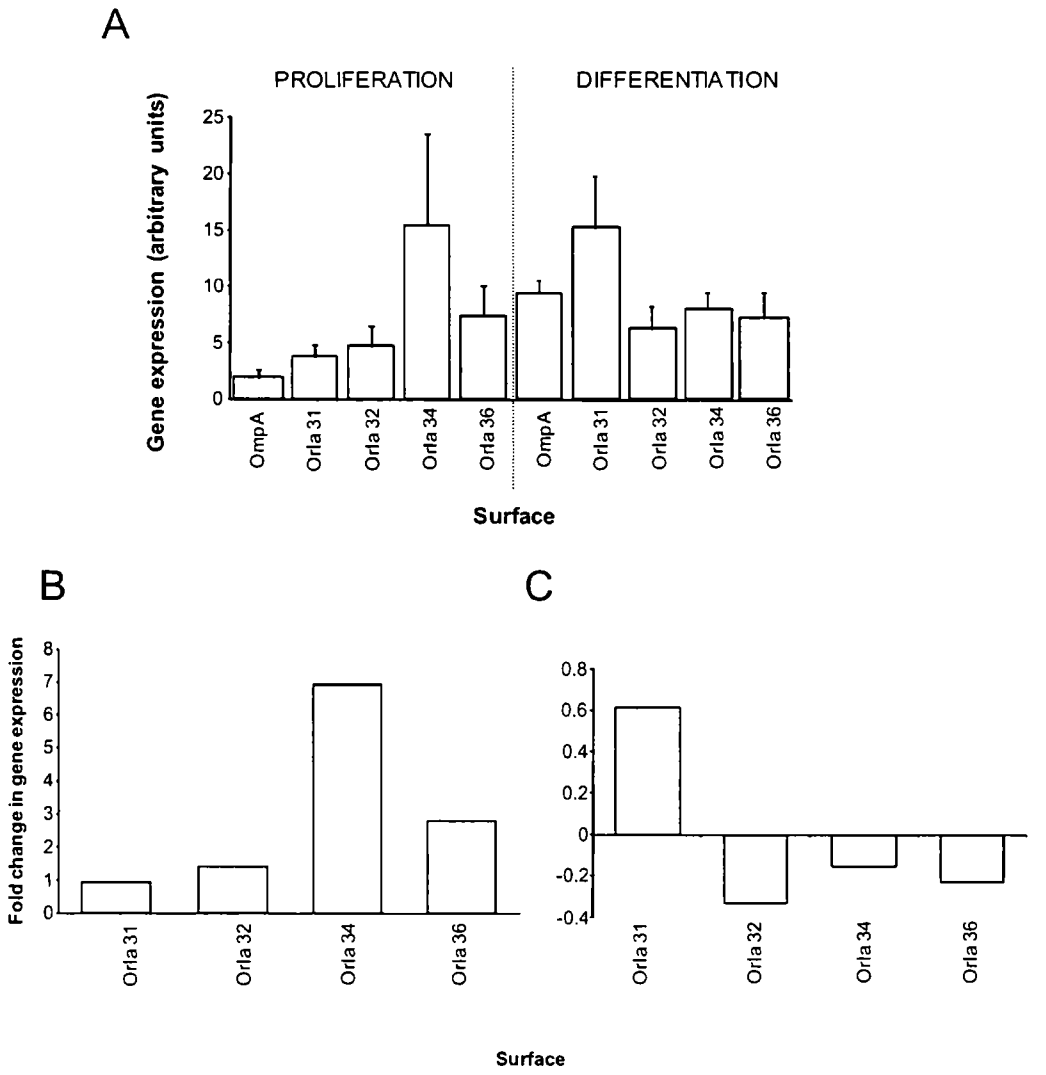

Figure 4.6 Neuronal differentiation of TERA2.cl-SP12 cells cultured on biomimetic surfaces presenting peptide motifs from ECM protein as assessed by tyrosine hydroxylase gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD.

4.3.7 Oct4 gene expression of embryonal carcinoma cells cultured on surfaces presenting motifs from extracellular matrix proteins

Upon retinoic acid induced differentiation, the pluripotency gene Oct4 decreased on all surfaces (Figure 4.7, A). Under proliferative conditions, all surfaces increased Oct4 gene expression when compared to the control levels, however, the increases were less than 1-fold for all surfaces tested (Figure 4.7, B). Under differentiation conditions, all surfaces behaved similar to the control surface, except Orla 31 and 36 which decreased gene expression relative to control levels by less than 1-fold (Figure 4.7, C). Statistical analysis proved none of the differences observed were significant.

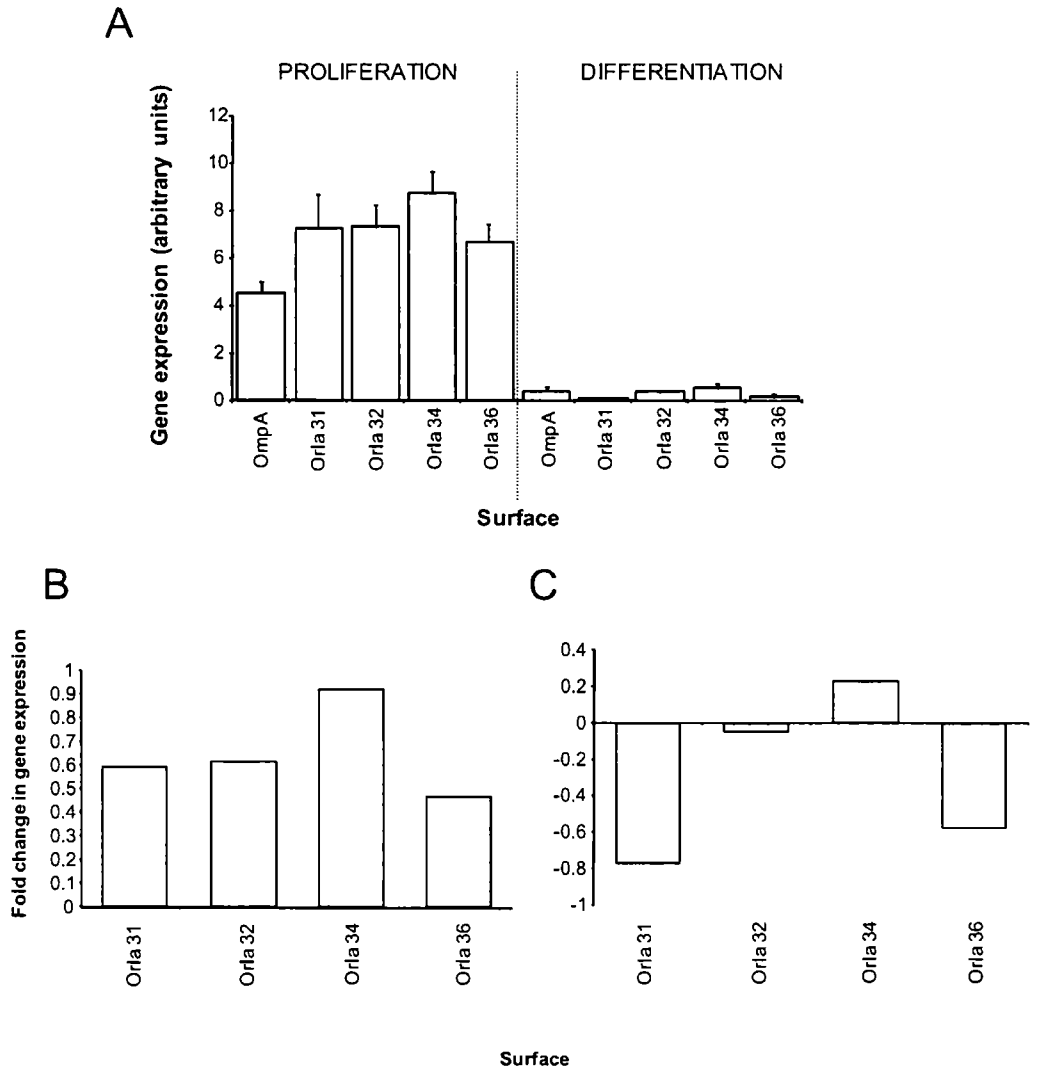

Figure 4.7 Pluripotency of TERA2.cl-SP12 cells cultured on ECM protein adsorbed surfaces as assessed by POUF51 gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD.

4.3.8 Ki67 gene expression of embryonal carcinoma cells cultured on surfaces presenting motifs from extracellular matrix proteins

Upon retinoic acid induced differentiation, the gene expression of proliferation marker Ki67 decreased on all surfaces (Figure 4.8, A). Under proliferative

conditions, the gene expression of the proliferative marker Ki67 decreased on all surfaces when compared to the control levels, however, the decreases were less than 1-fold for all the surfaces tested (Figure 4.8, B). Under differentiation conditions, the only surface that resulted in a large change was Orla 32, which increased Ki67 gene expression 1.5-fold, however, this was not a significant change when compared to control levels (Figure 4.8, C).

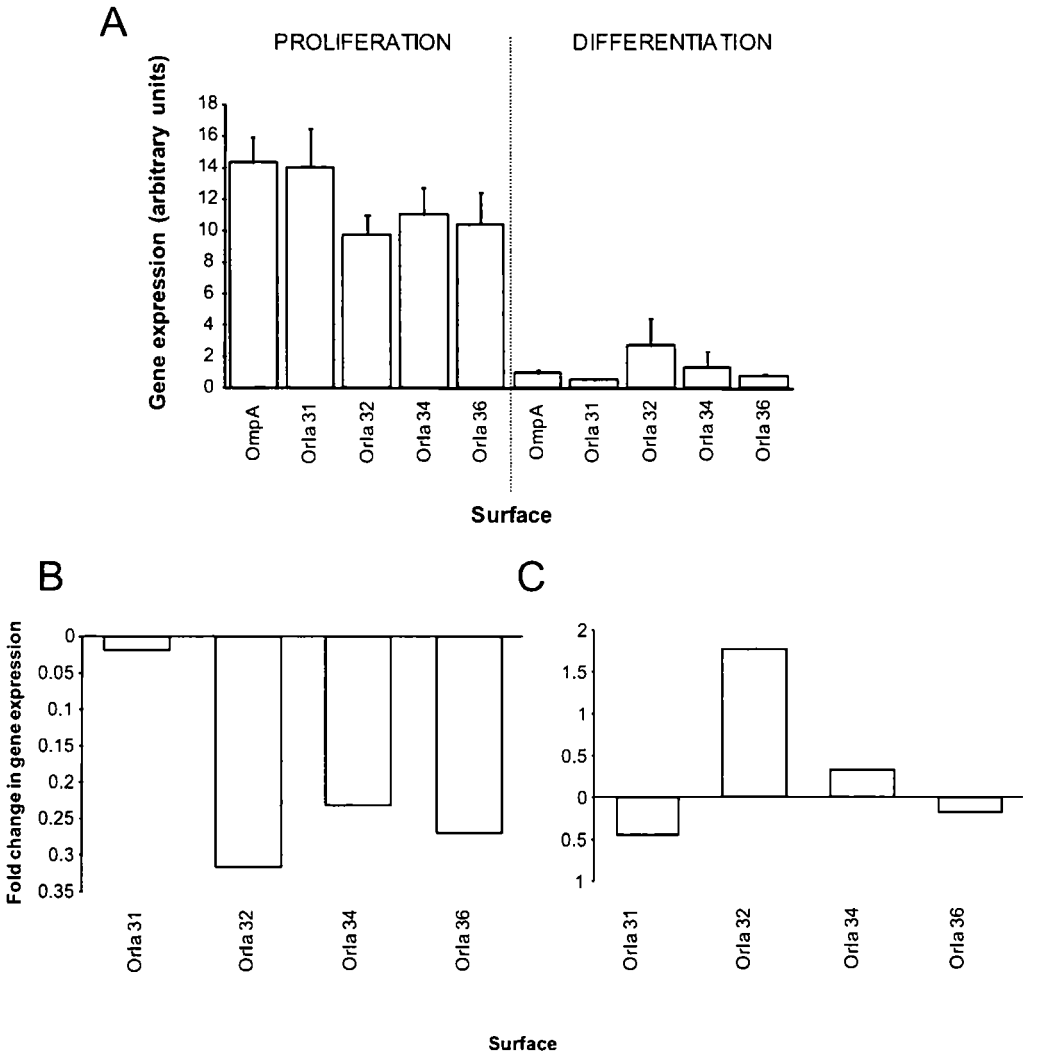

Figure 4.8 Proliferation of TERA2.cl-SP12 cells cultured on ECM protein adsorbed surfaces as assessed by Ki67 gene expression. A) The gene expression of TERA2.cl-SP12 cells cultured under proliferative conditions is compared to cells cultured under retinoic acid differentiation conditions. B) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under proliferative conditions. C) Fold change in gene expression relative to uncoated surfaces of Tera2.cl-SP12 cells cultured under retinoic acid induced differentiation. Data represents mean values of 3 repeats normalised to GAPDH, Error Bars: SD.

4.3.9 Primer validation

For all primer sequences used, a dissociation plot was obtained with one peak, this demonstrates that only one product was formed (Figure 4.9, A). To verify the amplified product was of the correct size, gel electrophoresis was conducted and the product size compared to the expected size (Figure 4.9, B). Other than the single band at the correct size, no additional bands were observed throughout the gel.

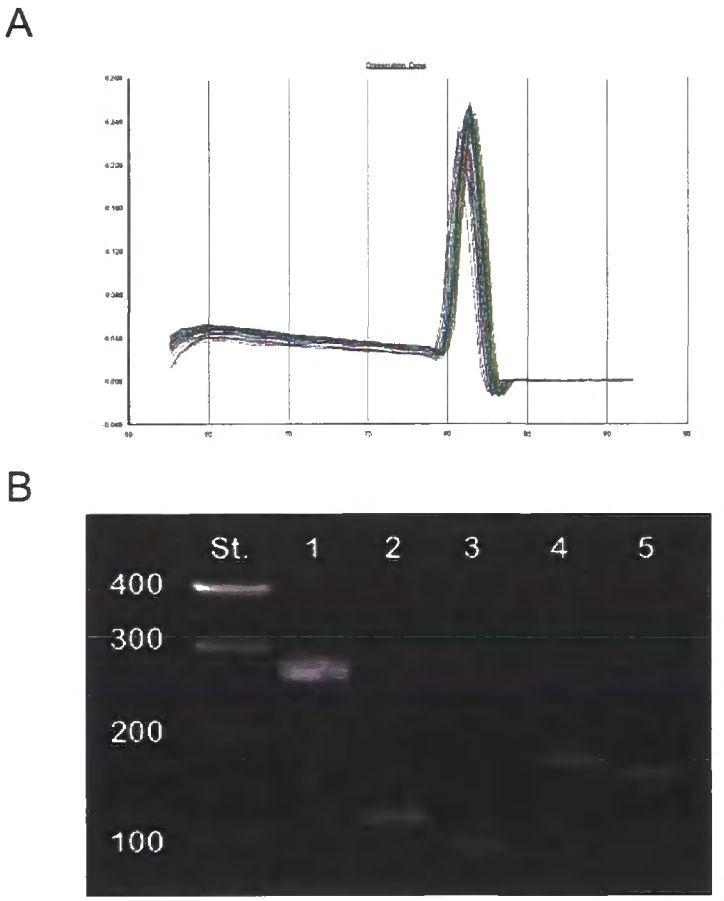

Figure 4.9 Primer validation. A) Sample dissociation plot showing only one melting point. B) Agarose gel electrophoresis confirming product size. 100bp standards were loaded (St.), GAPDH lane 1 (expected size 266bp), βIII tubulin lane 2 (expected size (108bp), tyrosine hydroxylase lane 3 (expected size 74pb), Ki67 lane 4 (expected size 155bp), Oct4 lane 5 (expected size 145bp).

Surface	Gene							
	βIII		TH		Oct4		Ki67	
	Pro	Diff	Pro	Diff	Pro	Diff	Pro	Diff
Collagen I	NC	NC	NC	NC	NC	NC	NC	NC
Collagen IV	NC	NC	NC	2	NC	NC	NC	NC
Fibronectin	NC	NC	NC	10*	NC	NC	2.5*	3.5**
Laminin	NC	NC	NC	NC	NC	NC	NC	NC
Matrigel™	NC	NC	NC	4	7	NC	2.5*	3**

Surface	Gene							
	βIII		TH		Oct4		Ki67	
	Pro	Diff	Pro	Diff	Pro	Diff	Pro	Diff
Orla 31 (Collagen I)	NC	NC	1	NC	NC	NC	NC	NC
Orla 32 (Collagen IV)	NC	2	1	NC	NC	NC	NC	1.5
Orla 34 (Fibronectin)	NC	NC	7	NC	NC	NC	NC	NC
Orla 36 (Laminin)	NC	NC	3	NC	NC	NC	NC	NC

Table 4.2 Summary of results for gene expression on surfaces coated with ECM components and biomimetic surfaces presenting motifs from ECM proteins. Tables show the fold increase in gene expression of: βIII tubulin (βIII); tyrosine hydroxylase (TH); Oct4 (Oct4); and Ki67 (Ki67) for surfaces that resulted in an increase of 1-fold or greater. *$p<0.05$, **$p<0.01$ indicate the significant levels, NC = no change.

4.4 Discussion

In this study, it is demonstrated that the gene expression of differentiation, pluripotency and proliferative markers is regulated by the ECM components that are presented to Tera2.cl-SP12 cells. In agreement with previous studies, retinoic acid induced differentiation resulted in an increase in mRNA expression of neuronal markers [361] and a decrease in the mRNA expression of the pluripotency marker Oct4 [362,363]. As expected, the response of the cells to the surface was dependent upon the ECM component presented. The response of Tera2.cl-SP12 cells to alternative ECM proteins was also seen to be dependent on the culture conditions in which the cells were grown. Different responses were observed for proliferative and differentiated cultures, for example, increases in gene expression for cells under proliferative conditions were not always observed for cells grown in differentiation conditions and vice versa.

The ECM is important in the differentiation of EC stem cells. Studies have demonstrated that upon differentiation EC stem cells increase their synthesis and secretion of laminin and collagen IV [80,364-366], by 72 hours, the level of laminin and collagen IV reach a maximum level [366]. Although both collagen IV and laminin are upregulated, it has been shown that these networks are independent of each other [367]. Conflicting reports exist for fibronectin, which has been shown to be synthesised by both undifferentiated and differentiated EC stem cells [368,369] with an upregulation following differentiation [370]. Some studies have shown that EC stem cells do not synthesise fibronectin until they differentiate [371]. This data indicates that ECM proteins are involved in the differentiation process. EC stem cells cultured on surfaces coated with alternative ECM components provide a model system to study how these affect differentiation.

The ECM component that influenced the greatest number of genes was Matrigel™. Given the large number of different components, including both proteins and growth factors, Matrigel™ is subjecting the cells to more inductive signals than single ECM proteins alone. Matrigel™ has been shown to increase both proliferation and differentiation, whereas, in the same assay, collagen I and laminin had no effect [191].

Matrigel™ is used for feeder free maintenance of ES cells and has been proposed to be better at maintaining the pluripotent phenotype when compared to conventional murine embryonic fibroblasts (MEFs) [372] [373]. This is supported herein as under differentiation conditions, cells cultured on Matrigel™ had a 7-fold increase in Oct4 gene expression when compared to control levels.

It has been proposed that the culture of stem cells in 3D gels promote self renewal and maintenance of a pluripotent phenotype [374]. Gel formation is not essential in maintaining an undifferentiated phenotype of stem cells as it has been demonstrated that: collagen I and gelatin (which do form gels) result in cell behaviour similar to laminin and fibronectin (which do not form gels) [375]. When Matrigel™ is diluted it loses its gel forming ability, in this section it was used at a concentration (3:1) that will form a gel. The volume of Matrigel™ that was used in this section was recommended by the manufacturer to produce a thin layer coating whilst maintaining a gel resulting in optimal formation to ensure that the interactions due to the 3D structure were kept to a minimum. Therefore the 3D structure could have helped maintain pluripotency and increase proliferation however this will have been minimal.

Similar to Matrigel™, fibronectin upregulated the gene expression of Ki67 and tyrosine hydroxylase, this was unexpected as fibronectin is not a component of Matrigel™, whereas, collagen IV and laminin, which are components of Matrigel™ did not have an effect similar to Matrigel™. However, as both the whole protein and the Orla 34 surface presenting the fibronectin motif PHSRN had a positive effect on EC stem cells. When investigating the attachment of PC12 cells, the surface Orla 34 was found to have the greatest effect on cell attachment. As this surface also has the greatest effect on differentiation and proliferation of EC stem cells, this suggests that this motif is presented in a manner, which is either superior to or more faithful to the natural motif found in the whole molecule of fibronectin.

There have been few investigations of how the peptide motifs tested herein affect cell behaviour in terms of neuronal differentiation, maintenance of pluripotency

and proliferation. There are some reports where peptides have been found to influence cell proliferation. RGDS is known to increase the proliferation of murine fibroblasts [376] and PHSRN in combination with RGD increases proliferation of human fibroblasts and HaCaT cells [377]. This data indicates that the peptide motifs can be used to control proliferation.

In comparison to whole proteins, presentation of individual motifs had little effect on the gene expression of Tera2.cl-SP12 cells. Given the complexity and the number of functional motifs presented by whole ECM proteins, it is not surprising that individual motifs did not have as great an effect as the whole proteins. Similar to the protein fibronectin, presentation of the fibronectin motif Orla 34 increased the gene expression of tyrosine hydroxylase. In Chapter 3, it was found that Orla 34 resulted in the greatest level of cell attachment of PC12 cells, this data demonstrates that the conformation of motif presentation could be an important factor for the observed results.

Studies have shown that, laminin, in comparison to collagen I, collagen IV, fibronectin and glass, significantly increased confluence during culture of EC cells. The observed effects were inhibited with the addition of soluble YIGSR, indicating that the motif YIGSR can control proliferation [186]. These results provide further evidence that peptides can be used to control proliferation, although these results are not supported herein as little effect was observed when cells were cultured on biomimetic surfaces presenting peptides. Although EC cells were used, it was a different cell line from the one used in this section, another difference is the method of motif presentation. In the study by Sweeney et al.[186] a linear YIGSR peptide in solution was used whereas, herein the peptides are presented in a loop tethered to a surface and for the reasons previously discussed this may account for the differences observed. A further factor that may influence the results is cell number, however all the data herein has been normalised to GAPDH and therefore the level of attachment will not affect this data.

When investigating tyrosine hydroxylase expression, all motifs investigated increased gene expression when cultured under proliferative conditions however these effects were not maintained following differentiation. Other groups have

noted similar effects. Following three days differentiation of ES cells, the level of differentiation was greater on fibronectin when compared to laminin, whereas following five days differentiation, the level of differentiation was greater on laminin than fibronectin [378].

In this study neither the whole laminin protein nor motif had an effect on tyrosine hydroxylase gene expression levels. Retinoic acid induced differentiation of EC stem cells is widely used, however, there are alternative methods for induction of neuronal differentiation, such as the application of sonic hedgehog with FGF8 [379], cyclic-AMP (cAMP) [80] or NGF [380]. Previously, in a study using calf adrenal chromaffin cells, culture of these cells on laminin coated surfaces has been shown to increase the level of tyrosine hydroxylase [347]. In the system tested herein, tyrosine hydroxylase gene expression does increase upon retinoic acid induced differentiation. It is possible that in embryonal carcinoma cells, the combination of laminin and retinoic acid is insufficient to increase tyrosine hydroxylase gene expression and an alternative soluble molecule such as FGF8 is needed to potentiate the effects of laminin.

Many cell processes involve a change in the ECM. A cell can change the ECM using matrix metalloproteinases (MMPs) [381]. A subgroup of MMPs are collagenases and these are involved in the remodeling of collagen. MMP-1 (collagenase-1) is known to cleave collagen. The entrance to the binding of MMP-1 is only 5Å, each of the three chains of collagen are 5Å in width, therefore MMP-1 cannot bind to the whole collagen triple helix. A proposed mechanism is that MMP-1 induces a local conformational change in the structure of collagen and unwinds the triple helix so that it can then bind to the individual chains to cleave the helix [382]. When peptide motifs are presented by the OmpA molecules this is in a conformation that is dissimilar to that found in situ. This could result in MMP being ineffective in remodeling the surface upon which the cells are growing and could alter the cell behaviour in comparison to adsorbed proteins, accounting for some of the observed differences. Contradictory to this, when rabbit synovial fibroblasts (RSF) are cultured on surfaces coated with the fibronectin fragment 120FN (containing the adhesion domain RGD) an increase in MMP expression is seen when compared to cells on surfaces coated with whole fibronectin molecules,

suggesting that a domain outside of fibronectins adhesion domain can suppress MMP expression. Binding to fibronectin is mediated by α5β1, the suppression of MMP activity was found to be controlled by the integrin α4β1 [383]. This study presents the possibility that culture of cells on surfaces coated with whole protein molecules could suppress the expression of MMPs, decreasing matrix remodeling and subsequently decreasing the ability of cells to remodel the surface.

The greatest effects were observed when EC stem cells were cultured on surfaces with adsorbed proteins and relatively small effects were observed when cells were cultured on surfaces presenting peptide motifs. As integrins interact with the peptide presenting surfaces and relatively small effects are observed this indicates that integrin binding plays a minor role in the differentiation and proliferation of EC stem cells under the conditions tested herein. For example, EC stem cells cultured on surfaces with adsorbed fibronectin show a significant increase in neuronal differentiation as assessed by TH expression and also a significant increase in proliferation as assessed by the proliferative marker Ki67. In contrast, cells grown on surfaces presenting the fibronectin motif PHSRN only show a minor increase in neuronal differentiation (an increase in TH expression) and no increase in proliferation is seen. Integrin binding should not be discounted as being involved in these processes however, when the motifs were presented in this conformation they were not sufficient to elicit a large effect. In support of the argument that integrin binding does not greatly affect EC stem cell differentiation a study conducted by Burdsal et al [384] showed that upon differentiation there was an upregulation in integrin expression. Following differentiation, β1 integrin expression was seen to increase by 1.5 folds and the α3 showed a 24 fold increase. Although differentiation induced a large increase in integrin expression, no corresponding increase in adhesion was observed for the differentiated EC stem cells. The authors concluded that although integrin expression was increased the lack of a resultant increase in attachment demonstrated that integrin binding mediated attachment could not account for the morphological difference observed between undifferentiated and differentiated EC stem cells. This supports the work herein where peptide surfaces whose effects are mediated via integrins have little effect compared to surfaces where the whole protein molecule is present,

containing multiple motifs that can control cell differentiation via non-integrin mechanisms.

Further support that integrin binding in not necessary for the control of differentiation comes from experiments where integrins have been deleted. Deletion of the β1 integrin from the F9 EC stem cell line results in a reduction in cell attachment and cell spreading on surfaces coated with collagen laminin and fibronectin. Under uninduced conditions the deletion of the β1 integrin does not affect the expression of the undifferentiated marker SSEA1, supporting the hypothesis that integrin binding does not affect differentiation. However, when the cells were induced to differentiate differences were observed following β1 integrin deletion. When control cells were treated with RA and dbcAMP, factors known to induce differentiation towards parietal endoderm the control cells showed a decrease in the undifferentiated marker SSEA1 whereas EC stem cells where the integrin β1 had been deleted were unresponsive to these inductive factors and maintained expression of the undifferentiated marker SSEA1 [385]. Under the conditions used by Stephens et al their study demonstrated that integrin engagement is not essential to maintain EC stem cells in the undifferentiated state. However this study does show that integrin engagement is necessary for certain factors to elicit their effects. Therefore it can be concluded that integrin engagement can affect EC stem cell differentiation however, this is dependent upon the inductive factors used. In terms of the experiments presented herein this suggests that RA induction of Tera2.cl.SP12 cells is not dependent upon integrin engagement however, under different differentiation inductive conditions integrin binding and therefore surfaces presenting ECM peptide motifs could have a greater effect.

Care should be taken when interpreting these results as although cell behaviour in the *in vitro* and *in vivo* environments is similar (as previously discussed), *in vitro* cell culture is highly simplified and not identical to the *in vivo* environment. Coating surfaces with ECM molecules may not be sufficient to elicit an effect on development and further factors are necessary, for example, coating surfaces with laminin does not produce dopaminergic-like neurons from neural stem cells however with addition of both heparin and bFGF, tyrosine hydroxylase expression

is observed. When each of these factors is used individually no effect is seen, thus demonstrating a need to combine factors to produce the required effects [386].

COMPARISON OF RESULTS GAINED TO THE HYPOTHESIS UNDER INVESTIGATION

It was predicted that as large teratomas are produced when EC stem cells are co-transplanted with Matrigel™, the culture of EC stem cells on surfaces coated with Matrigel™ would increase Ki67 gene expression. This was observed and indicates that the large teratomas produced could be due to increased proliferation of the transplanted cells. Fibronectin also induced an increase in Ki67 gene expression, however, when co-transplanted with EC stem cells no teratoma formation was observed. This indicates that increased proliferation alone is not sufficient to result in a teratoma. However, contrary to the predictions, a reduction in Ki67 gene expression was not observed for cells cultured on collagen I or laminin, which results in a reduction in teratoma size. EC stem cells co-transplanted with collagen I appeared to show a reduction in their differentiation potential. These results are part supported, following seven days retinoic acid induced differentiation, tyrosine hydroxylase expression is reduced on collagen I surfaces. However, a reduction is also observed for cells cultured on laminin coated surfaces. It was also predicted that cells cultured on collagen I would have an increased gene expression of Oct4 due to a decrease in differentiation; this was not observed when cells were cultured on protein coated surfaces. The peptide motifs from collagen I, collagen IV, fibronectin and laminin were investigated to demonstrate whether these motifs have a similar effect to the whole proteins. This was partly demonstrated, as EC stem cells cultured on the protein fibronectin and the fibronectin motif PHSRN resulted in the greatest gene expression of tyrosine hydroxylase when compared to the other surfaces tested.

CHAPTER 5 – GENERAL DISCUSSION

Stem cells hold great potential for the cure of disease, however, the translation of stem cells theoretical potential in the laboratory to practical clinical use has been limited. There is a need for greater laboratory research to be done using these cells in order understand how to control their behaviour and ultimately to achieve success in the cure of disease. This study shows how the ECM regulates cell behaviour, specifically demonstrating how ECM proteins and their motifs regulate cell behaviour.

It is demonstrated that *in vivo*, the environment into which human pluripotent stem cells are transplanted affects their differentiation and proliferation. The ECM is suggested as a key factor controlling stem cell behaviour *in vivo* and using co-transplantation of hEC stem cells with alternative ECM components it is demonstrated that the ECM is capable of controlling stem cell behaviour. Unexpectedly, when the transplantation of hEC stem cells is carried out in the presence and absence of growth factors little difference is noted between these two conditions, thus demonstrating that under these conditions, the ECM is more important than growth factors in controlling hEC stem cell proliferation and differentiation. Taken together, these results strongly indicate that ECM proteins are key factors in controlling stem cell behaviour.

To further investigate how the ECM controls cell behaviour, *in vitro* analysis is carried out and surfaces, composed of both adsorbed ECM proteins and peptide motifs, are tested. It is proven that peptide motifs can be presented from ECM proteins via biomimetic surfaces in a functional fashion to control cell attachment and neurogenesis. *In vivo*, cells are contacted by multiple different ECM proteins; to mimic this *in vitro* surfaces of peptide motifs were prepared from a number of different ECM proteins and presented simultaneously on a single surface. It was expected that surfaces presenting multiple motifs would allow greater control of stem cell behaviour, however, this was not the case and the results were counterintuitive. These results using ECM proteins and their peptide motifs demonstrated that *in vitro* analysis can be used to investigate and control cell

behaviour. Importantly, these results indicated that certain motifs were inactive in controlling cell attachment and neurogenesis.

To start to explain some of the observations from the *in vivo* experiments, *in vitro* analysis was carried out using surfaces with adsorbed ECM components and biomimetic surfaces presenting peptides from ECM proteins that had been demonstrated as capable of controlling cell attachment or neurogenesis. Using real-time PCR, some parallels were drawn between cells transplanted *in vivo* and those cultured *in vitro*. *In vivo*, teratomas produced with the co-injection of Matrigel™ were the largest of all teratomas produced. *In vitro*, culture of hEC stem cells on Matrigel™ coated surfaces, showed significant upregulation in the gene expression of the proliferative marker Ki67. This indicates that the large teratomas produced *in vivo* could be due to Matrigel™ increasing the rate of proliferation of hEC stem cells.

In vivo Matrigel™ was shown to increase the rate of teratoma formation. *In vitro*, Matrigel™ was found to upregulate the gene expression of the differentiation marker TH, the pluripotent marker Oct4 and the proliferation marker Ki67. *In vitro*, fibronectin upregulated the gene expression of TH and Ki67 (similar to Matrigel™) however, unlike Matrigel™, it did not up regulate Oct4. *In vivo*, fibronectin was unable to increase the success rate of teratoma formation. In agreement with Choo et al. [387], these results indicate that the upregulation of Oct4 gene expression and hence the pluripotent cell population is a key factor in teratoma production. Further evidence that Oct4 expression is key in teratoma formation comes from the work of Kleppner et al. [72], where they demonstrated that hEC stem cells differentiated with retinoic acid reduces the rate of teratoma formation.

SUMMARY OF KEY FINDINGS

- The anatomical location into which human pluripotent stem cells are transplanted influences their behaviour.

- Transplantation of human pluripotent stem cells intrahepatically produces large teratomas, which are composed of immature cell types.

- Co-transplantation of hEC stem cells with collagen I or laminin reduced the size of teratomas formed and reduced the maturity of differentiation with little positive staining for the mature neural marker NF200.

- Co-transplantation of hEC cells with Matrigel™ increased the success rate of teratoma formation; the size of teratomas formed; and did not affect the composition of the teratoma.

- Growth factors presented in Matrigel™ had little effect on: the success rate of teratoma formation; teratoma size; or teratoma composition.

- Biomimetic surfaces produced by Orla Protein Technologies can present peptides from ECM proteins to control: cell attachment; neurite outgrowth; and neuronal differentiation.

- *In vivo* observations that Matrigel™ increased the success rate of teratoma formation and teratoma growth rate were in part explained by *in vitro* analysis, where Matrigel™ increased gene expression of the pluripotent stem cell marker Oct4 and the proliferative marker Ki67.

FUTURE WORK

This work explores the use of teratomas as a model system to investigate how the ECM controls cell behaviour. Although the teratoma model is widely used to determine the potency of stem cells, little work has been conducted to assess the mechanisms underlying this system. Although, increasing proliferation rates through contacts with the ECM are proposed as resulting in rapid teratoma growth, this could be investigated further. *In vivo*, positive staining was obtained for the proliferative marker Ki67 in all teratomas produced however, this data was not quantitative. *In vivo*, quantitative data is difficult to obtain, however, flow cytometry could be used to analyse cells cultured on Matrigel™ *in vitro* to obtain the increase in Ki67 in comparison to that of control levels. Teratoma composition was analysed using a range of antibodies to undifferentiated and differentiated cell types known to be present in teratomas produced by human stem cells; this approach could be extended with the inclusion of previously untested markers, such as, specific keratin markers to provide a more in-depth analysis of the teratoma composition.

No teratoma formation was observed with PuraMatrix™. It is possible to incorporate ECM proteins into PuraMatrix™. To further investigate this system, laminin could be incorporated into the PuraMatrix™ hydrogel with the prediction of an increase in the success rate of teratoma formation. To further demonstrate how peptides from ECM proteins affect cell behaviour, these could be immobilised onto gold nanoparticles and co-transplanted with human stem cells to modulate teratoma formation. *In vitro*, the collagen IV motif, when presented via biomimetic surfaces (Orla 32) increased the expression of Ki67, it would be predicted that the inclusion of this would increase proliferation of the teratoma.

Investigations herein demonstrate that biomimetic surfaces can be used to control cell behaviour. The expression of βIII tubulin was used to indicate neuronal differentiation. This could be investigated in greater detail, although an increase in βIII expression was taken as indicating an increase in neuronal differentiation this

may not necessarily be the case. An increase in the percentage of cells expressing βIII could be due to: selective death; decreased proliferation; or inhibition of attachment of non-βIII positive cell types. This system could be investigated further with a more detailed analysis involving the investigation of these possible factors.

All data was analysed by hand and the sample sizes chosen were sufficient to make the analysis relevant but they were a sample and did not include the whole experiment, automated image analysis could be used to increase the sample size.

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