



**Purification, identification and characterisation of signals directing
Embryonic Stem (ES) cell differentiation**

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Abstract

Embryonic Stem (ES) cells are derived from the pluripotent cells of the 4.5 days post coitum (dpc) mouse blastocyst inner cell mass (ICM) and can be maintained indefinitely in culture in the presence of the cytokine leukaemia inhibitory factor (LIF) as the *in vitro* equivalent of their cells of origin. ES cells retain their pluripotentiality as defined by their ability to contribute progeny cells to all lineages within the embryo proper following re-introduction into the blastocyst environment. ES cells therefore provide an *in vitro* system from which to study the biology of pluripotent cells and identify the signals that control the early stages of mammalian development. However, while signals that prevent ES cell differentiation *in vitro* such as LIF and other members of the IL-6 cytokine family have been identified, signals that induce lineage specific ES cell differentiation *in vitro* have remained elusive.

In the embryo, ICM cells differentiate through the formation of an obligatory intermediate pluripotent cell population, termed primitive ectoderm, following blastocyst implantation. Primitive ectoderm is distinguished from the ICM by gene expression, differentiation potential and morphology. However, the signals that induce primitive ectoderm formation, maintenance and further differentiation remain unknown.

Recently, it has been reported that culture of ES cells in medium conditioned by the human hepatocellular carcinoma cell line HepG2 directs the formation of homogenous population of pluripotent cells termed early primitive ectoderm-like (EPL) cells. EPL cells are the *in vitro* equivalent of embryonic primitive ectoderm, as defined by morphology, gene expression and differentiation potential *in vivo* and *in vitro*. This indicates that EPL cell formation replicates a critical *in vivo* developmental event *in vitro*. However, the identity of the signals that induce EPL cell formation is unknown. The aim of this thesis was the purification and identification of the EPL cell inductive signals within this conditioned medium (MEDII) and localisation of these signals during EPL cell and primitive ectoderm formation. Prior to the

commencement of this work, it had been established that EPL cell formation requires a high and low molecular weight signal. The low molecular weight signal was purified and identified as the imino acid L-proline or one of a family of short (2-4 amino acid) peptides containing L-proline.

In this thesis, the high molecular weight signal was biochemically and biophysically characterised to design a suitable purification strategy, and purified using heparin affinity, anion exchange, hydrophobic interaction and gel filtration chromatography. The high molecular weight signal was identified as the extracellular matrix associated isoform of fibronectin (FN), cellular FN (cFN). Cellular FN displayed identical biochemical, biophysical and EPL cell inductive properties to the activity purified from MEDII and was shown to be present within the purified sample and at high levels in MEDII.

L-proline and cFN were confirmed as the EPL cell inductive factors through characterisation of EPL cells formed in the presence of these signals. EPL cells were defined by morphology, gene expression and differentiation potential. Further, L-proline and cFN were shown to have distinct roles in EPL cell formation where by L-proline is required for the induction of EPL cell formation while cFN is required for EPL cell equivalent morphology and gene expression, and has an additional role as an EPL cell maintenance factor. However, L-proline and cFN retained an ability to compensate for each other in a dose dependent manner during EPL cell formation, perhaps reflecting additional convergent roles.

Cellular FN was localised in the early embryo and embryoid bodies (EB's) temporally and spatially adjacent to primitive ectoderm and was also shown to be associated with ES and EPL cell ECM. Furthermore, *cFN* transcripts were shown to be expressed by both pluripotent cells and extra-embryonic visceral endoderm (VE) *in vivo* and *in vitro*. This indicates that EPL cells and primitive ectoderm are exposed to both endogenous and exogenous cFN. The role of exogenous cFN as a maintenance factor for EPL cells further suggests that EPL cell formation *in*

vitro closely mimics primitive ectoderm formation in the embryo and, more importantly, that *in vitro* pluripotent cell differentiation systems can be used to identify biologically relevant signals.

Statement of originality

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, it contains no material that has been published by any other person except where due reference is made. The author consents to the thesis being made available for photocopying and loan.

Michael David Bettess

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This Thesis is dedicated to the memory of my Grandfather

Arnold Wilson Welsh

(31/10/1907-19/8/1998)

Chapter 1

Introduction



Chapter 1

1.1 Background

Mammalian embryogenesis is a complex process whereby a single founder cell, the fertilised egg, undergoes a series of finely controlled and coordinated events to form all the complex tissues, organs and structures that constitute the embryo and mature organism. Understanding the mechanisms that drive this developmental process has been a focus of human interest and research for many years. Simple model systems such as the fruit fly *Drosophila melanogaster* and the amphibian *Xenopus laevis* have provided useful comparative information regarding the evolutionally conserved fundamentals of development. However, intrinsic differences between non-mammalian embryogenesis and the development of a mammalian organism within the uterine environment limit the use of these models for investigations into mammalian development. More specifically, a fundamental feature of early mammalian embryogenesis, not displayed in other organisms, is the maintenance of a population of cells that proliferate, differentiate, migrate and apoptose to form the entire organism. These cells are the pluripotent cells of the mammalian embryo. The biology of pluripotent cells is a major focus of this thesis.

The mouse has become the preferred experimental model for the study of mammalian development because of the relatively rapid life cycle, large litter size, short gestation time and similarities in developmental progression with other mammalian species, including man. However, the small size and inaccessibility of the embryo has hindered studies into mammalian embryogenesis during the earliest stages of development. Early studies using microscopy allowed detailed investigation of the morphological changes that occur during *in utero* development. In recent times, the advent of molecular biology has re-focused mammalian developmental research from an understanding of embryonic tissue origin and formation, to an understanding of the origin and biology of specific signals that control these events.

1.2 Early embryogenesis

Early mammalian embryogenesis has been best characterised in the mouse. The mouse is a well-researched model system for early mammalian development and is reviewed below and in Hogan *et al.*, 1994.

1.2.1 Pre-implantation development

Mammalian embryogenesis begins when fertilisation occurs, shortly after the sperm intercepts the egg in the ~~pallodia region of the uterus~~^{oviduct}. A single sperm penetrates the outer layers of the oocyte (cumulus mass and zona pellucida) and enters the cytoplasm, triggering the second meiotic division of the oocyte. This leads to extrusion of the second polar body. Nuclear membranes form around the chromosomes to create maternal and paternal haploid pronuclei that move toward the centre of the egg. At this stage DNA replication takes place. After breakdown of the nuclear membrane, the chromosomes assemble on the spindle and the first cleavage event of the embryo takes place some 20-24 hours post fertilisation (Figure 1.1).

At the 2-cell stage of development the mouse embryo undergoes the oogenic-embryonic transition, where the maternal mRNA is degraded, concomitant with transcriptional activation of the embryonic genome (Latham *et al.*, 1991). Continued division gives rise to the 4-cell embryo at 1.5 days post coitum (dpc) in which individual embryonic cells, or blastomeres, each have the same totipotent developmental potential as the fertilised egg (Kelly, 1977). Around 2.5 dpc the blastomeres of the 8-cell embryo cannot act as the precursor cell of an embryo by themselves but can contribute to all cell lineages when aggregated in chimeric morula (Kelly, 1977).

1.2.2 Blastocyst formation

Formation of the eight-cell embryo is followed by embryonic compaction, which occurs as a consequence of uvomorulin (E-cadherin) activation (Kemler *et al.*, 1977; Vestweber and Kemler, 1985; Sefton *et al.*, 1992). Individual blastomeres flatten upon each other such that cell-cell contact is maximised, establish gap-junction coupling, and develop a polarised phenotype both cytotocortically and cytoplasmically (Figure 1.1 F; Sutherland and Calarco-Gilliam, 1983; Johnson *et al.*, 1986; Sefton *et al.*, 1992).

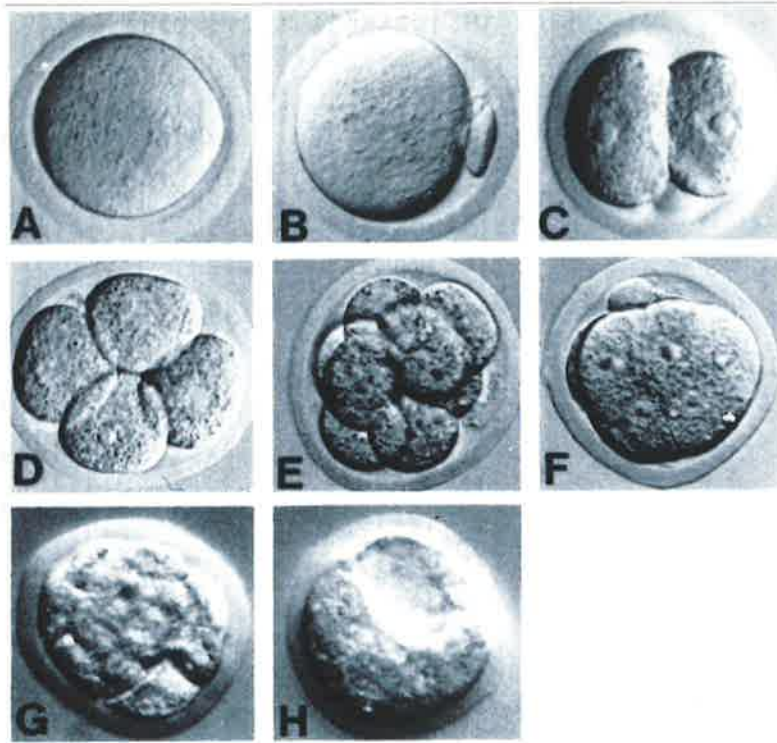
Establishment of cellular polarity is believed to be the basis for the first partitioning in cellular developmental potential (Johnson *et al.*, 1988). Cells at or near the centre of the embryo are progenitors for the inner cell mass (ICM), the pluripotent cell population from which the embryo proper is derived. Pluripotent cells are defined by their capacity to both generate and respond to all

Figure 1.1 From fertilised egg to blastocyst in the pre-implantation mouse embryo

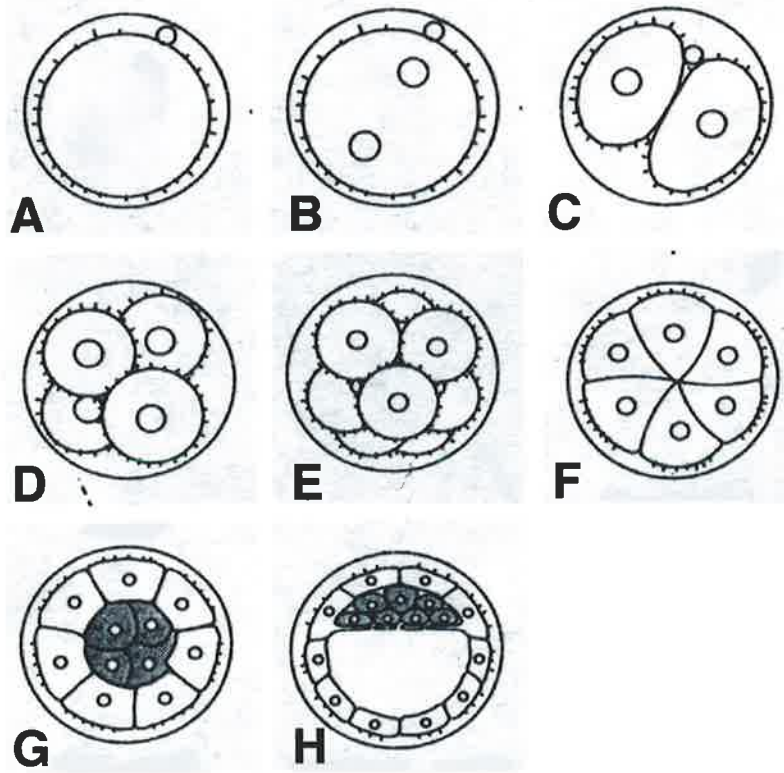
Photographic (i) and diagrammatic (ii) representations of the early stages of mouse preimplantation development. (A) Oocyte arrested in Metaphase II of Meiosis. (B) The fertilised egg with extruded polar body. (C) The two cell stage embryo. (D) The four cell stage embryo. (E) The eight cell stage embryo before compaction. (F) The late eight cell stage embryo after compaction. (G) The 16 cell stage embryo. (H) The 32 cell stage embryo.

Adapted from Maro *et al.* (1991).

i



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the signals that define mammalian embryogenesis, thus contributing progeny to all cell lineages of the embryo and adult, including the germ line. The outer blastomeres differentiate and establish the extra-embryonic trophoblast cell lineage, which forms an outer epithelial layer surrounding the ICM (Gardner, 1983). Passive accumulation of fluid within the blastocyst follows the specific movement of Na^+ and K^+ ions into the blastocoel, through the action of Na^+/K^+ pumps located within the basolateral plasma membrane of the mural trophoblast (Watson and Kidder, 1988; Watson *et al.*, 1990). This results in formation of the blastocoelic cavity (or blastocoel) and at this stage the embryo is referred to as the blastocyst (Figure. 1.1 H).

At about 4 dpc, shortly before implantation, a second developmental partitioning occurs as the cells of the ICM adjacent to the blastocoel, differentiate into a second extra-embryonic epithelial layer, the primitive endoderm (Figure. 1.1 H). At 4.5 dpc the mature blastocyst consists of two extra-embryonic lineages and the ICM. Proteolytic action results in the hatching of the blastocyst from the surrounding zona pellucida (Figure 1.2; Perona and Wassarman, 1986; Hogan *et al.*, 1994), and the embryo is now ready to implant into the uterine wall (Figure 1.3 A).

1.2.3 Post-implantation development

Implantation of the embryo is mediated by the mural trophoblast, ~~apposed to the ICM,~~ which first invades the uterine epithelium and the underlying basal lamina, and then penetrates the uterine stroma (Hogan *et al.*, 1994). The mural trophoblast cells cease cell division but continue DNA replication to form polyploid chromosome containing trophoblast giant cells (TGCs) around 4.75 dpc. These cells play an important role in induction of the decidual reaction during embryo attachment (Gardner and Johnson, 1972). Proliferation of the polar trophoblast adjacent to the pluripotent cells displaces the ICM into the blastocoelic cavity (Figure 1.3 B). Around the time of implantation, the pluripotent ICM cells commence a program of rapid proliferation as the estimated 20-25 ICM cells of the day 4.5 embryo give rise to approximately 660 pluripotent cells by 6.5 dpc and 8060 cells by 7.5 dpc (Snow, 1977). During this proliferative phase the cell cycle times drop as low as 4.4 hours (Snow, 1978). Rapid proliferation of pluripotent cells is accompanied by formation of a central pro-amniotic cavity, at approximately 5.0 dpc, converting the solid mass of pluripotent cells into an epithelial monolayer (refer Figure 1.3 B, C). Like all epithelia, this tissue is polarised

Figure 1.2 The blastocyst hatching from the zona pellucida

(A-B) late stage blastocyst. (C) Inner cell mass flattening (D-G) breach of the zona pellucida and escape of the trophectoderm cell layer. (H) Fully expanded blastocyst with spherical/ovoid form.

1. Inner cell mass
2. Blastocoel cavity
3. Trophectoderm pushing through hole in the zona pellucida wall
4. Boundary of breach in zona pellucida wall
5. Polar trophectoderm cell
6. Fully expanded blastocyst
7. Empty zona pellucida

Adapted from Kaufman, 1992

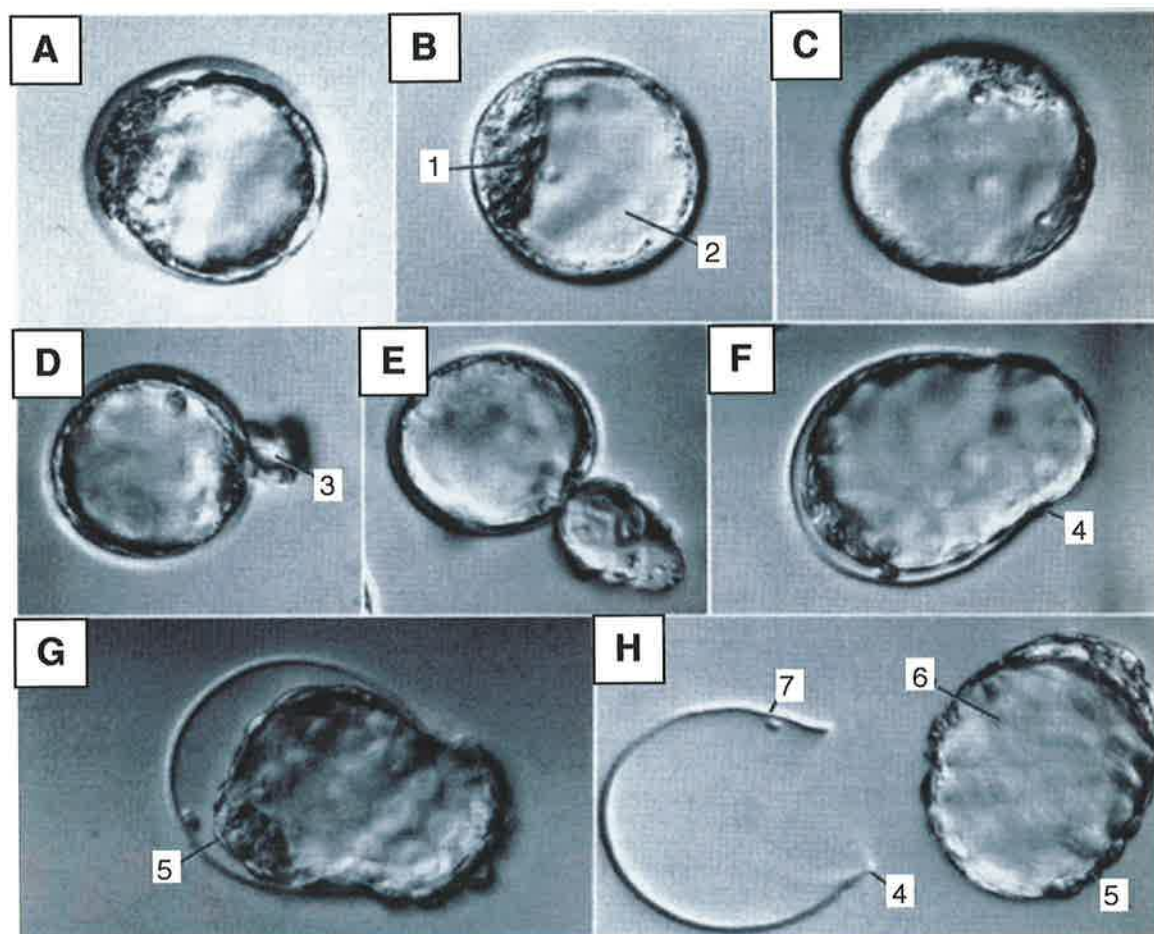
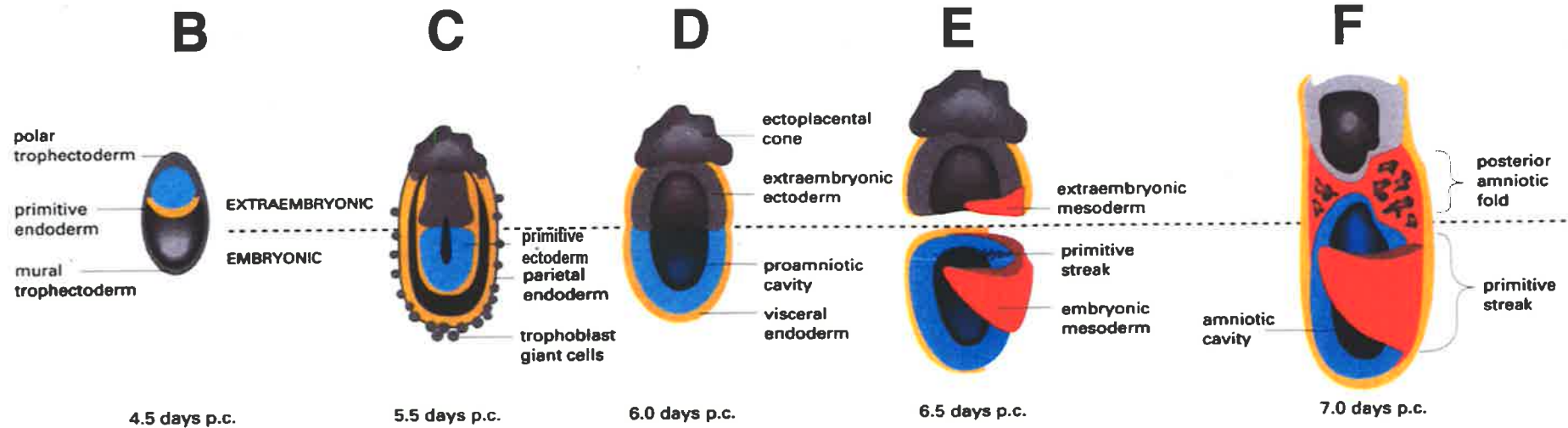
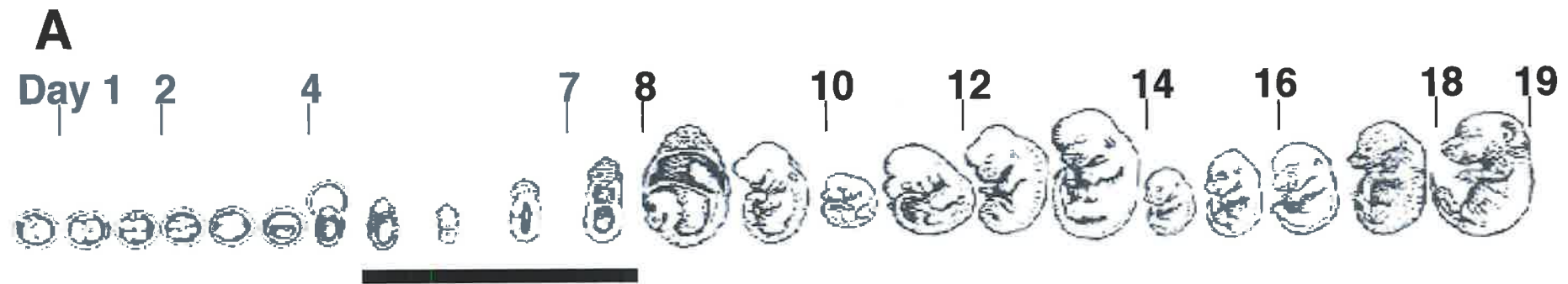


Figure 1.3 Schematic representation of mouse development from the late blastocyst stage to gastrulation.

Schematic representation of the 19 days of mouse *in utero* development (A). The days of development expanded below are represented above the black line. (B) The fully expanded blastocyst 4.5 dpc, just prior to implantation. The embryo is composed of three distinct tissues, trophectoderm (grey), primitive endoderm (yellow) and Inner cell mass (blue). (C) Early egg cylinder stage embryo post implantation, day 5.5 pc. Primitive endoderm cells that migrate onto the inner surface of the trophectoderm, differentiate to parietal endoderm (yellow). Proamniotic cavity formation is initiated. Appearance of primitive ectoderm (D) Late egg cylinder stage embryo at 6.5 dpc with a fully formed, pseudo stratified epithelial layer of primitive ectoderm surrounding inner proamniotic cavity. Primitive endoderm cells that remained in contact with the pluripotent cells have differentiated to form visceral endoderm (yellow). Outer extra-embryonic lineages have been removed for simplicity. (E-F) Gastrulation stage 6.5 (E) and 7.0 (F) day pc embryos. Mesoderm formation begins at the posterior most end of the primitive ectoderm with delamination and invagination of mesodermal cells between the primitive ectoderm and visceral endoderm. The mesoderm extends to the distal most tip of the embryo with the wings extending proximally towards the anterior most end of the egg cylinder.

Adapted from Hogan *et al*, (1994)



and is attached to a basement membrane extracellular matrix (ECM) through a basal cell membrane (Levio *et al.*, 1980; Coucouvanis and Martin, 1995, Edgar and Murray 2000). The reorganisation of the pluripotent cells to an epithelium is accompanied by alterations in gene expression, antigen presentation and developmental potential that define these pluripotent cells as a separate population termed the primitive ectoderm (Beddington, 1983; Rathjen *et al.*, 1999; Lake *et al.*, 2000). Primitive ectoderm is an important developmental intermediate as all the tissues of the embryo, and the embryonic mesoderm, arise from these cells during the process of gastrulation (Gardner and Rossant, 1979). The cup shaped primitive ectoderm defines the egg cylinder at this stage of development.

As the pluripotent cells proliferate and expand into the blastocoelic cavity, two distinct extra-embryonic lineages are derived from the primitive endoderm. The parietal endoderm is derived from primitive endoderm that migrates onto the inner surface of the trophectoderm and loses contact with the adjacent pluripotent cells (Gardner, 1983). The visceral endoderm (VE) arises from primitive endoderm cells that remain adjacent to the pluripotent cells of the egg cylinder, separated by the basement membrane ECM (Lavio *et al.*, 1980; Gardner, 1983; Hogan *et al.*, 1994: Figure 1.3 D and Figure 1.4).

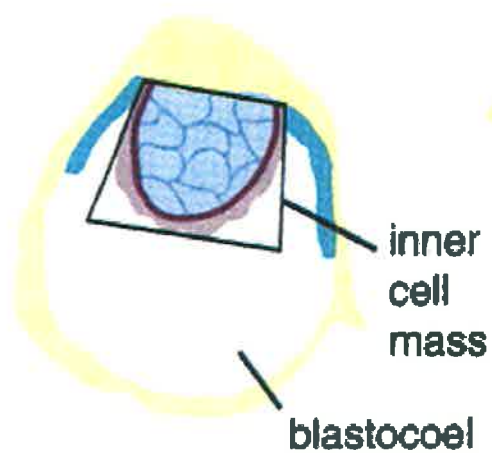
Recently, the interaction between embryonic pluripotent cells and extra-embryonic endoderm lineages has been a focus of research (reviewed in Bielinska *et al.*, 1999). A two-step mechanism for pro-amniotic cavitation, involving a VE derived signal, has been proposed (Coucouvanis and Martin, 1995). A diffusible 'death' signal, arising from the VE, induces cavitation through programmed cell death, or apoptosis, of the adjacent pluripotent cells. The primitive ectoderm cells making up the epithelial sheet avoid apoptosis through interaction with a secondary 'survival' signal associated with the basement membrane separating the VE and the primitive ectoderm (Figure 1.4; Coucouvanis and Martin, 1995). The mechanism that induces this cavitation is thought to be analogous to the biological mechanisms directing tube formation in other biological systems, such as angiogenesis (Coucouvanis and Martin, 1995). However, the identity of the signals that are directly responsible for inducing tube formation in the embryo and adult remain unknown.

Figure 1.4 Pro-amniotic cavitation of the early mouse embryo

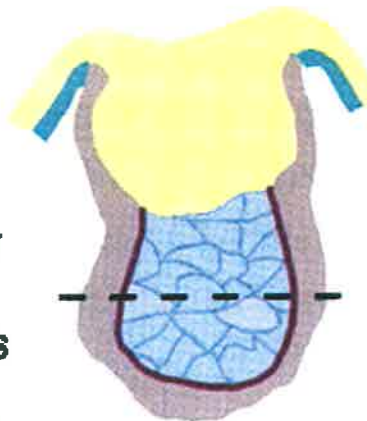
Schematic representation of peri-implantation development. The upper row depicts sagittal sections and the lower row transverse sections through the planes indicated by dotted lines. Pluripotent cells are shown in blue. (E 4.0) Fully expanded blastocyst stage embryo prior to implantation containing the outer layer of trophoblast surrounding the fluid-filled blastocoel. Primitive endoderm (depicted in legend as visceral endoderm) surrounds the ICM, separated by a layer of basement membrane extra-cellular matrix (ECM). (E 5.0) Implantation stage embryo. (E 6.0) Primitive ectoderm surrounds the central pro-amniotic cavity.

Adapted from Coucouvanis and Martin, 1995

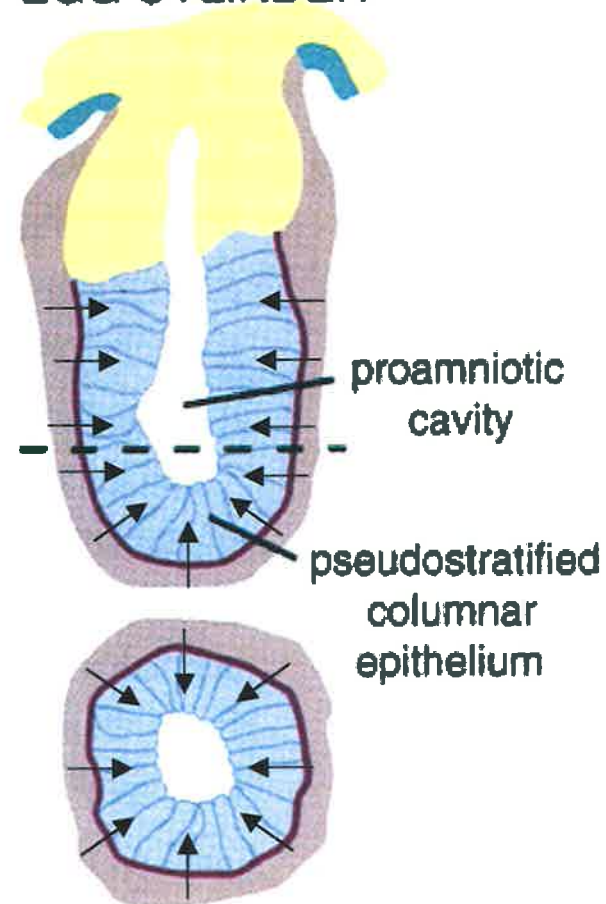
E 4.0
BLASTOCYST



E 5.0



E 6.0
EGG CYLINDER



- trophoblast
- parietal endoderm
- visceral endoderm
- primitive ectoderm
- A** basement membrane

→ death signal

1.3 Gastrulation

Gastrulation is the process during which the three primary germ layers, endoderm, mesoderm and ectoderm are derived from primitive ectoderm (Beddington, 1981, 1982, 1983; Lawson *et al.*, 1991; Tam and Behringer, 1998). These three germ layers, which have lost their pluripotent developmental potential, are the progenitor cells for the complex organs and cells of the embryo and adult organism. The generation of this vast array of cell lineages and structures during gastrulation requires a highly controlled, coordinated and complex series of cellular signalling events.

Gastrulation begins at 6.5 dpc when the proximal primitive ectoderm cells, on the posterior most side of the egg cylinder, lose their epithelial conformation and undergo a transition to mesenchyme (Tam and Behringer, 1998). A loss of cell-cell contact within the epithelial sheet and migration of cells through the basement membrane ECM (Viebahn, 1995; Hay, 1995) accompany this event. The accumulation and invagination of these cells between the primitive ectoderm and surrounding VE leads to formation of a transient structure, termed the primitive streak (Tam and Behringer, 1998; refer Figure 1.3 D). Appearance of the primitive streak defines the posterior end of the embryo and, as a consequence, the anterior-posterior axis of the embryo. As gastrulation proceeds, nascent mesoderm emerges from the primitive streak and migrates across the embryonic/extra-embryonic boundary to form the extra-embryonic mesoderm, or distally towards the tip of the egg cylinder. Mesoderm cells also migrate laterally to form the mesodermal wings that wrap around the embryo. By 8.0 dpc, mesoderm migration has reached the anterior pole and distal tip resulting in a layer of mesoderm spanning the length of the embryo. This is the definitive embryonic mesoderm germ layer of the early mouse embryo (refer Figure 1.3).

By 7.5 dpc, at the anterior most end of the streak, a specialised structure equivalent to the organiser of other vertebrate embryos is formed. This structure, termed the node, induces formation of a mixed population of definitive gut endoderm and mesoderm, termed mesendoderm (Beddington and Robertson, 1999). The mesendoderm contains the axial mesendoderm that populates the mid-line of the embryo to form the head process and notochord (Tam and Beddington, 1987; Lawson *et al.*, 1991; Beddington and Robertson, 1999). The mesendoderm arises in the distal posterior region of the egg cylinder at 7.5 dpc and then expands anteriorly (Tam and Beddington, 1992). The VE is

eventually displaced by the anteriorly migrating definitive endoderm around 8.5 dpc (Tam and Behringer, 1998).

The anterior primitive ectoderm cells are the prospective ectodermal germ layer cells that form the neur ectoderm (nervous system) and the epidermal cell lineages (Quinlan *et al.*, 1995). The primitive ectoderm cells fated to form ectoderm remain in contact with the ECM basement membrane and are adjacent to the VE during the initial stages of gastrulation. The proximity of anterior primitive ectoderm cells to anterior VE (AVE) during the early stages of gastrulation is significant as signals arising from the AVE have been implicated in directing the fate of the ectodermal germ layer (Thomas and Beddington, 1996; Bielinska *et al.*, 1999).

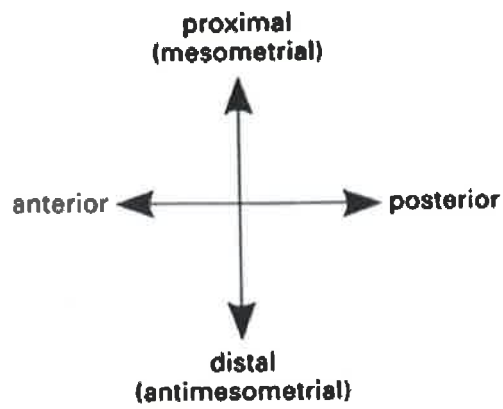
Clonal fate analysis has been used to identify regions of the primitive ectoderm that are developmentally restricted in the contribution of progeny cells to the three primary germ layers (Beddington, 1981, 1982; Tam and Beddington, 1987; Tam and Beddington, 1992; Lawson *et al.*, 1991; Quinlan *et al.*, 1995). These reports have been used to generate fate maps for primitive ectoderm cells within regions of the egg cylinder as represented in Figure 1.5. Transplantation of marked primitive ectoderm cells from pre-gastrulation or early post-gastrulation stage embryos to heterotopic sites revealed that the transplanted tissue can adopt the fate of its new location up to 8.0 dpc (Beddington, 1981; Beddington, 1983; Parameswaran and Tam, 1995; Tam and Zhou, 1996). These reports further suggest that primitive ectoderm is a homogeneous cell population not developmentally restricted at this stage, and that cell fate decisions governing primitive ectoderm differentiation are determined by position in the egg cylinder rather than pre-disposition of cell fate (Beddington, 1983; Parameswaran and Tam, 1995; Tam and Zhou, 1996).

Attainment of a threshold number of primitive ectoderm cells is a requirement for the initiation of gastrulation in the mouse embryo (Tam and Behringer, 1998). Mouse embryos with reduced numbers of pluripotent cells in the egg cylinder, due to ablation or inhibition of proliferation (Snow and Tam, 1979), delay the onset of gastrulation until this threshold has been reached. Mouse embryos that have defects in primitive ectoderm proliferation and fail to reach the cell number threshold either die or initiate a failed gastrulation process (Tam and Behringer, ¹⁹⁹⁷~~1998~~). However, initiation of gastrulation is not solely reliant on a critical mass of primitive ectoderm cells but requires additional inductive signals. This has been demonstrated by genetic ablation of the Wnt3

Figure 1.5 Fate maps of the primitive ectoderm before and during gastrulation

The embryo is shown for pre-streak, early streak and late streak development. The origin boundaries and overlap of prospective tissues are plotted as indicated in the legend.

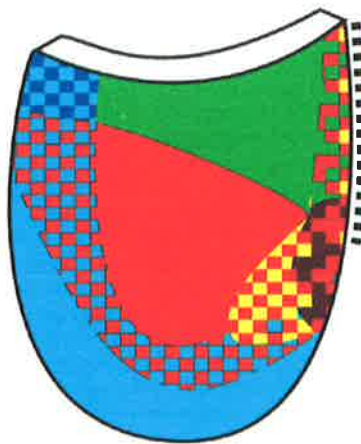
(Adapted from Hogan et al., 1995)



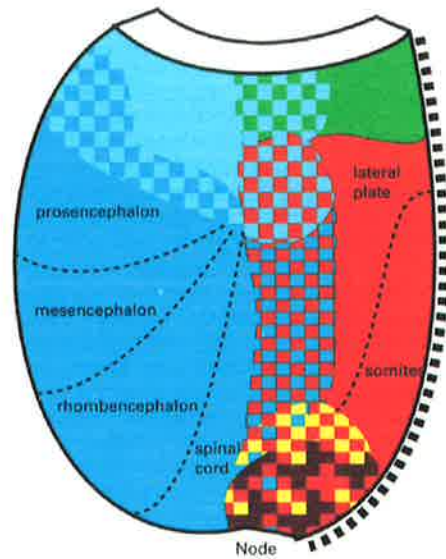
PRESTREAK
6.0 d.p.c.



EARLY STREAK
7.0 d.p.c.



LATE STREAK
7.5 d.p.c.



- Ectoderm
- Mesoderm
- Head process/Notochord
- Endoderm
- Extra-embryonic Mesoderm
- Amnion Ectoderm
- Surface Ectoderm
- Primitive Streak

secreted glycoprotein. *Wnt3* is expressed throughout the primitive ectoderm at 6.25 dpc with highest levels in the presumptive posterior primitive ectoderm, where gastrulation is initiated. Embryos lacking a functional copy of the *Wnt3* gene are phenotypically normal at 6.5 dpc but fail to induce gastrulation and continue to expand as an egg cylinder (Liu ^{Liu}~~Liu~~ *et al.*, 1999).

1.3.1 Formation of the embryonic axes

In many non-mammalian developmental systems the axes of an embryo are determined from a body plan imparted on the egg by localised maternal determinants (reviewed in Melton, 1991). Mammals however, have been thought to be an exception to this rule given that there is little evidence of maternal direction and the first morphological signs of polarity are observed after implantation. However, recent studies in the mouse embryo suggest pre-implantation events are important in the development of mammalian embryonic polarity (Gardner, 1997; Ciemerych *et al.*, 2000). The establishment of embryonic asymmetries in the pre-implantation embryo involve formation of the animal/vegetal axis prior to blastocyst formation and the embryonic/abembryonic axis post-blastocyst formation.

The animal/vegetal axis is established as the polar bodies are extruded during the first and second meiotic divisions in the egg. These polar bodies remain tethered to the same position on the oocyte membrane during blastomere division, a location which defines the animal pole of the embryo (Gardner, 1997). Recent reports have shown that blastomeres located near to or opposite the polar body, at the 8-cell stage, retain their same relative positions in the blastocyst (Ciemerych *et al.*, 2000). Therefore, these cells come to lie on either end of an axis of symmetry that has been shown, by lineage marker analysis, to correlate with the anterior-posterior axis of the future post-implantation embryo (Figure 1.6; Weber *et al.*, 1999).

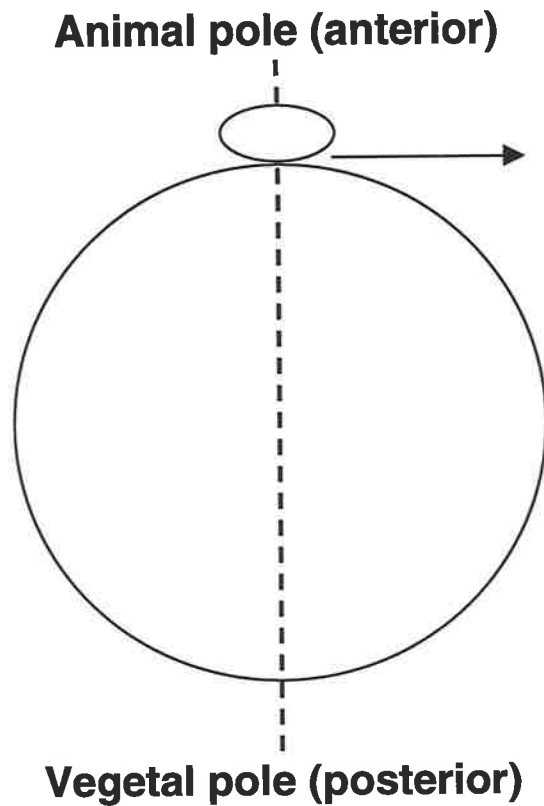
The embryonic/abembryonic axis is directed by the localisation of ICM to one half of the embryo following blastocyst formation. The embryonic/abembryonic axis is located perpendicular to the animal/vegetal plane and corresponds to the future dorsal/ventral axis of the embryo (Beddington and Robertson, 1999). In many animal eggs, sperm entry position provides a cue for embryonic patterning. However, no such role had been found in mammals until recently where it has been shown that the sperm entry position of the mouse egg can predict the plane of initial cleavage and

Figure 1.6 Diagrams illustrating polarity of the egg and blastocyst

(A) The animal/vegetal pole of the egg and embryonic/abembryonic pole of the blastocyst (B) are indicated by the dashed lines. Extruded polar bodies of the egg and blastocyst are shown, with the inner cell mass (ICM) of the blastocyst also represented.

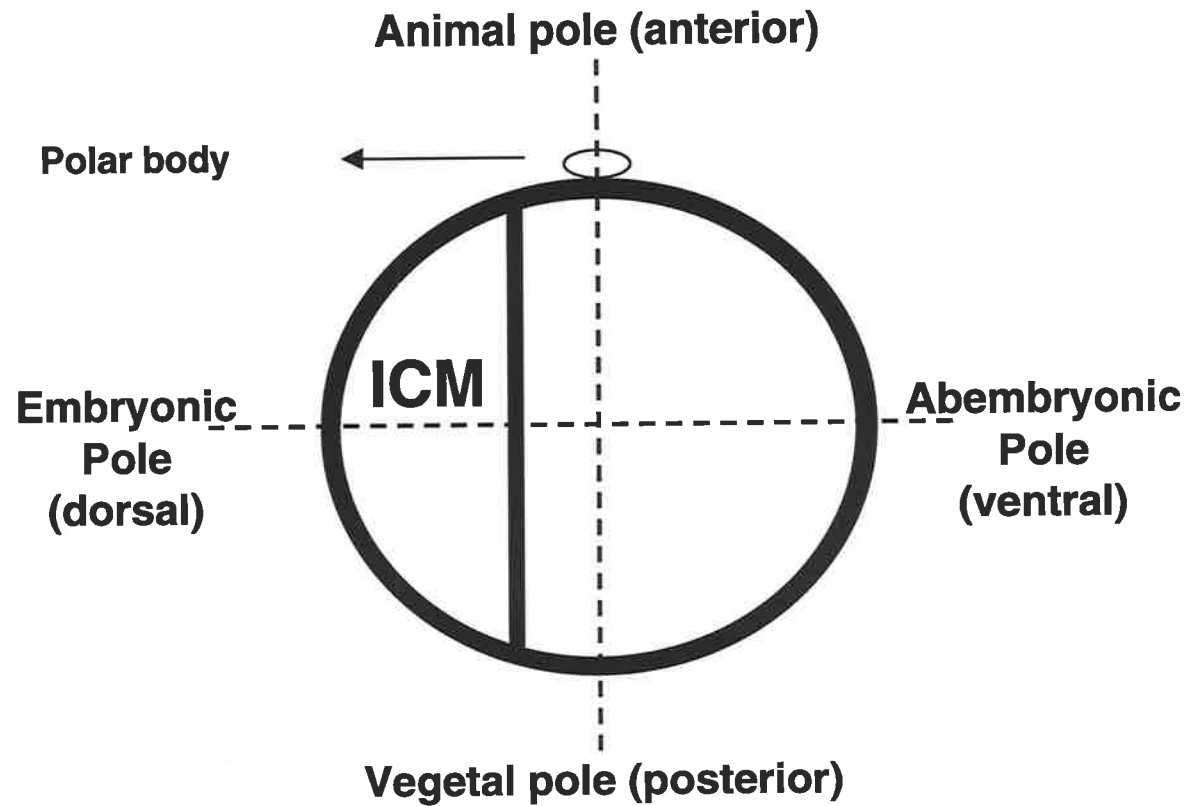
A

FERTILISED EGG



B

BLASTOCYST



define the embryonic and abembryonic halves of the future blastocyst (Piotrowska and Zernicka-Goetz, 2001). A consistent relationship between the structure of the blastocyst and the two-cell stage in the mouse has been implicated further using a non-invasive marking technique that revealed the embryonic/abembryonic axis of the blastocyst is normally perpendicular to the plane of first cleavage (Gardner, 2001). Therefore, during normal mouse development the axes of the blastocyst, which are implicated in establishing those of the foetus, are already specified by the onset of cleavage (Gardner, 2001). This suggests axes specification of the blastocyst depends on spatial patterning of the zygote (Gardner, 1997).

However, chimeric embryos constructed from 2-cell stage or 8-cell stage blastomeres develop into normal blastocysts and then fertile adult mice without axial duplication (Garner and McLaren, 1974; Gardner, 1988; Ciemerych *et al.*, 2000). Furthermore, the early degradation of maternal mRNA and activation of zygotic transcription, and the extensive pluripotent cell mixing prior to a relatively late gastrulation in comparison to other developmental systems (Gardner and Cockcroft, 1998), suggest the specification of mammalian embryonic axes requires additional determinants to those present within pre-implantation embryo (reviewed in Beddington and Robertson, 1999). A likely candidate in the establishment of embryonic asymmetry post-implantation is the AVE, which has been implicated in providing instructive cues for anterior specification in the mouse, prior to overt signs of primitive streak formation (reviewed in Beddington and Robertson, 1998). The first signs of anterior-posterior polarity are detectable prior to primitive streak formation by the asymmetric expression of *Hex*, *cerebrus-like* and other markers in the primitive endoderm (Rosenquist and Martin, 1995; Varlet *et al.*, 1997; Acampora *et al.*, 1998; Rhinn *et al.*, 1998; Shawlot *et al.*, 1998). *Hex* expressing cells migrate in an anterior/proximal manner from the distal tip of the embryo to become the AVE (Thomas *et al.*, 1998). This is the first genetic evidence of polarity within the embryo. The AVE is further defined by expression of *Hesx1* following the initiation of gastrulation at 6.5 dpc (Thomas and Beddington, 1996). *Hesx1* expressing AVE then induces *Hesx1* expression in the adjacent primitive ectoderm cells and supplies additional signals that direct their fate during gastrulation (Thomas and Beddington, 1996). These reports highlight the role of AVE as an organising centre in the establishment of anterior-posterior axis formation and primitive ectodermal cell fate.

Gene expression within the primitive ectoderm is also required for establishment of the anterior/posterior axis in the post-implantation mouse embryo. Prior to gastrulation, β -catenin, a member of the Wnt signalling pathway, is ubiquitously expressed throughout the embryo while *cripto*, a member of the EGF-CFC family of growth factors, is asymmetrically expressed in a proximal-distal gradient in the primitive ectoderm (Ding *et al.*, 1998; Xu *et al.*, 1999). Following the onset of gastrulation *cripto* is expressed in the primitive streak and newly formed embryonic mesoderm. A generated disruption of the β -catenin or *Cripto* allele yields mutant embryos that fail to initiate primitive streak formation, and markers of the AVE are located at the distal end of mutant embryos suggesting the anterior/posterior axis is not established (Haegel *et al.*, 1999; Ding *et al.*, 1998; Huelsken *et al.*, 2000). Furthermore, high level chimerism of wild-type ES cells (1.7.3), which colonise primarily embryonic lineages, after injection in to β -catenin^{-/-}blastocysts (Beddington and Robertson, 1989), results in initiation of primitive streak formation and anterior/posterior axis formation (Huelsken *et al.*, 2000). This indicates that β -catenin expression in the primitive ectoderm is required for the onset of gastrulation and definition of the anterior/posterior axis (Huelsken *et al.*, 2000).

In conclusion, gastrulation and embryonic axis formation are complex events that combine morphogenic movements and inductive interactions between germ layers. However, the complexity of these events has inhibited our understanding of the signals that direct these processes. A starting point from which to understand gastrulation is to determine the identity and cellular origin of factors that direct primitive ectoderm formation and maintenance *in vivo* and *in vitro*. As the cellular substrate of gastrulation, *in vitro* primitive ectoderm isolated from the embryo or generated *de novo* could be exploited to identify the signals that control these events *in vivo*. However, the origin and identity of the signals that initiate and direct primitive ectoderm formation and maintenance remains unknown.

1.4 Pluripotent cells *in vivo*

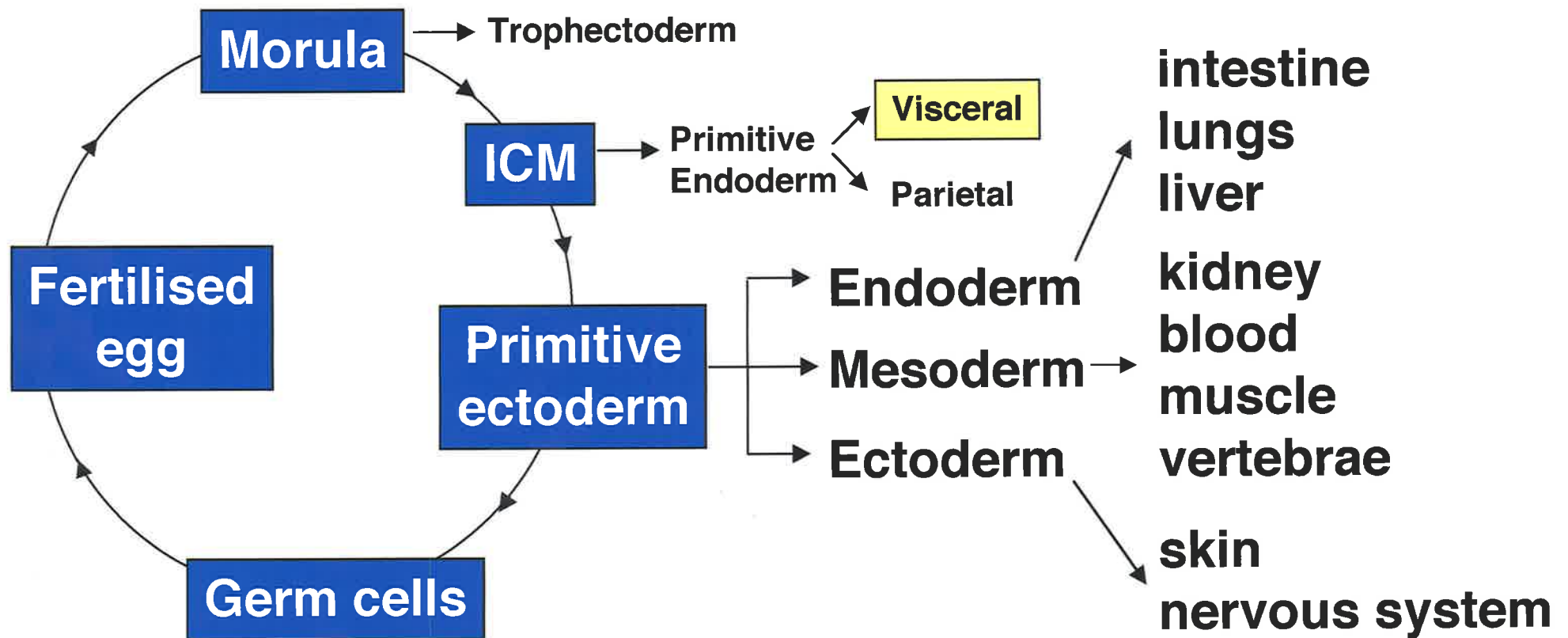
Mammalian embryogenesis is characterised by the controlled and coordinated proliferation, differentiation, apoptosis and migration of pluripotent cells. However, the initial pluripotent cell lineage of the embryo, the ICM, is not equivalent to the pluripotent primitive ectoderm lineage that differentiates during gastrulation. Instead, through a series of developmental events the ICM of the 3.5 dpc undergoes alterations in proliferation (Snow, 1977), morphology (Coucouvanis and Martin, 1995), gene expression (Rathjen *et al.*, 1999) and differentiation potential (Beddington, 1983; Rathjen *et al.*, 1999; Lake *et al.* 2000) to give rise to a distinct and homogeneous population of primitive ectoderm. The formation of primitive ectoderm is the first embryonic differentiation event within the early mammalian embryo and appears to be an obligatory progression in pluripotent cell development prior to terminal differentiation. The pluripotent cell life cycle is represented in Figure 1.7.

Pluripotent cells can be characterised *in vivo* and *in vitro* by the expression of markers such as alkaline phosphatase (Hahnel *et al.*, 1990, Rossant and Pederson, 1986), the SSEA-1 antigen (Solter and Knowles, 1978), fibroblast growth factor 4 (*Fgf4*) (Hébert *et al.*, 1990; Niswander and Martin, 1992; Rappolee *et al.*, 1994; Feldman *et al.*, 1995), and the germline-specific homeobox gene *Oct4* (Schöler *et al.*, 1989a; Schöler *et al.*, 1989b; Rosner *et al.*, 1990; Yeom *et al.*, 1991; Nichols *et al.*, 1998).

Gene analysis has been used to monitor pluripotent cell progression from the ICM (3.5 dpc) to primitive ectoderm of the egg cylinder (5.5 dpc) (Pelton, 2000). Morphologically, the ICM is first observed in the 3.5 dpc early blastocyst. Around 24 hours later the fully expanded blastocyst hatches from the zona pellucida. In this stage blastocyst the pluripotent cells are separated from the blastocoelic cavity by newly formed primitive endoderm. The late stage 4.75 dpc blastocyst is defined by the formation of primary trophoblast giant cells and an increase in pluripotent cell number. At 5.0 dpc, proliferating polar trophectoderm projects down upon the pluripotent cells, pushing the epiblast into the blastocoelic cavity to create the “epiblast bud”. Formation of a small pro-amniotic cavity defines the 5.25 dpc embryo, while pluripotent cells become polarised and reorganise into an epithelial sheet of primitive ectoderm by day 5.5 of embryogenesis.

Figure 1.7 Totipotent and pluripotent cells in mammalian development

Schematic representation of the pluripotent cell life cycle and the early stages of mammalian development. Pluriopotent and totipotent cell types are boxed in ~~red~~^{blue}. Differentiated tissues from each pluripotent cell population are indicated.



Cells of the 3.5 dpc and 4.5 dpc ICM express differentially regulated genes such as *Rex1* (Rogers *et al.*, 1991; Hosler *et al.*, 1993), *Psc1* and *Psc2* (Pelton *et al.*, 2000; Rodda *et al.*, 2001). The 4.75 dpc pluripotent cell population is defined by maintenance of *Oct4* and *Psc1* and a down-regulated expression of *Rex1* and *Psc2* in pluripotent cells (Pelton, 2000; Rodda *et al.*, 2001). These markers are absent from the embryonic region of 5.0 dpc embryos, indicating these markers are down regulated during differentiation (Pelton, 2000). *Psc1* expression is detectable in pluripotent cells at 5.0 dpc, immediately prior to pro-amniotic cavity formation, but is not re-expressed or detectable in extra-embryonic lineages (Pelton, 2000).

Psc3 is not expressed in blastocyst pluripotent cells at 3.5 dpc or 4.5 dpc but is expressed in the cells of the overlying extra-embryonic ectoderm and early primitive ectoderm of the 4.75 dpc embryo (Pelton, 2000). Following the initiation of pro-amniotic cavitation at 5.25 dpc, *Psc3* is expressed in all pluripotent cells but is not re-expressed in extra-embryonic lineages (Pelton, 2000). *Psc3* expression persisted within pluripotent cells following the initiation of pro-amniotic cavitation but is down-regulated following formation and elongation of the epithelial sheet of primitive ectoderm between 5.5 dpc-5.75 dpc.

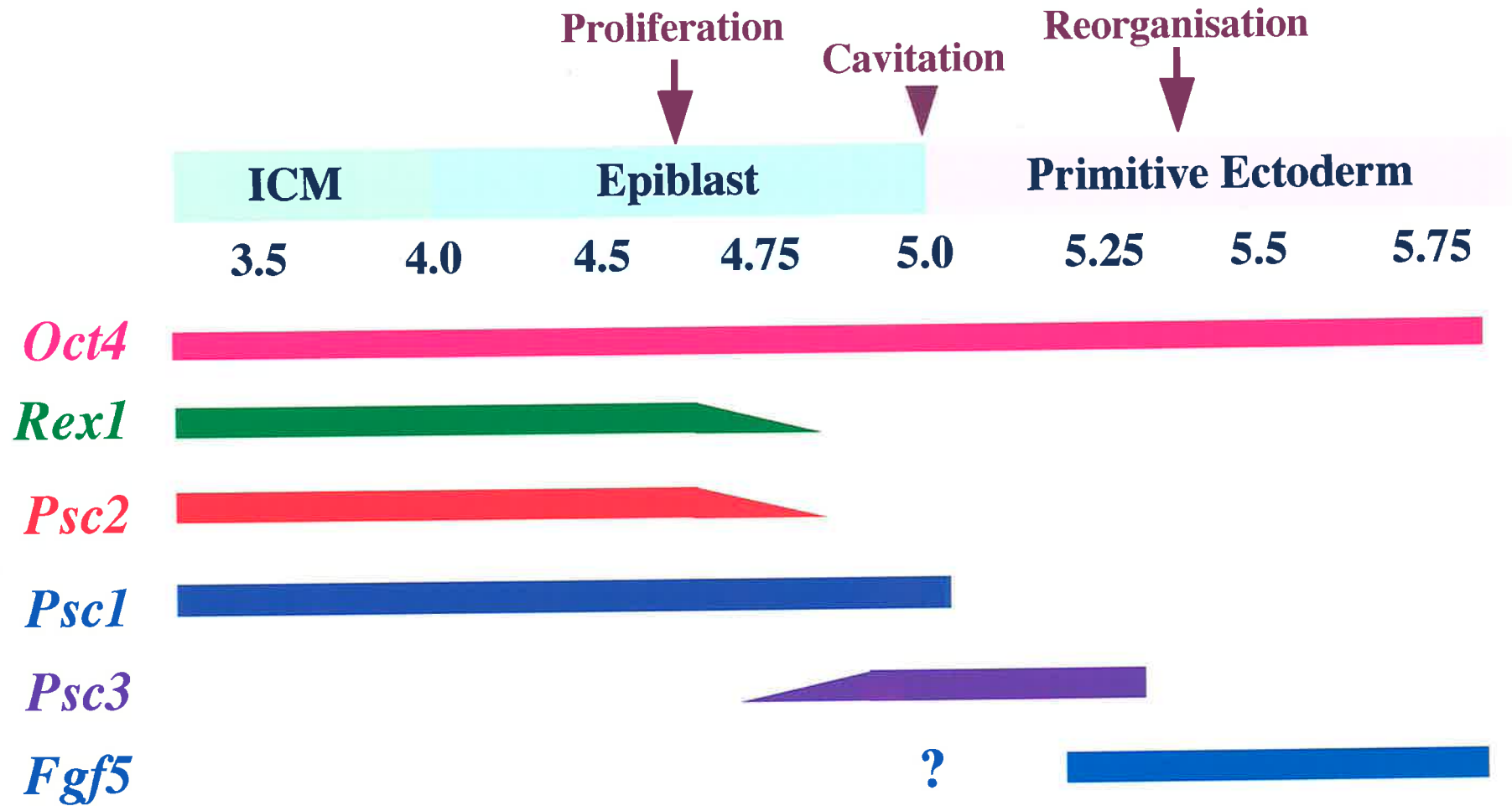
Fgf5 expression is not detected in blastocyst pluripotent cells but is detected in primitive ectoderm in 5.25 dpc embryos. *Fgf5* remains expressed in the cells of the embryo up to and during gastrulation but is not present in extra-embryonic lineages at these stages (Pelton, 2000). A representation of the expression patterns of genes differentially expressed in pluripotent cells in the early mouse embryo is represented in Figure 1.8.

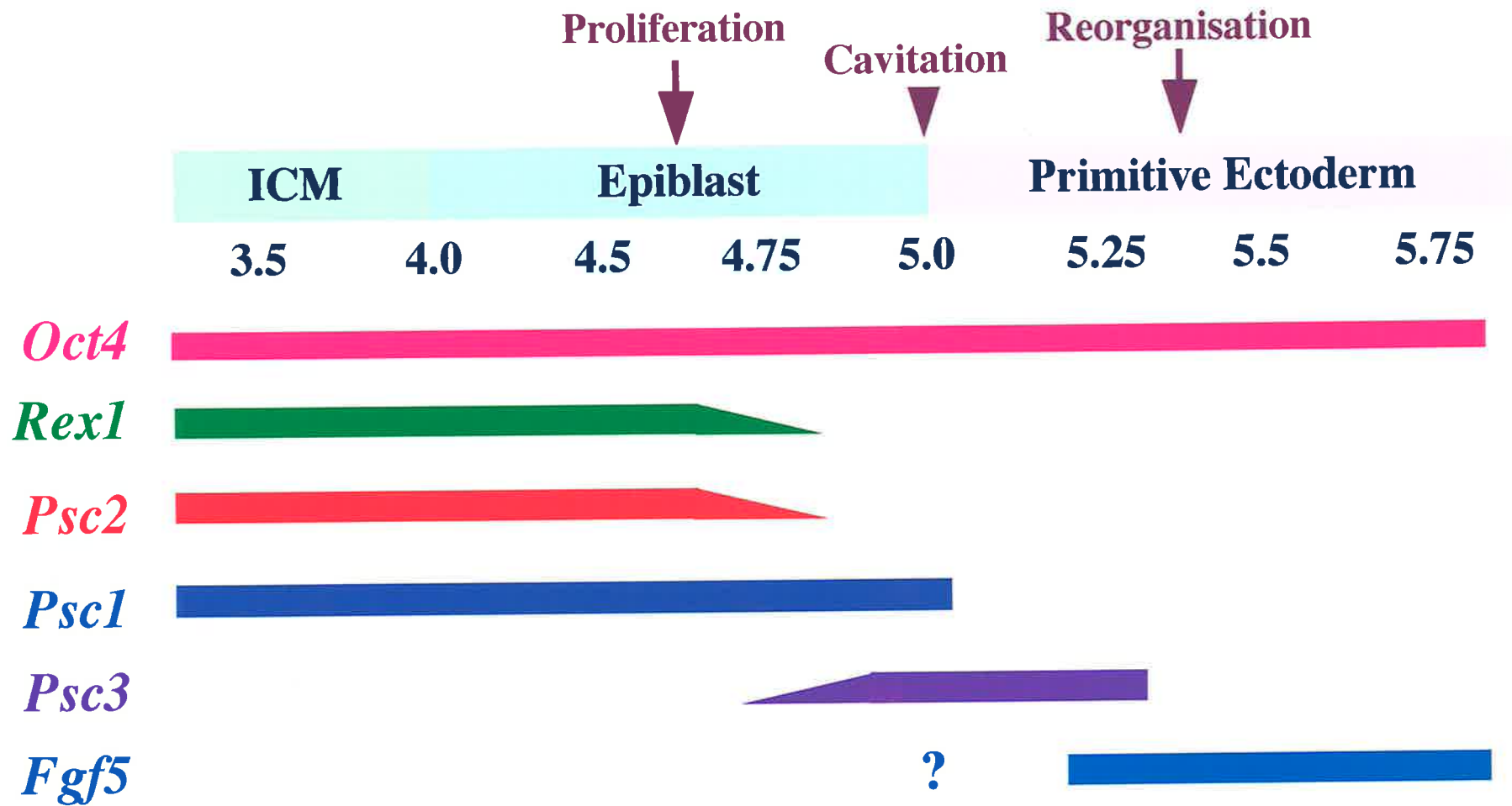
The presence of distinct patterns of gene expression within pluripotent cell sub-populations highlights the complexity of the developmental progression of pluripotent cells throughout early mouse development. These gene expression changes appear to correlate with key developmental events which occur during this stage of development, including blastocyst formation at 3.5 dpc, increased cellular proliferation at 4.75 dpc, pro-amniotic cavitation at 5.0 dpc and primitive ectoderm reorganisation into an epithelial layer at 5.25 dpc. Interestingly, no spatial heterogeneity has been detected within pluripotent cell populations suggesting distinct sub-populations are homogeneous.

(Pelton, 2000)

Figure 1.8 Marker gene expression in pluripotent cells of the early mouse embryo.

Schematic representation of temporal marker gene expression in the pluripotent cells of the early mouse embryo.





1.5 Signals required for pluripotent cell developmental progression *in vivo*

Introduction of specific and functional mutations into the mouse genome has uncovered signals required for pluripotent cell development and primitive ectoderm formation *in vivo*. Genes known to be required for pluripotent cell development *in vivo* are discussed below and in Table 1.1.

1.5.1 Extracellular factors controlling pluripotent cell development

1.5.1.1 TGF β growth Factor Family

The bone morphogenic proteins (BMP) constitute a subgroup of the transforming growth factor- β (TGF- β) family of growth factors. Transcripts for two BMP type I receptors *activin-like kinase receptor 3* (*ALK3*) and *ALK6*, and three type II receptors, *BMPRII*, *ActRIIA* and *ActRIIB* are expressed throughout the primitive ectoderm at 6.5 dpc (Manova *et al.*, 1995, Matzuk *et al.*, 1995; Mishina *et al.*, 1995; Gu *et al.*, 1998; Song *et al.*, 1999; Beppu *et al.*, 2000). Mice carrying functional genetic mutations in the *ALK3* (Mishina *et al.*, 1995), *ALK6* (Gu *et al.*, 1998) or the *BMPRII* receptor (Beppu *et al.*, 2000), show similar embryological defects including reduced proliferation of pluripotent cells, morphological disorganisation of the egg cylinder and inhibited or defective initiation of gastrulation. In addition, mice lacking functional copies of both type II BMP receptors *ActRIIA* and *ActRIIB* also show similar embryological defects (Song *et al.*, 1999). These reports highlight the role of BMP signalling in pluripotent cell development and differentiation.

Bone morphogenic protein 4 (*BMP4*) is initially expressed at low levels throughout the primitive ectoderm at 6.5 dpc. During gastrulation *BMP4* is expressed at higher levels within newly formed differentiated tissues (Winnier *et al.*, 1995). Mice carrying a functional genetic mutation in the *BMP4* gene exhibit reduced proliferation of the primitive ectoderm and failed gastrulation (Winnier *et al.*, 1995; Lawson *et al.*, 1999). This suggests that the *BMP4* growth factor and the BMP/activin signalling pathways are crucial for primitive ectoderm proliferation, consistent with the phenotype of mice lacking type I or type II BMP receptors (Mishina *et al.*, 1995; Gu *et al.*, 1998; Beppu *et al.*, 2000; Song *et al.*, 2000). In particular, *ALK3* and the type II receptors have been shown to bind *BMP4* and it has been proposed that *BMP4* mediates its signal through a

Gene	Gene Product	Embryonic cell expression	Mutant phenotype	Reference
<i>Fgf4</i>	Fibroblast growth factor	All pluripotent cells except PGC's and germline lineages	Defect in pluripotent cell proliferation following blastocyst implantation	Niswander and Martin, 1992; Rappollee <i>et al.</i> , 1994; Feldman <i>et al.</i> , 1995
<i>Fgfr2</i>	Tyrosine kinase linked FGF receptor	Low level expression in PE at 6,5 dpc	Defect in pluripotent cell proliferation and differentiation following blastocyst implantation	Orr-Urtegger <i>et al.</i> , 1991; Arman <i>et al.</i> , 1998
<i>Fgfr1</i>	Tyrosine kinase linked FGF receptor	Low level expression in PE at 6,5 dpc	Growth retardation by 6.5 dpc; delayed/defected mesoderm induction	Orr-Urtegger <i>et al.</i> , 1991 and 1993; Yamaguchi <i>et al.</i> , 1992; Deng <i>et al.</i> , 1994; Yamaguchi <i>et al.</i> , 1994
<i>Bmp4</i>	TGF- β growth factor	Low level pluripotent cell expression	Defects in pluripotent cell proliferation/survival and mesoderm induction. Arrest between 6,5 - 9,5 dpc	Winnier <i>et al.</i> , 1995; Hogan, 1996
<i>ALK6</i>	Type I activin-like kinase receptor	Low-level expression in PE and EEE from 6,5 dpc	Disorganisation of PE and EEE; arrest prior to gastrulation	Gu <i>et al.</i> , 1998
<i>ALK3</i>	Type I activin-like serine/threonine TGF- β kinase receptor	Expressed in PE and extra-embryonic cell lineages at 7,0 dpc	Defect in proliferation of PE prior to gastrulation. No mesoderm formation	Mishina <i>et al.</i> , 1995
<i>BMPRII</i>	Type II BMP receptor	Expressed throughout the PE at 6,5 dpc	Growth retardation of PE and failure to gastrulate. Resorbed 8,5 - 9,5 dpc	Beppu <i>et al.</i> , 2000
<i>ActRIIA</i>	Type II BMP receptor	Weak in EEE the PE between 5,5 - 7,5 dpc	Defect in mice lacking functional copies of <i>ActRIIB</i> ; growth arrest at 6,5 dpc and fail to gastrulate	Song <i>et al.</i> , 1999
<i>ActRIIB</i>	Type II BMP receptor	Weak in EEE and high in the PE between 5,5 - 7,5 dpc	Defect in mice lacking functional copies of <i>ActRIIA</i> ; growth arrest at 6,5 dpc and fail to gastrulate	Song <i>et al.</i> , 1999
<i>Smad4</i>	Intracellular effector molecule that mediates TGF- β signalling	Expressed uniformly in PE and extra-embryonic regions at 6,5 dpc	Lethal between 6,5 - 8,5 dpc; proliferation and differentiation defects in pluripotent cells in culture	Yang <i>et al.</i> , 1998
<i>EGFR</i>	Epidermal growth factor receptor	Unknown	Peri-implantation degeneration of pluripotent cells on CF-1 genetic background	Threadgill <i>et al.</i> , 1995; Siliba and Wagner, 1995
<i>Stat3</i>	Mediates biological actions in response to cytokines and growth factors	Unknown	Small PE degenerates around 6,5 dpc and are resorbed by 7,5 dpc. No mesoderm formation	Takeda <i>et al.</i> , 1997
<i>Cyclin A2</i>	Cell cycle regulator	Ubiquitous	Degeneration of pluripotent cells around 5,5 dpc	Murphy <i>et al.</i> , 1997
<i>Max</i>	Obligate dimerisation-DNA-binding partner for Myc oncogene family	Ubiquitous	Death 5,5 - 6,5 dpc Loss of pluripotent and extra-embryonic cell proliferation	Shen-Li <i>et al.</i> , 2001
<i>Brca1</i>	Breast cancer susceptibility gene	Unknown	Defects in pluripotent cell proliferation from 4,5 dpc; fail to form organised egg cylinders	Lui <i>et al.</i> , 1996; Hakem <i>et al.</i> , 1996; Ludwig <i>et al.</i> , 1997
<i>Brca2</i>	Breast cancer susceptibility gene <i>E.coli</i> RecA homologue	Ubiquitous at 6,5 dpc	Variable defects, impaired proliferation of PE by 5,5 dpc	Suzuki <i>et al.</i> , 1997; Ludwig <i>et al.</i> , 1997; Sharan <i>et al.</i> , 1997
<i>Rad51</i>	<i>E.coli</i> RecA homologue	Unknown	Defect in pluripotent cell proliferation by 5,5 dpc	Lim and Hasty, 1996
<i>Ref1</i>	DNA repair/redox induced activation of AP-1/DNA binding	Unknown	Embryo degeneration around 5,5 dpc	Xanthoudakis <i>et al.</i> , 1996
<i>muREC2/ RAD51L1</i>	<i>E.coli</i> RecA homologue	Unknown	Severe growth retardation at 5,5 dpc. No pro-amniotic cavity formation. Resorbed by 8,5 dpc	Shu <i>et al.</i> , 2001
<i>mRad50</i>	Double strand DNA break detection protein	Unknown	Cell proliferation defect at 6,0 dpc Thickened PE. Resorbed by 7,5 dpc	Luo <i>et al.</i> , 1999
<i>Blm</i>	A member of the RecQ family of RNA and DNA helicases	Unknown	Increased apoptosis in the pluripotent cells of the early post-implantation embryo leads to reduced embryo size. Death 13,5 dpc	Chester <i>et al.</i> , 1998

Gene	Gene Product	Embryonic cell expression	Mutant phenotype	Reference
<i>Mdm2</i>	Zinc finger protein. Binds p53	Ubiquitous	Death at 7.5 dpc. No proliferation of embryonic cells. Accumulation of p53	Montes de Oca Luna <i>et al.</i> , 1995; Jones <i>et al.</i> , 1995
<i>Tsg101</i>	Putative transcription factor	Ubiquitous	Death at 7.5 dpc. No proliferation of embryonic cells. Accumulation of p53	Ruland <i>et al.</i> , 2000
<i>Fug1</i>	Yeast RNAI homologue	Ubiquitous expression after 6.5 dpc	Impaired growth and reorganisation of the PE	DeGregori <i>et al.</i> , 1994
<i>Hdh</i>	Protein of unknown function	Expressed in PE day 6.5	Increased apoptosis in PE and abnormal gastrulation at embryonic day 7.5	Duyao <i>et al.</i> , 1995; Zeitlin <i>et al.</i> , 1995
$\beta 1$ -integrin	ECM cell surface receptor sub-unit	Unknown	Retarded pluripotent cell growth and defective EEE formation. Die 5.5 dpc	Stephens <i>et al.</i> , 1995; Fässler and Meyer, 1995
<i>LAMC1</i>	ECM component	Unknown	Absence of basement membranes. Retarded pluripotent cell growth and defective EEE. Die 5.5 dpc	Smyth <i>et al.</i> , 1998; Smyth <i>et al.</i> , 1999
<i>Fibronectin</i>	ECM component	Unknown	Defect in PE formation. Delayed gastrulation. Death around 10.5 dpc	George <i>et al.</i> , 1993; Georges <i>et al.</i> , 1996
<i>FAK</i>	Focal adhesion kinase signal transduction component	Unknown	Defect in PE formation. Delayed gastrulation. Death around 10.5 dpc	Furuta <i>et al.</i> , 1995
<i>Rac</i>	Rho family protein; regulates formation of the actin-based cytoskeleton, adherens junctions and focal adhesion complexes	Unknown	Irregular PE morphology. Increased apoptosis. Embryos die prior to 9.5 dpc due to defects during gastrulation	Sugihara <i>et al.</i> , 1998
<i>APC</i>	Wnt pathway signalling component	Ubiquitous	Degeneration of PE	Moser <i>et al.</i> , 1995
<i>Hnf4</i>	Basic HLH transcription factor	Primitive and VE	Death of PE around 6.5 dpc	Chen <i>et al.</i> , 1994; Duncan <i>et al.</i> , 1994; Duncan <i>et al.</i> , 1997
<i>vHnf1</i>	Homeobox gene	VE	Absence of extra-embryonic regions, highly disorganised embryonic region; death by 5.5 dpc	Barbacci <i>et al.</i> , 1999; Coffinier <i>et al.</i> , 1999
<i>Oct4</i>	Homeobox gene	All pluripotent cells	Essential for ICM pluripotency: ICM developmental restricted to trophectoderm	Rosner <i>et al.</i> , 1990; Schöler <i>et al.</i> , 1989a; Schöler <i>et al.</i> , 1989b; Yeom <i>et al.</i> , 1990; Nichols <i>et al.</i> , 1998
<i>Evx2</i>	Homeobox gene	VE	Degeneration of pluripotent and extra-embryonic cells around 5.0 dpc	Spyropoulos and Capecchi, 1994
<i>Lim1</i>	Homeobox gene	Proximal PE 6.5 dpc. Mesoderm and Primitive streak	Malformed egg cylinder. No node or fore- and midbrain	Shawlot and Behringer, 1995
<i>Otx2</i>	Homeobox gene	PE 6.5 dpc. Anterior ectoderm.	Malformed egg cylinder. Lacks head structures	Acampora <i>et al.</i> , 1995; Matsuo <i>et al.</i> , 1995; Ang <i>et al.</i> , 1996
<i>GATA6</i>	Zinc Finger transcription factor	First expressed in part of the ICM and trophectoderm. Then in parietal endoderm and the mesoderm.	Abnormal PE and VE formation. Die around 5.5 dpc.	Koutsourakis <i>et al.</i> , 1999

Table 1.1 Summary of gene expression and effect of loss of function mutation on pluripotent cells up to day 7.5 of mouse embryogenesis

Legend; PE; primitive ectoderm, VE; visceral endoderm, EEE; extra-embryonic ectoderm, EEE_n; extra-embryonic endoderm, ICM; inner cell mass.

heterodimeric complex of BMPRII and ALK3 (Beppu *et al.*, 2000). Therefore, this report implicates a cell-autonomous BMP ligand is required for pluripotent cell development *in vivo*.

The Smad family of proteins comprise intracellular mediators and regulators of TGF- β signalling. Activated Smad proteins translocate to the nucleus through association with Smad4, a common mediator of this pathway, to regulate the transcription of target genes (reviewed in Kretschmar and Massagué, 1998). Mice lacking the *Smad4* gene also show defects in pluripotent cell proliferation, egg cylinder formation and mesoderm induction similar to those described for the BMP receptors (Yang *et al.*, 1998). Collectively, these reports indicate a cell-autonomous requirement for BMP signalling during primitive ectoderm proliferation and differentiation.

1.5.1.2 The FGF growth factor family

Analysis of loss-of-function mutations in the Fgf receptor genes *Fgfr1* (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994) and *Fgfr2* (Arman *et al.*, 1998; Arman *et al.*, 1999), have implicated Fgf signalling in the control of pluripotent cell proliferation during peri- and post-implantation development respectively. *Fgfr2* null mutant embryos develop normally to the blastocyst stage but degenerate shortly after implantation due to failure of egg cylinder formation and reduced numbers of pluripotent cells, reflecting a possible proliferative defect. In addition, these mutants display impaired ICM proliferation when blastocysts are cultured *in vitro*. Mutation of *Fgfr1* (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994) also resulted in reduced numbers of pluripotent cells at 6.5 dpc, and delayed and failed gastrulation with disorganised egg cylinders, similar to defects in some BMP pathway members (1.5.2). These reports suggest FGFR activation is required for pluripotent cell development and differentiation *in vivo*.

Transcripts and protein product from the *Fgf4* gene have been detected in the ICM of 3.5 dpc blastocyst and the post-implantation pluripotent cell populations (Niswander and Martin, 1992; Rappolee *et al.*, 1994). The *Fgf4* gene is regulated by the pluripotent cell homeobox gene *Oct4*, in a *Sox2* dependent manner, indicating a possible pluripotent cell requirement for *Fgf4* expression (Yuan *et al.*, 1995; Ambrosetti *et al.*, 1997; Fraidenraich *et al.*, 1998). Mice lacking the *Fgf4* gene display impaired ICM proliferation, and *Fgf4*^{-/-}blastocysts cultured *in vitro* displayed enhanced trophectoderm outgrowth with a severe impairment in ICM proliferation. Addition of exogenous

FGF4 protein rescued ICM proliferation in mutant embryos, demonstrating that FGF4 is an autocrine or paracrine signal that promotes survival and growth of the pluripotent cells of the ICM (Feldman *et al.*, 1995). The role of FGF4 in early mouse development contrasts the role of FGF3, FGF5 and FGF8. Genes for these growth factors are expressed in pluripotent cells *in vivo* but are not required for pluripotent cell development (Mansour *et al.*, 1993; Hébert *et al.*, 1994; Crossley and Martin, 1995). The similar arrest phenotypes observed for *Fgf4* and *Fgfr2* functional mutants suggests the FGF4 signal transmitted by the FGFR2 in a common signal transduction pathway in pluripotent cells (Arman *et al.*, 1998) as FGFR2 has been shown to bind FGF4 (Ornitz *et al.*, 1996). Furthermore, transgenic expression of a pan-specific dominant negative FGF receptor (dnFGFR) results in formation of morphologically normal blastocysts until cell division ceases after the fifth cycle, just prior to implantation (Chai *et al.*, 1998). The ICM of these embryos contains one-third the number of normal cells, in the absence of increased apoptosis, indicating that FGF signalling directly effects pluripotent cell proliferation.

In conclusion, pluripotent cells are dependent on FGF signalling for the regulation of pluripotent cell development by the autocrine or paracrine interaction of FGF4 ligand with FGFR2. The distinct nature of the *Fgf4/Fgfr2* and *Fgfr1* null phenotypes indicates a possible switch from FGFR2 to FGFR1 signalling just prior to gastrulation.

1.5.1.3 The EGF receptor and signalling pathway

Homozygous null mutation of the epidermal growth factor receptor (*Egfr*) gene results in peri-implantation death due to degeneration of the ICM (Threadgill *et al.*, 1995; Siliba and Wagner, 1995). However, this phenotype is dependent on a CF-1 genetic background. An identical mutation on 129/Sv background induces embryo death at mid-gestation due to placental defects while mutants on a CD-1 background live for up to 3 weeks with abnormalities in skin, kidney, brain, liver, and the gastrointestinal tract. The multiple abnormalities associated with EGFR deficiency indicate this receptor is involved in a wide range of cellular activities and has a role during early embryonic development (Threadgill *et al.*, 1995; Siliba and Wagner, 1995). The less severe defects of *Egfr* mutation on CD-1 and 129sv backgrounds may be due to functional redundancy with unknown members of the *Egfr* family.

In summary, pluripotent cells require extracellular signalling and receptor activation from specific members of multiple growth factor families. These autocrine or paracrine signals direct pluripotent cell maintenance, proliferation and differentiation *in vivo*. The range of phenotypes and stages of developmental arrest in these embryos indicate a high level of complexity in pluripotent cell signalling events. This is consistent with the progressive alterations in pluripotent cell gene expression that define distinct pluripotent cell sub-types during early mouse embryogenesis *in vivo* (refer 1.4; Pelton, 2000).

1.5.1.4 Pluripotent cell-ECM signalling/attachment

Attachment to the embryonic basement membrane ECM separating primitive ectoderm from VE has been implicated in pluripotent cell survival (Stephens *et al.*, 1995; Coucouvanis and Martin, 1995; Murray and Edgar, 2000). Integrins are heterodimeric receptor complexes for extracellular matrix components and are important effectors of cell adhesion, differentiation, and migration during development (De Arcangelis and Georges-Labouesse, 2001). Mice carrying a targeted and functional mutation of the $\beta 1$ -*integrin* subunit gene die during early post-implantation development. Homozygous mutant embryos form phenotypically normal blastocysts and initiate implantation at 4.5 dpc. By 5.5 dpc mutant embryos have collapsed blastocoels and the ICM cells show retarded growth and defective extra-embryonic endoderm formation. These data suggest that $\beta 1$ -integrins, and ECM ligand activation, are required for normal growth and survival of mouse pluripotent cells (Stephens *et al.*, 1995; Fässler and Meyer, 1995). Similarly, mice lacking the laminin $\gamma 1$ subunit gene *LAMC1* show similar defects to $\beta 1$ -*integrin*^{-/-} mice (Smyth *et al.*, 1999; Aumailley *et al.*, 2000). These embryos fail to form a recognisable basement membrane ECM and lack deposition of additional ECM components, suggesting the loss of *LAMC1* may be an indirect effect through loss of cell-ECM binding and signalling through $\beta 1$ -integrin heterodimers (Smyth *et al.*, 1999).

Mice carrying homozygous genetic mutations for individual basal lamina components, present in the ECM surrounding pluripotent cells *in vivo*, generally show no embryonic defects, suggesting functional redundancy between ECM components during embryogenesis (Arikawa-

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FN distribution has also been studied in distinct stages of mouse blastocyst development using 2 cell embryos flushed from uteri and allowed to form blastocysts in culture. FN deposition was found to be absent from small blastocysts (3.1 days culture), but was present in localised dots or fibrils at cell boundaries in expanded blastocysts (3.8 days). Further development allowed FN deposition in irregularly shaped fibrils, preferentially at cell boundaries in expanded/hatching embryos (4.1 days), and in faint immunoreactive fibrils in hatched blastocysts (4.9 days). This deposition of FN was observed prior to, and in distinct patches from, collagen IV and laminin in early and later stage blastocysts respectively. This was proposed to indicate that a FN matrix can form independently of collagen IV/laminin in the blastocyst and may play a stabilisation role between the ICM and trophectoderm layers prior to assembly of a basement membrane in the embryo (Thorsteinsdottir, 1992).

Hirasawa *et al.*, 1999, Ryan *et al.*, 1999; Bonaldo *et al.*, 1998; Zheng *et al.*, 1995; Helbling-Leclerc *et al.*, 1995; Noakes *et al.*, 1995). An exception to this is FN, a component of the embryonic basement membrane separating VE and primitive ectoderm (Wartiarova *et al.*, 1979; George *et al.*, 1993; Peters and Hynes, 1996). The expression of FN in pluripotent cells *in vivo* is unknown but mice lacking functional copies of the *FN* gene form primitive ectoderm with greatly reduced cell numbers, a shortened anterior-posterior axis and a delayed onset of gastrulation (George *et al.*, 1993; Georges-Labouesse *et al.*, 1996). Mutant mice have deformed neural tubes and mesoderm derived tissues and are resorbed by 10.5 dpc. These defects are mimicked in mice lacking the integrin associated focal adhesion kinase (FAK), which mediates signalling through integrin binding ECM components (Furuta *et al.*, 1995). In particular, primitive ectoderm formation in *FAK*^{-/-} embryos is identical to *FN*^{-/-} embryos (Furuta *et al.*, 1995). These data suggest that FN is required for normal primitive ectoderm development via activation of receptor mediated signalling pathways through FAK. Together, these reports suggest the FN ligand is not required for the formation of primitive ectoderm, but is specifically required for normal primitive ectoderm maintenance and gastrulation.

A role for cell adhesion in pluripotent cell development is consistent with the phenotype of mice lacking a ubiquitously expressed member of the Rho protein family, *Rac1* that regulates formation of the actin-based cytoskeleton, the adherens junctions and focal adhesion complexes. RAC1 has recently been shown to associate with Uvomorulin *in vivo* (Akthar *et al.*, 2000 ;Akthar and Hotchin, 2001), suggesting it may have a role in cell-cell adhesion. Mice lacking a functional copy of the *Rac1* gene form an irregularly shaped and thickened primitive ectoderm with greatly reduced cell numbers, a high incidence of apoptosis and shortened anterior-posterior axis. Embryos die prior to 9.5 dpc due to defects during gastrulation (Sugihara *et al.*, 1998). Furthermore, primitive ectoderm cells isolated from *Rac1*^{-/-} mutant embryos and cultured *in vitro* showed reduced cell adhesion and migration on ECM substrate when compared to wild type explants (Sugihara *et al.*, 1998). Collectively, these reports identify the role of extracellular and intracellular cell adhesion components in the correct development, maintenance and differentiation of primitive ectoderm.

The *adenomatous polyposis coli* (*APC*) gene is involved in the ubiquitin mediated degradation of the cytoplasmically localised β -catenin, a component of the Wnt signalling pathway (Hinck *et al.*, 1994). β -catenin is also tethered at the cell membrane associated with cadherin based

cell-cell adhesion. Mice lacking the β -catenin gene form a normal primitive ectoderm and die due to a failure to gastrulate, similar to *Wnt3*^{-/-} mutant mice (Huelskens *et al.*, 2000; ^{Liu}~~Lui~~ *et al.*, 1999). However, mice lacking the *APC* gene die shortly after implantation due to death of the primitive ectoderm (Moser *et al.*, 1995).

Collectively, these reports indicate that regulation of cell-ECM and cell-cell adhesion/Wnt signalling pathway components are essential for primitive ectoderm maintenance and differentiation *in vivo*.

1.5.2 Intracellular factors controlling pluripotent cell development

1.5.2.1 The STAT family

The signal transducer and activator of transcription (STAT) proteins have been shown to mediate biological actions in response to many cytokines and growth factors. Many ligands activate the STAT3 protein, including Il-6 family members such as leukaemia inhibitory factor (LIF), and EGF (Takeda *et al.*, 1997). Following receptor activation STAT proteins are phosphorylated and form homo-or heterodimers and induce gene expression within the nucleus. Targeted disruption of the mouse *Stat3* gene leads to degeneration of the pluripotent cells. At 6.0 dpc the egg cylinder is smaller than wild-type embryos and by 7.5 dpc the mutant embryo has been resorbed with no overt sign of gastrulation (Takeda *et al.*, 1997). This defect may be associated with pluripotent cell growth as the *in vitro* culture of mutant blastocysts yields small ICM outgrowths (Takeda *et al.*, 1997).

1.5.2.2 Homeobox genes

Targeted disruption of the pluripotent cell-specific homeobox gene *Oct4* results in early embryonic lethality (Nichols *et al.*, 1998). *Oct4*^{-/-} embryos undergo normal cleavage development and form blastocysts. This occurs in the absence of the *Oct4* gene product, as maternal stores become depleted by the 2-cell stage. This suggests that *Oct4* is not required for growth or survival of the blastomeres. However, *Oct4* deficient embryos do not form an ICM. Growth of pre-implantation blastocysts in culture as embryo outgrowths results in the derivation of only extra-embryonic lineages such as trophoblastic giant cells, extra-embryonic ectoderm and parietal and VE cells. Thus,

Oct4 appears essential for the formation and pluripotency of the ICM as loss of *Oct4* results in divergence to the trophectoderm lineage (Nichols *et al.*, 1998).

1.5.2.3 Cell cycle control and the Myc family of transcription factors

Cell cycle progression is controlled by cyclin dependent kinases (Cdks) complexed with cyclins. A-type cyclins are involved at both G1/S and G2/M transitions of the cell cycle. During rodent embryo development, rapid G1/S and G2/M entry is required to maintain the high proliferation rate of pluripotent cells during post-implantation development (MacAuley, 1993). A homozygous functional mutation of the *cyclin A2* gene, the product of which is involved in G1/S and G2/M transitions, is lethal during mouse embryogenesis (Murphy *et al.*, 1997). The embryos develop normally until 5.5 dpc. However, by 6.5 dpc the embryo is significantly smaller than wild-type/heterozygous counterparts and does not have distinct embryonic or extra-embryonic regions. These defects coincide with the rapid pluripotent cell proliferation. Mutant embryos were resorbed by 7.5 dpc. Furthermore, mice lacking a functional copy of the *Cull1* gene, which encodes a ubiquitin ligase that regulates the abundance of cell cycle proteins including cyclins, arrest between 5.5-6.5 dpc with an accumulation of cyclin E in embryonic tissues (Dealy *et al.*, 1999; Wang *et al.*, 1999). Pluripotent cells in these mutant mice are still able to proliferate up to 6.5 dpc, suggesting cyclin E accumulation in these cells drives the cell cycle, but cannot support embryonic growth. These data highlight the requirement for cell cycle control during the proliferative phase of embryonic development.

The Myc family of basic region helix-loop-helix (bHLH) transcription factors are involved in cellular processes such as proliferation, differentiation and apoptosis (Hendriksson and Luscher, 1996; Facchini and Penn, 1998). During cellular proliferation, Myc promotes cell-cycle progression through the induction of cyclin gene expression. Myc is a direct transcriptional activator of *cyclin D2* (Bouchard *et al.*, 1999) and *Cull1* (O'Hagan *et al.*, 2000), and has been shown to initiate transcription of *cyclin A2* and *cyclin E* (Jansen-Durr *et al.*, 1993). These cyclin genes drive entry into S phase and initiate cell cycle progression and cell division. Members of the Myc family (c-Myc and N-Myc) are expressed in primitive ectoderm and serve important roles in mammalian development.

However, mutation of these genes does not affect early development, perhaps due to functional redundancy (Malynn *et al.*, 2000). Myc activity requires dimerisation to an obligatory bHLH partner, Max, prior to the activation of target genes (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991). *Max* is expressed ubiquitously in the embryo and mice carrying functional mutations in the *Max* gene die shortly after implantation (5.5-6.5 dpc) due to proliferative defects in the pluripotent and extra-embryonic cell lineages, in the absence of increased apoptosis (Shen-Li *et al.*, 2001).

Precise regulation of the G1/S phase checkpoint of the cell cycle is required for early mouse embryogenesis. This is observed in mice lacking the *mdm2* proto-oncogene that arrest at 5.5 dpc with little embryonic material and an accumulation of the p53 protein (Montes de Oca Luna *et al.*, 1995; Jones *et al.*, 1995). The p53 protein is a major component of the G1/S phase checkpoint (Taylor and Stark, 2001). The product of the *mdm2* gene forms a complex with p53 to down-regulate its cellular activity and allow S phase entry. Presumably, the arrest of these cells is due to blockage of G1/S phase transition by p53. Accordingly, *Mdm2*^{-/-} mice on a *p53*^{-/-} background develop normally and are viable (Montes de Oca Luna *et al.*, 1995; Jones *et al.*, 1995; Levillard *et al.*, 1999). Mice lacking a functional copy of the *tsg101* transcription factor displayed similar defects to *mdm2*^{-/-} mice. The *tsg101*^{-/-} mice were comparatively small at 5.5 and 6.5 dpc, had failed to develop further by 7.5 dpc and were resorbed 24 hours later without initiating gastrulation (Ruland *et al.*, 2000). TUNEL assays indicated that the small size of the embryo was not due to increased apoptosis and BrdU determined that cellular proliferation was poor. Mutant embryos also display an accumulation of p53 (Ruland *et al.*, 2000). Accordingly, the formation of *tsg101*^{-/-} mice on a *p53*^{-/-} mutant background gave partial rescue to 8.5 dpc (Ruland *et al.*, 2000).

In conclusion, these reports indicate that the regulation of checkpoint control mechanisms *in vivo* is integral for the proliferative phase of early mouse embryogenesis. Furthermore, the formation and post-implantation lethality of *Max*^{-/-} and *cyclinA2*^{-/-} embryos may be due to maternal stores of these proteins that rapidly dilute out during post-implantation proliferation or the essential requirement of Myc activity and cyclin gene expression for the rapid pluripotent cell proliferation during early mammalian development (Murphy *et al.*, 1997; Shen-Li *et al.*, 2001).

1.5.2.4 DNA repair

The DNA damage response pathway genes *Brca1* (Hakem *et al.*, 1996; Ludwig *et al.*, 1997) *Brca2* (Suzuki *et al.* 1997; Ludwig *et al.*, 1997; Sharan *et al.*, 1997), *Rad51* (Lim and Hasty, 1998), *muREC2/RAD51L1* (Shu *et al.*, 1999), *Rad50* (Luo *et al.*, 1999) and *Ref1* (Xanthoudakis *et al.*, 1996) are essential for primitive ectoderm development and gastrulation. While the exact cellular roles of these genes remain unknown they are known to be essential for cell proliferation and DNA recombination repair. Expression of the *Brca1*, *Rad51*, *Rad50*, *Ref1* and *muREC2/RAD51L1* genes during pluripotent cell development in the early mouse embryo has not been determined, but *Brca2* is expressed at low levels throughout the primitive ectoderm and additional embryonic and extra-embryonic tissues before and after gastrulation (Sharan *et al.*, 1997).

Mice lacking a functional *Brca1* gene form morphologically normal blastocysts prior to implantation. However, at 5.5 dpc these embryos have failed to form egg cylinders. At 6.5 dpc mutant embryos failed to undergo differentiation with increasing disorganisation and retarded proliferation of the pluripotent cells, as was determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation (Hakem *et al.*, 1996; Liu *et al.*, 1996). *Brca1* mutant mice are resorbed before day 8.5 of development. The reduced number of pluripotent cells in *Brca1*^{-/-}embryos do not result from elevated levels of apoptosis, as measured using TUNEL assays, but are a result of a proliferative defect. In support of this, cyclin E levels are reduced in mutant embryos (Hakem *et al.*, 1996) and *Brca1* mutant blastocysts displayed impaired ICM proliferation when cultured *in vitro* (Liu *et al.*, 1996). The aggregation of *Brca1*^{-/-}morula with wild-type tetraploid 4-cell stage embryos, where wild-type cell progeny can only contribute to extra-embryonic lineages, failed to rescue the *Brca1* phenotype indicating that the *Brca1* defect is pluripotent cell specific (Hakem *et al.*, 1996).

Mice carrying functional mutations in the *Brca2* gene display a similar phenotype to *Brca1*^{-/-} mice (Suzuki *et al.*, 1997). *Brca2*^{-/-}embryos have a reduced size by 5.5 dpc and displayed impairment in pluripotent cell proliferation *in vivo* and during *in vitro* blastocyst outgrowth. However, these defects in pluripotent cell function are apparent before detectable *Brca2* expression by *in situ* hybridisation. Gastrulation is not initiated in more severely affected embryos and is impaired in less severely affected embryos. In contrast to *Brca1*^{-/-}embryos, *Brca2*^{-/-}embryos do not

down-regulate cyclin E, can induce mesoderm formation and establish an anterior-posterior axis prior to death at day 8.5.

Rad51 is a mammalian gene highly homologous to the *E.coli RecA* gene, the product of which mediates double strand DNA repair (Lim and Hasty; 1996; Shu *et al.*, 1999). The *Rad51* gene product has been shown to associate with BRCA1 in mitotic cells (Scully, 1997) suggesting an interaction with the BRCA1 DNA repair pathway. Mice lacking functional copies of the *Rad51* gene arrest at the early egg cylinder stage at 5.5 dpc, similar to *Brcal* mutant embryos, and begin to be resorbed at 7.5 dpc (Lim and Hasty; 1996). Furthermore, while *Rad51* mutant embryos formed a pro-amniotic cavity, the failure to form mesoderm and reduced cellular proliferation were similar to *Brcal*^{-/-}embryos (Lim and Hasty, 1996). Direct measurement of primitive ectoderm proliferation *in vivo* by BrdU incorporation revealed reduced proliferation at day 5.5, 6.5, and 7.5 of embryogenesis in *Rad51*^{-/-}embryos. These defects suggest an additional role for *Rad51* in cell proliferation. A second mammalian gene with regions of homology to *E.coli RecA* is the *muREC2/RAD51L1* gene, the product of which mediates a radiation-inducible DNA damage response. Mouse embryos carrying homozygous and functional mutations in the *muREC2/RAD51L1* gene (Shu *et al.*, 1999) are severely retarded in growth around 5.5 dpc and are resorbed by 8.5 dpc, similar to *Rad51*.

The *Brcal*^{-/-}, *Brc2*^{-/-}, *Rad51*^{-/-} and *muREC2/RAD51L1*^{-/-} mice all show partial rescue on a p53 mutant background (Lim and Hasty, 1996; Hakem *et al.*, 1997; Ludwig *et al.*, 1997; Brugarolas and Jacks, 1997; Cressman *et al.*, 1999; Shu *et al.*, 1999). This indicates that the products of these genes may have additional roles in maintaining chromosomal fidelity through DNA damage-induced checkpoint control at the G1/S phase transition of the cell cycle.

Additional genes involved in DNA repair are also essential for early mouse embryogenesis. The products of the *Ref1* and *Rad50* genes mediate redox/DNA repair or DNA-strand exchange/repair functions respectively (Xanthoudakis *et al.*, 1996; Luo *et al.*, 1999). Mice lacking the *Ref1* gene are severely retarded in growth around 5.5 dpc and are resorbed by 8.5 dpc (Xanthoudakis *et al.*, 1996), while embryos lacking a functional copy of the *Rad50* gene form morphologically distinct embryos at 6.0 dpc that are overtly smaller, lack the distinct columnar morphology of primitive ectoderm and are resorbed around 7.5 dpc (Luo *et al.*, 1999). Furthermore, mouse embryos homozygous for a targeted mutation in the Bloom's syndrome gene (*Blm*), which

encodes a member of the RecQ family of RNA and DNA helicases, are developmentally delayed and die by 13.5 dpc. The growth retardation in mutant embryos is accounted for by a wave of increased apoptosis in the pluripotent cells of the early post-implantation embryo (Chester *et al.*, 1998). These reports highlight the requirement for cell growth and genome integrity during the proliferative phase of pluripotent cell development and primitive ectoderm formation *in vivo*.

Collectively, these results suggest that the *Brcal/2*, *Rad51*, muREC2/RAD51L1, *Ref1*, *Rad50* and *Blm* genes play essential roles in DNA damage induced checkpoint control and/or embryonic cell DNA fidelity during the proliferative phase of early embryonic development.

1.5.2.5 Genes of unknown function

Many house keeping genes functionally inactivated in the mouse genome lead to post-implantation embryonic lethality, often due to pluripotent cell defects (Deng and Behringer, 1995; Matsui *et al.*, 1996; Bosl *et al.*, 1997; Michaelson *et al.*, 1999; Hirsch *et al.*, 2000; Denzel *et al.*, 2000; Chen *et al.*, 2000; Howman *et al.*, 2000; Huo and Scarpulla, 2001). These defects are often the result of a loss or dilution of maternal transcripts that lead to loss of normal cell function. However, genes of unknown function, expressed in the primitive ectoderm, are also required for pluripotent cell development and further differentiation. These include the mouse homologue of the yeast RNA1 gene, *fug1*, ubiquitously expressed at 6.5 dpc in the early mouse embryo. Embryos lacking functional copies of the *fug1* gene arrest at 6.0 dpc, prior to detectable expression, and display delayed and poor egg cylinder formation with a failure to undergo gastrulation (*fug*) (DeGregori *et al.*, 1994).

The huntingtin gene (*Hdh*) produces a protein of unknown function implicated in the neurodegenerative disorder Huntington's disease. *Hdh* is expressed in primitive ectoderm at day 6.5 of development. Mice mutant in the gene encoding huntingtin (*Hdh*), display increased apoptosis in the primitive ectoderm and abnormal gastrulation at embryonic day 7.5 leading to resorption by day 8.5. Therefore, *Hdh* is critical early in embryonic development, prior to the emergence of the nervous system (Duyao *et al.*, 1995; Zeitlin *et al.*, 1995).

Functional gene ablation studies in mice therefore indicate that activation of multiple growth factor signalling pathways and cell-ECM attachment are required for pluripotent cell development

during early mouse embryogenesis. While ECM appears to direct pluripotent cell survival or maintenance, embryos blocked in growth factor/signalling pathway activation generally display a disruption in pluripotent cell proliferation and differentiation. The range of proliferative defects in these mice indicates the diverse growth factor requirements for pluripotent cell development *in vivo*. Furthermore, the rapid proliferation of these cells and their role as the progenitors for the embryo and adult requires strict maintenance of DNA fidelity. The early embryonic lethality and degeneration of pluripotent cells in mice lacking functional copies of genes required for pluripotent cell proliferation, DNA repair and/or checkpoint control indicates the importance of these events to the future development of the embryo.

1.6 VE and the control of pluripotent cell fate

1.6.1 Maintenance of pluripotent cell lineages

VE plays an active role in providing nutrients to the embryonic cell lineages (Bielinska *et al.*, 1999). In addition, analysis of several genes has implicated VE in the maintenance of pluripotent cells.

Evx1 is a homeobox gene expressed throughout the VE and the posterior most primitive ectoderm cells at 6.25 dpc (Spyropoulos and Capecchi, 1994). Ablation of *Evx1* results in degeneration of pluripotent and extra-embryonic cells around 5.0 dpc. Mutant embryos implant but fail to grow or undergo differentiation. The mutant embryos become disorganised and undergo resorption by 6.5 dpc (Spyropoulos and Capecchi, 1994). Defective developmental progression of the pluripotent cells is thought to result from perturbation of communication between the extra-embryonic VE and pluripotent cells (Spyropoulos and Capecchi, 1994).

Hnf4 and *vHnf1* are members of a complex regulatory network composed of a heterogeneous class of transcription factors in the hepatic nuclear factor (HNF) family. *Hnf4* and *vHnf1* are expressed throughout the VE from 5.5 dpc, with initiation of *Hnf4* expression within the primitive endoderm at 4.5 dpc. These genes are not expressed in pluripotent cell lineages (Chen *et al.*, 1994; Duncan *et al.*, 1994; Barbacci *et al.*, 1999; Coffinier *et al.*, 1999). Mice lacking functional copies of the *Hnf4* gene implant and form a morphologically normal VE but show disturbances in primitive

ectoderm formation including increased apoptosis by 6.5 dpc, and delayed onset of gastrulation to 9.5 dpc (Chen *et al.*, 1994; Duncan *et al.*, 1994). *Hnf4*^{-/-} mutant embryos fail to gastrulate normally and are resorbed by 10.5 dpc (Chen *et al.*, 1994; Duncan *et al.*, 1994; Duncan *et al.*, 1997). Mice lacking the *vHnf1* gene show more severe defects post-implantation with the absence of extra-embryonic regions, including parietal and VE, and a highly disorganised embryonic region with death by 5.5 dpc. However, aggregation of *Hnf4*^{-/-} or *vHnf1*^{-/-} ES cells with wild type tetraploid 4-cell morula results in the formation of a normal primitive ectoderm that enters gastrulation in an equivalent manner to wild type embryos (Duncan *et al.*, 1997; Barbacci *et al.*, 1999; Coffinier *et al.*, 1999). These reports are further supported by similar findings in mice lacking a functional copy of the gene for the zinc finger transcription factor GATA6. Mutant embryos die around 5.5 dpc and chimeric analysis has revealed this is caused by an indirect effect on the primitive ectoderm caused by defects within VE (Koutsourakis *et al.*, 1999). These reports suggest that primitive ectoderm is directly reliant on *Hnf4*, *vHnf1* and *GATA6* gene expression within the VE for maintenance and survival and indicate VE is a source of signals for PE survival.

1.6.2 Specification of pluripotent cell fate

In contrast to 6.25 dpc primitive ectoderm, VE displays positional specification prior to the visible onset of gastrulation. The divergent homeobox gene *Hex* is expressed in the distal primitive endoderm of the 4.5 dpc blastocyst. At 5.5 dpc *Hex* expression is restricted to a small patch of VE at the anterior tip of the egg cylinder (Thomas *et al.*, 1998). Lineage analysis has shown that *Hex* expression in the AVE is derived from cell migration rather than a spatial and cellular shift in *Hex* expression (Thomas *et al.*, 1998). The expression of *Hex* supports the identification of anterior-posterior axis establishment prior to, and independent of, primitive streak formation during early mammalian development, *Hex* expression is the earliest genetic marker of anterior-posterior asymmetry in the mouse embryo.

Recent embryological and genetic experiments in mice have suggested that the AVE of the early gastrulating embryo can function as a head organising centre. The VE antigenic marker VE-1, is first detected at 5.0 dpc, and is the first antigenic marker to demarcate differential cell populations within regions of this tissue. The immunoreactive VE cells cover the anterior most one third of the

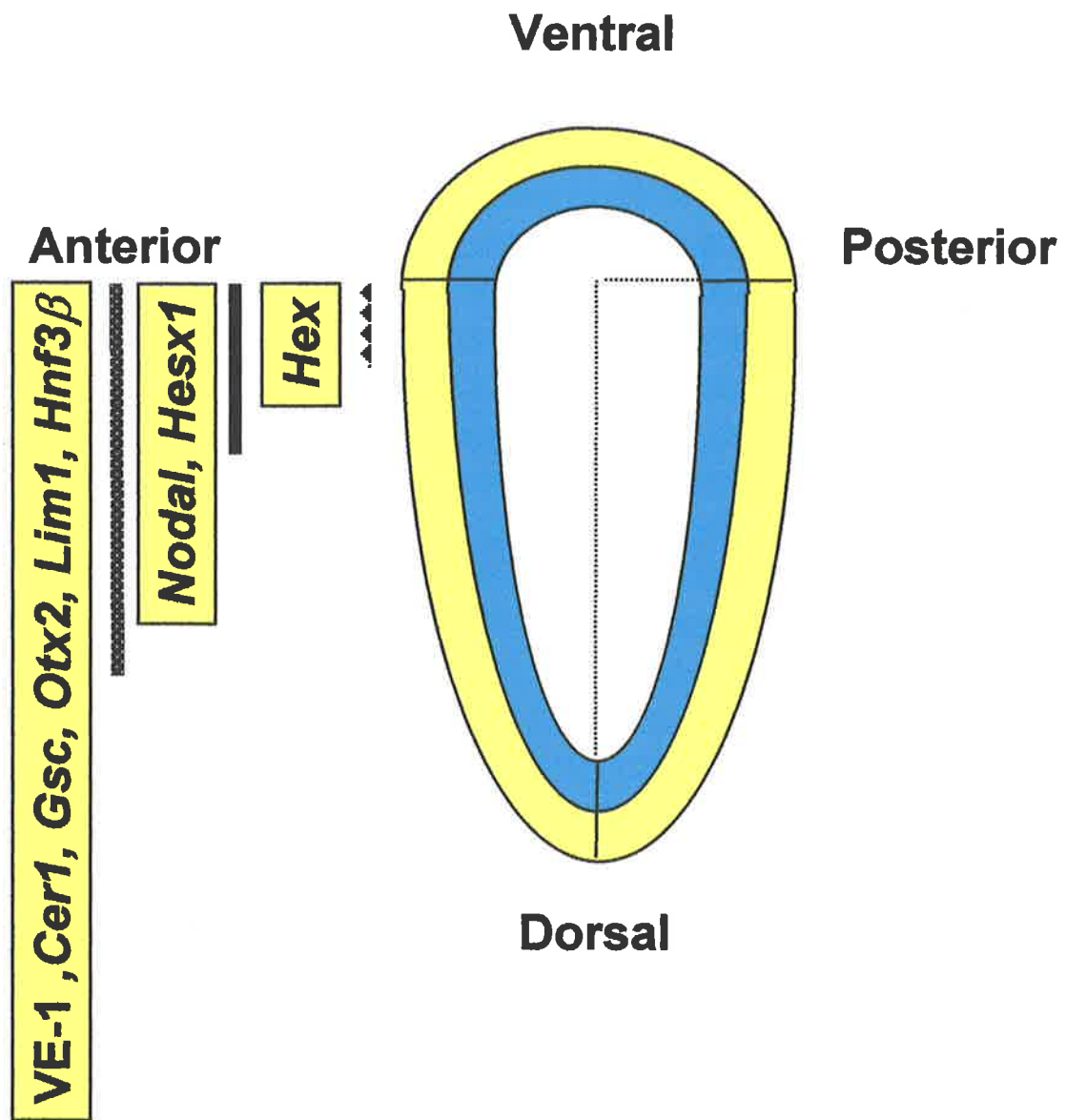
primitive ectoderm, directly opposite to the site of primitive streak ingressio (Rosenquist and Martin, 1995). Genetic markers differentially expressed in the VE include the anteriorly localised homeobox genes *Lim1* (Shawlot and Behringer, 1995; Belo *et al.*, 1997), *Otx2* (Acampora *et al.*, 1995), *Goosecoid* (Belo *et al.*, 1997), *Hesx1* (Thomas and Beddington, 1996), an additional member of the Hnf family, *Hnf3 β* (Ang *et al.*, 1994; Weinstein *et al.*, 1994; Belo *et al.*, 1997), the TGF- β family member *Nodal* and the homologue of the *Xenopus Cerebrus* gene, *Cerebrus like* (*Cer1*) (Belo *et al.*, 1997). All these genes display a restricted pattern of expression to the AVE (Figure 1.9).

The homeobox gene *Hesx1* is initially expressed in a small patch (10-20 cells) of the anterior most VE, adjacent to the embryonic/extra-embryonic boundary, first detectable with the onset of primitive streak formation at 6.5 dpc (Thomas and Beddington, 1996). As gastrulation proceeds, *Hesx1* expression is up regulated in the adjacent primitive ectoderm that is fated to give rise to forebrain in the developing embryo (Thomas and Beddington, 1996). Surgical removal of VE cells expressing *Hesx1* during the earlier stages of gastrulation prevents or restricts the expression of *Hesx1* in the adjacent ectoderm and forming neurectoderm. This is the first example of VE directly effecting gene expression in the adjacent ectoderm. Mouse embryos lacking the *Hesx1* gene show variable forebrain truncations ranging from mild to severe lack of forebrain tissue (Martinez-Barbera *et al.*, 2000). However, distinct from *Hnf* chimeric embryos, embryos composed of wild-type ES cells developing within *Hesx1*^{-/-}VE showed no evident forebrain abnormalities while *Hesx1*^{-/-} pluripotent cells, in wild-type blastocysts give rise to chimeras with forebrain defects similar to those observed in the *Hesx1*^{-/-}mutants. Therefore, while the AVE is responsible for defining the identity in the embryo, and in particular for the correct definition of the ectoderm cell fate (Thomas and Beddington, 1996), autonomous *Hesx1* expression is required in the primitive ectoderm and neurectoderm for normal forebrain formation (Martinez-Barbera *et al.*, 2000).

Lim1 and *Otx2* are co-expressed in the anterior most region of the VE with the forkhead-like transcription factor hepatocyte nuclear factor 3 beta (*Hnf3 β*) (Shawlot and Behringer, 1995; Acampora *et al.*, 1995; Belo *et al.*, 1997). The OTX2 gene product has been shown to directly associate with LIM1 and HNF3 β proteins (Dufort *et al.*, 1998 Perea-Gomez *et al.*, 1999) suggesting a commonality of action for these proteins during early development. Mice lacking a functional copy

Figure 1.9 Differential gene expression and antigen presentation in the prestreak/early streak stage AVE of the mouse embryo

Bars next to marker genes represent approximate proxial-distal extension of marker gene expression.



of the *Lim1*, *Otx2* or *Hnf3 β* genes all show a characteristic narrowing at the embryonic/extra-embryonic boundary at the early to mid-streak stages. Furthermore, *Lim1* and *Otx2* mutant mice display normal body formation in the absence of anterior head structures (Simeone *et al.*, 1993; Ang *et al.*, 1994; Ang and Rossant, 1994; Wienstein *et al.*, 1994; Matsuo *et al.*, 1995; Acampora *et al.*, 1995; Shawlot and Behringer, 1995). The similarity in phenotype of *Lim1*^{-/-} and *Otx2*^{-/-} mice may suggest a common signal pathway in head formation, while some *Hnf3 β* ^{-/-} embryos with more severe phenotypes show anterior brain truncations similar to *Lim1* and *Otx2* mutant mice (Ang and Rossant 1994). The requirement for *Hnf3 β* in VE for normal initiation of gastrulation from pluripotent primitive ectoderm cells was confirmed through the aggregation of *Hnf3 β* ^{-/-} ES cells with tetraploid wild type morula to generate chimeric embryos. Wild-type VE rescues primitive streak elongation in *Hnf3 β* ^{-/-} embryos (Dufort *et al.*, 1998). However, while rescue of node and notochord formation relies on a cell autonomous cell fate-signalling event. This report indicates a role for *Hnf3 β* in the AVE directing the correct differentiation of primitive ectoderm. Collectively, these reports indicate that *Lim1*, *Otx2* and *Hnf3 β* expression in the VE is required for the formation of anterior structures and work through a common signalling mechanism.

Nodal is a TGF β family member growth factor family. *Nodal* expression is first detected at low levels throughout the primitive ectoderm and overlying VE at 5.5 dpc (Conlon *et al.*, 1994; Varlet *et al.*, 1997). Prior to the onset of gastrulation, *nodal* exhibits graded expression in pluripotent cells with highest levels of expression in a broad zone in the posterior primitive ectoderm at the proximal boundary, and in a tightly restricted region of the anterior primitive ectoderm (Varlet *et al.*, 1997). As primitive ectoderm progresses, *nodal* expression becomes localised to the proximal posterior primitive ectoderm in an increasingly distal fashion (Varlet *et al.*, 1997). Prior to the onset of gastrulation (6.0 dpc), the *nodal* growth factor gene is also expressed in a morphologically thickened region of AVE and the underlying anterior primitive ectoderm (Varlet *et al.*, 1997). Mice lacking a functional *nodal* gene cannot initiate primitive streak formation and are arrested at the gastrulation stage of development (Conlon *et al.*, 1994). This defect could reflect *Nodal* expression in the primitive endoderm, primitive ectoderm or both cell lineages. To examine these possibilities chimeric embryos were derived from *nodal*^{-/-} ES cells injected into wild-type blastocysts, or wild type

ES cells injected into *nodal*^{-/-}blastocysts (Varlet *et al.*, 1997). Injected ES cells have been shown to colonise almost exclusively the embryonic lineages (Beddington and Robertson, 1989). Injection of wild type ES cells into *Nodal* mutant blastocysts allowed the formation of a primitive streak and the onset of gastrulation. The phenotype of chimeric embryos was dependent on the level of ES cell colonisation and embryos highly colonised by wild type ES cells formed normal posterior structures, but displayed anterior neural defects in later stage embryos, reminiscent of *Otx2*^{-/-}, *Lim1*^{-/-} or *Hnf3β*^{-/-} mutant embryos. Wild-type embryos injected with *Nodal*^{-/-}ES cells did not display any defects (Varlet *et al.*, 1997). This report reveals that *Nodal* expression in the primitive/VE is required for correct neural development in the mouse (Varlet *et al.*, 1997) and, in a collective manner, that signals arising from the AVE are required to impart anterior specification upon primitive ectoderm cells *in vivo*.

A role for VE in pluripotent cell differentiation has also been demonstrated *in vitro*. An uncharacterised factor expressed by the VE like cell line END2, induces the formation of muscle and neural lineages from aggregates of the primitive ectoderm-like P19 EC cell line (van den Eijden-van Raaij *et al.*, 1991; Mummery *et al.*, 1993). Activin A inhibited the activity of the END2 signal on P19 EC cell aggregates. Furthermore, an END2 cell derived signal has been shown to induce apoptosis within S2 EC cell aggregates (Coucouvanis and Martin, 1995). These reports further suggest that signals arising from the VE coordinate the maintenance and differentiation of primitive ectoderm.

In summary, numerous reports have demonstrated the crucial role of VE and VE derived signals in the maintenance, morphology and differentiation of pluripotent cells. However, the identity of the VE derived signals that direct primitive ectoderm maintenance and differentiation remain unknown.

1.7 Pluripotent cell lines *in vitro*

The identity and biological action of extracellular signals that direct the progression and differentiation of pluripotent cell populations *in vivo* is difficult to investigate due to the small size and inaccessibility of embryo at this stage of development. However, the ability to culture pluripotent

cells in culture provides an opportunity to identify these cellular signals with an unlimited source of pluripotent cells that differentiate *in vitro* in a manner that mimics early mammalian development in utero.

1.7.1 ICM Explants

Much of the early functional analysis of pluripotent cells *in vitro* was carried out using explants of 3.5 dpc-4.5 dpc mouse blastocyst ICM (Sherman, 1975). Furthermore, ICM cells can also be isolated from blastocysts by immunosurgery to remove the outer layer of trophectoderm (Hogan and Tilly, 1978). ICM explants further develop as attached cell cultures that differentiate into numerous and heterogenous cell lineages. They can also differentiate in suspension in a similar manner to embryoid bodies (EB's: 1.10.2; Hogan and Tilly, 1978). Many of these studies provided an early insight into the developmental potential of early embryonic cells (Gardner, 1983). However, stable pluripotent cell lines could not initially be established from ICM cultures.

1.7.2 Embryonal carcinoma (EC) cells

EC cells are derived from complex germ cell tumours termed teratocarcinomas, which contain a variety of endodermal, ectodermal and mesodermal tissues surrounding small nests of pluripotent cells, which sustain the tumour. EC cells are similar to ICM and primitive ectoderm cells with respect to morphology, cell surface antigen expression, ultrastructure and protein synthesis profiles (Martin, 1975; Martin, 1980). EC cells can be nullipotent, multipotent or pluripotent. Multipotent and pluripotent cells can give rise to a variety of differentiated cell types *in vitro* and *in vivo* when introduced into the blastocyst environment (Martin, 1981). However, apart from a single report (Stewart and Mintz, 1981), EC cells fail to colonise the germ line of chimeric mice. Thus, EC cells are not 'normal' pluripotent cells and have limited use as a model system for the investigation of pluripotent cell biology *in vivo* and *in vitro*. This may be due to the isolation of EC cells from mouse pluripotent cell tumours that have acquired karyotypic and developmental abnormalities *in vivo* or during *in vitro* culture (Martin, 1981).

Human pluripotent EC cell lines can also be derived from spontaneous testicular teratocarcinomas. These pluripotent cell lines can differentiate into tissues representative of the three embryonic germ layers and are also useful for understanding the mechanisms that control the differentiation of human pluripotent cells into a variety of embryonic and extra-embryonic cell types (Andrews, 1998). However, given the similar origin to mouse EC cells, human EC cells are likely to have similar developmental restrictions on their use as an *in vitro* model of development or source of therapeutically useful cells.

The restricted differentiation capacity of some EC cell lines can be exploited in specific *in vitro* pluripotent cell differentiation assays (Coucouvanis and Martin, 1995). S2 EC cells fail to form an external layer of VE or form a pro-amniotic cavity upon EB differentiation (Coucouvanis and Martin, 1995, 1999) while F9 EC cells are restricted to forming lineages of the primitive endoderm when differentiated by RA or di-butyryl cAMP (Strickland and Mahdavi, 1978; Hogan *et al.*, 1983; Mummery *et al.*, 1990). P19 EC cells give rise to muscle and neuronal lineages when differentiated as aggregates in the presence of dimethyl sulphoxide (DMSO) or RA respectively (Jones-Villeneuve *et al.*, 1982; McBurney *et al.*, 1982).

1.7.3 Embryonic Stem (ES) cells

ES cells are derived from the ICM of pre-implantation mouse blastocysts, and can be propagated in the pluripotential state indefinitely in culture (Evans and Kaufman, 1981; Martin, 1981). ES cells retain pluripotence even after genetic manipulation and long periods in culture, as determined by their ability to contribute to all the differentiated cell types of the embryo, including the germ line, when reintroduced into the mouse blastocyst by micro-injection. Members of the interleukin-6 family of cytokines act on ES cells to prevent spontaneous differentiation (Smith *et al.*, 1988) and bypass the requirement for culture with mitotically inactivated embryonic fibroblasts (that express LIF) as feeder cells. This converts ES cells into an experimentally accessible homogeneous population of cells (Smith *et al.*, 1988). ES cells are most closely related to the ICM cells of the 4.75 dpc embryo *in vivo* as determined by differentiation potential *in vitro* and *in vivo* and the expression of genetic and antigenic markers (Evans and Kaufman, 1981; Martin, 1981; Rathjen *et al.*, 1999). Brook and Gardner (1997) demonstrated that ES cells can be isolated from the pluripotent cells of

4.5 dpc blastocysts at double the frequency compared to 3.5 dpc blastocysts (Brook and Gardner, 1997) providing a potential *in vivo* equivalent for ES cells *in vitro*.

Pluripotent ES-like cells can also be isolated from primordial germ cells (PGC's) that colonise the genital ridge of 8.5 and 12.5 dpc 129 strain mouse embryos. PGC's cultured in the presence of the cytokine LIF and the growth factors bFGF and Steel factor, adopt a morphologically indistinguishable from ES cells and proliferate to form colonies of germ-line competent pluripotent cells, termed EG cells (Matsui, 1992; Resnick, 1992). The isolation of ES and EG cells lead to the proposition that pluripotent cell types may be inter-converted via a common pluripotent precursor (Rossant, 1993).

Recently, ES cells have been isolated from *in vitro* derived human blastocysts and human EG cells from embryonic gonads (Shamblott *et al.*, 1998). These cells differentiate *in vitro* into multiple somatic cell lineages. However, these cells are not equivalent to mouse pluripotent ES or EG cells in morphology and rate of proliferation. Thus, while human pluripotent ES and EC cells are derived from the embryo, display self-renewal, clonal propagation and differentiation into tissues of the three germ layers, they are difficult to maintain in culture and readily differentiate with a slow rate of growth. Further investigations into pluripotent cell signal requirements may allow pluripotent cell lines from human and additional mammalian species to meet the pluripotent cell criteria defined by mouse pluripotent cells (Shamblott *et al.*, 2000; Pera *et al.*, 2000).

1.8 Identification of cytokines that inhibit ES cell differentiation *in vitro*

In 1987 Smith and Hooper reported that medium conditioned by Buffalo Rat Liver (BRL) cells was able to prevent the spontaneous differentiation of EC and ES cell lines in culture. These cell lines were originally maintained on feeder layers of fibroblasts that provide a differentiation inhibiting activity (DIA). The DIA within BRL conditioned medium was purified and identified as being identical to the leukaemia inhibitory factor (LIF) cytokine (Smith *et al.*, 1988; Williams *et al.*, 1988; Moreau *et al.*, 1988). This was the first example of factor identification using ES cells. LIF acts through a signal transduction pathway, via interaction with a cytokine specific receptor that heterodimerises with the gp130 receptor subunit at the cell surface (Taga *et al.*, 1992; Piquet-Pellorce *et al.*, 1994). Additional cytokines of the IL-6 cytokine family that stimulate gp130 are also

able to prevent ES cell differentiation *in vitro* (Gearing and Bruce *et al.*, 1992; Conover *et al.*, 1993; Piquet-Pellorce *et al.*, 1994).

The effect of LIF on ES cells in culture is consistent with the expression of *LIF* transcripts in the early mouse embryo (Rathjen *et al.*, 1990b; Robertson *et al.*, 1993; Nichols *et al.*, 1996). *LIF* transcripts are expressed at the egg cylinder stage at low levels (days 4.5-7.5) in predominantly extra-embryonic tissues (Rathjen *et al.*, 1990b; Robertson *et al.*, 1993). This suggests that the ICM and primitive ectoderm populations are bathed in diffusible LIF, which is consistent with a role for this factor in pluripotent cell maintenance during early mouse embryogenesis (Smith, 1992). In addition, the LIF receptor is expressed in the pluripotent cells of the ICM (Nichols *et al.*, 1996) and exogenous expression of LIF in the mouse blastocyst has been shown to alter pluripotent cell differentiation *in vivo* (Conquet *et al.*, 1992). This is consistent with a role for LIF and its receptor in the maintenance of pluripotent cells of the early mouse embryo.

Mice lacking a functional *LIF* gene form normal blastocysts with an ICM and trophoblast that progress to term in wild type mothers suggesting that pluripotent cell populations of the early mouse embryo are not directly reliant on LIF activity and that gp130 may be activated by additional members of the IL-6 family (Stewart *et al.*, 1992; Ware *et al.*, 1995). However, targeted disruption of the low-affinity LIF receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death (Ware *et al.*, 1995) while mice lacking a functional copy of the *gp130* gene progress through the blastocyst stage of development and gastrulate normally before death at 12.5 dpc (Yoshida *et al.*, 1996). These observations suggest that mouse embryos have additional pathways for inhibiting pluripotent cell differentiation *in vivo*, distinct from gp130 and LIF. The ability of mouse 129 strain derived ICM cells to respond to LIF *in vitro* may explain why ES cells can be isolated from this strain of mouse with relative ease.

1.9 Pluripotent cell differentiation *in vitro*

1.9.1 Spontaneous and chemical differentiation

ES cells can be differentiated by the removal of LIF from the culture medium or the addition of chemical inducers such as retinoic acid (RA), dimethylsulphoxide (DMSO) or methoxybenzamide

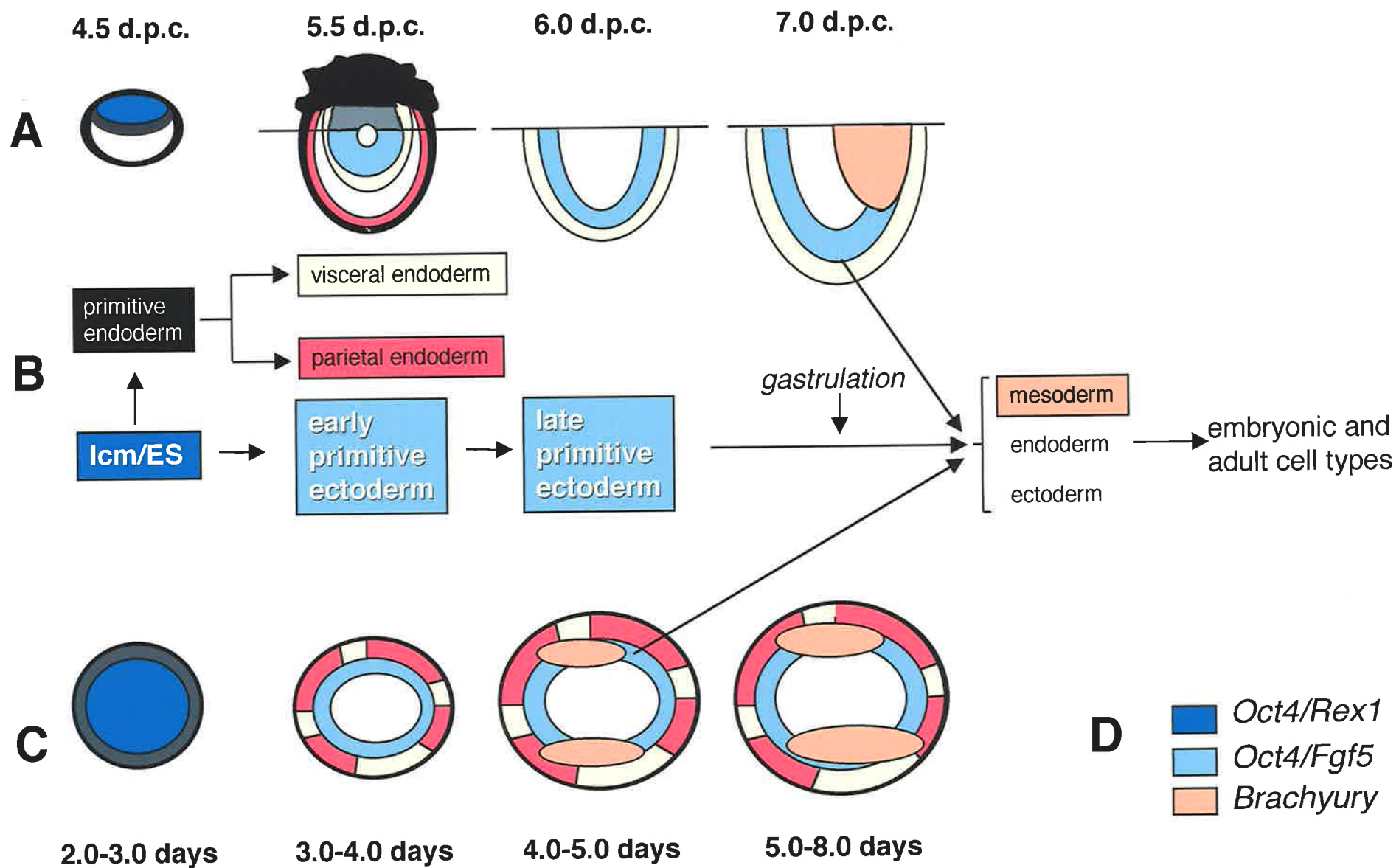
(MBA) (Smith, 1991). The removal of LIF from ES cells induces the formation of a number of poorly defined and heterogeneous cell populations that provide little information regarding pluripotent cell development *in vivo* (Smith, 1991). Furthermore, the differentiation of pluripotent cells under these conditions is often incomplete with formation of residual stem cell nests surrounded by terminally differentiated cells that may secrete LIF or other IL6 family cytokines into the surrounding culture medium (Rathjen *et al.*, 1990b). The differentiation of ES cells using RA generates fibroblast and parietal yolk sac-like cell lineages, while MBA induces a relatively uniform epithelial-like cell type (Smith, 1991). However, the restricted array of cell types produced, and the doubtful biological relevance of the pluripotent cell differentiation in the presence of these chemicals, suggest these methods have little use as developmental models of pluripotent cell differentiation.

1.9.2 Embryoid bodies (EB's)

ES and EC cells differentiate *in vitro* when cultured as aggregates in suspension, in the absence of LIF. Termed Embryoid Bodies (EB), these aggregates undergo a relatively ordered and coordinated program of differentiation reminiscent of mouse embryogenesis (Figure 1.10; Doetschman *et al.*, 1985; Shen and Leder, 1992; Lake *et al.*, 2000). The outer pluripotent cells differentiate to give rise to primitive endoderm. The primitive endoderm then further differentiates to form an outer layer consisting of both parietal and VE. Internal cells retain pluripotency, as determined by expression of *Oct4*, and undergo a cavitation event leading to the formation of a central cavity, analogous to pro-amniotic cavitation *in vivo* (Coucouvanis and Martin, 1995, 1999; Murray and Edgar, 2000). The appearance of primitive ectoderm is marked by up-regulated expression of the primitive ectoderm marker *Fgf5* (Hébert *et al.*, 1990; Hébert *et al.*, 1991; Haub and Goldfarb, 1991; Shen and Leder, 1992) and down-regulation of the ICM marker *Rex1* (Rogers *et al.*, 1991; Shen and Leder, 1992). With extended culture the primitive ectoderm differentiates into all three germ layers. The formation of these differentiated lineages also mimics pluripotent cell differentiation within the embryo where, for example, mesoderm formation is marked by up-regulated expression of the nascent mesoderm marker *brachyury*. These three events mimic the events before and after implantation in the mouse embryo (Evans and Kaufman, 1981; Martin, 1981; Doetschman *et al.*, 1985; Poirier *et al.*, 1991; Robertson, 1987; Coucouvanis and Martin, 1995).

Figure 1.10 Early mouse embryogenesis and embryoid body formation

Diagrammatic and schematic representation of (A) early mouse embryogenesis, (B) cell lineage and (C) ES cell embryoid body progression. Embryogenesis is shown from the ICM stage to formation of the three primary germ layers during gastrulation. Pluripotent cell populations are identified in blue. Early and late primitive ectoderm are distinguished to reflect alterations in gene expression and morphology. For simplicity, embryos at 6.0 and 7.0 days post coitum (dpc) are shown without extra-embryonic tissues except visceral endoderm.



Additional somatic cell lineages and structures, such as beating muscle and neurons, appear at later stages and can be identified by morphology and gene expression (Doetschman *et al.*, 1985; Sanchez *et al.*, 1991; Shen and Leder, 1992; Wang *et al.*, 1992; Keller *et al.*, 1993; Lints *et al.*, 1993; Brustle *et al.*, 1999). These differentiation events follow *in vivo* differentiation pathways assessed by the expression of marker genes and formation of intermediate cell types. However, the formation and differentiation of primitive ectoderm in EB's is stochastic and heterogeneous in contrast to the spatially and temporally organised differentiation observed *in vivo*.

1.9.3 EB's as a model of mouse development

The similarities between mouse embryo and EB development suggest that EB differentiation can be used as a model system for early mammalian development *in vitro*. Coupled with the genetic capacity of targeted ES cells carrying specific and functional mutations, this system is attracting increasing interest for the investigation of gene function during differentiation *in vivo* and *in vitro* (Rathjen *et al.*, 1998).

The helix-loop-helix transcription factor SCL plays an essential role in haematopoietic development (Robb *et al.*, 1995; Robb *et al.*, 1996). Mouse embryos in which the *scl* gene has been disrupted die around 9.0 dpc due to the failure of primitive haematopoietic development (Robb *et al.*, 1995). To assess which cell lineages are primarily effected through loss of *scl* expression during development, ES cells carrying homozygous null mutations of the *scl* locus were injected into blastocysts to generate chimeric mice. Analysis of these embryos demonstrated that the *scl*^{-/-}ES cells make a substantial contribution to all non-haematopoietic tissues but do not contribute to any haematopoietic lineage (Robb *et al.*, 1996). These observations reveal a crucial role for the SCL gene product in definitive haematopoiesis.

The differentiation of *scl*^{-/-}ES cells as EB's *in vitro* showed that the SCL transcription factor was also required for the formation of haematopoietic cells in culture. *Scl*-null EB's differentiated *in vitro* for up to 12 days express marker genes representative of mesoderm and endothelial cell formation but are also restricted in the formation of haematopoietic lineages (Elefanty *et al.*, 1997). Thus, genetic control of haematopoiesis is similar both in the embryo and during EB's differentiation.

In a similar fashion the ubiquitously expressed transcription factor *serum response factor* (*srf*) is essential for mouse embryogenesis (Arsenian *et al.*, 1998). The SRF transcription factor functions as a nuclear target of the Ras/mitogen activated protein kinase (MAPK) pathway. Embryos lacking a functional copy of the *Srf* gene stop developing at the onset of gastrulation, lacking detectable mesoderm (Arsenian *et al.*, 1998). This defect is also mimicked in *SRF*^{-/-}ES cells which fail to differentiate *in vitro* into mesoderm in EB's (Wienhold *et al.*, 2000).

However, *in vivo* defects are not always mimicked faithfully *in vitro* in EB's. Mice lacking a functional copy of the β 1-integrin gene develop a blastocyst with ICM but fail to develop pluripotent cell lineages and are resorbed soon after implantation (refer 1.5.4; Fässler and Meyer, 1995; Stephens *et al.*, 1995). In contrast, EB's derived from ES cells lacking β 1-integrin differentiate to form somatic cell lineages but are restricted in the formation of cardiac muscle cells and keratinocytes (Fässler *et al.*, 1996; Bagutti *et al.*, 1996). Similarly, mice lacking a functional copy of the *dystroglycan* gene, whose product is a cell surface receptor for the ECM component laminin, fail to form a basement membrane separating the primitive ectoderm and VE and fail to gastrulate with pluripotent cell death (Henry *et al.*, 1998). EB's derived from *dystroglycan* knockout ES cells differentiate in a similar manner to EB's lacking the *β 1-integrin* gene, and display restricted differentiation into cardiac muscle cells (Henry and Campbell, 1998).

Finally, EB's form many cell types present within the embryo but lack organisational intricacies associated with axis formation and morphogenesis. The disorganised structure of the EB leads to incorrect juxtaposition of cell lineages and stochastic tissue formation. This is distinct from the highly structured environment of cell differentiation within the mouse embryo.

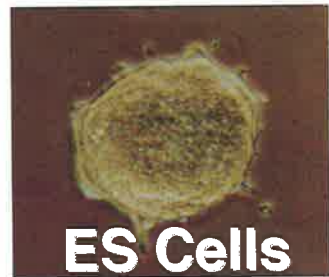
1.10 Early primitive ectoderm-like (EPL) cells

In 1999 it was reported that signals within a conditioned medium, taken as supernatant from the human hepatocellular carcinoma cell line HepG2 (MEDII), induce ES cells to form a morphologically distinct and homogeneous population of cells termed early primitive ectoderm-like (EPL) cells (Figure 1.11; Rathjen *et al.*, 1999). EPL cell formation has been observed from several different ES and EG cell lines (Rathjen *et al.*, 1999; I. Lyons per comm). EPL cells are most closely

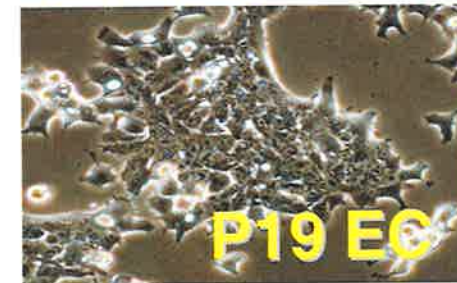
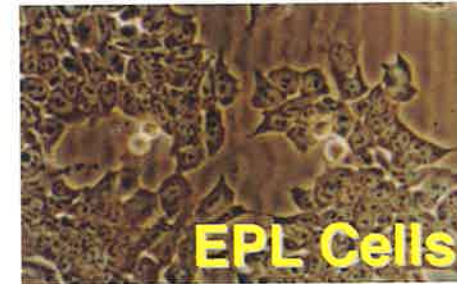
1.11 ES to EPL cell transition: an in vitro model of the ICM to primitive ectoderm transition

(A) Morphological alteration of ES cells to EPL cell following ES cell culture in the presence of MEDII. Morphology of the primitive ectoderm like-P19 EC cell line (B) Schematic representation of the ICM to primitive ectoderm transition *in vivo*.

A *In vitro*

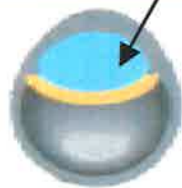


Med2
conditioned
medium



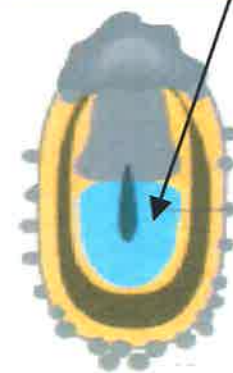
B *In vivo*

Inner cell
mass



Differentiation

Primitive
ectoderm



related to embryonic primitive ectoderm as identified by morphology, gene expression, differentiation potential and cytokine responsiveness (Rathjen *et al.*, 1999). This suggests that EPL cell formation mimics a critical *in vivo* developmental event *in vitro*. However, the identity of the EPL cell inductive signals within MEDII has not been reported. This thesis will focus on the purification and identification of the EPL cell inductive signals and the role of these signals in EPL cell formation.

1.11 Characterisation of EPL cells

1.11.1 EPL cell morphology

ES cells cultured in the presence of mitotically inactivated feeder cells, or culture medium supplemented with LIF, grow as compact 3-dimensional domed shaped colonies, similar to the cells of the ICM. In contrast, EPL cells grow in monolayer colony morphology with a distinct phenotype reminiscent of the primitive ectoderm epithelial layer *in vivo*. EPL cell morphology is reminiscent of the P19 embryonal carcinoma (EC) cell line (Figure 11.1 A; Robertson, 1986; Rathjen *et al.*, 1999), regarded as having equivalence to primitive ectoderm, as assessed by morphology, gene expression (Martin *et al.*, 1978; Robertson, 1986; Rogers *et al.*, 1991; van den Eijnden-van Raaij, 1991) and differentiation potential (Rossant and McBurney, 1982).

1.11.2 EPL cell gene expression

EPL cells differentiate spontaneously upon the removal of MEDII and LIF, to form a variety of differentiated cell types. This suggests that EPL cells are pluripotent and is consistent with the expression by EPL cells of the pluripotent cell markers alkaline phosphatase, SSEA-1 antigen, *Fgf4* and *Oct4* (Bettess, 1993; Rathjen *et al.*, 1999).

EPL cell formation has been assessed following the culture of ES cells in LIF supplemented (1000 U/ml) ES medium in the presence or absence of 50% MEDII. ES cells and EPL cells are cultured over an 8-day period with the passage and harvest of cells at 2-day intervals, with each consecutive 2-day harvest numbered consecutively until 8 days culture (p0-p3) (Rathjen *et al.*, 1999). Gene expression markers that distinguish ICM (*Rex1*, *Psc1* and *Psc2*) from primitive

ectoderm (*Fgf5* and *Psc3*) were used to identify the embryonic equivalents of ES cells and EPL cells. However, EPL cell formation *in vitro*, similar to primitive ectoderm formation *in vivo*, is a dynamic process where pluripotent cells adopt distinct pluripotent cell-subtypes, defined by gene expression, during the ICM to primitive ectoderm equivalent cell type.

Markers specific for trophectodermal, primitive endodermal or differentiated lineages were not expressed in ES or EPL cells. Furthermore, ES cells and EPL cells express *uvomorulin*, a gene not expressed in migratory germ cells (Rathjen *et al.*, 1999). The 3.5-4.5 dpc ICM expresses *Rex1* (Rogers *et al.*, 1991; Hosler *et al.*, 1993), *Psc1* and *Psc2* *in vivo* (Pelton *et al.*, 2000; Rodda *et al.*, 2001). These three marker genes are also expressed at high levels in ES cells. Culture of EPL cells for 2 days in the presence of LIF results in down-regulation of *Rex1* and *Psc2* and up-regulation of *Psc3* expression. This gene expression pattern is similar to the gene expression of pluripotent cells in the 4.75 dpc embryo *in vivo* (Pelton, 2000). EPL cells cultured for 4 days are most closely related to pluripotent cells of approximately 5.0 dpc as both pluripotent cell populations have down regulated *Rex1* and *Psc2*, express low levels of *Psc1* and high levels of *Psc3* and *Oct4* expression. EPL cells grown for 6 to 8 days are most closely related to primitive ectoderm cells at 5.25 dpc as both populations have down-regulated *Rex1*, *Psc1* and *Psc2* but express *Psc3* and *Fgf5*. All embryos in which pro-amniotic cavitation has commenced (around 5.25 dpc) fail to express *Psc1*, *Psc2* and *Rex1* but express high levels of *Fgf5* and *Psc3*. However, as primitive ectoderm elongation commences, *Psc3* is down regulated in the 5.5 dpc embryo. Therefore, gene expression analysis has revealed an alignment of ES cells with the ICM and EPL cells with the early primitive ectoderm, prior to 5.5 dpc (Table 1.2; Rathjen *et al.*, 1999; Pelton, 2000). Gene expression changes *in vivo* were closely correlated with the alterations in gene expression during the ES to EPL cell transition *in vitro* (Figure 1.12; section 1.4).

Interestingly, *Psc1*, *Psc2* and *Psc3* were originally identified by differential display PCR as genes specific for ES cell and EPL cell populations. *Psc1* and *Psc2* were isolated as ES cell markers while *Psc3* was defined as an EPL cell marker. Therefore, the expression of *Psc1* and *Psc2* in ICM/epiblast and *Psc3* in post-implantation primitive ectoderm prior to 5.5 dpc *in vivo* validates the formation of EPL cells from ES cells as a model for the progression of ICM to primitive ectoderm *in vivo*. (Rathjen *et al.*, 1999; Pelton, 2000).

Table 1.2 Gene expression patterns of ES cells and EPL cells compared to gene expression in the ICM and primitive ectoderm of the mouse embryo

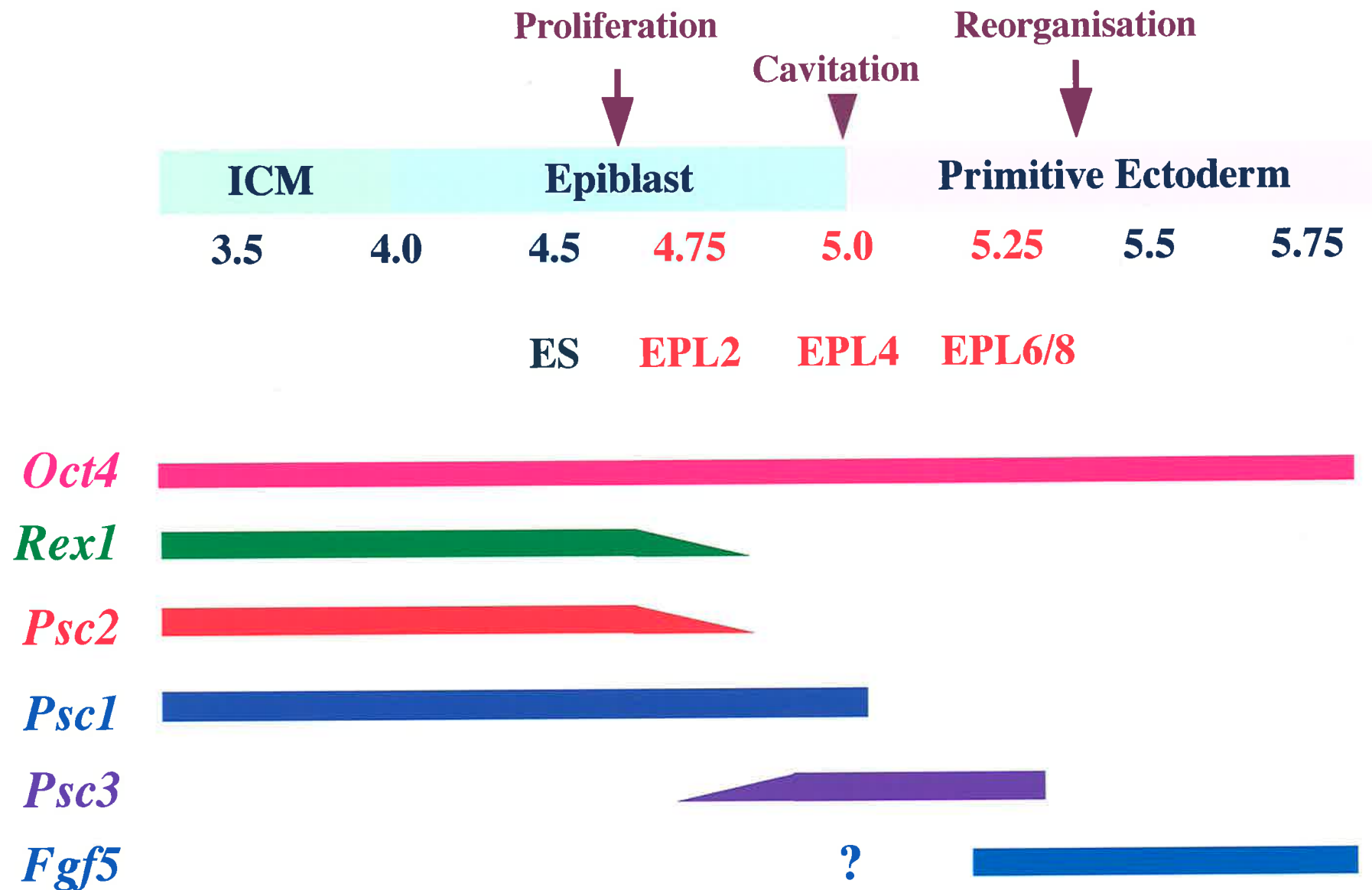
Diagnostic marker gene expression in pluripotent cell lines *in vivo* and *in vitro*. Markers differentially transcribed between pluripotent cell lineages have been highlighted. RNA was isolated from ES cells and EPL cells grown in the presence of MEDII for 2 to 8 days and analysed by northern blot for the expression of *Oct4*, *Uvomorulin*, *Fgf5*, *Rex1*, *Psc2*, *Psc1*, *Psc3*, *Alphafetoprotein*, *H19*, *Evx1* and *Brachyury*. *Gbx2* was detected by RNase protection. Alkaline phosphatase was detected by enzymatic activity. Embryonic expression patterns were determined by Rosner *et al.* 1990; Scholer *et al.*, 1990a; Yeom *et al.*, 1991 (*Oct4*); Hahnel *et al.*, 1990 (alkaline phosphatase); Sefton *et al.*, 1992 (*uvomorulin*); Haub and Goldfarb, 1991; Hebert *et al.*, 1991 (*Fgf5*); Rogers *et al.*, 1991 (*Rex1*); Bulfone *et al.*, 1993; Chapman *et al.*, 1997 (*Gbx2*); Pelton, 2000 (*Psc1*, *Psc2* and *Psc3*), Dziadek and Adamson, 1978 (AFP); Poirier *et al.*, 1991 (*H19*); Bastian and Gruss, 1990; Dush and Martin (1992) (*Evx1*) and Herrmann, 1991 (*Brachyury*).

Adapted from Rathjen *et al.* (1999)

	ES cells	ICM	EPL cells	Primitive ectoderm
<i>Oct4</i>	+	+	+	+
Alkaline phosphatase	+	+	+	+
<i>Uvomorulin</i>	+	+	+	+
<i>Fgf5</i>	-	-	+	+
<i>Psc3</i>	-	-	+	+
<i>Rex1</i>	High	+	Low	-
<i>Psc1</i>	+	+	-	-
<i>Psc2</i>	+	+	-	-
<i>Gbx2</i>	+	+	-	-
<i>AFP</i>	-	-	-	-
<i>H19</i>	-	-	-	-
<i>Brachyury</i>	-	-	-	-

Figure 1.12 The embryonic equivalent of ES and EPL cells

Schematic representation of temporal marker gene expression in the pluripotent cells of the early mouse embryo *in vivo* and ES and EPL cells *in vitro*.



1.11.3 EPL cell differentiation

Primitive ectoderm can be distinguished from cells of the ICM with respect to differentiation potential. Post-cavitation mouse pluripotent cells fail to contribute to chimeras when introduced into host blastocysts, suggesting that alterations in developmental potential may exist between pre- and post-pro-amniotic cavitated pluripotent cells (Rossant, 1977; Beddington, 1983). EPL cells re-introduced into a 4.5 dpc blastocyst also fail to contribute progeny cells to the embryo proper (Rathjen *et al.*, 1999). In this experiment, EPL cells were passaged twice in the absence of LIF prior to injection. Cells were cultured in the absence of LIF to prevent the re-introduction of residual ES cells to the blastocyst environment (Rathjen *et al.*, 1999).

In addition, the maintenance of the EPL cell cultures requires the continuous presence of MEDII. Removal of MEDII signals from EPL cell culture, in the continued presence of LIF, results in the reversion of these cells to an ES cell phenotype, gene expression and differentiation potential both *in vivo* and *in vitro* (Rathjen *et al.*, 1999). This event is further evidence that pluripotent cell types may be inter-converted via a common pluripotent precursor (Rossant, 1993).

EPL cells aggregate and differentiate as EB's when cultured in suspension in the absence of LIF. Morphological differences between ES and EPL cell EB's are evident within 4 days of aggregation. ES cell EB's comprise a homogeneous population of round and ordered aggregates with a visible outer layer of extra-embryonic endoderm, containing both visceral and parietal endoderm cells (Doetschman *et al.* 1985; Robertson, 1987; Lake *et al.*, 2000). EPL cell EB's appear disorganised with an apparent lack of exterior tissue layers (Lake *et al.*, 2000). Sections indicate that while ES cell derived EB's maintain a regular internal morphology of undifferentiated pluripotent cells (Martin *et al.*, 1977; Lake *et al.*, 2000), internal cells in EPL EB's were loosely packed and heterogeneous, possibly reflecting differentiation (Martin *et al.*, 1977; Lake *et al.*, 2000).

Differentiation of pluripotent cells can be monitored via alterations in gene expression both *in vivo* and during EB differentiation *in vitro* (Shen and Leder, 1992). The formation of late stage primitive ectoderm in ES cell and EPL cell derived EB's was assessed by northern blot analysis for expression of the *Fgf5*, *Oct4* and *Rex1* marker genes (1.11.2). High-level *Fgf5* and low-level *Rex1* expression are indicative of late stage primitive ectoderm formation. Late stage primitive ectoderm

was formed earlier in EPL cell derived EB's compared with ES cell EB's. In addition, the early onset of *Fgf5* expression and differentiation as detected by the loss of *Oct4* expression, indicated that pluripotent cell differentiation within EPL cell EB's was accelerated compared to ES cell EB's (Lake *et al.*, 2000).

Formation of early mesoderm in EB's was monitored by analysing expression of the *brachyury* gene which *in vivo* is expressed within the nascent mesoderm at the onset of gastrulation (Herrmann *et al.*, 1991; Keller *et al.*, 1993; Johansson and Wiles, 1995). *Brachyury* expression was observed 2 days post aggregation in EPL cell EB's, compared to the onset of expression 4 days post-aggregation in ES cell EB's (Lake *et al.*, 2000). The high level formation of mesodermal lineages in EPL cell derived EB's occurs in the absence of ectodermal lineages as determined by the absence of *Sox1* expression (Lake *et al.*, 2000). SOX1 is an HMG-box protein which is expressed in ectodermal cells committed to the neural fate at 7.5 dpc, and persists in cells destined to become neur ectoderm (Pevny *et al.*, 1998; Wood and Episkopou, 1999). Mesoderm enriched differentiation of EPL cells is demonstrated by the early onset and high level formation of beating cardiac muscle and haematopoietic cell types in EPL cell derived EB's when compared to ES cell derived EB's. Furthermore, mesoderm derived from EPL cell EB's can be directed towards a haematopoietic cell fate. Therefore, EPL cells differentiated as EB's readily give rise to mesoderm derived tissues that can be programmed to alternative cell fates (Lake *et al.*, 2000).

EPL cells are competent to form primitive endoderm, even though this is not the normal fate of primitive ectoderm *in vivo*. However, EPL cell derived EB's lack differentiated VE but form parietal endoderm. This is an interesting facet of EPL cell differentiation as VE and parietal endoderm are both derived from the primitive endoderm precursor (Hogan *et al.*, 1994). The absence of VE in EPL cell EB's suggest that either EPL cells are restricted in forming VE or that the EPL cell EB environment lacks a signal that allows VE formation. However, marked EPL cells mixed into ES cell EB's can contribute to VE (Lake *et al.*, 2000). Therefore, EPL cells retain the capacity to form VE but lack requisite inductive signals within an EPL cell EB environment (Lake *et al.*, 2000). This suggests that VE formation requires an inductive signal from adjacent pluripotent cells, consistent with the theories proposed by Gardner (1983) and Coucouvanis and Martin (1999).

Distinct from ES cell derived EB's, EPL cell EB differentiation is characterised by the early onset and high level formation of mesoderm at the expense of ectodermal lineages. The absence of VE and increased formation of mesoderm in EPL cell derived EB's suggests a relationship between pluripotent cell differentiation, VE derived signals and mesoderm formation. Therefore, signals derived from the VE are likely to inhibit mesoderm formation through the direction of pluripotent cell differentiation towards additional lineages. This is similar to mesoderm formation in the embryo where ectodermal cells fated for mesoderm pass through the primitive streak and lose contact with basement membrane to displace the surrounding VE, thus removing VE derived signals. In contrast, primitive ectoderm cells fated for ectoderm formation remain adjacent to VE in the anterior half of the embryo.

The lack of neural lineages again suggests a restriction in EPL cell developmental potential or a deficiency within the EPL cell EB environment. The chemical inducer RA has been used as a potent inducer of neural tissues in ES cell and P19 EC cell derived aggregates (Jones-Villeneuve *et al.*, 1982; Bain *et al.*, 1995). EPL cell aggregates were differentiated in the presence of RA and readily formed neural lineages (Lake *et al.*, 2000). This indicates that the absence of neural lineages formed during EPL cell derived EB's does not reflect a restriction in developmental capacity but indicates that an altered EB environment inhibits neuron formation, perhaps due to the absence of VE (Lake *et al.*, 2000). Consistent with this theory, EPL cells give rise to neurons when differentiated within ES cell EB's which contain VE (Rathjen *et al.*, 2001) and form exclusively neurons when differentiated in the presence of VE related signals derived from MEDII (Rathjen *et al.*, 2001; Rathjen *et al.*, *In manuscript*). These reports indicate that VE may secrete signals that induce the formation of ectodermal derived neural lineages (Lake *et al.*, 2000).

1.11.4 EPL cell cytokine responsiveness

1.11.4.1 FGF and TGF β growth factor families

Gastrulation is a highly complex and variant process between vertebrate species, yet the fundamental basics of gastrulation have remained conserved throughout evolution (Knoetgen *et al.*, 1999). This conservation is manifest in the observation that mouse, chicken and *Xenopus* embryos

have similar fate maps at the ectodermal stage, just prior to the initiation of gastrulation (Lawson *et al.* 1991, Quinlan *et al.*, 1995). During *Xenopus* embryogenesis, mesodermal lineages arise directly from the ectodermal tissues in response to specific signals from the underlying endoderm. *In vitro* functional assays have implicated members of the FGF and TGF- β families as inducers of ventral and dorsal mesoderm respectively (reviewed in Slack, 1994). The instability of primary primitive ectoderm culture prevents analysis of mammalian gastrulation using *in vivo* derived primitive ectoderm cells. However, while these signals have no effect on ES cell differentiation (Lake, 1996), the availability of EPL cells provides an *in vitro* assay to assess the responsiveness of mammalian primitive ectoderm to differentiation inductive signalling molecules identified in other model systems.

EPL cells cultured in the presence of exogenous mouse LIF and Activin A (50-150 ng/ml) or bFGf (10 ng/ml) growth factors differentiate to form distinct differentiated cell types. Basic FGF induced the homogeneous and directed formation of cell lineages with an appearance characteristic of fibroblasts. These colonies did not express the pluripotent cell marker alkaline phosphatase. Furthermore, the addition of other FGF family members (0.1-100 ng/ml), FGF4 and acidic FGF (but not *FGF5* (0.1-500 ng/ml)) resulted in the formation of morphologically indistinguishable fibroblasts. EPL cells cultured in the presence of exogenous mouse LIF and Activin A (50-150 ng/ml) also differentiate to form a fibroblastic differentiated cell type that is morphologically distinct from the cells induced by FGF activity. ^(Lake, 1996) The distinct morphology of fibroblastic cell lineages, induced by the activity of FGF and Activin A in EPL cell culture, may reflect the capacity of these growth factors to induce alternate mesoderm fates in *Xenopus* explant assays (reviewed in Slack 1990; Ruiz i Albata and Melton, 1990; Cooke, 1991; Amaya *et al.*, 1991, Hemmati-Brinvalou and Melton, 1992; Sive, 1993).

1.11.4.2 The LIF cytokine

The ES cell to EPL transition occurs in the presence or absence of exogenous mouse LIF. However, the addition of exogenous LIF retards the gene expression changes observed during EPL cell formation *in vitro* (Rathjen *et al.*, 1999; Lake *et al.*, 2000). This is similar to LIF inhibition of primitive ectoderm formation during EB differentiation *in vitro* (Shen and Leder., 1992) and the

inhibition of primitive ectoderm formation or differentiation in the mouse embryos constitutively expressing LIF (Conquet *et al.*, 1992).

Collectively, these reports suggest that the EPL cell inductive signals in MEDII induce ES cells to adopt the morphology, gene expression, differentiation potential and cytokine responsiveness, that mimics the biology of primitive ectoderm formation and maintenance *in vivo*.

1.12 Factors involved in EPL cell induction and maintenance

1.12.1 At least two separable biological signals induce EPL cell formation

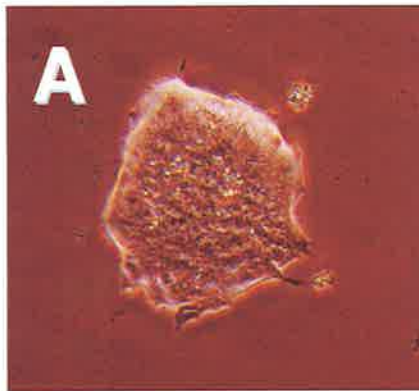
MEDII is derived from HepG2 cells in the presence or absence of serum. Separation of MEDII over a size exclusion filter (3-10 kd), reveals a requirement for at least 2 signals for EPL cell formation, consisting of a high molecular weight (>10kd) and low molecular weight (<3kd) components. The induction and maintenance of EPL cells occurs only in the presence of both signals and is scored by the distinct morphology of EPL cells when compared to ES cells (Figure 1.13; Rathjen *et al.*, 1999).

MEDII medium contains approximately 10 U/ml of human LIF (Rathjen *et al.*, 1999). This suggests that human LIF may also be involved in EPL cell induction and/or maintenance. The culture of EPL cells in the absence of exogenous mouse LIF and the presence of an anti-human LIF neutralising antibody results in EPL cell formation with increased spontaneous background differentiation compared to EPL cells cultured in MEDII alone (Rathjen *et al.*, 1999). EPL cell formation with low level background differentiation was restored by the addition of exogenous mouse LIF (Rathjen *et al.*, 1999). Mouse LIF does not cross react with the anti-human LIF antibody suggesting LIF activity is sufficient to inhibit the spontaneous differentiation of EPL cells. These data suggest that LIF has a role in the maintenance of EPL cells *in vitro*, but is not required for the induction of EPL cells. Therefore, the formation of EPL cells in the absence of LIF suggests that additional, unknown factors secreted by HepG2 cells induce the formation of EPL cells. These factors are the first described biologically derived signals to alter the developmental potential of ES cells in a homogeneous fashion.

Figure 1.13 Two separable bioactive signals induce EPL cell formation

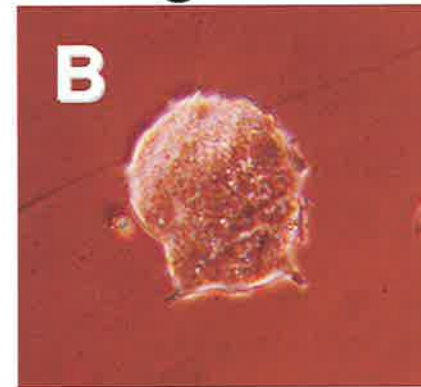
(A) ES cells cultured in 40 μ M L-proline (B) ES cells cultured in 50 μ g/ml of retentate protein fraction. (C) ES cells cultured in both 40 μ M L-proline and 50 μ g/ml of protein fraction.

Low Mw

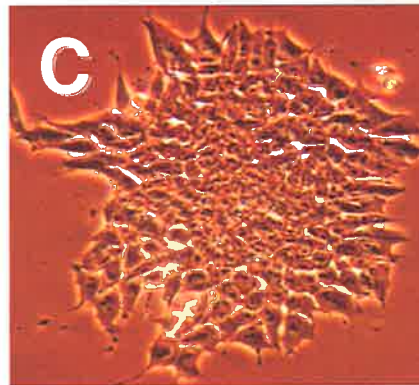
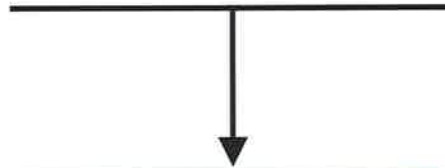


ES cells

High Mw



ES cells



EPL cells

1.12.2 Purification of the low molecular weight signal

Jennifer Washington purified the low molecular weight EPL cell inductive activity. 250 ml of the low molecular weight component, obtained from ultra-filtration of serum free MEDII, was applied to a Sephadex G10 gel filtration column pre-equilibrated in water. The active fractions from gel filtration were pooled, lyophilised, resuspended in 30:70 methanol:acetonitrile and applied to a normal phase column. (Waters Radial Pak LC 10 μ m cartridge (8mm I.D.)) Active fractions were lyophilised, resuspended in water and subjected to gel filtration on a Superdex Peptide PC 3.2/30 gel filtration column. Activity was detected in fractions eluting with a predicted molecular weight of <700D. The active peak was analysed via amino acid analysis and was shown to contain high levels of the imino acid L-proline and amino acid L-alanine when compared to non conditioned serum free media subjected to identical purification protocols. Purified samples of L-proline and L-alanine were assayed for EPL cell formation, in the presence of the high molecular weight fraction. These experiments determined that L-proline, but not L-alanine, had biological activity equivalent to the low molecular weight signal active in EPL cell induction in MEDII (Washington, unpublished data; Table 1.3). The minimum EPL cell inductive concentration of L-proline is 40 μ M in the presence of the high molecular weight signal as assessed by the induction of EPL cell morphology.

1.13 Biological activity and potential sources of L-proline during primitive ectoderm and EPL cell formation

The specificity of L-proline signalling during EPL cell formation can be demonstrated by the inability of other amino acids to induce ES cell differentiation (Table 1.3). However, the biochemical mode of action for L-proline remains unknown. It is known however, that a number of short L-proline containing peptides also induce morphological EPL cell formation at concentrations greater than or equal to free L-proline. Similarly, peptides that lack L-proline including peptides with known biological activity such as Arg-Gly-Asparagine (Buckley, 1999), were unable to induce the EPL cell morphology indicating a specific requirement for the L-proline molecule.

Free L-proline could also be ^{active} in EPL cell formation as a metabolite. A number of L-proline analogues have been assayed for morphological EPL cell formation from ES cells (Washington,

Table 1.3 Summary of the EPL cell inductive capacity of amino acids and peptides in the presence of the high molecular weight factor

The minimal concentration of an amino acid or peptide that caused morphological EPL cell formation in the presence of the high molecular weight fraction, is represented in bold brackets ie. (**40 μ M**). The concentration range of each signal examined for EPL cell formation is also shown. The peptide Arg-pro-lys-pro is the C-terminal fragment of the neuropeptide Substance P.

Data from Jennifer Washington

	(EPL)	concentration	
AMINO ACIDS			
L-proline	(40μM)	20-1000 μ M	
D-proline		30-3475	
L-alanine		390-3900	
L-lysine		55-5500	
PEPTIDES			
Pro-ala	(250)	20-1000	
Pro-gly	(250)	20-1000	
Ala-pro	(80)	20-1000	
Gly-pro	(40)	20-1000	
Ala-pro-gly	(40)	40-1000	
Gly-pro-ala	(40)	20-1000	
Gly-pro-OH-pro	(300)	40-5850	
Arg-pro-lys-pro	(40)	40-1000	(Substance P frag. 1-4)
Arg-gly-asp (RGD)		40-4800	

unpublished data). However, these analogues had either no observable effect on ES cell growth or differentiation, or inhibited ES cell growth at low concentrations and caused cell death at higher concentrations. This indicates that L-proline is not active as a metabolite in EPL cell formation.

Glycine-proline (Gly-Pro) and Alanine-Proline (Ala-Pro) are di-peptides that induce EPL cell formation at 40 μ M or 80 μ M respectively. Pro-Gly and Pro-Ala di-peptides also induce EPL cell formation but at a higher threshold concentration of 250 μ M. The activity of these signals may be due to peptide based signalling or the release of free L-proline by peptidase activity. The differing minimal active concentrations of these L-proline containing signals suggest either a structural requirement for peptide activity or the hydrolysis of di-peptide bonds. The relative concentrations required would suggest preferential hydrolysis of pre-proline di-peptides. However, Gly-Pro-Ala and Ala-Pro-Gly peptides also induce morphological EPL cell formation. The preferential hydrolysis of X-Pro bonds should release free Pro-Gly or Pro-Ala peptides for EPL cell formation at a concentration of 250 μ M. However, Gly-Pro-Ala and Ala-Gly-Pro peptides induce EPL cell formation at a minimal concentration equivalent to free L-proline (40 μ M). Given that the release of L-proline from these peptides would require hydrolysis of both Pro-X and X-pro bonds. These data suggest that L-proline is active in EPL cell formation from within these peptides, not due to the breakdown and release of free L-proline. The EPL cell inductive activity of these peptides is summarised in Table 1.3.

The distinct specific activities of EPL cell inductive peptides suggest a structural requirement for their action, suggesting L-proline could be acting through a receptor based signalling mechanism. The C-terminal 4 amino acids of the signalling peptide substance-P are also active in EPL cell formation at a minimal concentration equivalent to L-proline (Table 1.3). There is evidence within the literature to suggest this short peptide is active in receptor binding in other biological systems (Kato *et al.*, 1978; Repke *et al.*, 1987; Assem *et al.*, 1989) which further supports a receptor-based mechanisms for L-proline/peptide function during EPL cell formation.

The activation of receptor-based signalling by amino acid signals is not novel. Many modified amino acids have previously been shown to activate specific trans-membrane receptors (Danysz and Parsons, 1998, Dingledine *et al.*, 1999) and the amino acid glutamate and is a well-characterised neurotransmitter (reviewed in Bardoul *et al.*, 1998; Drescher, 2000).

A potential source of proline rich peptides *in vivo* is the basement membrane ECM that separates extra-embryonic endoderm and pluripotent cells and contains large amounts of collagen IV (Adamson and Ayers, 1979; Ashmarin *et al.*, 1998). During development, the basement membrane is continually re-modelled as ECM components are degraded and replaced within the ECM (Bernfield *et al.*, 1984; Furness, 1997). Collagen contains many tri-peptide repeats of Gly-X-Y, in which X is often Pro and Y is often hydroxyproline (OH-Pro), which provides collagen with its type II coiled structure and allows formation of the collagen triple helix (Haralson and Hassell, 1995). Protease specific degradation of collagens during development may yield small L-proline containing peptides. Gly-Pro-Y peptides have been shown to be active in EPL cell formation at a concentration equivalent to the minimal concentration of L-proline while the Gly-Pro-OH-Pro peptide induces morphological EPL cell formation at 300 μ M in the presence of the high molecular weight signal (Table 1.3). However, the cellular source of L-proline, or L-proline equivalent signals/active peptides, in early mouse development remains unknown.

1.14 Aims and Approach

The aims of this thesis were the purification and identification of the high molecular weight EPL cell inductive factor(s) from MEDII conditioned media. (1) The active component of the MEDII conditioned medium will be isolated and identified through traditional purification techniques. The morphological differences between ES cells and EPL cells was used as a rapid assay for the biological activity, the high molecular weight EPL cell inductive signal. Purification was preferred to expression cloning of HepG2 cell derived cDNA as the high molecular weight component may be derived from a large mRNA that would be difficult to isolate in a functional form as a cDNA. (2) To confirm correct purification of the EPL cell inductive signals, EPL cells formed in the presence of purified factors were investigated by techniques such as gene expression and differentiation potential. (3) Assessment of the biological source of these signals was investigated by screening both expression and/or secretion *in vitro* in candidate cell lines and *in vivo* in mouse embryos.

1.15 The significance of EPL cell formation: EPL cells as an *in vitro* model for early pluripotent cell development

The report of a conditioned medium, MEDII, which induces ES cells to form primitive ectoderm *in vitro*, is the first example of biologically relevant factors directing the differentiation of pluripotent cells in culture. Identification of EPL cells as the *in vivo* equivalent of primitive ectoderm, and the knowledge that these factors replicate an *in vitro* developmental event *in vitro*, provides an ideal opportunity from which to identify the signals that control the initial pluripotent cell differentiation event *in vivo* and *in vitro*.

Under ideal conditions pluripotent EPL cells may be directed in their differentiation decisions through culture in the presence of appropriate signals (Lake *et al.*, 2000; Rathjen *et al.*, 2001). Primitive ectoderm is the pluripotent cell substrate for mammalian gastrulation and, therefore EPL cell differentiation can be used as an *in vitro* model of gastrulation. This model system can then be exploited for identification of additional signals that induce lineage specific pluripotent cell differentiation *in vitro*, which is not currently possible using ES cells.

Primitive ectoderm and EPL cells are obligatory intermediates in ICM and ES cell differentiation *in vivo* and *in vitro* respectively. The response of EPL cells, but not ES cells, to differentiation inductive signals establishes EPL cell formation as an essential requirement for the further differentiation of pluripotent cells *in vitro*. Therefore, the use of known factors to induce EPL cell formation prior to further differentiation will benefit further analysis by provision of controlled and reproducible conditions for EPL cell culture in the absence of contaminating, differentiation inductive signals.

The recent isolation of human ES cells suggests that pluripotent cell biology can be applied to human medicine (Shamblott *et al.*, 1998; Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Pera *et al.*, 2000). Applications of this technology involve the isolation and genetic manipulation of human pluripotent cells, followed by lineage specific differentiation into the precursor of, or into, any somatic cell lineage appropriate for transplantation. In combination with gene modification technologies, any number and any kind of cell can be used as novel therapies for disease (Rathjen *et al.*, 1998; Wilmut *et al.*, 1998; Fuchs and Segre, 2000). However, human ES cells are not equivalent to mouse derived pluripotent cells.

Human ES cells proliferate slowly, are unstable in culture and have distinct feeder dependent cell culture conditions. The slow rate of self-renewal and instability of human ES cells prevents their use as a therapeutic tool. ^(Pera et al., 2000) Therefore, understanding mouse pluripotent cell biology *in vivo* and *in vitro* may lead to the establishment of cell culture conditions, that improve human ES cells proliferation in the absence of spontaneous differentiation, and allow the lineage specific differentiation of pluripotent cells *in vitro* for therapeutic application.

Chapter 2

Materials and Methods

Chapter 2

2.1 Abbreviations

Abbreviations are as described in "Instructions to authors": In addition,

A ₂₆₀	Absorbance at 260 nm
AP	Alkaline phosphatase
APS	Ammonium persulphate
ATP	Adenosine triphosphate
β-ME	β-Mercaptoethanol
BCIG	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
bp	Base pairs
BSA	Bovine serum albumin (Fraction V)
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
CM	Conditioned medium
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
FN	Fibronectin
cFN	Cellular Fibronectin
pFN	Plasma Fibronectin
EDTA	Ethylenediaminetetracetic acid
EtBr	Ethidium Bromide
DIG	Digoxigenin
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethyl formamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra acetic acid
EPL	Early primitive ectoderm-like
ES	Embryonic stem
EtOH	Ethanol (100%)
FCS	Fetal Calf Serum

FPLC	Fast phase liquid chromatography
g	Gram
GLB	Gel loading buffer
HBSS	Hanks buffered salt solution
HRP	Horse radish peroxidase
ITSS	Insulin-transferrin-sodium-selenite
kb	Kilobase pair
kd	Kilo dalton
L	Litre
M	Moles per litre
mM	Millimoles per litre
μ M	Micromoles per litre
μ FD	Micro farads
μ g	Microgram
μ m	Micron
μ l	Microliter
mAmps	Milliamperes
min	Minute
mg	Milligram
ml	Milliliter
mLIF	Mouse Leukaemia inhibitory factor
MOPS	3-{N-Morpholino} propane-sulfonic acid
MQ	Milli-Q
mRNA	Messenger RNA
MW	Molecular weight
NBT	4-nitroblue tetrazolium chloride
NP40	Nonidet P-40
OD ₆₀₀	Optical density at 600nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PMSF	Phenyl Methyl Sulfonyl Fluoride
RNA	Ribonucleic acid
RNAsin	Ribonuclease inhibitor
RNTP	Ribonucleotide phosphate
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate

TAE	Tris acetate EDTA
TFA	Trifluoroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethenediamine
tRNA	Transfer RNA
Tween-20	Polyoxyethylene-sorbitan Monolaurate
U	Units
UV	Ultraviolet
V	Volts

2.2 Tissue Culture

2.2.1 Materials

β-ME	Sigma Chemical Co.
cFN	Dr Jean-Paul Levesque, 2.5.5
Collagenase	Gibco BRL
ES DMEM	Gibco BRL
DMEM	Gibco BRL
DMEM, Phenol Red Free	Gibco BRL
Williams E	Gibco BRL
DMSO	Sigma Chemical Co.
F12 medium	Gibco BRL
FCS	Commonwealth Serum Laboratories
Gelatin	Sigma Chemical Co.
Gentamycin	Pharmacia and Upjohn
Hepes Buffer Powder	Gibco BRL
ITSS	Boheringer Manneheim
L-glutamine	Gibco BRL
Laminin (mouse)	Sigma Chemical Co.
Methanol	BDH
pFN (human)	Gibco BRL
PMSF	Sigma Chemical Co.
Sodium pyruvate Solution (100 mM)	Sigma Chemical Co.
Sodium Azide	Sigma Chemical Co.
Trypsin	Gibco BRL
Vitronectin (bovine)	Dr Zee Upton

2.2.2 Tissue culture plastic ware

6 well trays	Falcon
24 well trays	Falcon
96 well trays	Falcon
100 mm plates	Falcon
60 mm plates	Falcon
150 cm ² flasks	Falcon
75 cm ² flasks	Falcon
1700 cm ² plastic roller bottles	Corning
Pipettes (25 ml/10 ml/2 ml)	Falcon

2.2.3 Cell staining kits

Alkaline phosphatase staining kit (86-R)

Sigma Chemical Co.

2.2.4 Buffers

Borate: pH 9.0, 0.25 M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (50 ml), 1.0 M HCl (4.6 ml), MQ H_2O (44.4 ml)

Tris-HCl: pH 8.0, 0.1 M Tris-HCl (12.14 g in 1L MQ), adjusted with 1 M NaOH

PBS: pH 7.5, NaCl (8 g), KCl (0.2 g), KH_2PO_4 (0.2 g), Na_2HPO_4 (1.15 g) in 100 ml MQ H_2O

Citrate: pH 6.0, 0.1 M $\text{C}_6\text{H}_8\text{O}_7$ (36.85 ml), Na_2HPO_4 0.2 M (63.15 ml)

Acetic Acid: pH 5.0, 0.2 M NaOAc (70 ml) and 0.2 M HOAc (30 ml)

Citric Acid: pH 4.0, 0.1 M $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, 0.1 M (61.45 ml), Na_2HPO_4 0.2 M (38.55)

2.2.5 Solutions

L-glutamine: 100 mM L-glutamine in MQ H_2O , filter sterilised and stored as 10 ml aliquots at -20°C

ITSS: 1000x stock of 250 mg ITTS in 25 ml sterile MQ H_2O stored as 1 ml aliquots at -20°C

Trypsin-EDTA: Trypsin 1:250 (Difco) (1g), 10 ml versene solution (10x concentrate) to 100 ml in MQ H_2O .

PBS/ β -ME: 100 mM β -ME in sterile PBS. Fresh solution prepared fortnightly.

PBS/Gelatin: 0.2% (w/v) gelatin in PBS. Sterilised by autoclaving (20psi for 25 minutes at 140°C).

ITTS: Boehringer Mannheim.

Trypan blue: 0.4 g Trypan blue, 0.81 g NaCl, 0.06 g K_2HPO_4 in 100 ml MQ, pH 7.4 filter sterilised.

Versene solution(10x): 2 g EDTA, 80 g NaCl, 2 g K_2HPO_4 , 11.5 g Na_2HPO_4 in 1L MQ H_2O , filter sterilised and stored at 40°C .

HBSS: 0.8 g KCl, 0.12 g KH_2PO_4 , 16 g NaCl, 0.1 g Na_2HPO_4 , 2 g glucose, 4 ml 1% phenol red, .035 g NaHCO_3 in 2 litres

Sassa: 6.95 g NH_4Cl , 2.058 g Tris-HCl, 1 g KHCO_3 /litre, pH 8.5.

2.2.6 Cell culture medium

DMEM: (DMEM, Gibco BRL), pH 7.4, containing high glucose, supplemented with 10% FCS.

ES complete media: (DMEM, Gibco BRL), pH 7.4, containing high glucose, supplemented with 10% FCS, 40 mg/ml Gentamycin, 1 mM L-glutamine, 0.1 mM β -Mercaptoethanol (β -ME), and 1000 U of mLIF.

ES incomplete media: ES DMEM (Gibco BRL) in the absence of mLIF.

DMEM, Phenol red free: Phenol red free DMEM (Gibco BRL) pH 7.4, containing high glucose supplemented with 10% FCS, 40 mg/ml Gentamycin, 1 mM L-glutamine, 0.1 mM β -ME

Serum Free medium: Phenol Red Free DMEM (Gibco BRL) pH 7.4, containing high glucose supplemented with 40 mg/ml Gentamycin, 1 mM L-glutamine and 1 ml of ITTS.

2.2.7 Cell lines

All cells were maintained in tissue culture incubators at 37°C. ES cells in 10% CO₂, all other cell lines in 5% CO₂.

D3 ES: Derived from the ICM of the pre-implantation 129 strain mouse embryo blastocyst (Doetschman *et al.*, 1985; Kindly donated by Dr Lindsay Williams, Ludwig Institute, Melbourne).

HepG2: Human hepatocellular carcinoma (Knowles *et al.*, 1980; ATCC HB-8065).

Hep3b: Human hepatocellular carcinoma (Knowles *et al.*, 1980; ATCC HB-8064)

Hepa 1C1C7: Mouse hepatocellular carcinoma (Bernhard *et al.*, 1973; ATCC CRL-2026)

END2: Derived from retinoic acid differentiated P19 EC cell aggregates (Mummery *et al.*, 1985, 1991)

PYS2: Derived from ~~retinoic acid differentiated P19 EC cell aggregates~~ ^{mouse teratocarcinoma} (Lehman *et al.*, 1974; Mummery *et al.*, 1985, 1991)

COS1: Monkey Kidney cells (ATCC CRL-1650)

2.2.8 Miscellaneous

Ektachrome 100 slide film

Kodak

Freezing vials

Nunclon

Sterile bottles

Schott

PD10 gel filtration columns

Pharmacia

0.22 µm sterile bottle top filters

Corning.

0.22 µm sterile filters (Syringe based)

Millipore

0.22 µm Sartolab P-20 bell top sterile filters

Sartorius

Bacteriological Petrie dishes

Techno-plas

Microscopy

Nikon ELW D0.3 phase contrast

Peristaltic pump 7518-00

Cole Palmer instruments

Laminar Flow hood

Gelman Sciences

2.3 Tissue culture Methods

2.3.1 Gelatinised Tissue culture plates

All tissue culture dishes and plates used for ES and EPL cells were gelatinised with 0.2% (w/v) gelatin in PBS. Plates were covered with gelatin solution and left for at least 30 minutes at room temperature. The gelatin solution was removed and the plate washed in PBS immediately before use.

2.3.2 Determination of cell number

Cells were harvested as described (2.3.5) A 100 μ l aliquot of the single cell suspension was diluted in 900 μ l of trypan blue. Cell numbers were counted under 200 $\times$ phase contrast magnification using a haemocytometer. Trypan blue stained dead cells were omitted from the cell count.

2.3.3 Preparation of cell lines for storage

Cells were harvested to a single cell suspension as described in (2.3.5) with the following alterations. Cells were resuspended in 90% FCS and 10% DMSO. In general 1 confluent plate of ES cells was resuspended in 3 ml of FCS/DMSO and 1 ml aliquoted for freezing. Cells were placed in freezing racks and put at -80°C for 24 hours. Subsequently one vial was thawed to the efficiency of freezing. The frozen cells were then transferred to liquid nitrogen for long term storage.

2.3.4 Thawing stored cell lines

A vial of cells was removed from liquid nitrogen and placed in a 37°C water bath. As soon as the cells had thawed they were diluted in 10 ml of fresh culture medium and spun at 12,000 rpm for 5 minutes. Cells were then resuspended in the appropriate medium and placed into two to three gelatinised 100 mm dishes (ES cells) and a single 75 cm² flask (additional cell lines). Thawed cells were placed at 37°C, 10% CO₂ in a humidified incubator. The following day, medium is replaced with fresh medium.

2.3.5 Maintenance of ES and EPL cell lines

D3 ES cells were cultured, in the absence of feeders, on tissue culture grade plastic (Falcon) pre-treated with 0.2% gelatin/PBS. Semi-confluent ES cells plates were washed twice in PBS before the addition of trypsin (1 ml) at 37°C for 30 seconds. ES cells were removed from the dish and forced into a single cell suspension with pipetting and transferred into 4 ml complete ES cell medium. The cells were spun at 1200 rpm for 2 minutes, gently resuspended in 10 ml of ES complete medium and re-seeded at a cell density ranging from approximately 1.5x10³-3x10³ cell/cm² and maintained at 37°C in a humidified incubator at 37°C, 10% CO₂ as described in Smith (1991). ES cells were passaged every 3-4 days and maintained for a maximum of 30 passages before thawing of a replacement ES cells. ES cells were maintained in ES complete medium.

EPL cells were induced and maintained by the addition of ES cells (2×10^4 cells/cm²) to ES complete medium containing 50% MEDII or 50% ES sfMEDII supplemented with 10% FCS, 1 mM L-glutamine and 0.1 mM β -ME. Routine tissue culture was performed as described above for ES cells and in Smith (1991). EPL formation was apparent with the addition of between 10% and 80% MEDII/sfMEDII. Optimal culture conditions were observed at 50% (Rathjen *et al.*, 1999).

2.3.6 Maintenance of additional cell lines

END2 and PYS2 cell lines were maintained in 150 cm² flasks in DMEM phenol red free medium. The S2 EC cell line was maintained on γ -irradiated, mitotically inactivated, SLN feeder layers (CIS biointernational gamma radiation apparatus 18L 437C: 30 grays) cultured in DMEM phenol red free medium. Primary hepatic and hepatocellular carcinoma cell lines were maintained in incomplete ES DMEM lacking 0.1 mM β -ME. All cell lines were maintained in a humidified incubator at 37°C, 5% CO₂.

Cells were grown to confluence in 100 mm tissue culture trays or 150-75 cm² Flasks. Confluent cells were washed twice with PBS before the addition of 3 ml of trypsin to remove cells from the flask by knocking vigorously. Cells were spun down and resuspended in 10 mls of culture medium before transfer to a fresh tissue culture plate (gelatinised in 0.2% PBS/Gelatin) or flask. Cells were plated at dilutions from 1:10-1:80 of the 10 ml cell suspension. ES cells were supplied with fresh ES complete medium two days after passage and split every third day. PYS2, END2, S2 EC and hepatoma cell lines were passaged every fourth day. S2 EC cells were supplied with fresh culture medium daily.

2.3.7 Primary cultures

Primary liver cells were prepared using procedures based on those described by Giger and Myer (1981), with minor variations. Mice were sacrificed and immediately dissected to expose the liver. Each liver was perfused through the portal vein with 10 ml PBS, followed by 10 ml 0.05% collagenase in HBSS. The perfused liver was removed, macerated in 10 ml/liver of 0.05% collagenase/HBSS and incubated at 37°C for 30 minutes. Liver suspensions were regularly agitated by pipetting to aid liver disaggregation. Cells were washed twice in HBSS (10 ml) to remove collagenase, and contaminating erythrocytes were lysed in Sassa solution. Following two washes in William's E medium (1 ml/liver, Gibco BRL) the cells were seeded into gelatin treated tissue culture flasks in ES incomplete medium, lacking β -ME, and incubated at 37°C, 5% CO₂.

Primary avian hepatocytes were prepared from 17-18 day chick embryos. In brief, chick embryos were removed from the egg, decapitated and dissected to expose the heart and liver. The liver was perfused, via cannulation of the heart, with 10 ml of 0.9% NaCl containing 2 mM EDTA to remove blood cells, followed by 4 ml of 0.05% collagenase in HBSS. Livers were removed, pooled

in HBSS and transferred into fresh 0.05% collagenase/HBSS when all livers had been perfused and collected. Pooled livers were macerated with fine tipped scissors and incubated for 30 minutes with gentle shaking every 5 minutes and gentle pipetting using a 10 ml pipette every 10 minutes to aid cell disaggregation. Collagenase was removed by washing with HBSS (2 ml/liver). Contaminating erythrocytes were lysed in Sassa solution. Following two washes with Williams E (2 ml/liver) to remove cell debris and haemoglobin from lysed erythrocytes, the hepatocytes were resuspended in Williams E medium (1 ml/liver) and the yield determined. Typically 2×10^7 cells/liver were obtained and plated out in a 175 cm² tissue culture flask and cultured in 20 ml Williams E medium overnight before transfer to ES incomplete medium lacking β -ME at 37°C/5% CO₂.

2.3.8 Preparation of conditioned media

MEDII medium was obtained by seeding HepG2 cells (1×10^5 cells/ml) into incomplete ES DMEM lacking 0.1 mM β -ME. Conditioned medium was collected after 4 days, supplemented with 0.1 mM β -ME, and sterilised by filtration through a 0.22 μ m membrane. MEDII was stored at 4°C for 1-2 weeks or at -20°C for up to 6 months without apparent loss of EPL cell inductive activity.

Serum free MEDII was produced by seeding 2 confluent flasks of HepG2 cells into corrugated roller bottles containing 200 ml incomplete ES DMEM lacking 0.1 mM β -ME. Cells were seeded overnight at 37°C without rolling. Cells were washed twice in PBS and transferred into DMEM (pH7.4) containing high glucose but without phenol red, supplemented with 1 mM L-glutamine, 1 ml ITSS supplement (Boehringer Mannheim), and 110 mg/L sodium pyruvate. Serum free culture medium was conditioned for 4 days with rolling at 37°C before collection. Cell debris was removed by filtration through whatman paper and final sterilisation by filtration through a 0.22 μ m Sartolab P-20 bell top sterile filters. Serum free MEDII was stored as for MEDII. Conditioned media derived from primary hepatic cells and hepatic cell lines was collected after 4 days culture in ES incomplete medium, lacking β -ME, at 37°C in a 5% CO₂ humidified incubator. Conditioned media was supplemented with 1 mM L-glutamine and 0.1 mM β -Mercaptoethanol (β -ME) prior to filtration, sterilisation and use in ES/EPL cell assays.

2.3.9 Preparation of concentrated sfMEDII proteins (R)

Serum free MEDII (500 ml) was subjected to ultra-filtration using the Diaflo YM3 membrane and apparatus (Amicon) at 4°C under nitrogen pressure. The retained high molecular weight fraction was buffer exchanged to PBS using a PD10 gel filtration column to the manufacturers instructions, removing any remaining low molecular weight signal.

2.3.10 EPL cell induction assays for assessment of the high molecular weight activity

The activity of the high molecular weight EPL cell inducing signal was assessed by seeding 500 D3 ES cells into a 0.2% gelatin coated tissue culture wells (24 well tray) containing 1.0 ml ES DMEM, 1000 U mLIF, 40 μ M L-proline. R, R subjected to biochemical treatment, partially purified or purified (2.5.5) cellular fibronectin (FN) (10 μ g/ml) were assessed for EPL cell inductive capacity by supplementation into the culture medium. The cells were seeded for the morphological formation of EPL cells assessed by phase contrast microscopy throughout the culture period. After 5 days, cells were stained for alkaline phosphatase and visualised using the diagnostic kit 86-R (Sigma Chemical Co.).

2.3.11 Alkaline phosphatase staining

The alkaline phosphatase staining kit (86-R) was used according to the manufacturers specifications. Cells were fixed in an ethanol (13 ml), citrate solution (5 ml) and 37% formaldehyde (1.6 ml) solution for 5 minutes at room temperature. A diazonium salt solution was prepared by adding 0.2 ml sodium nitrate solution to 0.2 ml FRV-alkaline phosphatase solution. The solution was mixed gently by inversion and kept for 2 minutes in the dark at room temperature before the addition of 9 ml of MQ H₂O. Naphthol AS-BI Alkaline solution (0.2 ml) was added to the diluted diazonium salt solution. Fixed cells were rinsed gently with deionised water and incubated with the alkaline-dye solution for 15-30 minutes in the dark at room temperature. Cells were rinsed again with deionised water and placed at 37°C to dry.

2.3.12 Characterisation of the High molecular weight EPL cell inducing activity in MEDII

A single ml of R (1 mg) was subjected to a range of buffered pH solutions (Borate pH9.0, Tris-HCl pH8.5, PBS pH7.5, Citric acid pH6.0, NaAc pH 5.0 and Citrate pH4.0) by mixing R with 1 ml of buffers for a final concentration of 50 mM and incubate at room temperature for 1 hour. R (2 ml) was also exposed to variant temperatures (-20°C, 4°C, 56°C or 96°C), reducing agents β -ME and DTT (1 ml; 0.1 mM, 10 mM, 50 mM), 8 M Urea or 100 U of trypsin in 20 mM Tris-HCl pH 8.5, 20 mM CaCl₂ for 1 hour. Trypsin was inactivated post digestion with 100 μ M PMSF (Sigma Chemical Co.). All samples were subjected to buffer exchange into PBS using centricon 10 concentrating columns (Amicon). Samples were assessed for morphological EPL cell inducing activity (2.3.10, 2.3.11).

2.3.13 EPL cell induction by ECM components supplemented into ES DMEM

The EPL cell inductive activity of various ECM components was assessed by supplementation of soluble factors into the culture medium or the pre coating of tissue culture plastic

in $1 \mu\text{g}/\text{cm}^2$. ES cells were seeded for the morphological formation of EPL cells as described previously (2.3.10, 2.3.11). Pluripotent cell morphology assessed by phase contrast microscopy throughout the culture period. After 5 days, cells were stained for alkaline phosphatase and visualised using the diagnostic kit 86-R (Sigma Chemical Co.).

2.3.14 ECM cell coating assays

ECM components were attached to the surface of tissue culture plastic ($1 \mu\text{g}/\text{cm}^2$) from PBS and air-dried in a laminar flow hood. HepG2 cell derived ECM was isolated by the culture of HepG2 cells to confluence in 100 mm tissue culture plates pre-coated in 0.2% gelatin. Cells were scarified by culture in 0.02% (w/v) sodium azide, $100 \mu\text{M}$ PMSF in PBS for 6-18 hours and removed by pipetting to leave and intact ECM. ES cells were seeded (1×10^5 cells/ml) onto the HepG2 ECM in the presence or absence of $40 \mu\text{M}$ L-proline and assessed for EPL cell formation by morphology using a Nikon ELW D0.3 phase contrast microscope, 100x magnification.

2.3.15 Standard culture conditions for the assessment of EPL cell formation by morphology and gene expression

ES cells (1×10^5 cells/ml) were seeded into 100 mm gelatin coated (0.2%) tissue culture trays in ES complete medium. EPL cell formation was induced by the presence of 50% MEDII or sfMEDII in the culture medium. The EPL cell inductive capacity of the purified samples was assessed by supplementation into ES complete medium (2.3.1). The capacity of L-proline for EPL cell formation was examined by the supplementation of $40 \mu\text{M}$, $100 \mu\text{M}$ or $400 \mu\text{M}$ L-proline into ES complete medium in the presence or absence of supplemented R ($50 \mu\text{g}/\text{ml}$) or cFN ($10 \mu\text{g}/\text{ml}$). cFN and R were assessed for EPL cell formation from solution in the absence of L-proline. The EPL cell inductive capacity of cFN was also examined by pre-coating onto tissue culture plastic in the absence of L-proline. All cultures were passaged at a dilution of 1:10 and cells were passaged and harvested every 48hrs. The initial culture is termed passage 0 (p0) and cells were passaged and harvested every 48hrs up to p3. ES and EPL cells were re-fed every 24 hours after passage with 10 ml of appropriate culture medium.

2.3.16 Preparation of Embryoid Bodies (EB's)

ES and EPL cells cultured under standard conditions (2.2.5) to $p1^+$ or $p0^-$ in culture, where + denotes culture in the presence of mLIF and -denotes culture in the absence of mLIF, were used to make EB's by the following method. ES or EPL cells were removed from tissue culture plastic by trypsin (1 ml) and pipetted into a single cell suspension prior to being seeded into ES incomplete medium (1×10^5 cells/ml) in bacteriological petri dishes. ES cells failed to contact the bacteriological plastic dish and formed suspended, free floating, aggregates of EB's (1.9.9) in the absence of

exogenous mLIF. EB's were cultured over 4 days with harvest and re-feed of medium each day up to day 4.0. p0 designates the p1⁺ or p0 harvested cells while p1-p4 designates harvested EB's.

2.3.17 EB's formed in MEDII and END2/PYS2 cell line CM

D3 ES cell derived EB's were formed in the presence of incomplete ES DMEM containing 50% MEDII, END2, PYS2, sfMEDII or sfMEDII conditioned medium, stripped of cFN by gelatin sepharose chromatography (2.5.5). D3 ES cell derived EB's were also cultured in incomplete ES DMEM supplemented with the purified EPL cell inductive signals (400 μ M L-proline and/or 10 μ g/ml cFN). EB's were cultured for 7 days in the absence of exogenous LIF before seeding down 1 EB/well in gelatin coated (0.2%) 24 well trays. Cells were cultured in 50% ES incomplete DMEM/F12 medium.

2.3.18 Harvesting EB's for RNA preparation

Free floating EB's were transferred to a 20 ml tubes and allowed to settle. The bodies were washed 3 times in PBS and allowed to settle before decanting off the remaining PBS prior to storage at -80°C.

2.4 Protein Purification

2.4.1 Protein purification materials

Sephadex G-10 fine: Pharmacia

Sepharose Q fast flow: Pharmacia

Resource Q ion exchange column: Pharmacia

Phenyl sepharose: Pharmacia

Heparin Sepharose CL6B: Pharmacia

Resource Q ion exchange column: Pharmacia

Gelatin Sepharose: Pharmacia

2.4.2 Protein purification apparatus

Smart System Superose 6 gel Filtration column: Pharmacia

XK26/100 Gel filtration column: Pharmacia

XK50 column: Pharmacia

Centricon Concentrators: Amicon

Large scale Concentrator: Amicon

Large scale concentrator membrane filters: Amicon

Fast Phase Liquid chromatography machine (FPLC): Pharmacia

Sample collection carousel/FPLC: Pharmacia

LKB-Optical unit UV-1: Pharmacia

LKB-Control unit UV-1: Pharmacia

Bradford Reagent: Bio-Rad

Emax precision microplate plate reader: Molecular Dynamics

2.5 Protein Purification Methods

2.5.1 Determination of protein concentration by Bradford

Samples and standards were performed in triplicate. 10 μ l of BSA standards (1-0.1 mg/ml) were mixed with 200 μ l of 1 in 4 diluted Bradford Reagent (Bio-Rad) in a 96-well tray (Falcon). A_{280} was measured in an Emax precision microplate plate reader (Molecular Dynamics). Protein concentrations of samples were determined by calculation from the line of best fit of the standard curve.

2.5.2 Pilot purification

2.5.2.1 Anion exchange

Anion exchange column chromatography was performed using a liquid chromatography controller 500 gradient FPLC machine (Pharmacia) fitted with a 1 ml Resource Q column (Pharmacia). 2 mg of R was bound in 50 mM Tris-HCl, pH8.5, and elated with an increasing concentration of 1 M NaCl in 50 mM Tris-HCl, pH8.5, at a flow rate of 1 ml/minute over 40 minutes. Proteins were detected at A_{280} using LKB-Optical and control UV-1 control units. 1 ml fractions were collected across the gradient, desalted and concentrated using Centricon-10 units (Amicon) before assessment of EPL cell inductive activity by morphology based assay.

2.5.2.2 Hydrophobic interaction

Hydrophobic interaction column chromatography was performed using the FPLC apparatus (Pharmacia) fitted with a 1 ml Phenyl sepharose fast flow column (Pharmacia). 2 mg of R was bound in 1 M ammonium sulphate in 20 mM Tris-HCl, pH8.5, and elated with a decreasing concentration of salt to 20 mM Tris-HCl, pH8.5, at a flow rate of 1 ml/minute over 40 minutes. Proteins were detected at A_{280} . 1 ml fractions were collected across the gradient, desalted and concentrated using Centricon-10 units (Amicon) before assessment of EPL cell inductive activity by morphology based assay.

2.5.2.3 Heparin affinity

Heparin affinity column chromatography was performed using the FPLC apparatus (Pharmacia) fitted with a 1 ml heparin affinity fast flow column (Pharmacia). 2 mg of R was bound in 20 mM Tris-HCl, pH8.5, 150 mM NaCl and eluted with an increasing concentration in 20 mM Tris-HCl, pH8.5, at a flow rate of 1 ml/minute over 20 minutes to 500 mM NaCl and a final wash in 1 M NaCl. Proteins were detected at A_{280} . 1 ml fractions were collected across the gradient, desalted and concentrated using Centricon-10 units (Amicon) before assessment of EPL cell inductive activity by morphology based assay.

2.5.2.4 Gel filtration

Gel filtration column chromatography was performed using the Smart system™ micro-preparative purification system (Pharmacia) fitted with a 2.4 ml Superose 6 column (Pharmacia). 400 µg of protein (retained fraction) was loaded (4x50 µl) onto the Superose 6 column in 20 mM Tris-HCl, pH 8.5 in 150 mM NaCl and eluted at a flow rate of 25 µl/minute over 40 minutes. Proteins were detected at A280. 50 µl fractions were collected across the elution profile, desalted and concentrated using Centricon-10 units (Amicon) before assessment of EPL cell inductive activity by morphology based assay.

2.5.3 Purification Method 1:

2.5.3.1 Anion Exchange Chromatography

Serum free MEDII (4 Litres) was concentrated 13 fold to ultrafiltration using the Diaflo YM100 membrane and apparatus (Amicon) at 4°C under nitrogen pressure (55 psi). The retained high molecular weight fraction was exchanged to 20 mM Tris-HCl pH 8.5 prior to loading onto a 300 ml Q sepharose fast flow XK-50 column (Pharmacia) using a Liquid Chromatography controller 500 gradient FPLC machine (Pharmacia) at a flow rate of 5 ml/min. The column was re-equilibrated in buffer A (20 mM Tris-HCl pH 8.5). Bound proteins were eluted from the column in a step wise fashion using ~~alternate~~^{increasing} levels of buffer B (20 mM Tris-HCl pH 8.5, 1 M NaCl) through the column at a constant flow rate. The first step elution was in 200 mM NaCl using 80:20 (buffer A:buffer B) and was collected in a volume of 200 mls. The second step elution was in 500 mM NaCl (50:50) and sample was collected in 200 ml volume. The final elution step was the application of 100% buffer B (1 M NaCl). Eluted proteins were collected in 200 mls. Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 µM L-proline for EPL cell inductive morphology assay.

2.5.3.2 Phenyl sepharose Chromatography

The active fraction from anion exchange chromatography (180 mg) was treated with ammonium sulphate in 20 mM Tris-HCl pH 8.5 to a final concentration of 0.5M. The remaining sample (160 mg) was filtered through a 0.22 µm filter to remove precipitated protein material. The active sample was passed down a 40 ml phenyl sepharose fast flow, hydrophobic interaction column (Pharmacia, Sweden) at a flow rate of 5 ml/min. Bound protein was eluted from the column in a single step with the application of 100% buffer B (20 mM Tris-HCl pH 8.5) at a constant flow rate. Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 µM L-proline for EPL cell inductive morphology assay.

2.5.3.3 Heparin Affinity Chromatography

The active sample from the hydrophobic interaction purification was diluted in 20 mM Tris-HCl pH8.5 to 100 mM NaCl as assessed by conductivity. The sample was applied to a 25 ml heparin sepharose CL-6B affinity column (Pharmacia, Sweden). Bound proteins were eluted in a single step of 50 ml of 20 mM Tris-HCl pH8.5, 500 mM NaCl, containing 7.5 mg of protein. Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 μ M L-proline for EPL cell inductive morphology assay.

2.5.3.4 Superose 6 gel filtration: purification and size determination of the EPL cell inducing activity

A portion of the active sample from the heparin affinity chromatography purification (50 μ l) was applied to a micro preparative superose 6 gel filtration column, using Smart systemTM apparatus (Pharmacia, Sweden), in 20 mM Tris-HCl pH 8.5, 150 mM NaCl at a flow rate of 25 μ l/min. Purified protein samples were collected in 50 μ l volume and assayed for EPL cell induction. Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 μ M L-proline for EPL cell inductive morphology assay.

2.5.4 Purification Method 2:

2.5.4.1 Heparin Affinity Chromatography

A single litre of sfMEDII conditioned media was applied to a 50 ml heparin sepharose CL-6B column previously equilibrated in 20 mM Tris-HCl pH8.5 at a flow rate of 2 ml/min. After re-equilibration, step wise elution of bound proteins incorporated Tris-HCl pH8.5, 150 mM NaCl, 500 mM NaCl, and 1 M NaCl at a flow rate of 2 ml/min. Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 μ M L-proline for EPL cell inductive morphology assay.

2.5.4.2 Anion Exchange Chromatography

The 500 mM NaCl sample in 20 mM Tris-HCl pH8.5 was diluted to 150 mM in 20 mM Tris-HCl pH8.5 bound to a 1 ml Resource Q anion exchange column and eluted via a continuous gradient between 150 and 500 mM NaCl at a flow rate of 1 ml/minute for 60 minutes followed by a 5 minute wash in 1 M NaCl. The EPL cell inducing activity eluted between 200-300 mM NaCl. Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 μ M L-proline for EPL cell inductive morphology assay.

2.5.4.3 Superose 6 gel filtration: purification and size determination of the EPL cell inducing activity

Active samples from anion exchange were pooled and passed down a superose 6 gel filtration column using the smart system (Pharmacia) in 20 mM Tris-HCl pH8.5, 150 mM NaCl with activity eluting at a molecular weight between 460-1000 kd based on the previous elution of protein molecular weight markers. The purified activity was visualised as a highly purified band of approximately 250 kd on a silver stained SDS/reducing polyacrylamide gel (2.7.20). Eluted proteins were buffer exchanged to PBS using centricon 10 filtration columns and supplemented into ES complete medium + 40 μ M L-proline for EPL cell inductive morphology assay.

2.5.5 Affinity purification

Fibronectin affinity purification was performed by passing a single litre of sfMEDII over a 25 ml gelatin sepharose affinity chromatography matrix in an XK-50 column (Pharmacia) at a flow rate of 5 ml/min. After re-equilibration in PBS, FN was eluted from the column using 6 M Urea in PBS. Protein elution was detected at A280 and collected directly into dialysis tubing. Urea was removed from samples via dialysis in PBS (5x4 L at 4°C) prior to use in EPL cell induction assays. Protein yield was quantitated by Bradford (BioRad).

2.6 Molecular Biology

2.6.1 Radiochemicals

Radiolabelled chemicals used were purchased from Geneworks and are listed below:

α -32PdATP of specific activity 3000 Ci/mmol and radioactive concentration of 10 mCi/ml

α -32PdUTP of specific activity 3000 Ci/mmol and radioactive concentration of 10 mCi/ml

2.6.2 Chemicals

All chemicals were of molecular biological grade and the highest purity available.

Acrylamide (Protogel): Geneworks

Ammonium Acetate: BDH

APS: Sigma Chemical Co.

Agar: Sigma Chemical Co.

β -ME: Sigma Chemical Co.

Blue Dextran: Pharmacia

Brilliant Blue (Coomassie): Sigma Chemical Co.

Chloroform: BDH

DTT: Sigma Chemical Co.

EtBr: Sigma Chemical Co.

EDTA: Sigma Chemical Co.

Ficoll 400: BDH

Formaldehyde (37%): BDH

Formamide: BDH (De-ionised and stored at -20°C)

Glacial Acetic acid: BDH

Glycine: BDH

Hydrochloric acid: BDH

Isopropanol: Ajax Chemicals

Mineral oil: Sigma Chemical Co.

MOPS: Sigma Chemical Co.

Methanol: BDH

NBT: Boheringer Manneheim

Potassium acetate: BDH

Phenol: Wako

Potassium Acetate: BDH

PMSE: Sigma Chemical Co.

PEG 6000: BDH Chemicals

Tween 20: Sigma Chemical Co.

Ribonuclease A: Sigma Chemical Co.

Rifampicin: Boheringer Manneheim
Salmon sperm DNA: Boheringer Manneheim
Sepharose CL-6B: Pharmacia
Sephadex G-50 fine: Pharmacia
Sodium chloride: BDH
Sodium Hydroxide: BDH
NaDOC: Sigma Chemical Co.
Silver Nitrate: BDH
SDS: Sigma Chemical Co.
TEMED: Sigma Chemical Co.
tRNA: Boehringer Manneheim.
Tris-HCl: BDH
Triton X-100: BDH
Ultrapure Urea: Sigma Chemical Co.
Polyvinyl pyrolidone: BDH
Glycerol: BDH
Bromophenol blue: Sigma Chemical Co.
Xylene cyanol: Sigma Chemical Co.
Potassium Acetate: BDH
Sodium citrate: BDH

2.6.3 Enzymes

Restriction endonucleases supplied by New England Biolabs, Pharmacia and Geneworks.

Calf intestinal phosphatase: Roche
DNase I: Boheringer Manneheim
Klenow DNA polymerase: Geneworks
Pfu Turbo: Stratagene
Proteinase K: Merck
RNase A: Sigma Chemical Co.
RNasin: Geneworks
Superscript[™] II reverse transcriptase: Gibco/BRL
T7 RNA polymerase: Geneworks
T3 RNA polymerase: Geneworks
T4 DNA Ligase: Pharmacia

All enzymes were stored to the manufacturers specifications.

2.6.4 Buffers

Buffer 1: 100 mM Tris-HCl pH 7.4, 100 mM NaCl

Buffer 2: 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂

Buffer 3: Buffer 1, 1 mM EDTA.

CIP buffer (10x): 500 mM Tris-HCl pH 8.5, 1 mM EDTA)

Gel loading buffer(10x)(GLB): 50% glycerol, 0.4% bromophenol blue, 0.2% xylene cyanol, 10 mM EDTA.

MOPS buffer (10x): 800 ml MQ H₂O, 48.1 g MOPS (adjusted to pH to 7.0 with NaOH), 16.6 ml 3 M sodium acetate pH 5.2, 20 ml 0.5M EDTA (pH8.0).

PAGE Tank Buffer (5x): Tris-HCl (15 g), Glycine (72 g), SDS (5 g), in 1 L H₂O.

PBS(10x): NaCl (8 g), KCl (0.2 g), KH₂PO₄ (0.2 g), Na₂HPO₄ (1.15 g) in 100 ml.

Polymerase Chain Reaction (PCR) Buffer (10x): 100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, Gelatine .01%.

Protein gel loading buffer (2x): Tris-HCl pH6.8 (125 mM), Glycerol (20%), Bromophenol Blue (0.4%), β-ME (200 mM), H₂O to 1 ml, SDS (0.2%).

Reverse Transcription (RT) Buffer (10x): 500 mM Tris-HCl pH8.5, 60 mM MgCl₂, 400 mM KCl, 10 mM DTT.

SSC (20x): 3.0 M NaCl, 0.3 M sodium citrate, pH 7.4.

Substrate mix: 10 ml buffer 2, 40 μl NBT (75 mg/ml in 70% DMF), 40 μl BCIP (50 mg/ml in 100% DMF)

Transcription Buffer (10x): 40 mM Tris-HCl, pH8.0, 8 mM MgCl₂, 5 mM β-ME, CTP, ATP, GTP, 0.05 μg/μl and 10 μg/ml Rifampicin (Boheringer)

TBST Buffer (10x): NaCl 8 g, KCl 0.2 g, 25 ml 1 M Tris-HCl pH 7.5, and 1 ml Tween20

TAE (20x): Tris-HCl (96.4 g), sodium Acetate (32.8 g), EDTA (6.72 g), pH 8.2 in 1 L.

TE (1x): 10 mM Tris-HCl, pH 7.5, 1 mM EDTA

TFB1: 30 mM KAc, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂, 15% glycerol, pH 5.8

TFB2: 10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl₂, 15% glycerol, pH 6.5.

Tris-HCl buffer 1 M (pH 8.5): Tris-HCl (121.14 g), pH adjusted with 1 M HCl, in 1L.

Restriction enzyme buffer (10x): To manufacturers specifications (New England Biolabs, Pharmacia)

Tris-HCl pH6.8: Tris-HCl (6.05 g) in 40 ml H₂O, adjust pH with 1 M HCl to 100 ml H₂O. Filter through a 0.45 μm filter.

Tris-HCl pH8.8: Tris-HCl (91 g) in 300 ml H₂O, adjust pH with 1 M HCl to 500 ml H₂O. Filter through a 0.45 μm filter.

Western transfer buffer: 39 mM glycine, 48 mM Tris-HCl, 20% methanol, 0.037% SDS

2.6.5 Solutions

Acetate solution: 5 M Potassium Acetate (60 ml), 3 M Acetic acid (11.5 ml).

Coomassie Blue solution: 50% methanol, 0.05% Brilliant Blue, 10% acetic acid, 40% H₂O.

Destaining solution: 5% methanol, 7% acetic acid, 88% H₂O.

Denhardt's reagent (50x): 1% Ficoll 400, 1% BSA, 1% Polyvinyl pyrrolidone.

Developing solution: 6.0g sodium citrate, 25 µl of 37% formaldehyde solution, H₂O to 100 ml

Fixing solution: 50% methanol, 10% acetic acid, 40% H₂O.

Herring sperm DNA: 10 mg/ml

Megadeath solution: 0.1 M NaOH, 0.5% SDS, 10 mM Tris-HCl pH8.0, 1 mM EDTA

NaCl saturated TE solution: 1x TE (150 ml), Isopropanol (250 ml), saturated with NaCl.

PBT: 0.1% Triton X-100 in PBS

RNAse Free 100% Ethanol (EtOH):

Salmon sperm DNA: 10 mg/ml

Silver nitrate solution: 0.25g Silver nitrate, 50 µl of 37% formaldehyde in 250 ml H₂O.

Solution I: 50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA

Solution II: 0.2 M NaOH, 1% SDS

Solution III: 3 M KAc, 2 M HAc

tRNA: 20 mg/ml

TNM: Tris-HCl (pH 7.6, 30 mM), NaCl (150 mM) , MgCl₂ (15 mM), NP40 (0.4%)

TUNES: Tris-HCl, pH8.0, 7 M ultrapure Urea, 0.35 M NaCl, 1 mM EDTA, 2%SDS

2.6.6 Molecular biology kits

Megaprime kit: Amersham

Gigaprime-Random priming DNA oligolabelling kit: Geneworks

DIG RNA labelling kit: Boehringer Manneheim

Bresaclean: Geneworks

Supersignal: Pierce, USA

2.6.7 DNA molecular weight markers

Hind III/EcoR1 digest of phage Lambda DNA: Geneworks.

Fragment sizes in kb pairs: 21.7, 5.15, 5.0, 4.27, 3.38, 1.98, 1.92, 1.59, 1.37, 0.94, 0.83, 0.56.

Hpa II digest of pUC 19 DNA at 0.5 µg/ul: Geneworks.

Fragment sizes in bp: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26.

DNA fragment sizes and approximate concentrations were determined by also loading TAE agarose mini-gels with 500ng of marker DNA.

2.6.8 Protein molecular weight markers

Broad Range Kaleidoscope pre-stained SDS-PAGE Standards: BioRad

250kd, 150kd, 100kd 75kd, 50kd 37kd, 25kd.

Mark12 Wide range protein standards: Amrad

200kd, 116kd, 97kd, 66kd, 55kd, 36.5kd, 31kd, 21.5kd, 14.4kd, 6kd, 3.5kd, 2.5kd.

2.6.9 Cloning vectors

pBluescript KS+: Stratagene. pBluescript II KS+ plasmid DNA was linearised with appropriate restriction enzyme(s) and treated with CIP to inhibit re-ligation of the vector alone. Vector DNA was purified as described in section 2.7.4.

2.6.10 Cloned sequences

Fgf5 was kindly donated by Dr G Martin. This plasmid consisted of a fragment encoding the full length mouse *Fgf5* cDNA, cloned into the SmaI site of pBluescript KS+ (Hébert *et al.*, 1991).

Rex1 was kindly donated by Dr Neil Clarke. This plasmid contained 848bp of *Rex1* cDNA in the EcoRI site of pCRTMII.

Oct4 cDNA in pBluescript was a kind gift from Dr Hans Schöler. This clone contained a 462bp *Stu* I cDNA fragment spanning positions 491 to 953 of the *Oct4* cDNA sequence.

Brachyury (pSK75) was kindly provided by Dr Bernard G. Herrmann. The plasmid contained a 1764bp of *Brachyury* cDNA cloned into the EcoRI site of Bluescript SKII (Herrmann, 1991)

mGAP (pmGAP) contains 300bp of the *mGAP* cDNA sequence in pBluescript KS+ (Rathjen *et al.*, 1990).

EDA⁺ cFN cDNA in pBluescript contained a 462bp EcoRI cDNA fragment spanning positions 491 to 953 of the *EDA⁺* domain of cFN gene cDNA sequence.

2.6.11 Oligonucleotides

Internal forward (EDA1) and reverse (EDA2) PCR primers for the extra domain A (EDA) region of mouse cFN (Norton, 1995; accession number X93167) with 5' EcoRI linkers.

EDA1: 5'-ATGAATTCCTAAAGGACTGGCATTC-3'

EDA2: 5'-ATGAATTCCATATCATCGTCGAAG-3'

General sequencing primers:

T7: 5'-TAATACGACTCACTATAGGGAGA-3'

T3: 5'-ATTAACCCTCACTAAAGGGA-3'

2.6.12 Bacterial Strains

The *E.coli* DH5 α strain was used as a host for all recombinant plasmids.

DH5 α : *supE44* delta *lac* U169 (*phi80lacZ*deltaM15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*

2.6.13 Bacterial Growth Media

Growth media were prepared in double distilled water and sterilised by autoclaving. Antibiotics and other labile chemicals were added after the media solution had cooled to 50°C.

Luria broth: 1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH.

Psi broth: 2% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% MgSO₄, adjusted to pH 7.6 with KOH.

Solid Media: Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar.

Ampicillin (100 μ g/ml) was added for growth of transformed bacteria to maintain selective pressure for recombinant plasmids.

2.6.14 Miscellaneous materials

Mini-Protean II eletrophoresis apparatus: BioRad

Eppendorf/yellow capped tubes: Trace Scientific

Whatman 3MM chromatography paper: Whatman

Nucleic acid membrane (0.45 μ m): Amersham, Schleicher & Schuell, Immobilon

Centrifuge rotors: Sorvall, Beckmann, Jouan

Phosphorimager: Molecular Dynamics

Densitometer: Molecular Dynamics

Hybaid rotor: Hybaid

UltraHyb: Ambion

Curix 60 developing machine: AGFA

Curix blue HC-S plus medical X-ray film: AGFA

Cellophane:

2.7 Molecular Methods

2.7.1 Reverse transcription of total RNA

Reverse transcription was carried out with Superscript[™] II reverse transcriptase (Gibco/BRL) following the manufacturer's instructions. Briefly, 5 µg of cytoplasmic total D3 ES cell RNA and 500ng of oligonucleotide EDA2 were heated to 70°C for 10 minutes in a volume of 12 µl. The mixture was snap cooled on ice and incubated at 42°C for 50 minutes in a 20 µl reaction containing 500 nM dNTPs, 50 mM Tris-HCl pH8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 2.5 U Superscript[™] II. The reaction was diluted to 200 µl with H₂O and stored at -20°C.

2.7.2 Reverse transcriptase Polymerase chain reaction (PCR)

RT-PCR reactions were carried out using Pfu Turbo (Stratagene) according to the manufacturer's instructions. Briefly, 100 µl volume PCR reactions containing 2 µl of cDNA, 250 ng of each primer (EDA1 and EDA2), 200 µM dNTPs, 20 mM Tris-HCl pH8.8, 2 mM MgSO₄, 10 mM (NH₄)₂SO₄, 100 ng/ml BSA, 0.1% Triton X-100, and 2.5 U of Pfu Turbo polymerase were overlaid with mineral oil, heated to 94°C for 3 minutes, and cycled as follows in a PTC-100 Thermal cycler. Cycle 1-35 at 94°C, 1 minute, 52°C, 1 min, 72°C, 1 min. The final cycle, reactions were incubated at 72°C for 5 minutes to allow extension of partial PCR products.

2.7.3 Gel electrophoresis

Agarose gel electrophoresis (1% to 2% w/v agarose in 1x TAE, stored at 68°C) was carried out using horizontal minigels prepared by pouring 10 ml of agarose solution onto a 7.5 cm x 5.0 cm glass plate. Agarose mini-gels were allowed to set at room temperature prior to being submerged in 1x TAE in a gel electrophoresis system (BioRad). GLB was added to DNA/RNA samples to a final concentration of 5% glycerol, 0.04% bromophenol blue, 0.02% xylene cyanol and 1 mM EDTA (1x GLB). Samples were loaded into wells within the agarose gel and electrophoresed at 100 mA. DNA/RNA was visualised by staining gels with EtBr (10 µg/ml (w/v) in water) for 5 minutes and de-staining in water for 5 minutes. Gels were photographed under medium UV light using a tracktel thermal imager. Approximate molecular size of DNA fragments were assessed by comparison with EtBr with stained DNA molecular weight markers.

2.7.4 Isolation of DNA fragments from gels

Linear DNA fragments were run on appropriate percentage TAE agarose gels and visualised under long wavelength UV light. Bands were removed from preparative gels using a clean scalpel blade and purified from agarose using the Bresaclean kit (Geneworks) according to the manufacturers instructions.

2.7.5 Removal of 5' phosphate groups from vector DNA fragments

The 5' phosphate groups were removed from DNA ends by treatment with CIP prior to gel purification on a sepharose CL-6B spin column. This inhibited re-ligation of the vector backbone. Restriction digested vector DNA was precipitated in the presence of glycogen, washed in 70% ethanol, dried, and resuspended in 40 µl of MQ H₂O. The reaction was initiated by the addition of 5 µl of 10xCIP buffer and 1 µl (1 U/µl) of CIP. The reaction was complete after 30 minutes at 37°C for. Dephosphorylated vector DNA was electrophoresed on 1% TAE agarose gels (2.7.3) and purified (2.7.4) using the Bresaclean kit (2.7.4).

2.7.6 DNA Ligation reactions

DNA fragments were purified using the Bresaclean kit (Geneworks) following the manufacturers instructions. Linearised vectors used for synthesising riboprobes were purified by phenol/choloform extraction and EtOH precipitation.

2.7.7 Transformation of Competent Bacteria with Plasmid DNA

Preparation of RbCl₂ competent cells

Psi Broth (5 ml) was inoculated with a single colony of DH5₋ strain bacteria and grown overnight at 37°C with shaking. 500 µl of overnight culture was used to reinoculate 15 mls of Psi broth. The culture was grown at 37°C to an OD₆₀₀ of 0.6. 5 mls of bacteria were subcultured in 95 mls of Psi broth and grown to an OD₆₀₀ of 0.6 at 37°C with shaking. Cells were poured into 40 ml oakridge tubes and chilled on ice for 5 minutes prior to centrifugation at 6000rpm for 5 minutes at 4°C. The supernatant was aspirated and the cell pellet was resuspended 40 mls of TFB1, left on ice for 5 minutes and centrifuged at 6000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 4 mls of TFB2. After 15 minutes on ice 100 µl aliquots were snap frozen in a dry ice/EtOH bath and stored at -80°C

Transformation of Competent Cells

RbCl₂ competent *E.coli* bacterium were thawed on ice, mixed with approximately 10ng of plasmid DNA, and left on ice for 30 minutes. The cells were then heat shocked for 90 seconds at 42°C, and placed back on ice for 1 minute. L broth (2.6.15) was then added to the bacteria (800 µl) and left at 37°C for 30-40 minutes to allow cells to express antibiotic resistance after the heat shock. Cells were spun at 5000rpm for 5 minutes in an Eppendorf, the supernatant was removed leaving about 100-200 µl s in the bottom of the tube. Cells were resuspended in this volume and plated on required antibiotic selection agar at 37°C overnight.

2.7.8 Rapid small scale preparation of plasmid DNA

L broth (1.5 ml) containing 100 µg/ml of Ampicillin was inoculated with a single bacterial colony and grown overnight at 37°C in a rotating shaker. Each culture was poured into a 1.5 ml

Eppendorf tube and centrifuged at 14,000 rpm for 30 seconds. Centrifugation steps were carried out using an Eppendorf centrifuge 5145C. The majority of the medium was removed leaving around 100 µl. Bacterial pellets were resuspended in the remaining medium by vortexing and lysed by the addition of 300 µl of Megadeath solution. Cell debris was precipitated by mixing 155 µl of NaAc pH5.2 with the mixture and centrifugation at 14,000 rpm for 4 minutes. Nucleic acids were precipitated by mixing 1 ml of 100% EtOH with the supernatant. The sample was briefly vortexed, placed at -80°C for 30 minutes and centrifuged at 14,000 rpm for 4 minutes prior to removal of the supernatant. The nucleic acid pellet was washed by the addition of 400 µl of 70% ethanol followed by vortexing and a brief centrifugation. The remaining liquid was removed and the pellet was dried for 5-10 minutes at 37°C. Mini-prep DNA was resuspended in 20 µl of MQ H₂O containing 10 µg/ml RNase A.

2.7.9 Midi-preparation of Plasmid DNA

L broth (50 ml) containing 100 µg/ml Ampicillin in a 250 ml flask was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The culture was transferred to a 40 ml oakridge tube and centrifuged in a RC-5 Sorvall centrifuge and SS-34 rotor (Dupont) at 6000 rpm for 10 minutes. The supernatant was removed and the bacterial pellet was resuspended in 3 ml of Solution I. The suspension was gently mixed with 6 ml of fresh Solution II to lyse the cells. Following 5 minutes incubation on ice, cell debris was precipitated by the addition of 4.5 ml of Solution III. The solution was gently mixed by inversion, left on ice for 5 minutes, then mixed vigorously, left on ice for a further 15-20 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was mixed with 8 ml of Isopropanol in a clean oakridge tube, and nucleic acids were precipitated by centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant was drained and the pellet was dissolved in 400 µl of MQ H₂O. RNA was removed by incubation at 37°C for 30 minutes with 2 µl of RNase A (10 mg/ml). 8 µl of 10% SDS and 2 µl of Proteinase K (20 mg/ml) were added to the solution and incubated for a further 15 minutes at 37°C. The sample was extracted with an equal volume of phenol/chloroform and then with an equal volume of chloroform alone. The aqueous phase was precipitated by addition of 100 µl of 7 M NH₄Ac and 1 ml of 100% ethanol. DNA was precipitated for 20 minutes at -20°C then pelleted at 14,000 rpm in a bench-top centrifuge for 15 minutes. The DNA was washed in 400 µl of 70% ethanol, dried, and resuspended in 200 µl of MQ H₂O.

2.7.10 Double stranded DNA sequencing

A single µg of plasmid DNA was subjected to PCR based cycle sequencing in the presence of T7 or T3 oligonucleotide primer (100 ng) and 8 µl of Dye terminator ready reaction mix (Perkin Elmer) in a total volume of 20 µl. The PCR was cycled 24 times as follows.

Step 1: 96°C for 30 seconds

Step 2: 50°C for 15 seconds

Step 2: 60°C for 4 minutes

Reactions were then precipitated in 80 µl of 75% Isopropanol for 15 minutes at room temperature. Precipitated DNA was pelleted by centrifugation at 14,000 rpm for 15 minutes. Pelleted DNA was washed in 250 µl of 70% EtOH, centrifuged at 14,000 rpm for 5 minutes and dried by vacuum centrifugation. Reactions were analysed at the Institute for Medical and Veterinary Science Sequencing Centre, Adelaide, Australia and viewed on the Edit/view program (Perkin Elmer).

2.7.11 Restriction digestion analysis of plasmid DNA

Analytical digestion of DNA with restriction endonucleases was carried out from 1-24 hours at recommended temperatures. DNA was digested in 1x restriction enzyme buffer at 0.5 µgs of DNA to 1-2 U enzyme in a total volume of 20-100 µl. Enzymes were stored at -20°C and used to manufacturers specifications.

2.7.12 Synthesis of radioactive nucleic acid probes

Synthesis of radioactive DNA probes

DNA probes were generated using the Gigaprime kit (Geneworks) to the manufacturers specifications. Briefly, 100-500ng of purified cDNA was heated for 5 minutes at 100°C in a final volume of 6 µl. Sample was snap-cooled on ice for 2 minutes before the addition of 6 µl decanucleotides, 6 µl nucleotide buffer A and 5 µl of α -³²PdATP. Reaction was initiated with the addition of 1 µl Klenow and placed at 37°C for 15 minutes. Probe was purified by spin column in sepharose CL-6B. DNA probes were boiled for 2 minutes and snap-cooled on ice before adding to filters.

Synthesis of radioactive RNA probes

Plasmid DNA was linearised with appropriate enzymes and gel purified. Radioabelled riboprobes were assembled in 20 µl volume using the following reagents. Purified plasmid (0.5 µg in 4 µl), 2 µl of 10x transcription buffer (Boheringer Manneheim), 2 µl of DTT (100 mM), 1 U of RNasin (Geneworks), 0.5 µl UTP (300 µM), 3 µl dATP, dCTP, dGTP (3.3 mM), 6 µl of α -³²PdUTP (Geneworks) and 2 U of appropriate RNA polymerase. Total volume of 20 µl with MQ water and left at 37°C for 3 hrs to form the RNA probe template. After incubation total volume was made up to 100 µl with 10 µl 0.1 M EDTA, 60 µl MQ H₂O, and 10 µl of 20 mg/ml tRNA for precipitation. The riboprobe was precipitated with 11 µl of 3 M NaAc and 275 µl of RNase free EtOH at -80°C for 30 minutes. After centrifugation 14000 rpm, the probe was then resuspended in 100 µl of H₂O with 0.5 µl of RNasin. RNA probes were denatured by heating at 85°C for 2 minutes before being added to the filters.

2.7.13 Preparation of cytoplasmic RNA from tissues culture cells and EB's

Preparation of cytoplasmic RNA carried out by the methods of Edwards, Parfett, and Denhardt (1985). Briefly, cells or EB's were harvested by trypsinisation, washed in tissue culture grade PBS, and centrifuged at 1.2 rpm for 5 minutes prior to storage as pellets at -80°C. The cells were thawed out on ice and lysed by mixing thoroughly with in 2 mls of ice-cold TNM solution and 2 mls of TUNES solution, at room temperature. This solution was extracted twice with phenol/choloform (1;1) and the aqueous layer was transferred carefully, so as not to disturb the interface, to a corex tube where RNA was precipitated with 2.5 volumes of RNase free EtOH and 0.1 volumes of NaAc and left at -80°C for 30 minutes. After spinning at 10,000 rpm for 30 minutes, the RNA pellet was resuspended in 400 µl of sterile water, and transferred to an Eppendorf tube for re-precipitation as above. The RNA was pelleted at 14000 rpm for 15 minutes, resuspended in an appropriate volume of water, and the concentration was determined at A_{260} on a Cary 3 Bio UV visible spectrophotometer with Cary win UV software (Varian). RNA samples were stored at -20°C.

2.7.14 RNA gels for northern blot analysis

RNA samples were run on 1% agarose gels in (2.5gs of agarose in 210 mls MQ H₂O, 25 mls 10x MOPS, 15 mls 20% formaldehyde (4g paraformaldehyde in 20 mls of MQ H₂O) in 1x MOPS buffer at pH 4.5 poured with wells that held a maximum volume of 60ul. RNA samples were loaded with 20 µg of RNA as follows;

11.25 µl	RNA + MQ H ₂ O
5 µl	10xMOPS
8.75 µl	Formaldehyde (37%, pH above 4)
25 µl	Formamide (de-ionized)

Samples were mixed in an Eppendorf with vortexing before being spun to the bottom of the tube and heated at 65°C for 5 minutes, followed by snap cooling on ice. RNase free GLB (10x, 6ul) was then added and the samples were run at 6V/ cm gel length, with DNA molecular weight markers, until the first blue dye was three quarters to the bottom of the gel.

2.7.15 Transfer of Northern Blot

The part of the gel to be transferred was cut out and rinsed in water. RNA run next to lambda marker DNA was stained in EtBr (10 mg/ml in 200 ml water) for 45 minutes and de-stained in water overnight, and photographed. Two pieces of whatman paper were pre-wetted in 20x SSC and placed around a glass plate, positioned on a stand above a pool of 20x SSC buffer so that the ends of the paper were submerged in the transfer solution. The gel was then placed upside down on the whatman (avoiding bubbles) with parafilm surrounding the gel to avoid short-circuiting. A nitrocellulose filter, pre-wetted in 20x SSC, was then added carefully to the gel (avoiding bubbles) and 3 pieces of pre-wetted whatman were layered over the top, followed by 10 pieces of dried

whatman. A 5 cm stack of paper towels was added to absorb the 20x SSC solution. A second glass plate and a 800g weight were added, and this was allowed to blot overnight. The next day the filter was recovered from the blot, and the RNA was UV cross-linked to the nitrocellulose filter.

2.7.16 Hybridisation of radioactive probes to nylon filter Membranes

DNA probes

Hybridisation reactions were carried out in a Hybaid Hybridisation Oven at 42°C. Filters were prehybridised in UltraHyb (Ambion) for a minimum of 4 hours at 42°C in 10 ml prehybridisation solution/filter. Filters were probed overnight at 42°C and was washed at 42°C, using the following conditions;

- (1) 2x SSC, 0.1% SDS, for 15 minutes.
- (2) 0.2x SSC, 0.1% SDS, for 15 minutes.
- (3) 0.2x SSC, 0.1% SDS, at 68°C, for 15 minutes.

RNA probes

Northern filters were prehybridised for a minimum of 4 hours at 65°C in 10 ml UltraHyb (Ambion). Hybridisation reactions were carried out overnight at 65°C and washed at 65°C, using the following conditions

- (1) 2x SSC, 0.1% SDS, for 15 minutes.
- (2) 0.2x SSC, 0.1% SDS, for 15 minutes.
- (3) 0.2x SSC, 0.1% SDS, at 65°C, for 15 minutes.

All filters were sealed in clear plastic and filter exposed for 24 hours in a phosphorimaging cassette and processed on a Phosphorimager (Molecular Dynamics) (2.1.12).

2.7.17 Phosphorimaging analysis

Northern blot filters were exposed to storage in Phosphor screens (Molecular Dynamics) for 24-48 hours and analysed using the ImageQuant software package on a Phosphorimager (Molecular Dynamics). Quantitation of the expression levels of *Fgf5* and *Rex1* were performed with respect to *Oct4*. The quantitated value of *Oct4* was used to divide the numerical expression values of *Fgf5* or *Rex1*, derived by ImageQuant. ES cell expression of *Fgf5* and *Rex1* with respect to *Oct4* was normalised to 100%. The up-regulation or down-regulation of *Fgf5* and *Rex1* expression levels in EPL cells was calculated as a % of ES cell expression. Images were quantitated using this package and images were exported and manipulated as Adobe Photoshop picture files.

2.7.18 Synthesis of DIG labelled RNA probes

Plasmid DNA was linearised with appropriate enzymes and purified DIG labelled riboprobes were assembled in 20 µl volume using the following reagents. Purified plasmid (0.5 µg) was added to 4 µl of 10x transcription buffer, 1x DTT, 1 U of RNasin (Geneworks), and 2 µl of 10xDIG labelling mix (Boehringer Manneheim). 2 U of appropriate RNA polymerase was added in a total

volume of 20 µl and left at 37°C for 3 hrs to form the RNA probe template. After incubation total volume was made up to 100 µl with 10 µl 0.1 M EDTA, 60 µl H₂O, and 10 µl of 20 mg/ml tRNA for precipitation. The riboprobe was precipitated with 11 µl of 3 M NaAc and 275 µl of RNase free EtOH at -80°C for 30 minutes. After centrifugation 14000 rpm, the probe was then resuspended in 100 µl of H₂O with 0.5 µl of RNasin.

2.7.19 Dot blot test of DIG labelled probe activity

Nitrocellulose was soaked in 20x SSC and air-dried. A 1 µl sample of labelled DIG probe was serially diluted 10 fold to 1/10000, heated at 85°C for 2 minutes, chilled on ice and 1 µl of each dilution spotted onto the filter before UV cross linkage. The filter was washed for 1 minutes in Buffer 1, 30 minutes in Buffer 2 (blocking reagent), 1 minute in buffer 1, and 30 minutes with anti-DIG Fab antibody-alkaline phosphatase conjugate (1:5000 dilution). Unbound antibody was removed by washing 2x15 minutes in Buffer 1 and the membrane equilibrated in Buffer 3 for 2 minutes. Freshly made reaction solution (4.5 µl NBT to 1 ml of Buffer 3 then 3.5 µl of BCIP) was added to filter and left for 30 minutes in the dark. Reaction was terminated by washing the membrane in Buffer 4 for 5 minutes. Probes used for in situ hybridisation were reactive to a 1/1000 dilution.

2.7.20 Protein analysis by SDS/reducing PAGE

Gel were formed using Protogel polyacrylamide and made as follows using mini-Protean II apparatus:

Acrylamide (Protogel)	3.33 ml
Tris-HCl pH8.8 (1M)	3.75 ml
MQ H ₂ O	2.75 ml
10% SDS	100 µl
APS	50 µl
TEMED	10 µl

Separating gel was poured between glass plates (Biorad), separated by 0.75 cm spacers, to within 1.5 cm from the top of the glass plates. Gel was overlaid with MQ H₂O and allowed to set at room temperature.

Acrylamide (Protogel)	667 µl
Tris-HCl pH6.8 (1M)	625 µl
MQ H ₂ O	3.63 ml
10% SDS	50 µl
APS	25 µl
TEMED	10 µl

Excess water was removed from above the separating gel. The 4% stacker gel was poured between the glass plates with the addition of a 10 well teflon coated comb. The stacker gel was allowed to set at room temperature. Protein samples were obtained directly from conditioned medium, or chromatographic fractions, and were desalted in a centricon-10 unit (Amicon). Up to 10 μ l of sample was mixed with 10 μ l of 2x protein GLB (containing 200 mM β -ME), heated for 2 minutes at 95°C and electrophoresed at 100Volts. Gels were visualised by Coomassie or the silver staining method of Heukeshoven and Dernick (1985).

Coomassie Blue Stain

Gels were placed in Coomassie solution for 4 hrs and allowed to agitate slowly on an orbital shaker. The Coomassie solution was removed from the gel by washing in fixing solution until the gel became clear and protein bands were clearly visible. Gel was dried down between two cellophane membranes clamped down tightly onto a glass plate for 3-4 days.

Silver stain

Polyacrylamide gels were placed in fixing solution for 30 minutes before transfer to incubation solution for a further 10 minutes. Two MQ water washes of 5 minutes each returned the gel to the hydrophilic phase. The gel was washed for 30 minutes in silver nitrate solution before two quick water washes and visualisation of the silver stain in developing solution until bands are clearly visible. Reaction is stopped by the addition of 0.1 M EDTA pH8.0.

2.7.21 Western blot analysis

Gels for Western blots analysis were washed for 30 minutes in western transfer buffer and electroblotted on a mini-Protean II apparatus (BioRad) in this buffer to Protran nitrocellulose membrane (Schleicher & Schuell). Filters were blocked overnight in PBT/5% skim milk powder. Monoclonal antibody 3E2 (Sigma) or TV-1 (Chemicon), specific for the EDA region of human (3E2/TV-1) and mouse (TV-1) cFN, was diluted 1/1000 (3E2) or 1/400 (TV-1) in 1% skim milk powder, PBT and incubated with the filter at room temperature for two hours. Filters were washed 3 times in PBT at room temperature, then incubated for 2 hours at room temperature in anti-mouse alkaline phosphatase or anti-mouse HRP (Dako) diluted 1/1000 in 1% skim milk, PBT.

Filters probed with the HRP conjugated secondary antibody (Dako) were wash 3 times for 5 minutes with shaking in PBT prior to reaction with Supersignal chemiluminescent substrate for 1 minute (Pierce). Filters were placed between plastic sheets and exposed to Curix blue HC-S plus medical X-ray film (AGFA) for 5-30 seconds prior to development of signal in a Curix 60 X-ray film developing machine (AGFA).

Filters probed with the alkaline phosphatase conjugated secondary antibody (Dako) were washed 3 times in PBT at room temperature, then incubated for 2 hours at room temperature in goat anti-mouse alkaline phosphatase antibody (Dako) diluted 1/1000 in 1% skim milk, PBT. A 20

minute wash in western buffer 1 was followed by 2x 20 minute washes in buffer 2. The reaction was developed in western substrate mix in the dark and stopped by the addition of 10 ml buffer 3.

2.7.22 N-terminal protein sequencing

Gels for N-terminal sequencing analysis were washed for 30 minutes in western transfer buffer and electroblotted in a mini-Protein II apparatus (BioRad) in this buffer to PVDF nitrocellulose membrane (Immobilon). Proteins of the transferred gel were visualised by Coomassie and removed from the filter by clean scaple blade and subjected to Edman degradation in an N-terminal protein sequencer.

2.8 Histological analysis

2.8.1 Chemicals

BCIG: Sigma Chemical Co.

BSA: Boheringer Manneheim

EtOH:

Histoclear: Ajax Chemicals

Paraformaldehyde: MERCK Chemical Co.

Ribonuclease A: Sigma Chemical Co.

Salmon sperm DNA: Boheringer Manneheim

Levamisole: Sigma Chemical Co.

2.8.2 Solutions

Solution A: 0.82g $K_3Fe(CN)_6$, 1.05g $K_4Fe(CN)_6 \cdot 3H_2O$, 1 ml 1 M $MgCl_2$, 0.5 ml NaDOC, 1 ml NP40, 25 ml 0.1 M EDTA, MQ H_2O to 500 ml.

Buffer 1: 100 mM Tris-HCl pH 7.4, 100 mM NaCl

Buffer 2: Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$

Substrate mix: 10 ml buffer 2, 40 μ l NBT (75 mg/ml in 70% DMF), 40 μ l BCIP (50 mg/ml in 100% DMF)

Buffer 3: Buffer 1, 1 mM EDTA

2.8.3 Antibodies

Mouse (IgM) anti-human EDA⁺ cFN monoclonal: Sigma Chemical Co. (3E2)

Mouse (IgG) anti-mouse/human cFN monoclonal: Chemicon (TV-1)

Goat (IgG) anti-mouse AP-conjugate: (Dako)

2.8.4 Miscellaneous

Tissue-Tek cassettes: Sakura Finetek BV

Paraplast plus tissue embedding medium: Oxford Labware

OCT medium:

Superfrost glass slides: Biolab scientific

EG1120 tissue embedding machine: Leica

RM2135 microtome: Leica

Cryostat microtome: Leica

2.8.5 Histological sections of EB's and mouse embryos

~~Paraffin wax sections of~~ tissues for histological analysis were fixed in 4% PFA/PBS for 2 hours and dehydrated in 50% EtOH for 10 minutes and 70% EtOH for storage at 4°C. Processing

of the tissues for embedding involved an initial 1 hour wash in 70% and then 80% EtOH followed by 2 additional washes in 95% (1 hour) and 100% (2 hours) EtOH. Bodies were washed for 1 hour in HistoClear:EtOH (50:50) and twice for 2 hours in 100% HistoClear. Tissues were placed in metal moulds and washed with paraffin wax 3 times prior to embedding using an EG1120 tissue embedding machine (Leica). Samples were placed into Tissue-Tek cassettes (Sakura Finetek BV) and stored at 4°C. Sections (6-10µm) were cut on the RM2135 microtome (Leica) and collected on glass slides.

Cryosections were generated by washing EB's in ice cold PBS prior to fixation in 2.5% paraformaldehyde for 1 hour on ice. Incubations in cold 0.1 M glycine for 1 hour were followed by transfer to 0.6 M sucrose for 4 hours. EB's were positioned in OCT embedding medium in plastic moulds, frozen on dry ice and stored at -80°C until required. Frozen embedded sections were cut into 8µm sections using a cryostat microtome and applied to treated glass slides. Sections were air-dried overnight prior to immunohistochemistry analysis.

2.8.6 In situ hybridisation of histological sections

EB and mouse embryo histological sections were air dried and wax removed by two washes in 100% HistoClear. Slides were then washed for 2 minutes in 100%, 80% and 70% EtOH and washed in MQ H₂O for an additional 5 minutes. Slides were returned to PBS for 5 minutes and then post-fixed in 4% PFA/PBS solution for 10 minutes. Slides were washed twice for 5 minutes in freshly made 0.1% active DEPC/PBS (embryonic sections) or RIPA buffer (EB sections) and washed in 5x SSC for 10 minutes. Slides were placed in 30 µl pre-hybridisation solution (prepared at 52°C), covered gently in parafilm and incubated at 60°C for 1 hour. Probe was added to fresh prehybridisation mix and added to slides, under fresh parafilm, prior to placement in a humid chamber (50% Formamide and 2x SSC). Slides were incubated for 40 hours at 60°C and were then washed in 2x SSC at room temperature and 65°C for 30 minutes. Slides were washed in buffer1 at room temperature for 5 minutes and then in an anti-DIG/AP conjugated monoclonal antibody diluted 1/1000 in buffer1 + 0.5% blocking reagent for 2 hours. Slides were washed twice for 15 minutes in buffer1 and in buffer2 for 5 minutes. Substrate mix was added and left overnight. Reaction was stopped by immersing the slides in buffer 3.

2.8.7 Immunohistochemistry

Cell lines

Cells were fixed in 4% PFA/PBS for 1 hour, transferred to 50% EtOH for 10 minutes and stored at 4°C in 70% EtOH until required. Cells were washed 3 times in PBS for 5 minutes, 15 minutes in PBS 0.1% Tween 20 followed by 3 additional washes in PBS. Cells were blocked for 1 hour in 3% goat serum and 2% BSA in PBS. The TV-1 anti-mouse cFN monoclonal antibody was diluted 1:200 in blocking solution and incubated with cells for 2 hours at room temperature. The cells were washed 3 times for 5 minutes in PBS and once for 5 minutes in buffer1. Goat anti-mouse

IgG conjugated with AP was diluted 1:4000 in buffer1, supplemented with 3% Goat serum and 2% BSA, and incubated with cells overnight at 4°C. Cells were washed 2 times for 15 minutes in buffer1 followed by 5 minutes in buffer2. Immunostaining was observed by submerging cell in substrate mix supplemented with BCIP/NBT and 5 mM Levamisole. Reaction was left in the dark until suitable exposure was observed and stopped by transferring cells into buffer3. Cells were photographed using a Nikon microscope T300 microscope under 60x bright field magnification.

Tissue sections

Tissue sections were washed 3 times in PBS for 5 minutes followed by a 15 minute wash in PBS 0.1% Tween 20 and 3 additional washes in PBS for 5 minutes. Cells were blocked for 1 hour in 3% goat serum and 2% BSA in PBS. The TV-1 anti-mouse cFN monoclonal antibody was diluted 1:200 in blocking solution and incubated with cells for 2 hours at room temperature. The slides were washed 3 times for 5 minutes in PBS. Goat anti-mouse IgG conjugated TRITCI fluorescent label was diluted 1:50 in blocking solution and incubated with cells overnight at 4°C. Cells were washed 3 times for 5 minutes in PBS and mounted under glass slides in 80% glycerol. Immunostaining was observed and photographed under 20x phase contrast magnification using a Nikon microscope and fluorescent TRITCI red UV radiation

2.8.8 Containment Facilities

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

Chapter 3

Characterisation and partial purification of the high molecular weight EPL cell inductive signal

Chapter 3

3.1 Introduction

Signals within MEDII induce ES cells to form EPL cells in culture (Rathjen *et al.*, 1999). Purification and identification of these signals from MEDII is a pre-requisite for the formation, and further differentiation of EPL cells in the absence of contaminating factors contained within MEDII. In particular, purification and identification of these signals may prove advantageous in terms of EPL cell stability and homogeneity *in vitro* and furthermore, provide insights into pluripotent cell biology during embryogenesis (1.15).

The formation of EPL cells mimics the creation of primitive ectoderm within the early mouse embryo with respect to gene expression, differentiation potential and growth factor responsiveness. This suggests that signals directing EPL cell development are equivalent to the primitive ectoderm inductive signals *in vivo*. Partial purification and isolation of EPL cell inductive factors identified at least two signals required for EPL cell induction: a low molecular weight and a high molecular weight signal (Rathjen *et al.*, 1999). The low molecular weight signal was identified as the imino acid L-proline, or L-proline in the context of short peptides. However, the identity of an L-proline equivalent signal in the embryo remains unknown. Therefore, the expression and localisation of this signal cannot be mapped in the embryo. Identification of the high molecular weight signal however, may provide a more tractable system for analysing EPL cell inductive signal function *in vitro* and *in vivo*. The aim of the work presented in this chapter was the purification of the high molecular weight, EPL cell inductive signal from MEDII.

3.2 Morphology based cell assay for detection of the high molecular weight, EPL cell inducing signal

The high molecular weight fraction was prepared from 1 L of sfMEDII (2.2.2), that retains comparable EPL cell inducing activity to MEDII (Rathjen *et al.*, 1999), concentrated over a Y10 (10kd cut off) filtration membrane used to the manufacturers instructions (Amicon; 2.3.9). The retained fraction contains high molecular weight sfMEDII components, and was subjected to gel filtration over a G10 de-salting/gel filtration column, previously equilibrated in PBS, to the

manufacturers specifications (PD10, Pharmacia). The high molecular weight fraction was eluted in PBS, to remove remaining low molecular weight activity, and diluted in PBS to a final concentration of 1 mg/ml. This material was termed Retentate (R).

EPL cell inducing activity of the high molecular weight component was assessed by seeding 500 D3 ES cells into 0.2% gelatin coated 1.75 cm² tissue culture wells containing 1.0 ml ES DMEM supplemented with 1000 U mLIF and 40 µM L-proline (2.3.10). These conditions were established to facilitate detection of the high molecular weight signal during morphological EPL cell formation (Jenny Washington, data not shown). R (50 µg) was added to a 1 ml volume cell based assay in a maximum volume of 50 µl. Following the addition of R to cell assays, colony morphology was assessed throughout the 5-day culture period by phase contrast microscopy. After 5 days, pluripotent cells were stained for the expression the pluripotent cell marker alkaline phosphatase, using the kit 86-R (Sigma, Australia) (Figure 3.1).

A single unit of high molecular weight activity was defined as the minimum amount of the high molecular weight fraction required to induce > 95% pluripotent cell colonies to adopt the EPL cell morphology, in the presence of 40 µM L-proline. This was determined by titration assay to be 25 µg/ml of ES cell culture medium (data not shown). Therefore, the specific activity of the high molecular weight signal under these conditions, defined as the number of U/mg of protein in an assay volume of 1 ml, was 40 U/mg.

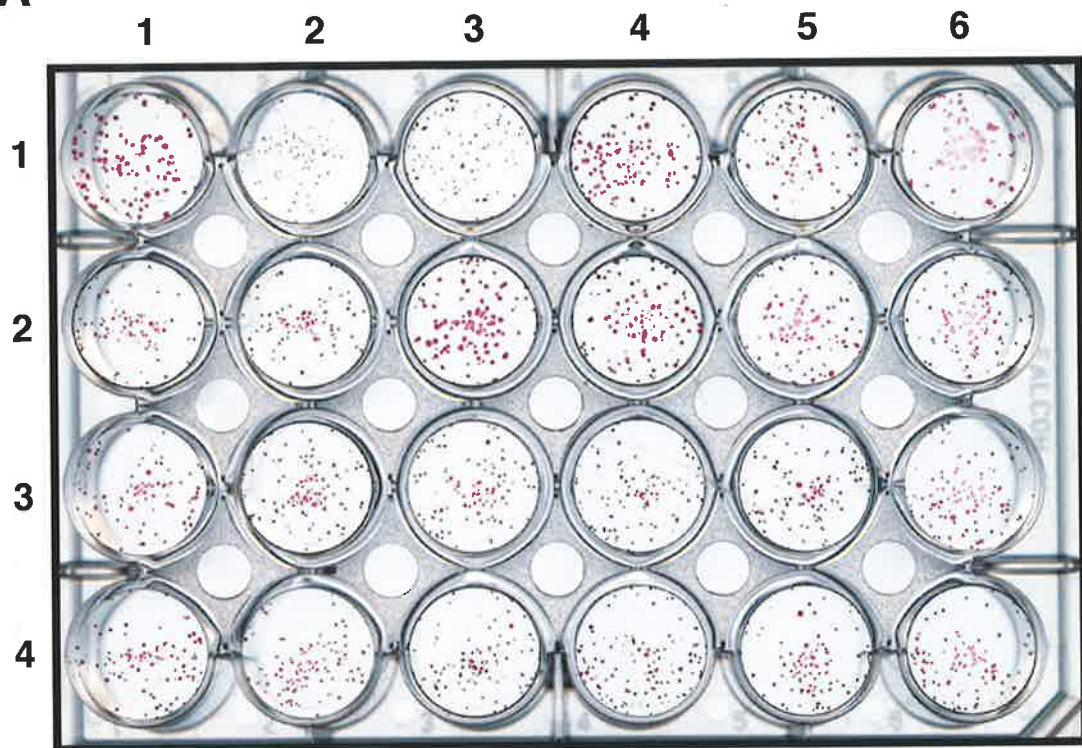
3.3 Characterisation of the biochemical properties of the high molecular weight signal

2 mls of R (containing 80 U of high molecular weight activity) was subjected to a range of temperatures (-20°C, 4°C, 25°C, 37°C, 56°C or 96°C) for 1 hour. Following these treatments the high molecular weight component was assayed for morphological induction of EPL cells in the presence of 40 µM L-proline (3.2; Figure 3.1). EPL cell inducing activity was lost following treatment of samples at 56°C or 96°C for 1 hour (Figure 3.2 C, D, E, F), but was not affected by treatment at 37°C or below (Figure 3.2 A, B, data not shown). Additionally, 1 ml of R (40 U) was treated with 100 µg of trypsin (Gibco, BRL) in 20 mM Tris-HCl, 20 mM CaCl₂ for 1 hour at 25°C

Figure 3.1 Representative cell based assay for identification of the high molecular weight EPL cell inductive factor

(A) Morphology based detection of EPL cell formation using the assay described in 3.2 after 5 days. (B) Schematic representation of assay parameters; +: EPL cell morphology > 95%; -: ES cell morphology >95%; +/-: approximately 50% ES/EPL cell morphology; EPL: D3 ES cells cultured in 50% MEDII; ES: D3 ES cells; R: sfMEDII proteins (50 µg/ml); P: 40 µM L-proline; Spont: D3 ES cells cultured in absence of LIF. In rows 2, 3 and 4, ES cells were cultured in 40 µM L-proline and partially purified samples of R. Pluripotent cells were stained with alkaline phosphatase following 5 days culture. Magnified images representative of the colony morphology of ES and EPL cells are depicted in Figures 3.2-3.4.

A



B

	1	2	3	4	5	6
1	EPL +	ES -	ES+R -	ES+R+P +	ES+P -	Spont
2	-	-	+	+	+/-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-

in a 2 ml volume. An approximate 25-fold excess of protease was used due to the presence of the anti-trypsin inhibitor alpha-1-antitrypsin in MEDII (Bouma *et al.*, 1989). The reaction was stopped by the addition of 100 μ M Phenyl Methyl Sulfonyl Fluoride (PMSF). PMSF was removed from the digested proteins by buffer exchange to PBS in centricon 10 columns (Amicon). 1 ml of R was incubated under identical conditions in the absence of protease as a control. Treatment of R with trypsin resulted in complete loss of EPL cell inducing activity in the presence of 40 μ M L-proline (3.2; Figure 3.2 E, F). These data suggest that the high molecular weight EPL cell inductive component(s) of MEDII is a heat and trypsin sensitive protein.

Determination of the biochemical/biophysical properties of the high molecular weight activity is important for the design of suitable purification conditions. To test the effect of pH on the high molecular weight EPL cell inductive capacity, 1 ml of R (40 U) was incubated for 1 hour in a 2 ml final volume, between pH4-9 at room temperature. Samples were incubated in the following buffers (50 mM Sodium Citrate (pH4.0), 50 mM Acetic acid (pH5.0), 50 mM Na_2HPO_4 (pH 6.0), PBS (pH7.4), 50 mM Tris-HCl (pH8.0) and 50 mM Sodium Borate (pH9.0). Samples were buffer exchanged to PBS using centricon-10 columns (Amicon) and assayed for EPL cell inducing activity (3.2). EPL cell inducing activity was observed between pH9.0-6.0, but was lost below pH6.0 in the presence of 40 μ M L-proline (Figure 3.3). These data indicate that the high molecular weight EPL cell inductive activity is acid sensitive but stable between pH6-9.

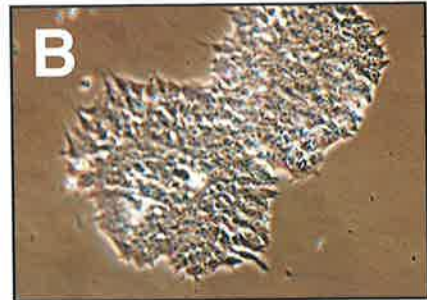
R (1 ml/40 U) was treated at a final concentration of 0.1 mM, 1 mM, 10 mM and 100 mM of the reducing agents Dithiothreitol (DTT) or β -Mercaptoethanol (β -ME) (Sigma, USA for 1 hour at room temperature in a final volume of 2 mls in PBS. Reducing agents were removed and samples returned to PBS in centricon 10 columns (Amicon), since reducing agents have deleterious effects on ES cell and EPL cell culture above 100 μ M (Smith, 1991), and samples were assayed for the high molecular weight activity (3.2). The active component in R was stable below 0.1 mM reducing agent, but destroyed at 10 mM or above (3.2; Figure 3.4). These data suggest that the high molecular weight EPL cell inductive signal is sensitive to reducing agents and may be composed of a molecule(s) linked by disulphide bonds.

Figure 3.2 The high molecular weight EPL cell inductive component of sfMEDII is sensitive to high temperature and the protease trypsin

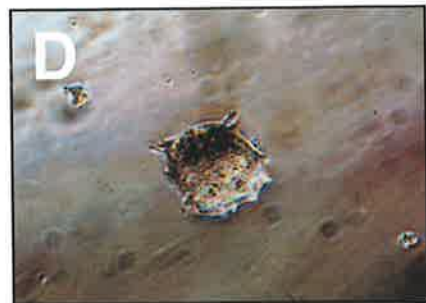
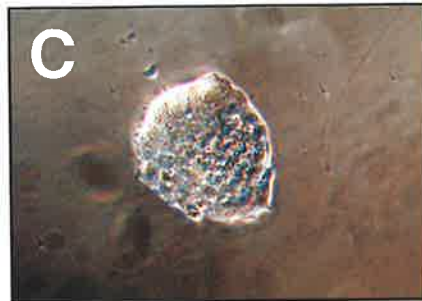
R (2 mg/80 U) was treated for 1 hour at 37°C (A,B), 56°C (B,C) or 96°C (C,D) prior to EPL cell morphology assay (3.2) in the absence (A,C,E) or presence (B,D,F) of 40 µM L-proline. R (1 mg/40 U) was treated with 100 µg of trypsin at 25°C for 1 hour (G,H) prior to EPL cell morphology assay (3.2) in the absence (G) or presence (H) of 40 µM L-proline. Treated R (2 or 1 mg respectively) was added to assay in maximum volume of 50 µl. Images were taken on a Nikon ELW D0.3 phase contrast microscope at 200x magnification after 4 days culture.

+ 40 μ M L-proline

37°C



56°C



96°C



Trypsin

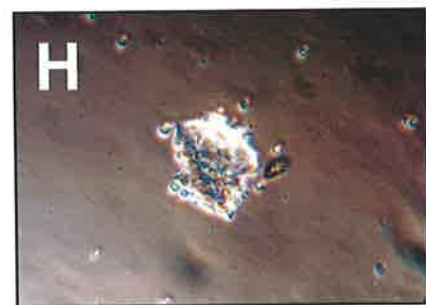
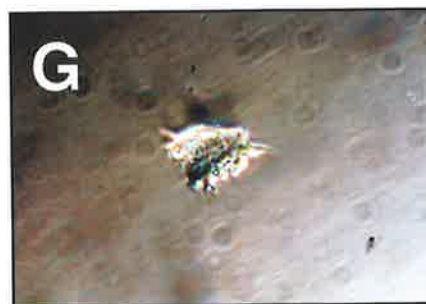


Figure 3.3 The high molecular weight EPL cell inductive component is sensitive to acidic pH

R (1 mg/40 U) was treated for 1 hour at room temperature at pH4.0 (A, B) pH5.0 (C, D) pH6.0 (E, F) pH8.0 and (G, H) and pH9.0 (I, J) prior to EPL cell morphology assay (3.2).¹ R (1 mg/40 U) was assayed post-treatment in the presence (B, D, F, H & J) or absence (A, C, E, G & I) of 40 μ M L-proline for EPL cell inductive activity. Images were taken on a Nikon ELW D0.3 phase contrast microscope at 200x magnification after 4 days culture.

+ 40 μ M L-proline

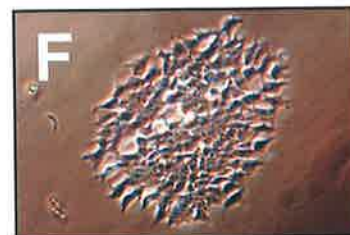
pH4.0



pH5.0



pH6.0



pH8.0



pH9.0

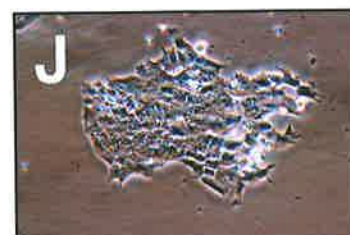
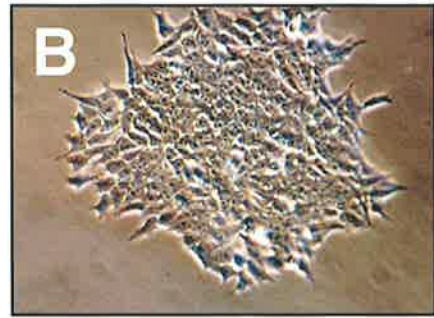


Figure 3.4 The high molecular weight EPL cell inductive component of MEDII is sensitive to reducing agents

R (1 mg/40 U) was treated for 1 hour at room temperature in 1mM (A,B) and 10mM (E,F) and 1mM (C,D) β -mercaptoethanol and 10mM (G,H) DTT prior to assessment of EPL cell inductive activity by morphology assay (3.2) in the presence (B,D,F,H) or absence (A,C,E,G) of 40 μ M L-proline. Images were taken on a Nikon ELW D0.3 phase contrast microscope at 200x magnification after 4 days culture.

+ 40 μ M L-proline

1mM DDT



1mM β -ME



10mM DDT



10mM β -ME



Collectively, these data suggest that the high molecular weight, EPL cell inductive signal within MEDII is a protein, sensitive to heat, acidic pH, reducing agents and the protease trypsin. These conditions define the biochemical parameters for the development of a purification strategy for this signal.

3.4 Binding of the high molecular weight activity to chromatographic stationary phase

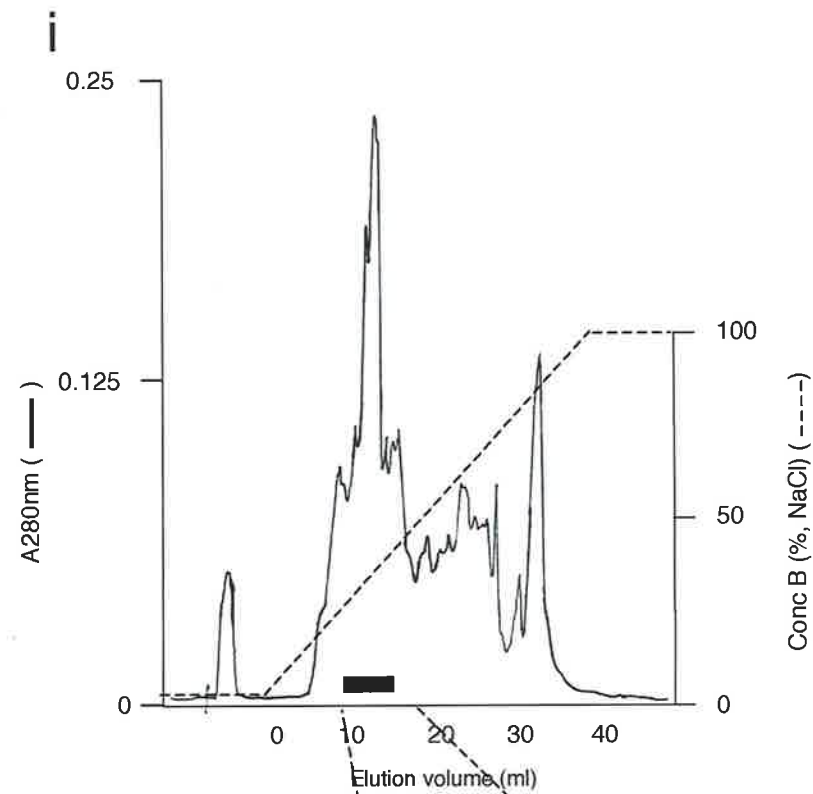
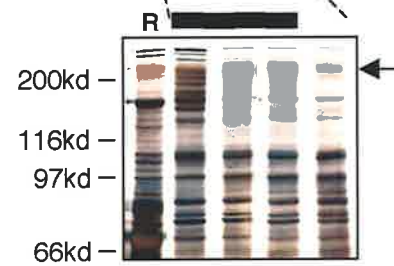
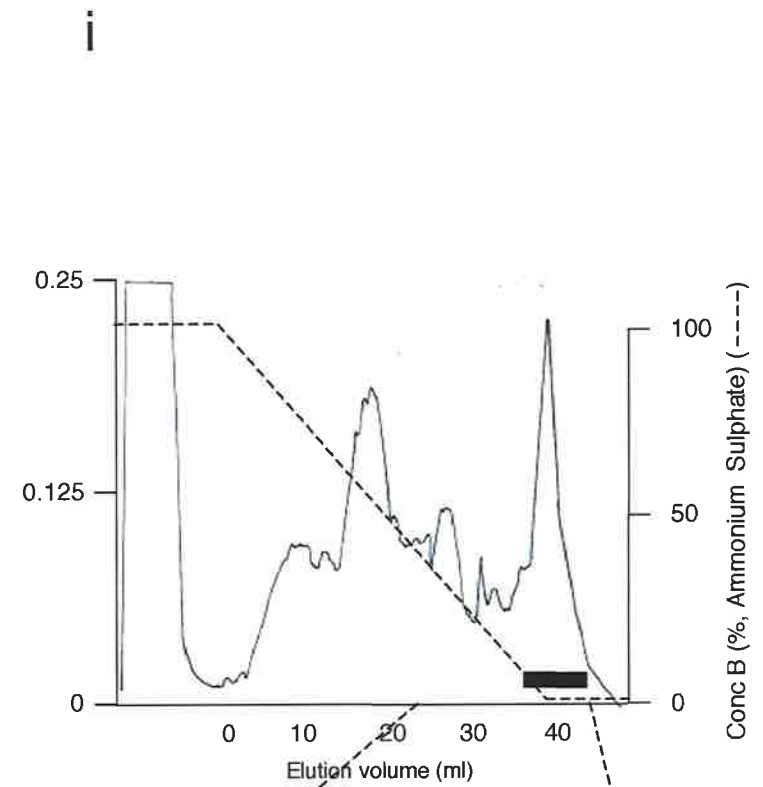
The high molecular weight EPL cell inductive signal was tested for its ability to bind to, and elute from, adsorbent chromatographic matrices. This analysis was performed to establish if the high molecular weight signal was particulate in nature and to provide a basis for further purification. All purifications were performed using 1 ml of R under conditions of basic pH (20 mM Tris-HCl pH8.5). Bradford reagent assay (BioRad) used to the manufacturers instructions quantitated protein yield. The specific activity of semi-purified material was determined by titration of known quantities of active samples to EPL cell morphology assay.

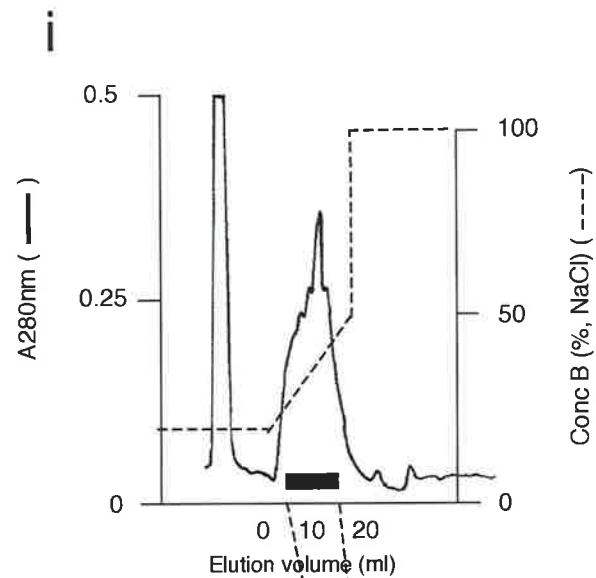
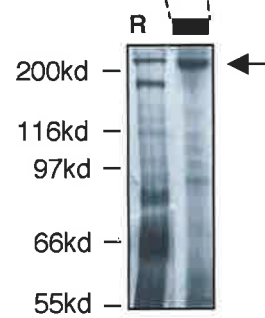
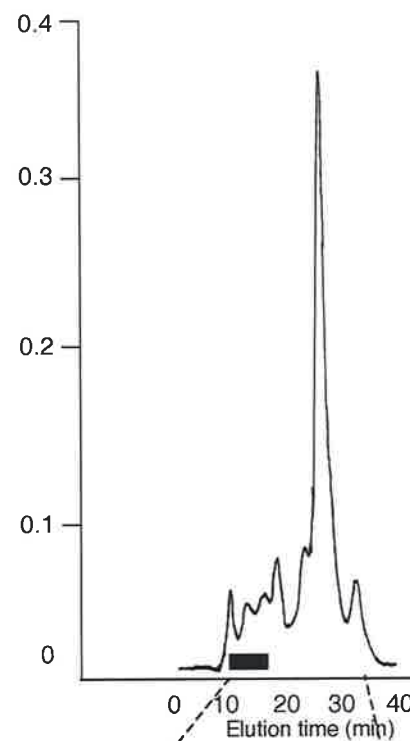
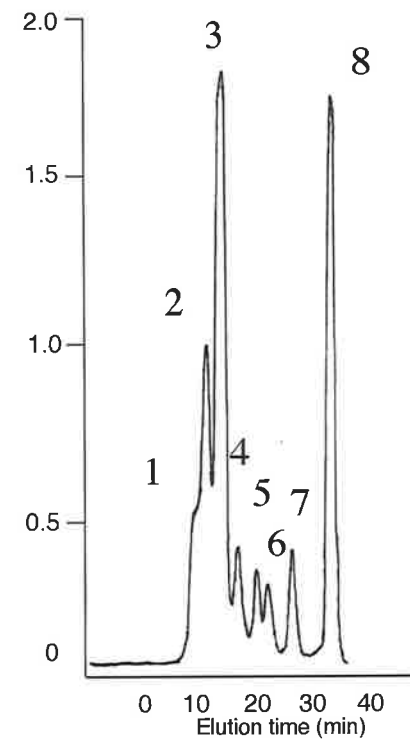
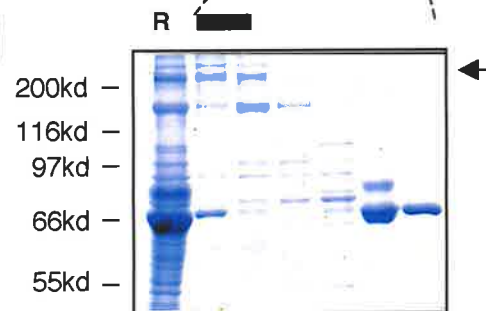
R (1 mg/40 U) was passed over a 1 ml anion exchange (MonoQ, Pharmacia; 2.5.2.1; Figure 3.5 A(i)) or 1 ml heparin sepharose CL6B affinity column (Pilot test column; Pharmacia; 2.5.2.3; Figure 3.5 B(i)) at a flow rate of 1 ml per minute, after dilution in an equal volume of 20 mM Tris-HCl pH8.5. Additionally, 1 ml of R was mixed slowly with an equal volume of 2 M ammonium sulphate in 20 mM Tris-HCl pH8.5 and centrifuged for 5 mins at 14,000 rpm prior to passage at a flow rate of 1 ml/minute over a 1 ml hydrophobic interaction column (Pharmacia) previously equilibrated in 20 mM Tris-HCl pH8.5, 1 M ammonium sulphate (2.5.2.2; Figure 3.5 B(i)). Proteins were eluted from anion exchange and heparin sepharose affinity columns by the graded or stepwise addition of 1 M NaCl in 20 mM Tris-HCl pH8.5 respectively and collected in 2 ml sample volumes (Figure 3.5 A,C (i)), while proteins bound to phenyl sepharose in 1 M ammonium sulphate in 20 mM Tris-HCl at pH8.5 were eluted by the graded addition of 20 mM Tris-HCl and collected in 2 ml sample volumes. Samples were buffer exchanged to PBS using centricon 10 columns (Amicon) prior to the addition of 1-50 μ l of sample (containing a maximum of 50 μ g) to cell assays for detection of the high molecular weight activity (3.2; Figure 3.5 B (i)). The EPL cell inductive

Figure 3.5 The high molecular weight EPL cell inductive component of sfMEDII can be purified using chromatographic media and has a molecular weight greater than 500kd

R (1 mg/40 U) was bound to, and eluted from, anion exchange (A), phenyl sepharose (B) and heparin sepharose (C) matrix or eluted from a superose 6 gel filtration chromatography column (D). Fractions (2 ml: A,B,C or 50 μ l: D) were assayed for the high molecular weight EPL cell inductive activity by morphology assay (3.2). Fractions containing the activity are represented in each case by the thick black bar.

- i) Proteins were detected at 280 nm. Dotted lines represent % of 1M NaCl in 20mM Tris-HCl pH 8.5 (A,C) or 1M ammonium sulphate in 20mM Tris-HCl pH 8.5 (B). High molecular weight standards separated under identical conditions on a superose 6 gel filtration column are shown in D (iii).
- ii) Column fractions were visualised by coomassie (10 μ g; (B,C (ii)) or silver (1 μ g; (A,D (ii)) stain of 10% SDS reducing-PAGE. The arrow indicates a 250kd molecular weight species present in all samples which contain the high molecular weight EPL cell inductive activity. Molecular weight markers (Amrad; 2.6.9) are indicated.

A**ii****B****ii**

C**ii****D****i****ii****iii**

- 1 Blue dextran (1000kd)
- 2 Thyroglobulin (660kd)
- 3 Ferritin (440kd)
- 4 Immunoglobulin (160kd)
- 5 Transferrin (77kd)
- 6 BSA (67kd)
- 7 Myoglobin (20kd)
- 8 Vitamin B12 (1355d)

activity eluted in 250-350 mM NaCl from anion exchange (Figure 3.5 A (i); Rathjen *et al.*, 1999), in 150-500 mM NaCl from the heparin affinity column (Figure 3.5 C (i)), and between 100-0 mM Ammonium Sulphate (Figure 3.5 B(i)). The elution of this activity from 3 separate purification matrices indicates the high molecular weight EPL cell inductive component is a single entity.

R (1 ml loaded in 10 sequential 50 μ l runs) was separated on a superose 6 gel filtration column equilibrated in 20 mM Tris-HCl, 150 mM NaCl using the Smart systemTM micro-preparative separation apparatus and collected in 50 μ l sample volumes (330 μ g; Pharmacia; 2.5.2.4). Samples (50 μ l/5 μ g) were used directly in EPL cell inductive assays (3.2). The EPL cell inductive activity eluted in the void volume of the superose 6 column (Figure 3.5 D(i)). An assay of an equivalent experiment is represented in Figure 3.1. High molecular weight standards passed down the Superose 6 column under identical conditions determined the elution time and approximate native molecular weight of the high molecular weight activity (Figure 3.5 D(iii)). Blue dextran (1000kd) and Thyroglobulin (660kd) standards eluted in the void volume but Ferritin (440kd) was retained on the column. These data suggest that high molecular weight EPL cell inductive signal is greater than 440kd in its native form.

Partially purified samples from the single purification steps were separated on SDS/reducing polyacrylamide gels and visualised by silver (2.7.20; 1 μ g/well; Figure 3.5 A,C (ii)) or Coomassie stain (2.7.20; 10 μ g/well; Figure 3.5 B,D (ii)). In general samples containing the high molecular weight signal were a mixture of proteins. However, the active fractions eluted from heparin sepharose were dominated by a high molecular weight species of about 250kd that was also present within fractions from the other pilot purifications that contain the high molecular weight activity. In particular this species was present at highest levels in the active samples derived from heparin affinity chromatography that contained the highest specific activity (Table 3.1).

The native molecular weight of the high molecular weight signal is greater than or equal to 500kd. However, polyacrylamide gels suggested the activity may be associated with a 250kd species. This suggests that the high molecular weight species may be a protein complex. To examine if the high molecular weight activity was composed of non-covalently associated proteins, 1 ml (100 μ g) of sample from the void volume of a superose 6 gel filtration, purified from 1 ml of R, was denatured

Table 3.1 Purification, specific activity and yield of the high molecular weight EPL cell inductive activity from sfMEDII proteins fractionated by pilot scale chromatographic purification

Samples containing the high molecular weight EPL cell inductive activity were determined by assay (3.2). Protein yields in active fractions were quantitated by Bradford assay (BioRad) used to the manufacturers instructions. Fractions containing activity were determined by cell assay (3.2). Specific activity was determined by titration of 10 μ g-0.1 μ g (max volume 50 μ l) of the high molecular weight signal from partially purified samples and minimum activity determined by cell based assay (3.2). Total units recovered is the multiple of the yield and specific activity. The activity recovered is % of units compared to R (1 ml/40 units). Fold purification is the increase in specific activity from the unpurified sample.

	Protein Yield (%)	Specific activity (Units/mg/ml)	Total Units	% Activity Recovered	Fold purification
sfMEDII		40	80		
Anion exchange	45	80	36	90	2
Phenyl Sepharose	20	200	26	66	5
Heparin affinity	10	400	18	45	10
Superose 6 GF	30	133	26	66	3.3

by the addition of guanidine HCl or urea to a final concentration of 6 M and 8 M respectively in a 2 ml volume. This sample was then concentrated by centricon (Amicon) and a 50 μ l was passed down a superose 6-column gel filtration column. The re-elution of these proteins in the void volume suggests that treatment of the active sample with these denaturants did not reduce the molecular size of the eluted activity (data not shown). This report suggests that the high molecular weight factor is not a complex of non-covalently associated proteins.

The specific activity of samples eluted from chromatographic medium was calculated by the titration of 10 μ g-0.1 μ g of partially purified sample into cell based assays in a maximum volume of 50 μ l. Specific activity was derived from the minimum amount of protein required to induce the EPL cell morphology in the presence of 40 μ M L-proline and is represented in Table 3.1.

The binding of the high molecular weight protein activity to specific stationary phase chromatographic medium provides information as to the nature of the high molecular weight EPL cell inductive factor. (1) Isolation of a single active species from a variety of columns indicates that the high molecular weight signal is a single entity. (2) The elution of this signal from superose 6 gel filtration column suggests that the signal has a molecular weight greater than 500kd. Failure of Urea or guanidine HCl to denature partially purified protein containing the high molecular weight signal suggests that this signal is not a non-covalently bound protein complex. However, denaturation experiments indicate the active sample may contain di-sulphide bonds consisted with the appearance of a species less than 500kd on polyacrylamide gels. (3) Binding of the active protein to anion exchange stationary phase matrix indicates the factor retains a net negative charge at pH8.5. (4) Interaction with phenyl sepharose matrix indicates that the EPL cell inductive factor contains hydrophobic regions. (5) The binding of the activity to the highly sulphated glycoprotein heparin suggests that the high molecular weight EPL cell inductive signal is a heparin binding enzyme, growth factor or ECM protein (Scopes, 1988).

Collectively, these data suggested that the high molecular weight EPL cell inductive activity within sfMEDII can be purified from solution through the sequential application of chromatographic purification protocols.

3.5 Purification of the high molecular weight signal: Method1 (2.5.3)

The biochemical properties of the high molecular weight EPL cell inducing signal were used for the development of a sequential purification procedure which incorporated filtration over a 100kd (Y100, Amicon) membrane followed by elution from anion exchange, hydrophobic interaction, heparin affinity and superose 6 gel filtration chromatography columns.

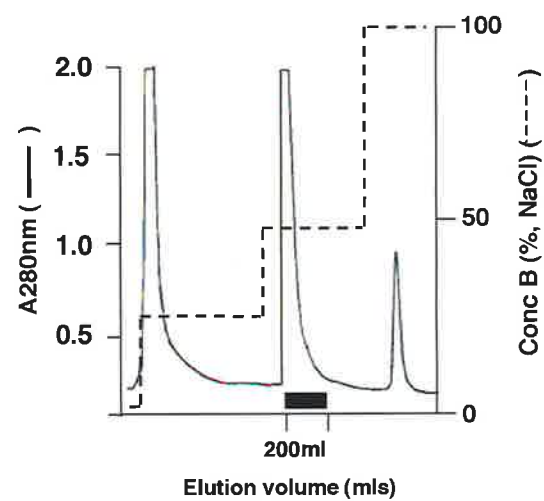
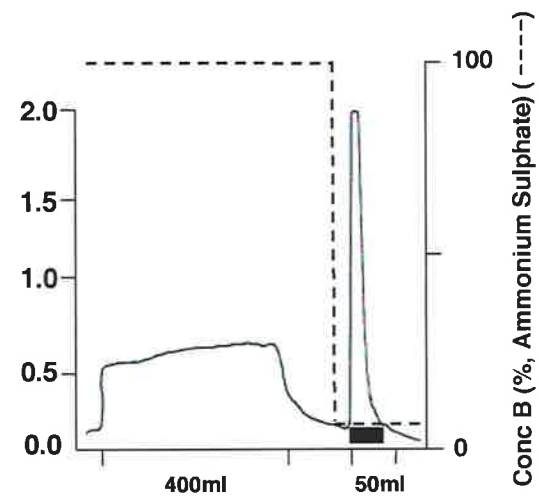
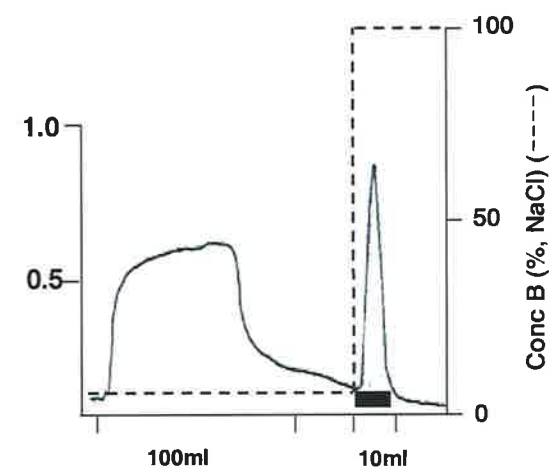
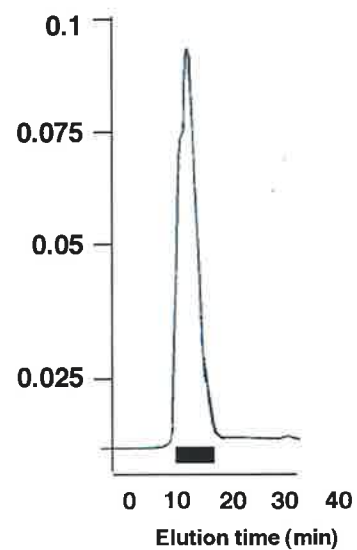
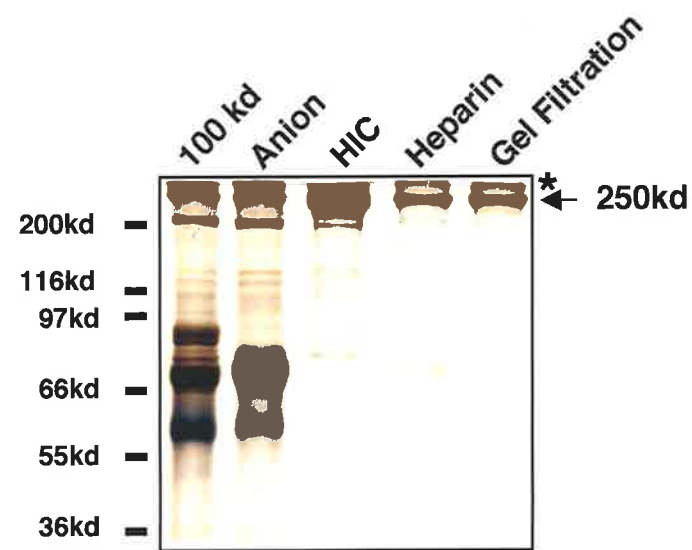
Four litres of sfMEDII containing 454 mg of HepG2 derived protein was concentrated 13 fold in a 400 ml concentrator (Y100; Amicon), with buffer exchange to 20 mM Tris-HCl, pH8.5 in a final volume of 300 ml. The concentrated sfMEDII proteins (360 mg) were passed over a 300 ml Q sepharose fast flow anion exchange XK-50 column pre-equilibrated in 20 mM Tris-HCl pH 8.5, at a flow rate of 5 ml/min (Pharmacia; 2.5.3.1). Re-equilibration of the column in 20 mM Tris-HCl pH8.5 was followed by stepwise elution of bound proteins. Weakly bound proteins were eluted by addition of 20 mM Tris-HCl pH8.5, 200 mM NaCl and collected in a volume of 200 ml. The retained proteins were eluted from the anion exchange column by the addition of 20 mM Tris-HCl, 500 mM NaCl and collected in a volume of 200 ml. A final elution in 20 mM Tris-HCl, 1 M NaCl was also collected in 200 ml (Figure 3.6 A). All samples used in cell assays (3.2) were returned to PBS by centricon 10 columns prior to use (Amicon). Proteins eluting in the 500 mM NaCl fraction were shown by cell assay to contain the EPL cell inducing activity (2.3.10). The specific activity was determined using titrations of this sample from 25-0.1 µg/well (3.2). The specific activity and fold purification of this step (Table 3.2) was reduced when compared to the pilot purification (Table 3.1).

The active fraction from anion exchange chromatography (180 mg) was carefully mixed 1:1 with 1 M ammonium sulphate in 20 mM Tris-HCl pH 8.5 and filtered through a 0.22 µm filter to remove precipitated protein material, prior to passage over a 40 ml phenyl sepharose fast flow, hydrophobic interaction column^(HIC) at a flow rate of 5 ml/min (Pharmacia; 2.5.3.2). Bound protein was eluted from the column in a single step in 50 ml of 20 mM Tris-HCl pH 8.5 (Figure 3.6 B). The 55.9 mg of eluted protein was prepared for cell assays as previously described and was shown by EPL cell morphology assay to contain the high molecular weight EPL cell inducing activity. The activity and yield (Table 3.2) were reduced when compared to the pilot purification (Table 3.1).

Figure 3.6 Purification of the high molecular weight factor: Method 1

Partial purification and gel electrophoresis of sfMEDII derived proteins buffer exchanged over a 100kd ultra-filtration membrane prior to sequential purification via anion exchange (A), hydrophobic interaction (B), heparin affinity (C) and superose 6-gel filtration (D) chromatography. High molecular weight EPL cell inductive activity was detected by cell based assay (3.2). Fractions containing the activity are represented in each case by the thick black bar. Proteins were detected at 280 nm. Dotted lines represent % of 1M NaCl in 20mM Tris-HCl pH 8.5 (A,C) or 1M ammonium sulphate in 20mM Tris-HCl pH 8.5 (B).

A reducing/denaturing 10% SDS-poly-acrylamide gel of the partially purified fractions containing the high molecular weight activity (3.2) (E). Arrow indicates the 250kd species. * indicates the high molecular weight aggregate that fails to enter the separating gel. Loadings: 100kd (5 μ g), Anion exchange (5 μ g), hydrophobic interaction (phenyl sepharose) (2 μ g), heparin affinity and superose 6 gel filtration (1 μ g). Molecular weight markers (Amrad; 2.6.9) are indicated.

A**B****C****D****E**

The active sample from hydrophobic interaction chromatography was diluted in 20 mM Tris-HCl pH8.5 to the conductivity of 100 mM NaCl in a final volume of 90 ml. The sample was applied to a 25 ml heparin sepharose CL-6B affinity column, pre-equilibrated in 20 mM Tris-HCl pH8.5, at a flow rate of 5 ml/min (Pharmacia; 2.5.3.3). The column was re-equilibrated to 20 mM Tris-HCl, 100 mM NaCl prior to the elution of bound proteins in a single step by the addition of 20 mM Tris-HCl pH8.5, 500 mM NaCl at a flow rate of 5 ml/min (Figure 3.6 C). The sample containing 7.5 mg of protein was collected in a 10 ml volume. The eluting fraction was shown by cell based assay to contain the EPL cell inductive signal at a specific activity and yield similar to the pilot purification (Table 3.2; Table 3.1).

The active sample from the heparin affinity chromatography purification (1 ml/ 750 µg) was concentrated and buffer exchanged to 20 mM Tris-HCl, 150 mM NaCl in a centricon 10 spin column (Amicon) and applied (50 µl) to a micro preparative superose 6-gel filtration column, using the Smart systemTM apparatus (Pharmacia; 2.5.3.4) previously equilibrated in 20 mM Tris-HCl pH 8.5, 150 mM NaCl. The superose 6 chromatograph revealed a single high molecular weight peak eluting in 200 µl of the void volume (Figure 3.6 D). Purified protein samples were assayed directly for EPL cell induction and shown to contain the high molecular weight EPL cell inductive activity with no net increase in specific activity.

Samples used in cell based assays containing the EPL cell inductive activity were also separated and visualised, via silver staining (1 µg/well) of a SDS/reducing polyacrylamide gel. This revealed purification from a complex mixture of proteins in sfMEDII to two major high molecular weight protein bands (Figure 3.6 E). The major purification occurred after hydrophobic interaction while the last two steps removed some low molecular weight impurities. The top band is a protein aggregate that fails to enter the separating gel while the lower band is a 250kd species. The presence of the 250kd high molecular weight species on a reducing gel and the sensitivity of the high molecular weight activity to reducing agents may indicate that this molecule represents subunits of a higher molecular weight complex.

Table 3.2 Purification of the high molecular weight EPL cell inductive factor:

Method 1

Sequential purification of sfMEDII proteins by 100kd cut off membrane filtration, anion exchange, phenyl sepharose, heparin affinity and superose 6 gel filtration. Protein yields in active fractions were quantitated by Bradford assay (BioRad) used to the manufacturers instructions. Fractions containing activity were determined by cell assay (3.2). Specific activity was determined by titration of 10 μ g-0.1 μ g (max volume 50 μ l) of the high molecular weight signal from partially purified samples and minimum activity determined by cell based assay (3.2). Total units recovered is the multiple of the yield and specific activity. The activity recovered is % of units compared to R (1 ml/40 units). Fold purification is the increase in specific activity from the unpurified sample.

	Protein (mg)	Specific activity (Units/mg/ml)	Total Units	% Activity Recovered	Fold purification
sfMEDII	454	40	18,160		
100kd	360	40	14,400	79	
Anion exchange	180	60	10,800	75	1.5
Phenyl Sepharose	55.9	150	8,385	77	3.75
Heparin affinity	7.5	500	3,750	45	12.5
Superose 6 GF	N/D	500	N/D	N/D	12.5

3.6 Purification of the high molecular weight signal: Method 2 (2.5.4):

A second purification of the high molecular weight activity incorporated sequential application of the chromatographic steps described in Method 1, without HIC, in an altered and more efficient order. Heparin sepharose affinity chromatography was used to adsorb the EPL cell inductive high molecular weight component directly from sfMEDII. One litre of sfMEDII containing 360 mg of protein was passed over a 25 ml Heparin sepharose column (Pharmacia; 2.5.4.1) equilibrated in 20 mM Tris-HCl pH 8.5. The majority of proteins within the conditioned medium failed to bind to the heparin sepharose and passed through the column. After re-equilibration in 100 ml of 20 mM Tris-HCl pH 8.5, bound proteins were eluted with a step-wise salt gradient, initiated with 20 mM Tris-HCl pH8.5, 150 mM NaCl, to remove non-specifically bound proteins attached through ionic interactions. The proteins retained on the column were eluted in 200 ml of 500 mM NaCl (25.5 mg) and a final elution in 200 ml of 1 M NaCl (Figure 3.7 A). Samples were buffer exchanged to PBS in centricon 10 columns (Amicon) and 10-0.1 µg titrations of each sample were assayed for EPL cell induction as described previously (3.2; 2.3.10). Proteins eluting in 500 mM NaCl (25.5 mg) contained the majority of the EPL cell inductive capacity as previously determined (data not shown).

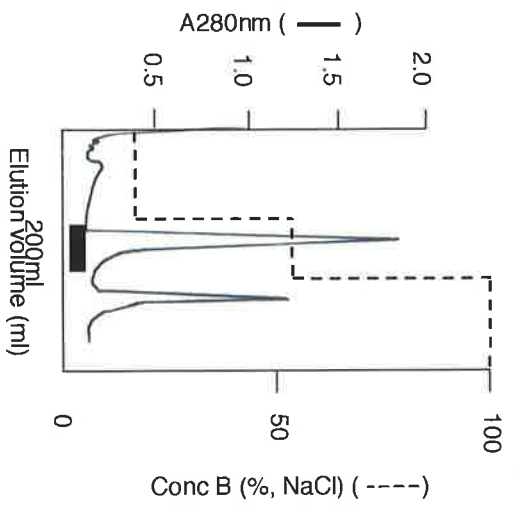
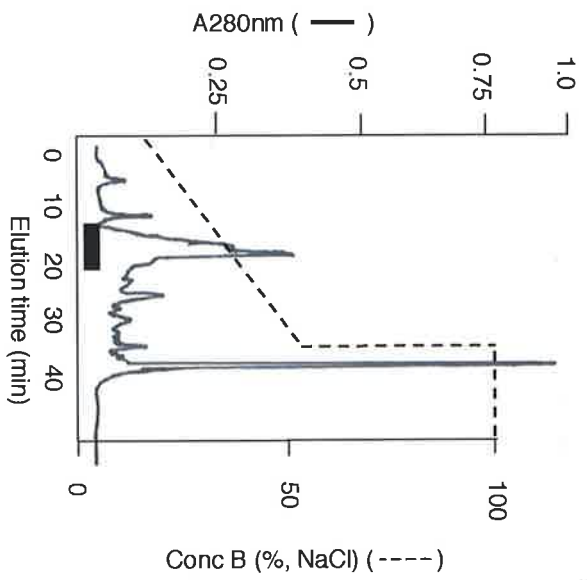
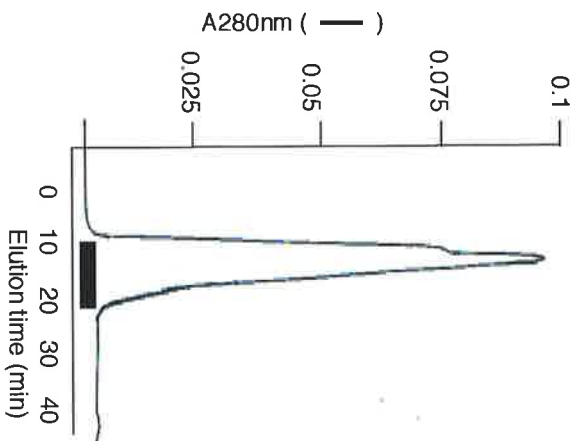
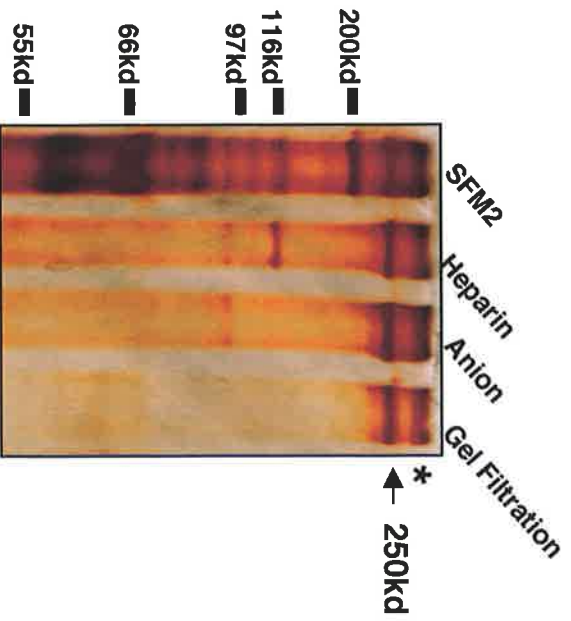
The active sample was diluted to 20 mM Tris-HCl pH 8.5, 150 mM NaCl (667 ml) prior to application to a 2 ml MonoQ anion exchange column (2 runs of 12.5 mg) at a flow rate of 1 ml/minute (Pharmacia, 2.5.4.2). The column was re-equilibrated in 20 mM Tris-HCl pH8.5, 150 mM NaCl. Proteins were eluted using a graded exchange into 20 mM Tris-HCl, 500 mM NaCl over 40 minutes, followed by a step-wise gradient to 1 M NaCl. Active samples and specific activity (10-0.1 µg/ml) were determined by morphology assay (3.2) after samples were de-salted in PBS through centricon concentrators. EPL cell inductive activity eluted between 250 mM-400 mM NaCl (2 mg) in a total volume of 10 ml (Figure 3.7 B). The active sample had a specific activity equivalent to the pilot purification (Table 3.3) but was greatly reduced in yield, perhaps due to the aggregation of proteins in some samples (data not shown).

The active sample (1 ml/200 µg) was further purified by sequential gel filtration chromatography purifications (using a 50 µl injection volume) over a superose 6 gel filtration

Figure 3.7 Purification of the high molecular weight factor: Method 2

Partial purification and gel electrophoresis of sfMEDII derived proteins. Heparin sepharose CL-6B (A), anion exchange (B) and superose 6 gel filtration chromatography (C). High molecular weight EPL cell inductive activity was detected by cell based assay (3.2). Fractions containing the activity are represented in each case by the thick black bar. Proteins were detected at 280 nm. Dotted lines represent % of 1M NaCl in 20mM Tris-HCl pH 8.5.

A reducing/denaturing 10% SDS-poly-acrylamide gel of the partially purified fractions containing the high molecular weight activity (3.2) from purification protocol method 2 (D). Arrow indicates the 250kd species. * indicates high molecular weight aggregate that fails to enter the separating gel. Loadings: sfMEDII (5 µg), heparin affinity (2 µg), anion exchange (2 µg) and superose 6 gel filtration (2 µg). Molecular weight markers (Amrad; 2.6.9) are indicated.

A**B****C****D**

column on the Smart System (Pharmacia; 2.5.4.3) in 20 mM Tris-HCl pH 8.5, 150 mM NaCl. The superose 6 chromatograph revealed a single high molecular weight peak, and shoulder, eluting in the void volume (Figure 3.7 C). 10-0.1 μ g of samples were assayed directly for EPL cell induction in the presence of 40 μ M L-proline (Table 3.3). Purified samples were shown to contain the high molecular weight EPL cell inductive signal by cell assay (3.2).

Semi-purified and centricon desalted samples (1 μ g), containing the high molecular weight activity were separated by SDS/reducing PAGE and visualised via silver staining. This revealed purification from a complex mixture of proteins to two high molecular weight protein bands apparently identical to those observed using purification method 1 (Figure 3.7 D). The 250kd band is therefore likely to represent the reduced subunits of a highly purified, high molecular weight protein species of greater than or equal to 500kd, which is active in EPL cell induction from ES cells.

3.7 N-terminal sequencing of the purified 250kd high molecular weight factor

The purified EPL cell active sample from Method 2 (1 μ g) was separated in a reducing/denaturing 8% polyacrylamide gel prior to electrophoretic transfer to a nitrocellulose membrane (Immobilon) at 400 mAmps for 2 hours in a wet western transfer apparatus (BioRad; 2.7.21). The transferred band was visualised by Coomassie stain (2.7.20), excised from the filter and processed by Ms Denise Miller, in the Department of Biochemistry at the University of Adelaide (2.7.22). As part of this process, the protein was eluted from the filter in tri-fluoro acetic acid (TFA, pH 2.0) and subjected to N-terminal Edman degradation on an applied Biosystems 492 protein sequencer. Results from these experiments detected the release of multiple N-terminal sequences did not produce a readable sequence (data not shown). The release of multiple N-termini may be due to the acid sensitive nature of the EPL cell inductive factor (3.3) or the presence of glycoproteins associated with the purified factor (3.3). This procedure failed to identify the 250kd high molecular weight species.

Table 3.3 Purification of the high molecular weight EPL cell inductive factor:
Method 2

Sequential purification of sfMEDII proteins by heparin affinity, anion exchange, and superose 6 gel filtration. Protein yields in active fractions were quantitated by Bradford assay (BioRad) used to the manufacturers instructions. Fractions containing activity were determined by cell assay (3.2). Specific activity was determined by titration of 10 μ g-0.1 μ g (max volume 50 μ l) of the high molecular weight signal from partially purified samples and minimum activity determined by cell based assay (3.2). Total units recovered is the multiple of the yield and specific activity. The activity recovered is % of units compared to R (1 ml/40 units). Fold purification is the increase in specific activity from the unpurified sample.

	Protein (mg)	Specific activity Units/mg/ml	Total Units	% Activity Recovered	Fold purification
sfMEDII	362	40	14,480		
Heparin Affinity	25.5	400	10,200	70	10
Anion exchange	2	500	1,000	9.9	12.5
Superose 6 GF	N/A	500	N/A	N/A	12.5

3.8 Discussion

Biochemical characterisation revealed the high molecular weight EPL cell inductive signal to be a single protein or protein complex with a native molecular mass greater than 500kd, sensitive to the protease trypsin, temperatures above 56°C, pH below 6.0 and reducing agents. Preliminary purification of the high molecular weight activity suggested an overall negative charge pH 8.5, and the presence of hydrophobic regions. Interaction with the highly sulphated glycoprotein heparin suggested the factor could be an enzyme, growth factor, nucleic acid binding protein, hormone receptor or a component of the ECM (Scopes, 1988).

This factor was purified by two methods using sequential binding and elution of the active protein from heparin affinity, anion exchange and hydrophobic interaction columns, followed by a final purification step using gel filtration. The purified fraction was separated by SDS-PAGE and visualised by silver stain to show two major bands of unknown ^{identity}~~origin~~. a high molecular weight aggregate that failed to enter the separating gel and a 250kd species, present in the active samples derived from each purification protocol. The relationship between the 250kd species and the native molecular weight of the EPL cell inductive factor (>500kd) is unknown, but it is possible that the high molecular weight signal is a complex of lower molecular weight molecules. This is not a non-covalent complex but potentially a complex linked by covalent di-sulphide bonds.

Chapter 4

Identification of the high molecular weight EPL cell inductive factor

Chapter 4

4.1 Introduction

Molecular identification of the purified high molecular weight EPL cell inductive signal (Chapter 3) is required for assessment of the cellular action and developmental relevance. In particular, identification of this signal may provide information regarding the mechanism of signal function, while cellular developmental activity can be deduced from the expression and localisation of the signal during early mouse development *in vivo* and EPL cell formation *in vitro*.

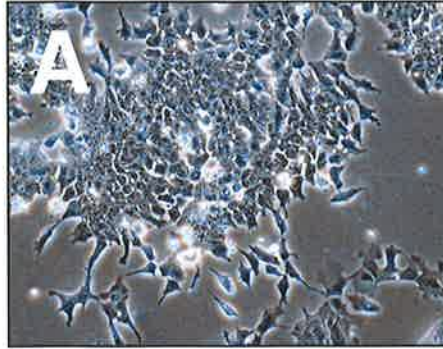
4.2 The high molecular weight EPL cell inductive factor can function when presented in an extracellular matrix (ECM) form

Several features of the high molecular weight EPL cell inductive activity are consistent with the function of this protein as a component of the ECM. These features include its large molecular size (>500kd), propensity to aggregate upon concentration (3.6) and its association with heparin sulphate proteoglycans (Scopes, 1988). Furthermore, basement membrane ECM components have been shown to have a key role in primitive ectoderm biology *in vivo* (Coucouvanis and Martin, 1995; Stephens *et al.*, 1995; Fässler and Meyer *et al.*, 1995). The loss of pluripotent cell attachment to the ECM *in vivo* and *in vitro* results in the loss of pluripotent cell maintenance (Coucouvanis and Martin, 1995; Stephens *et al.*, 1995; Fässler and Meyer *et al.*, 1995).

The adherence of ECM components to tissue culture plastic mimics the presentation of these factors in the context of ECM architecture (Papadopoulos *et al.*, 1990). To determine if HepG2 cell derived ECM is active in EPL cell formation, HepG2 cells were seeded onto gelatin (0.2%), and cultured to confluence. Cells were then killed and washed from the tissue culture plastic (2.3.13) to leave an intact HepG2 cell-derived ECM. This matrix was tested for EPL cell colony formation by seeding D3 ES cells (2×10^4 cells/cm²) in the presence of 40 μ M L-proline (Figure 4.1 A). HepG2 cells cultured on gelatin coated tissue culture plastic (0.2%), layed down an ECM that directed 100% of pluripotent cell colonies to adopt a morphology indistinguishable from EPL cells, in the presence of L-proline. Functional characterisation of these cells is described in chapter 5.

+ 40 μ M L-proline

**HepG2
ECM**



**Pure Factor
ECM**

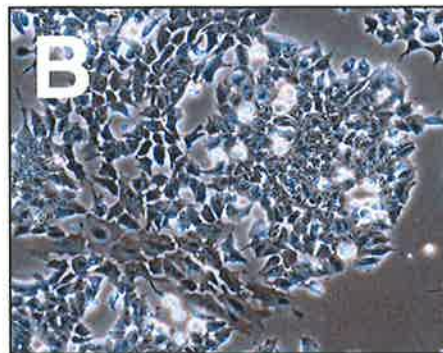


Figure 4.1 ES cells cultured on an ECM derived from HepG2 cells or the purified high molecular weight EPL cell inductive signal, adopt the EPL cell morphology in the presence of 40 μ M L-proline

D3 ES cells were seeded onto tissue culture plastic coated with an ECM derived from HepG2 cells (A) or 1 μ g/cm² purified high molecular weight factor (B) in the presence of 40 μ M L-proline. Cell morphology was assessed after 4 days. Images were taken on 200x magnification using phase contrast microscopy.

To assess the capacity of the high molecular weight factor to induce EPL cell morphology when presented as an ECM, tissue culture plastic was coated (2.3.13) with $1 \mu\text{g}/\text{cm}^2$ of the purified sample (3.6; Method 2) prior to the culture of D3 ES cells in the presence of $40 \mu\text{M}$ L-proline (Figure 4.1 B). Presentation of the purified factor to D3 ES cells as an ECM induced the morphological formation of EPL cells in 100% of colonies in the presence of $40 \mu\text{M}$ L-proline. These data suggest that the high molecular weight component may be an ECM or ECM associated protein.

4.3 ECM components and the induction of EPL cells from ES cells

In the embryo primitive ectoderm is induced, proliferates and is maintained upon a basement membrane that separates this pluripotent lineage from visceral endoderm. The diverse physiological roles of basement membranes are often determined by the nature of their constituents, architecture and cellular interactions (Henry and Campbell, 1998). Essential constituents of this basement membrane include collagen IV (Adamson and Ayres, 1979), laminin (Herken and Barracch, 1985) and nidogen (Miosge *et al.*, 2000). An additional component of this basement membrane is fibronectin (FN) (Wartiovaara *et al.*, 1979, Peters and Hynes, 1996). This suggests that FN may play a specific role during pluripotent cell biology.

HepG2 cells secrete the ECM components collagen IV, FN, laminin and vitronectin (Bouma *et al.*, 1989; Rescan *et al.*, 1991; Koli *et al.*, 1991; Kondoh *et al.*, 1999) and these components are presumably contained within MEDII conditioned medium. The EPL cell inductive capacity of ECM component, common to both MEDII and basement membrane of the day 6.5 mouse embryo, were assessed from solution, or when pre-coated onto tissue culture plastic, in the presence of $40 \mu\text{M}$ L-proline.

The EPL cell inductive capacity of mouse derived collagen IV (Sigma), bovine vitronectin (Sigma, Dr Zee Upton) and mouse laminin were assessed through supplementation into ES cell culture medium (2.3.13) ($10 \mu\text{g}/\text{ml}$), or by pre-coating onto tissue culture plastic (2.3.14) ($1 \mu\text{g}/\text{cm}^2$) in the presence of $40 \mu\text{M}$ L-proline. Results from these experiments demonstrated that collagen IV (Figure 4.2 A, B), vitronectin (Figure 4.2 C, D) and laminin (Figure 4.2 E, F) were inactive in the

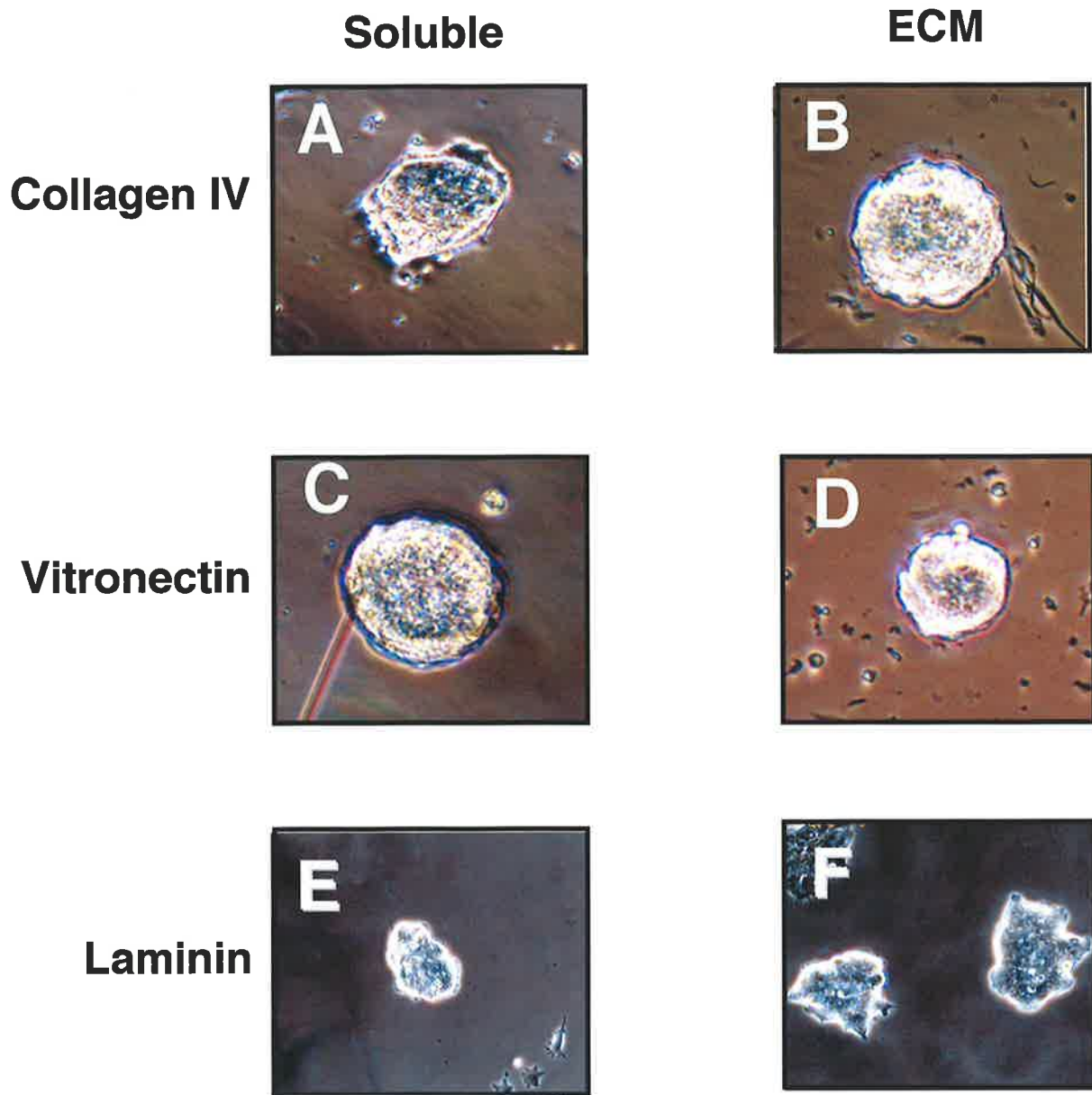


Figure 4.2 Collagen IV, Vitronectin and Laminin do not support morphological EPL cell formation

ES cells cultured in complete medium supplemented with 40 μM L-proline and 10 $\mu\text{g/ml}$ purified mouse collagen IV (A), bovine vitronectin (C) or mouse laminin (E) on tissue culture plastic previously coated with 0.2% gelatin. ES cells cultured in complete medium supplemented with 40 μM L-proline on tissue culture plastic coated with 1 $\mu\text{g/cm}^2$ of purified mouse collagen IV (B), bovine vitronectin (D) or mouse laminin (F). Morphology was assessed after 4 days. Images were taken at 200x magnification using phase contrast microscopy.

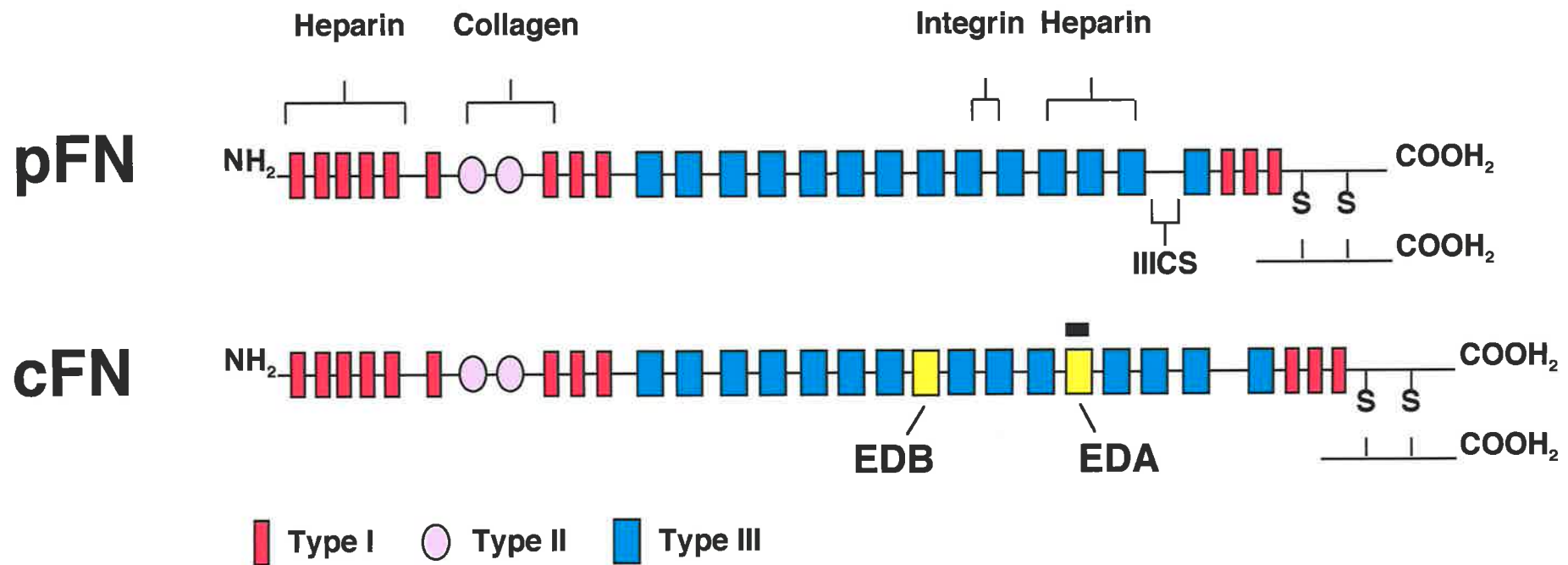


Figure 4.3 Schematic representation of the pFN and cFN protein

Fibronectin consists of repeating domains. Type I domains are shown in red, type II in ^{purple}blue and type III in ^{blue}green. The alternatively spliced extra type III domains, extra domain B (EDB) and EDA, are shown in yellow. Inclusion of one or both of these domains distinguishes cFN from pFN. The alternatively spliced variable (V) domain (IIICS in human) contains 3 regions of potential alternate splicing events that yield can yield 20 theoretical FN isoforms. FN exists as a heterodimer through di-sulphide bond formation at COOH₂-terminal end. The heparin and collagen binding domains and the receptor integrin binding site are indicated.

induction of EPL cell morphology from solution, or when presented to ES cells as an ECM. These data suggest that vitronectin, collagen IV and laminin are not equivalent to the purified EPL cell inductive factor within MEDII.

4.4 Cellular FN induces EPL cell formation in an equivalent manner to the purified high molecular weight inductive activity

FN is an important component of diverse cellular processes including embryogenesis, wound repair, inflammation, cell migration and adhesion (Peters and Hynes, 1996). The FN protein is composed of repeating modules of three types, termed type I, II and III (Hynes, 1986; Figure 4.3). Encoded by a single gene, the primary FN transcript is alternatively spiced in 3 regions which encode two extra type III domains, termed extra domain B (EDB) (termed EIIIB in mouse) and EDA (EIIIA), and the C-terminal IIICS or variable region (V in mouse) (Sekiguchi *et al.*, 1986). These three splice sites generate multiple FN isoforms that can be divided into two major subclasses, plasma FN (pFN) and cellular FN (cFN) (Figure 4.3; Beauvais-Jouneau *et al.*, 1997). The distinguishing feature between cFN and pFN is the inclusion of one or both of the extra type III domains, that localise cFN to the ECM by an unknown mechanism (Hynes, 1986).

Human plasma FN (pFN) was tested for EPL cell inductive capacity when supplemented into the ES complete medium (10 µg/ml) or when presented to ES cells as an ECM in the presence of 40 µM L-proline (Figure 4.4 A,B). Plasma FN (pFN) did not induce EPL cell morphology when presented to ES cells in solution (Figure 4.4 A), indicating that pFN cannot replicate the biological role of the MEDII high molecular weight factor. However, when presented as an ECM, pFN induced 100% of pluripotent cell colonies to adopt a morphology indistinguishable from EPL cells in the presence of 40 µM L-proline. (Figure 4.4 C).

The cFN subclass comprises secreted factors specifically recruited to, and active from, the ECM. Pure cFN, isolated from the conditioned medium of primary human stromal cells by gelatin sepharose affinity chromatography (Levesque *et al.* 1996; Figure 4.7 B), was used for the induction of EPL cells from ES cells. Cellular FN was presented to ES cells in solution (10 µg/ml), or as an ECM after pre-coating onto tissue culture plastic (1 µg/cm²), in either the presence or absence of L-

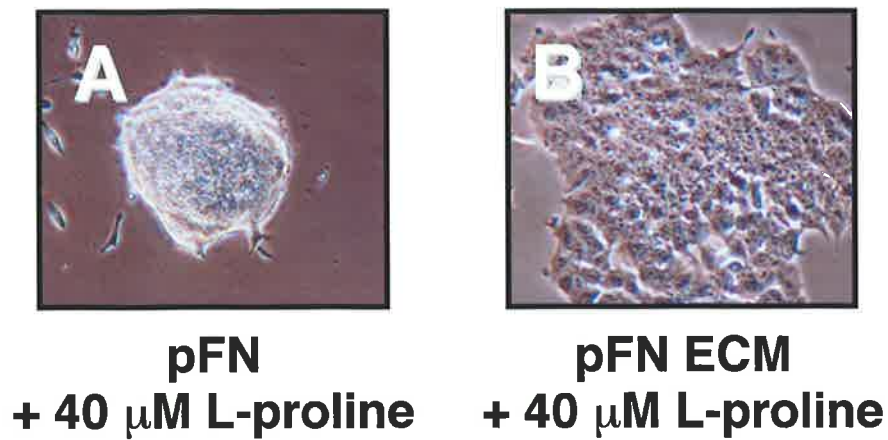


Figure 4.4 Morphology of ES cells cultured in the presence of human plasma FN (pFN)

ES cells were cultured in complete medium supplemented with soluble pFN (10 μ g/ml) on a pre-coated gelatin ECM (0.2%) in the presence of 40 μ M L-proline (A) or on a pre-coated ECM of human pFN (1 μ g/cm²) in the presence of 40 μ M L-proline (B). Images were taken at 200x magnification using phase contrast microscopy.

proline. As shown in figure 4.5, cFN induced the formation of cells morphologically indistinguishable from EPL cells in the presence but not absence of 40 μ M L-proline when presented in solution, or in the presence of L-proline when presented as an ECM. This is reminiscent of the action of the purified high molecular weight EPL cell inductive signal in MEDII.

4.5 cFN is the high molecular weight component of MEDII conditioned medium

The presence of cFN within the active and purified component of sfMEDII was confirmed by western blot analysis (2.7.21) using the monoclonal antibody 3E2 (Sigma; 2.8.3), as a specific probe for EDA⁺ human cFN. Purified sample, isolated by method 2 (3.6) was separated by 8% reducing/SDS PAGE with purified human pFN and stromal cFN as negative and positive controls respectively (Figure 4.6). Cellular FN was detected within the purified sample as a component of the aggregate that fails to enter the separating gel and as the major 250kd species. This indicates that the purified high molecular weight, EPL cell inductive sample within MEDII contains cFN.

FN can be purified specifically from solution due to its high affinity with denatured dermal collagen or gelatin (Engvall and Ruoslahti, 1977). Cellular FN was affinity purified from sfMEDII via adsorption to a 25 ml gelatin affinity chromatography column (Pharmacia). One litre of sfMEDII was passed over gelatin sepharose CL-6B, prior to re-equilibration to PBS. The bound cFN was eluted using 20 ml 6 M urea in PBS, collected into dialysis tubing and dialysed 4 times against 4 litres of PBS at 4°C for at least 5 hours per wash (Figure 4.7 A). The dialysed sample was filtered through a 0.22 μ m filter, and its purity was confirmed by silver staining of a 8% reducing SDS-PAGE (Figure 4.7 B). HepG2 derived cFN displayed identical EPL cell inductive properties, to the purified sample (refer 3.6; Figure 4.8, Figure 4.9 A) when presented to ES cells as an ECM, or in solution, in the presence of L-proline (Table 4.1). Importantly, cFN and the purified sample displayed identical specific activity in the induction of EPL cell morphology from ES cells in the presence of 40 μ M L-proline (Table 4.1). In addition, cFN was sensitive to heat (56°C) (Figure 4.9 B), trypsin digestion (Figure 4.9 C), acid pH (Figure 4.9 D) and reducing agents (Figure 4.9 E,F) as

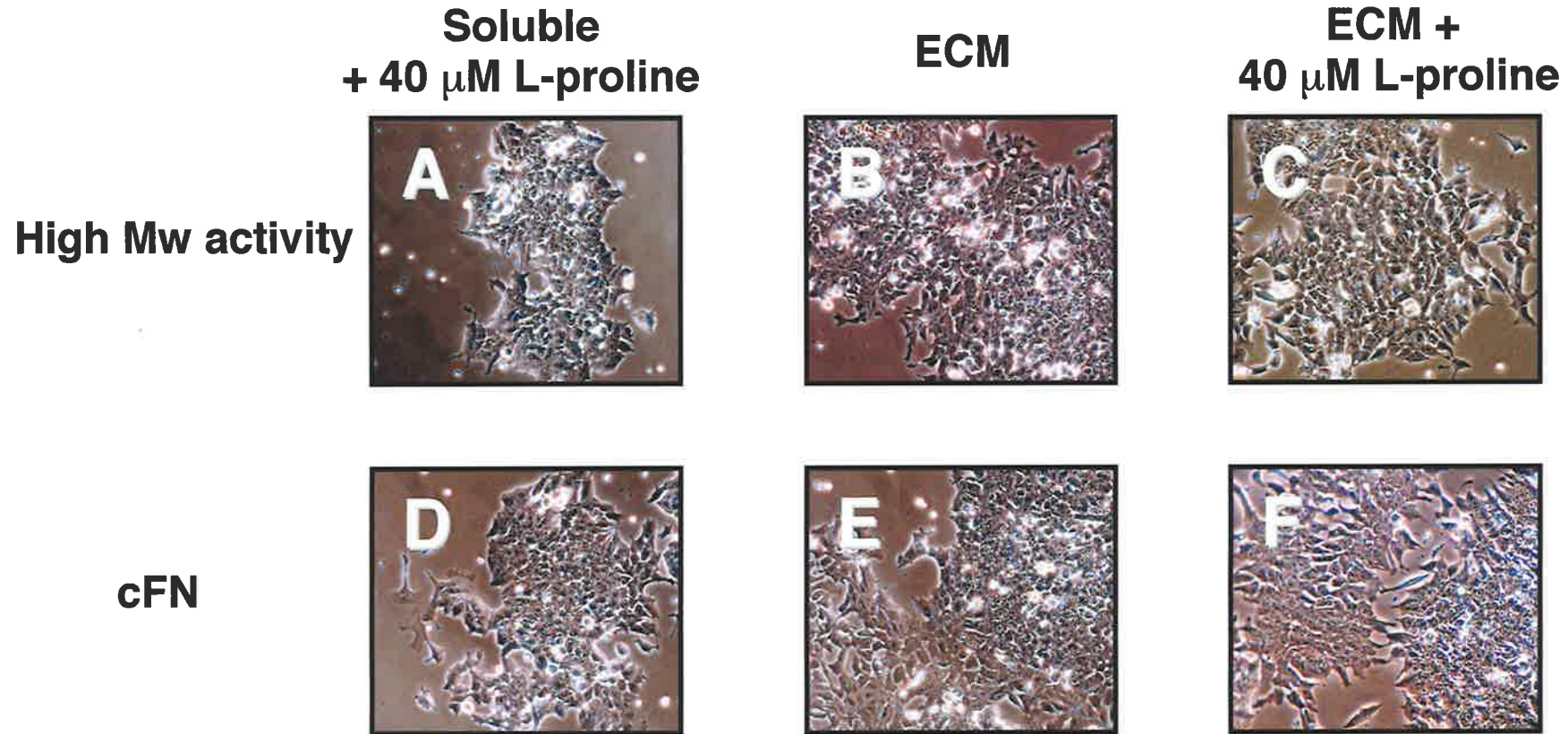


Figure 4.5 Morphology of ES cells cultured in the presence of 40 μ M L-proline and human cFN or the purified high molecular weight activity.

ES cells were cultured in the presence of medium supplemented with 40 μ M L-proline and the purified high molecular weight signal (10 μ g/ml) on a gelatin ECM (A) or cultured on the purified EPL cell inductive signal presented as an ECM (1 μ g/cm²) in the absence (B) or presence (C) of 40 μ M L-proline. ES cells were cultured medium supplemented with 40 μ M L-proline and soluble human cFN (10 μ g/ml) (D) or cFN presented as an ECM (1 μ g/cm²) in the absence (E) or presence (F) of 40 μ M L-proline. Morphology was assessed at day 4.0. Images were taken at 200x magnification using phase contrast microscopy.

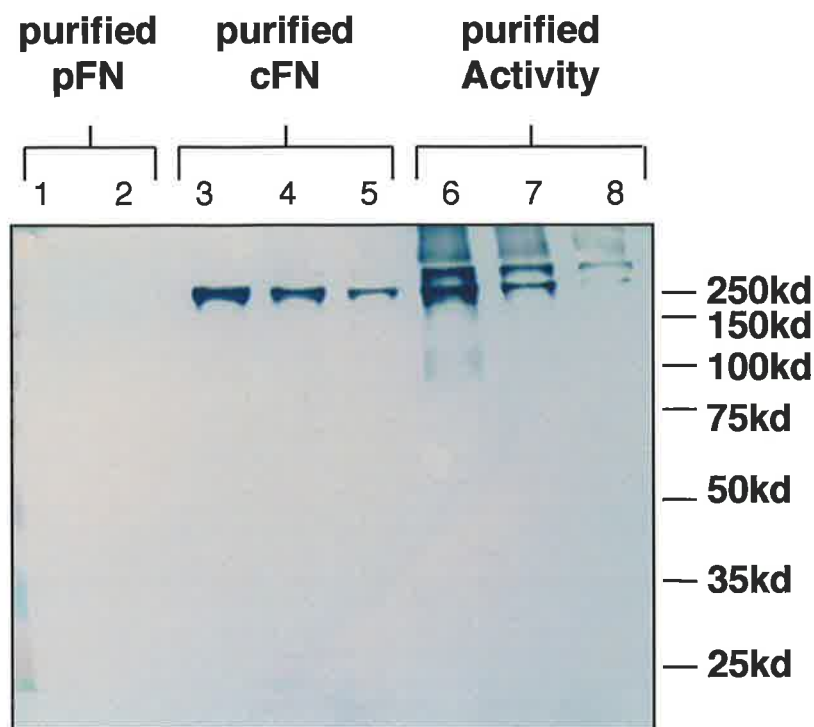


Figure 4.6 cFN is purified with the high molecular weight EPL cell inductive activity from SFMEDII

Western blot of purified human pFN (Sigma; Lane 1: 1.0 μ g, Lane 2: 0.5 μ g), human cFN (2.5.5; Lane 3: 1.0 μ g, lane 4: 0.5 μ g, lane 5: 0.25 μ g) and purified active samples (Lane 6: 1.0 μ g, lane 7: 0.5 μ g, Lane 8: 0.25 μ g) using the monoclonal antibody (3E2, Sigma) directed against the EDA domain of human cFN.

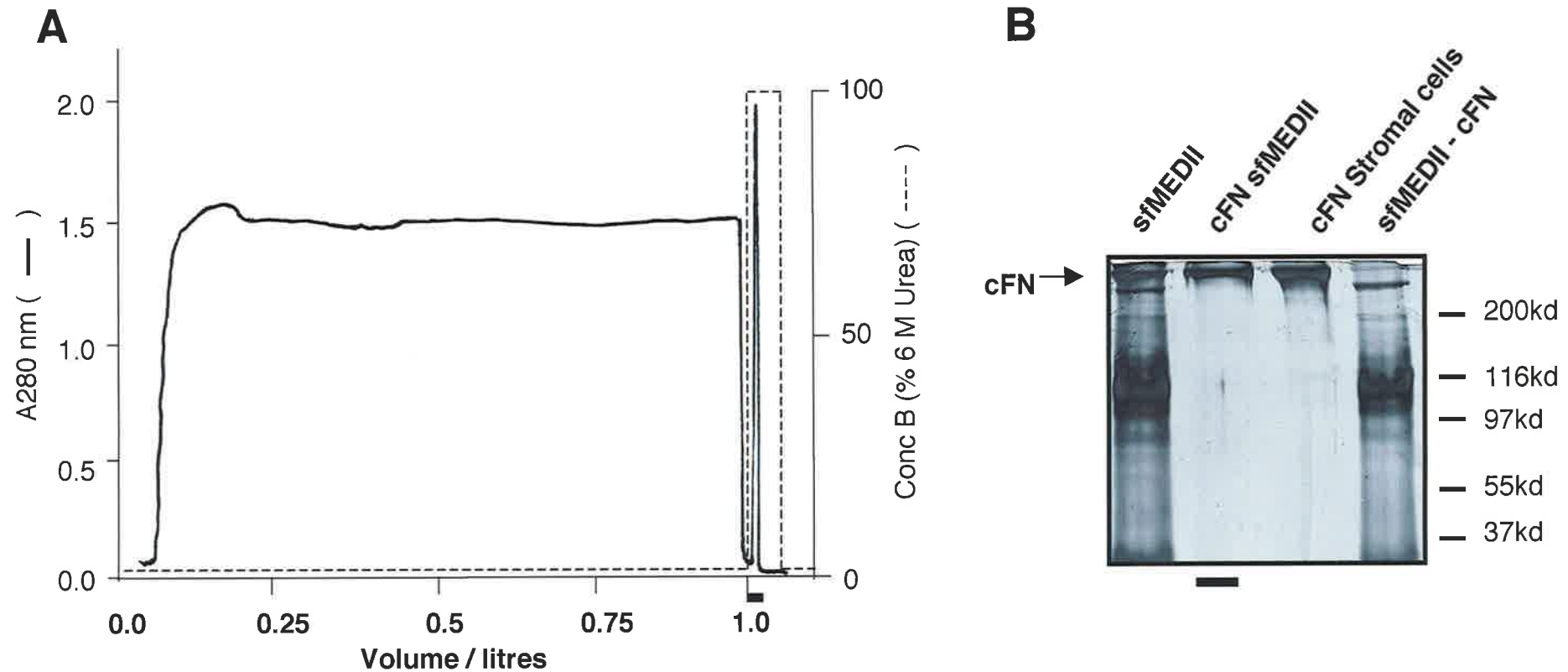


Figure 4.7 Purification of cFN from SFMEDII by gelatin sepharose affinity chromatography

sfMEDII (1 Litre) was bound to, and eluted from, a 25 ml gelatin sepharose column (A; Pharmacia). Bound proteins eluted in 20 mM Tris-HCl pH 8.5, 1 M NaCl. Fraction (20 ml) was assayed for the high molecular weight EPL cell inductive signal by morphology assay (3.2). Fraction containing the activity is represented by the thick black bar. Proteins were detected at 280 nm. Dotted lines represent % of 1 M NaCl in 20 mM Tris-HCl pH 8.5. Column fractions were visualised by silver stain (sfMEDII: 5 µg; cFN sfMEDII; 1 µg; cFN Stromal: 1 µg; sfMEDII - cFN; 5 µg) on a 10% SDS reducing-PAGE (B). The arrow indicates a 250kd molecular weight species present in all samples which contain the high molecular weight EPL cell inductive activity. Molecular weight markers (Amrad; 2.6.9) are indicated.

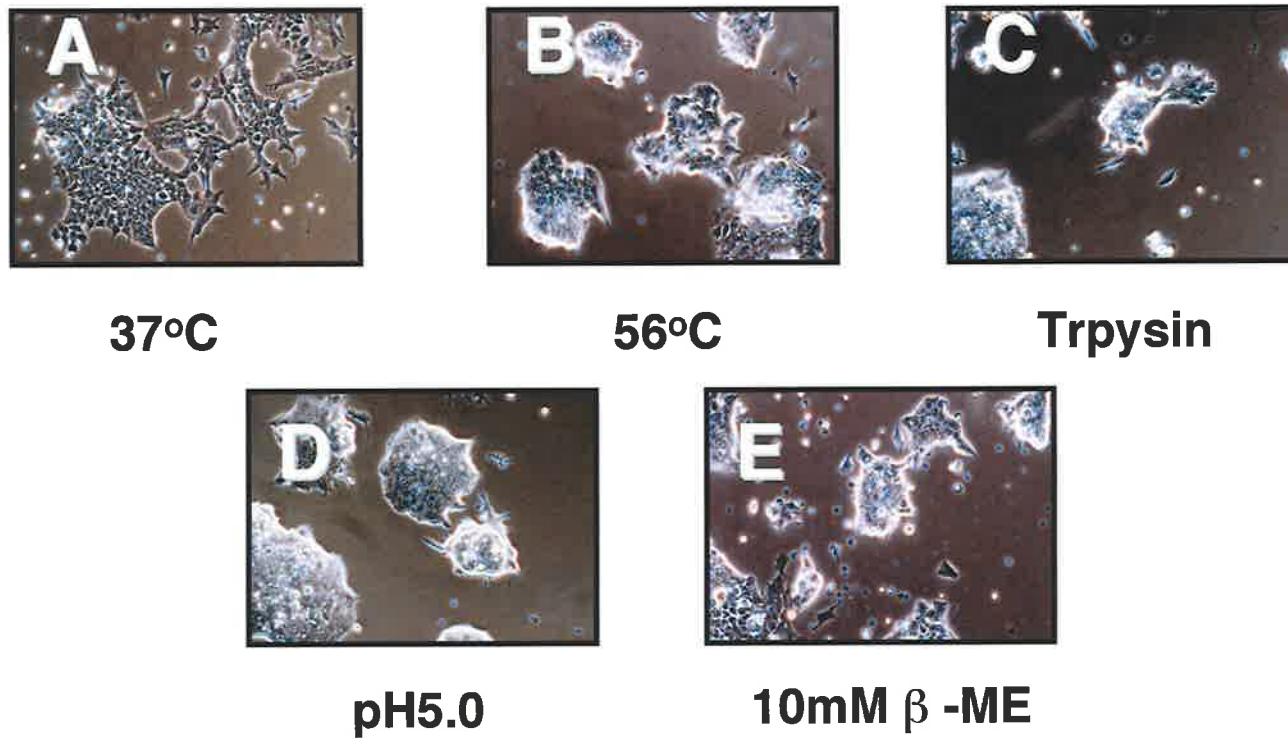
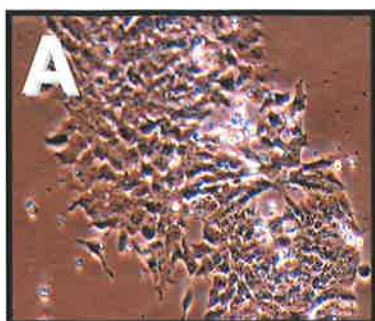
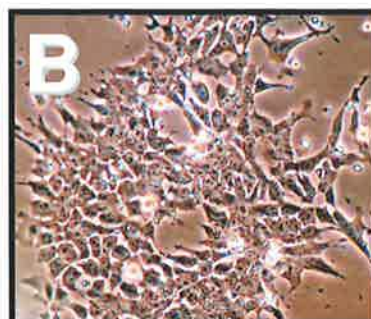


Figure 4.9 HepG2 cell derived cFN is sensitive to heat, acid, reducing agent and low pH equivalent to the purified high molecular weight EPL cell inductive signal

SFMedII derived cFN (40 U / 80 μ g), was supplemented with 500 μ g bovine serum albumin and exposed to heat (37°C (A), 56°C (B)), trypsin (25 U; C), 50 mM Citric acid pH5.0 (D) and the reducing agent β -ME (10 mM; E) prior to morphological assessment of EPL cell formation (3.2). Cells were maintained in ES complete medium supplemented with 40 μ M L-proline. Plates were pre-coated with gelatin ECM (0.2%). Images were taken at 200x magnification using phase contrast microscopy.



**HepG2 cFN
+ L-proline**



**HepG2 cFN ECM
+ L-proline**

Figure 4.8 sfMEDII derived human cFN induces EPL cell formation equivalent to the purified sample

sfMEDII derived cFN induces EPL cell formation from solution (10 $\mu\text{g/ml}$) (A) or as an ECM (1 $\mu\text{g/cm}^2$) (B) in the presence of 40 μM L-proline. Images were taken at 200x magnification using phase contrast microscopy.

Amendment: Assessment of cFN purity

The human cFN used in EPL cell inductive experiments in this thesis was purified, and assessed for purity, in an equivalent manner to other researchers which yields 99% pure FN, in the absence of lower molecular weight species, as assessed by silver stained reducing SDS-PAGE analysis (Engvall and Ruoslahti, 1977). Furthermore, human cFN purified from two separate sources induced morphological EPL cell formation from solution or as an ECM, while commercial human plasma FN (Sigma) was also active in this assay when presented as an ECM. The distinct origins of these FN molecules suggest that induction of the EPL cell morphology is associated with FN activity and is not likely to be due to FN-associated proteins or trace contaminants.

To assess further the purity of bioactive samples, HepG2 and stromal cell purified cFN samples were separated by 2-D gel electrophoresis (Biorad) and visualised by silver stain. 10 µg of gelatin sepharose purified cFN samples were focused over a pH 3-10 gradient overnight and then silver stained following 8% reducing SDS-PAGE separation as the second dimension. Additional non-FN species were detected by silver stain in stromal cell derived cFN but not in HepG2 cell derived cFN samples (data not shown). Therefore, any additional low molecular weight factors that co-purify with EPL cell inductive HepG2 cell derived cFN are below the level of detection in this assay.

These data do not however, distinguish additional proteins associated with cFN that are of similar native size (> 460 kd) and/or composed of similarly sized subunits (250 kd). To determine if additional high molecular weight protein species were associated with purified cFN, EPL cell inductive cFN was treated with 20 mg/ml CnBr in 0.1 M HCl for 6 hours (Forbes *et al.*, 1998). CnBr cleaves peptides N-terminal to each methionine residue yielding 12 diagnostic peptides from human FN of which 7 are expected to be resolved by silver stain on a tris-tricine 12.5% acrylamide gel. The mobility of these fragments is expected to vary according to the

	Protein (mg)	Yield (mg)	Specific activity (U/μg/ml)	Fold purification
Purified sample	362	7.5	500	12.5
sfMEDII	300	2	500	12.5
Stromal cell CM	N/A	N/A	500	N/A

Table 4.1 Specific activities of purified cFN and the high molecular weight factor

Samples containing the high molecular weight EPL cell inductive activity were determined by cell assay (3.2). Protein yields in active fractions were quantitated by Bradford assay (BioRad) used to the manufacturers instructions. Specific activity was determined by titration of 10 μ g-0.1 μ g (max volume 50 μ l) of the high molecular weight signal from partially purified samples and minimum activity determined by cell based assay (3.2). Total units recovered is the multiple of the yield and specific activity. The activity recovered is given as a percentage of units compared to starting material in sfMEDII. Fold purification is the increase in specific activity from the unpurified sample.

determined for the high molecular weight EPL cell inductive activity. Collectively, these data suggest that cFN is the high molecular weight EPL cell inductive factor within MEDII.

4.6 Discussion

Evidence presented in this chapter has shown that cFN, in concert with diffusible L-proline, is the high molecular weight EPL cell inductive component of MEDII. Cellular FN was verified as this factor based on its presence in the highly purified samples that contain the high molecular weight EPL cell inductive activity and its biological activity in EPL cell formation and cFN displays identical biochemical and biophysical properties to the purified factor.

ECM glycoproteins are often associated with additional signalling molecules, growth factors and cytokines (reviewed in Slater, 1996). This suggests that additional signals associated and purified with cFN may be mediating the morphological induction of EPL cells. However, affinity purification of cFN from gelatin sepharose requires the addition of 6 M urea and dialysis through high molecular weight cellulose tubing. This would suggest that non-covalently associated molecules were not directing EPL cell formation.

4.6.1 The ECM localised cFN is the high molecular weight EPL cell inductive signal

ES cells cultured on an ECM derived from HepG2 cells or cFN adopted a morphology indistinguishable from EPL cells. These data suggest that FN is active in the formation of the EPL cell morphology when presented to ES cells in an ECM. This is consistent with the ability of cFN, but not pFN, to induce EPL cell formation when presented in solution since the EDA and/or EDB type III domains direct cFN to the ECM during EPL cell formation (Hynes, 1986). The morphology of EPL cells therefore provides an *in vitro* assay that biologically distinguishes cFN from pFN. Previous attempts to detect a functional difference between cFN and pFN isoforms, isolated from the conditioned medium (CM) of fibroblasts, have been unsuccessful. This is due, in part, to the secretion of a mixture of heterodimeric forms of differing cFN isoforms, with respect to the presence or absence of EDA and/or EDB domains. Therefore, investigation into the role of

respective cFN EDA or EDB domains has been difficult. However, methods have been recently developed that allow the specific purification of EDA⁺ cFN through the use of heparin cellulose (3.6; Miyashita *et al.*, 1998) or gellan sulfate (Miyamoto *et al.*, 2001).

EDA⁺ cFN is predominantly expressed during biological processes involving substantial cell migration and proliferation, such as embryonic development, malignant transformation, and wound healing (Manabu *et al.*, 1997). Recombining EDA⁺ cFN fragments have been shown to modulate the cell-adhesive activity of FN in CHO cells and are more potent than EDB⁺ or ED⁻ FN fragments (Manabu *et al.*, 1997). However, cell spreading activity of EDA⁺ FN was not affected by antibodies recognizing the EDA domain but was abolished by antibodies against integrin $\alpha 5$ and $\beta 1$ integrin subunits and by addition of the $\alpha 5/\beta 1$ integrin binding site peptide Gly-Rag-Gly-Asparagine-Serine-Pro. This indicates that the EDA segment enhances the cell-adhesive activity of FN by potentiating the interaction of FN with the integrin $\alpha 5/\beta 1$ receptor. More specific functions for the EDA domain have also been determined, such as activation of the Toll-like receptor 4 in HEK 293 cells (Okamura *et al.*, 2001), and increased release of proteoglycan and production of matrix metalloproteinase 1 in chondrocytes (Saito *et al.*, 1999). Specific roles for the EDB domain of cFN have not been determined.

4.6.2 Pluripotent cells and ECM components *in vivo*

The role of cFN during embryogenesis and pluripotent cell biology has been investigated in mice lacking a functional copy of the *FN* gene. These mice progress normally to implantation but develop an abnormal phenotype, with reduced embryonic size, by 6.5 dpc. Mice lacking *FN* have a reduced cell number within the primitive ectoderm and a delayed onset of gastrulation (George *et al.*, 1996; Georges-Labrousse *et al.*, 1996). Embryonic phenotypic defects are associated with the presence of FN in the adjacent basement membrane in wild type embryos (Wartiovaara *et al.*, 1979; George *et al.*, 1996; Georges-Labrousse *et al.*, 1996). Therefore, cFN directly or indirectly affects the normal formation of primitive ectoderm *in vivo*. This is consistent with the proposed role of cFN in EPL cell formation.

Functional mutation of many basement membrane components in mice yields either neonatal defects or normal adult development (Arikawa-Hirasawa *et al.*, 1999, Ryan *et al.*, 1999; Bonaldo *et al.*, 1998; Zheng *et al.*, 1995; Helbling-Leclerc *et al.*, 1995; Noakes *et al.*, 1995). This suggests a high level of functional redundancy between basement membrane components. However, the FN mutant mouse embryo dies around 10 -11 dpc due to cell migratory defects during an abnormal gastrulation process (George *et al.*, 1993; Georges-Labrousse *et al.*, 1996). Importantly, this report indicates that FN is an essential factor for early mouse embryogenesis. This is further supported by the report of similar defects in mice lacking the gene for the major FN receptor subunit $\alpha 5$ *integrin*, the product of which heterodimerises with $\beta 1$ *integrin* to form the FN receptor within the embryo (Yang *et al.*, 1993). However, mouse embryos that lack a functional $\alpha 5$ *integrin* gene progress further in development than FN null mice, with more complete cell morphogenesis during gastrulation including formation of a notochord, somites, heart, brain and branchial arches, even though the embryo dies around day 10 -11. This additional morphogenesis may be due to the role of redundant FN receptors such as $\beta 1$ αV heterodimers (Yang and Hynes, 1996).

Interaction of $\alpha 5$ $\beta 1$ *integrins* with the FN ligand has been shown to transduce signals that regulate cell proliferation, differentiation, cell survival and apoptosis (Giancotti and Ruoslahti, 1990, Meredith *et al.*, 1993; Almeida *et al.*, 2000). Furthermore, mice lacking a functional copy of the *integrin* associated signalling kinase, focal adhesion kinase (FAK), display abnormalities reminiscent of FN deficient mice (Furuta *et al.*, 1995). These studies suggest that in addition to the role of FN as a matrix protein, this molecule can act as a ligand for the initiation of signal transduction cascades. However, morphological primitive ectoderm is formed in all these mutant embryos supporting the claim of functional redundancy between ECM components and suggesting that FN is not essential for primitive ectoderm formation *in vivo*. Therefore, the small size of primitive ectoderm in the mice lacking a functional FN gene suggests that FN is a maintenance signal for pluripotent cells or induces pluripotent cell proliferation or survival.

One ECM component required for embryonic development is the laminin $\gamma 1$ subunit, *LAMC1* (Smyth *et al.*, 1999; Aumailley *et al.*, 2000). Mice lacking the *LAMC1* gene progress normally to the expanded blastocyst stage at 4.5 dpc but die following implantation at 5.5 dpc. These embryos fail to form a recognisable basement membrane ECM and lack deposition of additional ECM components

(Smyth *et al.*, 1999). This supports the observation of pluripotent cell death in EB's when contact between the basement membrane ECM and primitive ectoderm is disrupted (Coucovanis and Martin, 1995; Murray and Edgar, 2000) and supports the requirement of ECM for primitive ectoderm formation. A similar phenotype is observed in mice lacking the gene for the ECM/FN receptor subunit, *$\beta 1$ integrin*. Mutant embryos fail to proliferate pluripotent cells and are resorbed following implantation at day 5.5 (Stephens *et al.*, 1995; Fässler and Meyer, 1995). The failure of embryogenesis is believed to be due to a lack of pluripotent cell binding to ECM ligands. Importantly, these defects are apparent even in the presence of presumably normal FN expression.

Collectively, these reports suggest that signals directing ECM architecture and pluripotent cell-ECM attachment/signalling are required for pluripotent cell formation, maintenance, differentiation and survival *in vivo*, and that the presence of cFN is insufficient to induce primitive ectoderm formation in the absence of an ECM architecture. This is consistent with the activity in EPL cell formation of cFN and not pFN.

Chapter 5

The roles of L-proline and cFN in EPL cell induction

Chapter 5

5.1 Introduction

L-proline and cFN were purified from MEDII conditioned medium based on their ability to induce the EPL cell morphology from ES cells in culture. Morphology based assays have been successful in identifying these, and additional bioactive signals from conditioned medium (Hooper and Smith, 1987; Smith *et al.*, 1988). However, morphological criteria are not sufficient to distinguish EPL cells from other pluripotent cell types that may have similar colony morphology. Therefore, while L-proline and cFN are required for induction of EPL cell morphology, it remains possible that additional unidentified factors within MEDII are required for EPL cell formation.

EPL cells have been most closely identified on the basis of gene expression and differentiation potential. EPL cell formation is characterised by up-regulation of the primitive ectoderm marker *Fgf5* and down-regulation of the ICM marker *Rex1*. Expression of the pluripotent cell marker *Oct4* is maintained (Rathjen *et al.*, 1999). EPL cells can also be identified by the early and high level onset of expression of the nascent mesoderm marker *brachyury* during EPL cell derived EB differentiation relative to ES cell derived EB's (Lake *et al.*, 2000).

In this chapter the gene expression and differentiation potential of ES cells, cultured in the presence and absence of purified signals, were investigated to identify the role of L-proline and cFN in EPL cell gene expression and differentiation potential.

5.2 Standard culture conditions for the assessment of ES and EPL cell gene expression

Gene expression during EPL cell formation was assessed by the culture of ES cells, seeded at high density (2×10^4 cells/cm²) in LIF supplemented (1000 U/ml) ES DMEM (ES complete medium) in the presence or absence of 50% MEDII, 50% sfMEDII or purified components. ES cells and EPL cells were cultured over an 8-day period with the passage (refer 2.3.5) and harvest of cells at 2 day intervals. ES cells or EPL cells cultured for 2 days were designated passage 0 (p0), with each consecutive 2-day harvest numbered consecutively until 8 days culture (p1-p3). These are standard conditions for assessment of EPL cell formation by gene expression (Rathjen *et al.*, 1999).

Cytoplasmic RNA was purified from harvested cells (p0-p3) and analysed by northern blot for expression of the pluripotent cell marker genes *Oct4*, *Rex1* and *Fgf5* (Rathjen *et al.*, 1999). The expression level of *Fgf5* and *Rex1* markers was measured relative to the *Oct4* pluripotent cell marker (2.7.17) and represented as a percentage of ES cell expression. The *Oct4* gene is expressed in pluripotent ES cells and EPL cells but not in background differentiated cells. Thus *Oct4* expression reflects the level of pluripotent cells within a population and was used as the standard for quantitation of *Fgf5* or *Rex1* expression levels. A loading control for total mRNA was provided by expression of the constitutive mouse *glyceraldehyde-3-phosphatase dehydrogenase (mGAP)* gene (Rathjen *et al.*, 1990b).

D3 ES cells cultured under standard conditions, in the absence of MEDII or purified components, maintained a morphology and gene expression profile similar to ES cells. This was characterised by the failure of ES cells (ESp0-ESp3) to up-regulate expression of the primitive ectoderm/EPL cell marker *Fgf5* or down-regulate the ICM/ES cell marker *Rex1*, whilst maintaining high levels of the pluripotent cell specific gene *Oct4* (Figure 5.1 (ii)).

5.3 Standard culture conditions for the assessment of ES and EPL cell derived EB differentiation

A striking feature of EPL cell EB differentiation is the early onset and high level formation of nascent mesoderm (Lake *et al.*, 2000). This differentiation event is mapped by a differentiated morphology of EPL cell EB's on day 4.0 of culture (1.11.3), and expression of the nascent mesoderm marker gene *brachyury*, first detected in EPL cell derived EB's by day 2.0 of culture (Lake *et al.*, 2000). Furthermore, this differentiation can also be detected by a clear morphological difference between ES cell and EPL cell derived EB's by day 4 of culture. At day 4, ES cell derived EB's are comprised of a relatively homogenous population of round and smooth cells. EPL cell derived EB's also appear to consist of a homogenous population of cells but are irregular and disorganised (Lake *et al.*, 2000). Therefore, ES cell and EPL cell derived EB's have a separate morphology and gene expression of *brachyury* at day 4.0 (Lake *et al.*, 2000: 1.11.3).

Previous studies into EPL/ES cell differentiation potential have used EPL cell cultures

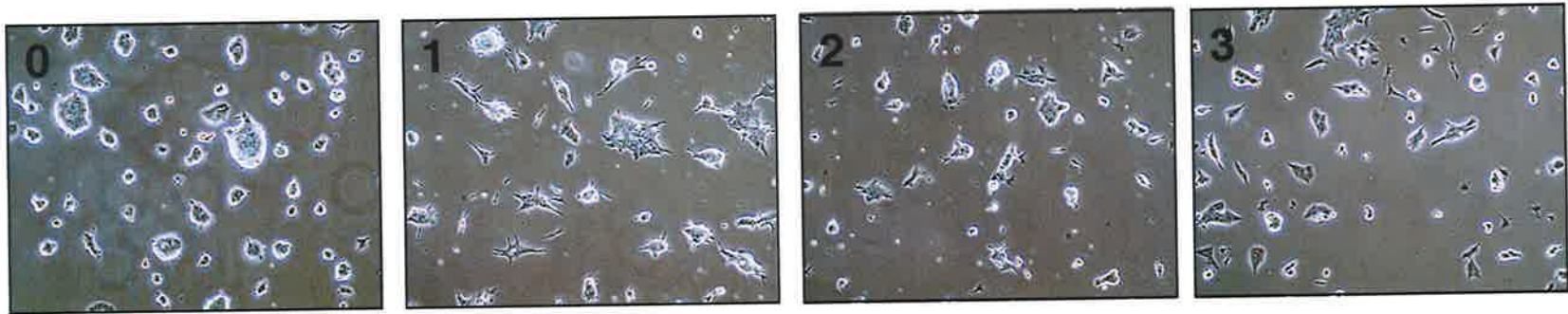
Figure 5.1 Culture of ES cells under standard conditions does not induce EPL cell gene expression

i) Morphological assessment of ES cells cultured under standard conditions of LIF supplemented DMEM (1000 U/ml) ii) Northern blot analysis of D3 ES cell RNA (20 µg). ES cells were cultured under standard conditions (2.2.5) and total RNA was collected from harvested cells. RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. The *Fgf5* transcripts are 2.7 and 1.8 kb (not expressed in ES cells) (Hébert *et al.*, 1990), *Rex1* 1.9 kb (Hosler *et al.*, 1989), *Oct4* 1.55 kb (Rosner *et al.*, 1990) and *mGAP* 1.5 kb.

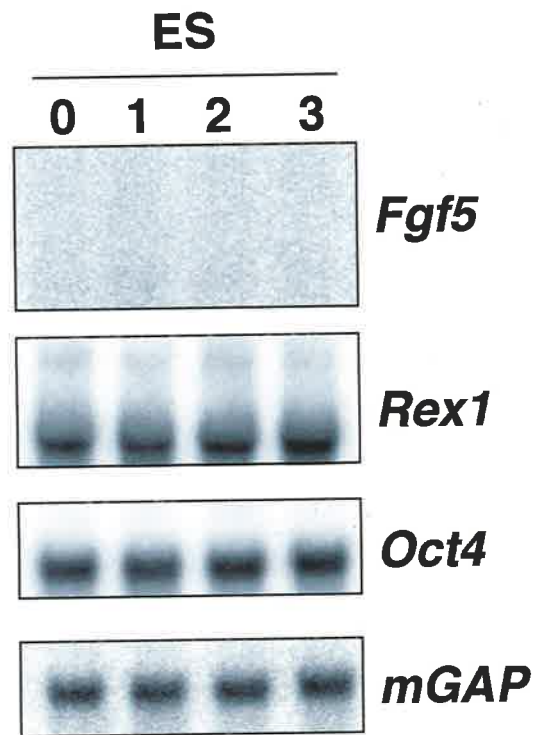
Amendment

i) Morphological assessment of ES cells seeded in LIF supplemented DMEM (1000 U/ml) for 2, 4, 6 and 8 days (0, 1, 2 and 3). Images were taken at 100 x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

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formed in the absence of exogenous LIF (to p1 of culture) prior to EB formation. However, MEDII contains approximately 10 U/ml of endogenous human LIF activity (Rathjen *et al.*, 1999). LIF is not an EPL cell inductive factor (Rathjen *et al.*, 1999), but exogenous addition of this cytokine has been shown to retard the onset of primitive ectoderm/EPL cell formation and differentiation *in vivo* and *in vitro* (Shen and Leder, 1992; Conquet *et al.*, 1992; Rathjen *et al.*, 1999). Therefore, EPL cell formation using purified factors must be performed in the presence of at least 10 U of LIF activity to replicate the conditions in MEDII. Rathjen *et al.* (1999) showed that EPL cells cultured in the presence of 1000 U/ml of exogenous mLIF for 4 days (p1⁺, where ⁺ denotes the presence of LIF) have equivalent gene expression to EPL cells maintained in the absence of exogenous mLIF, for 2 days (p0⁻, where - denotes the absence of LIF). However, the differentiation equivalence of these EPL cell cultures has never been assessed.

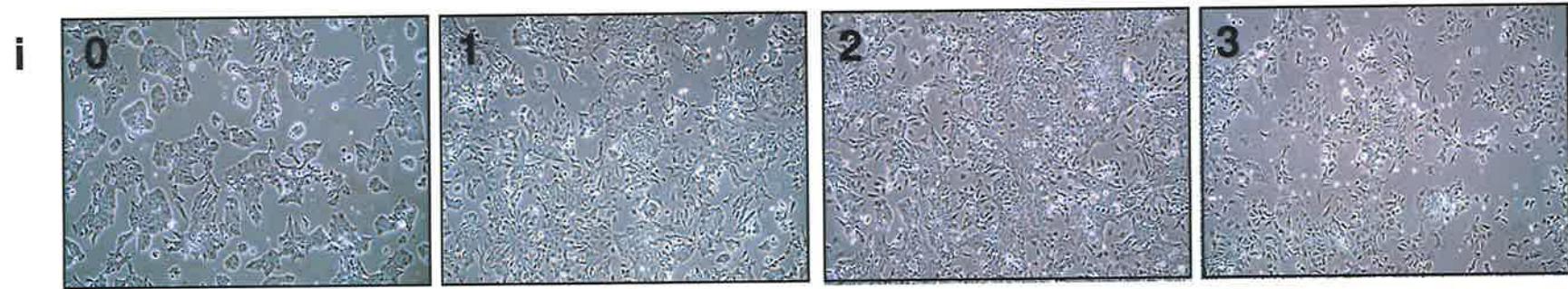
5.4 Gene expression of EPL cells induced by factors within MEDII and sfMEDII

D3 ES cells cultured under standard conditions (5.2) in the presence of 50% MEDII or sfMEDII adopted the EPL cell colony morphology (Figure 5.2 A (i), B (i)). Equivalent to previously published data (Rathjen *et al.*, 1999), these cells showed expression patterns of the *Fgf5*, *Rex1* and *Oct4* genes characteristic of EPL cell formation (Figure 5.2 A, B; Rathjen *et al.*, 1999). The maintenance of *Oct4* expression signifies maintenance of pluripotent cells within the EPL cell population. The up-regulation of *Fgf5* approximately 4.5-fold at p2 and 12.5-fold at p3 of culture indicates the formation of primitive ectoderm. The down-regulation of *Rex1* 4-fold at p0 to 8-fold at p3 signifies the progression of ES cells from an ICM like state towards the formation of EPL cells. These data indicate that sfMEDII contains signals that direct ES cells to adopt an EPL cell equivalent morphology and gene expression equivalent to MEDII produced in the presence of 10% FCS.

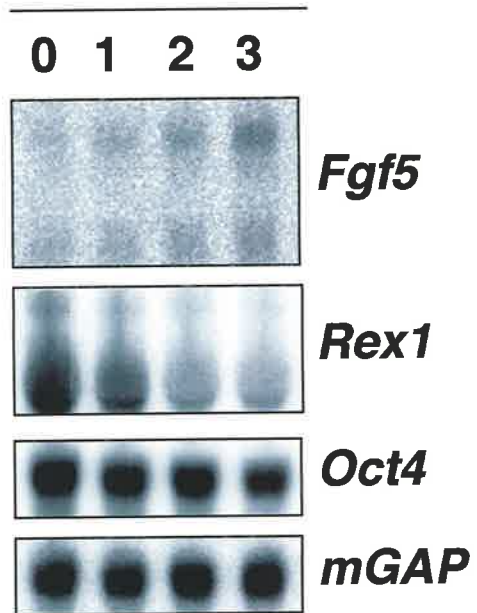
Figure 5.2 The EPL cell inductive capacity of MEDII and sfMEDII

Assessment of the EPL cell inductive capacity of MEDII and sfMEDII by morphology and gene expression. i) Morphological assessment of ES cells seeded in LIF supplemented DMEM (1000 U/ml) plus 50% MEDII (A) or 50% sfMEDII (B) for 2, 4, 6 and 8 days (0, 1, 2 and 3). Images were taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope. (ii) Northern blot analysis of EPL and sfEPL cell RNA (ii) 20 µg of total RNA was analysed for expression of *Fgf5*, *Rex1*, *Oct4* and *mGAP*. iii) Quantitative expression of the *Fgf5* and *Rex1* genes relative to expression of the pluripotent cell marker *Oct4*.

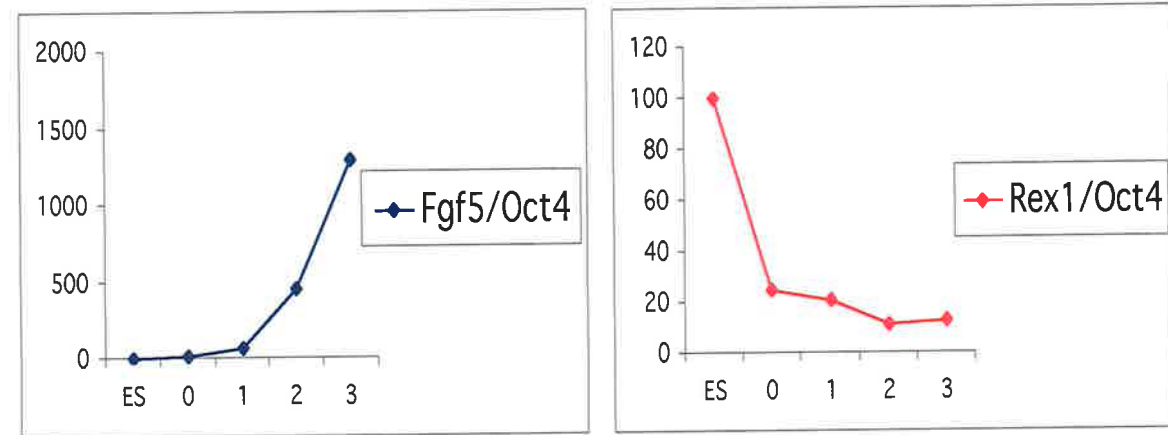
A



ii EPL

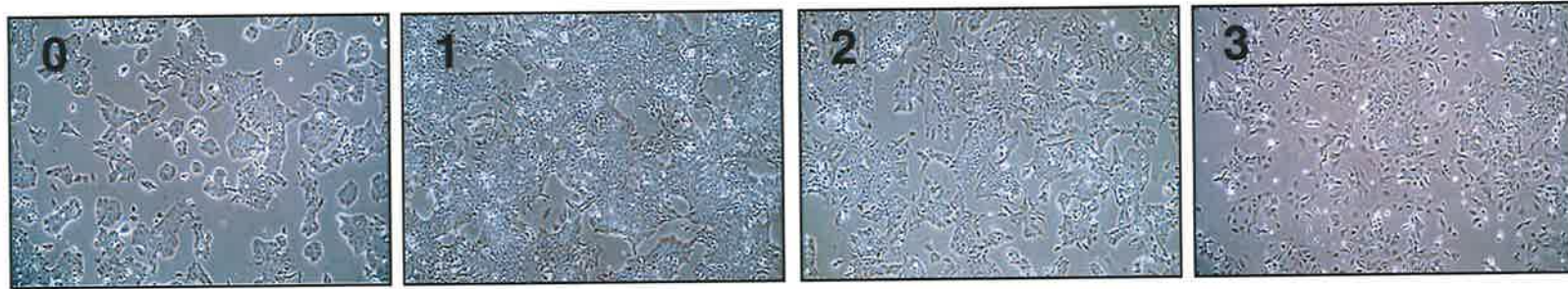


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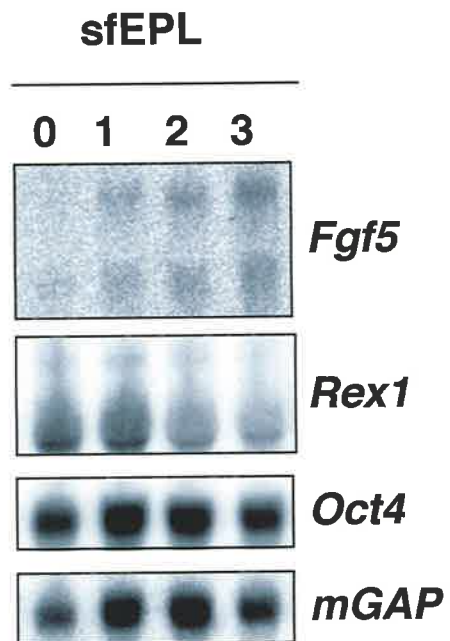


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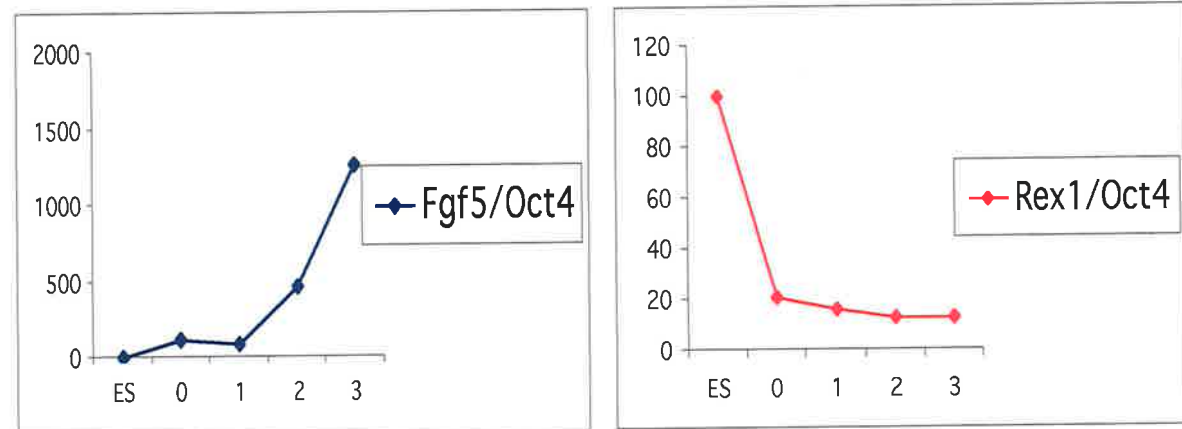
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iii



5.5 Differentiation of EPL cells induced by factors within MEDII and sfMEDII

To determine the differentiation characteristics of EPL cells formed in the presence or absence of LIF, EB's were aggregated from p0 and p1⁺ EPL cells. By day 4.0 of culture, EB's derived from p1⁺ EPL cells shared a similar morphology to EB's derived from p0 EPL cells and as described by Lake *et al.*, 2000 (Figure 5.3). Additionally, EB's derived from EPL cells formed in sfMEDII (p1⁺), were indistinguishable from p0 and p1⁺ EPL cell derived EB's. Northern blot analysis of these bodies showed induction of the *brachyury* marker gene at day 2.0 of culture in all EB's derived from EPL cells, distinct from ES cell derived EB's that induce *brachyury* gene expression on day 4.0 of culture (Figure 5.3). However, while there is some variability *brachyury* expression does start at day 2.0 and is effectively down regulated by day 4.0.

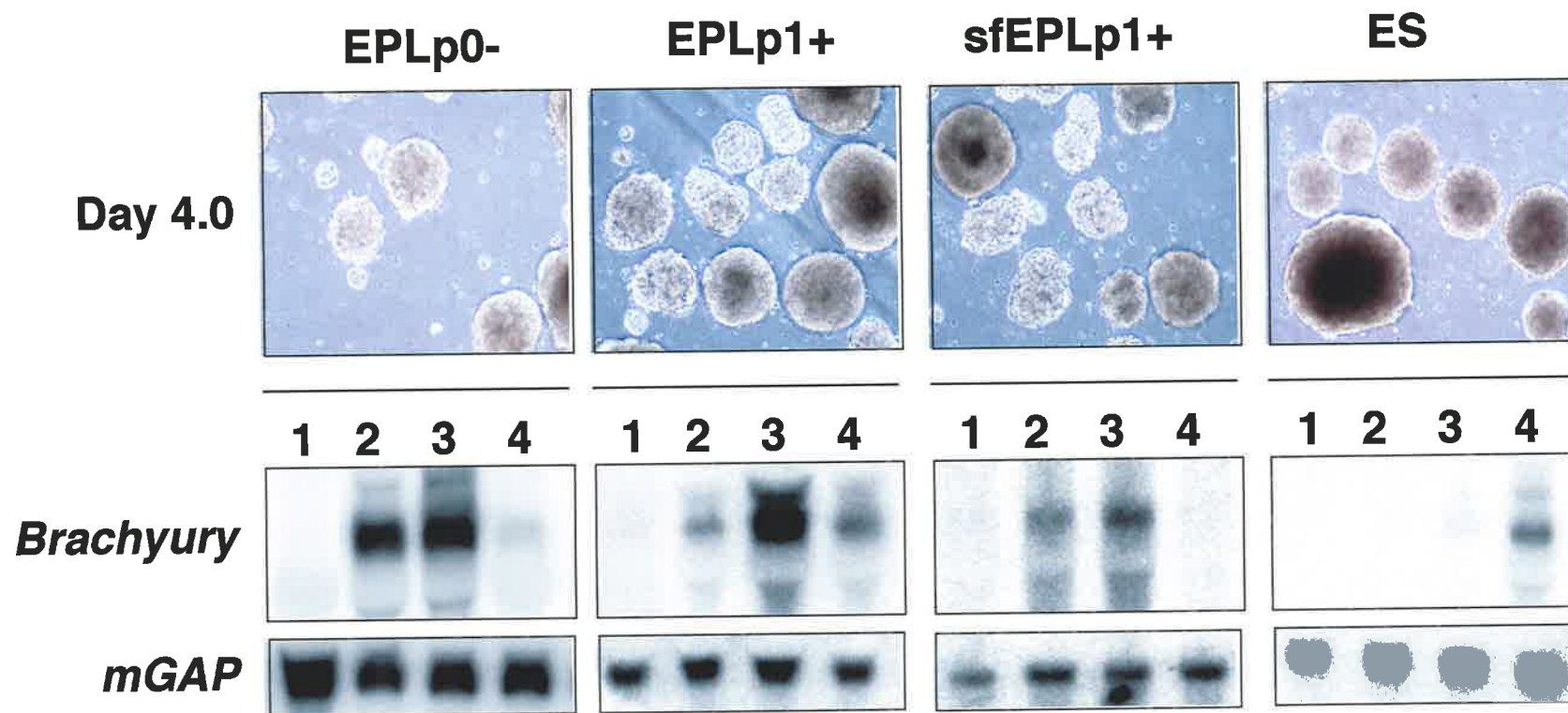
Collectively, these data indicate that EPL cells cultured under standard conditions in the presence of LIF for 4 days are equivalent to EPL cells cultured in the absence of LIF for two days. Furthermore, these data confirm that sfMEDII contains EPL cell inductive factors equivalent to MEDII. these observations allow determination of the EPL cell inductive capacity of L-proline and cFN under standard conditions in the presence of exogenous mLIF.

5.6 Concentration of inductive factors in MEDII

The level of low molecular weight activity within MEDII cannot be quantitated accurately because the molecular activity of this factor within MEDII has not been determined as it may comprise multiple, heterogeneous short peptides. However, 40 μ M L-proline was determined by titration to be lowest concentration of this signal that would support the morphological formation of EPL cells in response to the high molecular weight fraction (Washington, unpublished). Therefore, the low molecular weight signal during EPL cell formation would be investigated over a 10-fold concentration range of L-proline (40 μ M, 100 μ M or 400 μ M), supplemented into complete ES medium, to assess the EPL cell inductive capacity of the low molecular weight signal either alone or in the presence of cFN.

Figure 5.3 Mesoderm induction during differentiation of ES cell and EPL cell EB's

i) Morphology of day 4.0 EB's, derived from D3 ES cells cultured under standard conditions (2.2.5) in 50% MEDII in the absence of LIF for 2 days (EPLp0⁻), 50% MEDII in the presence of LIF for 4 days (EPLp1⁺), 50% sfMEDII in the presence of LIF for 4 days (sfEPLp1⁺) or standard D3 ES cells. EPL cell lineages were derived from D3 ES cells. ii) Northern blot analysis of 20 µg of total RNA isolated from D3 ES, EPLp0⁻, EPLp1⁺, sfEPLp1⁺ cells (p0), and EB's derived from these cells were probed for expression of the nascent mesoderm marker *Brachyury* between day 1.0 and 4.0 of differentiation. The *Brachyury* transcript is 2.1 kb (Hermann *et al.*, 1990; Herrmann, 1991). *mGAP* expression provides a loading control for total mRNA. Images taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.



The concentration of soluble cFN within MEDII was determined by comparing known amounts of gelatin sepharose purified human cellular FN (HepG2 derived; refer 4.5) against specific volumes of MEDII. Cellular FN (0.25-0.05 µg; as quantitated by Bradford assay performed to manufacturers specifications) was electrophoresed on an 8% reducing/denaturing polyacrylamide gel (2.7.20) with 5-1 µl of MEDII, and visualised by chemiluminescent western blot analysis (2.7.21) using the 3E2 anti-human EDA⁺ cFN monoclonal antibody (Sigma; 2.8.3). Quantitation of expression levels by densitometer (2.7.17; Molecular Dynamics) indicated that MEDII medium contained approximately 50 µg/ml of cFN (Figure 5.4). The specific activity of cFN, for the morphological formation of EPL cells in the presence of 40 µM L-proline, has been determined to be approximately 2 µg/ml (2.5.1). Thus, MEDII contains 25 U/ml of cFN for the induction of EPL cell morphology in the presence of 40 µM L-proline. The role of cFN during EPL cell formation was investigated at a concentration of 10 µg/ml (5 U/ml) in the presence of 40-400 µM L-proline.

5.7 ES cells cultured in the presence of cFN fail to adopt EPL cell gene expression and differentiation potential

To investigate the biological role of cFN during EPL cell formation, ES cells were cultured in ES complete medium supplemented with gelatin sepharose purified, MEDII derived, cFN (10 µg/ml; 2.3.10). Pluripotent cells in these cultures were identical in morphology to ES cells and did not adopt the EPL cell morphology (Figure 5.5 A). Northern blot analysis of these cells showed an identical pattern of gene expression to control D3 ES cells (Figure 5.5 B (i)). *Oct4* and *Rex1* expression levels remained consistent in the absence of *Fgf5* up-regulation (Figure 5.5 B (ii)). These data confirm that soluble cFN alone is not sufficient to induce EPL cell morphology or gene expression when presented in solution.

To determine if soluble cFN can direct ES cells to adopt EPL cell differentiation characteristics, ES cells were cultured in the presence of 10 µg/ml cFN as above and maintained to p1⁺ of culture prior to aggregation and differentiation as EB's. RNA was harvested from the parent p1⁺ culture and from each subsequent day of EB differentiation to day 4.0. The morphology of these EB's at day 4.0 was equivalent to ES cell derived EB's (Figure 5.5 C (i)) and northern blot analysis

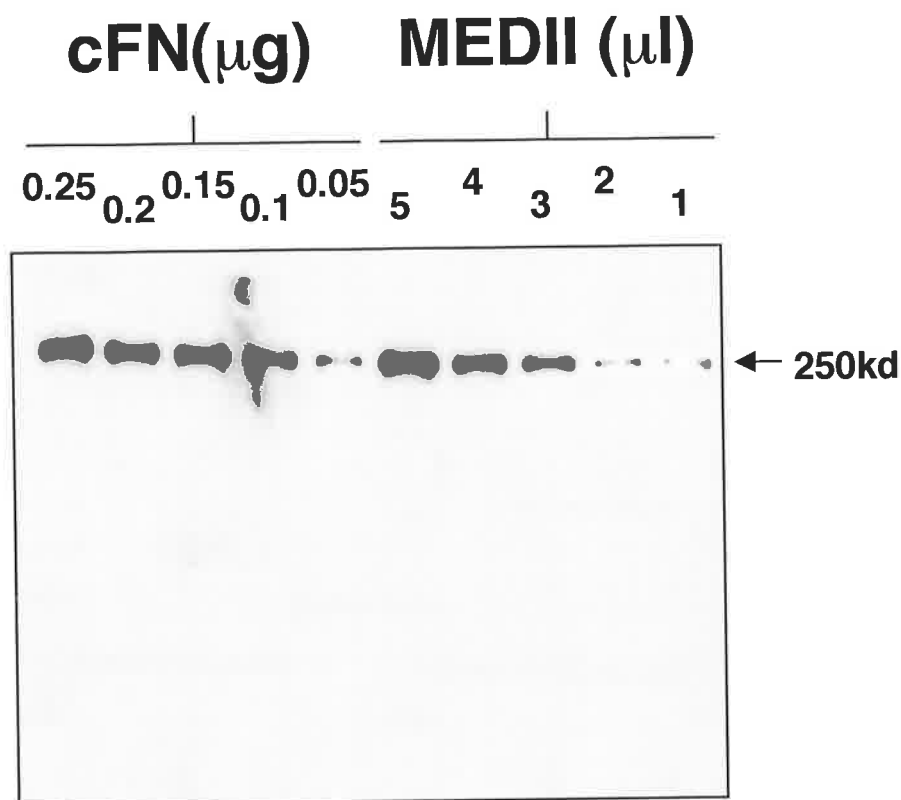
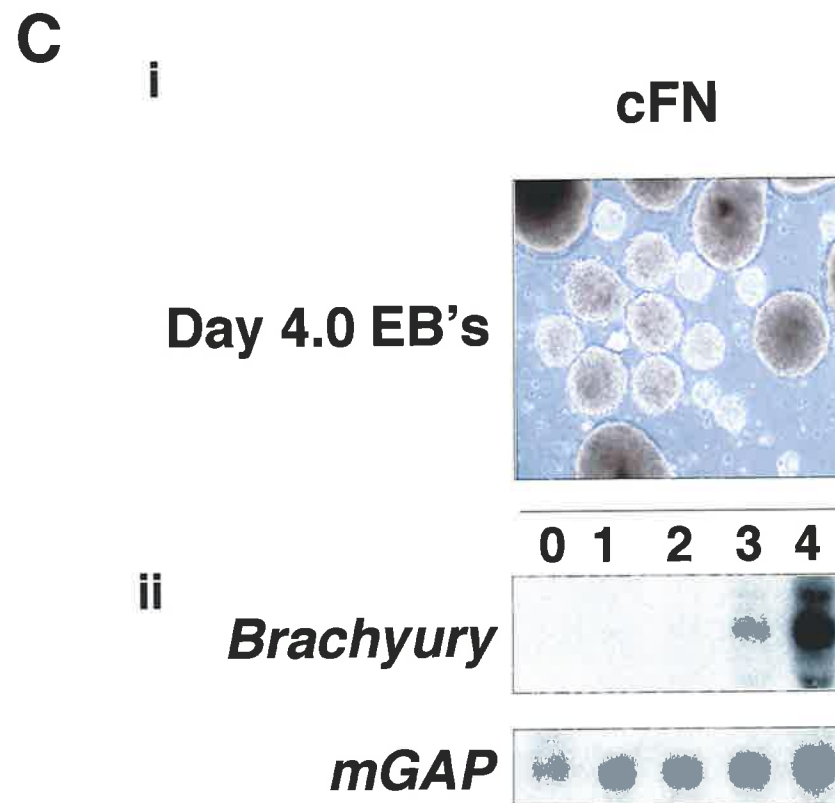
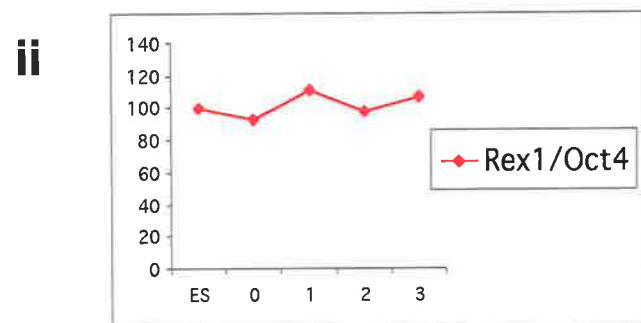
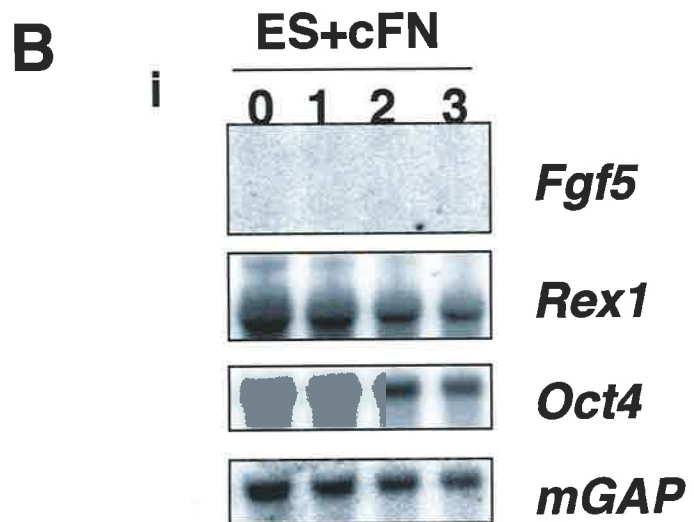
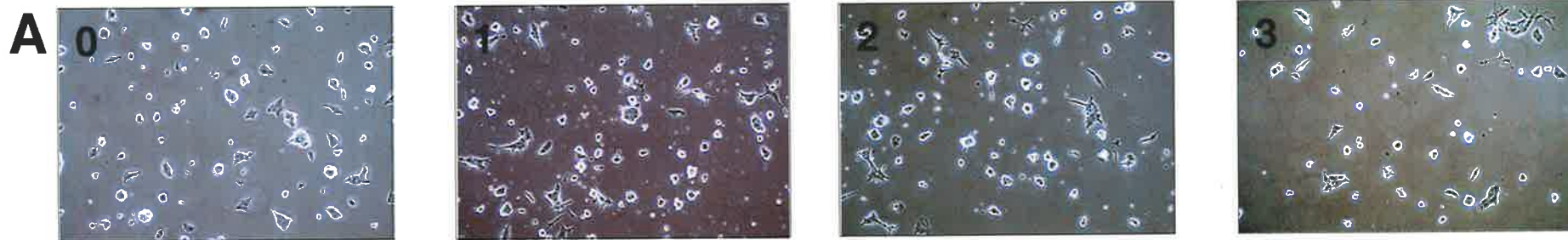


Figure 5.4 The concentration of cFN in MEDII

The concentration of cFN in MEDII was estimated by western analysis of known concentrations of MEDII derived cFN (Bradford, 2.5.1) against known volumes of MEDII medium. Standard dilutions cFN (0.25 μg, 0.2 μg, 0.15 μg, 0.1 μg & 0.05 μg) and MEDII (5 μl, 4 μl, 3 μl, 2 μl, 1 μl) were probed with the mouse anti-human EDA⁺ cFN monoclonal antibody 3E2 (Sigma) and visualised by a horse radish peroxidase anti-mouse secondary reacted with chemiluminescent substrate (Pierce) prior to exposure to film. cFN levels were analysed by volume integration on a densitometer (Molecular Dynamics). These data show that 0.5 μg of soluble cFN is present in 10 μl of MEDII.

Figure 5.5 Soluble cFN does not induce EPL cell formation in the absence of L-proline

Assessment of the EPL cell inductive capacity of cFN by morphology, gene expression and differentiation potential. A) Morphological assessment of ES cells cultured under standard conditions (2.5.5) in LIF supplemented DMEM (1000 U/ml) supplemented with cFN (10 µg/ml) for 2, 4, 6 and 8 days (0, 1, 2 and 3). Images were taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope. B) Northern blot analysis of ES + cFN cell RNA. i) 20 µg of total RNA, was analysed for expression of *Fgf5*, *Rex1*, *Oct4* and *mGAP*. ii) Quantitative expression of the *Fgf5* and *Rex1* genes relative to expression of the pluripotent cell marker *Oct4*. C) i) Morphology of day 4.0 EB's, derived from and ES + cFNp1⁺. ii) Northern blot analysis of 20 µg of total RNA isolated from EB's derived from these cells probed for expression of *Brachyury* between day 0-4 of differentiation. *mGAP* expression provides a loading control for total mRNA. Images were taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.



revealed expression of the *brachyury* marker gene initiated at day 4.0 of culture, identical to ES cell derived EB's (Figure 5.5 C (ii)). These results suggest that exogenous and soluble cFN fails to alter the differentiation potential of ES cells and is consistent with northern data that indicates soluble cFN cannot induce EPL cell formation in the absence of the low molecular weight signal.

To examine the effect of cFN presented as an ECM, ES cells were cultured under standard conditions on an exogenous ECM of HepG2 cell derived cFN ($1 \mu\text{g}/\text{cm}^2$) pre-coated onto tissue culture plastic. This ECM induced cultured pluripotent cells to adopt an EPL cell equivalent morphology (Figure 5.6 A (i)). However, northern blot analysis of these pluripotent cells indicated maintenance of ES cell equivalent gene expression (Figure 5.6 A (ii)). More specifically, these cells failed to up-regulate *Fgf5* or down-regulate *Rex1* marker genes while *Oct4* expression levels were maintained. These data indicate that ES cells cultured in the presence of cFN ECM retain characteristic ES cell gene expression. Importantly, these data suggest that an ECM of cFN is not an EPL cell inductive factor in the absence of L-proline.

To determine if ES cells cultured on a FN ECM adopt EPL cell differentiation capacity, D3 ES cells were cultured on an ECM of cFN ($1 \mu\text{g}/\text{cm}^2$), pFN ($1 \mu\text{g}/\text{cm}^2$), or collagen IV ($1 \mu\text{g}/\text{cm}^2$). ES cells maintained on an exogenous ECM until p1⁺ of culture formed EB's morphologically indistinguishable from ES cell derived EB's (Figure 5.6 B (i)). Northern analysis of these EB's detected *brachyury* expression initially in day 4.0 cultures, equivalent to characteristic ES cell differentiation (Figure 5.6 B (ii)).

Collectively, these data indicate that cFN ECM alone is not sufficient to induce EPL cell formation. Further, morphology alone is an insufficient indicator of EPL cell formation since culture of ES cells on a cFN matrix induced EPL cell morphology in the absence of EPL cell gene expression or differentiation potential.

5.8 Cellular FN and L-proline are necessary and sufficient for induction of EPL cell morphology, gene expression and differentiation potential

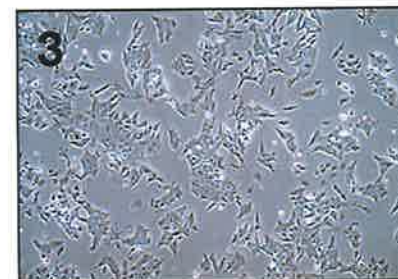
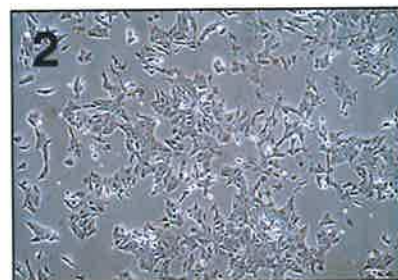
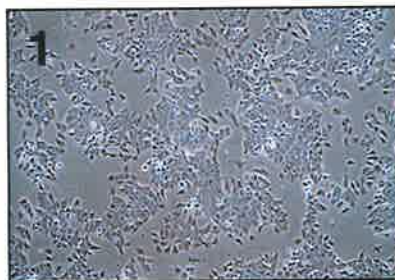
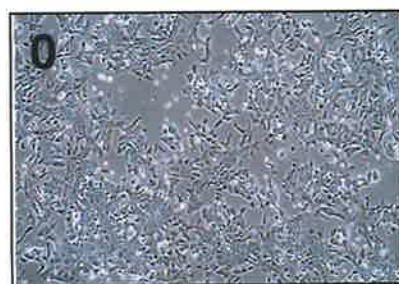
To assess the role of L-proline and cFN in EPL cell formation, D3 ES cells were cultured under standard conditions in medium supplemented with L-proline (40 μM , 100 μM or 400 μM) in

Figure 5.6 cFN ECM induces EPL cell morphology but not EPL cell gene expression in the absence of L-proline

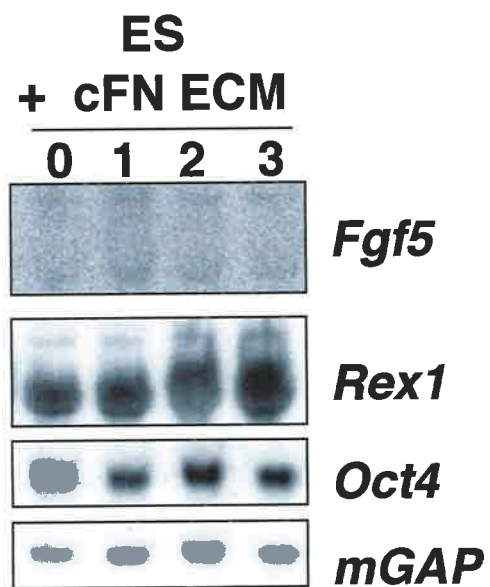
A) Morphological (i) and gene expression (ii) assessment of ES cells cultured in LIF supplemented DMEM (1000U/ml) on a sfMEDII derived cFN ECM ($1 \mu\text{g}/\text{cm}^2$) pre-coated onto tissue culture plastic. ES cells were cultured for 2, 4, 6 and 8 days (0, 1, 2 and 3). Northern blot analysis of 20 μg ES (+cFN ECM) cell RNA harvested from passage 0,1, 2 and 3, was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. Images were taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope. B) Mesoderm induction and differentiation in ES cell EB's is not affected by previous culture on ECM comprised of collagen IV, plasma FN or cFN. i) Morphology of EB's derived from D3 ES cells cultured for 4 days in contact with cFN ($1 \mu\text{g}/\text{cm}^2$), plasma FN ($1 \mu\text{g}/\text{cm}^2$) or collagen IV ($1 \mu\text{g}/\text{cm}^2$) ECM proteins, pre-coated on to tissue culture plastic, prior to EB formation. ii) Northern blot analysis of D3 ES cells cultured in contact with ECM proteins, and EB's derived from these cells cultured to day 4.0. 20 μg of total RNA was isolated and analysed for expression of *brachyury* between day 0-4 of differentiation. *mGAP* expression provides a loading control for total mRNA. Images were taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

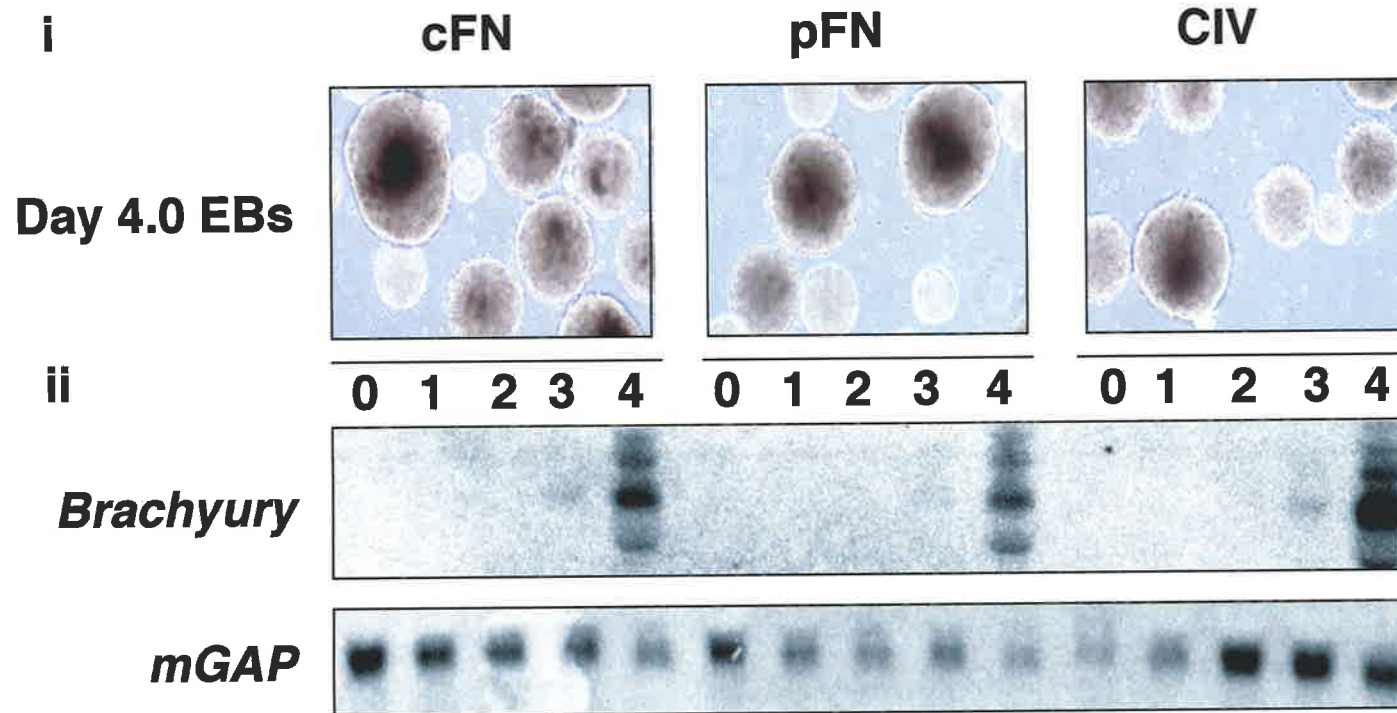
i



ii



B



the presence of soluble cFN (10 µg/ml). The morphology of these cells was indistinguishable from EPL cells throughout the culture period (Figure 5.7 A (i); Figure 5.8 A (i); Figure 5.9 A (i)). Northern blot analysis of D3 ES cells cultured in 40 µM L-proline and 10 µg/ml cFN revealed 5.3-fold and 6.6-fold induction of *Fgf5* expression to highest levels at p2 and p3 respectively (Figure 5.7 A (ii)(iii)). However, these cells failed to down-regulate expression of the ICM marker *Rex1* (Figure 5.7 A (ii)(iii)). These data indicate that 40 µM L-proline and 10 µg/ml cFN are not sufficient to induce characteristic EPL cell gene expression. This data also indicates an independent regulation of *Fgf5* and *Rex1* expression.

EB's derived from ES cells cultured in 40 µM L-proline and 10 µg/ml cFN to p1⁺ (designated day 0), shared an identical morphology with ES cell derived EB's at day 4.0 of culture (Figure 5.7 C (i)). Northern analysis of these EB's showed induction of *brachyury* expression equivalent to ES cell derived EB's at day 4.0 (Figure 5.7 C (ii)). These data confirm that culture of ES cells in 40 µM L-proline and 10 µg/ml cFN does not induce EPL cell gene expression and differentiation potential and that *Fgf5* up-regulation is not sufficient for EPL cell differentiation.

Northern blot analysis of D3 ES cells cultured in ES complete medium supplemented with 100 µM or 400 µM L-proline and 10 µg/ml cFN revealed 7-fold and 8-fold down-regulation of the *Rex1* by p2 relative to *Oct4* respectively (Figure 5.8 A (ii)(iii); Figure 5.9 A (ii)(iii)). Furthermore, these cells showed 6.3 and 11-fold induction of *Fgf5*, relative to *Oct4*, by p2 respectively (Figure 5.8 A (ii)(iii); Figure 5.9 A (ii)(iii)). Importantly, these cultures maintained or slightly up-regulated *Fgf5* expression levels at p3. This pattern of *Fgf5* and *Rex1* expression is reminiscent of EPL cell cultures formed in the presence of MEDII.

Quantitation of *Fgf5* expression relative to *Oct4* in ES cells cultured in 40 µM and 100 µM L-proline indicate that *Fgf5* was expressed at half maximal levels relative to control EPL cells (Figure 5.7 A, B (iii), Figure 5.8 A, B (iii), refer Figure 5.2 A, B (iii)). However, maintenance of pluripotent cells in 400 µM L-proline resulted in higher level expression of *Fgf5* (Figure 5.9 A, B (iii)). This suggests that pluripotent cells are responsive to L-proline in a concentration dependent manner.

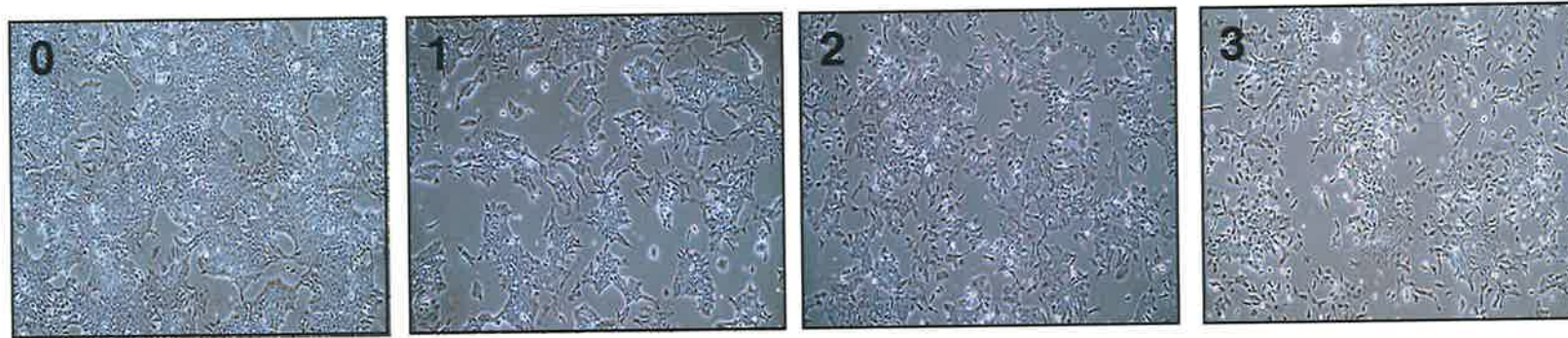
Figure 5.7 40 μ M L-proline induces EPL cell morphology in the presence of cFN, but not EPL cell gene expression

Morphological (i) and gene expression (ii) assessment of the EPL cell inductive capacity of (A) 40 μ M L-proline + cFN (10 μ g/ml) and (B) 40 μ M L-proline by morphology and gene expression. i) Morphological assessment of ES cells cultured under standard conditions of LIF (1000 U/ml) supplemented DMEM in the presence of 40 μ M L-proline and 40 μ M L-proline + cFN (10 μ g/ml) for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of A, B and C cell RNA analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

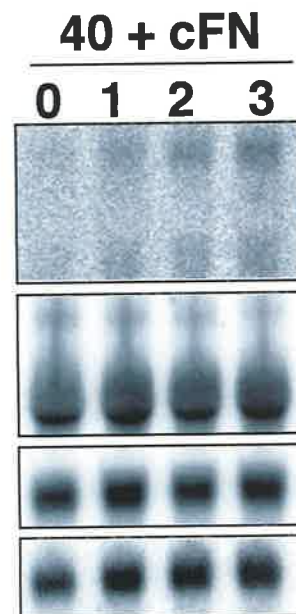
C) Mesoderm induction in EB's derived from ES cells cultured in 40 μ M L-proline for 4.0 days under standard conditions (2.5.5) prior to EB formation. i) Morphology of day 4.0 EB's derived from ES cells cultured in 40 μ M L-proline or 40 μ M L-proline + cFN (10 μ g/ml) to p1⁺ prior to EB formation. ii) Northern blot analysis of 20 μ g of total RNA, isolated from EB's derived from these cells, for expression of *brachyury* between day 0 and 4.0 of differentiation. *mGAP* expression provides a loading control for total mRNA. Images were taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

i



ii



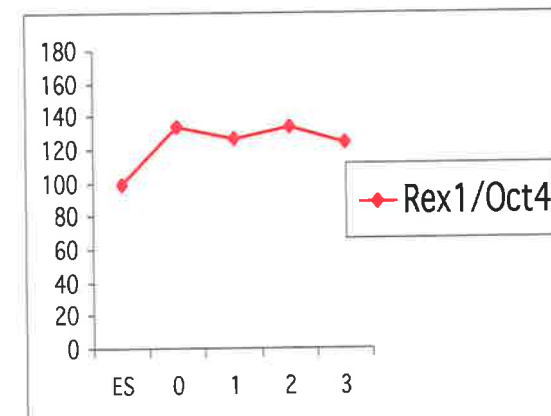
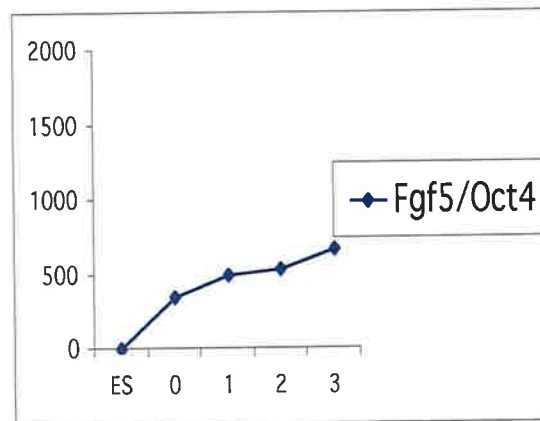
Fgf5

Rex1

Oct4

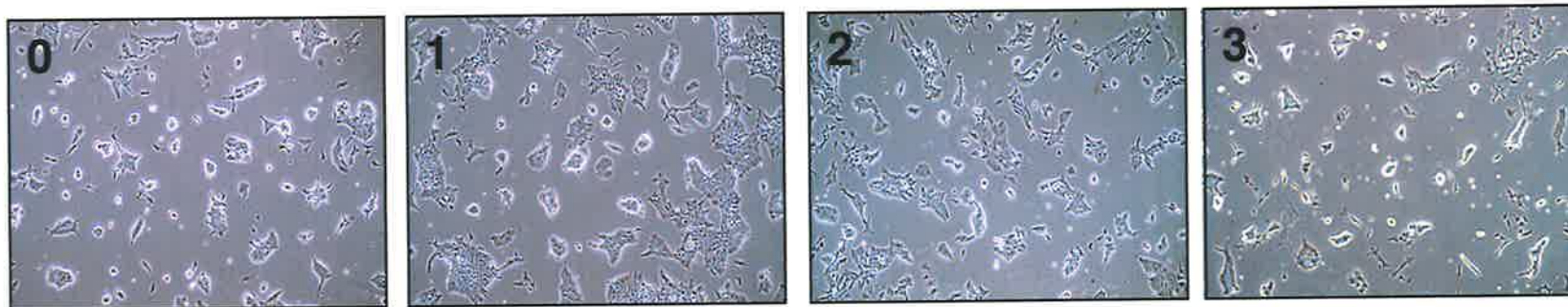
mGAP

iii

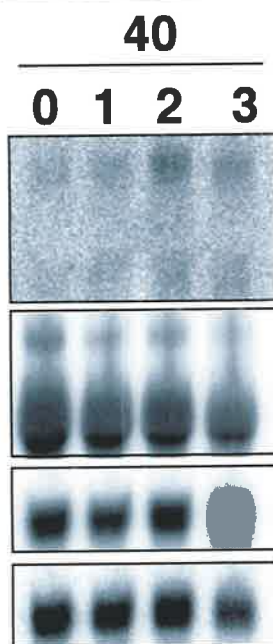


B

i



ii



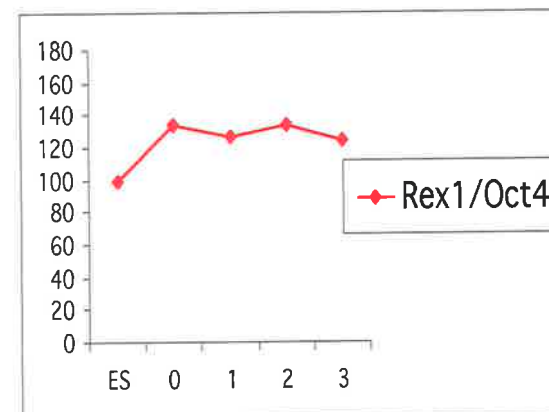
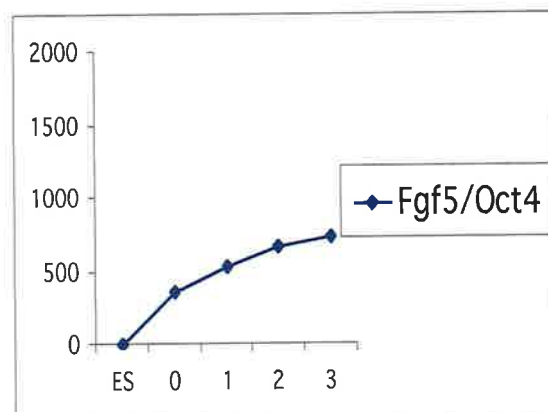
Fgf5

Rex-1

Oct4

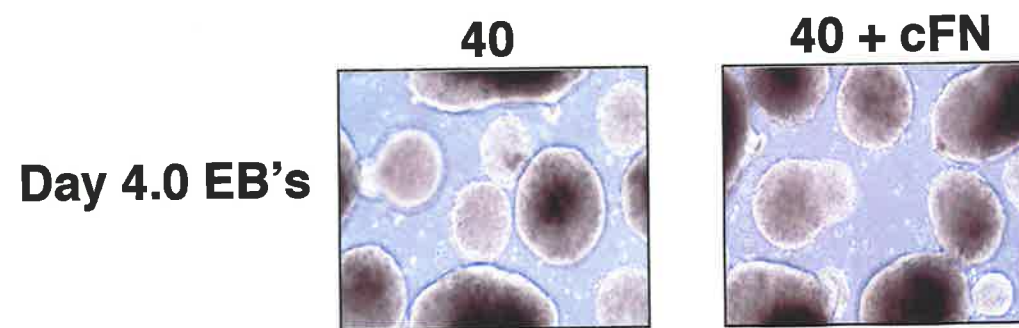
mGAP

iii



C

i



ii

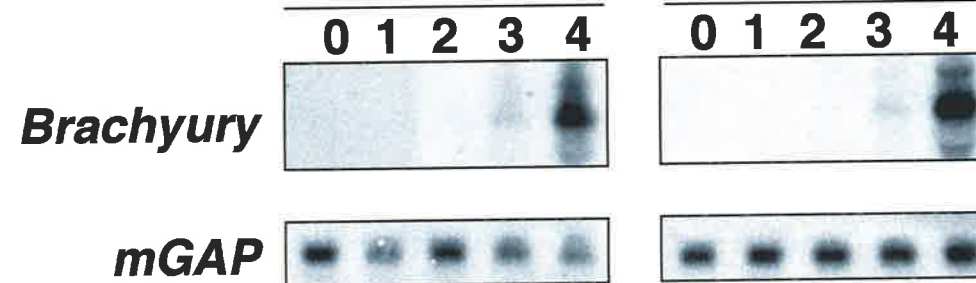
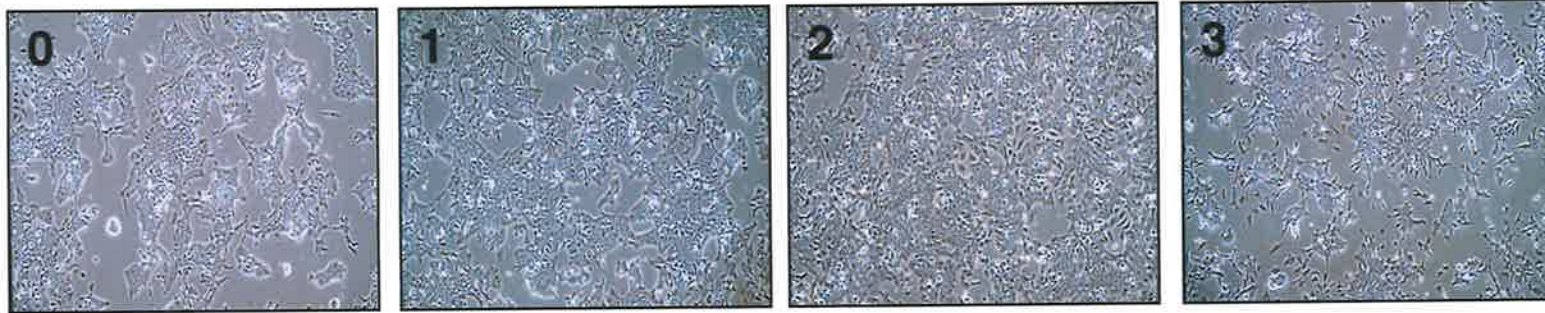


Figure 5.8 100 μ M L-proline induces EPL cell morphology and gene expression in the presence or absence of soluble cFN

Morphological (i) and gene expression (ii) assessment of the EPL cell inductive capacity of (A) 100 μ M L-proline + cFN (10 μ g/ml) and (B) 100 μ M L-proline by morphology and gene expression. i) Morphological assessment of ES cells cultured under standard conditions of LIF (1000 U/ml) supplemented DMEM in the presence of 100 μ M L-proline or 100 μ M L-proline + cFN (10 μ g/ml) for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of A, B and C cell RNA. 20 μ g of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images were taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope. C) i) Morphology of day 4.0 EB's derived from ES cells cultured in 100 μ M L-proline or 100 μ M L-proline + cFN (10 μ g/ml) for 4.0 days to p1⁺ prior to EB formation. ii) Northern blot analysis of 20 μ g of total RNA isolated from EB's, derived from these cells, for expression of *brachyury* between day 0 and 4.0 of differentiation. *mGAP* expression provides a loading control for total mRNA. Images were taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

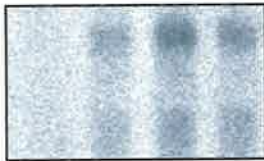
i



ii

100 + cFN

0 1 2 3



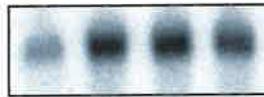
Fgf5



Rex1

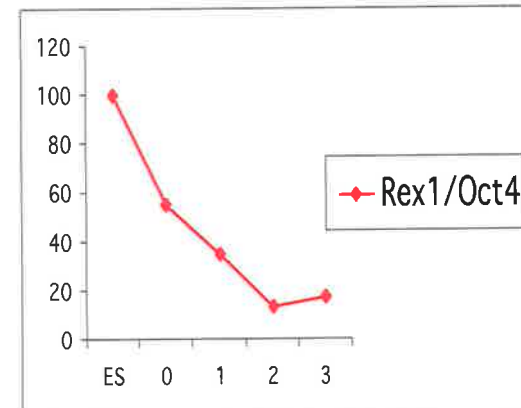
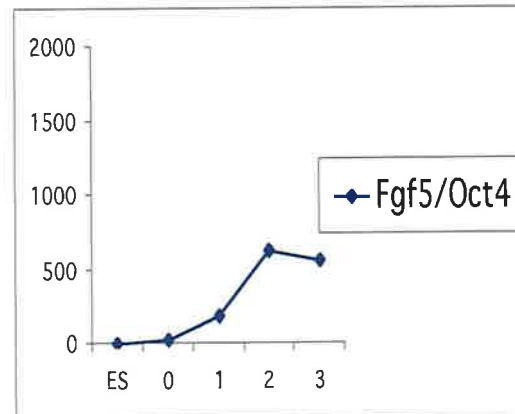


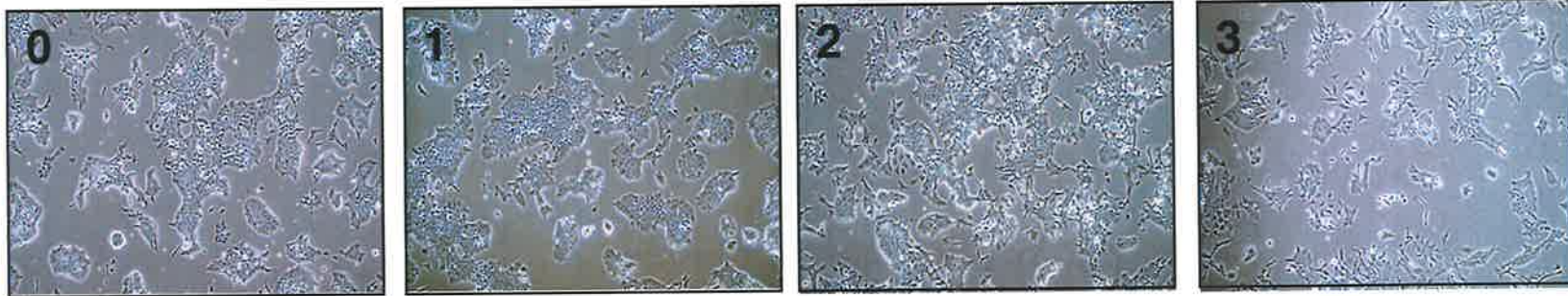
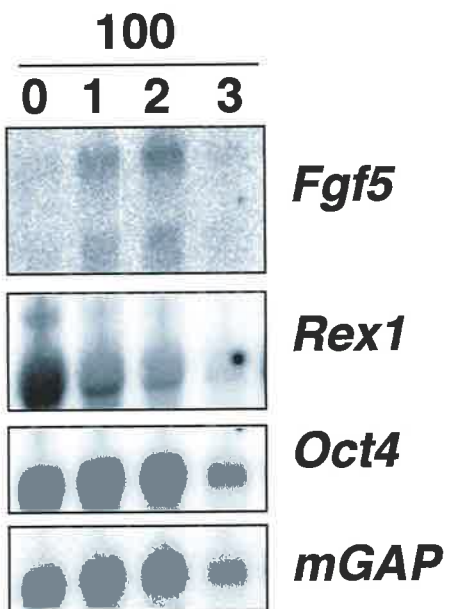
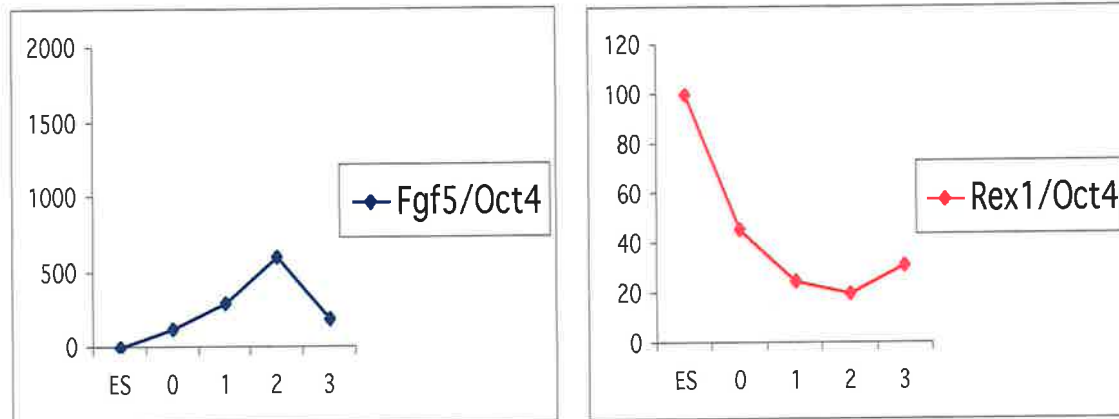
Oct4



mGAP

iii



B**i****ii****iii**

C

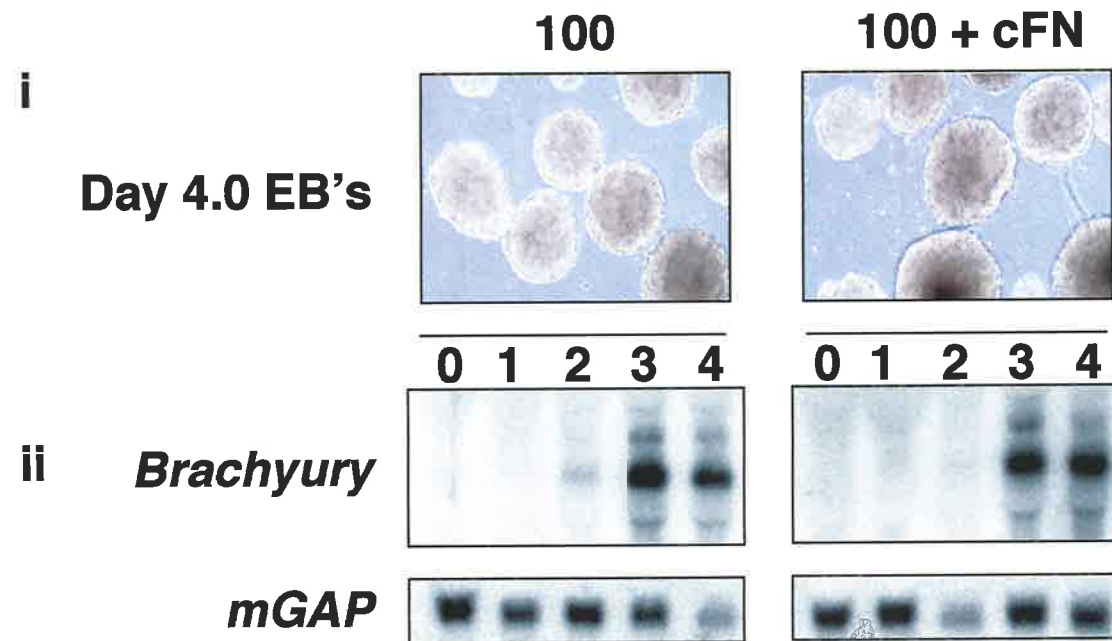
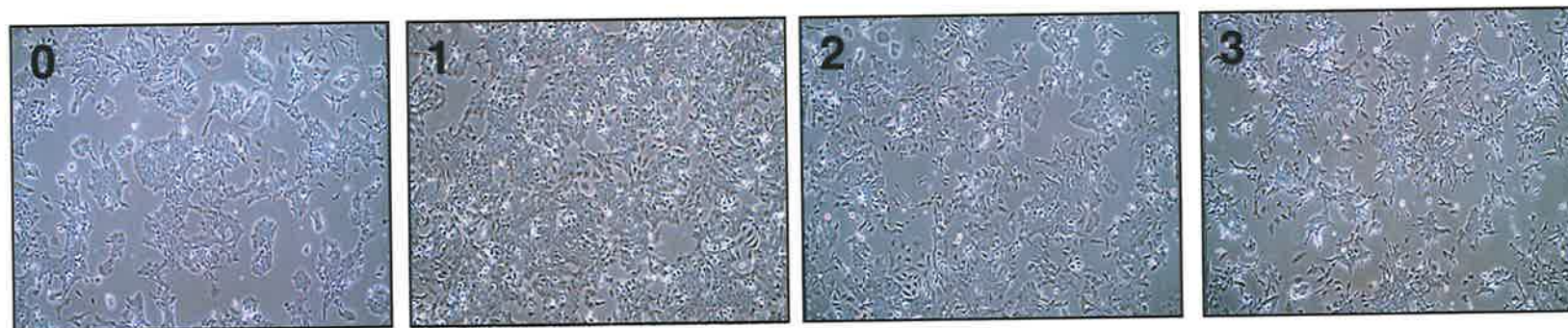


Figure 5.9 400 μ M L-proline induces EPL cell morphology and gene expression in the presence or absence of soluble cFN

Morphological (i) and gene expression (ii) assessment of the EPL cell inductive capacity of (A) 400 μ M L-proline + cFN (10 μ g/ml) and (B) 400 μ M L-proline by morphology and gene expression. i) Morphological assessment of ES cells cultured under standard conditions of LIF (1000 U/ml) supplemented DMEM in the presence of 400 μ M L-proline and 400 μ M L-proline + cFN (10 μ g/ml) for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of A, B and C cell RNA. 20 μ g of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope. C) Mesoderm induction in EB's derived from ES cells cultured in 400 μ M L-proline prior to EB formation. i) Morphology of day 4.0 EB's derived from ES cells cultured in 400 μ M L-proline or 400 μ M L-proline + cFN (10 μ g/ml) to p1⁺ prior to EB formation. ii) Northern blot analysis of 20 μ g of total RNA isolated from EB's, derived from these cells, for expression of *brachyury* between day 0-4 of differentiation. *mGAP* expression provides a loading control for total mRNA. Images were taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

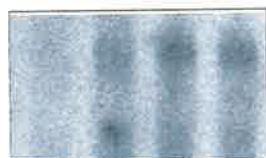
i



ii

400 + cFN

0 1 2 3



Fgf5



Rex1

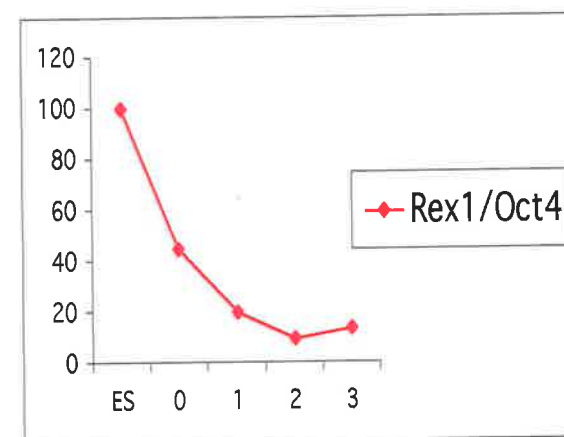
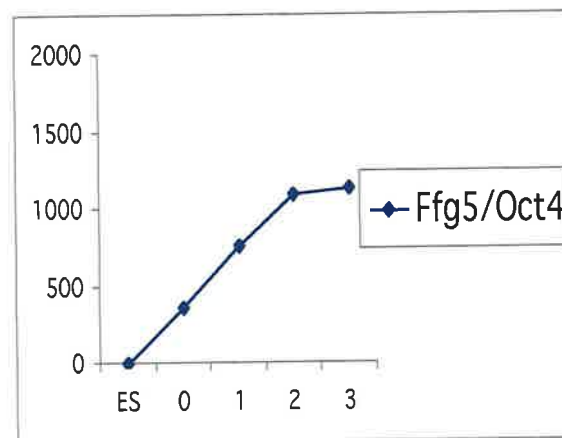


Oct4



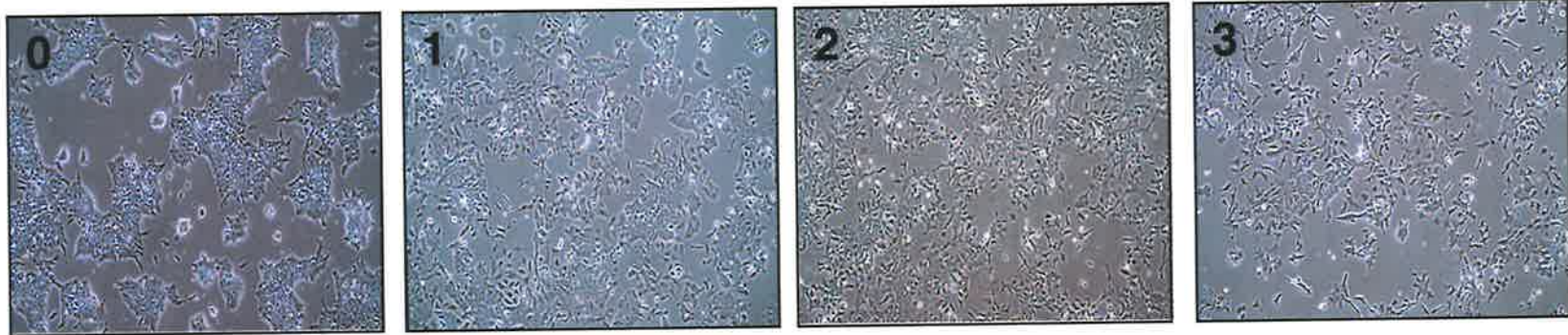
mGAP

iii

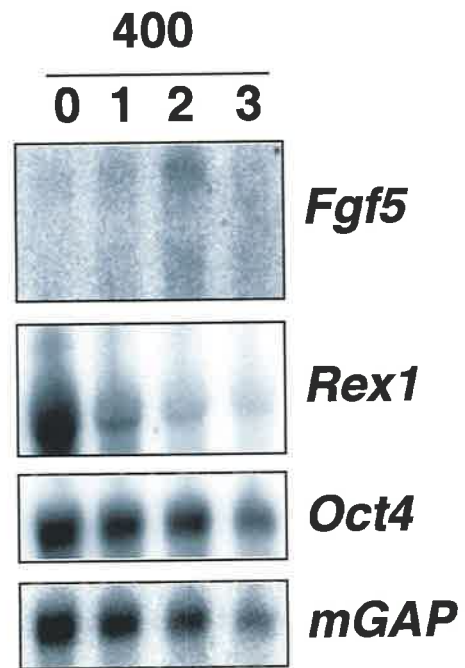


B

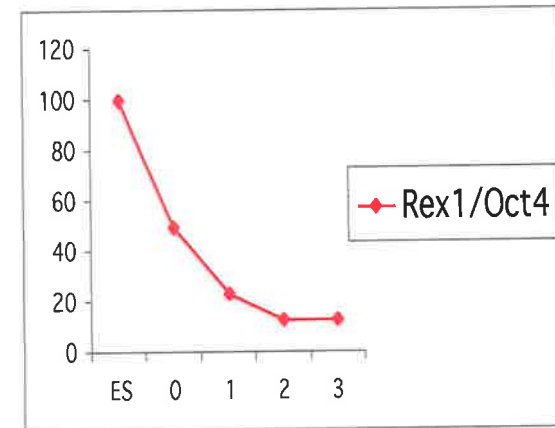
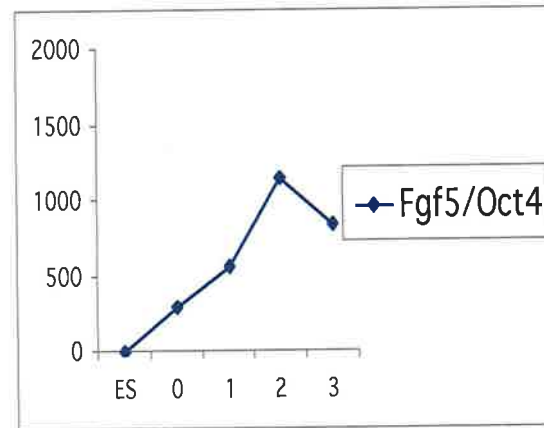
i



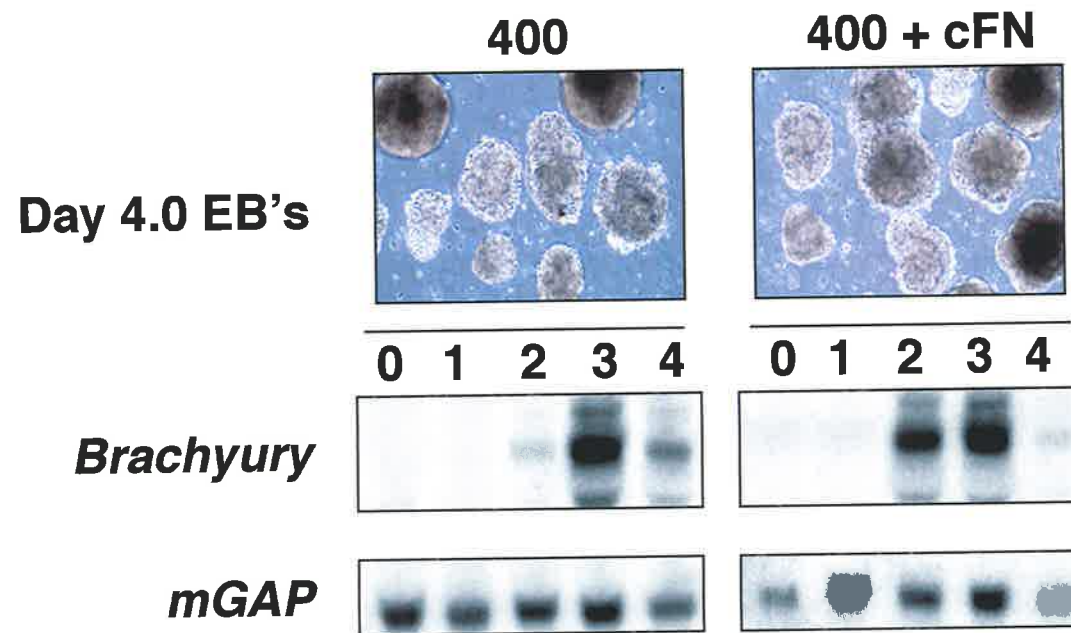
ii



iii



C



EB's derived from ES cells cultured in 100 μ M L-proline and 10 μ g/ml cFN to p1⁺ (designated day 0) gave rise to EB's that displayed an intermediate morphology between ES and EPL cell derived EB's at day 4.0 of culture (Figure 5.8 C (i)) where the disorganised morphology characteristic of EPL cell derived EB's was not observed at equivalent levels to control EPL cell derived aggregates. In support of this morphological distinction, expression of *brachyury* was initiated at day 3.0 of culture (Figure 5.8 C (ii)). ES cells cultured in 100 μ M L-proline and 10 μ g/ml cFN are therefore intermediate in differentiation capacity between MEDII induced EPL cells and ES cells.

ES cells cultured to p1⁺ in 400 μ M L-proline and 10 μ g/ml cFN gave rise to EB's that displayed an identical morphology to MEDII induced EPL cells by day 4.0 of culture (Figure 5.9 C (i)). Furthermore, EB's formed in this way induced *brachyury* expression at day 2.0 of culture, equivalent to EPL cell derived EB's (Figure 5.9 C (ii)). These data determined that ES cells cultured for 4 days to p1⁺ in 400 μ M L-proline and 10 μ g/ml of soluble cFN are identical in differentiation potential to MEDII induced EPL cells. However, it was also observed that in 400 μ M L-proline EB's retain slightly higher levels *brachyury* expression at day 4.0 when compared to MEDII induced EPL cells.

These data indicate that L-proline and cFN have equivalent biological activity to the EPL cell inductive signals within MEDII when presented to ES cells at a concentration of 400 μ M L-proline and 10 μ g/ml cFN.

5.9 A role for cFN in the induction of EPL cell morphology, gene expression and differentiation potential

To further assess the role of cFN in EPL cell formation, D3 ES cells were cultured under standard conditions in medium supplemented with L-proline (40 μ M, 100 μ M or 400 μ M) in the absence of soluble cFN (10 μ g/ml). ES cells cultured in the presence of 40 μ M L-proline and the absence of cFN adopted a morphology intermediate between ES cells and EPL cells (Figure 5.7 B (i); Figure 5.8 B (i); Figure 5.9 B (i)). Given that addition of cFN under these conditions induces EPL cell morphology, these data demonstrate the responsiveness of pluripotent cells to cFN in the

presence of 40 μ M L-proline and implicate cFN in directing EPL cell colony morphology, consistent with the ability of a cFN matrix to induce EPL cell morphology (5.6). Northern blot analysis of D3 ES cells cultured in 40 μ M L-proline and the absence of cFN revealed 6.6-fold and 7.3-fold induction of *Fgf5* expression to highest levels at p2 and p3 respectively (Figure 5.7 B (ii)(iii)). These conditions also failed to down-regulate expression of *Rex1* (Figure 5.7 B (ii)(iii)). These data indicate that 40 μ M L-proline does not induce characteristic EPL cell gene expression in the presence or absence of cFN. EB's derived from ES cells cultured in 40 μ M L-proline to p1⁺, in the absence of cFN, shared an identical morphology and differentiation with EB's derived from D3 ES cells cultured under identical conditions in the presence of 10 μ g/ml cFN (Figure 5.7 C (i)).

D3 ES cells cultured in ES complete medium supplemented with 100 μ M or 400 μ M L-proline in the absence of exogenous soluble cFN adopted a colony morphology indistinguishable from EPL cells (Figure 5.8 B (i) & Figure 5.9 B (i)). These data indicate that in higher concentrations of L-proline pluripotent cells can adopt the EPL cell morphology, presumably utilising components of the pre-existing gelatin matrix or, perhaps, an endogenous source of cFN. In addition, these data further indicate that, in these conditions, L-proline is sufficient for the formation of EPL cell colonies in a dose dependent manner.

Northern blot analysis of ES cells cultured in 100 μ M or 400 μ M L-proline, in the absence of cFN, displayed a respective 7-fold and 8-fold down-regulation of *Rex1* relative to *Oct4*. This gene expression pattern is most similar to MEDII induced EPL cells. These cells also showed a respective 6.2 and 11-fold induction of *Fgf5* by p2. This up regulation is reminiscent of MEDII induced EPL cells and ES cells cultured under identical conditions in the presence of cFN. Quantitation of *Fgf5* expression levels relative to *Oct4* in ES cells cultured in 40 μ M-400 μ M L-proline were similar to cells cultured in the presence of cFN until p2 (Figure 5.7 A, B (iii), Figure 5.8 A, B (iii), refer Figure 5.2 A, B (iii), Figure 5.9 A, B (iii)). This indicates that that the presence of exogenous soluble cFN has no effect on EPL cell gene expression up to p2. However, distinct from MEDII induced EPL cells or EPL cells formed in 100 μ M or 400 μ M L-proline and the presence of cFN, *Fgf5* expression was down regulated at p3 in the cultures maintained in the absence of soluble exogenous cFN (Figure 5.8 A, B (ii) and Figure 5.9 A, B (ii)). The persistence of *Fgf5* and *Oct4* expression in

EPL cell cultures containing soluble cFN suggests this signal can act as a maintenance factor for EPL cells. This proposition is further supported by the up regulated expression of *Fgf5* at p3 in MEDII induced EPL cells as, in contrast to the 10 µg/ml of soluble cFN presented to ES cells cultured in 100 µM-400 µM, MEDII contains 50 µg/ml of cFN. Therefore, the increased levels of *Fgf5* expression in p3 EPL cell cultures may reflect a dose dependent response of EPL cells to increasing levels of soluble exogenous cFN. Gene expression data in EPL cells and ES cells cultured in the presence or absence of L-proline and cFN are summarised in Table 5.1.

EB's derived from ES cells cultured in 100 µM-400 µM L-proline in the absence of cFN displayed a similar morphology and gene expression to EB's derived from cells cultured in the presence of 100 µM-400 µM L-proline and 10 µg/ml cFN (Figure 5.8 C; Figure 5.9 C). These data determined that ES cells cultured for 4 days in 100 µM L-proline are intermediate between ES cells and EPL cells in differentiation potential while ES cells cultured for 4 days in 400 µM L-proline are most similar in differentiation potential to MEDII induced EPL cells. However, EB's formed from EPL cells cultured in 400 µM L-proline and the absence of cFN maintain higher levels of *brachyury* expression at day 4.0 of culture. This is distinct from EB's derived from MEDII induced EPL cells or ES cells cultured in 400 µM L-proline and the presence of cFN. This indicates that EPL cell differentiation may be responsive to increased levels of exogenous cFN. However, some variation in *brachyury* expression has been detected in MEDII derived EPL cell differentiation (Figure 5.3). These differentiation experiments use cells derived from day 4.0 of culture and hence do not address EPL cell maintenance by cFN which is only visible by gene expression 8 days after the initiation of EPL cell culture.

In conclusion, the biological activity of L-proline suggests that this signal is equivalent to the low molecular weight EPL cell inductive component in MEDII. ES cells respond to L-proline irrespective of the presence or absence of cFN but cFN can act as a maintenance factor for EPL cell culture *in vitro*.

Table 5.1 Summary of morphology, gene expression and differentiation potential of ES cells, EPL cells and ES cells cultured in the presence or absence of MEDII and various concentrations of L-proline and 10µg/ml of cFN.

Arrows with *Fgf5* indicate up-regulated (↑), down-regulated (↓) or maintained (→) expression at p2 and p3 of culture under standard conditions. *Fgf5* without arrows indicate minimal alteration in expression levels throughout the culture period. Arrows with *Rex1* indicate maintenance (→) or down-regulation (↓) of *Rex1* expression through the culture period. Cellular FN used in assays at 10 µg/ml.

Culture Conditions	Morphology	Gene Expression		Differentiation
ES	ES	<i>Fgf5</i>	<i>Rex1</i> →	ES
MEDII	EPL	<i>Fgf5</i> ↑↑	<i>Rex1</i> ↓	EPL
cFN	EPL	<i>Fgf5</i>	<i>Rex1</i> →	ES
40 μM L-pro	EPL/ES	<i>Fgf5</i> ↑→	<i>Rex1</i> →	ES
40 μM L-pro + cFN	EPL	<i>Fgf5</i> ↑→	<i>Rex1</i> →	ES
100 μM L-pro	EPL	<i>Fgf5</i> ↑↓	<i>Rex1</i> ↓	EPL/ES
100 μM L-pro + cFN	EPL	<i>Fgf5</i> ↑→	<i>Rex1</i> ↓	EPL/ES
400 μM L-pro	EPL	<i>Fgf5</i> ↑↓	<i>Rex1</i> ↓	EPL
400 μM L-pro + cFN	EPL	<i>Fgf5</i> ↑→	<i>Rex1</i> ↓	EPL

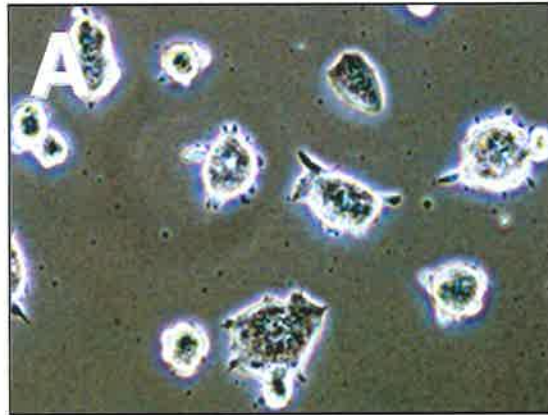
5.10 Correct presentation of cFN is required for induction of EPL cell morphology

Cellular FN directs EPL cell morphology when presented to ES cells as an ECM or in soluble form. The activity of cFN, but not pFN, when presented in soluble form implies that ECM integration is a requirement for EPL cell morphology. These assays are carried out in the presence of gelatin (0.2%) which may provide an architecture for ECM assembly and presentation of cFN to pluripotent cells.

ES cells were cultured in the presence of 400 μ M L-proline or MEDII on un-coated tissue culture plastic. Under these conditions, ES cells failed to adopt the EPL cell morphology (Figure 5.10). These results indicate a requirement for exogenous ECM architecture for the correct presentation of cFN during EPL cell formation and further suggest that EPL cell morphology is associated with the correct assembly of cFN into an ECM.

To assess the requirement for exogenous ECM for EPL cell gene expression and differentiation potential ES cells were cultured under standard conditions (2.3.15) in the absence of pre-coated gelatin (0.2%) in ES complete DMEM and 50% sfMEDII or 50% MEDII. Northern blot analysis of these cells showed up to 9.1-fold down-regulation of *Rex1* expression in a similar manner to control EPL cells (Figure 5.11 A, B (ii)). However, *Fgf5* expression levels were not increased in these pluripotent cell cultures (Figure 5.11 A, B (ii) & (iii)). These data indicate that an interaction between exogenous ECM and factors within MEDII is required for EPL cell colony morphology and expression of the EPL cell marker *Fgf5*. In particular, the correct assembly and presentation of cFN to pluripotent cells maybe a requirement for up-regulation of *Fgf5* expression at p2 and p3 of culture. Furthermore, these data confirm the independent regulation of *Fgf5* and *Rex1* expression during EPL cell formation.

Aggregates derived from p1⁺ EPL cells previously cultured in the absence of ECM formed EB's most similar to those derived from standard EPL cells (Figure 5.11 C (i)) as northern blot analysis of these bodies detected *brachyury* expression from day 2.0 of culture. However, *brachyury* expression persisted to day 4.0 of culture, which is distinct from EPL cell EB's and most similar to EB's derived from ES cells cultured in 100 μ M-400 μ M L-proline and the absence of, or



EPL



400 μ M L-proline

Figure 5.10 ES cells cultured in the absence of exogenous ECM fail to adopt the EPL cell morphology

ES cells cultured in the absence of pre-coated gelatin (0.2%) ECM (A,B) retain the ES cell morphology in the presence of (A) MEDII or (B) 400 μ M L-proline. Images were taken at 100x magnification using phase contrast microscopy.

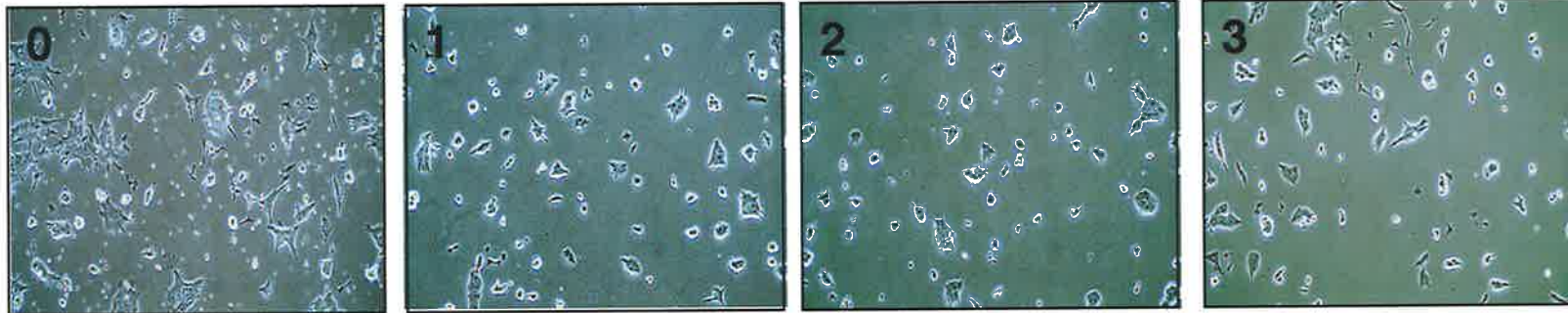
Figure 5.11 EPL cells cultured in the absence of exogenous ECM fail to adopt EPL cell morphology and gene expression

Assessment of the EPL cell inductive capacity of ES cells cultured under standard conditions in the absence of an exogenous gelatin ECM and the presence of (A) 50% sfMEDII or (B) 50% MEDII by morphology and gene expression. Morphological assessment of EPL cells cultured for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of sfEPL and EPL cell RNA. 20 µg of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

C) Mesoderm induction in EB's derived from EPL cell previously cultured in the absence of exogenous ECM. i) Morphology of day 4.0 EB's derived from EPL-ECM ($p1^+$) and sfEPL-ECM ($p1^+$) cells. ii) Northern blot analysis of 20 µg of total RNA isolated from EB's, derived from these cells, for expression of the nascent mesoderm marker *Brachyury* between day 0-4 of differentiation. *mGAP* expression levels were determined as a general loading control. Images taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

i



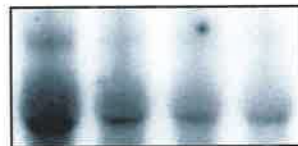
ii

sfEPL - ECM

0 1 2 3



Fgf5



Rex1

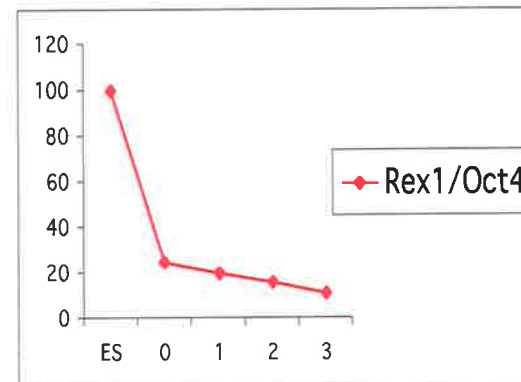
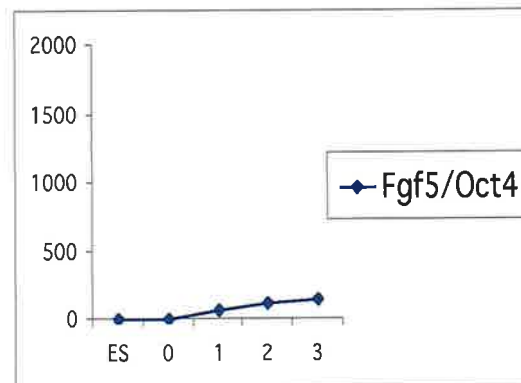


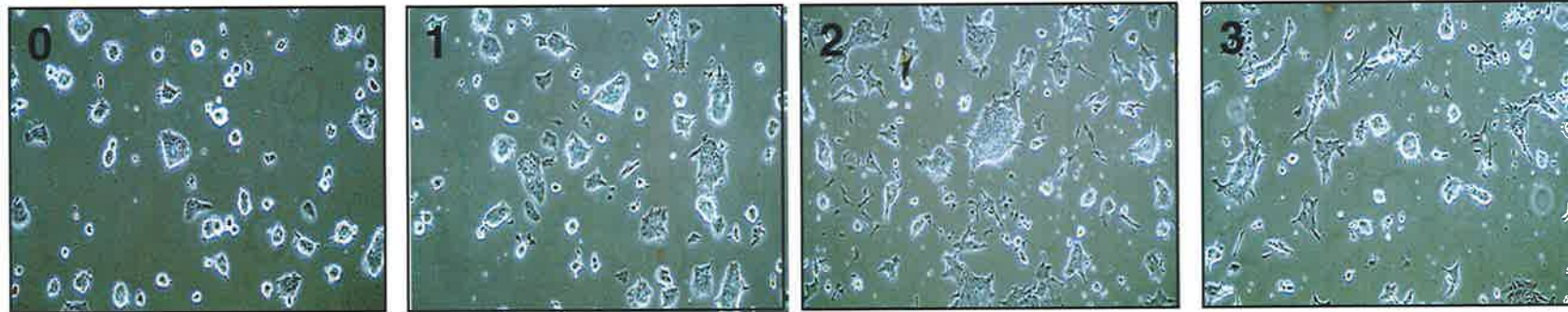
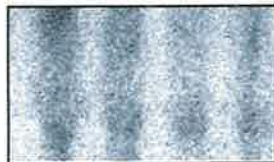
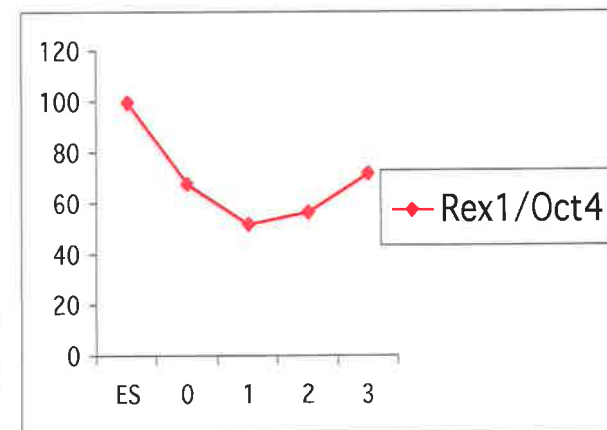
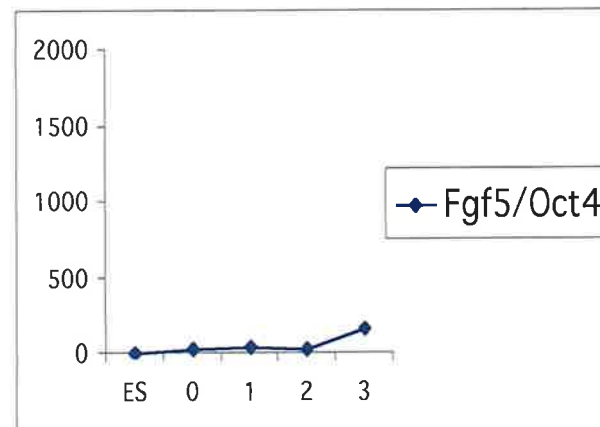
Oct4



mGAP

iii



B**i****ii** **EPL - ECM****0 1 2 3*****Fgf5******Rex1******Oct4******mGAP*****iii**

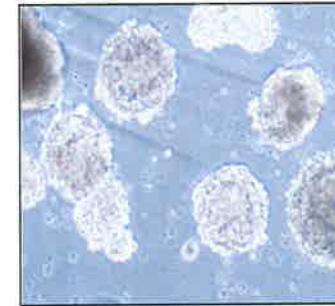
C

i

EPL - ECM

sfEPL- ECM

Day 4.0 EB's



ii

0 1 2 3 4

0 1 2 3 4

Brachyury



mGAP



reduced levels of, exogenous cFN when compared to MEDII. Therefore, the absence of exogenous ECM and cFN leads to a slight retardation in EPL cell differentiation (Figure 5.11 C (ii)). In conclusion, these data suggest that down-regulation of the *Rex1* gene is associated with the establishment of an EPL cell equivalent differentiation capacity while ECM signalling and up-regulation of the primitive ectoderm marker *Fgf5*, play a lesser role in EPL cell differentiation.

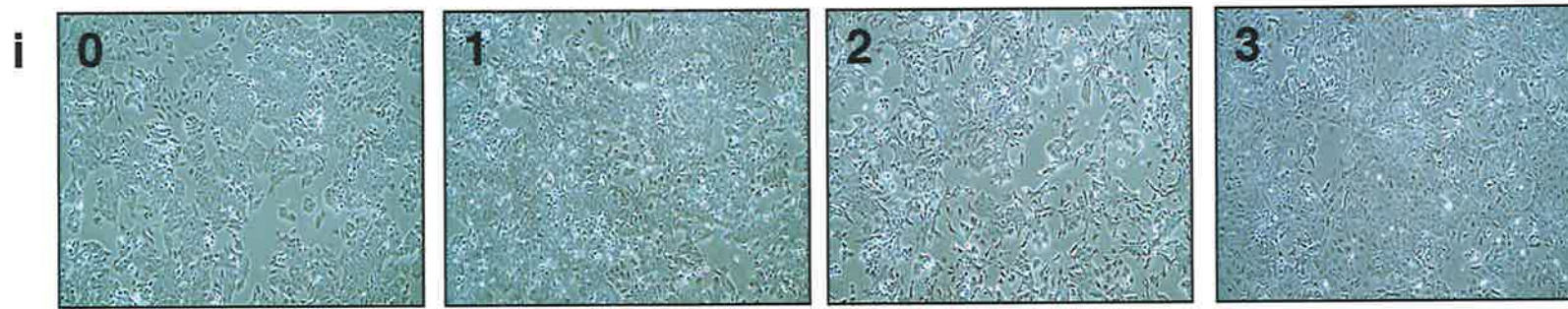
To examine whether interaction between cFN and an exogenous ECM architecture could restore EPL cell formation, D3 ES cells were cultured on a cFN ECM ($1 \mu\text{g}/\text{cm}^2$; 2.3.13), in the presence of 50% MEDII. These cells adopted a characteristic EPL cell colony morphology reflecting pluripotent cell attachment to cFN (Figure 5.12 (i)). Northern analysis of these cells indicated *Rex1* expression was down regulated 8-fold similar to control EPL cells (Figure 5.12 (ii)). *Fgf5* expression was up regulated 2.5-fold in p2 and 5-fold in p3 cultures of these cells, similar to EPL cells (Figure 5.12 (ii)). These data support the suggested role of cFN, in the form of an ECM, as a signal directing maintenance and *Fgf5* expression in EPL cell cultures. However, the reduced levels of *Fgf5* expression when compared to MEDII induced EPL cells may reflect either inefficient cFN presentation to pluripotent cells and/or the absence of additional exogenous ECM components. Furthermore, the activity of L-proline in these experiments suggests that MEDII contains the equivalent of approximately $400 \mu\text{M}$ L-proline.

5.11 An exogenous ECM of cFN and L-proline induces EPL cell equivalent morphology and gene expression

Previous results suggest that EPL cell formation requires a low molecular weight factor equivalent to L-proline and the presence of an ECM containing cFN. To confirm that this is sufficient for EPL cell formation D3 ES cells were seeded into ES complete medium supplemented with $40 \mu\text{M}$, $100 \mu\text{M}$ or $400 \mu\text{M}$ L-proline, in the absence of exogenous ECM or in contact with an ECM of pre-coated cFN ($1 \mu\text{g}/\text{cm}^2$). ES cells cultured in L-proline and the absence of ECM formed rounded domed shaped colonies identical to ES cells (Figure 5.13 A (i), Figure 5.14 A (i)), although some cell spreading was observed in $400 \mu\text{M}$ L-proline (Figure 5.15 A (i)), ES cells cultured in L-proline on a cFN ECM adopted colony morphology indistinguishable from EPL cells (Figure 5.13

Figure 5.12 EPL cells cultured in the presence of exogenous cFN ECM adopt EPL cell morphology and gene expression

i) Assessment of the EPL cell inductive capacity of ES cells cultured in the presence of an exogenous cFN ECM ($1 \mu\text{g}/\text{cm}^2$) and the presence of 50% MEDII by morphology and gene expression. Morphological assessment of EPL cells cultured for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of sfEPL and EPL cell RNA. 20 μg of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.



ii **iii**

EPL + cFN ECM

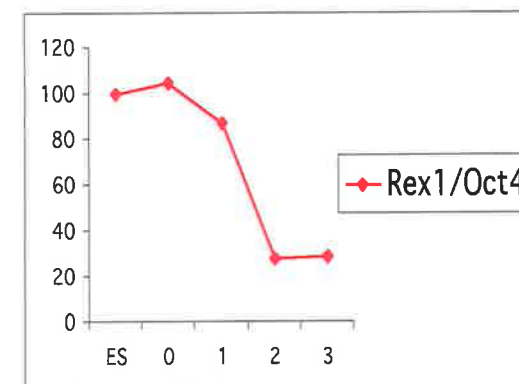
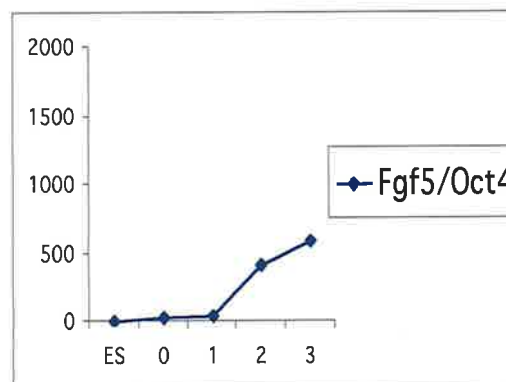
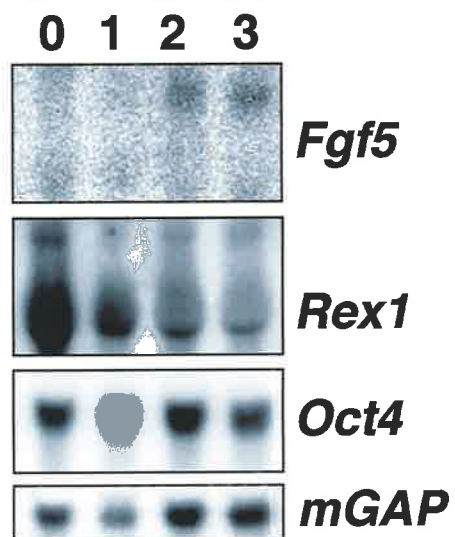
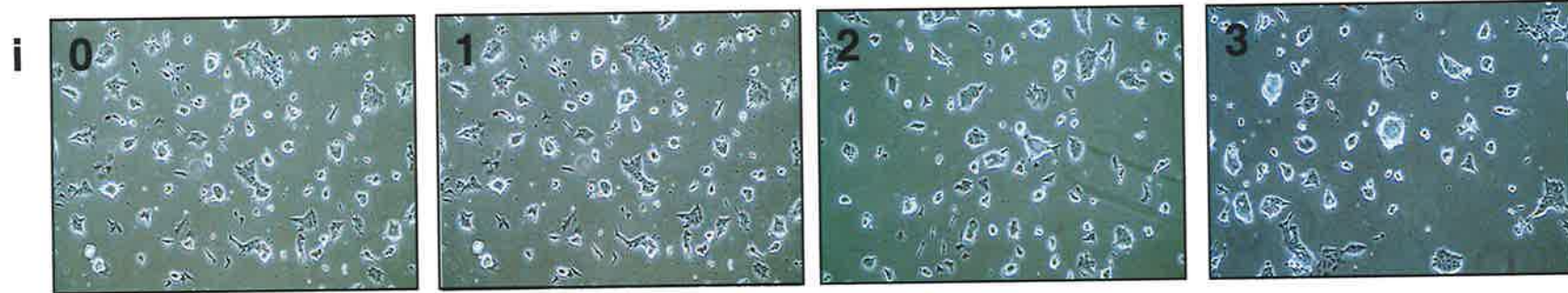


Figure 5.13 ES cells cultured in the presence of 40 μ M L-proline and the presence or absence of an exogenous cFN ECM fail to induce EPL gene expression

Morphology and gene expression of ES cells cultured in the presence of 40 μ M L-proline and the absence (A) or presence (B) of an exogenous cFN ECM (1 μ g/cm²). Morphological assessment of pluripotent cells cultured for 2, 4, 6 and 8 days (0, 1, 2 and 3) (i). Northern blot analysis of sfEPL and EPL cell RNA (ii). 20 μ g of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4* (iii). Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A



ii **40 - ECM**

0 1 2 3



Fgf5



Rex1

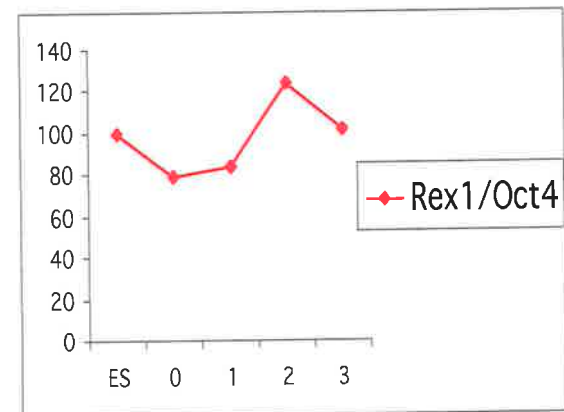
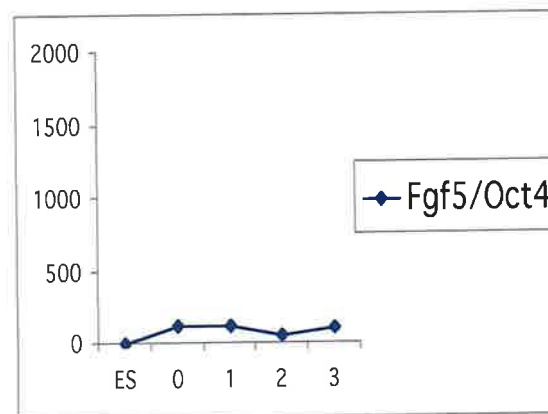


Oct4



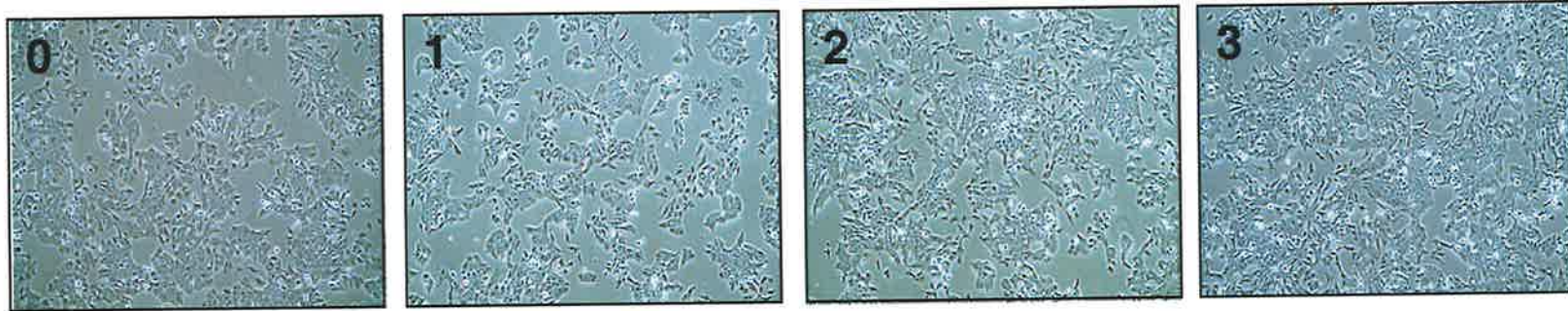
mGAP

iii



B

i



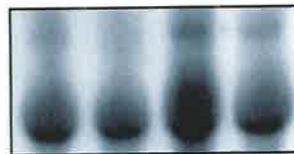
ii

40 + cFN ECM

0 1 2 3



Fgf5



Rex1



Oct4



mGAP

iii

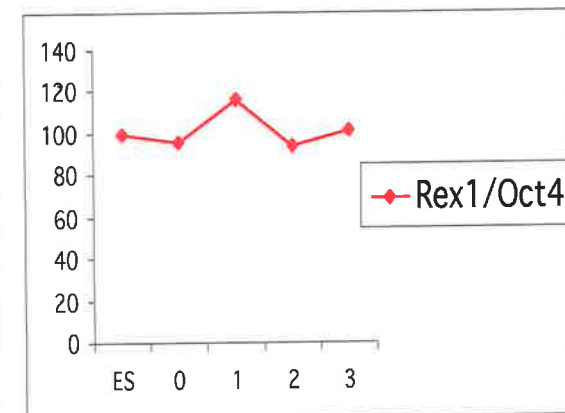
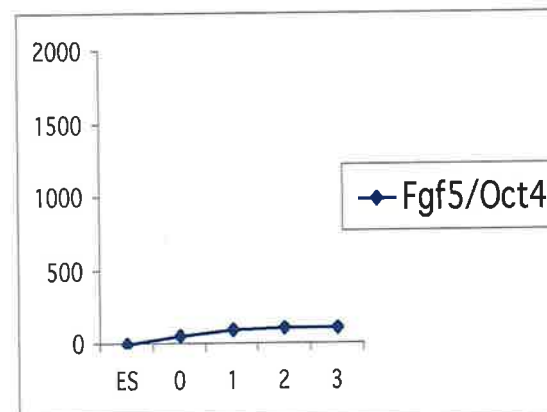
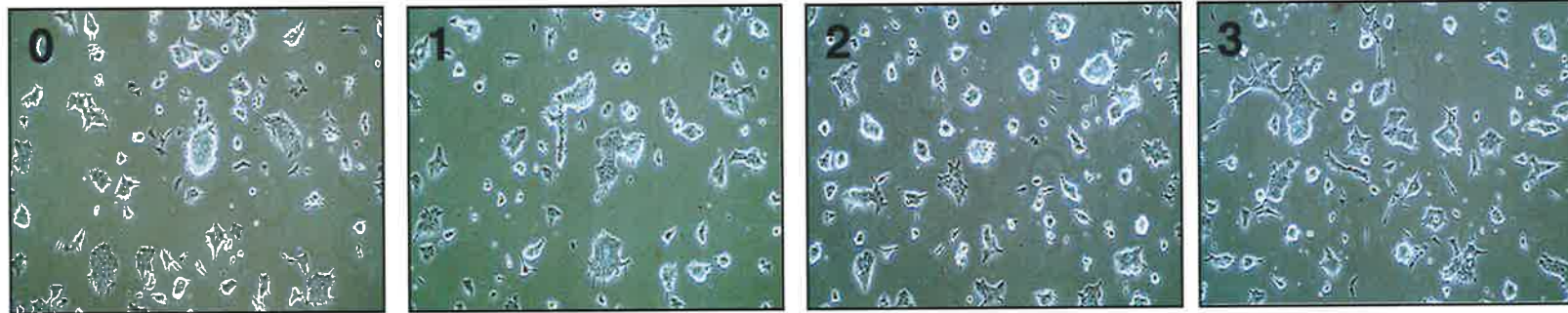


Figure 5.14 ES cells cultured in the presence of 100 μ M L-proline and the presence or absence of an exogenous cFN ECM fail to induce EPL gene expression

Morphology and gene expression of ES cells cultured in the presence of 100 μ M L-proline and (A) the absence or (B) presence of an exogenous cFN ECM (1 μ g/cm²). i) Morphological assessment of pluripotent cells cultured for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of sfEPL and EPL cell RNA. 20 μ g of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

i



ii

100 - ECM
0 1 2 3



Fgf5



Rex1

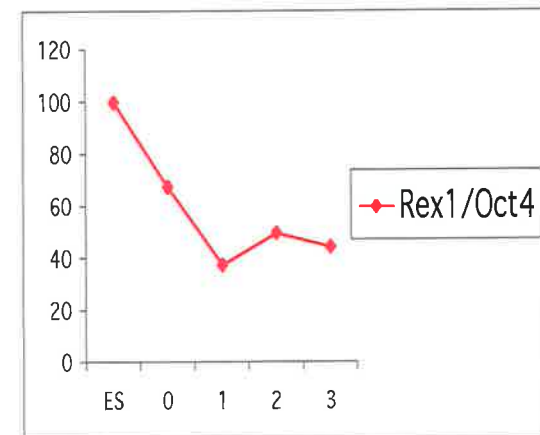
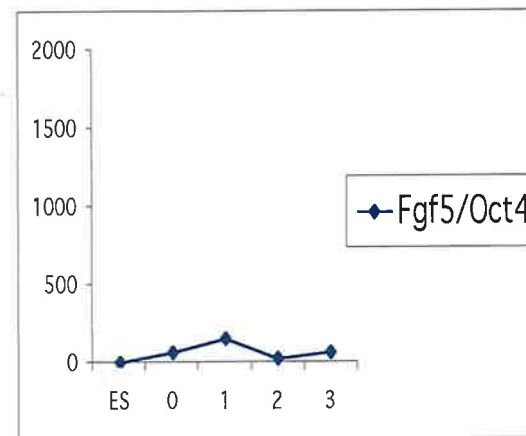


Oct4

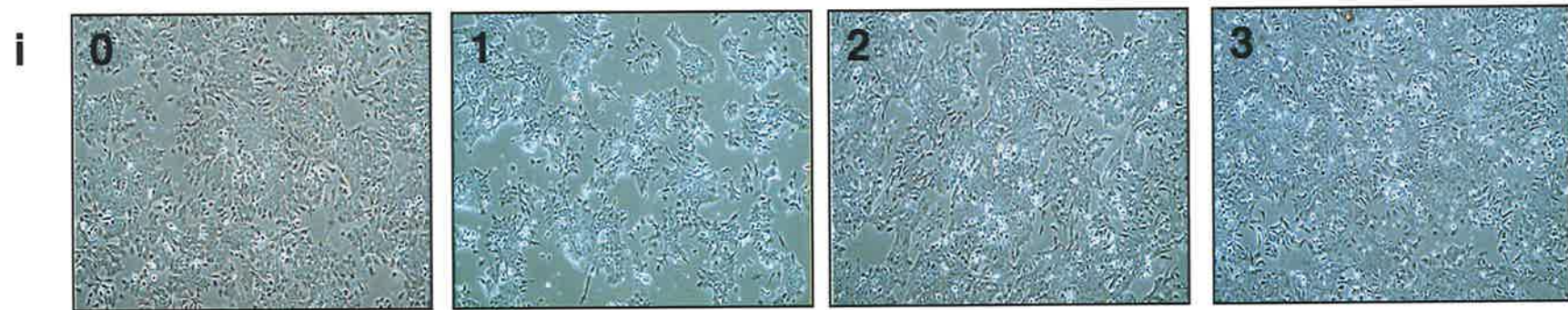


mGAP

iii



B



ii

100 + cFN ECM

0 1 2 3



Fgf5



Rex1



Oct4



mGAP

iii

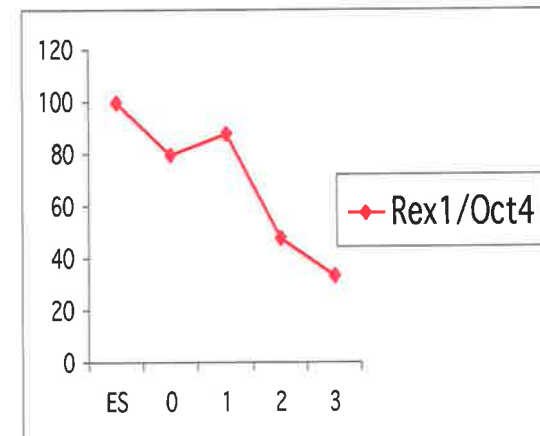
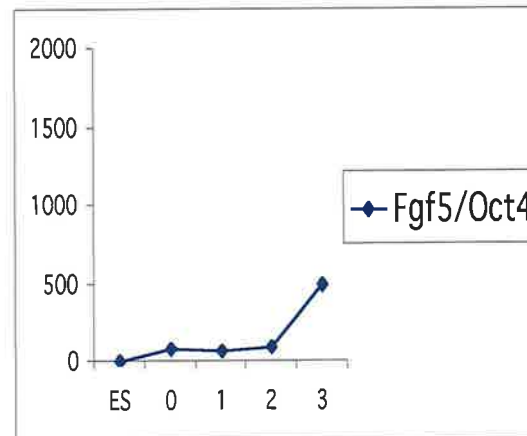
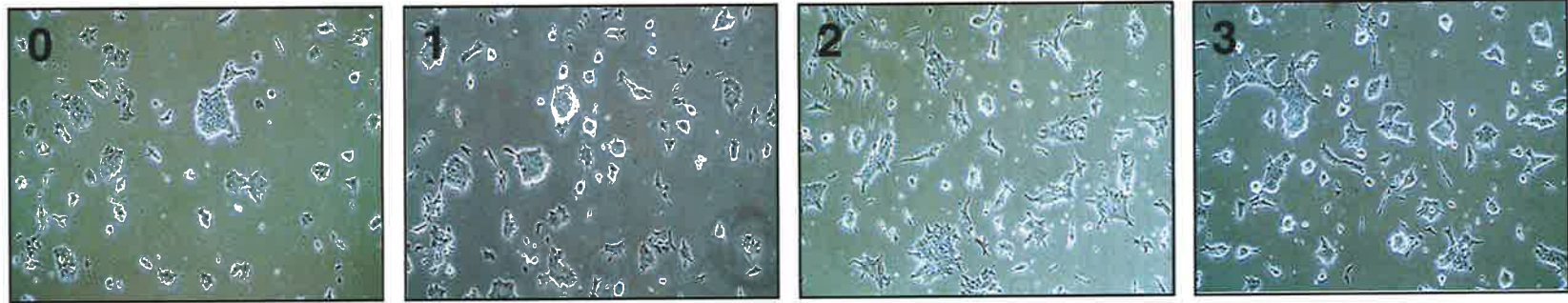


Figure 5.15 ES cells cultured in the presence of 400 μ M L-proline and the presence or absence of an exogenous cFN ECM fail to induce EPL gene expression

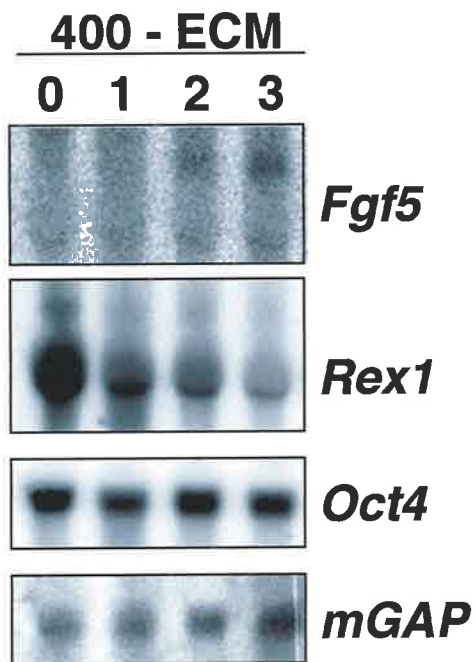
Morphology and gene expression of ES cells cultured in the presence of 400 μ M L-proline and (A) the absence or (B) presence of an exogenous cFN ECM (1 μ g/cm²). i) Morphological assessment of pluripotent cells cultured for 2, 4, 6 and 8 days (0, 1, 2 and 3). ii) Northern blot analysis of sfEPL and EPL cell RNA. 20 μ g of total RNA was analysed for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes. iii) Quantitation of marker gene expression levels for the *Fgf5* and *Rex1* relative to the expression of the pluripotent cell marker *Oct4*. Images taken at 100x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

A

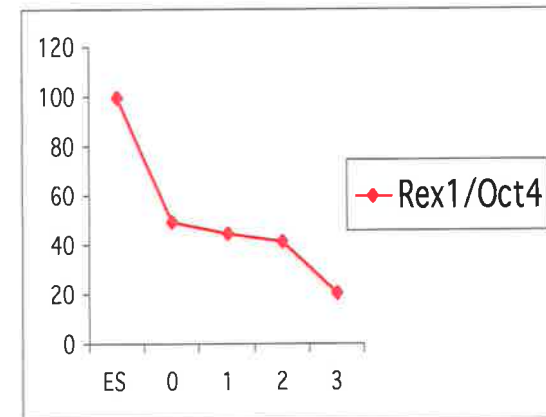
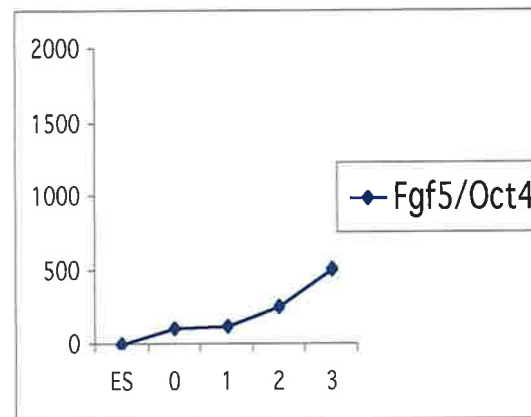
i



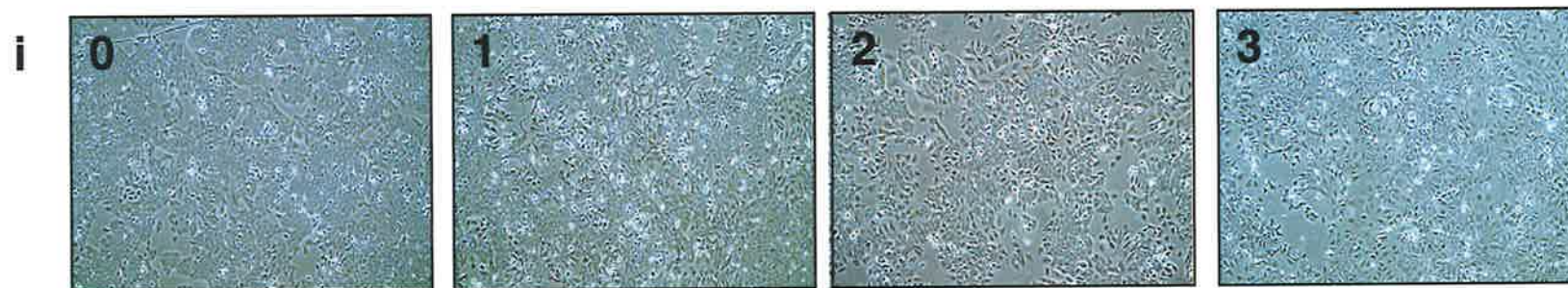
ii



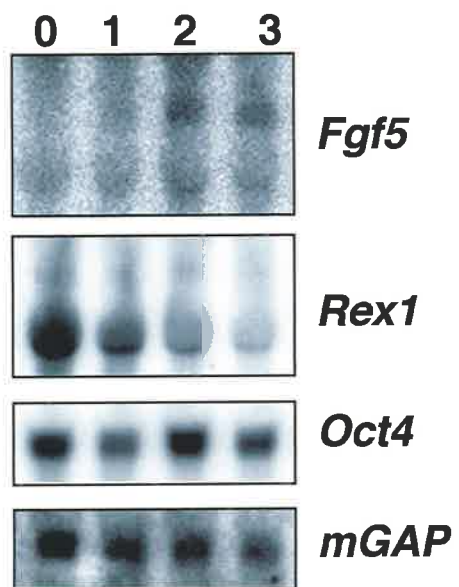
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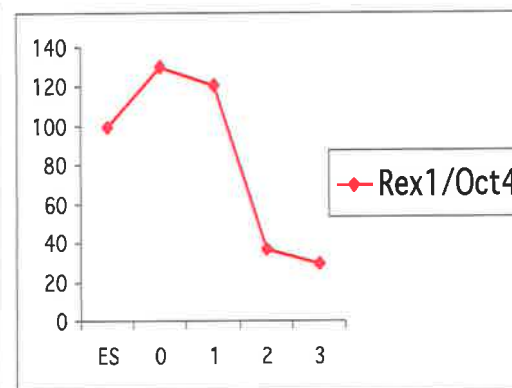
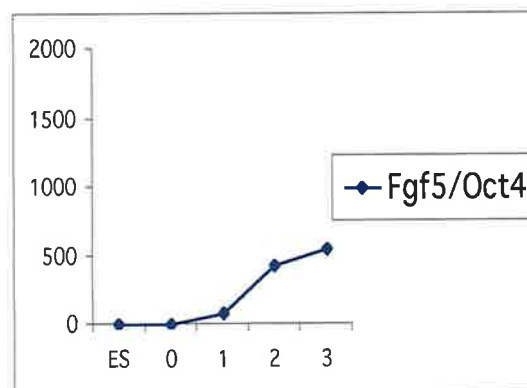
B



ii 400 + cFN ECM



iii



B (i), Figure 5.14 B (i) & Figure 5.15 B (i)).

Northern blot analysis revealed that ES cells cultured in 40 μ M L-proline, in the presence or absence of a cFN ECM, failed to down-regulate expression of *Rex1* relative to *Oct4* expression. This was consistent with the gene expression of ES cells cultured under identical conditions on gelatin ECM (refer 5.7). However, *Fgf5* expression was not increased when these cells were cultured in the absence of an exogenous ECM or when attached to an ECM of cFN (Figure 5.13 A, B (ii)). This contrasts with the increased expression of *Fgf5* in ES cells cultured in 40 μ M L-proline on a gelatin ECM and suggests an interaction between gelatin and cFN is important for optimal cFN presentation and correct EPL cell gene expression.

In ES cell cultures maintained in 100 μ M L-proline in the absence of exogenous ECM, *Rex1* expression was down regulated in the absence of *Fgf5* up-regulation (Figure 5.14 A (ii)). However, the culture of ES cells on an exogenous cFN matrix in the presence of 100 μ M L-proline, restored EPL cell morphology and gene expression as assessed by the down-regulation of *Rex1* and up-regulation of *Fgf5* (Figure 5.14 B (ii)). However, up-regulated *Fgf5* expression was not equivalent to MEDII induced EPL cells as they are retarded in *Fgf5* up-regulation to p3 of culture. ES cells cultured in 400 μ M L-proline in the absence of exogenous ECM formed ES cell like colonies. However, these cells showed marginally increased expression of *Fgf5*, delayed relative to control EPL cell cultures. In the presence of an exogenous matrix of cFN these cells adopted a morphology and gene expression most similar to EPL cells (Table 5.2). The early onset of *Fgf5* expression indicates cFN may induce expression of this gene in EPL cells cultured in 400 μ M L-proline.

5.12 Discussion

Experimental evidence presented in this chapter has defined cFN (presented to ES cells within an ECM architecture) and L-proline (or L-proline containing short peptides) as the EPL cell inductive signals within MEDII. These signals comprise a dose dependent two-signal model for EPL cell formation *in vitro* and, by analogy, are candidate signals for primitive ectoderm formation *in vivo*. Furthermore, this work has defined a relationship between EPL cell morphology, gene

Table 5.2 Summary of the by morphology, gene expression and differentiation potential ES cells and EPL cells cultured in the presence or absence of MEDII and various concentrations of L-proline, in the presence or absence of cFN ECM.

Arrows with *Fgf5* indicate up-regulated (↑), down-regulated (↓) or maintained (→) expression at p2 and p3 of culture under standard conditions. *Fgf5* without arrows indicate minimal alteration in expression levels throughout the culture period. Arrows with *Rex1* indicate maintenance (→) or down-regulation (↓) of *Rex1* expression through the culture period. Cellular FN used in assays at 10 µg/ml.

Culture Conditions	Morphology	Gene Expression		Differentiation
ES	ES	<i>Fgf5</i>	<i>Rex1</i> →	ES
MEDII	EPL	<i>Fgf5</i>	<i>Rex1</i> ↓	EPL
MEDII + cFN matrix	ES	<i>Fgf5</i> ↑↑	<i>Rex1</i> ↓	EPL
40 μM L-pro	ES	<i>Fgf5</i>	<i>Rex1</i> →	ES
40 μM L-pro + cFN Matrix	EPL	<i>Fgf5</i>	<i>Rex1</i> →	ES
100 μM L-pro	ES	<i>Fgf5</i>	<i>Rex1</i> ↓	EPL/ES
100 μM L-pro + cFN Matrix	EPL	<i>Fgf5</i> ↑	<i>Rex1</i> ↓	EPL/ES
400 μM + L-pro	ES	<i>Fgf5</i> ↑	<i>Rex1</i> ↓	EPL
400 μM L-pro + cFN Matrix	EPL	<i>Fgf5</i> ↑↑	<i>Rex1</i> ↓	EPL

expression and differentiation potential and has identified distinct regulatory pathways for the induction of EPL cell specific gene expression and maintenance *in vitro*.

5.12.1 The role of cFN during EPL cell formation

The formation of basement membrane ECM, and the attachment of pluripotent cells to it, is an essential requirement for pluripotent cell formation and maintenance *in vivo* (Stephens *et al.*, 1995; Fässler and Meyer, 1995; Smyth *et al.*, 1998) and survival *in vitro* (Coucouvanis and Martin, 1995). Interestingly, it is the formation of an ECM architecture, rather than the presence of individual components that allows normal pluripotent cell development (Henry and Campbell, 1998; Smyth *et al.*, 1998), as mice lacking functional copies of genes required for ECM assembly have pluripotent cell defects in the presence of presumably normal secretion of additional basement membrane components, including cFN (Henry and Campbell, 1998; Smyth *et al.*, 1999). This indicates that pluripotent cell development has a requirement for basement membrane assembly and architecture *in vivo*. Furthermore, the presence of a FN signal in ECM and activation of ECM signalling cascades through FAK have been associated with primitive ectoderm maintenance *in vivo* (George *et al.*, 1993; Furuta *et al.*, 1995; Georges-Labouesse *et al.*, 1996) perhaps via pluripotent cell survival or proliferation. These data indicate that ECM associated FN is a maintenance factor for primitive ectoderm *in vivo*.

In culture, the presentation of cFN in an ECM form is required for the distinctive colony morphology of EPL cells. This was demonstrated by retention of ES cell colony morphology when cultured in the presence of MEDII or L-proline (400 μ M) in the absence of exogenous ECM. However, a pre-coated gelatin ECM can maintain the EPL cell morphology in the absence of exogenous soluble cFN and the presence of MEDII or 400 μ M L-proline. This indicates that components of the gelatin ECM can compensate for cFN in EPL cell formation, or that gelatin provides a suitable scaffold for the incorporation of an endogenous source of cFN into the local ECM for presentation to pluripotent cells. The ability of D3 ES cells cultured in the absence of MEDII or L-proline to adopt an EPL cell morphology on FN but not other ECM components (4.3), indicates that FN presentation from an ECM is sufficient for morphological EPL cell formation.

Therefore, the domed shape colonies of EPL cell cultures in the absence of exogenous ECM (5.9) may be due to the lack of additional ECM substrata required for cFN presentation.

The induction of an EPL cell equivalent morphology from ES cells does not establish cFN as a signal required for EPL cell formation and/or maintenance. A role for this signal in EPL cell maintenance however, was confirmed by the capacity of both soluble and ECM associated cFN to maintain EPL cultures in the presence of L-proline as determined by the induction and maintenance of *Fgf5* and *Oct4* expression at p3 of culture. Therefore, EPL cells formed in L-proline regulate *Fgf5* expression in response to cFN which suggests that cFN signalling is required for EPL cell culture with respect to gene expression.

The presence of soluble exogenous cFN may also establish EPL cells with a differentiation potential most similar to MEDII induced EPL cells. EPL cells formed in the absence of exogenous cFN retain detectable levels of *brachyury* at day 4.0. In contrast, EPL cells formed in the presence of soluble exogenous cFN form EB's that contain lower levels of *brachyury* expression at day 4.0. Therefore, while some variation in *brachyury* expression at day 4.0 in EPL cell EB's does occur, these data suggest cFN is a signal required for EPL cell induction and maintenance. Therefore cFN plays a role in EPL cell morphology, gene expression and differentiation potential.

The major FN receptor of ES cells is the $\alpha 5/\beta 1$ integrin heterodimer (Yang and Hynes, 1996) which is located at specialised sites of cell-substratum contact termed focal adhesions (Furuta *et al.*, 1996). Stimulation of integrin receptors by ECM ligands such as FN activates specific signal transduction pathways through activation of FAK (Furuta *et al.*, 1996). *In vivo*, mice lacking functional copies of the *FN*, *$\alpha 5$ -integrin* or *FAK* genes display similar post gastrulation defects (George *et al.*, 1993; Yang *et al.*, 1993; Furuta *et al.*, 1996). These reports suggest that FN signalling mediated by attachment to integrin receptors, and activation of FAK, are required for during early embryogenesis. The role of cFN during EPL cell formation and the equivalence of EPL cells and primitive ectoderm further suggest FN signalling, perhaps through FAK activation, is required during EPL cell formation *in vitro*.

5.12.2 The role of the low molecular weight EPL cell inductive signal

L-proline or L-proline equivalent signals are essential for EPL cell formation. This is demonstrated by the attachment of ES cells to a FN matrix in the absence of L-proline equivalent signals and the adoption of an EPL cell equivalent morphology by these cells without the induction of EPL cell gene expression or differentiation potential. Therefore, L-proline activity is linked to EPL cell gene expression and differentiation potential. Furthermore, L-proline signalling can induce ES cells cultured in the presence of an exogenous ECM of gelatin or FN to adopt an EPL cell equivalent morphology. This suggests L-proline is also linked to the formation of the EPL cell morphology. However, the L-proline signal alone is not sufficient for the induction and maintenance of the EPL cell morphology as the culture of EPL cells in the absence of exogenous ECM leads to a retention of the ES cell morphology. Therefore, formation of the EPL cell morphology is reliant on the attachment of pluripotent cells to cFN, presented from within an ECM.

L-proline activity regulates expression of both the *Rex1* and *Fgf5* genes during EPL cell formation by distinct regulatory pathways. In low concentrations of L-proline (40 μ M) ES cells fail to down-regulate *Rex1* expression but can up-regulate *Fgf5* in the presence of an exogenous gelatin ECM indicating *Fgf5* expression is responsive to the presence of L-proline. However, increasing concentrations of L-proline (100 μ M-400 μ M) induce pluripotent cells to down-regulate *Rex1* expression equivalent to MEDII induced EPL cells and further up-regulate *Fgf5* expression. This indicates that *Rex1* and *Fgf5* expression levels respond to increasing concentrations of L-proline.

L-proline signalling drives ES cells to adopt EPL cell differentiation characteristics. This alteration in developmental potential appears to correlate with the down-regulation of *Rex1* expression. However, while *Rex1* is down regulated in ES cells cultured in 100 μ M-400 μ M L-proline in an equivalent manner to MEDII induced EPL cells, ES cells cultured in 100 μ M L-proline differentiate in a manner intermediate between ES and EPL cells. The formation of EPL cells in 400 μ M L-proline induces EB differentiation most similar to MEDII induced EPL cells. This indicates that while *Rex1* expression is down regulated in 100 μ M-400 μ M L-proline, the capacity of pluripotent cell to acquire an EPL cell equivalent differentiation potential requires higher levels of L-

proline activity. Therefore, EPL cells are responsive to L-proline signalling for the induction of EPL cell morphology gene expression and differentiation potential in a dose dependent manner.

The mode of L-proline action as a signalling molecule is unclear. The amino acid L-glutamate and numerous modified amino acids have been shown to activate specific, G-protein coupled trans-membrane receptors (Danysz and Parsons, 1998, Dingledine *et al.*, 1999). Activation of inhibitory (G_i) or stimulatory (G_s) G protein coupled receptors leads to activation or inhibition of adenylate cyclase (AC) which catalyses the formation of a second messenger, cAMP. Activation or inhibition of G protein coupled receptors can be assessed by increase or decrease of cAMP levels within the cell (reviewed in Birnbaumer *et al.*, 1990). ES cells cultured in the presence of L-proline (400 μ M) showed no alteration in cAMP levels (Hirst, unpublished data) suggesting L-proline does not activate AC coupled receptors and acts in a manner distinct from glutamate. However, the amino acid L-glutamate has also been shown to activate specific, phospholipase C (PLC) coupled receptors (Toms *et al.*, 1996) and has been associated with activation of the mitogen-activated protein kinase (MAPK) cascade in cultured rat cortical astrocytes (Abe and Saito, 2001). Furthermore, MAPK pathway activation has been recently associated with the differentiation of pluripotent cells *in vivo* and *in vitro* (Cheng *et al.*, 1998, Arsenian *et al.*, 1998; Burdon *et al.*, 1999; Wienhold *et al.*, 2000). The N-terminal tetra-peptide of the 11 amino acid neuropeptide Substance P is active in EPL cell formation. Substance P has well defined receptors (reviewed in, Hokfelt, 2001) but is inactive in EPL cell formation, however there is evidence to suggest the N-terminal peptide may activate a separate receptor based mechanism (Kato *et al.*, 1978; Repke *et al.*, 1987; Assem *et al.*, 1989). It therefore appears possible that the low molecular weight EPL cell inductive signal functions by receptor binding and activation of intracellular signalling pathways.

The EPL cell inductive activity of L-proline or short peptides containing L-proline, suggests that an *in vivo* source of L-proline signalling may be the breakdown of proteins into small peptides that drive pluripotent cell differentiation. L-proline is a constituent of many neuro- and vasoactive peptides and it has been suggested many of these peptides activate specific signal transduction pathways (Vanhoof *et al.*, 1995). During primitive ectoderm formation *in vivo*, constant re-modelling of the basement membrane involves degradation of ECM components and would yield multiple short peptides possibly containing L-proline, adjacent to embryonic pluripotent cells (refer 1.13). An

alternate source for an L-proline equivalent signal during primitive ectoderm formation and maintenance *in vivo* is secretion by the adjacent VE.

5.12.3 The mode of action of cFN and the low molecular weight EPL cell inductive signal

ES cells form EPL cells and respond to soluble cFN only in the presence of L-proline or L-proline equivalent signals. This response suggests that L-proline may either increase the attachment of pluripotent cells to the FN matrix, perhaps by increasing the expression of $\alpha 5$ integrin, or increases the incorporation of soluble cFN into the local ECM for ES/EPL cell attachment. Alternately, antagonism of integrin binding to additional ECM components by L-proline or L-proline containing peptides (possibly derived from the breakdown of ECM molecules), may also lead to increased FN attachment or these signals may activate alternate unknown responses in EPL cells.

L-proline signalling regulates *Rex1* expression while L-proline/cFN signalling regulates expression of *Fgf5* in EPL cells. The addition of soluble exogenous cFN to these cultures further up-regulates *Fgf5* expression. This indicates that L-proline regulates *Rex1* expression independent of cFN and that both L-proline and ECM/cFN signalling are required for *Fgf5* expression in EPL cells. The activity of these signals indicate a two-signal model for EPL cell formation.

Data presented in this chapter has shown that pluripotent cells are responsive to the EPL cell inductive signals in a dose dependent manner. The dose dependent action of these signals and overlap in gene regulation further suggests these signals can functionally compensate each other. Increasing L-proline concentrations or the presentation of cFN as an ECM stimulates morphological EPL cell formation and up-regulates expression of *Fgf5*. This indicates that during EPL cell formation L-proline and cFN signalling can activate similar regulatory pathways. However, the manner by which cFN/ECM and L-proline signalling interact and therefore the manner by which these signals compensate for each other is unknown, but may be linked to an ECM associated origin of both signals (5.11.2).

5.12.4 Regulation of pluripotent cell gene expression

The *Oct4* and *Oct6* gene products and a novel protein, *Rox1* (Ben-Shushan *et al.*, 1998), regulate *Rex1* expression in pluripotent cells. *Rex1* is rapidly down-regulated during pluripotent cell differentiation (Shen and Leder *et al.*, 1992) and EPL cell formation. However, primitive ectoderm and EPL cells retain *Oct4* expression levels during the down-regulation of *Rex1* expression (Schöler *et al.*, 1989a; Schöler *et al.*, 1989b; Rosner *et al.*, 1990; Yeom *et al.*, 1990; Poirier *et al.*, 1991; Nichols *et al.*, 1998). This suggests that additional factors controlling transcription from the *Rex1* promoter must be regulated during EPL cell/primitive ectoderm formation. The association of *Rex1* with ES/ICM cell equivalence suggests that L-proline (or L-proline equivalent) induced down-regulation of *Rex1* in pluripotent cells in a cFN/ECM independent manner and that this down-regulation is a key developmental progression for pluripotent cells *in vivo* and *in vitro*.

EPL cells cultured in the absence of exogenous ECM did not up-regulate expression of *Fgf5* but retain the capacity to differentiate in a manner more reminiscent of EPL cells than ES cells. This suggests that *Fgf5* is a cFN dependent response gene and that induction of *Fgf5* expression in these cells is not absolutely required for EPL cell formation. Mice lacking a functional copy of the *Fgf5* gene show only neo-natal defects, suggesting that primitive ectoderm formation in mice is also not inhibited by a lack of *Fgf5*. However, this effect may be due to functionally redundant Fgf growth factors that are present during pluripotent cell development *in vivo* and *in vitro* (Hébert *et al.*, 1990; Haub and Goldfarb, 1991; Hébert *et al.*, 1991; Rappolee *et al.*, 1994; Feldman *et al.*, 1995; Rappolee *et al.*, 1998; Deng *et al.*, 1994; Yamaguchi *et al.*, 1994; Feldman *et al.*, 1995; Haffner-Krausz *et al.*, 1999; Chen *et al.*, 2000). Therefore, L-proline and cFN have overlapping but distinct signalling activities on EPL cell gene expression.

5.12.5 The relationship between EPL cell morphology, gene expression and differentiation

An interaction between cell-ECM attachment and gene expression driven by distinct matrix components *in vitro* has been well documented in mammary epithelial cell lines (Streuli, 1993). Accordingly, pluripotent cell attachment to the ECM is linked to EPL cell morphology and the

control of *Fgf5* gene expression. This suggest that a relationship between morphology and gene expression in pluripotent cells. However, morphology does not always indicate gene expression within a cell population as ES cells cultured on an ECM of cFN are morphologically equivalent to EPL cells but retain ES cell gene expression and differentiation.

Gene expression in pluripotent cells can indicate identity and differentiation capacity. In particular, the culture of ES cells in higher concentrations of L-proline (100 μ M-400 μ M) or 50% MEDII, in the presence or absence of ECM, is sufficient to down-regulate *Rex1* expression. Pluripotent cells with down-regulated *Rex1* differentiate as EB's in a manner most reminiscent of EPL cells. In contrast, the culture of ES cells in the presence of 40 μ M L-proline on a gelatin ECM up-regulates *Fgf5* expression but fails to down-regulate *Rex1* and these cells differentiate in an identical fashion to ES cell derived EB's. Up-regulation of the *Fgf5* gene, in the absence of *Rex1* down-regulation therefore does not indicate EPL cell differentiation potential. Therefore, down-regulation of *Rex1* expression and retention of *Oct4* may be used as an indicator to the progression from ES cell to EPL cell as assessed by differentiation potential. This indicates a distinct relationship between gene expression and EPL cell differentiation capacity.

Finally, the capacity of EPL cells formed in the absence of exogenous ECM and with an ES cell equivalent morphology to differentiate in a manner distinct from MEDII induced EPL cells (or EPL cells differentiated in the presence of soluble cFN and exogenous ECM of gelatin), suggests a relationship between EPL cell morphology and differentiation potential. Furthermore, given that EPL cell morphology is linked to characteristic EPL cell gene expression, these data suggest a relationship exists between EPL cell morphology, gene expression and differentiation potential. While the relationship between these EPL cell characteristics requires further analysis, it is the control of EPL cell culture by L-proline and cFN that define these signals as EPL cell inductive.

5.12.6 Relationship of EPL cell formation to embryonic cavitation *in vitro*

The two-step mechanism for pro-amniotic cavitation in the vertebrate embryo proposed a diffusible VE derived 'death' signal which induces cavitation through programmed cell death, or apoptosis, of the adjacent pluripotent cells (Coucouvanis and Martin, 1995). The primitive ectoderm

cells making up the epithelial sheet avoid cell death through interaction with a secondary 'survival' signal associated with the basement membrane separating the VE and the primitive ectoderm (Figure 1.4; Coucouvanis and Martin, 1995). However, the identity of these signals has not been determined. The association of cFN with the basement membrane that contains the survival signal and the activity of L-proline as a freely diffusible signal align the biochemical nature of these signals with the signals active during cavitation *in vitro*. However, the capacity of the EPL cell inductive signals to direct cavitation in S2 EC cell derived EB's *in vitro* is has not yet been tested.

Chapter 6

**The cellular origins of EPL cell inductive
signals *in vivo* and *in vitro***

Chapter 6

6.1 Introduction

The cellular origin and relevance of the EPL cell inductive signals to primitive ectoderm formation *in vivo* and *in vitro* is yet to be established. However, liver derived cells such as HepG2 can be aligned with an extra-embryonic cell lineage located adjacent to the pluripotent cells of the post-implantation early mouse embryo, the VE. Liver and VE share similar protein secretion profiles and gene regulatory pathways (Rossant, 1995) and many genes initially identified in the liver are expressed in VE and are crucial for normal VE and primitive ectoderm formation and survival *in vivo* (Chen *et al.*, 1994; Duncan *et al.*, 1994; Barbacci *et al.*, 1999; Coffinier *et al.*, 1999). This suggests that hepatic cell cultures, such as HepG2, may act as an *in vitro* source of VE equivalent signals that induce primitive ectoderm formation *in vitro* (Rathjen *et al.*, 2001). Therefore, the EPL cell inductive signals may be associated by analogy with the VE.

In this chapter, additional hepatic cell cultures and the VE-like cell line END2 were examined for expression of the EPL cell inductive signals. Secretion of the low molecular weight signal was determined by EPL cell inductive activity, while the cellular origin and localisation of the cFN signal, in the early mouse embryo and *in vitro*, will be mapped using anti-sense RNA and specific anti-cFN monoclonal antibodies.

6.2 Conditioned media from hepatic cell lines and primary hepatic cultures induce morphological EPL cell formation

The main cell type within the liver is the hepatocyte, which secretes many soluble factors found within the blood plasma (Grieninger and Granick, 1978; Plant *et al.*, 1981). HepG2 cells secrete factors similar to *in vivo* hepatocytes and have been used as an *in vitro* model of hepatocyte function (Bouma *et al.*, 1989). Furthermore, additional hepatocellular carcinoma cell lines similar gene expression profiles to HepG2 cells (Kawai *et al.*, 2001), indicating that factor secretion profiles may also be similar. HepG2 and Hep3b hepatocellular carcinoma cell lines were originally isolated from cancerous lesions in adolescent humans (Knowles *et al.*, 1980). Hepalclc7 is a clonal derivative of the Hepal cell line originally isolated from the BW 7756 hepatoma carried in C57L

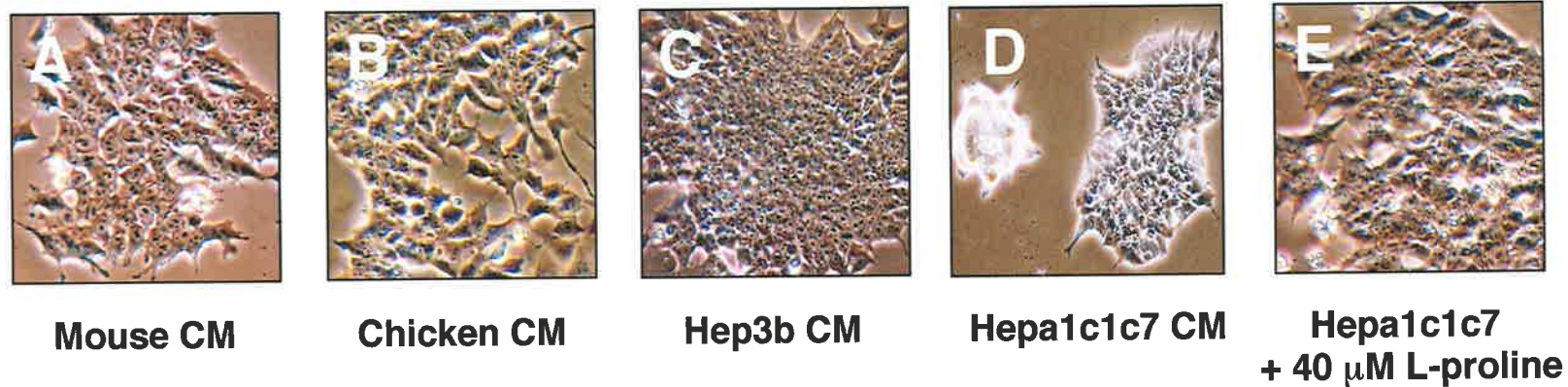


Figure 6.1 Morphology of ES cells cultured in hepatic and visceral endoderm CM

D3 ES cells were seeded onto gelatin coated (0.2%) tissue culture plastic and cultured in 1000U/ml LIF supplemented ES DMEM with 50% hepatic or END2 cell CM. EPL cell formation was assessed by morphology. ES cell cultured in 50% mouse (A) or chicken (B) primary liver cell culture CM or 50% human hepatocellular carcinoma Hep3b derived CM. ES cell cultured in 50% Hepa1c1c7 (D) and Hepa1c1c7 CM supplemented with 40 μ M L-proline (E). Images taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope.

mice (Bernhard *et al.*, 1973). Primary hepatocytes from mice and chickens can also be isolated in culture (2.3.7). However, these cells are difficult to maintain, cannot be passaged and are only stable in culture for up to 16 days (Giger and Myer, 1981). To determine the range of hepatic cell lines and cells that secrete the EPL cell inductive factors, CM derived from these cultures was assessed for the capacity to induce EPL cell formation from ES cells.

D3 ES cells were seeded at low density (250 cells/1.75 cm²) into gelatin coated (0.2%) 24 well tissue culture plastic (Falcon) and were assessed for the morphological induction of EPL cells. ES cells cultured in the presence of 50% conditioned medium (CM) derived from mouse (Figure 6.1 A) or chicken (Figure 6.1 B) primary hepatocytes (2.3.7) or Hep3b cells (Figure 6.1 C) adopted a morphology indistinguishable from EPL cells. This suggests CM derived from a range of hepatic cells has the capacity to induce morphological EPL cell formation.

Hepal1c1c7 CM induced partial EPL cell formation such that less than 95% of colonies adopted the EPL cell colony morphology (Figure 6.1 D). However supplementation of Hepal1c1c7 CM with 40 µM L-proline restored the EPL cell phenotype (Figure 6.1 E). These data suggest that Hepal1c1c7 cells secrete sufficient levels of a signal equivalent to cFN, but require the addition of exogenous L-proline into the culture medium to induce morphological EPL cell formation.

Collectively, these results imply that a range of hepatic cell cultures secrete signals biologically equivalent to the signals that induce morphological EPL cell formation in MEDII, although the levels of these signals may differ between various cell lines. Furthermore, the ability of hepatic cultures derived from different species to induce EPL cell formation indicates an evolutionary conservation of signal activity, consistent with the conservation of this stage of embryogenesis in mammals and birds (Tam and Behringer, 1998; Beddington and Robertson, 1999).

6.3 Secretion of EPL cell inductive signals by VE and parietal endoderm cells

A likely source of primitive ectoderm inductive signals *in vivo* is VE, which has been implicated in primitive ectoderm maintenance, survival and differentiation (Chen *et al.*, 1994; Duncan *et al.*, 1994; Spyropoulos and Capecchi, 1994; Duncan *et al.*, 1997; Barbacci *et al.*, 1999; Coffinier *et al.*, 1999; Bielinska *et al.*, 1999). More recently, a VE derived signal has also been implicated in

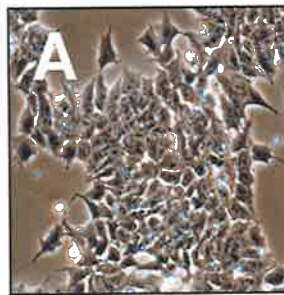
directing cavity formation and primitive ectoderm morphology in EB's *in vitro* (Coucouvanis and Martin, 1995).

An *in vitro* source of VE-like signals is the clonal cell line END2, derived from a retinoic acid differentiated mouse P19 EC cell derived EB (Mummery *et al.*, 1985, 1991). Previously, signals derived from END2 cells have been shown to induce the formation of muscle and neural lineages from aggregates of P19 EC cells (Mummery *et al.*, 1991), or direct pro-amniotic cavitation in S2 EC cell derived EB's (Coucouvanis and Martin, 1995). These reports suggest that END2 cells secrete signals that alter pluripotent cell biology.

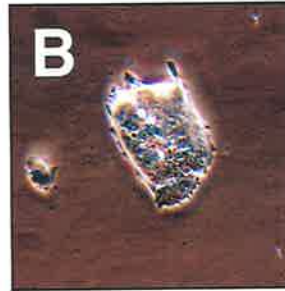
An *in vitro* source of parietal endoderm-like signals is the clonal cell line PYS2, also derived from a retinoic acid differentiated mouse P19 EC cell derived EB (Mummery *et al.*, 1985, 1991). However, while END2 and PYS2 cells are derived from a common origin (1.2.3) and may share common biological properties, PYS2 cells have not been shown to secrete factors that induce pluripotent cell differentiation and do not secrete the diffusible death signal active during embryonic cavitation (Coucouvanis and Martin, 1995).

Medium conditioned by END2 and PYS2 cells was assessed for the induction of EPL cells from ES cells seeded at low cell density (250 cells/1.75 cm²) in 50% CM. Signals in END2 CM directed ES cells to adopt an EPL cell equivalent while PYS2 CM failed to alter the ES cell equivalent morphology (Figure 6.2 A, B). This implies that END2 cells may secrete EPL cell inductive signals biologically equivalent to cFN and/or L-proline. Supplementation of PYS2 CM with 2 µg/ml of HepG2 cell derived cFN (2.3.16) induced formation of the EPL cell phenotype (Figure 6.2 C), suggesting that PYS2 cells secrete sufficient levels of a diffusible signal equivalent to L-proline for induction of EPL cell morphology. However, ES cells seeded at high density into ES complete DMEM (2x10⁴ cells/cm²) containing 50% PYS2 CM adopted the EPL cell colony morphology in the absence of exogenous cFN (Figure 6.2 D). This is distinct from the morphology of pluripotent cells cultured at low density in PYS2 CM and suggests that increased pluripotent cell density can circumvent the requirement for exogenous cFN.

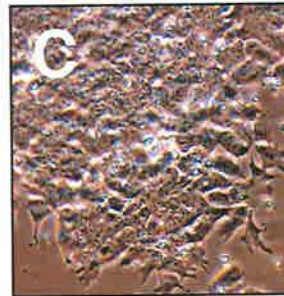
Northern analysis of high density ES cells cultured under standard conditions (5.2) in the presence of PYS2 and END2 CM showed down-regulation of the ICM marker *Rex1* and up-regulation of the primitive ectoderm marker *Fgf5*, with constant expression levels of *Oct4* (Figure



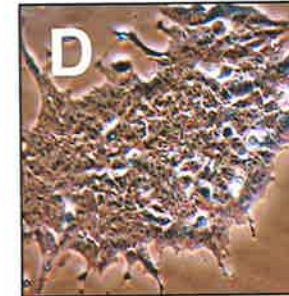
END2 CM



PYS2 CM



**PYS2
+ 2µg/ml cFN**



**PYS2
+ High ES cell density**

Figure 6.2 Morphology of ES cells cultured in CM derived from visceral endoderm and parietal endoderm like cell lines

D3 ES cells were seeded onto gelatin coated (0.2%) tissue culture plastic and cultured in 1000 U/ml LIF supplemented ES DMEM and 50% mouse END2 (A) or PYS2 (B) or PYS2 cell line derived CM supplemented with 2 mg/ml HepG2 cell derived cFN (C) or PYS2 or ES cells seeded at high density ($2 \times 10^4/\text{cm}^2$). Images were taken at 200x magnification using phase contrast microscopy.

6.3). However *Fgf5* expression was down regulated in p3 of EPL cells formed in END2 cell CM. This gene expression pattern was equivalent to EPL cells induced in the presence of 100 μ M-400 μ M L-proline and the absence of exogenous cFN activity.

The morphology of EB's derived from ES cells cultured in END2 and PYS2 CM to p1⁺ was similar, but not identical to, EPL cell derived EB's, and was more closely related to EB's derived from ES cells cultured in the presence of 100 μ M L-proline (refer 5.2.7). Northern blot analysis supported this observation with the onset of *brachyury* expression at day 3.0 of culture, intermediate between ES and EPL cell derived EB's (Figure 6.4, refer section 5.4.3), and equivalent to EB's derived from ES cells cultured in 100 μ M L-proline. These results are the first report of signals derived from PYS2 and END2 cells directing ES cell differentiation *in vitro*.

To confirm secretion of cFN by hepatic and primitive endoderm-like cell lines, 10 μ ls of CM derived from the cell lines in 6.2 and 6.3 were separated on an 8% SDS/reducing polyacrylamide gel and visualised by silver staining (Figure 6.5 A). A band running at the same molecular size as cFN was clearly visible in MEDII and at lower levels in Hepa1c1c7 CM. These observations support the action of a cFN equivalent activity in HepG2 and Hepa1c1c7 CM (6.2) and show that HepG2 cells secrete comparatively high levels of cFN into the culture medium. However, cFN was not clearly visible in the CM of END2, PYS2 and Hep3b cells by silver stain, but was detected as a secreted protein by western analysis using the TV-1 anti-mouse/human anti-cFN monoclonal antibody (2.8.3) (Figure 6.5 B). The level of cFN in HepG2 cell derived CM compared to Hep3b, END2 and PYS2 cell derived CM, suggests cFN may not be active at lower levels consistent with the lack of cFN activity in PYS2 cells.

In conclusion, END2 and PYS2 cells secrete low levels of cFN and a signal functionally equivalent to the low molecular weight EPL cell inductive signal. Accordingly, the low molecular weight EPL cell inductive factor in PYS2 and END2 cell CM induces intermediate EPL cell formation as assessed by gene expression and differentiation potential. Furthermore, PYS2 and END2 cells secrete similar levels of cFN. This level of cFN is inactive in PYS2 CM in the presence of ES cells seeded low density, but is active in END2 CM, suggesting that PYS2 cells secrete lower levels of the low molecular weight signal when compared to END2 cells.

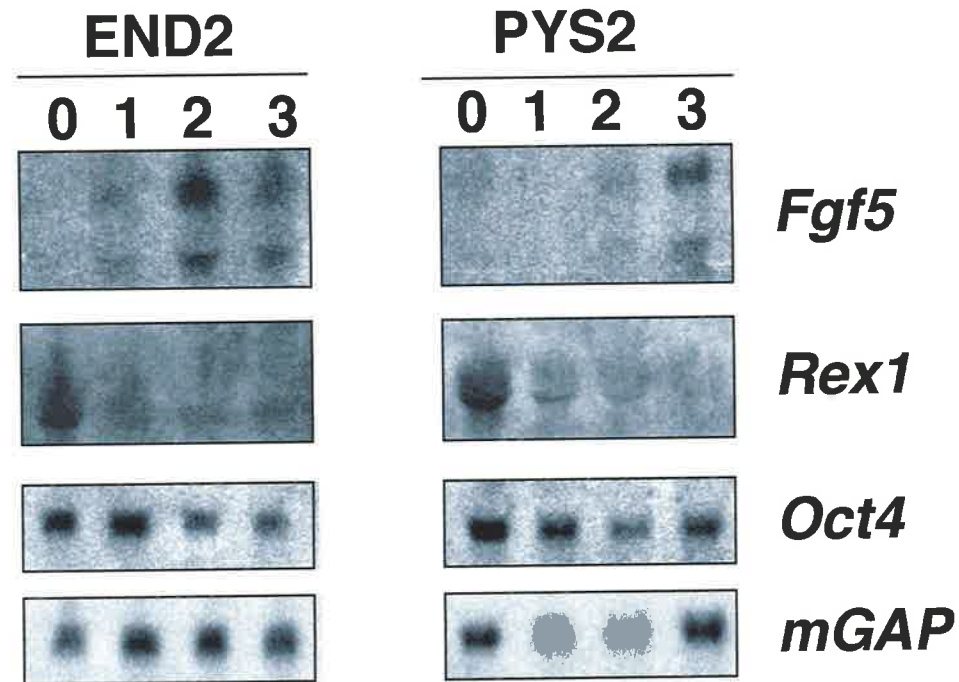


Figure 6.3 The EPL cell inductive capacity of signals within END2 and PYS2 CM: Gene expression

D3 ES cells were cultured under standard conditions of LIF supplemented DMEM (1000U/ml) plus 50% END2 or 50% PYS2 CM for 2, 4, 6 and 8 days (0, 1, 2 and 3). Northern blot analysis of 20 μ g of total RNA for expression of the *Fgf5*, *Rex1*, *Oct4* and *mGAP* marker genes.

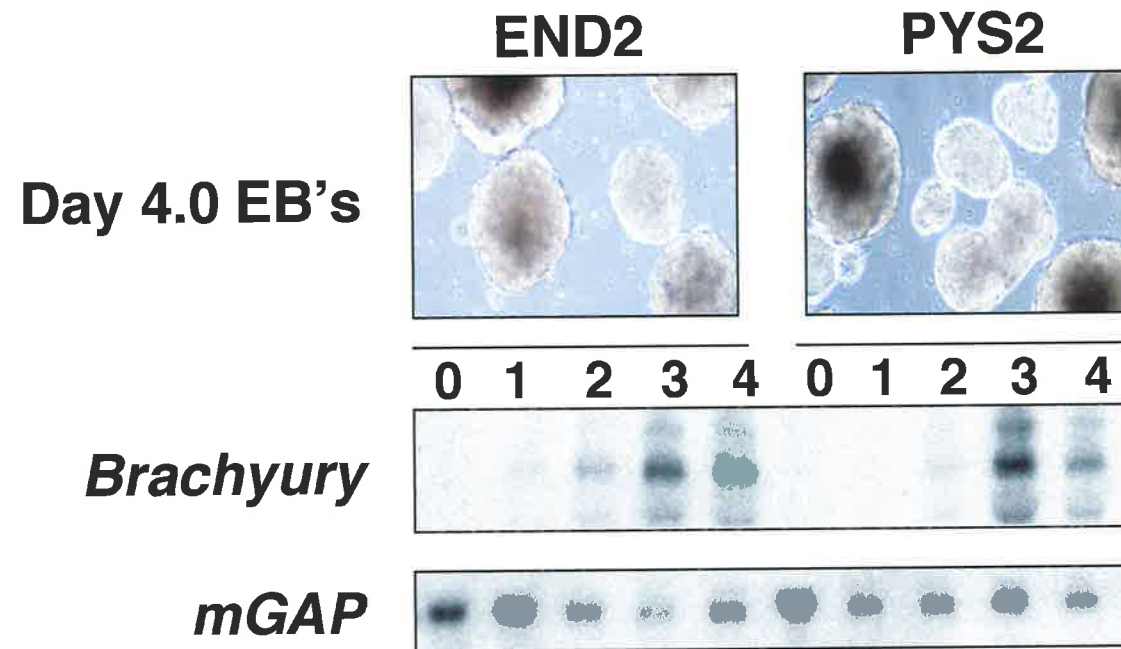


Figure 6.4 The EPL cell inductive capacity of signals within END2 and PYS2 CM: Differentiation

Mesoderm induction in EB's, derived from ES cells cultured in 50% END2 or PYS2 CM to p1⁺. (A) Morphology of day 4.0 EB's, derived from ES p1⁺ cultured in 50% END2 or PYS2 CM. (B) Northern blot analysis of 20µg of total RNA harvested from D3 ES p1⁺ cells cultured in 50% END2 or PYS2 CM (0) and EB's derived from these cells cultured for 4 days in suspension and the absence of LIF. Embryoid bodies were harvested daily (1, 2, 3 and 4 days). Harvested RNA was analysed for expression of the nascent mesoderm marker *Brachyury* between day 0-4 of differentiation. *mGAP* expression levels were determined as a standard loading control.

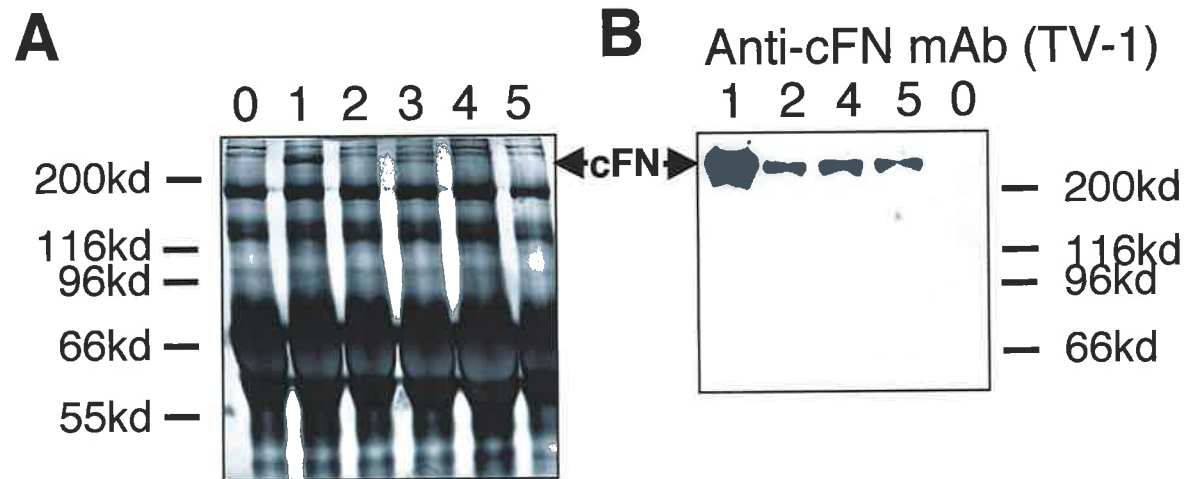


Figure 6.5 Hepatic and primitive endoderm-like cell lines secrete the EPL cell inductive signal cFN

(A) Culture medium and medium conditioned by hepatic and primitive endoderm-like cell lines were separated on an 8% SDS-reducing polyacrylamide gel (5 μ l) and visualised silver staining. (B) Western blot analysis of 10 μ l of culture medium and hepatic and primitive endoderm-like conditioned medium probed with the TV-1 anti-mouse/human cFN monoclonal (2.3.8). Cellular FN (250kd subunits) is indicated by the arrow. Culture medium (0), HepG2 (1) Hep3b (2), Hepa1c1c7 (3), PYS2 (4) and END2 (5). Morphology of END2 cells (C).

Importantly, these data indicate that a VE-like cell line secrete EPL cell inductive factors. This implicates VE as a source of primitive ectoderm inductive signals *in vivo*, and further suggests that HepG2 cells, with high level secretion of the low and high molecular weight signals, are an ideal source of VE-like signals for the induction of EPL cells *in vitro*.

6.4 Localisation of ECM associated FN during primitive ectoderm formation *in vivo* and *in vitro*

The localisation of ECM associated FN within the early mammalian embryo has been well characterised (Zetter and Martin, 1978; Wartiovaara *et al.*, 1979; George *et al.*, 1993; Peters and Hynes, 1996). These reports describe the onset of FN deposition at day 5.5 in the ECM surrounding the cells of the primitive endoderm that line the pluripotent cells of the pre-implantation blastocyst coincident with primitive ectoderm (Wartiovaara *et al.*, 1979). Cellular FN (EDA⁺ and EDB⁺) distribution within the embryo is maintained throughout the basement membrane ECM separating primitive ectoderm and VE during egg cylinder development (George *et al.*, 1993; Peters and Hynes, 1996). To confirm these results, transverse sections (2.7.4) of a 6.5 dpc mouse embryo were analysed for localisation of cFN using the anti-cFN monoclonal antibody TV-1 (2.8.8). Tissue sections were prepared in collaboration with Antonietta Nesci. Cellular FN localisation was visualised by an alkaline phosphatase reaction (2.6.20; Figure 6.6 A). Widespread localisation of cFN was detected in the decidua. Importantly, cFN was localised to the basement membrane ECM between primitive ectoderm and VE cell layers of the early embryo. These results support previously published data of cFN localisation within the basement membrane of the early mouse embryo, separating primitive ectoderm from VE.

EB's are a plentiful source of *in vitro* primitive ectoderm. The capacity of EB's to differentiate into more complex structures suggests that the signals directing primitive ectoderm formation *in vitro* are functionally equivalent to the primitive ectoderm inductive factors *in vivo*. The EB basement membrane has been previously implicated in directing pluripotent cell survival and primitive ectoderm morphology during cavitation in EB's (Coucouvanis and martin, 1995). The distribution of the cFN protein coincident with primitive ectoderm in EB's was investigated to

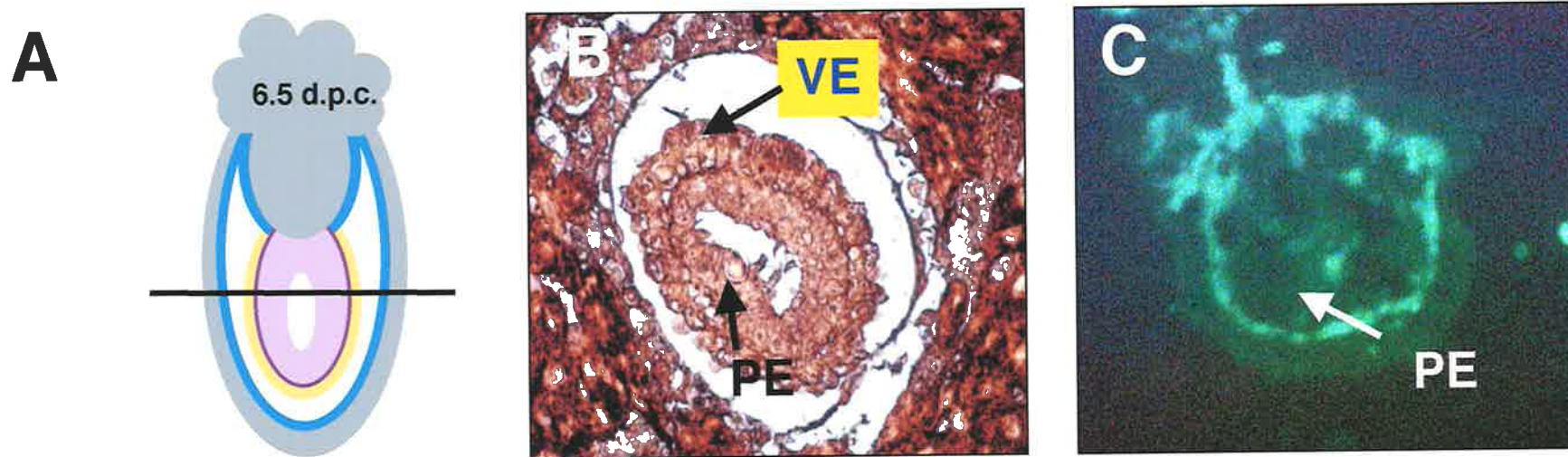


Figure 6.6 cFN is localised to the ECM surrounding primitive ectoderm during formation and differentiation *in vivo* and *in vitro*

Schematic representation of embryo sections from a 6.5 dpc mouse embryo (A). Immunohistochemistry of a 6.0 dpc advanced egg cylinder stage 129-strain mouse embryo. Paraffin wax embedded transverse section within the maternal decidua, stained for the localisation of FN with the TV-1 monoclonal antibody (Chemicon) and visualised by reaction with a goat anti-mouse AP conjugated secondary (B). Cryosection of D3 ES cell derived EB at day 4.0 of culture, stained for localisation of EDA⁺ cFN with the 3E2 monoclonal antibody (Sigma) and fluorescent FITC secondary (C). (B) Arrows indicates tissues in the mouse embryo. (VE) visceral endoderm. (PE) primitive ectoderm. Images taken at 200x magnification using phase contrast microscopy on a Nikon TE300 inverted microscope. Images taken at 200x objective using Hoffman interference contrast microscopy (B) and Hoffman interference fluorescence microscopy using a FITC filter (C).

determine whether this signal might also be present in the EB basement membrane (section 1.8). Cryosections (2.8.5) of day 4.0 ES cell derived EB's, known to contain high levels of primitive ectoderm (Lake *et al.*, 2000), were probed with the anti-EDA⁺ cFN monoclonal antibody, 3E2 (2.8.8). Cellular FN localisation was visualised via a secondary anti-mouse IgG monoclonal antibody, labelled with FITC (2.8.8). EDA⁺ cFN was detected within the basement membrane ECM separating the endoderm and primitive ectoderm lineages (Figure 6.6 B). Therefore cFN is localised adjacent to primitive ectoderm *in vivo* and *in vitro*, consistent with the proposed biological action of this molecule deduced from EPL cell formation.

6.5 Expression of EDA⁺ cFN transcripts in the early mouse embryo

The cellular origin of cFN during early mouse embryogenesis is unknown. To map the expression of EDA⁺ cFN transcripts in the early mouse embryo, a cFN specific nucleic acid probe was generated to regions within the EDA domain of the published cFN sequence (Perkinson and Norton, 1977; Gorski *et al.*, 1977; refer 2.5.5). RT-PCR (2.7.2) was performed on total RNA isolated from D3 ES cells using the 3' EDA2 and 5' EDA1 primers (2.6.13). ES cells were used as the source of RNA as these cells have been reported to express low levels of numerous transcripts (Varlet *et al.*, 1997; Chapman *et al.*, 1997). A fragment was cloned into the EcoRI site of pBluescript-KS+ vector (Stratagene) and sequenced in both directions to confirm the identity of the product (data not shown). This is the first report that ES cells express EDA⁺ cFN transcripts. Anti-sense and sense DIG labelled (Boehringer Mannheim) cFN RNA probes were generated *in vitro* using BamHI linearised plasmid with T7, and HindIII linearised template with T3 RNA polymerase respectively (2.7.12). An anti-sense *Oct4* probe (2.6.12) was generated by T7 RNA polymerase transcription on a HindIII linearised template (2.7.12). *Oct4* expression was used as a marker for the pluripotent primitive ectoderm (Schöler *et al.*, 1989a; Schöler *et al.*, 1989b; Rosner *et al.*, 1990; Yeom *et al.*, 1990). *In situ* hybridisation experiments were performed in association with Antonietta Nesci and Kathryn Hudson.

Serial transverse sections of a 6.5 dpc mouse embryo were removed from paraffin wax (2.8.7) and analysed for expression of *Oct4* and EDA⁺ cFN (Figure 6.7 A). *Oct4* expression was detected at high levels throughout the primitive ectoderm consistent with the known expression of

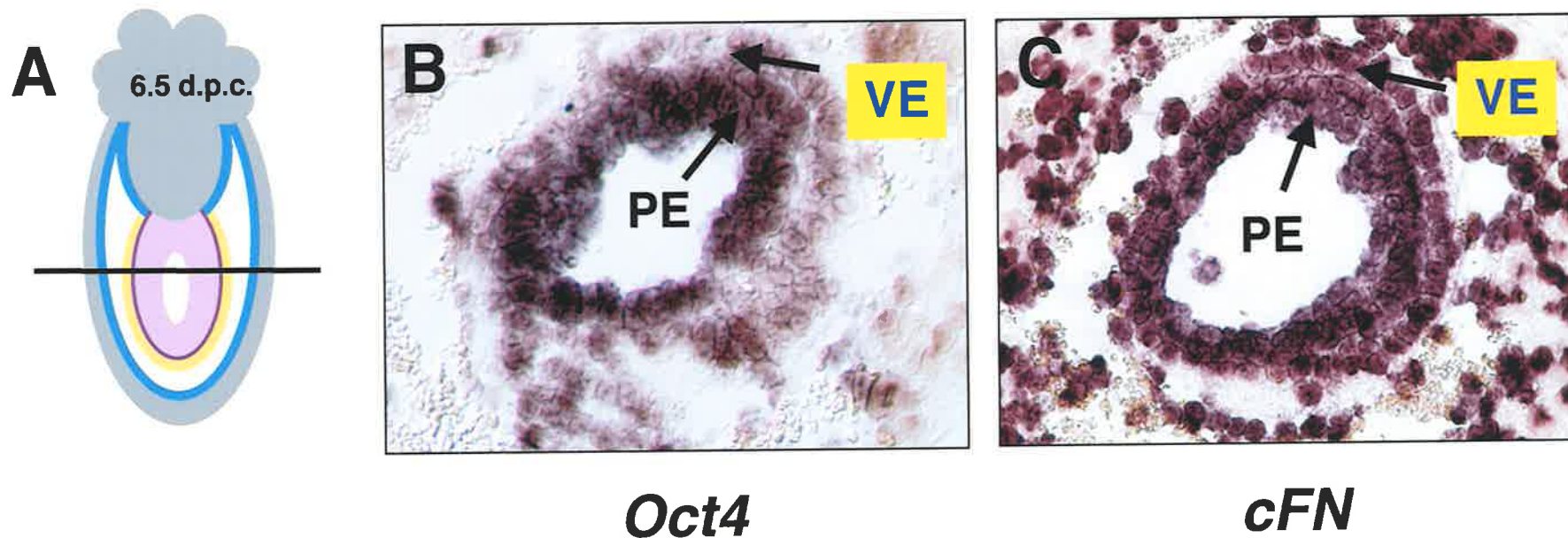


Figure 6.7 *cFN* is localised to the ECM surrounding primitive ectoderm during formation and differentiation *in vivo* and *in vitro*

Schematic representation of embryo sections from a 6.5 dpc mouse embryo (A). In situ hybridisation of a 6.5 dpc advanced egg cylinder stage 129-strain mouse embryo. Paraffin wax embedded transverse sections within the maternal decidua, probed for expression of *Oct4* (B) or *EDA+ cFN* (C) with DIG labelled anti-sense *Oct4* or *EDA+* RNA probes. Expression visualised by reaction with a mouse anti-DIG AP conjugated secondary monoclonal. Images taken at 200x objective using Hoffmann interference contrast microscopy.

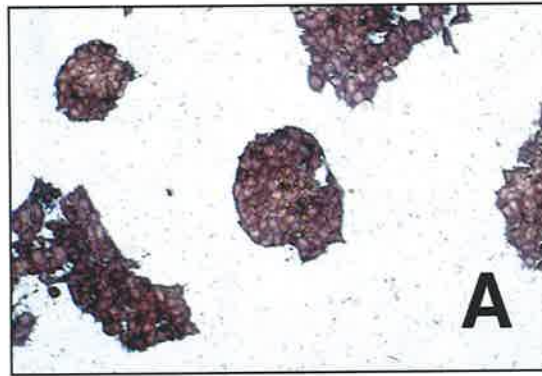
this gene in pluripotent cells (Schöler *et al.*, 1989a; Schöler *et al.*, 1989b; Rosner *et al.*, 1990; Yeom *et al.*, 1990). However, *Oct4* expression was also detected in sporadic cells or at low levels in the surrounding VE. This is not equivalent with the published data for expression of *Oct4* at this stage of development where expression of this marker is absent from the VE (Schöler *et al.*, 1989a). *EDA*⁺ *FN* expression was detected in the cells of the visceral endoderm and the pluripotent cells of the primitive ectoderm (Figure 6.7 B). Additional expression in specific cells within the surrounding decidua confirmed the specificity of detection. Sense transcripts for both probes failed to detect any expression (data not shown).

Collectively, these data suggest that VE and primitive ectoderm cell lineages contribute cFN to the embryonic basement membrane, and further suggest that during primitive ectoderm formation *in vivo* pluripotent cells are exposed to both endogenous and exogenous *EDA*⁺ cFN. This suggests that primitive ectoderm formation has a requirement for endogenous and exogenous FN.

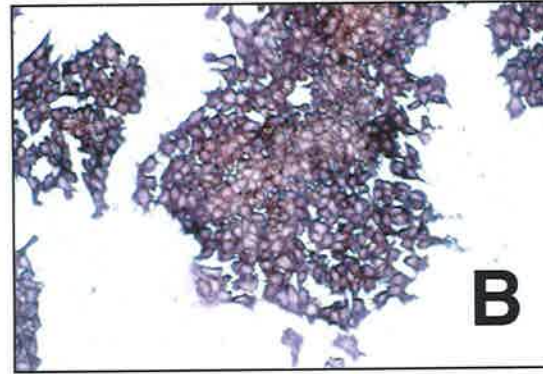
6.6 ES cells secrete endogenous cFN and localise it to the ECM

The endogenous expression of *cFN* by pluripotent primitive ectoderm cells *in vivo* and the detection of *EDA*⁺ transcripts within ES cell total RNA suggests that cFN may be secreted as an endogenous protein from ES cells and localised within the local ECM. This suggestion is supported by the reported localisation of FN on the surface of ES cell colonies (Henry and Campbell, 1998). However, the origin of this FN has not been determined. In culture, FN may be derived from exogenous pFN present within fetal calf serum, or an endogenous pool of secreted cFN or pFN. Cellular FN is not present within FCS (Varito *et al.*, 1987). Therefore, cFN associated with ES cells can only be derived from an endogenous pool.

To assess the origin of ES cell associated FN, D3 ES cells were cultured on gelatin (0.2%) in the presence or absence of MEDII. The cells were fixed and probed for incorporation of cFN into the local ECM, with the cFN specific TV-1 monoclonal antibody (2.8.3). Cellular FN localisation was visualised by immunohistochemical analysis using an alkaline phosphatase conjugated secondary antibody (2.8.3) (Figure 6.8). These results showed that endogenous cFN is present on the surface of ES cells *in vitro* and supports *in vivo* expression analysis that shows cFN expression



ES cells



EPL cells

Figure 6.8 endogenous cFN is secreted from, and localised to, the ECM of ES cells and EPL cells

ES cells cultured on a gelatin ECM (0.2%), and stained for localisation of endogenous cFN with the TV-1 monoclonal antibody (2.8.3). ES cells (A) and EPL cells (B) localised endogenous cFN to the local ECM. Images taken at 200x magnification using phase contrast microscopy.

in pluripotent cells (6.5). The presence of endogenous cFN on ES cell colonies therefore suggests that ES cells secrete an EPL cell inductive factor.

6.7 Expression and secretion of endogenous, pluripotent cell cFN is not altered by the presence of L-proline or MEDII

The formation of EPL cells in high level concentrations of L-proline, and the absence of exogenous cFN, suggests that EPL cell gene expression and morphology is acquired through cell attachment to endogenous cFN and/or the exogenous gelatin ECM. The role of endogenous cFN in EPL cell formation is further implicated by the formation of morphological EPL cells when ES cells are seeded at high cell density in 50% PYS2 CM. However, an alternate explanation is that L-proline can compensate for a lack of exogenous cFN by up-regulating the expression or secretion of the endogenous cFN pool.

To assess the effect of L-proline on *cFN* expression, ES cells were cultured under standard conditions in the presence or absence of MEDII or 400 μ M L-proline and analysed for the expression of *EDA*⁺ *cFN* transcripts by northern blot analysis using. Anti-sense radio-labelled RNA probes generated as in 6.5. Northern analysis demonstrated the 7.2 kb *EDA*⁺ cFN transcript was not significantly altered by the presence or absence of MEDII or 400 μ M L-proline (Figure 6.9 A, B, C).

To determine if the low molecular weight signal could regulate cFN secretion, western analysis (2.8.8) was performed on 10 μ ls of medium conditioned by ES cells and ES cells cultured in 400 μ M L-proline. Cellular FN was detected with the TV-1 antibody (2.8.3) and visualised by chemiluminescence (2.8.8) (Figure 6.9 D). These data showed that ES cells secreted cFN into the CM and that 400 μ M L-proline did not alter the endogenous secretion of this factor.

6.8 Discussion

The analogy between EPL cell and primitive ectoderm formation suggests that cFN and L-proline are equivalent to primitive ectoderm inductive signals *in vivo* and *in vitro*. In support of this proposition, cFN was shown to be localised and expressed in a spatial and temporal manner with

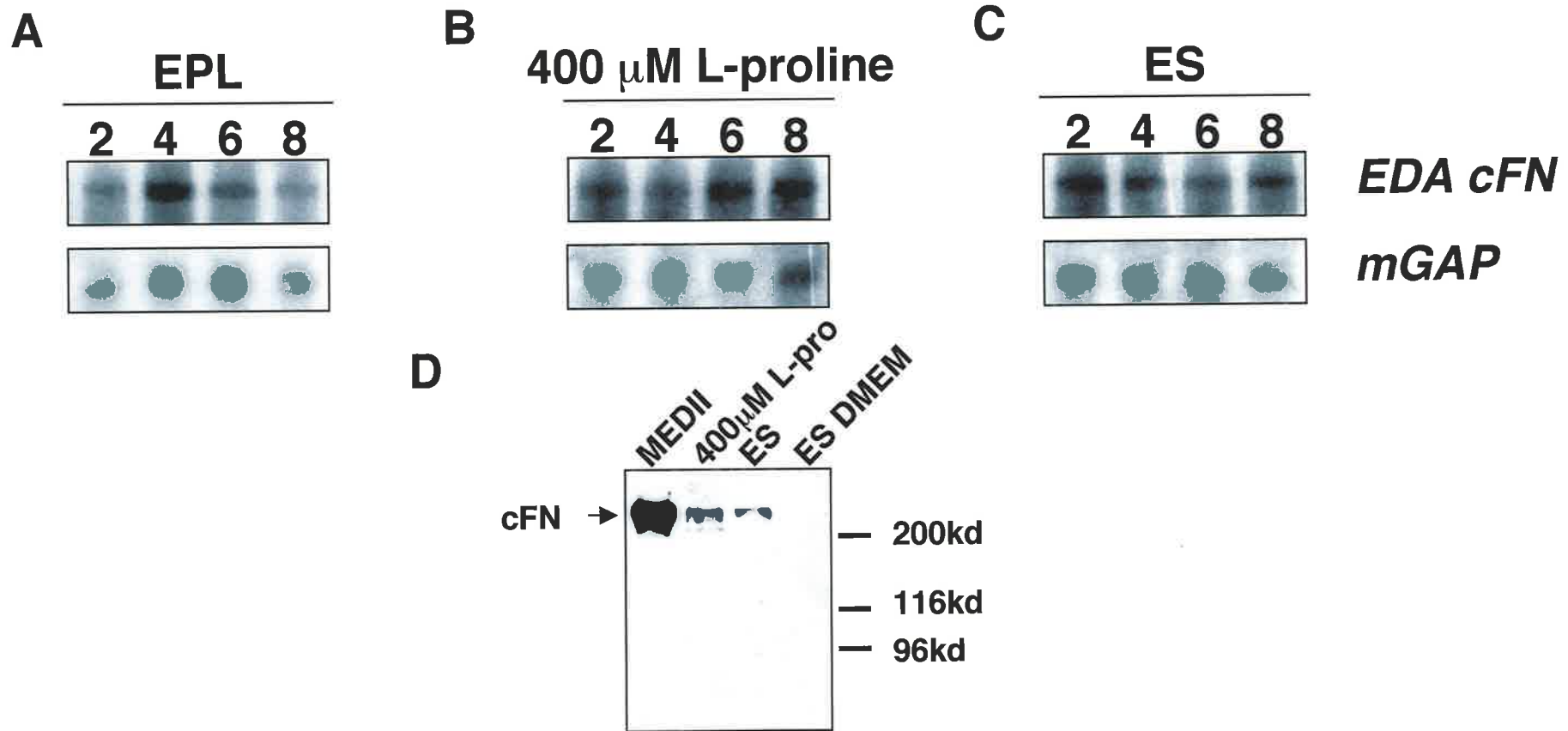


Figure 6.9 Northern blot analysis of *EDA+ cFN* expression, and western analysis of cFN secretion in EPL, ES and ES cells cultured in 400 μ M L-proline

Northern blot analysis of 20 μ g of total RNA, isolated from EPL cells (A), ES cells maintained in 400 μ M L-proline (B) and D3 ES cells (maintained for 2, 4, 6 and 8 days in culture) for the expression of *EDA+ cFN* and *mGAP*. cFN was probed with anti-sense RNA probe directed against the EDA domain of mouse cFN. The *FN* transcript is 7.2 kb (Kornblith *et al.*, 1986). *mGAP* was probed with a cDNA against mouse GAP (Rathjen *et al.*, 1990b). The *mGAP* transcript is 1.5 kb. Western blot analysis (D) of 10 μ l of MEDII, 400 μ M L-proline and ES cells conditioned medium for secretion of cFN using the TV-1 monoclonal antibody (Chemicon) to observe if L-proline up-regulates secretion of cFN.

primitive ectoderm *in vivo* and *in vitro* and a low molecular weight EPL cell inductive signal was shown to be secreted by the VE-like cell line END2. VE is located adjacent to primitive ectoderm, which implicates this lineage as a cellular origin of primitive ectoderm inductive signals *in vivo*. Furthermore, additional hepatic cell cultures that may act as an *in vitro* source of VE signals (Rathjen *et al.*, 2001) produce CM that contains cFN and induces the formation of morphological EPL cells from ES cells.

6.8.1 Expression and localisation of EPL cell inductive signals *in vivo* and *in vitro* supports EPL cell formation as a model of primitive ectoderm formation

Cellular FN is localised to the basement membrane ECM surrounding primitive ectoderm in the 6.5 day mouse embryo (Wartiovaara *et al.*, 1979). In addition, cFN is localised to the basement membrane ECM surrounding primitive ectoderm in day 4.0 EB's *in vitro*. This indicates that the cFN signal co-localises spatially and temporally with primitive ectoderm *in vivo* and *in vitro*. The cellular origin of cFN during the maintenance of primitive ectoderm development was shown by *in situ* hybridisation. The VE and primitive ectoderm of the embryo and distinct cells within the decidua expressed transcripts for *EDA*⁺ *cFN*. Interestingly, this suggests that pluripotent cells are exposed to both exogenous and endogenous cFN during primitive ectoderm formation and maintenance *in vivo*.

Equivalent to *in vivo* pluripotent cells, ES cells and EPL cells were shown to express *EDA*⁺ *cFN*, which is located in the local ECM of ES cells and EPL cells. This shows that ES cells express, and are associated with an EPL cell inductive signal in the absence of L-proline but do not adopt the EPL cell morphology unless there is sufficient levels of an L-proline equivalent signal, or cFN is presented to ES cells as an ECM (Chapter 5). The formation of EPL cell morphology in the presence of a higher concentration of L-proline (400 μ M) may be due to additional ECM factors rather than endogenous cFN as cFN expression and secretion are not altered.

VE-like cell lines secrete a low molecular weight EPL cell inductive signal *in vitro*. Therefore, the EPL cell inductive signals are present with in embryo both spatially and temporally during primitive ectoderm development. Therefore, a model for primitive ectoderm formation would suggest

that a low molecular equivalent signal is derived from VE while the cFN signal is derived from both VE and the pluripotent cells to allow primitive ectoderm formation.

6.8.2 Secretion of EPL cell inductive signals *in vitro* suggests conservation of primitive ectoderm formation

The secretion of EPL cell inductive signals is not a common event. The culture of ES cells in medium conditioned by 13 additional cell lines did not induce an EPL cell morphology or phenotypically alter ES cell culture (Rathjen *et al.*, 1999). The cellular specificity for the secretion of the EPL cell inductive signals, and the presence of these signals in the CM of hepatic cell lines or primary hepatocyte cultures derived from additional mammalian and avian species, implies that similar biological factors direct primitive ectoderm formation and maintenance in mammals and birds. This suggestion is consistent with the formation of primitive ectoderm equivalent tissues in these species, and the similar fate maps of primitive ectoderm equivalent tissues prior to gastrulation (Tam and Behringer, 1998).

6.8.3 Relationship between EPL cell inductive signals and embryonic cavitation signals *in vivo* and *in vitro*

VE is a candidate cellular source of signals that direct primitive ectoderm formation in the embryo and is crucial for the survival of adjacent pluripotent cells, providing uptake and delivery of nutrients, during early mouse embryogenesis (Bielinska *et al.*, 1999). In addition, signals arising from AVE have been implicated in directing pluripotent cell fate to neural lineages during gastrulation while, prior to this, VE has been implicated in pro-amniotic cavity formation following blastocyst implantation (reviewed in Bielinska *et al.*, 1999). The diffusible signal in the two-signal model for pro-amniotic cavity formation (Coucouvanis and Martin, 1995) is hypothesised to be secreted by VE.

The similar cellular origin and temporal expression/localisation of the EPL cell inductive signals during primitive ectoderm formation *in vivo* and in model systems *in vitro* suggests these signals may be active in pluripotent cell differentiation, morphogenesis and cavitation in the embryo.

END2 cells secrete a low molecular weight EPL cell inductive signal and a signal that induces embryonic differentiation, cavitation and morphogenesis (Coucouvanis and Martin, 1995). PYS2 cells also secrete the low molecular weight EPL cell inductive signal but do not secrete detectable levels of a signals that induce cavitation in S2 EC cell EB's (Coucouvanis and Martin, 1995; Murray and Edgar, 2000). It is therefore unclear whether the low molecular weight EPL cell inductive signal is equivalent to the diffusible death signal directing cavity formation during development of the primitive ectoderm *in vitro*. However, PYS2 cells have been shown to secrete lower levels of the low molecular weight signal than END2 cells, and this requires further investigation.

Cellular FN is also localised temporally and spatially with the survival signal active during pro-amniotic cavity formation in EB's and the early mouse embryo (Coucouvanis and Martin, 1995). While the role of cFN as a survival signal for pluripotent cells *in vitro* is yet to be investigated, END2 cells have been shown to secrete cFN in the absence of survival signal activity (Coucouvanis and Martin, 1995). This suggests that cFN is not equivalent to the survival signal for pluripotent cells. However, END2 cells secrete low levels of cFN that are inactive in maintaining EPL cells *in vitro* and therefore may be inactive as a survival signal. Furthermore, the assays of Coucouvanis and Martin (1995) were performed in a three dimensional matrix of dermal collagen. It is therefore possible that the cFN signal may not be presented correctly to pluripotent cells in these assays due to a lack of basement membrane formation and/or the high affinity of cFN for dermal collagen. Therefore, the equivalence of cFN to the ECM survival signal requires further investigation.

Chapter 7

Final Discussion

Chapter 7

7.1 Introduction

In vitro culture systems have been used to isolate and identify biologically relevant signals that direct the differentiation of multipotential haematopoietic stem cells *in vitro* (Metcalf, 1989; Metcalf, 1991). The therapeutic use of some of these signals in directing haematopoietic stem cell differentiation *in vivo* highlights the capacity of *in vitro* culture differentiation systems to isolate biologically relevant signals. Similarly, ES cells have also been assayed for signals that direct biological events during early mammalian development. However, while this system has been successful in identifying biologically relevant molecules, such as the IL-6 family of cytokines, which prevent pluripotent cell differentiation, these factors do not seem necessary for development *in vivo*. ES cell based assays have therefore not been successful at identifying biologically relevant signals that induce pluripotent cell differentiation.

The ability of ES cells to form EPL cells in the presence of MEDII is the first report of biologically derived signals directing ES cell differentiation. This suggests that signals within MEDII are replicating an *in vivo* developmental event *in vitro* and that the biological activity of EPL cell and primitive ectoderm inductive signals may be equivalent. MEDII contains two signals that direct EPL cell formation. Prior to the commencement of this study, the low molecular weight factor had been purified based on its ability to induce EPL cell equivalent colony morphology from ES cells, and identified as the imino acid L-proline or numerous short peptides (2-4 amino acids) that all contain a single, or multiple, L-proline residues. However, the biological activity and localisation of these low molecular weight signals *in vivo* and *in vitro* were unknown.

The aim of this thesis was the purification, identification and characterisation of the high molecular weight EPL cell inductive component in MEDII. Further, this signal was characterised during EPL cell formation in culture and primitive ectoderm formation *in vivo* and *in vitro*, to identify the cellular origin and biological role of this, and the L-proline equivalent, signals during early mammalian embryogenesis.

7.2 Purification and identification of the high molecular weight EPL cell inductive factor

Biochemical characterisation of the high molecular weight EPL cell inductive signal identified a trypsin, heat, acid and reducing agent sensitive protein dimer with a native molecular weight of approximately 500kd. This signal was purified from sfMEDII and was functional as an ECM component in the formation of EPL cells. This led to the identification of the high molecular weight signal as an isoform of the ECM component FN, cFN. Cellular FN displayed identical biochemical properties and biological activity to the purified factor during EPL cell formation from solution or when presented to ES cells in an ECM form. This indicated that cFN was the high molecular weight EPL cell inductive signal in MEDII.

7.3 cFN and L-proline induce EPL cell morphology, gene expression and differentiation potential from ES cells

L-proline and cFN were identified as the EPL cell inductive signals by their capacity to direct EPL cell formation from ES cells. EPL cells are characterised by a distinct epithelial colony morphology, gene expression and differentiation potential. In particular, EPL cell gene expression is characterised by up-regulation of *Fgf5*, down-regulation of *Rex1* and maintenance of *Oct4* expression while EPL cell differentiation is characterised by the early onset of *brachyury* expression following differentiation as EB's when compared to ES cell differentiation.

ES cells cultured in the absence of L-proline require an exogenous ECM of FN to adopt the EPL cell morphology. Furthermore, ES cells cultured in low concentrations of L-proline (40 μ M) required soluble exogenous cFN (10 μ g/ml) to adopt the EPL cell morphology. This indicated that cFN may play a role in EPL cell colony morphology in the absence of, or presence of low-level, L-proline. However, L-proline and cFN failed to induce characteristic EPL cell gene expression or differentiation potential at the low concentration. In contrast, ES cells cultured in 100 μ M-400 μ M L-proline adopted the EPL cell morphology and gene expression in the presence or absence of exogenous cFN, perhaps utilising an endogenous pool of cFN and the local gelatin ECM. However, endogenous cFN was not sufficient to maintain EPL cell gene expression at p3 of culture as *Fgf5*

expression was down-regulated. The maintenance of *Fgf5* expression at p3 in the presence of exogenous cFN (presented from solution or as an ECM) indicates that EPL cells are responsive to this signal and that cFN is an EPL cell maintenance factor.

The association of cFN with the ECM and presentation to pluripotent cells is crucial to the activity of this signal as an EPL cell maintenance factor. This was exemplified when ES cells were cultured in the presence of MEDII and the absence of exogenous gelatin ECM. These cells maintained an ES cell equivalent morphology and failed to up-regulate *Fgf5* expression, but down-regulated *Rex1* expression and adopted differentiation characteristics most similar to EPL cells. However, culture of pluripotent cells on an ECM of cFN in the presence of MEDII restored the EPL cell morphology and regulated expression of *Fgf5* at p2 and p3 of culture. Therefore, these data indicated that L-proline and cFN, in an ECM associated form, are the EPL cell inductive signals in MEDII and that cFN has a role in the morphology, gene expression and maintenance of EPL cells.

7.4 Secretion and expression of EPL cell inductive signals *in vivo* and *in vitro* implies VE is a source of primitive ectoderm inductive signals

Data presented in this thesis have shown that VE-like cell cultures secrete EPL cell inductive signals *in vitro*. This is not a common event for tissue culture cell lines (Rathjen *et al.*, 1999) and the analogy between EPL cells and primitive ectoderm therefore implicates VE as a cellular source of primitive ectoderm inductive signals in the embryo. In particular, it was shown that VE secretes the low molecular weight signal *in vitro* and that both VE and primitive ectoderm express cFN transcripts in culture and within the embryo. This suggests that primitive ectoderm and EPL cells are exposed to both endogenous and exogenous cFN. The requirement of exogenous cFN for EPL cell maintenance therefore suggests that primitive ectoderm formation in the embryo is further mimicked by EPL cell formation in culture and the activity of cFN *in vivo* and *in vitro*.

Cellular FN is localised to the basement membrane ECM separating primitive ectoderm from visceral endoderm (George *et al.*, 1996) which indicates that cFN is associated with primitive ectoderm in the embryo. More importantly, mice lacking FN form primitive ectoderm that is reduced

in cell numbers, possible reflecting a loss of pluripotent cell maintenance, equivalent to the role of cFN during EPL cell formation *in vitro*.

In summary, identification of the EPL cell inductive signals L-proline and cFN from MEDII has lead to the identification of the VE and pluripotent cells of the early mouse embryo as the potential cellular source of signals that provide para-and autocrine signals required for primitive ectoderm formation in culture and in the early mouse embryo.

7.5 Future work

7.5.1 EPL cells as a model of gastrulation and lineage specific differentiation:

Application to cell based gene therapy

The formation and maintenance of primitive ectoderm prior to differentiation during gastrulation suggests that the signals that induce primitive ectoderm formation are distinct from the signals that induce its further differentiation in the mouse embryo. Identification of the signals that direct primitive ectoderm differentiation has been hindered however, by an inability to stably culture this tissue *in vitro*. The culture of EPL cells as the *in vitro* equivalent of primitive ectoderm overcomes this restriction. Therefore, EPL cells may be used as a cellular substrate for the process of gastrulation and used to identify signals that direct pluripotent cell differentiation in a lineage specific manner (Lake, 1996).

Prior to the completion of this study, a further requirement for the establishment of this system was the establishment of a controlled environment for lineage specific differentiation in the absence of additional contaminating signals. The identification of cFN (preferably 25 µg/ml) and L-proline (400 µM) as the EPL cell inductive signals will allow EPL cell formation within a controlled environment to which specific factors may be added to direct the formation of differentiated cell lineages as defined by morphology and gene expression.

The identification of signals directing lineage specific pluripotent cell differentiation is crucial for the use of human ES cells for cell based gene therapy. Thus, human ES cells derived from a patient can be genetically modified (to correct a genomic abnormality or introduce a

therapeutic gene) and induced to form EPL cells in medium supplemented with L-proline, cFN and a serum that lacks contaminating factors that may interfere in the differentiation process. These ES cells can then be further differentiated in a lineage specific manner to the cell type of choice (ie haematopoietic stem cells) prior to transplantation back into the host patient for therapeutic benefit.

Since the completion of this study, MEDII medium has been shown to contain an additional signal that can direct the lineage specific differentiation of ES cells (Rathjen, unpublished data). Differentiated cells derived from the activity of this signal on EPL cells are currently being injected into host rodents to assess incorporation into host tissues prior to use in the treatment of a mouse animal model of disease (Hirst, unpublished data).

7.5.2 The equivalence of L-proline and cFN to the two-signal model of death and survival during cavitation in the vertebrate embryo

The biological nature, cellular origin and temporal and spatial expression of the EPL cell inductive signals are equivalent to the signals active during the process of cavitation during EB formation *in vitro*. This suggests that the signals active in these models may be equivalent. While this is yet to be determined, it is known that END2 cells secrete the diffusible death signal in the absence of survival signal activity (Coucounanis and Martin, 1995) and also secrete a signal equivalent to the low molecular weight EPL cell inductive signal in MEDII with low levels of soluble cFN. This suggests that the cavitation and EPL cell inductive signals may be secreted in a similar fashion from END2 cells.

The death signal from END2 cells induces apoptosis in adjacent S2 EC cells within the EB. This may be due to the absence of survival signal activity and/or the dose dependent response of pluripotent cells to this signal. END2 cells have been shown to secrete low levels of cFN and lower levels of the L-proline equivalent signal than HepG2 cells. Therefore, given the dose dependent response of pluripotent cells to L-proline and absence of survival signals activity, HepG2 cells may be a more suitable VE-like cell line to use as a source of signal directing embryonic cavitation in S2 EC cells. Additional work from this thesis will require repetition of the experiments of Coucounanis

and Martin (1995) using the purified EPL cell inductive components and HepG2 cells as external sources of VE-like signals.

END2 cells secrete cFN at low levels (6.3) in the absence of survival signal activity. This suggests that either cFN is not the survival signal or is the survival but is secreted at levels below those required for survival signal activity. Given the role of ECM in these model systems, the survival signal role of cFN ~~in this system~~ would be best examined using HepG2 cells as a source of cFN and an additional source of 3D matrix such as Methyl-cellulose to prevent any deleterious interactions within the dermal collagen matrix. These experiments will be used to align or distinguish the biology of these respective two-signal/ECM associated models that are active during primitive ectoderm formation and embryonic cavitation in the vertebrate embryo.

7.5.3 ES cells lacking cFN

An inherent problem with addressing the role of cFN during EPL cell formation is the endogenous secretion of this signal by pluripotent cells. While the role of FN during early mouse embryogenesis has been characterised by knockout technology (George *et al.*, 1993), no $FN^{-/-}$ ES cells have been isolated or derived from heterozygous ES cell clones. However, the presence of $FN^{-/-}$ ES cells would provide a cellular source for a comprehensive analysis for the role of endogenous and exogenous FN signalling during pluripotent cell culture and EPL cell formation. ES cells lacking FN can be assessed for EPL cell inductive capacity in response to L-proline in the presence or absence of exogenous cFN. These experiments would determine the requirement for cFN for EPL cell morphology, its role in the regulation of *Fgf5* expression and during the acquirement of EPL cell differentiation characteristics.

7.5.4 Receptor signalling by L-proline and cFN during EPL cell formation

The major FN receptor in the early mouse embryo is the $\alpha 5/\beta 1$ integrin heterodimer (Yang and Hynes, 1996), while in the absence of $\alpha 5$ *integrin* expression mouse cells use the $\alpha V/\beta 1$ heterodimer as a redundant receptor for the FN ligand (Yang and Hynes, 1996). While ES cells lacking the $\alpha 5$ *integrin* gene have not been generated, the absence of FAK activity in ES cells did not

interfere with differentiation into lineages of the three germ layers when cells were implanted subcutaneously into nude mice or when treated with retinoic acid *in vitro* (Ilic *et al.*, 1995). While this suggests that FAK is not essential for primitive ectoderm formation or pluripotent cell differentiation *in vitro*, it does not specify the role of this kinase, or FN signalling, during primitive ectoderm maintenance in EB's. The activity of FAK, or the αV and $\alpha 5$ integrin receptors, during EPL cell formation is unknown. Therefore ES cell lines lacking functional *FAK* genes, or created ES cell lines expressing dominant negative integrin receptors to the FN ligand, could be assessed for EPL cell formation and maintenance (Yang *et al.*, 1993; Furuta *et al.*, 1996). The morphology of EPL cells and regulation of *Fgf5* expression by FN during EPL cell formation provides an assay to monitor cFN activity. These experiments would determine if FN signalling is required for EPL cell formation and maintenance, and the pathway used by the FN ligand to mediate this response.

A receptor and signalling pathway for the low molecular weight EPL cell inductive signal has not been discovered. However, the pathway(s) linked to this signalling could potentially be identified using ES cells that carry functional mutations in genes, whose products are essential for intracellular signalling pathways that inhibit ES cell or pluripotent cell differentiation *in vivo* and *in vitro*. While association of low molecular weight EPL cell inductive signalling with G-coupled receptors is unlikely (5.11.2) the Ras pathway has been linked to pluripotent cell differentiation *in vivo* and *in vitro* (Cheng *et al.*, 1998, Arsenian *et al.*, 1998; Burdon *et al.*, 1999; Wienhold *et al.*, 2000). Mice lacking a functional gene for *Grb2*, the product of which is an association factor required for linking tyrosine kinase activity to intracellular MAPK signalling pathways, fail to develop beyond implantation due to defects in ICM survival and/or primitive endoderm differentiation (Cheng *et al.*, 1998). *Grb2*^{-/-}blastocysts show similar defects when cultured as outgrowths *in vitro* and *Grb2*^{-/-}ES cells appear normal but give rise to EB's that fail to form an outer layer of primitive endoderm and do not cavitate (Cheng *et al.*, 1998). The authors of this paper suggest that *Grb2* is involved in primitive endoderm specification during development and suggest that the lack of primitive endoderm prevents formation of embryonic lineages. However the authors of this paper fail to assess pluripotent cell development to primitive ectoderm or the formation of additional embryonic lineages (Cheng *et al.*, 1998). Therefore, it would be interesting to assess the ability of *Grb2*^{-/-}ES cells to form EPL cells in response to L-proline signalling. Given that EPL cell formation is an

obligatory intermediate in embryonic differentiation of pluripotent cells a block in L-proline signalling, perhaps through inhibition of the Ras pathway, should block EPL cell formation and inhibit ES cell differentiation. In support of this proposition, chemical inhibition of the Ras pathway member MEK1 increases self-renewal and inhibits ES cell differentiation *in vitro* (Burdon *et al.*, 1998). Formation and maintenance of EPL cells from ES cells bearing mutations in these other signalling pathways can therefore be used as an assay to elucidate the biochemical basis of L-proline activity.

Identification of the receptors and signalling pathways that transmit the cFN and L-proline signals in ES cells and EPL cells will allow further investigation into these pathways and the link between these pathways and the regulation of *Fgf5* and *Rex1* expression in EPL cells.

7.5.5 Collagen breakdown products as a source of L-proline equivalent signalling *in vivo* and *in vitro*

This thesis has identified two possible sources for L-proline equivalent signals during primitive ectoderm development *in vivo*. L-proline-like signals are secreted by VE and may be active immediately or derived from the degradation of the proline rich ECM component collagen IV, localised to the basement membrane ECM that separates VE and primitive ectoderm (Adamson and Ayers, 1979; Ashmarin *et al.*, 1998). Type IV collagen includes six genetically distinct polypeptides named $\alpha 1(\text{IV})$ through $\alpha 6(\text{IV})$. These isoforms are speculated to organize themselves into unique networks providing mammalian basement membranes specificity and inequality. The number of these genes has prevented creation of a knockout mouse lacking collagen IV and assessment of the role of this basement membrane component during pluripotent cell development.

The cellular origin of collagen IV *in vivo* and *in vitro* can be determined by *in situ* hybridisation and northern blot analysis of embryos and ES/EPL cells respectively. The degradation of collagen as a potential source of L-proline equivalent signalling *in vivo* can then be examined for its EPL cell inductive capacity by the degradation of purified collagen IV with a collagen IV specific gelatinase (gelatinase B), followed by assessment of peptide activity for EPL cell formation. The biology of collagen IV *in vivo* can be examined by investigation into gelatinase/collagenase

expression in the mouse embryo and then gene knockout studies against form of collagenases active at this time of development. In addition, antibodies directed against EPL cell inductive peptides derived from collagen IV can be cultured with blastocysts *in vivo* or *in vitro* to examine the effect on primitive ectoderm development.

In conclusion, results presented in this thesis have demonstrated that cFN and L-proline, or L-proline equivalent signals, direct EPL cell formation from ES cells *in vitro* and that this event mimics the formation of primitive ectoderm from the ICM during mouse development *in vivo*. This is the first example of biologically relevant signals, both in the embryo and in culture, that induce ES/ICM cell differentiation and supports the use of developmental *in vitro* differentiation systems as models for mammalian development. EPL cell formation using purified signals can be expected to have a major impact on the elucidation of signals that direct lineage specific pluripotent cell differentiation in mammals and the use of pluripotent or multipotent cells for *in vitro* culture and cell based gene therapy.

Chapter 8

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