

Review Article

The States of Pluripotency: Pluripotent Lineage Development in the Embryo and in the Dish

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The pluripotent cell lineage of the embryo comprises a series of temporally and functionally distinct intermediary cell states, the epiblast precursor cell of the newly formed blastocyst, the epiblast population of the inner cell mass, and the early and late epiblast of the postimplantation embryo, referred to here as early and late primitive ectoderm. Pluripotent cell populations representative of the embryonic populations can be formed in culture. Although multiple pluripotent cell states are now recognised, little is known about the signals and pathways that progress cells from the epiblast precursor cell to the late primitive ectoderm in the embryo or in culture. The characterisation of cell states is most advanced in mouse where conditions for culturing distinct pluripotent cell states are well established and embryonic material is accessible. This review will focus on the pluripotent cell states present during embryonic development in the mouse and what is known of the mechanisms that regulate the progression of the lineage from the epiblast precursor cell and the ground state of pluripotency to the late primitive ectoderm present immediately prior to cell differentiation.

1. Introduction

Establishment and development of the pluripotent cell lineage in the mouse embryo are a progressive process characterised by the sequential formation of a series of temporally and functionally distinct intermediary cell states. Cells fated to form the pluripotent lineage can be identified in the interior of the compacted morula of the mouse embryo by day 3, encapsulated within cells destined to establish the trophoctoderm. These cells establish the inner cell mass (ICM) of the blastocyst, which on day 3.5 comprises genetically discrete populations of epiblast precursor cells and primitive endoderm precursor cells. By 4.5 *days post coitum* (d.p.c.) these populations have segregated into spatially distinct regions of the ICM and cell identity has been fixed, forming the pluripotent epiblast and the extraembryonic primitive endoderm lineage (also known as the hypoblast). The epiblast proliferates rapidly and forms a pseudostratified epithelium of epiblast that has been designated primitive ectoderm. Primitive ectoderm can be distinguished from the preceding epiblast by morphology, gene expression, and differentiation potential. Analysis of the mouse embryo has revealed at least

four identifiable pluripotent cell populations, or states, that comprise the pluripotent lineage—the epiblast precursor cell, the epiblast of the ICM, and the early and late epiblasts of the postimplantation embryo, referred to here as early and late primitive ectoderm.

The understanding and characterisation of cell states in culture are most advanced in mouse where conditions for forming and maintaining distinct pluripotent cell states in culture are well established. Pluripotent cell lines have been isolated from the ICM, the later primitive ectoderm of mouse and the migrating germ lineage, mouse embryonic stem (ES) cells, epiblast stem cells (EpiSC), and embryonic germ (EG) cells, respectively. Inhibition of mitogen-activated protein kinases (ERK1/2) and wingless-related MMTV protein (Wnt) signalling can be used to derive ES cells in a naïve state of pluripotency from existing ES cell lines and from the blastocyst. Pluripotent cells in the naïve state, also known as ground state, are intrinsically self-maintaining if protected from inductive differentiation stimuli. Comparison of ground state ES cells with other pluripotent cell populations *in vitro* and the pluripotent lineage *in vivo* suggests that these cells are analogous to the newly formed pluripotent lineage in

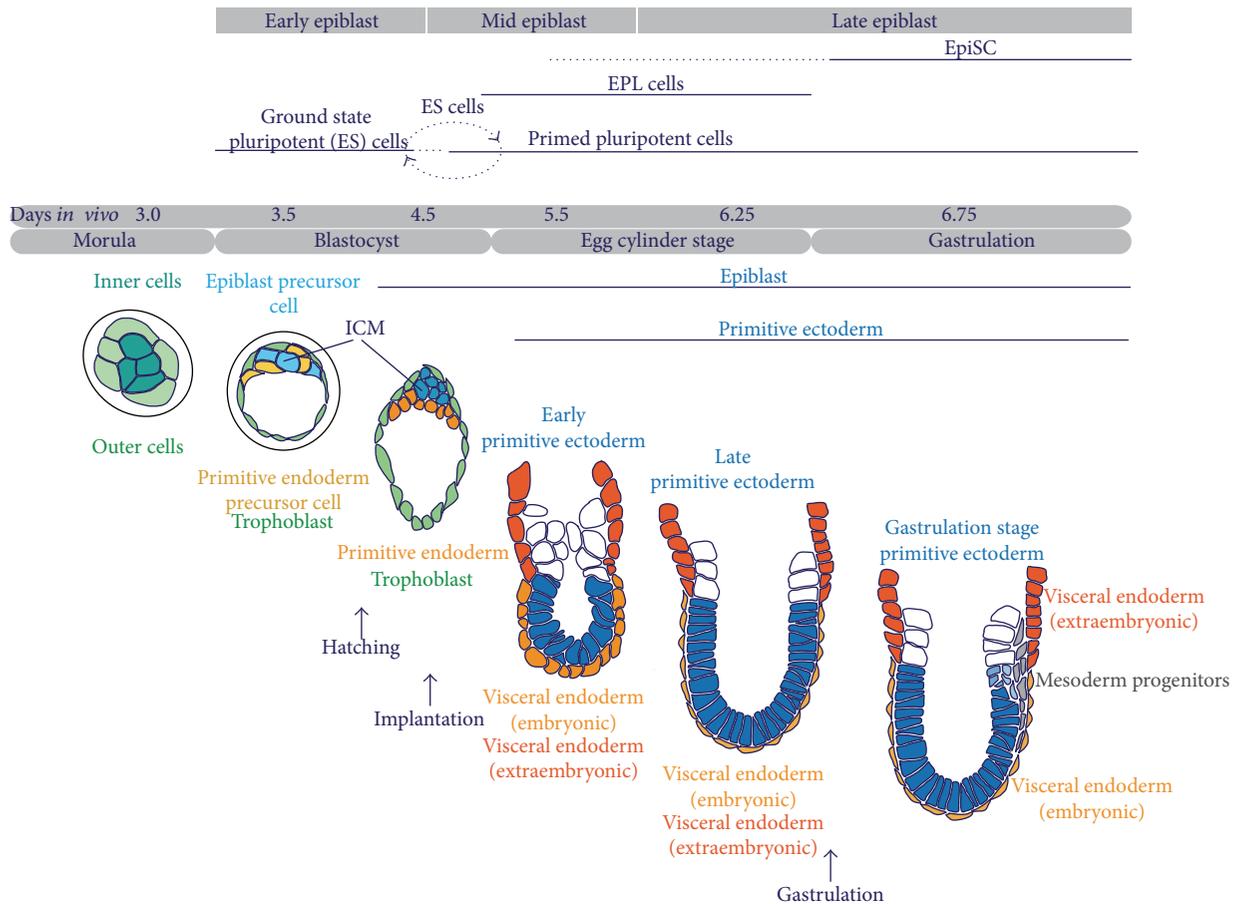


FIGURE 1: Aligning pluripotent cells in culture with the pluripotent lineage in the embryo. *Lower panel.* Embryogenesis in the mouse is shown from the 16-cell embryo, when the first distinction between inner and outer cells is seen, to the 6.75-day embryo, after pluripotent cell differentiation has commenced at gastrulation. Trophectoderm is not shown in either egg cylinder stage embryo representations. The pluripotent lineage can be followed in blue. *Upper panel.* The proposed alignment of cell states captured *in vitro* with developmental stages of the embryo. EPL cells represent the developmental continuum achieved within aggregates without passage. Differentiation in EPL cells is not progressive and it is not anticipated that populations representative of gastrulation stage primitive ectoderm will be formed. EpiSC have been derived from blastocysts to late gastrulation stage embryos (8.25-day embryos, not shown). Characterisation of EpiSC has shown the majority of these lines to be late epiblast, aligned with a population present in the late gastrula, although there is some evidence that lines derived from ES cells in culture can adopt a mid-epiblast identity, denoted with the dashed line. Cell states have been aligned with a proposed nomenclature that can be used to define pluripotent cell states functionally.

the embryo and differ from conventionally isolated ES cell lines. These later cells have been termed “primed” pluripotent cells. ES cells can be induced to form cells representative of the primitive ectoderm, either by culturing in medium containing L-proline, to form early primitive ectoderm-like (EPL) cells, or in medium supplemented with FGF2 and activin A, to form culture-derived EpiSC. EPL cells and EpiSC have characteristics that distinguish them from ground state ES cells, primed ES cells, and the ICM and that highlight similarity with the primitive ectoderm of the embryo. Although multiple pluripotent cell states are now recognised, little is known about the signals and pathways that progress cells from ground state pluripotency to late primitive ectoderm in the embryo or in culture.

This paper will focus on cell states that arise as the pluripotent lineage develops in the mouse embryo and those that can be captured in culture and review the literature on

the mechanisms regulating pluripotent cell state formation and maintenance. A reference diagram showing the stages of embryogenesis referred to throughout this review and defining the different pluripotent cell populations in culture and the terminology used can be found in Figure 1.

2. Inner versus Outer Cells: Differentiation in the 32-Cell Stage Embryo

2.1. Forming Trophectoderm. The eight cells, or blastomeres, that arise from the first three cleavage events of mouse embryogenesis are considered to be totipotent and unspecified, showing no differences in developmental potency and gene expression and being equally able to contribute to ICM and trophectoderm [1–4]. Immediately after the 3rd cleavage event, these cells form a cluster of loosely attached cells termed the morula (from the Latin for mulberry, *Morus*,

a lovely metaphorical extension), with each cell spherical and lacking intercellular junctions with neighbouring blastomeres. Within hours of cleavage the morula compacts [5], marked by the cells flattening against each other and obscuring intercellular boundaries (shown beautifully in [6]). With compaction comes polarisation of the blastomeres, each of which develops an apical surface exposed to the environment and distinguished by localisation of surface microvilli [6, 7], a localised concentration of actin-containing microfilaments, [8] and increased ligand binding capacity [9]. These changes are accompanied by an intracellular rearrangement of the microfilaments and basal localisation of mitochondria [6]. At this stage the cells establish gap junctions [10, 11]. Compaction is mediated by E-cadherin and associated proteins [12–17]. A detailed description of the mechanisms driving compaction is tangential to this review but can be found in Chen et al. [18].

The first differentiation event in the embryo is the formation of trophoblast on the outside of the embryo as a population distinct from the inner cells (the forerunner of the inner cell mass (ICM)). Tracing the origins of trophoblast and ICM has shown that all blastomeres of the 2-cell embryo and between 80 and 90% of blastomeres of 4-cell stage embryos contribute to both lineages, suggesting that there is little or no developmental instruction established early in embryogenesis that underlies the determination of ICM and trophoblast [19]. Polarisation of the blastomeres at the 8-cell stage has long been thought to underpin the establishment of the first two embryonic lineages. The cell polarity model dictates that asymmetric, or differentiative, division of a blastomere in the 4th cleavage division (from 8 cells to 16 cells) will yield two cells that can be discriminated on differential inheritance of apical or basal characteristics [20]. Nonpolarised, inner cells, from the basal portion of the blastomere, are encapsulated by a layer of outer, polarised cells formed from the complementary apical portions; these populations establish the ICM and trophoblast, respectively.

Simplistically, differentiative cleavage at the 4th cleavage division establishes the lineages. Lineage, allocation, however, appears to be a more progressive process. The phenotypic divergence of inner and outer cell populations at the 4th cleavage division is not accompanied by developmental restriction, with blastomeres of the 16-cell embryo maintaining totipotency. Outer cells of the 16-cell embryo can contribute to the ICM [21, 22]; transplantation studies have shown that blastomeres at the 16-cell stage can contribute to both inner and outer lineages [23] and aggregates of 16 isolated outer or inner blastomeres from 16-cell embryos can develop into normal, fertile mice after transfer into pseudo-pregnant recipients [24]. Contribution from the 5th cleavage division is thought to be required as not all 4th cleavage events are differentiative. Experiments enumerating cleavages at the 4th cleavage division suggest that 30% or 60% of 4th cleavage events are differentiative, [22, 25, 26]. It has been suggested that experimentally these estimations are impacted by the initial location of many cells destined for the inside of the embryo on or near the surface of the embryo [26]. Detailed morphological examination of 16-cell stage embryos suggests

that in approximately 70% of embryos all blastomeres have some contact with the external environment, albeit with only a small proportion of their surface for some cells [27].

On average, the ratio of outer cells to inner cells in a 16-cell embryo is approximately 10:6 [21, 24], with a range of 2 to 7 inner cells [21]. Increases in inner cell number are achieved by differentiative 5th cleavage events, with a higher occurrence of differentiative cleavage in 16-cell embryos with fewer inner cells [21, 22]. Later forming inner cells are preferentially located to regions of the ICM adjacent to the trophoblast [21]. These data have led to a model of inner cell allocation with two phases: qualitative differentiation between the lineages during the 4th cleavage event and quantitative regulation of lineage size during the 5th cleavage event [21]. Totipotency is lost and lineage identity is fixed by the 6th cleavage division, [22, 24, 28], although in a minority of 6th cleavage stage embryos a small number of differentiative cleavages have been detected [25].

Several genes have been implicated in the divergence of the ICM and trophoblast. In the TEAD/TEF transcription factor, TEAD4 is essential for establishing trophoblast-specific gene expression in outer cells and functional trophoblast stem cells and trophoblast. *Tea4*^{-/-} embryos fail to form a blastocyst and are unable to implant into the uterine wall [29, 30]. TEAD4 is present in all cells of the preimplantation embryo from the 4-cell stage but becomes differentially activated in inner and outer cells [31]. *Tea4* activation is regulated by Hippo signalling [31], and how differential Hippo activation is regulated in the embryo is not known. It has been proposed that the high degree of interaction between inner cells activates Hippo signalling, leading to phosphorylation of the TEAD4 coactivating protein Yap by Lats; phosphorylated Yap is excluded from the nucleus resulting in inactive TEAD4. In this model outer cells, with an exposed surface, would be less likely to activate Hippo signalling, resulting in translocation of Yap to the nucleus, activation of TEAD4, and initiation of trophoblast-specific gene expression, including *CDX2*, *GATA3*, *ELF5*, and *EOMES*. Analysis of lineage commitment in single blastocysts, however, suggests it is unlikely that cell:cell contact is the only factor regulating Hippo activation and propose that the polarisation of outer cells may inhibit Hippo signalling [32]. It is not yet clear if the activation of *Cdx2* and trophoblast-specific gene expression is through direct transcriptional activation by TEAD4 or TEAD4-mediated release of negative regulation of *Cdx2* [31]. Although the process of lineage determination in outer cells commences after the 4th cleavage event, evidenced by the preferential localisation of Yap in the nucleus of outer cells, the maintenance of totipotency in outer blastomeres of the 16-cell embryo suggests that these processes are fully reversible and that commitment of outer cells to a trophoblast fate is not initiated until after the 5th cleavage event. This is supported by transcriptional analysis of single cells which, despite revealing differences in expression of individual genes between blastomeres, was unable to separate two lineages at the 16-cell stage; resolution of trophoblast from the inner cell lineages was achieved at the 32-cell stage after the 5th cleavage event [4].

2.2. *Establishing the Inner Cell Mass.* Maintenance of the inner cell phenotype, and establishment of the pluripotent cell lineage from these cells, is dependent on a triad of pluripotency regulators: Oct4, NANOG, and SOX2. Details of the activity of these proteins in pluripotent cells can be found in a review by Chambers and Tomlinson [33]. Oct4 is found in all cells of the embryo, regardless of position, from conception until early blastocyst stage (64 cells). After this time Oct4 is gradually lost in the trophectoderm but maintained in the ICM [27]. Oct4 transcripts were detected in all cells to the 32-cell stage after which they were preferentially found in the ICM [4], suggesting genetic regulation of the locus underpins differential protein localisation in the embryo. *Oct4*^{-/-} embryos form blastocysts but the inner cells do not establish a pluripotent lineage and commit to trophectoderm [34]. Nanog is detected in all cells of the early blastocyst, after which it becomes progressively restricted to a subset of ICM cells which are distributed in a salt and pepper pattern throughout the cell mass [27]. As with *Oct4*, the transcription of *Nanog* is maintained at high levels in all cells until the 32-cell stage, and loss in trophectoderm is only detected at the 64-cell stage [4]. *Nanog*^{-/-} embryos also fail to establish a pluripotent lineage but inner cells in these embryos differentiate to the primitive endoderm lineage [35, 36]. Sox2 is detected in the blastomeres of developing embryos and in the cells of the ICM [37]. Sox2 can also be detected in the trophectoderm but in these cells the protein is found in the cytoplasm rather than the nucleus [37]. In contrast to *Oct4* and *Nanog* expressions, *Sox2* is not expressed in the early cleavage embryo [4] or in the trophectoderm of the blastocyst [37], suggesting that Sox2 in the early embryo is derived from a long-lived, maternally derived protein or transcript pool and not the product of zygotic transcription [37, 38]. *Sox2* expression increases between the 16-cell and 32-cell stages in the inner cells of the embryo [4]. *Sox2*^{-/-} embryos formed blastocysts but failed to elaborate the pluripotent lineage [37]. Determining roles for Sox2 in *Sox2*^{-/-} embryos is confounded by the presence of maternal protein/transcripts. Reducing *Sox2* transcript levels from the 2-cell stage using miRNA technology revealed an early role for Sox2 in trophectoderm formation [38].

Determination of the inner from the outer cells between the 16- and 32-cell stages has been proposed to result from differential expression of the trophectoderm determinant Cdx2. Although found in all blastomeres of the 16-embryo [4, 27, 39], bias in Cdx2 expression levels, and higher expression in outer cells, has been reported [27]. Cdx2 acts as a negative regulator of the activity of the core pluripotency transcription factors [40–43], providing a mechanism for promoting pluripotency in *Cdx2* low cells, and therefore preferentially in inner cells. In contrast, *Cdx2* high cells that are more likely to be outer cells are more likely to differentiate to trophectoderm. Cdx2 appears to exert control by interfering with the ability of the Oct4/Sox2/Nanog transcriptional complexes to activate the transcription of downstream targets, a class of genes required for pluripotency and that includes their own loci [43]. By the 32-cell stage Cdx2 transcript and protein is largely restricted to outer cells, relieving all inhibition of the

Oct4/Sox2/Nanog transcriptional complex in ICM. Although this mechanism likely explains the establishment of alternate transcriptional networks in inner and outer cells, questions still remain. Most notably, how is the transcription of *Oct4*, *Nanog* and *Cdx2* maintained in all blastomeres of the embryo prior to lineage segregation given the actions of Cdx2 on pluripotent gene expression?

3. Dividing the Inner Cell Mass: Forming Epiblast and Primitive Endoderm in the 64-Cell Stage Embryo

By transcriptional analysis, the inner cells of the 32-cell stage embryo represent a single cell population; by the 64-cell stage this population has diverged into two genetically discrete populations that are fated to form the pluripotent lineage, or epiblast, and the primitive endoderm [4]. Between the 32- and 64-cell stages, the embryo becomes a blastocyst, characterised by appearance of the blastocoelic cavity and the positioning of the inner cells, the ICM, to one pole of the embryo subjacent to the polar trophectoderm. As a consequence, cells within the ICM are differentially exposed to the blastocoelic cavity. For some time it was thought that a position adjacent to the cavity induced the differentiation of cells into the primitive endoderm lineage. This hypothesis, however, is not supported by recent analysis of the ICM population.

The expression of a number of genes/proteins in the ICM of the 64-cell embryo, including Nanog, GATA4, GATA6, SOX17, PDGFR α , FGF4 and FGFR2, acquires a salt and pepper distribution [4, 44–46]. Cells expressing Gata4, Gata6, Sox17, Pdgfra and Fgfr2 establish the primitive endoderm; although originally distributed throughout the ICM, these cells, termed here primitive endoderm precursor cells, coalesce into an epithelium at the blastocoel interface as a result of migration and cell sorting [25]. Any cells of a primitive endoderm identity that remain within the ICM are proposed to either acquire an epiblast precursor identity or die by apoptosis [25, 45]. Differential expression of Ephrins and the Slit receptor Robo2 between *in vitro* equivalents of the epiblast and primitive endoderm, respectively, may indicate a role for these pathways in cell segregation [47]. Changes in ICM morphology and a failure of epiblast and primitive endoderm segregation in the blastocysts exposed to inhibitors of Rho-associated kinase (ROCK) suggest a functional requirement for these kinases in lineage assortment [48]. Nanog-expressing cells, the epiblast precursor cells, become restricted by this process to the space between the polar trophectoderm and primitive endoderm and committed to the pluripotent lineage, shown functionally by the inability of these cells to generate primitive endoderm [49, 50]. The formation and coalescence of these cells mark the beginning of the pluripotent lineage. Pluripotentiality is the ability of a cell to act as the founder, or stem, cell for all the tissue found within the embryo and adult and many cell populations that are formed to support embryonic development. These cells differ from the blastomeres of the earlier embryo in that alone they cannot sustain full organismal

development. Pluripotent cells, through a combination self-renewal and differentiation capacity, undergo a program of development with the sequential formation of increasingly more specialised progenitors that eventuates in the formation of all the cells in the embryo and adult.

Determination of epiblast precursor cells and primitive endoderm precursor cells occurs in response to differential FGF signalling and receptor tyrosine kinase activation [26, 51]. Embryos without *Fgf4*, *Fgfr2*, or *GRB2* fail to form primitive endoderm suggesting that establishment of this tissue requires Fgf signalling [44, 52–54]. In embryos cultured in inhibitors of FGF/MAP kinase signalling cells of the ICM preferentially express Nanog and primitive endoderm precursor cells cannot be detected [4, 26, 50]. Conversely, ICM cells in embryos cultured in *Fgf4* preferentially form primitive endoderm precursor cells at the expense of epiblast precursor cells; this effect was dose dependent and seen at higher concentrations of *Fgf4* (>250 ng/mL) [26]. A model of lineage divergence dependent on differential expression of *Fgf4* and *Fgfr2* and differential activation of GRB2/MAP kinase signalling has been developed. *Fgf4*, which is transcriptionally activated by Oct4, Sox2, and Nanog [51, 55], is expressed throughout the morula but becomes restricted to epiblast precursor cells in the ICM [4, 51]. *Fgf4* from the epiblast precursor cells signals to primitive endoderm precursor cells through *Fgfr2* and increases *Gata6* expression via a GRB2/MAP kinase dependent mechanism [44, 51]. *Gata6* in the primitive endoderm precursor inhibits the expression of Nanog and Nanog, in the epiblast precursor cells, inhibits *Gata6* expression. Fgf signalling thereby provides a mechanism to establish and maintain two cell identities within the ICM.

The challenge remains to elucidate the processes that establish differential signalling within the ICM between the 32- and 64-cell stages. Inner cells that are recruited at the 4th cleavage division have been shown to be more likely to give rise to epiblast precursor cells [25]. Inner cells recruited in the 5th and 6th cleavages, by contrast, are biased towards the formation of primitive endoderm [25]. It has been speculated that inner cells recruited at the 4th cleavage division upregulate Sox2 which, in complex with Oct4, reinforces the expression of *Fgf4* and suppresses *Fgfr2* [4]. Cells recruited later are the daughters of outer cells that have commenced the processes of commitment to the trophoderm lineage and express *Fgfr2*. These cells respond to the *Fgf4* in the inner region of the embryo, downregulate the pluripotency regulators, and initiate expression of *Gata6* [4]. Neither early nor late arising inner cells have a fixed potential as primitive endoderm can be formed from early arising cells and epiblast precursors can be formed from late arising cells [25]. Although this model provides a mechanism for the generation of the two populations within the ICM, others have not been able to demonstrate the different potentials of early and late arising inner cells and favour a stochastic model of cell determination [26]. Treatment of the embryo with *Fgf4* has shown that all inner cells can be induced to form primitive endoderm [26]. This suggests a situation in which the level of *Fgf4* signalling in the embryo is balanced to induce a proportion of inner cells to form

primitive endoderm. Signal levels, coupled with intrinsic and stochastic variation between individual cells, will control the proportion of cells that upregulate *Gata6* expression but not their position within the ICM, generating the salt and pepper pattern of cell distribution seen.

Using the early embryo to understand how differentiation works, and in particular how the first lineages are established, has revealed little about inductive cues that function to establish the epiblast. Potentially, epiblast arises as a default state comprising those cells that are not determined as trophoderm or primitive endoderm. Alternatively, active signals are present in embryos that specify inner cells and which maintain the identity of the epiblast precursor during primitive endoderm formation.

4. Elaboration of the Pluripotent Lineage: From Early Epiblast to Late Primitive Ectoderm

At the time that the pluripotent cell lineage is established, the ICM comprises approximately eleven cells in the primitive endoderm epithelium and eight cells in the epiblast [25]. Epiblast begins to proliferate rapidly and the consequent excrescence fills the blastocoel. Measurement of pluripotent cell proliferation times in 5.5 d.p.c. and 6 d.p.c. embryos suggests cell cycle times of 11.5 and 9 hours, respectively, an extraordinarily rapid cell cycle for a mammalian cell [56–58]. This rapidity of cell cycle is achieved through adoption of an atypical cell cycle structure with over 50% of the cells at any one time being in S-phase and characterised by a short G1-phase and changes in the expression of key cell cycle regulators including cyclin A, cyclin E, and CDK2 [58]. Epiblast expands from 8 cells in the ICM to over 4000 cells in the primitive ectoderm in less than 3 days. Soon after the onset of proliferation cells in the centre of the pluripotent mass apoptose and surviving cells reorganise to form a pseudo-stratified epithelium of cells separated from the overlying primitive endoderm by a basement membrane [59]. Expansion and reorganisation of the pluripotent cells in mouse occurs around 5.0 d.p.c., concurrent with implantation of the embryo into the uterine wall. The epiblast of the postimplantation embryo has been termed primitive ectoderm. In the mouse primitive ectoderm, with the primitive endoderm derivative visceral endoderm, forms a bilaminar cup-like or cylindrical structure; embryos containing primitive ectoderm are referred to as egg cylinder stage.

The cells of the primitive ectoderm share with the epiblast of the ICM the quality of pluripotentiality yet can be distinguished from these cells in many ways. Developmentally, the potential of the primitive ectoderm is reduced compared to earlier cells with a progressive loss in the ability to contribute to the primitive endoderm lineage [49, 60]. Furthermore, primitive ectoderm, unlike epiblast of the ICM, is unable to contribute to the development of chimaeric animals after introduction into a host blastocyst [61–64]. The transcriptome of the primitive ectoderm is different, most notably in the loss of a number of ICM specific genes, such as *Zfp42* (*Rex1*) and *Tfcp2l1* [65], and, notably, downregulation

of *Nanog*, a key regulator of pluripotent cells in the ICM [66, 67]. Conversely, the expression of a number of genes, including *Fgf5* [68], increases with establishment of primitive ectoderm. The transcriptional regulation of *Oct4* has been shown to differ between the two populations [69], which, coupled with the downregulation of *Nanog*, suggests that the mechanisms underlying pluripotentiality change as cells progress from the ICM to the primitive ectoderm. Finally, these populations differ epigenetically; in female embryos the early epiblast and primitive ectoderms differ in the random inactivation of the X chromosome in the later but not the former [70–74] and a comparison of pre- and postimplantation pluripotent cells shows differences in DNA-methylation patterns [75].

The primitive ectoderm persists until 6.5 d.p.c., after which the cell population progressively loses pluripotency and differentiates to form one of the embryonic germ lineages, ectoderm, mesoderm, and endoderm. This developmental event, known as gastrulation, has been likened by John Gurdon to birth, death, and taxes, an obligatory process without which development does not progress and life cannot exist. Gastrulation initiates with the formation of the primitive streak a region at the prospective posterior embryonic-extraembryonic boundary that is characterised morphologically by localised breakdown of the basal lamina [76]. Prior to gastrulation Wnt signalling becomes restricted to the prospective site of the primitive streak and embryos lacking Wnt signalling fail to establish the primitive streak and gastrulate (reviewed in [77, 78]). As embryogenesis proceeds, the streak extends anteriorly along the posterior midline to the distal tip of the embryo. Differentiation of cells in the primitive streak occurs as a consequence of cells undergoing an epithelial to mesenchymal transition. Without contact with a basal lamina, cells can ingress and join the layer of emerging mesoderm. During ingression cells maintain the integrity of the epiblast epithelium [76]. Once within the streak, cells lose E-cadherin in response to FGF signals and become migratory, completing the epithelial to mesenchymal transition and forming mesoderm progenitors [76, 79]. Alternatively, cells within the primitive streak can intercalate with the visceral endoderm, establishing the definitive endoderm lineage [80]. It is not known if cells destined to become endoderm complete an EMT and subsequently undergo reepithelialisation or maintain epithelial characteristics throughout differentiation. A proportion of primitive ectoderm maintains contact with the remaining basal lamina; these cells lose pluripotency to form the progenitor populations of the ectodermal lineages. Pluripotency is eventually lost from the embryo proper with the onset of somitogenesis, as shown by an inability of embryonic explants to form teratomas or establish EpiSC lines [81, 82]. The loss of functional pluripotency coincided with chromatin modifications at the *Oct4* and *Nanog* loci that correlated with reductions in transcript levels within the embryos [81]. Prior to gastrulation (6.0–6.25 d.p.c.), a small population of cells in the proximal epiblast become specified to form the primordial germ lineage (primordial germ cells: PGCs). These cells can be recognised by the expression of *Blimp 2*, a transcription factor upregulated in response to BMP4

signalling emanating from the neighbouring extraembryonic ectoderm. PGCs cells migrate and colonise the allantois where they reside during gastrulation, protected from the inductive signals that induce differentiation. A discussion of the germ lineage is beyond this review and details of this process can be found in [83].

Surprisingly, given the recent prominence of pluripotent stem cell research, little is understood about the mechanisms that regulate the progression of the pluripotent lineage. Mouse mutations and *in vitro* assays suggest that signals emanating from the overlying primitive (visceral) endoderm are required for primitive ectoderm formation [59, 84, 85]. Analysis of the t^{w5} mutation in the mouse *t* complex, which prevents formation of the pluripotent cell epithelium in the postimplantation embryo, ascribed an essential role in development of the pluripotent lineage to the expression of *vacuolar protein sorting 52* (*Vps52*) in the visceral endoderm [86]. These studies highlight the importance of close association between the pluripotent cells and the extraembryonic endoderm for pluripotent lineage progression but give little understanding of the nature of the signals involved. Recapitulation of lineage progression *in vitro*, which will be described in the coming sections, provides an alternative approach to understanding this fundamental process in embryology and gaining insight into pluripotent cell biology.

5. Mouse Pluripotent Cells in Culture

5.1. Common or Garden ES Cells. The pluripotent cell population of the mouse blastocyst was first recognised by the ability of cells within the ICM to contribute to chimaeric animal formation when injected into host embryos and by the ability of the blastocyst to generate teratocarcinomas [87–89]. In 1981 two groups reported the isolation and maintenance of pluripotent cells from the mouse blastocyst [90, 91], and specifically from the epiblast component of the ICM [61]. These cell lines, termed embryonic stem (ES) cells, share many of the properties of the epiblast, including pluripotentiality. ES cells have been isolated repeatedly from the 129 laboratory, and more recently from other mouse strains [92–94]. ES cell lines have also been isolated from cultured blastomeres [95] and phenotypically similar cell lines, embryonic germ (EG) cells, have been isolated from the migrating germ lineage [96, 97].

Initially ES cells were maintained by co-culture with growth arrested mouse embryonic fibroblasts (MEF) or MEF cell lines [90, 91]; many mouse ES cell lines are still isolated and maintained on MEFs. Feeder layers could be replaced by medium conditioned by Buffalo Rat Liver (BRL) cells that contained a diffusible differentiation-inhibiting activity (DIA) [98]. DIA was subsequently shown to be identical to leukaemia inhibitory factor (LIF) [99, 100], an interleukin 6 (IL-6) class cytokine that was able to maintain pluripotentiality in ES cells in the absence of MEFs. The ability of LIF to sustain pluripotency in cells required supplementation of the medium with serum. LIF activates signal transducer and activator of transcription 3 (Stat3), a transcription factor that inhibits the differentiation of ES cells and promotes their self-renewal [101, 102]. LIF and Stat3 signalling can be replaced

by overexpression of the transcription factor *Tfcp2l1* (also known as *Ctrl1*), and *Tfcp2l1* has been suggested to act as the bridge between LIF and the pluripotency network [103, 104]. Serum induces the inhibitor-of-differentiation (Id) proteins, an activity that can be substituted by BMP4 [105]. Other members of the IL-6 cytokine family that signal through the gp130 receptor, which include Oncostatin M (OSM), Ciliary neurotrophic factor (CNTF), Cardiotrophin, and IL-6 with the soluble IL-6 receptor, are also able to maintain ES cells in culture [106–109]. Although LIF and the LIF receptor are expressed in a complementary pattern in the trophectoderm and ICM of the blastocyst [110], embryos deficient in LIF, the LIF receptor, gp130 and signalling components downstream of gp130 in mouse embryos develop past the blastocyst stage and have failed to show an essential role for this pathway in the maintenance of pluripotent cells in the embryo [111–115]. *STAT3*^{-/-} embryos arrest between 6.5 and 7.5 d.p.c. and show a defect in pluripotent lineage proliferation. The lineage, however, is formed in these embryos and embryos progress to the egg cylinder stage [115]. Signalling through gp130 has, however, been shown to be essential for resumption of embryonic development after developmental disruption, or diapause, which may explain the reliance of ES cells in culture on this pathway [116].

A variety of assays have been used to establish the pluripotentiality of ES cells. In the original reports ES cells were shown to be able to form teratocarcinomas containing derivatives of all three primary germ lineages [90, 91]; this assay had been used as a standard assay for pluripotentiality for some years to assess embryonal carcinoma cells [117]. It was also shown that, like EC cells, ES cells were able to differentiate in culture [90, 91]. ES cells have been shown to be able to colonise the ICM and participate in embryonic development, contributing to all lineages found in the embryo and adult, including the germ lineage, when reintroduced into a host blastocyst [118–120]. This property is shared with cells of the ICM [89] and, to a lesser extent, EC cell lines [121, 122]. The ability of ES cells to integrate into the ICM and participate widely in development demonstrates the functional normality of these cells and their equivalence with the epiblast of the ICM. ES cells are able to generate and respond to the signals that regulate embryogenesis, including those signals that regulate the progression of the pluripotent lineage and their subsequent differentiation, even after they have been maintained for extended periods in culture. The role that LIF plays in maintaining pluripotentiality in culture is likely to be replaced in the embryo by supportive, but as yet undefined, components of the embryonic environment.

Phenotypically, ES cells share many qualities with the epiblast of the blastocyst, including expression of the pluripotent regulatory network *Oct4*, *Nanog*, and *Sox2*, and a number of ICM-specific transcripts [65, 123] and a rapid cell cycle supported by a cell cycle structure analogous to that seen in the pluripotent cells of the embryo [58]. ES cells, like the epiblast, are reliant on the presence of *Oct4* and *Sox2*. Knock-down or knock-out of these gene products in ES cells leads to a loss of pluripotency and cell differentiation [124–126]. The loss of *Nanog* from ES cells has negative implications on cell viability [35], but cells have been shown to be able

to self-renew, albeit poorly, in the absence of *Nanog* [127]. The analogy between ES cells and the epiblast has led to ES cells being used as a surrogate for early epiblast to characterise stem cell self-renewal and differentiation. These cells in culture, however, do not grow as a homogenous population but exist in a metastable state. Heterogeneity has been revealed by the nonuniform expression in *Oct4*⁺ cells of *ZFP42*, *DPPA3*, *Nanog*, *PECAM1*, and *OTX2* [35, 127–131]. These genes mark interchangeable pluripotent cell states corresponding to an ICM-like state (*Zfp42*, *Nanog*, *Pecam1*, and *Dppa3*) and later pluripotent cell state (*Otx2*) that coexist and ensure self-renewal and perpetuation of pluripotency, and susceptibility to differentiation factors [132]. Cells expressing *Nanog* are thought to have a higher probability of self-renewal, reflecting the obligate self-renewal of ES cells engineered to express *Nanog* constitutively and that results in a cell population enriched in ICM-like cells [67, 132, 133]. Loss of *Nanog* expression rapidly induces changes in the population profile, and if perpetuated leads to increased differentiation [133]. The most reasonable explanation for heterogeneity in a population of ES cells is that the culture conditions for cell growth establish a disordered signalling environment which cannot support a homogenous population of cells [134]. In contrast, once the pluripotent lineage is established in the embryo no such metastability is detected.

5.2. EpiSC, Stem Cells from the Primitive Ectoderm. The technology used to establish ES cell lines from the blastocyst has not been able to establish pluripotent cell lines from primitive ectoderm. Early attempts to isolate pluripotent primitive ectoderm-derived cells from the embryo and embryoid bodies showed success but the derivation of cells from the embryo was limited by the culture environment [135]. Successful isolation of primitive ectoderm-derived cell lines, termed epiblast-derived stem cells (EpiSC), was achieved in a chemically defined medium supplemented with FGF2 and Activin A from 5.5 and 6.5 d.p.c. egg cylinder stage embryos [136, 137]. EpiSC lines have been established from the epiblast of blastocysts and embryos between 5.5 and 8.25 days of development, a window of time that coincides with the presence of pluripotent cell lineage in the embryo [81, 82, 138]. The growth of factors used to isolate these cells had been identified previously as able to maintain human pluripotent cells in culture [139–141] and have been found to require intracellular signalling through SMAD2 in both cell populations [142].

The pluripotentiality of EpiSC cells has been demonstrated by the formation teratocarcinomas containing a wide variety of tissue types, including representatives of all three germ lineages, and by multilineage differentiation in culture [136, 137]. The ability of a cultured cell to participate in embryonic development is considered a gold standard definition of pluripotentiality in cells derived from model animals, like mouse, where these experiments are ethically and technically possible. Embryonic primitive ectoderm and other primitive ectoderm-like cells in culture are unable to contribute to chimaera formation when injected into the blastocyst [61, 63, 64, 143, 144], despite genetic and functional demonstrations of pluripotency [143, 145–149]. Likewise, EpiSC are generally

unable to colonise the ICM and participate in embryonic development when introduced into a host blastocyst [136, 150]. EpiSC introduced into the blastocyst remained physically distinct from the ICM, suggesting that the inability to contribute is a consequence of differing adhesive properties between the cells of the ICM and EpiSC preventing assimilation [136]. EpiSC modified to overexpress E-cadherin, and treated with ROCK inhibitors, can form chimaeras after blastocyst injection [151]. The analysis of EpiSC, embryonic primitive ectoderm, and other primitive ectoderm-like cells suggests that the ability of a cell to integrate with the ICM is not a defining property of pluripotency. Recently it has been shown that EpiSC, but not ES cells, can be grafted into the primitive ectoderm of postimplantation embryos where they will disperse and differentiate appropriately [152]. This suggests EpiSC are pluripotent, functionally equivalent to the primitive ectoderm, and that the lack of chimaera formation following conventional blastocyst reflects cellular differences rather than a loss of pluripotency.

However sharing the quality of pluripotency, ES cells, and EpiSC differs in a number of key respects which reflect their origins. This has been reviewed recently [153, 154]. Most notably, an X chromosome in XX EpiSC is inactivated whereas XX ES cells do not exhibit X inactivation [155]; the gene expression of EpiSC mirrors that of the primitive ectoderm with a reduction in ICM-specific gene expression and increased expression of later epiblast markers [136, 137] and the genome organisation of ES cells and EpiSC differ suggesting the populations are epigenetically distinct [156]. EpiSC preferentially use the proximal enhancer to drive *Oct4* expression [136] and show lower expression of *Sox2* and *Nanog* [36, 150] suggesting that the maintenance of pluripotency in these cells is distinct from ES cells. Moreover, EpiSC can be derived and maintained from *Nanog*^{-/-} epiblast or *Nanog*^{-/-} ES cells, suggesting, *Nanog* is not required for pluripotency in the primitive ectoderm [81]. These qualities all distinguish pre- and postimplantation epiblast, correlate ES cells and EpiSC with their respective embryonic origins, and suggest that these cells are distinct populations and not an equivalent cell that acquires different characteristics in response to dissimilar culture conditions. More recently, proteomic analysis has identified a number of differentially expressed surface proteins that distinguish ES cells and EpiSC and reveal differences in signalling receptors and proteins involved in cell adhesion and cell migration [47]. A subset of these proteins was analysed in the embryo and shown to be differentially expressed between epiblast and primitive ectoderm. Metabolic differences, particularly in the use of carbohydrates, have also been shown to exist between ES cells and EpiSC, with EpiSC proposed to be exclusively glycolytic and ES cells generating energy through glycolysis and oxidative phosphorylation [157]. Finally, EpiSC do not easily revert to, or acquire characteristics of, the ES cell state when cultured in medium that supports ES cell renewal [155, 158], although some EpiSC lines appear to revert more readily than others [159]. Comparative analysis of a panel of independent EpiSC lines has suggested variability between lines and the maintenance in culture of EpiSC cell lines representative of earlier and later primitive ectoderm; the ability to readily

revert to an earlier state was embodied in cells representative of earlier primitive ectoderm [159]. By contrast, a recent study isolating EpiSC lines from the early primitive ectoderm of the pregastrula embryo (6 d.p.c.) to primitive ectoderm of late gastrula stage mouse embryos (8.5 d.p.c.) has shown that EpiSC in culture are transcriptionally and developmentally similar and aligned with anterior primitive ectoderm of late gastrula stage embryos [82].

5.3. Manipulating Pluripotency in Culture: The Ground State. Since recognition that LIF and gp130 signalling is unlikely to underpin pluripotency in the embryo, many attempts have been made to develop culture conditions for ES cells that more closely reflect the embryonic environment. The majority of these approaches will not be covered here as they have little to add to the discussion of pluripotent cell states. An exception to this is the recent demonstration by Austin Smith and colleagues of the culture of ES cells in serum-free medium supplemented with inhibitors of ERK/FGF signalling and glycogen synthase kinase-3 (GSK3) signalling, a medium termed 2i or 3i depending on the inhibitors used [160, 161]. ES cells, isolated from blastocysts into 3i medium, maintained pluripotency in culture and were able to contribute to chimaera development after injection into host blastocysts [160]. The ability of this medium to sustain ES cells suggests that pluripotency in culture can be achieved by eliminating ERK signalling and preventing this pathway from priming pluripotent cells for differentiation. 2i and 3i culture conditions have been used to derive ES cell lines from hitherto refractive laboratory strains of mice [160, 162] and rats [163], suggesting that the previous failure to generate ES cells reflects an insufficiency of culture conditions and not a requirement for specific genetic or epigenetic backgrounds. *Stat3*^{-/-} cells can be established and cultivated in 3i medium [160], demonstrating that, in line with the embryological evidence, the requirement for LIF/STAT signalling in pluripotency is specific to culture. Pluripotent cells cultured in ERK signalling inhibitors show compromised cell growth and viability that is alleviated by the addition of CHIR99021, a specific inhibitor of glycogen synthase kinase 3 (GSK3). GSK3 inhibition has been shown to increase the pool of β -catenin in the cell, which in turn promotes pluripotency [164]. This function of β -catenin does not require β -catenin-mediated transcription. In the maintenance of pluripotency, β -catenin has been suggested to act through the formation of multiple protein-protein interaction complexes, one of which sequesters Tcf3 and inhibits activity, and another one that tethers Oct4 to a complex at the cell membrane, potentially preventing the association of Oct4 in differentiation promoting complexes [164]. Alternative formulations of inhibitor medium have been developed by others, substituting ERK inhibition by inhibition of SRC kinases or inhibition of calcineurin signalling [165, 166]. SRC kinase has been shown to be required for the differentiation of ES cells to primitive ectoderm [167, 168]. ERK inhibitors, SRC kinase inhibitors, and inhibition of calcineurin signalling, therefore, share a common function and prevent differentiation within the context of inhibitor-based medium formulations; this is likely through the prevention of cSRC activation, a process mediated by calcineurin-NFAT and ERK1/2 [166].

The relationship between pluripotent cells cultured in 2i medium and pluripotent cells of the embryo has been investigated. Inhibition of ERK signalling from the 8-cell stage embryo to the blastocyst does not prevent the formation of epiblast or affect the ability of epiblast-derived cells to contribute to further development when the ERK embargo is lifted [50]. ES cells cultured in 2i or 3i medium have been proposed to be equivalent to the newly formed epiblast of the blastocyst and representative of cells, that is, in the “ground state” of pluripotency, a term coined to reflect the potency of this cell in comparison to what precedes and follows it in development and the positioning of the cell at the base of all embryonic lineages.

5.4. Manipulating Pluripotency in Culture: Early Primitive Ectoderm-Like Cells. Soon after the initial isolation of mouse ES cells, it was recognised that their broad differentiation potential could be harnessed to understand the regulation of differentiation events in the embryo, to produce populations of somatic cells for research, and to characterise lineage progression and the formation of differentiation intermediates. It was also recognised early that human equivalents of the mouse ES cell and derivatives would have enormous potentials as a source of cells with clinical relevance [169, 170].

EPL cells, a product of early attempts to control the differentiation of ES cells in culture, were first described in 1999 [143]. EPL cells are formed from ES cells cultured in medium supplemented with medium conditioned by the human hepatocellular carcinoma cell line HepG2 (MEDII) [143]. Expression of *Oct4*, *Sox2*, and alkaline phosphatase [143] and a differentiation potential in culture that includes the formation of populations of the mesoderm, endoderm and ectoderm, lineages [143, 147–149, 171] identify EPL cells as pluripotent, although these cells, like EpiSC and native primitive ectoderm, do not participate in chimaera formation when injected into a blastocyst [143]. The alteration in colony morphology [143], loss of ICM and ES cell-specific markers *Rex1*, *CRTR-1*, *PSCI*, *SPPI* and *GBX2*, upregulation of the primitive ectoderm markers *Fgf5* and *PRCE* [65, 143, 148, 172], increased proliferation rate [143], and a restricted ability to form cell populations characteristic of the primitive endoderm lineage [148, 149] are consistent with the formation of primitive ectoderm and discriminate these cells from ES cells.

Comparisons of EPL cells and EpiSC have not been reported except within the context of a comprehensive study of genome organisation in pluripotent cells [156]. Not surprisingly, however, EPL cells share properties with EpiSC, including an epithelial morphology, increased expression of primitive ectoderm markers when compared to mouse ES cells, and a differentiation potential that encompasses the three primary germ layers [136, 137, 147–149, 172]. There are differences between these populations. Comparison of the chromatin configuration of EPL cells and EpiSC shows EpiSC, but not EPL cells, to have undergone autosomal lyonisation [156]. EpiSC express *Nanog* at levels equivalent to or higher than mouse ES cells [136, 137], whereas *Nanog* expression is diminished with EPL cell formation [172]. *Nanog* expression in the embryo is lost with primitive ectoderm formation

and reexpressed in the late primitive ectoderm prior to gastrulation [35, 66, 67]. These data suggest that EPL cells represent a *Nanog*^{low}, preautosomal lyonisation primitive ectoderm, and EpiSC, the *Nanog*-expressing post-autosomal lyonisation primitive ectoderm. Lastly, when cultured in medium that supports ES, cells EPL cells readily revert to an ES cell state [143]. In contrast, the ability of EpiSC to revert is seen only in those lines representative of an earlier pluripotent cell state [159]. In a recent review EPL cells were defined as an intermediary state between ES cells and EpiSC [173]. This is consistent with the expression of the early postimplantation primitive ectoderm marker *Esp11* by EPL cells [65].

6. Mechanisms That Regulate Pluripotent Cell Progression: What We Have Learnt *In Vitro*

Pluripotency in ES cells, and in the epiblast of the embryo, is maintained through the orchestrated actions of three transcription factors, Oct4, Sox2, and Nanog. Coregulatory and autoregulatory mechanisms ensure the maintenance of active concentrations of these factors within the ES cell, reinforcing the pluripotent state, expression of cofactors and effector proteins required for pluripotency, and, perhaps most importantly, suppression of differentiation regulators, including those that specify the trophectoderm and primitive endoderm lineages [174]. Paradoxically, perhaps, Oct4 and Sox2 also ensure the expression of *Fgf4*. FGF signalling, through the Ras-Erk pathway, is not required for the propagation of undifferentiated cells in culture; *Fgf4*^{-/-} and *Erk*^{-/-} ES cells can be maintained but are unable to commit to differentiation [175]. The suppression of Erk signalling is critical to maintaining cells in the ground state of pluripotency [160]. These observations suggest that autocrine Erk signalling initialises pluripotent lineage progression in culture. Oct4 and Sox2, through regulation of effectors of pluripotency and progression, generate a balance in the pluripotent cell of renewal activity and differentiation activity and ensure that ES cells are poised to exit self-renewal and commit to lineage specification.

The ability to recapitulate the formation of primitive ectoderm from ICM with the formation of EPL cells from ES cells in culture provides a manipulable system to understand the signals regulating this process. EPL cell formation is induced by the amino acid L-proline, either as exogenously added amino acid at concentrations >100 μM or as a component of the conditioned medium MEDII [176–178]. The activity associated with L-proline appears specific, as other amino acids tested and analogues of proline did not exhibit the bioactivity, with the exception of ornithine [176–178]. Transport of L-proline into the cell by the amino acid transporter SNAT2 is required [177]. Many, but not all, L-proline-containing peptides are active, and activity is consistent with the ability of free l-proline to be liberated from the peptide through the actions of extracellular proteinases [176].

Primitive ectoderm can also be formed from ES cells within embryoid bodies (EBs) [135, 179]. In EBs, as in the embryo, the signals regulating primitive ectoderm formation

originate from the overlying primitive endoderm [59, 84, 180–182]. In EBs this signal has been characterized as a small, diffusible signal [59, 181], and it is tempting to speculate that it may be L-proline. L-proline uptake by SNAT2 can be inhibited by competitive concentrations of other amino acid substrates of SNAT2; inhibition of L-Proline uptake during EB differentiation prolonged the expression of ES cell markers, consistent with a requirement for L-proline uptake in primitive ectoderm formation (author unpublished). Although preliminary, these data are consistent with a role for L-proline in the regulation of primitive ectoderm formation within an *in vitro* model of early embryonic development.

How L-proline induces EPL cell formation from ES cells is not fully understood. Amino acids are canonically sensed in cells by two pathways mediated by mTOR or GCN2 [183, 184] that regulate biosynthetic activity across a number of pathways. mTOR activity has been shown to be necessary for L-proline activity but not sufficient, as addition of other activators of the mTOR signalling pathway failed to alter the ES cell phenotype [176]. This implies the involvement of additional, proline-specific pathways. L-proline is an unusual amino acid, the only secondary amino acid that is incorporated into proteins. The distinctive structure of l-proline, with the alpha nitrogen contained within a pyrrolidine ring, precludes metabolism by the normal amino acid metabolic enzymes. The central enzyme in proline metabolism is proline dehydrogenase (PRODH or POX) which converts L-proline to Δ^1 -pyrroline-5-carboxylate (P5C) and generates superoxide (ROS) [185, 186]. A competitive inhibitor of proline dehydrogenase (PRODH or POX), 3,4-dehydro-L-proline (DHP), inhibited the activity of L-proline on ES cells [178]. In addition, L-proline activity was inhibited by well characterized ROS scavengers, including glutathione, N-acetyl-L-cysteine (NAC), and ascorbic acid [178]. Ornithine, the other amino acid reported to differentiate ES cells to primitive ectoderm [178], can be converted to L-proline through the formation of P5C by ornithine aminotransferase (OAT) and reduction of P5C to L-proline by P5C reductase. These observations suggest that the biological activity associated with L-proline requires L-proline metabolism.

A requirement for Src family kinases in the formation of primitive ectoderm from ES cells was shown by the ability of broad specificity inhibitors to prevent ES cell differentiation on LIF withdrawal [168]. Using an elegant chemical genetics approach the formation of primitive ectoderm from ES cells in culture was shown to require signalling through cSRC and that inhibiting this transition effectively inhibited further differentiation [167]. The relationship between activation of this pathway and L-proline metabolism has not been established but it is of note that SRC kinase can be activated by increased intracellular ROS [187].

An alternative approach to understanding the progression of the pluripotent lineage and primitive ectoderm formation has been to look for transcription factors that regulate ES cell progression. Otx2 is differentially expressed between ES cells and EPL cells/EpiSC [131, 172]. Otx2 expression is seen in about 50% of Oct4⁺ common or garden ES cells (generally those with lower Nanog expression) whereas all

Oct4⁺ EpiSC expressed Otx2. Expression in ES cells cultured in 2i medium is much lower [188]. Manipulation of Otx2 levels suggested that this transcription factor regulates the balance between ICM-like cells and primitive ectoderm-like cells in common or garden ES cell populations, with Otx2^{-/-} cells resembling cells cultured in 2i medium and Otx2-overexpressing cells acquiring characteristics of EpiSC and primitive ectoderm [131]. How signalling by L-proline, mTOR, and cSRC integrates with transcriptional regulation by Otx2 and pluripotency regulation is not known.

7. How the Lineage Develops: A Model

If we imagine the pluripotent lineage as the sequential progression of cells from newly formed epiblast in the ground state of pluripotency in the preimplantation blastocyst to epiblast of the late primitive ectoderm, it is possible from *in vitro* analysis of pluripotent cells to begin to identify intermediary cell states and mechanisms regulating cellular transitions. Ground state pluripotent cells are characterised by the ubiquitous expression of Nanog and by a stable pluripotent cell transcription network. This state is achieved in culture by shielding cells from inductive environmental cues and preventing signalling through ERK1/2 and activation of cSrc. The nature of the shield in the embryo, if it exists, is not known. *In vitro*, increased ERK signalling results in cells becoming primed or able to respond to differentiation signals [175] and deletion of *Erk2*^{-/-} biases cells towards self-renewal [189]. Increasing ERK activity likely occurs in response to an accumulation of endogenously producing FGF4 that activates an autocrine response within the cells and suggests that a threshold exists beyond which the ground state of pluripotency is unsustainable.

The identity of the first primed cell population is not clear. In the embryo this cell is likely to be ephemeral, forming but responding immediately to environmental cues to form primitive ectoderm. Potentially this cell has been captured in culture, the *Nanog*⁺, *Rex1*⁺, and *Dppa3*⁺ cell components of ES cells populations cultured conventionally in LIF. Differences have begun to emerge in that distinguish ground state ES cells with ES cells cultured in LIF/serum [190, 191], and we have defined functional differences in the ability of these two populations to respond to L-proline (Boon Siang Nicholas Tan and Joy Rathjen, unpublished observations). ES cells cultured in LIF are also distinct from EPL cells, responding to BMP4 with self-renewal rather than differentiation [171] and maintaining the expression of *Nanog* [172]. The transitory nature and inherent instability of this initial primed cell explain the metastable state adopted in ES cell cultures in LIF, with ES cells within the mix sporadically responding to increased ERK signalling, despite the inhibitory presence of LIF/STAT3 signalling, and responding to environmental cues (most likely L-proline within the medium) to form an early primitive ectoderm-like cell. The early stages of ES cell differentiation can be reversed [133, 143]. Under the influence of LIF within the medium, a proportion of the early primitive ectoderm-like cells will revert to the primed

ES cell state. Thus, in ES cells cultured in LIF, pluripotent cells will be continually cycling between a primed ES state and an EPL cell state as the balance of LIF/STAT3 and ERK signalling fluctuates. Reversion is unlikely to be complete in the presence of LIF, so a proportion of cells will commit to lineage differentiation. These cells would accumulate within the population if regular passaging was not used to select against them.

Primitive ectoderm-like cells comprise a component of the population present at any one time in ES cells cultured in LIF. The prevalence of these cells will depend on many factors, the composition of the medium, the quality of the serum or serum replacer, and the number of differentiated cells within the population (which is a function of the time since passage). Primitive ectoderm-like cells will also be selected against at the point of passage, as differentiation of EPL cells can be triggered by disrupting cell to cell contacts [192] and later primitive ectoderm-like cells are much less likely to establish colonies after reduction to a single cell suspension (author unpublished) [137]. The poorer propagation ability of these cells is probably reflected in the poor colony forming ability of ES cells, with only between 25 and 50% of cells/passage establishing colonies [143, 193].

The balance of cells within the metastable state can be influenced by culturing ES cells without LIF, a documented antagonist of primitive ectoderm formation [143, 194], and in medium supplemented with MEDII to form effectively pure populations of EPL cells. The developmental progression that is seen when EPL cells are formed within cell aggregates in MEDII without passage suggests that EPL cells do not represent a static cell population but a self-perpetuating continuum that stretches from the earliest primitive ectoderm to primitive ectoderm that is committed to differentiation [147, 171]. It is tempting to speculate that maturation of EPL cells within aggregates is driven by the ever increasing expression of *Fgf5* by the cells [147, 171] and the accumulation of FGF5 in the environment. This is consistent with a role for ERK signalling maturation of the lineage [195]. In adherent culture with regular passage EPL cells can be maintained, although achieving this is extremely difficult and the cells are prone to differentiate. This is likely a consequence on the reliance of EPL cells on cell to cell contacts for stability and the ability of EPL cell dissociation to trigger differentiation [192].

The ability to capture embryonic primitive ectoderm as EpiSC suggests that with appropriate culture conditions cells late within the developmental continuum of primitive ectoderm can be stabilised in culture. EpiSC can also be established from ES cells through the manipulation of culture medium [155]. Although it is possible that the culture conditions induce differentiation, it is more likely that EpiSC establishment occurs as a function of culture. Changing ES cell medium to one supplemented with FGF2 and Activin A removes LIF, resulting in differentiation towards somatic lineages. Cells will transit through a primitive ectoderm-like state, which can be captured and stabilised in the new medium.

8. Terminology

A difficulty that is encountered when comparing cell populations in culture with those present in the pluripotent lineage of the embryo is the inconsistent use of terminology. In the embryo epiblast refers to the entirety of the pluripotent lineage, which implies that pluripotent cells in culture are all epiblast derived or epiblast like. Yet only those cells initially derived from the 5.5 and 6.5 d.p.c. epiblast, EpiSC, are attributed to this tissue. In the mouse embryo the biological distinction between pre- and peri/postimplantation epiblast is denoted by the use of primitive ectoderm for the later population; this has been reflected in the terminology for EPL cells but is not used to denote other primitive ectoderm-derived or primitive ectoderm-like cells. ES is a term that reflects the stem-like properties and differentiation potential of the cells in culture but has come to refer in mouse to cells derived from, or analogous to, the epiblast of the blastocyst. The adoption of embryonic stem to denote the human pluripotent cells in culture has been questioned recently as although these cells are blastocyst-derived they are demonstrably distinct from mouse ES cells/early epiblast and more like EPL cells/EpiSC/primitive ectoderm [154]. The ad hoc and inconsistent use of terminology has resulted in a need for a comprehensive knowledge of the field and of the quirks of cell nomenclature before the nuances of similarity and differences between cells in culture and the embryo can be appreciated. It is not reasonable to expect either field to alter terminology at this point in history, but a clear and concise understanding of the embryonic terminology and a consistent use of terms to describe cell states in the embryo need to be developed to allow accurate cross referencing between the lineage *in vivo* and cells *in vitro*. Others have suggested the use of preimplantation epiblast and early and late postimplantation epiblast to define the subpopulations present in the embryo [173]. This nomenclature is applicable to embryonic development of mouse and human, but falls down when used to describe the epiblast of mammalian species in which implantation is not coincident with epiblast epithelialisation. Establishment of pluripotent cells from these species, such as pig, encourages the development of a more general terminology. The author would propose a simple solution to the issue, based on the use of early, middle and late epiblast (Figure 1). Early epiblast in the embryo comprises pluripotent cells of the blastocyst, present prior to the epithelialisation of the epiblast and in culture cells in the ground state of pluripotency. Middle epiblast in the embryo is the epiblast of the peri-implantation epiblast and the newly formed epithelialized epiblast of the primitive ectoderm, present before autosomal lyonisation and X-inactivation in the female. In culture this describes EPL cells and EpiSC, derived from the embryo or from ES cells, which can readily revert to an ES cell-like state. Late epiblast of the embryo is present immediately prior to gastrulation and persists as the pluripotent cell population in the gastrulating embryo and has undergone autosomal lyonisation and X-inactivation in the female. In culture late epiblast is represented by EpiSC and potentially by EPL cells which have been cultured as

aggregates for 5 days, which no longer readily revert to an ES cell state. Straddling the early to middle epiblast boundary are primed ES cells, and their alignment with either population depends on the composition of the metastable state.

9. Human ES Cells: Where Do They Fit?

That human blastocysts contained pluripotent cells that could be cultured *in vitro* was first recognised by Bob Edwards, but it was not until the last years of the twentieth century that these cells were isolated as cell lines [196, 197]. More than any others, these cells triggered the realisation that pluripotent cells existed in multiple states in culture and an ongoing debate about their identity. They are derived from the early epiblast but in culture they are different from primed ES cells and do not show hallmarks of ground state pluripotency. They grow in large, epithelial-like colonies, culture very poorly when dissociated into single cells unless ROCK inhibitors are present [198], and are not maintained by LIF but require, like EpiSC, a medium supplemented with FGFs and Tgf β s [139–141]. Recent advances in human ES cell culture include multiple medium formulations that support the cells in chemically defined and xeno-free environments [141, 199–201]. Like EPL cells human, ES cells respond to BMP4 stimulation with differentiation rather than maintenance [171, 172, 202, 203] and like EpiSC they have an almost exclusively glycolytic metabolism [157]. Although continuing culture of human ES cells in the ground state of pluripotency has proven difficult, there are reports to suggest that the cells can revert to an early epiblast phenotype [204–207] and be maintained in the naïve or ground state [208]. *Prima facie*, and contrary to their origin, these cells appear to adopt in culture a middle-epiblast identity. Analysis of successful human ES cell isolation events suggests that the pluripotent cell mass in the human blastocyst continues development *in vitro* prior to the outgrowth of cell lines, and that the origin of the cell lines is more accurately described as a post-ICM intermediate with similarity to the epiblast [209]. Establishment of lines from a more advanced stage of the pluripotent lineage likely accounts for the middle-epiblast identity of these cells in culture. At odds with this are multiple reports demonstrating that human ES cells form trophectoderm when BMP4 is added (reviewed in [210]), a characteristic that is not consistent with middle-epiblast of the mouse or the lineage restrictions that occur within the cleavage stage mouse embryo. Potentially, this difference in potency results from species specific differences in early embryonic development, reflecting, for example, the different capabilities of mouse and human to interrupt development and establish diapause [211]. Or perhaps, like primed ES cells, these cells do not align with the developmental continuum but represent a metastable population that maintains pluripotency in cells cycling through more primitive and more advanced states and which maintains a subpopulation of cells that can form trophectoderm. It is clear that human ES cells in culture form complex populations comprised of multiple cell types including subpopulations of cells showing characteristics of early lineage progenitors [212, 213]. Finally, and much more radically, perhaps pluripotency in human ES

cells is not stable and trophectoderm formation is evidence of a loss of regulatory control.

10. Concluding Remarks

In 2013 it is unusual to write a review on pluripotency that does not include a section on reprogramming and iPS cells [214]. I make no apologies for this; this review is about pluripotent cell states in the embryo and in culture and including discussion of reprogrammed cells was beyond my scope. Although the ability to form iPS cells appears, at this point in time, to tell us little about the regulators that drive cells from early to middle and late-epiblasts, these cells hold enormous potential to refine our understanding of a cell state and pluripotency in the future. Reprogramming has the potential to highlight functionally the pathways that are critical to pluripotent cells in culture, not just those that maintain pluripotency but also those that impose physiological control on the cells. It is well documented that many abortive reprogramming events occur, indicative of a strong selective pressure against nonoptimal cellular networks. This selective pressure ensures a limited number of outcomes from the diverse starting population generated in a reprogramming experiment and will reflect networks essential to a pluripotent cell state in culture.

The embryo tells us that the pluripotent cells exist as a lineage, a continuum of cell populations that differ genetically, epigenetically, and functionally but which share a common quality of pluripotency. With the exception of the early epiblast, which is present in embryos that are suspended in diapause, there is little evidence of a stable pluripotent cell state during embryogenesis. This raises questions—does a pluripotent cell state exist in the embryo that is stable and sustainable after a cell has undergone the ground state to primed transition, or does the transition to a primed cell initiate an inevitable sequence of events that guarantee differentiation? Embryologically an inevitable program of differentiation makes sense. Pluripotency is a dangerous state and when uncontrolled results in tumours; ensuring differentiation by eliminating stable states that could persist and seed teratocarcinomas would act as an effective control mechanism to protect the early embryo. The ramifications of a continuum, though, are that the maintenance of primed cells in culture requires the establishment of a metastable population with cells cycling between earlier and later cell states, a process which responds to selective pressures exerted by the culture medium and cell passage, and which produces populations specific to culture, as seen in common or garden ES cell culture. A metastable state could be equally true of EpiSC, in which population diversity has been demonstrated functionally [150] and genetically [159], and human ES cells, in which many subpopulations have been defined genetically [212]. In maintenance conditions it is likely that EPL cells also adopt a metastable state cycling between early and later primitive ectoderm.

Pluripotent cells in culture are, and will continue to be, powerful tools for understanding the lineage and provide unique windows into pluripotency but, with the exception of ground state ES cells, have to be used with the knowledge

that they are likely representative of a transient moment of embryogenesis captured in culture. This does not limit the current pathways being pursued to exploit the clinical applications of human ES cells. These considerations do, however, suggest research directions. In an ideal world pluripotent cell lines would be isolated from the newly formed epiblast into conditions that support the ground state of pluripotency and maintained as stocks in ground state. Priming pluripotent cells and the transition to primitive ectoderm in current differentiation paradigms are generally left to chance but these processes are regulated in the embryo and can be regulated in culture. Understanding the embryological pathways and recapitulating them within differentiation protocols will provide superior pluripotent cell substrates for further differentiation. In my opinion primitive ectoderm-like cells will always be a better place to start any differentiation protocol to the somatic lineages.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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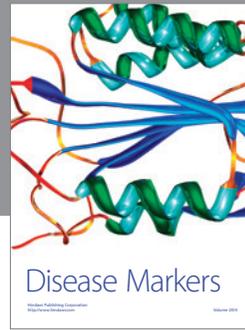
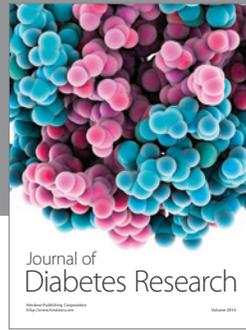
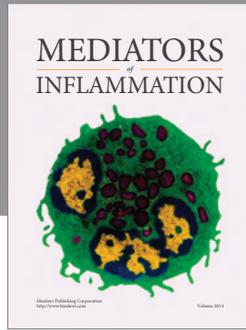
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