Fibronectin peptides and murine embryonic stem cells : An in vitro study

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Abstract

Murine embryonic stem cells are cells that are obtained from the inner cell mass of the embryo blastocyst at day 3.5. These cells have the novel capability of differentiating into almost any cell lineage in the mammal and this characteristic has resulted in them being referred to as pluripotent.

Fibronectin is involved in a number of different functions including differentiation, growth, migration and the adhesion of cells. In addition it is well known to interact with the extracellular matrix. Fibronectin also has an essential role to play in early development of the embryo as studies have shown that knocking out either fibronectin or its primary receptor eg $\alpha5\beta1$ integrin receptor is lethal to the developing embryo.

The section of fibronectin identified as being essential for binding and activation of $\alpha5\beta1$ integrin receptor has been identified as the 9th and 10th type III fibronectin domain. This peptide of fibronectin has subsequently been generated. In this study, the effect of this fibronectin peptide on murine embryonic stem cells was examined. In particular it was identified that the generation of 2-D orientated fibronectin peptide displays in no way enhances the cell attachment potential of the fibronectin peptides. The optimal conformation of the peptides for the attachment of embryonic stem cell colonies was identified as being the dimeric form of the peptide. The fact that these fibronectin peptides have no effect on the proliferation of embryonic stem cells was also determined.

There was an apparent morphological change in embryonic stem cells grown on the fibronectin peptides. These embryonic stem cells were flatter and reminiscent of differentiated embryonic stem cells. This occurred even when the embryonic stem cells were cultured in the presence of LIF, the cytokine essential to maintain embryonic stem cells in a state of self renewal. Alkaline phosphatase staining confirmed that while there was a morphological change the embryonic stem cells maintained their undifferentiated state when grown on fibronectin peptides in the presence of LIF. Finally Quantitative

Polymerase Chain Reaction was carried out in an attempt to ascertain why there appeared to be a morphological change in embryonic stem cells grown on fibronectin peptides as opposed to embryonic stem cells grown on gelatin controls. In the presence of LIF, there was a transient increase in the number of Oct-4, Brachyury and Nestin transcripts. Up regulation of these genes is known in some cases to come from a pro differentiation signal. However concomitant with these transcriptional up regulations, the levels of Nanog, the gene well known to resist and/or reverse changes in gene expression states in embryonic stem cells also increased upon growth on fibronectin peptides.

It is possible that in the presence of LIF, Nanog increases to inhibit the differentiation inducing signals produced by interaction of the fibronectin peptides with the α5β1 integrin receptor. When grown in complete media containing foetal calf serum in the absence of LIF, there were no obvious transcriptional changes that gave an indication to which, if any, lineage the fibroenctin peptides may be instructing the embryonic stem cells to differentiate into.

Upon using serum replacement media as opposed to foetal calf serum and allowing the embryonic stem cells to differentiate more obvious transcriptional changes were observed. ES cells grown on the fibronectin peptides appeared to be inhibited from increasing the levels of transcripts with known roles in cardiac and neuroectodermal differentiation. However there was a transient increase in the levels of FoxA2, a gene involved in the differentiation of embryonic stem cells into endodermal tissue. This increase was shortly followed by a significant decrease in the levels of transcripts when grown on the fibronectin peptides as opposed to gelatin controls.

Importantly it appears that allowing the differentiation of embryonic stem cells to occur in a monolayer instead of the classically used embryoid bodies could potentially be used as an alternative model to study embryonic stem cell differentiation.

Contents

List of tables

List of figures

8

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9

Declaration

I declare that all of the data presented in this thesis was generated by me personally unless stated otherwise in the text.

All data contained within should be considered not to be significant unless specifically stated otherwise.

Abbreviations

2-D	2-dimensional
5x	five times magnification on the microscope
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
BHK fibroblasts	Baby hamster kidney fibroblasts
Bp	Base pairs
BMPs	Bone morphogenetic proteins
BRL	Buffalo rat liver
cDNA	Complimentary deoxyribose nucleic acid
CTNF	Ciliary Neurotrophic Factor
Dpc	Days post coitum
DMEM	Dulbecco's modified eagle's medium
E14	murine embryonic stem cell line
ECM	Extracellular matrix
ES cell	Embryonic stem cell
Et al	and others
FIII8	8th type III fibronectin domain
FIII9	9th type III fibronectin domain
FIII10	10th type III fibronectin domain
FIII9-FIII10	peptide composed of the 9th and 10th type III fibronectin domain
FAK	Focal adhesion kinase
FCS	Foetal calf serum
Fgf5	Fibroblast growth factor 5
FN	Fibronectin
X g	number of g force units of acceleration
G418	Geneticin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

Gly (G)	Glycine
HCl	Hydrochloric acid
HDFs	Human dermal fibroblasts
HEFs	Human embryonic fibroblasts
His (H)	Histidine
ICM	Inner cell mass
IgG	Immunoglobulin G
IL-6	Interleukin-6
iPS	Induced pluripotent stem cells
kDA	kilodalton
Klf4	Kruppel-like family of transcription factor 4
KO	Knockout
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
MEFs	Mouse embryonic fibroblasts
MEM	Non essential amino acids
ml	Millilitre
μl	Microlitre
mM	Millimolar
mRNA	messenger ribonucleic acid
n	number of samples
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTC	Non template control
O.D.	Optical density
OSM	Oncostatin-M
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHAAA	Mutated pentapeptide of the FIII9 domain
PHSRN	pentapeptide of the FIII9 domain
Pro (P)	Proline

QPCR	Quantitative polymerase chain reaction
Leu (L)	Leucine
RGD	tripeptide of the FIII10 domain
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
Ser (S)	Serine
siRNA	Small interfering ribonucleic acid
Sox2	SRY(sex determining region Y)-box 2
STAT3	Signal transducer and activator of transcription 3
STAT3F	Dominant negative Signal transducer and activator of transcription 3
STAT3ER	Conditionally active signal transducer and activator of transcription 3 fusion protein
TBST	Tris-buffered saline tween-20
TE buffer	Tris-EDTA buffer
Tm	Melting temperature
Tris-HCl	Tris (hydroxymethyl) aminomethane HCl
Th17	T helper 17 cells
TTP	Tail tip fibroblasts
U/ml	Units per millilitre
Undiff control	Undifferentiated ES cell control
VKNEED	hexapeptide of the FIII8 domain

Chapter 1 Introduction

1.1. The identification of embryonic stem cells

Embryonic stem (ES) cells are continually growing cells derived from the blastocyst of the developing embryo that have the ability to generate all three of the primary germ layers ie the mesoderm, endoderm and ectoderm. (Smith 2001, Murry et al 2008). Under the appropriate culture conditions ES cells can undergo self renewal and maintain their full differentiation capacity through prolonged in vitro culture. (Smith 2001, Murry et al 2008). Despite their ability to differentiate into all 3 of the germ layers, as ES cells on their own are unable to result in the generation of a blastocyst and cannot differentiate into certain cell lineages such as the trophoectoderm under normal conditions, ES cells are best described as a pluripotent cell lineage, meaning they have the capacity to differentiate into a wide range of, but not all cell types. (Smith 2001).

The derivation of ES cells was first reported in 1981 (Evans et al 1981, Martin et al 1981) when it was shown that ES cells could be obtained from the inner cell mass (ICM) of the developing embryo (Evans et al 1981, Martin et al 1981). Kaufman and colleagues (Evans et al 1981) overcame the difficulty of isolating the epiblast through the induction of a phase in the developing embryo known as diapause, which occurs before the blastocyst implants itself into the uterine wall (Smith 2001). Though diapause is a physiologically normal process in rodents it can be enforced through the ovariectomisation of pregnant mice (Smith 2001). This delay in implantation enabled Evans et al 1981 to obtain blastocysts from the pregnant mice. Martin et al 1981 identified that ES cells obtained from the ICM could be maintained in an undifferentiated state when co-cultured with feeder cells. The reason for the effective culturing of ES cells with feeder layers was then identified as the actions of a cytokine produced by the feeder cells, namely Leukaemia Inhibitory Factor (LIF) (Smith et al 1988, Williams et al 1988) as discussed later.

14

1.2. Generation of mice chimeras from embryonic stem cells

The most useful characteristic of ES cells in terms of scientific research is the ability to use these cells to generate genetically manipulated mice designed to look at the specific role(s) that any one or multiple genes play in the life of a mammal. Generation of so called "knockout models" can often provide an insight into the function a gene can play in a range of contexts. An example of this within our own lab was the generation of mice deficient in the atypical chemokine receptor D6 that identified the role of D6 in the resolution of inflammation in a model of psoriasis (Jaimeson et al 2005). Today the use of ES cell generated mice is a standard method to test an *in vitro* hypothesis in an animal model that provides a more physiologically relevant insight into the particular aspect one is investigating. If ES cells are cultured appropriately, they maintain the ability to re-integrate into the developing embryo and generate genetically manipulated mice.(Smith 2001)

The initial study that identified the ability of ES cells to produce genetically altered mice was first published in 1984 (Bradley et al 1984). This study examined the ability of pluripotent cells, derived from the fertilized embryo, to generate germ-line chimeras. Through use of blastocyst injection Robertson and colleagues (Bradley et al 1984) demonstrated the ability of ES cells derived from fertilized embryos to contribute to the generation of live mice with no apparent developmental defects. ES cells injected into blastocysts was then investigated in order to identify which tissues in particular ES cells contributed to (Beddington et al 1989). This study subsequently showed that injected ES cells tended to contribute to the formation of the extraembryonic mesoderm and the foetus. ES cells were also capable of inducing the formation of the parietal or visceral endoderm . (Beddington et al 1989). This study concluded that ES cells were reminiscent of the ICM (Beddington et al 1989), which is the current view held today.

One of the more remarkable properties of ES cells is their ability to be maintained as a homogeneous population that is undifferentiated even during sustained culturing (Smith 2001). Providing they are cultured under controlled conditions ES cells can be expanded indefinitely while maintaining their pluripotent characteristics (Smith 2001). It should be noted however that the ability of ES cell populations to remain in an undifferentiated state in long term culture is not a natural property of these cells. ES cells require to be grown in media containing specific nutrients, metabolites and polypeptides to ensure a homogeneous, pluripotent population (reviewed in Smith 2001). In vivo the appearance of cells with ES cell properties is only transient so it is perhaps not surprising that cells derived from this pluripotent population require a certain level of manipulation in order for them to remain in an undifferentiated state (reviewed in Smith 2001).

In the 1970s pioneering EC cell biologists observed the necessity of culturing EC cells on feeder layers of growth arrested embryonic fibroblasts (Martins and Evans 1975). It was several years later that this initial premise was shown to be only partially correct.

In 1988 a landmark discovery was made as it was identified that the cytokine Leukaemia Inhibitory Factor (LIF) was capable of maintaining ES cells in an undifferentiated state (Smith et al 1988, Williams et al 1988). Smith et al 1988 used biochemical purification to identify that LIF was the agent from Buffalo rat liver (BRL) conditioned media responsible for the ES cells retaining their pluripotent state after long term culture. The conditioned medium was then purified to obtain LIF that was found to have a molecular weight of 20,000kDa (Smith et al 1988), which was in agreement with another group that had cloned LIF (Moreau et al 1988). Williams et al 1988 complemented this study by looking for the presence of LIF receptors on the surface of ES cells. After noting the presence of LIF receptors on the ES cell surface this group then confirmed the ability of recombinant LIF to maintain ES cells in a state of self-renewal (Williams et al 1988). They subsequently observed that at concentrations of LIF between 1,000-5,000 units/ml ES cells could

maintain their undifferentiated phenotype (Williams et al 1988). Both human and mouse LIF were tested and both gave similar results. Concentrations of LIF below 1,000U/ml however resulted in a reduced number of undifferentiated colonies. This paper further showed that ES cells maintained long term in the presence of LIF still retained their developmental potential (Williams et al 1988).

Further investigation into the ability of feeder cells to maintain an ES cell's undifferentiated state led to the discovery that LIF is produced by feeder cells in both a diffusible and an extracellular matrix associated form (Rathjen et al 1990). The two forms of LIF are encoded by alternative transcripts that produce active proteins with distinct biological functions. Interestingly the majority of LIF was localised within the extracellular matrix. It appears that ES cells grown on feeders would stimulate the production of diffusible LIF into the medium while in contact with the extracellular matrix associated form of LIF (Rathjen et al 1990).

The role of LIF in vivo was subsequently identified as a requirement in order for the implantation of the blastocyst to occur (Stewart et al 1992). LIF deficient embryos were produced through the generation of LIF knockout ES cells. (Stewart et al 1992). The subsequent protein produced was non functional as feeder cells produced from these embryos failed to maintain ES cells in an undifferentiated state (Stewart et al 1992). Mice generated from these LIF deficient embryos were reduced in weight in comparison to their wild type counterparts and the female LIF deficient mice were unable to implant (Stewart et al 1992). This paper then observed that while the embryos developed to the blastocyst stage LIF produced by the uterine endometrial glands was a requirement in order for blastocyst implantation to occur (Stewart et al 1992).

With the essential role for LIF in ES cell propagation in vitro, and the physiological role in vivo determined, steps were then taken to examine whether other factors could promote ES cell self-renewal. In 1994 Yoshida et al identified that Oncostatin-M (OSM), Ciliary Neurotrophic Factor (CNTF) or Interleukin-6 (IL-6) in combination with soluble IL-6 receptor could inhibit ES cell differentiation. All these cytokines signal through gp130

therefore gp130 was deemed to be essential for ES cell self-renewal. Through use of gp130 receptor deficient mice a role for this receptor in the development of embryos was observed as this mutation was lethal at about day 12.5 postcoitum (Yoshida et al 1996). In particular serious myocardial and haemopoietic defects were observed (Yoshida et al 1996). The function of gp130 signalling in the context of ES cells was further clarified by the finding that gp130 KO embryos cannot continue development after diapause. Gp130 signalling was subsequently found to be required for maintenance of the epiblast (Nichols et al 2001). In parallel to this work a series of studies identified that LIF mediated its effects on ES cells through the LIF receptor complex (Gearing et al 1991, Gearing et al 1992, Davis et al 1993). In addition to these studies potential transcription factor(s) were being examined. In due course it was revealed that the latent transcription factor, Signal transducer and activator of transcription 3 (STAT3) was required for ES cell self renewal (Niwa et al 1998, Matsuda et al 1999). To delineate the role STAT3 has on ES cell function one study made use of supertransfection to transfect ES cells with a dominant negative STAT3 (STAT3F) (Niwa et al 1998). These transfected ES cells were morphologically very similar to differentiated ES cells grown in the absence of LIF (Niwa et al 1998). The role of STAT3 on ES cell self renewal was confirmed by the production of a conditionally active STAT3 fusion protein (STAT3ER) (Matsuda et al 1999). Upon stable transfection of STAT3ER into ES cells it was observed that even in the absence of LIF these ES cells could be maintained in an undifferentiated state (Matsuda et al 1999). Therefore STAT3 was shown to be an essential transcription factor for the maintenance of ES cells in a state of self renewal. It is interesting that STAT3 is involved in the maintenance of ES cell self renewal as it is tends to be involved in the differentiation of a variety of cell types. One such example of STAT3's role in the differentiation of cells is its role in the much hyped, much publicised, new subset of CD4+ T-cells commonly known as Th17s (Stepkowski et al 2008).

1.4. Roles of specific genes in the control of ES cell pluripotency

Although there are extrinsic components essential for the maintenance of ES cell self-renewal, several genes have been identified as playing a major role in the maintenance of ES cells in an undifferentiated state. Arguably the most fundamentally important of these is Oct-4. Oct-4 is a member of the POU transcription factor family and is essential for the

maintenance of ES cell pluripotency (Nichols et al 1998). Oct-4 functions by interacting with the 8 base pair sequence ATGCAAAT, hence Oct meaning octamer (Smith et al 2001).

The role of Oct-4 in ES cells was first identified in 1990. Rosner et al 1990 cloned Oct-4 then tested its ability to function as a transcription factor. This paper showed that mRNA synthesis was upregulated by Oct-4 if stimulated appropriately. Oct-4 mRNA expression was detected in undifferentiated ES cells but importantly found to be lacking in differentiated ES cells (Rosner et al 1990). Oct-4 was then observed to be present in the ICM and trophoectoderm, though at much lower levels at 3.5 days post coitum (dpc) (Rosner et al 1990). Oct-4 mRNA expression in the trophoectoderm was undetectable at 4.5 dpc (Rosner et al 1990). In contrast, mRNA expression of Oct-4 in cells not derived from the trophoectodermal lineage maintained their expression of Oct-4. (Rosner et al 1990). This was clear evidence of differing spatial distribution of Oct-4 expression indicating a specific role in embryo development (Rosner et al 1990). Most importantly this paper noted that all early embryo cells expressing Oct-4 maintained pluripotency, a feature not found in differentiated ES cells (Rosner et al 1990).

These initial studies were taken a step further through use of Oct-4 specific antibodies (Palmieri et al 1994). This showed that Oct-4 protein expression is found in undifferentiated ES cells but not in retinoic acid induced differentiated ES cells. ES cells induced to differentiate by the removal of LIF also showed substantially reduced Oct-4 protein expression compared to undifferentiated ES cells. This was the first evidence that differences found in Oct-4 mRNA expression had consequences for ES cells in terms of functional protein levels. This was also the first report to observe that Oct-4 protein expression increased in the primitive endoderm, and markedly reduced upon trophoectodermal lineage differentiation. This report was an early indication that Oct-4 had a role to play in the lineage commitment of ES cells (Palmieri et al 1994). This was subsequently confirmed by Nichols et al 1998 as discussed below.

19

The evidence suggesting a role for Oct-4 in ES cell pluripotency was firmly established by a comprehensive report involving the use of Oct-4 deficient ES cells (Nichols et al 1998). Oct-4 deficient embryos were unable to produce pups, suggesting a role for Oct-4 in development. In vitro growth of Oct-4 deficient ES cells resulted in a population of trophoblast cells with a complete absence of any discernable ICM (Nichols et al 1998). As ES cell lines are obtained from the ICM of the blastocyst this was strong evidence that Oct-4 was essential in order to maintain an ES cell's pluripotent status.

Shortly after this breakthrough the same group confirmed initial evidence from Palmieri et al 1994 that Oct-4 is closely involved in determining lineage commitment of ES cells (Niwa et al 2000). If Oct-4 expression levels increased or decreased by up to 0.5 fold of normal expression levels, self-renewal would be maintained. However if Oct-4 levels increased between 0.5 fold to just under 2 fold, the ES differentiated into an endodermal and mesodermal population (Niwa et al 2000). Finally if Oct-4 protein levels decreased by more than 50% of normal a trophoectodermal population was formed. Niwa et al 2000 also provided evidence that the self-renewal properties of LIF were not linked to Oct-4 as ES cells with enforced Oct-4 expression still differentiated in the absence of LIF (Niwa et al 2000). The exact molecular mechanisms of Oct-4 in ES cells was further clarified by the finding that both the POU DNA binding domain and either the C-terminal or N-terminal transactivation domains are required to maintain ES cell self renewal (Niwa et al 2002). Mutation of both transactivation domains or the POU DNA binding domain resulted in differentiated ES cell colonies. This paper concluded that the transactivation domain's proline rich properties in conjunction with Oct-4's POU DNA binding domain were both required for ES cell self renewal to be maintained (Niwa et al 2002).

In recent years a second intrinsic factor required to maintain ES cell self-renewal was identified and referred to as Nanog (Chambers et al 2003, Mitsui et al 2003). It is similar to Oct-4 in that this gene codes for a protein which contains a homeodomain. Nanog however is classed as a divergent homeodomain protein as it differs substantially from other well known homeodomain proteins.(Chambers and Smith 2004). It was observed that Nanog was expressed in ES cells but was lacking in other cell lines including hematopoietic, fibroblast and parietal endoderm (Chambers et al 2003). This was a clear indication that

20

whatever Nanog's function, it was confined to pluripotent cells. Indeed, upon ES cell differentiation the levels of Nanog mRNA dropped significantly (Chambers et al 2003). This finding was the first evidence that Nanog may have a role in maintaining the undifferentiated state of ES cells.

As Nanog appeared to have a role in maintaining ES cell pluripotency the relationship between Nanog and LIF mediated gp130 signalling was examined (Chambers et al 2003, Mitsui et al 2003). ES cells overexpressing Nanog were grown in the absence of LIF and examined for evidence of differentiation. It was observed that in the absence of LIF ES cells induced to constitutively express Nanog could maintain their pluripotent state as the ES cells maintained their characteristic undifferentiated morphology and did not flatten out as is common during ES cell differentiation (Chambers et al 2003, Mitsui et al 2003). Even after sustained passaging the ES cells maintained their undifferentiated state. When induced to differentiate through use of chemical inducers such as retinoic acid it was observed that nanog overexpressing ES cells were largely resistant to differentation and maintained ES cell pluripotency (Chambers et al 2003). Wildtype ES cells cultured with the same inducers differentiated extensively in a matter of days. (Chambers et al 2003). However it must be remembered that in wild type ES cells Nanog on its own is not able to prevent ES cell differentiation in the absence of LIF. Nanog must be constitutively expressed in order for LIF impendent suppression of ES cell differentiation to occur. This fact must be taken into account before making any judgement about the possible role(s) of Nanog. It was also observed that the activation of STAT3 was unaltered during passaging indicating that Nanog could prevent ES cell differentiation by a pathway that was not reliant on LIF mediated-gp130 signalling (Chambers et al 2003, Mitsui et al 2003).

To gain further insights into the apparent role of Nanog in the pluripotency of ES cells Nanog deficient ES cells were generated (Mitsui et al 2003). Upon disruption of Nanog the levels of Oct-4 and rex1(another pluripotency marker but not discussed further) were significantly lower compared to wild type ES cells. In addition Nanog deficient ES cells were observed to upregulate several endodermal markers including both visceral and parietal endodermal markers as well as endodermal transcription factors (Mitsui et al 2003). These data were clear evidence that Nanog had a role in maintaining ES cell self-

renewal and preventing unrestricted differentiation into endodermal tissues (Mitsui et al 2003). The generation of Nanog null embryos gave an indication of the in vivo role for Nanog (Mitsui et al 2003). These Nanog null embryos lacked any discernable epiblast and the ICM obtained from these embryos was unable to remain in an undifferentiated state and very quickly differentiated into endodermal tissue (Mitsui et al 2003).

From these initial studies (Chambers et al 2003, Mitsui et al 2003) it was clear that Nanog is a vital intrinsic factor required to maintain the pluripotency of ES cells. It is interesting when one considers the functions of Oct-4 and Nanog as both have differing phenotypes when overexpressed or when their function is disrupted. Overexpression of Oct-4 results in endodermal/mesodermal differentiation (Niwa et al 2000) whereas constitutive expression of Nanog results in LIF-independent ES cell self-renewal (Chambers et al 2003, Mitsui et al 2003). It may be that a function of Nanog is to counteract the pro-differentiation properties associated with increased levels of Oct-4. If Oct-4 gene expression is disrupted then ES cells will differentiate into trophoectodermal cells (Niwa et al 2000) in contrast to Nanog null cells that will form an endodermal population (Chambers et al 2003, Mitsui et al 2003). The effects of Oct-4 and Nanog in addition to LIF mediated signaling are detailed in figure 1.1. below
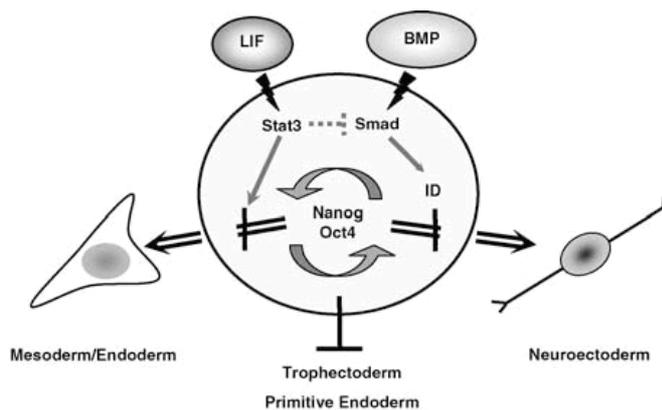
Figure 1.1. Signalling mechanisms of ES cell self renewal. Expression of Oct-4 and Nanog ensures ES cell pluripotency is maintained. In addition, BMP in combination with LIF act together to inhibit ES cell differentiation. BMPs induces the production of Id's to inhibit neural differentiation. LIF prevents non-neural differentiation through activation of STAT3. (Chambers and Smith 2004)

22

It is clear therefore that both transcription factors are required to inhibit ES cell differentiation into different lineages but they do so independently of each other. It could be argued that this adds security to the maintenance of ES cell pluripotency by having several mechanisms by which pluripotency is maintained. In terms of a physiological role, these genes could aid in the differentiation of ES cells into specific lineages during development. For example, ES cells required to differentiate into a trophoectodermal population of cells would inhibit their expression of Oct-4 thus allowing differentiation into this lineage. Similarly ES cells could produce an endodermal population if Nanog expression was inactivated.

Our understanding of Nanog's function has recently been updated by the finding that, in direct contrast to previous reports (Chambers et al 2003, Mitsui et al 2003), Nanog does not appear to be essential in order maintain the pluripotency of ES cells. Upon excision of the Nanog gene it was observed that all Nanog-/- cell lines remained undifferentiated as shown by their ability to form alkaline phosphatase positive colonies if grown in the presence of LIF (Chambers et al 2007). This is in contrast to previous data suggesting that Nanog null embryos lost their pluripotent state (Mitsui et al 2003). This finding also provided evidence that LIF mediated ES cell self-renewal is not dependent on Nanog (Chambers et al 2007) which would confirm earlier suspicions that the two mechanisms are independent of each other (Chambers and Smith 2004). This study did note however that upon differentiation of Nanog null ES cells there was an increase in endodermal markers eg GATA4 and GATA6 in comparison to wild type ES cells (Chambers et al 2007). This is evidence to confirm the hypothesis that one function of Nanog is to prevent differentiation of ES cells into an endodermal population (Chambers and Smith 2004).

This confirmed a potential role for Nanog in the inhibition of endodermal differentiation suggested in previous studies (Mitsui et al 2003). It was also shown in this study that Nanog was not required for ES cells to be integrated into the epiblast (Chambers et al 2007). This study concluded by suggesting that Nanog's main function is to result in germ cell production and to try to prevent a loss of pluripotency by downregulation of genes that would otherwise cause ES cells to differentiate (Chambers et al 2007). However Austin

23

Smith and colleagues maintained their view that Nanog was not essential to maintain ES cell pluripotency as although ES cells did have a greater tendency to differentiate in the absence of Nanog, ES cells could still maintain their undifferentiated state (Chambers et al 2007).

Two papers published this year have given us a greater understanding of the function of Nanog by reporting that Nanog must homodimerize in order for ES self-self renewal to be maintained (Wang et al, 2008, Mullin 2008 et al). It was identified that a tryptophan rich region of the C-terminal domain was essential for Nanog homodimerization to occur (Wang et al 2008, Mullin et al 2008). It is interesting to consider the requirement for the C-terminal domain in Nanog function when one considers that Oct-4 can make use of either its C-terminal or N-terminal domain in order to maintain its function as discussed previously (Niwa et al 2002). It is possible that the function of Nanog is more strictly controlled than Oct-4.

Although there is much evidence to suggest that Oct-4 and Nanog have differing functions in ES cell biology as highlighted by the differing phenotypes of ES cells over expressing or deficient for the above genes there is increasing evidence to suggest they may cooperate to repress ES cell differentiation (Loh et al 2006, Liang et al 2008). Through use of a variety of screening techniques it was suggested that Nanog in fact may control the expression levels of Oct-4 and Sox2, another marker of pluripotency(Loh et al 2006) and that Oct-4 and Nanog will bind to one another then form interactions with complexes of repressor proteins thus controlling ES cell function (Liang et al 2008). Additionally Nanog has been reported to cooperate with STAT3 to ensure ES cell self-renewal providing a link between two pathways that were previously thought to be distinct (Torres et al 2008). This paper also observed that Nanog would inhibit NFκB signalling which promotes ES cell differentiation (Torres et al 2008). It seems that the multiple mechanisms of ES cell self-renewal, both intrinsic and extrinsic, may turn out to be more functionally related than first thought. Further research into this area will undoubtedly provide more fascinating insights into the maintenance of ES cell pluripotency.

1.5. Induced pluripotent stem (iPS) cells – A new possibility for ES cells in regenerative medicine

As ES cells have the potential to differentiate into almost any cell lineage coded for in the mammalian genome the possibility of harnessing this ability has often being cited as vindication for the study of human ES cells. Spinal cord injuries, diabetes and parkinson's disease are a few of the human ailments that could be treated through use of human ES cells. However as human ES cells, just like murine ES cells, are derived from the blastocyst of an embryo there have been several moral and ethical objections from leading religious and political figures to the use of human embryos for any kind of research (Jaenisch et al 2004). In addition the recent human-animal hybrid proposal has also been met with similar opposition (Karpowicz et al 2004, Hyun et al 2007). Therefore studies were carried out in order to determine whether the useful properties of ES cells could be generated from other sources.

In 2006 it was shown that cells with strikingly similar properties to ES cells could be generated from somatically derived cell lines. The initial pioneering study carried out by Yamanaka and co-workers provided strong evidence that differentiated cell lines could in fact be re-programmed resulting in a reversion to the cell's previously pluripotent state (Takahashi et al 2006). This study carried out an initial screen of potential genes to be used in reverting mouse embryonic or adult fibroblast cultures and identified 24 as possible factors that could play a role in this.

To identify which of those genes could be used to return the above cell lines to a pluripotent state a novel assay was designed. This involved the insertion of a β-galactosidase and neomycin resistance gene fusion product through homologous recombination into a gene expressed in mouse ES cells but not in differentiated cells, Fbx15 (Takahashi et al 2006). These Fbx15-knockin ES cells were found to be unaffected even in the presence of high concentrations of G418. However differentiated somatic cells derived from Fbx15-knockin cells were unable to survive in standard concentrations of G418. This confirmed that only ES cells expressing the above construct could survive in G418 conditions. Then through use of retroviral transduction the genes highlighted to have

a role in ES cell pluripotency were introduced into Fbx15-knockin generated mouse embryonic fibroblasts (MEFs).

Upon growth in G418 none of the ES cells with individual genes introduced survived, clear evidence that it would take more than one individual gene in order to return the MEFs to an ES cell like pluripotent state (Takahashi et al 2006). Upon the retroviral transduction of all 24 potential genes colonies that could grow in the presence of G418 were subsequently observed. These cells resembled ES cells in terms of expression of ES cell genes and also cell doubling times. These initial 24 genes were reduced through individual withdrawal trials down to 4 genes deemed essential in order for ES pluripotency to be reactivated (Takahashi K et al 2006). These genes were Oct-4, Sox2, Klf4 and c-Myc. Removal of any of these retrovirally induced genes resulted in a massive reduction in the number of ES cell like colonies observed in the presence of G418 (Takahashi et al 2006).

Yamanaka and co-workers (Takahashi et al 2006) then introduced the four genes deemed essential for iPS derivation into tail tip fibroblasts (TTP) and again were able to derive colonies that could resist G418 conditions. This confirmed that this technique could be used on somatic tissues as opposed to embryonic cell lines. These iPS cells were observed to have similar though not identical expression levels of genes compared to ES cells and were also able to generate mesodermal, endodermal and ectodermal tissue (Takahashi et al 2006). In addition these iPS cells were found to stain positively for alkaline phosphatase, and were also unable to maintain their apparent pluripotent state in the absence of feeder cells even in the presence of LIF (Takahashi et al 2006). The last point is interesting and highlights a difference between iPS cells and ES cells as ES cells can be maintained in an undifferentiated state in the absence of feeders in the presence of LIF (Smith et al 2001). Therefore iPS cells appear very similar in many ways to ES cells but it seems that there may be subtle differences that must be investigated further.

Shortly after the initial publication by Yamanaka and coworkers (Takahashi et al 2006) similar studies confirmed their findings that retroviral transduction of Oct-4, c-Myc, Klf4 and Sox2 could return a somatic cell line such as TTPs to a pluripotent state (Okita et al

26

2007, Wernig et al 2007). Both used similar approaches to the initial publication (Takahashi et al 2006) but instead of selecting for Fbx15 these studies used either Nanog (Okita et al 2007) or Nanog or Oct-4 (Wernig et al 2007). These studies (Okita et al 2007, Wernig et al 2007) confirmed many of the findings from Yamanaka and coworkers paper (Takahashi et al 2006) but also highlighted minor differences between the iPS cells generated using different protocols. In particular it was noted that use of Nanog to select for the iPS cells resulted in the production of iPS cells that could be maintained in an undifferentiated state in the presence of LIF even if not co-cultured with feeder cells (Okita et al 2007, Wernig et al 2007).

This differed from iPS cells selected for using Fbx15 that required both culturing with feeder cells and the presence of LIF in the media (Takahashi et al 2006). In one of these papers a note of caution was issued regarding the use of iPS cells for regenerative therapies due to the possibility that use of retroviral transduction could inadvertently lead to upregulation of oncogenes and the onset of cancers (Wernig et al 2007). This study also suggested that a greater understanding of the exact pathways of somatic cell reprogramming at the molecular level would be advantageous and could lead to the identification of molecules that could reprogram cells without the need for retroviral transduction (Wernig et al 2007).

The potential uses of iPS cells in treating human disease are obvious, with the possibility of generating patient specific tissues that would negate the possibility of immune rejection as is often seen using conventional therapies. Shortly after the discovery of iPS cell production using mouse cell lines it was shown by several groups that this could also be achieved using human cell lines (Takahashi et al 2007, Yu et al 2007, Park et al 2007, Lowry et al 2008). Three of these papers used induced ectopic expression of the four genes described previously (Takahashi et al 2006) as being essential for the programming of mouse iPS cells in human cell lines including human embryonic fibroblasts (HEFs) and adult human dermal fibroblasts (HDF) (Takahashi et al 2007, Park et al 2007, Lowry et al 2008). As in murine studies this resulted in a reprogramming of the cells to a more pluripotent phenotype in terms of their function and morphology. These reprogrammed

27

cells were subsequently confirmed as human iPS cells through a variety of tests (Takahashi et al 2007, Park et al 2007, Lowry et al 2008).

The fourth paper published during this initial phase of human iPS work by James A Thomson and colleagues showed that another combination of genes could be used to generate iPS, specifically Oct-4, Sox2, Nanog and LIN28 (Yu et al 2007). The iPS cells derived from this process however seemed to fit all the criteria required to be classed as iPS cells (Yu et al 2007). One serious problem that could hold back the use of iPS cells in regenerative therapies was the tumour formation observed in some mice through reactivation of the c-Myc retrovirus. (Okita et al 2007). A modified protocol was therefore used to generate iPS cells that omitted the use of c-Myc in reprogramming somatic cells into iPS cells (Nakagawa et al 2008). The iPS cells generated displayed all the characteristics of iPS cells produced through use of the c-Myc retrovirus but did not result in tumour formation when used to generate mice. It was noted however that without the c-Myc retrovirus there was a marked reduction in the ability to generate iPS cells (Nakagawa et al 2008).

The issue of retroviral reactivation may have been solved with the recent report that iPS cells could potentially be generated without the use of this technique. In another report from Yamanaka and co-workers (Aoi et al 2008) it was shown that the generation of iPS cells from liver cells and stomach cells have no requirement for site specific retroviral integration. This opens up the possibility that iPS cells could be generated without the use of a mechanism now well known to result in the formation of tumours (Aoi et al 2008). iPS formation has also been reported through the reprogramming of adult neural stem cells, the first publication using an ectodermally derived cell line to do so (Kim et al 2008). This study showed that retroviral transduction of Oct-4 and Klf4 was sufficient to generate iPS cells from neural stem cells (Kim et al 2008). This study confirmed the observation of a previous report (Nakagawa et al 2008) which observed that iPS cells could be generated without the use of the c-Myc retrovirus therefore reducing the chances of spontaneous tumor formation (Kim et al 2008).

It is clear that iPS cells have the potential to do a great deal for regenerative medicine. With iPS cells the moral and ethical issues that come with the use of human ES cells would

28

largely be avoided. In addition the potential for immunorejection would be addressed as iPS cells could be produced from the recipient's own somatic tissue. It must be noted however that although there is much enthusiasm in the scientific community about the potential uses of these cells this enthusiasm must be tempered with a measure of scepticism and thorough study to ensure that iPS cells are indeed the exceptionally useful cells they appear to be. In particular the possibility of tumorigenicity must be thoroughly investigated, if possible a different type of gene transfer system should be used due to the possibility of retroviral mediated tumour formation.

1.6. Fibronectin – A multi-functional protein

Fibronectin (FN) is a protein that has several functions in vivo. Of particular importance is the role it plays in the development of embryos. Deletion of FN from mouse embryos resulted in the death of the embryo clearly demonstrating an essential role in the early stages of development (George et al 1993). It has also been shown that, during development, embryos upregulate genes involved in the production of FN, therefore they have the ability to manufacture FN in a cell autonomous manner (Shirai et al 2005). FN tends to be found in a dimeric form in vivo consisting of two subunits of approximately 250kDa each that are linked via a covalent interaction close to the C-terminus (Pankov and Yamanaka 2002). The FN subunits are composed of 12 type I, 2 type II and between 15-17 type III repeating units. Not only are type III repeating units the most numerous of the repeating units they are also the largest being measured at approximately 90 residues each (Pankov and Yamanaka 2002). The type III repeating units also lack any disufide interactions which differs from type I and type II repeating units. Type II repeating units contain two intra-chain disulfide bonds and are estimated at 60 amino acids long and type I repeating units also contain two disulfide bonds and are believed to be 40 amino acids long (Pankov and Yamanaka 2002).

 The function of FN is mediated via interactions with members of a family of cell surface receptors known as integrins that are described in greater detail further on in the introduction. In brief integrins are heterodimeric receptors composed of two different subunits that are involved in mediating the interaction of the intracellular cytoskeleton with the extracellular matrix (ECM) (Hynes 2002). FN can bind to 12 separate integrins and

appears to be the most promiscuous adhesion molecule identified to date in terms of integrin ligation (reviewed in Plow et al 2000). This ability to interact with multiple integrins implies that FN is involved in many different interactions in vivo therefore it is perhaps not surprising that disruption of the gene coding for FN is embryonic lethal. (Hynes 2002).

Much study has been done to identify the sequences in FN essential for binding to integrins. The sequence that has received the most attention resides within the 10th type III FN (FIII10) domain and is a short sequence of amino acids, Arg-Gly-Asp (RGD). This small tripeptide sequence was identified after initial studies into the adhesive properties of FN localized the cell binding region of the FN monomers to a specific region of the protein (Ruoslahti et al 1981, Ehrismann et al 1982). Peptides were then synthesized spanning the region responsible for cell attachment and one area in particular was identified as essential in order for cell adhesion to occur (Pierschbacher et al 1983). Further analysis pinpointed a sequence of four amino acids Arg-Gly-Asp-Ser as being responsible for the adhesive properties of FN (Pierschbacher et al 1984). A follow up study revealed that while the serine residue was dispensable the ability to adhere cells was lost upon substitution of the arginine, glycine or aspartic acid residues (Pierschbacher MD et al 1984).

It was then recognized that while the FIII10 domain is essential for integrin binding another sequence, a so called "synergy" site, was required for optimal binding to the classical FN receptor $\alpha5\beta1$(obara et al 1988, Nagai et al 1991). Although cell adhesion to FIII10 could occur it was reduced in comparison to whole FN (Mardon and Grant 1994). Soon after these reports the synergy sequence Pro-His-Ser-Arg-Asn(PHSRN) was shown to reside on the 9th type III FN domain (FIII9) (Aota et al 1994). This paper made the intriguing observation that the amino acid most critical for the function of the synergy site was arginine in position 1379 of the sequence. As the RGD sequence on the FIII10 domain also contains an arginine it is possible the arginine residues in these positions serve some essential role in terms of integrin binding.

It was observed in another study that the FIII9 domain did not adhere cells very well and required attachment to the FIII10 domain in order to mimic the adhesive properties shown by whole FN (Mardon and Grant 1994). In addition, cells grown on FIII10 alone tended to be rounder in terms of morphology in contrast to cells grown on FIII9-FIII10 domains that had a more spread morphology similar to cells grown on whole FN (Mardon and Grant 1994). These differences in morphology raised the possibility that the FIII9 and FIII10 domains carry out distinct functions in cell adhesion.

As both the FIII9 and FIII10 domain sequences are found on a loop it was suggested that the exact spatial relationship between the FIII9 and FIII10 domains was important in order for effective ligation to integrin receptors to occur. This hypothesis was confirmed in 1997 by a study showing that if the region linking the FIII9 and FIII10 domains was increased by as little as two amino acids the ability of the FIII9-FIII10 domain to induce cell spreading was markedly decreased (Grant et al 1997). In addition signaling via FAK phosphorylation was substantially reduced. Increasing the linker region also led to a reduction in FIII9-FIII10 domain's cell adhesion ability but to a lesser extent than cell spreading. This study concluded that the exact structural conformation of FIII9-FIII10 domain was essential in order for optimal cell adhesion/spreading and signaling mechanisms to occur (Grant et al 1997). Although FIII10 can adhere cells in the absence of FIII9, albeit not as efficiently as whole FN, it is clear that for events downstream of the initial cell attachment to occur, the spatial relationships between the integrin and FIII9-FIII10 domain must be precise (Grant et al 1997).

Further study of the interaction of FN with the $\alpha5\beta1$ integrin receptor led to the finding that another sequence could enhance the binding of FN to cells by stabilizing the FIII9 domain (Altroff et al 2001). The sequence VKNEED on the eighth type III Fn domain (FIII8) was identified to be responsible for stabilizing the FIII9 domain. An FIII8-FIII9-FIII10 peptide was shown to have greater cell adhesive and cell spreading properties compared with FIII9-FIII10 peptides (Altroff et al 2001). This study also noted that either the FIII8 domain on its own or the FIII8 domain attached to the FIII9 domain (FIII8-FIII9) had no cell attachment potential demonstrating the necessity of the FIII10 domain for cell adhesion to occur (Altroff et al 2001). The FIII8 domain was also shown to restore the

31

biological activity of FIII9-FIII10 peptides that had their PHSRN sequence mutated to PHAAA (Altroff et al 2001). This mutagenesis would otherwise result in a loss of the biological activity of the FIII9-FIII10 peptide providing evidence to suggest that the synergy site is only essential when using a FIII9-FIII10 peptide (Altroff et al 2001).

One issue that became apparent during the study and design of these FIII9-FIII10 peptides was the instability of the FIII9 domain even when attached to the FIII10 domain. In order to investigate this matter further, a number of different FIII9-FIII10 domains were constructed with mutations in their sequence to determine whether it was possible to enhance the stability of these FIII9-FIII10 peptides (Walle et al 2002). This study determined that a substitution mutation of a Leucine residue, at position 1408 in the sequence, with a Proline residue improved the stability of the FIII9 domain. It was deduced that this mutation inhibited the ability of the FIII9 domain to unfold therefore improving its conformational stability (Walle et al 2002). It was then ascertained that this mutation conferred greater cell adhesion/spreading ability on the FIII9-FIII10 peptides compared to wild type FIII9-FIII10 domains (Walle et al 2002). The structure of the FIII9-FIII10 cell adhesion domains is shown below.
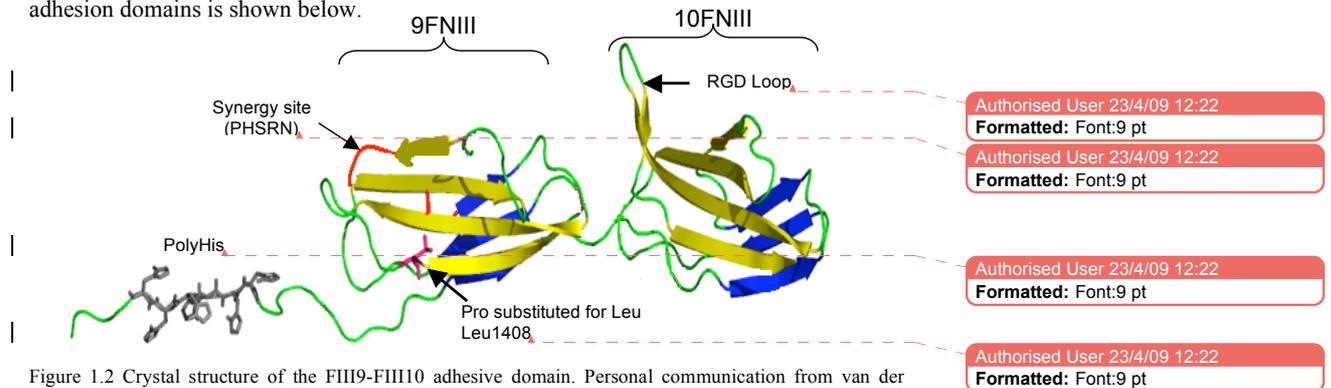
Figure 1.2 Crystal structure of the FIII9-FIII10 adhesive domain. Personal communication from van der Walle and colleagues.

The role of the stability of the FIII9-FIII10 domains in terms of the peptides biological functionality was dissected in more detail through mutation of the synergy site in conjunction with the leucine to proline mutation at position 1408 (Altroff et al 2003). This study first confirmed earlier findings that a leucine to proline mutation at position 1408 in the sequence increased the cell adhesion and cell spreading ability of the FIII9-FIII10

peptide compared to wild type FIII9-FIII10 peptides (Walle et al 2002, Altroff et al 2003). FIII9-FIII10 domains with mutations in this region quickly experienced a marked reduction in their cell adhesion potential and in particular their cell spreading ability (Altroff et al 2003). However the ability of these FIII9-FIII10 mutants to promote $\alpha5\beta1$ meditated cell adhesion and spreading returned to wild type levels upon mutation of Leucine to Proline at position 1408 in the sequence highlighting the potential of this mutation to enhance the biological activity of the FIII9-FIII10 domain (Altroff et al 2003). The leucine to proline substitution was also shown to stabilize the structure of the FIII9 domain when mutated in the synergy site (Altroff et al 2003).

The advantages of using FIII9-FIII10 peptides with a substitution mutation of leucine 1408 to proline for cell attachment are clear and in 2008 a study was published by our collaborators using these very peptides (Kreiner et al 2008). This study used the mutant FIII9-FIII10 domain described previously (Walle et al 2002) attached to a hinge motif taken from an IgG molecule and a mutated zipper derived from leucine amino acids (Kreiner et al 2008). The helices were used to allow the construction of dimeric, trimeric and tetrameric peptides to determine the optimal conformation required for cell attachment (Kreiner et al 2008).

A
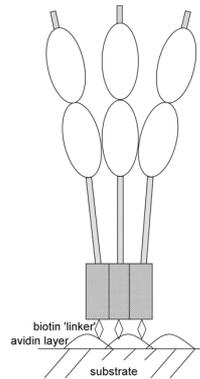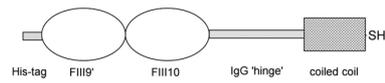
B

Figure 1.3 A. Diagram of FIII9-FIII10 monomer B. Schematic representation of 2-D polyvalent display utilizing the FIII9-FIII10 subunit. Kreiner et al 2008 and BBSRC grant proposal awarded to van der Walle CF, Mardon HJ, Graham GJ 2007.

To allow control over the assembly of the mutant FIII9-FIII10 domains, the chimera's C-terminal cysteine was biotinylated to ensure the chimera's became attached to tissue culture plastic coated with streptavidin. This resulted in the generation of 2-dimensional (2-D) polyvalent displays (Kreiner et al 2008). The peptides generated did not contain the FIII8 domain, as although this sequence does provide enhanced cell attachment/spreading potential compared with FIII9-FIII10 peptides, it was far harder to generate therefore it was more economically sound to use the FIII9-FIII10 peptides as the properties of both peptides appear very similar. (Van der Walle personal communication).

These studies have shown FN to be a useful protein for cell attachment and cell spreading studies. The FIII9-FIII10 peptides used by Van der Walle and co-workers (Kreiner et al 2008) have shown that this particular sequence of FN is sufficient for the attachment of cells expressing the $\alpha5\beta1$ integrin receptor. These studies on the precise requirements for cell attachment are important as they could improve the efficiency of cell adhesion

34

therefore use of these peptides could be a more financially sound method of attaching cells as opposed to using standard FN.

This concludes the discussion of the structural determinants required for FN's adhesive properties. The next section will focus on integrin receptors and their role in mediating the effects of FN in the biological context.

1.7. Integrins – The mediators of fibronectin function

The effects of adhesion molecules such as FN are mediated through their interaction with integrins, a family of cell adhesion receptors (Hynes et al 2002, Humphries et al 2006). Integrins are glycoproteins composed of two different subunits, termed α and β subunits which are linked via a non covalent interaction. The α and β subunits are composed of relatively small cytoplasmic domains in comparison to their much larger extracellular domains and both subunits are type I transmembrane proteins (Hynes et al 2002, Humphries et al 2006). Currently there are 24 different combinations of the 18 α and 8 β subunits that can lead to functional integrin receptors with some subunits such as the αV subunit and the β1 subunit appearing in 5 and 12 different integrin receptors respectively (Hynes 2002, Humphries et al 2006). Some integrin subunits however, such as the α1 subunit, are only found in combination with one other specific subunit, in this case the β1 subunit (Hynes 2002, Humphries et al 2006). Integrins have been known for some time to play key roles in a number of different physiological functions such as the movement of leukocytes and the development of embryos. In addition integrin receptors have been linked to the development of cancer and several other human ailments and are also used to deliver anti-inflammatory drugs (Hynes 2002). It has also been noted that integrin receptors can be used by a number of bacterial and viral agents in order to gain access to the host (Hynes 2002).

Due to the number of different roles that integrins can participate in, the focus of the next section will be on integrins in the context of cell attachment studies, as discussed

previously, and their role in developmental biology, as these subject areas most closely relate to the project described later in the thesis.

In order to investigate the role(s) the α5β1 integrin receptor has in the development of embryos, α5 deficient embryos were generated (Yang et al 1993). As α5β1 is one of the primary integrin receptors ligated by FN it should provide some insights into the importance of this interaction in vivo. The result was that disruption of α5 led to embryonic lethality, suggesting that it played some essential role in the development of embryos (Yang et al 1993). Defects in embryo formation were apparent from days 8.5 of gestation onwards with death occurring between 10 to 11 days of gestation.

In α5 deficient embryos a host of abnormalities were observed such as, neural tube and mesodermal defects, and also extraembryonic and vascular irregularities indicating a fundamental role of the α5β1 integrin receptor in development from the 8th day of gestation onwards (Yang et al 1993). However this study highlighted the functional redundancy of integrins as ES cells obtained from α5 null embryos retained the ability to migrate on FN (Yang et al 1993). This was an unusual finding as it was previously believed that this ability was exclusively due to the interaction of FN with α5β1 integrin receptor. It could therefore be concluded from this study that, while the integrin α5β1 plays a key role in the development of embryos, its ability to migrate on FN could be carried out by other integrins (Yang et al 1993). These data were early evidence of the now well known functional redundancy of certain integrins. In hindsight this may not come as a total surprise as there are several examples of receptor families in biology that will mediate similar effects through several receptors. A good example of such a receptor family is the chemokine receptor family (Rot A and von Adrian 2004).

This study was complimented by another looking at the effect of inactivating the gene encoding FN on embryo development. Just as with α5 deficient embryos the inactivation of FN resulted in early embryonic lethality (George et al 1993). In another striking similarity to the previously described study (Yang et al 1993), abnormalities in FN null embryos were first observed on day 8 of gestation. However substantial embryoid death was occurring at this timepoint (George et al 1993, Georges-Labouesse et al 1996). This

36

accelerated death of the embryos upon deletion of FN showed that FN plays a more integral role in embryo development than the α5β1 integrin receptor (George et al 1993, George Labouesse et al 1996). This was further evidence that integrins other than α5β1 could be involved in embryo development and that signaling of FN via α5β1 could be partially compensated with other integrins. α5β1 however remains crucial to the development of embryos due to the observed embryonic lethality upon α5 deletion (George et al 1993). This study also concurred with the observations by Yang et al 1993 in that defects in mesodermal development and vasculature and extraembryonic abnormalities were all observed (George et al 1993). Interestingly this study observed that FN null embryos successfully carried out implantation and began the process of gastrulation but noted that irregularities in development were first observable after the onset of gastrulation (George et al 1993).

Another study provided strong evidence that FN null embryos die due to the essential role that FN plays in the formation of blood vessels and the embryonic heart (George et al 1997). This observation is compatible with the previous studies (Yang et al 1993, George et al 1993) as heart tissue is a mesodermally derived. The subtle roles for various integrins capable of binding to FN in embryo development was then extensively dissected through a series of single and double integrin gene inactivations (Yang et al 1999). It was subsequently elucidated that, in general, integrins that bind to FN during embryonic development each play a unique, subtle role in the developmental process although there is a degree of functional overlap between integrin receptors (Yang et al 1999). The role of the FN=α5β1 interaction was further clarified by a study that provided strong evidence that the FN binding to α5β1 was essential in order for early angiogenesis and vasculogenesis to occur (Francis et al 2002). These studies have clearly shown an essential role for the interaction of FN with the α5β1 integrin receptor in early embryo development. Both FN and α5β1 integrin receptor appear essential for this process as functional deletion of either results in embryonic lethality (Yang et al 1993, George et al 1993).

37

1.8. Project outline

The purpose of this project was to examine the effects of the FIII9-FIII10 domains described in Kreiner et al 2008 on ES cell function and morphology. The following experiments will be carried out in this study,

1. ES cells will be grown in a monolayer on tissue culture plates coated with the FIII9-FIII10 peptides. In particular the optimal conformation of the FIII9-FIII10 peptides (ie monomer, dimer, trimer, tetramer) shall be investigated, then its ability to adhere cells compared to whole fibronectin and gelatin controls determined.

2. The ability of the FIII9-FIII10 derivative's ability to induce ES cell proliferation will be examined by growing ES cells on either the FIII9-FIII10 derivatives or gelatin then counting the number of cells.

3. A LIF titration will be carried out to look at the effects of altering concentrations of LIF on the possible effects the FIII9-FIII10 derivatives have on ES cells.

4.The differentiation potential of the FIII9-FIII10 peptides will be examined through use of alkaline phosphatase staining, a common marker of undifferentiated ES cells (Jackson et al 2002).

5.Finally Quantitative Polymerase Chain Reaction (QPCR) will be carried out in order to look more closely at any morphological changes that may be occurring with the reasoning that if there is a phenotypical change it is possible that the transcript levels may be altered.

This method of investigating the properties of various stimuli on ES cells differs from traditional work in that the ES cells will be grown in a monolayer as opposed to the generation of embryoid bodies. There is some evidence that ES cells can differentiate down a particular lineage upon growth in a 2-D environment (Nishikawa et al 1998, Ying et al 2003, Hayashi et al 2007) without the requirement of embryoid body formation. The ability to separate out specific precursor lineages using monolayer ES cells gives some advantages to this method of ES cell growth over embryoid body formation. There is some

evidence that differentiation into certain lineages is actually more optimal using monolayer cultures as opposed to embryoid bodies (Nishikawa 1998).

Chapter 2 Materials and methods

Tissue culture

2.1. Embryonic stem cell culture

ES cell line E14 was cultured in tissue culture flasks in knockout DMEM media to which
was added 15% ES-tested foetal bovine serum (STEM CELL TECHNOLOGIES), 2mM
L-glutamine (Invitrogen), 2mM penicillin/streptomycin (Invitrogen), β-mercaptoethanol
(SIGMA-ALDRICH), 1000units/ml LIF/ESGRO (CHEMICON INTERNATIONAL), non
essential amino acids (GIBCO) and gentomycin (SIGMA). These ES cells were split when
confluency reached approximately 70%. This media is referred to as "complete media" in
the text. ES cells were fed daily. ES cells allowed to differentiate using serum replacement
media for QPCR were cultured in media containing; Knockout Serum Replacement for ES
Cell Culture (Invitrogen), 2mM L-glutamine (Invitrogen), 2mM penicillin/streptomycin
(Invitrogen), β-mercaptoethanol (SIGMA-ALDRICH), non essential amino acids (GIBCO)
and gentomycin (SIGMA). ES cells were fed daily.

2.2. Coating six-well plates with whole FN or FIII9-FIII10 peptides

The same method was used to coat six-well plates whether whole FN (SIGMA, 0.1%
solution, from bovine plasma) or FIII9-FIII10 peptides (provided by Van der Walle and
colleagues, University of Strathclyde) were used. In brief, whole FN or FIII9-FIII10
peptides were diluted to the appropriate concentration required using protein buffer which
was composed of the following; 300mM NaCl, 10mM Na_2HPO_4. The protein buffer was
adjusted to pH7, then filter sterilized. Upon dilution of the stock FIII9-FIII10
derivative/whole FN solution with protein buffer the solution is once again filter sterilized.
The diluted FIII9-FIII10 peptides/whole FN were then coated onto the six-well plate and
left to incubate on a shaking platform to allow uniform coating of the plate surface. After
40 minutes to allow attachment of FIII9-FIII10 peptides/whole FN the FIII9-FIII10
peptide/whole FN was removed from the plate and three 5-minute washes were carried out

using wash buffer (300mM NaCl, 10mM Na_2HPO_4 , 0.05% tween solution adjusted to pH7.8 then filter sterilized). The appropriate number of cells were then seeded and left to incubate for the appropriate period of time depending on the desired time point being tested. The media in the cultures was changed daily.

2.3. Giemsa staining

Giemsa concentrate (BDH Laboratory Supplies) was diluted with water 1 in 10 to obtain a 10% giemsa solution. Cells to be stained first had their media aspirated following which they were washed with PBS. The ES cells were then fixed with methanol (Riedel-de Haen) for ten minutes, the fixative removed and the cells allowed to air dry for 30 minutes. 10% giemsa stain was then added to the cells and incubated for 10 minutes. The giemsa stain was then displaced from the ES cells by placing the giemsa stained cells under slow running water. After 10 minutes displacement of the giemsa stain the cells were left to dry. Once dry the cells were mounted on glass slides.

2.4.a Colony counting method

Colonies were counted by taking counts of 10 fields of view of the microscope using a 5x magnification. Care was taken to avoid the peripheral edges of the plate as a disproportionate number of colonies are found in this area. (unpublished observations).

2.4b Colony counting method-cloning efficiency

Colonies were stained using giemsa after 6 days using protocol 2.3. Then the total numbers of colonies were counted by dotting the colonies on the reverse side of the six well plate with a marker pen. Cloning efficiency was then calculated using the following equation, Number of colonies counted/Number of cells seeded per well x 100.

2.5. Alkaline phosphatase staining

Alkaline phosphatase (CHEMICON INTERNATIONAL) stain was carried out in accordance with instructions included in the kit. Briefly, ES cells were grown on six-well plates coated in either gelatin or FN peptide for five days. ES cells were cultured either with complete media containing LIF or media with all the constituents required for ES cell culture with the exception of LIF. After five days culturing the media was removed and the ES cells fixed with 4% paraformaldehyde. Fixative was left on ES cells for no longer than 1 minute as over fixing results in a loss of alkaline phosphatase stain. Fixative was removed and the ES cells washed with 1x rinse buffer (TBST: 20mM Tris-HCl, pH 7.4, 0.15M NaCl, 0.05% Tween-20). Alkaline phosphatase stain (constituents provided in kit, require to be made up before hand) was then added to the wells, (2ml/well). Wells were then incubated in the dark for 10 minutes. Alkaline phosphatase staining solution was then removed and ES cells rinsed with 1x rinse buffer. PBS is finally added to prevent the stained cells from drying out. Colonies were counted using the method outlined in section 2.4.a. The percentage of alkaline phosphatase positive and negative ES cells was then calculated.

2.6. Generation of 2-D polyvalent displays

100μl of avidin (SIGMA) solution (25μg/ml in PBS) was added to each well then left to incubate overnight at room temperature. The avidin solution was then aspirated and 3-five minute washes with wash buffer carried out. 200μl of 1% Pluronic F108 blocking solution (SIGMA) was then added to each well and incubated for two hours at room temperature. The Pluronic F108 (SIGMA) was then removed, then washed with wash buffer 3 times for five minutes. To create the 2-D displays the FN derivatives were first biotinylated using the method outlined in section 2.7. Once the FN derivatives have been biotinylated, and the six well plates coated with avidin and washed as described above the biotinylated FN derivatives were coated on the six well plates using the protocol outlined in section 2.2.

2.7. Biotinylation of FN peptides

Biotinylation of FN peptides was carried out according to the instructions of the Maleimide-PEO$_2$-Biotin kit (Thermo Scientific). In brief, the concentration of the FN derivatives required was calculated using the calculation described in the protocol (Thermo Scientific). The FN derivative was then dissolved to the required concentration in PBS. Just before use 190μl PBS was added to 2 mg Maleimide-PEO$_2$-Biotin which would produce a 20mM stock solution. The appropriate volume of Maleimide-PEO$_2$-Biotin was added to the protein solution as calculated using the calculation outlined in the protocol (Thermo Scientific) and the solution mixed. The protein/Maleimide-PEO$_2$-Biotin solution was then allowed to incubate for three hours on ice. Biotinylation was then confirmed using the HABA method outlined in section 2.8.

2.8.a. HABA method

24.2mg of HABA was dissolved in 9.9ml ultrapure water to which was added 100μl 1M NaOH. 600μl of the HABA solution and 10mg of avidin (SIGMA) was then added to 19.4ml PBS. 900μl of HABA/avidin solution was then pipetted into a 1ml cuvette and the absorbance measured at 500nm. If mixing the solution has been done correctly the A$_{500}$ of the solution should be between 1.3 to 0.9. To the 900μl HABA/avidin solution 100μl of the biotinylated protein was then added, mixed well and the absorbance measured. The number of biotin molecules successfully attached to the FN derivatives was then calculated using the calculations detailed in the protocol from Thermo Scientific.

2.8.b. Labelled protein purification

To remove any excess biotin protein purification was carried out using zeba desalt spin columns (Thermo Scientific). The bottom of the 5ml desalt spin column was removed, the cap loosened and placed in a collection tube. The spin column was then spun in a centrifuge at 1,000 x g for 2 minutes to remove the storage solution contained within the column. A mark was placed on the side of the column where the resin is slanted upwards.

The column was then placed with the mark facing outwards for the remainder of the spins. 2.5ml buffer (PBS) was added to the column and centrifuge at 1,000 x g for 2 minutes. This wash step was repeated a further three times. Finally the spin column was placed in a new collection tube, the protein sample added then spun again at 1,000 x g for 2 minutes and the sample collected.

Molecular biology

2.9. RNA extraction

RNase free pipettes obtained from Starlabs were used for all of the molecular biology techniques outlined below. RNA was extracted using the RNeasy mini kit following the protocol outlined in the RNeasy Mini Handbook (Qiagen) used for monolayer cultures. There were several options for lysis of the cells and RNA extraction depending on the method of growth. The following method was cited for the initial stages. 600μl RLT buffer (10μl β-mercaptoethanol added per 1ml RLT buffer) was added to the cells grown in a monolayer. The lysate was then homogenized by aspirating then passing through a 0.9mm diameter blunt 20 gauge needle that had an RNase free syringe attached. From here on the protocol used is exactly as described in the RNeasy Mini Handbook (Qiagen).

2.10. Reverse transcriptase (RT) reaction

The RT reaction was carried out in accordance with the protocol specified for the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene) with minor alterations. In short, 3μg RNA was made up to 15.7μl with RNase-free water and 1.0μl oligo(dT) (Stratagene) primer was added to the RNA/water solution. The PCR machine was then programmed to run the samples for 5 minutes at 65°C, then at 25°C for 10 minutes. After primer annealing the following was added to each reaction, 2μl 10x AffinityScript RT Buffer (Stratagene), 0.8μl dNTP mix (Stratagene), 0.5μl RNase Block Ribonuclease Inhibitor (Stratagene), 1μl AffinityScript Multiple Temperature RT (Stratagene). The PCR machine was then set to run at the following temperatures, 42°C for

44

55 minutes, 95°C for 5 minutes. The cDNA generated was then placed on ice and then stored at -20°C.

2.11. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

In order to test the specificity of the primers designed for use in SYBR green QPCR RT-PCR was carried out. To pre-made RT-PCR mastermix (rovalab) 2μl cDNA, 1μl forward/reverse primer mix (primers obtained from VH Bio) and 2μl RNase-free water were added (Ambion). The PCR run was then set as follows, 1. 95°C for 3 minutes, then 2. 95°C for 15 seconds, 3. 60°C for 20 seconds, 4. 72°C for 40 seconds, 5. Steps 2-4 were then repeated for 40 cycles, 5. 72°C for 7 minutes and finally the reaction terminates at 4°C.

2.12. QPCR

SYBR green was used to determine the absolute number of transcripts being produced by the cells. The numbers obtained were normalized to GAPDH. (protocol adapted from McKimmie CS PhD thesis 2005, McKimmie CS and Fazakerley JK 2005)

2.13. Designing gene specific primers

Two sets of primers specific for the same gene are required for this method of QPCR, a set of standard primers used for the generation of standard cDNA templates and a set of QPCR primers that would be used in the QPCR reaction itself.
The cDNA standards are a larger stretch of cDNA that contain the stretch of cDNA the QPCR primers will amplify which is essential for the quantification of the absolute levels of transcripts present to be accurately measured. Primer 3 software (http://frodo.wi.mit.edu/) was used for primer design. The ideal criteria for the design of primers are described below:

- GC content between 40%-65% (optimal content 50%)

- Length of sequence between 18-23 base pairs (bp)

- Primer melting temperature (Tm) between 59.5°C - 61°C (optimal temp 60°C)

- Maximum self complementarity : 2

- Maximum 3' self complementarity : 1 (This must remain constant)

- The size of the amplified QPCR primer product should be 150bp or less

- Sequences containing more than 3 C or G bases in a row are to be avoided

- A maximum of two C or G bases in the final five bases at the primer's 3' end

If the above parameters are followed closely it should result in the generation of primers specific for the gene of interest that are unlikely to result in the formation of hair-pin loops or primer dimers. It is preferable that primers are generated from sequences within 1500bp of the 3' end of the mRNA. If no specific primers are generated using the above criteria it is recommended that the Tm is relaxed to between 58°C - 62°C. The GC content can also be altered to 35% - 70%. Thirdly the maximum self complementarity can be relaxed to 3. It should be stressed however that the maximum 3' self complementarity should not be altered and remain at 1. The standard primers are generated in the same fashion as the QPCR primers.

Once both QPCR primers and standard primers have been designed they must be tested to ensure that they are specific for the gene of interest and they do not bind to alternative splice variants of the same gene or indeed to another gene. Primer specificity was determined through carrying out RT-PCR using cDNA from a source that is known to express the gene being analysed. The products of the PCR were separated using gel electrophoresis through a 2% agarose gel to which was added 1.5μl of ethidium bromide. Primers were used providing that upon visualization via gel electrophoresis a single band was observed that is approximately the correct size predicted from primer 3 design. A type IV hyperladder that measures between 100 – 1,000bps was used to determine if the primer product was the correct size. If two or more bands were visualized on the gel it was an indication that these primers were not appropriate for use in QPCR due to either non specific binding to another gene product/splice variant of the same gene or the formation of primer dimer.

If either primer dimer occurs or smears of low molecular weight cDNA are visible it is possible to optimize the primers to be specific for one particular product through reducing the concentration of the primer or increasing the annealing temperature. If no bands are observed although it is accepted this cDNA is known to express the gene of interest, then reducing the temperature annealing takes place at or increasing the concentration of the primers used can often result in the production of PCR product. Although it is possible to remove low molecular weight smears through optimizing the conditions at which the PCR takes place, if cDNA is detected with a molecular weight greater than the gene of interest it is often the result of non specific binding to another gene and new primers are often required.

2.14. Production of cDNA standards

In order to accurately quantify the levels of a particular gene in a sample a standard curve must be generated. Therefore cDNA templates were generated by carrying out RT-PCR with the standard primers and purifying the cDNA obtained on a PCR purification column according to instructions laid out in the protocol (Qiagen QIAquick columns). The standard generated was then serially diluted by a factor of 10. The QPCR primers were then used to amplify the particular sequence of interest and the serial dilutions are used as the template for the QPCR. Through use of cDNA standards it is possible to quantify the absolute transcript levels of the gene of interest.

To quantify the mass of each standard PCR product the optical density (O.D.) was examined using a spectrophotometer then the following calculation carried out;

O.D. x dilution factor x 50

Once the mass of the standard was determined the following calculations are carried out to determine the copies of each transcript. An example calculation is given below using mouse Oct-4 as an example:

Oct-4 PCR product has a mass of 6.78×10^{-8} g/μl and a base pair size of 901. At first, the molecular weight of this PCR product was determined.

Average molecular weight for double stranded nucleotide = 660 daltons

Molecular weight of double stranded DNA = 660 daltons x length of standard in bp

Molecular weight of double stranded DNA = 660 x 901

Molecular weight of double stranded DNA = 594,660 daltons

Number of moles of standard = mass in grams per μl / Molecular weight of DNA

Number of moles of standard = 6.78×10^{-8} / 594,660 = 1.1403×10^{-13} moles per μl

In order to determine the copy number from the number of moles Avagadro's constant is used..

Copies DNA per μl = Avagadro's constant x moles per μl

Copies DNA per μl = (6.02×10^{23}) x (1.1403×10^{-13} moles)

Copies DNA per μl = 6.863×10^{10} copies per μl

Standards of varying concentrations were used to obtain a good standard curve ranging from 1×10^{-5} to 1×10^{-9} dilutions of the original standard. Purified concentrated standard was first diluted to a concentration of 1×10^{-2} using 10mM TE buffer and stored for all future dilutions. Any further dilutions of the 1×10^{-2} standard used nuclease free water (Ambion) to dilute the standard. An example of a standard curve graph is shown in figure 2.1.
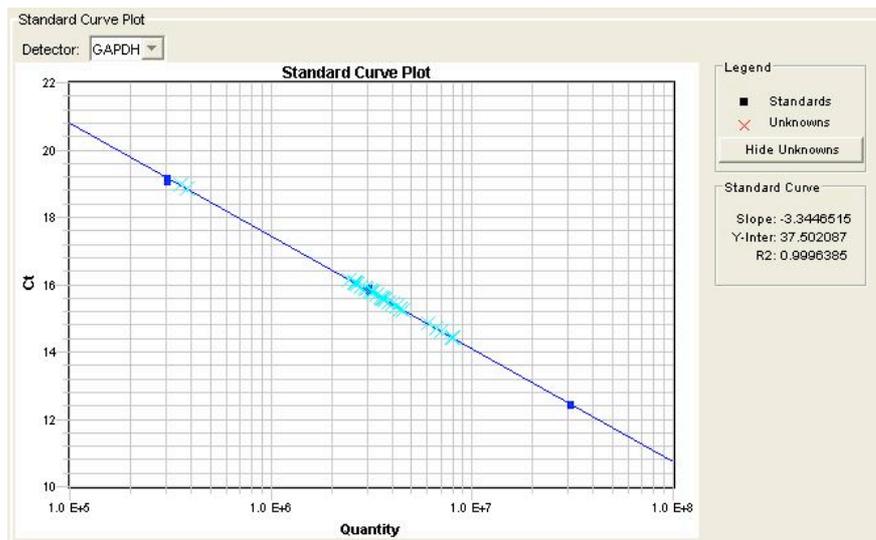
Figure 2.1. Example of a standard curve used for absolute quantification of transcripts

Figure 2.1 is an example of an accurate standard curve that can be used for the absolute quantification of transcripts as it fulfills the following requirements,

-The r^2 value is >0.98. If the r^2 is less than 0.98 then the data was excluded from the results.

-The slope should be 3.3. This means that the PCR product has been amplified every 3.3 cycles by a factor of 10. This infers that reaction efficiency is 100%
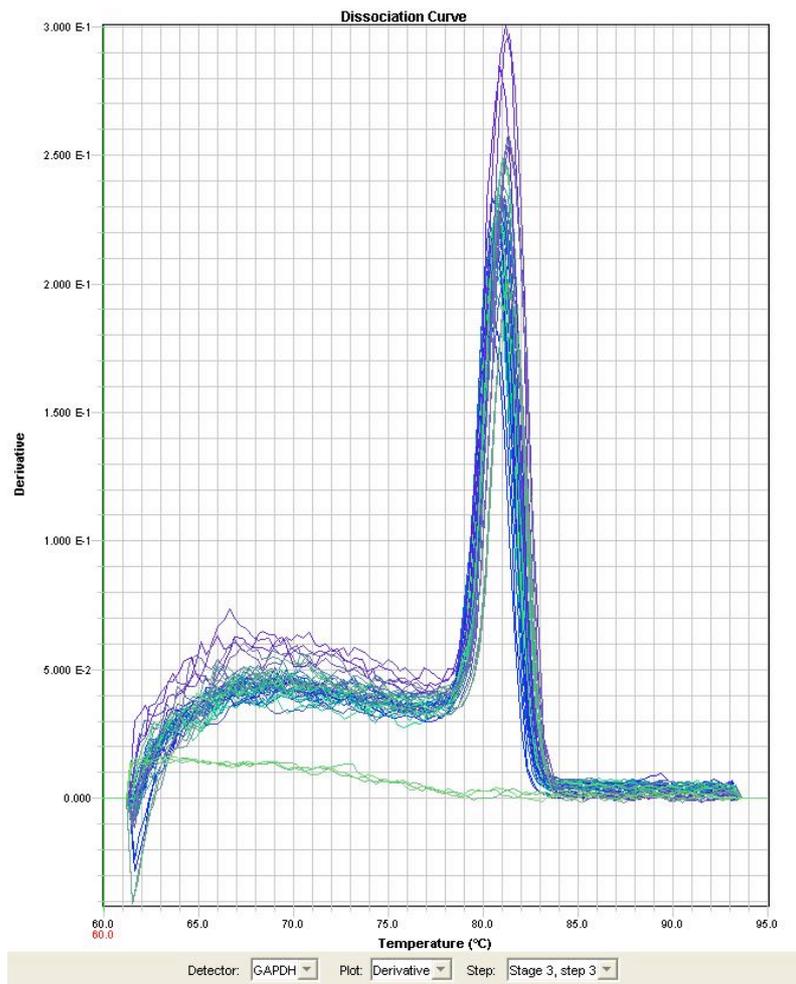
Figure 2.2 Dissociation curve

Figure 2.2 is an example of a dissociation curve. This is used to determine whether or not the primers were specific for the gene of interest. If this was the case there would be one peak as the gene of interest is amplified which is the case in figure 2.2. It also allows the user to visualize that every sample has been amplified. The non-amplified flat lines

observed near the bottom of the amplification plot are the non template control which of course will not be amplified.

2.15. SYBR green QPCR protocol

All samples were used in triplicate during the QPCR assay. A complete QPCR solution (called master-mix from here on) was made up immediately prior to preparing the samples for QPCR. 20% more master-mix than required was produced to ensure that there would be enough if any errors in aliquoting the master-mix occurred. For each individual triplicate reaction the following was required;

- 0.8μl QPCR primer mix (initial stock of 60μl of both forward and reverse QPCR primers mixed together, obtained from VH Bio)
- 8μl nuclease free water (Ambion)
- 10μl Power SYBR green mix (Applied Biosystems)

To each well 6μl of either a cDNA sample or cDNA standard was added plus 54.5μl of QPCR master-mix. This QPCR master-mix/sample or QPCR master-mix/standard solution was pipetted up and down five times to ensure proper mixing. Pipetting up and down had to be uniform for all the samples. Failing to do this often resulted in wide variations in terms of results. After mixing the master-mix/sample solution 19.8μl of the master-mix/sample solution was aliquoted into a well in a 96-well reaction (MicroAmp) plate (Applied Biosystems), then 19.8μl aliquoted into the adjacent two wells. 19.8μl was used to ensure that there is enough to successfully aliquot triplicates of the same volume and concentration to each well. To examine the detection potential of the QPCR assay and to determine the possibility of contamination a non-template control (NTC) was incorporated into the experiment. For NTC samples 6μl of nuclease free water was added to 54.5μl of master-mix then 19.8μl aliquoted into triplicates. The limit of detection of this QPCR assay is the result of one of two reasons; the amplification of non-specific primer-dimer which can occur in a sample with little to no cDNA template such as the NTC or the backgound contamination level. Once the samples have been aliquoted into the Microamp plates it was spun at 400 x *g* for 15 seconds to ensure that the sample was all on the bottom

of the well. Bubbles in the wells are not a cause for concern as they will be removed through heating that will occur in the QPCR assay.

2.16. Normalizing cDNA samples to GAPDH

In order to compare gene transcript levels between samples the levels of cDNA were normalized. To normalize the samples housekeeping genes were used. Housekeeping genes are genes that are expressed in all cells, have a role in the metabolism of the cell and have no apparent role in cell differentiation. The housekeeping gene used to normalize the samples in the QPCR assays described in this text was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This gene codes for a glycolytic enzyme that is involved in catalyzing the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate.

To calculate the absolute transcripts normalized to GAPDH the following calculation was carried out;
Copy number of gene of interest/Copy number of GAPDH housekeeping gene x 10^5.
This will give the copy number of the gene of interest per 100,000 GAPDH. The copy number of GAPDH was determined using the O.D. method as described previously. It is important that the correct housekeeping gene is chosen as there is a tendency for cultured cell line's transcript levels to be affected by one's test in comparison to one's control. This was tested prior to the commencement of QPCR and it was confirmed that the transcript levels of GAPDH were unaffected by growing ES cells on either surface coating. Therefore GAPDH was considered as a suitable housekeeping gene. While it would be prudent to use several housekeeping genes if different tissues were tested as 1 ES cell line ie E14's were used only 1 housekeeping gene was required.

Chapter 3 Results

3.1. Comparison of the adhesive ability of 2-D polyvalent displays with non-biotinylated FIII9-FIII10 derivatives

The first question to be answered in this project was whether or not the specific orientation of cell support displays in any way significantly enhanced the adhesive properties of the FIII9-FIII10 derivatives. It has been argued that control over aspects such as the orientation and density of the ligand must be addressed if adhesive support systems are to be as functionally useful as possible (Kreiner et al 2008). Therefore an experiment was designed to test if fixing the FIII9-FIII10 derivatives in a specific conformation through generation of 2-D polyvalent displays enhanced the number of colonies that would form compared when the FIII9-FIII10 derivatives were distributed in a random fashion.

In order to test the potential of these 2-D polyvalent displays for enhancing the growth of ES cells, FIII9-FIII10 derivatives were biotinylated at the C-terminal cysteine residue using the biotin-PEO-malemide kit and immobilized on six well plates coated with streptavidin. In addition six well plates were also coated with FIII9-FIII10 derivatives without prior biotinylation to examine the importance of orientation for any effects observed. ES cells were also grown on gelatin coated six well plates, a common substrate for ES cell growth and finally on avidin coated plates to look at the potential positive or negative effect this substrate has on ES cell growth. The dimeric form of the FIII9-FIII10 derivatives was chosen for this experiment over the other conformations as FN most often is found composed of two monomers which both contain the FIII9-FIII10 domains essential for cell adhesion and spreading (Mardon and Grant 1994). In addition one would expect that the dimeric version of the FIII9-FIII10 derivatives would very closely mimic the effect the other derivatives have due to the close similarity of their amino acid sequences. As shown in figure 3.1, there appeared to be no significant improvement upon using 2-D orientated polyvalent displays on the attachment potential of the dimeric FIII9-FIII10 derivatives in comparison to dimeric FIII9-FIII10 derivatives that had not been attached in a specific conformation. This calls into question the notion that the specific orientation of cell growth surfaces in any way enhances their adhesive potential (Kreiner et

al 2008, Koo et al 2002, Irvine et al 2002, Maheshwari et al 2000). There was almost a complete absence of ES cell colonies of any kind on plates coated with avidin. Evidently this surface is not supportive of ES cell growth.
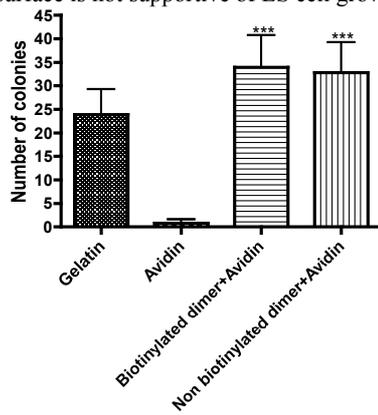
Figure 3.1 Comparison of biotinylated and non biotinylated FIII9-FIII10 dimer's adhesive potential. 300µg/well of either biotinylated or non biotinylated FIII9-FIII10 dimer, 0.1% gelatin or 25µg/well Avidin were coated on a six well plate. ES cells were grown for 3 days in complete medium, stained with giemsa and 10 counts/well taken at 5x magnification. 1,000 ES cells seeded/well. Comparison of gelatin and the biotinylated or non biotinylated FIII9-FIII10 dimer coated plates to adhere ES cell colonies were tested using an unpaired student's t test used. P<0.0001 = * , n = 10**

There were two observations that were worth closer examination however. First, plates coated with either the 2-D orientated polyvalent displays or the non biotinylated FIII9-FIII10 derivatives supported a significantly greater number of ES cell colonies when compared to gelatin. This is potentially useful as these FIII9-FIII10 derivatives could be used as an alternative surface for ES cell growth. It was also noted that in terms of morphology the ES cells colonies grown on the FIII9-FIII10 derivatives were markedly different when compared with ES cells grown on gelatin coated dishes. ES cells grown on gelatin had the classical round appearance of undifferentiated ES cells. In contrast ES cells grown on the FIII9-FIII10 derivatives, regardless of biotinylation had a more flattened

morphology, which is a characteristic of differentiated ES cells as shown in figure 3.2. These morphological changes were all the more interesting when one considers these ES cells were cultured in the presence of LIF.
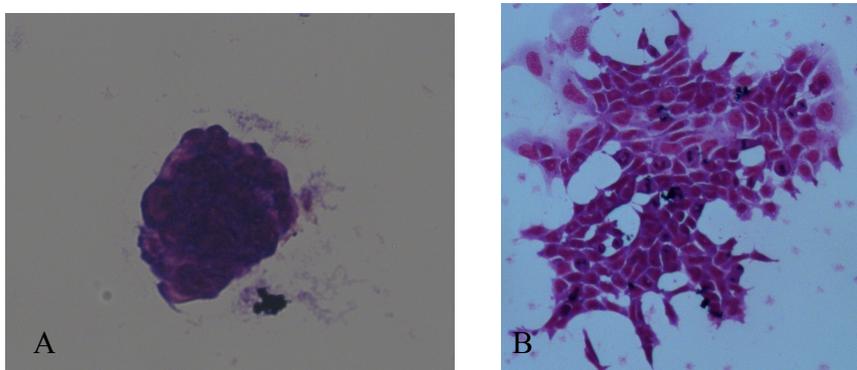
Figure 3.2 ES cells grown in complete media for 3 days then stained with 10% giemsa on A. Gelatin coated six well plates or B. FIII9-FIII10 derivative coated six well plates. Magnification x20. Complete media was used to culture the ES cells grown on gelatin or FIII9-FIII10 derivatives. Media was changed daily.

The steps taken to determine whether or not ES cells grown on the FIII9-FIII10 derivatives were differentiating even in the presence of LIF through use of QPCR are dealt with later in the text. However, initially, data relating to the adhesive properties of the FIII9-FIII10 derivatives are presented.

3.2. Optimal conformation of FIII9-FIII10 derivatives for ES cell adhesion

With a lack of any discernable advantage in terms of attaching colonies of ES cells using 2-D orientated displays as opposed to using non-biotinylated FIII9-FIII10 derivatives the generation of orientated 2-D polyvalent displays was abandoned and attention was focused on the effects of the FIII9-FIII10 derivatives on ES cell function and morphology. The FIII9-FIII10 derivative was available in four states, ie monomeric, dimeric, trimeric and tetrameric. Therefore steps were taken to determine which of the derivatives had the greatest adhesive potential. To this end a protein titration was carried out using the four

derivatives at various concentrations with ES cells grown on them for 3 days. After 3 days growth the ES cells were stained using giemsa and the colonies counted using the colony counting method detailed in section 2.3 of materials and methods.
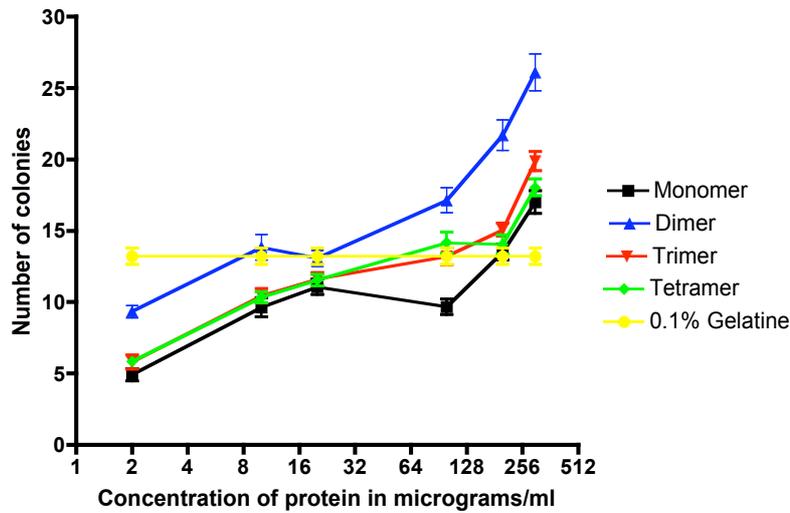
Figure 3.3 Comparison of ES cell colony supporting potential of all four FIII9-FIII10 derivatives and 0.1% gelatine control at the following concentrations; 2µg/ml, 10µg/ml, 20µg/ml, 100µg/ml, 200µg/ml, 300µg/ml n =30. 1,000 ES cells seeded/well. Complete media was used to culture the ES cells grown on gelatin or FIII9-FIII10 derivatives. Media was changed daily.

As can be observed from figure 3.3 the optimal FIII9-FIII10 derivative in terms of its ability to support ES cell colony growth appears to be the dimeric form. Although not statistically significantly more efficient at supporting ES cell colony growth compared to the other derivatives the dimer appears consistently better than the other derivatives, even if only subtly so. It appears that the optimal concentration for supporting ES cell colony growth has not been established in this assay. A dose response curve is clearly visible in figure 3.3 with no apparent saturation point as the number of ES cell colonies does not plateau at the concentrations of FIII9-FIII10 derivatives used. In order to confirm that the dimeric form of the FIII9-FIII10 derivative was indeed the most efficient at supporting ES cell colony growth a second experiment was set up in order to determine the cloning efficiency of ES cells on all four FIII9-FIII10 derivatives. The cloning efficiency is simply a percentage of the number of ES cells seeded that form colonies. Six-well plates were

therefore coated with one of the four FIII9-FIII10 derivatives, or gelatin, then ES cells were seeded and allowed to grow for six days. After six days the media was removed and the ES cells stained using giemsa as detailed in materials and methods section 2.3. and cloning efficiency determined as described in materials and methods section 2.4b.
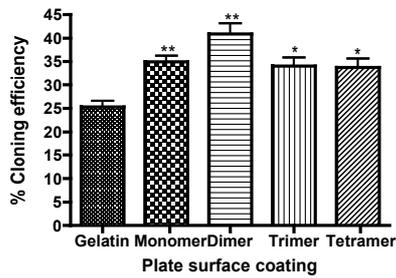
Figure 3.4 Cloning efficiency of all four FIII9-FIII10 derivatives compared to gelatin coated dishes. 50μg/well of the derivatives were used and 0.1% gelatin used as a control. 1,000 ES cells seeded/well. Complete media was used to culture the ES cells grown on gelatin or FIII9-FIII10 derivatives. Media was changed daily. An unpaired student's t test was carried out comparing the adhesive abilities of the gelatin control with each of the FIII9-FIII10 derivatives. P<0.006 = **, P<0.05 = * n = 6

The results of the cloning efficiency experiment presented in figure 3.4 confirm the data shown in figure 3.3 in that the FN derivative most effectively support ES cell growth when in dimeric form. It confirms that although the dimer is not significantly better at supporting ES cell colonies compared to the monomeric, trimeric or tetrameric forms of the derivatives it is consistently more efficient. In addition it should also be noted that at 50μg/ml concentration, all of the FIII9-FIII10 derivatives supported a statistically greater number of ES cell colonies than gelatin controls.

3.3. Effect of FN derivative on ES cell proliferation

There was a visible alteration of the morphology of the ES cells grown on the FIII9-FIII10 derivatives. An experiment was also set up to investigate whether or not the derivative had any effect on the proliferation of ES cells. To do this six well plates were coated with one of the four FIII9-FIII10 derivatives, or gelatin, and compared at different time points to examine any potential effect these proteins may or may not have on ES cell proliferation.

Gelatin
Monomer
Dimer
Trimer
Tetramer

Number of cells (x10⁴) per well

Number of days post cell seeding
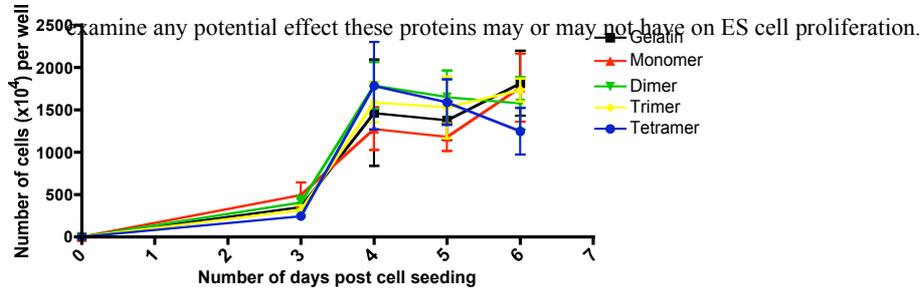
Figure 3.5 Proliferation of ES cells cultured on the various FIII9-FIII10 derivatives. 100μg/well of any one of the FIII9-FIII10 derivatives were added to each well. 20,000 ES cells seeded/well. Complete media was used to culture the ES cells grown on gelatin or FIII9-FIII10 derivatives. Media was changed daily. No significant difference in the number of cells were observed when ES cells were plated on either gelatin or the FIII9-FIII10 derivatives.

It is very clear from figure 3.5 that the FIII9-FIII10 derivatives play no role in the proliferation of ES cells as there is no difference in the number of cells, either between the different forms of the FIII9-FIII10 derivatives or the gelatin controls. Figure 3.5 follows the stereotypical lag, log and plateau phases of cells placed in a new environment. Initially there is a lag phase while the ES cells become attached and form colonies after they have been newly plated. Then at day 3 there is a substantial increase in cell numbers regardless of the media plated. Then as the ES cell confluency increases the level of proliferation drops in an attempt to maintain equilibrium with the environment the ES cells find themselves in. This data has been confirmed by a publication from another group looking

58

at the effect of various ECM components on ES cells (Hayashi et al 2007). Although this study used whole FN the effect was the same in that there was no enhanced proliferation of ES cells when grown on whole FN or indeed any of the ECM components tested (Hayashi et al 2007).

3.4. Comparison of the adhesive abilities of dimeric FIII9-FIII10 derivative with whole FN

As the dimeric form of FIII9-FIII10 had a modest but noticeably greater ability to allow the growth of ES cell colonies the next question to ask was whether or not these FIII9-FIII10 derivatives have greater potential in this regard than whole FN. This is of interest as FN in its intact form is commercially expensive to make and purify. If the FIII9-FIII10 derivative could support the growth of a greater number of colonies or cells than whole FN then it could have some commercial advantages in that it would be cheaper to generate and purify these FIII9-FIII10 derivatives as opposed to whole FN. In addition one of the commercial problems with whole FN is the difficulty to ensure its purity. (Chris Walle, personal communication).

If these FIII9-FIII10 peptides were shown to be adequate for supporting cell adhesion then it would be a way of controlling the exact structure of the FN being used. Therefore the ability of dimeric FIII9-FIII10 and whole FN on a microgram for microgram and mole for mole basis was compared in order to determine the ES cell supportive potential of the FIII9-FIII10 peptides as compared with conventional whole FN. When the FIII9-FIII10 derivative and whole FN were compared using an equal number of micrograms it is clear the FIII9-FIII10 derivative has a significantly greater ability to support colony growth as shown in figure 3.6. This effect was noted for both the number of colonies counted and also in terms of cloning efficiency. A second point to be made was that both the FIII9-FIII10 dimer and whole FN enabled a significantly greater number of cells to attach when compared with gelatin controls.
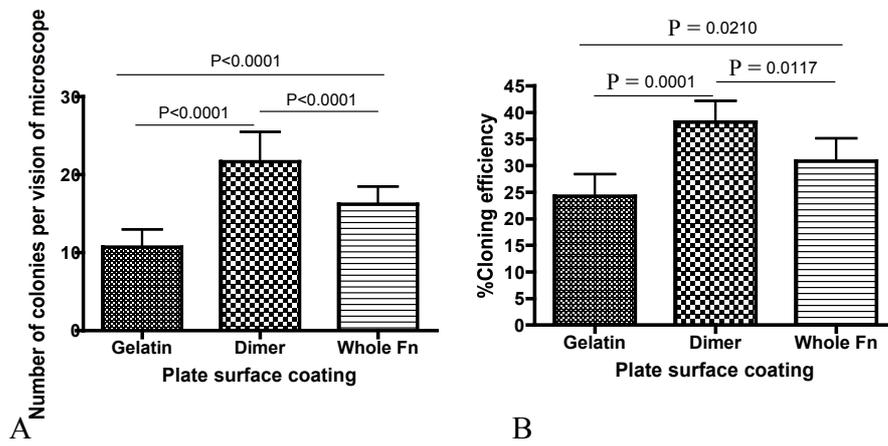
Figure 3.6 Comparison of the colony supporting ability of the dimeric FIII9-FIII10 and whole FN at equal microgram concentrations. In figure 3.6A ES cells were grown on either 50µg/well of dimeric FIII9-FIII10 or whole FN or 0.1% gelatin for 3 days, stained with giemsa and counted. In figure 3.6B ES cells were grown for 6 days on the dimeric FIII9-FIII10, whole FN or gelatin, stained with giemsa then the cloning efficiency determined. Dishes were coated with 0.1% gelatin or 50µg/well of dimeric FIII9-FIII10 or whole FN diluted in protein buffer. 1000 ES cells/well seeded. In both experiments complete media was used to culture the ES cells grown on gelatin or dimeric FIII9-FIII10. Media was changed daily.

If the FIII9-FIII10 dimer is compared to whole FN on a mole for mole basis the opposite effect is seen to that observed in figure 3.6. It is clear from figure 3.7 that whole FN has markedly greater colony supportive abilities than that of the FIII9-FIII10 dimer when used at the same molar concentration. This trend is seen once more when the cloning efficiency is tested. There is no difference in the number of colonies supported by gelatin or the FIII9-FIII10 dimer due to the low concentration of the FIII9-FIII10 dimer required in order to set up a mole for mole comparison with intact FN. Intact FN has a molecular weight of 440-500kDa compared to the FIII9-FIII10 derivatives molecular weight of 29.7kDa. Therefore 2.97µg/ml of FIII9-FIII10 derivatives has the same number of molecules as 50µg/ml intact FN and these were the concentrations of the two proteins used for this experiment.
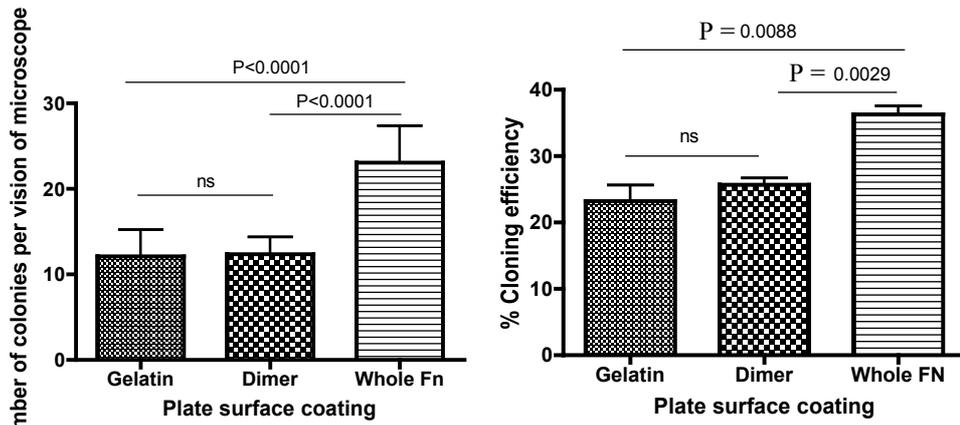
A   B

Figure 3.7 Comparison of the colony supporting ability of the dimeric FIII9-FIII10 and whole FN at equal mole for mole concentrations. In figure 3.7A ES cells were grown on either the dimeric FIII9-FIII10 derivative or whole FN for 3 days, stained with giemsa and counted. In figure 3.7B ES cells were grown for 6 days on either the dimeric FIII9-FIII10 derivative, whole FN or gelatin, stained with giemsa and the cloning efficiency determined. Dishes were coated with 0.1% gelatin or an equal mole for mole concentration of dimeric FIII9-FIII10 (2.97μg/ml) or whole FN (50μg/ml) diluted in protein buffer. 1,000 ES cells/well seeded. In both experiments complete media was used to culture the ES cells grown on gelatin or the FIII9-FIII10 dimer. Media was changed daily.

3.5. Effect of LIF titration of ES cell morphology

As ES cells grown in six well plates coated with the FIII9-FIII10 dimer appeared to show some morphological changes characteristic of differentiating ES cells, even though these ES cells were grown in the presence of LIF, the question was asked whether this apparent phenotypical differentiation could be altered through increasing the concentration of LIF. Therefore ES cells were grown on dimeric FIII9-FIII10 in the presence of media containing either the standard LIF concentration (1,000units LIF/ml) required for long term ES cell culture, or 5,000 or 10,000 units of LIF/ml. These cells were cultured then giemsa stained after 3 days growth and first colony formation analysis was performed in order to determine whether or not increasing the concentration of LIF in the media had any effect on the number of colonies formed. Figure 3.8 clearly displays there is no effect on the number of colonies formed on increasing the concentration of LIF in the media. This was not at all unexpected as LIF's most characterised role is the maintenance of ES cells in
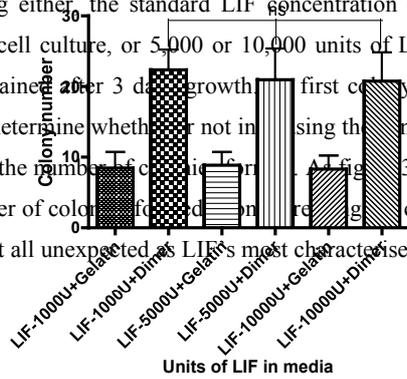
an undifferentiated state (Smith et al 2001) whereas there is little evidence that LIF plays any role in supporting ES cell colony formation.
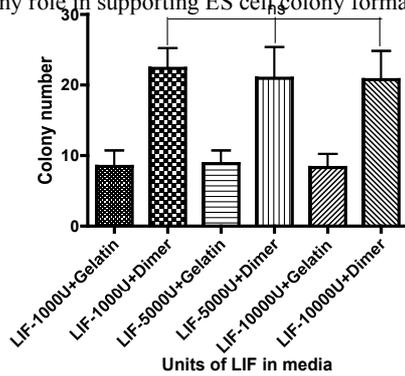
Figure 3.8 Effect of LIF titration on colony numbers. 1,000 ES cells/well were seeded and grown in media containing 1,000, 5,000 or 10,000 units of LIF/ml on either 50μg/well FIII9-FIII10 dimer or 0.1% Gelatin. The media used was changed daily. The colony counting technique described in materials and methods was used to count the colonies, n = 30

Although increasing the concentration of LIF in the media had no effect on ES cell colony numbers upon closer inspection of the colonies it was apparent that there were some peculiar morphological differences. There appeared to be different types of colonies forming in terms of their phenotype. Some colonies were made up of a tight cluster of cells which is characteristic of undifferentiated ES cell colonies and were round in shape whereas other colonies had a more dispersed morphology. Examples of these colonies are given in figure 3.9. The ratios of these "tight" and "dispersed" colonies seemed to change depending on the concentration of LIF used. Therefore the number of tight and dispersed colonies were counted using the colony counting technique described previously (materials and methods section 2.4.).

A   B
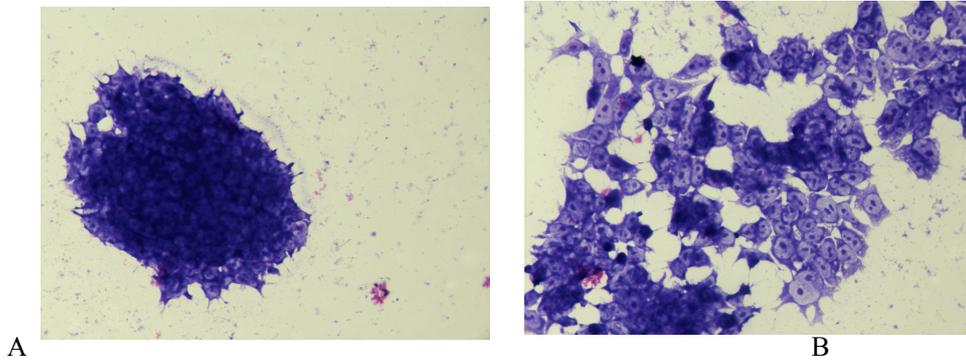
Figure 3.9.A. Tight colonies, 3.9.B. Dispersed colonies. Magnification x20. ES cells grown on 50μg/well FIII9-FIII10 dimers for 3 days then stained with 10% giemsa. Media was changed daily.

Upon counting the numbers of tight and dispersed colonies at the various concentrations of LIF used it was noted that the ratio of both types of colonies did indeed alter when LIF concentration was altered.
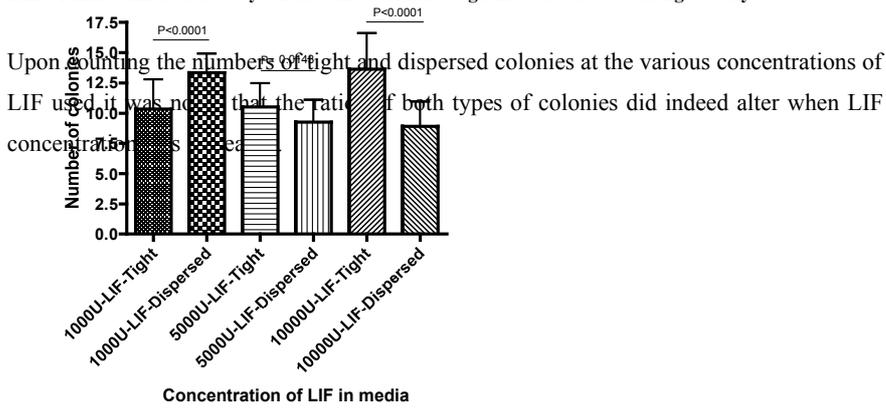
Figure 3.10 Changes in the number of tight and dispersed colonies upon titration of LIF. Six well plates were coated with FIII9-FIII10 dimer at 50μg/well. 1,000 ES cells were seeded per well and grown in complete media containing 1,000,5,000 or 10,000 units of LIF/ml. n = 30. Media was changed daily.

63

Figure 3.10 shows that at 1,000 Units of LIF/ml the colony phenotype that tends to dominate is the dispersed phenotype. This is consistent with the partially differentiated phenotype observed when ES cells were first grown on FIII9-FIII10 dimer as discussed earlier. Increasing the concentration of LIF to 5,000 Units of LIF/ml reduced the number of dispersed colonies concomitant with an increase in the number of tight colonies. It appears that at 5,000 units of LIF/ml the undifferentiated phenotype of ES cell colonies is now more dominant than the differentiated morphology. Increasing the concentration of LIF used in the media to 10,000 Units of LIF/ml has a clear effect on the numbers of tight and dispersed colonies with significantly more tight colonies being observed compared with dispersed colonies. This could be a sign that any potential differentiating signals that the FIII9-FIII10 dimer triggers in ES cells may be inhibited by increased concentrations of LIF drowning the signal out. This is very possible as even at standard LIF concentrations of 1,000 Units of LIF/ml there are ES cells that maintain a more undifferentiated morphology and look similar to tight colonies suggesting that LIF signalling via the LIF receptor is dominant over the FN-integrin receptor interaction. A final point of note to be taken from this experiment is the overall number of colonies observed remains consistent regardless of the concentration of LIF used, though the phenotypes of these clonies do alter with altered LIF concentration.

3.6. Alkaline phophatase staining

As there appeared to be some phenotypic indication that ES cell differentiation was occurring due to the flattened morphology of some of the ES cells plated onto the FIII9-FIII10 dimer, alkaline phosphatase staining was carried out to determine whether or not ES cell differentiation was taking place. Alkaline phosphatase is a marker expressed on ES cells when they are cultured in an undifferentiated state. Upon differentation of ES cells alkaline phosphatase ceases to be produced. Undifferentiated ES cells will stain pink for alkaline phosphatase staining whereas differentiated ES cells will be clear upon staining for the presence of alkaline phosphatase. ES cells were therefore grown as a monolayer on

gelatin or the FIII9-FIII10 dimer and cultured in complete medium in the presence or absence of LIF.
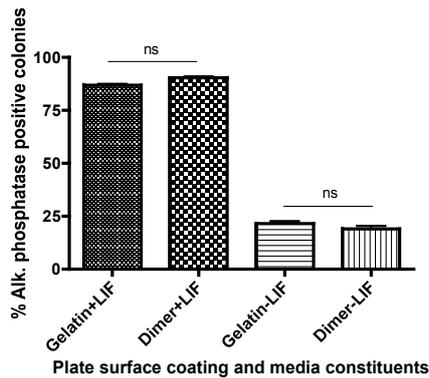
Figure 3.11 Alkaline phosphatase staining. ES cells were grown on either 50μg/well FIII9-FIII10 dimer or 0.1% gelatin for 5 days. Alkaline phosphatase staining was carried out after 5 days of culturing. Alkaline phosphatase positive and negative colonies were counted using the colony counting method described in materials and methods. N = 30. 1,000 ES cells seeded/well.

The results obtained, shown in figure 3.11, were contrary to expectations. Although there appeared to be a flattening of the ES cells, which is a morphological indication that ES cell differentiation may be occurring, no difference was observed in alkaline phosphatase positive staining between the gelatin or dimeric FIII9-FIII10 grown ES cells. ES cells grown in the presence of LIF were approximately 90% alkaline phosphatase positive which is the percentage expected of an undifferentiated ES cell population. Upon removal of LIF the alkaline phosphatase positive ES cell number markedly reduced confirming the role of LIF in the maintenance of ES cells in an undifferentiated state (Smith 2001).

A

B

C

D
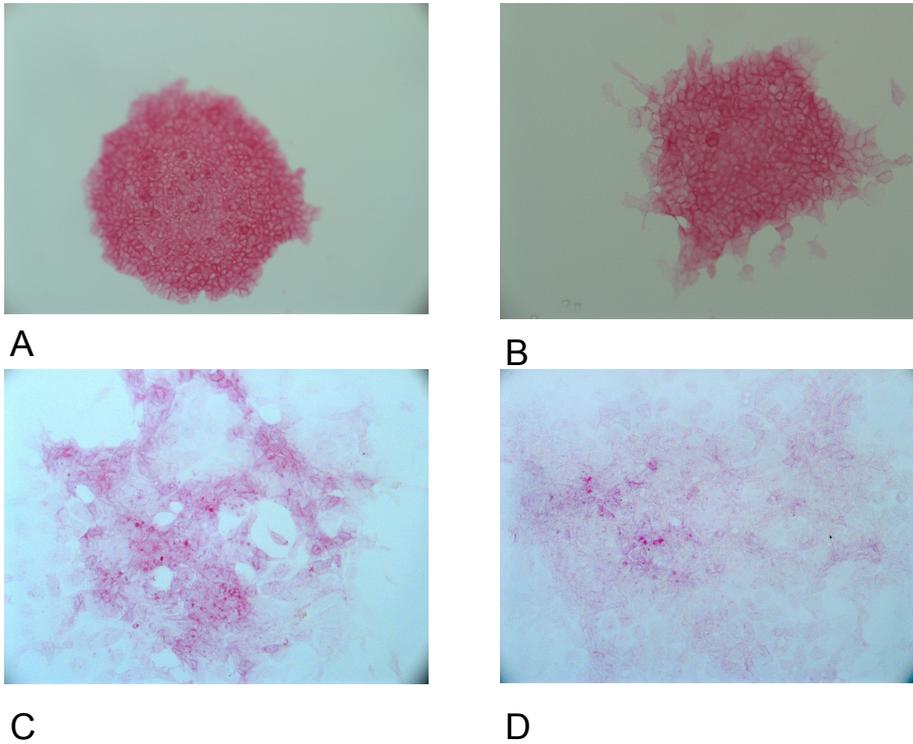
Figure 3.12 Alkaline phosphatase stained ES cells grown on A. Gelatin B. FIII9-FIII10 dimer in complete media containing 1,000 Units/ml LIF or C. Gelatin D. Fn dimer in complete media minus LIF Media changed daily. 1,000 ES cells seeded/well. x20 magnification.

As shown in figure 3.12, ES cells grown in the presence of LIF on gelatin or the FIII9-FIII10 dimer are both highly alkaline phosphatase positive However upon detailed analysis of the images it becomes apparent that while ES cells grown on gelatin are uniformly round, ES cells grown on the FIII9-FIII10 dimer have a more flattened morphology at the colony edges. It is possible that the there is a layer of more flattened ES cells at the bottom of the ES cell colony which are receiving a differentiation stimulus from the FIII9-FIII10 dimer. However upon formation of the colony, ES cells grow on top of the layer of

flattened ES cells thus do not receive the differentiation stimulus. Removal of LIF results in a substantial reduction in the percentage of alkaline phosphatase positive colonies and those, which do remain positive, are not uniformly positive as seen in figure 3.12. There appeared to be no major differences in morphology when LIF is removed and the ES cells allowed to differentiate on either gelatin or the FIII9-FIII10 dimer.

3.7. Transcriptional analysis of ES cells grown on the FIII9-FIII10 dimer

The alkaline phosphatase data presented in figure 3.11 suggested that the morphological changes observed through use of giemsa stain may have been just that, i.e. morphological with no apparent differentiation occurring in terms of loss of alkaline phosphatase expression. However the possibility could not be ruled out altogether that the FIII9-FIII10 dimer could aid in the differentiation of ES cells and it remained possible that the interaction of LIF binding via the LIF receptor was simply dominant the FIII9-FIII10 dimer's pro-differentiation signals. To investigate this possibility a transcriptional analysis of a variety of genes expressed by ES cells was undertaken. Therefore, ES cells were grown on gelatin or the FIII9-FIII10 dimer in the presence or absence of LIF, their RNA extracted, converted to cDNA then QPCR carried out.

The time points studied were 3 days and 6 days after culturing on the different surface coatings and media. Day 3 was chosen as a suitable time for analysis as this was the time point at which morphological differences between ES cells grown on gelatin or the FIII9-FIII10 dimer were initially identified. Secondly, in this assay day 3 appears to be important as figure 3.5 shows that proliferation of ES cells starts at this time point. Day 6 was chosen as the second time point of investigation as when using this particular assay the ES cells are roughly 70-80% confluent after 6 days of culture. To leave it any longer would risk over-confluency. The SYBR green standard curve method was used for QPCR as this assay has the advantage of determining the absolute numbers of transcripts whereas Taqman will only generate the fold change of any transcript levels. Although the absolute number of transcripts is often not essential the ability to look at these levels then if you wish convert to fold change was the factor that helped us decide to use this method. Primers designated QPCR primers and Standard primers were both generated for the same

67

genes of interest as both are required for the absolute quantification of ES cell transcripts as detailed in section 2.13 of materials and methods.

In order to carry out SYBR green QPCR we had to generate our own primers specific for the genes of interest as outlined in materials and methods (materials and methods sections 2.12., 2.13.). We took a wide selection of genes that were indicative of differentiation into the three germ layers. However, the majority of genes assayed for were mesodermal in origin as there is literature suggesting that the interaction of FN with ES cells has a role in mesodermal development, see section 1.7. The genes assayed for, and their primer sequences, are detailed in box 1.

QPCR primers

Gene	Lineage marker	QPCR1 (Forward primers 5'-3')	QPCR2(Reverse primers 5'-3')	Product size
Pou5f1/Oct4	Pluripotency	atcactcacatcgccaatca	aaggtgtccctgtagcctcatac	136
Nanog	Pluripotency	ctctcctcgcccttcctct	accttgttctcctcctcctca	123
Ehox	Pluripotency	ggataacagcaaccaagaggac	cgcacaccagataccagcac	138
Flk1/kdr	Mesodermal	agaaaaggagatgcccgact	acacaccgaaagaccacaca	100
Nkx2.5	Mesodermal	cgagcctggtagggaaagag	gggtgtgaaatctgagggacag	123
Tal 1/Scl	Mesodermal	cttgccttcctatcctgtgg	gctttgcttgccctaatgtc	109
Gata-1	Mesodermal	aggaaggggaagagcaacaacac	gagagaagaaaggactgggaaag	153
Brachyury	Mesodermal	ccaaagaaagaaacgaccaca	gaacaagccaccccatt	87
CD34	Mesodermal	cctacttcctttgtgctctcctc	tccaccattctccgtgtaataag	113
Foxa2	Endodermal	tgagtggagactttgggagag	agggacacagacaggtgagac	86
Nestin	Ectodermal	acctttgggtctctggaaaaa	agggttcactgtcatctgctc	138

Standard Primers

Gene	Lineage marker	Standard 1 (Forward primers 5'-3')	Standard 2 (Reverse primers 5'-3')	
Pou5f1/Oct4	Pluripotency	gtgaagttggagaaggtggaac	caaaatgatgagtgacagacagg	901
Nanog	Pluripotency	ggtgtcttgctctttctgtgg	gcacttcatcctttggttttg	624
Ehox	Pluripotency	gcatcaaaacaccaactacctac	gcttcactcacacctatccatctt	504
Flk1/kdr	Mesodermal	cggggcaagagaaatgaa	tgagagatgaggaaggagcaa	2000
Nkx2.5	Mesodermal	ctccgccaacagcaacttc	gggttctcatttcttacaacagg	446
Tal 1/Scl	Mesodermal	ctctgccttgcctttctcc	cttctttgtgcctgtgctg	1757
Gata-1	Mesodermal	cagattccacaggtttcttttcc	gctccacagttcacacactctct	544
Brachyury	Mesodermal	cggtgctgaaggtaaatgtgt	gtgtgtggaggggagagaga	605
CD34	Mesodermal	gatggctgttgggaagaaaa	agaggcgagagaggagaaatg	628
Foxa2	Endodermal	acatcaccacagccaccac	gccataaataaagcacgcaga	782
Nestin	Ectodermal	ccctcaaccctcaccactc	Tgtctccatacctccttcattca	1922

Box 1. Primer sequences.

Primer optimisation

Once potential primer sequences had been identified they were purchased and then tested to determine whether or not they would be suitable for QPCR, i.e. are they specific for the

gene of interest. This was carried out using RT–PCR with cDNA known to express these genes at the transcript level. The cDNA used was a mix of all of the cDNA samples obtained from ES cells grown on gelatin or the FIII9-FIII10 dimer on days 3 and 6. All of the genes described in box 1 are well known to be expressed within ES cells, so it was felt that this would be appropriate.

Gel A
1 Type IV hyperladder
2 Foxa2 QPCR
3 Foxa2 Std
4 Brachyury QPCR primer dimer
5 Brachyury Std
6 Nanog QPCR
7 Nanog Std
8 CD34 QPCR
9 CD34 Std
10 Type IV hyperladder

Gel B
1 Type IV hyperladder
2 GATA-1 QPCR
3 GATA-1 Std
4 SCL QPCR
5 GATA-1 Std

Gel C
1 Type IV hyperladder
2 Nestin QPCR
3 Nestin Std

Gel D
1 Brachyury QPCR
2 Type IV hyperladder
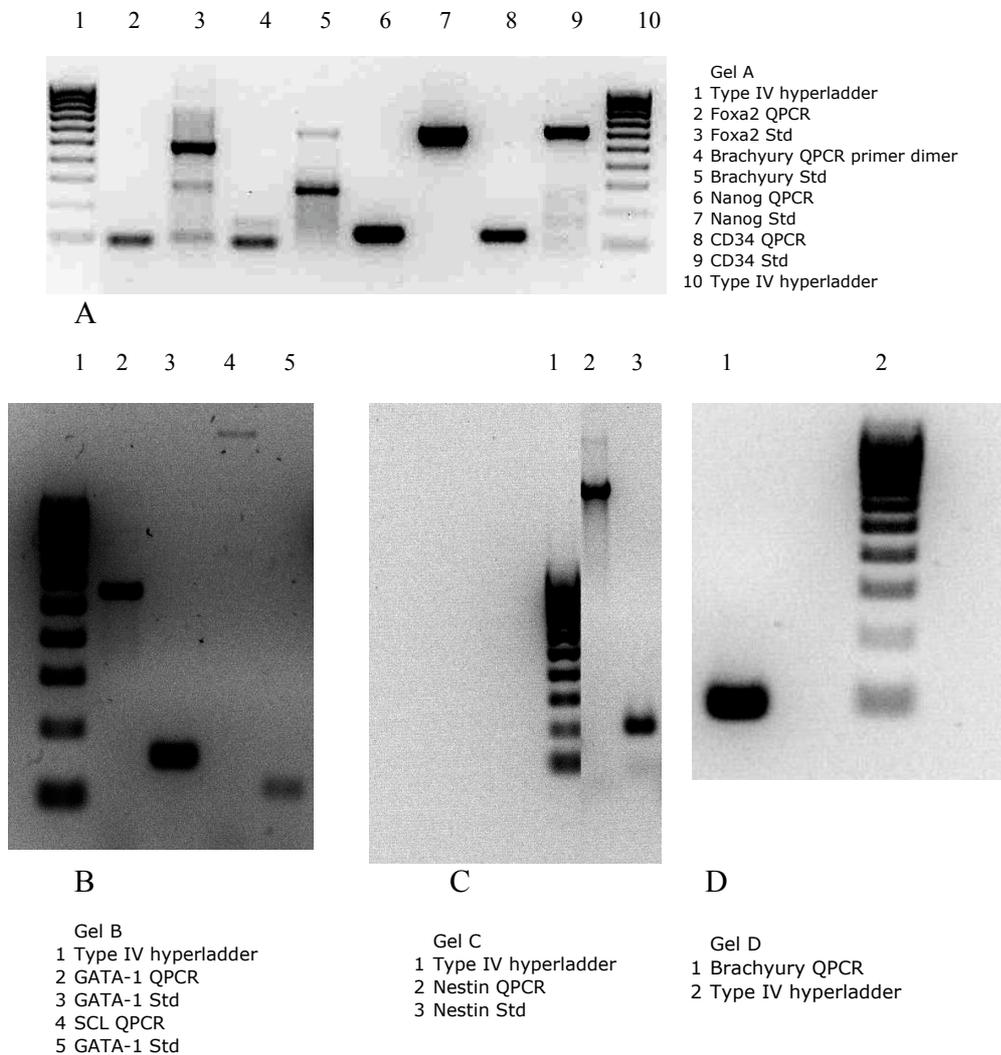
Figure 3.13 PCR products of QPCR and

Standard primer pairs (continued overleaf)

69

E   F
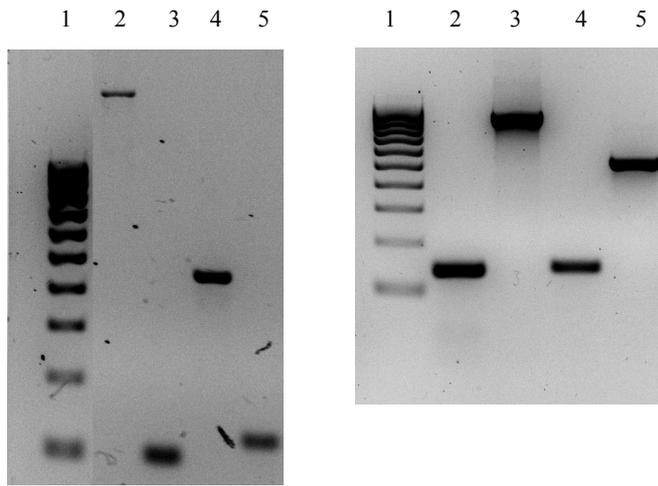
Gel E   Gel F
1 Type IV hyperladder   1 Type IV hyperladder
2 Flk-1 Std   2 Oct-4 QPCR
3 Flk-1 QPCR   3 Oct-4 Std
4 NKX2.5 Std   4 Ehox QPCR
5 NKX2.5 QPCR   5 Ehox Std

Figure 3.13 continued

A type IV hyperladder which allows assessment of the size of bands between 1,000bps to 100bps was added to each gel to confirm that the PCR product being formed was the correct length. This, in conjunction with only one band forming per lane, would reinforce the specificity and accuracy of the PCR primers being used. In gel A there are three wells in which multiple bands are found. These bands were intentionally left in so that certain aspects of PCR could be discussed. Lanes 3 and 5 of gel A have one strong band and several much lighter bands. The darkest, most intense band is the desired product whereas the lighter bands are evidence of the primers binding non-specifically to other genes and/or the formation of primer dimer structures. The PCR products in these wells were used to generate the standards to quantify the numbers of transcripts being produced when normalised to GAPDH. Providing the QPCR primers used for the same gene were specific (ie one band on the gel) this would allow these primer pairs to be used as the QPCR primers. These QPCR primers would then bind to a much smaller region of the same gene that is found within the product generated by the standard primers. Lane 4 of gel A

(Brachyury) contains one very strong band but also another much lighter band that is evidence of non-specific binding or primer dimer. As the primer set being used for this PCR was a QPCR primer set these primers were deemed unsuitable for QPCR. Therefore another set of QPCR primers were used that would amplify a region of the product generated by the Brachyury standard primer set. The product formed from this new set of primers is shown in gel D, lane 1. The sequence and size of the PCR products are listed within box 1. The non-specific brachyury QPCR primers were omitted from table 1 to avoid confusion.

The first gene to be analysed was Oct-4. As discussed in the introduction Oct-4 is essential for the maintenance of pluripotency of ES cells (Smith A 2002). In addition to being essential for maintaining ES cells in an undifferentiated state it can also play a role in the differentiation of ES cells (Niwa et al 2000). Therefore analysis of this gene was an ideal place to
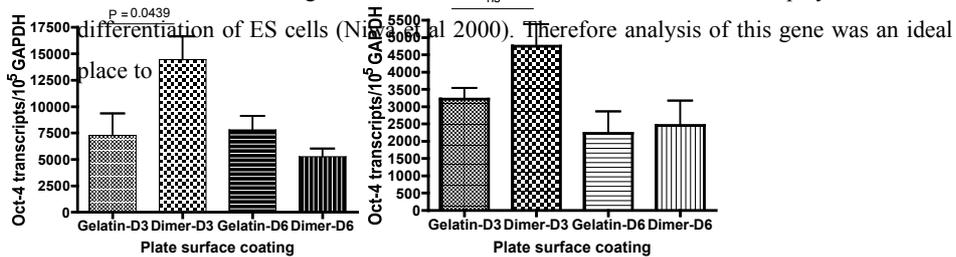
A   B

Figure 3.14 QPCR analyses of Oct-4 transcript levels grown on either 0.1% gelatin or 100μg/well dimeric FIII9-FIII10 in media that either, A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells seeded/well.

Interestingly when ES cells were grown on dimeric FIII9-FIII10 there was an increase in the number of transcripts being produced at day 3. This was of potential interest as if the transcript numbers were converted to fold change there was just under a two fold increase

in the number of transcripts in ES cells grown on the FIII9-FIII10 dimer compared to ES cells grown on gelatin. There is evidence that an increase in Oct-4 levels is involved in the differentiation of ES cells into the mesodermal/endodermal lineage (Niwa et al 2000). ES cells grown on the FIII9-FIII10 dimer in the absence of LIF also had an increase in the number of transcripts on day 3 compared to gelatin controls. However this increase, while being consistent, was not significant although the trend is similar to ES cells grown in the presence of LIF.

A final point of note was the general reduction of in the level of transcripts detected upon removal of LIF. This is a general feature of the the assays carried out using FCS complete medium. It is possible this could be due to the transcriptional leakage (Hurst et al 2004, Yanai et al 2006). ES cells are well known for their ability to form almost any type of cell or tissue, therefore they are very transcriptionally active when maintained in the presence of LIF (Graham GJ, personal communication). When LIF is removed and ES cells allowed to differentiate many genes are switched off at the transcriptional level only to become activated again at a later time. It is possible this phenomenon is responsible for the differences in transcript levels observed.

The second gene to be assayed for was Nanog, which has also been identified as a key regulator of ES cell pluripotency (Chambers et al 2003, Mitsui et al 2003) as discussed in the section 1.4.
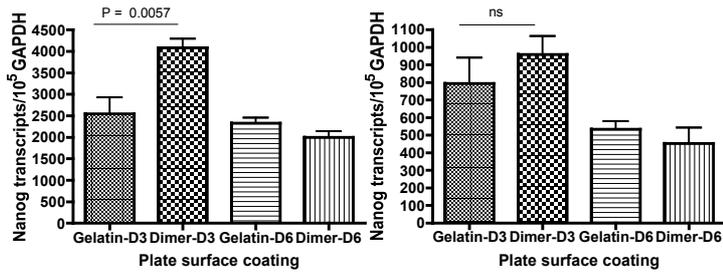
A   B

Figure 3.15 QPCR analyses of Nanog transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/well dimeric FIII9-FIII10 in media that either, A. contained LIF or B lacked LIF. N = 6. 20,000 ES cells seeded/well.

Similar to the trend observed with Oct-4 levels when grown on the FIII9-FIII10 dimer there is an increase in the number of nanog transcripts on day 3 compared to the gelatin controls. There are no major changes in nanog transcripts in gelatin grown ES cells on either day similar to the lack of change in Oct-4 transcripts in gelatin grown ES cells. ES cells grown in the absence of LIF showed no major alterations in transcript levels on ES cell grown on the two surfaces. There is a slight upregulation of nanog transcripts when grown on the FIII9-FIII10 dimer on day 3 compared to the gelatin controls but this does not reach signficance.

The transcriptional profile of Ehox was then examined upon growing ES cells on gelatin or the FIII9-FIII10 dimer. Ehox is a gene that at present has been found in mouse but not human ES cells and is found in undifferentiated ES cells (Jackson et al 2002).
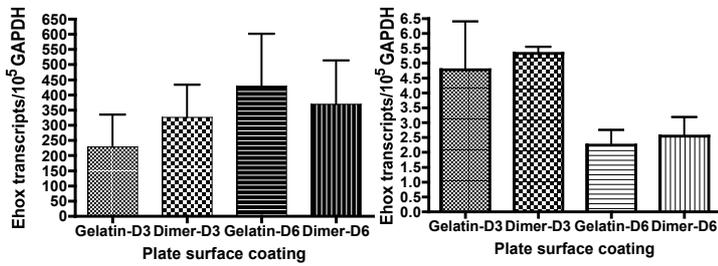
A   B

Figure 3.16 QPCR analyses of Ehox transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in media that either A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

In the presence of LIF the levels of Ehox transcripts were unchanged as can be seen in figure 3.16 when grown on either the FIII9-FIII10 dimer or gelatin coated surfaces. There is a general increase in the levels of Ehox from day 3 to day 6 but there does not appear to be any difference between the ES cells grown on the FIII9-FIII10 dimer compared to ES cells grown on gelatin. Upon removal of LIF the levels of Ehox drop to very low levels in comparison to ES cells grown in the presence of LIF, almost to the absolute limit of detection ability of the assay. The different surfaces that the ES cells are grown on do not appear to make any difference in terms of transcript level fluctuations. By day 6 the levels of Ehox are further reduced. This fits with the evidence that suggests Ehox is present in undifferentiated ES cells but is reduced upon the onset of differentiaton (Jackson et al 2002). The QPCR data described so far is summarized in table 1 below.

Gene	In the presence of LIF Day 3	Absence of LIF Day 3	In the presence of LIF Day 6	Absence of LIF Day 6
Oct-4	Increased	Unchanged	Increased (ns)	Unchanged
Nanog	Increased	Unchanged	Unchanged	Unchanged
Ehox	Unchanged	Unchanged	Unchanged	Unchanged

Table 1. Changes in the level of pluripotency gene's transcript levels of ES cells grown on the FIII9-FIII10 dimer compared to gelatin controls.

Brachyury is a gene expressed early during mesodermal differentiation of ES cells and is considered to be one of the earliest known markers of mesoderm differentiation (Herrmann et al 1990, Wilkinson et al 1990). Upon carrying out QPCR for this gene in the presence of LIF it was noted that the levels of Brachyury increased if grown on the FIII9-FIII10 dimer in comparison to gelatin grown ES cells.
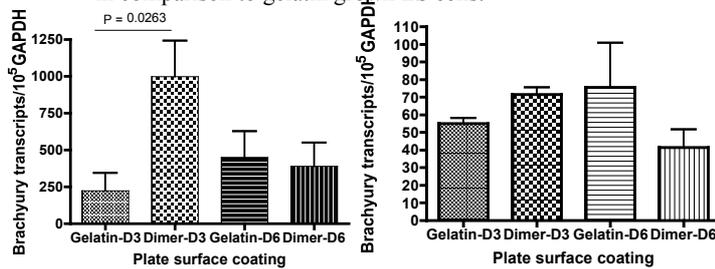
A   B

Figure 3.17 QPCR analyses of Brachyury transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/well dimeric FIII9-FIII10 in media that either, A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

Rather surprisingly upon the removal of LIF the levels of Brachyury did not significantly alter when grown on either gelatin or the FIII9-FIII10 dimer. There is a very slight increase in transcript levels when grown on the FN dimer as opposed to gelatin controls on day 3 but this does not reach significance. On day 6 the trend is the opposite with a slight increase in Brachyury in ES cells grown on gelatin but again this does not reach significance.

With the transient upregulation of brachyury noted, several other genes with known roles in mesodermal differentiation were examined. GATA-1 is one of the most studied haematopoietic transcription factors with established functions in erythroid and megakaryocyte development (Orkin 1998). It is apparent that in either in presence or absence of LIF the FIII9-FIII10 dimer has no significant effect on the GATA-1

transcriptional profile of ES cells at these time points as figure 3.18 demonstrates. This leads us to believe that if there is any differentiation inducing properties of the FIII9-FIII10 dimer it does not program ES cells to differentiate into erythroid/megakaryocyte lineages. It is also possible that the ES cells grown on the FIII9-FIII10 dimer may be differentiating into erythroid/megakaryocytic lineages but the time points chosen for this assay were too early to detect this
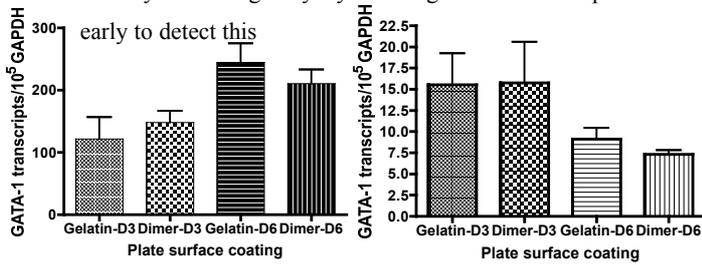
A   B

Figure 3.18 QPCR analyses of GATA-1 transcript levels expressed by ES cells grown on either 0.1% gelatin or 100µg/well dimeric FIII9-FIII10 in media that either, A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

A marker of hemopoiesis (Bonnet 2002), CD34 was then assayed for. Quite strikingly, in the presence of LIF, growing ES cells upon the FIII9-FIII10 dimer significantly suppressed the number of transcripts generated when compared to gelatin grown ES cells as displayed in figure 3.19. This reduction in the transcriptional profile of CD34 is apparent on both day 3 and day 6. Removal of LIF and growth on the various surfaces results in a consistent but non-significant increase in CD34 levels when grown on the FIII9-FIII10 dimer as opposed to gelatin grown ES cells.
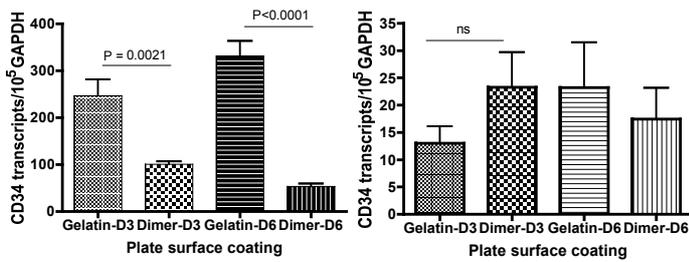
A   B

**Figure 3.19 QPCR analyses of CD34 transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/well dimeric FIII9-FIII10 in media that either, A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.**

SCL is a transcription factor required for early differentation of ES cells into haematopoietic stem cells (Lecuyer et al 2004, Park et al 2005). This gene was examined to look for any indications of differentiation into this lineage. In figure 3.20 there appears to be no changes in SCL transcripts when grown on either gelatin or the FIII9-FIII10 dimer in the presence or absence of LIF. There is a reduction in transcript numbers on day 6 in both gelatin grown and FIII9-FIII10 dimer grown ES cells in the presence or absence of LIF but again does not appear to be as a result of the plate surfaces. It is possible that this notable reduction in SCL levels on day 6 is due to the ES cells sensing confluency that alters their transcriptional profile.
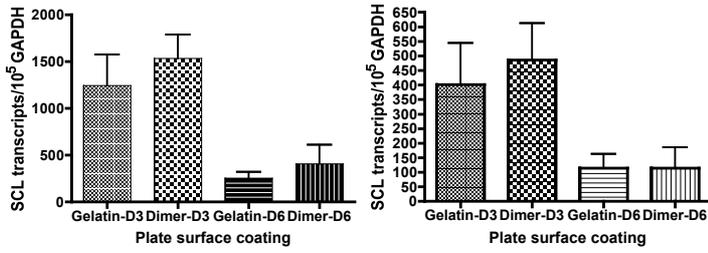
A   B

Figure 3.20 QPCR analyses of SCL transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/well dimeric FIII9-FIII10 in media that either, A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

To look for any evidence of endothelial cell differentiation, Flk-1 a gene well known to be involved in the differentiation of ES cells into this lineage (Shalaby et al 1995, Shalaby et al 1997) was examined. ES cells grown on FIII9-FIII10 dimer in either the presence or absence of LIF resulted in more than the number of transcripts that was reproducible valid for each condition as shown in 3
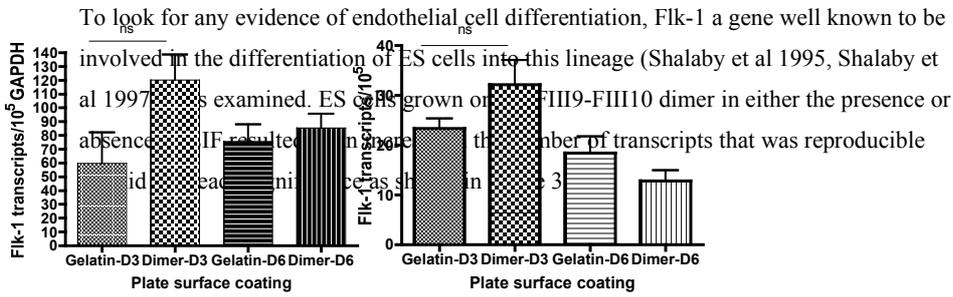

A   B

Figure 3.21 QPCR analyses of Flk-1 transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in media that either A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

In order to identify any possible role the FIII9-FIII10 dimer may have in the induction of cardiomyocyte differentiation, the gene NKX2.5 was assayed for. NKX2.5 is a transcription factor involved in the development of heart formation (Murry et al 2008) and would therefore be an indicator of the ability of the FIII9-FIII10 dimer to induce cardiac development. Figure 3.22 clearly shows that at the time points looked at there was no obvious effect on the levels of NKX2.5 when ES cells were grown on either the FIII9-FIII10 dimer or gelatin. When LIF is removed there is a small increase in ES cells grown on the FIII9-FIII10 dimer although this increase is not significant. Secondly in the absence of LIF there is a sharp decrease in the levels of NKX2.5 in ES cells grown on both surfaces between day 3 and day 6.
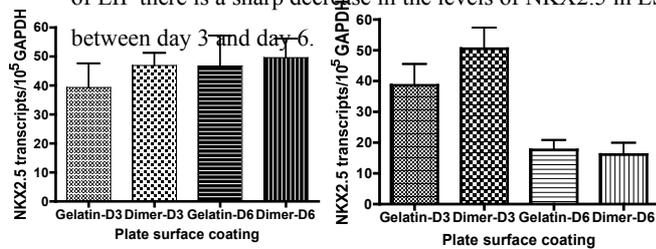

A   B

Figure 3.22 QPCR analyses of NKX2.5 transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in media that either A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

Changes in mesodermal transcripts grown on FIII9-FIII10 dimer in comparison to gelatin control ES cells are shown in table 2.

	Presence of LIF	Presence of LIF	Absence of LIF	Absence of LIF
Gene	Day 3	Day 6	Day 3	Day 6
Brachyury	Increased	Unchanged	Unchanged	Unchanged
GATA-1	Unchanged	Unchanged	Unchanged	Unchanged
CD34	Decreased	Decreased	Increased (ns)	Unchanged
SCL	Unchanged	Unchanged	Unchanged	Unchanged
Flk-1	Increased (ns)	Unchanged	Increased (ns)	Unchanged
NKX2.5	Unchanged	Unchanged	Unchanged	Unchanged

Table 2. Changes in the levels of mesodermal gene transcript levels of ES cells grown on the FIII9-FIII10 dimer compared to gelatin controls.

In order to look for indications that endodermal differentiation may be occurring, QPCR was carried out to look at the effect, if any, of the FIII9-FIII10 dimer on ES cell transcript levels of Foxa2. This gene was chosen due to its established role in endodermal differentiation, often in conjunction with Sox17 (Kanai-Azuma et al 2002, Friedman and Kaestner 2006). Foxa2 is widely expressed in the definitive endoderm and in structures derived from the endoderm such as pancreas, liver and lung (Friedman and Kaestner 2006). The results in figure 3.23 show that Foxa2 does not have any role in the morphological changes initially observed either in presence or absence of LIF. There is an increase observed in the Foxa2 transcripts going from day 3 to day 6 although there is no difference between the ES cells grown on the FIII9-FIII10 dimer and gelatin.

A   B

Figure 3.23 QPCR analyses of Foxa2 transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in media that either A. contained LIF or B. lacked LIF.

N = 6. 20,000 ES cells/well.

During this study we attempted to generate primers specific for Sox 17 but were unable to do so as the primers designed could not be optimized to bind specifically to the gene of interest. Instead multiple bands were observable upon carrying out electrophoresis of the RT-PCR products on an agarose gel then visualizing the bands through use of ethidium bromide

In order to look for some evidence of ectodermal differentiation, neuro-ectodermal differentiation specific QPCR was used to examine whether the gene Nestin was affected by growth on the FIII9-FIII10 dimer compared with gelatin controls. Nestin is a gene expressed by neuroepithelial stem cells (Lendahl et al 1990) and today is a commonly used marker to identify neuro-ectodermal populations (Conti et al 2005).
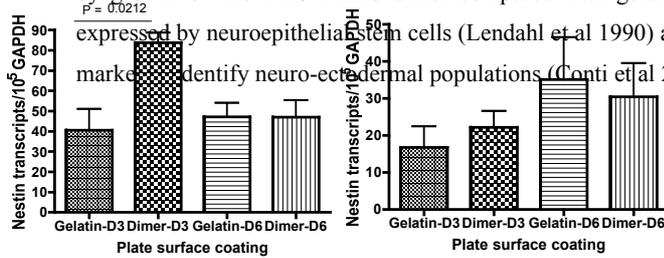
A   B

Figure 3.24 QPCR analyses of Nestin transcript levels expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in media that either A. contained LIF or B. lacked LIF. N = 6. 20,000 ES cells/well.

In the presence of LIF there is an increase in the levels of Nestin in ES cells grown on the FIII9-FIII10 dimer on day 3 compared to gelatin controls. There is then a subsequent down regulation of the levels of Nestin on day 6 compared to day 3. This down regualation of Nestin grown on the FIII9-FIII10 dimer from day 3 to 6 is similar to the down regulation

81

observed when Oct-4 and Brachyury were assayed for. In the absence of LIF there appears to be a very general increase in Nestin transcripts when moving from day 3 to day 6 although this increase is not significant. However the FIII9-FIII10 dimer appears to play little role in this upregulation as the levels of Nestin transcripts are very similar when grown on either gelatin or FIII9-FIII10 dimer. The results of the final two assays examining for endodermal and ectodermal differentiation are described succinctly below.

	Presence of LIF	Presence of LIF	Absence of LIF	Absence of LIF
Gene	Day 3	Day 6	Day 3	Day 6
Foxa2	Unchanged	Unchanged	Unchanged	Unchanged
Nestin	Increased	Unchanged	Unchanged	Unchanged

Table 3. Changes in the levels of endodermal (Foxa2) and ectodermal (Nestin) gene transcript levels of ES cells grown on the FIII9-FIII10 dimer compared to gelatin controls.

Based on the results of the QPCR assays there appeared to be some indications that the FIII9-FIII10 dimer may have some pro differentiation effects when ES cells were grown upon them in a monolayer. However, the Foetal Calf Serum (FCS) in ES cell complete media is known to contain factors that have some pro differentiation effects. In order to more closely examine any possible differentiation stimuli the FIII9-FIII10 dimer may be conferring to the ES cells, serum replacement media optimized for ES cell culture was used in place of complete FCS containing media.

ES cells were seeded into six well plates coated with either gelatin or FIII9-FIII10 dimer, then left to grow in the absence of LIF. It was thought that this would optimize any differentiation potential the FIII9-FIII10 dimer may have. In this assay additional time points were used and an undifferentiated control added in order to more closely study any early transcriptional changes that may occur when growing ES cells on either gelatin or the FIII9-FIII10 dimer.

A

B
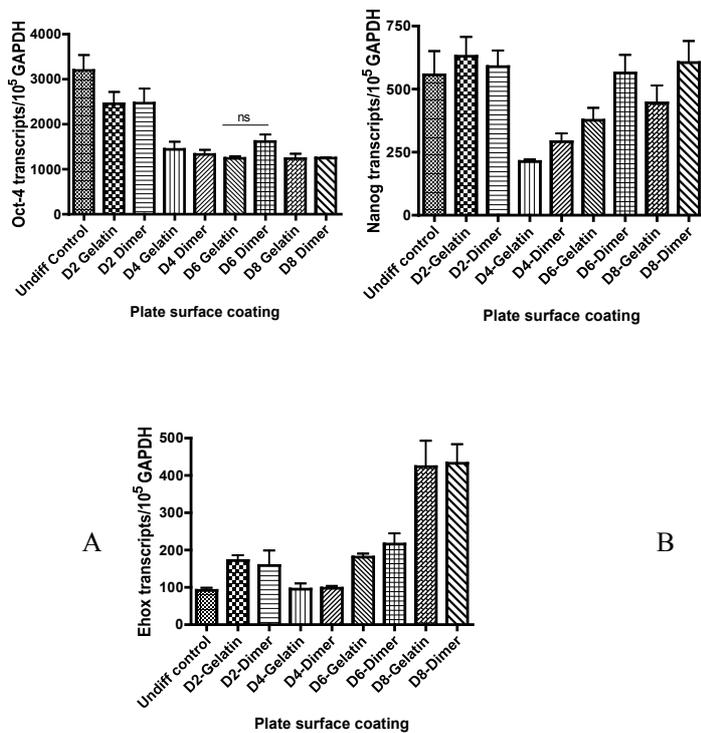
C

Figure 3.25 QPCR analyses of ES cell pluripotency markers, A. Oct-4 B. Nanog C. Ehox transcripts expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in serum replacement medium. N = 3.

In figure 3.25A the levels of Oct-4 are shown to drop upon removal of LIF when compared to undifferentiated ES cells but are found to persist at low but noticeable levels. This fits with previous observations that Oct-4 does remain at a low level in differentiating ES cells (Smith 2001). In contrast to observations using FCS medium in the presence of LIF there appears to be no real increase in the levels of Oct-4 when grown on the FIII9-FIII10 dimer as opposed to gelatin. There is a small increase in Oct-4 levels in ES cells grown on the FIII9-FIII10 dimer on day 6 but this does not reach significance.

The result observed in figure 3.25B was unexpected as, although there is an initial drop in Nanog levels as would be expected upon the removal of LIF (Chambers et al 2003), the levels of Nanog are found to recover and increase. This is particularly noted when ES cells are grown on the FIII9-FIII10 dimer. By day 8 the levels of Nanog generated by ES cells grown in a monolayer on the FIII9-FIII10 dimer have returned to the same level as those observed in the undifferentiated ES cell controls.

Growth of ES cells in the absence of LIF, in serum free medium, on either gelatin or FIII9-FIII10 dimer appears to have no direct effect on the levels of Ehox produced. There is a small increase followed by decrease in the levels of Ehox transcripts. This is followed by a substantial increase in the levels of Ehox on day 8. This result is in agreement with the initial study of this gene (Jackson et al 2002). The results of the assays looking at pluripotency markers using serum replacement media are summarized below.

Gene	General pattern of transcriptional profile	Specific effect of FIII9-FIII10 dimer compared to gelatin controls
Oct-4	Steady down regulation.	No effect.
Nanog	Initial down regulation, followed by steady up regulation to levels similar to those observed in undifferentiated controls.	Slightly higher transcript levels observed although not significant.
Ehox	Low levels initially, with the levels of transcripts increasing several fold on the last time point.	No effect.

Table 4. Changes in the levels of pluripotency gene transcript levels of ES cells grown on the FIII9-FIII10 dimer compared to gelatin controls.

The various genes used to analyze whether the FIII9-FIII10 dimer induces ES cell differentiation into any one particular cell lineage/cell type(s) using serum replacement medium were then assayed for.

84
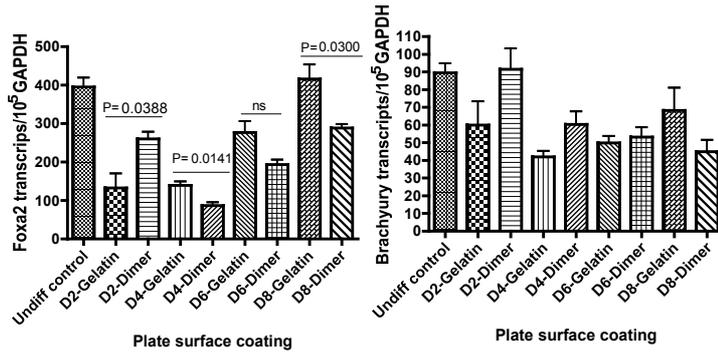
A   B

Figure 3.26 QPCR analyses of, A. Foxa2 B. Brachyury transcripts expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in serum replacement medium. N = 3.

In figure 3.26A it is clear that the FIII9-FIII10 dimer has some effect on the levels of Foxa2 transcripts. After two days of growth in serum replacement medium in the absence of LIF there is a transient and significant upregulation in Foxa2 transcripts produced by ES cells grown on the FIII9-FIII10 dimer as opposed to gelatin controls. At every other time point the opposite is observed with a lower level of Foxa2 transcripts generated by ES cells on the FIII9-FIII10 dimer compared to gelatin grown ES cells. Upon assaying for Brachyury an up regulation was observed in ES cells grown on the FIII9-FIII10 dimer compared to gelatin grown ES cells on days 2 and 4 although this increase does not reach significance. On day 6 there is no observable difference in the transcripts produced by ES cells on either surface and on day 8 there is a reduction in the level of transcripts produced by FIII9-FIII10 dimer grown ES cells when compared to gelatin grown ES cells. This again does not reach significance.

A   **B**
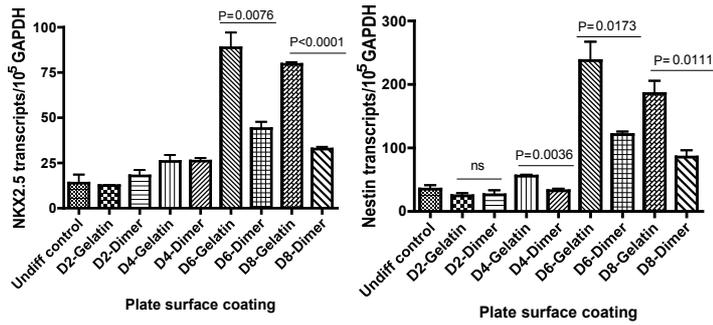
Figure 3.27 QPCR analyses of, A. NKX2.5 B. Nestin transcripts expressed by ES cells grown on either 0.1% gelatin or 100μg/ml dimeric FIII9-FIII10 in serum replacement medium. N = 3.

Growth of ES cells on the FIII9-FIII10 dimer appears to have a significant effect on both the levels of NKX2.5 and Nestin transcripts as upregulation of both are limited upon growth on the FIII9-FIII10 dimer when compared to gelatin grown ES cells controls. The FIII9-FIII10 dimer affects the level of Nestin transcripts earlier than NKX2.5. Nestin upregulation is limited on day 4 on the FIII9-FIII10 dimer whereas NKX2.5 shows initial signs of inhibition on day 6.

A

B
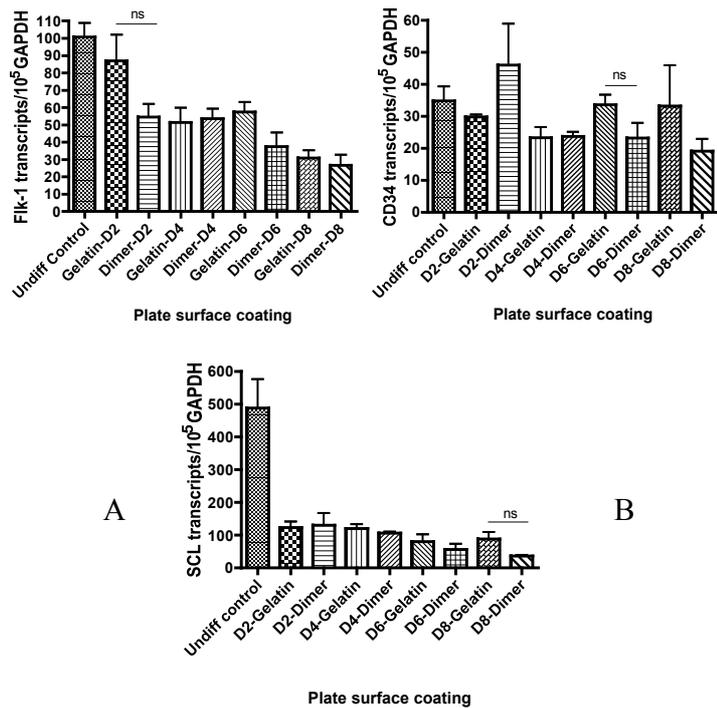
C

Figure 3.28 QPCR analyses of, A. Flk-1 B. CD34 C. SCL transcripts expressed by ES cells grown on either 0.1% gelatin or 100µg/ml dimeric FIII9-FIII10 in serum replacement medium. N = 3.

From figure 3.28A we can say with reasonable confidence that neither the FIII9-FIII10 dimer nor gelatin has any discernable role in the induction of a FLK-1 positive population of ES cells. There is a general reduction in the levels of FLK-1 transcripts upon removal of LIF. There also appears to be no effect of the FIII9-FIII10 dimer on CD34 transcripts as no

real trend appears here. Finally the levels of SCL appear unaffected by the growth of ES cells on either gelatin controls or the FIII9-FIII10 dimer. There is a considerable reduction in SCL transcripts upon removal of LIF but no difference occurs between the two surfaces in the absence of LIF. The data observed during the QPCR assays carried out to look for differentiation of ES cells are summarized in table 5 below.

Gene	General pattern of transcriptional profie	Specific effect of FIII9-FIII10 dimer compared to gelatin controls
Foxa2	Initial down regulation compared to undifferentiated ES cell controls. Followed by up regulation of transcripts	Significant increase in transcripts on day 2, followed by a reduction in the level of transcripts on days 4, 6, and 8. Days 4 and 8 show significant down regulation.
Brachyury	No obvious pattern	Increased levels of Brachyury transcripts on days 2 and 4 but does not reach significance. A reduction in the transcripts on day 8 but again is not significant.
NKX2.5	No change initially in the levels of transcripts compared to ES cell controls. Followed by changes depending on the plate surface coating.	Significant reduction in transcripts on days 6 and 8.
Nestin	No change initially in the levels of transcripts compared to ES cell controls. Followed by changes depending on the plate surface coating.	Significant reduction in transcripts on days 4, 6 and 8.
Flk-1	General reduction in transcript levels.	No significant effect.
CD34	No real pattern, slight overall reduction in transcript numbers compared to undifferentiated ES cell controls.	No significant effect.
SCL	Several fold reduction in transcript levels compared to undifferentiated ES cell controls.	No significant effect.

Table 5. Changes in the markers of differentiation gene transcript levels of ES cells grown on the FIII9-FIII10 dimer compared to gelatin controls.

Chapter 4 Discussion

4.1. Cell adhesive properties of the FIII9-FIII10 derivatives

The data presented in figure 3.1 indicated that there was a negligible effect on the cell attachment potential of FIII9-FIII10 derivatives when the protein is displayed in a specific orientated conformation. Although there appeared to be a small increase in the number of colonies formed when orientated 2-D polyvalent displays are used there was no significant difference compared to using FIII9-FIII10 surfaces furnished in a random manner. Although there are studies suggesting that factors such as the clustering and concentration of ligands are required to optimize cell adhesion (Maheshwari et al 2000, Koo et al 2002, Irvine et al 2002, Kreiner et al 2008) the data presented in figure 3.1 show quite the opposite. It is possible that the specific conformation of ligands could enhance cell adhesion of specific cell lines such as BHK fibroblasts used in Kreiner et al 2008 and that the lack of effect observed in this study was specific to ES cells. It is unlikely however that the generation of 2-D polyvalent displays is in any way an advantage to the growth of ES cells in monoculture and therefore this was removed from the remainder of the project

As stated earlier in the results section there were a number of interesting results come came out of the experiment presented in figure 3.1. The FIII9-FIII10 dimer had a greater ability to support ES cell colonies than gelatin, a commonly used surface for the culturing of ES cells. Therefore it is possible that these FIII9-FIII10 derivatives would be of potential use as a surface medium for ES cell culture. The second and more intriguing observation was the phenotypic changes observed upon culturing ES cells on FIII9-FIII10 derivatives as opposed to gelatin as shown in figure 3.2. ES cells grown on gelatin tended to from small round colonies whereas ES cells grown on the FIII9-FIII10 derivatives were predominantly flatter and more spread in morphology.

This difference in the morphology of ES cells grown on the FIII9-FIII10 derivatives as compared with those grown on gelatin was of interest as there is some literature suggesting

that ES cells grown on Fibronectin in a monolayer tend to flatten out and differentiate (Nishikawa et al 1998, Ying et al 2003, Hayashi et al 2008). With the majority of work carried out on delineating the mechanisms of ES cell differentiation using embryoid bodies the use of monolayer ES cells could be an alternative method of ES cell differentiation. Indeed there is evidence that ES cell differentiation along certain lineages proceeds more efficiently in monolayers as opposed to embryoid bodies (Nishikawa et al 1998). What made this change in morphology all the more interesting is that these ES cells were cultured in complete medium containing 1000U/ml of LIF. Even though LIF is essential for the maintenance of ES cells in an undifferentiated state (Smith et al 1998, Williams et al 1998) there appeared to be partial differentiation of the ES cells grown on the FIII9-FIII10 dimer.

4.2. Optimal conformation of FIII9-FIII10 derivatives for ES cell adhesion

The fact that the dimeric form of the FIII9-FIII10 derivative had the greatest ability to support the growth of ES cell colonies was an unexpected finding when you consider that one molecule of the dimer will have a smaller number of the FIII9-FIII10 adhesive sites compared to the trimer or tetramer which will have 3 and 4 FIII9-FIII10 adhesive sites per molecule respectively. The experiment shown in figure 3.3 and confirmed by the cloning efficiency experiment in figure 3.4 showed that the FIII9-FIII10 derivative, which consistently achieved the highest levels of ES cell colony formation, was the dimer.

It is reasonable to hypothesize that the dimeric form presents the FIII9-FIII10 adhesive sites in a manner that enables the ES cells to more easily interact with them and subsequently bind. It must be stressed however that although the dimeric form of the FIII9-FIII10 derivative is consistently more efficient at ES cell adhesion compared to the other conformations they are not statistically significant. Therefore the difference is marginal but consistent. This result goes against a previous report by our collaborators suggesting that the tetrameric form of the FN derivative was the most efficient at adhering cells (Kreiner et al 2008). However in the report by Kreiner et al 2008 BHK fibroblasts were used in the cell adhesion assays. It is possible that the reason our results differ from that of Van der Walle and colleagues (Kreiner et al 2008) was simply due to the fact that different cell

lines were used. The two studies do agree however that there is no one conformation of the FIII9-FIII10 derivative that is statistically the most efficient at cell adhesion.

4.3. Effect of FIII9-FIII10 derivatives on ES cell proliferation

The lack of any discernable effect on proliferation of ES cells upon growth on the FIII9-FIII10 derivatives in comparison to gelatin grown ES cells is no surprise as it is in agreement with the study carried out by Hayashi et al 2007. It is interesting to note however that in the report by Hayashi et al 2007, the growth curve observed is gradual whereas figure 3.5 in the current study shows a marked increase in cell numbers between days 3 and 4. The result by Hayashi et al 2007 are perhaps at odds with the literature as ES cells are well known for their ability to proliferate and expand when in controlled culture conditions (Smith et al 2001). This rapid increase in cell numbers is the reason that close monitoring of ES cell cultures and regular cutting back of cell numbers is required to maintain ES cells in a pluripotent state (Smith et al 2001). Upon plating of ES cells one would expect a period of cell attachment where there is a minimal increase in cell numbers. As ES cells become accustomed to their environment an increase in cell numbers would then be expected to occur as observed in this study. Although whole FN was used in the study by Hayashi et al 2007 it is unlikely that this would be a factor contributing to the differences in result when both studies are compared.

4.4. Comparison of the adhesive abilities of the dimeric FIII9-FIII10 with whole FN

It was not surprising to see that when used at equal microgram concentrations the FIII9-FIII10 dimer had far superior cell attachment abilities compared with whole FN. When one considers that whole FN has a molecular weight of between 440-500kDA and the FIII9-FIII10 dimer's molecular weight is only 29.7kDA, if used at the same quantity in micrograms there would be a far larger number of FIII9-FIII10 adhesive sites in 50μg of the FIII9-FIII10 dimer than in whole FN.

However this experiment was important as if the FIII9-FIII10 dimer has a greater potential to support ES cell colony formation compared with whole FN at the same concentration

91

this would be a good argument for its use instead of whole FN for basic cell adhesion and for the reasons stated in the results section.

The ability of the FIII9-FIII10 dimer and whole FN to support ES cell colonies was then examined with both proteins used at an equal mole for mole concentration. This simply means that the same number of FIII9-FIII10 binding sites would be found in six well plates coated with either the FIII9-FIII10 dimer or whole FN. One might expect that if the same numbers of binding sites were available, a similar number of ES cel colonies would form. Interestingly this was not seen upon plating the proteins in this manner, and whole FN was statistically more efficient at supporting the growth of ES cell colonies compared with the FIII9-FIII10 dimer as shown below in figure 3.7.

This trend is consistent when the numbers of ES cell colonies are examined at day 3 using the colony counting technique and is in agreement with the cloning efficiency data. Whilst of intellectual value this result was again not totally unexpected. The FIII9-FIII10 dimer is designed to bind with high affinity to the $\alpha5\beta1$ integrin receptor but whole FN can bind to multiple integrin receptors including $\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, $\alpha8\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, $\alpha v\beta6$ and $\alpha IIb\beta3$ and all of these have been identified on ES cells (Yang JT et al 1999). Although the $\alpha5\beta1$ integrin receptor appears to be the primary receptor for interaction with FN (or at least the best studied) it would not be a surprise if the additive effect of whole FN binding to multiple integrin receptors would enhance its ES cell binding ability when both proteins are used at equal molar concentrations.

Secondly the FIII9-FIII10 dimer may not be a perfect mimic of FN found in the ECM, which intact FN more closely resembles. This is a logical suggestion as the derivatives are only a small peptide fragment of the whole protein. Finally the cell binding domain may be more stable when found in the context of the whole protein and may have a preferred orientation upon absorption to plastic surfaces for integrin binding. All of these possibilities could explain the results observed. However the pharmaceutical industry would have to justify the costs and the purity of manufacturing whole FN instead of using

the FIII9-FIII10 dimer. There is no doubt that these FIII9-FIII10 dimers have some potential uses for basic cell attachment purposes.

4.5. Effect of LIF titration on ES cell morphology

The change in morphology of ES cells grown on the FIII9-FIII10 dimer when the concentration of LIF was increased, i.e. from a dispersed phenotype to a tight phenotype was an indication that something may be going on at the molecular level. It is possible that when ES cells were adhering to the FIII9-FIII10 dimer they were being given signals to start to differentiate even in the presence of LIF. The presence of LIF in this situation would inhibit any differentiation of the ES cells although due to the potential pro-differentiation signals being received by the cells from the FIII9-FIII10 dimer there is an initial morphological change. However as the concentration of LIF was increased this pro differentiation signal was further subdued and the ES cells reverted to a phenotype more similar to that seen when ES cells are grown on gelatin plates. It is clear from figure 3.10 that the titration of LIF did have an effect on these "tight" and "dispersed" colonies as the ratios of both colony types clearly change as the cytokine is increased in concentration. It would have been interesting to carry out QPCR analysis of ES cells grown on the FIII9-FIII10 dimer in the presence of increasing concentrations of LIF to look for any changes in transcript levels that could be responsible for the observed change in morphology. However it was felt at the time that to repeat this experiment would be too expensive in terms of the use of LIF to justify carrying out.

4.6. Alkaline phosphatase staining

The use of the alkaline phosphatase assay provided strong evidence that while there appeared to be morphological changes occurring on ES cells grown in a monolayer on the FIII9-FIII10 dimer, these cells in the presence of LIF were maintaining their undifferentiated, pluripotent state. It was clear that any pro-differentiation signals the ES cells may have been receiving from the FIII9-FIII10 dimer were being inhibited by LIF signalling via the gp130 receptor chain. Upon removal of LIF the expected loss of the majority of alkaline phosphatase staining occurred. There was no obvious difference

93

between ES cells cultured in the absence of LIF grown on either gelatin or the FIII9-FIII10 dimer. Any effect the FIII9-FIII10 dimer had on ES cell differentiation did not result in any marked reduction in alkaline phosphatase activity compared with ES cells grown on gelatin.

4.7. Transcriptional analysis in the presence and absence of LIF in serum containing media

The transient increase in Oct-4 levels was of interest as a publication from Austin Smith's laboratory elegantly showed that if Oct-4 expression was increased by just under two fold it resulted in the production of an early endodermal/mesodermal population of cells (Niwa et al 2000). It must be stated clearly at this point however that transcriptional changes are merely an indicator as to what may be going on and one cannot draw strong conclusions from them. Therefore it is possible that this upregulation of Oct-4 could result in the formation of an early mesodermal/endodermal population but protein expression studies would have to be carried out in order to confirm such a hypothesis. On day 6 the increased levels of Oct-4 transcripts observed on day 3 had reduced to the levels of ES cells grown on gelatin. It should also be noted that ES cells grown on gelatin maintain their levels of Oct-4 with no real increase or decrease in transcript numbers which is an indication that these ES cells remain essentially undifferentiated.

Upon growth of ES cells on the two surfaces in the absence of LIF a similar trend is observed in that there is an increase in transcript numbers on day 3 in ES cells grown on the FIII9-FIII10 dimer compared to gelatin. However, it should be noted that although this increase is consistent it does not reach significance ($P = 0.0556$). Similarly to ES cells grown in the presence of LIF there is a no real increase in numbers of Oct-4 transcripts in ES cells grown on gelatin in the absence of LIF. It should be remembered however that although there is an increase in Oct-4 transcripts, which may indicate a possible mesodermal/endodermal population forming, the ES cells grown on the FIII9-FIII10 dimer remain undifferentiated as shown by the alkaline phosphatase study in figure 3.11.

This increase in Oct-4 transcript numbers was an indication that the FIII9-FIII10 dimer did indeed have some ES cell differentiation inducing properties. It should be remembered however that in the presence of LIF ES cells maintain their pluripotent state as seen through the alkaline phosphatase positivity of the colonies in figure 3.12. even though there is an increase in Oct-4 levels. As an increase in Oct-4 levels is suggestive of ES cell differentiation into the mesodermal/endodermal lineages we looked for other markers that might provide further evidence that the FIII9-FIII10 dimer were attempting to push ES cells down this lineage. The upregulation of Brachyury in ES cells grown on the FIII9-FIII10 dimer at day 3 in the presence of LIF was further evidence indicating that ES cells grown on the FIII9-FIII10 dimer were trying to differentiate into an early mesodermal population.

When LIF is removed and the ES cells allowed to differentiate on either gelatin or FIII9-FIII10 dimer coated surfaces there is a small increase in Brachyury levels when grown on the FIII9-FIII10 dimer although this is not significant. Similar to the levels of Oct-4 the elevated levels of Brachyury transcripts observed on day 3 induced by the FIII9-FIII10 dimer are reduced to levels very similar to those observed on gelatin on day 6. These results suggest that there may be some mesodermal inducing properties of the FIII9-FIII10 dimer but in the presence of LIF the ES cells are maintaining their undifferentiated phenotype in spite of the transcriptional fluctuations observed. On day 6 in the absence of LIF there is a decrease in the levels of Brachyury observed in ES cells grown on the FIII9-FIII10 dimer. This is perhaps strange when one considers the enhanced levels of Brachyury observed in ES cells grown on the FIII9-FIII10 dimer on day 3 in the presence of LIF. Though it is merely speculation it is possible that in the absence of LIF ES cells grown on the FIII9-FIIII10 dimer increase the levels of Brachyury as early as 1 or 2 days after plating and what we are seeing on day 3 are the levels of Brachyury decreasing as the signal to differentiate into an early mesodermal population has been received by the cell.

Concomitant with the increase in levels of Oct-4 and Brachyury there is also an increase in the levels of Nanog on day 3 when grown in the presence of LIF. It is perhaps not surprising that Nanog up regulates in ES cells grown upon the FIII9-FIII10 dimer as Nanog is known for its ability to reverse the differentiation of ES cells and return the cells

95

to an undifferentiated state if grown in the presence of LIF (Suzuki et al 2006a, Suzuki et al 2006b). The studies carried out by Belmonte and colleagues (Suzuki et al 2006a, Suzuki et al 2006b) demonstrated that ES cells stimulated to differentiate using bone morphogenetic proteins (BMPs) resulted in the upregulation of Brachyury which occurred in tandem with an upregulation of Nanog. This dual up-regulation of Nanog and Brachyury has been noted in this study with the difference in stimulating this upregulation being the use of a peptide of FN, which binds with high affinity to $\alpha5\beta1$ integrin receptor (van der Walle et al 2002).

Belmonte and co-workers then identified that STAT3 and Brachyury would bind to an element involved in the regulation of Nanog that resulted in elevated levels of Nanog to counteract the pro-differentiation effects of BMPs (Suzuki et al 2006b). Importantly in 2007, Austin Smith's group showed that one of the major functions of Nanog was to maintain ES cell pluripotency by preventing the upregulation of pro-differentiation genes (Chambers et al 2007). It is possible that growth of ES cells on the FIII9-FIII10 dimer signals ES cells to begin differentiating but in the presence of STAT3 mediated LIF signalling results in the upregulation of Nanog, which reverses the pro-differentiation effects of the FIII9-FIII10 dimer. This reversal of ES cell differentiation is confirmed in the high percentage of alkaline phosphatase staining observed when ES cells are grown on the FIII9-FIII10 dimer in the presence of LIF.

If this co upregulation of Brachyury and Nanog in ES cells grown on FIII9-FIII10 dimer in the presence of LIF had been identified earlier in the project it would have been of interest to use small interfering RNA (siRNA) to partially inhibit Nanog and observe whether the FIII9-FIII10 dimer could maintain elevated levels of Brachyury and induce ES cell differentiation. Inhibiting the function of Nanog completely would not be desirable however as this would result in the formation of an endoderm population (Mitsui et al 2003) regardless of the potential effects of the FIII9-FIII10 dimer. In addition, over expression of Nanog in ES cells then growing them on the FIII9-FIII10 dimer with subsequent monitoring of the levels of Brachyury would also be of interest. It is possible that over expressing Nanog could inhibit the transient increase in Brachyury levels

normally observed on day 3 in ES cells grown on FIII9-FIII10 dimer in the presence of LIF.

It is clear that growing ES cells either on gelatin or the FIII9-FIII10 dimer in the presence of LIF does not alter Ehox. There are no major changes in the levels of Ehox regardless of the substrate grown on with the exception of a general increase in the levels of Ehox on day 6 as opposed to day 3. This increase in the levels of Ehox is not significant therefore the levels of Ehox in this setting are clearly maintained. Upon removal of LIF there is a general reduction of the levels of Ehox. This is in agreement with Jackson et al 2002 who identified that Ehox is expressed in undifferentiated ES cells and upon the induction of differentiation is reduced. This paper then goes on to state that the levels of Ehox begin to increase again several fold as differentiation continues. It is possible this increase is not observed as the assay is not carried out over a sufficiently long period of time. However this effect is observed later in this study using serum replacement media with additional, later time points.

It appears that the haematopoietic transcription factor GATA-1 (Orkin 1998) is not altered upon growth of ES cells on either the FIII9-FIII10 dimer or the gelatin controls in the presence of LIF. There is an increase from day 3 to day 6 but there are no changes between surfaces. Removal of LIF does not appear to alter the lack of effect of the FIII9-FIII10 dimer has on ES cell differentiation compared with the gelatin controls as though there is a general reduction in the levels of GATA-1, this occurs both in the gelatin control and FIII9-FIII10 dimer grown cells. Therefore it appears, certainly at the time points examined, that these ES cells are not being induced to differentiate into the erythrocyte/megakaryocyte lineage due to the general reduction in the levels of GATA-1. It is possible that this is due to the growth of the ES cells in a monolayer. Secondly the time points used may be too early to observe any changes in GATA-1 that could be evidence of erythrocyte/megakaryocyte differentiation.

Possibly the most surprising result obtained during the course of this study was the marked reduction in the level of CD34 upon growth of ES cells on the FIII9-FIII10 dimer in the

presence of LIF compared to gelatin controls. As can be seen from figure 3.19, the levels of CD34 transcripts increase from day 3 to day 6 on gelatin, in contrast to ES cells grown on the FIII9-FIII10 dimer in which there is a significant reduction (P = 0.0003) in the number of CD34 transcripts from day 3 to day 6. The reason for this is unknown. It is possible that the presence of STAT3 mediated LIF signaling, in addition to signals generated via the FIII9-FIII10 dimer binding to the α5β1 integrin receptor, results in a signaling cascade that reduces the levels of CD34. Further study would be required to identify why this peculiar result has occurred.

Upon removal of LIF there is a non significant increase in the levels of CD34 in ES cells grown on the FIII9-FIII10 dimer compared to gelatin controls. It is perhaps strange that this would occur as growth of ES cells on the FIII9-FIII10 dimer in the presence of LIF resulted in a marked reduction in the levels of CD34. One possibility is that the combination of signals from LIF signaling via the LIF receptor and the FIII9-FIII10 dimer signaling via the α5β1 integrin receptor results in a down regulation of CD34. However in the absence of LIF receptor signaling, the interaction of the FIII9-FIII10 dimer with the α5β1 intgrin receptor results in an up regulation of CD34 transcripts.

Upon assaying for changes in the transcriptional levels of SCL, another transcription factor involved in haematopoesis (Lecuyer et al 2004, Park et al 2005) it was found that there was no obvious effect of the FIII9-FIII10 dimer on the levels of SCL. However there was a significant reduction (P<0.02) in the levels of SCL on day 6 compared to day 3 in ES cells grown on both surfaces in the presence of LIF. In the absence of LIF the trend is the same with a notable reduction in SCL transcripts from day 3 to day 6. This does not reach significance but is a consistent finding. One explanation of this marked reduction in the levels of SCL on day 6 is that SCL could be sensitive to approaching confluency. In this assay, on day 6, ES cells are approximately 70%-80% confluent and it is possible that this effects the levels of SCL.

Analysis of the effect of growing ES cells on the FIII9-FIII10 dimer revealed that in the presence or absence of LIF there was a consistent, non-significant increase in the levels of Flk-1 transcripts. The levels of Flk-1 in ES cells grown on gelatin did not alter substantially on day 3 or 6 whether LIF was present or absent indicating that at least at these time points there appeared to be little in the way of gelatin inducing a Flk-1 positive population of cells. Initially the possibility that the FIII9-FIII10 dimer may be a mild inducer of endothelial cell differentiation, which Flk-1 is known to have a role in (Shalaby et al 1997) was considered. Although the fact that the increase in Flk-1 transcripts is not significant suggests that it might not be biologically important.

The observed increase in the levels of Nestin was intriguing as it indicated that the FIII9-FIII10 dimer may have some ectodermal inducing properties. This result is in agreement with another publication also investigating the effects of FN on ES cell function (Hayashi et al 2007). The publication by Ashashima and colleagues however reports a less than 0.5 fold increase in Fgf5 transcripts (primitive ectoderm marker) as evidence for this. In addition they state that ES cells grown in serum free medium in the presence of LIF have substantially reduced alkaline phosphatase levels when compared to gelatin controls. This result is not surprising as Austin Smith's group previously showed that serum free cultures of ES cells cannot maintain their pluripotent identity even in the presence of LIF (Ying et al 2003) and begin neural differentiation. This paper showed that it was bone morphogenetic proteins (BMPs), found in the serum that acted in concert with LIF to maintain the pluripotent state of ES cells (Ying et al 2003). Therefore the results from Ashashima and colleagues do not definitively confirm that it is the effect of the fibronectin on the ES cells, not simply the removal of BMPs from the media, that is causing the neural differentiation phenotype.

From the QPCR results shown in figure 3.22 it appears that FIII9-FIII10 dimer plays no observable role in either elevating or decreasing NKX2.5 transcripts as there is no difference between ES cells grown on gelatin or the FIII9-FIII10 dimer on day 6. The levels of NKX2.5 are elevated very slightly when moving from day 3 to day 6 but this is not due to the surfaces the ES cells are grown on. In the absence of LIF there is a substantial decrease in the levels of NKX2.5 on either gelatin or the FIII9-FIII10 dimer. The reduction of NKX2.5 transcripts in the absence of LIF was at odds with conventional wisdom, as ES cells are well known to differentiate into cardiomyocytes when LIF is

removed. However this occurs when ES cells are differentiated as embryoid bodies, the in vitro version of the embryo. It is possible that when plated on a monolayer ES cells do not have the capacity to form cardiomyocyte populations and therefore do not up regulate NKX2.5.

There was no identifiable effect of the FIII9-FIII10 dimer on the levels of Foxa2, an endodermal marker. When ES cells were plated on either gelatin controls or the FIII9-FIII10 dimer no real differences were observed in the presence or absence of LIF. In addition the levels of Foxa2 dropped in both conditions suggesting that whatever the effect of the FIII9-FIII10 dimer was, it did not involve the gene Foxa2, At least not at these time points.

4.8. Transcriptional analysis using serum replacement media

As stated in the results section, the presence of potential differentiating agents in the media led to the use of serum replacement media in place of foetal calf serum complete media. Removal of LIF and growth of ES cells on either gelatin or the FIII9-FIII10 dimer led to a gradual reduction in the levels of Oct-4 in serum replacement media. There was an increase in the levels of Oct-4 on day 6 when ES cells were grown on the FIII9-FIII10 dimer but this did not reach significance. This calls into question whether or not there is a mesodermal/endodermal inducing quality of the FIII9-FIII10 dimer as previously thought when significant increases in the levels of Oct-4 were observed in ES cells grown on the FIII9-FIII10 dimer in the presence of LIF. This assay is informative however as it clearly shows that the ES cells are differentiating as there is a gradual reduction of Oct-4 although the levels of Oct-4 do remain at a low level as has been previously documented when LIF is removed (Smith AG 2001).

It is clear that growth of ES cells on the FIII9-FIII10 dimer in no way affects the levels of Ehox as no differences were observed between ES cells grown on gelatin or the FIII9-FIII10 dimer. The changes observed in the levels of Ehox between time points however are in agreement with the initial report describing the identification of this gene (Jackson et al

100

2002). Graham and colleagues (Jackson et al 2002) used embryoid bodies to show that low levels of Ehox are observed in undifferentiated ES cells but these levels increase five fold as the bodies differentiate further. In the assay detailed in figure 3.25C the levels of transcripts up regulate on day 8 to levels similar to those observed by Jackson et al 2002. This result is interesting from another point of view as it appears that, at least in terms of early transcriptional events, growing ES cells on a monolayer could be used as an alternative to embryoid bodies. The gene expression profiles are similar to that observed by Jackson et al 2002 with the only real difference in that the up regulation of Ehox occurs two days later using the monlayer approach.

The reduction in the level of Nanog transcripts on both surfaces in serum replacement medium was to be expected as ES cells are known to lose Nanog expression upon culturing in the absence of LIF (Chambers et al 2003). Rather surprisingly it was noted that from day 4 onwards the levels of Nanog began to increase on both surfaces. It is possible that, in the absence of LIF, Nanog transcripts are up regulated in an attempt to resist ES cell differentiation but in the absence of STAT3 mediated LIF signaling, functional Nanog protein cannot be produced therefore ES cell differentiation continues uninhibited. This is a possibility as Nanog and STAT3 signalling have been reported to act in concert to inhibit differentiation (Torres et al 2008).

The increase in the levels of Foxa2 transcribed by ES cells grown on the FIII9-FIII10 dimer compared with gelatin controls cultured in serum replacement medium is an indication that the ES cells could be receiving an endodermal/mesodermal differentiation signal. It should be noted that the levels of Foxa2 grown on the FIII9-FIII10 dimer intitially increase on day 2, then reduce notably on day 4. The levels of Foxa2 transcripts then steadily rise on day 6 and day 8. The levels of Foxa2 are static between days 2 and 4 when ES cells were grown on gelatin. The levels then begin to rise on days 6 and 8. As ES cells are known to differentiate into all 3 germ layers in the absence of LIF (Smith 2001) it is possible that the FIII9-FIII10 dimer gives the ES cells an earlier, perhaps more specific signal to differentiate into this particular lineage.

Although the levels of Brachyury do increase in ES cells grown on the FIII9-FIII10 dimer on days 2 and 4 this does not reach significance, indicating that although Brachyury may play a role in the effects observed through use of the FIII9-FIII10 dimer it may not be the primary gene involved. This is at odds with ES cells grown in the presence of LIF in complete media on the FIII9-FIII10 dimer when a significant increase in the levels of Brachyury was noted earlier in the results. It is possible that in the absence of LIF using serum replacement medium reduces the impact of Brachyury on this process. Secondly in the serum replacement assay a day 3 time point was not used which may have shown significant up regulation of Brachyury as observed in ES cells grown on the FIII9-FIII10 dimer.

It is apparent that the FIII9-FIII10 dimer inhibits the differentiaton of ES cells into certain lineages as both NKX2.5, a cardiac transcription factor (Murry et al 2008) and the neuroepithelial stem cell marker Nestin (Lendahl et al 1990), are both significantly reduced upon growth of ES cells on the FIII9-FIII10 dimer when compared to gelatin grown ES cells. The reductions in the levels of Nestin are a particular surprise as, in the presence of LIF in Foetal calf serum media, the levels of Nestin were elevated when grown on the FIII9-FIII10 dimer. It is possible that STAT3 signaling in conjunction with the signals received through the interaction of the ES cells with the FIII9-FIII10 dimer result in up regulation of Nestin and Nanog, similar to the mechanism which Brachyury and Nanog can be up regulated in the presence of LIF (Suzuki et al a,b 2006).

At the time points used in this assay there is no effect on the levels of Flk-1 transcripts by growth of ES cells on either gelatin or the FIII9-FIII10 dimer in serum replacement media as there is a uniform reduction in the levels of Flk-1 transcripts. It must be concluded therefore that in this particular assay there is no stimulus for endothelial differentiaton due to the reduction in Flk-1 transcripts. In media containing foetal calf serum there is a transient increase in the levels of Flk-1 as discussed earlier. Again it is possible that the foetal calf serum in addition to the FIII9-FIII10 dimer provides an environment that is favorable to an increase in Flk-1 transcripts.

The levels of CD34 are also unperturbed upon growth of ES cells on either gelatin or the FIII9-FIII10 dimer in the serum replacement medium based assay. Also no effect on the levels of SCL transcripts were observed when ES cells were grown on gelatin or the FIII9-FIII10 dimer although there was a substantial reduction in the levels of transcripts when compared with undifferentiated controls. It is possible this occurs as growth in the absence of LIF on either gelatin or the FIII9-FIII10 dimer is not suitable for the up regulation of SCL at the time points tested. This would fit as both SCL and CD34 are hematopoietic markers and in this current assay there is little evidence to suggest that the ES cells are differentiating along this lineage.

To conclude, there is evidence presented in this thesis that indicates that the FIII9-FIII10 dimer is the most efficient of all the conformations of the FIII9-FIII10 derivatives in supporting ES cell growth. There are potential advantages in using these FIII9-FIII10 derivatives in terms of cell attachment over standard fibronectin as there is greater control over the purity and structure as discussed in the introduction. In addition these FIII9-FIII10 proteins would be cheaper to manufacture than standard fibronectin. In terms of differentiation potential, there appears to be some signs that transcriptionally, the FIII9-FIII10 dimer has some mesodermal/endodermal effects. However these proteins on their own are not adequate to generate specific cell lineages and would be required to be used in conjunction with numerous other factors.

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