Notice 1

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Influence of the extracellular matrix on the *in vitro* differentiation of mouse embryonic stem cells into neurons

A thesis submitted for the degree of

Doctor of Philosophy

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Abstract:

Stem cell maintenance and differentiation are regulated by local inductive cues; one potential source of inductive cues is the extracellular matrix. The fundamental hypothesis of the thesis was that differentiation of embryonic stem cells can be manipulated by substrates onto which cells are plated thus, our first experimental chapter explores the impact of the extracellular matrix proteins, laminin, fibronectin and type IV collagen on neural induction of E14Tg2a mouse embryonic stem cells (mESCs) plated as adherent monolayers. In-cell Western assays were carried out to determine coating efficiency of selected matrix proteins. Cell viability and proliferation was estimated using the MTT assay. In addition, the effect of matrix proteins on neurite outgrowth of mESCs-derived neurons was evaluated by counting the number of primary neurites (using βIII tubulin labelling) and neural progenitor (nestin). Total neurite length per neuron was quantified on neurons derived from the differentiated mESCs. Immunocytochemistry against tyrosine hydroxylase and βIII tubulin was used to observe the progression of neuronal maturation of cells plated onto matrix proteins. The MTT assay was used to determine the effect of each matrix on adhesion of mESCs, 24 hours after replating. The results showed that laminin and fibronectin were associated with increase in cell adhesion and they also enhanced the proliferation rate of mESCs when compared to type IV collagen. Laminin significantly increased the number of βIII tubulin positive cells 96 hours after re-plating, but failed to result in a larger numbers of positive catecholaminergic neurons.

Second experimental chapter focused on effect of the immobilization of growth factors [sonic hedgehog (Shh), fibroblast growth factor 8b (FGF8) and fibroblast growth factor basic (FGF2)] on the generation of midbrain dopaminergic neurons from mESCs. A combination of matrix proteins components was produced by incubating laminin, fibronectin, type IV collagen and heparan sulphate in a ratio of 1:1:1:0.1. This mixture was used to immobilize growth factors within matrix proteins via non-covalent interactions. In-cell Western assays were carried out to identify stable immobilization of growth factors onto the matrix proteins. The effect of matrix proteins with and without growth factors on neural differentiation was investigated by determining the expression of the AMP^R gene (encoding β-lactamase) in *Lmx1a*-AMP mESCs. Immunocytochemistry was used to observe the progression of neuronal maturation under different conditions. FGF2 and FGF8b were successfully immobilized on a combination of selected matrix proteins but the immobilized factors failed to demonstrate any significant effect on the proportion of cells expressing *Lmx1a*. This is likely to be explained by the unexpected high proportion of *Lmx1a* expressing cells produced even in the absence of specific growth factors.

In the last experimental chapter the focus was on investigation of the signaling pathways that regulate differentiations. New tools to promote the differentiation of stem cells into particular cell types can be generated by identifying specific cues in the microenvironments and deciphering how neighbouring cells and the extracellular matrix control developmental fate. It is well established that complex interactions between soluble and extracellular matrix molecules regulate intracellular signaling and differentiation.

Many of these signaling events involve one or more kinases including; focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt, hereafter called Akt), and mitogen-activated protein kinases (MAPK) – such as those also known as Extracellular regulated kinases (ERKs). However, the relevance of a given signaling molecule in mediating the pro-survival signaling induced by extracellular matrix appears to be cell–type specific. Therefore, it is desirable to determine which of these intracellular signaling pathways might be involved in the effects of extracellular matrix on survival and differentiation of mouse embryonic stem cells. This chapter explores the role of small molecule inhibitors of FAK, PI3K, Akt and MAPK-ERK on adhesion and proliferation of cells produced by neural differentiation of embryonic stem cells. The focus of the chapter is on early events in neural induction making use of *Sox1*-eGFP reporter ESCs. This cell line expresses the eGFP reporter under control of the *Sox1* promoter, one allele expressing eGFP and the other *Sox1*. The work showed that plating onto laminin (but not gelatin or poly-D-lysine) activated PI3K-Akt and MAPK-ERK survival signaling pathways. Plating of cells on laminin in the presence of CHIR99021, or in combination with PF562271, resulted in significant conversion of viable embryonic stem cells to *Sox1+* neural stem/progenitor cells, indicating the role of laminin in the generation and survival of neurons.

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Declaration

I hereby declare that the material presented for examination in this thesis has been carried out solely by the candidate under the supervision of Dr. John M Haynes and Professor Colin W Pouton. This thesis contains no material which has been accepted for the award of any other degree at Monash or another university.

Signature of Candidate: .....

Date: .....

Conference Presentations

- Pankaj Gulati, Colin W Pouton and John M Haynes. Examining the Role of Various Extracellular Matrices upon the differentiation of Mouse Embryonic Stem Cells into Neurons. 5th The Australian Health and Medical Research Congress, Melbourne, Australia 2010 (Poster).

- Pankaj Gulati, Colin W Pouton and John M Haynes. Examining the Role of Various Extracellular Matrices upon the differentiation of Mouse Embryonic Stem Cells into Neurons. 3rd International Congress on Stem Cells and Tissue Formation, Dresden Germany, 2010 (Poster).

- Pankaj Gulati, Warren Raye, Colin W Pouton and John M Haynes. Examining the Role of Various Extracellular Matrices upon the differentiation of Mouse Embryonic Stem Cells into Neurons. ISSCR 7th Annual Meeting, Barcelona, Spain, 2009 (Poster).

- Pankaj Gulati, Warren Raye, Colin W Pouton and John M Haynes. Examining the Role of Various Extracellular Matrices upon the differentiation of Mouse Embryonic Stem Cells into Neurons. Monash Institute of Pharmaceutical 3rd Annual Symposium, Melbourne, Australia, 2009 (Poster).

- Pankaj Gulati, Warren Raye, Colin W Pouton and John M Haynes. Examining the Role of Various Extracellular Matrices upon the differentiation of Mouse Embryonic Stem Cells into Neurons. Cancer Research Council 2nd Annual Symposium, Melbourne, Australia, 2009 (Poster).

- Pankaj Gulati, Warren Raye, Colin W Pouton and John M Haynes. The effect of substrate (matrix) composition on the differentiation of neural stem cells into dopaminergic neurons. Australian Neuroscience Society inc. 28th Annual Meeting, Hobart, Australia, 2008 (Poster).

Abbreviations

%	Percent
°C	Degree Celsius
µ	Micro
h	hour(s)
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cells
CNS	Central nervous system
DA	Dopamine
DAPI	4', 6-Diamidine-2-Phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK1/2	Extracellular-regulated kinases 1 and 2
ES	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FAT	Focal adhesion terminal
FCS	Fetal calf serum
FGF2	basic Fibroblast growth factor

FGF8b	Fibroblast growth factor 8b
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GDNF	Glial-derived neurotrophic factor
GSK 3β	Glycogen synthase kinase
HSPG	Heparan sulphate proteoglycan
iPS	Induced pluripotent stem cells
JNK	Jun N-terminal kinases
LIF	Leukaemia inhibitory factor
MAPK	Mitogen activated protein kinases
mDA	Midbrain dopaminergic neurons
MDCK	Madin-darby caninie kidney epithelial cells
mESCs	Mouse embryonic stem cells
NC	Neural crest
NE	Norepinephrinergic
NGF	Nerve growth factor
NSCs	Neural stem cells
NSPC	Neural stem precursor cell
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
PI3K	Phosphoinositide 3-kinases
PKB/Akt	Protein kinase B
PLL	Poly-L-lysine
PNS	Peripheral nervous system
SAPK	Stress activated protein kinases

SEM	Standard error of the mean
SGZ	Subgranular zone
SSC	Side scatter
SVZ	Subventricular zone
VEGF	Vascular endothelial growth factor

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Chapter 1 General Introduction

1.1. Stem cells

1.1.1. Definition

Stem cells are defined by two basic properties, indefinite self-renewal and an ability to differentiate into one or more specialized cell types i.e. pluripotency (Lerou *et al.*, 2005). Self-renewal ensures the maintenance of the stem cell pool (Mountford, 2008). When a stem cell divides into two daughter cells, both will be identical to the parent cell. In asymmetrical division, at least one daughter cell differentiates (Young *et al.*, 2004). Stem cells that are capable of differentiating into only one cell type are known as unipotent, whereas, stem cells that can differentiate into more than one cell type may be either multipotent or pluripotent (Prowse *et al.*, 2011).

Stem cells can be divided into two categories: adult and embryonic stem cells (Wu *et al.*, 2007). Adult stem cells are tissue-specific (Mountford, 2008; Wu *et al.*, 2007), for example, skin stem cells can only differentiate into the cell types that comprise skin, while hematopoietic stem cells are limited to the hematopoietic system. The distinguishing feature between embryonic stem cells and adult stem cells is the fact that embryonic stem cells are truly pluripotent, i.e. they can differentiate into any cell type of the three embryonal germ layers: endoderm, ectoderm and mesoderm (Hwang *et al.*, 2007; Rippon *et al.*, 2004; Singec *et al.*, 2007). Thus, embryonic stem cells can give rise to any cell type in the body. The multi-cellular aggregates or embryoid bodies (EBs) generated in the initial stages of differentiation of most embryonic stem cell lines also provide a model which mimics the events during early development (Qutachi *et al.*, 2013a).

1.1.2. Establishment of embryonic stem cell lines

Mammalian embryonic stem cells were isolated for the first time from the inner cell mass (ICM) of a mouse blastocyst (Martin, 1981). The cells of the ICM were then plated on a feeder layer of mouse embryonic fibroblasts (MEF). MEFs produced a soluble factor, later identified as leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988), that is required to maintain the embryonic stem cells in an undifferentiated state in *in vitro* cultures (Martin, 1981). Upon differentiation, the cells of the ICM had the potential to develop into any or all tissues of the body (Evans *et al.*, 1981; Rippon *et al.*, 2004). Furthermore, *in vivo* differentiation potential was confirmed by injecting these newly derived embryonic stem cells subcutaneously into athymic mice generating a teratoma, a multicellular mass of differentiated cells (Martin, 1981).

The most definitive demonstration of the pluripotency of these murine embryonic stem cells however was the ability to generate chimeric mice (Nagy *et al.*, 1990). Successful derivation of human embryonic stem cell lines was accomplished in 1998, and again in 2000, by harvesting the inner cell mass of blastocyst stage human embryos produced by *in vitro* fertilization (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998). The human embryonic stem cells are similar to murine embryonic stem cells in their ability to differentiate into embryoid bodies *in vitro*, and form teratomas *in vivo* after implantation into immunodeficient mice. In contrast to mouse embryonic stem cells (mESCs) the human embryonic stem cells are unresponsive to LIF and require culture on MEF feeder layers in the presence of basic fibroblast growth factor (FGF2), or on matrigel or laminin in MEF-conditioned medium (Itskovitz-Eldor *et al.*, 2000; Laslett *et al.*, 2003). A study by Mahlstedt et al. (2010) showed that physicochemical modification of polystyrene surfaces used in growing human embryonic stem cells helped maintain pluripotency (Mahlstedt *et al.*, 2010).

1.1.3. Neural induction and application of stem cells

The main aim of this thesis is to increase our understanding of the influence of extracellular matrix on the differentiation of mESCs into neurons. Neural induction is the initial step in the generation of vertebrate nervous system. Neural tissue is derived from the embryonic ectoderm, which also gives rise to the epidermis layer. Therefore, each cell in the embryonic ectoderm undergoes a process of fate determination. The classical approach of neural induction entailed the incubation of embryonic stem cell derived aggregates with retinoic acid in the presence of serum (Bain *et al.*, 1996). The study by Ying showed that neural fate emerges in absence of serum or added growth factors in adherent monolayer (Ying *et al.*, 2003a).

Co-culture of embryonic stem cells with stromal cell line PA6 in the presence of serum replacement has also been shown to be an efficient method of neural induction (Kawasaki *et al.*, 2000). Hence, it can be stated that neural induction is controlled by a unique set of signaling cues that includes paracrine factors but also signaling induced by physical contact with neighbouring cells and the extracellular matrix (Jin *et al.*, 2005). Thus, the extracellular matrix plays a critical role in cell adhesion, migration, proliferation and may be also involved in differentiation of embryonic stem cells into different lineages (Chen *et al.*, 2007a).

Moreover, the establishment of pluripotent human embryonic stem cells has raised tremendous optimism for their potential to treat diseases for which currently no cures are available. Diseases resulting from a loss of function of a single cell type, such as Parkinson's disease or Type I diabetes, are the most amenable to embryonic stem cell-derived therapies (Mountford, 2008; Pouton *et al.*, 2007; Wu *et al.*, 2007). In these cases, the pluripotency of embryonic stem cells could be exploited to generate dopaminergic neurons and pancreatic beta cells, respectively, or any other desired cell type depending on the disease.

Furthermore, the high proliferative capacity of embryonic stem cells could allow therapeutic cells and organs to be generated on an as-needed basis, relieving the current limitation of a shortage of donor organs and tissues for transplantation (Pouton *et al.*, 2007; Robinton *et al.*, 2012). Several detailed differentiation protocols are available to promote the development of embryonic stem cells into neurons (Nefzger *et al.*, 2012; Watmuff *et al.*, 2012). Therefore, by understanding the role of matrix proteins in regulating differentiation of embryonic stem cells we can gain insights into the early development of neurons and eventually control the behaviour of these cells *in vitro* and facilitate production of deserved cells types for *in-vivo* application.

1.2. Extracellular matrix

The extracellular matrix is a complex mixture of glycoproteins, collagens and glycosaminoglycans, which forms a meshwork of fibres embedded in a gel-like intercellular material known as ground substance in which the cells and fibres of connective tissue are embedded; it is composed largely of glycosaminoglycans, water, and ions. The important functions of extracellular matrix are, (i) it interacts with cells via cell surface receptors such as integrins; (ii) serves as a reservoir for growth factors and; (iii) provides a substrate for cell attachment and spreading (Watt *et al.*, 2000). Glycoproteins in general are defined as fibrous extracellular matrix component that reinforces ground substance and resists its expansive forces. One function of the matrix is to provide sites for attachment that guide migrating cells into defined pathways and sway the extent and direction of their movements (Campbell *et al.*, 1985; Lu *et al.*, 2012).

Matrix and cell interactions affect differentiation of cells and are required for the maintenance of the proper tissue architecture of the developing embryo (Lu *et al.*, 2012; Zagris, 2000). Molecules of extracellular matrix self-assemble into the immediate cellular environment by linking each other through multiple binding regions. Specific matrix receptor molecules in the plasma membrane help cells into an organized multifunctional network (Liesi, 1990). Investigation at the molecular and biochemical level has led to the classification of extracellular matrix into four major classes that are; collagens, glycosaminoglycans, structural glycoproteins and elastin (Liesi, 1990; Lu *et al.*, 2012).

1.2.1. Collagens

Collagen is one of the most abundant proteins in the animal kingdom, representing approximately one third of all proteins in tissues (van der Rest *et al.*, 1991). The collagen family is a highly diverse group of proteins as shown in Table 1.1 (Gelse *et al.*, 2003; Olsen, 1995; Prockop *et al.*, 1995). The most abundant collagens form extracellular fibrils or network-like structures, but the others fulfil a variety of biological functions such as cell proliferation, migration and apoptosis (Gelse *et al.*, 2003). Structure of type IV collagen is discussed in detail in section 1.4.1..

Types	Tissue distribution
Fibril-forming collagens **Type I, II, III, V and XI**	Bone, dermis, tendons, ligaments, cartilage and skin
Basement membrane collagens **Type IV**	Basement membranes
Microfibrillar collagens **Type VI**	Widespread: dermis, cartilage, placenta, lungs and vessel wall
Anchoring fibrils **Type VII**	Skin, dermal– epidermal junctions; oral mucosa and cervix
Hexagonal network forming collagens **Type VIII and X**	Endothelial cells, descemet's membrane and hypertrophic cartilage
Fibril-associated collagens **Type IX, XII, XIV, XX and XXI**	Blood vessel wall, cartilage, vitreous humor, cornea, dermis, tendon, vessel wall, placenta, lungs and liver
Transmembrane domain **Type XIII and XVII**	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs and liver
Multiplexins **Type XV, XVI and XVIII**	Smooth muscle cells, kidney, pancreas amnion and keratinocytes

Table 1.1: Shows various types of collagen as they belong to the major collagen families.

1.2.2. Glycoproteins

Laminin, fibronectin, tenascin and entactin are glycoproteins, which are involved in cell and tissue adhesion processes at specific developmental stages. These are multi-domain molecules which interact with one another, with other extracellular matrix molecules and with cell surfaces and are thought to be responsible for arranging the collagen, proteoglycan and cells into an ordered structure (Aumailley *et al.*, 2005; Bernardes *et al.*, 2009; Orsini *et al.*, 2012; Yurchenco *et al.*, 1993). The structures of fibronectin and laminin are discussed in detail in 1.4.2. and 1.4.3..

1.2.3. Glycosaminoglycans and proteoglycans

Glycosaminoglycans such as hyaluronate, chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate are components of extracellular matrix which have been implicated in the control of cell proliferation, migration, differentiation and maintenance of morphogenetic structures. They occur as large polymers of repeating disaccharides and are covalently linked to protein to form the proteoglycans. Heparan sulphate is ubiquitously present on the cell surface and in extracellular matrix including basement membrane. It is synthesized as an alternating copolymer of hexuronic acid and glucosamine (Whitelock *et al.*, 2005). Heparan sulphate proteoglycans (HSPGs) consist of heparan sulphate chains that interact with a variety of proteins such as heparin-binding growth and differentiation factors, morphogens, extracellular matrix components, protease inhibitors, protease, lipoprotein lipase and various pathogens (Habuchi *et al.*, 2000; Iozzo, 1998).

1.3. Integrins

Integrins are transmembrane proteins that mediate interaction between the cell and extracellular matrix. Many matrix proteins in vertebrates are recognized by multiple integrins: for example, at least 8 integrins bind fibronectin, and at least 5 bind laminin. About 20 integrin heterodimers, made from one of 9 types of β subunits and one of 14 types of α subunits, have been identified (Powell *et al.*, 1997; Watt, 2002). The interaction of cells with extracellular matrix proteins is crucial for activation of various biological processes, including cell adhesion, spreading, proliferation, differentiation, apoptosis, gene induction, embryogenesis and wound healing (Figure 1.1 A) (Giancotti *et al.*, 1999; Hynes, 2002; Juliano, 2002; Lee *et al.*, 2004). Interaction of extracellular matrix proteins with cells is mediated by transmembrane receptors, of which the integrins constitute the most important class (Bosman *et al.*, 2003; Boudreau *et al.*, 1999b; Gumbiner, 1996).

These heterodimers transmit extracellular signals when in contact with matrix proteins, by way of one or more potential signaling cascades involving focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), nuclear factor (NF)-kB, and mitogen-activated protein kinases (MAPK). However, we still cannot explain how these signals facilitate adhesion and proliferation (Figure 1.1 B and C) (Cho *et al.*, 2000; Daley *et al.*, 2008; Frost *et al.*, 1999).

The complexity of the extracellular surroundings can be appreciated by examining the temporal expression of patterns of extracellular matrix components and their corresponding cell surface receptors in the developing central and peripheral nervous systems. The expression of extracellular matrix proteins increases as neural progenitors differentiate, migrate and their neuronal axons elongate, but expression begins to taper off towards the end of the development program (Flaim *et al.*, 2005).

Cell-cell or cell-extracellular matrix interactions play a crucial role in controlling stem cell differentiation, neural development, axon outgrowth and synapse formation (Cooke *et al.*, 2010; Lathia *et al.*, 2007). The extracellular matrix proteins have not been extensively investigated for their influence on differentiation of mESCs into neurons, whereas, their effect has been explored in detail on cells extracted from mouse and chick brain. These experiments have shown that extracellular matrix provides an external factor in guiding and encouraging conversion of cells into neurons (Lein et al., 1996, Kowtha et al., 1998). The hypothesis is that different extracellular matrix proteins help in controlling the differentiation of cells through their numerous domains, carbohydrate moieties and isoform specific affinities for cell integrins (Kowtha *et al.*, 1998; Obremski *et al.*, 1995; Westermann *et al.*, 1989).

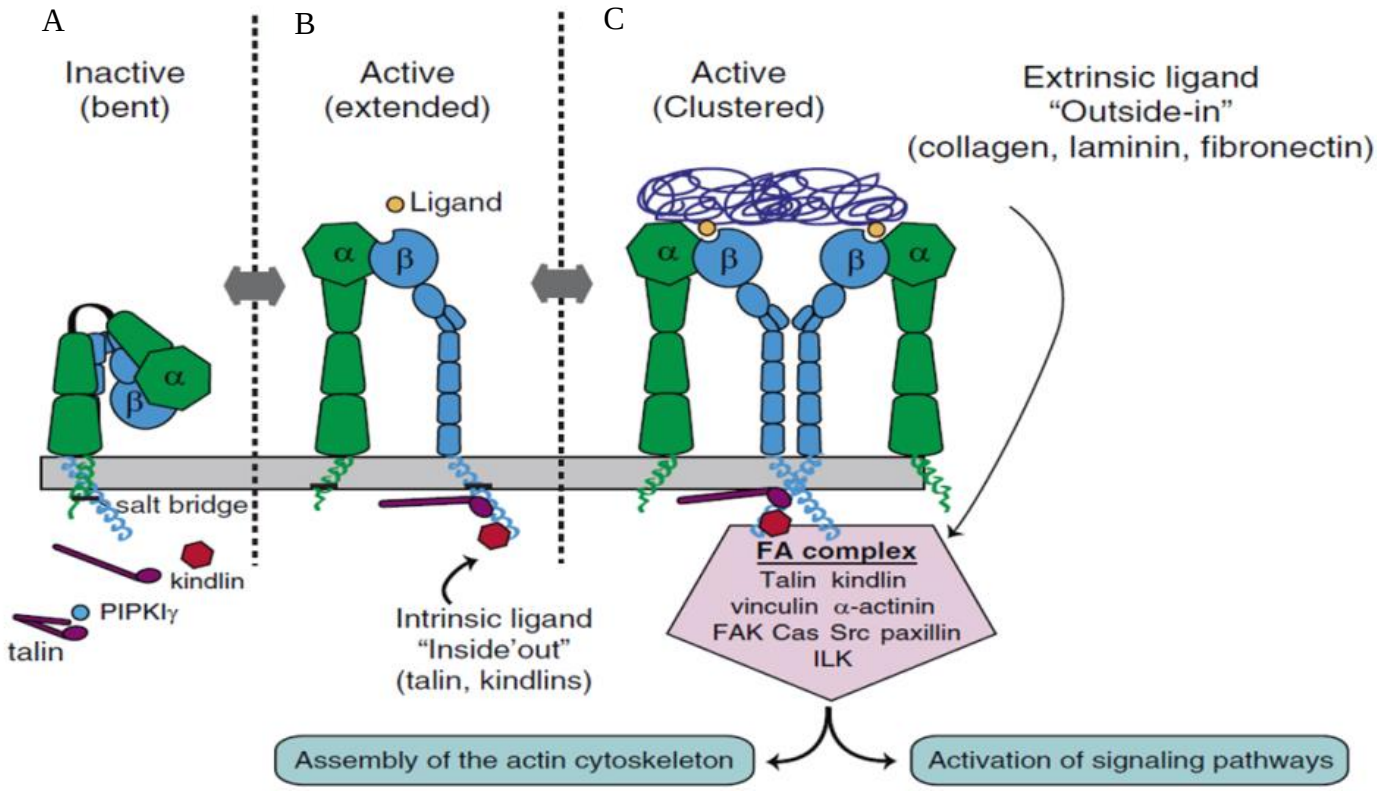


Figure 1.1: Structure of an integrin cell-surface matrix receptor (from Srichai *et al.*, 2010).

The binding of an integrin to its ligand is dependent on extracellular divalent cations (for example Ca^{2+} and Mg^{2+}), which bind to the integrins α subunit. The integrin then binds with its ligand in the extracellular matrix which initiates the intracellular end of the β subunit to bind to talin and α-actin (Figure 1.1 A). Intracellular attachment proteins then aggregate at the cytoplasmic end of the integrin, enabling the integrin to link with the cell's actin filaments. Cells connect to the extracellular matrix at focal adhesions, forming tight connections between the cell membrane and the extracellular matrix as well as the cell membrane and the cytoskeleton (Figure 1.1 B). Focal adhesions are assembled following the aggregation of signaling molecules and actin-anchoring proteins. These proteins create the structural link, which enables stress fibers to connect to the membrane and the integrins (Figure 1.1 C)

1.4. Structural overview of selected matrix proteins

As type IV collagen, laminin and fibronectin have been used in the experimental part of this thesis, these molecules are described in details below.

1.4.1. Type IV Collagen

Type IV collagen is a nonfibrillar collagen and composed of three *a* chains that are coiled around each other in a triple-helical conformation (Figure 1.2). It makes up about 50% of all basement membranes and is first expressed at embryonic stage day 4.5 (E4.5) in mice (Myllyharju *et al.*, 2004; Veit *et al.*, 2006). Nonfibrillar collagens differ from connective tissue fibrillar collagens by the presence of globular or rod like, noncollagenous domains (NC domains). In mammals, six distinct genes encode for six distinct chains of type IV collagen, known as α-chains (α1 – α6). Although there could be many potential combinations, the chains interact and assemble with a remarkable specificity to form only three distinct heterotrimers of α1α1α2, α3α4α5, and α5α5α6. The α1(IV) and α2(IV) chains, which were first to be described, and are thus, called ‘‘classical’’ chains, are present in the basement membrane of all tissues, whereas, the other four chains have restricted tissue distribution during development. For example the α3(IV), α4(IV), and α5(IV) chains are present in the glomerular basement membrane of the kidneys, lungs, testes and eyes, whereas, the α 5(IV) and α 6(IV) chains are found in the basement membrane of skin, smooth muscles and the kidneys (Hudson, 2004; Hudson *et al.*, 2003; Khoshnoodi *et al.*, 2008).

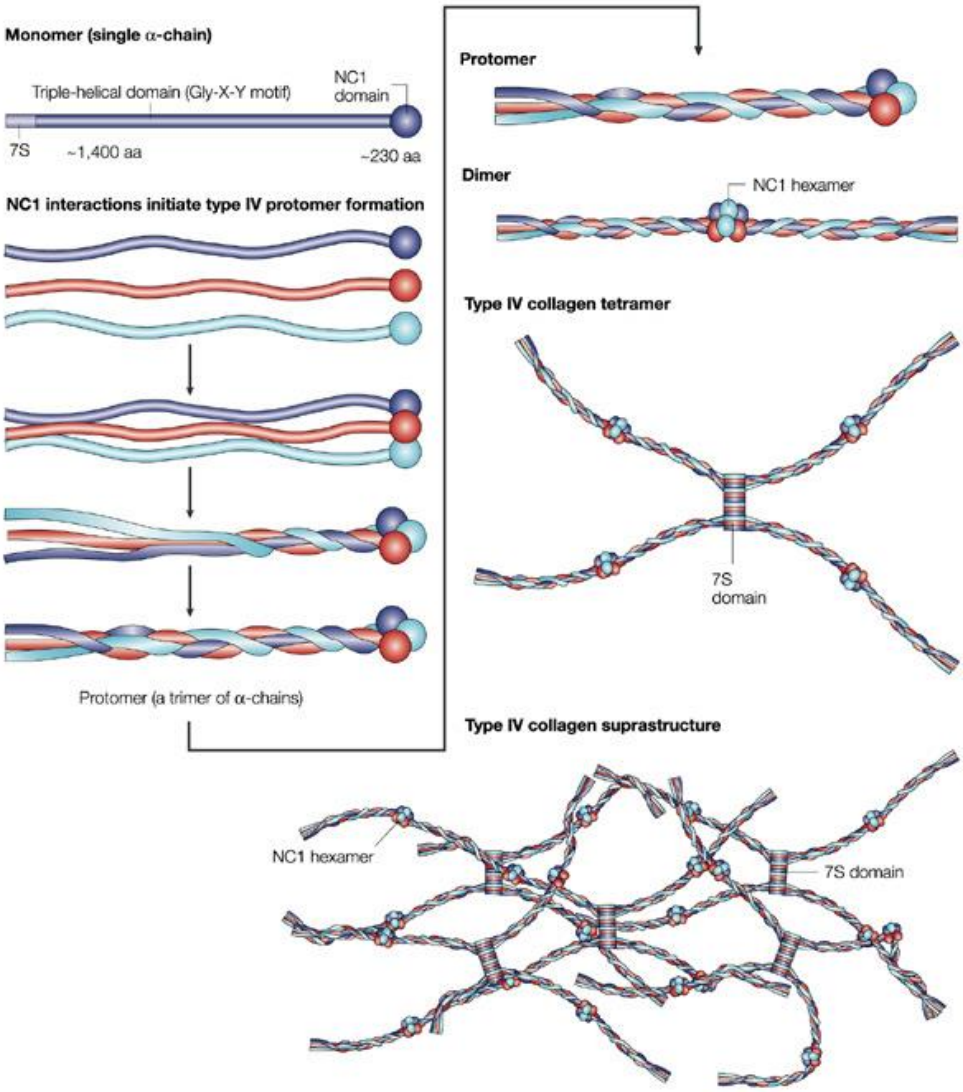


Figure 1.2: Structure of Type IV collagen (from Kalluri, 2003).

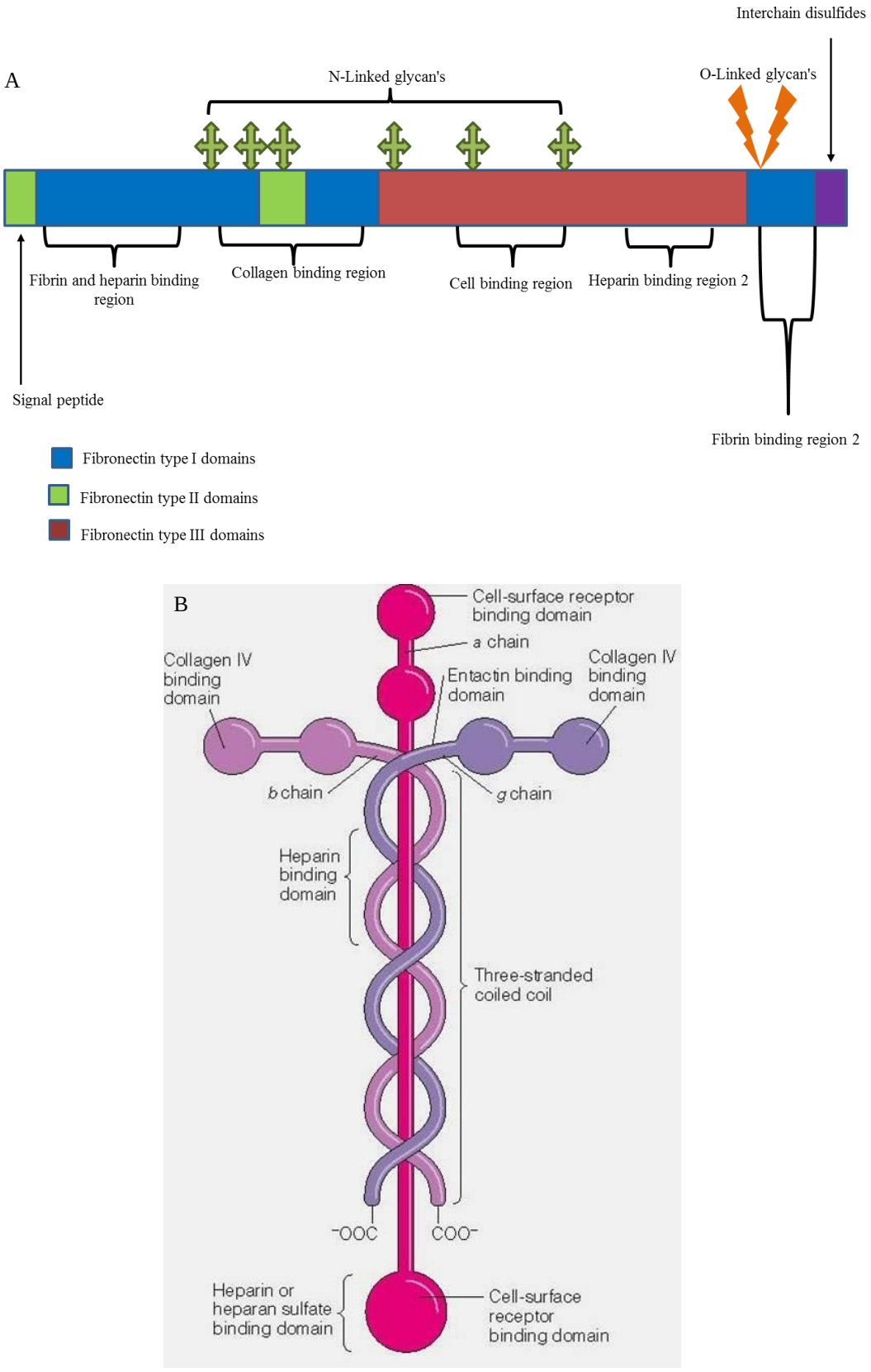


Figure 1.3: Structures of (A) Fibronectin and (B) Laminin (from Pankov *et al.*, 2002; Timpl *et al.*, 1994).

1.4.2. Fibronectin

Fibronectin, a large (~440kDa) glycoprotein, is a common adhesive protein of the interstitial matrix (Pankov *et al.*, 2002) (Figure 1.3 A). There are 20 different fibronectin isoforms in humans, generated by alternative splicing of a single fibronectin gene. The majority of the splicing patterns are cell-type specific, and give rise to a number of different cellular fibronectin isoforms, which are found immobilized in extracellular matrix. Another type of fibronectin is plasma fibronectin (pFN), which is primarily made by hepatocytes and secreted as a soluble form into the blood plasma (Owens *et al.*, 1982). Cells interact with fibronectin predominantly via integrin receptors, and this interaction signals a sequence of events including changes in gene expression, cell survival, proliferation, differentiation and migration (Danen *et al.*, 2001). Out of the known integrins, the following pairs, α3β1, α4β1, α8β1, αIIbβ3, αvβ1, αvβ3, αvβ5 and αvβ6, have been shown to attach with fibronectin (Danen *et al.*, 2001; Martino *et al.*, 2009; Meland *et al.*, 2010; Sonnenberg, 1993).

1.4.3. Laminin

Laminin is one of the major components of basement membrane that has a molecular weight of M_r - 850,000. It is a multidomained, cross-shaped glycoprotein that is structured in meshwork of basement membranes (Figure 1.3 B), such as epithelial lining, adjacent blood vessels, nerves and underlying pial sheaths of the brain. It has been reported to occur in sites other than basement membranes during early stages of development and is localized to specific types of neurons in the central nervous system (CNS) during both embryonic and adult stages (Zagris, 2000).

Laminin protein is composed of three different polypeptide chains, termed alpha, beta and gamma. At present, 5 alpha, 3 beta, and 3 gamma chains are known for mouse and human laminin (Yurchenco *et al.*, 1993). All chains are glycosylated and few chains have been shown to have glycosaminoglycan side chains. The first laminin isoform was discovered 26 years ago and additional isoforms were simultaneously being identified under different nomenclatures (Timpl *et al.*, 1994). 18 years ago, a unifying nomenclature for the trimers was introduced. It found widespread acceptance because it was rational and allowed simplified transfer of information (Burgeson *et al.*, 1994).

A more simplified trimer nomenclature was proposed using the numbers associated with each subunit. Both names are provided in Table 1.2 (Aumailley *et al.*, 2005). In the nomenclature presented, a simple change makes it easier to identify the laminin isoforms and name newly identified molecules. In the past, laminin trimers were designated as laminin 1 to 15 in their order of discovery, with no direct relationship to chain numbers (Table 1.2). Each trimer is composed of three genetically distinct chains termed α, β, and γ. According to the previous nomenclature a trimer could either be

identified by the Arabic numeral (e.g. 10), or by its chains (e.g. α5β1γ1) (Burgeson *et al.*, 1994).

Laminin trimers were named exclusively on the basis of chain composition, either according to Table 1.2, or without Greek letters; for example 211 for α2β1γ1, can equally be used with no loss of information therefore, 211 is more informative than laminin 4 and obviates the need to memorize the chain composition (Aumailley *et al.*, 2005). In the developing CNS, the presence of laminin has been observed in regions where tracts are growing. Laminin is not only associated with the basement membrane of developing Pia Mater and blood vessels, but is also found in small, punctuate particles in the extracellular matrix where tract formation occurs. It also plays a role in the migration of neural crest cells into the bowel and stimulates their early differentiation into neurons (Luckenbill-Edds, 1997).

Standard	Abbreviated	Previous
α1β1γ1	111	1
α2β1γ1	211	2
α1β2γ1	121	3
α2β2γ1	221	4
α3Aβ3γ2	332	5A
α3Bβ3γ2	3B32	5B
α3Aβ1γ1	311	6A
α3Aβ2γ1	321	7A
α4β1γ1	411	8
α4β2γ1	421	9
α5β1γ1	511	10
α5β2γ1	521	11
α2β1γ3	213	12
α4β2γ3	423	14
α5β2γ2	522	-
α5β2γ3	523	15

Table 1.2: Nomenclature of laminin.

1.5. Role of extracellular matrix proteins in neurogenesis

Neurogenesis is the process of generation and differentiation of neurons from neural stem cells. The mammalian CNS is generated from neural stem cells residing in the deepest layer of the neural tube facing the ventricular lumen This area is called the ventricular zone. Neural stem cells are defined as cells that exhibit the capacity to differentiate into one of three cell types of the CNS (namely neurons, astrocytes and oligodendrocytes) and also have the capacity to generate sufficient numbers of cells to form an adult brain by self-renewal. Neurogenesis occurs predominantly during embryonic stages is completed before birth in most parts of the mammalian CNS (Hantaz-Ambroise *et al.*, 1987; McKay, 1997).

The initial research was done on investigating the effect of extracellular matrix proteins like laminin, fibronectin and collagens to purified neurons obtained from chick embryo. Plastic substrata coated with fibronectin and laminin were tested by culturing dissociated neurons from embryonic chick dorsal root sympathetic ganglia (peripheral neurons), spinal cord and retina (CNS neurons). Laminin was able to attach and induce extension of neurites from both central and peripheral neurons, whereas, fibronectin only had these effects on peripheral neurons (Hall *et al.*, 2008).

Neurite length, number of neurites initiated, and extent of neurite branching on fibronectin and laminin coated surfaces were evaluated and compared with similar measurements of neuronal responses to poly-L-lysine-treated plastic. Poly-L-lysine provided an adhesive surface for neurite elongation, but fibronectin and laminin appeared to promote more rapid neurite elongation, indicating that glycoproteins play an important role during specific developmental stages (Rogers *et al.*, 1983).

Carbonetto and Cochard (Carbonetto *et al.*, 1987) took neurons from embryonic chick sympathetic ganglia and dorsal root ganglia (DRG) to evaluate whether extracellular matrix adhesive proteins like fibronectin, collagens (types I, III, IV) and laminin are important determinants of nerve regeneration. Their findings showed that neurons grew poorly on substrata containing glycosaminoglycans and in culture medium lacking nerve growth factor. DRG neurons extended nerve fibers only on laminin and not on fibronectin, collagen or polylysine. The neuron-like rat pheochromocytoma cell line, (PC12) was utilised for studying interaction with extracellular matrix components including laminin, type IV collagen and fibronectin. A cell attachment assay showed that PC12 cells adhere readily to laminin or type IV collagen but poorly to fibronectin (Tomaselli *et al.*, 1987).

DRG neurons of purified chick embryo were used to study cooperative actions of extracellular matrix proteins laminin and fibronectin in the presence of nerve growth factor. Only 20% of cells survived in presence of nerve growth factors whereas, in presence of laminin or fibronectin survival increased to 80% and was accompanied by extensive neurite outgrowth. The same authors reported that increased levels of potassium in the presence of laminin helped in survival and neurite outgrowth, suggesting cooperative action between matrix and nerve growth factor (Millaruelo *et al.*, 1988).

Neuronal precursor cells present in chick DRG were excised to study conditions required for initial differentiation and long-term survival. Cells isolated at embryonic day 6 (E6) failed to attach when plated on surfaces coated with polyornithine alone or with bovine serum albumin (BSA). When the same cells plated onto surfaces coated with a combination of polyornithine with laminin or fibronectin, attachment as well as development of neurons was observed (Ernsberger *et al.*, 1988).

As a variety of intrinsic and environmental factors are known to direct growth of axons to their peripheral targets, localization of a variety of extracellular matrix molecules within the chick trigeminal mesenchyme has been studied using indirect immunofluorescence. The study suggested that laminin is implicated in the guidance of trigeminal peripheral axons and it might be produced in localized patches by peripheral nervous system (PNS) components (Moody *et al.*, 1989). Mesenchymal cells of the chick tail bud were used to evaluate the role of different extracellular matrix proteins on differentiation. Griffith and Sanders showed that laminin and laminin-containing substrata (Matrigel) were found to promote the differentiation of neural crest derivatives (neurons and melanocytes) and neuroepithelial cells; type I collagen promoted both myogenesis and chondrogenesis; while type IV collagen promoted myogenesis only (Griffith *et al.*, 1991).

Sarthy (1993) investigated whether type IV collagen, which is associated with laminin in basement membrane, is expressed by neuronal and non-neural cells during retinal development. Immunostaining of type IV collagen showed it was expressed at embryonic day 12 (E12) in the lens, embryonic (hyaloid) blood vessels and internal limiting membrane (ILM) of the retina. At embryonic day 17 (E17), immunostaining was reduced in the ILM, whereas, the lens and hyaloid were strongly stained and type IV collagen was barely detected in the ILM of postnatal retinas. These workers concluded that high levels of type IV collagens were present during the early development when most of the axonal growth occured (Sarthy, 1993).

Therefore, it can be summarized from the above findings that matrix proteins not only serve as attachment molecules but also helped in survival and development of neurons isolated from chick embryo and out of tested matrix proteins laminin turned out to be best as compared to fibronectin, type IV collagen and chemically defined substrates like polyornthine and polylysine. Studies in early 1990 shifted with specific focus on laminin extracted from plant and animal sources. The following findings also supported the fact that laminin presence was most beneficial as compared to other extracellular matrix proteins.

Lectin concanavalin A and laminin-like substrates extracted respectively from plants and leech CNS. The extracted substrates were used to evaluate their effect on Retzius neurons extracted from the leech. Neurons showed broad flat growth cones and thick bundles of process on lectin concanavalin A whereas, on laminin-like substrate, fine straight processes with numerous branches were observed thus, suggesting that nature of the growth substrate can also influence morphology of neurons (Ross *et al.*, 1988).

Dean et al. (1990) studied the role of carbohydrate moieties of laminin in cell migration and neurite elongation. To test the hypothesis, lectins were used to block carbohydrate moieties of laminin. Wheat germ agglutinin or *Griffonia simplicifolia* agglutinin blocked the binding of the neuron-like rat PC12 onto plates coated with laminin. When concanavalin A was used, cell binding was not affected but neurite outgrowth was prevented. Non-glycosylated and glycosylated laminins were purified by immunoaffinity from tunicamycin treated cultures of a mouse embryonal carcinoma derived cell line. When PC12 cells were plated on surfaces coated with purified non-glycosylated laminin, no effect was noted on binding of cells but neurite outgrowth was impaired when compared to glycosylated laminin. Thus, it can be inferred that once cells bound to laminin the carbohydrate residues of that glycoprotein must be available to enable the cells to spread or to extend neurite processes (Dean *et al.*, 1990).

Focus of investigation shifted in 1990s to neuronal cells extracted from mice or rat to decipher the effects of extracellular matrix onto adhesion, migration and development of neurons. Neuronal cells of embryonic olfactory epithelium (OE) were extracted from mice to study the role of extracellular matrix on migration, motility, and adhesion of cells. Migration of OE neuronal cells was found to be dependent on substratum-bound extracellular matrix molecules, and was specifically stimulated and guided by laminin (or the laminin-related molecule merosin) in preference to fibronectin, type I collagen, or type IV collagen. Time-lapsed video microscopy of OE cells showed higher motility on surfaces coated with laminin as compared to fibronectin. Adhesion of OE neuronal cells was measured quantitatively but no correlation, either positive or negative, between the migratory preferences of cells and the strength of cell-substratum adhesion was observed (Calof *et al.*, 1991).

The effect of type IV collagen on morphological development of embryonic rat sympathetic neurons was observed *in vitro*. Within 24 hours, type IV collagen accelerated the outgrowth processes resulting in increased number and length of neurites (Timpl et al. 1989). Effects of type IV collagen on the morphological development of embryonic rat sympathetic neurons *in vitro* was done by plating cells on surface coated with noncollagenous portion of type IV collagen and intact collagen. Similar outgrowth processes was observed in both the conditions thus, suggesting that the neurite promoting ability of type IV collagen resides within the noncollagenous domain (Lein *et al.*, 1991).

In the last twenty years, focus of research in the field of extracellular matrix shifted to neurons isolated either from mice/rat. Laminin which helped in cell attachment and neuronal process elongation *in vitro* was used to test whether the same properties can be demonstrated in adult brain. To test the hypothesis, fetal neurons rich in serotonin (5-HT), dopamine (DA) or norepinephrine (NE) neurons were transplanted into the motor cortex or hippocampus region of adult brain. Tracts used for microinjection were sealed with laminin or a mixture of laminin and type IV collagen. The density of serotonergic, dopaminergic and norepinephrinergic neurons measured was substantially higher in tracts treated with laminin or mixture of laminin and type IV collagen as compared to vehicle control. The study failed to observe any stimulatory activity when laminin was injected alone suggesting that laminin assists and guides process outgrowth of various subtypes of neurons during early developmental stage but not in adult brain (Eldridge *et al.*, 1989).

Growth patterns of axons and dendrites differ with respect to their number, length, branching, and spatial orientation; therefore, it is likely that these processes differ in their growth requirements. To validate this hypothesis, cultured rat sympathetic neurons were examined in the presence of large structural proteins of extracellular matrix, matrix-associated growth factors and neurotrophins. Purified substrata proteins laminin and type IV collagen supported axonal growth, and osteogenic protein-1 selectively stimulated dendritic growth. In contrast, nerve growth factor modulated the growth of both types of processes. Hence specific interactions of processes with the extracellular environment may be critical determinants of cell shape in neurons (Lein *et al.*, 1996).

Rat cortical progenitor cells were extracted to analyse the effect of type IV collagen on proliferation and differentiation. Ali et al. (1998) showed that when cells are exposed to type IV collagen, their proliferation and glial cell differentiation are inhibited, while neuronal differentiation is enhanced. When cultures were exposed in conjunction with FGF2, glial cell differentiation was induced and neuronal differentiation remained enhanced. This study indicated that cortical progenitor cells responded differently to local environmental signals (Ali *et al.*, 1998).

Most of the adhesive interactions between cells and extracellular matrix are mediated by integrins, by recognizing short linear amino acid sequences of matrix proteins. The well-known Arginine-Glycine-Aspartate (RGD) motif is likely to be the most common cause of integrin binding. Mollusc neurons were extracted to investigate the effect of selected matrix components on neurite outgrowth and adhesion. Adhesion was observed in the case of native laminin and type IV collagen but not in case of fibronectin. Subsequently denatured fibronectin was observed to enhance cell adhesion, and this was blocked by RGD peptides, suggesting that denaturation uncovers RGD binding sites in fibronectin. Neurite outgrowth was induced on laminin and denatured fibronectin, but not on type IV collagen, demonstrating that mollusc neurons could attach to various substrate but all of them were not capable of inducing neurite outgrowth (Wildering *et al.*, 1998).

Laminin 5 (laminin-332) and laminin 1 (laminin111) are major components of the extracellular matrix of the blood brain barrier (BBB) which interacts with integrins on both endothelial cells and astrocytes (Chen *et al.*, 2003a; Chen *et al.*, 2003b; Indyk *et al.*, 2003; Wagner *et al.*, 2000).

Neural crest (NC) is a model system widely used to investigate the multipotency during vertebrate development. Environmental factors are known to control NC cell fate and though the influence of extracellular matrix in NC cell migration is well documented, there is little information in the literature about its role in differentiation. Costa-Silva et al. (2009) showed that fibronectin promoted differentiation into smooth muscle cell phenotypes without affecting differentiation into glia, neurons and melanocytes. NC cells isolated from quail trunk were grown on fibronectin and type IV collagen showed that fibronectin increases the survival of NC cells and increases the proportion of unipotent and oligopotent NC progenitors endowed with smooth muscle potential. Thus, it can be postulated that fibronectin promotes NC cell differentiation along the smooth muscle lineage (Costa-Silva *et al.*, 2009).

One of the earliest studies to decipher the effect of extracellular matrix proteins on neurons extracted from human's source was carried out in 1982. Human fetal sensory neurons were used to investigate the effect of mouse nerve growth factors (NGF) on neurite length, density and rate of growth. Neurons were grown on surfaces coated with collagen, laminin and fibronectin. It was observed that collagen was associated with maximal neurite length and density when exposed to 1 ng/ml of NGF. Neurite length was reduced in absence of NGF and blocked by addition of NGF antibodies. Absence of NGF could be partly compensated for by increasing the concentration of matrix protein used for coating. Time course experiments showed that neurite growth was more pronounced on laminin followed by fibronectin and was least developed on collagen. Thus, it can be concluded that laminin enhances neurite growth of human sensory neurons in synergy with NGF (Baron-Van Evercooren *et al.*, 1982).

A study by Hirose et al. (1993) was reported to understand the effect of extracellular matrix proteins on embryonic stem cells derived from mouse. Matrix proteins such as fibronectin, laminin, heparan sulphate proteoglycan, type I collagen, type IV collagen, and type VIII collagen were investigated for their neurite outgrowth potential by *in vitro* assay of cholinergic neuronal cell lines and primary cultured neurons from embryonic mouse brain. Findings showed that all the matrix proteins had high neurite promoting activity but collagens only showed high neurite-promoting activity in neurons from prenatal, not postnatal mouse brain. These workers postulated that collagens only contribute to neurite extension of CNS neurons in the developing brain (Hirose *et al.*, 1993).

Rat embryos were used for extraction of DRG cells, which were seeded onto plates coated with poly-L-lysine, laminin, poly-L-lysine combined with laminin, or type I collagen. Phase-contrast microscopy was used to monitor cell survival and neurite outgrowth. The results showed that DRG neurons grew slowly with high survival rate on poly-L-lysine combined with laminin. However, those cells grown on type I collagen were clustered with thicker and longer neurites showing their growth pattern was influenced by presence or absence of particular substrata. It was concluded that surfaces coated with poly-L-lysine and laminin were the best option for study of single neurons (Guo *et al.*, 2004).

Kohno et al. (2005) investigated the effect of laminin on organelle transport and its relationship to neurite growth using dissociated mouse DRG neurons. A time-lapse study demonstrated that many small-diameter branches were formed after the addition of laminin. Addition of laminin resulted in a decrease in organelle movement in the neurite shaft and growth cone which resulted in slow growth cone advancement. This suggests that laminin inhibits the elongation of primary neurites, but promotes branching and elongation of branches (Kohno *et al.*, 2005).

In a study by Chen et al. (2007a) human skin fibroblast cells exposed to ascorbic acid showed increased attachment to plastic due to enhanced collagen synthesis. In contrast, inhibition of collagen synthesis by *cis*-hydroxyproline decreases the rate of attachment of fibroblasts to the plastic surface and increased rate of their detachment by trypsin, thus, showing that collagen helps in adhesion of cells onto at least one type of plastic surface (Chen *et al.*, 2007a).

There is considerable interest in the treatment of both peripheral and CNS disorders with neural prosthetic implants, for this form of treatment to be successful effective integration on implantation will be required. Research has been carried out in past few years to understand the effect of extracellular matrix proteins such as fibronectin, laminin and type IV collagen on adhesion and differentiation of mESCs. Embryonic mouse neural stem cells (NSCs) isolated from E14 mice, were grown on plates coated with laminin in presence of epidermal growth factor (EGF) and FGF2 in neurobasal media (Balasubramaniyan et al., 2004).

After 7 days of *in vitro* cell cultures, 20% of the NSCs developed into morphologically and biochemically fully mature neurons, with extensive dendrites and multiple synaptic contacts. When these neurons were tested by electrophysiology experiments to assess maturity on day 22, none of the neurons had developed the characteristics of a functional neuron. These findings indicate that differentiation assessed by morphological appearance does not correlate with electrophysiological maturation of mouse NSCs into neurons. The processes of neurogenesis and subsequent maturation may be regulated differently (Balasubramaniyan *et al.*, 2004).

Extracellular matrix and cell-adhesive proteins like gelatin, type IV collagen, laminin and polylysine, were immobilized on a polystyrene surface. A layer-by-layer technique was used based on hydrophobic and electrostatic interactions between oppositely charged macromolecules. The mESC line D3 was tested for adhesion and growth. The cells grew best on surfaces coated with gelatin and type IV collagen assemblies. Effect on growth of non-differentiated or differentiated cells was evaluated but no significant differences were observed (Brynda *et al.*, 2005).

Goetz et al. (2006) showed how environmental interactions alone can modify the development of neurogenic precursor cells. They evaluated the influence of natural and synthetic proteins including laminin, fibronectin, gelatin and poly-L-ornithine on embryonic stem cell-neurogenesis. They found highest densities of neural precursors when cells were grown in combination of laminin and poly-L-ornithine and reported that more non-neural phenotypes were present on gelatin (a mixture of water-soluble collagen components) and fibronectin (Goetz *et al.*, 2006b).

Embryonic stem cell transplantation represents a potential means for the treatment of degenerative diseases like Parkinson's disease (Robinton *et al.,* 2012; Watmuff *et al.,* 2012). A critical aspect of stem cell transplantation is to have appropriate embryonic stem cell migration from the site of insertion to the mid brain. Surfaces coated with or without type I collagen, type IV collagen, matrigel, fibronectin or laminin were tested for generation of embryoid bodies from mESC lines. Among the matrix proteins tested, type IV collagen showed maximum migration enhancing effect. In addition, pre-treatment of undifferentiated or differentiated mESCs with type IV collagen resulted in improved engraftment and growth after transplantation into the subcutaneous tissue of nude mice (Li *et al.,* 2010).

The influence of the above selected matrix proteins can be summarised as fibronectin is crucial for embryogenesis (George *et al.,* 1993) and is involved in the developing nervous system (Reichardt *et al.,* 1991). Fibronectin promotes both the survival and migration of neural crest cells and migration of neurons to different cortical regions (Henderson *et al.,* 1997; Testaz *et al.,* 2001). Neurite extension and synapse formation in the developing brain is also known to be promoted by fibronectin (Einheber *et al.,* 1996; Sheppard *et al.,* 1995). Laminin plays an important role in neural development (Reichardt *et al.,* 1991). Laminin 1 is known to promote cell survival and migration in the developing nervous system (Perris *et al.,* 2000) and both laminin 1 and 2 promote neurite outgrowth (Colognato *et al.,* 2000).

In the mature brain laminin 10 is found in the hippocampus and protects hippocampal neurons from excitotoxic lesion. Indyk et al. (2003) reported that laminin 10 (laminin-511) found in the mature brain hippocampus region protects hippocampal neurons from excitotoxic lesions, and laminin α2 subunit is found in close association with dendritic spines in the hippocampus, and may be involved in synaptogenesis (Indyk *et al.*, 2003). Laminin 5 is a major component of the extracellular matrix of the blood brain barrier, where it can be found in the basement membrane of the blood vessels where it interacts with integrins on both endothelial cells and astrocytes (Hagg *et al.*, 1997; Wagner *et al.*, 2000). Type IV collagen plays crucial role in development and regeneration of the PNS (Timpl *et al.*, 1984).

Type IV collagen is reported to be synthesized by glial cells and is present in both fibre tracts and ganglia of the PNS and it is one of important components of basement membrane where it provides mechanical stability (Hirose *et al.*, 1993; Olsen, 1995; Tryggvason *et al.*, 1993). Studies of extracellular environment from different tissues and organisms have led to the identification of common signaling molecules including growth factors, cell-cell signaling molecules, adhesion molecules and extracellular matrix molecules. Combinations of these are likely to provide strategies to maintain stem cell populations and enhance differentiation of cells into subtypes of neurons. In addition, the extracellular matrix proteins influence the outgrowth of neuronal process. The role of extracellular matrix proteins has been studied mainly in the PNS where they have been shown to promote the migration of neural crest derivatives, the outgrowth and guidance of neurites and the induction of proteins involved in neurotransmitter metabolism (Frade *et al.*, 1996; Luckenbill-Edds *et al.*, 1995).

The ability of embryonic stem cells to generate neural cell types *in vitro* offers a powerful tool to study how the cell-extracellular matrix interactions regulate neural stem cell specification and lineage choice (Mruthyunjaya *et al.*, 2010; Timpl *et al.*, 1994; Uemura *et al.*, 2010). Findings have shown that the local microenvironment or stem cell niche, regulates self-renewal and fate of stem cells in developing tissues or organs (Fuchs *et al.*, 2000; Ma *et al.*, 2008). Within the niche, the extracellular matrix, plays a critical role in regulating stem cell differentiation into different lineages, as well as in cell migration and proliferation (Chen *et al.*, 2007a). Therefore, based on the above information the decision was made to investigate the influence of type IV collagen, fibronectin and laminin on differentiation of mESCs.

1.6. Experimental outline

The experimental work of this thesis was divided into three chapters to understand the influence of extracellular matrix protein on differentiation of mESCs.

1.6.1. Experimental study 1 (Chapter 2: Matrix proteins and their influence on neural induction):

Extracellular matrix proteins as described in (Section 1.2) have history of biological use, mainly to enhance adhesion of cells to plastic surfaces. They are now being reported to play roles in proliferation and differentiation of stem cells (Divya *et al.*, 2007; Ma *et al.*, 2008). It is generally understood that stem cells prefer to grow on a biological rather than a synthetic surface thus, chapter 2 examines *in vitro* the influence of extracellular matrix proteins on differentiation of mESCs into neurons.

Therefore, for this study, the surface of the tissue culture plates was coated with extracellular matrix proteins and seeded with mESCs to understand their effects on cell viability, proliferation and differentiation. Preliminary experiments determined the concentration of selected matrix proteins for optimal coverage of the culture plate surface. Immunolabeling was then carried out with βIII tubulin and nestin to understand the effects of matrix proteins on neural induction and to determine if differentiation of mESCs in to particular neuronal subtypes could be favoured by particular components of the extracellular matrix.

1.6.2. Experimental study 2 (Chapter 3: Immobilization of growth factors onto surface coated with extracellular matrix components):

Lmx1a is a key intrinsic determinant of midbrain dopaminergic neurons, expressed in response to the ventralizing activity of sonic hedgehog (Shh) (Andersson *et al.*, 2006). FGF8b and FGF2 have also been reported to play crucial roles in generation of midbrain dopaminergic neurons (Lee *et al.*, 2000; Raye *et al.*, 2007). Significantly, when growth factors are present in immobilized form it reduces their biodegradation, increasing their availability (Masters, 2011). Working on these principles decided to investigate whether, the bioactivity of growth factors like FGF2, FGF8b and Shh could be increased if they were immobilized onto combinations of selected matrix proteins, thereby increasing differentiation of *Lmx1a*-eGFP mESCs into midbrain dopaminergic neurons.

1.6.3. Experimental study 3 (Chapter 4: Identifying the signalling pathways through which mESCs attach and differentiated on matrix proteins found in basal lamina):

The attachment and binding of the extracellular matrix proteins to integrins initiates intracellular signaling (Figure 1.1), which is essential for key functions, including, migration, differentiation and proliferation. Integrins modulate cellular functions by enabling cell-cell connections and cell-extracellular matrix connections. FAK has been implicated to play a crucial role in activating the downstream signaling pathway such as extracellular regulated kinase 1/2 (ERK1/2) MAPK (Forrest *et al.*, 2009) and PI3K-protein kinase B (PKB/Akt, hereafter called Akt) pathway (Parsons, 2003) (discussed in sections 4.1.3.-4.1.8.).

ERK1/2 pathway is most widely implicated in developmental functions regulating growth and differentiation (Newbern *et al.*, 2011). Whereas, PI3K-Akt pathway mediates anti-apoptotic effects and survival of cells (Ojeda *et al.*, 2011). The findings from experimental study one and two pointed out that the presence of laminin was beneficial with respect to adhesion, proliferation and differentiation of mESCs into neurons as compared to other selected matrix proteins. Chapter 4, analyses the signaling events caused by interaction of mESCs with extracellular matrix components which in turn might have an influence on mESC survival and differentiation.

For this study, it was decided to use an alternative genetic reporter mESCs line (*Sox1*-eGFP mESCs) which was grown in '2i' medium to attain ground state (discussed in section 4.2.1.). This reporter cell line allowed identification of early stages of neural differentiation. This study was conducted systematically, beginning by investigating the effect of laminin, gelatin and poly-D-lysine on differentiation towards a *Sox1+* population and further developed by exposing the cultures to inhibitors of kinases that have been reported to play role in cell adhesion, survival and differentiation in presence of above selected substrates.

Chapter 2 Matrix proteins and their influence on neural induction

2.1. Introduction

The inner cell mass of mouse and primate blastocysts have mainly been used to derive pluripotent embryonic stem cells. Embryonic stem cells exhibit indefinite replicative potential and an ability to differentiate into derivatives of all three germ layers, i.e. endodermal, mesodermal and ectodermal cell types, including neurons (Chen *et al.*, 2007b; Evans *et al.*, 1981). The pluripotent nature of embryonic stem cells is of great interest because they can serve as a potential source for homogenous and large neuronal cell populations for biochemical approaches. Laminin, fibronectin, collagens and glycosaminoglycans, the known extracellular matrix proteins, are all present during neuronal migration and axon pathway formation in the embryonic brain. It has been suggested that neurons of CNS use laminin and fibronectin as a substratum for attachment and neurite extension (Liesi, 1990).

Embryonic stem cells have the ability to differentiate into various cell types and specially generation of neural cell types *in vitro* (Robinton *et al.*, 2012) therefore, this offers a tool to study how cell-extracellular matrix interaction regulate neural stem cell specification. The challenge is now to understand how embryonic stem cells interact with complex extracellular matrix components that lead to generation of neurons (Czyz *et al.*, 2001; Goetz *et al.*, 2006a). Extracellular matrix proteins are known to modify the morphology of cells, for example, differentiation of cells of mammary glands is induced when they come into contact with type 1 collagen, whereas, fibronectin and laminin induce extensive spreading and proliferation and enhance survival of granulosa cells (Williams *et al.*, 2008).

2.1.1. Midbrain differentiation

Several protocols have been described so far for the differentiation of dopaminergic neurons from embryonic stem cells via the formation of embryoid bodies (EBs) (Lee *et al.,* 2000; Okabe *et al.,* 1996). Raye et al. (2007) demonstrated that addition of Shh and FGF8b helped in differentiation of mESCs into midbrain phenotypic dopaminergic neurons (Raye *et al.,* 2007). Parkinson's disease is a neurodegenerative disorder characterized by selective degeneration of the dopaminergic (DA) neurons in the substantia nigra (A9) of the midbrain (Olanow *et al.,* 1999). The development of midbrain specific neurons has been investigated in detail and currently there are two aspects by which dopaminergic neurons generation can be influenced. The first one is through modified culture conditions (Kelly *et al.,* 2005; Ye *et al.,* 1998) and second is these dopaminergic neurons are generated from embryonic stem cells by introduction of transcriptional factors such as *Nurr1*, *Pitx3* and *Lmx1a* through genetic modifications (Baizabal *et al.,* 2009; Friling *et al.,* 2009).

2.1.2. Growth factors

FGF2 can help in differentiation of mESCs into neuroepithelial cells and when further differentiated in presence of Shh and FGF8 they generated dopaminergic neurons (Lee *et al.,* 2000). FGF2 can promote embryonic stem and neural stem cells proliferation. Additionally, FGF2 play many roles in the injured spinal cord, including promotion of neural progenitor proliferation, neuronal survival and enhancement of functional recovery (Willerth *et al.,* 2007).

Ye et al. (1998) showed that by blocking signaling pathway of FGF8, a morphogen involved in the patterning of isthmus and Shh, a ventralizing molecule, resulted in absence of midbrain dopaminergic neurons (Ye *et al.*, 1998). It has been suggested that the generation of dopaminergic neurons *in vivo* is dependent on co-operation between FGF8 and Shh, a cell patterning molecule first described in drosophila (Pringle *et al.*, 1996).

The potential of embryonic stem cells to differentiate along the retinal ganglion cell lineage was evaluated by exposure to FGF2. FGF2-induced embryonic stem cells derived neural progenitors (ES-NPs) were able to generate RGC-like cells *in vitro* upon differentiation (Jagatha *et al.*, 2009). Therefore, it can be hypothesised that during development, cells encounter these intersecting growth factors along the anterior–posterior (FGF8) and dorsal–ventral (Shh) axes, they help differentiation into dopaminergic neurons (Okabe *et al.*, 1996; Pringle *et al.*, 1996). Dopaminergic neurons can be detected as early around embryonic day 10 (E10) in the mouse and they arise in close proximity to two important organizing centres, floor plate and the isthmus. Thus, it can be hypothesized that Shh signaling molecule secreted by the floor plate of the isthmus plays crucial role in development of dompaminergic neurons (Goridis *et al.*, 2002). Shh specifies the entire ventral midbrain in a concentration and position dependent manner, and its absence leads to abnormal size of midbrain (Agarwala *et al.*, 2001).

By using a chemically defined system to direct human embryonic stem cells to neuroepithelial cells, Yan et al. (2005) showed that *Sox1*-expressing neuroepithelial cells when treated with FGF8 and Shh, there was an efficient production of dopaminergic neurons (Yan *et al.*, 2005). When differentiating mESCs were exposed to exogenous Shh on day 4 to 6 of differentiation, it led to an increase in dopaminergic neurons (Barberi *et al.*, 2003). When embryonic stem cells derived neural precursor cells (NPCs) were exposed *in vitro* to FGF8 and Shh, it increased the proportion of *Lmx1a*+ dopaminergic neurons (Baizabal *et al.*, 2009).

2.1.3. Chapter aims

- Investigation of the roles of three extracellular matrix proteins, fibronectin, type IV collagen and laminin, on mESCs viability.

- Neural progenitor expansion and neurite out growth in medium devoid of growth factors/differentiating agents were also explored.

- In addition mESCs were terminally differentiated in the presence of growth factors that have been reported to induce formation of dopaminergic neurons, to investigate whether selected matrix proteins had specific effects on differentiation of mESCs.

2.2. Materials and Methods

2.2.1. Maintenance of mESCs

E14Tg2a, a sub clone of the E14 line derived from the inbred mouse strain 129/Ola was used. E14Tg2a cells were procured from the American cell culture type (ATCC) and had undergone 8 passages. The cell line was routinely cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Australia) supplemented with 10% embryonic stem cell qualified fetal calf serum (Invitrogen, Australia), 2 mM L-glutamine (Invitrogen, Australia), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, Australia) and 10^3 Units/ml murine LIF (Millipore, Massachusetts) at 37°C with 5% CO_2. Medium was changed every 48 hours until cultures approached 75-80% confluence and were subsequently passaged. Flasks, dishes and wells were pre-incubated with 0.1% gelatin solution in 1X phosphate buffered saline (1X PBS) with final concentration of 2.7 mM potassium chloride, 137 mM sodium chloride, 10 mM sodium phosphate and 1.76 mM potassium phosphate (for at least 15 min on hot plate set at 37^{o}C prior to use, to coat the surface of plasticware with a gelatin film.

For passaging, medium was aspirated and cells were washed once with sterile 1X PBS. Accutase (Sigma-Aldrich, Australia) was added at 1 ml per 7.5 cm^2 surface area to give a thin film over the cells and the cultures were incubated for 5 min at 37°C. Culture medium containing serum was added to stop the enzymatic action. Cells were pelleted in a conical tube (200g for 5 minutes). After re-suspension in fresh culture medium (with supplements) mESCs were counted using a haemocytometer and seeded at 2 x 10^4 cells/cm^2 onto plates (surface coated with 0.1% v/v gelatin).

For long term storage, embryonic stem cells were frozen down in cryo-vials (Corning, Australia) in a mixture of 50% fetal calf serum, 40% culture medium and 10% dimethyl sulfoxide (DMSO) (Sigma, Australia) in an isopropanol filled "Mr Frosty" (Nalgene labware, New York) for at least 90 minutes (at -80°C) and then transferred to liquid nitrogen.

2.2.2. Neural differentiation

For neural induction an adherent monolayer approach was used (Figure 2.1). mESCs were seeded on gelatin-coated tissue culture plates/flask at 4.5 x 10^3 cells/cm^2 in normal mESCs maintenance medium. 24 hours later, cells were washed once with 1X PBS and switched to serum free N2B27 medium for 5 days. N2B27 medium is a 1:1 mixture of Neurobasal medium supplemented with N2: DMEM/F12 medium supplemented with B27, insulin and bovine serum albumin V. On day 5, adherent cells were dissociated to produce a single cell suspension with Accutase solution (as described under "Maintenance of mESCs" in section 2.2.1) and seeded at $5x10^3$ cells/cm^2 onto surfaces coated with selected matrix proteins including laminin (5 µg/cm^2, Invitrogen, Australia), fibronectin (5 µg/cm^2, BD Bioscience, Australia) and type IV collagen (1 µg/cm^2, BD Bioscience, Australia) in N2B27 supplemented with morphogens 20 ng/ml FGF2 (R&D Systems, USA), 20 ng/ml heparin sulphate (Sigma-Aldrich, Australia), 100 ng/ml FGF8b (R&D Systems, USA) and 500 ng/ml Shh (R&D Systems, USA). After 3 days (on day 8), the medium was replenished with fresh N2B27 supplemented with morphogens. On day 6, after replating (day 11), cells were terminally differentiated in N2B27 medium supplemented with 10 ng/ml glial-derived neurotrophic factor (GDNF) (R&D Systems, USA) and 200 µM ascorbic acid (Sigma-Aldrich, Australia) for protection of neurons and terminal differentiation. During this phase the medium was changed every other day until day 21.

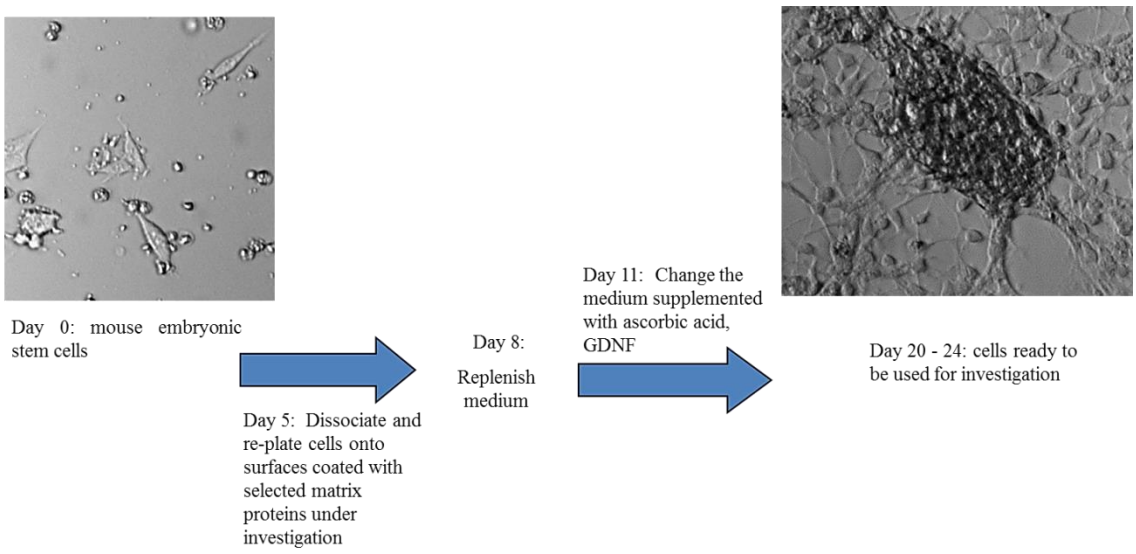


Figure 2.1: Diagrammatic overview of neural differentiation

2.2.3. In-cell Western assay

As no information was available on the mass of each matrix protein required to attain uniform coating of individual well, a simple experiment on coating efficiency was performed. The assay was carried out by coating wells of ninety-six well plates with selected matrix proteins at 1, 5 and 10 µg/cm^2. Each experiment was performed in quadruplicate, including negative controls which were not exposed to primary antibodies. Proteins coated surfaces were fixed by incubation with solution of 4% paraformaldehyde in 1X PBS (w/v) for 30 minutes at room temperature. For permeabilization, 100 µl of 0.1% TritonX-100 (Sigma-Aldrich, Australia) in 1X PBS was added to each well for 30 minutes. Wells coated with matrix proteins were blocked with 100 µl of blocking buffer (Li-cor Biotechnology, USA) for 90 minutes at room temperature. Mouse anti-laminin (Sigma-Aldrich, Australia), rabbit anti-fibronectin (Millipore, Australia) and mouse anti-type IV collagen (Abcam, UK) were diluted 1:200 in blocking buffer and incubated at 4ºC with volume of 50 µl per well.

Unbound primary antibody was removed by washing wells (5 times) with 200 µl 0.1% Tween20 (Sigma-Aldrich, Australia) in 1X PBS with shaking. The secondary antibody, IRDye 800CW donkey anti-mouse and IRDye 680LT donkey anti-rabbit (Li-cor Biotechnology, USA) were diluted 1:1000 in blocking buffer. Unbound secondary antibody was removed by washing each well (5 minutes) with 0.1% Tween20 (Sigma-Aldrich, Australia) in 1X PBS (with gentle shaking) 5 times. Wells were scanned for emission at 700 nm and 800 nm with the Odyssey Infrared Imaging system (ODY-18090) (Li-cor Biotechnology, USA). Values from the 700 nm and 800 nm channel corresponded to the amount of primary antibody bound to the wells. Control wells not exposed to primary antibodies was used for background compensation. The background fluorescence values at 700 nm and 800 nm were averaged and subtracted from the respective values of wells incubated with primary antibodies. Coating efficiency was estimated by comparing the integrated fluorescence intensity of individual wells measured using the Odyssey.

2.2.4. Microculture Tetrazolium (MTT) Assay

The CellTiter 96® AQueous One Solution (Promega, Australia) was used to determine the effects of different selected matrix protein concentrations on cell viablity and proliferation. The MTS tetrazolium compound (Owen's reagent) was reduced enzymatically by cells into a soluble coloured formazan product. This conversion is accomplished by NADPH or NADH-dependent reductase enzymes in metabolically active cells. Ninety-six well plates were coated with selected matrix proteins (in triplicate) at 1, 5 and 10 µg/cm^2.

mESCs were seeded at a density of 5000 cells/well in presence of serum free N2B27 medium. A time course experiment was carried out and numbers of viable cells were measured every 24 hours up to 96 hours after seeding onto assay plates. 20 µl of CellTiter 96® AQueous One Solution Reagent was dispensed into each well of the ninety-six well assay plate and incubated for four hours at 37^{o}C. Absorbance was read at 490 nm using a plate reader. Wells coated with selected concentrations of matrix in presence of N2B27, but without mESCs served as negative control. Values at wavelength 490 nm absorbance values were averaged and subtracted with absorbance of negative control. The absorbance value/fraction of gelatin (Y axis) was plotted versus concentration of the selected matrix proteins (X axis).

2.2.5. Immunocytochemistry

All incubation steps took place at room temperature unless stated otherwise. For fixing, cells were incubated for 30 minutes in 4% paraformaldehyde. Subsequently, cells were incubated with 1X PBS containing 0.5% TritonX-100 (Sigma-Aldrich, Australia) for another 30 minutes. Non-specific antibody binding was blocked with 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, Australia) in 1X PBS for one hour at room temperature, this was followed by overnight exposure (at 4°C) with primary antibodies either mouse-anti-β III tubulin (1:1000) (Sigma-Aldrich, Australia), mouse anti-nestin (1:100) (Millipore, Australia) and rabbit-anti-tyrosine hydroxylase (1:200) (Millipore, Australia) diluted in 1X PBS (containing 1% BSA). After 12 hours of incubation cells were washed 4 times with 1X PBS containing 1% BSA, for 5 minutes each, then incubated with secondary antibodies: donkey anti-mouse Alexa Fluor 488 (1:1000) (Invitrogen, Australia) and Texas Red®-X donkey anti-rabbit 595 (1:1000) (Invitrogen,

Australia) for 120 minutes. Finally, cells were washed 4 times with 1X PBS containing 1% BSA for 5 minutes each and then covered with 1X PBS for imaging.

2.2.6. Imaging and counting of βIII tubulin, Nestin and tyrosine hydroxylase immunopositive cells

Images were taken with a CoolSNAP$_{FX}$ camera (Photometrics, Arizona) attached to an Eclipse TE-2000E microscope (Nikon, Japan). To quantify the number of βIII tubulin, Nestin and tyrosine hydroxylase positive cells, images were captured using a 10 times objective. Four sites/well were analysed by imaging up and down, left and right hand corners respectively of each well, in duplicate from three separate experiments. βIII tubulin, nestin and tyrosine hydroxylase positive cells were then counted manually (see Figure 2.11.A for typical field of view and counts).

2.2.7. Quantification of neurite outgrowth

Neurite outgrowth was analysed using the NIH ImageJ analysis software using a NeuronJ plugin for the multi-platform free image-processing program (Meijering *et al.*, 2004). Neurite tracer processes pairs of neuronal and nuclear marker images to obtain skeletons of neuronal extensions and masks of neuronal nuclei. Neurites were quantified from images of neurons immunostained for βIII tubulin. A neurite was defined as a process having longer than the width of one cell body terminating in a growth cone, and neurites were recorded using trace function. Total neurite length was measured and divided by the number of neurons observed in the field.

For these analyses, each captured image was identified as a sampling unit and four sites/well were analysed by imaging up and down, left and right hand corners respectively of each well, in duplicate, from three separate experiments (Figure 2.8. B typical example of outgrowth).

2.3.8. Statistical analysis

All results are presented as mean ± standard error of the mean (SEM) of at least three separate experiments. Statistical analyses were performed on raw data using PRISM v5.00 (GraphPad Software, USA). Data were analysed by Student's *t* test or one-way analysis of variance or two-way analysis of variance (ANOVA) with post-hoc Bonferroni's test. In all cases, $P < 0.05$ was considered to be significant.

2.3. Results

2.3.1. Coating evaluation

Wells of tissue culture plates were coated with selected matrix proteins at 1, 5 and 10 µg/cm^2, after incubation for three hours at 37^{o}C (Figure 2.2 A), remaining solution was aspirated and taken up for assay as described under materials and methods. As we observed similar pattern with all selected matrix proteins below is an example of fibronectin (Figure 2.2 A, B and C). Figure 2.2 B shows better coverage of the surface being coated with selected matrix protein as the concentration of matrix proteins was increased (Figure 2.2 C).

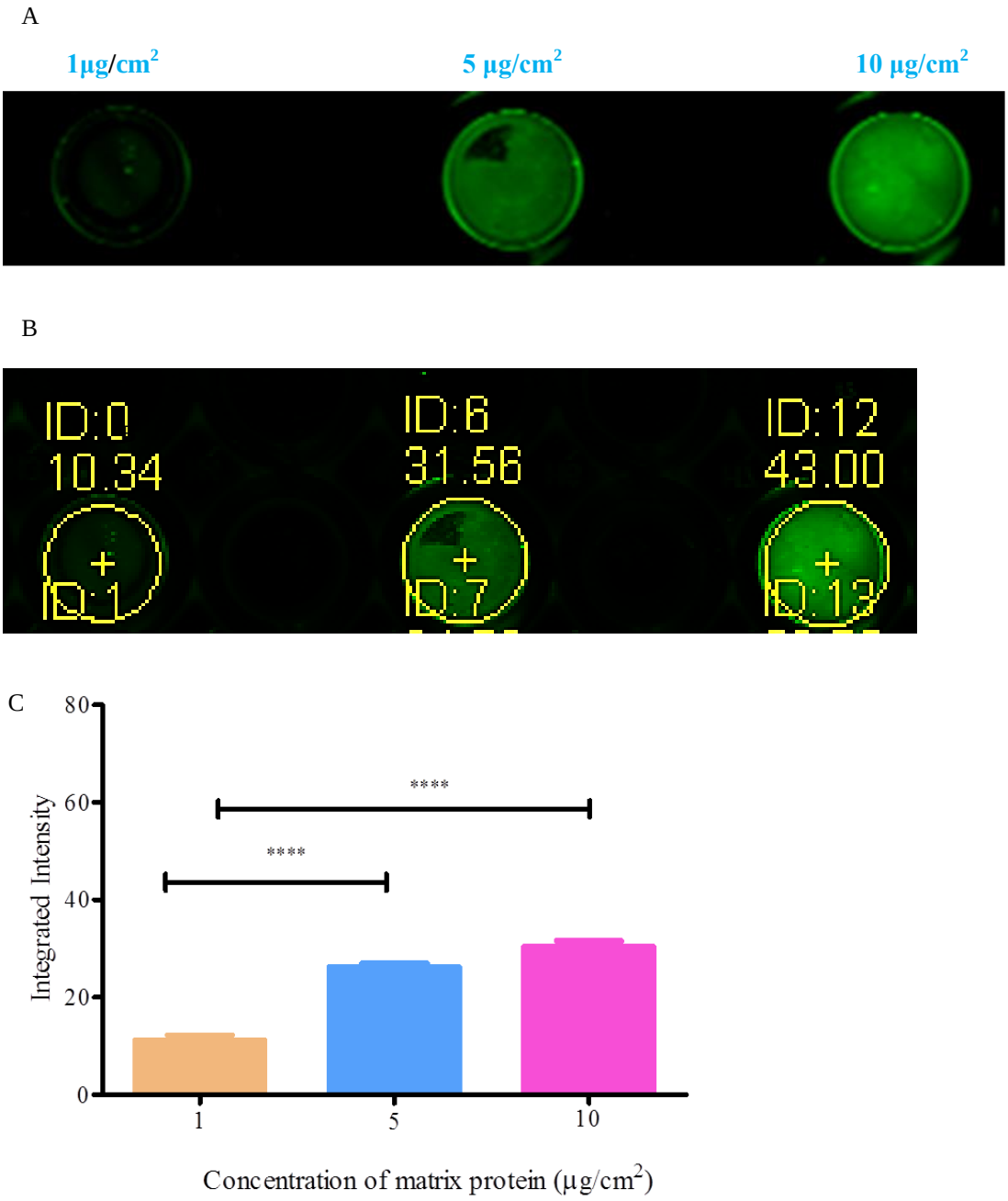


Figure 2.2: In-cell Western to evaluate coating procedure. (A) Fibronectin coating of 48 well plate, (B) How the integrated intensity of individual well was calculated using the Odyssey Infrared Imaging system (ODY-18090) and (C) Integrated intensity measured at 700 nm for fibronectin by Odyssey Infrared Imaging system (ODY-18090). Values are expressed as mean ± SEM of three independent experiments. **** $P < 0.001$, indicate a significant difference between each individual concentration of matrix protein, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

2.3.2. Morphological appearance of the mESCs during initial neural induction on different matrix proteins

The morphological appearance of mESCs maintained on gelatin (0.1%v/v) varied considerably in comparison to mESCs cultured on extracellular matrix proteins. The colonies formed when cultured on gelatin (0.1%v/v) were more of aggregates (Figure 2.3 A). Cells cultured on surface coated with laminin and fibronectin showed colonies of more uniform in size and shape as compared to cells cultured on surface coated with type IV collagen (Figure 2.3 B, C and D). Colonies growing on type IV collagen were scattered around the surface whereas, on laminin and fibronectin they were closely knit.

mESCs cultured on laminin for 96 hours started showing sign of differentiation such as (Figure 2.4 D), which was not observed in case of gelatin (Figure 2.4 A). Therefore, it can be postulated that presence of gelatin did not help in differentiation. mESCs cultured on fibronectin and type IV collagen showed mixture of differentiated and undefined phenotypes (Figure 2.4 B and C). From these observations, it shows that laminin might promote neural differentiation.

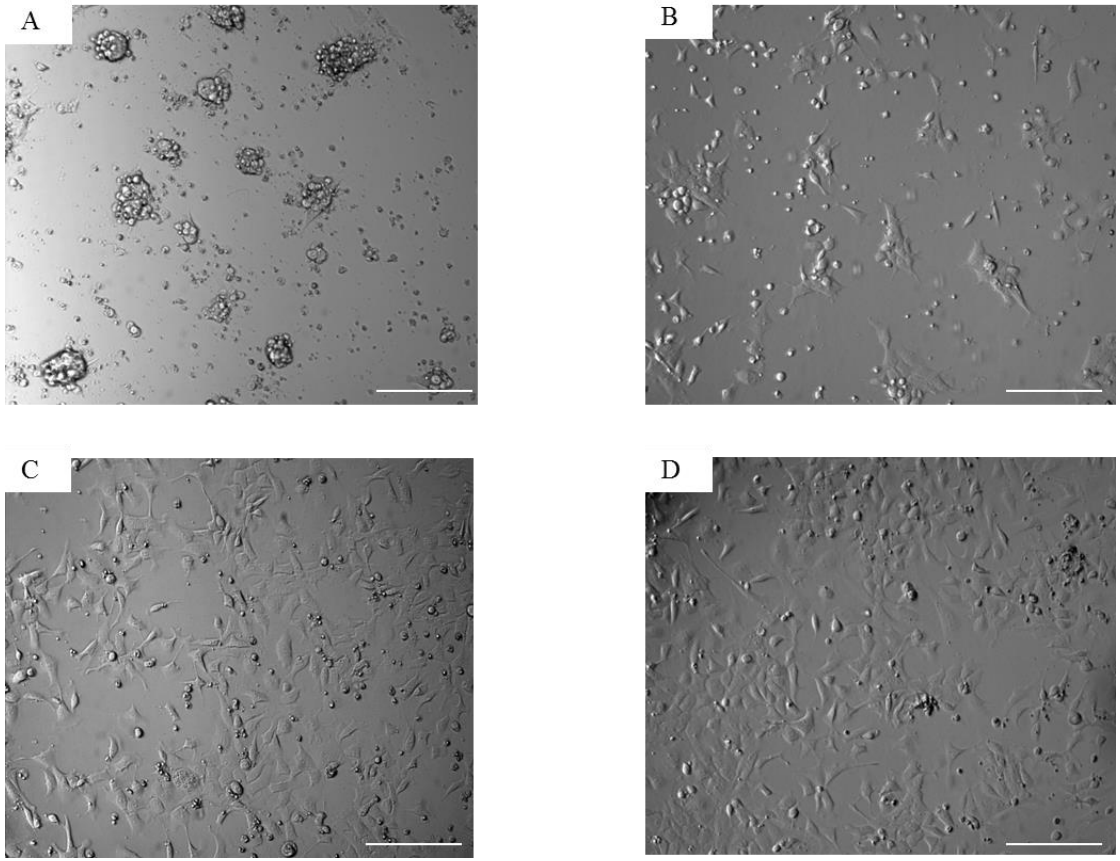


Figure 2.3: Photomicrographs of mESCs after 24 hours of exposure to neural differentiation medium and replating onto different surfaces. Wells were coated with the following selected matrix proteins (A) gelatin 0.1% (v/v), (B) type IV collagen (1 μg/cm^2), (C) fibronectin (5 μg/cm^2) and (D) laminin (5 μg/cm^2). Scale bar is 100 μm.

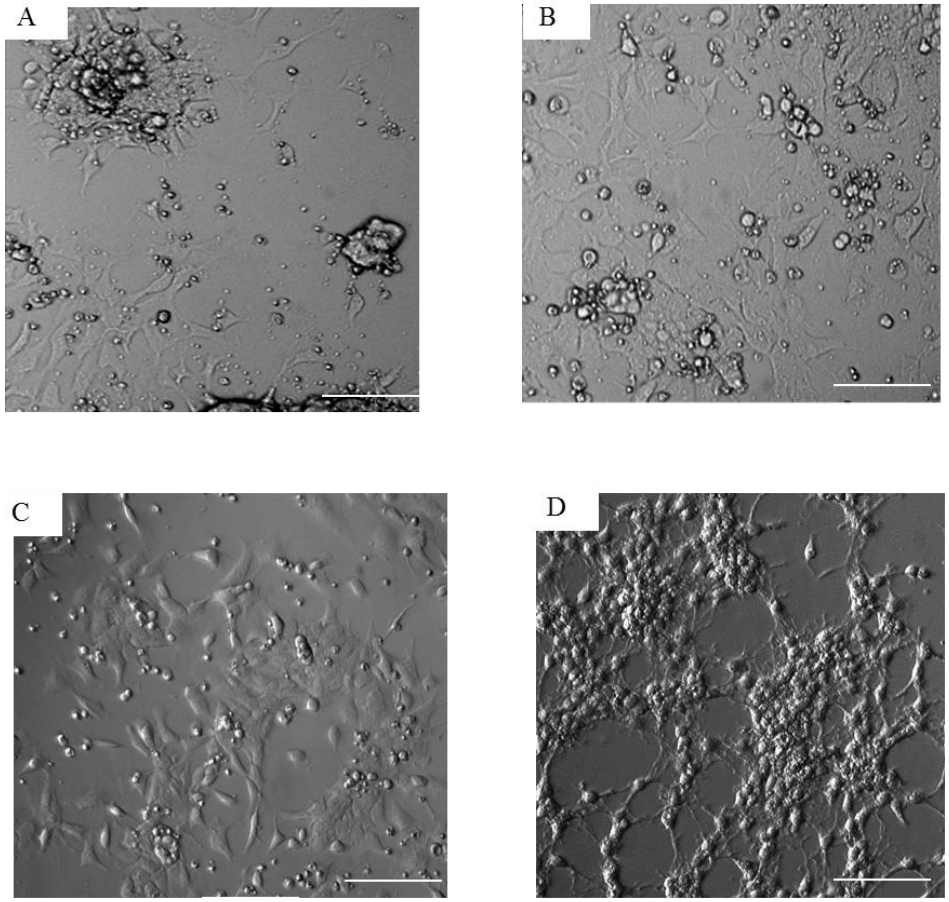


Figure 2.4: Photomicrographs of mESCs after 96 hours of exposure to neural differentiation medium and replating onto different surfaces. Wells were coated with the following selected matrix proteins (A) gelatin 0.1% (v/v), (B) type IV collagen (1 μg/cm^2), (C) fibronectin (5 μg/cm^2) and (D) laminin (5 μg/cm^2). Scale bar is 100 μm.

2.3.3. Microculture Tetrazolium (MTT) Assay

Cells responses to biological, chemical or mechanical changes in their intracellular or extracellular environment can contribute to cell proliferation or death. Thus, MTT assay was performed to show how different matrix proteins influence mESC viability and proliferation. Photomicrographs taken 24 hours after replating onto surfaces coated (Figure 2.3) with type IV collagen had few cells in contrast many cells were evident in plates coated with laminin or fibronectin. The preference is still same after 96 hours (Figure 2.4).

An MTT assay was carried out on mESCs 24 hours after replating. This assay showed that viability in 96 plate wells coated with type IV collagen was not significantly enhanced and a concentration-dependent effect on cell viability was not observed, as seen in case of cells plated on surfaces coated with laminin or fibronectin (Figure 2.5). Thus, based on the analysis 24 hours and 96 hours (Figure 2.6) after replating, the most appropriate concentrations of matrix proteins were selected for further investigation, namely 1 $\mu g/cm^2$ for type IV collagen and 5 $\mu g/cm^2$ respectively for both laminin and fibronectin. Since the major aim this thesis was to study the role of the three matrix proteins on neural differentiation it was decided to normalise the data against mESCs grown on gelatin. The reduction in cell viability after 24 hours of replating (Figure 2.6) could be explained by either cell detachment/death or by changes in cell metabolic activity over time (Hughes *et al.*, 2012; Lawton *et al.*, 2013; Li *et al.*, 2012; Marcos-Campos *et al.*, 2012).

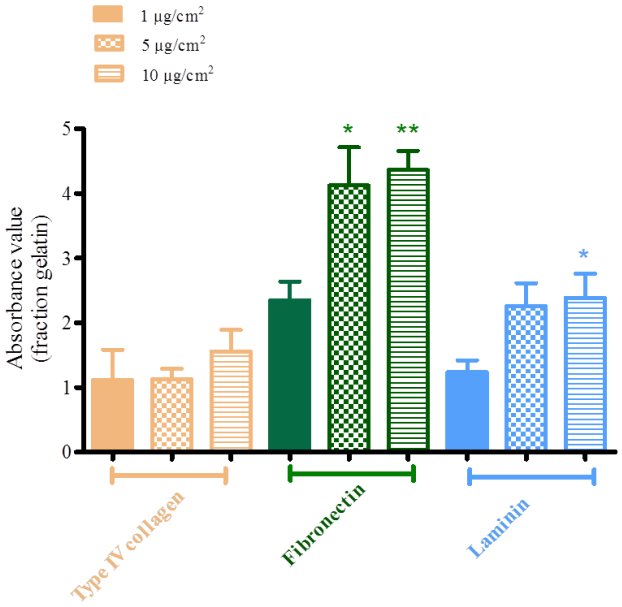


Figure 2.5: Effects fibronectin, laminin and type IV collagen effects on cell adhesion 24 hours after re-plating. Values are expressed as mean ± SEM of three independent experiments. * $P < 0.05$ and ** $P < 0.01$, indicate significant difference between each individual matrix, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

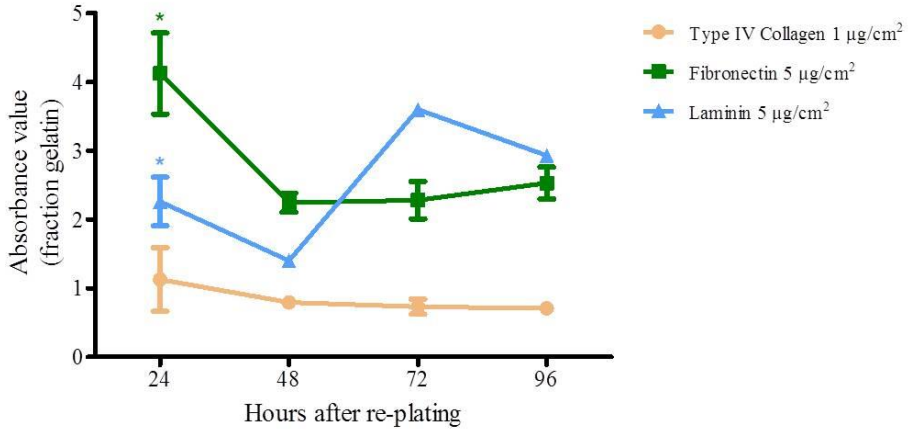


Figure 2.6: Effects of type IV collagen, fibronectin and laminin on proliferation of cells derived from mESCs. Values are expressed as mean ± SEM of three independent experiments. * $P < 0.05$, indicate significant difference between laminin and type IV collagen, fibronectin and type IV collagen, one-way ANOVA with post-hoc Bonferroni's multiple comparison test.

2.3.4. Neural progenitor expansion and differentiation into neurons

To examine the effect of matrix on the adhesion and survival after replating of mESC-derived neural progenitors, 5000 cells/well were seeded onto matrix coated wells and after 24 hours, the number of nestin positive cells was quantified, from the images captured from four corners of the well i.e. top and bottom left and right hand corners of respectively of the well. Figure 2.7 A-C shows representative images indicating that many of the cells were immunopositive for nestin and had a similar appearance on all surfaces after 24 hours. A comparison of positive cell counts at this time showed that on average the cell number was not significantly different after plating on the three selected matrix proteins (Figure 2.7 D).

The effect of selected substrates on neurite outgrowth of mESC-derived neurons was performed by quantifying number of primary neurites and total neurite length per neuron. The mESCs-derived neurons were identified using βIII tubulin immunocytochemistry (Figure 2.8 A and B). Generally, 24 hours after replating, the proportion of cells immunopositive for βIII tubulin was less than 6% indicating that the differentiation was predominantly at the progenitor stage. The numbers of βIII tubulin-positive cells 24 hours after replating was independent of surface, but the number of βIII tubulin-positive neurons increase from 11/view to 31/view on laminin after 96 hours whereas, it went from 10/view to 20/view, 8/view to 19/view respectively on fibronectin and type IV collagen (Figure 2.8 C and D). Total neurite length was calculated as described in section 2.2.7. Type IV collagen did show an increase in the number of neurons but substantially increased neurite length as compared to fibronectin and laminin (Figure 2.10 A and B). Therefore, laminin enhanced conversion of neuronal progenitor positive cells into neurons (Figure 2.8) but failed to stimulate neurite outgrowth.

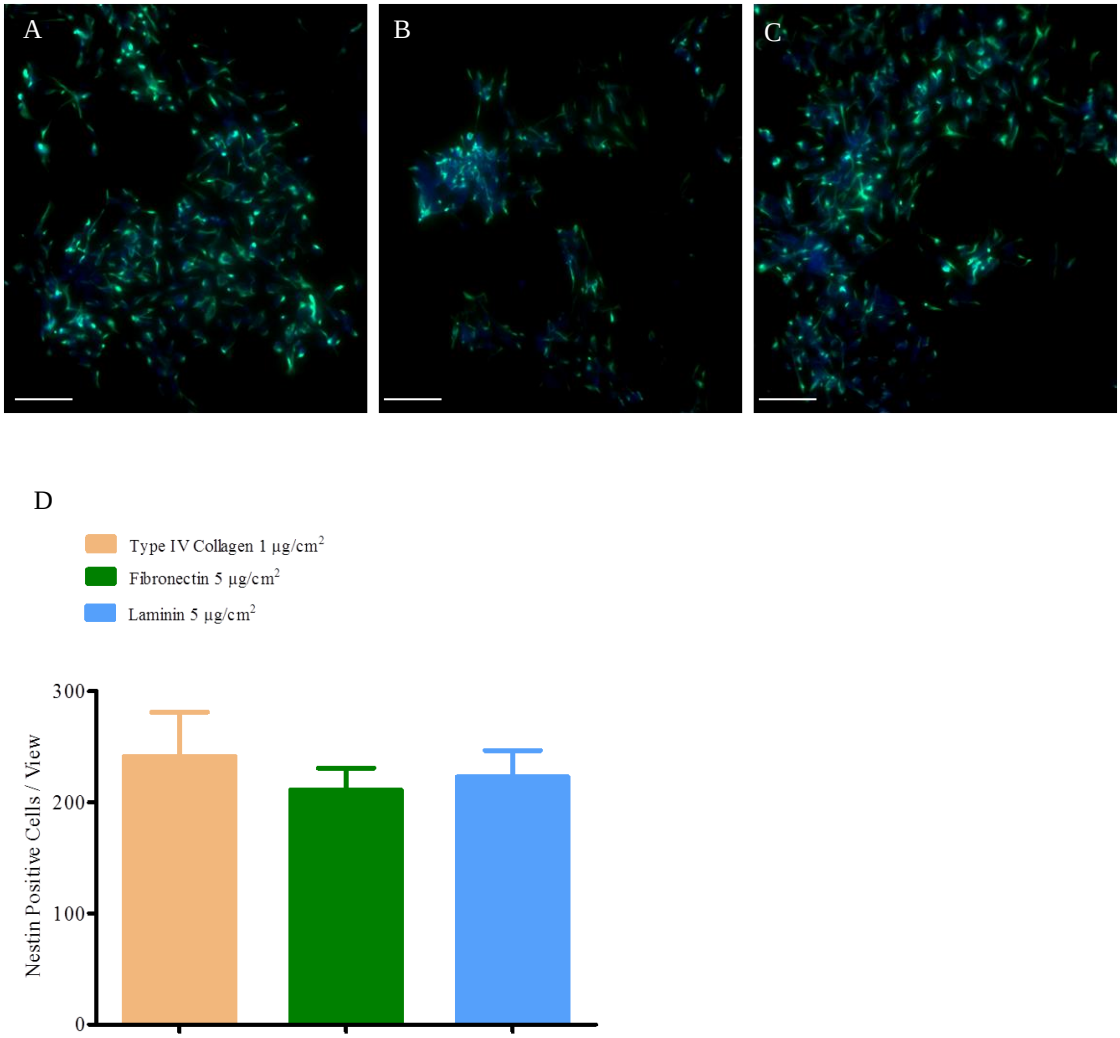


Figure 2.7: Expansion of mESCs-derived nestin positive neural progenitors. (A) laminin (5 µg/cm^2), (B) fibronectin (5 µg/cm^2) and (C) type IV collagen (1 µg/cm^2) showing nestin positive cells in green and nuclei stained with DAPI in blue at 24 hours after re-plating. Bar plots summarizing the effect of different substrates on number of nestin positive cells (D). Values are expressed as mean ± SEM of three independent experiments. No significant difference was observed between tested conditions $P < 0.05$, one-way ANOVA. Scale bar is 100 µm.

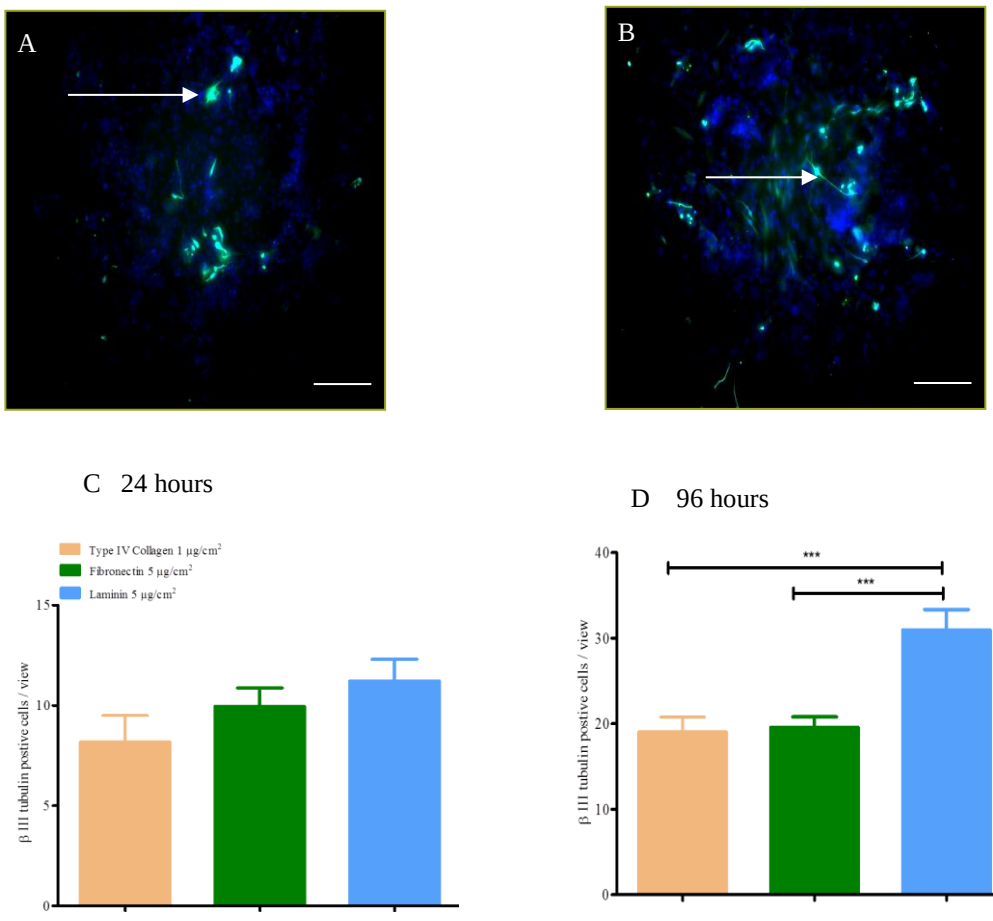


Figure 2.8: Neuronal cells derived from mESCs. (A) laminin (5 µg/cm^2), (B) fibronectin (5 µg/cm^2) showing βIII tubulin positive cells (white arrow) in green and nuclei stained with DAPI in blue at 96 hours after replating. βIII tubulin staining (C, D; cells counted after 24 and 96 hours of re-plating respectively). Values are expressed as mean ± SEM of three independent experiments. *** P < 0.001, indicates a significant difference between individual matrix proteins, one-way ANOVA post-hoc Bonferroni's multiple comparison test. Scale bar is 100 µm.

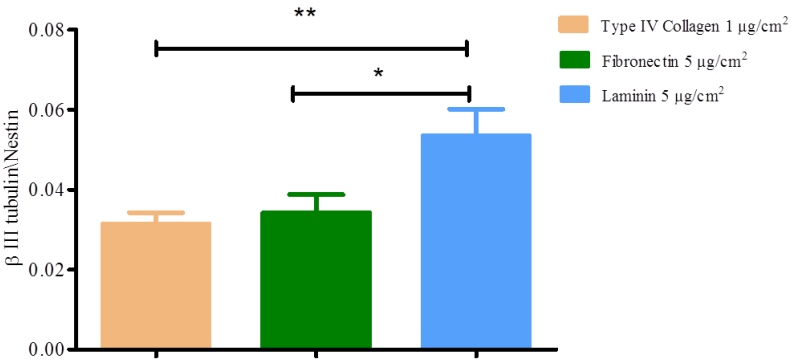


Figure 2.9: Bar plots summarizing the effect of selected matrix proteins on the ratio of βIII tubulin/Nestin population 24 hours after replating on day 5 (i.e day 6). Values are expressed as mean ± SEM of three independent experiments. Statistical differences for ratio between laminin, fibronectin and type IV collagen * $P < 0.05$ and ** $P < 0.01$, indicate a significant difference between matrix proteins under investigation one-way ANOVA with post-hoc Bonferroni's multiple comparison test.

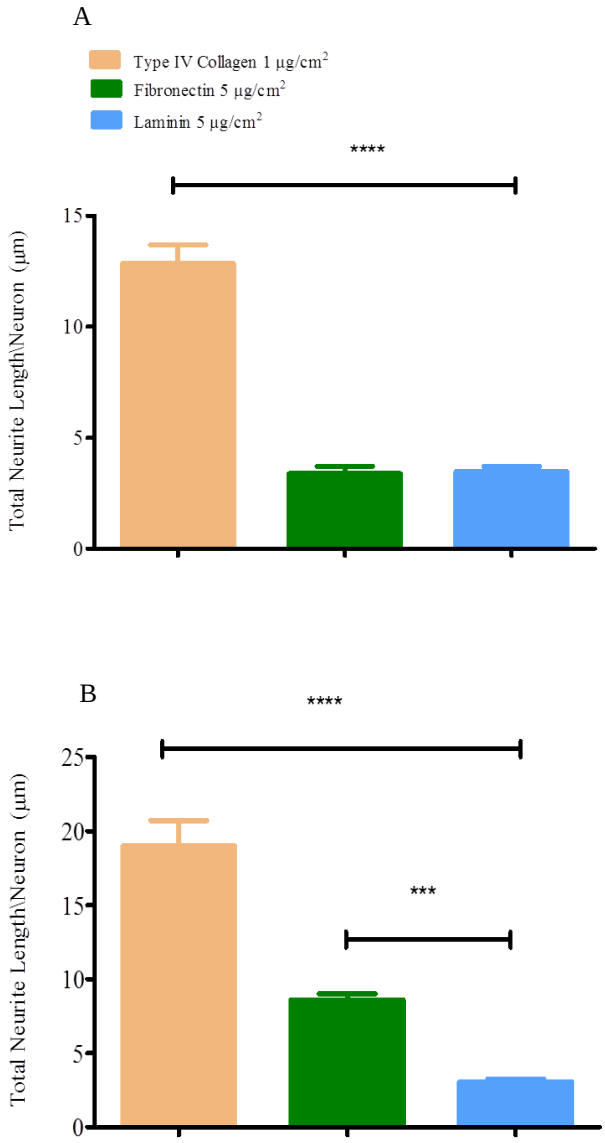


Figure 2.10: Bar plots summarizing the effect of different substrates on total neurite length per neuron. (A) 24 hours and (B) 96 hours. Values are expressed as mean ± SEM of three independent experiments. Statistical differences for total neurite length per neuron between laminin, fibronectin and type IV collagen are significant *** $P < 0.001$ and **** $P < 0.0001$, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

2.3.5. Quantification of tyrosine hydroxylase positive cells

To ascertain the effect of selected substrates on differentiation of mESCs into dopaminergic or noradrenergic neurons, differentiated mESCs were labelled at day 21 after replating (i.e day 26) with tyrosine hydroxylase (a catecholaminergic marker) and βIII tubulin. Number of immunopositive cells was counted manually in images captured as explained in section 2.3.4. selected fields (Figure 2.11 and 2.12). No significant difference was observed between tested conditions $P < 0.05$, one-way ANOVA.

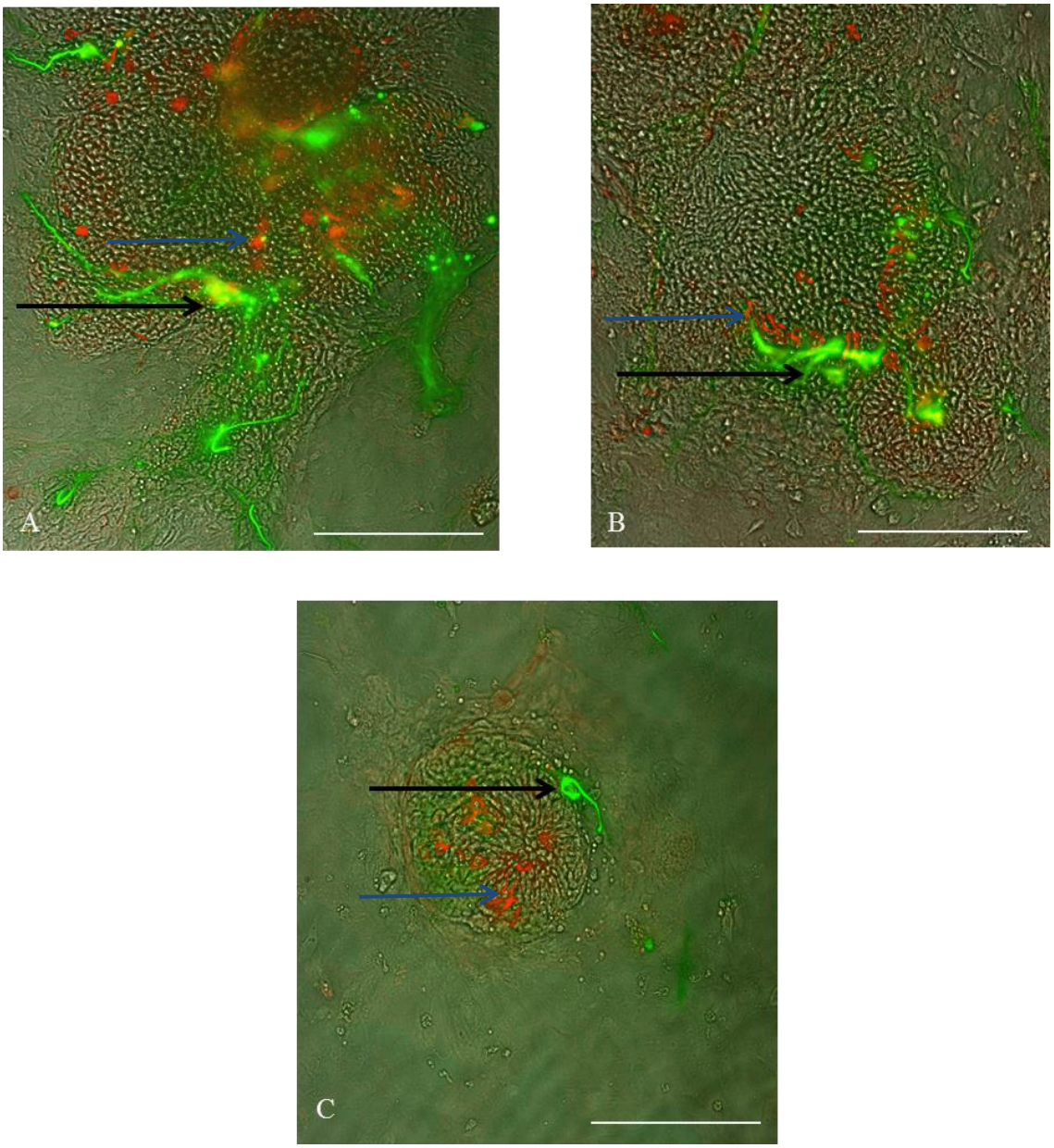


Figure 2.11: Neuronal cells derived from differentiated mESCs. (A) laminin (5 µg/cm^2), (B) fibronectin (5 µg/cm^2) and (C) type IV collagen (1 µg/cm^2) showing tyrosine hydroxylase positive cells (blue arrow) in red and βIII tubulin positive cells (black arrow) in green at day 21 after re-plating on day 5. Scale bar is 100 µm.

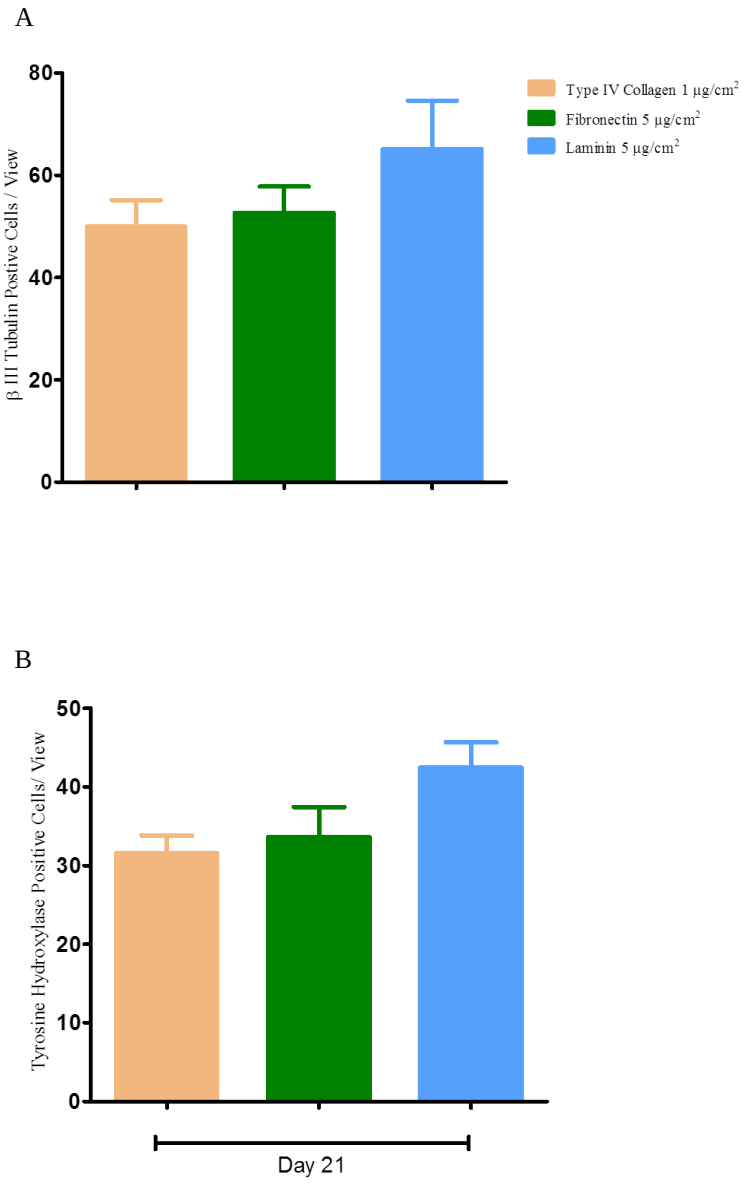


Figure 2.12: (A) βIII tubulin and (B) tyrosine hydroxylase; cells counted after 21 days. Values are expressed as mean ± SEM of three independent experiments. No significant difference was observed between tested conditions $P < 0.05$, one-way ANOVA.

2.5. Discussion

This study investigated the effects of three matrix proteins commonly found in the basement membrane. mESCs attached and proliferated on surfaces coated with matrix proteins after replating cells after 5 days of neural induction on gelatin in N2B27 medium. Given that survival of cells plated onto plastic plates is poor, it is clear that matrix proteins play a crucial role in the attachment and proliferation of neural progenitors. This is particularly important when cells are plated as monolayers and is supported by previous studies (Gospodarowicz *et al.*, 1980; Hall *et al.*, 1987; Tomaselli *et al.*, 1987). Other published studies have shown that extracellular matrix proteins including laminin and fibronectin stimulate adhesion and process outgrowth and these studies support my observation that adhesion of mESC-derived neural stem cells was promoted by laminin or fibronectin, rather than type IV collagen or gelatin (Majumdar *et al.*, 2003; Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Rogers *et al.*, 1985).

The role of proteins like laminin, fibronectin, and type IV collagen has been extensively studied in the PNS where they promote neurite outgrowth (Duband *et al.*, 1982; Thiery *et al.*, 1982). In the CNS, extracellular matrix proteins and in particular laminin and fibronectin are likely to play a role in neuronal differentiation, regeneration and neurite elongation (Carri *et al.*, 1988). This study evaluated the effect of matrix proteins on the growth of neural progenitors by quantifying number of nestin positive cells 24 hours after re-plating. Comparison of cell counts showed no significant expansion over the first 24 hours on any of the matrix proteins.

When incidence of βIII tubulin positives was determined, laminin had the most pronounced effect. The number of βIII tubulin positive cells doubled after 96 hours on laminin after re-plating as compared to other matrix proteins under investigation. Results from the above study showed that laminin is particularly effective and in line with the information already in public domain.

Lein et al. (1991) reported that type IV collagen is synthesized by glial cells and is present on ganglia in the peripheral nervous system, where it provides tensile strength as a structural component of endoneural basement. When embryonic rat sympathetic neurons were seeded onto surfaces coated with type IV collagen and laminin, it was observed that type IV collagen but not laminin enhanced sympathetic neurite outgrowth. This growth promoting activity was associated primarily with the NC1 domain. Tucker and Erickson (1984) reported that collagen enhanced outgrowth of neural crest cells in absence of fibronectin (Tucker *et al.*, 1984).

Fibronectin is known to enhance neurite extension in neural crest cells (Sheppard *et al.*, 1995). There are several binding domains in fibronectin that may regulate this property. This concept was tested by plating human neuroblastoma cells onto laminin, and after overnight incubation, sizable percentages of cells showed extended neurites thus, showing that laminin presence is helpful in neurite extension (Lewandowska *et al.*, 1989; Mugnai *et al.*, 1988). Avian trunk neural crest cells bound to fibronectin which facilitated rapid migration indicating that fibronectin is important for neural crest cell adhesion and migration (Rovasio *et al.*, 1983).

Extracellular matrix proteins are assumed to play a crucial role in migration of trunk neural crest. To test this hypothesis, neural tubes from stage 12 chick embryos were suspended within gelling solutions of either basement membrane or rat tail collagen. Extensive crest migration was reported in basement membrane gel, as compared to rat tail collagen. In basement membrane gels, the laminin cell-binding peptide (YIGSR) completely inhibited migration of neural crest away from the neural tube, suggesting that laminin is the migratory substratum. Indeed, laminin, as well as collagen and fibronectin, are present in the embryonic extracellular matrix. Thus, it is possible that other extracellular matrix proteins, in addition to or instead of fibronectin, may serve as migratory substrata for neural crest *in vivo* (Bilozur *et al.*, 1988).

In our study, quantification of the primary neurites and total neurite length per neuron showed that laminin induced a significantly greater number of neurites as compared to fibronectin and type IV collagen 96 hours post plating, but it was type IV collagen which induced the most significant neurite elongation, thus suggesting that laminin failed to enhance neurite elongation although it doubled the number of neurons. Both laminin and fibronectin are synthesized in the CNS during development where they influence neuronal migration and axon guidance (Smith *et al.*, 1987). Liesi (1990) showed that brain cells bound preferentially to glial laminin, indicating that laminin plays crucial role in attachment of cells and neurite extension (Liesi, 1990). Laminin and fibronectin induced pronounced neurite outgrowth of cultured human fetal sensory neurons in presence of nerve growth factor. By increasing the amount of matrix, the effect of withdrawal of nerve growth factor on neuron growth was reduced (Baron-Van Evercooren *et al.*, 1982).

Embryonal carcinoma PCC4uva cells have the ability to differentiate into neurons when exposed to mixture of retinoic acid and dbcAMP. This *in vitro* model system was used to study the effects of laminin on early neural differentiation. When compared to type IV and I collagen or fibronectin, laminin was a more effective enhancer of neuronal differentiation and neurite growth (Sweeney *et al.*, 1990). These authors reported that when cells were treated with antibodies against laminin, the laminin induced effects were nullified. This supports the hypothesis that laminin plays a crucial role in influencing early differentiation of cell populations (Sweeney *et al.*, 1990).

Flanagan et al. (2006) plated human neural stem precursor cells (NSPCs) on different substrates including poly-L-ornithine, fibronectin, laminin or Matrigel, to compare the response of these cells with those of mouse NSPCs. These authors showed that laminin enhanced migration of human and mouse NSPC-derived neurons, as well as their migration, expansion, differentiation and neurite outgrowth. Laminin was more effective than poly-L-ornithine, fibronectin or Matrigel (Flanagan *et al.*, 2006). Differentiation of human embryonic stem cells into neural progenitors and neurons was reported to be stimulated by laminin, to a greater extent than fibronectin or type I collagen. Ma et al. (2008) also observed that robust neuroectodermal precursors were generated by neural rosettes on surfaces coated with laminin (Ma *et al.*, 2008). In this study it was observed that laminin stimulated neuronal differentiation but not neurite outgrowth. Whereas, type IV collagen stimulated neurite outgrowth as compared to laminin. This observation could be specific to the effects on neurons obtained from mESCs.

Stem cells isolated from human embryos or foetal tissues have the potential to provide neurons to ameliorate neural disorders, and foetal tissue has been used in attempts to replace lost neurons in Parkinson's disease. However, one major obstacle is that the majority of cells do not differentiate into neurons when grafted into areas which are outside the known areas of neurogenesis, namely the subgranular zone (SGZ) and subventricular zone (SVZ) of the adult CNS and grafts do not exhibit constitutive neurogenesis *in vivo* under normal conditions (Jiao *et al.*, 2008). Priming foetal human neural stem cells with laminin *in vitro* generated a pure population of neurons and enhanced generation of cholinergic neurons in grafted cells (Tarasenko *et al.*, 2004; Wu *et al.*, 2002). Working with these principles, it was explored whether priming with selected matrix proteins, laminin, fibronectin or type IV collagen could enhance development of dopaminergic neurons *in vitro*, but no significant difference between the selected matrix proteins was observed.

2.6. Conclusion

I have demonstrated using a defined adherent culture system, that differentiation of neurons from mESCs was significantly enhanced by laminin to a greater extent than fibronectin or type IV collagen. Laminin stimulated neural progenitor generation, expansion and differentiation into neurons, but did not enhance neurite outgrowth of mESCs derived neurons. This finding is consistent with data showing that laminin is crucial for neurogenesis. Thus, the interaction between stem cells and matrix proteins appeared to be an early inductive signal to help regulate neural specification of mESCs. This finding may smooth the progress of early mouse CNS development which could be replicated for human CNS development for potential application in the management of neurological diseases.

Chapter 3 Immobilization of growth factors onto surfaces coated with extracellular matrix components

3.1. Introduction

3.1.1. The transcription factor, *Lmx1a*

Transcriptional factors directing dopaminergic neurons maturation are not yet completely understood but several key molecular events have been identified in developing mouse midbrain. One of critical importance is the expression of the LIM homeobox transcription factor (*Lmx1a*), which is essential for development of dopaminergic neurons (Cai *et al.*, 2009). In the past few years it has been shown that by combining expression of *Lmx1a* and forkhead box protein A2 (*Foxa2*), dopaminergic neurons could be differentiated from human fibroblasts (Andersson *et al.*, 2006; Pfisterer *et al.*, 2011). *Lmx1a* targeted by siRNA in the chick embryo inhibited the formation of mature dopaminergic neurons (Millonig *et al.*, 2000). Friling et al. (2009) also showed that increased expression of *Lmx1a* in mESCs provided a robust strategy for efficient production and enrichment of dopaminergic neurons (Friling *et al.*, 2009).

Lmx1a was discovered in the *Dreher* (*dr*) mouse, mutant with many developmental defects, including cerebellum and dorsal spinal cord (Millonig *et al.*, 2000). Positional cloning of the gene responsible for the *dr* phenotype identified a 1.8kb cDNA clone located on chromosome 1, which shared significant identity throughout the coding region with the hamster Lmx1.1 (94% nucleotide identity, 98% amino-acid identity). This gene was termed *Lmx1a*, the mouse homolog of the hamster Lmx1.1 (Millonig *et al.*, 2000). Follow-up studies showed that *Lmx1a* is widely expressed in the brain (Failli *et al.*, 2002).

3.1.2. *Lmx1a* is sufficient and required for midbrain dopaminergic neurons (mDA) development *in vivo*

Over the past few years, there has been an explosion of research focusing on the development of strategies to steer embryonic stem cells towards desired fates, with the hope for an embryonic stem cells based replacement therapy for diseases such as Parkinson disease (Barberi *et al.*, 2003; Kawasaki *et al.*, 2002). One potential advantage of using embryonic stem cells derived midbrain dopaminergic neurons is the unlimited supply of cells for transplantation, but the drawbacks with current differentiation techniques are the efficiency and purity (Prowse *et al.*, 2011).

Lmx1a is expressed both in neural progenitors and in post mitotic midbrain dopaminergic neurons. However, the generation of ectopic midbrain dopaminergic neurons is known to be limited to ventrolateral regions with the greatest frequency occurring ventrally. It was shown by Lin et al. (2009) that *Lmx1a* alone is not sufficient to induce ectopic dopaminergic neuron development in the lateral region of ventral midbrain thus indicating that other factors are required to act in parallel to *Lmx1a*. One such candidates is *Foxa1*/2, which cooperates with *Lmx1a* through a feed forward loop during the induction of midbrain dopaminergic neurons (Lin *et al.*, 2009).

By contrast, in dorsal regions, *Lmx1a* participates in a different developmental program regulated by bone morphogenetic proteins (BMPs) to specify a functional roof plate (Chizhikov *et al.*, 2004). Therefore, the activity of *Lmx1a* is context dependent. In addition, a mixture of cell types, including serotonin (5-HT) neurons and γ-aminobutyric acid (GABA) neurons is often present in the differentiating embryonic stem cell culture (Reubinoff *et al.*, 2001). It has been reported that serotonin neurons

mediate dyskinetic side effects in Parkinson's patients with neural transplants (Politis *et al.*, 2010). By contrast, GABAergic neurons send out long projections to their normal targets and effect behavioural improvement (Thompson *et al.*, 2008). Previous stem cell differentiation protocols have relied on exogenous growth factors, such as Shh, FGF8 and Wnts. Numerous studies published from year 2000 to 2005 showed that combination of Shh, FGF8 and FGF2 gave a high percentage of dopaminergic neurons (Barberi *et al.*, 2003; Buytaert-Hoefen *et al.*, 2004; Kim *et al.*, 2002). Thus, based on the above studies it was decided to evaluate whether, *Lmx1a* could be induced by growth factors such as Shh, FGF8b and FGF2 and matrix proteins.

3.1.3. Chapter aims

Strong binding to heparan is a characteristic feature of the fibroblast growth factors. Complex formation protects fibroblast growth factors from heat and acid inactivation and can also act as bioactive reservoirs for signaling factors (Gospodarowicz *et al.*, 1986; Vazin *et al.*, 2010; Vlodavsky *et al.*, 1990). Heparan sulphate one of glycosaminoglycans occur naturally and can bind ionically to the amine groups on growth factors via its sulphate and carboxylate side chains (Ashikari-Hada *et al.*, 2004). Heparan-containing delivery systems have been used to immobilize high affinity heparan binding growth factors to protect them from degradation (Capila *et al.*, 2002; Edelman *et al.*, 1992; Hou *et al.*, 2010; Kreuger *et al.*, 2006; Sakiyama-Elbert *et al.*, 2000b; Sakiyama-Elbert *et al.*, 2000c; Skop *et al.*, 2013; Vazin *et al.*, 2010).

Thus, working on these principles I tried to explore whether heparan sulphate could immobilize growth factors like FGF and Shh onto surfaces coated with laminin, fibronectin and type IV collagen. Thus, I wanted to investigate

- Immobilized growth factors were evaluated for physical stability and availability by in-cell Western assay. The effectiveness of modified substrata was investigated by determining the proportion of *Lmx1a+* cells by flow cytometry at various stages during *in vitro* differentiation.

- Effects of individual matrix proteins on the proportion of *Lmx1a+* expressing cells.

3.2. Materials and Methods

The experimental procedures used in this chapter have already been explained in Chapter 2, sections 2.2.1. - 2.2.7. unless detailed below.

3.2.1. Substrata preparation

Sterile round glass cover slips were placed into a 48 well plate. A mixture of laminin (Invitrogen, Australia), fibronectin (Invitrogen, Australia), type IV collagen (Invitrogen, Australia) and heparan sulphate (Sigma-Aldrich, Australia) in ratio of 1:1:1:0.1 along with 100 ng Shh (Sigma-Aldrich, Australia), 50 ng FGF8b (R&D Systems, USA) and 10 ng FGF2 (R&D Systems, USA) were incubated at 37^{o}C for 3 hours. After 3 hours incubation, remaining mixture was aspirated carefully. The rationale behind this strategy was to generate a basement membrane-like substrata, comprises of laminin, fibronectin, type IV collagen and heparan sulphate (Laurie *et al.*, 1982), to which addition of biologically relevant growth factors, might result in an improved microenvironment to support neural differentiation. It has been reported that a mixture of laminin, type IV collagen and heparan sulphate, incubated at 35^{o}C in a ratio of 1:1:0.1, generates a basement membrane like substrata (Grant *et al.*, 1989). To this we decided to add fibronectin on its reported role in differentiation of embryonic stem cells into the glia cells and neurons (Liu *et al.*, 2010).

3.2.2. In-cell Western assay

Every experiment was performed in quadruplicate, including negative controls which were not exposed to primary antibodies. Growth factors immobilized onto matrix were fixed by incubation with 4% paraformaldehyde in 1X PBS for 30 minutes at room temperature. For permeabilization, 200 µl of 0.1% TritonX-100 (Sigma-Aldrich, Australia) in 1X PBS was added to each well for 30 minutes. Surface coated wells were blocked with 200 µl of blocking buffer (Li-cor Biotechnology, USA) for 90 minutes at room temperature with gentle shaking. Mouse anti-FGF2 (Sigma-Aldrich, Australia), goat anti-FGF8b (R&D Systems, USA) and goat anti-Shh (R&D Systems, USA) were diluted 1:100, 1: 400 and 1: 200 respectively in blocking buffer and incubated overnight at 4^{o}C in a volume of 100 µl per well.

Unbound primary antibody was removed by washing wells (5 times) with 200 µl 0.1% Tween20 (Sigma-Aldrich, Australia) in 1X PBS. Wells were then exposed to secondary antibody; IRDye 800CW donkey anti-mouse and anti-goat (Li-cor Biotechnology, USA) were diluted 1:1000 in blocking buffer for two hours at room temperature. Unbound secondary antibodies were removed by washing each well (5 minutes) with 0.1% Tween20 (Sigma-Aldrich, Australia) in 1X PBS (by gentle shaking 5 times). Each individual well of 48 well plate coated with and without growth factors were scanned for emission at 800 nm with the Odyssey Infrared Imaging system (ODY-18090, Li-cor Biotechnology, USA).

Values from the 800 nm channel corresponded to the amount of primary antibody bound to the wells. Control wells not exposed to primary antibodies were used for background compensation. The obtained values at 800 nm were averaged and subtracted from the respective values of wells incubated with primary antibodies and normalization dye. Graphs were plotted using the integrated intensity measurements for each well.

3.2.3. *Lmx1a*-β-lactamase reporter mESCs

An *Lmx1a*-AMP-IRES-eGFP-FneoF targeting vector was constructed for homologous recombination, designed such that, the AMP-IRES-eGFP-FneoF construct replaced exon 1 of the *Lmx1a* gene. This targeting vector was introduced into mouse E14Tg2a ESCs by electroporation with a GenePulser XCellTM instrument (BioRad, USA) and cells targeted at one allele were isolated by Dr Stewart Fabb (Monash Institute of Pharmaceutical Sciences).

3.2.4. Effect of combined matrix proteins sequestered with growth factors on differentiation of *Lmx1a* reporter cells

The mESCs reporter cell line *Lmx1a*-eGFP was differentiated under three different conditions and *Lmx1a* reporter gene expression was quantified on days 8, 12, 16 and 20 of differentiation.

Condition 1: Cells were plated onto combined matrix proteins in N2B27 medium supplemented with 500 ng/ml Shh, 10 ng/ml recombinant FGF2 and 100 ng/ml FGF8b for a total of 6 days with medium replenished on day 3.

Condition 2: 100 ng/well Shh, 10 ng/well FGF2 and 50 ng/well FGF8b were pre-immobilized onto the same combination of matrix proteins prior to plating. Cells were incubated for 6 days in N2B27 medium, which was replenished without growth factors on day 3 of differentiation.

Condition 3: 10 ng/well FGF2 and 50 ng/well FGF8b were pre-immobilized onto the combination of matrix proteins prior to plating. Cells were incubated for 6 days with medium replenished without growth factors on day 3.

Cells were terminally differentiated after incubation by each of the above three conditions by exposing them to N2B27 medium supplemented with 10 ng/ml glial-derived neurotrophic factor (GDNF) (R&D Systems, USA) and 200 μM ascorbic acid (Sigma-Aldrich, Australia) from day 6 onwards for final differentiation. During the terminal differentiation the medium was changed every other day until day 21.

3.2.5. Influence of individual matrix proteins on *Lmx1a* reporter cells

The embryonic stem cell reporter cell line was seeded at $5x10^3$ cells/cm^2 onto surfaces coated with 0.1 % v/v gelatin (Sigma-Aldrich, Australia), 0.1 mg/ml poly-D-lysine (PDL, Sigma-Aldrich, Australia), laminin (5 μg/cm^2, Invitrogen, Australia), fibronectin (5 μg/cm^2, BD Bioscience, Australia) or type IV collagen (1 μg/cm^2, BD Bioscience, Australia) in N2B27 medium without any growth factors. *Lmx1a* promoter-driven expression was quantified by flow cytometry (FACS Canto II) on days 3, 5, 7 and 9 of neural induction to investigate the effect of individual matrix proteins.

3.2.6. β-lactamase staining of live cells

For quantitation using flow cytometry, the LiveBLAzer™ FRET – B/G Loading Kit (Invitrogen, Australia) was used to produce fluorescence in AMP^+ cells. Cells were trypsinized and pelleted. Approximately 0.5 million cells were resuspended in 0.5 ml of 1X PBS and 100 µl of 6X Live Blazer substrate loading solution. β-lactamase activity was detected by incubating the cell suspension with CCF2-AM in dark conditions for two hours. The cells were exposed to light at 406 nm. The substrate emits around 530 nm (green) however, if beta-lactamase reporter gene expression is activated it leads to cleavage of the substrate, thereby disrupting the fluorescence resonance energy transfer (FRET) and resulting in emission at 460 nm (blue) following excitation at 406 nm. After incubation cells were pelleted, resuspended in 2 ml of 1X PBS, pelleted a second time, resuspended in 2 ml of 1X PBS and cleared of clumps with a 70 µm strainer (BD Biosciences, Australia). As a negative control, wild type mESCs were used having been submitted to treatment with the LiveBlazer substrate over the same time period as sample cells. Flow cytometry was conducted by setting gates such that 99.0-99.5% precent of wild type cells were registered as *Lmx1a*-AMP negative. For each analysis 10,000 events were counted.

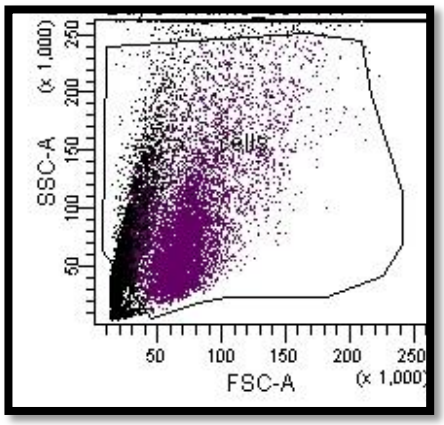
3.2.6.1 Gating procedure

1. Cells have traditionally been gated according to physical characteristics (Pimton *et al.*, 2011). For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter (FSC). Also, dead cells have lower forward scatter and higher side scatter (SSC) than living cells.

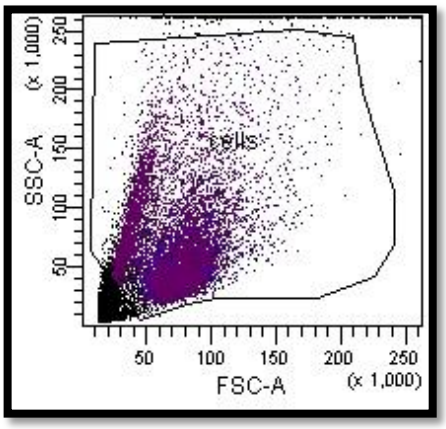
2. Cells were treated with SYTOX red dye. SYTOX red dead cell stain is a high-affinity nucleic acid stain that easily penetrates cells with compromised cell membranes but will not cross uncompromised cell membranes. The advantage of SYTOX red is that it's excited at 635 nm thus, leaving the 488 nm laser free.

3. On the density plot below of (A) wild type cells, (B) *Lmx1a* reporter cell line, each dot/point represents an individual cell that has passed through the instrument.

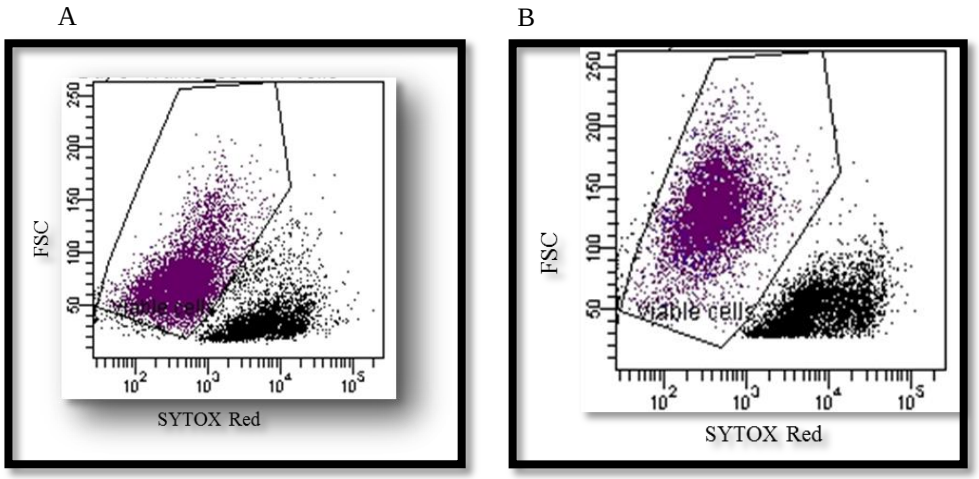
A



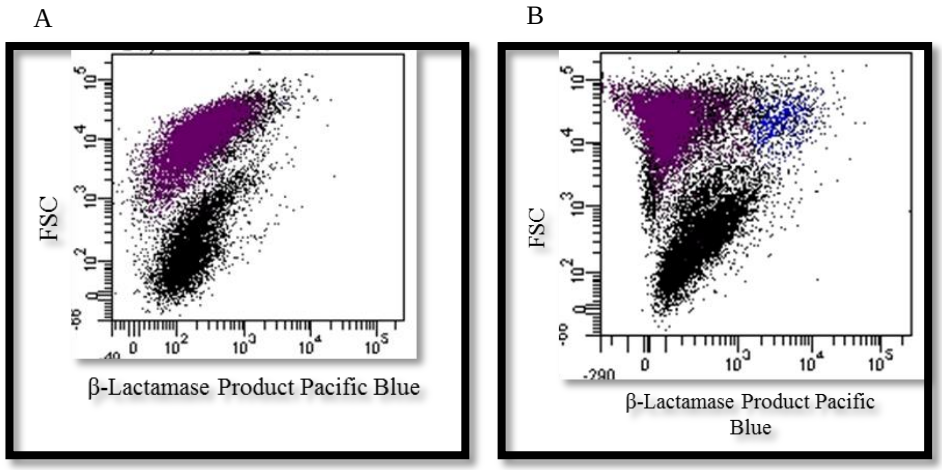
B



4. Density plot was plotted between FSC and SYTOX red to capture viable cells. Where cells in purple are viable and in black are dead cells. (A) Wild type cells, (B) *Lmx1a* reporter cell line.



5. Density plots of the above viable cells were done between FSC and β-lactamase product pacific blue. Pacific blue is the channel used for detecting *Lmx1a* + cells (Blue colour) and *Lmx1a* - cells (Purple colour). (A) Wild type cells, (B) *Lmx1a* reporter cell line.



6. Statistics view of (A) Wild type cells, (B) *Lmx1a* reporter cell line.

A

Population	#Events	%Parent
All Events	27,475	####
cells	10,000	36.4
Viable cells	6,017	60.2
Lmx1a+	12	0.2
NOT(Lmx1a+)	6,005	99.8

B

Population	#Events	%Parent
All Events	17,615	####
cells	10,000	56.8
Viable cells	5,580	55.8
Lmx1a+	421	7.5
NOT(Lmx1a+)	5,159	92.5

3.2.7. Statistical analysis

All results are presented as mean ± SEM of at least three separate experiments. Statistical analyses were performed on raw data using PRISM v5.00 (GraphPad Software, USA). Data were analysed by Student's *t* test or one-way analysis of variance or two-way analysis of variance (ANOVA) with post-hoc Bonferroni's test. In all cases, $P < 0.05$ was considered to be significant.

3.3. Results

3.3.1. Growth factor immobilization studies

With reference to previous literature, three different growth factors were selected for study based on their ability to promote embryonic stem cell differentiation and survival (Cohen *et al.*, 2010). The initial objective was to confirm whether the selected antibodies reacted with their expected targets. Figure 3.1 indicates that the selected antibodies for FGF8b and Shh were specific in nature and showed no cross reactivity whereas, FGF2 antibody (a polyclonal antibody) showed some cross reactivity with FGF8b and Shh.

The next challenge was to determine the concentration of growth factors to be selected. Coating experiments using solutions of growth factors in ascending concentrations were carried out. For FGF2 the concentrations were 10, 25 and 50 ng/well, for FGF8b 50, 100 and 200 ng/well and for Shh 50, 100 and 200 ng/well were used (Figure 3.2). The bar graphs (Figure 3.3) show concentration-dependent coating for FGF8b and Shh, but not FGF2. Therefore, it was decided to use the following concentrations of growth factors for further experiments FGF2 10 ng/well, FGF8b 50 ng/well and Shh 100 ng/well.

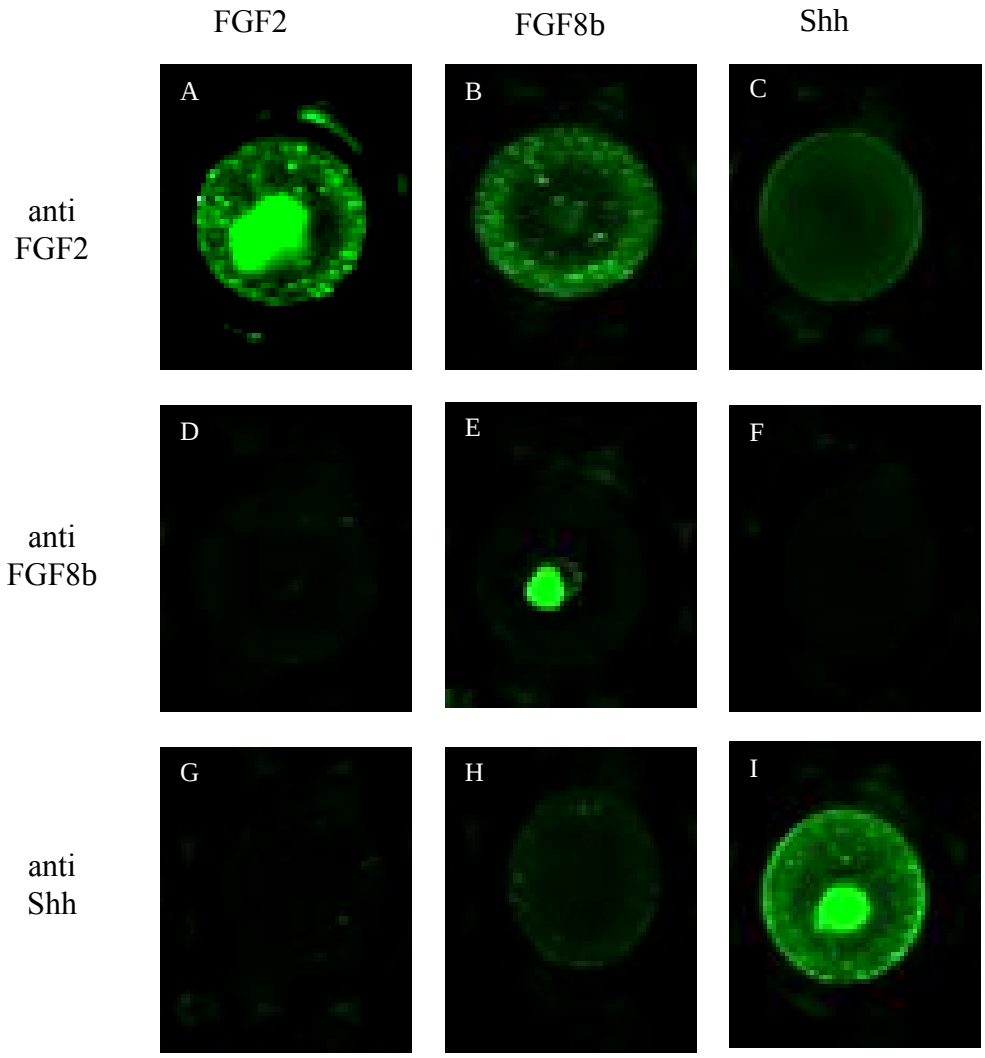


Figure 3.1: In-cell Western to evaluate cross reactivity of antibodies of selected growth factors. Immunoreactivity against (A-C) FGF2, (D-F) FGF8b and (G-I) Shh.

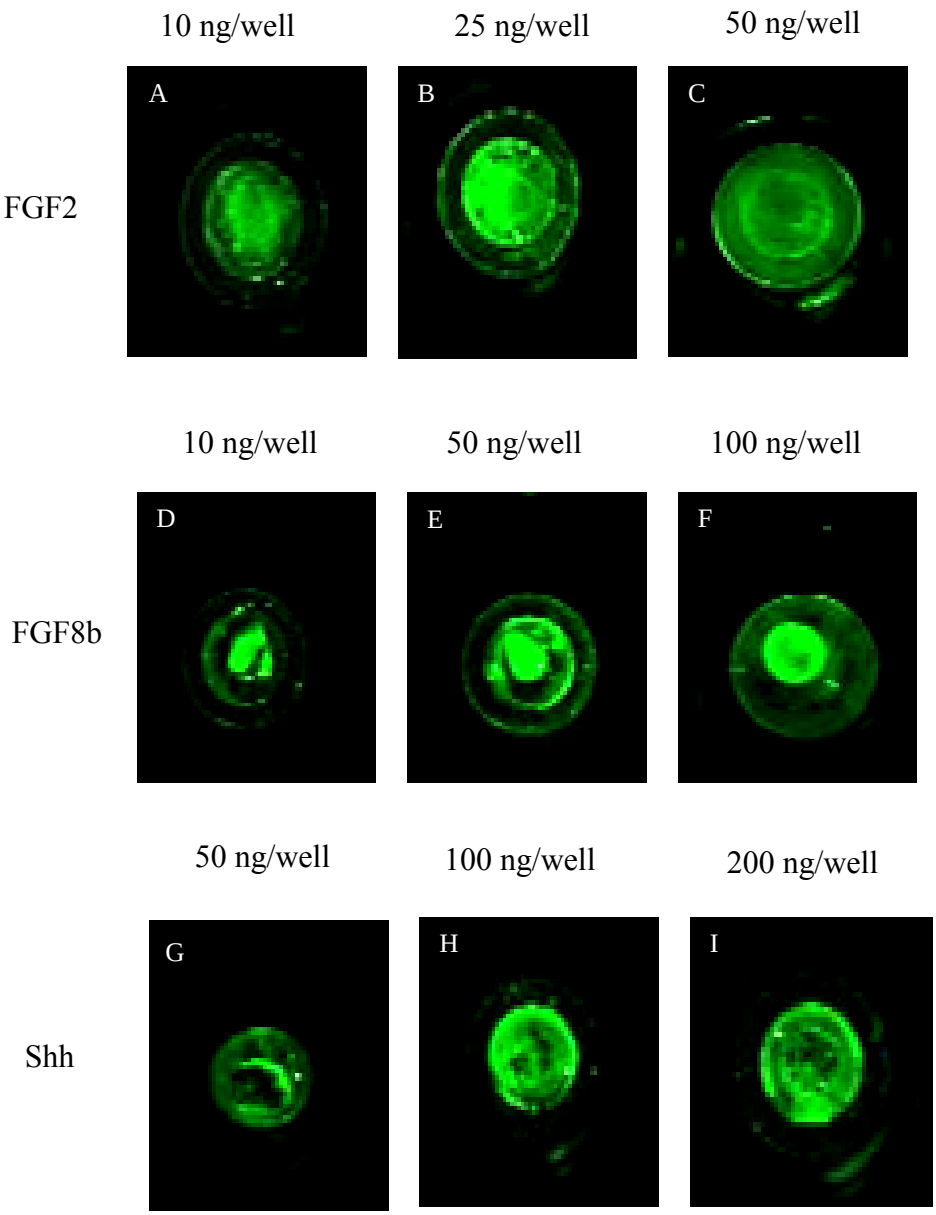


Figure 3.2: In-cell Western to evaluate immobilization of selected growth factors individually onto combinational matrix proteins. (A-C), FGF2, (D-F), FGF8b and (G-I), Shh.

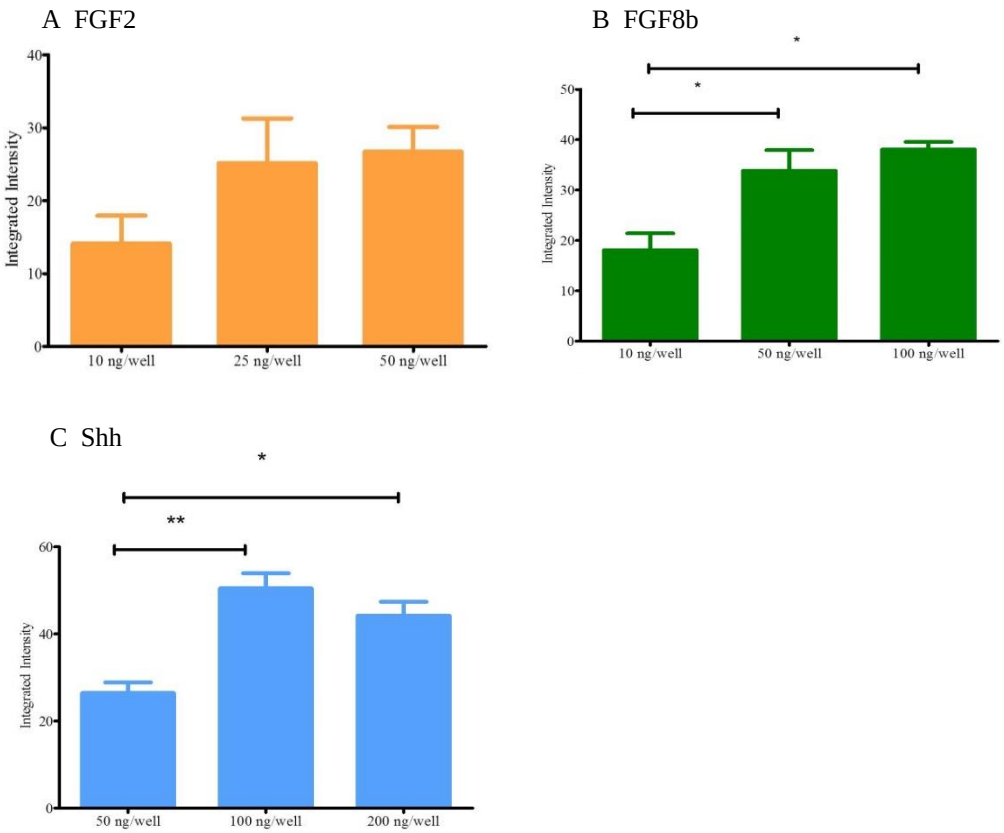


Figure 3.3: Bar plots summarizing integrated intensity quantified for growth factors after loading with solutions at different concentrations. Values are expressed as mean ± SEM of three independent experiments. Statistical differences for individual selected growth factors are significant * $P < 0.05$ and ** $P < 0.01$, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

3.3.2. Immobilization efficiency

The next challenge was to determine the increase or decrease in availability of growth factors after their immobilization as compared to traditional method of external addition (i.e. in the culture medium) during differentiation. FGF2 and FGF8b were added to the culture medium at 10 and 100 ng/well respectively. Bar graphs (Figure 3.4 G and H), show that the amount of growth factors available to cells at the surface of the substrate at the starting point of differentiation step was greater in case of pre-immobilized factors compared to external addition. In practice, this represented a reduction in the mass of FGF8b needed when pre-immobilization was used.

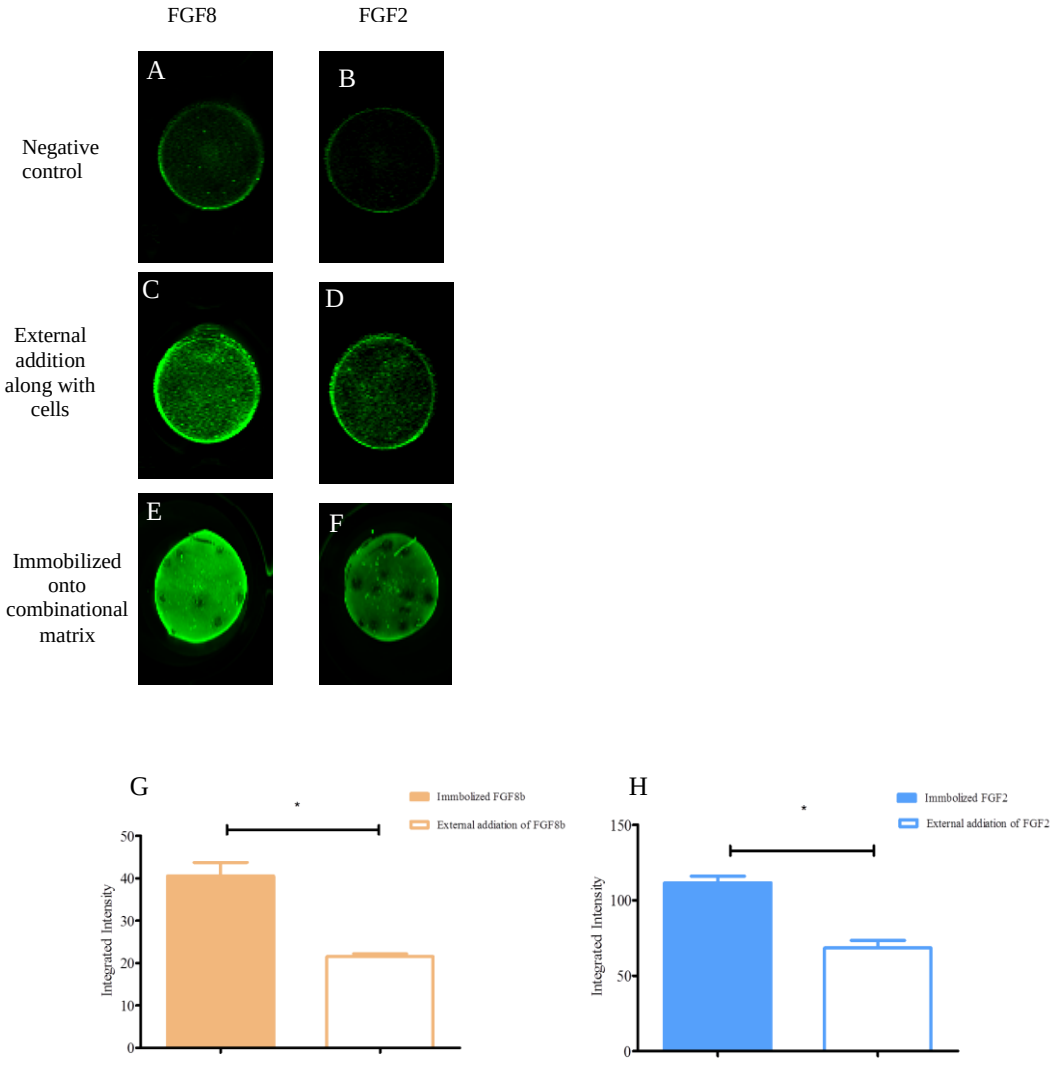


Figure 3.4: In-cell Western to evaluate immobilization efficiency. (A and B) negative control, (C) FGF8b (100 ng/well), (D) FGF2 (10 ng/well) incubated with mESCs respectively, (E) FGF8b (50 ng/well) and (F) FGF2 immobilized (10 ng/well) respectively onto combination of selected matrix proteins. Bar plots (G and H) summarizing integrated intensity measured at 800 nm. Values are expressed as mean ± SEM of three independent experiments. Statistical differences for immobilization and direct incubation was analysed by student *t*-test unpaired, * $P < 0.05$.

3.3.3. Determination of physical stability of surfaces with immobilized FGF2 and FGF8b

The stability of the adhesion of FGF2 and FGF8b to the matrix proteins was determined, after their immobilization onto the mixture of matrix proteins, by analysing the amount bound to substrata over time. The assay was carried out on day 0, 1, 4 and 6 after immobilization onto matrix proteins. Modified substrata wells were exposed to N2B27 medium, which was replenished every day with fresh medium, and the assay was carried out as described in section 3.2.2. From Figure 3.5 it can be postulated that after immobilization, growth factors were stable for six days on modified substrata. Given, that in a standard protocol the external addition of growth factors is for six days, any cellular response which we observed due to immobilized factors can be compared to standard protocol of addition of growth factors.

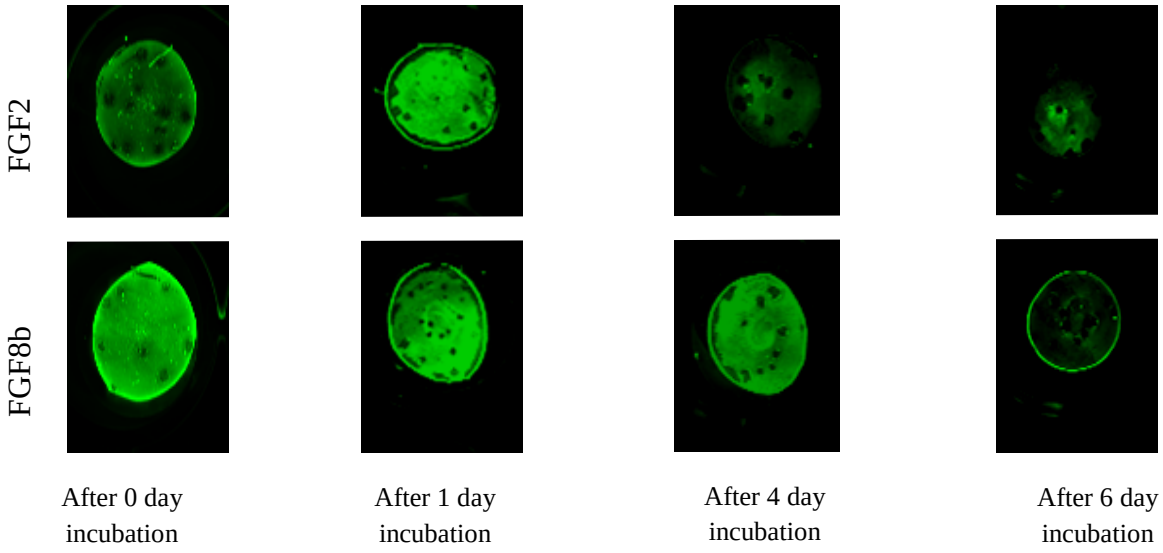


Figure 3.5: In-cell Western was performed on Day 0, 1, 4 and 6 to determine stability profile of selected growth factors immobilized onto combinational matrix proteins.

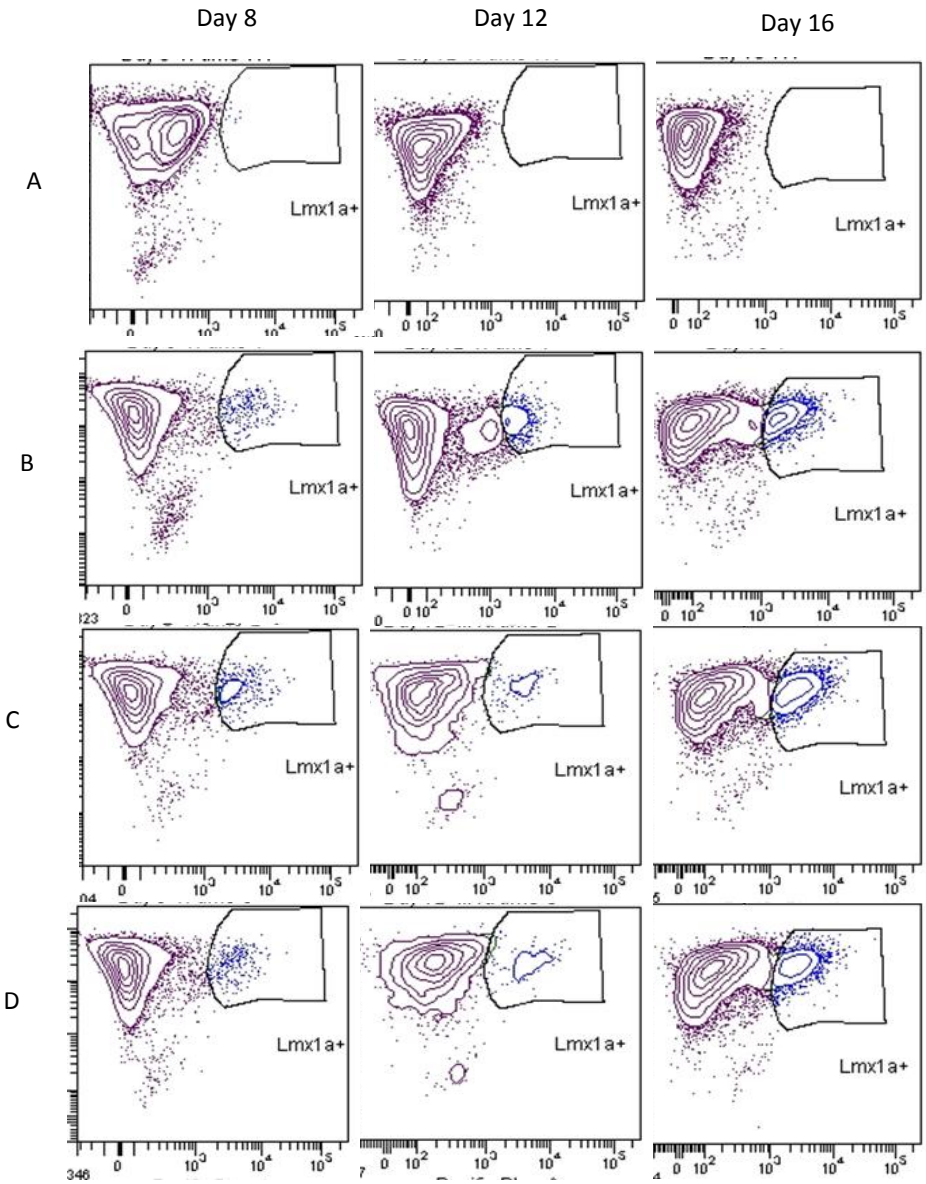
3.3.4. Flow cytometry analysis of reporter gene expression during differentiation on combinations of matrix proteins sequestered with growth factors

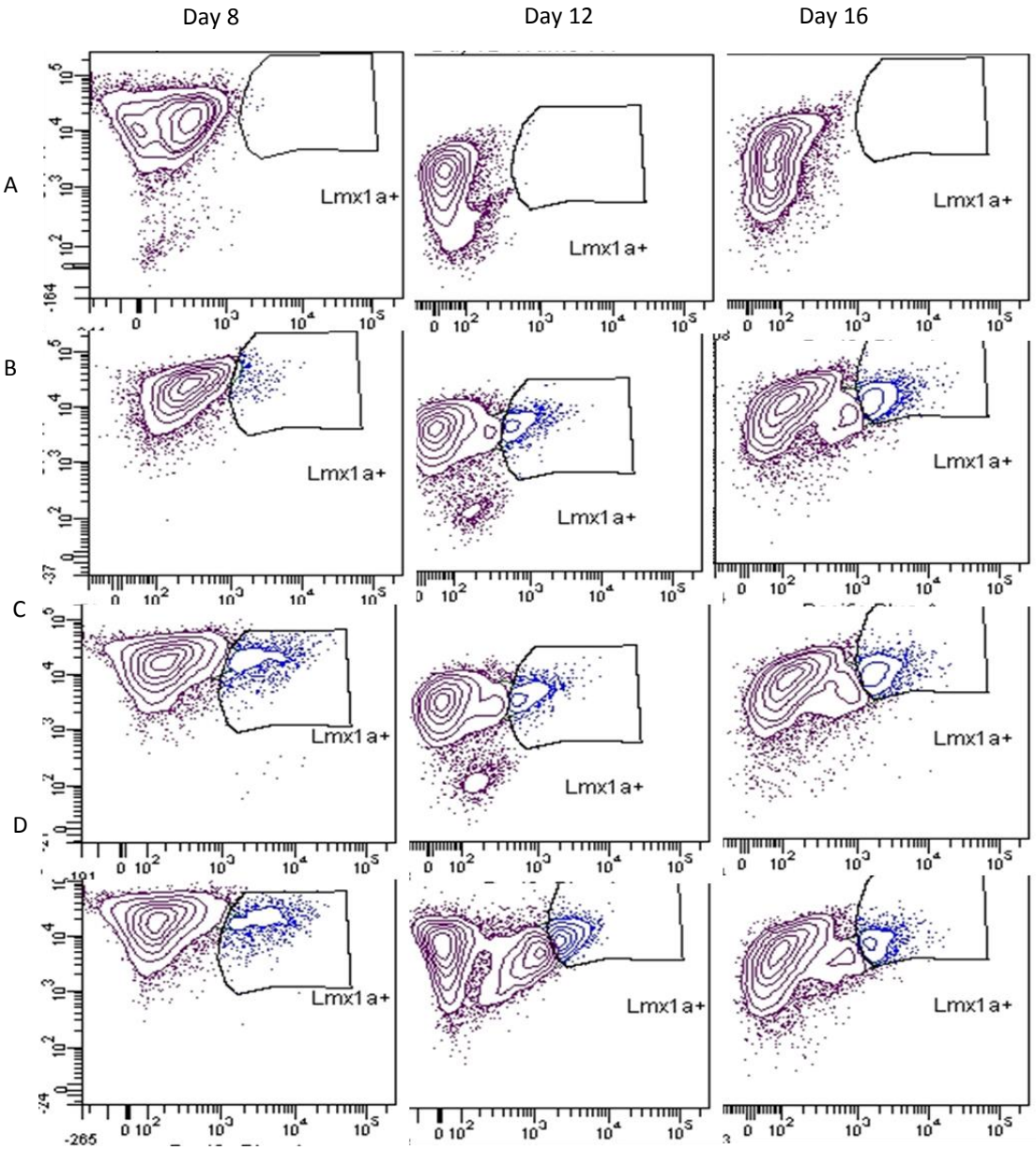
The effect of immobilized growth factors on *Lmx1a*+ populations up until day 20 of differentiation was examined. Results are shown in Figure 3.6 and 3.7. Figure 3.6 A-D shows representative FACS plots and Figure 3.7 shows quantitative data. The data indicate that compared to external addition of growth factors, immobilized factors did not result in any significantly different effects on *Lmx1a*+ cells quantified on day 8, 12, 16 and 20 of differentiation.

3.3.5. Quantification of tyrosine hydroxylase positive cells

Cells differentiated as per three conditions described in section 3.2.4. were probed with anti-tyrosine hydroxylase (catecholaminergic marker) and the number of tyrosine hydroxylase positive cells were manually counted on day 21 of differentiation (Figure 3.8 and 3.9). For these analyses, each captured image was identified as a sampling unit and data from four sites/well were imaged, in duplicate wells, from three separate experiments was used. By day 21 of differentiation, cells displayed reactivity to tyrosine hydroxylase (Figure 3.8) and number of tyrosine hydroxylase positive cells counted after external addition of growth factors like Shh, FGF8b and FGF2 was found to be 48.0/view. When the same set of growth factors were immobilized onto mixture of matrix proteins, number of tyrosine hydroxylase positive cells was 41.0/view and in the third condition tested where FGF8b and FGF2 were immobilized, tyrosine hydroxylase

positive cells found to be 50.0/view. We failed to observe any significant difference observed between the three groups (One way ANOVA).





β-Lactamase Product Pacific Blue

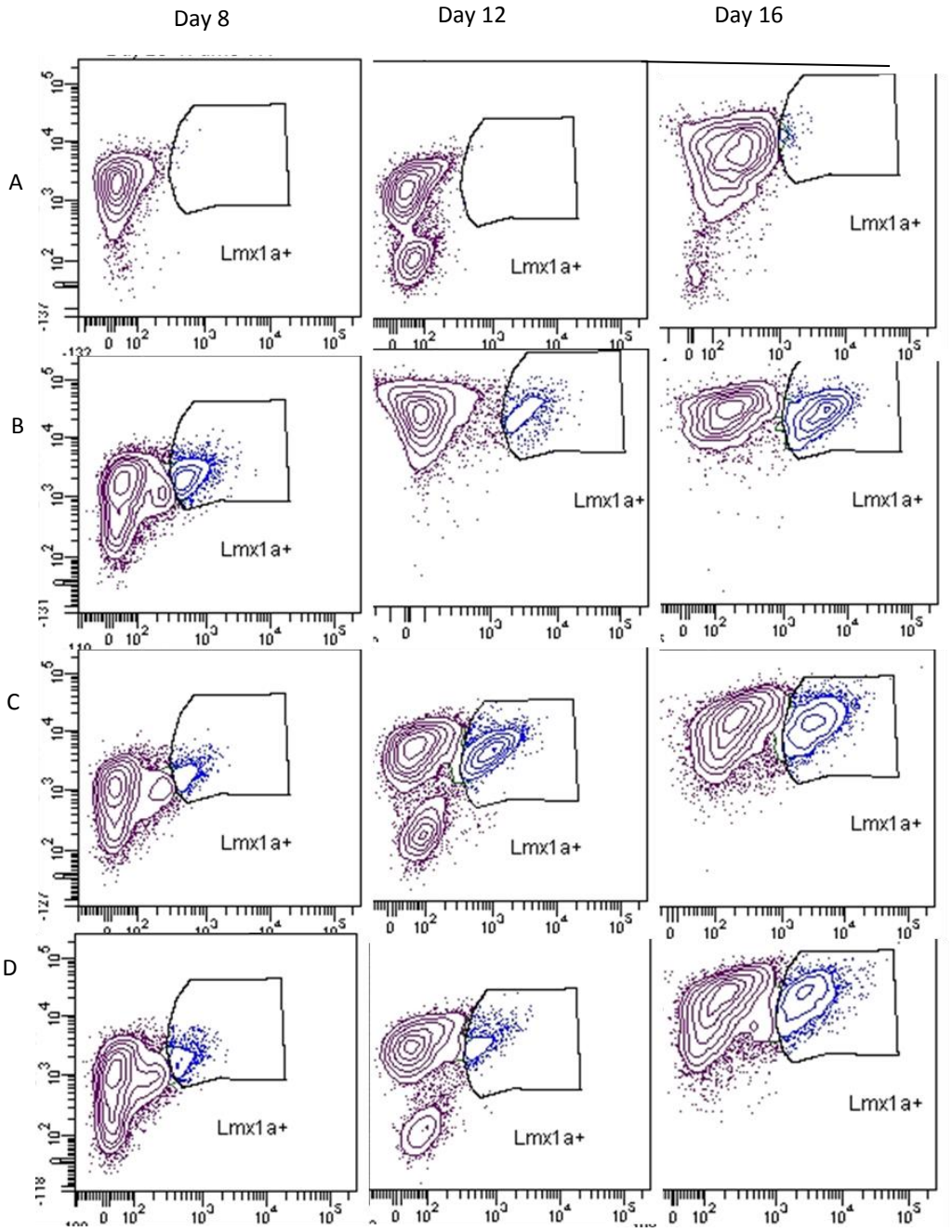


Figure 3.6: Representative FACS data showing *Lmx1a*+ population differentiated under three different conditions from day 8, 12 and 16. (A) Wild type cells (E14Tg2a), (B) *Lmx1a* knock-in mESCs differentiated with external addition of Shh, FGF8b and FGF2 for six days and (C) *Lmx1a* knock-in mESCs differentiated where Shh, FGF8b and FGF2 immobilized onto combinational matrix proteins and (D) *Lmx1a* knock-in mESCs differentiated where FGF8b and FGF2 immobilized onto combinational matrix proteins.

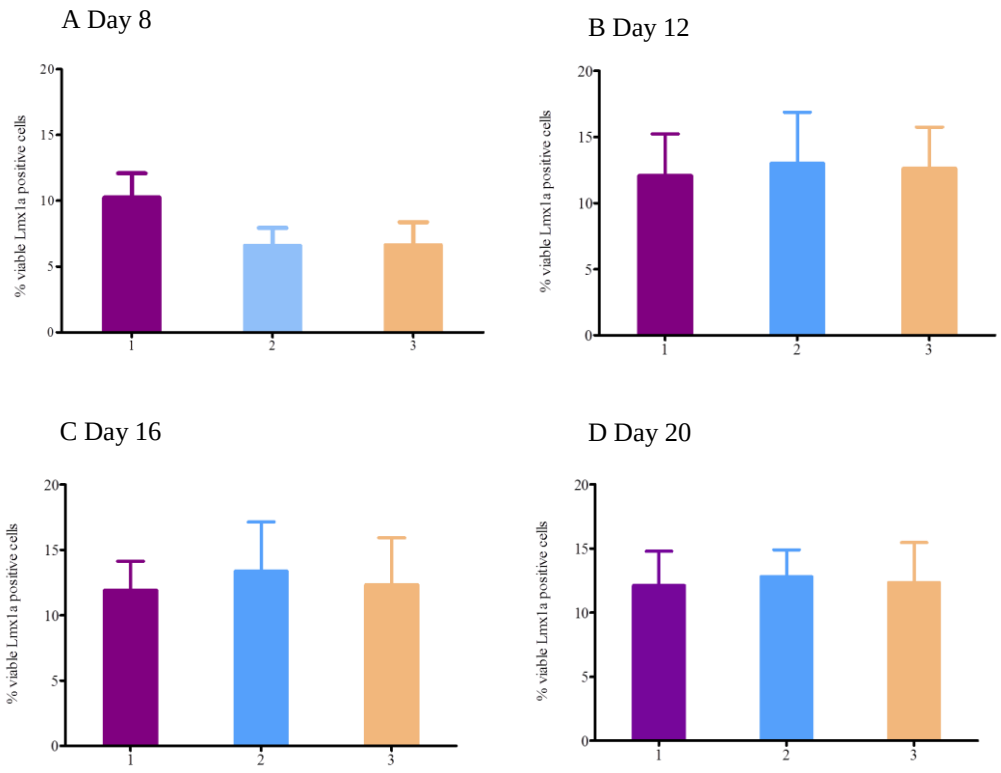


Figure 3.7: Bar plots summarizing the effect of three different conditions. (1) External addition of Shh, FGF8b and FGF2 for six days, (2) Shh, FGF8b and FGF2 immobilized onto combinational matrix proteins and (3) FGF8b and FGF2 immobilized onto combinational matrix proteins on percentage of *Lmx1a+* cells measured on day 8, 12, 16 and 20. Values are expressed as mean ± SEM of three independent experiments. No significant difference was observed between tested conditions $P < 0.05$, one-way ANOVA.

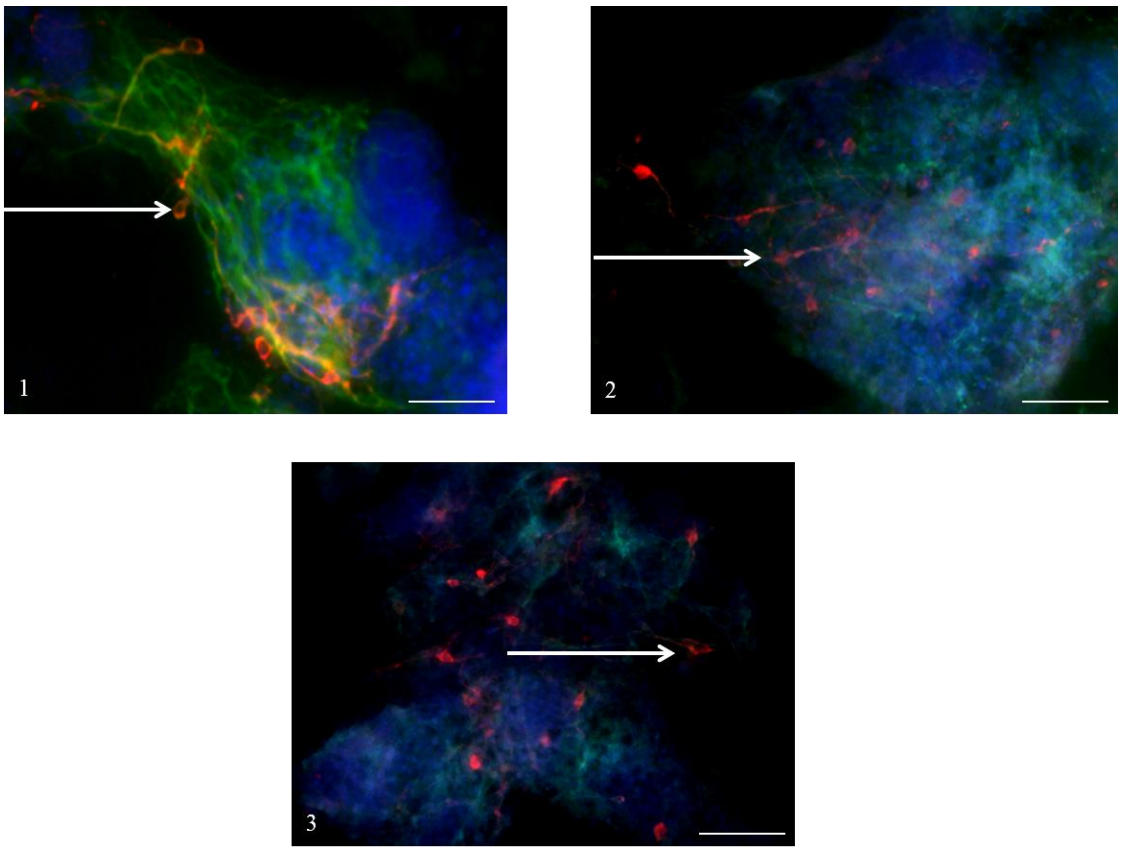


Figure 3.8: Immunofluorescence images showing representative fields of tyrosine hydroxylase (white arrow) positive cells (red), βIII tubulin positive cells (green) and nuclei stained with DAPI (blue) differentiated using one of three different conditions. (1) External addition of Shh, FGF8b and FGF2 for six days, (2) Shh, FGF8b and FGF2 immobilized onto combinational selected matrix proteins and (3) FGF8b and FGF2 immobilized onto combinational selected matrix proteins for 21 days. Scale bar is 50 µm

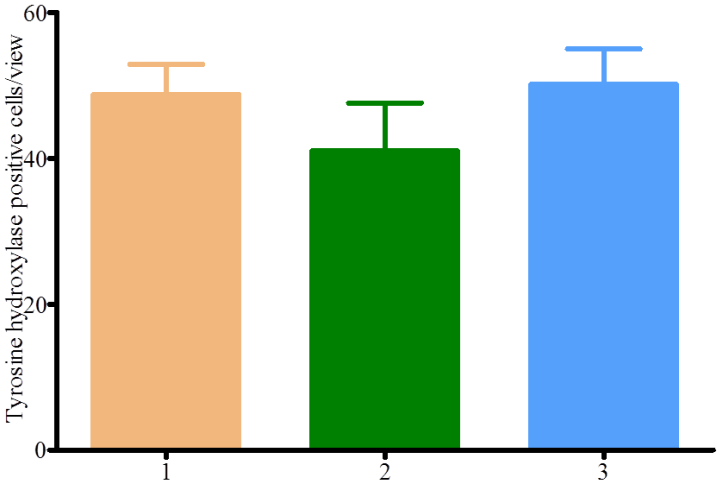


Figure 3.9: Bar plots summarizing the effect of three different conditions tested. (1) External addition of Shh, FGF8b and FGF2 for six days, (2) Shh, FGF8b and FGF2 immobilized onto combinational matrix proteins and (3) FGF8b and FGF2 immobilized onto combinational matrix proteins to quantify tyrosine hydroxylase positive cells. Values are expressed as mean ± SEM of three independent experiments. No significant difference between tested conditions $P < 0.05$, one-way ANOVA.

3.3.6. Influence of individual matrix proteins on *Lmx1a+* cells

The percentage of *Lmx1a+* cells was quantified on day 3, 5, 7 and 9 of neural induction on surfaces coated with gelatin (0.1% v/v), PDL (0.1 mg/ml), laminin (5 µg/cm^2), fibronectin (5 µg/cm^2) or type IV collagen (1 µg/cm^2). Figure 3.11 (A-D) shows that laminin enhanced the *Lmx1a+* population on day 5 when compared to fibronectin, type IV collagen or gelatin, but this effect was not observed on days 3, 7 or 9 of neural induction. Therefore, it can be inferred that the presence or absence of individual matrix proteins have no direct impact on the proportion of *Lmx1a+* cells; however it was observed that it is necessary to have a suitable substrate for stable cell attachment.

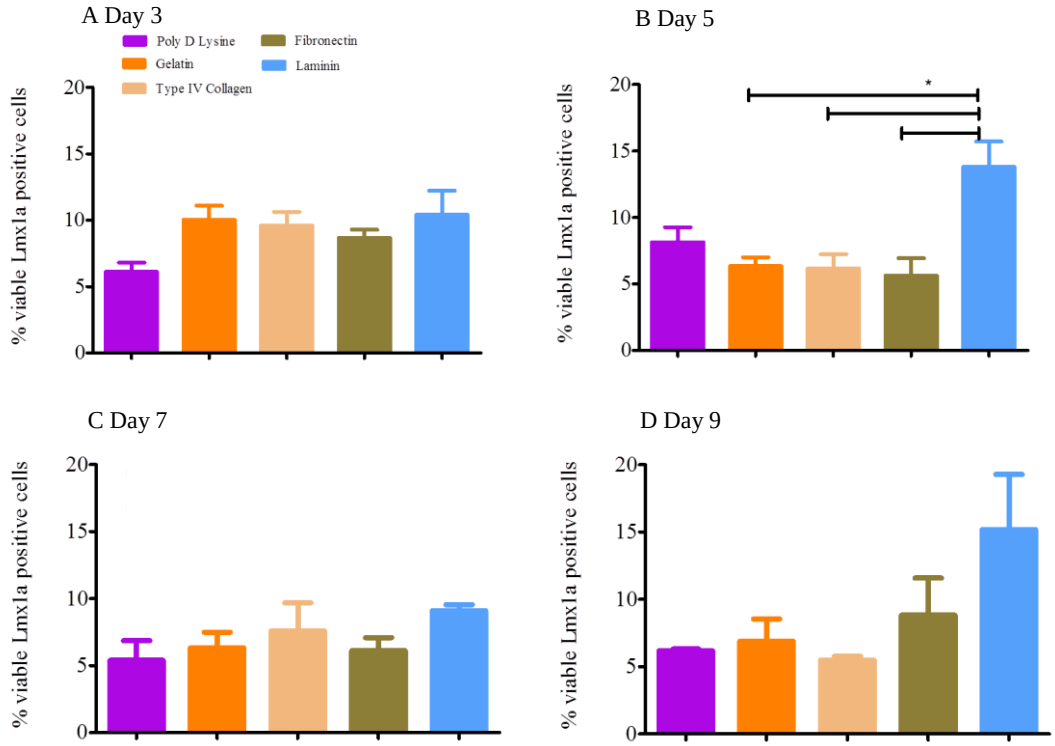


Figure 3.10: Bar plots summarizing the effect of selected matrix proteins on *Lmx1a*+ population. Values are expressed as mean ± SEM of three independent experiments. Statistical differences for % *Lmx1a*+ cells between laminin, fibronectin, type IV collagen and gelatin are significant * $P < 0.05$, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

3.4. Discussion

Growth factors are one of the critical factors regulating a wide variety of cellular functions, including proliferation, migration and differentiation (Masters, 2011). A significant issue with respect to availability of growth factors to cells during induction/differentiation stages is that growth factors are prone to rapid degradation in physiological conditions or inactivation due to enzymatic action (Masters, 2011). The current strategy of delivering growth factors to the site of action through conjugated /three dimensional biomaterial system requires expertise and significant funding (Masters, 2011).

An alternative strategy is to use either covalent or non-covalent immobilization methods to display growth factors on either chemically defined polymers or matrix proteins. These strategies can prolong growth factor availability and reduce the mass of growth factor required. Furthermore, this strategy has a major advantage which allows soluble and matrix bound factors to perform the natural functions they perform in the *in vivo* environment (Driessen *et al.*, 2003; Ferrara, 2010). Making use of this principle, the present study explored whether growth factors including FGF2, reported to have roles in renewal and proliferation (Levenstein *et al.*, 2006). FGF8b and Shh, which have been shown to induce differentiation of neurons towards midbrain dopaminergic phenotypes (Ye *et al.*, 1998), could be immobilized onto combinations of selected matrix proteins including laminin, fibronectin, type IV collagen and heparan sulphate.

It was decided to exploit the natural binding sites of matrix proteins facilitate binding of selected growth factors. For example, the α chains of laminin contain binding sites for heparan sulphate (Hozumi *et al.*, 2009; Kouzi-Koliakos *et al.*, 1989) and bind to type IV

collagen via the globular regions of either of its four arms (Charonis *et al.*, 1985; Rao *et al.*, 1985) (Figure 3.11). Fibronectin is reported to contain two heparan binding domains which are thought to interact with heparan sulphate proteoglycans (Mosher *et al.*, 1981). Instead of binding directly to the targeted growth factors onto the selected matrix proteins it was decided to indirectly bind them making use of their strong binding to heparan (Habuchi *et al.*, 1992; Roghani *et al.*, 1992; Turnbull *et al.*, 1992). The perceived advantage of binding growth factors in a deliberate manner is that it may retain the growth factors on the substratum, restricting loss of factors into the medium, thereby increasing their activity (Chang *et al.*, 2011; Flaumenhaft *et al.*, 1990; Foxall *et al.*, 1995; Nissen *et al.*, 1999).

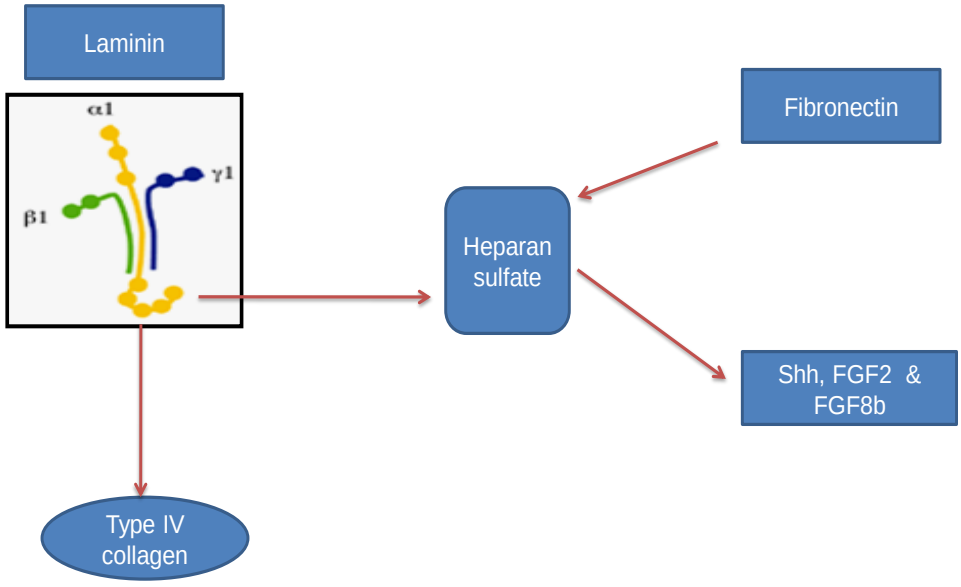


Figure 3.11: Schematic diagram of immobilization of growth factors (from Grant et al., 1989, Hozumi et al., 2009, Chang et al., 2011).

Grant et al. (1989) showed that when laminin, type IV collagen and heparan sulphate proteoglycan are incubated in ratio of 1:1:0.1, this combination yields basement membrane like structure (Grant *et al.*, 1989). Working on the above principles, it was possible to immobilize the three growth factors, Shh, FGF8b and FGF2. It was also possible to show that the surfaces were more immunoreactive to relevant antibodies when FGF8b and FGF2 were pre-immobilized onto the surface (Figure 3.5). This is consistent with previous research which showed that immobilization of nerve growth factors increases their availability (Kapur *et al.*, 2003).

Similarly, heparin coated alginate gels were used to deliver FGF2 (Tanihara *et al.*, 2001). Vascular endothelial growth factor (VEGF) and FGF2 have also been delivered by utilising either collagen gels or cross-linking poly (lactide-co-glycolide) (PLG) with heparin (Jeon *et al.*, 2006; Steffens *et al.*, 2004). Although this type of strategy has been used in the above mentioned papers, there are surprisingly few studies of this type, making use of intact heparin/heparan as an integral component of the growth factor delivery vehicle. The use of this strategy to enhance neural differentiation of embryonic stem cells has not been described previously.

After showing that growth factors could be immobilized onto matrix, the next objective was to explore whether this promising platform could be used to improve control of stem cell differentiation. There are some interesting reports with other systems that relate to the current study. *In vivo* LIF is present in both diffusible and matrix bound forms, and is known to activate pathways that support the self-renewal of mESCs (Rathjen *et al.*, 1990; Williams *et al.*, 1988). Interestingly immobilization of LIF has been used to improve embryonic stem cells propagation by Alberti et al. (2008). These authors were able to support pluripotency of mESCs for two weeks without extra addition of LIF (Alberti *et al.*, 2008). Differentiation has been studied using immobilized VEGF. This protein plays a critical role in regulating angiogenesis and endothelial cell function. It has also been postulated that signaling by VEGF is a rate limiting step in initiating angiogenesis (Ferrara *et al.*, 2003).

As the half-life of VEGF is very short and is capable of inducing undesirable side effects, such as supporting tumour growth, controlled delivery of VEGF has been investigated by immobilizing VEGF on modified collagens (Chiu *et al.*, 2010; He *et al.*, 2011). The presentation of VEGF in an immobilized state also has physiological importance, as living tissue contains both matrix-bound and soluble forms of VEGF. Moreover, immobilized VEGF has been found to successfully stimulate the proliferation of endothelial cells (Shen *et al.*, 2008; Zisch *et al.*, 2001). Rahman et al. (2010) also showed that by immobilizing VEGF into an agarose hydrogel they were able to induce differentiation of mESCs into blood progenitor cells. The technique was more effective than exposure of mESCs to soluble VEGF (Rahman *et al.*, 2010)

NGF which influences the proliferation, differentiation, survival and death of neuronal cells (Heese *et al.*, 2007) has been coupled onto different matrix proteins to examine whether its availability could be improved. β-NGF was successfully delivered by immobilizing onto a heparin-containing fibrin based system, and was able to enhance neurite extension of cells derived from dorsal root ganglia (Sakiyama-Elbert *et al.*, 2000a). A fibrin based delivery system was developed which was able to immobilize growth factors based on its ability to interact with heparin binding sites within the system. Its efficiency was evaluated by measuring delivery of NGF on peripheral nerve regeneration in a rat having sciatic nerve defect. The result showed that bound NGF was able to enhance peripheral nerve regeneration (Lee *et al.*, 2003).

Results of this study from section 3.3.4., 3.3.5. and 3.3.6. showed that individual matrix proteins tested were consistently better than controls but there was no statistically significant increase in expression of either *Lmx1a* or tyrosine hydroxylase when embryonic stem cells were differentiated on matrix proteins pre-incubated with FGF8, FGF2 and Shh. This was a surprising result in the light of the published studies described (Section 3.1.2.) showed importance of growth factors like Shh/FGF8 and *Lmx1a* gene plays a crucial role in generation of dopaminergic neurons. However, it can be argued that research groups have also shown that growth factors like Shh/FGF8 are not very effective at directly dopaminergic induction (Gaspard *et al.*, 2008; Nefzger *et al.*, 2012). Moreover, it has been shown *Lmx1a* can be expressed by forebrain cells (Nefzger *et al.*, 2012) and this might mask any induction of midbrain dopaminergic neurons. It has been reported that the yield of tyrosine hydroxylase positive neurons can be enhanced by increasing neural induction time of embryonic stem cell-derived neural progenitors with growth factors like FGF8b and Shh (Koch *et al.*, 2009b; Zeng *et al.*,

2011). These are the probable reasons why our strategy of studying the effect of immobilized growth factors failed to show any positive effects. When the individual matrix proteins were evaluated against one another, to investigate which was the best substrate for supporting neural development, only laminin showed a significant effect on the proportion of cells that were *Lmx1a+* population. This difference was significant only on day 5 (Figure 3.10 B). The observed advantage of using laminin is in line with the reported findings that laminin is known to assist and guide process outgrowth of various subtypes of neurons during early development stage as compared to other selected matrix proteins including fibronectin and type IV collagen (Eccleston *et al.*, 1985; Eldridge *et al.*, 1989; Hantaz-Ambroise *et al.*, 1987).

3.5. Conclusion

The present study has shown that stable and bioactive concentrations of FGF2, FGF8b and Shh can be immobilized onto combinations of matrix proteins. Though there is gradual dissociation and loss of the growth factors over a 5-7 day period, pre-immobilization might be expected to be a more efficient means of presenting growth factors to the embryonic stem cells. By utilising this strategy we were able to reduce the mass required to half in case of FGF8b. The methodology of immobilization described above is quite simple, and it has the potential to be used for many other growth factors type to test their stepwise control of signaling during distinct stages of stem cell neural fate conversion.

Chapter 4 Identifying the signaling pathways through which mESCs attach and differentiate on matrix proteins found in basal lamina

4.1. Introduction

4.1.1. Extracellular matrix and integrins

In the past few years it has become clear that extracellular matrix is an extremely dynamic structure that it is an essential regulator of embryonic biological functions (Bosman *et al.*, 2003). Earlier, extracellular matrix was viewed as passive and inert scaffold which acted as a stabilising factor. Cell adhesion to matrix proteins is mainly mediated by heterodimeric transmembrane receptors which belong to the integrin family consisting α and β subunits (Hynes, 2002). Each subunit has a large extracellular domain, a transmembrane domain and generally short cytoplasmic domains (Burridge *et al.*, 1988; Geiger *et al.*, 2001). At least 16 different α and 8 different β subunits have been identified, forming more than 20 integrins (Geiger *et al.*, 1992; Powell *et al.*, 1997; Rosales *et al.*, 1992). The extracellular domain binds extracellular matrix proteins with a specificity that is determined by the pairing of the different α and β subunits. Some integrins, such as α5β1, the "classic" fibronectin receptor, interact only with a single extracellular matrix protein; however, more commonly, a given integrin like $\alpha_V\beta_3$ will recognize several extracellular matrix proteins including thrombospondin, laminin and fibrinogen (Bosman *et al.*, 2003; Hynes, 2002; Vinogradova *et al.*, 2000).

4.1.2. The role of extracellular matrix in cell survival

Numerous factors, including the enzyme cytochrome P450 2E1 (i.e., CYP2E1), small proteins (i.e., cytokines) involved in cell communication and oxidative stress, can trigger apoptosis. A particular form of apoptosis, associated with response to inappropriate cell/extracellular matrix interactions, is termed 'anoikis'. Anoikis is a Greek word meaning "homelessness" (Douma *et al.*, 2004; Frisch *et al.*, 2001). Cell anchorage is essential for the survival of most cell types (Thomas *et al.*, 1999) and it has been reported that proteins such as fibronectin or collagen suppresses apoptosis of mammary epithelial cells (Boudreau *et al.*, 1995), indicating that a given cell type may require specific extracellular matrix proteins to promote cell survival (di Summa *et al.*, 2012; Lam *et al.*, 2012; Wang *et al.*, 1999).

Integrins are known to keep cells alive via adhesion that involves activation, by tyrosine phosphorylation (Giancotti, 2000; Pullan *et al.*, 1996), of FAK (Kornberg *et al.*, 1992a; Kornberg *et al.*, 1992b). Phosphorylated FAK leads to activation of various downstream signaling molecules including PI3K, protein kinase B (PKB/Akt) and mitogen activated protein (MAP) kinases, also known as ERKs (Kornberg *et al.*, 1992b). These kinases have been implicated in integrin-mediated protection of cells against various apoptotic stimuli and in control of gene expression (Craddock *et al.*, 2001; Ivankovic-Dikic *et al.*, 2000; Parsons, 2003).

A schematic representation of this signaling pathway is also given here. When a cell first comes in contact with matrix proteins, integrins expressed at the cell surface bind with appropriate ligands in the extracellular matrix. This activation initiates the intracellular end of the β subunit of integrin to bind to Talin and α-actin. Cells connect to the extracellular matrix at focal adhesions, forming tight connections between the cell membrane and the extracellular matrix as well as the cell membrane and the cytoskeleton. The aggregations of focal adhesions lead to activation of downstream signaling molecules like FAK. The phosphorylated FAK in turn activates PI3K and MAPK-ERK kinases (discussed in detail below) which are known to be implicated in range of cellular functions including migration, cell proliferation, and survival.

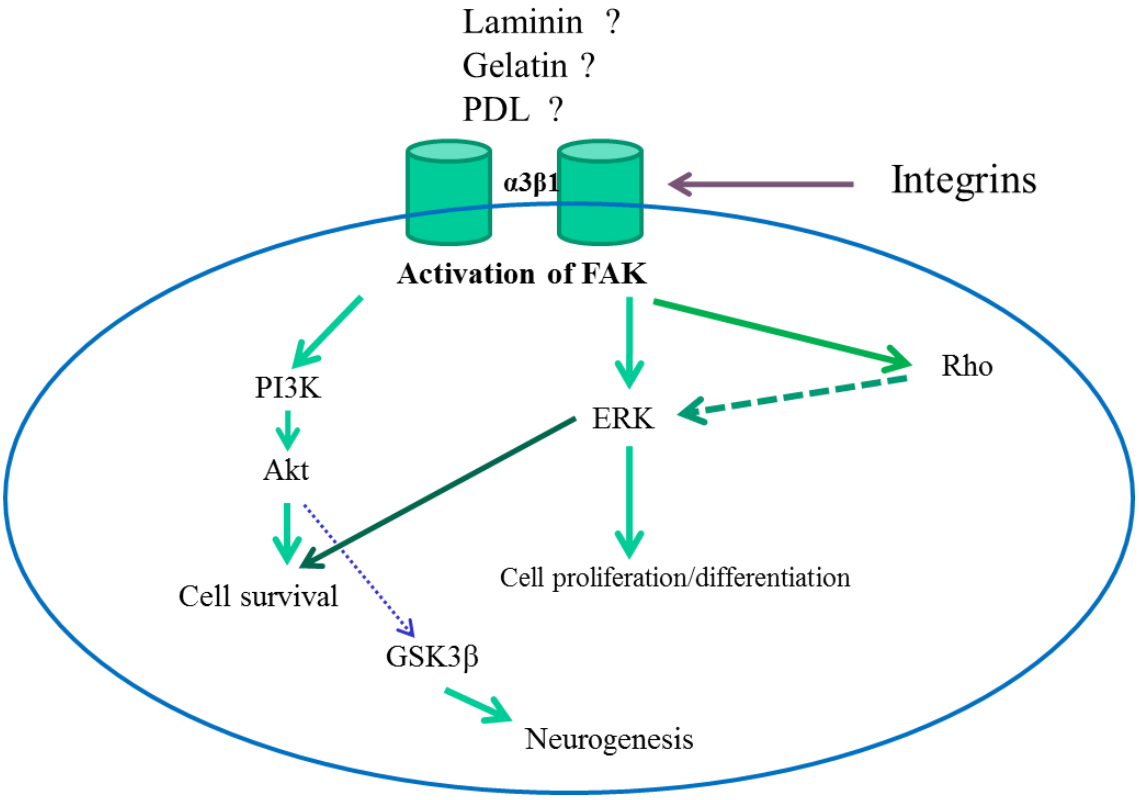


Figure 4.1: Schematic representation of signaling pathways that may be involved in cell survival, proliferation and differentiation of mESCs.

4.1.3. Focal adhesion kinase (FAK)

FAK is expressed in most tissues and cell types and is evolutionarily conserved in mammalian species as well as lower eukaryotic organisms (Henry *et al.*, 2001; Palmer *et al.*, 1999). FAK is a non-receptor tyrosine kinase, which consists of a central tyrosine kinase (catalytic) domain flanked by large N- and C-terminal non-catalytic domains (Girault *et al.*, 1999; Schaller *et al.*, 1995). The N-terminal region contains a FERM domain which mediates interactions of FAK with proteins associated with the plasma membrane. The C-terminal domain of FAK is designated as "FAT", for Focal Adhesion Terminal (Figure 4.2) (Martin *et al.*, 2002; Sieg *et al.*, 1999).

FAKs are expressed in different tissues and play a major role in focal adhesion (Hanks *et al.*, 1992; Hynes *et al.*, 1992). Initially, it was thought that only fibronectin was capable of phosphorylating the tyrosine residues (Hanks *et al.*, 1992) but subsequently it was observed that other matrix proteins such as laminin, type IV collagen and vitronectin also possess this ability (Guan *et al.*, 1992). Upon integrin ligation and clustering, FAK is phosphorylated at multiple sites within the kinase and the C-terminal domains (Calalb *et al.*, 1992; Schlaepfer *et al.*, 1994) (Figure 4.2). Tyr-397 is a major site of FAK auto-phosphorylation both *in vivo* and *in vitro* (Calalb *et al.*, 1996; Schaller *et al.*, 1994; Schlaepfer *et al.*, 1999).

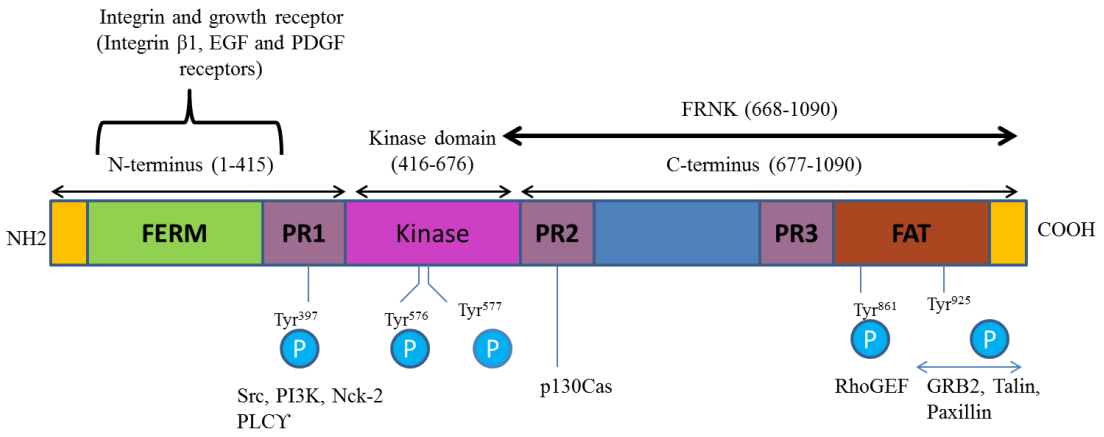


Figure 4.2: Linear structure of FAK showing its major domains, motifs and site of tyrosine phosphorylation (from Lal *et al.*, 2009).

The important tyrosine phosphorylation sites of FAK are shown above. The N-terminal domain has the Tyr-397 autophosphorylation site. Phosphorylated Tyr-397 is reported to interact with Src and PI3K proteins. The kinase domain has the Tyr-576/577 which plays a critical role in FAK activity. The C-terminal domain has two phosphorylation sites Tyr-861 and Tyr-925. Integrin association occurs through binding of paxillin and talin at the FAT region in C-terminal domain.

4.1.4. The role of FAK in spreading and survival of cells

FAK plays a crucial role in regulating cell adhesion, survival and proliferation (Ilic *et al.*, 1995a; Owens *et al.*, 1995). Fibroblasts derived from FAK knock-out mice migrate and spread slowly onto surfaces coated with different matrix proteins (Ilic *et al.*, 1995a). An opposite effect was observed when FAK was overexpressed in Chinese hamster ovary (CHO) cells (Cary *et al.*, 1996).

Reconstitution of FAK-deficient cells with wild type FAK restores cell migration (Sieg *et al.*, 1999). FAK-deficient cells exhibited a decrease in directional guidance as they did not respond to external forces whereas, FAK expressing cells responded by reorienting their movement and forming prominent focal adhesions (Wang *et al.*, 2001).

FAK plays a critical role in suppressing anoikis and apoptosis caused by diverse stimuli. It has been shown by mutating FAK Tyr-397 that phosphorylation of Tyr-397 plays a critical role in the cell survival function of FAK (Chan *et al.*, 1999; Frisch *et al.*, 1996; Sonoda *et al.*, 2000). Thus, it can be hypothesised that downstream signaling molecules such as PI3K, Akt and ERK might be involved in regulation of cell migration and survival.

4.1.5. Phosphatidylinositol 3-kinase (PI3K)

PI3Ks were first discovered over 25 years ago (Vanhaesebroeck *et al.*, 2005). They belong to a family of lipid kinases which are divided into three subgroups Class I, II and III based on their sequence homologies and substrate specificities (Arcaro *et al.*, 2000). Class I PI3K is the best characterized subgroup and is activated in response to growth factor and cytokine signaling. Class I PI3Ks comprise p110 catalytic subunit and a regulatory adapter subunit: p85. The p85 subunit includes an SH3 domain and two SH2 domains, which act as mediators between p85 and tyrosine phosphorylated sequences, such as those on phosphorylated FAK proteins (Cantley, 2002; Escobedo *et al.*, 1991).

Activation of a range of downstream signaling cascades are facilitated primarily by phosphoinositide products of PI3K activity, i.e. PI (3, 4, 5) P3 (phosphatidylinositol 3, 4, 5-trisphosphate). Overall, PI3Ks have been implicated in a wide array of physiological processes such as proliferation, development, growth and migration (Cantley, 2002).

4.1.6. Protein kinase B (PKB)

PKB commonly known as Akt, was identified in 1991 (Fayard *et al.*, 2005). It is thought to be a product of cellular homologue of v-Akt oncogene; so far three isoforms have been identified in mammalian cells: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Fayard *et al.*, 2005). Even though they are encoded by distinct genes present on different chromosomes, they share 80% of their amino acid sequence and similar domain structures (Kumar *et al.*, 2005). Akt isoforms are known to play major role in signaling effects like cell survival, metabolism and proliferation (Manning *et al.*, 2007; Yang *et al.*, 2003).

PI3K, a key regulator of fundamental cellular responses, promotes the phosphorylation of an array of protein kinases including Akt. Both PI3K and Akt are activated by numerous growth factors and insulin. Akt binds to second messenger of PI3K through its PH domain (Thomas *et al.*, 2002). The interaction between the two molecules does not directly activate Akt; instead it is believed to induce a conformation change in Akt which promotes phosphorylation at specific sites, particularly Threonine 308 and Serine 473, by phosphoinositide-dependent kinase 1 and 2 (PDK1 and PDK2), respectively (Hodgkinson *et al.*, 2002; Manning *et al.*, 2007).

4.1.7. MAP kinase extracellular-regulated kinases 1 and 2 (ERK1/2)

The MAP kinases comprise a large family of signal-regulated serine/threonine kinases. Three main MAP kinases cascades have been characterized: the ERK1/2 pathways, the Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) cascade and the p38 pathway (Pearson *et al.*, 2001). The MAP kinase ERK pathway can be activated by many different stimuli, including growth factors, cytokines and extracellular matrix. This pathway has been implicated in a wide range of cellular functions like migration, cell proliferation and survival (Peyssonnaux *et al.*, 2001; Roux *et al.*, 2004). In general, ERK activation is considered to be a pathway leading to increased cell survival and protection against several different pro-apoptotic stimuli, including death receptor-mediated activation and survival factor removal (Wada *et al.*, 2004). MEK1 deficient fibroblasts migrate more slowly than wild-type cells on a fibronectin substrate, but behave normally on a collagen substrate. These findings are consistent with a requirement for the ERK pathway for stimulation of migration by extracellular matrix and cell survival (Giroux *et al.*, 1999).

However, ERK1 disruption causes minimal phenotypic change, and has no effect on cell survival (Pagès *et al.*, 1999), but this may be explained by the ability of ERK2 to compensate for the loss of ERK1. However, ERK activity has also been reported to promote apoptosis in neuronal cells (Lu *et al.*, 2006). It has been proposed that the duration and/or amplitude of ERK signals may affect decisions related to cell fate (Lu *et al.*, 2006; Zugasti *et al.*, 2001). In addition, it has been reported that the protective effects of ERK2 against distinct apoptogenic stimuli are dependent on its cellular localization (Ajenjo *et al.*, 2004).

4.1.8. Glycogen synthase kinase 3 (GSK3)

GSK3, was identified in 1980 by Embi et al. (Embi *et al.*, 1980). GSK3s are serine/threonine kinases that were initially identified as key regulatory enzymes in glucose metabolism (Woodgett *et al.*, 1984). GSK3α and GSK3β are two known isoforms, encoded by separate genes but they share 95% homology in the kinase domain (Cohen *et al.*, 2004; Wada, 2009). Seventeen years ago, GSK3 was shown to be involved in the Wnt-β-catenin signaling pathway (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce *et al.*, 1995). Subsequently, β-catenin was identified as a substrate of GSK3. GSK3-mediated phosphorylation triggers β-catenin destabilization (Yost *et al.*, 1996), marking phosphorylated β-catenin for degradation, and reducing the activity of β-catenin in the cell nucleus. Thus, these findings established a central role for GSK3 in Wnt-β-catenin signaling. Wnt proteins are now known to play crucial role in neural development (Ding *et al.*, 2000), neurogenesis, neuronal migration, neuronal polarization and axon growth and guidance (Cohen *et al.*, 2004; Hur *et al.*, 2010; Wada, 2009), thus one would expect small molecule GSK3 inhibitors to influence these processes.

4.1.9. Chapter aims

Cell adhesion molecules play an important role in holding the stem cells within their specific niche and thereby allowing cell-cell and cell-extracellular matrix interactions. These interactions as are essential to prevent 'anoikis', a form of apoptosis induced by the lack of sufficient cell-extracellular matrix contact. Currently, tissue engineering strategies typically involve the development of extracellular matrix scaffolds that promote cell survival and differentiation (Arnaoutova *et al.*, 2012; Kollmer *et al.*, 2012; Zhang *et al.*, 2012a).

Sox1 is a specific marker for neuroectoderm *in vivo* (Wood *et al.*, 1999) and has been used to identify induction of neural differentiation (Aubert *et al.*, 2003; Ying *et al.*, 2003b). Thus, working on this principle I decided to use mESCs expressing *Sox1*, which is the marker for early neural development to investigate the following:

- If selected matrix proteins like laminin improve cell survival as compared to gelatin (0.1%v/v) and PDL?
- If CHIR99021 (a small molecule GSK3β inhibitor) enhances neurogenesis or not?
- Whether laminin is able to activate signaling pathways indicated in cell survival?

4.2. Materials and Methods

The experimental procedures used in this chapter have already been explained in Chapter 2, sections 2.2.1.-2.2.7. and Chapter 3, sections 3.2.1.-3.2.6. unless detailed below.

4.2.1. Generation of 'ground state' *Sox1*-eGFP reporter mESCs

*Sox*1-eGFP reporter mESCs were described by Austin Smith's research group (Silva *et al.*, 2008; Ying *et al.*, 2008) and were obtained as a gift. Smith and colleagues (2008) showed that iPS cells could be derived more efficiently with the help of 2i medium. 2i is a mixture of the following inhibitors; CHIR99021/GSK3 inhibitor (3 μM) and PD0325091/MAPK/ERK inhibitor (1 μM) (EMD Calbiochem, USA) (Silva *et al.*, 2008). Working with ground state pluripotency cells offer the distinct advantage that molecular dissection of signaling pathways involved in cell survival, such as those provided by interaction with matrix proteins, can be studied with more confidence as the starting point is more reproducible (Silva *et al.*, 2008).

Thus, working on these principles, I decided to treat *Sox1* knock in mESCs with 2i and LIF for four passages before using them for experiment to understand the effect of matrix proteins on cell survival and differentiation. *Sox1*-eGFP cells were maintained in medium containing 2i and 10^3 Units/ml murine LIF (Millipore, Massachusetts) for four passages and taken up for assay (Silva *et al.*, 2008; Ying *et al.*, 2008).

4.2.2. Inhibitors selected for study

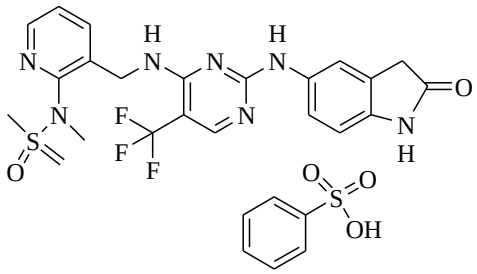
The following kinase inhibitors were used for the study and their chemical structures are shown in figure 4.3

Kinase inhibitor	Biological activity	Supplier	Inhibitory concentration (IC_{50})	Concentration used
PF562271: potent reversible inhibitor of FAK and Pyk-2	It is an orally bioavailable small molecule and ATP-competitive focal adhesion kinase (FAK) inhibitor with potential antineoplastic and antiangiogenic activities. FAK inhibitor PF-00562271 inhibits the tyrosine kinase FAK, and to a lesser extent, proline-rich tyrosine kinase (PYK2), which may inhibit tumor cell migration, proliferation, and survival (Hao *et al.*, 2009; Stokes *et al.*, 2011).	Gift from Cancer Therapeutics CRC, Melbourne, Australia	FAK = 1.5 nM Pyk2 = 14 nM phospho-FAK = 5 nM	500 nM

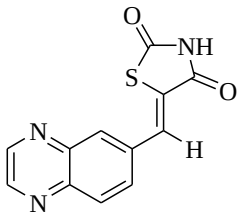
CHIR99021: is the most selective inhibitor of glycogen synthase kinase 3β (GSK3β)	It is the most selective inhibitor of glycogen synthase kinase 3β (GSK3β) reported so far and it does not inhibit cyclin-dependent kinases (CDKs). CHIR99021 has been shown in long term expansion of murine embryonic stem cells in conjunction with MEK/MAPK inhibitor (Kiyonari *et al.*, 2010).	Cellagen Technology, United States of America	7 nM	3 μM
648450-29-7: an inhibitor of phosphatidylinositol 3 kinase γ (PI3Kγ)	It is a potent and selective inhibitor of PI 3-kinase γ (PI 3-Kγ). Displays 30-fold selectivity over PI 3-Kδ and PI 3-Kβ and 7.5-fold selectivity over PI 3-Kα. Suppresses the progression of joint inflammation and damage in both lymphocyte-independent and lymphocyte-dependent mouse models of rheumatoid arthritis (Camps *et al.*, 2005; Peng *et al.*, 2010).	EMD Calbiochem, United States of America	8 nM, 60 nM, 270 nM, 300 nM for p110-γ, α, β and δ-isoforms, respectively.	1 μM

612847-09-3:Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2 a compound that potently and selectively inhibits Akt1/Akt2 activity	A cell-permeable and reversible quinoxaline compound that potently and selectively inhibits Akt1/Akt2 activity.	EMD Calbiochem, United States of America	58 nM, 210 nM, and 2.12 µM for Akt1, Akt2, and Akt3, respectively.	1 µM
PD0325901: MEK1/2 Inhibitor III	PD0325901 is a selective MEK1 and MEK2 inhibitor. PD0325901 inhibits MEK activity in mouse colon 26 cells. PD0325901 inhibits the growth of melanoma cell lines in vitro and in vivo; induces G1-phase cell cycle arrest and apoptosis in a mouse xenograft model. PD0325901 also inhibits production of proangiogenic cytokines such as VEGF (Sheth *et al.*, 2011; Torti *et al.*, 2012)	EMD Calbiochem, United States of America	0.33 nM	1 µM

Table 4.1: Overview of the kinases used in the study



PF562271



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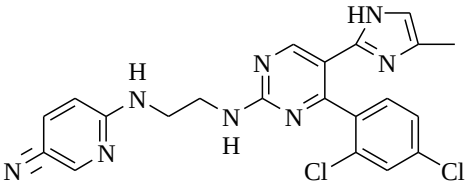
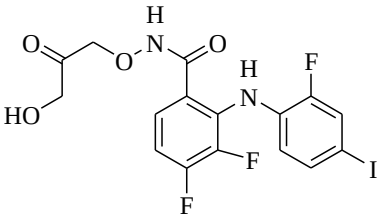


CHIR99021



PD0325901

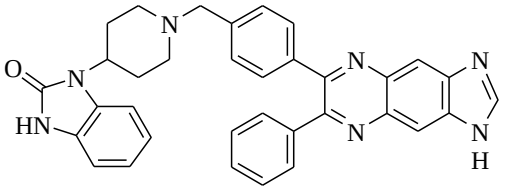


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Figure 4.3: Chemical structures of five kinases inhibitors

4.2.3. Neural induction

For neural induction, an adherent monolayer approach was used as described in section 2.2.2. Cells were seeded at 5.0 x 10^3 cells/cm^2 onto tissue culture well individually coated with following substrates - gelatin (0.1%v/V), PDL (0.1 mg/ml), laminin (5 µg/cm^2, Invitrogen, Australia) and grown in serum-free N2B27 medium for 6 days. N2B27 medium is a 1:1 mixture of neurobasal medium supplemented with N2: DMEM/F12 medium supplemented with B27, insulin and bovine serum albumin V. To understand the effect of PF562271 the cells were exposed from day 0-6 of neural induction. Whereas, cells exposed to downstream signaling molecular inhibitors were exposed for first 48 hours of neural induction only.

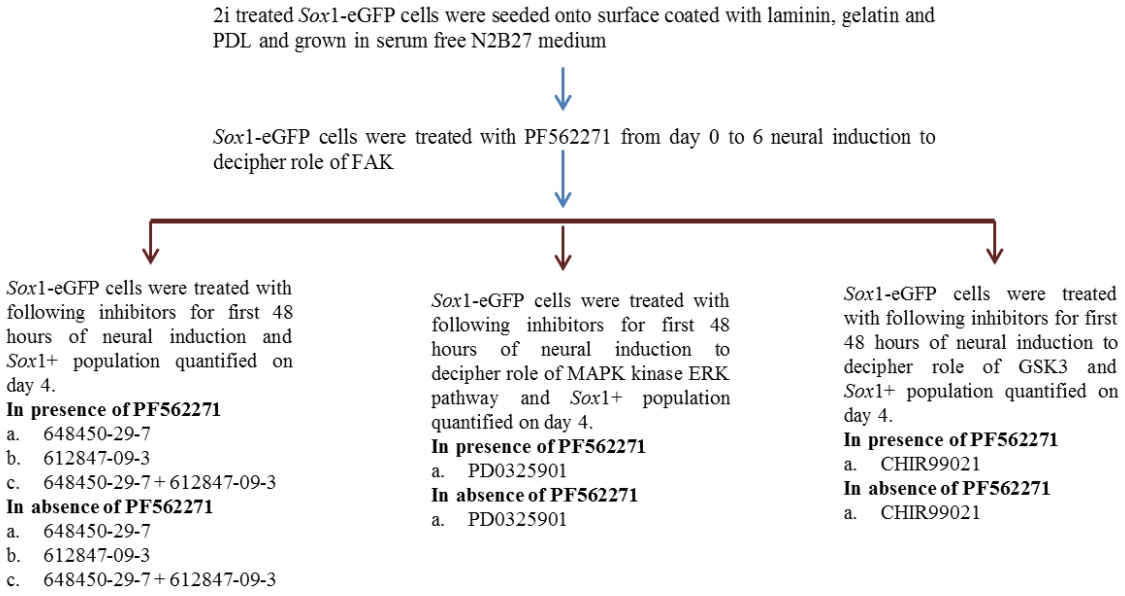

Figure 4.4: Flow chart of the experimental.

4.2.4. Trypan blue staining

Trypan blue is a membrane impermeable dye used to discriminate between viable and non-viable cells. Cell aliquots were mixed with trypan blue dye by preparing a 1:1 dilution of the cell suspension using a 0.4% trypan blue solution in 1X PBS. Non-viable cells appear blue whereas viable cells will be unstained under microscope.

4.2.5. Flow cytometry

Single cell suspension analysis was performed with a FACS Canto II analyser (BD Science, Australia). Before analysis, cell suspensions were cleared of clumps by passing through a 70 µm strainer (BD Biosciences, Australia) and SYTOX red dye was added to identify dead cells. The mechanism of action of SYTOX red dye has been explained on page number 82. For the gating of GFP positive cell populations, wild type cells were submitted to comparable differentiation conditions and used as negative controls. Gates were set so that 99.0-99.5% of wild type registered as GFP negative. For each analysis 10,000 events were counted.

4.2.5. Statistical analysis

All results are presented as mean ± SEM of at least three separate experiments. Statistical analyses were performed on raw data using PRISM v5.00 (GraphPad Software, USA). Data were analysed by Student's *t* test or one-way analysis of variance or two-way analysis of variance (ANOVA) with post-hoc Bonferroni's test. In all cases, $P < 0.05$ was considered to be significant.

4.3. Results:

4.3.1. Effect of laminin on cell survival

In chapter 2, it was shown that by using adherent culture system, differentiation of neurons from mESCs was significantly enhanced by laminin as compared to fibronectin or type IV collagen. Based on these findings laminin was used as the relevant substrate to identify signaling pathways implicated in survival and differentiation of mESCs. Gelatin and PDL were used as control substrates.

In the initial experiments, when the analysis was carried out on day 4 of neural induction, PDL appeared to be able to significantly enhance the percentage of *Sox1+* cells in the population as compared to gelatin and laminin. On day 6, there appeared to be more *Sox1+* cells in the gelatin-coated plates (Figure. 4.5 A). There were no obvious explanations for these observations. However, there were concerns that there were *Sox1*-GFP+ cells present on days 1, 2 and 3, very early in the differentiation (Figure 4.5 A). This indicated that the embryonic stem cells used in my study may not have been fully pluripotent. It has been reported previously that embryonic stem cells grown in LIF and serum may become partially differentiated.

Passaging the cells often causes loss of partially differentiated cells, but the appearance of *Sox1+* cells so early suggested that this was not occurring in the experiments shown in Figure 4.5 A. To improve the quality of the starting point for differentiation experiments, it was decided to generate 'ground state' pluripotent stem cells. These were formed by treating mESCs with 2i as described in section 4.2.1. When 2i treated cells were subjected to neural induction on the selected substrates there were no *Sox1+* cells present on day 1, and a more reproducible step change in the percentage of *Sox1+* cells was observed on day 4. The differentiation took place over a similar time span for all three substrates, and no significant differences in percentage *Sox1+* cells plated onto different substrates were observed Figure 4.5 B.

Sox1-eGFP mESCs were exposed to the FAK inhibitor PF562271 for all six days of neural induction. This was done to determine if the activation of FAK is essential for cell adhesion or not? When routinely passaged mESCs underwent neural induction in presence of PF562271, by day 2, no cells remained adhered to PDL (Figure 4.6 A). To confirm this effect, the same experiments were repeated on 2i treated cells. Figure 4.6 B showed that activation of FAK is essential especially for adhesion to a chemically defined substrate such as PDL. No cells remained adhered to the substrate coated with PDL by day 4 of neural induction in the presence of PF562271.

Variable effects were observed in case of routinely passaged *Sox1* knock-in mESCs (Figure 4.5 A and Figure 4.6 A) but inspection of the plates and the data suggested that the experiments were more reliable after mESCs were treated with 2i. Therefore, it was decided to perform further experiments with mESCs treated with 2i to decipher downstream signaling pathway involved in cell adhesion, survival and proliferation. When 2i treated mESCs were grown on selected substrates, it was observed on day 4 of neural induction, laminin showed slightly better neural conversion of viable cells to *Sox1+* as compared to gelatin (Figure 4.7).

In the same way, on day 4 of neural induction in presence of PF562271 (Figure 4.8) no cells remained adhered to the substrate coated with PDL. Thus, day 4 of neural induction was selected to measure *Sox1+* (Fraction viable cells) to decipher the signaling pathways involved in survival of extracellular matrix through FAK which has been reported to interact with a number of cytoskeletal and signaling proteins, including Src, PI3K, Grb2, p130Cas and paxillin (Cary and Guan, 1999, Hanks et al., 2003).

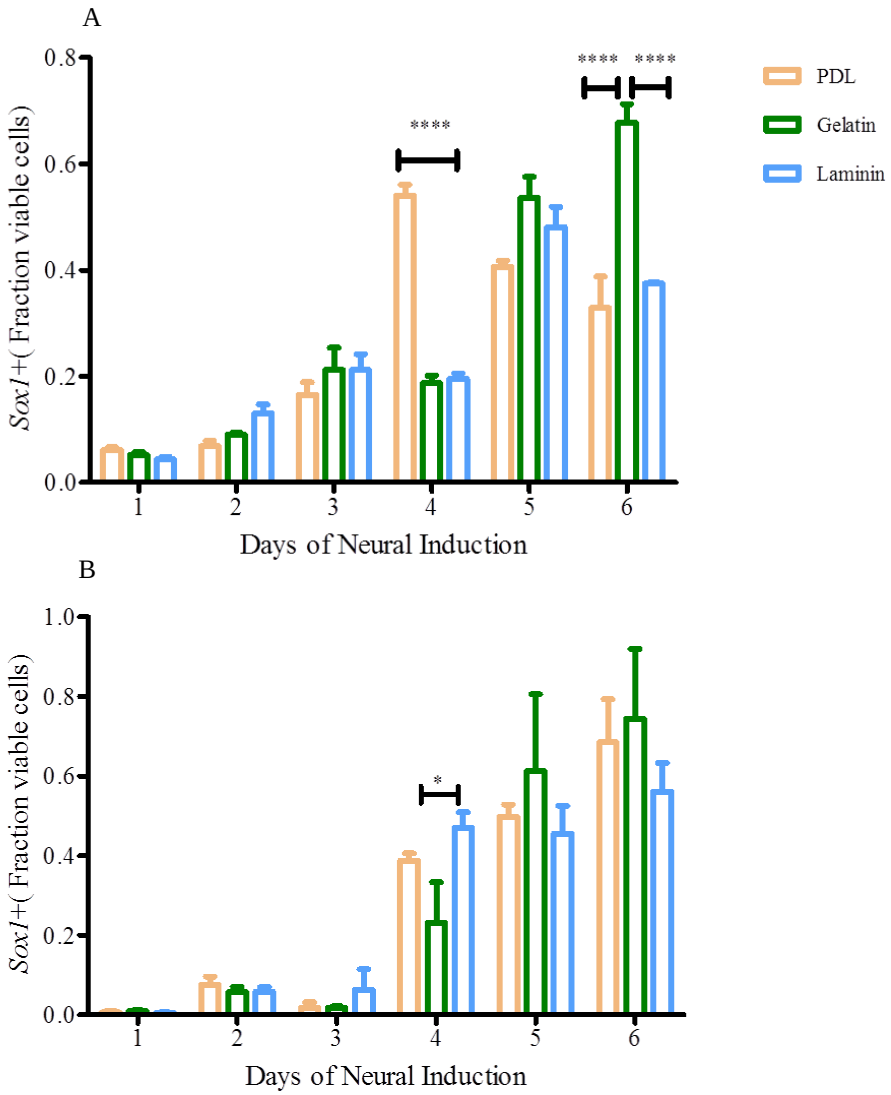

Figure 4.5: The effect of selected substrates on *Sox1+* (Fraction viable cells) quantified from day 1-6 of neural induction. (A) Routinely passaged *Sox1* knock-in mESCs and (B) 2i treated *Sox1* knock-in mESCs. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences between substrates tested individually are significant * P < 0.05 and **** P < 0.0001, two-way ANOVA post-hoc Bonferroni's multiple comparison test.

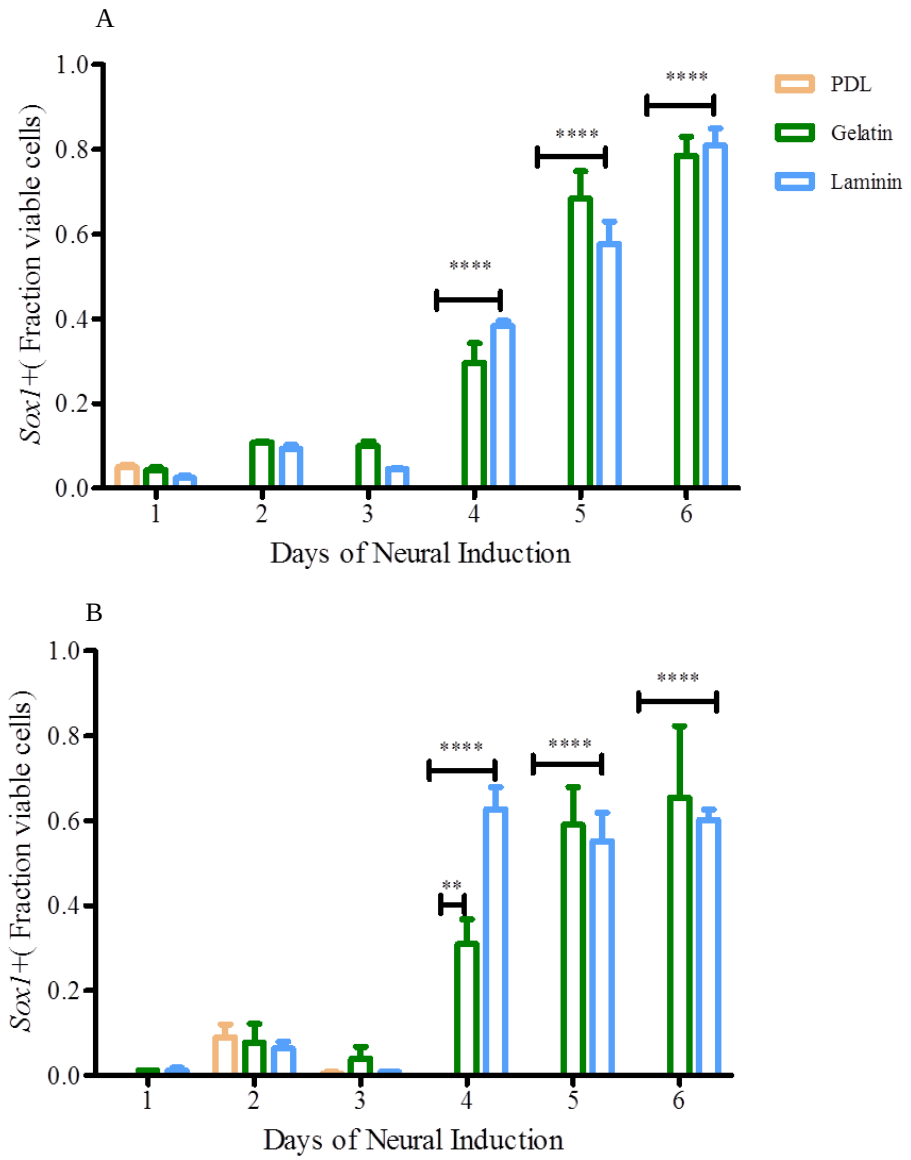

Figure 4.6: The effect of selected substrates in presence of PF562271 on *Sox1+* (Fraction viable cells) quantified from day 1-6 of neural induction. (A) Routinely passaged *Sox1* knock-in mESCs and (B) 2i treated *Sox1* knock-in mESCs. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences between substrates tested individually in presence of PF562271 are significant **P < 0.01 and **** P < 0.0001, two-way ANOVA post-hoc Bonferroni's multiple comparison test.

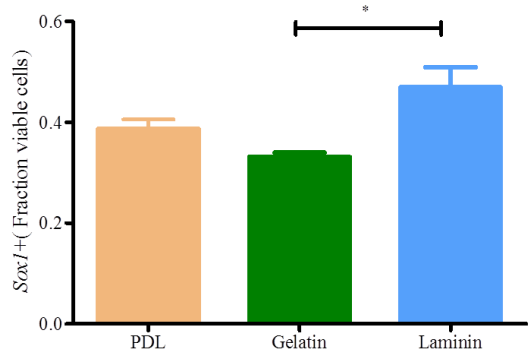

Figure 4.7: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences between substrates tested in absence of PF562271 are significant * P < 0.05, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

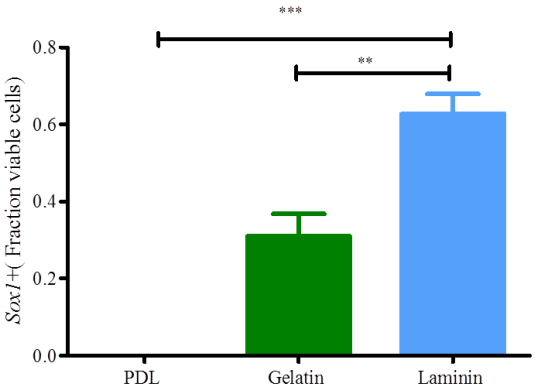

Figure 4.8: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of PF562271. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences between substrates tested in presence of PF562271 are significant ** P < 0.01 and *** P < 0.001, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

4.3.2. Role of the PI3K-Akt pathway in cell survival

4.3.2.1. Effect of PI3K inhibitor

Sox1+ (Fraction viable cells) was determined on day 4 of neural induction after cells were exposed to PI3Kγ inhibitor 648450-29-7. The effect of this inhibitor was independent of the plate-coating (Figure 4.9). When effects of matrix proteins on *Sox1+* in the presence of 648450-29-7 (Figure 4.9) to that in absence of inhibitor (Figure 4.7) were compared, we observed that *Sox1+* dropped in all three matrix proteins from 0.4, 0.3 and 0.5 to 0.1, 0.1 and 0.2 respectively in case of PDL, gelatin and laminin. When modified mESCs were subjected to combination of 648450-29-7 and PF562271, there were distinct differences between cells plated on different substrates. Cells growing on surfaces coated with laminin had a significantly larger fraction of *Sox1+* cells, as compared to cells plated on surfaces coated with gelatin or PDL (Figure 4.10). This result suggested that laminin has the ability to counteract 648450-29-7 and PF562271.

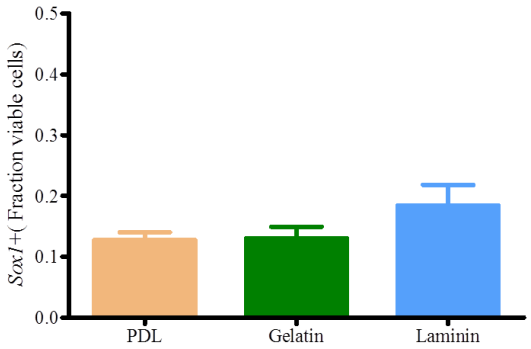

Figure 4.9: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of 64850-29-7. Values are expressed as mean ± SEM of 3 independent experiments. There was no significant difference observed between the substrates one way ANOVA.

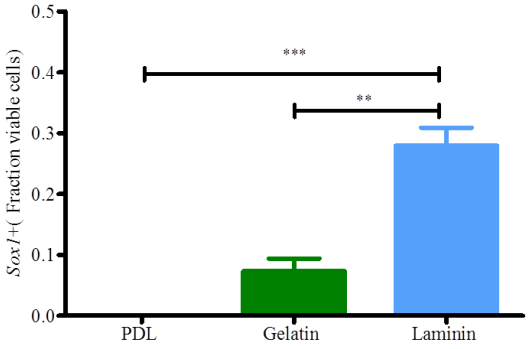

Figure 4.10: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of 64850-29-7 and PF562271. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences in cells grown on different substrates in presence of 64850-29-7 and PF562271 are significant ** $P < 0.01$ and *** $P < 0.001$, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

4.3.2.2. Effect of Akt inhibitor

In the presence of 612847-09-3, the conversion of viable cells to *Sox1+* was slightly enhanced on PDL as compared to gelatin and laminin. Moreover, this effect was compared without any inhibitors (Figure 4.7) and it was observed that *Sox1+* dropped from 0.4 (Figure 4.7) to 0.2 (Figure 4.11) in case of PDL. Whereas, in the case of gelatin it dropped threefold (0.3 to 0.1) but the most drastic drop was seen in case of laminin where nearly fivefold drop was recorded (0.5 to 0.1) This can be attributed to the fact that PDL might have the ability to antagonise the action of 612847-09-3 (Figure 4.11). Figure 4.12 shows that when cells were subjected to combination of 612847-09-3 and PF562271, the effect of the FAK inhibitor on cells plated on PDL showed the dominant effect, resulting in detachment of the cells. Cells plated on gelatin and laminin exposed to combination of 612847-09-3 and PF562271 showed a drop in *Sox1+* to 0.1 from 0.3 and 0.5 respectively as gelatin and laminin were able to antagonise action of PF562271 but not of 612847-09-3.

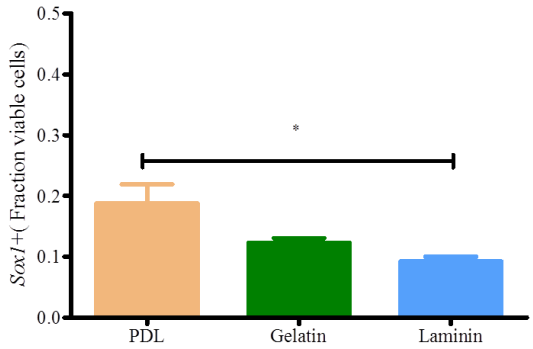

Figure 4.11: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of 612847-09-3. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant * P < 0.05, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

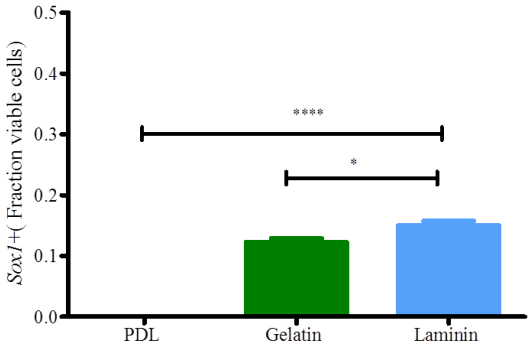

Figure 4.12: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of 612847-09-3 and PF562271. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant * P < 0.05 and **** P < 0.0001, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

4.3.2.3. Effect of PI3K and Akt inhibitor in combination

When cells were exposed to combination of 648450-29-7 and 612847-09-3 (Figure 4.13) or along with PF562271 (Figure 4.14), we observed that only laminin significantly enhanced the conversion of viable cells to *Sox1+* as compared to gelatin and PDL. *Sox1+* dropped tenfold from 0.4 (Figure 4.7) to 0.04 (Figure 4.13) when plated on surfaces coated with PDL in presence of both 648450-29-7 and 612847-09-3. Whereas, *Sox1+* dropped fourfold from 0.3 (Figure 4.7) to 0.07 (Figure 4.13) on gelatin and two and half fold when plated on laminin from 0.5 (Figure 4.7) to 0.2 (Figure 4.13); in presence of both the inhibitors 648450-29-7 and 612847-09-3.

The above findings were in harmony that PDL is not able to inhibit action of 648450-29-7 but is able to inhibit 612847-09-3 action. Gelatin and laminin showed ability to antagonise the action of inhibitors when used in combination with PF562271 (Figure 4.14). Thus, it can be concluded from the above findings that PDL requires activation of FAK for the attachment of cells but not in case of gelatin and laminin. The property of PDL countering 612847-09-3 needs to be studied in more detail.

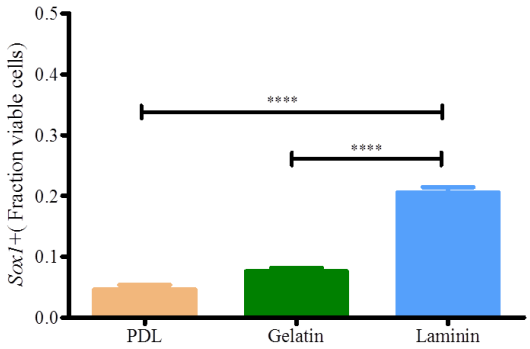

Figure 4.13: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of 612847-09-3 and 648450-29-7. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant **** P < 0.0001, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

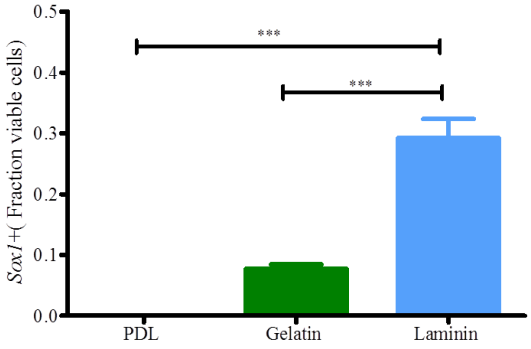

Figure 4.14: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of 612847-09-3, 648450-29-7 and PF562271. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant *** P < 0.001, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

4.3.3. Role of the MAP kinase ERK pathway on cell survival

When cells were incubated with the MEK inhibitor, PD032590, laminin slightly enhanced conversion of viable cells to *Sox1+* as compared to gelatin but moderately as compared to PDL (Figure 4.15). When incubated with combination of PD032590 and PF562271 in presence of selected substrates, we observed laminin replicated the same effect (Figure 4.16). It was observed that cells when plated on surface coated with PDL there were no cells attached by day 4 when they were exposed individually to PD032590 or in combination with PF562271. The other interesting finding can be inferred from above (Figure 4.16) is that gelatin which had so far shown ability to antagonize action of 648450-29-7 and 612847-09-3 failed to counter the presence of PD032590 in combination with PF562271. *Sox1+*/Viable cell population drastically dropped in presence of laminin to 0.04 (Figure 4.15) from 0.5 (Figure 4.7). Whereas, in the presence of PD032590 with PF562271 it dropped down to 0.05 (Figure 4.16) from 0.6 (figure 4.8) when grown on laminin. Therefore, it can be assumed that laminin has the ability to counter PD032590 when used in combination with PF562271 but this needs to be confirmed whether, this property is exclusive to laminin or shown by other glycoproteins.

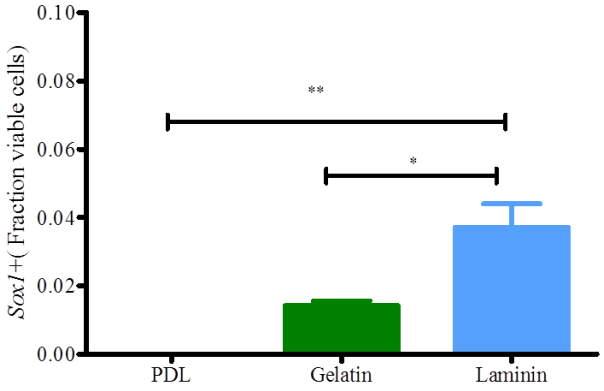

Figure 4.15: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of PD0325901. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant * P < 0.05 and ** P < 0.01, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

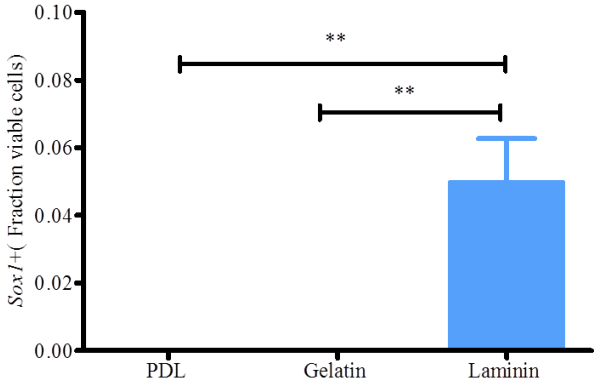

Figure 4.16: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of PD0325901 and PF562271. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant ** P < 0.01, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

4.3.4. GSK3 and neurogenesis

GSK-3β inhibition protects neurons from cell death by the stabilization of two GSK-3 targets, β-catenin and Tau (Cross *et al.*, 2001), indicating that GSK-3β inhibition is a crucial tool for neuronal apoptosis. Neurogenesis is tightly connected to apoptosis, as nearly half of the neuronal progenitor pool undergoes apoptosis caused by neuronal network formation processes and synaptic selection processes (Feliciano *et al.*, 2012; Jessberger *et al.*, 2008).

Thus, working on the same principle we wanted to investigate whether the selected substrates in presence of CHIR99021 (GSK-3β inhibitor) enhance neurogenesis or not. Cells when exposed only to CHIR99021, laminin showed significant conversion of viable cells to *Sox1+* as compared to gelatin but moderate effect as compared to PDL (Figure 4.16). Even though laminin showed promising results, PDL cannot be completely ignored as it was better than gelatin. This promising ability of PDL was lost when cells were incubated with combination of CHIR99021 and PF562271 (Figure 4.18). Thus, both the figures (4.17 and 18) showed that laminin turned out to be the substrate which enhanced neurogenesis followed by gelatin and PDL.

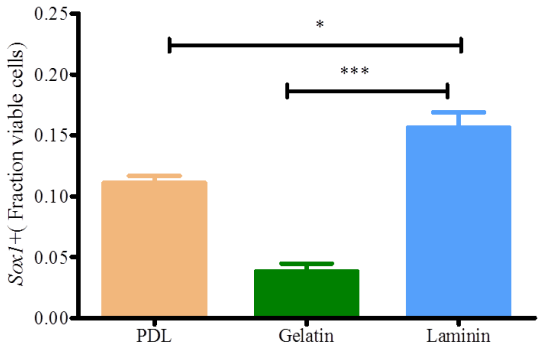

Figure 4.17: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of CHIR99021. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant * P < 0.05 and *** P < 0.001, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

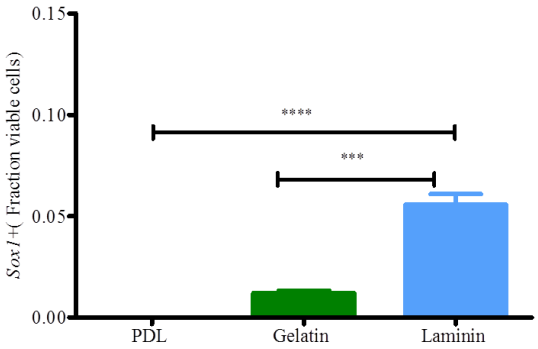

Figure 4.18: *Sox1+* (Fraction viable cells) determined on day 4 of neural induction to decipher the effect of different substrates in presence of CHIR99021 and PF562271. Values are expressed as mean ± SEM of 3 independent experiments. Statistical differences of cells grown on different substrates are significant *** P < 0.001 and **** P < 0.001, one-way ANOVA post-hoc Bonferroni's multiple comparison test.

4.4. Discussion

4.4.1. Ground state pluripotent stem cells and survival signaling from extracellular matrix

Induced pluripotent stem cells (iPS) were first generated by Yamanaka and co-workers group in 2006 by introducing specific genes *Klf4*, *Sox2* and *Oct3/4* into adult mouse cells (Takahashi *et al.*, 2006). They showed that iPS cells were reprogrammed to resemble embryonic stem cells (Ebben *et al.*, 2011). iPS technology is now commonly used in stem cell laboratories and has reshaped our understanding of pluripotency. Austin Smith and co-workers, identified small molecule kinase inhibitors that were able to maintain embryonic stem cells, in a pluripotent state in the absence of LIF and serum (Ebben *et al.*, 2011; Silva *et al.*, 2008). Growth of mESCs in this medium, now known as 2i, contains inhibitors of MAPK signaling and GSK3β. Embryonic stem cells grown in 2i were described as reaching a ground state, which appears to be a more definitive pluripotent state than mESCs grown in LIF and serum.

FAK is a 125 kD non-membrane bound protein tyrosine kinase that phosphorylates upon activation of most integrins. The mechanism by which integrins activate FAK is not known in detail but the process requires an intact action cytoskeleton since cytochalasin D treatment prevented FAK activation (Shattil *et al.*, 1994). Studies, using FAK-deficient cells have displayed decrease in motility when plated on surfaces coated with fibronectin thus, indicating that FAK is necessary for cell adhesion and migration (Ilic *et al.*, 1995a).

Thus, it was interesting to explore what happens when *Sox1* knock-in mESCs are exposed to PF562271. It can be inferred from (Figure 4.6 B) that addition of PF562271 leads to blockage of attachment of cells on surfaces coated with PDL which was not observed in case of laminin and gelatin. Therefore, it can be inferred from these findings that activation of FAK is essential for growth on PDL. It can also be inferred that matrix proteins like laminin and gelatin either have ability to counter presence of PF562271 or they can activate Src which also has been implicated to play role in cell adhesion (Graf *et al.*, 2013; Kaplan *et al.*, 1995; Livshits *et al.*, 2012).

4.4.2. PI3K-Akt pathway in cell survival

It has reported that when cells are plated on surfaces coated with laminin or fibronectin, FAK is activated (Chen and Guan, 1996, Suh and Han, 2010, Xu et al., 2010). This involves phosphorylation of the Tyr-395 residue of FAK, through which it activates PI3K, which in turn activates Akt. Cells adhering on surfaces coated with fibronectin experience an accumulation of PI3K-generated phosphorylated lipids, resulting in activation of Akt activity (Chen and Guan, 1996, Chen et al., 1996). Dystroglycan, cell surface receptor for laminin when blocked causes disruption in the PI3K/Akt signaling pathway. It has been reported that addition of a PI3K inhibitor leads to decreased phosphorylation of Akt and its downstream effector GSK3, which in turn leads to detachment of cells (Langenbach and Rando, 2002).

To investigate whether the PI3K-Akt pathway plays a role in the induction of *Sox1+* we incubated 2i treated knock in mESCs with 648450-29-7, 612847-09-3 individually or in combination with PF562271. In presence of 648450-29-7 (Figure 4.9), we did not see any significant effect on *Sox1+* population. Whereas, in presence of 648450-29-7 along with PF562271, laminin significantly enhanced conversion of viable cells to *Sox1+* as compared to gelatin and PDL (Figure 4.10). We did observe quite contrasting results, when cells were plated on surfaces coated with PDL, in presence of 612847-09-3; it was able to enhance cell viability and conversion to *Sox1+* cells with respect to laminin and gelatin (Figure 4.11). This action could be attributed to PDL, which possibly has the ability to activate Akt and needs to be further investigated with other commercially available Akt inhibitors.

Yao and Cooper (1995) provided the first evidence indicating that PI3K is implicated in the suppression of apoptosis. It was later reported that by using the following inhibitors (LY294002 and Wortmannin) against PI3K, they were able to reduce the effects of NGF on cell survival and showed that both PI3K and Akt are necessary for survival of NGF dependent sympathetic neurons (Crowder *et al.*, 1998; Yao *et al.*, 1995). Thus, in this study it was interesting to investigate the fraction of cells that became *Sox1+* in the presence of both 648450-29-7 and 612847-09-3 (Figure 4.13-4.14) or along with PF562271.

We observed that only laminin was able to help in survival of cells and enhanced conversion of viable cells to *Sox1+* with respect to gelatin and PDL. Several groups have shown that pro-survival signaling arises from extracellular matrix and/or integrins. Upon detachment from the extracellular matrix, epithelial cells are known to enter programmed cell death, which is halted by plating cells onto extracellular matrix, which leads to rapid elevation of the PI3K lipid product and Akt activity (Khwaja *et al.*, 1997). Findings from above (Figure 4.13 to 4.14) showed that when cells were exposed to both 648450-29-7 and 612847-09-3 better conversion of viable cells to *Sox1+* on surfaces coated with laminin was observed as compared to gelatin and PDL.

The pro-survival signaling originating from extracellular-integrin interaction or FAK activation does not always involve the PI3K-Akt pathway (Almeida *et al.*, 2000). Pathways such as JNK (Almeida *et al.*, 2000) and MAPK-ERK (Gu *et al.*, 2002) have also been reported to mediate the pro-survival signaling from matrix. Therefore, it can be inferred from this finding that laminin could potentially activate alternative survival signaling pathway, which were not activated by PDL.

4.4.3. Activation of the MAP kinase-ERK pathway

The mechanism of activating of ERK through phosphorylation of FAK is not yet clear. One theory is that binding of α6β4 to laminin-5 leads to tyrosine phosphorylation of Shc which leads to stimulation of the ERK through downstream activators. This was not observed when laminin came in contact with α3β1 and α2β1 or when collagen was used in place of laminin (Doehn *et al.*, 2009; Duan *et al.*, 2010; Mruthyunjaya *et al.*, 2010; Schlaepfer *et al.*, 1997), suggesting that the aforementioned interaction is quite specific in nature.

ERK elicits specific biological responses including cell survival and protection against several different pro-apoptotic stimuli (Wada *et al.*, 2004). ERK signaling also plays a role in neurite outgrowth, possibly via the extracellular matrix (Wang *et al.*, 2011). Several groups have shown that ERK pathways provide protection against anoikis (Manohar *et al.*, 2004). In the present study, cells exposed to laminin were more able to promote cell survival and expression of *Sox1+*, than cells exposed to gelatin and PDL. This suggests that laminin may activate alternative signaling pathways for cell survival that are not activated by gelatin or PDL. Some evidence for this hypothesis may lie in findings like extracellular matrix-induced survival of Madin-Darby Canine Kidney Epithelial (MDCK) cells involves PI3K-Akt pathway but not MAP-kinase ERK pathway (Khwaja *et al.*, 1997), and laminin 5 induced breast tumour cell survival and anchorage is independent of both PI3K and ERK pathways (Zahir *et al.*, 2003).

It has also been also shown that ligation of α6β4 to laminin-5 leads to tyrosine phosphorylation of Shc, recruitment of Grb2, activation of Ras leading to stimulation of the ERK pathway (Ajenjo *et al.*, 2004; Boudreau *et al.*, 1999a; Mainiero *et al.*, 1997). This was specific for α6β4, as ligation of α3β1 and α2β1 or collagen did not cause these events (Boudreau *et al.*, 1999a; Mainiero *et al.*, 1997) thus indicating that only ligation of specific integrins can activate the ERK pathway in a given cell type. We also tried to understand when integrins ligates to their cognate extracellular matrix ligands if it leads to activation of ERK? Different theories have been proposed, highlighting the complexity of integrin signaling (Doehn *et al.*, 2009; Duan *et al.*, 2010; Mruthyunjaya *et al.*, 2010; Schlaepfer *et al.*, 1997). The role of FAK in extracellular matrix-induced ERK activation is controversial.

FAK is capable of activating MAPK through recruitment of Grb2 (102), Shc or Src (Doehn *et al.*, 2009; Duan *et al.*, 2010; Mruthyunjaya *et al.*, 2010; Schlaepfer *et al.*, 1997), but other mechanisms independent of FAK that result in ERK activation have also been described (Doehn *et al.*, 2009; Duan *et al.*, 2010; Mruthyunjaya *et al.*, 2010; Schlaepfer *et al.*, 1997).

In addition, paradoxical effects of the PI3K-Akt pathway on extracellular matrix-induced activation of the ERK pathway have also been reported. For example, it has been shown that β1 integrin-induced ERK activation in monocytes is dependent on PI3K activity (Reyes-Reyes *et al.*, 2001) while others have reported that PI3K-Akt and ERK pathways act independently from each other (Danilkovitch *et al.*, 2000). Therefore, it can be stated that the role of FAK and/or of PI3K in integrin-induced ERK activation may be cell-type and/or context-dependent.

4.4.4. Activation of GSK3β by matrix proteins

Analysis using various markers of progenitors, intermediate progenitor cells (IPCs) and post mitotic neurons showed that deletion of GSK3 genes markedly enhanced the proliferation of progenitor cells, while suppressing neuronal differentiation (Hur *et al.*, 2010). The above findings (Figure 4.17 to 4.18) showed that when cells were incubated individually with CHIR99021 or in combination with PF562271, better conversion of viable cells to *Sox1+* was achieved on laminin as compared to gelatin or PDL. It can be inferred that laminin not only has the ability to help in cell survival, but also plays a crucial role in neurogenesis.

Numerous studies have demonstrated the importance of β-catenin-mediated Wnt signaling in proliferation and fate determination during cerebral cortical development (Gulacsi *et al.*, 2008) and GSK3 activation leads to increase in phosphorylation of β-catenin (Hur *et al.*, 2010). Therefore, it needs to be further elucidated whether laminin has the ability to activate β-catenin on its own.

4.5. Conclusion

This study has demonstrated survival of neural stem cells and induction of *Sox1+* was significantly enhanced by laminin as compared to gelatin or PDL. Laminin stimulated cell survival and neurogenesis. This finding is consistent with data showing that laminin and other matrix proteins have the ability to activate various downstream kinase pathways involved in cell survival. Therefore, it can be summarized that laminin, has the ability to activate both PI3K/Akt and ERK pathway to help in survival of cells. This effect may be specific to cell type, but mESCs survived and differentiated towards neural lineages more efficiently on laminin than gelatin or PDL. Thus, the interaction between mESCs and key matrix proteins appeared to be an early inductive signal to help regulate neural specification of mESCs. This finding which has not been shown by any other group so far as per my information will help to gain better knowledge of both the intracellular pathways involved in cell survival and factors regulating these signaling pathways. These results are particularly relevant in providing an insight into signaling pathway activation which leads to increase in survival of stem cells and could be beneficial especially before transplantation of cells.

Chapter 5 Thesis summary and future directions

5.1. Introduction

The conclusion and recommendation outlined in this chapter refer to the outlines of the study, described in Section 1.6. I acknowledge that although the discussion of results was already detailed in the preceding chapters, a chapter to summarize the results and discussion of the project is also needed.

This chapter provides a review of the work presented in this thesis and discusses its implications. Immunocytochemistry, in-cell Western, quantification of primary neurite, neural progenitor numbers, and total neurite length per neuron were undertaken to examine the influence of matrix proteins on differentiation of mESCs into neurons. These experiments were followed by In-cell Western to examine whether it was possible to immobilize growth factors, including Shh, FGF2 and FGF8b, onto combination of matrix proteins. It was of interest to examine whether immobilized growth factors enhanced *Lmx1a* expression in mESCs. Lastly, a series of small molecule enzyme inhibitors were used to investigate the signaling pathways involved in the survival and neural induction of embryonic stem cells using a *Sox1*-eGFP mESC reporter cell line.

5.2. Influence of extracellular matrix proteins on neurogenesis

In chapter 2, the effect of laminin, fibronectin and type IV collagen on the influence of adhesion, proliferation and neuronal differentiation of mESCs were investigated. The preliminary results confirmed that matrix proteins could be coated uniformly onto tissue culture plates. As the concentration of proteins in the coating solution was increased, there was an increase in surface area coated. The adhesive properties of the basement membrane are usually ascribed to the glycoproteins. Laminin is the most abundant glycoprotein in the basement membrane and it has structural as well as a regulatory roles (Timpl *et al.*, 1994; Yurchenco *et al.*, 1993). The findings that laminin enhanced stem cell adhesion and proliferation is consistent with the work of Eccleston et al. (1985), who plated dissociated adult neurons onto surfaces coated with purified glycoproteins of the extracellular matrix (Eccleston *et al.*, 1985). There may, however, be a difference in the thickness and distribution of the extracellular matrix coating on the surface of well and this may have affected mESCs adhesion and proliferation.

Further studies are necessary to investigate by using technique like scanning electron microscope. Detailed surface analysis by scanning electron microscope at the micron level would help us in understanding surface characteristics of matrix proteins under investigation such as whether these have a smooth or rough surface topography. It is generally understood that cells prefer to grow on a rough surface, as the filopodia require surface grooves to attach (Li *et al.*, 2013). However, other research suggests on the contrary. A study by Gordin et al. (2004) found that cell growth was better on a smooth rather than rough surface when tested on the same base material (Gordin *et al.*, 2004).

Considering the studies in this area, it appears that the relationship between cell attachment, growth and surface properties are dependent on the method of testing (*in-vivo* or *in-vitro*), the cell type and the surface characteristics therefore, it would be necessary to undertake specific cell studies to draw any conclusions on the ability of cells to attach and grow on the surface of the extracellular matrix proteins.

Immunocytochemistry from chapter 2 show that none of the selected matrix proteins enhanced the number of nestin positive or βIII tubulin positive cells 24 hours after plating. Whereas, 96 hours after plating laminin increased the number of βIII tubulin positive cells. Although, it is not completely clear from this study how and why laminin enhanced cell number. These studies do indicate that laminin contributes to neural induction and these effects can be seen after 96 hours of plating. To understand, the way in which laminin stimulated the neural induction; further time lapse studies could be done using immunofluorescent staining with different neural markers. It is also necessary to determine if the same effect of laminin coating is observed on different cell types.

It has been reported that laminin enhances the densities of serotonergic, dopaminergic and norepinephrinergic neurons as compared to control, when used as a substrate for transplantation of brain cells (Eldridge *et al.*, 1989). Thus, working on the same principle it was decided to test whether selected matrix proteins including laminin, fibronectin and type IV collagen are able to enhance generation of dopaminergic neurons from embryonic stem cells.

The results presented in chapter 2 sections 2.3.5. showed no significant differences. The findings in the past decade have demonstrated that laminin enhances generation of dopaminergic neurons from human neural stem cells (Ostenfeld *et al.*, 2004; Yu *et al.*, 2007). Human neural stem cells responded to the priming effect of laminin and this resulted in an increase in the percentage of cholinergic neurons (Tarasenko *et al.*, 2004; Wu *et al.*, 2002). Therefore, the results obtained in our study suggest that there may be differences in the effects of matrix proteins on embryonic stem cells and indicate the need to carry out future studies using human embryonic stem cells.

5.3. Growth factor immobilization

Growth factors are proteins that transmit signals in order to regulate cellular activities such as differentiation and proliferation (Babensee *et al.*, 2000). They play critical roles in cell regeneration (e.g. nerve regeneration) or repair of tissues (e.g. bone and cartilage). Growth factor effects are concentration dependent (Babensee *et al.*, 2000). Growth factors such as FGF2, BMP and NGF, if presented in bioactive form, can also influence the cells to produce and secrete other specific growth factors or cytokines, which can often be part of either wound healing or specific events in development (Babensee *et al.*, 2000; Luginbuehl *et al.*, 2004; Masters, 2011). Physical adsorption, physical entrapment and covalent attachment are three common ways of attaching growth factors onto surfaces (Masters, 2011; Wood *et al.*, 2010).

Physical adsorption utilizes weak nonspecific intermolecular interactions such as hydrogen bonding, hydrophobic interactions, Van der Waals forces, and valence electron interactions (Vladkova, 2010; Zhang *et al.*, 2012b). *In vivo* and *in vitro* heparin has been shown to help in binding, preserving the stability and biological activity of the growth factors (Arisaka *et al.*, 2013; Lee *et al.*, 2012a; Seo *et al.*, 2012). Growth factors have been incorporated into the scaffold matrix either by bulk encapsulation, specific or nonspecific surface adsorption or by adding microspheres encapsulating them (Wood *et al.*, 2010). Thus, working on these principles tried to covalently immobilize growth factors onto heparin and the results generated in chapter 3 showed that the growth factors like FGF2 and FGF8b were successfully immobilized which also increased the effectiveness of FGF8b and FGF2, as indicated in section 3.3.2 and 3.3.3..

Although these results show that physical adsorption is an effective way to immobilize growth factors to the surface, coating only provides a transient modification of the material surface (Vladkova, 2010). The inability to control the growth factor conformation and orientation upon the adsorbing substrate, factor desorption (wash-off), diffusion kinetics, and inaccessibility to large molecules on the material surfaces are the drawbacks of this method (Laurencin *et al.*, 2009; Lee *et al.*, 2012b).

More refined approaches are available for the immobilization via chemical methods or onto plasma-treated surfaces can be used to introduce active functional groups (Laurencin *et al.*, 2009). Plasma treatments under a wide range of reacting gases types (ammonia, nitrogen, hydrogen, oxygen, and ozone) have been employed to introduce various functional groups (e.g., carboxyl, hydroxyl, carbonyl, ether, peroxide, and amine) onto the material surface (Vladkova, 2010). Chemically inert polydimethylsiloxane (PDMS) was simultaneously mixed with acrylic acid (AA) and 2-hydroxyethyl methacrylate (HEMA) employing so-called "two-step plasma treatment", followed by collagen immobilization to study the cellular responses to modified surfaces. Such surface design significantly increased the number of the adhered and proliferated cells (Almutairi *et al.*, 2012; Vladkova, 2010; Zhou *et al.*, 2012).

Yoon et al. (2006) also fabricated macroporous poly lactide-co-glycolide (PLGA) scaffolds using blending mixture of PLGA and NH2-PEG-PLGA to generate surface amine groups for heparin immobilization (Yoon *et al.*, 2006). Interestingly heparin immobilized microspheres also release bioactive FGF2 in a sustained manner and exhibit pronounced angiogenic effect in an animal model (Kim *et al.*, 2006).

Embryonic stem cells are pluripotent cells, which means they can be expanded indefinitely, and if exposed to appropriate environmental conditions, can be differentiated into target cell types like neurons and cardiomyocytes (Luginbuehl *et al.*, 2004). In the past few years, a considerable volume of research has been carried out to utilise the potential of embryonic stem cells to help in the treatment of nerve injury, however most findings failed to demonstrate a dramatic increase in functional recovery (Johnson *et al.*, 2010; Wood *et al.*, 2010) following cell transplant.

One of the hypothesis for the failure of this approach is the cells were not exposed to an appropriate microenvironment before transplant hence there has been a strong focus on delivery of growth factors either through fibrin scaffolds, heparin alginate gels, cross-linking with collagen, or by integrating poly (lactide-co-glycolide) as a scaffold (Luginbuehl *et al.*, 2004; Masters, 2011).

My attempts to provide growth factors in an immobilized form did not produce any strong desired effects. Several mechanisms could explain this effect firstly; presence of different extracellular matrix proteins could modulate the cellular response of the immobilized growth factors. To illustrate this theory, a group in Japan tried using a combinatorial approach the addition of growth factors and extracellular matrix proteins, but they found that the cellular response to immobilized growth factors differed depending upon the extracellular matrix coating present on the culture substrates. For instance, the differentiation of neural stem cells to astrocytes was promoted on fibronectin coated substrates modified with immobilized NGF, but inhibited when the NGF was immobilized to laminin coated substrates (Nakajima *et al.*, 2007; Saik *et al.*, 2011). Secondly, the temporal aspects of exposure may have been sub-optimal. The six day exposure may have been too short. Evidence indicates that increasing the exposure time during neural induction from 6 to 10 days alters the outcome substantially (Colleoni *et al.*, 2010; Koch *et al.*, 2009a; Zeng *et al.*, 2011).

In order to fabricate biomimetic materials that can promote long-term survival, a stable immobilization of such bimolecular motives to the substrate surface is critical to maintain the bioactivity and ultimately proper function (Kollmer *et al.*, 2012; Rogers *et al.*, 2012; Skop *et al.*, 2013). The development of a biomimetic microenvironment conducive to cell adhesion, proliferation, differentiation and host tissue integration requires a more in-depth investigation of the mechanisms of protein adsorption and reorganization, as well as of the bio adhesion and cell/biomaterial and cell/extra cellular matrix interactions, cell signaling and cell growth biology.

5.4. Cell matrix interactions

Several reports have shown that integrins activate several signaling pathways that regulate pro-survival effects (Discher *et al.*, 2009; Roberts *et al.*, 2008). These signaling pathways involve the, FAK, PI3K, Akt and the MAP kinase ERK. However, the relevance of a given signaling molecule in mediating the pro-survival signaling induced by extracellular matrix appears to be cell type specific. It was therefore necessary to investigate which of these intracellular signaling pathways might be involved in the effects of extracellular matrix on survival and differentiation of *Sox1* eGFP mESCs.

In chapter 4, my initial findings indicated that the effects of selected substrates on the *Sox1+* population during days 1 to 6 of neural induction was that; PDL significantly enhanced the number of cells expressing *Sox1,* as compared to gelatin and laminin, but by day 6, there were more *Sox1+* cells on gelatin surface. Subsequently, cells were incubated with an inhibitor of FAK. This enzyme is known to play a major role in adhesion (Hanks *et al.*, 1992; Hynes *et al.*, 1992).

In the presence of the FAK inhibitor, no cells remained attached to plates coated with PDL, suggesting that activation of FAK is essential for cells to adhere onto surfaces coated with PDL. We have observed that passaging of mESCs with LIF and serum over time does not maintain cells in the pluripotent state and it's supported by findings that cells become more specialized and restricted in their developmental potential (Graf *et al.*, 2009; Murry *et al.*, 2008; Sato *et al.*, 2004).

Therefore, to make sure that initial findings obtained were due to blockage of the FAK signaling pathway, I decided to generate ground state pluripotent stem cells as described in section 4.2.1. 2i treated *Sox1* eGFP mESCs were therefore, used to investigate the signaling pathways involved in supportive effects of extracellular matrix proteins. I showed that laminin and gelatin (which is a mixture of collagens) (Wu *et al.*, 2002) were able to offer protection from the FAK inhibitor PF562271, which was not seen in case of PDL. This finding with PDL needs to be further investigated with other chemically defined substrates like peptide acrylate and poly [2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH), which have been reported to sustain long term human embryonic stem cell growth in several different culture medium (Villa-Diaz *et al.*, 2010).

FAK is known to play a critical role in survival of cells by enhancing their adhesion onto substrates (Ilic, 2006; Ilic *et al.*, 1995b; Owens *et al.*, 1995), particularly inactive substrates. Natural matrix proteins may be able to activate alternative pathways through which cells remained adherent. To investigate the roles of the PI3K-Akt signaling module and the MAP kinase ERK pathway in the pro-survival effect of the matrix proteins, these enzymes were blocked in turn using known pharmacological inhibitors.

Inhibition of the PI3K-Akt pathway showed that laminin enhanced conversion of *Sox1+* cells as compared to gelatin and PDL. One unexpected and particularly interesting result was the finding that when cells were incubated with 612847-09-3, conversion of viable cells to *Sox1+* cell was moderately enhanced on PDL as compared to gelatin and laminin. The survival effect of gelatin and PDL was greatly abrogated when ERK pathway was inhibited.

These findings suggested that laminin has the ability to activate both PI3K/Akt and ERK pathway to help in survival of cells. This is consistent with the role of specialized integrin-extracellular matrix ligand interactions regulate many cell functions, including survival, proliferation, motility, morphology, and differentiation in a cell/tissue-specific and time-dependent manner. α2β1 mediated cell attachment to type I collagen stimulates the tyrosine phosphorylation of FAK and, subsequently, the activation of ERK and MAPK that has been implicated in the control of osteoblast-specific gene expression and matrix mineralization (Fu *et al.*, 2013; Xu *et al.*, 2012). Integrin α5β1 is suggested to be involved in cell matrix adhesion (Sekine *et al.*, 2012). Research groups have demonstrated that by increasing micro scale roughness of substrates promoted greater α5β1 binding and FAK phosphorylation in osteoblasts on fibronectin adsorbed surfaces (Rechenmacher *et al.*, 2013). Given the crucial and overlapping roles, multiple integrins play in cell signaling and functional pathways it would be worthwhile to explore whether β1 integrin was required for the responses shown by laminin in chapter 4.

The results in chapter 4 showed that laminin in presence of CHIR99021 enhanced conversion of viable cells to *Sox1+* with respect to gelatin and PDL. Blocking GSK3β activity is analogous with activation of Wnt. Hence, the role of matrix proteins in activating the Wnt pathway will provide us with new tools to help control stem cell survival and differentiation (Munji *et al.*, 2011). Migration of neural crest cells is an intricate process that requires the delamination of cells from an epithelium and movement into an extracellular matrix. It has been reported that the canonical Wnt signaling pathway is needed for neural crest induction, while the non-canonical Wnt pathway is required for neural crest migration (Munji *et al.*, 2011).

By performing time-lapse analysis of cell movement and cell protrusion in neural crest cultured on a laminin substrate, it would be possible to clarify whether the effects of CHIR99021 are due to canonical or non-canonical Wnt signaling that controls neural crest migration. This experiment lays the groundwork for more detailed studies that could examine various matrix proteins, time points, and cell densities that may contribute to the above effect from cells specifications. Although 2-D coatings have been used in this study, an abundance of clinical applications rely on 3-D scaffolds for nerve regeneration (Blumenthal *et al.*, 2013; Khaing *et al.*, 2012). Studies have suggested that cells behave quite differently in 3-D environments than 2-D (Berneel *et al.*, 2012; Buttery *et al.*, 2011; Lin *et al.*, 2012; Qutachi *et al.*, 2013b). Examination of these substrate specific effects in 3-D physical environments would be an important initial step towards application of the substrate specific strategy for nerve regenerative purpose.

5.5 Conclusion

In summary, this research was undertaken to determine the potential effect of matrix proteins for use in differentiation of stem cells into neurons. The results from this study indicate that matrix proteins are important for differentiation, although further *in vitro* testing is required to gain a more comprehensive understanding of the matrix effects on differentiation of stem cells in neurons, and whether they have ability to enhance the differentiation of specific subtypes of neurons. The effects of laminin need to be further investigated with other matrix proteins like fibronectin, type IV collagen and vitronectin. It can be concluded from this study that laminin which is most conducive for generation of neurons and has the complex ability to promote cell survival either through activation of PI3K-Akt or MAPK-ERK pathway.

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