Embryonic stem cell differentiation and the analysis of mammalian development

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ABSTRACT   Molecular and cellular analysis of early mammalian development is compromised by the experimental inaccessibility of the embryo. Pluripotent embryonic stem (ES) cells are derived from and retain many properties of the pluripotent founder population of the embryo, the inner cell mass. Experimental manipulation of these cells and their environment *in vitro* provides an opportunity for the development of differentiation systems which can be used for analysis of the molecular and cellular basis of embryogenesis. In this review we discuss strengths and weaknesses of the available ES cell differentiation methodologies and their relationship to events *in vivo*. Exploitation of these systems is providing novel insight into embryonic processes as diverse as cell lineage establishment, cell progression during differentiation, patterning, morphogenesis and the molecular basis for cell properties in the early mammalian embryo.

KEY WORDS: *Embryonic Stem cells, Primitive ectoderm, Embryonic development, Differentiation, Morphogenesis*

Pluripotent Cells and Early Mouse Development

One of the distinguishing features of early mammalian development is the maintenance of a population of developmentally plastic, pluripotent stem cells which give rise to all cells that constitute the mature organism. Around 10-20 cells, which appear to be developmentally equivalent, make up the inner cell mass (ICM), located at one end of the 3.5 d.p.c. (days post coitum) blastocyst. Mammalian development entails the regulated proliferation of these cells and allocation of descendants to specific cell lineages following differentiation.

Around 4.0 d.p.c., ICM cells lining the blastocoelic cavity differentiate to extraembryonic primitive endoderm. Pluripotence is retained by internal ICM cells, referred to as the 'epiblast', now surrounded by extraembryonic endoderm and trophectoderm. At about the time the embryo implants into the uterine wall these pluripotent cells begin to proliferate rapidly such that the 20-25 cells present at 4.5 d.p.c. expand to give rise to about 660 cells by 6.5 d.p.c. and 8060 cells by 7.5 d.p.c. (Snow, 1977). Concurrent with pluripotent cell proliferation, primitive endoderm cells migrate along the pluripotent cell surface and differentiate into one of two cell types. Primitive endoderm cells that remain in contact with the pluripotent cells differentiate into visceral endoderm while those primitive endoderm cells that migrate onto the blastocoelic surface of trophectoderm differentiate into parietal endoderm (Hogan *et al.*, 1994).

Formation of the proamniotic cavity within the pluripotent cell population is first visible at 5.0 d.p.c. and is accompanied by reorganisation of the cells into a pseudostratified columnar epithelial sheet. Concomitant with this process, the pluripotent cells become polarised, and undergo changes in gene expression (Pelton *et al.*, 2002) and developmental potential (Beddington, 1983a; Lake *et al.*, 2000) suggesting that this morphologically distinct pluripotent cell population, termed primitive ectoderm, is derived from the ICM by differentiation. Primitive ectoderm provides the substrate for gastrulation, which is initiated at around 6.5 d.p.c.. This process transforms the pluripotent monolayer into a multi-layered embryo consisting of the 3 primary germ layers, mesoderm, endoderm and ectoderm.
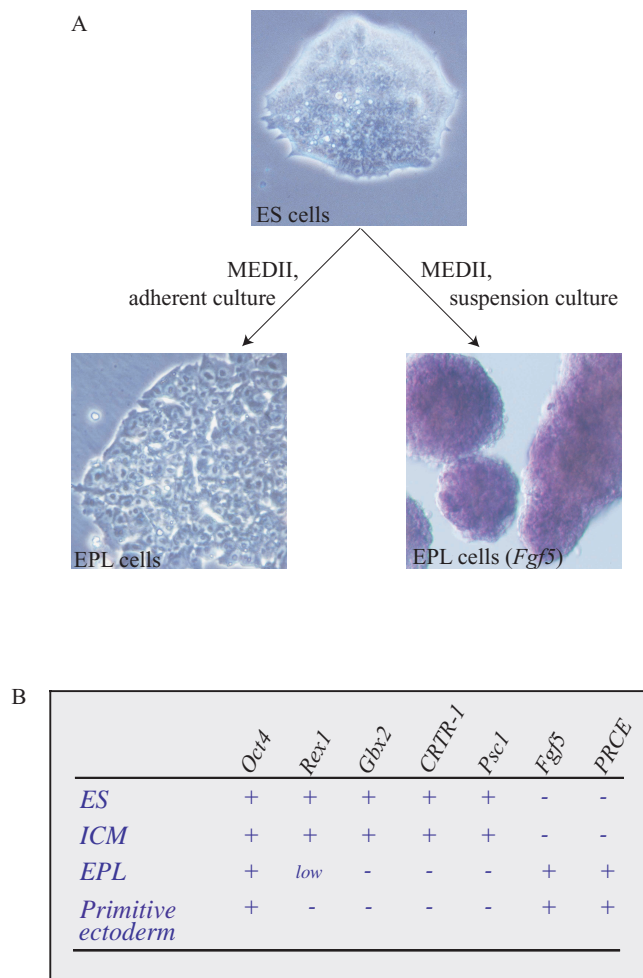
While coordinated regulation of pluripotent cell proliferation, differentiation and morphogenesis underpins development of the mammal, relatively little is known of the signalling mechanisms that regulate these processes at a cellular or molecular level. Accumulating evidence implicates visceral endoderm as a source of signals controlling primitive ectoderm formation, maintenance and differentiation. Firstly, deletion of *Evx1* and *Hnf4*, transcription factors expressed in the visceral endoderm of the pre-gastrulation

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embryo, results in deterioration of the pluripotent cells around 5.0 d.p.c. (Spyropoulos and Cappechi, 1994) and increased apoptosis of the primitive ectoderm by 6.5 d.p.c. (Duncan *et al.*, 1994) respectively. These observations identify visceral endoderm as a source of signals that mediate formation and maintenance of the primitive ectoderm. Recombination of *Hnf4*^{-/-} embryos with *Hnf4*^{+/+} visceral endoderm rescues this defect resulting in formation of a normal embryo capable of gastrulation (Duncan *et al.*, 1997), thereby confirming that the cellular defect in *Hnf4*^{-/-} embryos is associated with extraembryonic cell lineages. Expression of the paired-like homeobox gene, *Hesx1*, in the anterior visceral endoderm at day 6-6.5 d.p.c. is followed by expression in the adjacent primitive ectoderm within 24 hours (Thomas and Beddington, 1996). This apparent patterning of the anterior primitive ectoderm is induced from *Hesx1* expressing visceral endoderm and is required for appropriate specification since *Hesx1*^{-/-} embryos show forebrain deficiencies (Martinez-Barbera *et al.*, 2000).

A

ES cells

MEDII, adherent culture

MEDII, suspension culture

EPL cells

EPL cells (*Fgf5*)

B

	Oct4	*Rex1*	*Gbx2*	*CRTR-1*	*Psc1*	*Fgf5*	*PRCE*
ES	+	+	+	+	+	-	-
ICM	+	+	+	+	+	-	-
EPL	+	*low*	-	-	-	+	+
Primitive ectoderm	+	-	-	-	-	+	+

Fig. 1. **Formation of EPL cells from ES cells. (A)** *ES cells form EPL cells when cultured in MEDII, either in adherent culture for 2 days or suspension culture for 4 days. EPL cells formed in suspension culture were identified by* in situ *hybridisation with digoxygenin labelled anti-sense probes directed against* Fgf5. **(B)** *Comparison of gene expression in ES and EPL cells in culture and ICM and primitive ectoderm of the embryo (Chapman* et al., *1997; Rathjen* et al., *1999; Pelton* et al., *2002).*

While the role of visceral endoderm as a source of inductive signals for pluripotent cells is emerging from close analysis of gene expression and knockout phenotypes, the molecular identity of these signals has proven elusive, probably due to experimental limitations associated with the early mouse embryo.

Embryonic Stem (ES) Cells: Their Origin, Properties and Uses

Pluripotent embryonic stem (ES) cells are derived from outgrowths of the pluripotent cells of the pre-implantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981) and can be maintained as a homogeneous population of undifferentiated cells indefinitely in culture. ES cells share many properties with the pluripotent cells of the ICM, including expression of the pluripotent cell marker, *Oct4*, and expression of the more restricted markers *Rex1*, which is expressed by the ICM but not later embryonic pluripotent cell populations, and *uvomorulin*, which is not expressed by pluripotent cells of the primordial germ cell lineage (Rathjen *et al.*, 1999). Further, ES cells retain the pluripotent developmental potential of the ICM, as demonstrated *in vivo* by contribution to all tissues of the embryo and adult after reintroduction to mouse blastocysts (Bradley *et al.*, 1984), and *in vitro* by the formation of embryoid bodies (EBs), in which a broad range of differentiated cell types representative of the three germ layers of the mouse embryo are formed (Doetschman *et al.*, 1985; Lake *et al.*, 2000). ES cells differ from their founding population in their cytokine requirements. Maintenance of pluripotence in mouse ES cells is reliant on activation of the gp130 receptor subunit, normally achieved by addition of members of the IL-6 family of cytokines to the culture medium (reviewed in Rathjen and Rathjen, 2001). As yet, no role for gp130 signalling has been determined in the establishment or maintenance of the pluripotent cell population *in vivo* (Yoshida *et al.*, 1996), although a role for gp130 activation in embryonic diapause has been described (Nichols *et al.*, 2001).

As a highly proliferative cell population that maintains, in culture, the properties of a normal pluripotent cell, ES cells provide a unique experimental resource for the analysis of mammalian development. In this review we discuss exploitation of the ES cell system to investigate cellular properties associated with pluripotent cells, and to model cellular events that occur *in vivo*, such as differentiation and cell fate specification.

Modelling Embryonic Differentiation: Formation of Primitive Ectoderm from ES Cells

Modelling the first embryonic differentiation event, formation of a population of pluripotent cells analogous to primitive ectoderm, can be achieved through differentiation of ES cells as embryoid bodies (EB) (Shen and Leder, 1992) or by co-culture of ES cells with MEDII, a medium conditioned by the human hepatocellularcarcinoma cell line, Hep G2 (Rathjen *et al.*, 1999). Within EB, primitive ectoderm exists transiently as a differentiation intermediate which arises during the spontaneous differentiation of ES cells, and coexists with a number of other cellular lineages, including derivatives of the extraembryonic endodermal lineage and populations representative of the embryonic germ lineages. The resulting differentiation environment is complex and deregulated, precluding maintenance, analysis or controlled differentiation of the primitive ectoderm.

Addition of MEDII to ES cells in adherent culture results in the formation of a homogeneous population of pluripotent cells, termed early primitive ectoderm-like (EPL) cells, which exhibit many properties consistent with embryonic primitive ectoderm but distinct from ICM and ES cells (Fig. 1A). EPL cell colonies form as epithelial sheets, rather than the characteristic domed colony structure of ES cells in culture, and express *Oct4* and the primitive ectoderm marker *Fgf5*, but not the ICM marker *Rex1*. A more detailed analysis of these markers, and others isolated on the basis of differential expression in ES and EPL cells, indicated that formation of EPL cells *in vitro* and primitive ectoderm *in vivo* followed a similar progression (Fig. 1B; Pelton *et al.*, 2002). Rigorous demonstration of pluripotence is complicated by the failure of both embryonic primitive ectoderm and EPL cells to contribute to embryonic development after introduction into host blastocysts (Beddington, 1983a; Rathjen *et al.*, 1999). However, consistent with the expression of pluripotent cell markers, the extensive differentiation potential of EPL cells has been demonstrated *in vitro* by the formation of multiple cell types following spontaneous differentiation and the generation of effectively homogeneous populations of differentiated derivatives by directed differentiation (Lake *et al.*, 2000; Rathjen *et al.*, 1999; Rathjen *et al.*, 2002). ES cells also differentiate to EPL cells when cultured as cellular aggregates in suspension in medium supplemented with MEDII (Fig. 1A; Rathjen *et al.,* 2002). The formation of EPL cells occurs in the presence or absence of exogenous LIF, but as would be predicted from *in vitro* and *in vivo* experiments (Conquet *et al.*, 1992; Shen and Leder, 1992), the acquisition of primitive ectoderm characteristics is delayed in the presence of gp130 signalling.

Formation and maintenance of EPL cells in culture is dependent on the continued presence of MEDII, which can be separated into two distinct biological activities, a high molecular weight component of greater than 100 kDa and a low molecular weight activity of less than 3 kDa (Rathjen *et al.*, 1999). Analysis of the fractions reveals that the low molecular weight activity is a small peptide while the high molecular weight fraction has been identified as a component of the ECM. In the absence of other signalling sources, withdrawal of MEDII from EPL cells results in loss of pluripotence and differentiation. However, when EPL cells are cultured in the absence of MEDII in medium supplemented with LIF, pluripotence is maintained and an ES cell morphology, gene expression and developmental potential is established. The ability to revert to an ES cell phenotype has been proposed to be a common feature of pluripotent cells in culture (Matsui *et al.*, 1992; Rossant, 1993).

Visceral endoderm and the liver share similarities with respect to function and gene expression, despite their diverse embryological origin (Rossant, 1995). Formation of primitive ectoderm from ES cells in response to medium conditioned by a liver derived cell line is therefore not unexpected, and suggests a functional similarity between MEDII *in vitro* and visceral endoderm signalling *in vivo*. Consistent with this, the small and large active components of
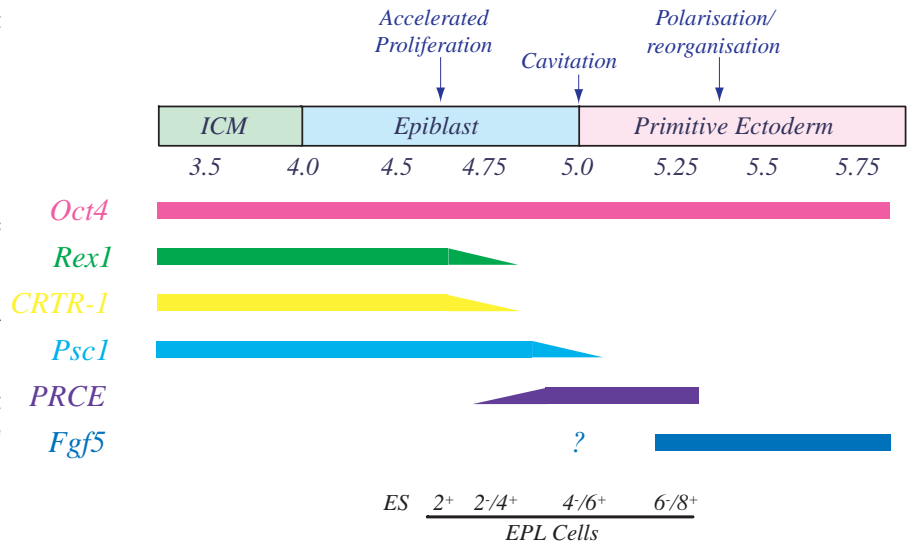
Fig. 2. **Summary of pluripotent cell gene expression *in vivo* and *in vitro*.** *The pluripotent cell populations present within the early mouse embryo are aligned with approximate time points in days post coitum (d.p.c.), the embryonic events characteristic of this developmental stage and gene expression in pluripotent cell populations* in vivo. *ES cells and EPL cells grown for 2, 4 or 6 days in MEDII in the presence (+) or absence (-) of LIF are aligned on the basis of gene expression* in vitro. Fgf5 *expression was not determined in 5.0 d.p.c. embryos.*

MEDII are expressed respectively by the visceral endoderm-like cell line END-2, and within the basement membrane supporting the pluripotent cells during primitive ectoderm formation (Bettess, 2001).

As a population of cells equivalent to embryonic primitive ectoderm, EPL cells represent an important obligate intermediate cell population in the differentiation of ES cells in culture. Establishment of EPL cells in culture appears to follow closely the generation of primitive ectoderm *in vivo* and represents an important first step in the development of protocols for controlled, lineage specific differentiation of pluripotent cells in culture. EPL cell formation and differentiation therefore provides a system for the identification of differentially expressed genes and transient intermediate cell populations during pluripotent cell progression *in vivo*, and the investigation of germ layer selection, differentiation, and patterning of somatic lineages.

Distinct Subpopulations of Pluripotent Cells are Formed during Developmental Progression of Pluripotent Cells

Although cellular criteria have been used to identify two pluripotent cell populations within the early embryo (ICM and primitive ectoderm), other evidence suggests that primitive ectoderm formation from ICM proceeds via temporally distinct intermediate pluripotent cell populations (Pelton *et al.*, 1998). Utilising the ES to EPL transition as an *in vitro* model for ICM to primitive ectoderm formation, it has been possible to correlate differential gene expression in pluripotent cells *in vitro* and *in vivo* to provide a molecular basis for the definition of transient pluripotent cell subpopulations, and identify genes that may play important roles in pluripotent cell developmental progression.

Differential display PCR on ES cells and EPL cells maintained in culture for up to 8 days was used to identify novel differentially

regulated genes expressed in synchronous fashion throughout the population. Expression of *CRTR-1* appeared to be coordinately regulated with expression of *Rex1*, a zinc finger protein regulated by *Oct4* expression (Ben-Shushan *et al.*, 1998). Both genes are expressed in ES cells and ICM, and downregulated *in vitro* in EPL cells cultured for 2 days and *in vivo* between 4.5 d.p.c. and 4.75 d.p.c. This is a time associated with the onset of accelerated pluripotent cell division in the embryo. Down regulation of *CRTR-1* and *Rex1* coincided with up regulation of *PRCE* expression. Both *in vitro* and *in vivo* *PRCE* expression was transient and down regulated in EPL cells cultured past day 4, and after 5.25 d.p.c. of embryonic development. *Psc1* was expressed in ES cells and ICM and down regulated *in vitro* in EPL cells cultured for 4 days and *in vivo* between 5.0 and 5.25 d.p.c.. *Fgf5,* which is not expressed in ES cells or ICM, is up-regulated to high levels in EPL cells cultured for 4 days and throughout the embryonic primitive ectoderm, although the exact timing of *Fgf5* up-regulation has not been established. *Fgf5* expression is maintained in the pluripotent cells before down-regulation associated with loss of pluripotence at gastrulation.

All transcripts were expressed throughout the pluripotent cell populations and no evidence was found for spatial heterogeneity within this pool. Molecular homogeneity of pluripotent cells in the pre-gastrulation embryo is consistent with the fate of heterotopic transplanted primitive ectoderm from pre-streak or early primitive streak embryos, which assumes a fate appropriate to the transplanted location (Beddington, 1983b; Parameswaran and Tam, 1995; Tam and Zhou, 1996). In the absence of spatial heterogeneity within the pluripotent population, patterning of the embryo must therefore be established by signals originating from extraembryonic cell types, consistent with the results emerging from embryonic analysis (Beddington and Robertson, 1999).

While the availability of differentially expressed pluripotent cell markers within embryonic populations refines knowledge of the temporal progression of pluripotent subpopulations *in vivo* (Fig. 2), it is not clear whether these represent distinct cell types which can be maintained stably in appropriate conditions. An alternative explanation is that they represent transient intermediates which are developmental equivalents of transformed EC cells isolated from teratocarcinomas. Changes in gene expression may reflect alterations in the competence of the pluripotent cells to respond to embryonic signalling, a possibility consistent with the temporal correlation between changes in gene expression and cellular events *in vivo* such as proliferation and cavitation. Analysis of the promoters of differentially expressed genes may allow definition of downstream signalling pathways operating in the embryo at these times.

EPL Cell Differentiation *In Vitro* provides a Model for Formation of the Primary Germ Layers of the Mammalian Embryo

The aggregation of EPL cells, in a manner analogous to embryoid body formation by ES cells, results in differentiation as evidenced by loss of pluripotence and formation of differentiated cell populations (Lake *et al.*, 2000). However, unlike ES cell EBs, in which cell populations representative of all three primary germ layers and the extraembryonic endoderm can be detected, the repertoire of cells produced from EPL cells EBs is more restricted

	ES cell EB	EPL cell EB	EBM
early primitive ectoderm	pluripotent	pluripotent	pluripotent
late primitive ectoderm	pluripotent	pluripotent	pluripotent
visceral endoderm	extraembryonic endoderm		
parietal endoderm	extraembryonic endoderm	extraembryonic endoderm	
mesoderm progenitors	mesoderm	mesoderm	
beating cardiocytes	mesoderm	mesoderm	
blood	mesoderm	mesoderm	
neurectoderm	ectoderm		ectoderm
neurons	ectoderm		ectoderm
glia	ectoderm		ectoderm
neural crest	ectoderm		ectoderm
surface ectoderm*	ectoderm		ectoderm
endoderm	endoderm		

* surface ectoderm induced from EBM by addition of BMP4 (J.Rathjen and C.Long, personal communication.)

Legend:
- pluripotent cells
- extraembryonic endoderm
- mesoderm
- ectoderm
- endoderm
- not determined
- not observed
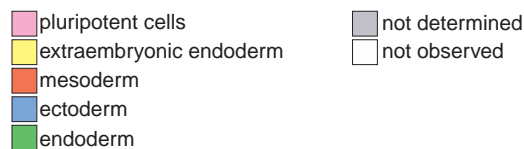
Fig. 3. Lineages formed during differentiation in ES cell EB, EPL cell EB and EBM. *Cell populations were determined on the basis of the following: low and high levels of* Fgf5 *expression (early and late primitive ectoderm); expression of alpha-feto protein and* SPARC *(visceral and parietal endoderm); expression of* brachyury, Nkx 2.5 *and* Flk1, *and phagocytosis of opsonised beads (mesoderm progenitors, beating cardiocytes and macrophages); expression of* Sox1, *Tubulin ß III isoform, Glial Fibrilliary Acidic Protein,* Sox10 *and* keratin 18 *(neurectoderm, neurons, glia, neural crest and surface ectoderm); expression of* SPARC *and morphology (endoderm).*

(Fig. 3). *Brachyury*, a marker of nascent mesoderm is detected earlier and at a much higher level in EPL cell EBs compared to ES cell EBs, but *Sox1*, a marker for the neural precursor population, is not detected at any stage of differentiation. These differences in the formation of progenitor cell populations are reflected in the formation of terminally differentiated cell populations, with the formation within EPL cell EBs of high levels of beating cardiocytes, a mesodermal derivative, but not neurons, an ectodermal derivative.

The lack of ectoderm formation within EPL cell EBs does not reflect a restricted differentiation potential as neurectoderm/neuron formation from EPL cells can be induced by differentiation within an ES cell EB environment, the potent neural inducer retinoic acid or supplementation of the differentiation environment with MEDII (Lake *et al.*, 2000; Rathjen *et al.*, 2001). The most notable difference between ES cell EBs and those formed from EPL cells is in the extraembryonic endoderm lineage, in particular a deficiency of the primitive endoderm derivative visceral endoderm in EPL cell EBs (Lake *et al.*, 2000). It has been suggested that this deficiency may be responsible for the lack of ectodermal lineage formation in EPL cell EBs (Rathjen *et al.*, 2001; Rathjen *et al*, 2002).

In vivo, mesoderm differentiates from primitive ectoderm positioned along the posteriodistal axis. These cells detach from the epithelial monolayer and transverse the primitive streak, an area of localised ECM breakdown. EPL cell EBs, formed from a single cell suspension of EPL cells differentiated in the absence of visceral endoderm, therefore simulate the loss of pluripotent cell:cell and

cell:ECM contacts which accompanies mesoderm formation. The possibility that this simulated epithelial to mesenchymal transition (EMT) underlies enhanced mesoderm formation is consistent with studies of the behaviour of cells ablated in the *Fgfr1* receptor during gastrulation. *Fgfr1$^{-/-}$* cells are unable to enter the primitive streak during gastrulation and fail to contribute to the mesodermal lineage, suggesting that primitive ectoderm EMT is required for formation of mesoderm (Ciruna and Rossant, 2001; Ciruna *et al.*, 1997). Differentiation within EPL cell EBs therefore appears to provide a model for the investigation of mesoderm lineage formation during gastrulation.

Within the embryo, the ectodermal lineage differentiates from anteriodistal primitive ectoderm, a population of pluripotent cells that maintains cell:cell and cell:ECM interactions and remains in close contact with the visceral endoderm during gastrulation. Maintenance of cell:cell and cell:ECM contacts and visceral endoderm signalling during EPL cell differentiation can be achieved by formation and differentiation of EPL cells within cellular aggregates in suspension culture in the presence of MEDII (EBMs). Differentiation of EPL cells in this environment results in suppression of both visceral endoderm and mesoderm lineage formation, and formation of a homogeneous population of neurectoderm (Fig. 3; Rathjen *et al*, 2002). The resulting neurectoderm population comprises a stratified epithelium with morphology, gene expression and differentiation potential consistent with embryonic neural tube. Expression analysis of the marker genes *Oct4*, *Rex1*, *Sox1* and *Gbx2* during formation of neurectoderm in these aggregates suggests that differentiation recapitulates formation of this lineage in the embryo, with the sequential elaboration of primitive ectoderm, definitive ectoderm, neural plate and neural tube (Rathjen *et al*, 2002). The correspondence between ectoderm/ neurectoderm induction *in vivo* and *in vitro*, both in morphology and sequential elaboration of developmental intermediates, suggests that directed differentiation of EPL cells provides a model system for analysis of the establishment of the ectodermal and neurectodermal lineages.

The formation of EPL cells from ES cells provides a homogeneous population of primitive ectoderm-like cells in culture, representing the immediate precursor cell of the three primary germ layers. The ability to direct differentiation of these cells to homogeneous populations of differentiated derivatives relies on the absence of alternate cell populations, such as visceral endoderm, which act as a source of endogenous differentiation inducing signals (Lake *et al.*, 2000; Rathjen *et al.*, 2001; Rathjen *et al*, 2002). The fact that differentiation of EPL cells appears to recapitulate establishment of the germ layers during development validates this system for the analysis of embryonic decisions following loss of pluripotence. Experimentally, the ability to modify the differentiation environment in con-

trolled fashion provides an opportunity to dissect the roles of individual signalling pathways during differentiation, and to discriminate between the activity of soluble signalling molecules, ECM and the cellular environment in both the induction and suppression of alternate cell fates.

Pluripotent Cell Differentiation and Specification/Patterning of Somatic Lineages

Allocation of ectodermal cells to the surface ectoderm or neurectoderm lineages appears to be determined by respective activation or suppression of BMP signalling pathways (Hemmati-Brivanlou and Melton, 1997). In lower vertebrates this appears to be mediated via antagonists of the BMP family of signalling molecules, emanating from Spemann's organiser. Although an analogous structure is formed during embryogenesis in birds (Henson's node) and mammals (node), increasing evidence suggests that these organiser structures and BMP antagonists in higher vertebrates do not play an equivalent role in neural induction (Blum *et al.*, 1992; Cho *et al.*, 1991; Rivera-Perez *et al.*, 1995; Yamada *et al.*, 1995; Streit *et al.*, 2000; Streit *et al.*, 1998; Klingensmith *et al.*, 1999). While direct analysis of ectodermal specification in the mammalian embryo has not yet been achieved, BMP4 treatment of ectodermal precursors derived by directed
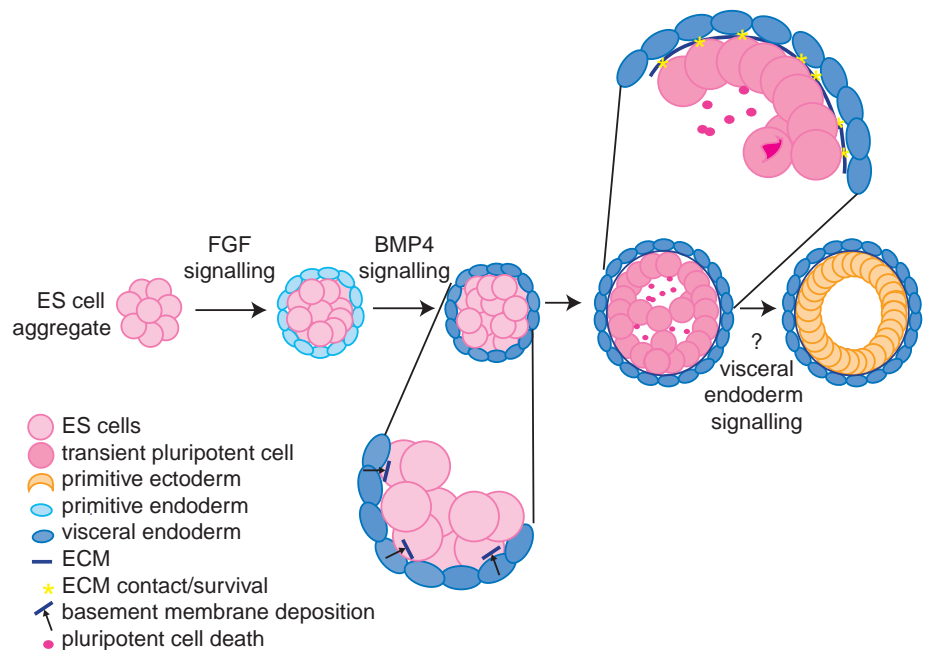
Fig. 4. Proposed mechanism of cavitation in ES cell embryoid bodies. *After aggregation of ES cells, outer cells differentiate to primitive endoderm followed by visceral endoderm, events that require FGF and BMP signalling respectively. While visceral endoderm is shown here as homogeneous, parietal endoderm has also been demonstrated to form during this stage of EB differentiation. With establishment of the extraembryonic endoderm lineage, a basement membrane is deposited between the pluripotent cells and the endoderm. Basement membrane is proposed to induce an alteration in the pluripotent cell state such that pluripotent cells in contact with the basement membrane survive and reorganise to a columnar epithelium of primitive ectoderm, whereas those cells centrally located and separate from the ECM undergo cell death, forming a cavity. It is unclear if the acquisition of gene expression characteristic of primitive ectoderm within the pluripotent cells occurs in response to signalling from the ECM or requires additional signals from the visceral endoderm.*
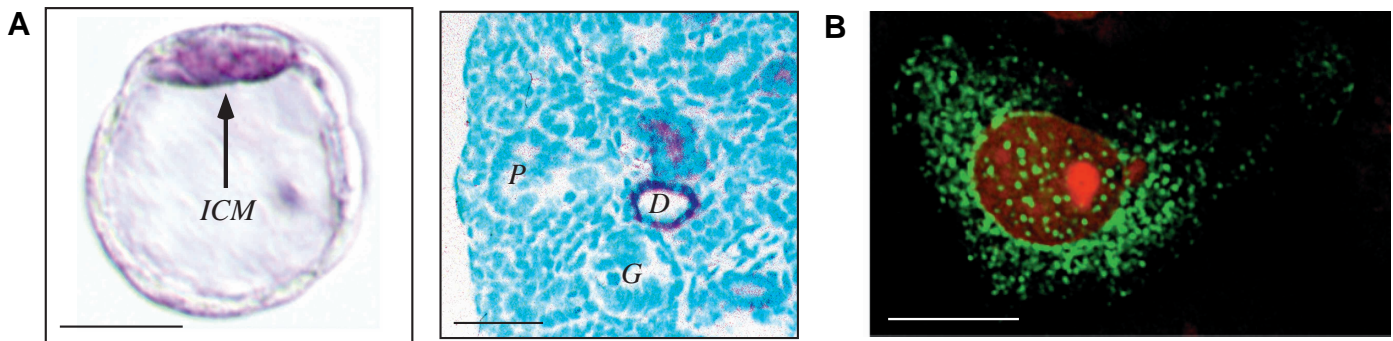
Fig. 5. **Molecular analysis of genes differentially expressed by pluripotent cells. (A)** *Whole mount* in situ *hybridisation of a 4.5 d.p.c. mouse blastocyst (left) and sectioned 16.5 d.p.c. embryonic kidney (right) using a digoxygenin labelled* CRTR-1 *specific anti-sense riboprobe. Scale bars represent 10 μm and 100 μm respectively. ICM, inner cell mass; D, distal convoluted tubule; P, proximal convoluted tubule; G, glomerulous.* **(B)** *Confocal microscopy of a COS-1 cell transiently transfected with EGFP-Psc1. Scale bar indicates 10 μm. The nucleus of the cell is stained with propidium iodide (red). Nuclear speckles and cyto-speckles are visible within the nucleus and cytoplasm respectively.*

differentiation of ES (Kawasaki *et al.*, 2000) or EPL (C. Long, personal communication; Rathjen *et al.,* 2002) cells has been shown to promote surface ectoderm formation at the expense of neurogenesis. This suggests that while the cellular basis of ectodermal specification in the mammalian embryo may differ from equivalent events in lower vertebrates, the molecular regulation of neural induction may be conserved.

During embryogenesis the neural tube acquires a complex pattern of gene expression along the anterior/posterior and dorsal/ventral axes in response to positional information emanating from neighbouring cell populations such as the notochord and visceral endoderm, and signalling centres such as the node and anterior visceral endoderm (AVE) (Echelard *et al.*, 1993; Liem *et al.*, 1997; Thomas and Beddington, 1996). Expression of diverse positionally restricted genes can be detected within ES cell EBs, despite the lack of positional organisation or establishment of anterior/posterior and dorsal/ventral axes, presumably as a result of signalling from the visceral endoderm and potentially from exposure of cells to inappropriate cell populations and signalling molecules generated as a consequence of the chaotic environment. (Rathjen and Rathjen, 2001). In contrast, formation of neurectoderm from EPL cells results in a population of cells which do not express markers characteristic of fore- or hind-brain, trunk neural tube, or dorsal/ventral patterning, but do express markers characteristic of early midbrain (Rathjen *et al.*, 2002). The general lack of positional specification within EBM, which is perhaps not unexpected given the lack of visceral endoderm within these aggregates, suggests that rather than midbrain, EBM comprise a population of naive, or unpatterned, neurectoderm characterised by the expression of pan-specific neural markers and the broadly expressed early midbrain markers. The formation of neurectoderm by EBM differentiation therefore separates neural induction from the manifestation of positional specification, processes which during embryogenesis occur concomitantly and have not been uncoupled experimentally.

Pluripotent Cell Differentiation can be used as a Model of Morphogenesis

Establishment of the primitive ectoderm in both the embryo and EBs occurs concurrently with the formation of a cavity, termed the proamniotic cavity during embryogenesis, in the pluripotent cell mass. The mechanism of cavity formation has been studied *in vitro* using the differentiation of pluripotent cells as EBs, a system more tractable to analysis than the embryo. Although cavitation *in vivo* occurs when the epiblast comprises less than 120 cells (Snow, 1977), many less than are present in an EB at cavitation, the mechanism of cavity formation is suggested to be conserved between these systems (Coucouvanis and Martin, 1995). Cavity formation in EBs is initiated with the formation of multiple foci of cell death which merge to a single, centrally located cavity surrounded by a monolayer of primitive ectoderm cells. EB cavitation has been suggested to result from integrated action of a diffusible 'death' signal secreted by the visceral endoderm and a survival signal associated with the ECM separating the endoderm and primitive ectoderm cell populations (Coucouvanis and Martin, 1995). Consistent with this, disruption of FGF signalling in EBs, by expression of a dominant negative FGF receptor, resulted in both loss of the extraembryonic endodermal lineage and disruption of cavitation (Chen *et al.*, 2000). Further, the expression of *Bmp2* and *Bmp4* by pluripotent cells at the time of visceral endoderm formation and cavity formation was shown to be required for both processes, as disruption of BMP signalling inhibited expression of visceral endoderm markers and prevented cavity formation (Coucouvanis and Martin, 1999).

Although the above experimental approaches demonstrate a requirement for extraembryonic endodermal lineages in EB cavitation, the role of a death signal in this process has been questioned. EBs formed from *LAMC1*$^{-/-}$ ES cells, which fail to establish a basement membrane, are deficient in cavitation despite the formation of extraembryonic endoderm. Cavitation can be restored in these aggregates by addition of ECM components to the differentiation environment (Murray and Edgar, 2000). The failure of cavitation, but not the deficiency in extraembryonic endoderm, in EB formed from ES cells expressing the dominant negative FGF receptor can also be restored by supplementation of the differentiation environment with ECM (Li *et al.*, 2001). Cavitation and formation of primitive ectoderm is observed during the differentiation of ES cells as EBM, despite these aggregates being deficient in the extraembryonic lineage (Rathjen *et al.*, 2002). Within EBM, cavitation proceeds via the formation of a single, centrally located cavity rather than the formation of multiple, discrete foci of death as seen in EB. MEDII is proposed to provide a source of visceral endoderm-like signalling and has been demonstrated to contain

ECM components, such as cellular fibronectin (Bettess, 2001), suggesting that the role of visceral endoderm in cavitation is deposition of a basement membrane.

The ability of ECM components to reconstitute cavitation in pluripotent cells in the absence of extraembryonic endoderm suggests that both pluripotent death and survival are induced by the basement membrane (Fig. 4). The role of ECM deposition may be to alter the pluripotent state such that direct association with ECM components within the basement membrane is required for cell survival. It is unclear if differentiation of the surviving cells to primitive ectoderm is also induced by the basement membrane as establishment of the identity of the pluripotent cells at the time of the formation of a columnar epithelial of cells has only been established in EB and EBM, in which additional visceral endoderm-like signalling may be present.

Functional Analysis of Genes Differentially Expressed during Pluripotent Cell Differentiation

ES cells provide a valid model system for investigation of the properties associated with pluripotent cells. *In vitro* manipulation of ES cells has been used to investigate the molecular basis of pluripotence (Niwa *et al.*, 2000), unusual regulation of cell cycle progression (Savatier *et al.*, 1996), telomere maintenance (Armstrong *et al.*, 2000), the cellular basis of nuclear reprogramming (Tada *et al.*, 2001) and signalling pathways associated with loss of pluripotence (Burdon *et al.*, 1999). The close relationship between EPL cells and embryonic primitive ectoderm suggests that ES cell differentiation can also be used to analyse the developmental progression of embryonic cells, with the function of differentially expressed pluripotent cell specific genes providing novel insight into the molecular and cellular basis of alterations in cell biology during differentiation.

CRTR-1 is a Developmentally Regulated Transcriptional Repressor

The *CRTR-1* cDNA encodes a 481 amino acid open reading frame homologous with the CP2 family of transcription factors (Rodda *et al.*, 2001). Conservation between CRTR-1 and other mammalian family members is extensive and includes regions implicated in DNA binding and protein oligomerisation but not transcriptional activation.

Members of the *CP2* family are generally expressed ubiquitously (Jane *et al.*, 1995). By contrast, *CRTR-1* is expressed specifically in the pluripotent cells of the 3.5-4.75 d.p.c. mouse embryo and at high levels in the epithelial monolayer lining the embryonic and adult kidney distal convoluted tubules (Fig. 5A). While both pluripotent cells and the DCT lining are epithelial in origin, *CRTR-1* expression is not a general property of such cells as other epithelial cells including the lining of kidney proximal convoluted tubules do not express detectable *CRTR-1*. Both sites of *CRTR-1* expression *in vivo* are associated with cavitation (Horster *et al.*, 1997) suggesting a role for the protein in precursor cell survival or apoptosis (Coucouvanis and Martin, 1995). Additional sites of lower level expression have been identified by ribonuclease protection and include embryonic intestine, limb, lung, skin and adult placenta, testis, stomach and small intestine. These have not been resolved at the cellular level.

Members of the CP2 family bind a consensus DNA sequence consisting of a direct bipartite repeat, CNRG-N$_6$-CNRG, and activate transcription (Lim *et al.*, 1993). CRTR-1 is the first member of the family shown to repress transcription when bound to a heterologous promoter in a variety of cell lines including kidney (293T and COS-1 cells) and ES cells. The transcriptional repression activity was found to be localised to the N-terminal 52 amino acids which are both necessary and sufficient for CRTR-1-mediated transcriptional repression (Rodda *et al.*, 2001).

CRTR-1 has also been demonstrated to interact with other family members including CP2 and LBP-1a by yeast two hybrid and GST-pull down analysis (S. Rodda and P. Rathjen, unpublished data). Inter-family interactions between CP2 family members such as CP2 and LBP-1a (Yoon *et al.*, 1994) suggest the formation of multiprotein complexes which regulate gene expression. The properties of CRTR-1 are therefore consistent with a role as a developmentally regulated, dominant repressor of target genes activated by CP2 family members, associated with embryological events such as differentiation and cavitation.

Psc1 is a Developmentally Regulated SR Protein with a Novel Role in RNA Metabolism

The 1,005 amino acid open reading frame of *Psc1* contains an RNA binding domain and an arginine, serine rich region of 25 amino acids including four RS dipeptide repeats, identifying it as a member of the SR-like family of proteins. *Psc1* is expressed temporally throughout the pluripotent cell pool within the early embryo and at elevated levels in embryonic brain and adult lung and placenta (Schulz, 1996).

SR proteins contribute to spliceosome formation and have been implicated in both constitutive splicing and alternate splice site selection (for review see Caceres *et al.*, 1998). Consistent with this, SR proteins localise to 20 to 50 discrete regions within the nucleus termed nuclear speckles, which contain snRNPs, non snRNP's and other splicing factors (Mintz and Spector, 2000). The SR protein SC35 (Fu and Maniatis, 1990) colocalises with GFP-Psc1 to nuclear speckles in transiently transfected Cos-1 cells. In addition, GFP-Psc1 localises to a large number of discrete, punctate regions throughout the cytoplasm, termed cytospeckles, which do not contain SC35 (Fig. 5B). Although nuclear-cytoplasmic shuttling has been reported for SR proteins such as SF2/ASF, SRp20 and 9G8 (Caceres *et al.*, 1998) and U2AF (Gama-Carvalho *et al.*, 2001), these proteins do not show punctate cytoplasmic localisation of the kind associated with cytospeckles. Psc1 therefore differs from other SR proteins with respect to its developmental regulation and regionalised cytoplasmic distribution.

Psc1 containing cytospeckles are reminiscent of RNA granules which traffic via the cytoskeleton (Bassell *et al.*, 1999), colocalise with microtubules and contain translational machinery such as ribosomal sub units and elongation factors (Hazelrigg, 1998). Consistent with this, real time analysis of GFP-Psc1 transfected cells shows that Psc1 containing cytospeckles are motile within the cytoplasm. In contrast to RNA granules however, cytospeckles undergo nuclear import although nuclear export has not been observed (Kavanagh, 1998).

The presence of both RNA and protein interaction motifs in the Psc1 sequence suggests that Psc1-containing speckles in the nucleus and the cytoplasm are likely to be RNA-protein complexes.

Developmental regulation of *Psc1* expression suggests that regionalised cytoplasmic localisation and/or nuclear transport of specific RNA and/or protein cargo's is likely to be of developmental significance. While the cellular relevance of cytospeckles is unknown, cytoplasmic localisation of RNA might allow transcripts to be targeted for site specific localisation, degradation or translation, which could be used to establish asymmetry within the cell. Similar localisation of the *D. melanogaster* neuroblast transcript, *prospero*, (Li *et al.*, 1997) and the *S. cerevisaie* transcript *ASH1* (Long *et al.*, 1997) provide a mechanism for differentiation in the developing embryo.

PRCE functions as a Developmentally Regulated Mammalian Separin

The *PRCE* cDNA encodes a 2118 amino acid open reading frame (Pelton, 2000) that shares C terminal homology with the separin domain (Leismann *et al.*, 2000) of three fungal separin proteins, ESP1 from *S. cerevisiae* (Baum *et al.*, 1988; McGrew *et al.*, 1992), cut1 from *S. pombe* (Uzawa *et al.*, 1990), and bimB from *A. nidulans* (May *et al.*, 1992). These proteins are members of the CD group of cysteine proteases, a class that also includes caspases (Uhlmann *et al.*, 2000), which mediate chromosome segregation at the onset of anaphase through cleavage of the cohesin subunit complex.

Expression of *PRCE* is developmentally regulated. It is expressed specifically in the pluripotent cells of the mouse embryo from 4.75 to 5.25 d.p.c. and later in mouse adult tissues including bone marrow, testis and intestine (Pelton, 2000; Pelton *et al.,* 2002). These tissues contain stem cell and/or progenitor cell pools and are associated with rapid cell proliferation. Biochemical properties of the PRCE protein are consistent with the proposed function in cell division. In fungi, separin activity is confined to anaphase in two ways: via sequestration of the protein in the cytoplasm before mitosis and by the formation of tight inhibitory complexes with securin proteins. Cohesin cleavage at the onset of anaphase is achieved by activation of separin proteases following APC mediated ubiquitination and degradation of the securins. Consistent with this, GFP-PRCE sub-cellular localisation is cytoplasmic within the majority of transfected cells, but also observed at the centrosome, the site of microtubule nucleation required for mitotic spindle formation. Furthermore, PRCE interacts with and colocalises the mouse securin PTTG, and over expression of PRCE inhibits cell cycle progression in somatic cells (T. Pelton and P. Rathjen, unpublished data). PRCE therefore appears to be a novel developmentally regulated mammalian separin.

Manipulation of separin/securin function can lead to aneuploidy (Jallepalli *et al.*, 2001). In this regard, it is interesting that PRCE is expressed in pluripotent cells which exhibit unusual genomic stability *in vitro* (Pera *et al.,* 2000). Further, regulated expression of a separin protease in rapidly dividing cells suggests that progression through an anaphase checkpoint in the cell cycle can be used to accelerate cell division in a developmental context. Differential expression of cell cycle regulators may therefore provide opportunities to alter the properties of embryonic cell populations such as pluripotent cells which have an unusual cell cycle structure (Savatier *et al.*, 1996).

Summary

Understanding the events of early mammalian embryogenesis at the molecular and cellular level is hampered by difficulties associated with experimental inaccessibility of the embryo. Furthermore, unlike many developmental processes which can be investigated using analogy with the development of more tractable experimental models, early mammalian embryogenesis involves the elaboration and differentiation of a unique population of pluripotent cells which are not present in embryos of lower vertebrates. *In vitro*, model systems for early development exploiting pluripotent cells in culture have been available for more than 15 years, but until recently difficulties in controlling the differentiation of these cells has limited their application to the questions of mammalian development. Utilising conditioned medium that appears to recapitulate the signalling activities of extraembryonic endoderm in culture, we have demonstrated the homogeneous differentiation of ES cells to a second, pluripotent cell population, EPL cells, which show equivalence to embryonic primitive ectoderm. EPL cell formation *in vitro* provides a model system for characterisation of pluripotent cells, which has led to the identification of discrete transient cell states formed during establishment of primitive ectoderm *in vivo*. Further, the availability of a homogeneous population of primitive ectoderm, the immediate precursor population of the three primary germ layers, has allowed the development of model systems for directed differentiation and cell fate specification during gastrulation. Analysis of EPL cell differentiation has facilitated the uncoupling of events that occur concomitantly during embryogenesis, such as induction and patterning of the neural lineage, and dissection of the signalling environments that control pluripotent cell differentiation, allowing the relative roles of soluble signalling molecules, ECM associated activities and cellular environment to be assessed. The ability to generate essentially homogeneous populations of cells representing differentiation intermediates provides a unique opportunity to study both the developmental potential and gene expression profile of cells that exist only transiently in the embryo.

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