MOLECULAR MECHANISM OF L-PROLINE INDUCED EPL-CELL FORMATION

A thesis submitted to the University of Adelaide for the

Degree of Doctor of Philosophy

By

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SUMMARY

During early embryogenesis pluripotent cells of the inner cell mass (ICM) give rise to a second pluripotent cell population known as the primitive ectoderm an obligate developmental intermediate and the substrate for gastrulation. The ICM and primitive ectoderm are distinguished on the basis of morphology, gene expression and differentiation potential. However, the signals and mechanisms involved in the transition form ICM to primitive ectoderm are not understood. Culture of ES cells in the presence of a conditioned medium MEDII leads to a transition of ES cells to a population of pluripotent primitive ectoderm-like (EPL) cells that are the *in vitro* equivalent of the primitive ectoderm. In terms of EPL cell formation the bioactive component of MEDII was identified as L-proline. In this thesis the molecular mechanism by which L-proline induces EPL-cell formation was elucidated.

As well as L-proline, short L-proline containing peptides were also shown to induce EPL-cell formation but different peptides displayed different abilities to induce the transition with some inducing the complete transition and others inducing morphology changes only. The mechanism of L-proline induced EPL-cell formation was shown to be independent of NK receptors. The mechanism of L-proline induced EPL-cell formation, as deduced from the results presented in this thesis, was suggested to involve the internalisation of L-proline via the SAT2 amino acid transporter into ES cells as competitive inhibitors of SAT2 prevented EPL-cell formation. MAPK signalling via the action of MEK1 was implicated in L-

proline induced EPL-cell formation as inhibitors of MEK1 prevented EPL-cell morphology, gene expression and differentiation potential in the presence of L-proline.

PI3K signalling was implicated in L-proline-induced EPL-cell morphology since PI3K inhibitor LY294002 maintained domed colonies in the presence of L-proline but failed to maintain an ES-cell gene expression profile and differentiation potential. Both MAPK and PI3K signalling were suggested to lie down-stream of L-proline action since treatment of ES cells with L-proline induced the activation of ERK1/2 and Akt down-stream effectors of MAPK and PI3K signalling respectively. A gene potentially involved in the PI3K-mediated morphology change was *Lefty2*. Therefore, the mechanism of L-proline induced EPL-cell formation appears to involve internalisation of L-proline and at least two signalling pathways down-stream of L-proline, which regulate different components of the transition.

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.

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CHAPTER 1:

GENERAL INTRODUCTION

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1.1 MOUSE EMBRYOLOGY

1.1.1 Pre-implantation development

Cell division of the zygote takes place 20-24 h following fertilization. It is at this two-cell stage that activation of the embryonic genome begins and the degradation of most maternal mRNA occurs (Telford *et al.*, 1990; Latham *et al.*, 1991). At the four-cell stage 1.5 days post coitum (dpc), individual cells are known as blastomeres and have totipotent developmental potential (Figure 1.1). Composite embryos made from a donor blastomere and genetically distinct blastomeres demonstrated donor-cell contribution throughout embryonic and extraembryonic tissues of the resulting embryo (Kelly, 1977).

At the 8-cell stage (2.5 dpc) uvomorulin, which is expressed throughout development up to the blastocyst stage, becomes phosphorylated leading to the compaction of the embryo (Fleming *et al.*, 1992; Sefton *et al.*, 1992). Uvomorulin was demonstrated as critical for compaction as targeted mutation of this gene led to de-compaction of the morula (Riethmacher *et al.*, 1995). The formation of tight junctions at the outer (apical) surfaces of the cells surrounding the compacted morula results in the establishment of distinct outer and inner cell populations (Figure 1.1) (Becker *et al.*, 1992). The cells on the inside of the compacted embryo go on to form the inner cell mass

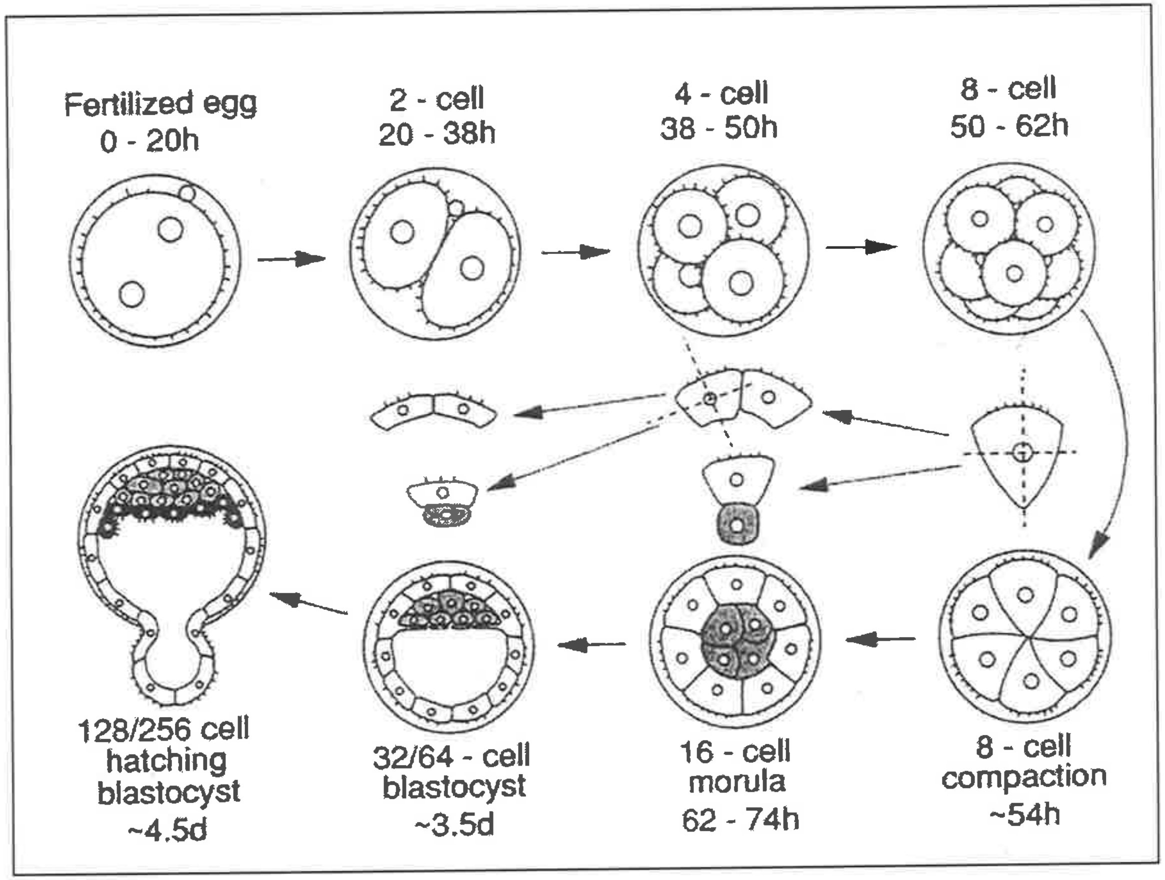
Figure 1.1

Schematic illustration of mouse pre-implantation development

Cell division of the zygote takes place 20-24 h following fertilization and leads to the formation of four blastomeres by 38-50 h. Compaction of the embryo is initiated at the 8-cell stage leading to the establishment of distinct outer and inner cell populations. The cells on the inside of the compacted embryo go on to form the inner cell mass (ICM) while the outer cells develop into a monolayer of extraembryonic trophectoderm. The accumulation of fluid by diffusion at ~3.5 dpc leads to the formation of the blastocyst consisting of a fluid-filled cavity surrounded by a layer of trophectoderm, with the cells of the ICM at one pole. At ~4 dpc, the cells of the ICM lining the blastocoelic cavity differentiate into extra-embryonic primitive endoderm. Around 4.5 dpc, the combined effect of the increasing fluid volume in the blastocyst and weakening of the zona pellucida by proteolytic enzymes leads to the hatching of the blastocyst.

Reproduced from Fleming *et al.*, 1992

Figure 1.1



Fertilized egg
0 - 20h
2 - cell
20 - 38h
4 - cell
38 - 50h
8 - cell
50 - 62h
128/256 cell
hatching
blastocyst
~4.5d
32/64 - cell
blastocyst
~3.5d
16 - cell
morula
62 - 74h
8 - cell
compaction
~54h

As development continues, Na+/K(+)-ATPase located on the plasma membrane of the trophectoderm cells is activated, resulting in increased concentration of these two ions in the interstitial spaces of the morula (Watson and Kidder, 1988; Watson, 1992).

(ICM), a population of pluripotent cells that is the precursor of all future lineages of the embryo including the germ line. The surrounding outer cells develop into a monolayer of extraembryonic trophectoderm that initially surrounds the developing embryo and ultimately develops into the placenta (Figure 1.1) (Gardner, 1983). As development continues, Na^+ and K^+ pumps located on the plasma membrane of the trophectoderm cells are activated, resulting in increased concentration of these two ions in the interstitial spaces of the morula (Manejwala *et al.*, 1989). Accompanying passive diffusion of water results in formation of the blastocoelic cavity (Figure 1.1) with tight junctions and desmosomes between the trophectodermal cells inhibiting the escape of fluid. At this stage of development the embryo is known as a blastocyst and consists of the fluid-filled blastocoelic cavity surrounded by trophectoderm, with the cells of the ICM at one pole. The layer of trophectoderm that overlies the ICM is known as polar trophectoderm while the cells that line the cavity are called mural trophectoderm.

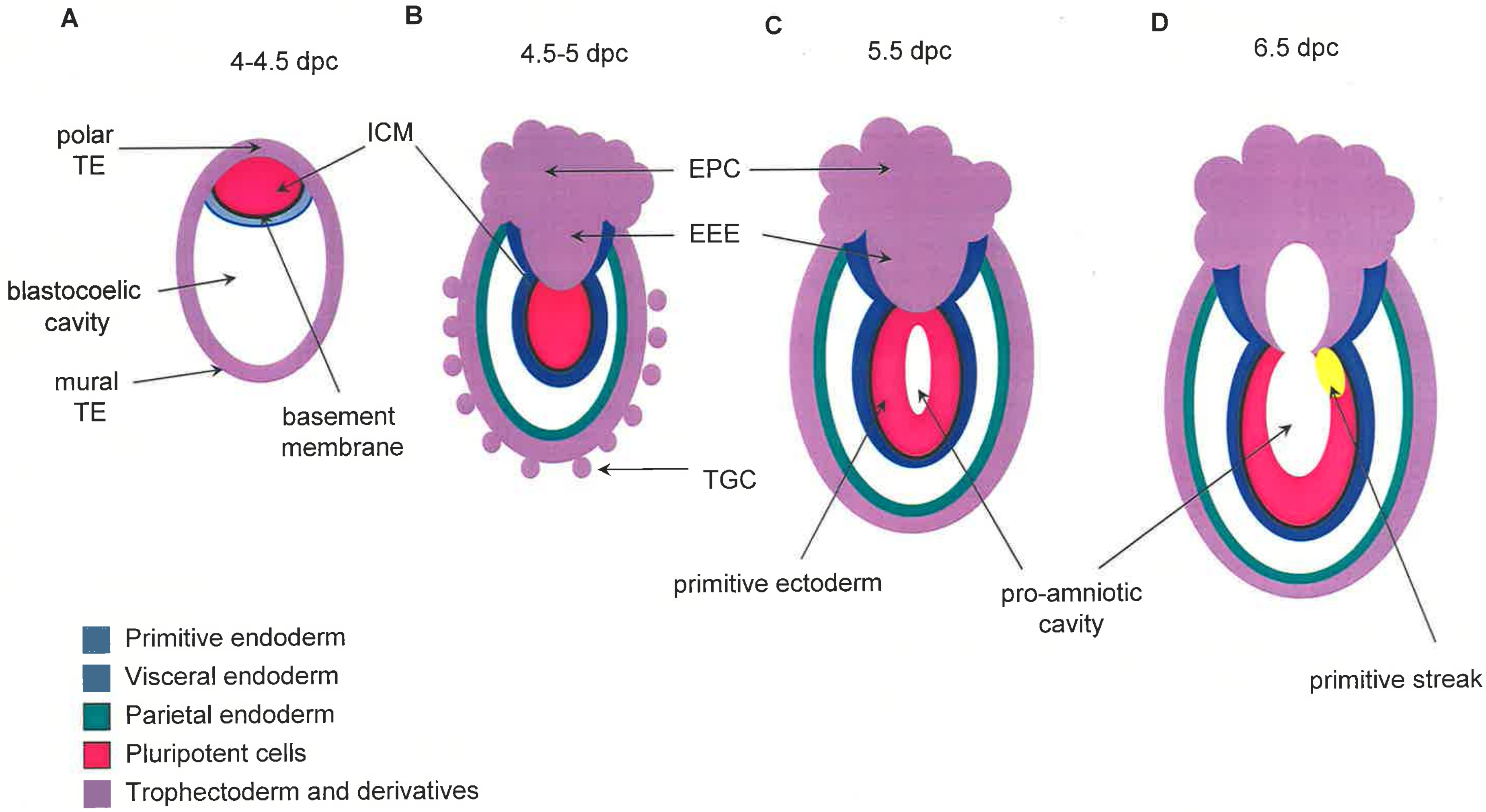
At ~4 dpc, the cells of the ICM lining the blastocoelic cavity differentiate into extra-embryonic primitive endoderm (Figure 1.2 A). The classical model of primitive endoderm (PE) formation assumes ICM cells are homogeneous and have the potential to become epiblast or PE depending on their position; the cells located on the surface differentiating to PE. *In vitro*, embryoid body differentiation supported this model as cells located on the outside of bodies differentiate to PE (Becker *et al.*, 1992; Murray and Edgar, 2001). However, recent evidence has suggested the ICM is not a homogeneous population and the induction of PE is not simply a positionally specified event. ICM cells of

Figure 1.2

Schematic illustration of post-implantation development in the mouse

(A) Following implantation of the embryo at ~4.5 dpc, cells of the ICM lining the blastocoelic cavity have differentiated into extra-embryonic primitive endoderm and at this stage the embryo consists of three distinct lineages; trophectoderm (TE), primitive endoderm and pluripotent ICM cells. The layer of TE that overlies the ICM is known as polar TE while the cells that line the cavity are called mural TE. The primitive endoderm secretes basement membrane proteins. (B) At 4.5-5 dpc, primitive endoderm gives rise to two distinct extra-embryonic lineages, the parietal and visceral endoderm. The parietal endoderm is formed from the primitive endoderm cells that lose contact with the adjacent pluripotent cells and line the mural TE while the visceral endoderm is established from primitive endoderm that remains adjacent to the pluripotent cells and separated from them by the basement membrane. The ICM is overlayed by extra-embryonic ectoderm (EEE) and ectoplacental cone (EPC) derived from the polar TE, and the blastocyst is surrounded by trophoblast giant cells (TGC) derived from mural trophectoderm. (C) By ~5.5 dpc, the pro-amniotic cavity has formed as the solid mass of ICM cells rearranges to form a pseudostratified epithelial sheet of primitive ectoderm that lines the egg cylinder. (D) Gastrulation begins at ~6.5 dpc with the formation of the primitive streak.

Figure 1.2



A
4-4.5 dpc
B
4.5-5 dpc
C
5.5 dpc
D
6.5 dpc
polar TE
ICM
blastocoelic cavity
mural TE
basement membrane
EPC
EEE
TGC
primitive ectoderm
pro-amniotic cavity
primitive streak
Primitive endoderm
Visceral endoderm
Parietal endoderm
Pluripotent cells
Trophectoderm and derivatives

the early blastocyst express markers of the epiblast and PE in non-overlapping domains (Chazaud *et al.*, 2006). Lineage tracing experiments also showed that individual labelled ICM cells could contribute to the epiblast or PE lineage but rarely to both indicating predetermination of cell fate (Chazaud *et al.*, 2006). Single cell microarray analysis results also support the heterogeneous nature of ICM cells with individual cells having distinct epiblast-like or PE-like gene expression (Kurimoto *et al.*, 2006). Thus, formation of PE may involve the cell sorting of epiblast and primitive endoderm progenitors within the ICM population that have pre-determined developmental potential (Yamanaka *et al.*, 2006).

The basis of this heterogeneous nature of ICM cells may involve FGF mediated receptor tyrosine kinase (RTK) signalling and the expression of GATA transcription factors. Loss of a down-stream adaptor protein Grb2, that links RTK signalling to the MAPK cascade, prevents PE establishment in the null embryos (Cheng *et al.*, 1998). Mice null for FGF4 and FGF-receptor 2 also fail to form PE (Feldman *et al.*, 1995; Wilder *et al.*, 1997; Arman *et al.*, 1998). Similarly, over-expression of a dominant negative FGFR inhibits formation of PE in differentiating embryoid bodies (Li *et al.*, 2001). Over-expression of GATA6, a transcription factor normally expressed within the PE, is able to rescue this phenotype (Li *et al.*, 2004). Therefore, Grb2 mediated RTK signalling in a subset of ICM cells may recruit them to form PE, a fate decision that is reinforced by the expression of markers such as GATA6.

1.1.2 Peri- and Post-implantation development

1.1.2.1 Blastocyst implantation

Around 4.5 dpc, the combined effect of the increasing fluid volume in the blastocyst and weakening of the zona pellucida by proteolytic enzymes leads to the hatching of the blastocyst. In the mouse, implantation occurs at ~5 dpc. A number of hormones and cytokines secreted by the uterine wall are required for implantation including oestrogen, progesterone and leukaemia inhibitory factor (LIF) (Mantalenakis and Ketchel, 1966; Bhatt *et al.*, 1992). The role of LIF appears to be the initiation of uterine extracellular matrix (ECM) breakdown by the activation of ECM metalloproteinases to enable trophectoderm invasion of the uterine wall (Harvey *et al.*, 1995). During implantation, the polar trophectoderm invades the uterine epithelium and penetrates uterine stroma. The proliferation rate of the polar trophectoderm is greater than that of the mural trophectoderm and this results in the displacement of the ICM into the blastocoelic cavity (Gardner and Papaioannou, 1975). At the time of implantation, the pluripotent cells of the ICM undergo rapid proliferation and the 20-25 cells of the ICM at 4.5 dpc expand to about 660 cells at 6.5 dpc (Snow, 1977). The proliferation of the pluripotent cells leads to a reduction in size of the blastocoelic cavity while at the same time the primitive endoderm gives rise to two distinct extra-embryonic lineages, the parietal and visceral endoderm (Figure 1.2 B). The parietal endoderm is formed from the primitive endoderm cells that lose contact with the adjacent pluripotent cells due to their migration to the inner surface of the mural trophectoderm (Gardner, 1983). The visceral endoderm is established from primitive endoderm that remains adjacent the pluripotent cells and separated from them by a basement membrane previously formed by components secreted from the primitive endoderm (Gardner, 1983).

1.1.2.2 Cavitation and primitive ectoderm formation

At about 5 dpc, pro-amniotic cavity formation is initiated by a mechanism that is proposed to involve a two-signal-mediated process (Coucouvanis and Martin, 1995). A diffusible "death" signal originating from the visceral endoderm is proposed to induce the apoptosis of the pluripotent cells while a second non-diffusible signal, associated with the basement membrane, provides a survival signal thereby allowing the solid mass of ICM cells to form a pseudostratified epithelial monolayer of pluripotent primitive ectoderm that lines the egg cylinder (Figure 1.2 C, D). Cavitation was later shown to be dependent on BMP signalling (Coucouvanis and Martin, 1999). Treatment of S2 embryoid bodies (which normally fail to cavitate) with exogenous BMP2, 4 or 7 induced cavitation. The formation of visceral endoderm was shown to be dependent on BMP since only after treatment of S2 embryoid bodies with BMP was differentiated visceral endoderm present and cavitation induced. However, the importance of BMP in cavitation was not simply via induction of visceral endoderm differentiation since expression of dominant negative BMPR1b in the embryonic ectodermal cells, in the presence of differentiated visceral endoderm, prevented cavitation (Coucouvanis and Martin, 1999). This suggests BMP is acting to induce cavitation, in part by inducing the differentiation of the visceral endoderm and also via a separate effect on programmed cell death.

The formation of primitive ectoderm is accompanied by changes in gene expression, antigen presentation and developmental potential (Rathjen *et al.*, 1999; Lake *et al.*, 2000). Gene expression changes (Figure 1.3) include the down-regulation of transcription factor *Rex1* which is expressed in the ICM up to 4.5 dpc but is no longer detected following primitive ectoderm formation at 6 dpc (Rogers *et al.*, 1991), while the homeobox gene *Gbx2* is similarly present at 3.5-4.0 dpc but not at 6 dpc (Chapman *et al.*, 1997). The expression of *CRTR1* and *Psc1* is detected in 3.5 dpc ICM with the expression ceasing by 5 dpc (Pelton *et al.*, 2001). The expression of *Nanog* is detected at the compacted morula stage, maintained in the ICM of the blastocyst and down-regulated by the implantation stage (Chambers *et al.*, 2003).

The expression of *Fgf5* is detected only following primitive ectoderm formation as it is not present at 3.5 dpc but is detected between 5.25-5.5 dpc (Haub and Goldfarb, 1991). The expression of pluripotence markers *Oct4* (Scholer *et al.*, 1990), alkaline phosphatase (Hahnel *et al.*, 1990) and *uvomorulin* (Sefton *et al.*, 1992) is maintained following primitive ectoderm formation indicating that cells of the ICM and the primitive ectoderm are both pluripotent cell populations.

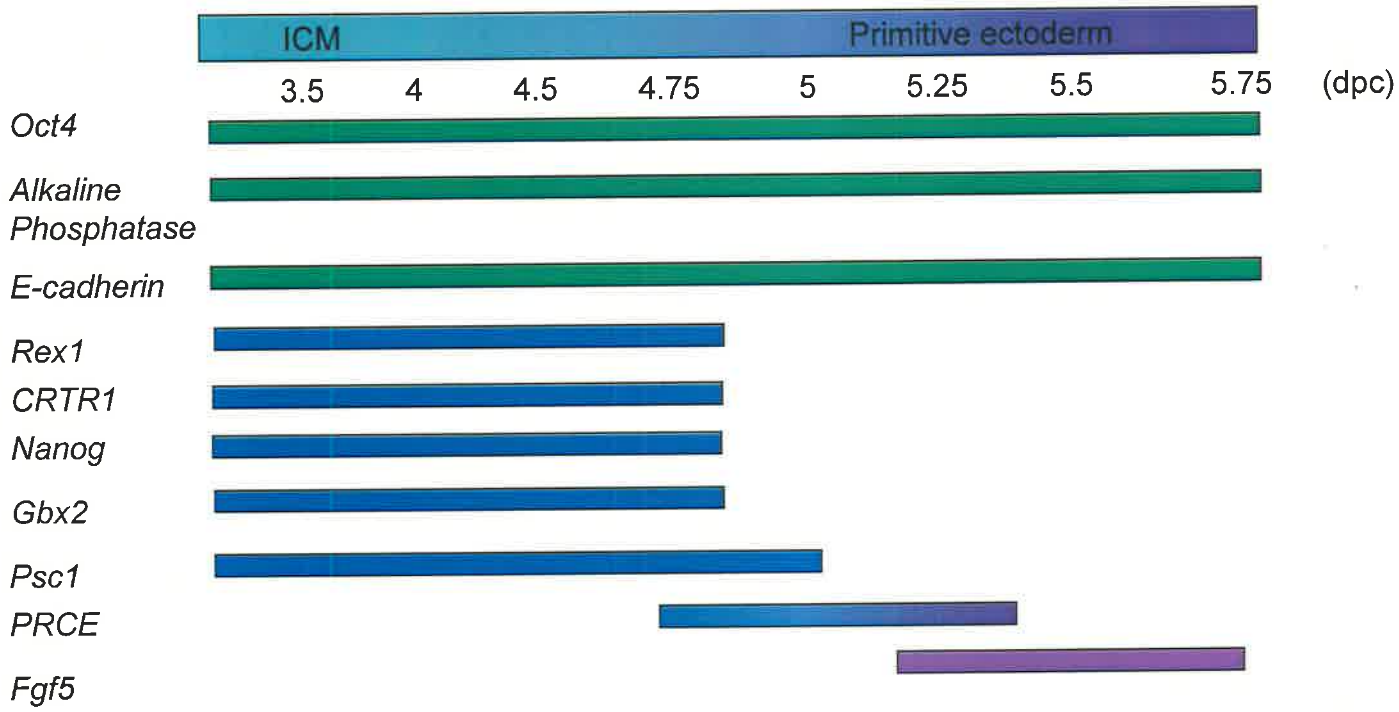
Developmental potential differences between the pluripotent cells of the ICM and the pluripotent cells of the primitive ectoderm have also been demonstrated (Rossant, 1977; Beddington, 1983). Introduction of pluripotent cells from post-cavitation embryos into host blastocysts failed to result in

Figure 1.3

Gene expression profiles of ICM and primitive ectoderm cells

Expression patterns of mRNAs differentially expressed between ICM and primitive ectoderm cells. *In vivo* expression patterns determined by Scholer *et al.*, 1990; Yeom *et al.*, 1991 (*Oct4*), Hahnel *et al.*, 1990 (alkaline phosphatase), Sefton *et al.*, 1992 (*E-cadherin*), Haub and Goldfarb, 1991; Herbert *et al.*, 1991 (*Fgf5*), Rogers *et al.*, 1991 (*Rex1*), Bulfone *et al.*, 1993; Chapman *et al.*, 1997 (*Gbx2*), Pelton *et al.*, 2000 (*Psc1, PRCE*), Pelton *et al.*, 2002 (*CRTR1*), Chambers *et al.*, 2003; Hart *et al.*, 2004 (*Nanog*).

Figure 1.3



ICM
Primitive ectoderm
3.5
4
4.5
4.75
5
5.25
5.5
5.75
(dpc)
Oct4
Alkaline Phosphatase
E-cadherin
Rex1
CRTR1
Nanog
Gbx2
Psc1
PRCE
Fgf5

chimera formation unlike cells from pre-cavitation embryos that were able to contribute to all future cell lineages when introduced into host blastocysts (Gardner, 1971).

1.1.2.3 Signalling involved in pluripotent cell progression in vivo

Establishment and maintenance of pluripotence has received considerable attention and is summarised in Section 1.4. Expression of transcripts for type I BMP receptors *activin-like receptor 3 (ALK3)* and *ALK6* and type II receptors *BMPRII*, *ActRIIA* and *ActRIIB* is detected within 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). Similarly *BMP4* is also expressed throughout the primitive ectoderm at 6.5 dpc (Winnier *et al.*, 1995). Mice null for *ActRIIA* and *ActRIIB* (Song *et al.*, 1999) or expressing mutant forms of *ALK3* (Mishina *et al.*, 1995), *ALK6* (Gu *et al.*, 1998) or *BMP4* (Winnier *et al.*, 1995; Lawson *et al.*, 1999) exhibit reduced proliferation of pluripotent cells, morphological disorganisation of the egg cylinder as well as gastrulation defects ranging from inhibited to delayed gastrulation. This implicates BMP4 signalling in regulating proliferation of the primitive ectoderm as well as developmental progression of pluripotent cells *in vivo*. Further evidence for the involvement of BMP signalling in the development of pluripotent cells comes from mice null for the BMP down-stream effector *Smad4*. *Smad4*$^{-/-}$ mice display defects in pluripotent cell proliferation, establishment of the egg cylinder and gastrulation, particularly in the induction of mesoderm, similar to those evident with mutations in BMP receptors (Yang *et al.*, 1998).

Signalling via the fibroblast growth factor (Fgf) receptors also appears to be involved in regulating pluripotent cell fate during early development. Embryos null for *Fgfr2* develop normally to the blastocyst stage but fail to survive following implantation exhibiting impaired egg cylinder development and decrease in the number of pluripotent cells (Arman *et al.*, 1998). *Fgfr1*$^{-/-}$ embryos similarly display proliferative defects with reduced cell numbers evident at 6.5 dpc, disorganisation of the egg cylinder and gastrulation defects (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994). Collectively the data suggest that Fgf mediated signalling is involved in the proliferation and differentiation of pluripotent cells.

Expression of *Fgf4* is detected at the blastocyst stage in the cells of the ICM and following implantation the expression is maintained in cells of the epiblast (Rappolee *et al.*, 1994). Homozygous mutant *Fgf4*$^{-/-}$ embryos die immediately after implantation due to the inability of the pluripotent cells to survive (Feldman *et al.*, 1995). The requirement of *Fgf4* appears to be in part indirect as its expression is required for the formation of extraembryonic ectoderm from polar trophoblast cells, with *Fgf4*$^{-/-}$ and *FgfR2*$^{-/-}$ mutants unable to form this cell type (Feldman *et al.*, 1995; Arman *et al.*, 1998). Fgf4 has therefore been implicated in the signalling between the pluripotent cells of the ICM and polar trophectoderm to mediate the formation of extraembryonic ectoderm. *Fgf4* is also required for the formation of the egg cylinder as *Fgf4*$^{-/-}$ embryos do not form primitive ectoderm following implantation nor do cells from the ICM proliferate *in vitro* unless Fgf4 is added exogenously (Feldman *et al.*, 1995). This implicates Fgf4 as an autocrine or paracrine factor that facilitates the survival and proliferation of the pluripotent cells.

1.1.2.4 Role of visceral endoderm in induction of primitive ectoderm

The transition of ICM cells to primitive ectoderm appears to require signals from the visceral endoderm (Spyropoulos and Capecchi, 1994; Duncan *et al.*, 1997; Koutsourakis *et al.*, 1999). The homeobox gene *Evx1* is expressed throughout the visceral endoderm prior to its expression in the primitive ectoderm at ~6.25 dpc (Spyropoulos and Capecchi, 1994). *Evx1*$^{-/-}$ embryos show deterioration of the pluripotent and extra-embryonic cells by 5.0 dpc. The mutant embryos were able to undergo implantation but showed inhibited growth and differentiation and were resorbed by 6.5 dpc. The impaired development is believed to result from the interruption of the communication between visceral endoderm and the pluripotent cells, even though a direct role for *Evx1* has not been established (Spyropoulos and Capecchi, 1994).

Further support for the role of visceral endoderm in the formation of primitive ectoderm was provided by other gene deletion experiments. Deletion of *Hnf4*, a transcription factor expressed in the primitive ectoderm and visceral endoderm between 4.5 and 7.5 dpc, resulted in embryos with increased apoptosis within the primitive ectoderm by 6.5 dpc, delayed gastrulation and death around 9.5-10.5 dpc (Chen *et al.*, 1994). The *Hnf4*$^{-/-}$ phenotype was rescued following formation of tetraploid chimeras with *Hnf4*$^{-/-}$ embryos and *Hnf4*$^{+/+}$ visceral endoderm (Duncan *et al.*, 1997). This suggests that the visceral endoderm may be the source of signals required for the correct development of the primitive ectoderm.

A phenotype similar to that seen with *Hnf4*$^{-/-}$ embryos was observed following targeted disruption of the transcription factor *GATA6* that is expressed within the visceral endoderm from 6.5 dpc. *GATA6*$^{-/-}$ embryos demonstrated lethality early during embryonic development due to death of the primitive ectoderm cells (Koutsourakis *et al.*, 1999). The defect appears to result from impaired differentiation of the visceral endoderm demonstrated by low expression of visceral endoderm markers *Hnf4* and *GATA4* at 6.5 dpc (Morrisey *et al.*, 1998).

1.1.2.5 Involvement of ECM in epithelialization of the primitive ectoderm

Visceral endoderm and the extracellular matrix (ECM) separating the visceral endoderm from the pluripotent cells appear to provide additional signals that regulate the epithelial phenotype associated with primitive ectoderm formation.

The cells of the primitive endoderm express many ECM components leading to the deposition of a basement membrane that separates them from the pluripotent cells of the ICM. Laminin is a key component of ECM during early embryogenesis: knockouts of the *LAMC1* gene, encoding the laminin-γ1 subunit, result in early embryonic lethality due to the inability to establish the basement membrane (Smyth *et al.*, 1999). The presence of this basement membrane *in vivo* has in turn been associated with the establishment of epithelialization of the pluripotent cells in embryoid bodies (EBs) made from ES cells in suspension culture (Murray and Edgar, 2000; Li *et al.*, 2001). During EB differentiation, the requirement for the presence of an outer layer of endoderm in epithelialization could be overcome by exogenous addition of laminin (Li *et al.*, 2001). Furthermore, The inability to establish a basement membrane was rescued in EBs derived from *$LAMC1^{-/-}$* ES cells through the addition of exogenous laminin (Murray and Edgar, 2000). The molecular mechanism by which laminin induces the formation of the epithelial layer was shown to be independent of two putative laminin receptors β1-integrin or α-dystroglycan. Studies with recombinant proteins established that a heparin-binding domain of laminin was required, thus implicating the involvement of heparin sulphate proteoglycan in this process (Li *et al.*, 2001).

However, even though epithelialization was shown to be dependent on the presence of a basement membrane, the differentiation of the cells to a primitive ectoderm fate was not induced as their gene expression profile was consistent with that of ES cells and not primitive ectoderm (Li *et al.*, 2001). Thus, there appears to be an uncoupling between the morphology and the differentiation of the cells.

1.1.2.6 Gastrulation of the mouse embryo

Primitive ectoderm is an obligate developmental intermediate from which the three germ layers ectoderm, endoderm and mesoderm are derived during gastrulation (Pelton *et al.*, 1998). The process of gastrulation begins at ~6.5 dpc, with the formation of the primitive streak (Figure 1.4). The primitive

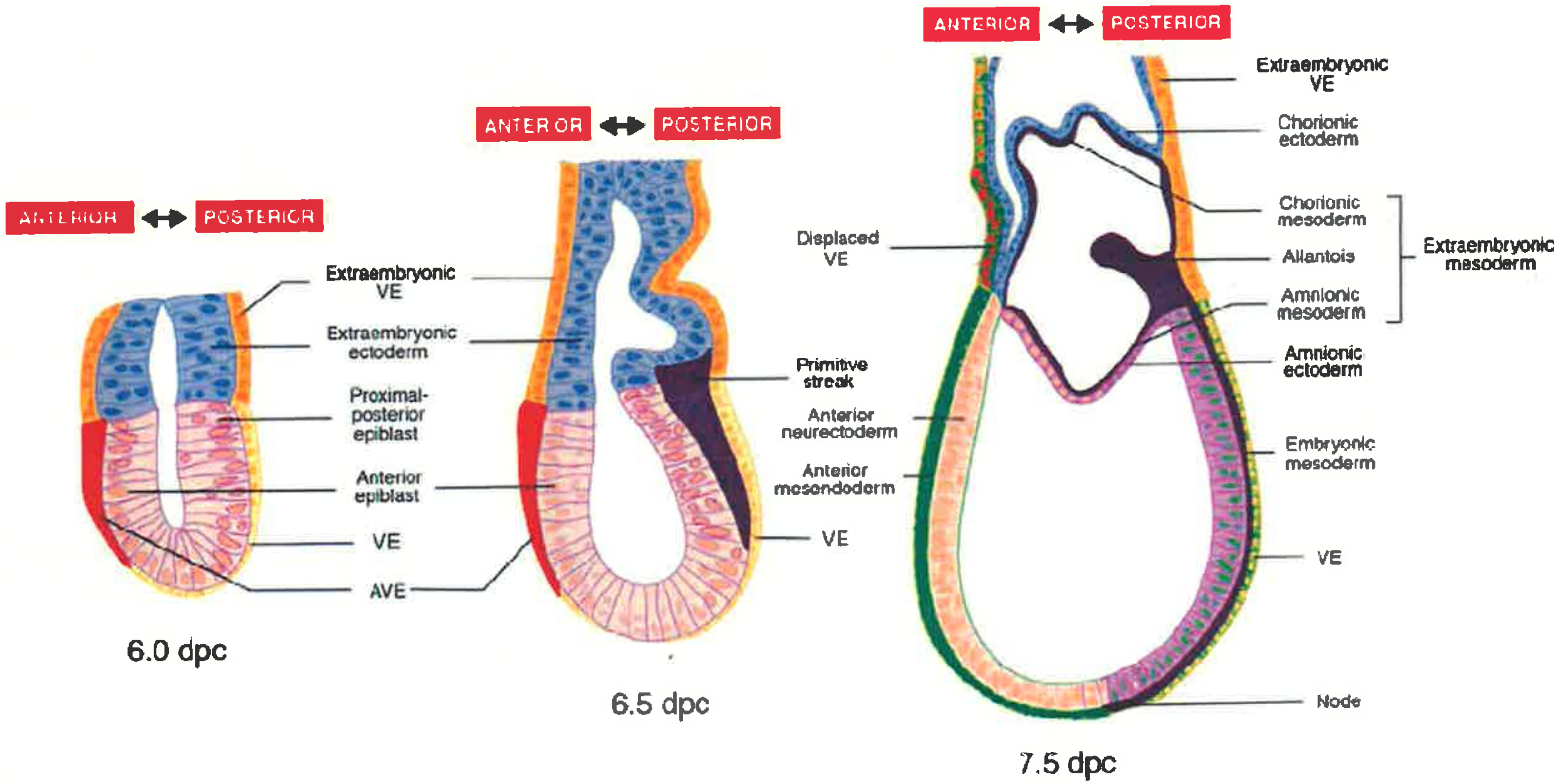
Figure 1.4

Illustration of cell lineages formed during gastrulation

The process of gastrulation begins at ~6.5 dpc, with the formation of the primitive streak. The primitive streak is first observed on the proximal posterior side of the embryo where there is thickening of the proximal extra-embryonic/embryonic junction. The cells located within the streak region undergo an epithelial-to-mesenchyme transition and as gastrulation progresses the streak extends distally. At 7.5 dpc, the most distal tip of the streak is defined as the node. The inductive environment surrounding the node results in formation of both mesoderm and endoderm while the inductive environment in more proximal regions of the streak lead to induction of mesoderm. Extra-embryonic mesoderm is derived from the nascent mesoderm that migrates across the extra-embryonic ectoderm/primitive ectoderm boundary while the definitive endoderm is established from the cells that traverse the streak and incorporate within the visceral endoderm progressively displacing the visceral endoderm anteriorly and proximally. Extra-embryonic, lateral, paraxial and axial mesoderm are established sequentially from progressively more distal regions of the streak. Primitive ectoderm located in the anterior region and remaining in contact with the underlying visceral endoderm during early stages of gastrulation gives rise to neurectoderm and surface ectoderm.

Reproduced from Baron *et al.*, 2005.

Figure 1.4



ANTERIOR
POSTERIOR
Extraembryonic VE
Extraembryonic ectoderm
Proximal-posterior epiblast
Anterior epiblast
VE
AVE
6.0 dpc
ANTERIOR
POSTERIOR
6.5 dpc
Displaced VE
Primitive streak
Anterior neurectoderm
Anterior mesendoderm
VE
ANTERIOR
POSTERIOR
Extraembryonic VE
Chorionic ectoderm
Chorionic mesoderm
Allantois
Amnionic mesoderm
Extraembryonic mesoderm
Amnionic ectoderm
Embryonic mesoderm
VE
Node
7.5 dpc

streak is first observed on the proximal posterior side of the embryo where there is thickening of the proximal extra-embryonic/embryonic junction, and is characterised by the localised breakdown of extracellular matrix, expression of Wnt3a and nuclear localisation of β-catenin (Hogan *et al.*, 1994; Liu *et al.*, 1999; Mohamed *et al.*, 2004). The cells located within the streak region undergo an epithelial-to-mesenchyme transition and as gastrulation progresses the streak extends distally reaching the distal tip of the embryo by 7.5 dpc. At 7.5 dpc, the most distal tip of the streak is defined as the node, a structure equivalent to the organiser in other vertebrate embryos (Beddington and Robertson, 1999). The nascent mesoderm emerging from the primitive streak migrates across the extra-embryonic ectoderm/primitive ectoderm boundary to form the extra-embryonic mesoderm (Parameswaran and Tam, 1995). On the other hand, the definitive endoderm is established from the cells that transverse the streak and incorporate within the visceral endoderm progressively displacing the visceral endoderm anteriorly and proximally (Tam *et al.*, 1993; Hogan *et al.*, 1994). Studies in Xenopus lavais and Danio rerio suggest the cell population formed within the streak is a precursor for both the mesodermal and endodermal lineages termed mesendoderm (Rodaway and Patient, 2001). Similarly, a mesendoderm appears to be present in the mouse embryo as loss of Mixl1 affects the differentiation into mesodermal and endodermal cells (Hart *et al.*, 2002). The induction of this mesendodermal precursor to the mesoderm or endoderm lineages appears to be controlled by different inductive environments. The inductive environment surrounding the node results in formation of both mesoderm and endoderm while the inductive environment in more proximal regions of the streak lead to induction of mesoderm. The type of mesoderm formed is dependent on where and when the cells exit the streak (Tam and Beddington, 1992). Extra-embryonic, lateral, paraxial and axial mesoderm are established sequentially from progressively more distal regions of the streak. Embryonic mesoderm cells further differentiate to give rise to haematopoietic lineages, muscle, vertebrae, dermis and kidney during development. Derivatives induced by the node include axial mesoderm that will populate the midline of the embryo give rise to the notochord and head processes and definitive endoderm (Tam and Beddington, 1987; Lawson *et al.*, 1991; Beddington and Robertson, 1999). Primitive ectoderm located in the anterior region and remaining in contact with the underlying visceral endoderm during early stages of gastrulation give rise to neurectoderm and surface ectoderm (Quinlan *et al.*, 1995; Tam, 1989). Signals emanating from the anterior visceral endoderm (AVE) appear to be involved in specifying fate of the ectodermal lineage (Thomas and Beddington, 1996; Bielinska *et al.*, 1999).

1.1.2.7 Establishment of polarity during mouse development

The establishment of polarity within the embryo determines the body plan of the developing animal. The first patterning is observed within the oocyte with the second meiotic division and the extrusion of the polar body, which remains tethered during development, marking the animal pole and thus establishing the animal-vegetal axis (Figure 1.5 A) (Zernicka-Goetz, 2002). The second factor contributing to the establishment of polarity within the embryo is postulated to be the site of sperm entry during fertilization (Piotrowska and Zernicka-Goetz, 2001; Hiiragi and Solter, 2004). The first cleavage plane

Figure 1.5

Establishment of polarity during early mouse embryogenesis

(A) Pre-implantation development of a mouse embryo to the blastocyst stage. (1) Fertilized egg with the male (blue star) and female (pink star) pro-nuclei marked and the extruded polar body marking the animal pole. (2) Two cell embryo with the first cleavage plane determined by the location of the polar body. (3) Eight cell embryo. (4) Blastocyst-stage embryo. The blue line marks the embryonic-abembryonic axis with the ICM located at the embryonic and blastocoelic cavity at the abembryonic pole. The yellow line marks the animal-vegetal axis. (B) Schematic diagram of mouse embryo at the blastocyst and primitive ectoderm stage illustrating the tilting of the polar trophectoderm and the ectoplacental cone respectively. A, anterior; P, posterior; Ab, abembryonic; Em, embryonic. Reproduced from Zernicka-Goetz, 2002. (C) Schematic illustration of the movement of anterior visceral endoderm gene expression patterns (red) and posterior gene expression patterns (blue) during the establishment of the anterior-posterior axis at 5.5-6.5 dpc. The posterior inductive signals emanating from the extra-embryonic ectoderm are indicated (orange arrows). Reproduced from Zernicka-Goetz, 2002.

Fig. 1.5 B

ICM – pink

Blastocoelic cavity – light blue

Primitive ectoderm – dark blue

Ectoplacental cone – green

Figure 1.5

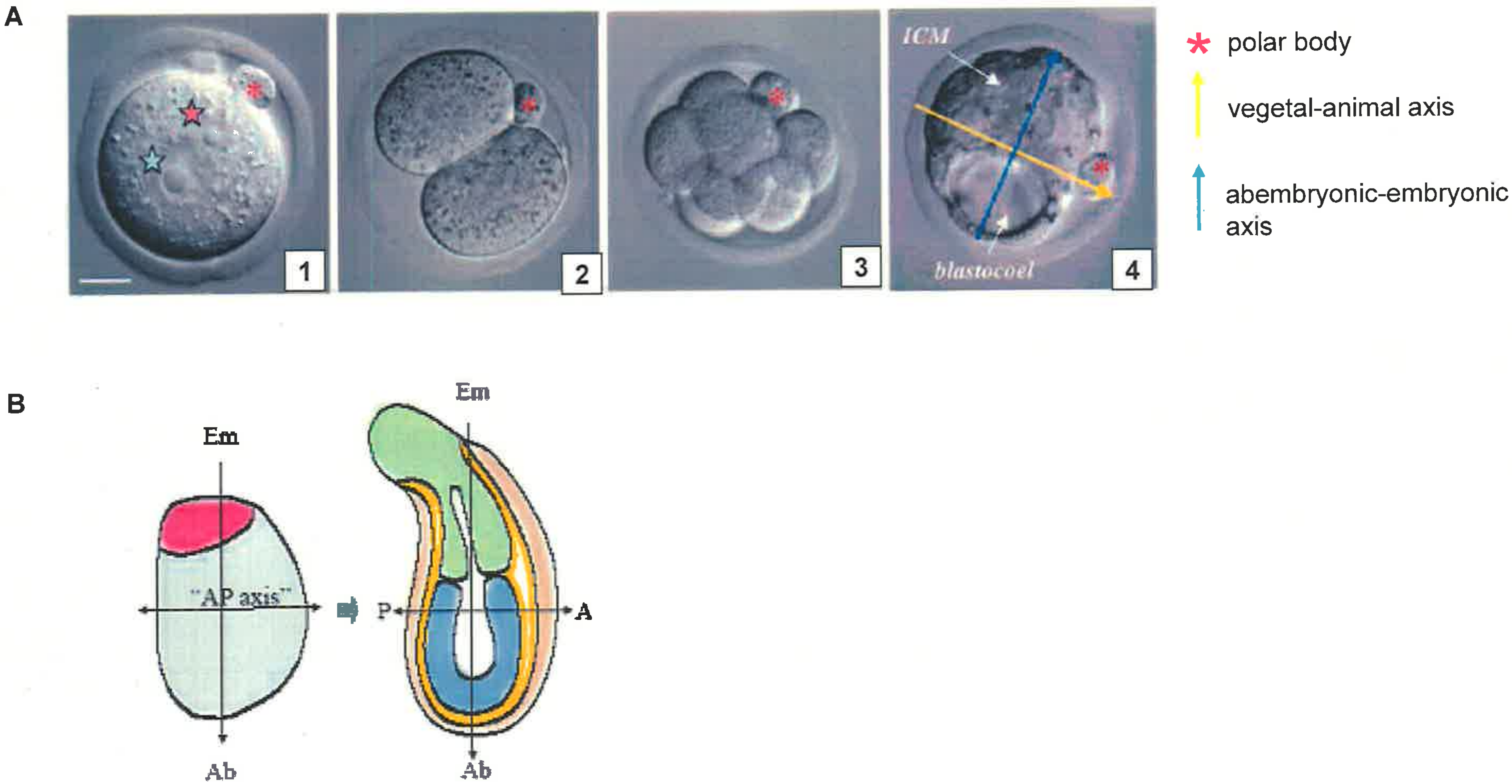
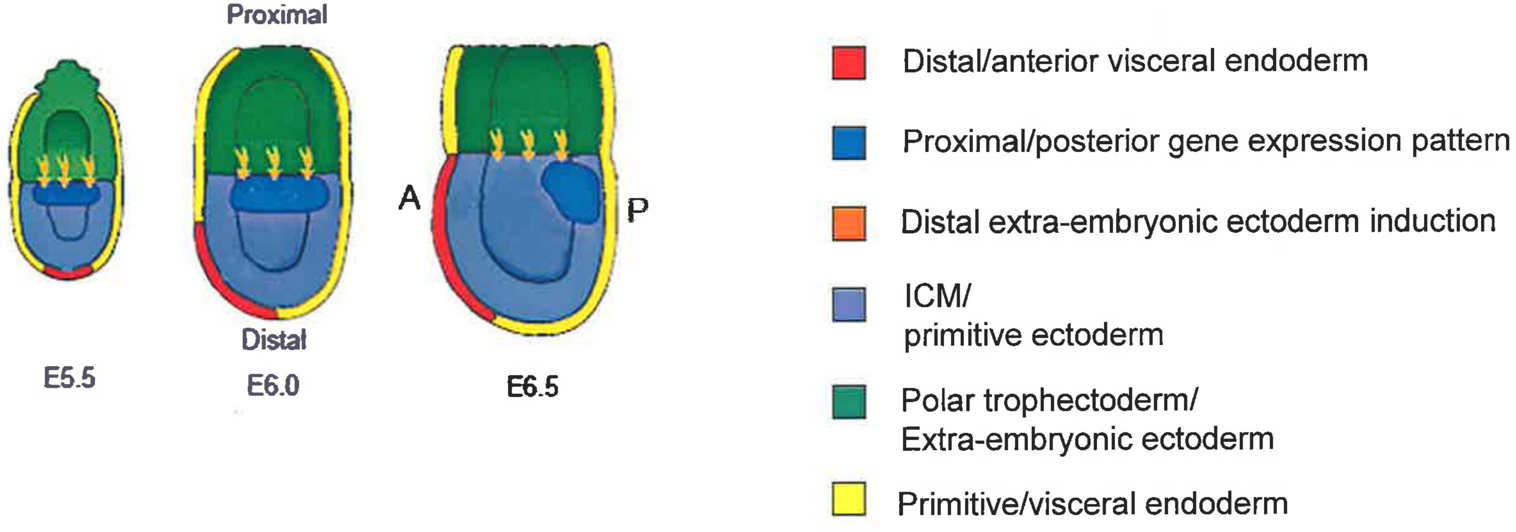

A
ICM
blastocoel
1
2
3
4
polar body
vegetal-animal axis
abembryonic-embryonic
axis
B
Em
"AP axis"
Ab
Em
P
A
Ab

Figure 1.5

C


Proximal
Distal
A
P
E5.5
E6.0
E6.5
Distal/anterior visceral endoderm
Proximal/posterior gene expression pattern
Distal extra-embryonic ectoderm induction
ICM/
primitive ectoderm
Polar trophectoderm/
Extra-embryonic ectoderm
Primitive/visceral endoderm

appears to be determined by the location of the polar body and the position of sperm entry. Following the first cleavage, the two resulting blastomeres exhibit asynchronous division with the earlier dividing blastomere being the one inheriting the sperm entry site (Piotrowska and Zernicka-Goetz, 2001). However, the work of Gardner and Davies contradicts these findings suggesting that the first cleavage plane is not dictated by the site of sperm entry and position of polar body but rather that it aligns to the plane of bilateral symmetry of the zygote (Gardner and Davies, 2003; 2006). The orientation of the first cleavage plane also determines the embryonic-abembryonic axis in the blastocyst (Figure 1.5 A, B) (Gardner, 2001). The embryonic-abembryonic axis is evident within the blastocyst with the ICM located at the embryonic pole and blastocoelic cavity at the abembryonic pole. At this time, the embryo is considered to be bilaterally symmetrical (Gardner, 1997). However, the location of the ICM is slightly tilted with respect to the embryonic-abembryonic axis (Figure 1.5 B). Following formation of the primitive ectoderm, the embryo has a proximal-distal axis, which is aligned with the previous embryonic-abembryonic axis. Again, slight asymmetry is evident with the ectoplacental cone being tilted with respect to the proximal-distal axis (Zernicka-Goetz, 2001). The direction of the tilt does not correlate with the anterior or posterior region of the embryo (Gardner *et al.*, 1992).

Cell-lineage tracing studies have shown that as the primitive ectoderm is formed the visceral endoderm cells from the animal pole move distally with growth of the egg cylinder while the cells from the vegetal pole move to more proximal positions (Weber *et al.*, 1999). Therefore, asymmetric distribution of visceral endoderm cells precedes gastrulation and appears to be involved in the determination of the anterior-posterior axis. Asymmetric distribution of visceral endoderm cells is also accompanied by changing gene expression patterns within the visceral endoderm. The expression of *Hex* in the visceral endoderm shifts from the distal tip (5.5 dpc) to the anterior region (6.5 dpc) due to the active unilateral migration of the AVE cells (Thomas *et al.*, 1998; Srinivas *et al.*, 2004). *Hex* is one of a group of genes initially expressed at the distal tip and later at the anterior pole of the embryo within a region known as the anterior visceral endoderm (AVE) (Figure 1.5 C). The anterior visceral endoderm is crucial for the development of anterior structures as loss of the AVE resulted in embryos that lack forebrain (Thomas and Beddington, 1996). This suggests that the AVE is involved in specification of the anterior region of the embryo and possibly in the set up of the anterior-posterior axis. Posterior character of the embryo is induced proximally by BMP4 expressed by the extra-embryonic ectoderm (Figure 1.5 C) (Lawson *et al.*, 1999). Concomitant with the movement of the anterior-associated gene expression from the distal to anterior region, the posterior-associated gene expression moves from the proximal region to the posterior of the embryo (Zerincka-Goetz, 2004).

1.2 EMBRYONIC STEM CELLS AND THEIR DERIVATIVES

1.2.1 Mouse ES-cell derivation and properties

Embryonic stem (ES) cells are derived from the ICM of the pre-implantation mouse blastocyst (4-4.5 dpc) and can be propagated indefinitely in culture (Martin, 1981; Evans and Kaufman, 1981). Mouse ES cells have been readily isolated from 129 strain and less frequently from C57BL/6 strain blastocysts (Rathjen and Rathjen, 2001). Most of the established ES cell lines are male (XY) since the female (XX) karyotype appears unstable with one of the X chromosomes frequently lost (Rastan and Robertson, 1985). Isolation of ES cells was originally achieved by plating expanded blastocyst-stage embryos, either intact or following microsurgical isolation of the ICM, onto inactivated feeder cells in medium supplemented with β-mecaptoethanol and foetal calf serum. Following culture for several days, the ICM-derived cells were disaggregated and replated onto fresh feeders. Culture of the disaggregated cells leads to propagation of some colonies with undifferentiated morphology, which can be isolated and replated to establish ES-cell lines (Robertson, 1987).

Subsequently it was found that conditioned medium from feeder cells could also support the self-renewal of ES cells in the absence of feeders (Smith and Hooper, 1987). The bioactive factor was later identified as LIF, an agonist of gp130 signalling (Smith *et al.*, 1988; Williams *et al.*, 1988; Nichols *et al.*, 1990; Yoshida *et al.*, 1994). Self-renewal can also be maintained by culture in the presence of other IL-6 family members including oncostatin M (Gearing and Bruce, 1992), ciliary neurotrophic factor (Conover *et al.*, 1993) and cardiotrophin-1 (Pennica *et al.*, 1995).

ES cells have been shown to express ICM-specific markers including *Rex1*, *CRTR1*, *Gbx2* and *Nanog* (Rogers *et al.*, 1991; Chapman *et al.*, 1997; Pelton *et al.*, 2002; Chambers *et al.*, 2003). ES cells in culture also remain pluripotent and express markers of pluripotence such as alkaline phosphatase (Hahnel *et al.*, 1990) and *Oct4* (Scholer *et al.*, 1990). The pluripotence of ES cells has been demonstrated by their ability to contribute to all cell types of the embryo including the germ line following injection into the blastocyst (Gardner and Rossant, 1979; Beddington and Robertson, 1989). ES cells give rise to teratocarcinomas containing cells derived from mesoderm, ectoderm and endoderm following injection under the kidney capsule or into the brain (Evans and Kaufman, 1983). Pluripotence has also been demonstrated *in vitro* since ES cells in culture can differentiate into cell types derived from all three-germ lineages (Smith, 2001; Gadue *et al.*, 2005).

1.2.2 Human ES cells

Human ES cell (hES) lines have also been isolated from *in vitro* fertilised pre-implantation embryos (Thomson *et al.*, 1998; Pera *et al.*, 2000; Reubinoff *et al.*, 2000) or *in vitro* cultured blastocysts (Stojkovic *et al.*, 2004). Fundamental characteristics of mouse ES cells are also shared by hES cells; including expression of the pluripotence markers *Oct4* and *alkaline phosphatase*, ICM markers *Nanog*, *Rex1*, *Sox2*, telomerase activity and formation of teratomas in immunodeficient mice (Thomson *et al.*, 1998; Richards *et al.*, 2002; Henderson *et al.*, 2002; Bhattacharya *et al.*, 2004; Ginis *et al.*, 2004). Human ES cells also proliferate and maintain their karyotype in prolonged continuous culture (Amit *et al.*, 2000). Differences observed between mES cells and hES cells include expression of cell surface markers such as TRA-1-60, TRA1-81, TRA-2-49, TRA2-54, GCTM-2, SSEA-3 and SSEA-4 (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Pera *et al.*, 2000;

Draper *et al.*, 2002; Henderson *et al.*, 2002). Human ES cells also exhibit slower proliferation with the doubling time of mES cells and hES cells being 12-15 h and 30-35 h respectively (Amit *et al.*, 2000). Morphological differences can also be noted with mES colonies growing as tight domed clusters and hES cells exhibiting less compact aggregation. Differentiation via embryoid body formation leads to more cystic bodies in the case of hES cells (Thomson *et al.*, 1998).

Molecular differences in maintenance of self-renewal in culture are also observed between mES cells and hES cells. LIF signalling mediated by the gp130 receptor is sufficient for maintaining the undifferentiated state of mES cells but not hES cells in culture (Smith *et al.*, 1988; Williams *et al.*, 1988; Yoshida *et al.*, 1994; Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Although the LIF/STAT3 pathway is present and can be activated in hES cells it is not sufficient for maintaining pluripotence of the cells (Daheron *et al.*, 2004). The identity of the signal/s provided by murine embryonic fibroblast (MEF) feeder layers that maintain hES cells in a self-renewing state remain poorly defined although signalling via TGFβ/Activin/Nodal, Fgf and Wnt pathways have been implicated (Section 1.4.5).

1.2.3 In vitro differentiation of mES cells

ES cells can be differentiated *in vitro* by the withdrawal of LIF, by induction with chemical agents such as retinoic acid (Smith, 1991), through the formation of embryoid bodies (EBs) (Doetschman *et al.*, 1985) or by lineage-specific differentiation protocols (Rathjen *et al.*, 2002; Pelton *et al.*, 2002; Ying *et al.*, 2003). Differentiation of ES cells via LIF withdrawal is spontaneous and leads to the generation of a variety of cell lineages at various stages of terminal differentiation alongside a number of residual ES-cell colonies (Rathjen *et al.*, 1990; Smith, 2001). Chemical induction of differentiation with methoxybenzamide produces cells with epithelial-like morphology while retinoic acid treatment induces fibroblast-like cells and neurons (Smith, 2001; Aouadi *et al.*, 2006). Chemical induction results in the formation of only a small number of cell types and the biological relevance of these cell types is not known. However, retinoic acid-induced differentiation of ES cells has provided an *in vitro* method for delineating some questions regarding molecular mechanisms of differentiation. Retinoic acid induced differentiation of ES cells into neurons and inhibited cardiomyogenesis, a process that was shown to be coupled to the activity of p38 mitogen-activated protein kinase (MAPK) (Aouadi *et al.*, 2006): Activity of p38 MAPK kinase acted as a switch between differentiation to cardiomyocytes (p38 on) and neurons (p38 off) (Aouadi *et al.*, 2006). The retinoic acid-induced differentiation system was also used to determine the involvement of the orphan nuclear receptor GCNF in the repression of pluripotency genes *Oct4*, *Nanog*, *Sox2* and *Fgf4* (Gu *et al.*, 2005). *GCNF*$^{-/-}$ ES cells, unlike wild-type ES cells, were not able to repress the expression of pluripotency genes following retinoic acid treatment (Gu *et al.*, 2005).

Differentiation of ES cells through the formation of EBs enables the formation of cell types arising from all three germ layers. EBs are formed by the aggregation of ES cells in suspension in media lacking LIF. The differentiation of EBs appears initially to follow the temporal differentiation of the early mouse embryo but with little or no spatial organization (Doetschman *et al.*, 1985; Robertson, 1987; Rathjen and Rathjen, 2000). Following formation of aggregates, the cells on the outside of the body differentiate into primitive endoderm, which after a few days further differentiates to visceral and parietal endoderm. The remaining cells undergo a developmental program involving proliferation, differentiation and apoptosis of the centrally located cells leading to the formation of a monolayer of a columnar epithelium adjacent to the outer endoderm layer and surrounding a central cavity. This is followed by progressive differentiation and loss of pluripotence, as evidenced initially by the loss of *Oct4* expression and the appearance of mesoderm markers like *Brachyury* and *Goosecoid* followed by formation of terminally differentiated cell types derived from all three germ layers (Doetschman *et al.*, 1985; Hamazaki *et al.*, 2001; Keller *et al.*, 1993; Lumelsky *et al.*, 2001; Strubing *et al.*, 1995; Wilkinson *et al.*, 1990).

The use of modified EB differentiation protocols has facilitated the formation of populations of differentiated cell types belonging to all three primary germ layers including neural lineages (Strubing *et al.*, 1995; Kawasaki *et al.*, 2000; Lee *et al.*, 2000; Wichterle *et al.*, 2002), hematopoietic cells (Wiles and Keller, 1991; Nakano *et al.*, 1996; Nishikawa *et al.*, 1998) and endodermal lineages (Hamazaki *et al.*, 2001; Lumelski *et al.*, 2001; Hori *et al.*, 2002).

ES cells differentiated via modified EB protocols into glial precursors were shown to differentiate into myelinating oligodendrocytes and astrocytes *in vivo*

Therefore, EB-based differentiation protocols have various limitations in part as the formation of primitive ectoderm is accompanied by the formation of visceral endoderm.

following transplantation into the spinal cord of 1-week-old myelin-deficient rats, an animal model for the hereditary human myelin disorder Pelizaeus-Merzbacher disease (Brustle *et al.*, 1999). ES cells differentiated into pancreatic islet-like cells were grafted subcutaneously in the shoulder of diabetic mice. The implanted cells vascularized, remained immunoreactive to insulin and formed aggregates morphologically similar to normal pancreatic islets (Lumelsky *et al.*, 2001). This demonstrates that ES-cell derived differentiated progeny have the potential to serve a functional role *in vivo*. However, the formation of differentiated cells types via EBs does not occur synchronously or homogeneously but rather occurs in the presence of contaminating cell types which can result in inappropriate signalling and cell-cell interactions (Rathjen and Rathjen, 2000; Smith, 2001). Therefore, EB-based differentiation protocols have various limitations in part since formation of visceral endoderm accompanies formation of the obligatory intermediate pluripotent cell population primitive ectoderm from ES cells. Visceral endoderm is a cell population implicated as a source of inductive signals that regulate subsequent cell specification (Spyropoulos and Capecchi, 1994; Duncan *et al.*, 1997; Koutsourakis *et al.*, 1999). The physiological relevance of the cells produced may also pose a problem since EB differentiation is inherently disorganised and doesn't involve synchronous generation of temporal intermediates and progenitors in a manner recapitulating embryogenesis. The presence of multiple cell populations and complex interplay of signalling involved in EB differentiation makes the study of signalling and the determination of molecular mechanisms difficult.

1.2.4 In vitro model of early embryogenesis – Homogeneous formation of early primitive ectoderm-like cells from ES cells

During EB differentiation, the formation of primitive ectoderm arises as a result of spontaneous differentiation, is transient, and exists within the EB along with other cell types including extraembryonic endodermal lineages (Smith, 2001). Therefore, the differentiation environment within EBs is complex and poorly organised. ES cells can be converted into a homogenous population of early primitive ectoderm-like (EPL) cells when cultured in the presence of a conditioned medium, MEDII, derived from the hepatocellular carcinoma cell line HepG2 (Figure 1.6 A) (Rathjen *et al.*, 1999). Just as ES cells represent an *in vitro* equivalent of ICM cells, EPL cells have been shown to be an *in vitro* equivalent of primitive ectoderm cells of the 5.5 dpc embryo as shown by morphology, gene expression and differentiation potential (Rathejn *et al.*, 1999; Section 1.2.3.1-1.2.3.4).

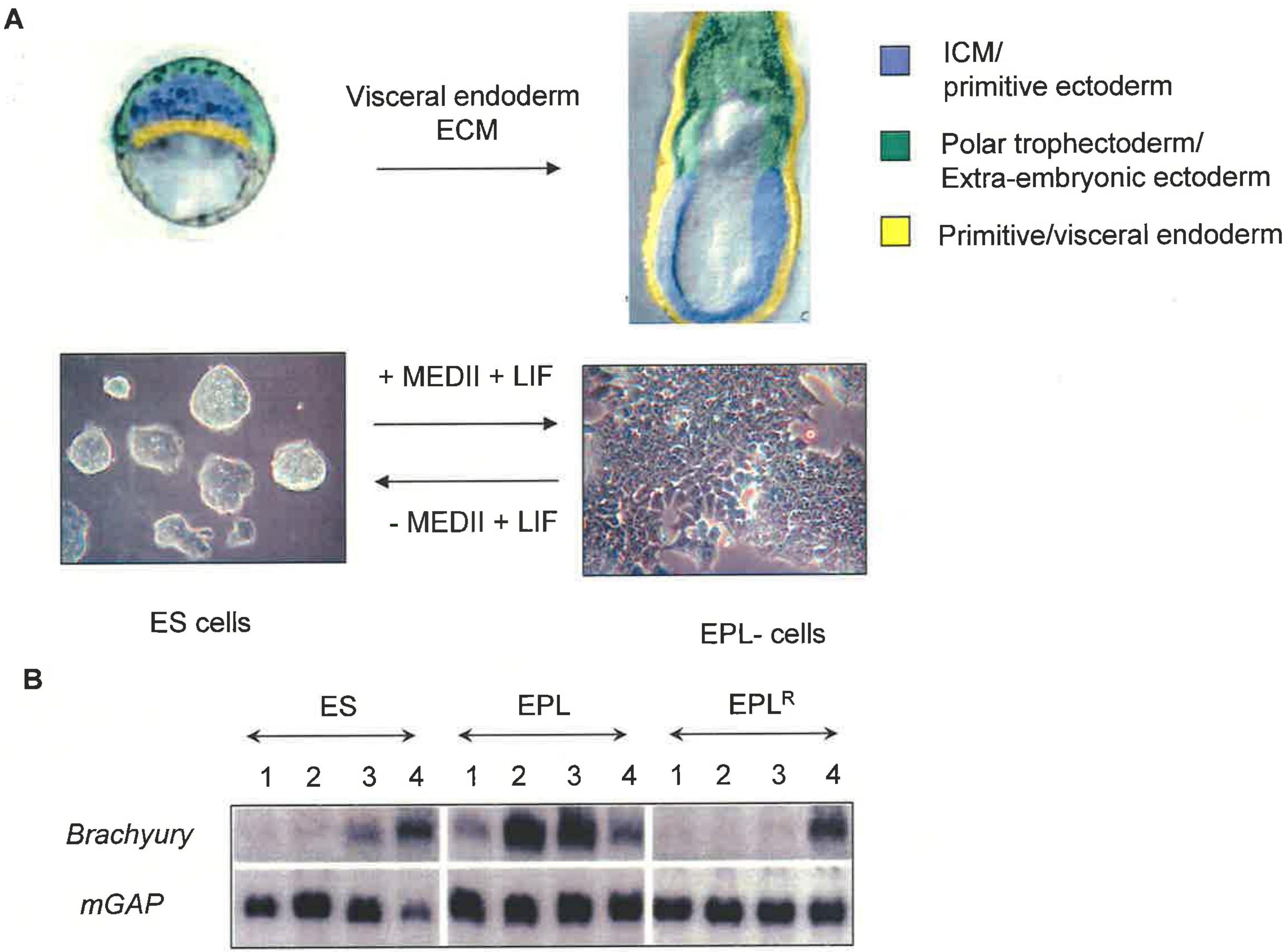
Maintenance of EPL cells requires the continuous presence of MEDII. Removal of MEDII and LIF following EPL-cell formation leads to differentiation of the cells and formation of cell types derived from all three germ layers (Lake *et al.*, 2000; Rathjen *et al.*, 2001; Rathjen *et al.*, 2002). Removal of MEDII in the presence of LIF leads to reversion of the EPL cells back to an ES-cell state (Figure 1.6 B) (Rathjen *et al.*, 1999; Lake *et al.*, 2000). This reversion was demonstrated by morphology, gene expression and differentiation potential *in vitro* and the ability of the reverted cells to contribute to all tissues of the embryo following introduction into mouse blastocysts (Rathjen *et al.*, 1999; Lake *et al.*, 2000). EPL cells, like cells of the primitive

Figure 1.6

Transition from ES to EPL cells: In vitro model of ICM-to-primitive ectoderm conversion

(A) Illustration of ICM-to-primitive ectoderm and ES-to-EPL cell conversion. *In vivo* embryo images reproduced from Zernicka-Goetz, 2002. The ES-to-EPL cell transition can be induced by the addition of the conditioned medium MEDII in the presence of LIF. ES cells are the *in vitro* equivalent of the ICM and EPL cells are the *in vitro* equivalent of the primitive ectoderm. The transition is uniform and homogeneous and results in the formation of a morphologically distinct cell type. (B) Production of EPL cells requires the continuous presence of inductive factors within MEDII. Withdrawal of MEDII in the presence of LIF leads to reversion of EPL cells to an ES-cell state. The figure is of a Northern blot of embryoid bodies derived from ES cells, EPL cells or reverted EPL cells (EPL^R) on days 1-4 of differentiation. Blots were probed for expression of nascent mesoderm marker *Brachyury*. *GAPDH* was used as a loading control. Reproduced from Lake *et al.*, 2000.

Figure 1.6


A
Visceral endoderm
ECM
ICM/
primitive ectoderm
Polar trophectoderm/
Extra-embryonic ectoderm
Primitive/visceral endoderm
+ MEDII + LIF
- MEDII + LIF
ES cells
EPL- cells
B
ES
EPL
EPL^R
1 2 3 4 1 2 3 4 1 2 3 4
Brachyury
mGAP

ectoderm, are unable to form chimeras following introduction into a host blastocyst (Gardner, 1971; Rossant, 1977; Beddington, 1983; Rathjen *et al.*, 1999). EPL-cell formation from ES cells is homogeneous thus providing a population that is suited for directed differentiation and the study of molecular mechanisms involved in differentiation (Section 1.2.3.3).

Signals from the visceral endoderm are important in the formation of primitive ectoderm *in vivo* (Spyropoulos and Capecchi, 1994; Duncan *et al.*, 1997; Koutsourakis *et al.*, 1999; Section 1.1.2.3). The formation of EPL cells in response to MEDII is likely of embryological relevance since hepatocytes and visceral endoderm, although having distinct embryological origin, exhibit similar gene expression profiles (Meehan *et al*, 1984). Consistent with this, conditioned medium from a visceral endoderm-like cell line, END2, is also able to induce the ES-to-EPL cell transition (Bettess, 2001). Formation of EPL cells in response to MEDII suggests functional similarity between visceral endoderm, and its primitive ectoderm inducing action *in vivo,* and MEDII and its induction of EPL cells *in vitro*.

1.2.4.1 EPL-cell morphology

In vivo, cells of the ICM and the primitive ectoderm can be distinguished on the basis of morphology and the same is true for ES and EPL cells *in vitro*. Morphology changes associated with the mouse ES-to-EPL transition include a change from domed-shaped ES colonies to a monolayer with clearly discernable cells containing visible nuclei and nucleoli (Figure 1.6 A). EPL cells are morphologically similar to P19 embryonal carcinoma cells (McBurney and Rogers, 1982), which are reported to be similar to the cells of the post-implantation primitive ectoderm (Rogers *et al.*, 1991). However, morphology alone is not sufficient to unambiguously identify EPL-cell formation as gene expression and differentiation potential changes associated with EPL-cell formation can occur in the absence of morphology changes, and conversely morphology changes have been detected without changes in gene expression and differentiation potential (Bettess, 2001). For example, growth of ES cells on a cellular fibronectin matrix results in EPL-cell morphology but an ES-cell gene expression profile is retained (Bettess, 2001). Similarly, culture of ES cells in the presence of MEDII on tissue culture plastic in the absence of a matrix such as gelatin results in EPL-cell gene expression profile but colony morphology remains ES-like (Bettess, 2001).

1.2.4.2 EPL-cell gene expression

As with ES cells, EPL cells express markers of pluripotence such as *Oct4*, *SSEA-1* and alkaline phosphatase activity (Solter and Knowles, 1978; Johnson *et al.*, 1977; Rosner *et al.*, 1990; Rathjen *et al.*, 1999; Lake *et al.*, 2000). Consistent with their pluripotent status, EPL cells do not express markers of differentiated cell types or extraembryonic lineages. For example, the cells do not express visceral endoderm marker *alphafetaprotein* (Dziadek and Adamson, 1978) or mesoderm markers *brachury* (Wilkinson *et al.*, 1990) and *goosecoid* (Blum *et al.*, 1992).

EPL cells can be distinguished from ES cells on the basis of gene expression (Figure 1.7 A). For example, ES cells express ICM markers *Rex1* (Rogers *et*

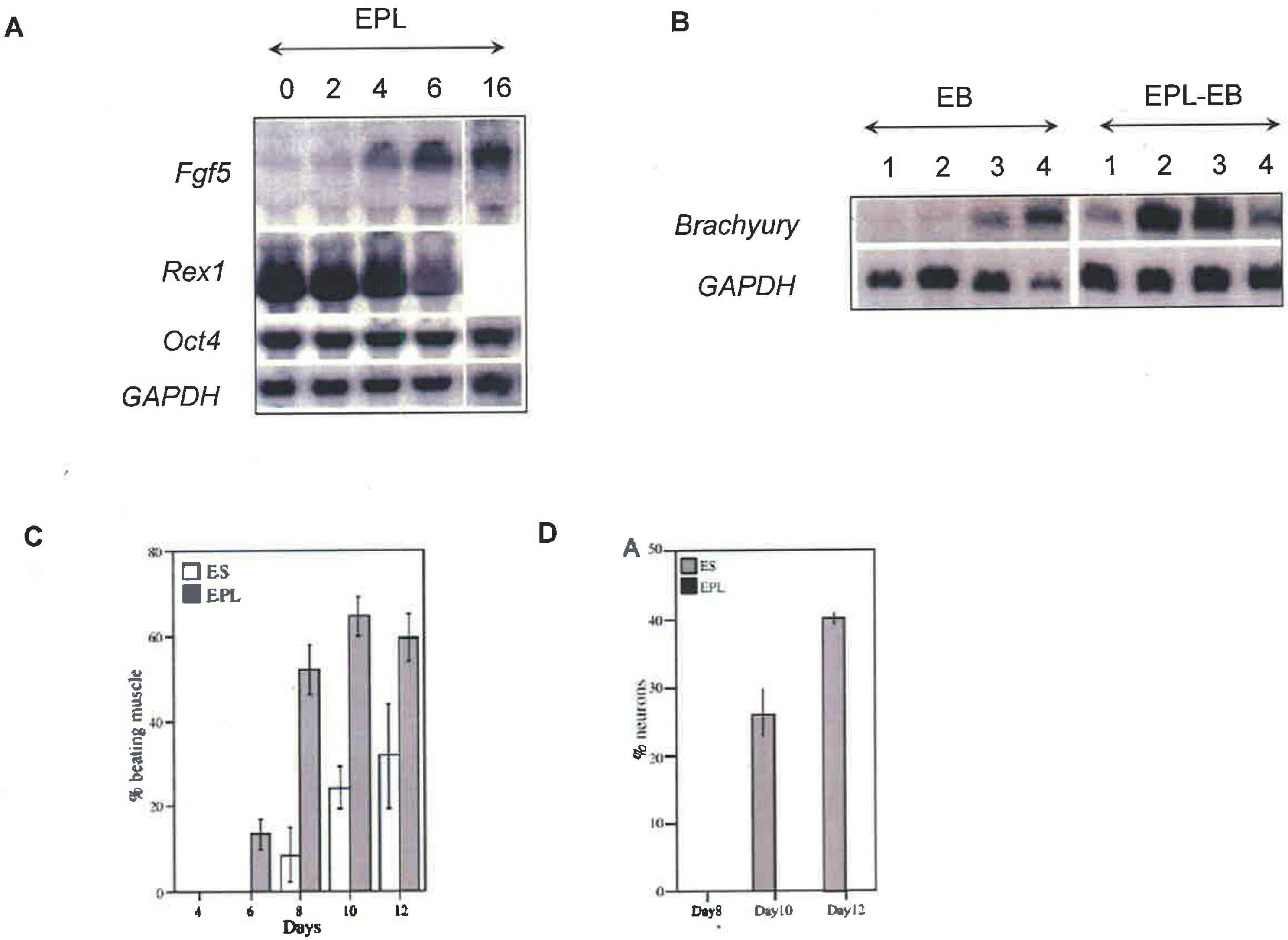
Figure 1.7

Gene expression profile and differentiation potential of EPL cells

(A) Northern blot analysis of total RNA (20 µg) derived from ES- and EPL-cells cultured in MEDII + LIF for 0, 2, 4, 6 or 16 days was analysed for expression of *Rex1*, *Fgf5* and *Oct4*. (B) Northern blot analysis of total RNA (20 µg) derived from ES- and EPL-cell embryoid bodies (EPL-EB) differentiated for up to 4 days. The blot was probed for the expression of mesoderm marker *Brachyury* with *GAPDH* used as a loading control. (C, D) Terminal differentiation of EPL-EB. (C) The percentage of ES- and EPL-EBs containing beating muscle during days 4-12 of differentiation. (D) The percentage of ES- and EPL-EBs forming neurons during days 8-16 of differentiation.

Reproduced from Lake *et al.*, 2000.

Figure 1.7


A
EPL
0 2 4 6 16
Fgf5
Rex1
Oct4
GAPDH
B
EB
EPL-EB
1 2 3 4 1 2 3 4
Brachyury
GAPDH
C
ES
EPL
% beating muscle
0
20
40
60
80
4 6 8 10 12
Days
D
A
ES
EPL
% neurons
0
10
20
30
40
50
Day8 Day10 Day12

al., 1991), *Gbx2* (Chapman *et al.*, 1997) *CRTR1*, and *Psc1* (Rathjen *et al.*, 1999; Pelton *et al.*, 2002) but expression of these is down-regulated in EPL cells similar to the down-regulation *in vivo* following the formation of the primitive ectoderm from the ICM. Conversely, the formation of EPL cells leads to up-regulation of the primitive ectoderm marker *Fgf5* (Haub and Goldfarb, 1991; Rathjen *et al.*, 1999). These differences in gene expression thus correlate to changes seen *in vivo* with the transition from ICM to the primitive ectoderm. A recent microarray comparing the gene expression profiles of ES and EPL cells has identified a number of new candidate EPL-cell markers including the demethylase *Dnmt3b1* and Nodal inhibitor *Lefty2* (Rathjen, unpublished data). Northern blot and quantitative PCR analysis confirmed *Dnmt3b1* and *Lefty2* as being specifically up-regulated in EPL cells (Rathjen and Lonic, unpublished data).

1.2.4.3 EPL-cell differentiation potential

Unlike cells of the ICM, primitive ectoderm cells are unable to contribute to chimera formation following introduction into a host blastocyst (Gardner, 1971; Rossant, 1977; Beddington, 1983). Similarly EPL cells, unlike ES cells, are also unable to give rise to chimeras when introduced into a 4.5 dpc blastocyst (Rathjen *et al.*, 1999).

ES and EPL cells can also be distinguished based on their differentiation potential when cultured as aggregates (Figure 1.7 B). In culture medium comprised of DMEM, β-Me and in the absence of LIF, a morphological differences between bodies derived from ES cells (EBs) and EPL cells (EPL-

EBs) are evident from day 4 of differentiation with the EBs composed of aggregates with an outer layer of (extraembryonic) parietal and visceral endoderm while EPL-EBs lack this outer layer (Martin *et al.*, 1977; Lake *et al.*, 2000). Furthermore, while EBs produce cell populations representative of all three germ layers as well as the extraembryonic endoderm (Rathjen and Rathjen, 2000; Smith, 2001) EPL-EBs form a more restricted number of cell types. *Brachyury*, a marker for nascent mesoderm, is detected earlier in EPL-EBs compared to EBs (Figure 1.7 B), while the neural marker *Sox1* is not detected in EPL-EBs but is in EBs (Lake *et al.*, 2000; Rathjen *et al.*, 2001; Rathjen *et al.*, 2002). Consistent with this, EPL-EBs produce a high percentage of beating cardiocytes (Figure 1.7 C) and hematopoietic cell types (both mesoderm derivatives) but fail to form neurectoderm (Figure 1.7 D) and extraembryonic visceral endoderm indicating that EPL-EBs form mesoderm at the expense of ectoderm (Lake *et al.*, 2000; Rodda *et al.*, 2002).

The absence of visceral endoderm in EPL-EBs is believed to be crucial for the enrichment in mesoderm that is seen. Within the embryo, visceral endoderm signals are likely involved in the recruitment of the pluripotent cells to non-mesodermal lineages whereas cells that are destined to become mesoderm migrate through the primitive streak and lose contact with the basement membrane and visceral endoderm (Lake *et al.*, 2000; Rathjen *et al.*, 2002; Baron, 2005). Conversely, cells that go on the form neurectodermal lineages remain adjacent to the visceral endoderm (Tam, 1989; Quinlan *et al.*, 1995). Therefore, EPL cells can be formed from ES cells in the presence of visceral endoderm signalling provided by MEDII but once EPL cells are formed, and

MEDII is removed, EPL-EBs differentiate to an enriched population of mesoderm (Rathjen *et al.*, 1999; Lake *et al.*, 2000).

EPL cell aggregates cultured in the presence of MEDII adopt an alternate cell fate with the uniform formation of *Sox1, Sox2, Nestin, N-CAM*-positive neurectoderm (Figure 1.8 A, B) (Lake *et al.*, 2000; Rathjen *et al.*, 2002). The differentiation occurs in the absence of any extraembryonic endoderm, and mesoderm indicated by absence of AFP and *Brachyury* expression respectively in EBMs (Figure 1.8 C) (Rathjen *et al.*, 2002). The differentiation occurs in a manner recapitulating embryogenesis, with sequential homogeneous formation of cells equivalent to primitive ectoderm, neural plate and neural tube (Rathjen *et al.*, 2002; Rodda *et al.*, 2002).

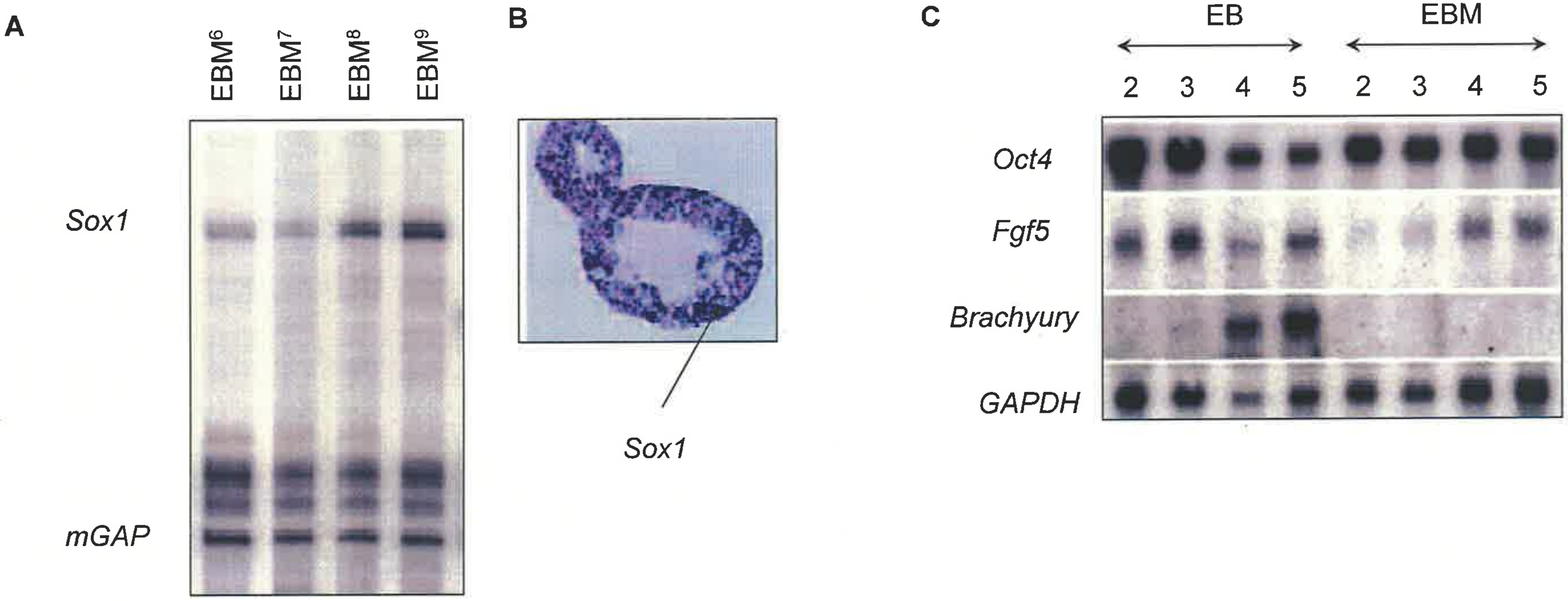
Differentiation via an EPL-cell intermediate provides a methodology for lineage-specific differentiation of pluripotent cells to progenitors or terminally differentiated cell types in a controlled manner (Rathjen *et al.*, 1999; Lake *et al.*, 2000; Rathjen *et al.*, 2002; Rodda *et al.*, 2002). This differentiation is achieved in a manner that parallels embryogenesis, with the synchronous formation of temporal intermediates and progenitors. In particular, the absence of visceral endoderm and other contaminating cell populations provides an environment free from problems associated with activation of inappropriate signalling pathways and enables the generation of large quantities of defined and pure progenitor populations in response to exogenous signals that may be useful in analysis of mechanisms during

Figure 1.8

Differentiation of EBs in MEDII (EBMs) leads to formation of homogeneous neurectoderm

(A) RNase protection analysis of 15 µg RNA isolated from EBM^{6-9} analysed for the expression of neural marker *Sox1* relative to *GAPDH*. (B) Whole-mount *in situ* hybridisation of EBM^{9} analysed for the expression of *Sox1*. (C) Northern blot analysis of total RNA (20 µg) isolated from day 2-5 EBs or EBMs probed for *Oct4*, *Fgf5* and *Brachyury*. *GAPDH* was used as a loading control. Reproduced from Rathjen *et al.*, 2002.

Figure 1.8


A
EBM6
EBM7
EBM8
EBM9
Sox1
mGAP
B
Sox1
C
EB
EBM
2
3
4
5
2
3
4
5
Oct4
Fgf5
Brachyury
GAPDH

embryogenesis and for implantation in cell based therapies (Lake *et al.*, 2000; Rathjen *et al.*, 2002).

1.2.4.4 Cytokine responsiveness of EPL cells

During gastrulation, various cytokine factors are involved in the specification of embryonic lineages. For example, growth factors Nodal and Wnt3, expressed in the posterior region of the primitive ectoderm, have a role in induction of the primitive streak (Conlon, 1994; Mohamed *et al.*, 2004; Rivera-Perez and Magnuson, 2005; Kemp *et al.*, 2005). On the opposite side of the embryo, anterior visceral endoderm expresses the Wnt inhibitor Dkk1 and the Nodal inhibitors, Cerberus-like and Lefty1, ensuring the correct positioning of the primitive streak on the posterior side (Kemp *et al.*, 2005). The *in vivo* substrate on which these signals act is primitive ectoderm. However, primary cultures of primitive ectoderm cells are unstable (Rathjen *et al.*, 2003) and so not readily suited for analysis of gastrulation inductive signals *in vitro*. Therefore, the availability of an *in vitro* equivalent of primitive ectoderm, EPL cells, potentially enables dissection of such differentiation-inductive signals. Proof of principle of EPL cells as a system suitable for the analysis of gastrulation-inductive signals is provided by their response to BMP. BMP is known to be involved in the formation of mesodermal lineages *in vivo* (Winnier *et al.*, 1995; Johansson and Wiles, 1995). Treatment of ES cells with BMP4 does not lead to induction of any embryonic lineages but instead enhances self-renewal (Ying *et al.*, 2003; Qi *et al.*, 2004; Harvey, unpublished results). Conversely, treatment of EPL cells with BMP4 (10 ng/ml) results in increased formation of mesoderm (Harvey, unpublished data). The ES-to-EPL cell transition occurs in the presence or absence of LIF but the presence of LIF is associated with delay of associated gene expression changes (Rathjen *et al.*, 1999; Lake *et al.*, 2000). A similar effect is observed with EB differentiation *in vitro* where the presence of LIF inhibits the formation of primitive ectoderm (Shen and Leder, 1992) and *in vivo* where mouse embryos constitutively expressing LIF show inhibition of primitive ectoderm formation (Conquet *et al.*, 1992).

The study of events during early embryogenesis is hindered by the small size and inaccessibility of the embryo. Thus, the formation of EPL cells an *in vitro* equivalent of the primitive ectoderm provides a model system, which enables the study of events and molecular mechanisms involved in early development. Mechanisms and signalling involved in processes such as the progression of pluripotent cell populations as well as gastrulation can be studied in a system free of contaminating cell types and confounding signals. The production of a homogeneous population of EPL cells also provides a clean substrate for directed differentiation where differentiation can be tightly controlled to produce physiologically relevant cells in large quantities.

1.2.5 Identification of MEDII derived factors involved in EPL-cell formation

Fractionation of serum free MEDII over a size exclusion 3-10 kDa membrane revealed the presence of two signals, one a low molecular weight (3 kDa) eluate (E) and a high molecular weight (>10 kDa) retentate (R) (Rathjen *et al.*, 1999). The induction of EPL cell morphology occurred in the presence of both the high and low molecular weight components (50% E + 50 μg/ml R) but not in the presence of R alone (Rathjen *et al.*, 1999; Washington, unpublished data). Sequential fractionation of E was performed over a Sephadex G10 gel filtration column, normal-phase HPLC column and Superdex gel filtration column. Fractions eluting at a predicted size of <700 Da exhibited EPL inductive activity. The active peak was subject to amino acid composition analysis and shown to have elevated levels of L-alanine and L-proline (300-400 μM) compared to non-conditioned serum free medium. Morphological activity assays in the presence of R determined that L-proline but not L-alanine was able to induce EPL cell morphology. The minimal concentration of L-proline required was 40 μM (Washington, unpublished data).

Addition of 40 μM of L-proline alone to ES cells induced EPL cell morphology of about 50% colonies with the remainder retaining ES morphology. Upon addition of R a homogeneous conversion was induced with 100% of colonies exhibiting EPL cell morphology. Cells cultured in the presence of 40 μM L-proline ± R did not down-regulate *Rex1* but did up-regulate *Fgf5* while aggregates derived from these cells induced expression of *Brachyury* on day 4. This indicates that induction of EPL cell morphology and up-regulation of *Fgf5* in the presence of 40 μM L-proline ± R does not correlate with induction of EPL cell differentiation potential (Washington, unpublished data). Culture of ES cells in high concentrations of L-proline 200-400 μM led to homogeneous induction of EPL cell morphology as well as gene expression and differentiation potential changes that were consistent with the cells being of EPL cell fate (Bettess, 2001). This indicates that L-proline, the bioactive component within MEDII, is sufficient to induce the ES-to-EPL cell transition in terms of morphology, gene expression and differentiation potential at high concentrations. At low concentrations component R is required for a homogeneous morphological transition (Washington, unpublished data). Fractionation and biochemical characterisation identified component R to be cellular fibronectin (Bettess, 2001). Apart from L-proline, short L-proline containing peptides were also able to induce EPL-cell morphology and are further discussed in Chapter 3.

Although L-proline is sufficient to induce EPL cell formation the mode of action of L-proline in the ES-to-EPL cell transition is not known. Similarly, the signalling pathways required for the transition have also not been identified. The delineation of the molecular mechanism involved in the formation of EPL cells would potentially allow greater control over the maintenance of pluripotence and switching to directed differentiation in a homogeneous and synchronous manner.

Since the ES-to-EPL cell transition appears to recapitulate the *in vivo* differentiation of ICM to primitive ectoderm and enables lineage specific differentiation (Rathjen *et al.*, 1999; Lake *et al.*, 2000; Rathjen *et al.*, 2002), this model of early embryogenesis provides a unique opportunity to study the molecular mechanisms of developmental decisions controlling pluripotence, the transition between two pluripotent populations, the loss of pluripotence and directed differentiation to each of the three germ layers and beyond. Manipulation of these molecular mechanisms would then provide a means for directing differentiation of homogeneous EPL-cell populations to alternate cell fates in a manner which mimics developmental cues functional *in vivo*. This would potentially overcome problems associated with activation of inappropriate signalling pathways due to mixed cell populations produced in EB differentiation and lead to homogeneous cell populations, which would be suitable for transplantation studies.

1.2.6 Remodelling of basement membranes – a potential source of L-proline/short L-proline-containing peptides

In vivo, the availability of L-proline may be provided by basement membrane remodelling. Remodelling of basement membranes occurs in various cellular processes such as embryo morphogenesis, blastocyst implantation, angiogenesis, tissue remodelling and tumour metastases (Matrisian, 1992; Birkedal-Hansen, 1995). A major protein component of basement membranes (60-90%) is proline-rich collagen IV (Van Der Rest and Garrone, 1991). Basement membrane remodelling involves degradation of cross-linked insoluble collagen fibres. The matrix metalloproteinases (MMPs), specifically the type IV collagen proteases, MMP-2 and MMP-9, are involved in the degradation of collagen IV (Matrisian, 1992; Birkedal-Hansen, 1995). The protease MMP-9 is expressed in early development with transcripts detected in peri-implantation mouse blastocysts (Brenner *et al.*, 1989; Behrendtsen *et al.*, 1992). MMP-9 is essential for implantation as treatment of mouse blastocyst outgrowths with MMP-9 neutralizing antibodies blocks invasion and degradation of basement membranes (Librach *et al.*, 1991; Behrendtsen *et al.*, 1992). Consistent with this, MPP-9 was shown by *in situ* hybridisation to be localized to trophoblast cells of the 7.5 dpc embryo supporting a role in implantation (Harvey *et al.*, 1995). Therefore, degradation of collagens during development by specific proteases, within the epiblast or visceral endoderm may be a source of free L-proline and short L-proline-containing peptides during the formation of primitive ectoderm.

1.3 SIGNALLING PATHWAYS INVOLVED IN ES-CELL SELF-RENEWAL, PLURIPOTENCE AND DIFFERENTIATION

1.3.1 Signalling mechanisms involved in self-renewal and differentiation of ES cells

Self-renewal is defined as proliferation accompanied by the suppression of differentiation and is essential for maintenance of pluripotence (Smith, 2001). Maintenance of mouse ES cells in a pluripotent, self-renewing state requires culture of cells in the presence of members of the Interleukin 6 (IL-6) family, which activate intracellular signalling via the gp130-LIFRβ (Leukaemia inhibitory factor receptor β) complex (Figure 1.9) (Yoshida *et al.*, 1994). LIF is mainly used for the maintenance of ES cells in culture (Williams *et al.*, 1988) but other IL-6 family members including oncostatin M (Gearing and Bruce, 1992), cilliary neurotrophic factor (Conover *et al.*, 1993) and cardiotrophin-1 (Pennica *et al.*, 1995) are also able to maintain self-renewal. Activation of the gp130-LIFRβ complex leads to the activation of several down-stream signalling pathways (Figure 1.9) including MAPK (Boulton *et al.*, 1994; Yin *et al.*, 1994; Takahashi-Tezuka *et al.*, 1998; Burdon *et al.*, 1999), PI3K (Boulton *et al.*, 1994; Takahashi-Tezuka *et al.*, 1997; Paling *et al.*, 2004) and STAT3

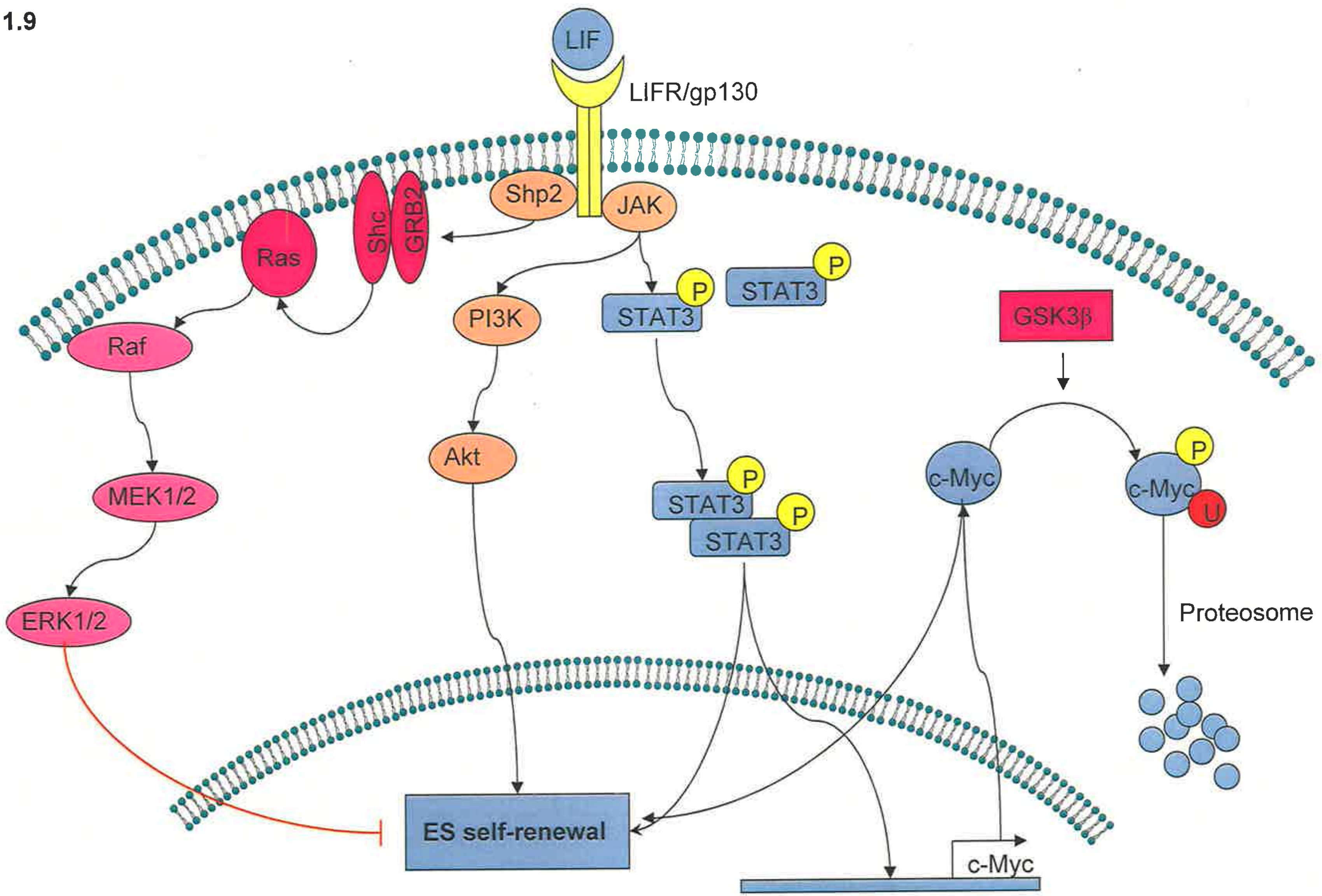
Figure 1.9

LIF-mediated signalling

Activation of the gp130-LIFRβ complex by LIF leads to the activation of several down-stream signalling pathways including MAPK, PI3K and STAT3. Following LIF binding, the JAK kinases associated with gp130-LIFRβ are activated and phosphorylate tyrosine residues on gp130 and LIFRβ. The phosphorylated residues form docking sites for Src homlogy 2 (SH2) domains of STAT3 which localises them to the receptor complex and leads to their phosphorylation on Tyr705. Tyrosine phosphorylated STAT3 dimerises and translocates to the nucleus. Within the nucleus STAT3 is able to bind to the Myc promoter and induces its expression. STAT3 actions facilitate self-renewal in ES cells. In the absence of active STAT3 the Myc protein is targeted for degradation via a GSK3β-dependent phosphorylation of residue T58, which targets Myc to the proteosome. Phosphorylation of a tyrosine residue on gp130 proximal to the membrane is required for activation of SHP2. Phosphorylation of Tyr118 on Shp-2 produces a binding site for the Grb2 adapter protein. The complex formed by Grb2 and Sos guanine-exchange factor then activates Ras and initiates the MAP kinase cascade leading to the sequential activation of Raf, MEK1 and ERK1/2. The MAPK signalling cascade is associated with differentiation of ES cells.

Adapted from Cartwright *et al.*, 2004.

Figure 1.9


LIF
LIFR/gp130
Shp2
JAK
Shc
GRB2
Ras
Raf
MEK1/2
ERK1/2
PI3K
Akt
STAT3
P
STAT3
P
GSK3β
STAT3
P
STAT3
P
c-Myc
c-Myc
P
U
Proteosome
ES self-renewal
c-Myc

(Stahl *et al.*, 1994; Niwa *et al.*, 1998; Matsuda *et al.*, 1999). STAT3 activity is required for the maintenance of mouse ES cells in culture (Niwa *et al.*, 1998; Matsuda *et al.*, 1999).

The receptors gp130-LIFRβ, having no intrinsic kinase activity, are constitutively associated with Janus-associated kinases (JAK) (Stahl *et al.*, 1994). Following ligand stimulation, auto-phosphorylation of the JAK1/2 occurs on Tyr1022/Tyr1023 of JAK1 (Wang *et al.*, 2003) and on Tyr1007/Tyr1008 of JAK2 (Feng *et al.*, 1997; Frank *et al.*, 2002). Activated JAK1 subsequently phosphorylates tyrosine 683 (Ohtani *et al.*, 2000) on gp130 and tyrosine residues on LIFRβ (Murakami *et al.*, 1993; Stahl *et al.*, 1994). Residues Tyr759/767/814/905/915 on gp130 are also phosphorylated and form docking sites for Src homlogy 2 (SH2) domains of STAT3 (Yamanaka *et al.*, 1996; Ohtani *et al.*, 2000).

1.3.1.1 STAT3 signalling in ES cells

Once bound, activation of STAT3 depends on phosphorylation of its most carboxyl-terminal tyrosine residues (Stahl *et al.*, 1995; Yamanaka *et al.*, 1996). The phosphorylation of STAT3 on Tyr705 (Darnell *et al.*, 1994; Ihle, 1995) leads to its dimerisation and translocation into the nucleus (Niwa *et al.*, 1998). STAT3 has been demonstrated to be crucially important in the self-renewal of ES cells mediated by LIF. Niwa *et al.* (1998) developed a dominant-negative-tetracycline-inducible STAT3 (STAT3F), in which Tyr705 was mutated to phenylalanine. The induction of STAT3F expression by withdrawal of tetracycline in ES cells led to differentiation of the cells (Niwa *et* *al.*, 1998). The need for activated STAT3 in LIF-mediated maintenance of ES cells was also demonstrated using an estradiol-inducible STAT3. The STAT3 coding region was fused to the ligand-binding domain on the estrogen receptor and expressed in ES cells. The transfected ES cells could be maintained in an undifferentiated state in the absence of LIF if the synthetic estrogen receptor ligand 4-hydroxytamoxifen (4OHT) was present (Matsuda *et al.*, 1999). STAT3 is thus necessary and sufficient for maintenance of undifferentiated mouse ES cells.

Although LIF is required *in vitro* for the maintenance of mouse ES cells, *in vivo* there does not seem to be a requirement for LIF in the unarrested development of the pre- and peri-implantation embryo even though the ligand and receptors are expressed in the embryo at that time (Nichols *et al.*, 1996). Mice carrying mutations in LIF, LIF receptor or gp130 develop beyond peri-implantation stages (Ware *et al.*, 1995; Yoshida *et al.*, 1996). The requirement for LIF-mediated signalling at the blastocyst stage of mouse development may be restricted to a role in diapause, a state of arrested blastocyst development, which can arise in lactating mice. Normal development and implantation of the embryo is restored following elevation of maternal estrogen levels and this can occur after a delay of up to 4 weeks. *gp130*$^{-/-}$ mouse embryos are unable to maintain the epiblast during diapause and are not able to resume normal development following diapause (Nichols *et al.*, 2001). Successful isolation of ES cells occurs more efficiently from blastocysts derived from female mice in diapause and this may be the reason for the requirement of LIF to maintain ES cells *in vitro* (Smith, 2001). Unlike in

All residue numbers given refer to mouse protein sequences.

mouse ES cells LIF/STAT3 signalling is not involved in the maintenance of self-renewal in human ES cells (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Daheron *et al.*, 2004).

1.3.1.2 MAPK signalling in ES cells

As well as the activation of STAT3, LIF treatment also activates MAPK signalling downstream of the stimulated gp130-LIFRβ receptor complex (Figure 1.9) (Takahashi-Tezuka *et al.*, 1998). The presence of LIF promotes phosphorylation of a tyrosine residue of gp130 proximal to the membrane (Stahl *et al.*, 1995; Yamanaka *et al.*, 1996). This leads to the phosphorylation of Tyr118 on Shp-2 (Burdon *et al.*, 1999) and the creation of binding site for the Grb2 adapter protein. The complex formed by Grb2 and Sos guanine-exchange factor then activates Ras and initiates the MAP kinase cascade leading to the sequential activation of MEK1 by Raf-1-mediated phosphorylation of Ser217/221 (Zheng and Guan, 1994; Pages *et al.*, 1994; Xu *et al.*, 1995) and ERK1/2 by MEK1-mediated phosphorylation of Thr202/Tyr204 (Sturgill *et al.*, 1988; Payne *et al.*, 1991). The activation of MAPK signalling acts to promote differentiation rather than self-renewal. Elimination of the Shp-2 binding site on gp130 (Burdon *et al.*, 1999), knockouts of *Grb2* (Cheng *et al.*, 1998) and *Shp2* (Qu and Feng, 1998) and culture in the presence of MEK1 inhibitors all lead to enhanced LIF-induced self-renewal of ES cells. Therefore, the balance between LIF-activated STAT3 and MAPK signalling in part determines the ability of mES cells to remain in a self-renewing state. The role of LIF signalling in the maintenance of pluripotence in other pluripotent populations such as the primitive ectoderm has not been investigated.

1.3.2 Myc is a down-stream effector of LIF

The transcription factor Myc was shown to be highly expressed in ES cells and down-regulated with differentiation (Cartwright *et al.*, 2005). Chromatin immunoprecipitation (ChIP) analysis revealed that STAT3 binds to the Myc promoter in ES cells (Cartwright *et al.*, 2005). Functional studies with ES cells expressing chimeric STAT3-ER, which allows maintenance of the undifferentiated state in the absence of LIF if 4OHT is present (Matsuda *et al.*, 1999), indicated that 4OHT induced Myc expression in the same manner as was seen with LIF. It was thus concluded that Myc was a direct transcriptional target of STAT3 (Cartwright *et al.*, 2005).

The down-regulation of Myc mRNA occurred by day 2 of EB differentiation and was seen to precede the down-regulation of the pluripotence marker *Oct4* on day 4, suggesting a role in maintenance of self-renewal or very early differentiation (Cartwright *et al.*, 2005). Apart from the inhibition of Myc mRNA expression with differentiation, Myc protein was also targeted for degradation. GSK3β-dependent phosphorylation of residue T58, which targets Myc to the proteosome, increased following LIF withdrawal (Cartwright *et al.*, 2005). Over expression of Myc^{T58A}-ER in ES cells maintained them in a self-renewing state in the presence of 4OHT: The expression of pluripotence markers AP and *Oct4* and ES markers *Rex1* and *SSEA1* was maintained in the absence of LIF (Cartwright *et al.*, 2005). This suggests Myc is a down-stream effector of LIF/STAT3 that maintains ES cells in a self-renewing state.

1.3.3 PI3K regulates LIF-activated ERK activity

Another group of kinases known to be activated downstream of LIF-activated gp130 receptors is the phosphoinositide-3-kinase (PI3K) of the class I_A (Figure 1.9) (Boulton *et al.*, 1994; Takahashi-Tezuka *et al.*, 1998). LIF-induced PI3K signalling is important in maintenance of ES cells as a loss of PI3K signalling has been associated with a loss of self-renewal ability (Paling *et al.*, 2004). Culture of ES cells in the presence of the PI3K inhibitor LY294002 (5 µM) reduced the number of domed colonies expressing the pluripotence marker alkaline phosphatase (AP) and increased the incidence of flattened colonies of irregular shape which failed to stain uniformly for AP, indicative of the presence of differentiated cells (Paling *et al.*, 2004). Although the inhibition of PI3K signalling did not alter the total levels of Oct4 and STAT3 protein or the amount of phosphorylated STAT3 as detected by Western blot, there was up-regulation of STAT5, an early marker of differentiation (Paling *et al.*, 2004). Incubation of cells with LY294002 also enhanced the level of phosphorylation of ERK within 10 min following LIF treatment. As previously discussed, MAPK signalling down-stream of LIF is involved in induction of differentiation (Burdon *et al.*, 1999). Consistent with this, co-treatment with LY294002 and the MAPK inhibitor U0126 increased the number of undifferentiated AP-positive colonies (Paling *et al.*, 2004). Therefore, the ability of ES cells to self-renew appeared to be due, in part, to the PI3K-mediated inhibition of ERK activity.

1.3.4 Wnt signalling maintains ES self-renewal in the absence of LIF

Although LIF-induced STAT3 signalling is crucial for the self-renewal of mouse ES cells, this pathway does not appear to be involved in self-renewal of human ES (hES) cells (Humphrey *et al.*, 2004). A microarray screen performed to identify other potential pathways involved in self-renewal of mouse ES cells identified the canonical Wnt pathway as a potential candidate (Sato *et al.*, 2004). Canonical signalling by the Wnt pathway is induced following the binding of the Wnt protein to the Frizzled receptor (Wodarz & Nusse, 1998). Down-stream signalling leads to the phosphorylation and inactivation of GSK-3 allowing the nuclear accumulation of β-catenin (Amit *et al.*, 2002; Liu *et al.*, 2002). The Wnt signalling cascade can also be activated artificially through the use of specific GSK-3 inhibitors such as 6-bromoindirubin-3'-oxime (BIO) (Sato *et al.*, 2004).

Use of a reporter mES cell line, in which a modified version of yellow fluorescent protein was regulated by a Wnt-responsive promoter, showed that Wnt signalling is active in undifferentiated ES cells and not differentiated counterparts (Sato *et al.*, 2004). Likewise, the expression of ES/ICM marker *Rex1*, as measured by a luciferase reporter, was up-regulated following treatment with BIO, an effect that was overcome by the expression of a dominant-negative TCF-3 which specifically blocks Wnt target genes (Sato *et al.*, 2004). Cells stably expressing the *Rex1* reporter were tested for the ability of BIO treatment to prevent differentiation of the cells in the absence of LIF over 5 days without passage. Whereas BIO-treated colonies maintained high expression of the reporter and a domed morphology in the absence of LIF, the untreated cells presented a flattened morphology and low expression of *Rex1* reporter indicative of differentiation (Sato *et al.*, 2004).

1.3.5 BMP4-induced Id activity complements LIF-mediated self-renewal by inhibiting neural differentiation

Although the presence of LIF promotes the maintenance of mES cells in an undifferentiated, self-renewing state LIF alone is insufficient for this purpose. Following the withdrawal of serum, LIF alone fails to inhibit neural differentiation as measured by the emergence of *Sox1* positive cells in culture (Ying *et al.*, 2003). BMP is a factor that is known to act as an antagonist of neural differentiation in ES cells and neural specification within the embryo (Wilson & Hemmati-Brivanlou, 1995; Wilson & Edlund, 2001). The co-treatment of cells with BMP4 (10 ng/ml) and LIF (10 ng/ml) in the absence of serum increased the incidence of pure ES-cell colonies that could be continually passaged as measured by the expression of SSEA-1, alkaline phosphatase, transcripts for *Nanog* and *Oct4*, and the lack of expression of mesoderm or neurectoderm markers. This function of BMP4 is not a property of all TGFβ superfamily members since TGFβ1 had no effect on ES-cell maintenance. The use of LIF and BMP4 in serum-free conditions also allowed the *de novo* derivation of ES cells from blastocysts as well as clonal propagation of ES cells (Ying *et al.*, 2003).

GFP-expressing ES cells cultured in BMP4 and LIF injected into blastocysts resulted in chimeras. The effect of BMP4 on ES cell self-renewal was not due to altered activity of the LIF-initiated STAT3 pathway or suppression of the LIF-initiated MAPK cascade, as levels of phosphorylated STAT3 and ERK1/2 remained unaltered between LIF and LIF+BMP treatments. However, treatment with BMP4 was shown to lead to increased phosphorylation of the transcription factors Id1 and Id3. To clarify the role of Ids in self-renewal, Id-expressing constructs were transfected into ES cells. While there was no effect on the cells in the presence of serum, the self-renewal of Id-expressing ES cells was maintained in neural-inducing media without the requirement for BMP4 (Ying *et al.*, 2003). It appears that the function of Ids may be not to maintain self-renewal *per se* but to prevent neural differentiation and thus allow LIF to function as a self-renewal factor in the absence of serum (Figure 1.10 A). Consistent with this, Id-transfected cells in the presence of serum following LIF withdrawal differentiated in the same manner as untransfected counterparts. In N2B27 medium, which, following LIF withdrawal, led to neural differentiation of untransfected cells, Id-transfected cells had neural differentiation suppressed.

1.3.6 BMP4 down-regulates LIF-induced p38 and ERK activity

In contradiction to the findings of Ying *et al.* (2003), Qi *et al.* (2004) showed BMP4 to have a role in self-renewal via the inhibition of p38 and ERK activity (Figure 1.10 B) (Qi *et al.*, 2004). In this case, BMP4 was identified as a candidate for promoting self-renewal as its expression was up-regulated in mouse embryonic fibroblast STO cells which, when used as feeder layers, maintained mouse ES cells compared to lines that were unable to maintain ES cells, ES cell-resisting STO (RSTO) cells. ES-cell self-renewal could be

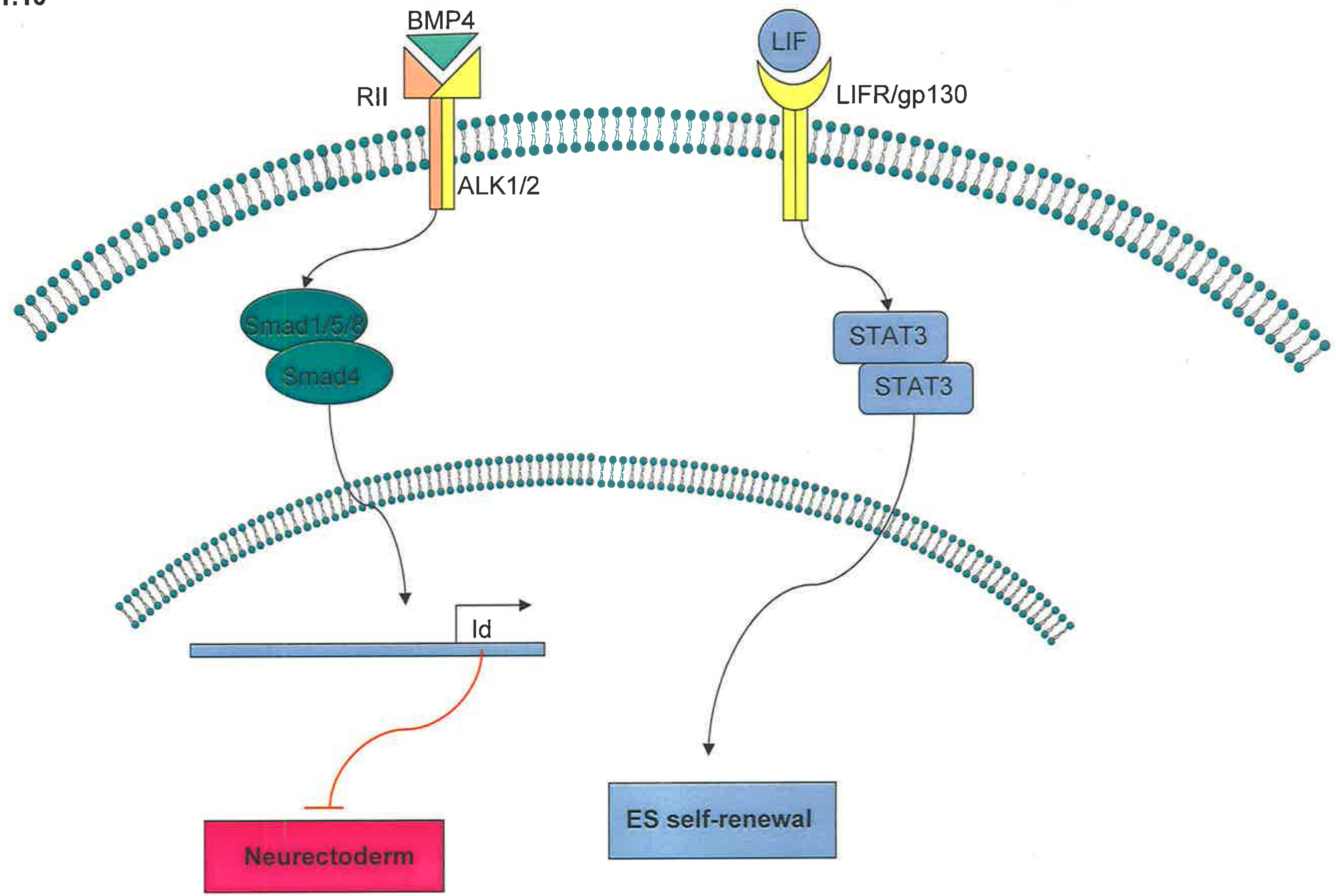
Figure 1.10

BMP4 supports self-renewal of embryonic stem cells

(A) Treatment of ES cells with BMP4 induces Id proteins that act to prevent neural differentiation and allow LIF to function as a self-renewal factor in the absence of serum. Adapted from Ying *et al.*, 2003. (B) Treatment of ES cells with BMP4 induces the expression of *XIAP*, a bridging protein that couples the BMP receptor ALK3 to p38 signalling via TAK1. Over-expression of *XIAP* prevents the ability of ES cells to maintain self-renewal. Inhibition of ES-cell self-renewal following over-expression of *XIAP* does not occur in $Alk^{-/-}$ ES cells indicating that ALK3-mediated activation of XIAP is required to inhibit self-renewal. Even though XIAP acts to stimulate p38 and MAPK in ES cells the net effect of BMP4 is to promote ES cell self-renewal by an unknown mechanism since BMP4 treatment of ES cells led to down-regulation of p38 and ERK activity. Adapted from Qi *et al.*, 2004.

Figure 1.10

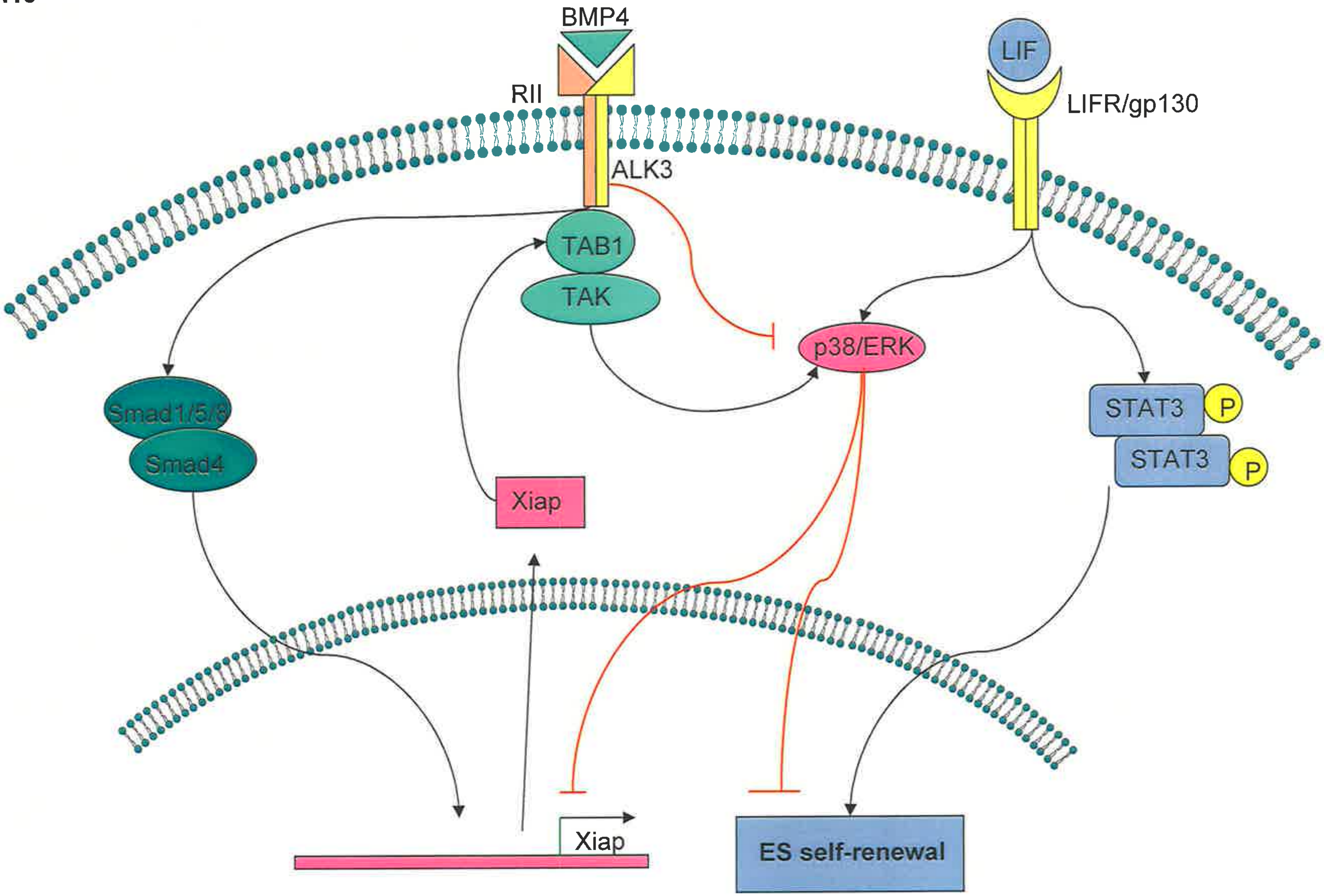
A



BMP4
RII
ALK1/2
LIF
LIFR/gp130
Smad1/5/8
Smad4
STAT3
STAT3
Id
Neurectoderm
ES self-renewal

Figure 1.10

B


BMP4
RII
ALK3
LIF
LIFR/gp130
TAB1
TAK
p38/ERK
Smad1/5/8
Smad4
Xiap
STAT3
P
STAT3
P
Xiap
ES self-renewal

maintained on RSTOs provided BMP4 was provided exogenously (Qi *et al.*, 2004).

A microarray screen of ES cells cultured in COS-BMP4 conditioned medium (highly permissive for self-renewal) compared to those cultured in COS-BMP8bh conditioned medium (which was poorly permissive) identified a group of differentially expressed genes. One of the genes shown to be up-regulated by BMP4 was *XIAP*. *XIAP* is a bridging protein that couples the BMP4 receptor, ALK3, to the down-stream p38 MAPK cascade via the action of TAK1 (Kimura *et al.*, 2000; Birkey-Reffey *et al.*, 2001; von Bubnof and Cho, 2001). *XIAP* was shown to be relevant to ES-cell maintenance as over-expression of *XIAP* disrupted self-renewal. However, inhibition of self-renewal following over-expression of *XIAP* did not occur in *Alk3*$^{-/-}$ ES cells suggesting that ALK3 is required to mediate this effect.

Even though BMP4 can stimulate p38 and MAPK via XIAP, the net effect of BMP4 is to promote ES-cell self-renewal. BMP4 treatment of ES cells led to down-regulation of p38 and ERK activity by 5 min following treatment. The continued culture of ES cells in the presence of the non-permissive medium COS-BMP8bh was possible when chemical inhibitors of p38 (SB203580) and ERK (PD98059) were incubated with the cells thus mimicking the activity of BMP4 (Qi *et al.*, 2004). Therefore, BMP4 appears to contribute to ES cell self-renewal by inhibiting p38 and ERK activity by an unidentified mechanism.

1.3.7 cYes is involved in ES-cell maintenance independent of LIF

The Src family of kinases also appear to play a role in ES-cell maintenance. Inhibition of Src signalling inhibits self-renewal as evidenced by inhibition of *Oct4*, alkaline phosphatase and *Nanog* expression (Anneren *et al.*, 2004). This inhibition does not appear to depend on LIF-initiated JAK/STAT or ERK signalling. Over-expression of constitutively active Src and Hck kinases in ES cells resulted in enhanced self-renewal at reduced LIF concentrations (Boulter *et al.*, 1991; Ernst *et al.*, 1996). A member of the Src tyrosine kinase family, cYes, was identified as a candidate, as its mRNA was found to be enriched in embryonic, haematopoietic and neural stem cells compared to their differentiated counterparts (Anneren *et al.*, 2004). cYes was shown to be present in its activated phosphorylated state in both mouse and human ES cells and its activity was down-regulated following LIF withdrawal and differentiation in mouse ES cells (Anneren *et al*, 2004). cYes phosphorylation was induced by both LIF and serum treatment in mouse ES cells. The Src-mediated pathway was shown to be independent of JAK/STAT and ERK as a Src inhibitor (SU6656, 5 μM) had no effect on the activity of JAK/STAT3 or ERK in the presence of LIF.

1.3.8 Signalling involved in self-renewal of human ES cells

1.3.8.1 TGFβ/Nodal/Activin signaling

Maintenance of the pluripotent status of human ES cells has been shown to require signaling via the TGFβ/Nodal/Activin branch of TGFβ signalling (Vallier *et al.*, 2005; James *et al*, 2005). Human ES cells growing in MEF conditioned medium (CM) displayed elevated levels of phosphorylated

Smad2/3, effectors of TGFβ/Nodal/Activin signalling, localised in the nucleus (Vallier *et al.*, 2005; James *et al.*, 2005). The phosphorylation and nuclear localisation of Smad2/3 was lost following differentiation (Vallier *et al.*, 2005; James *et al.*, 2005). Likewise, over-expression of Nodal in hES cells maintained prolonged expression of pluripotence marker genes and reduced induction of neuroectoderm markers in hES cells cultured in chemically defined medium (CDM) that normally induces neural differentiation (Vallier *et al.*, 2004). Treatment of hES cells growing in CM with SB431542, an inhibitor of SMAD2/3 phosphorylation by type 1 TGFβ receptors (Laping *et al.*, 2002), or Activin inhibitor follistatin prevented self-renewal as demonstrated by the down-regulation of pluripotence markers such as *Oct4* and *Nanog* (Vallier *et al.*, 2005; James *et al.*, 2005). However, follistatin treatment didn't induce the differentiation of Nodal over-expressing hES cells indicating that Nodal and Activin act independently to maintain markers of pluripotence (Vallier *et al.*, 2005).

In another study microarray analysis of two hES cell lines exhibiting differential ability to maintain self-renewal identified higher levels of Nodal/Activin, Fgf, Wnt, and Hedgehog (Hh) expression in the line exhibiting self-renewal advantage, implicating these molecules in the maintenance of the undifferentiated state (Xiao *et al.*, 2006). Further more Activin A was found to be required for maintenance of hES cell self-renewal and pluripotence and expression of *Oct4*, *Nanog*, *Nodal*, *Wnt3*, Fgf2 (Xiao *et al.*, 2006).

Treatment of hES cells with BMP seems to have a pro-differentiation effect unlike the effect of BMP on mES cells that promotes self-renewal. Smad1,5, effectors of BMP signaling, have low levels of phosphorylation in self-renewing hES cells which are increased following differentiation (James *et al.*, 2005). Consistent with this, hES cells grown in CM in the presence of BMP had decreased levels of *Oct4* expression and adopted differentiated morphology (Xu *et al.*, 2002; James *et al.*, 2005). The induction of Smad1,5 phosphorylation could be counteracted by the addition of Activin (James *et al.*, 2005).

1.3.8.2 Fgf signaling

Fgf signalling has recently been demonstrated as important in maintaining hES cells in a self-renewing state (Amit *et al.*, 2004; Wang *et al.*, 2005; Grebner *et al.*, 2006; Levenstein *et al.*, 2006). Culture of hES cells in absence of feeders or fibroblast conditioned medium resulted in differentiation but the addition of high concentrations of Fgf2 inhibited this differentiation and maintained the cells in a self-renewing state as demonstrated by morphology, expression of *AP*, *Oct4*, *SSEA*-4 and *Tra1-60* (Wang *et al.*, 2005; Levenstein *et al.*, 2006). The effectiveness of Fgf2 in maintaining self-renewal was comparable to that observed with fibroblast conditioned medium. Cells cultured in Fgf2 maintained pluripotence, demonstrated by the ability to form teratomas in immunodeffient mice (Levenstein *et al.*, 2006).

Analysis of the respective roles of FGF and TGFβ signaling demonstrated that Fgf could not rescue differentiation induced by inhibiting TGFβ/Activin/Nodal signalling using SB431542 (Vallier *et al.*, 2005) suggesting that TGFβ signalling is necessary for mediating Fgf maintenance of pluripotence in hES cells. Similarly, TGFβ signaling alone was unable maintain pluripotence in long term cultures, suggesting that both FGF and TGFβ signalling are required for the maintenance of pluripotent human ES cells in culture. Two modes of action for Fgf in the maintenance of pluripotence have been demonstrated. Fgf has been shown to synergise with the BMP antagonist Noggin in the repression of BMP signalling and maintenance of self-renewal in the absence of feeders (Xu *et al.*, 2005). Fgf2 also acts through modulating the expression of TGFβ ligands that act on hESCs to maintain the undifferentiated state (Grebner *et al.*, 2006).

1.3.8.3 Canonical Wnt/β-catenin signalling

The undifferentiated state of hES cells was maintained in the presence of BIO, an inhibitor of GSK3β and an activator of canonical Wnt signalling (Sato *et al.*, 2005), a situation similar to that previously described for mouse ES cells (Section 1.3.4). Undifferentiated morphology, *Oct4*, *Rex1* and *Nanog* expression were comparable between hES cells grown in CM or BIO. Similarly treatment of hES cells with recombinant Wnt3a resulted in maintenance of colonies with compact undifferentiated morphology and high expression of *Oct4* (Sato *et al.*, 2005).

However, findings of Dravid *et al.*, (2006) contradict the above results suggesting that Wnt/β-catenin signalling is not sufficient for the maintenance of pluripotent state of hES cells in the absence of feeders. The addition of Wnt antagonists Frizzled-Related Protein2 and Dickoppf-1 to hES cells growing on feeders did not abolish the formation of AP positive colonies which retained expression of *SSEA-4* in extended culture (Dravid *et al.*, 2006). Similarly addition of Wnt3a to hES cells grown on non-supportive feeders did not allow maintenance of undifferentiated hES cells.

1.4 TRANSCRIPTION FACTOR NETWORKS CONTROLLING PLURIPOTENCE

1.4.1 Oct4 and maintenance of ES-cell state

The POU family transcription factor *Oct4* is a key regulator of pluripotence. It was first identified in embryonal carcinoma (EC) cells as a transcription factor that bound an element responsible for the undifferentiated phenotype (Okamoto *et al.*, 1990). *Oct4* is also expressed in ES cells (Nichols *et al.*, 1998) and EPL cells (Pelton *et al.*, 2002) and in the equivalent *in vivo* populations, ICM and primitive ectoderm (Niwa, 2001). *In vivo*, *Oct4* is also expressed in other pluripotent cells including oocytes, early cleavage-stage embryos and germ-line cells and its expression is strongly down-regulated in nearly all other cell types (Scholer *et al.*, 1990; Palmieri *et al.*, 1994; Saijoh *et al.*, 1996; Pelton *et al.*, 2002). The requirement of *Oct4* for pluripotence and normal development was demonstrated by gene deletion studies. *Oct4*$^{-/-}$ embryos do not develop because the ICM differentiates to trophectoderm. *Oct4* expression is reduced in the pluripotent cells differentiating to trophectoderm and is briefly elevated in cells differentiating to primitive endoderm (Palmieri *et al.*, 1994). Consistent with this, changes in *Oct4* expression levels in ES cells can be used to direct their differentiation to these lineages: Conditional mutants with 50% increase in levels of Oct4 expression resulted in ES-cell differentiation to endoderm and mesoderm lineages, while a 50% decrease induced the formation of trophectoderm (Figure 1.11) (Niwa *et al.*, 2000).

The 5' untranslated region of the Oct4 gene contains a proximal promoter and at least two enhancer elements, the proximal enhancer and the distal enhancer, which regulate the expression of *Oct4*. The distal enhancer appears to be required for expression in ES and ICM cells while the proximal enhancer is important for the expression of Oct4 in the primitive ectoderm (Minucci *et al.*, 1996; Yeom *et al.*, 1996). Upon differentiation, and loss of pluripotence, *Oct4* expression is down-regulated. This down-regulation is mediated, in part, by the binding of germ cell nuclear factor (GCNF) to the proximal promoter region of *Oct4* and GCNF is absent in cells of the germ line where *Oct4* expression is maintained (Fuhrmann *et al.*, 2001).

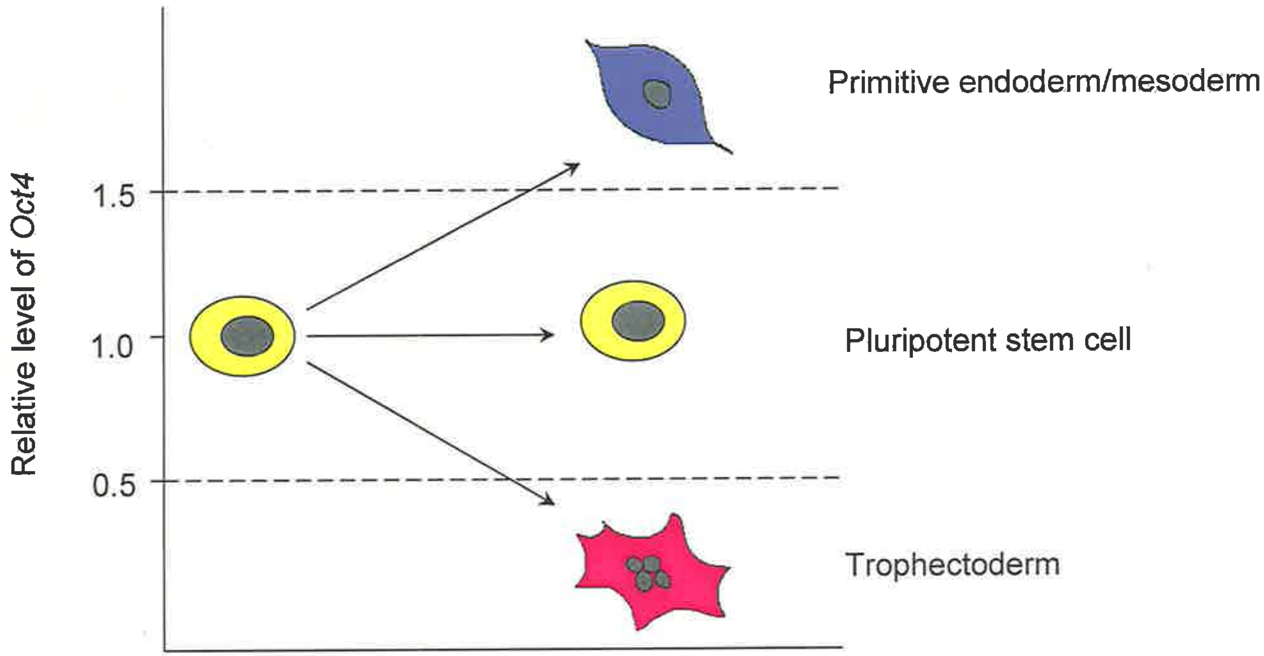
Oct4 is a transcription factor that functions as both an activator and a repressor of gene expression depending on the co-factors present (Niwa, 2001). Oct4 binds sequences containing the octamer motif ATGCAAAT as well as other AT-rich sequences (Saijoh *et al.*, 1996, Okamoto *et al.*, 1990) but high affinity binding is influenced by the presence of co-factor binding sites, such as those for Sox2. For example, Oct4 and Sox2 induce the

Figure 1.11

Oct4 levels direct differentiation of ES cells

Changes in *Oct4* expression levels in ES cells are able to direct differentiation to different lineages. Maintenance of steady levels of Oct4 allows self-renewal of ES cells. Conditional mutants with 50% increase in levels of Oct4 expression result in ES-cell differentiation to endoderm and mesoderm lineages, while a 50% decrease induces the formation of trophectoderm. Adapted from Niwa *et al.*, 2000.

Figure 1.11



Relative level of *Oct4*
1.5
1.0
0.5
Primitive endoderm/mesoderm
Pluripotent stem cell
Trophectoderm

expression of the *Fgf4* gene by acting synergistically at the *Fgf4* promoter (Yuan *et al.*, 1995; Ambrosetti *et al.*, 1997).

Although *Oct4* is required for pluripotence it is not sufficient to maintain it as its expression does not circumvent the requirement for LIF: ES cells constitutively expressing *Oct4* from a transgene still differentiate upon the withdrawal of LIF (Niwa *et al.*, 2000).

1.4.2 Sox2

At the blastocyst stage *Sox2* expression is detected in the cells of the ICM and maintained in the cells of the epiblast as well as the extraembryonic ectoderm and germ cells (Rappolee *et al.*, 1994; Avilion *et al.*, 2003). Homozygous mutant *Sox2*$^{-/-}$ embryos display an embryonic lethal phenotype with death occurring immediately after implantation (Avilion *et al.*, 2003). Embryos appear to require *Sox2* expression for the maintenance of epiblast cell identity as, in the absence of *Sox2*, the cells differentiate into trophectoderm or extraembryonic endoderm (Avilion *et al.*, 2003). ES cell lines can not be derived from *Sox2*$^{-/-}$ embryos suggesting a role for Sox2 in maintaining pluripotence of ICM cells (Avilion *et al.*, 2003).

1.4.3 Foxd3

Foxd3 is required for maintenance of the pluripotent cells of the embryo. *Foxd3*$^{-/-}$ embryos show abnormalities at around 6.5 dpc with a reduced size of the epiblast and lack of primitive streak culminating in lethality by 13.5 dpc (Hanna *et al.*, 2002). The expression of *Foxd3* was necessary for derivation of ES cells as no *Foxd3*$^{-/-}$ lines could be established. The ICM of *Foxd3*$^{-/-}$ blastocysts cultured *in vitro* failed to proliferate and expand suggesting that *Foxd3* is required to maintain the ICM. These data indicate that *Foxd3* plays a crucial role in the maintenance of the epiblast and self-renewal of ES cells (Hanna *et al.*, 2002). The *Foxd3*$^{-/-}$ phenotype could not be rescued by the exogenous addition of Fgf4 and the abnormalities were not a result of effects of *Oct4* and *Sox2* expression since normal expression of these two genes was maintained in blastocysts. However, *Foxd3* and *Oct4* have been shown to physically associate at defined regions of promoters and therefore appear to co-operate in regulating gene expression (Guo *et al.*, 2002). It has been suggested that a lack of *Foxd3* may result in the inability of the cells of the epiblast and ICM to respond to *Fgf4* (Hanna *et al.*, 2002).

1.4.4 Nanog

Nanog is the only gene shown to be involved in ES-cell self-renewal without the need for an active LIF/STAT pathway. Within the embryo, *Nanog* expression was detected in the compacted morula and the ICM and was down-regulated by the time of implantation (Chambers *et al.*, 2003). Over-expression of *Nanog* allowed clonal propagation of ES cells in the absence of LIF. *Nanog* was shown to work independently of the JAK/STAT pathway as *Nanog* over-expressing cells maintained normal levels of STAT3 phosphorylation and could be maintained in the presence of a JAK/STAT inhibitor under conditions that led to differentiation of the parental ES cells (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). ES cells over-expressing Nanog also appeared to be resistant to retinoic acid-induced differentiation while

Nanog$^{-/-}$ embryos show peri-implantation arrest and are not able to maintain pluripotent cells with ICM lineage differentiating to parietal endoderm-like cells (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). This indicates that *Nanog* expression promotes self-renewal and represses differentiation.

Nanog over-expression did not have an effect on ERK activation, as phospho-ERK levels were unchanged between normal and *Nanog* over-expressing cells (Chambers *et al.*, 2003). Also, inhibition of MAPK signalling following LIF stimulation did not alter *Nanog* mRNA levels. This suggests that MAPK signalling is not involved in *Nanog*-mediated maintenance of ES cells.

However, *Nanog* over-expression could not compensate for the absence of *Oct4*. *Oct4*$^{-/-}$ ES cells, maintained by the presence of a doxycycline-responsive *Oct4* transgene, could not be maintained with over-expressed *Nanog* in the absence of doxycycline. This suggests that *Nanog* is able to act independently of *STAT3* in maintaining self-renewal but not independently of *Oct4* (Chambers *et al.*, 2003).

Inactivation of one *Nanog* allele, which led to 50% reduction in expression of Nanog protein, resulted in ES cells that could not be maintained in the undifferentiated state in the presence of LIF. However, self-renewal could be rescued by heterologous expression of Nanog. Therefore, *Nanog* expression levels appear to be crucial for self-renewal of ES cells (Hatano *et al.*, 2005).

The expression of Nanog in pluripotent cells has been shown to require the presence of conserved Oct4 and Sox binding sites in its promoter (Kuroda *et al.*, 2005). Factors shown to bind these sites in embryonal carcinoma F9 cells and embryonic germ cells from 12.5 dpc embryos included Oct4 and Sox2. However, extracts from ES cells showed binding of an unidentified Sox-element binding protein together with Oct4 on the Nanog promoter (Kuroda *et al.*, 2005). The factor p53 is also able to regulate the expression of *Nanog* by binding the Nanog promoter and suppressing transcription following DNA damage, an effect that appears to require recruitment of co-repressor mSin3a. This suggests that p53 assists in maintenance of ES-cell genetic stability by inducing differentiation to cell types sensitive to p53-dependent apoptosis (Lin *et al.*, 2004).

1.4.5 Transcription factor interactions maintaining the pluripotent state

Oct4, Nanog, Sox2 and FoxD3 are transcription factors required for maintenance of ES cells and cells of the ICM (Section 1.4.1-1.4.4) and they exhibit co-regulation, thus establishing a network required for pluripotence. Oct4 regulates the expression of Nanog in a biphasic manner with steady state levels inducing and elevated levels inhibiting Nanog expression (Rodda *et al.*, 2005; Pan *et al.*, 2006). FoxD3 on the other hand is a positive regulator of Nanog expression (Pan *et al.*, 2006). Nanog and FoxD3 both act as inducers of Oct4, while Oct4 behaves as a negative regulator of its own expression (Pan *et al.*, 2006). BothOct4 and Nanog bind the *Sox2* promoter and reduction of Oct4 or Nanog levels leads to concomitant down-regulation of Sox2 expression (Tomioka *et al.*, 2002; Catena *et al.*, 2004; Loh *et al.*, 2006). The suppression of Nanog by Oct4 explains the reason behind the identical endodermal differentiation phenotype observed with Oct4 over-expression or loss of Nanog.

1.5 AIMS AND SIGNIFICANCE

As described in this chapter, self-renewal of ES cells is quite well understood in terms of the factors required, signalling pathways activated and molecular mechanisms involved. On the other hand, signalling cascades involved in the formation and maintenance of EPL cells have not been as intensively investigated. Similarly, the transition of ICM to primitive ectoderm is quite poorly understood with the available information largely restricted to differences in gene expression profiles between the two cell types while the molecular mechanisms involved in the transition remain largely elusive. The *in vitro* model of primitive ectoderm formation, the ES-to-EPL cell transition, enables us to tackle these questions and delineate the molecular mechanism involved in the formation of EPL cells. The transition is known to require the presence of the bioactive component in MEDII, L-proline, but the molecular mechanism by which L-proline induces this transition has not been investigated.

The aim of this thesis was to investigate mechanisms involved in the L-proline-induced EPL-cell formation and identification of inhibitors of the transition. This involved the investigation of the mode of action of L-proline and the analysis of signalling pathways involved in the induction of EPL-cell morphology, gene expression and differentiation potential. This thesis also investigated the ability of small L-proline-containing peptides, which have been shown to induce EPL-cell morphology, to induce associated gene expression and differentiation potential.

Delineating the mechanisms of action and the signalling pathways involved in the ES-to-EPL cell transition has the potential to provide greater understanding of the mechanism controlling this early stage of development. The work also provides information that will allow control over the directed production of homogenous populations of specific cell types that have applications in the cell-based treatments of various disease conditions. Identification of inhibitors of the transition will allow greater control over the maintenance of ES cells, which may be particularly useful in human ES-cell work since human ES cells are prone to spontaneous differentiation. Thus, the mapping and manipulating of L-proline-dependent mechanisms of action can potentially provide a means for controlling the maintenance of pluripotence and switching to directed differentiation in a homogenous and synchronous manner. The manipulation can be done by directly targeting the identified signalling pathways involved in the establishment of EPL cells or by utilising the small, non-toxic, organic inhibitors of the transition also identified in this thesis.

CHAPTER 2:

MATERIALS AND METHODS

Abbreviations

APS – ammonium persulfate solution

β-Me – beta mecaptoethanol

BMP – bone morphogenic protein

Dnmt3b – DNA methyltransferase 3b

EBM $^{1-9}$ – embryoid body *made in* MEDII 1-9 days of differentiation

EPL – early-primitive ectoderm-like

ES – embryonic stem

ICM – inner cell mass

MEF – mouse embryonic fibroblast

NK – Neurokinin

4OHT – 4-hydroxytamoxifen

PAT – proton/amino acid transporter

SAT – sodium/amino acid transporter

CHAPTER 2: MATERIALS AND METHODS

2.1 ABBREVIATIONS

Abbreviations are as described in "Instructions to authors" (1978) *Biochem. J* 169:1-27. Additional abbreviations are as follows:

Ala	L-alanine
AP	Alkaline phosphatase
β-Me	β-mecaptoethanol
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	Base pairs
BSA	Bovine serum albumin (fraction V)
cDNA	Complementary DNA
CM	Conditioned medium
DIG	Digoxygenin
DMEM	Dulbecco's Modified Eagles Medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetracatic acid
EPL	Early primitive ectoderm-like
ES	Embryonic stem
EtBr	Ethidium bromide

EtOH	Ethanol 100%
FCS	Foetal calf serum
Gly	glycine
kb	kilobase
LIF	Leukaemia inhibitory factor
M	Moles per litre
MeAIB	α-(Methylamino)isobutyric acid
mM	Millimoles per litre
μM	Micromoles per litre
μl	Microlitre
min	Minute
mg	Milligram
ml	Millilitre
MOPS	3-{N-Morpholino} propane-sulfonic acid
MQ	Milli-Q
Mr	Relative molecular weight
mRNA	Messenger RNA
MW	Molecular weight
NBT	4-nitroblue tetrazolium chloride
NP40	Nonidet P-40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMSF	Phenyl Methyl Sulfonyl Fluoride

Pro	L-proline
RNA	Ribonucleic acid
RNAsin	Ribonuclease inhibitor
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SP	Substance P
SPCOOH	Substance P free acid
SP(1-7)	Substance P N-terminal 1-7 residues
sec	Second
TAE	Tris acetate EDTA
TEMED	N, N, N', N'-tetramethyl-ethenediamine
tRNA	Transfer RNA
Tween-20	Polyoxyethylene-sorbitan monolaurate
U	Units
UV	Ultra violet
V	Volts

2.2 MOLECULAR MATERIALS

2.2.1 Cell culture reagents

DMEM (Gibco)

FCS (Commonwealth Serum Laboratories)

β-mecaptoethanol (Sigma)

Trypsin/EDTA (Sigma)

2.2.2 Amino acids and peptides

All amino acids and peptides purchased from Sigma

Ala-pro (L-alanine-L-proline)

Gly-pro (glycine-L-proline)

Pro-ala (L-proline-L-alanine)

Pro-gly (L-proline-glycine)

SPCOOH, SP(1-7)

α-(Methylamino)isobutyric acid

glycine, L-lysine, L-proline, L-serine

2.2.3 Chemical inhibitors

L-732,138 [N-Acetyl-L-tryptophan3,5-bis(tirfluromethyl)benzyl ester] (Sigma)

PD098059 [2-(2-Amino-3-methylphenyl)-4H-1-benzopyran-4-one] (Sigma)

U0126 [1,4-Diamno-2,3-dicyno-1,4-bis(o-aminophenylmercapto)butadine] (Sigma)

LY-294002 [2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride] (Sigma)

2.2.4 Antibodies

Anti-digoxigenin-AP, Fab fragment (Roche)

Anti-β-actin (C-11): sc-1615 (Santa Cruz Biotechnology)

Anti-phospho-Akt (Ser473) rabbit antibody (Cell Signaling Technology)

Anti-phospho-Stat3 (Tyr705) (3E2) mouse mAb (Cell Signaling Technology)

Anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) rabbit antibody (Cell Signaling Technology)

2.2.5 Kits

Omniscript reverse transcriptase kit (Qiagen)

MinElute gel extraction kit (Qiagen)

QIAprep miniprep (Qiagen)

Quantum Prep Plasmid Midiprep Kit (Bio Rad)

Platinum PCR SuperMix (Invitrogen)

pGEM-T Easy Vector System I (Promega)

2.2.6 Enzymes

BamHI, EcoRI, HindIII, PstI, XbaI, XhoI (New England Biolabs)

T7, T3, SP6 RNA polymerases, *E. coli* DNA polymerase I (Klenow fragment), RNasin (Roche)

2.2.7 Plasmids

mGAP: Mouse *glyceraldehyde 3-phosphate dehydrogenase* cDNA clone in pGEM3Z contained a 300 bp *HindIII/PstI* fragment from the 5' end of the mouse gene (Rathjen *et al.*, 1990)

Oct4: The *Oct4* cDNA clone in pBluescript contained a 462 bp *StuI* cDNA fragment of positions 491 to 953 of the *Oct4* cDNA sequence (Scholer *et al.*, 1990).

Rex1: An 848 bp *Rex1*-containing fragment in pCR^{TM}II cloned into *XhoI* site (Hosler *et al.*, 1989).

CRTR1: Full-length *CRTR1* open reading frame (1446 bp) cloned into *HindIII/SacI* site of pGEMT provided by Stephen Rodda.

2.2.8 Markers

2.2.8.1 DNA markers

1 kb+ ladder (Invitrogen)

Band sizes (kb): 100, 200, 300, 400, 500, 650, 850, 1000, 1650, 2000, 5000

2.2.8.2 Protein markers

Benchmark™ prestained protein ladder (Invitrogen)

Band sizes (Mr in kDa): 6, 14.8, 19.4, 25.9, 37.1, 48.8, 64.2, 82.2, 115.5, 181.8

2.2.9 General reagents

^{32}P α dATP (Perkin-Elmer)

Agarose (Sigma)

AP substrate (Amersham Biotech)

BCIP (Sigma)

Bradford (Bio Rad)

Complete mini–protease inhibitor cocktail tablets (Roche)

DIG RNA labeling mix, 10X (Roche)

DMSO (Sigma)

ECF substrate (Amersham Biotech)

Ionomycin (Sigma)

NBT (Sigma)

Oligo-dT_{12-18} primer (Invitrogen)

Phosphatase inhibitor cocktail 1 (P2850) and 2 (5726) (Sigma)

Platinum SYBR Green qPCR SuperMix UDG (Invitrogen)

RNAwiz (Ambion)

SUPERase-In (Ambion)

Tween-20 (Sigma)

2.3 BUFFERS AND SOLUTIONS

Acetate buffer:	3 M Kac, 2 M HOAc, pH 5.8
1° antibody solution:	1X TBST, 5% (w/v) BSA
2° antibody solution:	1X TBST, 5% (w/v) non-fat dry milk
AP buffer:	100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris HCl pH 9.5, 0.1% (v/v) Tween-20
Blocking solution:	1X TBST, 5% (w/v) non-fat dry milk
β-mecaptoethanol/PBS:	100 mM β-mecaptoethanol in 14 ml PBS (made fresh every 14 days)
CHURCH buffer:	0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA, 50 μg/ml ssDNA (Hering sperm DNA)
Development solution:	AP buffer (10 ml) + 33 μl NBT (75 mg/ml), 66 μl BCIP (50 mg/ml)
Ficoll:	1X MOPS, 18.5% formaldehyde, 50% formamide, 4% Ficoll400, bromophenol blue
10 X GLB:	50% (v/v) glycerol, 0.1% (w/v) SDS, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol
In situ post hybridisation buffer:	2X SSC, 50% (v/v) formamide, 0.1% (v/v) Tween-20

LB:	1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0
5X ligation buffer:	250 mM Tris HCl pH 7.5, 25% (w/v) PEG 6000, 50 mM $MgCl_2$, 5 mM rATP, 5 mM DTT
Lysis buffer:	50 mM Tris HCl pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 10 mM EDTA, 100 µl phosphatase inhibitor cocktail 1/2, 1 complete mini protease inhibitor tablet
10X MOPS:	0.4 M morpholinopropanesulfonic acid (free acid), 0.1 M sodium acetate, 10 mM EDTA adjusted to pH to 7.2 with NaOH
PBS:	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM $NaHPO_4$, pH 7.4
PBS/gelatin:	0.2% (w/v) gelatin in PBS
PBT:	PBS + 0.1% (v/v) Tween-20
4% PFA:	20% (w/v) PFA pH 7 in 70% (v/v) ethanol
Post-hybridisation wash:	2X SSC, 0.1% SDS
Pre- hybridisation wash:	1X SSC, 0.1% SDS
2X SDS load buffer:	125 mM Tris HCl pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 5% (v/v) β-mecaptoethanol
SDS-PAGE buffer:	25 mM Tris-Glycine, 0.1% (w/v) SDS
20X SSC:	3 M NaCl, 0.3 M sodium citrate
Strip-solution:	0.2X SSC, 0.1% SDS

TAE:	40 mM Tris acetate, 20 mM NaAc, 1 mM EDTA, pH 8.2
TBS:	25 mM Tris HCl pH 8, 150 mM NaCl
TBST:	TBS + 0.1% (v/v) Tween-20
TEN buffer:	40 mM Tris HCl pH 7.4, 1 mM EDTA, 150 mM NaCl
Transcription buffer:	40 mM Tris HCl pH 8, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT
Trypan blue:	0.4 g trypan blue, 0.6 g KH_2PO_4, in 100 ml MQ H_2O
RIPA:	150 mM NaCl, 1 mM EDTA, 50 mM Tris HCl pH 8, 1% (v/v) NP-40, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS
10X Versene buffer:	80 g NaCl, 2 g KCl, 2 g EDTA, 2 g KH_2PO_4, 11.5 g N_2HPO_4
Western transfer buffer:	39 mM glycine, 48 mM Tris HCl pH 8.3, 0.037% (w/v) SDS, 20% (v/v) methanol

2.4 OLIGONUCLEOTIDES

DNA primers were synthesised by Geneworks Ltd.

2.4.1 Sequencing primers

Downstream sequencing primer	ATTTAGGTGACACTATAGAA
Upstream sequencing primer	TAATACGACTCACTATAGGG

2.4.2 Conventional PCR primers

Actin	F	ATGGATGACGATATCGCTG
	R	ATGAGGTAGTCTGTCAGGT
NK1R	F	CGTGGTTGTG TGTACCT TCG
	R	ACAGTACCAGAAGCTCAACG
NK2R	F	TCATCTGGTGGTGTTTGTCC
	R	TCCCCAGGATGAAGTAGAGG
NK3R	F	TCTCTGGGGAGGAGAGATCC
	R	GGGGTTGTACATGGTTGAGC
SAT1	F	GATGGATGCTTCTCCTGA
	R	CCAGGATAATGCCAATGA
SAT2	F	GTCCCTTGTCCTCATTCTTCC
	R	AACGTCAGGATGGGTACTGC
PAT1	F	ACATCAGCATGTTCGTCAGC
	R	CAGCTGCGACATAGAACTGG
PAT2	F	CTGGACCTTCCTGAGAGTGC
	R	TCCCCATAGTCCATGAAAGG
PROT	F	CCCCCTCTTCTTTCTTGAGC
	R	CACCCCATTACCACTCTTGG
Dnmt3b1	F	CGACAGGCTTGGGCTG
	R	GTGGGCCCACTCCAGC

2.4.3 Quantitative PCR primers

Actin	F	CTGCCTGACGGCCAGG
	R	GATTCCATACCCAAGAAGGAAGG

Brachyury	F	TGCTGCCTGTGAGTCATAAC
	R	GCCTCGAAAGAACTGAGCTC
CRTR1	F	ATGTGAGGCCAAAGATGACC
	R	TGTGCTGAGGACAAAACAGG
Dnmt3b1	F	CGACAGGCTTGGGCTG
	R	GTGGGCCCACTCCAGC
Lefty2	F	TGTATTCTCAGTGAGCTT
	R	GCAGTCCCTGACATGGTA
Oct4	F	CCCAGGCCGACGTGG
	R	GATGGTGGTCTGGCTGAACAC
Rex1	F	TGCCTCCAAGTGTTGTCCC
	R	ATTCATGTTGTCTTAGCTGCTTCC

2.5 TISSUE CULTURE MEDIA

DMEM:	Dulbecco's Modified Eagle Medium (1X) 4.5 g/l D-glucose and sodium pyruvate
ES complete medium:	DMEM, 10% (v/v) FCS, 1000 U LIF, 0.1 mM β-Me
ICβ medium:	DMEM, 10% (v/v) FCS, 0.1 mM β-Me

2.6 TISSUE CULTURE METHODS

2.6.1 LIF production

COS-1 cells were transfected with mouse LIF expression plasmid pDR10 as described by Smith (1991) with the following modifications. Transfection was performed via electroporation using Bio Rad Gene Pulsar at 270 V and a capacitance of 250 μD. Transfected cells were plated at $7x10^4$ cells /cm^2 in

DMEM, pH 7.4, containing high glucose and supplemented with 10% FCS. Medium was collected and assayed for LIF expression as described by Smith (1991).

2.6.2 MEDII production

HepG2 cells (Knowles *et al.*, 1980) were maintained in culture in DMEM supplemented with 10% FCS and passaged to confluence. For production of conditioned medium (MEDII) HepG2 cells were seeded into DMEM supplemented with 10% FCS at a density of $5x10^4$ cells/cm^2. Medium was collected after 5 days, sterilized by filtration through a 0.22 μm membrane and supplemented with 0.1 mM β-Me before use. Medium was stored for up to 2 weeks at 4°C.

2.6.3 ES-cell culture

Feeder-independent D3 ES (Doetschman *et al.*, 1985) cells were cultured as described in Rathjen and Rathjen (2003) in the presence of 1000 U/ml mouse leukemia inhibitory factor (LIF). Briefly, cells were seeded at 10^6 cells in 10 ml ES complete medium in 10 cm tissue culture dishes, re-fed on day 2 and passaged on day 3 using Trypsin/EDTA (Sigma).

2.6.4 EPL-cell formation

Prior to EPL-cell formation the ES cells were passaged once on gelatin. ES cells were seeded at $3x10^5$ cells per 3 ml ES complete medium supplemented with 200 μM L-proline or 50% MEDII ± 1000 U/ml LIF in 6 cm tissue culture dish (pre-coated with gelatin for 30 min and washed with PBS). Cells were fed daily with ES complete medium supplemented with 200 μM L-proline or 50% MEDII ± 1000 U/ml LIF and passaged on days 2 and 4 with samples collected for analysis on days 0, 2, 4 and 6.

2.6.5 Morphology assay

ES cells were seeded at low density (1000 cells) into 1 ml ES complete medium alone or supplemented with 200 μM L-proline, or the peptide being tested at the concentration indicated, in gelatin-coated wells of 24 well trays. The cells were cultured for 5 days following which the morphology of the colonies was assessed and the colonies photographed under phase contrast using 100X or 200X magnification.

2.6.6 Embryoid body formation

ES cells were seeded at 3.5×10^5 cells per 3 ml ES complete medium alone or supplemented with 200 μM L-proline or 50% MEDII ± 1000 U/ml LIF in 6 cm tissue culture dishes (pre-coated with gelatin for 30 min and washed with PBS). Cells were fed daily and passaged on day 2. On day 4, the cells were trypsinised to a single-cell suspension and seeded at 3×10^5 cells into 3 ml ICβ medium in 3 cm bacterial dishes. Bodies were cultured for 4 days with a re-feeding on day 2.

2.7 MOLECULAR BIOLOGY TECHNIQUES

2.7.1 Agarose gel electrophoresis

Electrophoresis of DNA or RNA was performed using 2% (w/v) agarose gels in TAE using horizontal mini gels prepared by pouring 10 ml agarose on to 7.5

cm x 5 cm glass slides. Electrophoresis was carried out at 80-100 V. RNA samples were loaded with Ficoll load buffer while DNA samples were loaded with GLB. Gels were stained with ethidium bromide, visualised under UV and photographed.

2.7.2 Restriction endonuclease digestion

Plasmid DNA was digested in the buffer suggested by the manufacturer and 2 U enzyme per 1 µg DNA for 2 h at the appropriate temperature. Complete digestion was determined by agarose gel electrophoresis.

2.7.3 Sequencing

Sequencing reactions were performed using 4 µl Big Dye mix, 500-1000 ng plasmid DNA, 100 ng sequencing primer in 20 µl reactions in 0.5 ml PCR tubes. The cycling parameters were 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. The reaction was cycled 25 times on a PT200 thermal cycler. Following the completion of the sequencing reaction the DNA was ethanol precipitated, dried and sent for analysis at the Institute of Medical and Veterinary Science DNA sequencing facility.

2.7.4 Reverse transcription

Preparation of cDNA was performed using Omniscript reverse transcriptase kit and oligo-dT. RNA (1 µg) was combined with 10X RT buffer, dNTP mix (5 mM each dNTP), Oligo-dT primer (10 µM), RNase inhibitor (10 U/µl) and Omniscript reverse transcriptase in a 20 µl reaction on ice. The reaction was mixed by vortexing for 5 s and centrifuging briefly to collect residual liquid. The reactions were incubated at 37 °C for 60 min.

2.7.5 Polymerase chain reaction (PCR)

PCR was performed using 1 µl cDNA, 200 ng each of specific forward and reverse primers and 12 µl platinum PCR supermix. Each of the primers was optimised to ensure amplification was in the linear range by determining the cycle number at which the product can first be detected. PCR was performed on PT200 thermal cycler.

2.7.6 Quantitative PCR

Quantitative PCR was performed using 1 µl cDNA, 5 µM each of specific forward and reverse primers and 12 µl SYBR Green mix. Primer efficiency was determined for each primer set using qGene software by performing PCR on serial diluted template. PCR was performed on triplicates with cycling parameters of 96 °C for 30 sec, 60 °C for 30 sec, 72 °C for 45 sec. The reaction was cycled 40 times. PCR was performed on PT200 thermal cycler. The generated threshold values were analysed by qGene software.

2.7.7 Transformation of competent bacterial cells

For transformation, competent DH5α cells were thawed on ice and 50 µl cells was aliquoted into a pre-chilled Eppendorf and mixed with 5 µl plasmid (~5 ng). The cells were incubated on ice for 30 min and then heat shocked at 37°C for 5 min. LB (500 µl) was added and the cells further incubated with shaking at 37°C for 1 h. The cells were pelleted by centrifugation at 3000 g and 450 µl supernatant discarded. The cells were resuspended in 50 µl LB and plated on LB coated plates containing 100 mg ampicillin and incubated overnight at 37 °C.

2.7.8 DNA preparation

2.7.8.1 Small-scale preparation

Performed using QIAprep miniprep kit (Qiagen). LB (3 ml) was inoculated with plasmid DNA and incubated in a rotator overnight at 37 °C. The bacterial cells were pelleted and resuspended in 250 µl Buffer P1 and Buffer P2 added and mixed gently. Solution was neutralised by addition of 350 µl Buffer N3. The precipitate was removed by centrifugation for 10 min at 14000 rpm. The supernatant was applied to a QIAprep column and centrifuged for 60 s. The bound DNA was washed with subsequent additions of buffer PB and PE and centrifugation. The DNA was eluted with 50 µl water into a clean Eppendorf.

2.7.8.2 Large-scale preparation

Performed using Quantum Prep Plasmid Midiprep Kit (Bio rad). LB broth (3 ml) was inoculated with plasmid DNA and incubated in a rotator for 8 h at 37 °C. Of this inoculate, 100 µl was added to 25 ml LB broth in a 250 ml flask and incubated overnight at 37°C in a rotary shaker. Following incubation, the cells were pelleted by centrifugation for 15 min at 6000 rpm and the supernatant discarded. The cells were resuspended by vortexing in 5 ml Cell Resuspension Solution. Following this, 5 ml of Cell Lysis solution was added and the solution mixed by inverting the tube 8 times. Neutralisation Solution was added and mixed by inverting the tube 8 times. The white precipitate was

Following addition of 500 μl of water the DNA was eluted from the column via centrifugation.

pelleted by centrifugation for 10 min at 8000 rpm, the supernatant transferred into a clean tube and mixed with 1 ml Quantum Prep matrix by shaking. The matrix was pelleted by centrifugation for 2 min at 8000 rpm and the supernatant discarded. The matrix was washed in 600 µl wash buffer twice prior to addition to a spin column. The spin column was placed in a collection tube and centrifuged for 30 s, the wash buffer discarded and matrix rinsed in a further 500 µl of wash buffer. Centrifugation following addition of 500 µl of water to the column eluted the DNA.

2.7.8.3 Gel extraction of DNA

Performed using MinElute gel extraction kit (Qiagen). Following agarose-gel electrophoresis DNA bands were visualised under long-wave UV and excised. The agarose was resuspended in 3 vol buffer QG to 1 vol of gel by incubation at 50 °C with vortexing. Isopropanol (1 gel volume) was added to the dissolved agarose and the tube inverted several times. The solution was applied to a MinElute column in a 2 ml collection tube and centrifuged for 1 min. The flow through was discarded and the bound DNA washed with subsequent additions of 500 µl of buffer QG and 750 µl buffer PE and centrifugation for 1 min. The flow through was discarded and the column centrifuged for an additional 1 min. The spin column was transferred to a clean 1 ml collection tube and the DNA eluted in to 10 µl of MQ water by incubation for 1 min followed by centrifugation for 1 min.

2.8 CELL FIXATION

Cells were washed 3X with PBS and then incubated with 4% PFA in ethanol for 15 min at room temperature. Fixation solution was then removed and cells dehydrated with subsequent additions of 50%, 70% and 90% ethanol diluted in PBS. The fixed and dehydrated cells were stored in 90% ethanol at 4 °C until use.

2.9 RNA ANALYSIS

2.9.1 RNA extraction

RNA extraction was performed using RNAwiz extraction reagent. Cells were resuspended in RNA isolation reagent and incubated at room temperature for 5 min. Chloroform, 0.2X starting volume, was added to the cell homogenate the samples mixed vigorously for 20 s and incubated at room temperature for 10 min. The mixture was centrifuged at 14,000 rpm for 15 min at 4 °C. The top phase was transferred into a clean Eppendorf and mixed with 1X starting volume of isopropanol and incubated at room temperature for 15 min. This was followed by centrifugation at 14,000 rpm for 15 min at 4 °C to pellet the RNA. The supernatant was removed and the pellet washed with 1X starting volume of ice cold 75% ethanol followed by centrifugation for 5 min at 14,000 rpm at 4°C. Following centrifugation the supernatant was again discarded and the pellet allowed to air dry prior to resuspending in RNase free water. RNA concentration was determined using spectrometer readings at OD_{260}.

2.9.2 Northern blot

2.9.2.1 Northern gel

RNA samples were prepared by combining 5 µg RNA with an equal volume of 2X Ficoll loading buffer, denaturing the RNA by heating at 95ºC for 5 min and then snap cooling the samples on ice. Samples were loaded onto 1.5% agarose gels containing 1X MOPS buffer, 0.6 M formaldehyde and electrophoresed at 80-95 V in buffer containing 1X MOPS and 0.2 M formaldehyde. Following electrophoresis the gels were soaked in water 2X 10 min to remove the formaldehyde.

2.9.2.2 Northern blot transfer

The wicks were set up by placing 3 pieces of Whatman filter paper (pre-wetted in 20X SSC) over a glass gel stand in a Pyrex dish filled with 20X SSC and adding another 3 pieces of Whatman paper perpendicular to the existing sheets ensuring the edges were dipping into the 20X SSC. The gel was placed on the wet Whatman papers and a nitrocellulose membrane placed on top followed by 3 sheets of wet Whatman paper and 6 sheets of dry Whatman paper. Parafilm was used to seal the edges of the stack. Paper towels were added to make a stack ~10 cm high. The whole transfer set up was covered in Gladwrap to prevent drying out and a glass weight placed on top. The transfer was allowed to proceed overnight (minimum 16 h). Following the transfer, the membrane was washed in 2X SSC and UV cross-linked. The membrane was stored in 2X SSC at 4 ºC until probing.

2.9.2.3 Preparation of radioactive RNA probes

Probes were prepared using Decaprime kit. 25 ng DNA template was combined with 2.5 µl of Decaprime solution and heated at 95 °C for 5 min and then snap cooled on ice. 5µl 5X reaction buffer (dATP), 5 µl α-^{32}PdATP (50 µCi), 1 µl exonuclease-free Klenow was added to the solution along with RNase free water to a final volume of 25 µl. The contents was gently mixed and then incubated at 37 °C for 10 min. Following this 25 µl of RNase free water was added and the contents passed though a G50 column by centrifugation for 1 min at 3000 rpm. The isolated RNA probe was denatured at 95 °C for 5 min and snap cooled on ice before being added to the hybridisation solution.

CRTR1: A 736 bp fragment was generated by digestion with XhoI/HindIII

GAPDH: A 300 bp GAPDH fragment was obtained by digestion with HindIII/PstI

Lefty2: A 288 bp fragment generated by PCR with *Lefty2* specific primers (Section 2.4.3)

Oct4: A 462 bp fragment was obtained by digestion with XhoI/HindIII

Rex1: An 848 bp fragment was generated by digestion with EcoRI

2.9.2.4 Probing the membrane

Membranes were placed into hybridisation tubes and incubated with 1X SSC and 0.1% SDS for 30 min at 65°C. Following washing, CHURCH buffer was added and incubated with the membrane for 1-4 h. Northern probes were prepared using the DECAprimeII kit. Following synthesis, the probes were denatured by heating at 95°C for 5 min and then added directly to the CHURCH buffer in the hybridisation tube. The membrane was hybridised with the probe overnight at 65 °C. Following the overnight incubation, the hybridisation buffer was removed and the membrane washed once with 2X SSC and 0.1% SDS at room temperature and then another 2X at 65 °C. The membrane was then sealed in a plastic bag with 2X SSC and exposed to Fuji screen overnight in a developing cassette. The bands were visualised by scanning the Fuji screens in a Bio Rad FX scanner and the intensity of the bands quantitated using Quantity One software.

2.9.2.5 Stripping the membrane

Membranes were stripped by incubation with boiling stripping solution containing 0.2X SSC and 0.1% SDS for 30 min or until no radioactivity could be detected.

2.9.3 Preparation of DIG-labelled in situ probes

Using cleaned linearised template DNA the transcription reaction was set up by combining 1 µg of template with 5X transcription buffer, DTT, 10X DIG label mix, 1 U RNAasin and 2 U RNA polymerase T7 or S6. The reactions were incubated at 37 °C for 2 h. The transcription reaction (20 µl) was made up to 100 µl with water to which 10 µl 3M sodium acetate, pH 5.2 and 250 µl ethanol was added. The solution was incubated at –20°C for 15 min followed by centrifugation at 14,000 rpm for 15 min at 4 °C. The pellet was washed with 70% ethanol, dried and resuspended in 50 µl RNase-free water. For *in situ* hybridisation, 600 ng/ml DIG labelled probe was used.

***Oct4*:** The sense template was generated by Xho digestion and transcripts polymerised with T7 RNA polymerase. The anti-sense template was generated by EcoRI digestion and transcripts produced with T3 RNA polymerase.

***Rex1*:** Sense and anti-sense riboprobes were produced using BamHI or XbaI linearised templates and T7and Sp6 RNA polymerase respectively.

2.9.4 In situ hybridisation

Fixed and dehydrated cells were re-hydrated on ice by sequential additions of 90%, 70%, 50% and 0% ethanol diluted in PBS. Cells were removed from ice and rinsed 3X 5 min in PBT. Cells were permeabilised with 3X 20 min washes in RIPA buffer and fixed for 20 min in 4% PFA, 0.2% gluteraldehyde in PBT. Cells were then washed 3X 5 min in PBT, 5 min in 1:1 ratio of PBT and hybridisation solution and then 5 min in hybridisation solution only. Hybridisation solution containing denatured 100 µg/ml salmon-sperm DNA and tRNA was added to cells sealed in a box humidified with towels soaked in 1:1 ratio of formamide and water. The cells were incubated at 65 °C for 1-5 h. Denatured DIG-probes were then added to the hybridisation solution with salmon sperm DNA (100 µg/ml) and tRNA (100 µg/ml) and added to the sense or anti-sense wells. The cells were returned to the humidified box and incubated overnight at 65 °C. The cells were washed 3X 30 min in post-hybridisation wash buffer at 65 °C, allowed to cool to room temperature and washed 3X 5 min in TBST, then 1 h in TBST with 10% FCS. AP-antiDIG antibody (1:2000 in TBST) and 10% FCS was added to cells and incubated overnight at 4 °C in a box humidified with towels soaked in water. The cells were washed 3X 5 min with TBST at room temperature and further 2 hours in TBST using at least 3 changes of TBST followed by 3X 10 min washes in AP buffer. The development solution, containing BCIP and NBT was added to cells and incubated in the dark for 60 min to 24 h. The development reaction was stopped by washing 3 X 5 min in PBT with 1 mM EDTA.

2.10 PROTEIN ANALYSIS

2.10.1 Lysis of ES and EPL cells

Cells grown in 6 cm dishes were washed once with ice-cold PBS, harvested on ice with TEN buffer and centrifuged 3000 g 2 min, 4 °C. Cell pellets were lysed in 60 µl lysis buffer for 30 min at 4 °C with gentle rocking. Lysates were centrifuged 14000 rpm 10 min, 4 °C and the supernatants were stored at -80 °C until use.

2.10.2 Bradford assay

Protein concentration was determined using the Bradford assay. A 1/10 dilution of sample or BSA standards was added to a 1/5 aqueous dilution of Bradford reagent (40 µl reagent + 160 µl water). The colour reaction was allowed to proceed for 5 min prior to analysis reading OD_{280}. For SDS-PAGE analysis 10 µg of protein was used.

2.10.3 SDS-PAGE analysis

SDS-polyacrylamide gel (10%), containing 1X Tris-SDS buffer, 0.1% APS and 0.1% TEMED, were poured between glass plates using 1 mm spacers, overlayed with MQ water and allowed to polymerise for 30 min. Following polymerisation, the water was removed and a stacker gel (4%), containing 2.5 X Tris-SDS buffer, 0.1% APS and 0.1% TEMED, was poured. 10 or 15 well combs (1 mm) were inserted and the gel allowed to polymerise for 30 min. Prior to loading samples (10 µg protein) were boiled in 2X SDS-load buffer containing 10% β-mecaptoethanol at 100 °C for 3 min and electrophoresed using a Bio Rad minigel apparatus in SDS-PAGE buffer at 140 V. Following electrophoresis, proteins were transferred to nitrocellulose membrane by Western blot.

2.10.4 Western blot analysis

Proteins were transferred from the SDS-polyacrylamide gels to nitrocellulose in Western transfer buffer using a stack consisting of; fibrous pad, 3 Whatman filter papers, gel, nitrocellulose membrane, 3 Whatman filter papers, fibrous pad. All components were pre-soaked in Western transfer buffer. The transfer was performed at 100 V and 350 mA using Biorad minigel Western transfer apparatus. Following transfer, the membrane was blocked by incubation in 5% milk in TBST for 1 h at room temperature or in 5% BSA in TBST overnight at 4 °C if phospho-specific antibodies were to be used. Membrane was incubated with appropriate dilution of primary antibody for 2 h at room temperature, or overnight at 4 °C if phospho-specific antibodies were to be used. Membrane was then washed 3X 5 min in TBST prior to addition of 1/2000 dilution of AP-conjugated secondary antibody and incubated for 1 h at room temperature. Membrane was then washed 1X 10 min and 3X 5 min in TBST. The bands were developed by applying an AP substrate solution to the membrane and incubating for 5 min prior to scanning in a Bio Rad FX scanner.

2.11 ALKALINE PHOSPHATASE STAINING OF CELLS

Cells were seeded into 1 ml medium at 1000 cells per well in 24 well trays. Following 5 days of culture, the cells were washed 3X with PBS and fixed with 500 µl AP-fixation buffer (5 ml citrate buffer, 1.65 ml formaldehyde, 13.35 ml methanol) for 10 min at room temperature and washed 5X with water prior to addition of 500 µl development solution (AP-buffer 10 ml, BCIP 33 µl, NBT 66 µl). Development was allowed to continue for 30 min in the dark and reactions were stopped by washing the cells with water.

CHAPTER 3:

THE INDUCTION OF EPL-CELL FORMATION BY SHORT L-PROLINE-CONTAINING PEPTIDES

CHAPTER 3: THE INDUCTION OF EPL-CELL FORMATION BY SHORT L-PROLINE CONTAINING PEPTIDES

3.1 INTRODUCTION

3.1.1 EPL-cell induction by L-proline and short L-proline-containing peptides

It has previously been established that L-proline is a bioactive component within MEDII that is responsible for the transition of ES-to-EPL cells as measured by morphology, gene expression and differentiation potential (Bettess, 2001). The effect of L-proline appears to be stereospecific since D-proline was not able to induce the morphology change. Various L-proline analogues, which contained modifications of the L-proline structure in the amino region, carboxyl region or the ring structure, were also unable to induce the transition (Table 3.1). Short peptides that did not contain L-proline were likewise unable to induce the morphology change (Table 3.1). On the other hand, various short L-proline-containing peptides including ala-pro, gly-pro, pro-ala, pro-gly and fragments of the neuropeptide substance P (SP) such as SP(1-7) were identified as having the ability to induce EPL-cell morphology. However, the gene-expression profile and differentiation potential of these cells was not determined. Cell morphology alone is not sufficient to establish the formation of EPL cells since it has previously been shown that EPL-cell morphology can occur in the absence of gene expression and differentiation potential changes and *vice versa* (Bettess, 2001; Section 1.2.3.1). Therefore, to determine whether the short L-proline-containing peptides are able to

Table 3.1 Summary of the EPL-cell inductive capacity of amino acids and peptides

Morphological assessment of the ES-to-EPL transition in the presence of various compounds.[1]

Compound	*Concentration range (µM)*	*Activity (µM)* [2]	*Other effects*
AMINO ACID			
L-proline	20–1000	40	
D-Proline	30–3475	–	
L-Alanine	390–3900	–	
L-Lysine	55–5500	–	
Sarcosine	10–1120	–	cell death (50)
PROLINE ANALOG			
L-prolinamide	1–1000	–	
N-acetyl-L-proline	64–636	–	
N-*t*-BOC-L-proline	230–4650	–	
pyrrolidine	10–1000	–	
cis-4-hydroxy-L-proline	80–460	–	cell death
trans-4-hydroxy-L-proline	270–550	–	
3,4-dehydro-L-proline	1–500	–	cell death (100)
L-azetidine-2-carboxylic acid (AZET)	10–1000	–	cell death(100)
L-pipecolic acid	390–15500	–	
PEPTIDE			
Pro-ala	20–1000	50	
Ala-pro	20–1000	80	
Ala-pro-gly	40–1000	40	
Pro-OH-pro	20–1000	40–80	
Pro-gly	20–1000	50	
Gly-pro	20–1000	40	

Gly-pro-ala	20–1000	40	
Gly-pro-OH-pro	40–5850	300	
Gly-pro-arg-pro *(inhibitor of fibrin polymerization)*	40–1000	80	
Gly-pro-gly-gly *(inhibitor of dipeptidyl peptidase IV)*	1–1200	50	
Val-ala-pro-gly	40–1000	40	
Arg-gly-asp (RGD) *(cell attachment domain of fibronectin)*	40–4800	–	
Substance P (RPKPQQFFGLM–NH_2)	0.005–500	–	cell death (50)
Substance P free acid *(RPKPQQFFGLM–COOH)*	7–110	40	
Substance P_{1-4} (RPKP)	40–1000	40	
Bradykinin (RPPGFSPFR)	10–100	40	
Neurokinin A (HKTDSFVGLM–NH_2)	10–100	–	
Senktide (Succinyl-DFMFGLM–NH_2)	10–100	–	

[1] *ES cells were cultured for 5 days in the presence of the various compounds over the concentration range shown then assessed for transition to EPL cells*

[2] *Concentration at which alkaline phosphatase-positive, EPL-like colonies are first observed. A dash indicates no activity over the concentration range tested.*

(Washington *et al.*, submitted)

induce EPL-cell formation, gene expression and differentiation potential of these cells needed to be determined.

3.1.2 Neuropeptide Substance P

Since morphology assays identified the ability of SP fragments to induce EPL-cell morphology, information regarding known mechanisms of SP action may be useful in understanding the mechanism functioning to induce EPL-cell formation.

3.1.2.1 Substance P expression

SP is a peptide belonging to the tachykinin family of neurotransmitter peptides. It is encoded for by the *PPT-A* gene and is synthesised from three distinct mRNA transcripts αPPT-A, βPPT-A and γPPT-A. SP is synthesised as a precursor protein and requires enzymatic processing and C-terminal amidation before it is biologically active (Harrison and Geppetti, 2001). Immunohistochemistry has revealed that SP is primarily expressed within the peripheral and central nervous systems (Khawaja and Rogers, 1996) while early embryonic expression has not been investigated. Activity of neuropeptides is modulated by enzymes that hydrolyse them to inactive products or products with altered biological activity. Metabolism of Substance P has been shown to involve a number of enzymes including Substance P-endopeptidase, angiotensin converting enzyme (ACE), neutral endopeptidase 24.11 (NEP), dipeptidyl-aminopeptidase (DPAP) and post proline endopeptidase (PPEP) (Khawaja and Rogers, 1996; Snijdelaar *et al.*, 2000).

3.1.2.2 *Signalling mediated by SP acts via the Neurokinin family of receptors*

The classic mode of action of SP is via neurokinin (NK) receptors, members of the G protein-coupled family of receptors (Khawaja and Rogers, 1996). There are three known NK receptors, NK1R, NK2R and NK3R, each expressed in the central and peripheral nervous system. SP has the greatest affinity for NK1R (K_d=0.05 nM) but is able to bind all three receptors (Harrison and Geppetti, 2001). The three NK receptors are linked to G_q and G_{11}, pertussis toxin-insensitive G-proteins, which lead to the activation of phospholipase C (PLC) and the production of 1,4,5-inositoltriphosphate (IP_3) and diacylglycerol (DAG) (Harrison and Geppetti, 2001). IP_3 and DAG elevate intracellular calcium levels through release of calcium from intracellular stores and influx through membrane Ca^{2+} channels (Khawaya and Rogers, 1996).

In some cells, the NK receptors have been shown to effect adenylate cyclase activation via G_s (stimulatory) and G_i (inhibitory) proteins (Khawaja and Rogers, 1996).

3.1.2.3 *Substance P - NK1 receptor interaction*

Mutagenesis and radiolabelling studies have identified a hydrophobic pocket, between transmembrane domain (TMD) TMDII and TMDVII of the NK1 receptor, where SP is believed to be able to insert itself and make contacts with the loops on the extracellular side of the receptor (Harrison and Geppetti, 2001). A computer simulation of SP binding to the NK1 receptor has been performed based on mutagenesis data (Huang *et al.*, 1994) and photolabelling (Lequin *et al.*, 2002). Results of the simulation showed that the N-terminal part of SP (NH_2-Arg-Pro-Lys-Pro) is pointed toward TMDI while the C-terminal portion (Gly-Met-Leu-NH2) is directed to TMDII and TMDVI and into the core of TMDVII (Pellegrini *et al.*, 2001).

3.1.2.4 Biological activity of substance P fragments

Various SP N-terminal and C-terminal metabolites have been shown to have biological function, often in a manner opposite to the parent peptide (Khan *et al.*, 1995). Following *in vivo* administration of SP into rats, the major metabolites detected were SP(1-7) and SP(1-4) (Michael-Titus *et al.*, 2002). The SP degradation involved neutral endopeptidase (NEP) and aminopeptidase in the primary cleavages and ACE in the secondary cleavages (Michael-Titus *et al.*, 2002).

Behavioural tests on rats indicated that injection of the C-terminal fragment, SP(5-11), into the periaque grey induced anxiogenic behaviour while the N-terminal fragment, SP(1-7), produced opposing anxiolytic effects. Work comparing the effect of infusing these peptides on the concentration of amino acids in the spinal cord of the rat demonstrated that SP(1-7) decreased the release of excitatory amino acids whereas SP(5-11) stimulated release (Skilling *et al.*, 1990).

In one case, the degradation of SP to SP(1-4) acted as a negative feedback mechanism. Myeloid progenitor proliferation is positively regulated by SP and inhibited by SP(1-4). SP(1-4) was demonstrated to induce the expression of

TGF-β1 which accounted for part of the inhibitory effect. The remaining effect is speculated to be due to steric hindrance since modelling suggests both SP and SP(1-4) are able to bind to the same pocket within NK1. Therefore, SP(1-4) binding may compete with the binding of SP and antagonize signalling effects associated with SP binding (Joshi *et al.*, 2001).

SP(1-7) induces dopamine release following injection into rat brain during morphine withdrawal (Zhou and Nyberg, 2002). Intracerebroventricular injection of SP(1-7) led to the up-regulation of NMDA receptor subunit NR2A mRNA (Zhou *et al.*, 2000). In the rat spinal cord, SP(1-7) induced NK1 receptor mRNA and protein expression. The effect was stereo specific since the isomer containing D-Proline, D-SP(1-7) was unable to mimic the induction (Velazquez *et al.*, 2002).

3.1.3 Actions of Substance P at micromolar concentrations

3.1.3.1 SP and Mast cells

SP is also involved in inflammatory responses and treatment of mast cells with SP leads to the release of histamine from the cells via an exocytotic process known as degranulation. Histamine release by mast cells requires micromolar concentrations of SP, unlike effects mediated through NK1R that generally require nanomolar concentrations to elicit a cellular response. RT-PCR revealed that mast cells do not possess NK1 receptors. Thus, the action of SP on these cells may be via an alternative mechanism possibly independent of cell-surface receptors. The receptor-independent mode of action is supported by the fact that fluorescent labelled SP was able to translocate into live mast cells in a receptor- and energy-independent manner (Lorenz *et al.*, 1998). The degranulation in response to SP was inhibited by GDPβS and pertussis toxin indicating the involvement of $G_{i/o}$ (Lorenz *et al.*, 1998). Further support for an NK1R-independent action of SP was provided by knockout studies. *NK1R*$^{-/-}$ knockout mice were still able to release histamine from mast cells following treatment with SP (Saban *et al.*, 2002). However, in both studies the compensatory action of NK2R and NK3R cannot be ruled out since SP is also able to bind these receptors.

SP receptor/s and the known signalling pathways associated with the receptor/s may be involved in the ES-to-EPL transition. In contrast to SP(1-7), the endogenous, C-terminally amidated form of SP(1-11) as well as C-terminal fragment SP(5-11) were unable to induce the ES-to-EPL transition. As previously discussed, N-terminal fragments of SP have been demonstrated to have biological effects that are distinct from C-terminal fragments and the parent peptide (Skilling *et al.*, 1990, Khan *et al.*, 1995, De Araujo *et al.*, 1999, 2001). Therefore, the mode of action of the N-terminal fragments in other systems may provide clues as to their function in pluripotent cell differentiation. The free acid form of SP ($SPCO_2H$) shows binding affinity for NK1R and biological function similar to that seen with the N-terminal fragments and distinct from SP (Dietl *et al.*, 1989; Beaujouan *et al.*, 2000). Therefore, a second goal of the work presented in this chapter was to determine the ability of SP(1-7) and $SPCO_2H$ to induce EPL-cell gene expression and differentiation potential, and to identify the potential involvement of NK receptors in the ES-to-EPL cell transition.

Concentration of L-proline in serum free MEDII was 479 pmol, compared to 26 pmol in unconditioned DMEM.

3.2 RESULTS

3.2.1 Formation of EPL cells in the presence of L-proline or 50% MEDII

Induction of EPL-cell morphology by L-proline has previously been established (Bettess, 2001) and was confirmed in my hands. The concentration-dependent effects of L-proline on pluripotent cell morphology were assessed by seeding ES cells at low density (1000 cells per well) and culturing for 5 days in ES complete medium (containing 1000 U/ml LIF) in the presence of 40-400 µM L-proline. EPL-cell morphology was evident at 40 µM, although at this concentration the transition was not homogenous with ~40% colonies still maintaining a domed shape characteristic of ES cells. Concentrations of 80-400 µM, however, led to homogenous establishment of a monolayer of cells with an EPL-cell morphology, which stained positive for alkaline phosphatase (AP) consistent with maintenance of pluripotence (Figure 3.1).

ES cells grown either in ES complete medium plus 40 µM or 200 µM L-proline or in 50% MEDII + 1000 U/ml LIF were analysed for gene expression changes associated with EPL-cell formation. The formation of EPL cells is characterised by the maintained expression of pluripotence markers AP and *Oct4* and the down-regulation of ES-specific markers *Rex1* and *CRTR1* (Pelton *et al.*, 2000). The morphology of the cells grown for 2, 4 and 6 days in ES complete medium supplemented with 40 µM or 200 µM L-proline or 50% MEDII + 1000 U/ml LIF was consistent with that of EPL cells (Figure 3.2 A).

Figure 3.1

Concentration-dependent induction of EPL-cell morphology by L-proline

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) in the presence of the indicated concentrations of L-proline. Following four days of culture, the cells were photographed under phase contrast (100X magnification), fixed (2.8) and stained for expression of the pluripotence marker alkaline phosphatase (2.10). The experiment was performed in duplicate three times and results from a representative experiment are shown.

Figure 3.1

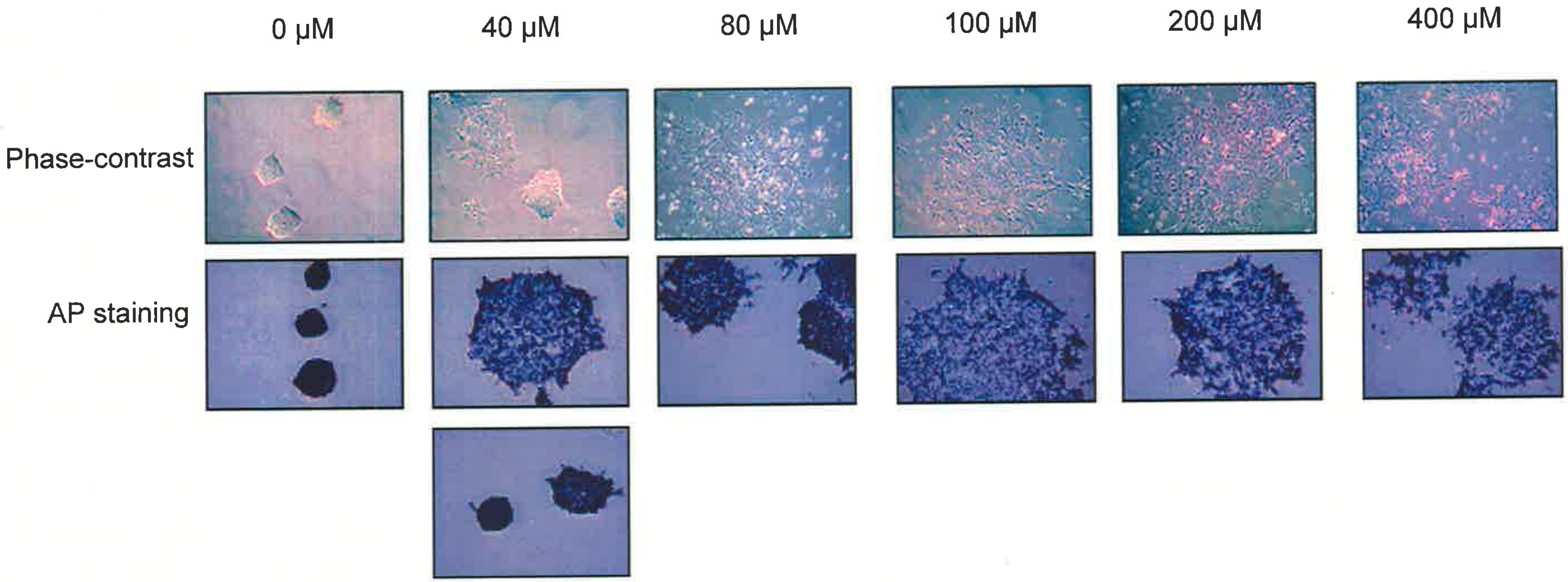


Figure 3.2

Induction of EPL-associated gene expression by L-proline

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) supplemented with 40 µM (♦), 200 µM L-proline (■) or 50% MEDII (▲) and grown for 6 days with passage every 2 days. (A) Morphology of the cells grown for 2, 4 and 6 days in the conditions indicated at (200X magnification). (B) Quantitative PCR (2.7.6) was performed to determine expression of (A) *Rex1*, (B) *CRTR1* and (C) *Oct4* relative to *β-actin*. Normalised expression of each gene in ES cells was assigned a value of 1 and the expression level in day 2-6 cells is shown relative to this. Error bars represent SD of triplicates. The experiment was performed three times and results from a representative experiment are shown.

Figure 3.2

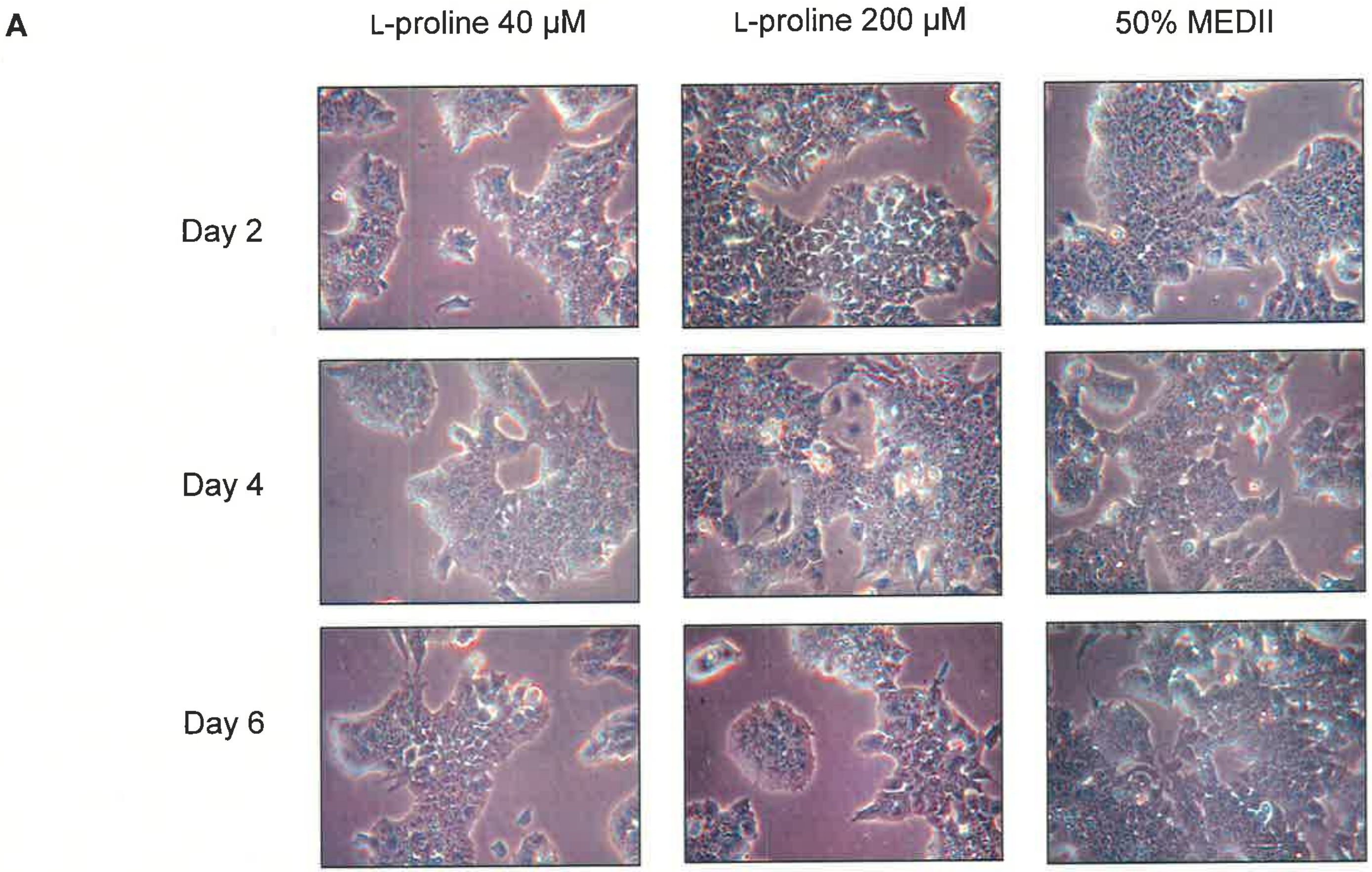
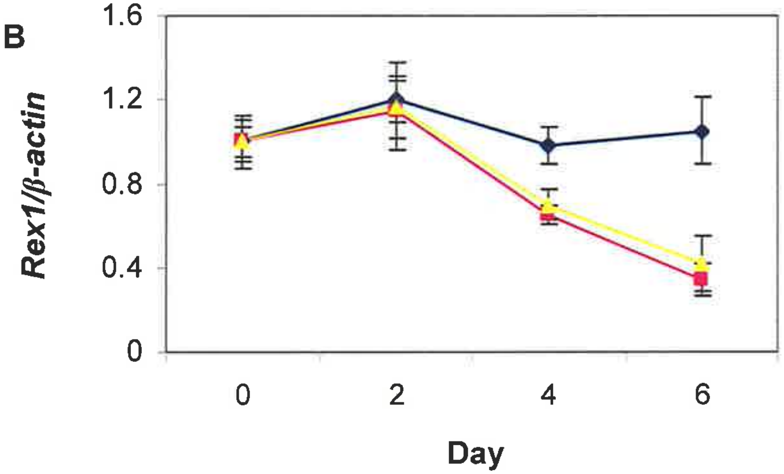
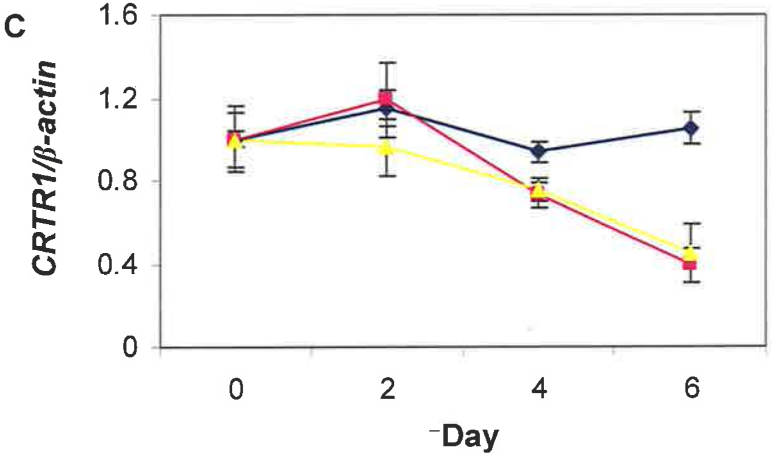


Figure 3.2

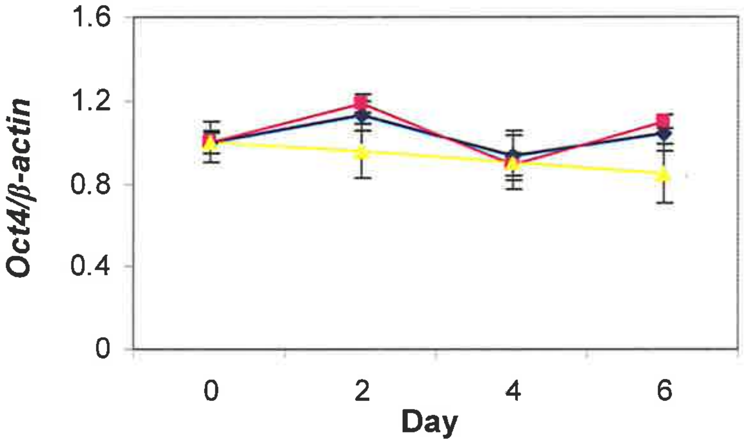
B



C



D



Quantitative PCR analysis showed that cells grown in 50% MEDII + 1000 U/ml LIF or ES complete medium plus 200 μM but not 40 μM L-proline down-regulated expression of the ES markers *Rex1* and *CRTR1* (Figure 3.2 B, C) while maintaining the expression of *Oct4* (Figure 3.2 D).

To demonstrate that the levels of *Oct4* detected by Northern blot resulted from homogeneous expression within the culture, *in situ* hybridisation was performed on ES cells maintained for 4 days or cells cultured in the indicated concentration of L-proline for the same time period. Fixed cells were hybridised with 600 ng anti-sense or sense *Oct4* RNA probe. All cells expressed *Oct4* under each of the conditions on day 4 (Figure 3.3 A). To demonstrate the down-regulation of *Rex1* detected by Northern blot was also homogeneous within the culture, *in situ* hybridisation was performed on ES cells maintained for 4 days or cells cultured in 200 μM L-proline in ES complete medium or 50% MEDII + 1000 U/ml LIF for the same time period. Fixed cells were hybridised with 600 ng anti-sense or sense *Rex1* RNA probe. Day 4 ES cells maintained expression of *Rex1* while cells grown in L-proline or 50% MEDII showed uniform down-regulation of *Rex1* (Figure 3.3 B). This confirms that the transition induced by L-proline results in a homogeneous population of pluripotent EPL cells. As determined by Bettess (2001) EPL cell state is most clearly characterised by the down-regulation of *Rex1* and maintenance of *Oct4*. *Fgf5* which was previously used as a positive EPL-cell marker (Rathjen *et al.*, 1999) was not as reliable in defining EPL cells; its expression oscillated during extended EPL-cell culture and its expression was

Figure 3.3

***In situ* hybridisation analysis of *Oct4* and *Rex1* expression in pluripotent cells grown in the presence of L-proline**

ES cells were seeded in ES complete medium (containing 1000 U/ml LIF) supplemented with 0, 200, 400 μM L-proline or 50% MEDII. Cells were refed daily and passaged every two days. On day 4 cells were fixed (2.8) and hybridised (2.9.4) with 600 ng sense or anti-sense DIG-labelled RNA probes (2.9.3) for (A) *Oct4* or (B) *Rex1*. Probe was detected using alkaline phosphatase activity with an AP-anti-DIG antibody. Cells were photographed under phase contrast (100X magnification). The experiment was performed in duplicate twice and a representative result is shown.

Figure 3.3

A

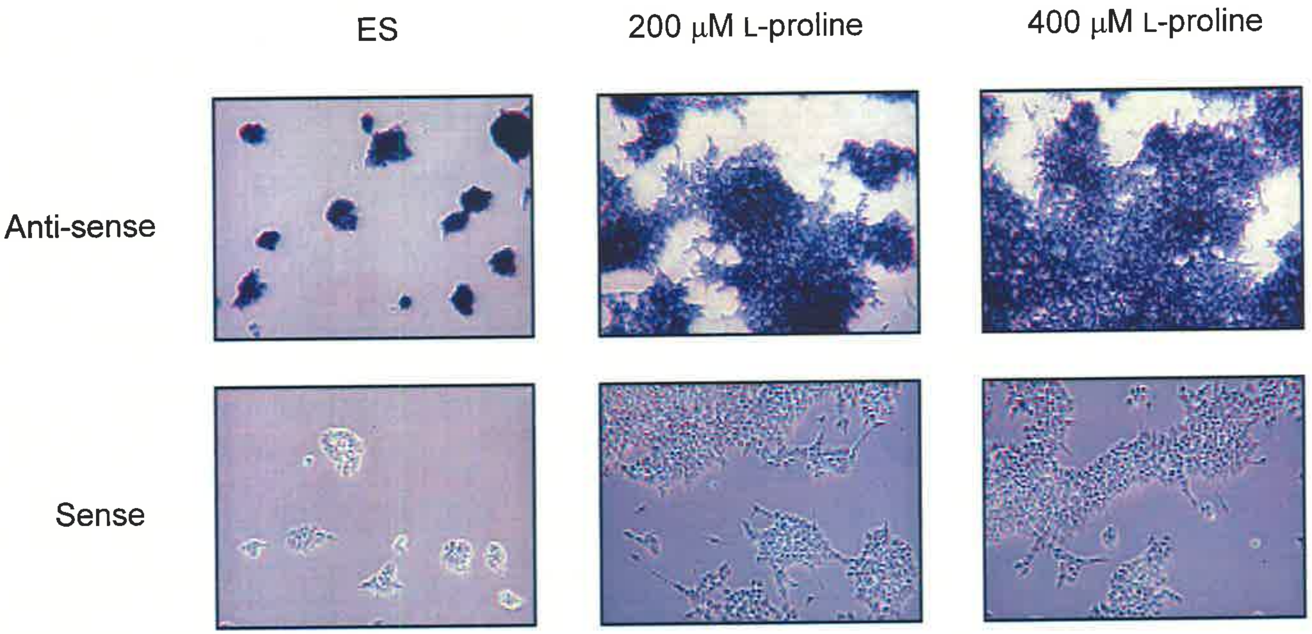
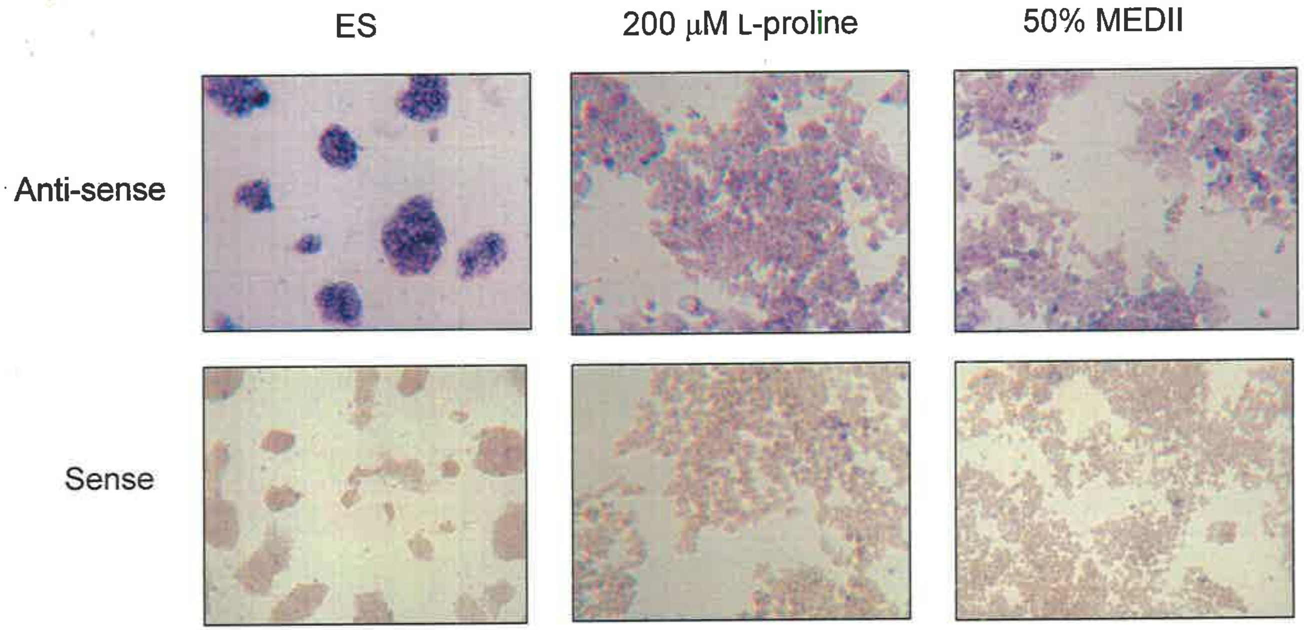


Figure 3.3

B



not required for induction of EPL-cell differentiation potential (Bettess, 2001; White, unpublished data).

To assess the differentiation potential of the cells, embryoid bodies (EBs) were formed from pluripotent cells cultured in ES complete medium, ES complete medium supplemented with 200 µM L-proline or 50% MEDII + 1000 U/ml LIF. ES and EPL cells exhibit different differentiation potentials with EPL-cell derived bodies forming mesoderm earlier, as measured by the up-regulation of the nascent mesoderm marker *Brachyury* on day 2-3 of EB differentiation compared to the induction on day 4 that is seen in ES-cell derived EBs (Lake *et al.*, 2000). Cells were cultured for 4 days prior to the formation of aggregates. Figure 3.4 shows the morphology of ES cells grown for 4 days in ES complete medium alone (Figure 3.4 A) or supplemented with 200 µM L-proline (Figure 3.4 B) or 50% MEDII + 1000 U/ml LIF (Figure 3.4 C) and the morphology of the aggregates derived from these cells after 4 days. Northern blot analysis of the bodies showed that *Brachyury* expression was up-regulated on day 3 in bodies made from cells cultured in ES complete medium supplemented with L-proline or 50% MEDII + 1000 U/ml LIF compared to day 4 in ES-cell derived EBs (Figure 3.4 D).

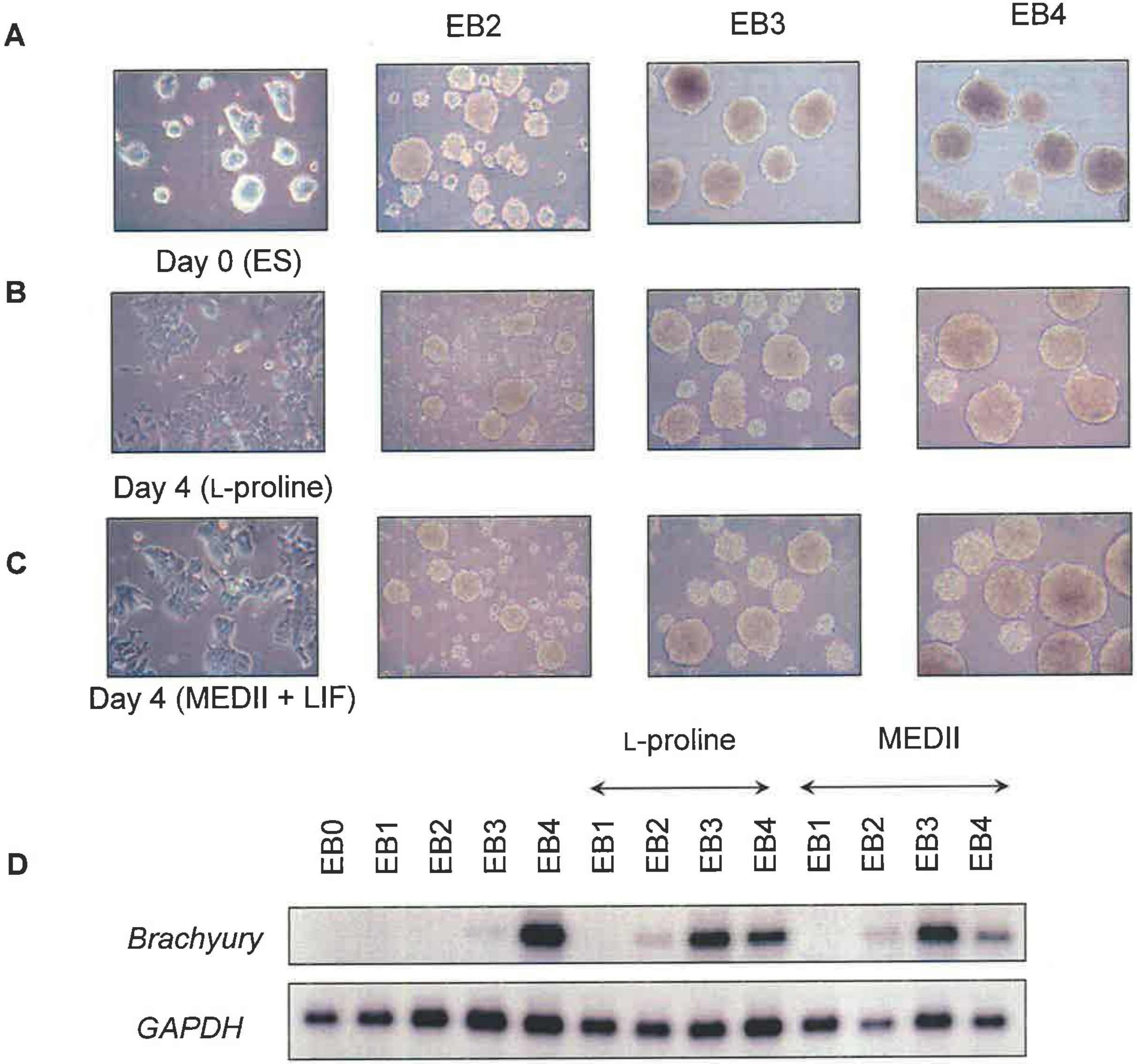
EPL-cell formation in MEDII + LIF is characterised by conversion of compact, domed colonies to a monolayer of cells, down-regulation of expression of *Rex1* and *CRTR1*, maintenance of *Oct4* expression and up-regulation of *Brachyury* in bodies 24-48 h earlier than in bodies derived from ES cells

Figure 3.4

Differentiation potential of ES cells grown in the presence of L-proline or 50% MEDII

Cells were grown for 4 days with daily re-feeding and passage on day 2 in ES complete medium (containing 1000 U/ml LIF) or in ES complete medium supplemented with 200 μM L-proline or 50% MEDII+LIF. On day 4, bodies were formed (2.6.6) by seeding single cells into ICβ medium and grown for 4 days. (A-C) Phase contrast photographs (100X magnification) of (A) ES cells grown in ES complete medium and day 2, 3 and 4 EBs derived from these cells, (B) ES cells grown in ES complete medium supplemented with 200 μM L-proline and day 2, 3, 4 aggregates derived from these cells and (C) ES cells grown in 50% MEDII+LIF day 2, 3, 4 aggregates derived from these cells. (D) Northern blot analysis (2.9.2) of total RNA (10 μg) isolated from day 1, 2, 3 and 4 aggregates analysed for expression of mesoderm marker *Brachyury*. *GAPDH* was used as a loading control. All experiments were performed three times and representative results are shown.

Figure 3.4


A
EB2
EB3
EB4
Day 0 (ES)
B
Day 4 (L-proline)
C
Day 4 (MEDII + LIF)
L-proline
MEDII
D
EB0
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
Brachyury
GAPDH

(Rathjen *et al.*, 1999; Bettess, 2001; Pelton *et al.*, 2002). Here it is confirmed that culture of ES cells in 200 μM L-proline + LIF results in the formation of cells equivalent to those obtained in MEDII + LIF.

3.2.2 Selected L-proline containing peptides induce the ES-to-EPL transition

Morphology assays indicated that ala-pro (80 μM), gly-pro (40 μM), SP(1-7) (40μM) and $SPCO_2H$ (40 μM) peptides could induce EPL-cell morphology (Table 1.2; Washington *et al.*, submitted). With L-proline, the concentration at which morphology changes were initially seen was not sufficient to induce EPL-cell gene expression or differentiation potential (Bettess, 2001). Therefore, ES cells exhibit concentration-dependent responses to L-proline. Low concentrations of L-proline induce EPL-cell morphology and higher concentrations also induce changes in gene expression and differentiation potential (Bettess, 2001).

The peptides gly-pro, ala-pro, SP(1-7) and $SPCO_2H$ were tested for their ability to induce changes in gene expression and differentiation potential at higher concentrations. The morphology of cells cultured in ES complete medium alone or in ES complete medium supplemented with 200 μM gly-pro, 400 μM ala-pro, 200 μM SP(1-7) or 200 μM $SPCO_2H$ was comparable to that of the cells grown in ES complete medium plus L-proline or 50% MEDII + 1000 U/ml LIF (Figure 3.5 A).

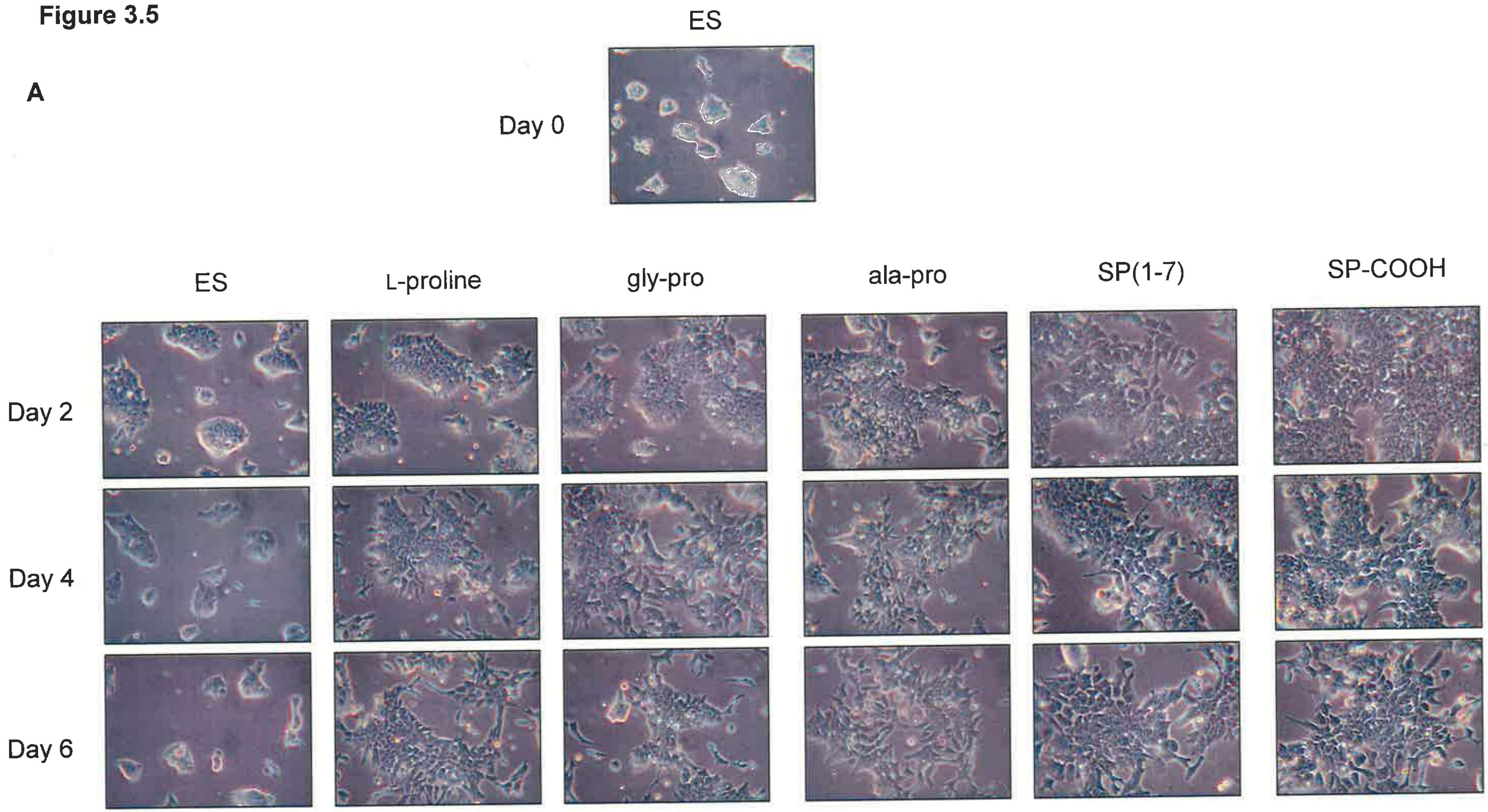
Figure 3.5

Small L-proline-containing peptides induce EPL-cell morphology and gene expression

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) (♦) supplemented with 200 µM L-proline (■), 400 µM ala-pro (■), 200 µM gly-pro (■), 200 µM SP(1-7) ( ), 200 µM $SPCO_2H$ (X) or 50% MEDII (○). Cells were grown up to 6 days, re-fed daily and passaged on days 2 and 4. (A) Phase contrast photographs (200X magnification) of cells grown for up to 6 days in the conditions indicated. (B) Northern blot (2.9.2) analysis of total RNA (10 µg) isolated from cells grown in the presence of 200 µM L-proline, 400 µM ala-pro or 200 µM gly-pro. The blots were sequentially probed for the expression of *CRTR1*, *Rex1* and *Oct4*. *GAPDH* was used as a loading control. (C-D) Quantification (using volume integration Quantity one software) of Northern blot results for *Rex1* (C) and *CRTR1* (D). Relative expression of marker gene versus *GAPDH* in ES cells was assigned a value of 1 and expression in day 2-6 cells is shown relative to this. (E) Quantitative PCR (2.7.6) of *Rex1* expression. Relative expression of marker gene versus *β-actin* in ES cells was assigned a value of 1 and expression in day 2-6 cells is shown relative to this. Error bars represent SD of triplicates. (F) Phase contrast photographs (100X magnification) of day 6 cells fixed (2.8) and stained for the expression of the pluripotence marker alkaline phosphatase (2.11). All experiments were performed three times and representative results are shown.

Figure 3.5

A



ES
Day 0
ES
L-proline
gly-pro
ala-pro
SP(1-7)
SP-COOH
Day 2
Day 4
Day 6

Figure 3.5

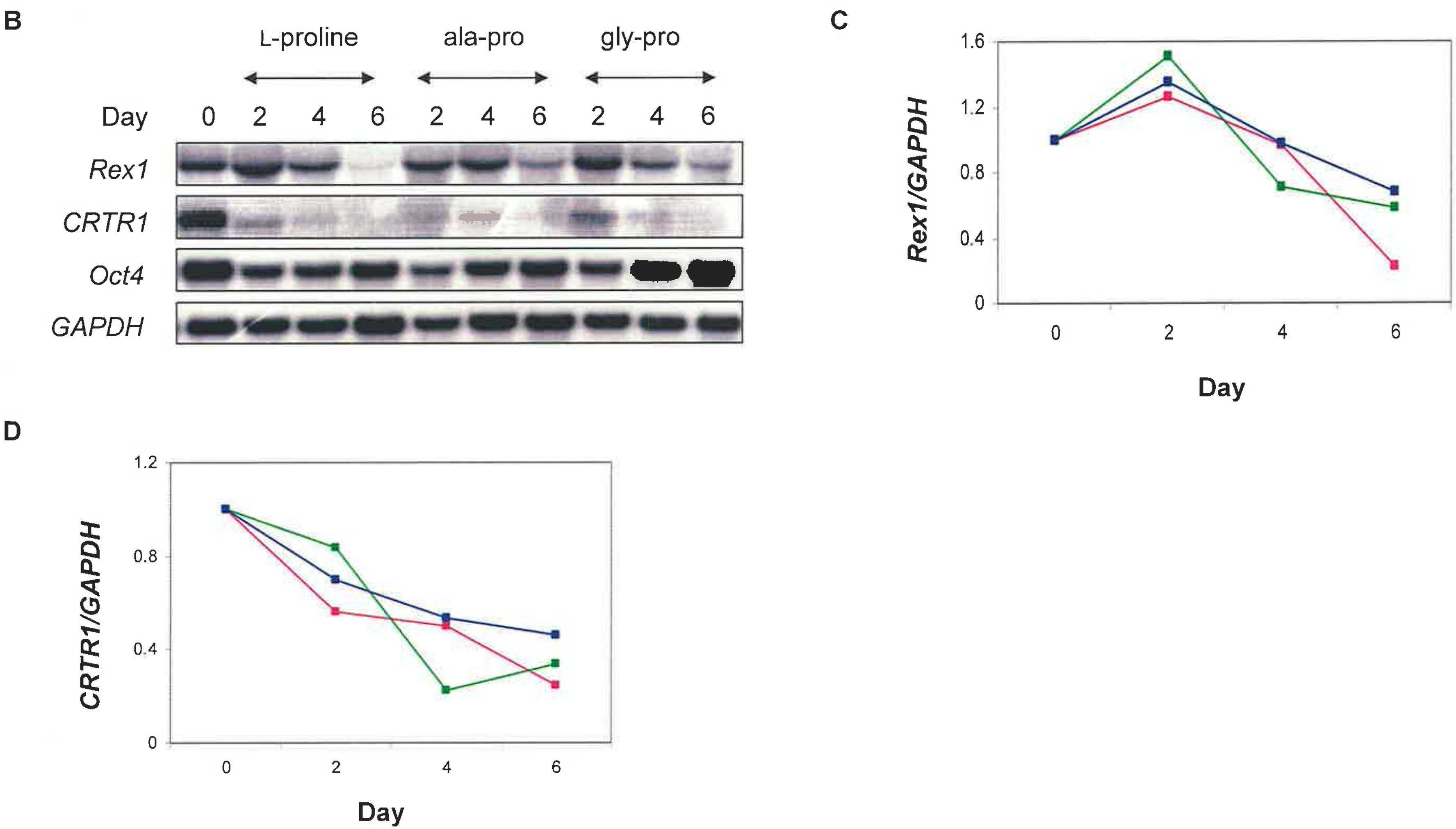
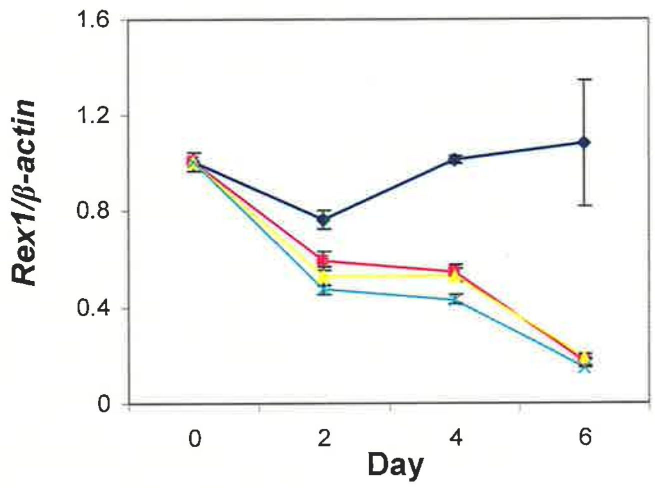
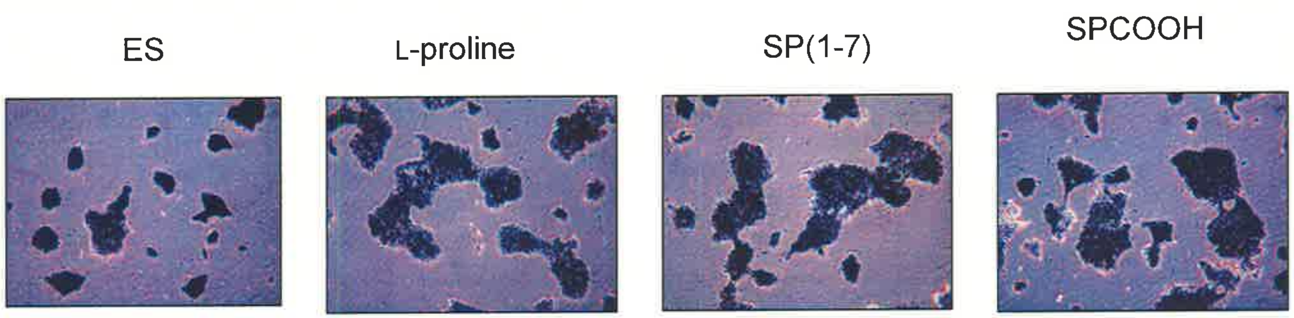

B
L-proline
ala-pro
gly-pro
Day
0 2 4 6 2 4 6 2 4 6
Rex1
CRTR1
Oct4
GAPDH
C
Rex1/GAPDH
1.6
1.2
0.8
0.4
0
0
2
4
6
Day
D
CRTR1/GAPDH
1.2
0.8
0.4
0
0
2
4
6
Day

Figure 3.5

E



F



Northern blot analysis of cells grown in the presence of gly-pro or ala-pro showed down-regulation of the ES-cell markers *Rex1* and *CRTR1* similar to that for cells grown in L-proline and maintenance of the expression of the pluripotence marker *Oct4* (Figure 3.5 B, C, D). Results from quantitative PCR analysis of cells treated with 200 µM SP(1-7) or 200 µM $SPCO_2H$ showed that *Rex1* expression was down-regulated to a similar extent as in cells grown in the presence of 200 µM L-proline (Figure 3.5 E). Alkaline phosphatase staining of the cells on day 6 confirmed that cells cultured under the various conditions maintained pluripotence (Figure 3.5 F).

To determine the effect of the peptides on the differentiation potential of the cells, embryoid bodies were made from ES cells or cells grown in ES complete medium plus 200 µM gly-pro, 400 µM ala-pro, 200 µM SP(1-7), 200 µM $SPCO_2H$ or 200 µM L-proline for four days. The morphology of the bodies on days 2 and 4 is shown in Figure 3.6 A. The induction of *Brachyury* expression in the bodies derived from cells cultured in the presence of 200 µM gly-pro, 400 µM ala-pro, 200 µM SP(1-7) and 200 µM $SPCO_2H$ occurred on day 3 consistent with EPL-cell differentiation potential (Figure 3.6 B, C).

3.2.3 Pro-ala and pro-gly fail to induce the ES-to-EPL cell transition

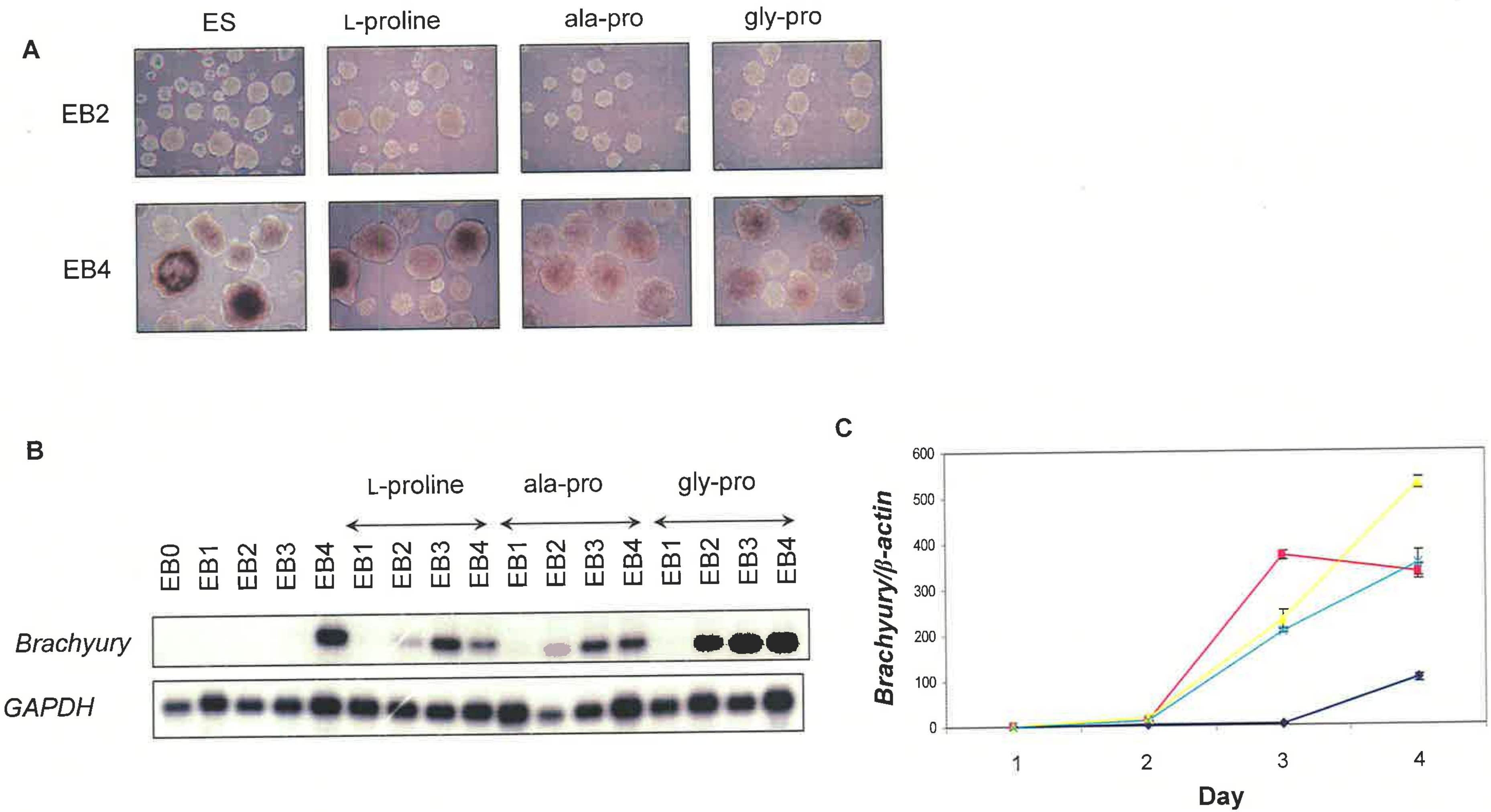
The induction of EPL-cell morphology by culturing ES cells in the presence of pro-ala and pro-gly was first evident at 50 µM for both peptides. ES cells grown in the presence of 250 µM pro-ala or pro-gly also adopted EPL-cell morphology (Figure 3.7 A) but failed to down-regulate the expression of *Rex1* (Figure 3.7 B) and bodies derived from the ES cells cultured for 4 days in the

Figure 3.6

Induction of EPL cell differentiation potential by short L-proline-containing peptides

ES cells were cultured in ES complete medium (containing 1000 U/ml LIF) supplemented with 200 μM L-proline, 200 μM gly-pro, 400 μM ala-pro, 200 μM SP(1-7) or 200 μM $SPCO_2H$ for 4 days with daily re-feeding and passage on day 2. On day 4, aggregates were formed (2.6.6) by seeding single cells into ICβ medium. Embryoid bodies were grown for 4 days. (A) Phase contrast photographs (100X magnification) of day 2 and 4 EBs. (B) Northern blot (2.9.2) analysis of total RNA (10 μg) isolated from day 1, 2, 3 and 4 day bodies analysed for expression of the mesoderm marker *Brachyury*. *GAPDH* was used as a loading control. (C) Quantitative PCR (2.7.6) of *Brachyury* expression on day 1-4 bodies derived from cells cultured for 4 days in ES complete medium (♦) or supplemented with 200 μM L-proline (■), SP(1-7) (▲) or $SPCO_2H$ (**X**). Expression of *Brachyury* in EB1 was assigned a value of 1 and the expression level in day 2-4 bodies is shown relative to EB1 expression. Error bars represent SD of triplicates. Experiments were performed two times and representative results are shown.

Figure 3.6



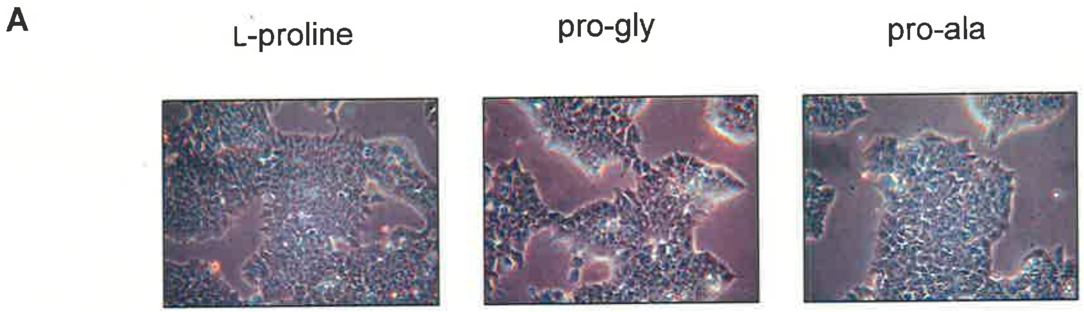
A
ES
L-proline
ala-pro
gly-pro
EB2
EB4
B
L-proline
ala-pro
gly-pro
EB0
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
Brachyury
GAPDH
C
Brachyury/β-actin
600
500
400
300
200
100
0
1
2
3
4
Day

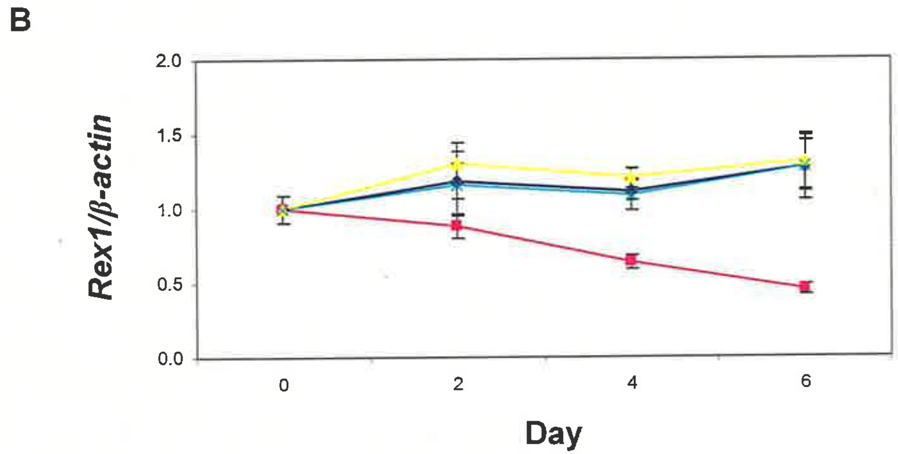
Figure 3.7

Peptides pro-ala and pro-gly induce EPL-cell morphology but not gene expression changes

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) supplemented with 200 μM L-proline, 250 μM pro-ala or 250 μM pro-gly. Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. (A) Phase contrast photographs (200X magnification) of day 4 cells. (B) Quantitative PCR (2.7.6) of *Rex1* expression in ES cells grown in ES complete medium (♦) or supplemented with 200 μM L-proline (■), 250 μM pro-ala (X) or 250 μM pro-gly (▲). Relative expression of *Rex1* versus *β-actin* in ES cells was assigned a value of 1 and expression in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. The experiments were performed two times and a representative result is shown.

Figure 3.7





presence of pro-ala or pro-gly showed up-regulation of *Brachyury* expression on day 4, consistent with ES-cell differentiation potential (Figure 3.8). Bodies made from ES cells grown in 1.25 mM pro-gly also showed induction of *Brachyury* expression on day 4 (Figure 3.8). Therefore, pro-gly and pro-ala were capable of inducing EPL-cell morphology but not the associated gene expression and differentiation-potential changes.

3.2.4 Neurokinin receptors do not appear to be involved in the ES-to-EPL cell transition

SP(1-7) and $SPCO_2H$, which were able to induce EPL-cell morphology, gene expression and differentiation potential changes may signal through neurokinin receptors (Khawaja and Rogers, 1996). The expression of NK1R, NK2R and NK3R was investigated in pluripotent cells using RT-PCR with primers specific for the individual receptors and cDNA from pluripotent cells. Mouse brain cDNA was used as a positive control for the expression of NK1R and NK3R (Mantyh *et al.*, 1988; Buell *et al.*, 1992; Baker *et al.*, 2003) while for NK2R mouse intestine cDNA was used (Tsuchida *et al.*, 1990; Vannucchi *et al.*, 2000). As shown in Figures 3.9 A and B, expression of NK1R and NK3R was detected only in the positive controls while NK2 receptor was expressed in ES cells (Figure 3.9 C). Sequencing of the PCR fragment confirmed that the band detected corresponded to NK2R.

Specific receptor antagonists were used to investigate the functional involvement of NK2R in the ES-to-EPL cell transition and to confirm that NK1R and NK3R were not involved. Many selective peptide antagonists have

Figure 3.8

Peptides pro-ala and pro-gly do not induce EPL cell differentiation potential

ES cells were cultured in ES complete medium (containing 1000 U/ml LIF) supplemented with 250 μM or 1.25 mM pro-gly, 250 μM pro-ala or 200 μM L-proline for 4 days with daily re-feeding and passage on day 2. On day 4, aggregates were formed (2.6.6) by seeding single cells into ICβ medium and culturing for 4 days. Total RNA (10 μg) isolated from day 1, 2, 3 and 4 bodies was analysed for expression of mesoderm marker *Brachyury* by Northern blot (2.9.2). *GAPDH* was used as a loading control. The experiment was performed two times and a representative result is shown.

Figure 3.8

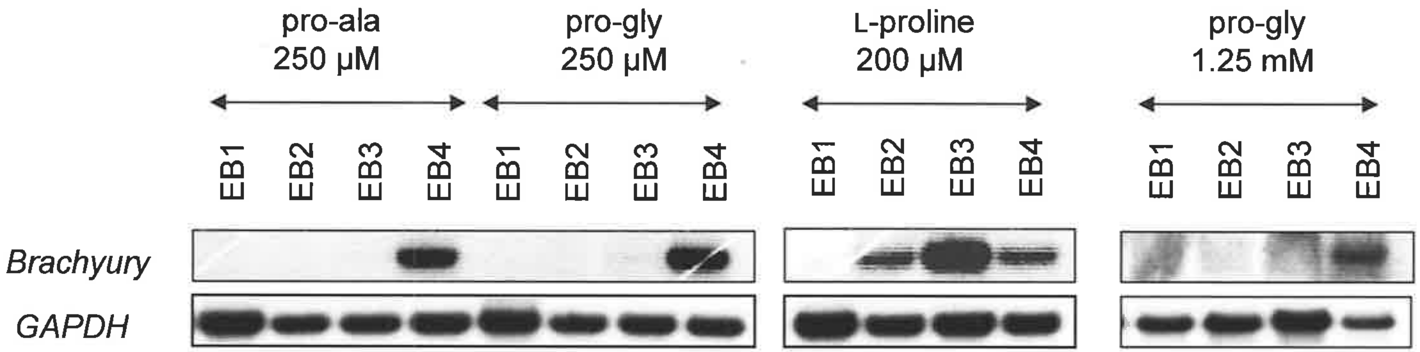
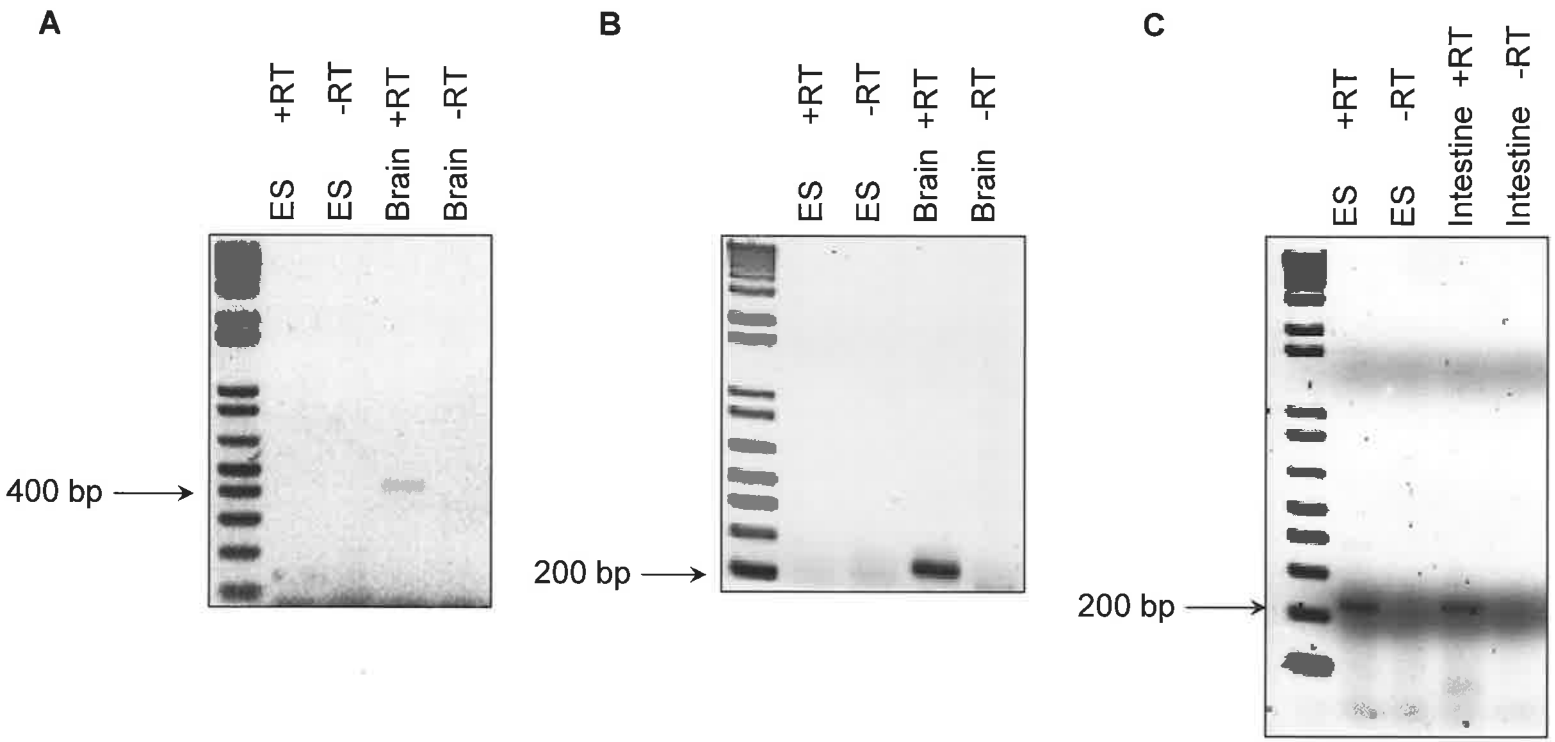

pro-ala
250 µM
pro-gly
250 µM
L-proline
200 µM
pro-gly
1.25 mM
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
Brachyury
GAPDH

Figure 3.9

ES cells express NK2R but not NK1R and NK3R

First strand cDNA synthesis (2.7.4) was performed in the presence (+RT) or absence (-RT) of reverse transcriptase. PCR was performed (2.7.5) on ES-cell, mouse-brain and intestine cDNA (used as a positive control) using primers (2.4.2) specific for (A) NK1R, (B) NK3R and (C) NK2R. PCR products were separated by electrophoresis (2.7.1) alongside a 1 kb ladder on 2% agarose gels, stained with ethidium bromide and visualised by FX scanning. Expected sizes of fragments are: NK1R, 436 bp; NK3R, 241 bp; NK2R 230 bp (relevant marker sizes are indicated). Experiment was performed four times and a representative result is shown.

Figure 3.9


A
ES +RT
ES -RT
Brain +RT
Brain -RT
400 bp
B
ES +RT
ES -RT
Brain +RT
Brain -RT
200 bp
C
ES +RT
ES -RT
Intestine +RT
Intestine -RT
200 bp

been developed for the individual NK receptors (Regoli *et al.*, 1994; Harrison and Geppetti, 2001). L-732,138 is a selective antagonist for NK1R at concentrations up to 1 µM (Cascieri *et al.*, 1994). SR 142801 is a selective antagonist of NK3R at concentrations below 1 µM. At concentrations above 1 µM, SR 142801 also binds NK2R and blocks NK2R-dependent responses (Emonds-Alt *et al.*, 1995). Therefore, to investigate the functional involvement of neurokinin receptors, ES cells were cultured in EPL-cell inductive conditions in the presence of various concentrations of the NK-receptor antagonists.

To test for the involvement of NK1R ES cells were cultured in the presence of 1 µM or 10 µM NK1R antagonist L-732,138. Quantitative PCR demonstrated that neither 1 µM or 10 µM of L-732,138 was able to prevent down-regulation of *Rex1* when ES cells were cultured in ES complete medium plus 200 µM L-proline (Figure 3.10 A). ES cells cultured in the presence of 10 µM inhibitor alone also maintained *Rex1* levels consistent with the cells remaining ES like (Figure 3.10 A). The cells cultured in the presence of 1 µM or 10 µM L-732,138 maintained their pluripotence since on day 6 all the cells stained positively for alkaline phosphatase (Figure 3.10 B).

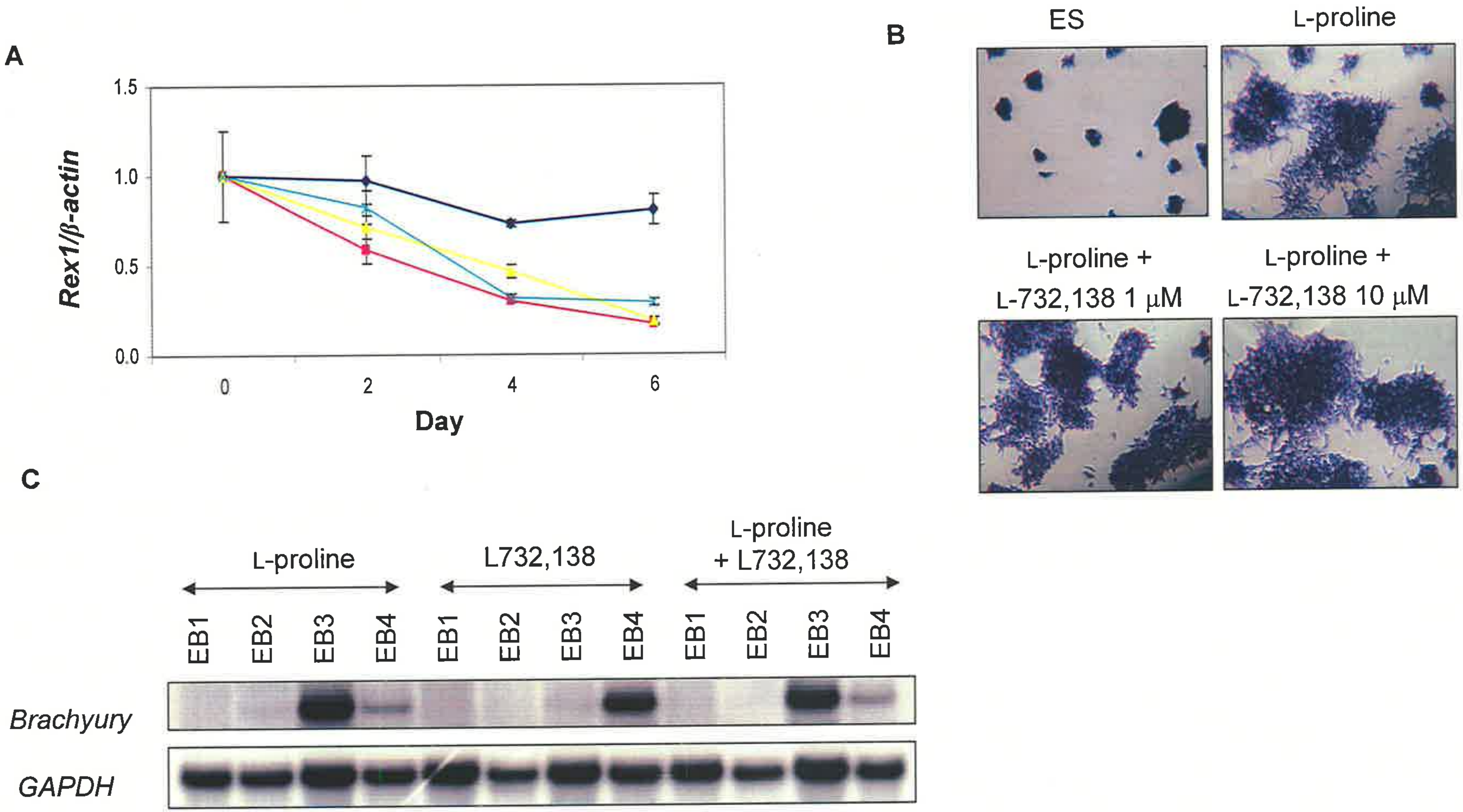
To test the differentiation potential of pluripotent cells cultured in the presence of L-732,138, RNA was extracted from embryoid bodies formed from cells cultured for 4 days in ES complete medium supplemented with 200 µM L-proline, 10 µM L-732,138 or both and analysed via Northern blot for the expression of *Brachyury*. The bodies derived from cells treated with L-

Figure 3.10

NK1R antagonist L-732,138 does not prevent L-proline induced EPL cell gene expression and differentiation potential

(A-C) ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) supplemented with 200 μM L-proline in the presence or absence of 1 μM or 10 μM L-732,138. Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. On day 4, aggregates were formed (2.6.6) by seeding single cells into ICβ medium and culturing for up to 4 days. (A) Quantitative PCR (2.7.6) of *Rex1* expression in cells grown in ES complete medium supplemented with 200 μM L-proline (■), 1 μM L-732,138 and 200 μM L-proline ( ), 10 μM L-732,138 (♦) or 10 μM L-732,138 and 200 μM L-proline (X). Relative expression of *Rex1* versus *β-actin* in ES cells was assigned a value of 1 and expression in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. (B) Phase contrast photograph (100X magnification) of alkaline phosphatase activity (2.11) following fixation and staining of day 6 cells. (C) Northern blot (2.9.2) analysis for expression of *Brachyury* using RNA (10 μg) isolated from day 1, 2, 3 and 4 embryoid bodies derived from cells grown for 4 days in the presence of 200 μM L-proline, 10 μM L-732,138 or both. *GAPDH* was used as a loading control. The experiments were performed two times and results from a representative experiment are shown.

Figure 3.10


A
1.5
1.0
0.5
0.0
Rex1/β-actin
0
2
4
6
Day
B
ES
L-proline
L-proline +
L-732,138 1 μM
L-proline +
L-732,138 10 μM
C
L-proline
L732,138
L-proline
+ L732,138
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
EB1
EB2
EB3
EB4
Brachyury
GAPDH

732,138 alone up-regulated *Brachyury* expression on day 4 while cells cotreated with the antagonist and L-proline up-regulated *Brachyury* on day 3 consistent with their EPL-cell like gene expression (Figure 3.10 C). These results confirm that NK1R is not involved in the ES-to-EPL cell transition since the transition could occur in the presence of a NK1R-specific antagonist. The activity of the antagonist was confirmed by inhibition of SP-induced Ca^{2+} mobilisation in COS cells expressing NK1R (Holland, personal communication).

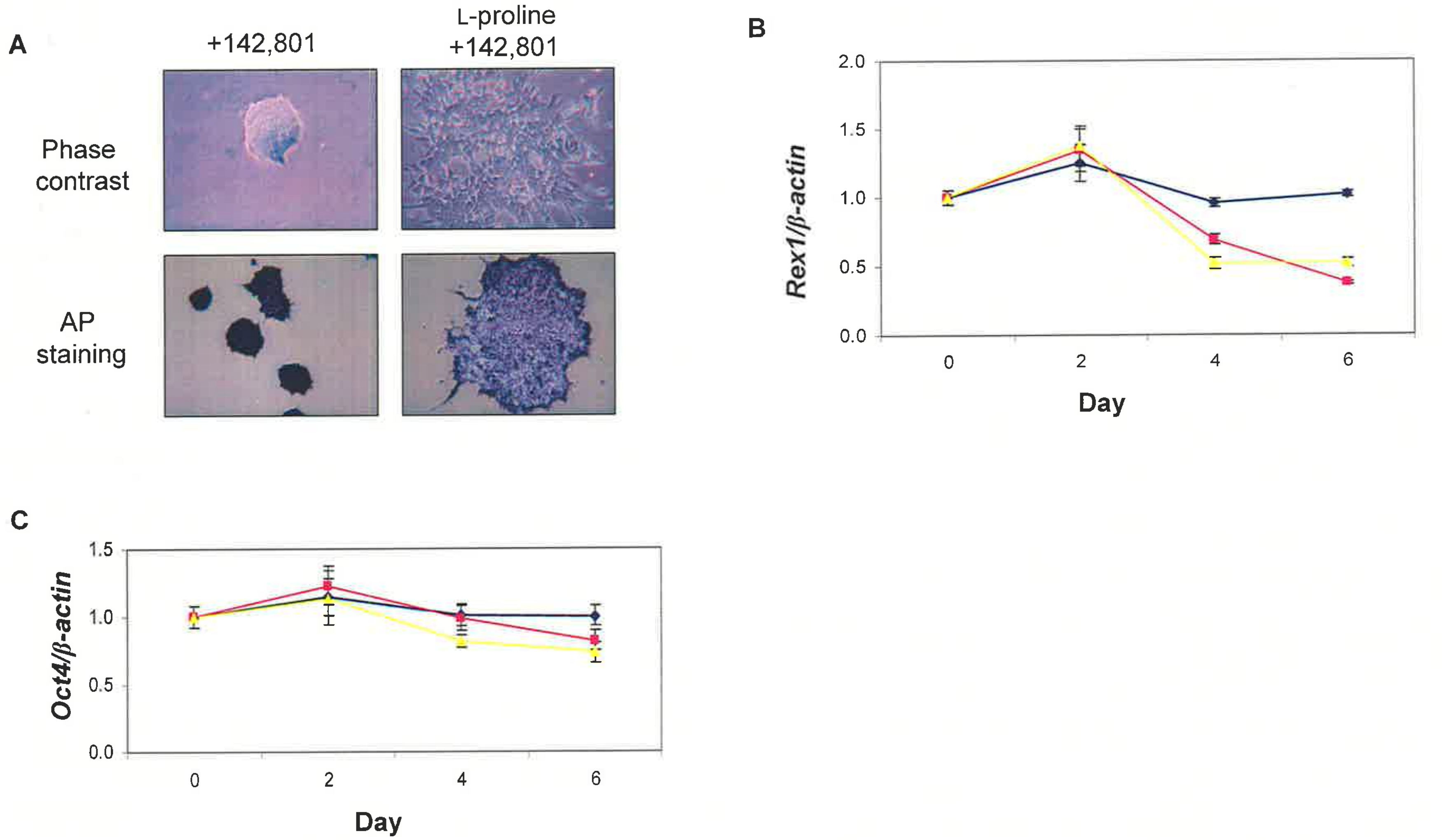
To determine the involvement of NK2R and NK3R in the ES-to-EPL cell transition 1 μM SR 142801 was used (Emonds-Alt *et al.*, 1995). Morphology assays indicated that 1 μM SR 142801 was unable to prevent L-proline-induced EPL-cell morphology in the presence of 200 μM L-proline (Figure 3.11 A). The pluripotence of the cells was not affected as they stained for alkaline phosphatase (Figure 3.11 A) and maintained *Oct4* expression (Figure 3.11 C). To determine the effect on gene expression, ES cells were cultured in the presence or absence of 1 μM SR 142801. The results show that the presence of the inhibitor did not prevent the down-regulation of *Rex1* induced by L-proline (Figure 3.11 B). The functionality of the inhibitor was confirmed by inhibition of Ca^{2+} mobilisation following activation of NK2R or NK3R expressed in CHO cells (Edmonds-Alt, personal communication). These results indicate that NK2R and NK3R are not involved in the L-proline-induced EPL-cell formation.

Figure 3.11

The NK2R and NK3R antagonist SR 142801 is not able to prevent L-proline induced EPL cell formation

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) supplemented with 1 µM SR 142801 (♦), 200 µM L-proline (■) or both (▲). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. On day 4, the cells were either photographed under phase contrast (100X magnification), fixed and stained for the expression of the pluripotence marker alkaline phosphatase (2.11) or cultured for a further two days. (A) Phase contrast photograph (100X magnification) of alkaline phosphatase staining (2.11) of day 4 cells. (B-C) Quantitative PCR (2.7.6) of (B) *Rex1* and (C) *Oct4* expression of cells grown in 1 µM SR 142801 (♦), 200 µM L-proline (■) or both (▲). Relative expression of marker gene versus *β-actin* in ES cells was assigned a value of 1 and expression in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. Experiments were performed two times and a representative result is shown.

Figure 3.11


A
+142,801
L-proline
+142,801
Phase
contrast
AP
staining
B
2.0
1.5
1.0
0.5
0.0
Rex1/β-actin
0
2
4
6
Day
C
1.5
1.0
0.5
0.0
Oct4/β-actin
0
2
4
6
Day

3.3 DISCUSSION

3.3.1 Specific short L-proline containing peptides induce EPL-cell formation

The results from this chapter indicate that short L-proline containing peptides ala-pro, gly-pro, SP(1-7) and $SPCO_2H$ are, like L-proline, able to induce the formation of EPL cells as determined by morphology, gene expression and differentiation potential (Figures 3.5, 3.6). In contrast, the peptides pro-ala and pro-gly, although possessing the ability to induce EPL-cell morphology were not able to induce EPL-associated changes in gene expression and differentiation potential at the concentrations tested (Figures 3.7, 3.8). These results also demonstrate an uncoupling of EPL-cell morphology from the remaining EPL-cell attributes, gene expression and differentiation potential changes. This suggests the presence of two signalling pathways that are responsible for morphology determination on the one hand and differentiation on the other.

3.3.2 Mode of action of EPL-cell inductive factors

The fact that SP fragments SP(1-7) and $SPCO_2H$ are able to induce EPL-cell morphology, gene expression and differentiation potential (Figures 3.5, 3.6) suggests the involvement of NKR signalling in the ES-to-EPL cell transition. Their ability to induce the ES-to-EPL-cell transition does not involve the classical mode of action via NK receptors since NK1R and NK3R are not expressed on ES cells (Figure 3.9) and NK2R, although present (Figure 3.9), was shown not to be functionally involved, since the presence of a NK2R inhibitor SR142801 was unable to prevent EPL-cell formation in response to

L-proline (Figure 3.11). Therefore, it appears that the actions of short L-proline-containing peptides in EPL-cell formation do not involve, at least exclusively, the known SP effector pathways.

Surprisingly SP, unlike SPCOOH and SP(1-7) did not induce EPL-cell formation but rather caused cell death (Washington, unpublished data). Since ES cells express NK2R, SP may be binding this receptor and activating signalling resulting in cell death. Thus, ES cells may not be able to form EPL-cells in the presence of SP, by responding to the free L-proline released by proteolysis of SP, as they may have already been induced to undergo apoptosis.

It is possible that the EPL-inductive capacity of the short peptides is due to their break down to release free proline. The efficacy of proline-containing peptides may depend on the location of proline within the intact peptide or the rate of proteolysis of the peptide to release free proline. As discussed in Section 4.1.4 specific peptidases are able to cleave imino acid bonds and hydrolyse L-proline-containing peptides (Dehm and Nordwig, 1970; Priestman and Butterworth, 1985; Lorey *et al.*, 2002; Maes *et al.*, 2005). The location of L-proline within the peptide as well as the surrounding residues influences the rate of proteolysis. For example, X-prolyl-aminopeptidase, prefers an N-terminal glycine in the bond attacked (Dehm and Nordwig, 1970). X-prolyl-aminopeptidase has low efficiency in the cleavage of Ala-Pro-Gly (6%) and high cleavage efficiency for Gly-Pro-Hyp and Gly-Pro-Ala (100%) (Dehm and Nordwig, 1970). Similarly, potential expression of prolyl peptidases with a preference for X-Pro in pluripotent cells may explain why ala/gly-pro peptides and not pro-ala/gly peptides are able to induce EPL-cell formation.

Previous studies have shown that the SP peptide, even though it is C-terminally amidated, is broken down quickly in cell suspension and in lysates of cells with most of it degraded after 30 min (Lorenz *et al.*, 1998). Studies investigating the metabolism of SP in the rat striatum also produced similar results with the disappearance of the peptide and increased concentrations of L-proline evident by 20 min (Michael-Titus *et al.*, 2002). The SP fragments used in our experiments were SPCOOH, the free acid form, and SP(1-7), both of which lack C-terminal amidation and thus possibly are more susceptible to digestion than SP (Yang *et al.*, 1999; Gentilucci and Tomelli, 2004). Thus, there remain at least two possibilities: one that these peptides are broken down to free L-proline, which then acts as an inductive agent, and the other that the mode of action involves binding to an unidentified receptor or internalisation followed by induction of signalling. Internalisation cannot be discounted since SP has been shown to be taken up into live cells (Lorenz *et al.*, 1998). Furthermore, a related fluorescently labelled peptide SGYKGRPKP, which contains the first 4 residues of SP (RPKP), is rapidly taken up into ES cells and induced EPL-cell morphology (Forwood and Morris, unpublished data).

3.3.3 Potential involvement of L-proline/short L-proline-containing peptides in pluripotent cell progression *in vivo*

Although L-proline and small L-proline-containing peptides induce EPL-cell formation *in vitro*, the role for these factors *in vivo*, in the establishment of primitive ectoderm, is not known. *In vivo*, primitive ectoderm is established when the cells of the inner cell mass rearrange into a columnar epithelial structure that lines the egg cylinder (Section 1.1.2.2). At this time, a layer of visceral endoderm lines the blastocoelic side of the pluripotent cells with a basement membrane separating the two cell populations. The main component of basement membranes is proline-rich collagen IV (Van Der Rest and Garrone, 1991). Extracellular matrices such as basement membranes undergo continuous remodelling through the action of proteases and this remodelling leads to the production of free proline and short peptides of the form gly-pro-X (Telejko *et al.*, 1992). Thus, high local concentrations of L-proline and proline-containing peptides may be present at the time the ICM undergoes the transition to primitive ectoderm.

Studies by Coucouvanis and Martin (1995) suggested that cavitation and the formation of the columnar ectoderm *in vitro* involves the interplay between two signals: one a signal originating from the visceral endoderm which induces apoptosis of the inner cells and a second signal associated with the basement membrane required for survival of the columnar ectoderm cells. In addition, Coucouvanis and Martin (1995) showed that the same combination of signals was responsible for cavitation of rat embryos. This hypothesis is consistent with our findings that the *in vitro* establishment of primitive ectoderm-like populations requires L-proline or L-proline-containing peptides, known breakdown products of collagen resulting from ECM remodelling, which may have equivalence to one of the factors suggested by Coucouvanis and Martin (1995). Further evidence for the involvement of ECM components in the formation of primitive ectoderm was provided by work with *Fgfr2*$^{-/-}$ ES cells. Embryoid bodies formed from *Fgfr2*$^{-/-}$ ES cells were unable to cavitate, did not form columnar ectoderm and failed to undergo further differentiation (Li *et al.*, 2001). The *Fgfr2*$^{-/-}$ ES cells were also shown to be unable to establish a BM and did not express collagen IV or laminin-1. The ability of the embryoid bodies to differentiate and establish a columnar ectoderm was restored by the introduction of purified ECM components (Li *et al.*, 2001). Various collagen breakdown products have also been demonstrated to have biological activity in other cell systems. These short peptides have been shown, for example, to act as chemo-attractants for neutrophils (Weinberger *et al.*, 2005), activate alveolar macrophages (Laskin *et al.*, 1994) and act as inhibitors of fibrinogen/thrombin clotting (Maruyama *et al.*, 1993).

3.3.4 Summary

In summary, L-proline and short L-proline-containing peptides have been demonstrated to induce the formation of the *in vitro* equivalent of primitive ectoderm, EPL cells. The transition induced by L-proline and the L-proline-containing peptides was homogeneous with uniform AP and *Oct4* staining and uniform down-regulation of *Rex1* expression demonstrated following EPL-cell formation. The mechanism operating in our *in vitro* system may have *in vivo* biological relevance since ECM components have been suggested to be involved in the establishment of primitive ectoderm *in vivo* (Coucouvanis and Martin, 1995; Li *et al.*, 2001) and have various other biological activities in other systems (Maruyama *et al.*, 1993; Laskin *et al.*, 1994; Weinberger *et al.*, 2005). The mechanism of action, in terms of the site of action and down-stream signalling cascades employed by L-proline and the peptides, will be further explored in the later chapters.

CHAPTER 4:

INVOLVEMENT OF AMINO ACID TRANSPORTERS IN THE ES-TO-EPL CELL TRANSITION

CHAPTER 4: INVOLVEMENT OF AMINO-ACID TRANSPORTERS IN THE ES-TO-EPL CELL TRANSITION

4.1 INTRODUCTION

The imino acid L-proline is able to induce the ES-to-EPL cell transition (Bettess, 2001; Chapter 3). However, the mechanism by which this occurs is not known. Thus, it has not been established whether the functional role of L-proline in EPL-cell formation is on the cell surface or inside the cell following internalisation. Amino acid internalisation is mediated by a large number of plasma membrane amino-acid transporters.

4.1.1 Transport of L-proline into cells

Various transporters located in the plasma membrane carry out the uptake of amino acids into cells. Some amino-acid transporters act to accumulate specific amino acids from the extracellular environment into cells (secondary active transporters) while others facilitate amino-acid exchange (tertiary active transporters). Due to the fact that intracellular concentrations of amino acids are usually in excess of those in the extracellular fluid, uptake is an active process mediated, for example, by transmembrane Na^+ gradients maintained through the activity of the Na^+/K^+ ATPase, a primary active transporter. Thus, secondary active transporters couple the influx of amino acids to the thermodynamically favourable movement of ions such at Na^+ or H^+ down a concentration gradient established by the primary active transporters (Hyde *et al.*, 2003). Tertiary active transporters utilise the amino-acid concentration gradients established by the secondary transporters to accumulate their substrate amino acids by coupling their influx with the efflux of other amino acids (Hyde *et al.*, 2003). Amino-acid transporters are classified into 'systems' based on their substrate specificity. L-proline transport is known to be mediated by four systems; system A, system IMINO, system PROT and system PAT (Table 4.1) (Hyde *et al.*, 2003).

4.1.1.1 System A

In most cell types, L-proline transport into cells occurs via system A. System A consists of three subtypes SAT1 (Wang *et al.*, 2000), SAT2 (Yao *et al.*, 2000; Sugawara *et al.*, 2000) and SAT3 (Hatanaka *et al.*, 2001). System A is a Na^+-dependent transporter that facilitates the uptake of small neutral amino acids and whose activity is sensitive to pH changes (Hyde *et al.*, 2001). System A is also able to facilitate the transport of amino acids with N-methyl substitutions such as N-methylaminoisobutyric acid (MeAIB) (Hyde *et al.*, 2003).

The SAT1 transporter has been cloned from rat (Varoqui *et al.*, 2000) and human (Wang *et al.*, 2000). Expression studies indicate that in the rat, SAT1 is expressed predominantly in the brain while in human it is expressed in brain, heart and placenta. The K_m for MeAIB, the model system A substrate, is 890 μM in human. Unlike SAT1, the SAT2 isoform has a ubiquitous expression pattern (Yao *et al.*, 2000; Sugawara *et al.*, 2000). Early embryonic expression of SAT1 and SAT2 is unknown but system A activity has been detected in embryogenesis from the late blastocyst stage (Zuzack *et al.*,

TABLE 4.1 PROPERTIES OF L-PROLINE TRANSPORTERS

System	Protein	Gene	Substrate*	Notes	References
A	SAT1 SAT2 SAT3	*SLC38A1* *SLC38A2* *SLC38A4*	G, A, S, C, P, Q, N, H, M, T, Y, V, MeAIB G, A, S, C, P, Q, N, H, M, MeAIB G, A, S, C, P, N, H, M, K, R	Na^+ dependent Cationic amino acid transporter	Wang *et al.*, 2000; Yao *et al.*, 2000; Sugawara *et al.*, 2000; Hatanaka *et al.*, 2001
IMINO	SIT1/XT3s1	*SLC6A20*	P, MeAIB	Na^+ dependent	Kowalczuk *et al.*, 2005; Takanaga *et al.*, 2005
PROT	PROT	*SLC6A7*	P	Na^+ dependent	Fremeau *et al.*, 1992; Shafqat *et al.*, 1995
PAT	PAT1 PAT2	*SLC36A1* *SLC36A2*	P, G, A, MeAIB, GABA P, G, A, MeAIB, GABA	H^+ coupled transport	Boll *et al.*, 2002; Chen *et al.*, 2003

*Substrates are L-amino acids unless indicated otherwise

1985). The substrate K_m values also indicate that SAT2 has higher affinity for its substrates than SAT1, with values in the range 200-500 µM (Yao *et al.*, 2000).

With both SAT1 and SAT2, inhibition of substrate transport was evident in the presence of a molar excess of small neutral amino acids such as L-alanine, L-serine, L-proline, glycine and MeAIB, which compete with the substrate for passage through the transporter. Cationic amino acids such as L-lysine had no effect since they cannot be transported by SAT1 or SAT2 (Varoqui *et al.*, 2000; Wang *et al.*, 2000; Yao *et al.*, 2000; Sugawara *et al.*, 2000).

The third member of the system A family SAT3 has properties which are different from the other two members. SAT3 is exclusively expressed in the liver and although it is able to transport MeAIB the K_m of 6.7 mM indicates that MeAIB is not a high affinity substrate (Hatanaka *et al.*, 2001). SAT3 is also able to transport cationic amino acids such as L-lysine and L-arginine and its affinity for the charged amino acids is much greater than for neutral amino acids (Hatanaka *et al.*, 2001).

4.1.1.2 System PAT

The PAT system consists of four subtypes PAT1-4 with PAT1 and PAT2 the only proteins cloned and functionally characterised (Boll *et al.*, 2002; Wreden *et al*, 2003). PAT transporters couple the transport of small neutral amino acids with the transport of H^+ into the cell. In the mouse, PAT1 transporter is expressed in a number of tissues with highest levels detected in the small intestine, kidney, colon and brain while PAT2 is mainly found in the lung and heart (Boll *et al.*, 2002; Wreden *et al.*, 2003). Early embryonic expression of PAT1 and PAT2 is unknown. Substrates of PAT1 include glycine, L-alanine, L-proline and γ-aminobutyrate with K_m values ranging from 2.8-7.5 mM. L-proline is the preferred substrate with a K_m of 2.8 mM. PAT2 substrates include small α-amino acids and D-proline with K_m values of 100-700 μM (Boll *et al.*, 2002; Chen *et al.*, 2003).

4.1.1.3 System IMINO

The IMINO system has been functionally defined for many years but the gene and protein product remained elusive. Recently, two groups reported cloning a transporter with IMINO properties from rat (Takanaga *et al.*, 2005) and mouse (Kowalczuk *et al.*, 2005). The distribution of IMINO transcript included the brain, lung, kidney, thymus, spleen and intestine. Early embryonic expression of the IMINO transporter is unknown. The IMINO transporter only efficiently transports L-proline with K_m of 200-300 μM (Kowalczuk *et al.*, 2005; Takanaga *et al.*, 2005). Other (poorly transported) substrates include L-pipecolate, hydroxyproline, proline methyl ester, betaine and MeAIB (Kowalczuk *et al.*, 2005).

4.1.1.4 System ASC

In some cell types, part of the L-proline transport is achieved by another neutral amino acid transporter, system ASC (Baker *et al.*, 1999). In C6 glioma cells, L-proline transport has been demonstrated to occur via a saturable, Na^+-

dependent mechanism that involves system A and ASC (K_m=200 μM). The transport was inhibited by proline derivatives and analogues including hydroxyproline, and was stereo-specific, as D-proline could not be transported (Zafra *et al.*, 1994). Two system ASC transporters have been identified: ASCT1 cloned from a human cortex cDNA library and a human hippocampus cDNA library (Arriza *et al.*, 1993; Shafqat *et al.*, 1993) and ASCT2 isolated from a mouse testis cDNA library (Utsunomiya-Tate *et al.*, 1996). Northern blot analysis showed ASCT1 to be expressed in all tissues tested with highest levels detected in the brain, skeletal muscle and pancreas (Arriza *et al.*, 1993). Early embryonic expression of ASCT1 and ASCT2 is unknown. Substrates transported by ASCT1 included L-alanine, L-serine, L-threonine and L-valine (Arriza *et al.*, 1993). Northern blot analysis of ASCT2 revealed expression in lung, skeletal muscle, kidney, large intestine and adipose tissue (Utsunomiya-Tate *et al.*, 1996). Substrates for ASCT2 include small neutral amino acids, which exhibit very high affinity (K_m=20 μM), with lower affinity (K_m=280-520 μM) for long-chain amino acids (Utsunomiya-Tate *et al.*, 1996). Neither ASCT1 nor ASCT2 transport methylated amino acids such as MeAIB (O'Kane *et al.*, 2004).

4.1.1.5 System PROT

Apart from the common transporters, specific L-proline transporters exist in the brain (Hyde *et al.*, 2003). The PROT system is a brain-specific high-affinity transporter system (K_m=5-10 μM), is Na^+-dependent and has been shown to transport L-proline analogues (Shafqat *et al.*, 1995). More recently, this transporter has been localized at glutamatergic neurons (Renick *et al.*, 1999; Crump *et al.*, 1999) suggesting a signalling role for proline at these synapses.

4.1.2 Amino-acid transporters and signalling

Many cell types are able to sense amino acid levels and respond to them. However, the molecular mechanisms involved are not well understood. For example, the activity of mTOR kinase is known to be up-regulated by increased amino-acid levels, particularly leucine, in adipocytes (Lynch, 2001) but it is not known whether this is due to a direct effect on mTOR kinase or whether there is an upstream amino-acid sensor. Recently, it has been theorised that amino-acid transporters may themselves have the ability to act as "receptors" that sense the concentration of a particular amino acid and activate appropriate signalling pathways in response. There are four main suggestions as to how amino-acid transporters may act to initiate cellular signalling (Hyde *et al.*, 2003):

- The amino-acid transporter may undergo a conformational change following transport of the amino acid that leads to an induction of signalling.

- The signalling may be a secondary event resulting from changes in cell physiology such as altered pH or ion concentration since most amino-acid transporters couple transport to the translocation of ions such as Na^+ down a concentration gradient.

- Following internalisation into the cytoplasm, the amino acid may be, in its natural or modified form, recognised by an intracellular receptor that initiates signalling.

- The external concentration of an amino acid may be sensed by a specific 'amino-acid receptor' located in close proximity to the amino-acid transporter such that the transporter regulates the concentration of the amino acid around the receptor and thus the extent of signalling.

Amino acid-dependent signalling has been demonstrated in the case of mTOR-dependent phosphorylation of the S6 ribosomal protein. The protein S6 was phosphorylated in response to elevated amino-acid levels in an mTOR-dependent manner (van Sluijters *et al.*, 2000). The S6 protein is involved in the translation of mRNA coding for translational machinery proteins. Eukaryotic initiation factor 4E (eIF4E) binding protein-1 (4E-BP1) is also phosphorylated in response to elevated leucine levels and to a lesser extent by other amino acids. The phosphorylation was rapamycin-sensitive indicating the involvement of mTOR (van Sluijters *et al.*, 2000).

System A has been suggested to be able to function as both an amino-acid transporter and a sensor which is able to respond to changes in the external environment since amino-acid deprivation leads to an increase in activity of SAT2 transporter. This increased activity was shown to be due to increased transcription of the transporter and a greater number of transporter proteins on the cell surface (Hyde *et al.*, 2001; Ling *et al.*, 2001). As yet there are no amino-acid dependent signalling pathways that have been linked directly down-stream of SAT2 in this adaptive regulation.

For amino-acid transporters to function directly as signalling initiators, the transporter proteins need to be coupled to down-stream signalling pathways. There are various proteins that have been suggested as being involved in amino-acid initiated signalling by acting as bridging proteins including LIM (Lin-11, Isl-1 and Mec-3) domain proteins, heat shock proteins, cytoskeletal proteins and integrins (Hyde *et al.*, 2003). Ajuba is a LIM domain-containing protein that has been shown to associate with the transporter EAAT2. This protein also contains SH3 domains and is able to associate with Grb2 *in vitro* and *in vivo* which may provide a means of activating MAPK signalling. However, at the moment there is no evidence for EAAT2-dependent activation of MAPK signalling (Hyde *et al.*, 2003).

The first demonstration of interaction of a transporter with signalling intermediates, which led to altered function of the transporter, was shown for the GABA transporter GAT1 (Deken *et al.*, 2000). A component of the synaptic vesicle docking machinery syntaxin 1A was demonstrated to bind directly to the N-terminal domain of GAT1 and via this interaction decrease the rate of substrate internalisation. The inhibition of the syntaxin-GAT1 interaction by GAT1 substrates enabled GABA internalisation (Quick, 2002). Further regulation of the transporter occurred by the substrate-induced tyrosine phosphorylation of the transporter protein that led to decreased rate of internalisation and greater expression at the membrane (Whitworth and Quick, 2001).

4.1.3 Amino acids and embryonic development

Developmental studies have suggested that amino acids are involved in early embryogenesis in roles distinct from simply being the building blocks of proteins. *In vitro*-fertilised mouse eggs that are allowed to develop to the blastocyst stage require essential and non-essential amino acids in the culture medium. Exposure of the developing embryos to medium lacking in non-essential amino acids, for as little as five minutes, leads to a decrease in the number of embryos reaching the blastocyst stage (Gardner *et al.*, 1996). These observations led to investigations of amino-acid transporter systems operating during early development and results showed that the expression and function of amino-acid transporter systems appears to be developmentally regulated with particular transporters appearing at specific stages of development (Van Winkle, 2001). For example, prior to the blastocyst stage, the embryo does not express the glutamate transporter X_{AG}^- and is not able to accumulate this amino acid into cells (Van Winkle and Dickinson, 1995). Another transporter that is developmentally regulated is system N, which mediates glutamine transport. Functional studies demonstrated that there is a transient increase in the accumulation of glutamine at the four to eight cell stages of development, which correlates with the transient appearance of system N (Van Winkle and Dickinson, 1995, Van Winkle and Campione, 1996). Therefore, it appears that the accumulation of particular amino acids during embryonic development is regulated at least in part by the developmentally regulated expression of the transporters mediating their uptake.

The requirement for various amino acids during development is generally not well understood and at present not associated with initiation of intracellular signalling cascades. Most studies suggest that signalling resulting from changes in amino-acid concentrations are due to changes in cell volume or osmolarity (Van Winkle, 2001; Hyde *et al*, 2003). However, more direct effects of amino acids on cells have been identified. Amino acids have been implicated as having an essential role in blastocyst implantation through work studying the formation of blastocyst outgrowths *in vitro* (Martin *et al.*, 2003). For implantation to occur, trophoblast cells must undergo an epithelial-to-mesenchyme transition which results in various cellular changes including alterations in cellular motility, composition of apical membranes and adhesion complexes (Sutherland, 2003). The amino acids leucine and arginine have been shown to be essential in the above process as blastocysts cultured *in vitro* in medium lacking these amino acids fail to form outgrowths (Gwatkin, 1969). The action of amino acids was shown to be dependent on mTOR signalling since incubation of blastocysts with mTOR inhibitor rapamycin inhibited outgrowth formation (Martin and Sutherland, 2001).

Amino acid-dependent activation of mTOR appears to involve the leucine transporter system $B^{0,+}$ (Martin *et al.*, 2003). In ovariectomized mice, implantation of blastocysts can be induced by treatment with estrogen. Treatment with estrogen was shown to result in elevated Na^+ levels in uterine secretions which would mediate increased transport of leucine via the Na^+-coupled system $B^{0,+}$ and the activation of mTOR signalling (Martin *et al*, 2003). *In-vitro* evidence supported this role of system $B^{0,+}$ with blastocysts unable to undergo outgrowth in low Na^+ medium having their invasive property rescued by increasing Na^+ concentrations (Van Winkle, 1981). Therefore, these results suggest that amino acid-dependent signals regulate the differentiation of trophoblasts to an invasive cell type and indicate the involvement of the amino-acid transporter in the process.

4.1.4 Breakdown of short L-proline-containing peptides

The mechanism by which short L-proline-containing peptides induce EPL-cell morphology has not been characterised. It may result from a breakdown of these peptides to yield free L-proline. A number of peptidases have been identified that are able to release free proline from peptides (Table 4.2).

4.1.4.1 Proline-dependent peptidases

Peptidases have been identified which specifically cleave imino acid bonds within peptides (Table 4.2) (Dehm and Nordwig, 1970; Priestman and Butterworth, 1985; Lorey *et al.*, 2002; Maes *et al.*, 2005). One such peptidase is X-prolyl-aminopeptidase isolated from swine kidney homogenates (Dehm and Nordwig, 1970). Although the enzyme is able to cleave peptides of various lengths, provided that the second N-terminal position in the substrate is a proline residue, the highest cleavage rates were observed with tripeptides. Enzymatic assays also revealed that X-prolyl-aminopeptidase

Table 4.2 Summary of L-proline proteases and their preferred substrates

Enzyme	Bond attacked	Specific substrate	References
Imidodipeptidase (prolidase)	X-Pro	Gly-Pro	Bergmann *et al.*, 1932 Davis and Smith, 1957
X-prolyl-aminopeptidase	X-Pro- - -	Gly-Pro-Hyp	Dehm and Nordwig, 1970
Iminodipeptidase (prolinase)	Pro-X	Hyp-Gly	Grabmann *et al.*, 1932
Proline iminopeptidase	Pro-X- - -	poly-Pro	Sarid *et al.*, 1962
Aminopeptidase cleaving Gly-Pro-β-naphthylamide	X-Pro- - -	Gly-Pro-β-NA	Hopsu-Havu *et al.*, 1968
Carboxypeptidase P	- - -Pro-X	Z-Pro-Ala	Dehm and Nordwig, 1970
Lysosomal carboxypeptidase	- - -X-Pro	Z-Leu-Pro	Fruton and Bergman, 1939

Enzyme	Bond attacked	Specific substrate	References
Dipeptidyl peptidase II (DPPII)	X-Pro- - - X-Ala- - -	Ala-Pro-pNA Ala-Pro-4Me2NA Arg-Pro-Lys-Pro	McDonald and Schwabe, 1980; Eisenhauer and McDonald, 1986; Araki *et al.*, 2001; Maes *et al.*, 2005
Dipeptidyl peptidase IV (DPPIV)	X-Pro- - -	Neuropeptide Y, substance P	Hopsu-Havu *et al.*, 1966; Henis *et al.*, 1988; Mentlein et al., 1993; Oravecz *et al.*, 1997
Angiotensin converting enzyme (ACE)	- - - X-Y	Bradykinin, Substance P, angiotensin I, enkephalins	Yang *et al.*, 1971, Filipovic *et al.*, 1978; Stewart *et al.*, 1981; Andrade *et al.*, 1998
Neutral endopeptidase	- - - Z	Enkephalins, atrial natriuretic factor, substance P	Matsas *et al.*, 1983; Malfryo *et al.*, 1987; Erdos and Skidgel, 1989
Post proline endopeptidase	Pro-X	Substance P	Kato et al., 1980; Andrews et al., 1980

prefers glycine to be the N-terminal amino acid residue in the bond attacked, as the rate of cleavage of Ala-Pro-Gly was 6% of that for Gly-Pro-Hyp and Gly-Pro-Ala. The enzyme was unable to hydrolyse the bond if hydoxyproline was substituted for proline (Dehm and Nordwig, 1970). This enzyme has been suggested to be involved in the secondary catabolism of collagen, which is made up of repeating Gly-Pro-X sequences (Van Der Rest and Garrone, 1991).

Prolinase, also known as iminodipeptidase, is able to cleave proline-containing bonds and has been isolated from a number of animal tissues (Grabmann *et al.*, 1932; Sarid et al., 1962; Akrawi and Bailey, 1976; Priestman and Butterworth, 1985). Human kidney prolinase was shown to cleave peptides Pro-Leu, Pro-Val and Pro-Phe at a rate of 40-48% and peptides Pro-Gly and Pro-Ala at a rate of 9-13% of the control peptide Gly-Leu (Priestman and Butterworth, 1985). The enzyme exhibited no activity towards Pro-Gly-Gly, a known proline iminopeptidase substrate, and Gly-Pro a known substrate for prolidase (Priestman and Butterworth, 1985).

Dipeptidyl peptidase II (DPPII) has been identified and isolated in a number of mammalian tissues. DPPII functions to hydrolyse oligopeptides, in particular tripeptides, to release N-terminal X-Pro or X-Ala dipeptides (McDonald *et al.*, 1968; Mentlein and Struckhoff, 1989; Eisenhauer and McDonald, 1986). The affinity of human DPPII was greater for the sequence X-Pro than for X-Ala (Maes *et al.*, 2005).

4.1.5 L-proline and EPL cells

To begin delineating the molecular mechanism by which L-proline induces EPL-cell formation it was necessary to determine whether L-proline was exerting its EPL-cell inducing abilities at the cell surface, potentially by binding a receptor located in the cell membrane, or whether transport of L-proline into the ES cell was required. If L-proline internalisation was necessary, it was likely that one or a combination of amino-acid transporters known to transport L-proline was involved. As previously discussed (Section 4.1.1), there are a number of amino-acid transporters that have been identified as mediating L-proline transport in various cell types. Transporters such at system A members, SAT1 (Wang *et al.*, 2000), SAT2 (Yao *et al.*, 2000) and SAT3 (Hatanaka *et al.*, 2001) and PAT1 (Boll *et al.*, 2003) and PAT2 (Chen *et al.*, 2003) also transport other small neutral amino acids. Others such as PROT (Fremeau *et al.*, 1996; Shafqat *et al.*, 1995) exclusively transport L-proline. In this chapter, the involvement of amino-acid transporters in the ES-to-EPL cell transition is investigated.

4.2 RESULTS

4.2.1 Expression of L-proline transporters on ES and EPL cells

There are a number of amino-acid transporters that are able to mediate L-proline transport into cells (Table 4.1). The ability of L-proline to induce EPL-cell formation, as assessed by morphology, gene expression and differentiation potential, was previously shown to require a minimum concentration of ~200 μM. The expression of the various amino-acid transporters, known to transport L-proline in other cell types, was investigated in pluripotent cells via RT-PCR with primers specific for the individual transporters and cDNA from pluripotent cells. Expression in ES cells is of particular interest since these cells are responsive to extracellular L-proline (Chapter 3).

Figure 4.1 A shows that *PAT2* and *SAT1* transcripts were not expressed in ES cells, but a band of the correct size was detected in the positive controls for both transporters. Transcripts for *SAT2*, *PROT* and *PAT1* were expressed in ES and EPL cells (Figure 4.1 B).

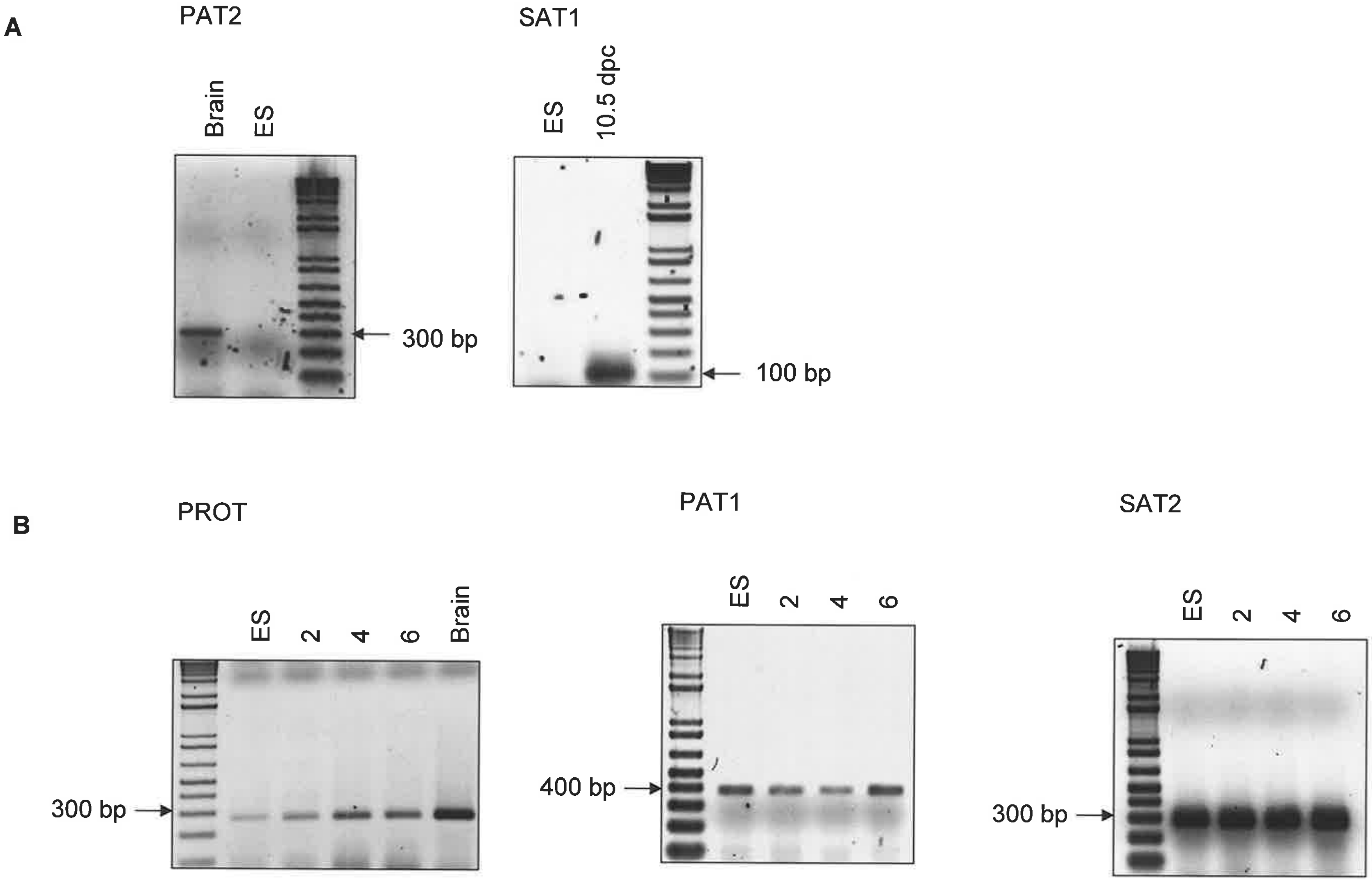
SAT2 was detected in pluripotent cell samples after 28 cycles while *PAT1* was detected after 35 cycles and *PROT* after 40 cycles. All primers were optimised to be within the linear range for amplification. Therefore, *SAT2* expression appears to be greater in ES and EPL cells compared to *PROT* and *PAT1*. The material in the bands was isolated and sequenced to confirm the identity.

Figure 4.1

Pluripotent cells express transcripts for amino acid transporters SAT2, PAT1 and PROT but not SAT1 and PAT2.

RT-PCR (2.6.4-5) was performed using primers (2.4.2) specific for SAT1, SAT2, PAT1, PAT2 and PROT on mRNA isolated (2.9.1) from ES cells and EPL cells (IMINO sequence not available at the time of the experiment). Mouse brain cDNA or 10.5 dpc embryo cDNA was used as a positive control. The linear range for amplification of the individual transporters was determined for each primer set (2.6.5). PCR (2.7.5) was performed for 28 cycles for SAT2, 35 cycles for SAT1 and PAT1 and 40 cycles for PAT2 and PROT. PCR products were electrophoresed (2.7.1) on 2% agarose gel with 1 kb ladder, stained with ethidium bromide and visualised by FX scanning. Expression of (A) PAT2, SAT1 in ES cells, mouse brain or 10.5 dpc embryo, and (B) PROT, PAT1 and SAT2 in ES cells or cells cultured for 2, 4 and 6 days in the presence of 200 μM L-proline (relevant size markers are indicated). Expected fragment sizes: PAT1 393 bp, PAT2 308 bp, SAT1 120 bp, SAT2 291 bp and PROT 270 bp. Experiments were performed three times and representative results are shown.

Figure 4.1


A
PAT2
Brain
ES
300 bp
SAT1
ES
10.5 dpc
100 bp
B
PROT
ES
2
4
6
Brain
300 bp
PAT1
ES
2
4
6
400 bp
SAT2
ES
2
4
6
300 bp

4.2.2 Investigation of functional involvement of L-proline transporters in the ES-to-EPL cell transition

Transport of a particular amino acid through a specified transporter can be blocked using a molar excess of another substrate that acts as a competitive inhibitor (Christensen, 1989; Baker *et al.*, 1999). The involvement of the individual transporters expressed on ES cells, PROT, PAT1 and SAT2, in the ES-to-EPL transition was initially monitored by performing morphology assays in the presence or absence of a molar excess of competitive inhibitors over L-proline. SAT2 transports L-proline, L-alanine, L-serine, glycine and MeAIB while PAT1 transports L-proline, L-alanine, glycine, MeAIB but not L-serine and PROT transports L-proline exclusively (Table 4.1). L-lysine functions as a negative control since it is not transported by SAT2, PAT1 or PROT.

ES cells cultured for five days in ES complete medium supplemented with 200 μM L-proline form flattened EPL-cell colonies. However, the culture of cells with L-proline and either 5 mM MeAIB, or 10 mM glycine prevented the morphology change (Figure 4.2). Culture of ES cells in the presence of L-proline and 10 mM L-serine also prevented the formation of colonies with EPL-cell morphology (Figure 4.2). However, culture of ES cells in the presence of L-proline and 5 mM L-lysine did not prevent establishment of EPL-cell morphology (Figure 4.2). The ability of glycine, L-serine and MeAIB to block L-proline-induced EPL-cell morphology indicates that transport of L-proline into ES cells is required for establishment of EPL cell morphology. System PROT does not appear to be involved since glycine, L-serine and MeAIB do not act as competitive inhibitors of L-proline transport through this

Figure 4.2

Molar excess of glycine, MeAIB and L-serine but not L-lysine inhibits L-proline-induced EPL-cell morphology

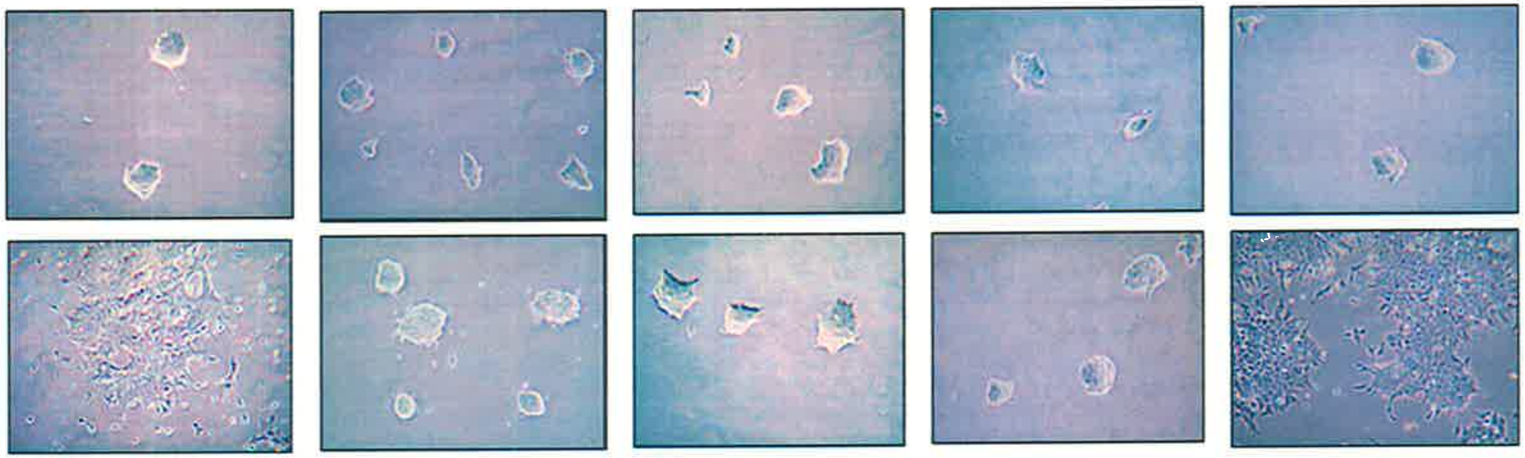
ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) in the presence of 200 μM L-proline with or without 10 mM glycine, 5 mM MeAIB, 10 mM L-serine, or 5 mM L-lysine. Following five days of culture, the cells were photographed under phase contrast (100X magnification). The experiment was performed in duplicate four times and a representative result is shown.

Figure 4.2

SAT2 substrate:	yes	yes	yes	no
PAT1 substrate:	yes	yes	no	no
	+ glycine	+ MeAIB	+ L-serine	+ L-lysine

ES complete medium

+ L-proline



Therefore, the transporter that appears to be involved in L-proline dependent EPL-cell formation is SAT2.

transporter. The ability of L-serine to inhibit L-proline-mediated EPL-cell formation indicates that PAT1 is not involved since L-serine does not act as a competitive inhibitor of L-proline uptake through this transporter. Therefore, the transporter that appears to be responsible for the uptake of L-proline into ES cells is SAT2. The ability of glycine, L-serine and MeAIB to prevent EPL-cell morphology was not a general amino-acid effect since excess L-lysine, a cationic amino acid not transported by any of the three transporters, did not prevent induction of EPL-cell morphology. The results therefore indicate that competitive inhibitors of SAT2 are able to prevent the ES-to-EPL cell morphology change mediated by L-proline (Figure 4.2). Transporters ASC and IMINO were not included in our expression analysis (Section 4.2.1) but can be ruled out as being functionally involved in the transition since they are both not able to transport N-methylated amino acids such as MeAIB and MeAIB was able to act as a competitive inhibitor of the transition.

4.2.3 *Dnmt3b* is a novel EPL-cell marker

Microarray analysis identified that *Dnmt3b1* was up-regulated following formation of EPL cells (Figure 4.3 A). This was confirmed by PCR analysis of EBM series with *Dnmmt3b1* shown to be up-regulated in EBM3 (primitive ectoderm/EPL) and EBM6 (definitive ectoderm) compared to EBM0 (ES) and EBM9 (neurectoderm) (Figure 4.3 B). *Dnmt3b1* was also up-regulated in EPL cells formed in adherent culture in response to L-proline by day 4 (Figure 4.3 C). As previously discussed (Section 1.2.4.2) *Fgf5* was shown to be an unreliable marker of the EPL-cell state with expression oscillating during culture of EPL cells and *Fgf5* expression was not being absolutely required for

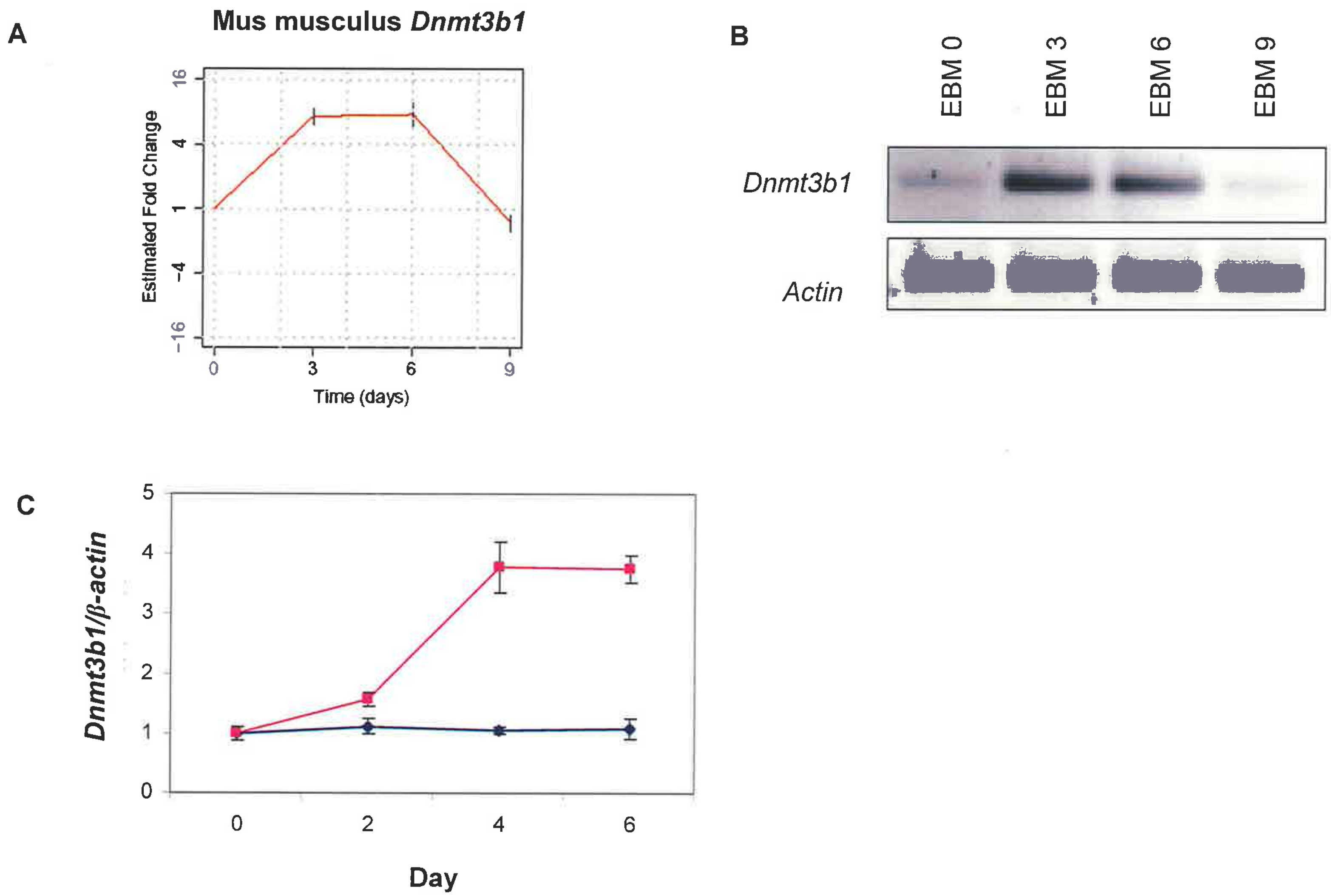
Figure 4.3

Dnmt3b1 expression is up-regulated in EPL cells

(A) *Dnmt3b1* expression as determined by microarray analysis of RNA from EBM0 (ES/ICM), EBM3 (EPL/primitive ectoderm), EBM6 (definitive ectoderm) and EBM9 (neurectoderm).

(B) Validation of *Dnmt3b1* microarray expression data by RT-PCR (2.7.4-5) analysis. ES cells were seeded into MEDII and cultured for 9 days in suspension. Total RNA was isolated (2.9.1) from bodies and analysed for the expression of *Dnmt3b1* relative to β-actin. The linear amplification range for each primer set was determined (2.7.5). (C) *Dnmt3b1* up-regulation in adherent EPL cells. ES cells were seeded into ES complete medium (♦) supplemented with 200 μM L-proline (■). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Quantitative PCR (2.7.6) was performed to determine expression of *Dnmt3b1* relative to that of *β-actin*. Normalised expression in ES cells was assigned a value of 1 and the expression in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. The experiment was performed three times and a representative result is shown.

Figure 4.3


A
Mus musculus *Dnmt3b1*
Estimated Fold Change
16
4
1
−4
−16
0
3
6
9
Time (days)
B
EBM 0
EBM 3
EBM 6
EBM 9
Dnmt3b1
Actin
C
Dnmt3b1/β-actin
5
4
3
2
1
0
0
2
4
6
Day

establishment of EPL-cell differentiation potential (Bettess, 2001, Washington, unpublished data). Therefore, future analysis utilised *Dnmt3b1* as a positive EPL-cell marker.

4.2.4 Competitive inhibitors of L-proline transporter SAT2 prevent EPL-cell gene expression

To determine the gene expression profile of cells in the presence of L-proline and a molar excess of glycine, L-serine, MeAIB or L-lysine, RNA extracted from the cells cultured for up to 6 days was analysed for the expression of the ES-cell specific marker *Rex1*, EPL-cell marker *Dnmt3b1* and the pluripotence marker *Oct4*. Culturing ES cells in ES complete medium in the presence of 200 µM L-proline and excess glycine, serine or MeAIB resulted in maintained expression of *Rex1* and *Oct4* and low expression of *Dnmt3b1*, indicative of an ES cell associated gene expression profile (Figure 4.4 A-C and 4.5 A-C). Excess lysine in the presence of 200 µM L-proline was not able to maintain ES cell-associated gene expression with the cells down-regulating *Rex1* and up-regulating *Dnmt3b1* (Figure 4.4 A-B and 4.5 A-B) consistent with the formation of EPL cells. The presence of glycine, L-serine, MeAIB or L-lysine alone in the medium did not affect the expression of these marker genes compared to ES cells (Figures 4.4 A-C and 4.5 A-C). These data indicate that competitive inhibitors of the SAT2 transporter maintained the ES-cell state in terms of gene expression and prevented the formation of EPL cells in the presence of L-proline.

Figure 4.4

Molar excess of glycine prevents L-proline-induced EPL-cell gene expression

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) alone (♦), supplemented with 200 μM L-proline (■) or 20 mM glycine (▲) or with 200 μM L-proline and 20 mM glycine (X). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Quantitative PCR (2.7.6) was performed to determine expression of (A) *Rex1*, (B) *Dnmt3b1* and (C) *Oct4*. *β-actin* was used as a control. Relative expression of each gene versus *β-actin* in ES cells was assigned a value of 1 and the expression level in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. Experiments were performed three times and a representative result is shown.

Figure 4.4

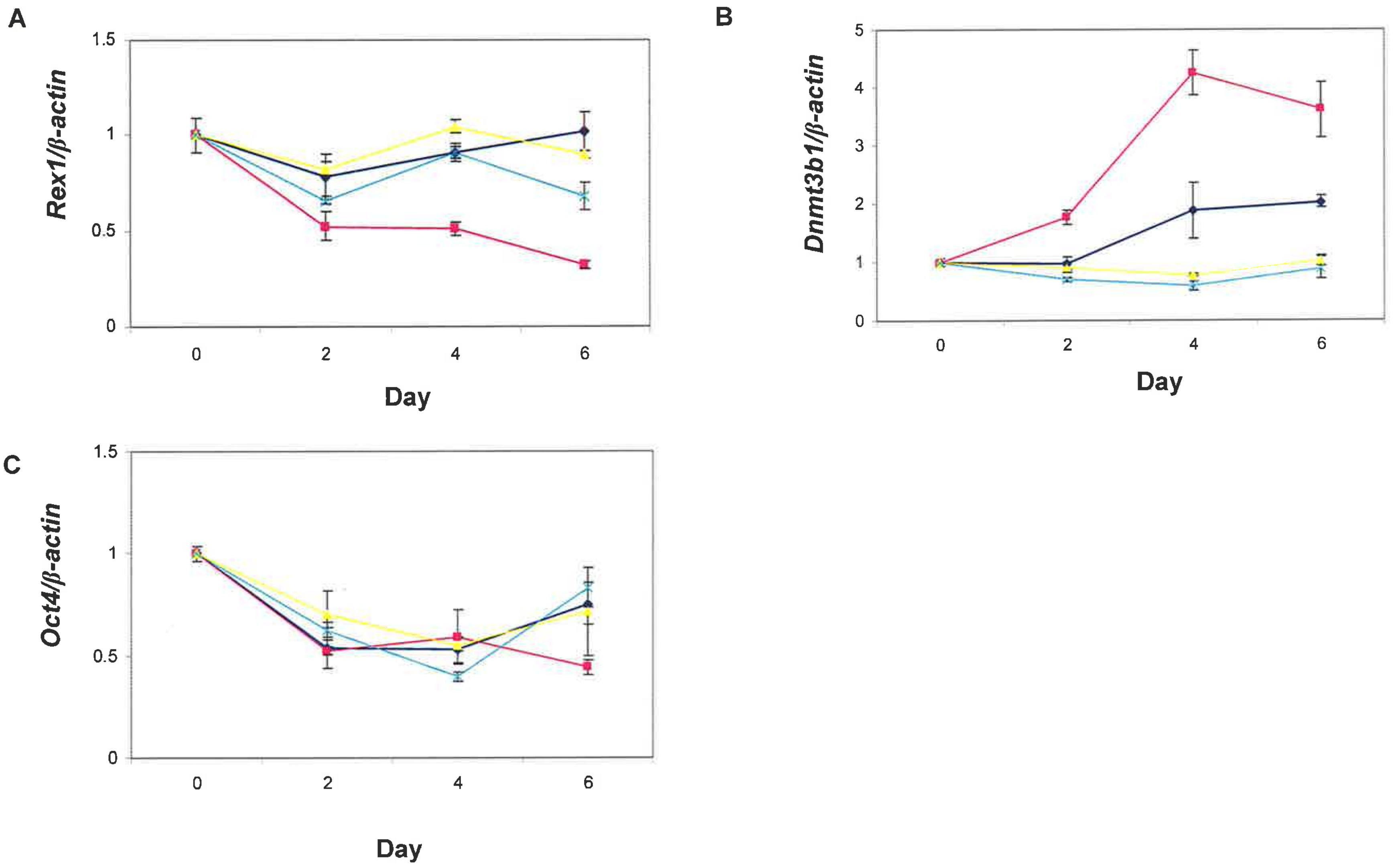
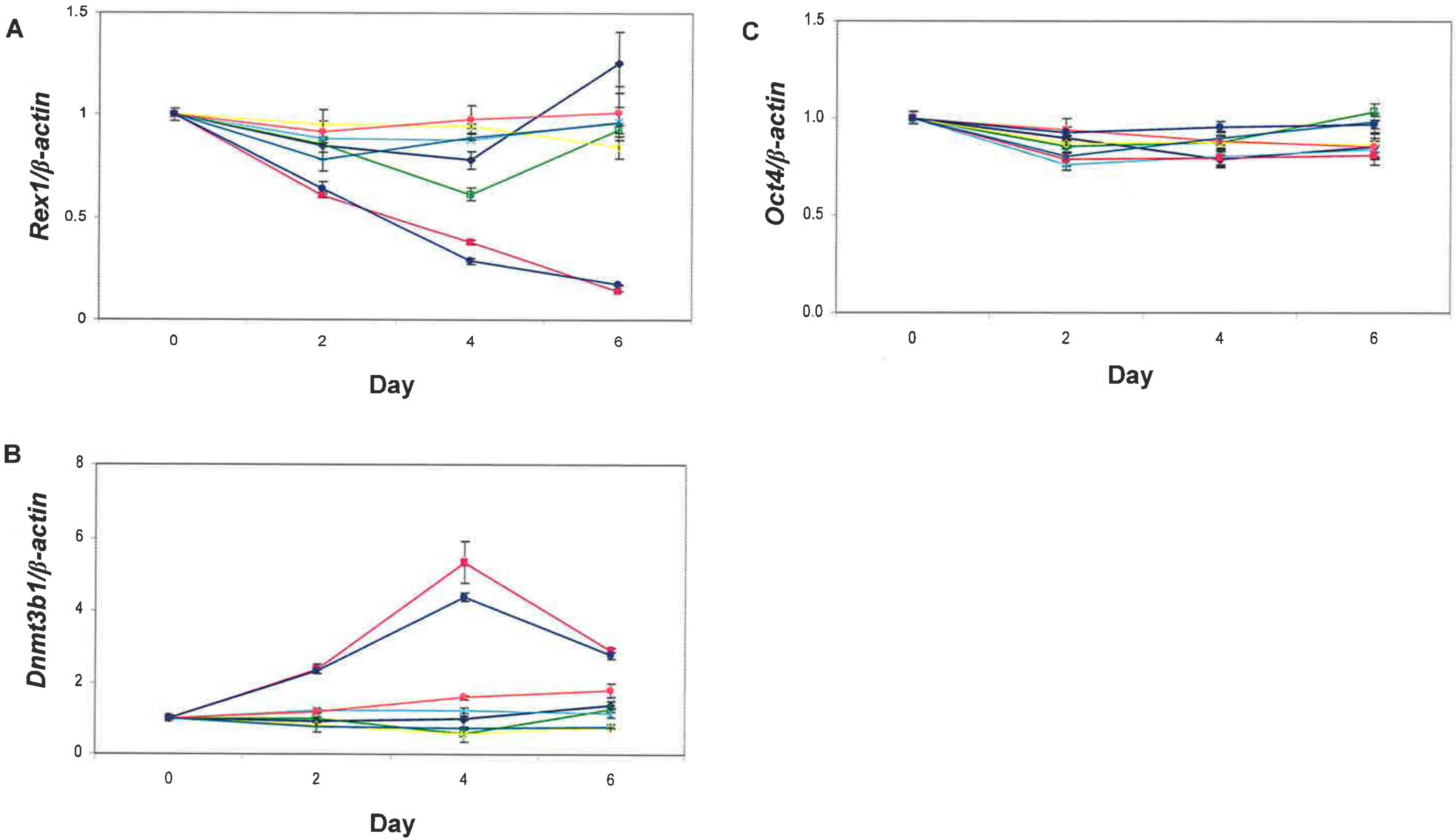

A
1.5
1
0.5
0
Rex1/β-actin
0
2
4
6
Day
B
5
4
3
2
1
0
Dnmt3b1/β-actin
0
2
4
6
Day
C
1.5
1
0.5
0
Oct4/β-actin
0
2
4
6
Day

Figure 4.5

Molar excess of MeAIB and L-serine but not L-lysine inhibits L-proline induced EPL cell gene expression

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) alone (♦), supplemented with 200 μM L-proline (■), 5 mM MeAIB (□), MeAIB and L-proline (◌), 10 mM L-serine (◊), L-serine and L-proline (ı), 5 mM L-lysine (X) or L-lysine and L-proline (●). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Quantitative PCR (2.7.6) was performed to determine expression of (A) *Rex1*, (B) *Dnmt3b1* and (C) *Oct4*. *β-actin* was used as a control. Relative expression of each gene versus *β-actin* in ES cells was assigned a value of 1 and the expression level in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. Experiments were performed three times and representative results are shown.

Figure 4.5


A
1.5
1
0.5
0
Rex1/β-actin
0
2
4
6
Day
C
1.5
1.0
0.5
0.0
Oct4/β-actin
0
2
4
6
Day
B
8
6
4
2
0
Dnmt3b1/β-actin
0
2
4
6
Day

4.2.5 Competitive inhibitors of L-proline transporters prevent L-proline-induced EPL-cell differentiation potential

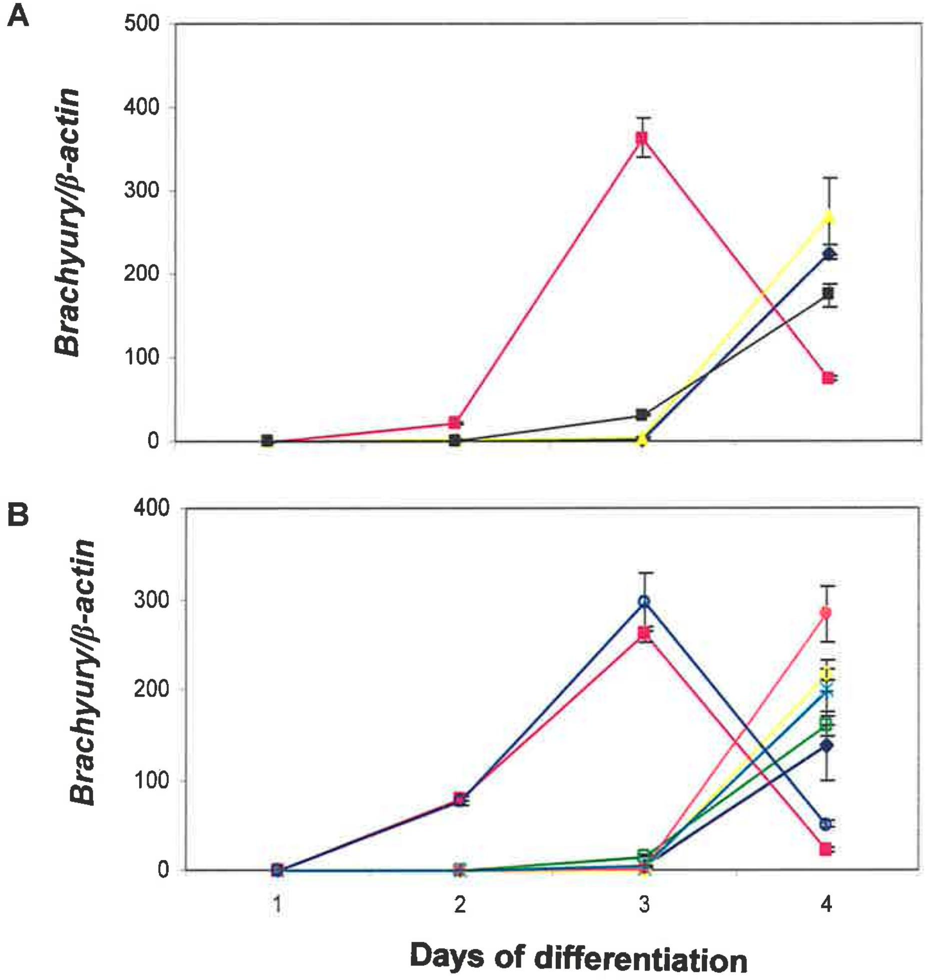
The effect on differentiation potential of cells grown for 4 days in ES complete medium supplemented with L-proline in the presence or absence of 20 mM glycine, 10 mM L-serine, 5 mM MeAIB or 5 mM L-lysine was investigated. Figure 4.5 shows the quantitative PCR results for *Brachyury* expression in the embryoid bodies made from cells grown under these conditions. Bodies made from ES cells grown in ES complete medium up-regulated expression of *Brachyury* on day 4 consistent with ES-cell differentiation potential (Figure 4.6 A, B). Bodies made from cells grown in the presence of L-proline up-regulated expression of *Brachyury* on day 3, consistent with EPL-cell differentiation potential. Bodies made from cells grown in the presence of L-proline and SAT2 competitive inhibitors glycine (Figure 4.6 A), serine or MeAIB (Figure 4.6 B) up-regulated *Brachyury* expression on day 4 indicating ES-cell differentiation potential. The presence of glycine, L-serine, MeAIB or L-lysine alone in ES complete medium did not affect the differentiation potential of the ES cells with bodies derived from these cells still up-regulating *Brachyury* on day 4. The effect observed in the presence of SAT2 competitive inhibitors was specific and not a general amino acid effect since bodies made from cells grown in the presence of L-proline and L-lysine up-regulated *Brachyury* expression on day 3. These results indicate that ES cells cultured in the presence of L-proline and excess SAT2 competitive inhibitors maintained a differentiation potential consistent with that of ES cells.

Figure 4.6

Molar excess of glycine, MeAIB and L-serine but not L-lysine inhibits EPL cell differentiation potential

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) alone (♦), supplemented with 200 µM L-proline (■), 20 mM glycine ( ), L-proline and glycine (■), 5 mM MeAIB (□), MeAIB and L-proline ( ), 10 mM L-serine ( ), L-serine and L-proline (ı), 5 mM L-lysine (X) or L-lysine and L-proline (O). Cells were grown for 4 days with daily re-feeding and a passage on day 2. On day 4, embryoid bodies were formed (2.6.6) by seeding single cells into ICβ medium. Embryoid bodies were grown for up to 4 days and collected for analysis daily. Collected embryoid bodies were analysed by quantitative PCR (2.7.6) for the expression of *Brachyury*. *β-actin* was used as a control. Relative *Brachyury* expression versus *β-actin* in EB1 was assigned a value of 1 and the expression level in day 2-4 bodies is shown relative to this. Error bars represent the SD of triplicates. Experiments were performed two times and representative results are shown.

Figure 4.6


A
B
500
400
300
200
100
0
Brachyury/β-actin
1
2
3
4
Days of differentiation

4.2.6 SAT2 substrates block EPL-cell inductive capacity of ala-pro and gly-pro

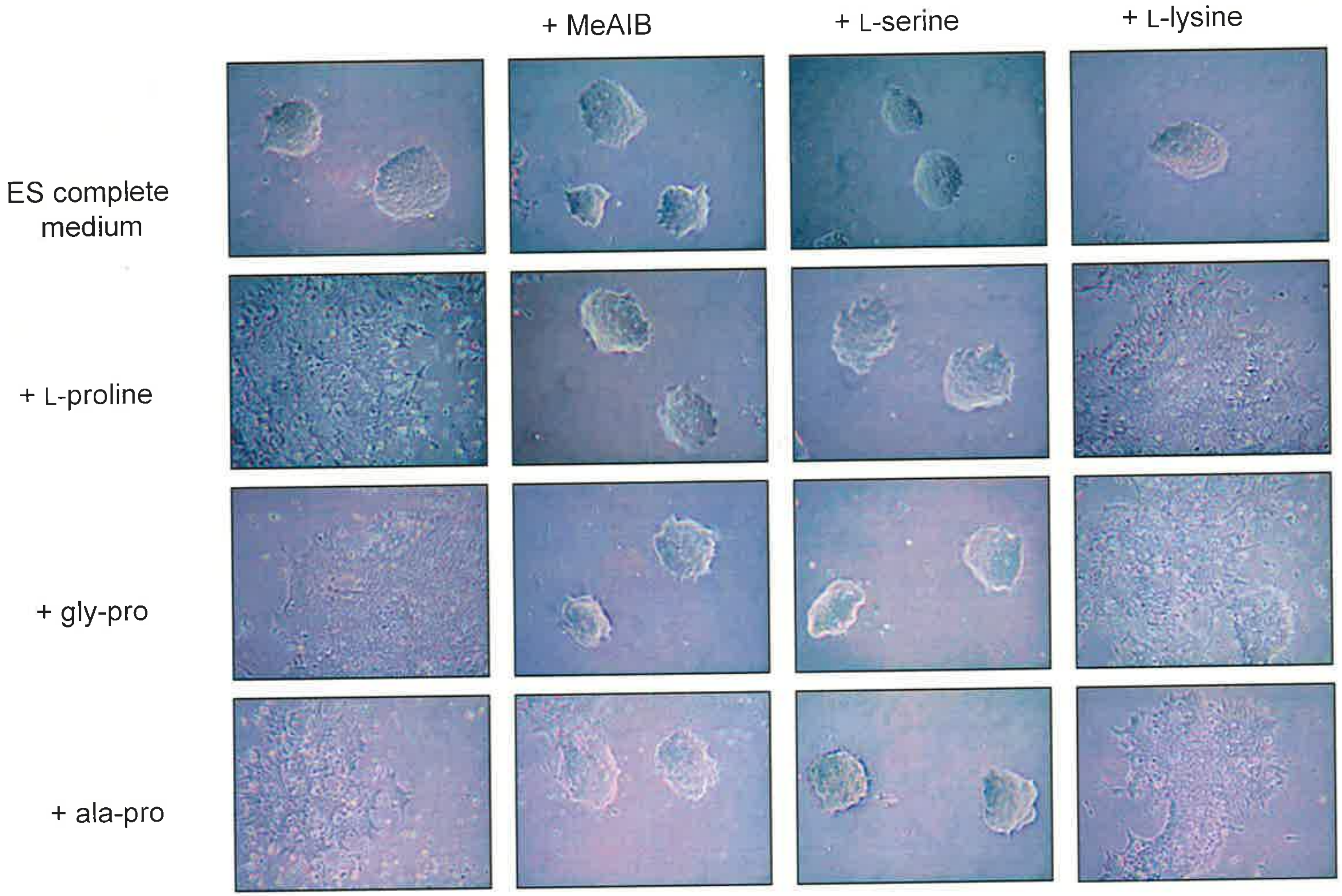
In Chapter 3 it was demonstrated that the ES-to-EPL cell transition could be induced by free L-proline and also by some L-proline-containing peptides. The results in this chapter indicate that internalisation of L-proline, via an amino acid transporter, appears to be necessary for the transition as determined by results from experiments using competitive inhibitors of amino-acid transporters. However, amino-acid transporters such as SAT2 transport only single amino acids and not peptides. The possibility exists that the peptides such as gly-pro and ala-pro, which were shown to induce the transition, do so by first being broken down to release free L-proline, which is then internalised via the transporter. If this is the case, then SAT2 competitive inhibitors such as glycine, L-serine and MeAIB should prevent the EPL-cell inductive ability of these L-proline containing peptides. In order to test this hypothesis, morphology assays were performed with cells cultured in the presence of 400 μM ala-pro and 200 μM gly-pro with or without 5 mM MeAIB, 10 mM serine or 5 mM lysine for five days. Figure 4.7 shows that in the presence of either peptide and MeAIB or L-serine colonies remained ES like while in the absence of these SAT2 competitive inhibitors the peptides induced EPL-cell morphology. L-lysine did not affect the EPL-cell inductive capacity of the peptides. Therefore, it appears that, at least with the ala-pro and gly-pro, their EPL-cell inductive capacity is due to breakdown of the peptides to free L-proline, which is then internalised via SAT2.

Figure 4.7

SAT2 transporter inhibitors prevent EPL-cell morphology induced by gly-pro and ala-pro

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) in the presence of 200 μM gly-pro, 400 μM ala-pro or 200 μM L-proline as a control with or without 5 mM MeAIB, 10 mM L-serine or 5 mM L-lysine. Following five days of culture, the cells were photographed under phase contrast (100X magnification). The experiment was performed in triplicate three times and a representative result is shown.

Figure 4.7


+ MeAIB
+ L-serine
+ L-lysine
ES complete medium
+ L-proline
+ gly-pro
+ ala-pro

4.3 DISCUSSION

4.3.1 Transporter SAT2 is required for the formation of EPL cells in response to L-proline and short L-proline-containing peptides

Semi-quantitative RT-PCR showed the SAT2 transporter to be highly expressed in both ES and EPL cells (Figure 4.1). The functional involvement of SAT2 in the L-proline mediated ES-to-EPL cell transition was indicated by the fact that SAT2 competitive inhibitors glycine, L-serine and MeAIB were able to prevent the transition (Figures 4.2, 4.4, 4.5, 4.6). L-lysine, an amino acid that is not a substrate of SAT2, did not have an effect on the L-proline-induced transition indicating that the effect was not a general response to all amino acids but is specific to competitive inhibitors of SAT2 (Figures 4.5, 4.6 B). SAT2 is the only amino acid transporter expressed in ES cells known to transport all four substrates; L-proline, glycine, L-serine and MeAIB.

The results from this chapter strongly indicate a requirement for L-proline uptake into ES cells for the induction of EPL-cell formation in terms of morphology, gene expression and differentiation potential: Culture of ES cells in the presence of L-proline plus glycine, L-serine or MeAIB prevented establishment of flattened EPL-cell colonies (Figure 4.2), maintained expression of ES-specific marker *Rex1* and prevented up-regulation of the EPL-cell marker *Dnmt3b1* (Figures 4.3, 4.4). Likewise, embryoid bodies derived from cells cultured in L-proline and glycine, L-serine or MeAIB had differentiation potentials consistent with ES cells, up-regulating the mesoderm marker *Brachyury* on day 4 (Figure 4.5).

The establishment of SAT2 amino-acid transporter involvement in the ES-to-EPL cell transition allows the explanation of some previously noted observations (Table 1.2). Morphology assays indicated that D-proline unlike L-proline was not able to induce EPL-cell formation (Washington, unpublished data). Also, various L-proline analogues including those with carboxy terminal modifications such as L-prolinamide, amino terminal modification like N-acetyl-L-proline and those with altered ring structures like pipecolic acid were unable to induce EPL-cell formation (Washington, unpublished data). System A transport is stereo-selective, as D-proline, unlike L-proline, is not transported (Zafra *et al.*, 1994). In addition, the transport of fluorescently labelled L-proline analogues was shown to be 50-70% lower compared to transport of L-proline (Langen *et al.*, 2002) indicating the selectivity of the natural ligand for the transporter. Therefore, the inability of D-proline and proline analogues to induce EPL-cell formation may be due to the limited ability of SAT2 to mediate their transport into cells.

4.3.2 Potential roles for SAT2 in EPL-cell formation

ES and EPL cells also express the L-proline transporters PAT1 and PROT (Figure 4.1) in addition to SAT2 (Renick *et al.*, 1999; Crump *et al.*, 1999; Boll *et al.*, 2002; Chen *et al.*, 2003). However, the activity of these two transporters is not sufficient to mediate the transition since when L-proline uptake via the SAT2 transporter is selectively blocked using L-serine, which does not inhibit PAT1 or PROT, the ES state is maintained. This suggests that the rate of influx of L-proline into cells might be important for EPL-cell formation. Semi-quantitative RT-PCR suggested that SAT2 was more highly expressed in ES and EPL cells compared to PAT1 and PROT (Figure 4.1). SAT2 also has a higher affinity for L-proline (K_m=200 μM) than PAT1 (K_m=2.8 mM). Together these are consistent with the possibility that SAT2 may mediate a more rapid accumulation of L-proline into ES cells. The rapid accumulation may be required for L-proline to reach a threshold concentration within cells necessary for activation of signalling cascades in a manner analogous to activation of mTOR signalling via elevated L-leucine levels (Lynch, 2001). Accumulation of L-proline may also be required for the synthesis of specific proline-rich proteins that are required for the transition. A slower rate of accumulation of L-proline into ES cells may be insufficient to reach the threshold concentration before L-proline is diverted to other functions within the cell.

L-proline needs to be constantly present in the medium to maintain the EPL-cell state. This is because ES medium contains LIF that will revert EPL cells back to ES cells in the absence of the EPL-inductive factor (Rathjen *et al.*, 1999; Lake *et al.*, 2000). This may support the rate/threshold hypothesis since continued presence of extracellular L-proline may allow its continual accumulation and maintenance above the required threshold.

A possible down-stream target of L-proline action is the amino acid-associated signalling effector mTOR. *mTOR*$^{-/-}$ mice show arrested development following implantation at embryonic day 5.5 (Gangloff *et al.*, 2004). At this stage, wild type embryos have an elongated egg cylinder surrounding a pro-amniotic cavity as well as defined primitive ectoderm, extra-embryonic ectoderm and differentiated visceral and parietal endoderm. The *mTOR*$^{-/-}$ embryos were severely reduced in size, did not display clear regions of primitive ectoderm or extra-embryonic ectoderm and showed disorganised visceral endoderm cells (Gangloff *et al.*, 2004). This suggests that mTOR may be involved in the transition of ICM cells to primitive ectoderm cells and extraembryonic ectoderm. Recent work from the lab shows that rapamycin, which specifically inhibits mTOR, is able to prevent the L-proline-mediated ES-to-EPL transition (Hamra, unpublished results).

4.3.3 Short L-proline containing peptides – mode of action may involve breakdown

The fact that L-proline internalisation appears to be necessary for the ES-to-EPL cell transition raised the question as to how L-proline-containing peptides were functioning to induce formation of EPL cells. Peptides cannot be transported into cells via SAT2, which is only able to transport small neutral amino acids (Yao *et al.*, 2000; Sugawara *et al.*, 2000). At least three possibilities exist to explain the involvement of proline-containing peptides in EPL-cell formation. Firstly, it is possible that the peptides were functioning to induce the transition via a different mechanism to free L-proline, which did not require internalisation into the cell. Secondly, the peptides may be internalised via a different mechanism, possibly via peptide transporters such as PEPT1/2 (Saito *et al.*, 1997; Fujita *et al.*, 2004) and once inside were able to induce the same signals as L-proline to enable EPL-cell formation. Thirdly, the peptides may be broken down extracellularly via the action of peptidases to release free L-proline which could then be internalised via SAT2 and induce

EPL-cell formation. Results from morphology assays demonstrated that SAT2 substrates MeAIB and L-serine were both able to prevent EPL-cell morphology induced by the peptides gly-pro, ala-pro (Figure 4.7) and MEDII (Washington, unpublished data), suggesting that the mode of action of the peptides is consistent with the third possibility and involves their extracellular breakdown to free proline, which is then internalised by the transporter SAT2 to exert its effect. However, to confirm this gene expression and differentiation potential analysis would also need to be performed.

4.3.4 Potential role for L-proline induction of primitive ectoderm *in vivo*

Although L-proline has been shown to induce the formation of EPL cells *in vitro* the role of this amino acid *in vivo* in the formation of primitive ectoderm is not known. As previously discussed (Chapter 3.3), the embryonic environment would likely have high local concentrations of free L-proline present at the time of primitive ectoderm formation provided by basement membrane breakdown. This together with the finding that system A is nearly absent in the mouse embryo prior to the blastocyst stage followed by increasing dependence on system A from the late blastocyst stage (Zuzack *et al.*, 1985) is consistent with the findings in this chapter that the system A member SAT2 is required for L-proline internalisation necessary for EPL-cell formation. An important property of the SAT2 transporter is that it is known to be regulated by growth factors. In smooth muscle cells SAT2 activity was increased due to increased transcription of the *SAT2* gene induced by TGFβ (Ensenat *et al.*, 2001). In skeletal muscle cells, insulin signalling did not affect transcription but led to enhanced recruitment of the SAT2 protein to the plasma membrane from the endosomal compartment (Hyde *et al.*, 2002). Other studies have also implicated cAMP and MAPK signalling in modifications of system A activity (Hatanaka *et al.*, 2001; Lopez-Fontanals *et al.*, 2003). The involvement of growth factor signalling on system A activity during development has not been investigated. It is therefore possible that high levels of SAT2 expression at this stage of development, together with high local concentration of L-proline resulting from collagen turnover results in the accumulation of L-proline into ICM cells to induce the formation of primitive ectoderm.

4.3.5 Summary

In summary, the results from this chapter indicate that the transition from ES to EPL cells first requires L-proline internalisation into ES cells via the system A transporter SAT2. Furthermore, some of the L-proline-containing peptides (gly-pro and ala-pro) that are able to induce EPL-cell formation (Chapter 3) appear to act as a source of free L-proline. The determination of this first part of the molecular mechanism involved in the transition has also highlighted the first step at which the transition can be controlled, through the use of competitive inhibitors of the SAT2 transporter such as MeAIB, glycine and L-serine.

The use of SAT2 substrates glycine, L-serine and MeAIB as competitive inhibitors of EPL-cell formation may provide a means of maintaining of ES cells in culture. This may be particularly relevant to human ES cells that are prone to spontaneous differentiation in culture. The removal of these competitors and the addition of L-proline may, compared to the complex conditioned medium MEDII, provide a cheap and reliable means of forming EPL cells which can then be used as the starting population for further directed differentiation. Of particular interest is the production of cells that could be used in preclinical animal models of disease and in the clinic itself.

CHAPTER 5:

INVESTIGATION OF SIGNALLING PATHWAYS INVOLVED IN THE ES-TO-EPL CELL TRANSITION

CHAPTER 5: INVESTIGATION OF SIGNALLING PATHWAYS INVOLVED IN THE ES-TO-EPL CELL TRANSITION

5.1 INTRODUCTION

5.1.1 LIF-activated MAPK signalling

The binding of LIF to the gp130-LIFRβ receptor complex leads to the activation of several down-stream signalling pathways including MAPK (Boulton *et al.*, 1994; Yin and Yang, 1994; Burdon *et al.*, 1999), PI3K (Boulton *et al.*, 1994; Takahashi-Tezuka *et al.*, 1998; Paling *et al.*, 2004) and STAT3 (Stahl *et al.*, 1994; Niwa *et al.*, 1998; Matsuda *et al.*, 1999) (Figure 1.9). Signalling via STAT3 and PI3K is required for the maintenance of mouse ES cells in culture (Niwa *et al.*, 1998; Matsuda *et al.*, 1999; Paling *et al.*, 2004) while MAPK signalling inhibits self-renewal and promotes differentiation (Burdon *et al.*, 1999; Schmitz *et al.*, 2000). Neither gp130 nor LIFRβ possess intrinsic kinase activity but are constitutively associated with Jak family non-receptor tyrosine kinases (Stahl *et al.*, 1994). Following ligand binding, the gp130/LIFRβ receptors dimerise which leads to the activation of the associated Jaks that in turn phosphorylate the receptors forming docking sites for SH2 containing proteins (Boulton *et al.*, 1994; Stahl *et al.*, 1994). One of the proteins recruited to the activated gp130-LIFRβ receptor complex is Shp-2 (Fukada *et al.*, 1996). Shp-2 binds to the phosphorylated tyrosine 118 on the gp130 receptor via its N-terminal SH2 domain. Shp-2 is consequently phosphorylated at tyrosine 118 leading to the recruitment of Grb2 (Hibi and Hirano, 2000). The complex formed by Grb2 and Sos guanine-exchange factor then activates Ras and initiates the MAPK cascade leading to the sequential activation of MEK1 by Raf-1 phosphorylation of Ser217/221 (Zheng and Guan, 1994; Pages *et al.*, 1994; Xu *et al.*, 1995) and ERK1/2 by MEK1 phosphorylation of Thr202/Tyr204 (Sturgill *et al.*, 1988; Payne *et al.*, 1991).

5.1.2 Inhibitors of MAPK pathway component MEK1

In recent years many small, cell-permeable protein kinase inhibitors have been developed (Davies *et al.*, 2000). Protein kinase inhibitors are useful tools for studying the biological roles of kinases and for the identification of signalling cascades involved in various biological processes (Cohen, 1999). The inhibitor U0126 inhibits MEK1 action (Favata *et al.*, 1998). *In vitro*, 10 μM U0126 was found to reduce MEK1 activity to 56% while other kinases tested were not significantly affected (Davies *et al.*, 2000). Only when used at 1000-fold higher concentrations were small reductions in kinase activity evident on other kinases in the panel (Davies *et al.*, 2000). U0126 functions by suppressing the activation of MEK1 by Raf and not by affecting the kinase activity of MEK1 itself (Davies *et al.*, 2000). U0126 has been shown to inhibit MAPK signalling via MEK1 in many cell systems (DeSilva *et al.*, 1998; Davies *et al.*, 2000).

Another MEK1 inhibitor is PD098059 (Dudley *et al.*, 1995; Alessi *et al.*, 1995). *In vitro*, PD098059 was shown to have an IC_{50} of 2-7 μM for MEK1 while the related kinase MEK2 was inhibited at much greater concentrations ($IC_{50} \approx 50$ μM) (Alessi *et al.*, 1995). PD098059 was functional in cell-based systems as it was shown to suppress MEK1 activation, by a number of different agonists, in Swiss 3T3 cells by 80-90% (Alessi *et al.*, 1995). Similar to U0126, PD098059 prevents the activation of MEK1 by binding the inactive form of the kinase and thus blocking its phosphorylation by Raf (Alessi *et al.*, 1995, Davies *et al.*, 2000, Mody *et al.*, 2001). U0126 and PD098059 also inhibit the activation of ERK5 by MEK5 (Kamakura *et al.*, 1999). However, the effect of the inhibitors on ERK5 and MEK5 require higher concentrations than for the inhibition of MEK1 (Mody *et al.*, 2001). Western blot analysis revealed that inhibition of EGF-stimulated activation of ERK5 and MEK5 by PD098059 required concentrations of ~100 μM while ERK1/2 activation was inhibited by 50 μM. Similarly, 10 μM U0126 was required for inhibition of MEK5 and ERK5 while ERK1/2 inhibition occurred at 3 μM (Mody *et al.*, 2001).

5.1.3 EPL cells and MAPK signalling

The culture of ES cells in the presence of 200 μM L-proline induces the formation of EPL cells (Chapter 3) and the induction requires the uptake of free L-proline into ES cells via the SAT2 amino acid transporter (Chapter 4). However, the molecular mechanism by which L-proline induces the transition between the two pluripotent cell types has yet to be established.

While MAPK activity is associated with pluripotent cell differentiation, the stage at which this is operative has not been identified and in particular the involvement of MAPK signalling in the formation of EPL cells has not previously been established. This chapter investigates the involvement of MAPK signalling in the formation of EPL cells by utilising specific chemical inhibitors to components of the MAPK signalling cascade and assessing their effect on the establishment of EPL-cell fate. Also, activation of down-stream components of MAPK signalling, in response to L-proline, are assessed through phospho-specific antibodies.

5.2 RESULTS

5.2.1 Analysis of MAPK involvement in EPL-cell morphology

Using the morphology assay, ES cells were cultured in ES complete medium for 5 days ± 200 μM L-proline in the presence or absence of the MEK1 inhibitors. The colonies cultured in ES complete medium alone were domed and compact consistent with their identity as ES cells (Figures 5.1 A) while the colonies grown in the presence of 200 μM L-proline formed an epithelial monolayer where individual cells were visible, indicative of EPL cell formation (Figures 5.1 A). The presence of MEK1 inhibitors, PD098059 and U0126, in ES complete medium alone had no effect on the morphology of ES cell colonies or proliferation indicating that the inhibitors were not toxic (Figure 5.1 A, B). The inhibitors were functional as they inhibited phosphorylation of ERK1/2 following LIF treatment (Figure 5.1 C). This supports previous work that indicated MAPK signalling is not required for the maintenance of ES cells (Qu and Feng, 1998; Burdon *et al.*, 1999; Schmitz *et al.*, 2000). However, the cells co-treated with 200 μM L-proline and either MEK1 inhibitor did not form colonies with EPL-cell morphology as did those grown in L-proline but instead retained the characteristic colony morphology of ES cells (Figure 5.1 A). The inhibitor U0126 also prevented EPL-cell morphology induced by 50% MEDII + 1000 U LIF (Figure 5.1 A). However, the presence of the inhibitors PD098059 and U0126 did not inhibit the elevated proliferation rate observed in EPL cells

Figure 5.1

MEK inhibitors prevent L-proline- and MEDII-induced EPL-cell morphology

(A) ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) in the presence of 200 µM L-proline ± 12.5 µM PD089059 or 5 µM U0126 or 50% MEDII ± 5 µM U0126. Following five days of culture, the cells were photographed under phase contrast (100X magnification). The experiment was performed in triplicate four times and a representative result is shown. *(B) ES (♦) or EPL (■) cells were seeded at 10^4 cells per 100 µl ES complete medium in a 96 well tray in the presence or absence of 12.5 µM or 25 µM PD098059, 5 µM U0126 or DMSO vehicle. Cells were grown for 48 h, treated with 10 µl WST-1 reagent, incubated for 2 h and absorbance measured at 480 nm. Error bars represent the SD of triplicates. The experiment was performed three times and a representative result is shown. (C) Cells were grown in ES complete medium for 2 days and serum/LIF starved in DMEM, 0.1% FCS, 0.1 mM βMe without LIF for 4 h prior to treatment. Cells were then left untreated, treated with 1000 U/ml LIF, or co-treated with LIF plus 12.5 µM or 25 µM PD098059, 5 µM U0126 or DMSO for 10 min. 10 µg total protein was analysed by Western blot (2.10.4) for phospho-ERK1/2. β-actin was used as a loading control. The experiment was performed two times and a representative result is shown.

* Data kindly provided by Fernando Felquer

Figure 5.1

A

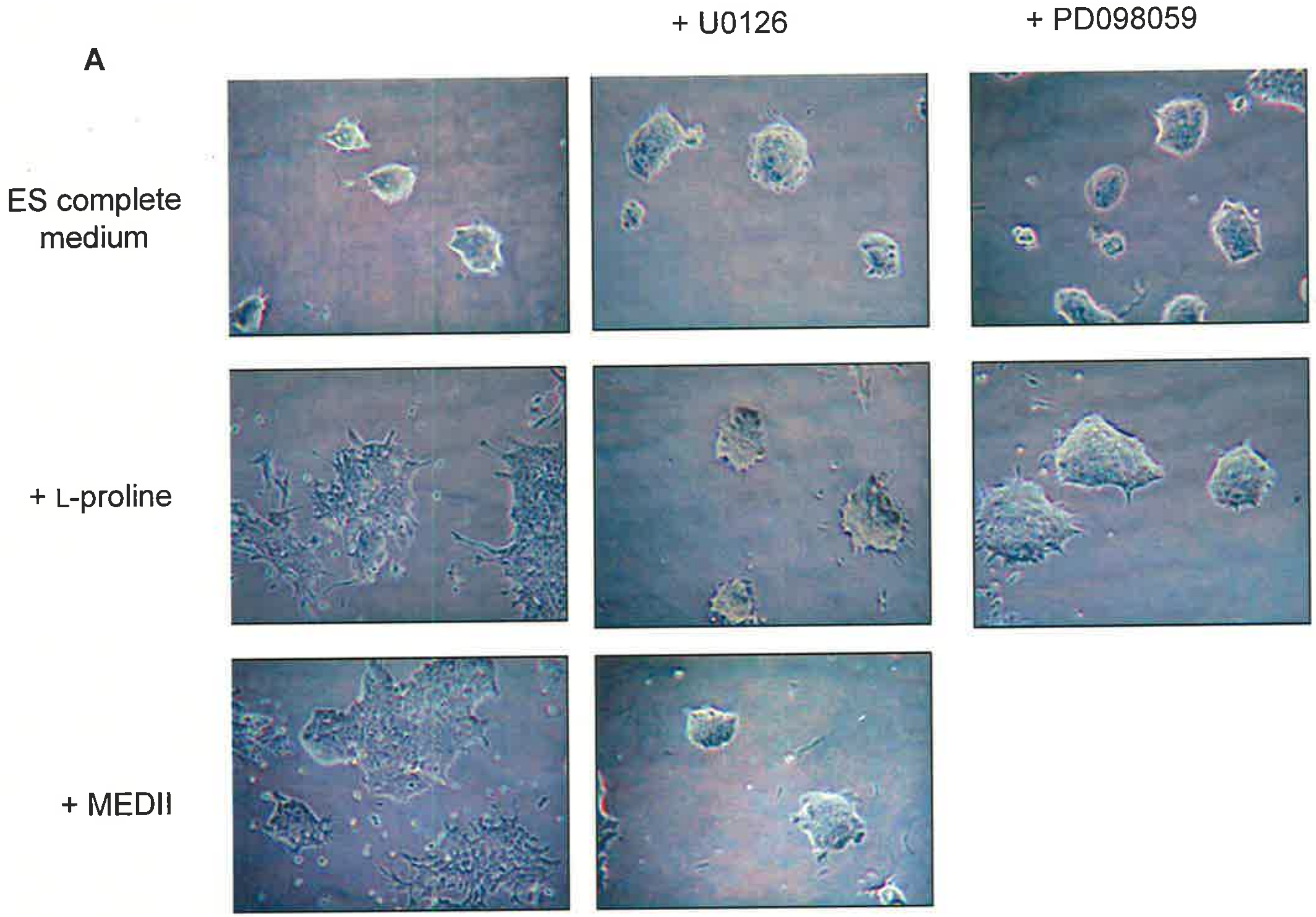
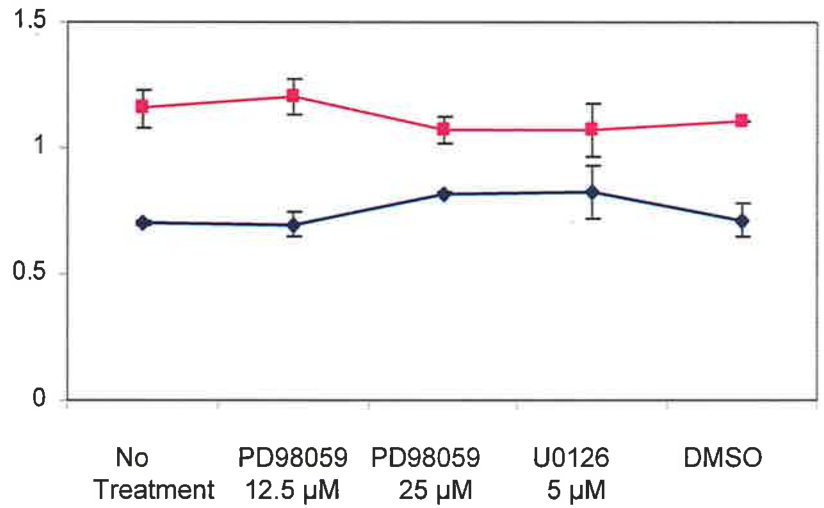
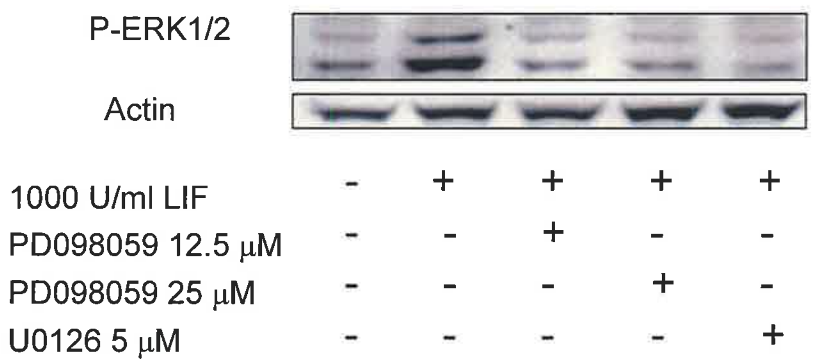


Figure 5.1

B


1.5
1
0.5
0
No Treatment
PD98059 12.5 μM
PD98059 25 μM
U0126 5 μM
DMSO

C


P-ERK1/2
Actin
1000 U/ml LIF - + + + +
PD098059 12.5 μM - - + - -
PD098059 25 μM - - - + -
U0126 5 μM - - - - +

compared to ES cells (Figure 5.1 B) suggesting that the elevated proliferation rate observed in EPL cells is not controlled by MAPK signalling.

5.2.2 Effect of MEK1 inhibition on gene expression during the ES to EPL cell transition

The effect of the MEK1 inhibitors on gene expression changes associated with EPL-cell formation was investigated by growing cells for six days in ES complete medium ± 200 μM L-proline with or without the inhibitors (PD098059 or U0126). The ES cells cultured in the presence of the inhibitors alone did not change their gene expression profile in terms of the genes analysed while cells grown in the presence of 200 μM L-proline down-regulated *Rex1*, up-regulated *Dnmt3b1* and maintained expression of *Oct4*, indicative of the formation of EPL cells (Figure 5.2 A-C). The cells grown in medium containing 200 μM L-proline and either 25 μM PD098059 or 5 μM U0126 maintained *Oct4* and *Rex1* levels and did not up-regulate the expression of *Dnmt3b1* (Figure 5.2 A-C). Therefore, the cells cultured in L-proline containing the inhibitors maintained a gene expression profile consistent with that of ES cells, suggesting that inhibition of MAPK signalling prevented L-proline-induced EPL-cell formation.

5.2.3 Differentiation potential of cells cultured in the presence of L-proline and MEK1 inhibitors

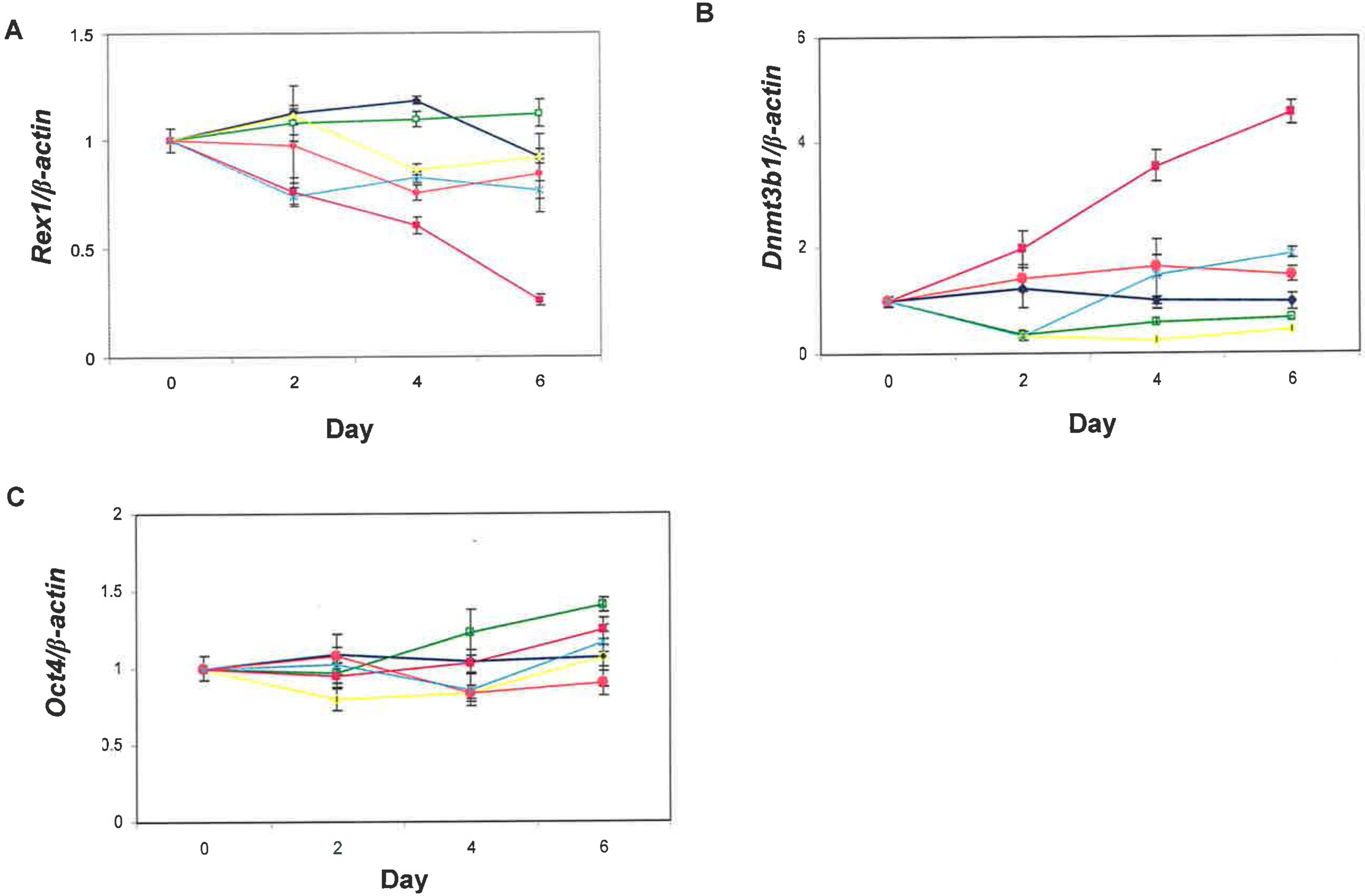
Quantitative PCR analysis was used to determine the levels of *Brachyury* expression in embryoid bodies over 4 days of differentiation. Bodies formed from ES cells up-regulated *Brachyury* on day 4, as expected, and the

Figure 5.2

MEK inhibitors U0126 and PD098059 prevent L-proline-induced EPL-cell gene expression

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) (♦) or supplemented with 200 μM L-proline (■), 12.5 μM PD098059 (□), L-proline and PD098059 (●), 5 μM U0126 (◊) or L-proline and U0126 (X). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Quantitative PCR (2.7.6) was performed to determine expression of (A) *Rex1*, (B) *Dnmt3b1* and (C) *Oct4* relative to *β-actin*. Normalised expression of each gene in ES cells was assigned a value of 1 and the expression level in day 2-6 cells is shown relative to this. Error bars represent SD of triplicates. All experiments were performed three times and a representative result is shown.

Figure 5.2



A
Rex1/β-actin
1.5
1
0.5
0
0
2
4
6
Day
B
Dnmt3b1/β-actin
6
4
2
0
0
2
4
6
Day
C
Oct4/β-actin
2
1.5
1
0.5
0
0
2
4
6
Day

presence of MEK1 inhibitors alone did not affect this timing (Figure 5.3). In cells grown in the presence of 200 μM L-proline for 4 days prior to body formation the expression of *Brachyury* was up-regulated on day 3, consistent with the cells being of EPL identity. However, for cells grown in ES complete medium containing L-proline and either PD098059 or U0126 for 4 days prior to body formation, the up-regulation of *Brachyury* occurred on day 4 consistent with MEK1 inhibition preventing the adoption of EPL differentiation potential.

5.2.4 Analysis of the effect of L-proline on activation of ERK1/2 and STAT3

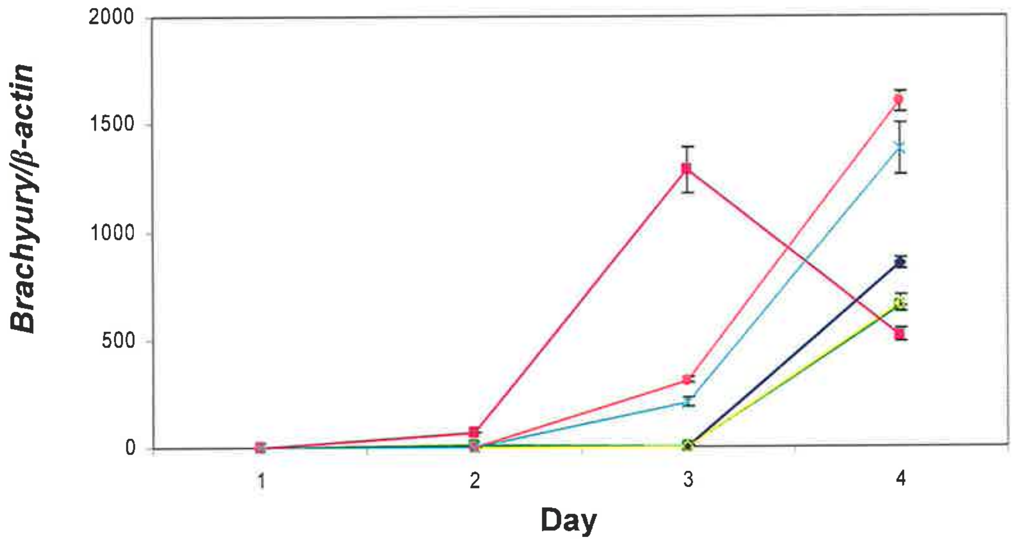
The previous results indicate a requirement for an active MAPK signalling cascade in the formation of EPL cells. The role of the MAPK pathway in relation to the action of L-proline is not known. There are at least two possibilities: (i) that the MAPK pathway is a parallel pathway that is required in the transition which, in combination with the L-proline-induced signals, leads to EPL-cell formation or (ii) that MAPK signalling is activated by L-proline. In order to determine whether L-proline is exerting a direct effect on MAPK signalling, ES cells were treated with 200 μM L-proline and Western blot analysis used to ascertain the effect of these treatments on the phosphorylation of ERK1/2, which lies immediately down-stream of MEK1 (Sturgill *et al.*, 1988; Payne *et al.*, 1991). Activation of ERK1/2 was assessed in two ways: (i) ES cells were grown in ES complete medium and then stimulated with 200 μM L-proline for up to 120 min (ii) ES cells were starved in 0.1% serum medium without LIF for 4 h and then stimulated with 200 μM L-

Figure 5.3

MEK inhibitors U0126 and PD098059 inhibit EPL cell differentiation potential

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) (♦) or supplemented with 200 μM L-proline (■), 12.5 μM PD098059 (□), L-proline and PD098059 (●), 5 μM U0126 ( ) or L-proline and U0126 (X). Cells were grown for 4 days with daily re-feeding and a passage on day 2. On day 4, embryoid bodies were formed (2.6.6) by seeding single cells into ICβ medium. Embryoid bodies were grown for 4 days. Collected bodies were analysed by quantitative PCR (2.7.6) for the expression of *Brachyury* relative to *β-actin*. Normalised *Brachyury* expression in EB1 was assigned a value of 1 and the expression level in day 2-4 bodies is shown relative to this. Error bars represent SD of triplicates. The experiment was performed two times and a representative result is shown.

Figure 5.3



proline for up to 15 min. Figure 5.4 A shows that treatment of ES cells growing in ES complete medium with 200 µM L-proline stimulated ERK1/2 phosphorylation by 30 min with phosphorylation maintained to at least 120 min. Treatment of serum- and LIF-starved ES cells with 200 µM L-proline led to transient phosphorylation of ERK1/2 with a peak at 5 min (Figure 5.4 B). The kinetics of ERK phosphorylation are faster following serum and LIF starvation since baseline phosphorylation has been down-regulated under these conditions and will allow an immediate response following treatment.

Serum- and LIF-starved ES cells treated with LIF alone induced phosphorylation of STAT3 by 15 min (Figure 5.4 C). L-proline did not inhibit this LIF-induced STAT3 phosphorylation. These results indicate that L-proline activates ERK1/2 in ES cells but does not have an effect on STAT3 phosphorylation.

5.3 DISCUSSION

5.3.1 MAPK signalling is required for EPL-cell formation

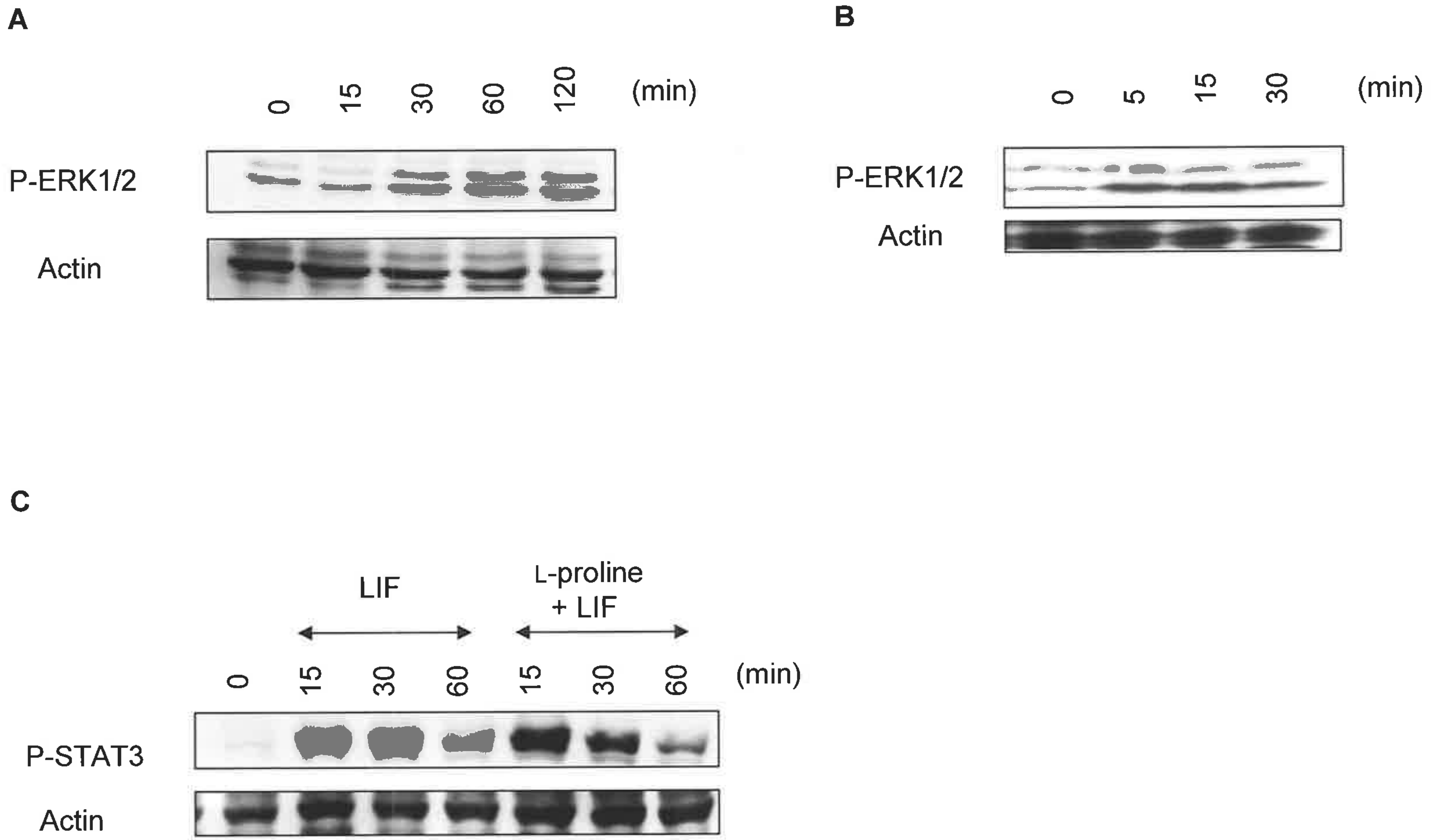
The results described in the chapter show that an active MAPK signalling pathway is required for the ES-to-EPL cell transition. Inhibition of MAPK signalling through the use of MEK1-specific inhibitors PD098059 and U1026 prevented the establishment of EPL-cell morphology (Figures 5.1), gene expression (Figure 5.2 A-C) and differentiation potential (Figure 5.3). The involvement of MAPK signalling appears to lie directly down-stream of L-proline, as treatment of ES cells with L-proline rapidly induced the activation of ERK1/2 in cells grown in the absence of serum (Figure 5.4 B). For ES cells

Figure 5.4

L-proline induces phosphorylation of ERK1/2 in pluripotent cells but does not inhibit STAT3 activation by LIF

(A) ES cells were seeded at $3x10^5$ and grown in ES complete medium (containing 1000 U/ml LIF) for 2 days and then treated with 200 μM L-proline for 0, 15, 30, 60 or 120 min. Cells were lysed (2.10.1) and 10 μg protein analysed via Western blot (2.10.4) for the presence of phospho-ERK1/2 (2.2.4). β-actin (2.2.4) was used as a loading control. The experiment was performed three times and a representative result is shown. (B-C) Cells were grown in ES complete medium for 2 days and serum/LIF starved in DMEM, 0.1% FCS, 0.1 mM βMe without LIF for 4 h prior to treatment with (B) 200 μM L-proline for 0, 5, 15 and 30 min or (C) 1000 U/ml LIF or 1000 U/ml LIF and 200 μM L-proline for 0, 15, 30 and 60 min. Cells were lysed (2.10.1) and 10 μg protein analysed via Western blot (2.10.4) for the presence phospho-STAT3 (2.2.4). β-actin was used as a loading control. The experiment was performed three times and a representative result is shown.

Figure 5.4


A
0
15
30
60
120
(min)
P-ERK1/2
Actin
B
0
5
15
30
(min)
P-ERK1/2
Actin
C
LIF
L-proline
+ LIF
0
15
30
60
15
30
60
(min)
P-STAT3
Actin

grown under standard culture conditions (serum plus LIF), the addition of L-proline was still able to elevate levels of ERK1/2 phosphorylation even though LIF is capable of activating this signalling intermediate. This suggests that ERK1/2 activation by L-proline is over and above that by LIF alone and may act to tip the balance toward ERK1/2-mediated EPL-cell formation and away from STAT3-mediated maintenance of ES cells.

The possibility also existed that L-proline was inducing EPL-cell formation by negative regulation of STAT3 signalling, another LIF-induced pathway. However, this was not the case, as L-proline treatment had no effect on LIF-induced STAT3 activity (Figure 5.4 C).

MAPK signalling has previously been associated with the differentiation of ES cells and loss of pluripotence (Burdon *et al*., 1999; Schmitz *et al*., 2000). Here it is demonstrated that active MAPK signalling is required in the transition of one pluripotent cell population to another, the first obligatory step in differentiation to embryonic lineages. Therefore, the role of MAPK in differentiation may not initially be a pro-differentiation signal but a signal that is required for the establishment of a second pluripotent cell population that is then competent to respond to differentiation signals.

5.3.2 *In vivo* evidence supporting a role of MAPK signalling in establishment of primitive ectoderm

In vivo, the establishment of primitive ectoderm has been hypothesised to require ECM components as well as signals from the visceral endoderm but the identity of these signals is not known (Spyropoulos and Capecchi, 1994; Coucouvanis and Martin, 1995; Duncan *et al.*, 1997; Koutsourakis *et al.*, 1999; Murray and Edgar, 2000, Li *et al.*, 2001). The MAPK signalling pathway has not previously been implicated in the formation of primitive ectoderm *in vivo*.

Immunohistochemistry with phospho-ERK1/2 antibodies (Corson *et al.*, 2003), showed that the strongest staining in 5.5 dpc embryos was within the extra-embryonic ectoderm (Corson *et al.*, 2003) with other regions of signalling identified at the distal tip of the epiblast (5.5 dpc), allantoic bud and blood island mesoderm (7.5 dpc). Speckled staining was also evident within the primitive ectoderm (Corson *et al.*, 2003). ERK activity was not investigated in development earlier than 5.5 dpc.

Knockout mice have been made for *ERK1* and *ERK2*. *ERK1* does not appear to be required during early development since *ERK1*$^{-/-}$ mice were shown to be viable and fertile with the main abnormality being impaired thymocyte proliferation and maturation (Pages *et al.*, 1999). This abnormality could not be compensated for by ERK2 as ERK2 was expressed and functional in these mice but was not able to rescue the thymocyte deficiency phenotype. *ERK2*$^{-/-}$

mice were shown to have a developmental phenotype with lethality observed at embryonic day 6.5 (Yao *et al.*, 2003). When mutant embryos were examined more closely it was noted that at 5.5 dpc, while the wildtype embryos formed the egg cylinder with organised embryonic and extra-embryonic cell types, the mutant counterparts were smaller, oval in shape and did not display proximal-distal polarity and lacked the ectoplacental cone (Saba-El-Leil *et al.*, 2003). By 6.5 dpc the disorganisation within the *ERK2*$^{-/-}$ embryos was more evident with the inability of the presumptive primitive ectoderm to form a single layer of pseudostratified epithelia, although the internally located disorganised cells still stained positive for *Oct4*. The internally localised cells were not profiled for expression of markers of the primitive ectoderm. Visceral and parietal endodermal cell types were identified within the mutants indicating that differentiation to these cell types was not prevented by the absence of *ERK2* (Saba-El-Leil *et al.*, 2003). The most severe effect in *ERK2* null mice was the complete lack of extra-embryonic ectoderm and the ectoplacental cone indicating that *ERK2* is crucial in the establishment of these cell types (Saba-El-Leil *et al.*, 2003). The epiblast phenotypes were not investigated further but the fact that the primitive ectoderm did not appear to form correctly is supportive of the possibility that MAPK signalling via ERK2 is involved in the *in vivo* establishment of this cell type. The *in vivo* results discussed above suggest that even though ERK1 and ERK2 are both direct down-stream effectors of MEK1 they have individual roles. Therefore, characterisation of mice null for both ERK1 and ERK2 may prove informative in terms of clarifying a role in primitive ectoderm formation.

5.3.3 Summary

In summary, the results in this chapter have established the requirement for an active MAPK signalling pathway in the formation of EPL cells in this *in vitro* model of primitive ectoderm formation. Active MAPK signalling required for EPL-cell formation was shown to lie down-stream of L-proline as L-proline treatment rapidly activated ERK1/2. The involvement of MAPK signalling in EPL-cell formation may have embryonic relevance since ERK2 knockout mice are unable to form primitive ectoderm correctly. The identification of the involvement of this pathway in EPL-cell formation provides a means for manipulating the state of cells in culture via direct manipulation of signalling pathways.

CHAPTER 6:

INVESTIGATION OF THE ROLE OF PI3K SIGNALLING THE ES-TO-EPL CELL TRANSITION

CHAPTER 6: INVESTIGATION OF THE ROLE OF PI3K SIGNALLING IN THE ES-TO-EPL CELL TRANSITION

6.1 INTRODUCTION

6.1.1 PI3K signalling

The phosphoinositide-3-kinase (PI3K) family is a group of enzymes, which transduce their signals via lipid second messengers. Signalling via the PI3K pathway is involved in many cellular processes including growth, differentiation, survival, proliferation, migration and metabolism (Katso *et al.*, 2001). PI3K members phosphorylate phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns(4)P), phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to produce phosphatidylinositol-3-phosphate (PtdIns(3)P), phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3).

PI3K family members are classified into three classes based on the structural features and enzymatic activity of the catalytic subunit. Class I PI3K are able to phosphorylate PtdIns(4)P and PtdIns(4,5)P_2. They are coupled to upstream signalling by binding adaptor proteins. The type of adaptor protein (regulatory subunit) bound further distinguishes the class I proteins to subclass A or B (Vanhaesebroeck *et al.*, 1997). Class I_A members consist of one catalytic subunit (p110α, p110β or p110γ) and one regulatory subunit (p85α, p85β or p55γ). Class I_A PI3K are recruited to the activated receptors by binding the phosphorylated tyrosine residues on the receptors via the SH2 domains of the PI3K regulatory subunits. This translocation facilitates the enzymatic activity of PI3K by bringing it in close proximity to its membrane-localised lipid substrates (Cantley, 2002). Class I_A members are also able to interact with Ras proteins. *In vitro*, PI3K-mediated signalling has been demonstrated following incubation of GTP-Ras with p110α-p85α (Rodriguez-Viciana *et al.*, 1996). *In vivo*, co-expression of mutant Ras proteins with p110α-p85α indicated that Ras is able to regulate PI3K-mediated signalling (Rodriguez-Viciana *et al.*, 1994; Marte *et al.*, 1997).

Class I_B PI3K contains only one member and the enzyme is composed of a p110γ catalytic and a p101 regulatory subunit. This enzyme does not associate with SH2 domain-containing adaptors and is thus not activated by tyrosine kinase signalling. Instead, the class I_B member is activated by G protein-coupled receptors (Vanhaesebroeck *et al.*, 1997).

Class II enzymes consist of three forms CIIα, CIIβ and CIIγ. *In vitro*, class II enzymes are able to phosphorylate PtdIns(4)P but not PtdIns(4,5)P_2 while the single class III PI3K (Vps34) phosphorylates PtdIns. Class II PI3K have been activated by growth factors including EGF and PDGF (Arcaro *et al.*, 2000; Wheeler and Domin, 2001), insulin (Brown et al., 1999; Urso *et al.*, 1999), stem cell factor (Arcaro *et al.*, 2002), chemokines (Turner *et al.*, 1998), integrin (Zhang *et al.*, 1998; Paulhe *et al.*, 2002) and TNFα and leptin (Ktori *et al.*, 2003).

Signalling down-stream of PI3K is mediated by pleckstrin homology (PH) domain-containing proteins which are recruited by binding PtdIns(3,4,5)P_3. Two effectors activated by PI3K signaling are Akt and PDK1. Binding PtdIns(3,4,5)P_3, via their PH domains, brings Akt and PDK1 in close proximity thus enabling the activation of Akt by PDK1-mediated phosphorylation of Thr308 and Ser471 on Akt (Alessi *et al.*, 1996). Activated Akt is a pro-survival signal as it phosphorylates and inactivates pro-apoptotic factors such as Bad (Cardone *et al.*, 1998) and Forkhead transcription factors (Brunet *et al.*, 1999). Akt is also involved in (i) the control of glycogen synthesis by negatively regulating glycogen synthase kinase 3α and β (Cross *et al.*, 1995; Hajduch *et al.*, 1998) and (ii) cell growth through activation of its down-stream effector mTOR by phosphorylation of serine 2448 and inactivation of the mTOR inhibitor TSC2 (Nave *et al.*, 1999; Manning *et al.*, 2002; Inoki *et al.*, 2002). Signalling via mTOR activates protein synthesis through the activation of p70S6 kinase, an activator of translation, and inhibition of eukaryotic initiation factor 4E-BP1, an inhibitor of translational initiation (Manning *et al.*, 2002; Inoki *et al.*, 2002).

6.1.2 PI3K and survival

Signalling via the PI3K pathway has been associated with ES cell proliferation and apoptosis (Sun *et al.*, 1999; Hallmann *et al.*, 2003; Gross *et al.*, 2005). Serum has been suggested to contain factors that promote survival of ES cells in culture (Ying *et al.*, 2003; Gross *et al.*, 2005). Signalling via the PI3K pathway appears to be crucial for this survival since inactivation of PI3K activity through the use of a chemical inhibitor LY24002 induced apoptosis in

ES cells in culture in the presence of serum (Gross *et al.*, 2005). In support of these findings, it was shown that ES cells null for *Pten*, a negative regulator of PI3K signalling, exhibited enhanced proliferation with elevated levels of PtdIns(3,4,5)P_3 and Akt phosphorylation and the ability to proliferate in the absence of serum (Sun *et al.*, 1999). Also, ES cells null for the regulatory subunit p85α of class I_A PI3K demonstrated growth retardation, elevated levels of apoptosis and alterations in the cell-cycle progression with a G_0/G_1 cell-cycle arrest (Hallmann *et al.*, 2003). Together, the evidence suggests a role for PI3K signalling in the progression of ES cells through the cell cycle as well as providing survival cues.

6.1.3 PI3K signalling and ES cell self-renewal

PI3K signalling has also been implicated in the self-renewal of ES cells since incubation of ES cells with a PI3K inhibitor LY294002 induced formation of cells with a flattened morphology which failed to stain uniformly for alkaline phosphatase (Paling *et al.*, 2004). PI3K inhibition was associated with elevated levels of activated ERK1/2 in the treated cells. The induction of flattened morphology by PI3K inhibitor could be overcome by co-incubation with MEK inhibitors (Paling *et al.*, 2004). This suggests that the balance between activity of PI3K and MAPK pathways may be important in maintaining self-renewal in ES cells with PI3K acting as a pro-self-renewal signal and MAPK as an anti-self-renewal signal.

There are parallels between these results and the requirements for EPL-cell formation. Formation of EPL cells is associated with adoption of a flattened morphology and this transition can be inhibited through the use of MEK1 inhibitors, as discussed in Chapter 5. Therefore, it is possible that a component of L-proline signalling in pluripotent cells involves modification of signalling via the PI3K pathway.

Further evidence for a role of PI3K signalling in self-renewal of ES cells was demonstrated by the ability of transfected myristoylated Akt (myr-Akt) to support self-renewal of ES cells in the absence of LIF (Watanabe *et al.*, 2006). Following removal of the active Akt construct, via deletion of myr-Akt, the ES cells were still able to differentiate to cells representing a variety of lineages (Watanabe *et al.*, 2006). Similarly, expression of a 4-hydroxy-tamoxifen-inducible myr-Akt-Mer fusion protein resulted in maintenance of mouse ES cells in the absence of LIF and primate ES cells in the absence of feeders (Watanabe *et al.*, 2006).

6.1.4 Lefty2, an inhibitor of Nodal signalling

Lefty proteins (Lefty1, 2) are members of the TGFβ family and act as antagonists of Nodal signalling. Lefty proteins inhibit Nodal signalling as well as TGFβ- and BMP4-initiated responses (Meno *et al.*, 1997; Bisgrove *et al.*, 1999; Branford *et al.*, 2000; Ulloa and Tabibzadeh, 2001). The mechanism of Lefty2 inhibition of Nodal signalling has been shown to include two mechanisms: (i) interacting directly with Nodal and preventing Nodal's interaction with its receptor and (ii) preventing the assembly of the active signalling receptor complex by interacting with the EGF-CFC family of receptors required for Nodal signalling (Chen and Shen, 2004). Apart from their negative regulatory role, Lefty proteins have been shown to stimulate MAPK signalling: both Lefty and its precursor were shown to activate ERK1/2 activity (Ulloa *et al.*, 2001). However, the biological relevance of this signalling is not known.

Embryos null for ALK4, the receptor capable to binding either Nodal or Activin, arrest at the egg cylinder stage and are unable to undergo gastrulation (Gu *et al.*, 1998). The mutant embryos had reduced numbers of epiblast cells, epiblasts that were disassociated from the visceral endoderm and disorganised extra-embryonic ectoderm (Gu *et al.*, 1998). This suggests that signalling via the ALK4 receptor, potentially by Nodal and modulated by Lefty proteins, is involved in the ability of the embryo to form primitive ectoderm.

In the embryo Lefty1 and Lefty2 are expressed transiently on the left side of the gastrulating embryo (Meno *et al.*, 1996). At the primitive streak stage (embryonic day 7) Lefty2 and not Lefty1 is expressed in the emerging mesoderm while later at embryonic day 8 Lefty1 is expressed within the left half of the prospective floor plate while Lefty2 is expressed within the left side of the lateral plate mesoderm (Meno *et al.*, 1998). Lefty proteins are involved in left-right axis determination as well as mesoderm formation (Scheir, 2003). *Lefty2*$^{-/-}$ mice show expansion of the primitive streak and thus excessive mesoderm production, the opposite phenotype of *Nodal*$^{-/-}$ embryos, which fail to form mesoderm (Conlon *et al.*, 1994; Meno *et al.*, 1999). *Lefty1*$^{-/-}$ mice, on the other hand, show a disrupted left-right axis (Meno *et al.*, 1997). Neither of the two null phenotypes displayed defects in the production of primitive ectoderm. However, redundancy between the two proteins may be responsible for masking such an effect.

More recently the Nodal and Lefty proteins have been implicated in maintenance of human ES cells (hES). *Nodal* and *Lefty1/2* expression were shown to be down-regulated upon induction of differentiation with an expression profile that preceded the loss of pluripotence factors *Oct4* and *Nanog* (Besser, 2004). The expression of *Nodal* and *Lefty* also correlated with high levels of Smad2/3 phosphorylation, a down-stream effector of Nodal signalling, under conditions that maintained the undifferentiated cell type (Besser, 2004). Other work demonstrated that active ALK4 signalling is required for maintenance of the undifferentiated cell state as introduction of a chemical inhibitor SB431542 induced differentiation of hES cells as determined by morphology change and down-regulation of *Oct4* and *Nanog* expression (James *et al.*, 2005). The same effect was not evident in mouse ES cells: even though undifferentiated ES cells had high levels of Smad2/3 phosphorylation, the cells maintained *Oct4* levels irrespective of the presence of the inhibitor, provided LIF was present (James *et al.*, 2005).

6.1.5 Lefty2 expression is up-regulated following EPL-cell formation

EPL cells can be formed in adherent culture (Chapter 2; Rathjen *et al.*, 1999) and can also be formed in suspension culture (Rathjen *et al.*, 2002). Culture of ES cells in suspension in the presence of MEDII leads to the formation of bodies (EBMs) that undergo differentiation with sequential homogeneous formation of populations equivalent to the primitive ectoderm, neural plate and neural tube (Rathjen *et al.*, 2002). Day 3 EBMs represent a population of EPL cells. A microarray study, (J. Rathjen et al., unpublished) comparing day 0 (ICM/ES), 3 (primitive ectoderm/EPL), 6 (definitive ectoderm) and 9 (neurectoderm) EBMs was carried out to identify markers of the individual cell populations. A number of genes were identified as being up-regulated within the EPL-cell population compared to all other populations. One of the genes identified as being significantly up-regulated was Lefty2 (Figure 6.1). Analysis of the microarray results demonstrated that Lefty2 was up-regulated in EBM3 ~2 fold compared to EBM0, 6 and 9 (Figure 6.1 A).

6.2 RESULTS

6.2.1 The effect of inhibition of PI3K signalling on pluripotent cell morphology

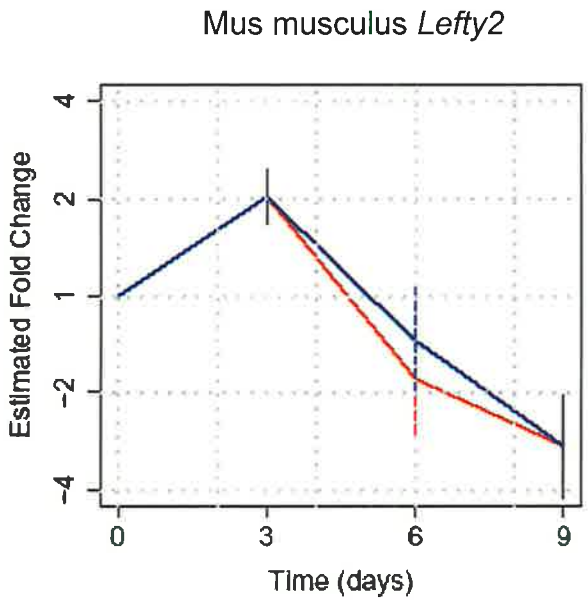
The involvement of PI3K signalling in the ES-to-EPL cell transition was initially assessed through the use of a chemical inhibitor of PI3K, LY294002, in morphology-based assays. The inhibitor LY294002 acts as a competitive inhibitor of the ATP binding site of PI3K catalytic subunits (Vlahos *et al.*, 1994). All PI3K family members show similar sensitivity to LY294002 and evidence of involvement of PI3K in a biological system can be derived by treating cells with concentrations between 5 μM and 20 μM LY294002 (Vanhaesebroeck and Waterfield, 1999). Incubation of ES cells for five days in ES complete medium in the presence of 5 μM LY294002 did not have an effect on the morphology of the cells with the colonies maintaining their domed shape (Figure 6.2 A). At this concentration of inhibitor, there did not appear to be an effect on the proliferation of the cells (Figure 6.2 B) which is

Figure 6.1

Lefty2 expression is up-regulated in EPL cells

Lefty2 expression as determined by microarray analysis of RNA from EBM0 (ES/ICM), EBM3 (EPL/primitive ectoderm), EBM6 (definitive ectoderm) and EBM9 (neurectoderm).

Figure 6.1



Mus musculus *Lefty2*
Estimated Fold Change
4
2
1
-2
-4
0
3
6
9
Time (days)

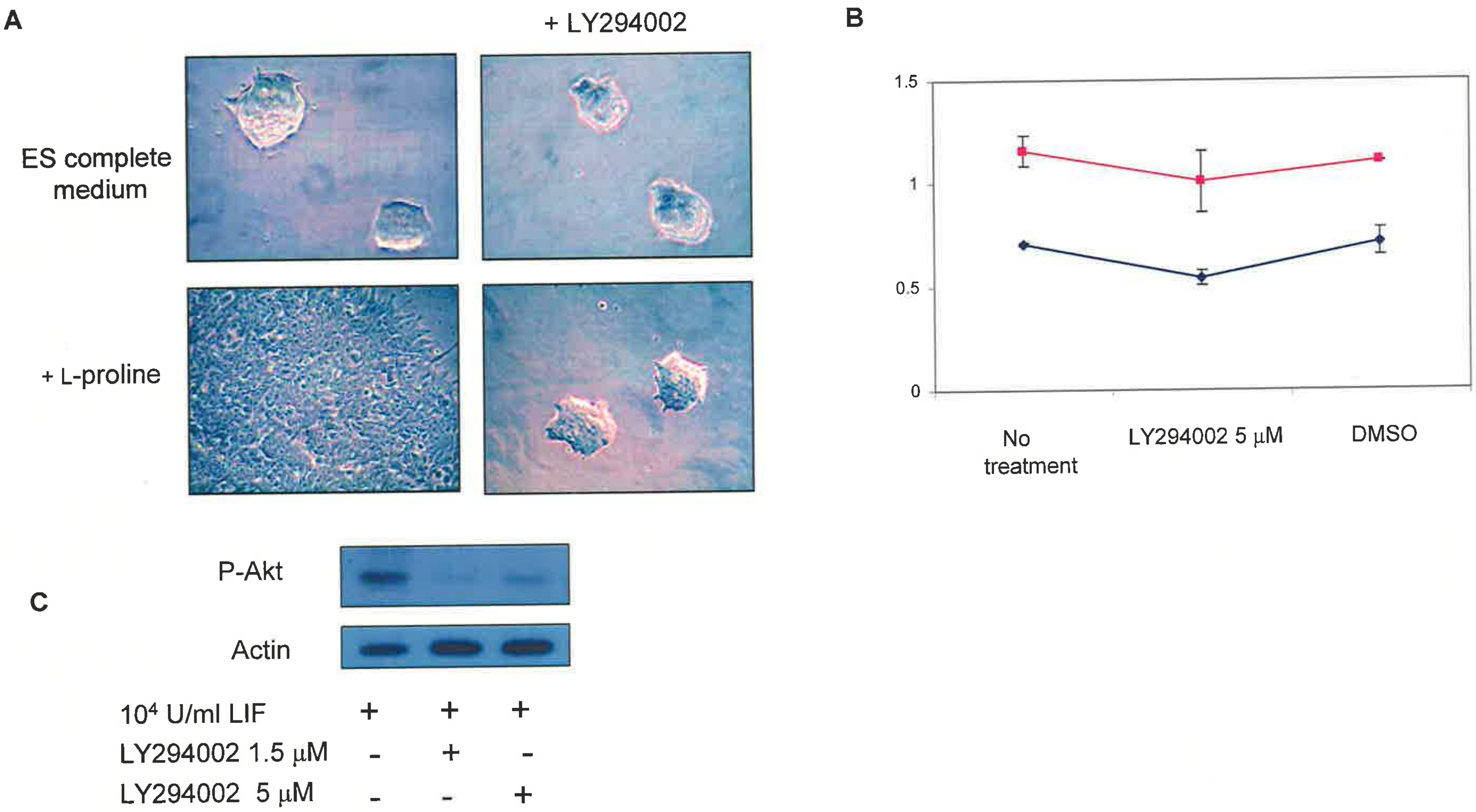
Figure 6.2

PI3K inhibitor LY294002 prevents L-proline-induced EPL-cell morphology but does not affect proliferation

(A) ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) ±200 μM L-proline with or without 5 μM LY294002. Following five days of culture, the cells were photographed under phase contrast (100X magnification). The experiment was performed in duplicate four times and a representative result is shown. *(B) ES (♦) or EPL (■) cells were seeded at 10^4 cells per 100 μl ES complete medium in a 96 well tray in the presence or absence of 5 μM LY294002 or DMSO vehicle. Cells were grown for 48 h, then incubated with 10 μl WST-1 reagent for 2 h and absorbance measured at OD 480 nm in a microplate ELISA reader. Error bars represent the SD of triplicates. The experiment was performed three times and a representative result is shown. (C) Cells were grown in ES complete medium (containing 1000 U/ml LIF) for 2 days and then starved in DMEM, 0.1% FCS, 0.1 mM βMe for 4 h prior to treatment. Following starvation, cells were treated with 10^4 U/ml LIF or co-treated with LIF and 1.5 μM or 5 μM LY294002 for 10 min. 10 μg total protein was analysed by Western blot for phospho-Akt (2.2.4). β-actin was used as a loading control. The experiment was performed three times and a representative result is shown.

*Data kindly provided by Fernando Felquer.

Figure 6.2


A
+ LY294002
ES complete medium
+ L-proline
B
1.5
1
0.5
0
No treatment
LY294002 5 µM
DMSO
C
P-Akt
Actin
10⁴ U/ml LIF + + +
LY294002 1.5 µM - + -
LY294002 5 µM - - +

consistent with published results showing that effects on proliferation or apoptosis are evident at concentrations of ≥25 μM LY294002 (Jirmanova *et al.*, 2002; Gross *et al.*, 2005). In the presence of the PI3K inhibitor EPL cells maintained their faster proliferation rate as compared with ES cells suggesting that PI3K signalling is not involved in mediating the increase of proliferation rate characteristic of EPL cells. The co-culture of ES cells in the presence of 200 μM L-proline and 5 μM inhibitor resulted in inhibition of the formation of EPL-cell morphology while the cells grown in L-proline in the absence of the inhibitor formed a monolayer consistent with the formation of EPL cells, as expected (Figure 6.2 A). The inhibitor LY294002 was functional as it inhibited Akt phosphorylation induced by LIF (Figure 6.2 C).

6.2.1.1 The gene expression profile of pluripotent cells cultured in the presence of PI3K inhibitor

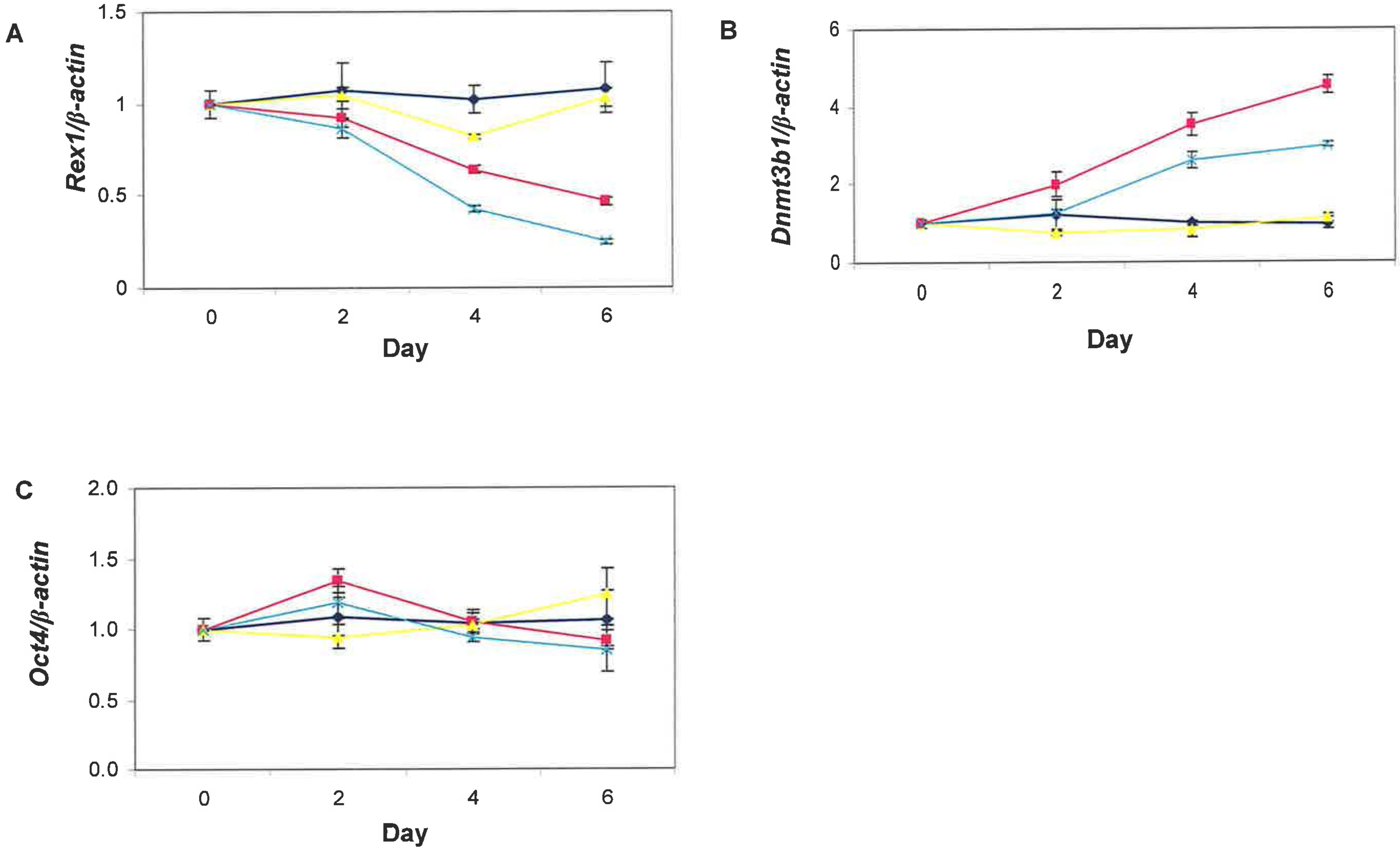
The results from the morphology assays indicated that PI3K signalling plays a role in the formation of EPL cells, at least in terms of morphology. The effect of the PI3K inhibitor on gene expression was assessed following culture of ES cells for up to 6 days in ES complete medium ±200 μM L-proline with or without 5 μM LY294002. The results indicated that the cells grown in the presence of ES complete medium supplemented with 5 μM LY294002 alone maintained an ES-cell gene expression profile with maintenance of *Rex1* (Figure 6.3 A), low levels of *Dnmt3b1* expression (Figure 6.3 B) and continued expression of *Oct4* (Figure 6.3 C). This supports the morphology results indicating that in the presence of LY294002 ES-cell self-renewal is maintained. The cells grown in the presence of L-proline and LY294002

Figure 6.3

PI3K inhibitor LY294002 does not inhibit L-proline-induced EPL-cell gene expression

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) (♦) supplemented with 200 μM L-proline (■) or 5 μM LY294002 (▲) or both (X). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Quantitative PCR (2.7.6) was performed to determine expression of (A) *Rex1*, (B) *Dnmt3b1* and (C) *Oct4* relative to that of *β-actin*. Expression of each gene in ES cells was assigned a value of 1 and the expression level in day 2-6 cells is shown relative this. Error bars represent the SD of triplicates. The experiment was performed three times and a representative result is shown.

Figure 6.3



A
1.5
1
0.5
0
Rex1/β-actin
0
2
4
6
Day
B
6
4
2
0
Dnmt3b1/β-actin
0
2
4
6
Day
C
2.0
1.5
1.0
0.5
0.0
Oct4/β-actin
0
2
4
6
Day

showed a gene expression profile comparable to that of the cells grown in L-proline alone with the down-regulation of *Rex1* (Figure 6.3 A), up-regulation of *Dnmt3b1* (Figure 6.3 B) and maintenance of *Oct4* (Figure 6.3 C). Therefore, even though the induction of EPL-cell morphology was inhibited by LY294002, the inhibitor did not prevent the induction of EPL-associated gene expression.

6.2.2 Effect of PI3K inhibition on the differentiation potential of ES and EPL cells

The results described above suggest that in the L-proline-induced ES-to-EPL cell transition the inhibition of PI3K signalling is able to uncouple the morphology change associated with the transition from gene expression changes. The effect of the PI3K inhibitor LY294002 on the differentiation potential associated with EPL-cell cell formation was also investigated. Figure 6.5 shows that in embryoid bodies made from ES cells grown in the presence of LY294002, the bodies maintained a differentiation potential consistent with that found in ES cells with induction of *Brachyury* expression observed on day 4. In bodies derived from ES cells grown in the presence of L-proline and LY294002, *Brachyury* expression was up-regulated on day 3, as is seen for the cells grown in L-proline (Figure 6.4). These data suggest that the inhibition of PI3K signalling inhibited EPL-cell morphology but not changes in gene expression or differentiation potential.

6.2.3 Akt is rapidly activated by L-proline in ES cells

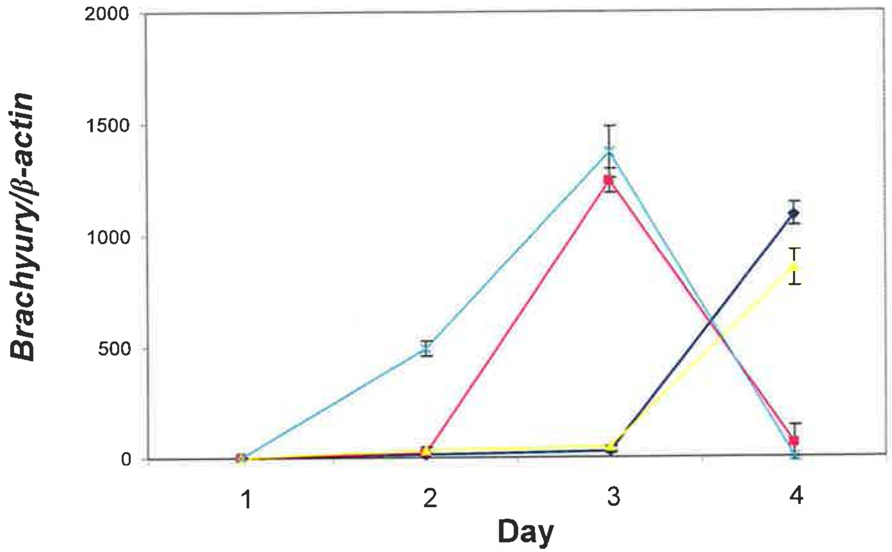
The previous results indicate a requirement for an active PI3K signalling cascade in the formation of EPL-cell morphology. The role of the PI3K

Figure 6.4

PI3K inhibitor LY294002 does not inhibit L-proline-induced EPL-cell differentiation potential

ES cells were seeded into ES complete medium alone (containing 1000 U/ml LIF) (♦) supplemented with 200 μM L-proline (■) or 5 μM LY294002 (▲) or both (X). Cells were grown for 4 days, re-fed daily and passaged on day 2. On day 4, bodies were formed by seeding single cells into ICβ medium (2.6.6). Bodies were grown for 4 days and collected for analysis daily. Collected bodies were analysed by quantitative PCR (2.7.6) for the expression of the mesoderm marker *Brachyury* relative to that of *β-actin*. Normalised expression of *Brachyury* in EB1 was assigned a value of 1 and the expression level in day 2-4 bodies is shown relative to this. Error bars represent the SD of triplicates. The experiments were performed three times and representative results are shown.

Figure 6.4



pathway in relation to the action of L-proline is not known. There are two main possibilities: (i) that the PI3K pathway is a parallel pathway that is required in the transition and, in combination with the L-proline-induced signals, leads to EPL-cell morphology or (ii) that PI3K signalling is activated directly by L-proline. In order to determine whether L-proline induces PI3K signalling, ES cells, either growing in ES complete medium or serum starved for 4 h in 0.1% serum and no LIF, were treated with 200 µM L-proline for up to 120 min. Western blot analysis was used to ascertain the effect of these treatments on the phosphorylation of Akt, which lies immediately down-stream of PI3K (Alessi *et al.*, 1996). Figure 6.5 shows that treatment of ES cells with 200 µM L-proline induced Akt phosphorylation with a peak evident at 30 min. This suggests that L-proline directly activates signalling mediated by PI3K/Akt.

6.2.4 *Lefty2* expression is up-regulated upon EPL-cell formation

Microarray analysis identified that *Lefty2* was specifically up-regulated following formation of EPL cells (Section 6.1.5). This was confirmed by Northern blot analysis of EBM series with *Lefty2* shown to be up-regulated in EBM3 (primitive ectoderm/EPL) compared to EBM0 (ES), 6 (definitive ectoderm) and 9 (neurectoderm). *Lefty2* expression in EBM aggregates was significantly up-regulated by 24 h following culture in MEDII (Figure 6.6 A). *Lefty2* was also up-regulated in EPL cells formed in adherent culture (Rathjen *et al.*, 1999) in response to L-proline by day 6. This correlates with down-regulation of *Rex1* but the maintenance of Oct4 expression, a profile characteristic of EPL cells (Figure 6.6 B).

Figure 6.5

L-proline activates the PI3K down-stream effector Akt

ES cells were grown in ES complete medium (containing 1000 U/ml LIF) for 2 days and then treated with 200 µM L-proline or starved in DMEM, 0.1% FCS, 0.1 mM βMe for 4 h prior to addition of L-proline. Cells were collected following 0, 15, 30, 60 and 120 min of treatment, lysed (2.10.1) and analysed by Western blot (2.10.4) for the presence of phospho-Akt (2.2.4). β-actin was used as a loading control. The experiment was performed three times and a representative result is shown.

Figure 6.5

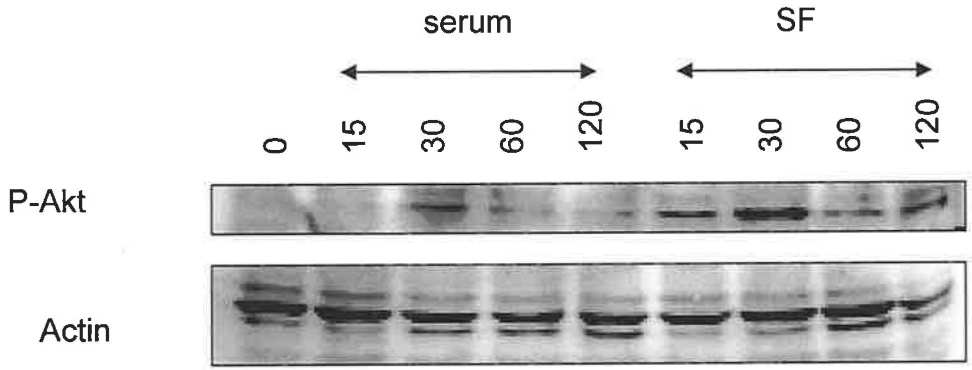


Figure 6.6

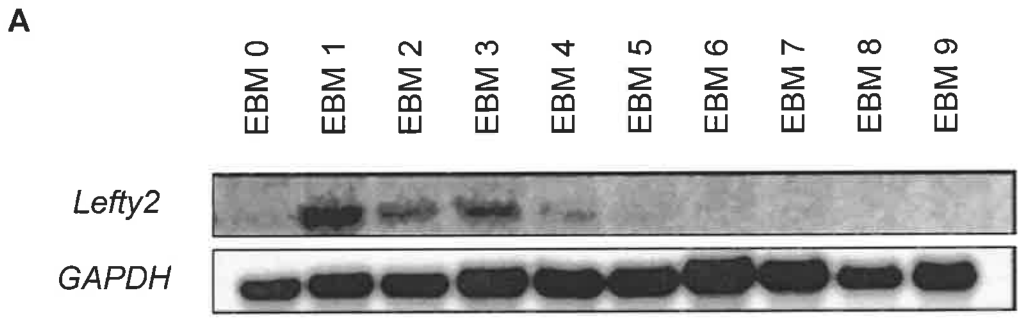
Lefty2 expression is up-regulated upon EPL-cell formation

(A) Validation of *Lefty2* microarray expression data by Northern blot analysis (2.9.2). ES cells were seeded into MEDII and cultured for 9 days in suspension. Total RNA (10 µg) was isolated from bodies and analysed for the expression of *Lefty2* relative to that of *GAPDH*. (B) *Lefty2* up-regulation in adherent EPL cells. ES cells were seeded into ES complete medium supplemented with 200 µM L-proline. Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Total RNA (10 µg) was analysed by Northern blot (2.9.2) for expression of *Lefty2* relative to that of *GAPDH.* The experiments were performed two times and representative results are shown. (C) ES cells were seeded into MEDII and cultured for 9 days in suspension culture. Quantitative PCR (2.7.6) was performed to determine expression of *Lefty2* (□) and *Nodal* (▲) relative to that of *β-actin*. Normalised expression of each gene in EBM0 was assigned a value of 1 and the expression level in bodies on days 1-9 is shown relative to this. Error bars represent the SD of triplicates. The experiment was performed three times and a representative result is shown.

EPL cells

Definitive ectoderm

Figure 6.6


A
EBM 0
EBM 1
EBM 2
EBM 3
EBM 4
EBM 5
EBM 6
EBM 7
EBM 8
EBM 9
Lefty2
GAPDH

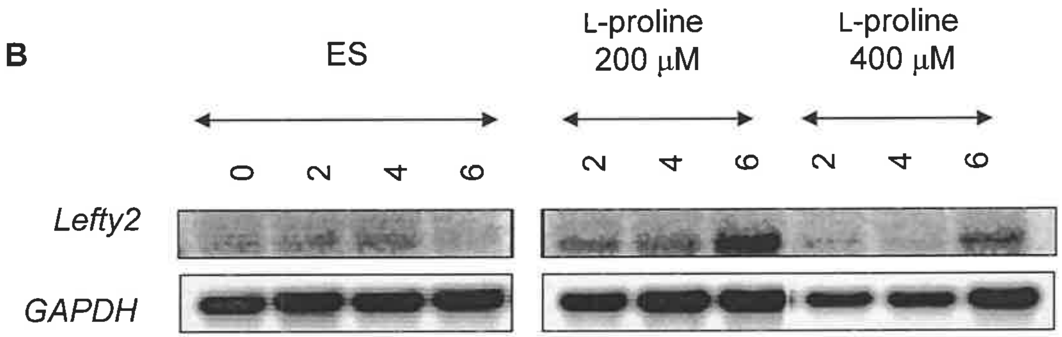
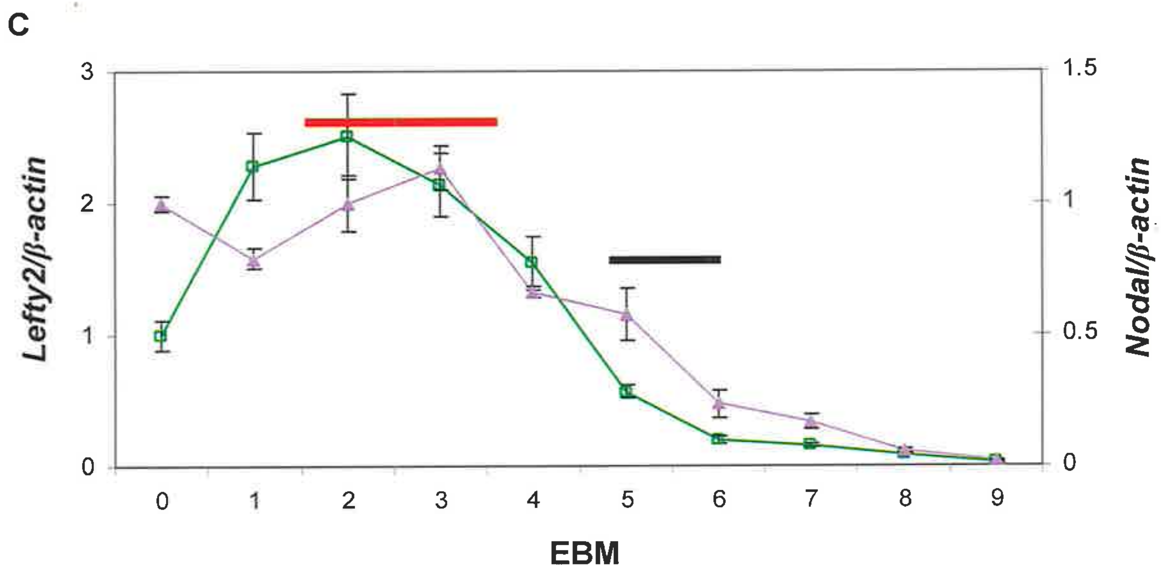

B
ES
L-proline 200 μM
L-proline 400 μM
0
2
4
6
2
4
6
2
4
6
Lefty2
GAPDH

Figure 6.6



The expression of *Nodal* and *Lefty2* correlates with the results of Besser (2004) in that the expression of the two genes is down-regulated with loss of pluripotence; that is, when EPL cells differentiate to a definitive ectoderm population in the EBM series (Figure 6.6 A, B, C). However, *Lefty2* was specifically up-regulated upon formation of pluripotent EPL cells in contrast to *Nodal* which was expressed in both ES and EPL-cells.

6.2.5 The connection between PI3K signalling and EPL-cell morphology may involve Lefty2

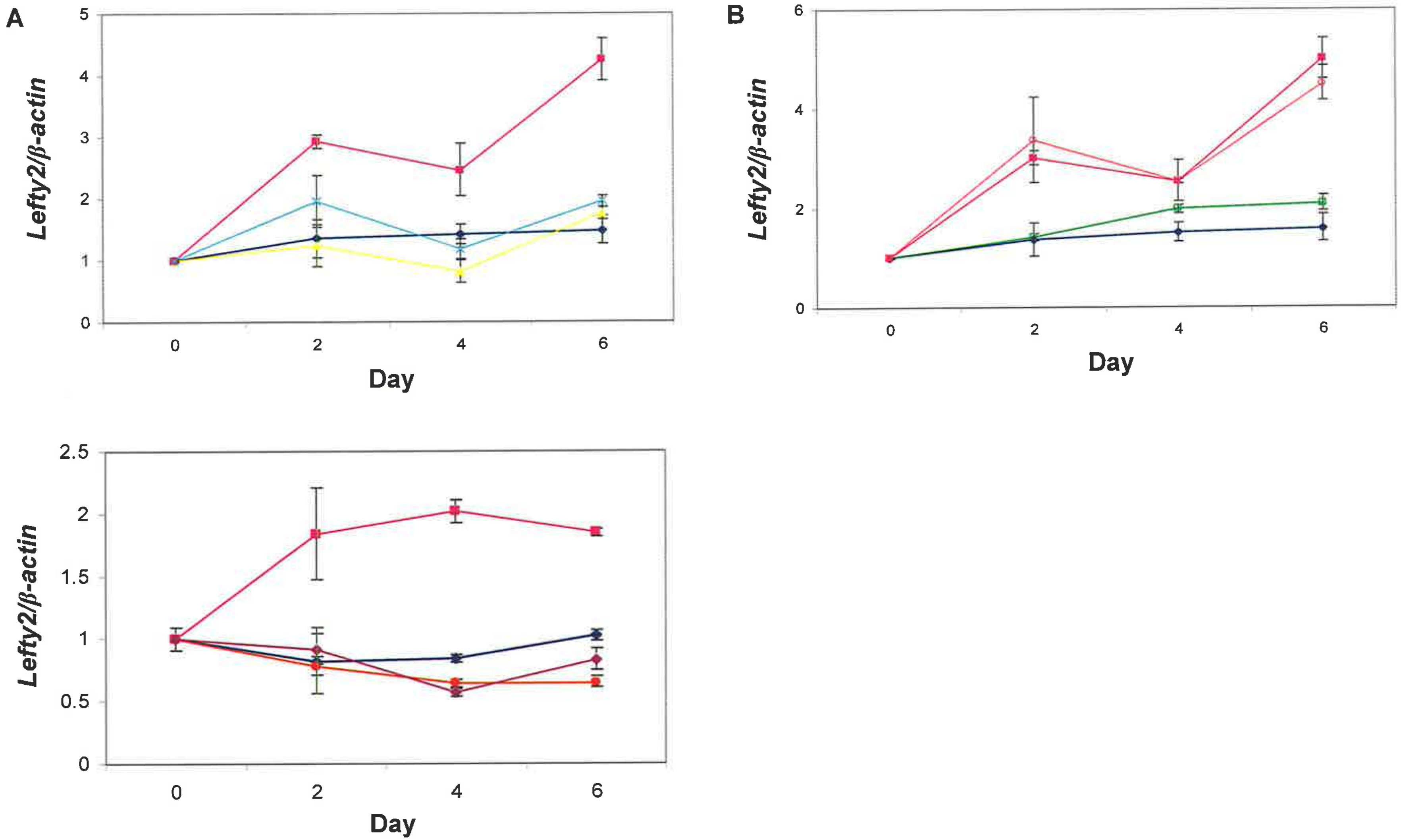
As previously discussed (Section 3.2.3), morphology change and gene expression/differentiation potential can be uncoupled in the conversion of ES cells to EPL cells. The results in this chapter suggest that this uncoupling involves signalling via the PI3K pathway in that inhibiting PI3K activity prevents L-proline-mediated EPL-cell morphology while not affecting the gene expression and differentiation potential changes associated with the formation of EPL cells. Quantitative PCR analysis for the expression of *Lefty2* in ES cells treated with L-proline ± LY294002 showed that *Lefty2* up-regulation was inhibited in the presence of the inhibitor (Figure 6.7 A). However, work with the MEK1 inhibitor U0126 suggested that the *Lefty2* up-regulation is not a consequence of MEK1 signalling since treatment with U0126 was able to prevent EPL-cell-associated morphology, gene expression and differentiation potential but did not lead to the inhibition of *Lefty2* up-regulation (Figure 6.7 B). *Lefty2* up-regulation was, however, shown to lie down-stream of L-proline action as inhibition of L-proline uptake into ES cells by incubation in excess glycine prevented *Lefty2* induction (Figure 6.7 C). The results implicate *Lefty2*

Figure 6.7

PI3K inhibitor LY294002 and excess glycine prevent L-proline-induced Lefty2 expression while MEK inhibitor U0126 does not

ES cells were seeded into ES complete medium (containing 1000 U/ml LIF) (♦) supplemented with 200 μM L-proline (■) or (A) 5 μM LY294002 (▲), L-proline and LY294002 (x), (B) 5 μM U0126 (□), L-proline and U0126 (○), or (C) 10 mM glycine (●), L-proline and glycine (♦). Cells were grown for up to 6 days, re-fed daily and passaged on days 2 and 4. Quantitative PCR (2.7.6) was performed to determine expression of *Lefty2* relative to that of *β-actin*. Expression in ES cells was assigned a value of 1 and the expression in day 2-6 cells is shown relative to this. Error bars represent the SD of triplicates. Experiments were performed three times and representative results are shown.

Figure 6.7


A
5
4
3
2
1
0
Lefty2/β-actin
0
2
4
6
Day
B
6
4
2
0
Lefty2/β-actin
0
2
4
6
Day
C
2.5
2
1.5
1
0.5
0
Lefty2/β-actin
0
2
4
6
Day

as being involved in the induction of EPL-cell morphology down-stream of PI3K signalling.

6.3 DISCUSSION

The results from this chapter support the findings discussed in Chapter 3 which indicated that EPL-cell morphology can be uncoupled from the gene expression and differentiation potential of the cells. This uncoupling has led to the identification of PI3K as a second signalling pathway that plays a role in the morphological changes accompanying the formation of EPL cells. MAPK signalling has previously been implicated in alterations in pluripotent cell morphology, gene expression and differentiation potential (Chapter 5). Therefore, two signalling pathways have been identified down-stream of L-proline, which regulate different components of the transition. Neither PI3K nor MAPK signalling was involved in mediating the accelerated proliferation of EPL-cells which suggests that another pathway may be important in regulating changes in proliferation rate during the ES-to-EPL cell transition.

Morphology assays showed that incubation of ES cells in the presence of 5 µM LY294002 did not have an effect on the morphology of the cells with the colonies maintaining their domed shape (Figure 6.2 A). There did not appear to be any induction of a flattened morphology, which contradicts the results of Paling *et al.* (2004), which showed that incubation of ES cells with LY294002 induced formation of cells with a flattened morphology that failed to stain uniformly for alkaline phosphatase. The reason for this difference is not clear: The concentration of the inhibitor used (5 µM) and time of culture (5 days) were the same between the experiments. However, the ES cell lines used were different (D3 versus E14g2a used by Paling *et al.*) and Paling *et al.* routinely cultured their cells in the absence of serum and in the presence of 0.1 mM non-essential amino acids prior to their self-renewal assays. Therefore, the differences observed may be cell line or culture medium specific.

The concentration of the PI3K inhibitor used, 5 μM LY294002, did not appear to affect the proliferation rate of the ES cells or EPL cells (Figure 6.2 B) which is consistent with the results of Paling *et al.* and other published results showing effects on proliferation or apoptosis are evident at concentrations ≥25 μM LY294002 (Jirmanova *et al.*, 2002; Gross *et al.*, 2005). These results also suggested that accelerated proliferation rate in EPL cells is controlled by a pathway other than PI3K since the presence of LY294002 did not reduce the proliferation observed.

The co-culture of ES cells with L-proline and LY294002 prevented the induction of EPL-cell morphology while the cells grown in L-proline alone formed an epithelial monolayer as expected (Figure 6.2 A). The PI3K inhibitor did not prevent the induction of EPL-associated gene expression (Figure 6.3) or differentiation potential (Figure 6.4) suggesting that PI3K signalling is involved exclusively in the induction of EPL-cell morphology. The involvement of PI3K signalling is likely to lie down-stream of L-proline action since time-course treatments of ES cells treated with L-proline showed rapid activation of

Akt (Figure 6.5). A possible effector down-stream of Akt may be amino acid-associated signalling effector mTOR (Section 4.3.1).

PI3K signalling has recently been shown to be present in the pre-implantation embryo. Western blot analysis and immunofluorescence showed the expression of PI3K and Akt from the one-cell to the blastocyst stage. The proteins were shown to be phosphorylated in response to insulin treatment (Riley *et al.*, 2005). A role for PI3K signalling was also demonstrated in blastocyst hatching since incubation of mouse embryos in the presence of the inhibitor LY294002 resulted in lowered rates of hatching (Riley *et al.*, 2005). from the zona pellucida, required for implantation of the embryo (Hartshorne and Edwards, 1991). Although the most well-defined role for the PI3K signalling cascade is in cellular survival by mediating signalling inhibiting apoptosis, it has also been associated with actin remodelling and morphology changes in various cell types (Datta *et al.*, 1999; Qian *et al.*, 2003; Brachmann *et al.*, 2005). In *Zebrafish* embryos, PI3K signalling initiated by PDGF was shown to be required for the polarization of mesendodermal cells and the formation of processes (Montero *et al.*, 2003). Similar results were seen with *Xenopus* embryos where inhibitors of PI3K signalling prevented PDGF-induced mesoderm spreading (Symes and Mercola, 1996). In cultured sensory neurons, PI3K was shown to be required for the elongation and branching of axons (Markus *et al.*, 2002). Thus, it is possible that the induction of EPL-cell morphology requires cytoskeletal rearrangements initiated by PI3K signalling.

Previously (Chapter 5), it has been established that the induction of EPL-cell fate requires active signalling via the MAPK pathway as inhibition of MEK1 prevented EPL-cell formation. However, the inhibition of MEK1 was not able to prevent the induction of *Lefty2* expression suggesting *Lefty2* may be necessary but not sufficient for EPL-cell formation. Fig. 6.7 B Here it was identified that inhibition of PI3K signalling was able to prevent the establishment of EPL-cell morphology and prevented up-regulation of *Lefty2* expression but without affecting the expression of *Rex1* and *Dnmt3b1* or the differentiation potential of the cells. This suggests that the induction of *Lefty2* expression lies down-stream of PI3K signalling and that this signalling cascade is involved only in the morphology change. These results and those from Chapter 5 suggest that the induction of EPL-cell morphology requires both an active MAPK pathway as well as signalling via PI3K. Signalling via PI3K leads to the induction of *Lefty2* expression and MAPK signalling is not required for that induction (Figure 6.7). It would be interesting to determine whether *Lefty2* in fact has a direct involvement in the establishment of EPL cell morphology through treatment of ES cells with Lefty2 protein to see if this is sufficient to induce the morphology change.

CHAPTER 7:

FINAL DISCUSSION

CHAPTER 7: FINAL DISCUSSION

7.1 INTRODUCTION

The aim of this thesis was to elucidate the molecular mechanism by which L-proline induces EPL-cell formation. This required determination of the mode of action of L-proline as well as the identification of signalling pathways affected by L-proline to induce the ES-to-EPL cell transition.

The ability of L-proline and short L-proline containing peptides to induce EPL-cell formation in terms of morphology, gene expression and differentiation potential was confirmed (Chapter 3). The mechanism of L-proline induced EPL-cell formation was shown to be independent of NK receptors due to the lack of NK1R and NK3R expression on ES cells and the inability of an NK2R inhibitor to prevent the transition (Chapter 3).

Amino acid transporter SAT2 was implicated in L-proline induced EPL-cell formation since SAT2 competitive inhibitors glycine, L-serine and MeAIB were able to prevent the transition while L-lysine, an amino acid that is not a substrate of SAT2, was not (Chapter 4). SAT2 is the only amino acid transporter expressed in pluripotent cells known to transport all four substrates; L-proline, glycine, L-serine and MeAIB. The SAT2 competitive inhibitors were also able to prevent EPL-cell morphology induced by peptides gly-pro and ala-pro suggesting that their mode of action involves their breakdown to free proline, which is then internalised by the transporter SAT2 to induce EPL-cell formation (Chapter 4). Further confirmation for the requirement of peptide breakdown in EPL-cell formation will require the determination of gene expression and differentiation potential of ES cells treated with gly-pro and ala-pro in the presence or absence of SAT2 competitive inhibitors. Recent work has also demonstrated that glycine is able prevent EPL-cell morphology induced by MEDII (Washington, unpublished data). This was the first time the repertoire of L-proline transporters present in mouse ES cells was described and the first demonstration of the involvement of an amino acid transporter in the induction of EPL-cell formation.

MAPK signalling via the action of MEK1 was implicated in L-proline induced EPL-cell formation as inhibitors of MEK1, PD098059 and U0126, were able to prevent EPL-cell morphology, gene expression and differentiation potential in the presence of L-proline (Chapter 5). MAPK signalling was suggested to lie down-stream of L-proline action since treatment of ES cells with L-proline induced ERK1/2 activation in short- and long-term treatments (Chapter 5). L-proline did not appear to affect STAT3 signalling with L-proline treatment failing to have an effect on LIF-induced STAT3 phosphorylation (Chapter 5). This was the first demonstration of the involvement of MAPK signalling in the transition between two pluripotent populations and the first time L-proline was shown to directly induce signalling in pluripotent cells.

PI3K signalling was shown to be involved in L-proline-induced EPL-cell morphology but not gene expression or differentiation potential since the PI3K inhibitor LY294002 maintained domed colonies in the presence of L-proline but failed to maintain an ES-cell gene expression profile and differentiation potential (Chapter 6). PI3K signalling was also suggested to lie down-stream of L-proline action since treatment of ES cells with L-proline induced the activation of Akt, a down-stream target of PI3K (Chapter 6). A gene potentially involved in the PI3K-mediated morphology change was *Lefty2*, whose expression is up-regulated following EPL-cell formation and prevented in the presence of an inhibitor of PI3K but not MEK1 (Chapter 6). This result also confirmed that PI3K activation did not lie down-stream of MEK1. These results were the first description of multiple signalling pathways lying down-stream of L-proline action and the initial demonstration that the activation of EPL-cell morphology is mediated by an additional pathway distinct from that responsible for EPL-cell gene expression and differentiation potential.

7.2 A MODEL FOR L-PROLINE INDUCED EPL-CELL FORMATION

In the *in vitro* model of primitive ectoderm formation, the transition from ES cells to EPL cells requires the presence of the imino acid L-proline. From the results described in this thesis, the mechanism of L-proline action is as follows (Figure 7.1):

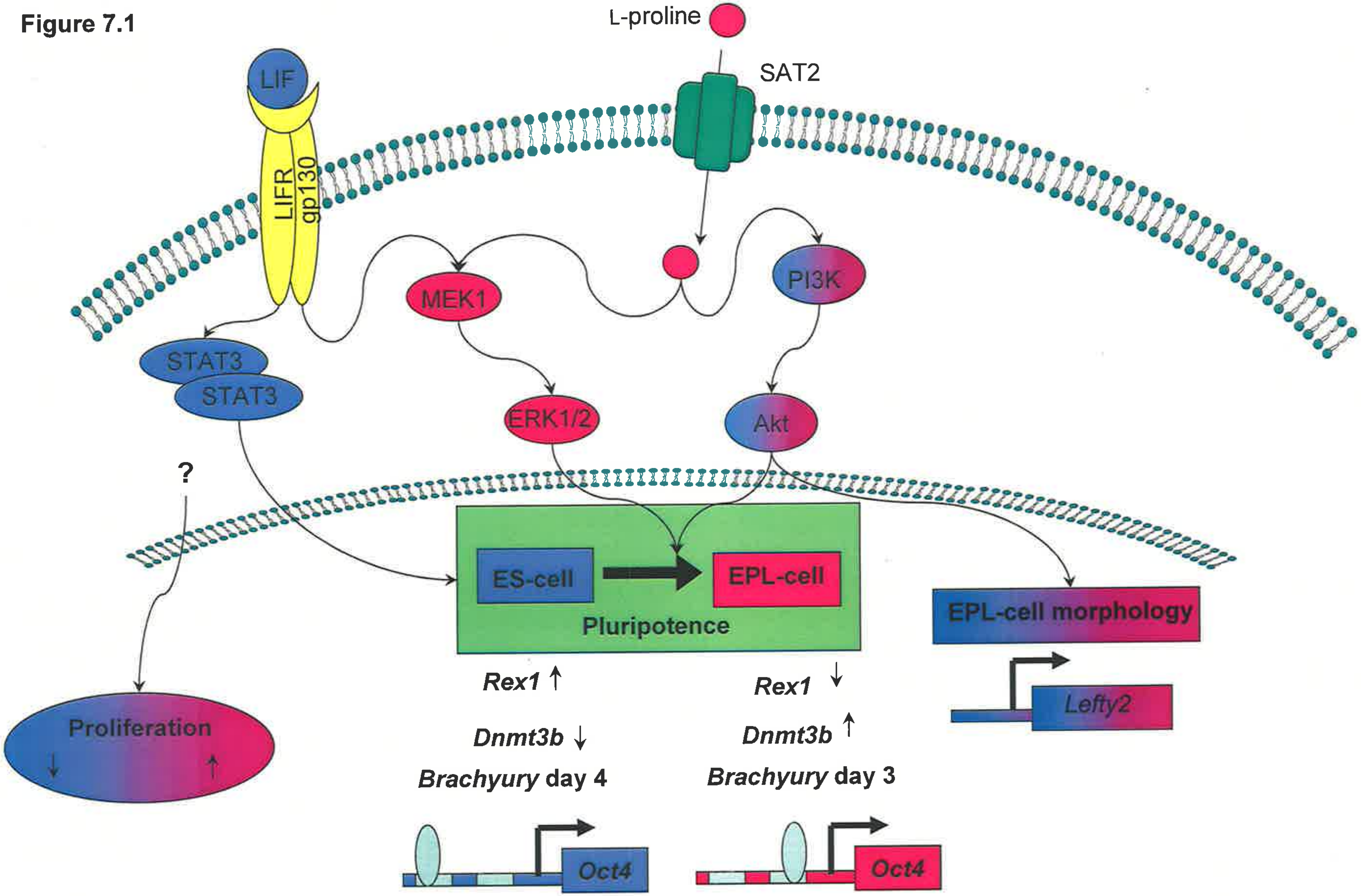
L-proline is internalised into ES cells from the medium via the action of the amino acid transporter SAT2. Following internalisation L-proline acts to induce the activation of MAPK signalling via MEK1, which leads to the activation of ERK1/2. This elevates MAPK activity above that achieved by LIF alone. The activation of MAPK signalling is essential for EPL-cell morphology, gene expression and differentiation potential. PI3K signalling is also activated by L-proline leading to the activation of the down-stream effector Akt. This

Figure 7.1

Potential mechanism of L-proline induced EPL-cell formation

L-proline is transported into ES cells from the medium via the action of the amino acid transporter SAT2. Inside the cell L-proline induces the activation of MAPK signalling via MEK1, which leads to the activation of ERK1/2. This elevates MAPK activity above that achieved by LIF alone tipping the balance from STAT3 mediated self-renewal to EPL-cell formation. The second pathway activated by L-proline is PI3K signalling leading to the activation of the down-stream effector Akt. This pathway is essential for the establishment of EPL-cell morphology. The activity of this pathway potentially leads to the induction of *Lefty2* that may have a role in the morphology change.

Figure 7.1



L-proline
SAT2
LIF
LIFR
gp130
MEK1
PI3K
STAT3
STAT3
ERK1/2
Akt
?
ES-cell
EPL-cell
Pluripotence
EPL-cell morphology
Lefty2
Proliferation
Rex1 ↑
Dnmt3b ↓
Brachyury day 4
Rex1 ↓
Dnmt3b ↑
Brachyury day 3
Oct4
Oct4

pathway is necessary for establishment of EPL-cell morphology since PI3K inhibitor LY294002 prevented EPL-cell morphology, but not sufficient as MEK1 inhibitors were also able to prevent EPL-cell morphology. The activity of this pathway potentially leads to the induction of *Lefty2* that may have a role in the morphology change.

7.3 L-PROLINE ACTION MAY BE EMBRYOLOGICALLY RELEVANT

At the late blastocyst stage, prior to primitive ectoderm formation, the cells of the ICM are surrounded by a basement membrane, which separates the pluripotent cells from the visceral endoderm. During embryonic development basement membranes, rich in proline abundant collagen IV, undergo continuous remodelling through the action of proteases. This remodelling leads to the production of free proline and small peptides of the form gly-pro-X (Telejko *et al.*, 1992).

The matrix-metaloprotease MMP-9 is involved in the degradation of collagen IV (Matrisian, 1992; Birkedal-Hansen, 1995) and is expressed in peri-implantation mouse blastocysts (Brenner *et al.*, 1989; Behrendtsen *et al.*, 1992). Therefore, the environment surrounding the ICM is likely to contain high local concentrations of L-proline and L-proline-containing peptides. Cells of the ICM at the time of primitive ectoderm formation may be able to internalise L-proline via SAT2 since system A is up-regulated in the mouse embryo from the late blastocyst stage (Zuzack *et al.*, 1985). In the *in vitro* model of primitive ectoderm formation, following internalisation L-proline induces the activation of MAPK and PI3K signalling. Although MAPK signalling has not previously been implicated in the formation of primitive ectoderm *in vivo*, circumstantial evidence points to a possible role. Regions of active ERK1/2 signalling have been identified within the primitive ectoderm throughout development (Corson *et al.*, 2003). Complementary to this, *ERK2*$^{-/-}$ mice are embryonic lethal at 6.5 dpc and show inability of the presumptive primitive ectoderm to form a single layer of pseudostratified epithelia (Yao *et al.*, 2003). This suggests involvement of ERK2 signalling in the establishment of primitive ectoderm *in vivo*. PI3K signalling is active in the pre-implantation mouse blastocyst (Riley *et al.*, 2005). The role of PI3K in the morphology change associated with EPL-cell formation has precedent since PI3K signalling has been associated with actin remodelling, morphology changes and polarization in various other cell types (Datta *et al.*, 1999; Qian *et al.*, 2003; Montero *et al.*, 2003; Brachmann *et al.*, 2005).

7.4 CONFIRMATION OF SAT2 INVOLVEMENT IN EPL-CEL FORMATION AND THE IMPORTANCE OF L-PROLINE INTERNALISATION

SAT2 competitive inhibitors L-alanine, glycine and L-serine are substrates of SAT2 as is L-proline. The ability of these amino acids as well as MeAIB to prevent EPL-cell formation suggests that internalisation of L-proline via SAT2 is necessary for EPL-cell formation or that the interaction of L-proline with SAT2 triggers signalling that induces EPL cell formation.

To confirm the involvement of SAT2 in EPL-cell formation the SAT2 transporter could be knocked down in ES cells using siRNA targeting

(Hannon, 2002; Zamore, 2002). If SAT2 knock-down prevents the transition in the presence of L-proline this would suggest that SAT2 is required for L-proline mediated EPL-cell formation. However, this does not distinguish between a role for SAT2 in mediating internalisation or actively inducing signalling.

If the ability of L-proline to induce EPL-cell formation in SAT2 knock down cells is reestablished following overexpression of another L-proline transporter, such as PROT, the role of SAT2 in mediating EPL-cell formation is likely to involve internalisation of L-proline.

7.5 CONFIRMATION OF MAPK AND PI3K SIGNALLING INVOLVEMENT IN EPL-CELL FORMATION

The results in this thesis showing the ability of MEK1 inhibitors to prevent EPL-cell formation and the activation of ERK1/2 phosphorylation by L-proline suggested the involvement of MAPK signalling in EPL-cell formation. Further confirmation of MEK1 involvement in this process could be provided by siRNA targeting the MEK1 transcript in ES cells. ES cells with knocked down MEK1 levels should behave like U0126 inhibitor-treated cells by failing to form EPL cells in response to L-proline treatment.

L-proline treatment leads to induction of ERK1/2 phosphorylation, consistent with *in vivo* evidence that ERK2 is important for primitive ectoderm formation (Saba-El-Leil *et al.*, 2003; Yao *et al.*, 2003). Thus, it would be appropriate to analyse ES cell lines derived from *ERK2*$^{-/-}$ mice to determine if ERK2 is crucial for EPL-cell formation. If ERK2 is required for the transition then treatment of *ERK2*$^{-/-}$ ES cells with L-proline would not induce EPL-cell formation and the cells would be expected to maintain ES cell like morphology, gene expression and differentiation potential.

While the experiments described above will confirm the requirement of MEK1 signalling for EPL-cell formation they do not define whether MEK1 activation is sufficient for the formation of EPL cells. This could be tested through the establishment of ES cell lines with inducible expression of a constitutively active form of MEK1. MEK1 is activated by phosphorylation of Ser218 and Ser222 by Raf (Zheng and Guan, 1994; Pages *et al.*, 1994; Xu *et al.*, 1995). Mutation of these sites to acidic residues such as Asp or Glu results in constitutively active MEK1 (CA-MEK1) (Mansour *et al.*, 1994). To achieve conditional induction the CA-MEK1 tetracycline-regulatable system (tet-off) developed by Bujard and colleagues (Gossen and Bujard 1992) and modified for use in ES cells by Niwa *et al.*, (Niwa *et al.*, 1998) could be used. Following withdrawal of tetracycline the effect of CA-MEK1 expression on EPL-cell formation could be assessed. If MEK1 activity is sufficient for EPL-cell formation then the ES cells expressing CA-MEK1 following withdrawal of tetracycline would adopt EPL-cell gene expression and differentiation potential. These results would define whether MEK1 activity is sufficient for formation of EPL cells.

In ES cells, LIF activates the 'self-renewal' STAT3 pathway and the 'differentiation' MAPK pathway down-stream of the gp130-LIFRβ complex but the net effect of LIF action is the maintenance of self-renewal via the STAT3 pathway (Figure 7.2 A). Activation of MAPK signalling via L-proline may tip the balance from self-renewal, mediated by LIF activated-STAT3, to differentiation due to the higher net activation of MAPK signalling leading to the transition from ES to EPL cells (Figure 7.2 B). This requirement for the LIF-activated MAPK activity in conjunction with the L-proline activated MAPK activity could be tested using ES cells expressing estradiol-inducible STAT3 (Matsuda *et al.*, 1999). These ES cells maintain their undifferentiated state in the absence of LIF if 4OHT is present. Thus, these ES cells can be tested for their ability to form EPL cells in response to L-proline in the presence of a range of concentrations of 4OHT and the absence of LIF. These cells would have the activated STAT3 component of LIF signalling but not LIF-induced MAPK signalling. Under these conditions it might be expected that EPL-cell formation would require higher concentrations of L-proline (>200 μM) to induce the transition as the total levels of MAPK activation in these cells would be lower due to the absence of LIF activated MAPK activity (Figure 7.2 C). This system can be exploited to understand the connection between signalling strength and cell response in terms of differentiation.

It may also be interesting to elucidate downstream targets of MAPK signalling and potentially investigate the effect of MAPK signalling on expression of these genes. For example, the effect of MAPK signalling on the switch in enhancer usage in the *Oct4* promoter could be investigated. *Oct4* expression in the ICM and ES cells requires the distal enhancer while expression in the primitive ectoderm and EPL cells requires the proximal enhancer (Section

Figure 7.2

Relative levels of MAPK activity control the transition between ES and EPL cells

(A) Culture of ES cells in the presence of LIF induces the activation of STAT3 and MAPK signalling down-stream of the gp130-LIFRβ complex. In the absence of any other signals the level of MAPK signalling activated by LIF is not enough to tip the balance to differentiation and the net effect of LIF is the maintenance of ES self-renewal via the action of STAT3. (B) In the presence of L-proline MAPK signalling is still activated by LIF as well as by L-proline leading to a higher level of MAPK activity in ES cells. This elevated level of MAPK activity is enough to tip the balance from STAT3 mediated self-renewal to EPL-cell formation. (C) When ES cells expressing estradiol-inducible STAT3 are cultured in the presence of 4OHT they can maintain their self-renewal in the absence of LIF. The absence of LIF means that the component of MAPK signalling mediated by LIF is also absent. Under these conditions treatment of ES cells with L-proline (200 μM) would still lead to activation of MAPK activity but the overall level of activity would not be high enough to tip the balance to induce EPL-cell formation.

Figure 7.2

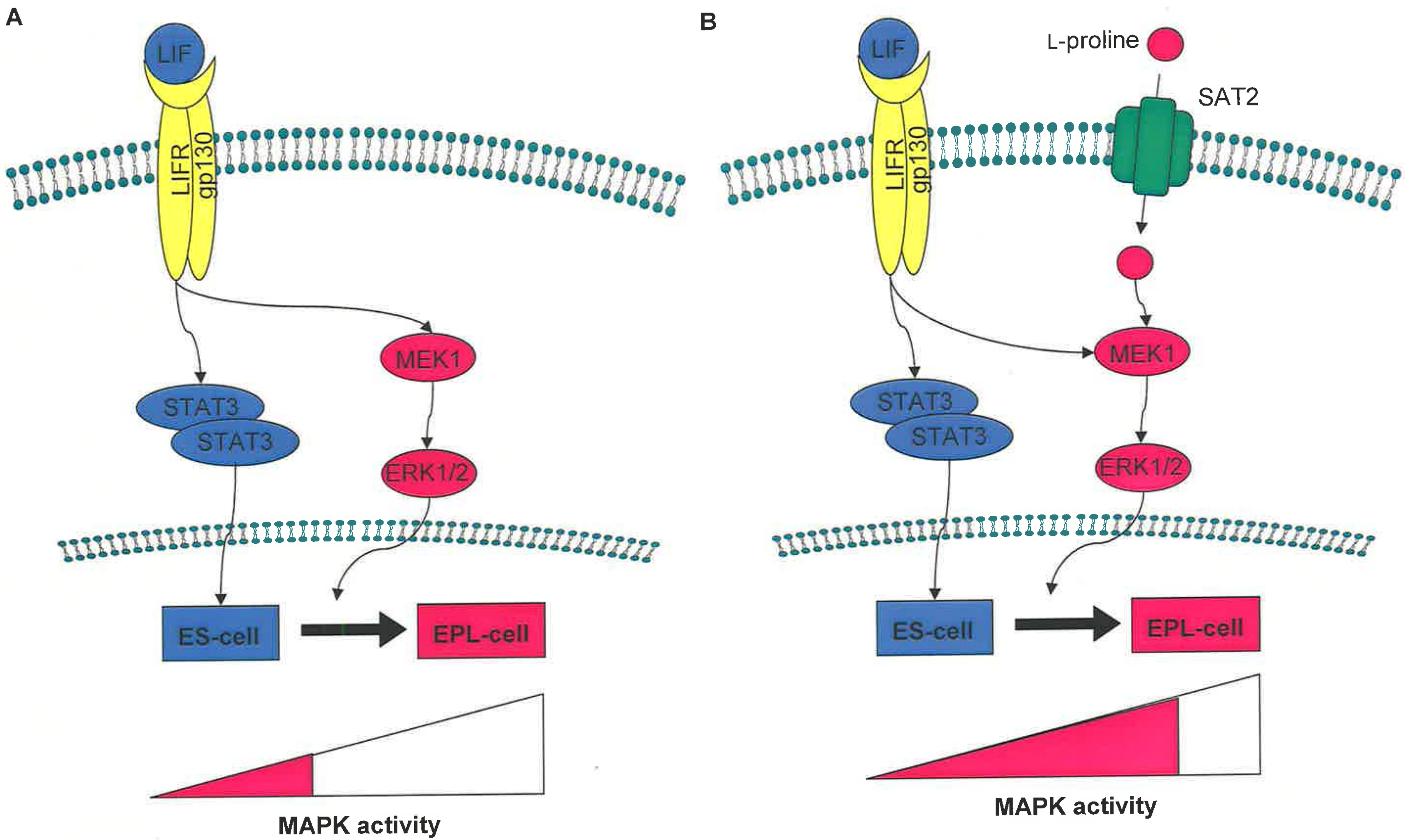
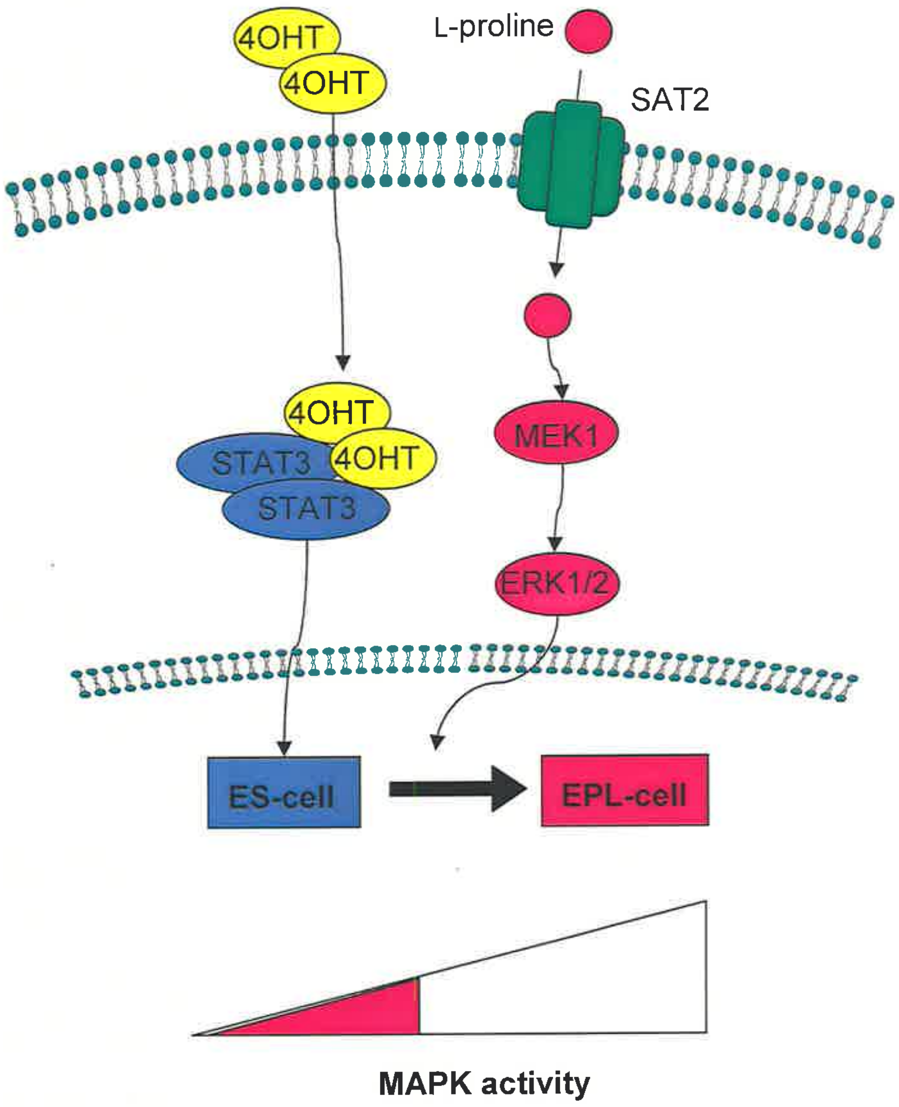

A
LIF
LIFR
gp130
MEK1
STAT3
STAT3
ERK1/2
ES-cell
EPL-cell
MAPK activity
B
LIF
L-proline
SAT2
LIFR
gp130
MEK1
STAT3
STAT3
ERK1/2
ES-cell
EPL-cell
MAPK activity

Figure 7.2

C



4OHT
4OHT
L-proline
SAT2
4OHT
STAT3
4OHT
STAT3
MEK1
ERK1/2
ES-cell
EPL-cell
MAPK activity

1.4.1; Minucci *et al.*, 1996; Yeom *et al.*, 1996). LRH1 (liver receptor homologue 1), whose transcript is expressed during early development and in ES cells, has been shown to bind the *Oct4* promoter within the proximal enhancer and proximal promoter (Gu *et al.*, 2005; Gao *et al.*, 2006). LRH1 was required for *Oct4* expression within the primitive ectoderm as *LRH1*$^{-/-}$ embryos lacked *Oct4* expression within the primitive ectoderm but had normal expression within the ICM (Gu *et al.*, 2005). Consistent with this, *LRH1*$^{-/-}$ ES cells down-regulated *Oct* faster during differentiation (Gu *et al.*, 2005). MAPK signalling may have a role in the regulation of *Oct4* expression via the action of LRH1 as LRH1 has been shown to be activated by ERK phosphorylation in HeLa cells (Lee *et al.*, 2006). *Oct4* reporter constructs could be introduced in to *ERK2* null cells, inducible constitutive MEK1 ES cells or wildtype ES cells treated with MEK1 inhibitors to investigate the relationship between enhancer usage and MAPK signalling. Similarly, promoter regions of genes differentially expressed between ES or EPL cells such as *Nanog* (Chambers *et al.*, 2003), *Rex1* (Rogers *et al.*, 1991) or *Dmnt3b* (Rathjen, unpublished data) could be cloned upstream of a reporter to enable investigation of role of MAPK signalling in regulation of their expression.

The involvement of PI3K and Lefty2 in the morphology change alone could be confirmed by selectively inhibiting PI3K signalling or Lefty2 in ES cells via siRNA respectively and monitoring any effects on morphology in the presence of L-proline. The involvement of *Lefty2* in L-proline-induced EPL-cell morphology down-stream of PI3K signalling could be further clarified by over-expression of *Lefty2* in ES cells. If *Lefty2* is sufficient for EPL-cell morphology then its over-expression will lead to induction of EPL-cell morphology in the absence of any signal generated by L-proline.

7.6 ACTIVATION OF MAPK AND PI3K SIGNALLING BY L-PROLINE POTENTIAL MECHANISMS

The imino acid L-proline has not previously been associated with activation of signalling cascades. Therefore, the mechanism by which L-proline is able to induce the activation of ERK1/2 and Akt remains elusive. It is possible that the transport of L-proline through the SAT2 transporter causes the transporter itself to induce signalling via mechanisms previously described in Chapter 4:

i) Passage of the amino acid induces a conformational change in the transporter, inducing signalling.

ii) The signalling may be a secondary event resulting from changes in cell physiology such as altered pH or ion concentration.

iii) Following internalisation, the amino acid is recognised by an intracellular receptor that initiates signalling.

iv) The external concentration of an amino acid is sensed by a specific 'amino acid receptor' located in close proximity to the amino acid transporter such that the transporter regulates the concentration of the amino acid around the receptor and thus the extent of signalling activated.

If hypothesis i) is correct then the transporter must in some way be able to discriminate between different amino acids and the strength of signalling because other amino acids also transported by SAT2, including L-alanine, glycine and L-serine, do not induce EPL-cell formation. It is possible that the rate of transport of the different substrates is the key trigger in activation of signalling. However L-alanine and L-proline are transported at similar rates but induce different cellular responses in pluripotent cells.

In terms of hypothesis ii), similar changes in Na^+ concentration would occur during transport of any of the SAT2 substrates L-alanine, glycine and L-serine since they are all transported at similar rates. Given that only L-proline is able to induce EPL-cell formation, the mechanism of signalling has to be able to discriminate between the different SAT2 substrates.

The data presented in this thesis could be explained by hypothesis iv). L-proline could be binding a receptor on the membrane or an allosteric site on the transporter itself but there is no known receptor with such properties.

As it is difficult to see how an amino acid alone would be able to activate a signalling cascade, the most likely scenario appears to involve hypothesis iii), the binding of L-proline to an intracellular receptor, which is then able to induce the activation of MAPK and/or PI3K signalling. Receptors that specifically bind amino acids including glycine and glutamate have been identified in the central nervous system (Palacios *et al.*, 1981; Monahan *et al.*, 1990). Glycine is a cofactor ligand at NMDA receptors, a class of excitatory ion channel receptors (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). It is therefore possible that a specific L-proline receptor exists that is able to bind this imino acid and activate MAPK and/or PI3K in ES cells.

7.7 IDENTIFICATION OF AN L-PROLINE RECEPTOR

As discussed in section 7.6 L-proline is most likely binding an intracellular receptor to initiate intracellular signalling. Thus, to delineate the function of L-proline in EPL-cell formation, identification of this receptor is important. Methods for identification of the receptor could include passing ES cell lysates or membrane preparations over L-proline immobilised on a column. The proteins bound to the column could be eluted and analysed by mass spectrometry in order to identify the receptor (Figure 7.3). A candidate receptor has been identified using this approach (Forwood and Morris, unpublished data).

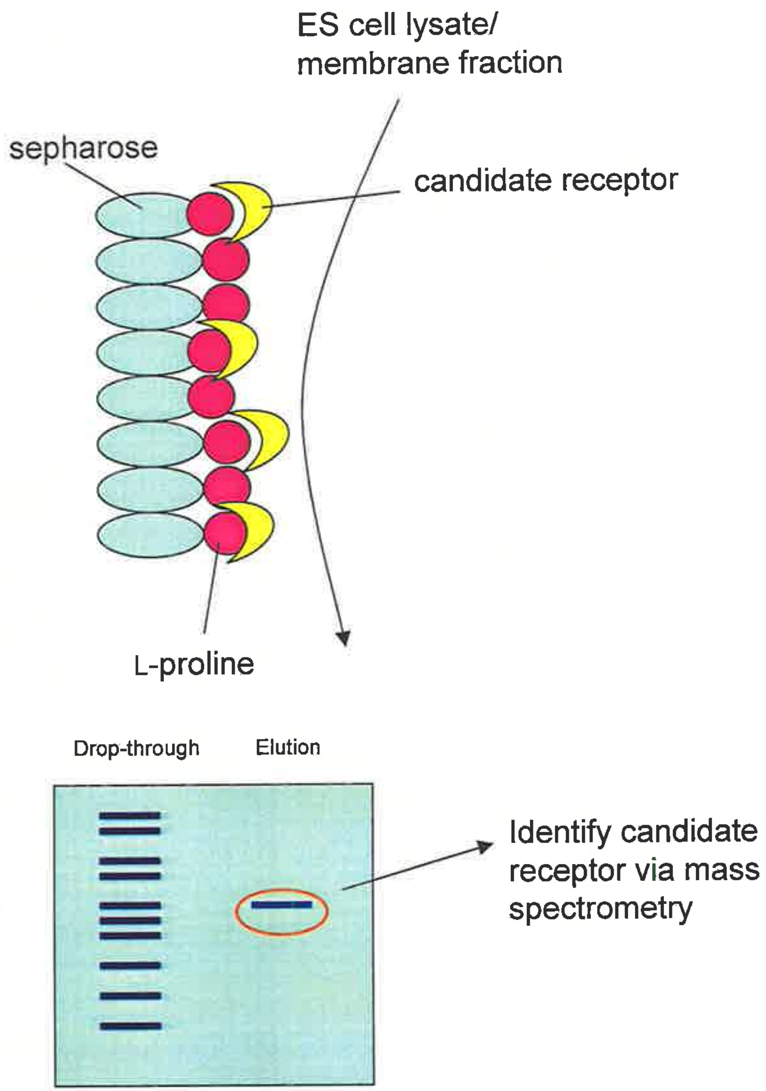
Another possible method for identification of the receptor could involve immunoprecipitation. We know that ERK1/2 and Akt are activated following L-proline treatment and that inhibition of MEK1 and PI3K prevents correct EPL-cell formation. However, we do not know the up-stream components of the cascades involved. Since the MAPK and PI3K signalling pathways are well characterised there are a number of potential up-stream candidates that may be involved in activating MEK1 and PI3K respectively. These include Shc (Pelicci *et al.*, 1992), Grb (Lowenstein *et al.*, 1992), IRS (Sun *et al.*, 1991; 1995), FRS (Kouhara *et al.*, 1997) and Gab (Holgado-Madruga et al., 1996;

Figure 7.3

Identification of the L-proline receptor

The L-proline receptor could be identified by preparing ES cell lysates or membrane fractions and passing them over L-proline immobilised on a column of sepharose beads. The proteins bound to the column could be eluted, run on a SDS-PAGE and the bands of interest excised and subject to mass spectrometry analysis in order to identify the receptor.

Figure 7.3


ES cell lysate/
membrane fraction
sepharose
candidate receptor
L-proline
Drop-through
Elution
Identify candidate
receptor via mass
spectrometry

Zhao *et al.*, 1999). Performing immunoprecipitation with antibodies for these adaptor proteins, following stimulation of ES cells with L-proline, may enable identification of a coimmunoprecipitated L-proline receptor. Once identified the receptor could be validated for involvement in EPL-cell formation by knockdown using siRNA.

7.8 IMPLICATIONS OF THIS WORK FOR MAINTENANCE OF PLURIPOTENCE AND DIRECTED DIFFERENTIATION

The elucidation of the mechanism of L-proline action and the signalling pathways involved in the formation of EPL cells has identified targets of intervention for inhibition of the transition. Amino acids that act as competetive inhibitors of SAT2 transporter (glycine, L-serine, MeAIB) could be used as inhibitors of the ES-to-EPL cell transition as could inhibitors of MAPK signalling. The identification of these inhibitors provides alternative or additional mechanisms that can be utilised in the maintenance of ES cells in culture. This may be particularly relevant in human ES cell work as human ES cells display great propensity for spontaneous differentiation (Sathananthan and Trounson, 2005). Further more, ES cells can be induced to form a homogeneous population of EPL cells simply by the addition of L-proline. This will provide a homogeneous population as a starting material for directed differentiation. Further differentiation of EPL cells to alternative fates can be induced by the controlled addition of exogenous signals. The differentiation of EPL cells occurs in the absence of visceral endoderm signalling thus enabling the production of cell types in a controlled manner. Problems associated with activation of inappropriate signalling pathways can also be avoided allowing the generation of defined and pure populations of cells that may be useful in analysis of molecular mechanisms of development and cell based therapies.

Thus, the manipulation of L-proline-dependent mechanisms of action identified in this thesis can potentially provide a means for controlling the maintenance of pluripotence and switching to directed differentiation in a homogenous and synchronous manner. The identified signalling pathways can be targeted directly with small, non-toxic, organic inhibitors of the transition.

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