Early cell fate decisions in the mouse embryo

Néstor Saiz and Berenika Plusa

Faculty of Life Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK

Correspondence should be addressed to B Plusa; Email: berenika.plusa@manchester.ac.uk

Abstract

During mammalian preimplantation development, the fertilised egg gives rise to a group of pluripotent embryonic cells, the epiblast, and to the extraembryonic lineages that support the development of the foetus during subsequent phases of development. This preimplantation period not only accommodates the first cell fate decisions in a mammal's life but also the transition from a totipotent cell, the zygote, capable of producing any cell type in the animal, to cells with a restricted developmental potential. The cellular and molecular mechanisms governing the balance between developmental potential and lineage specification have intrigued developmental biologists for decades. The preimplantation mouse embryo offers an invaluable system to study cell differentiation as well as the emergence and maintenance of pluripotency in the embryo. Here we review the most recent findings on the mechanisms controlling these early cell fate decisions. The model that emerges from the current evidence indicates that cell differentiation in the preimplantation embryo depends on cellular interaction and intercellular communication. This strategy underlies the plasticity of the early mouse embryo and ensures the correct specification of the first mammalian cell lineages.

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Introduction

Animals develop surrounded by extraembryonic membranes, which protect the embryo and mediate the uptake of nutrients and gases as well as the disposal of waste products. Amniotes have the most complex set of membranes, which allow them to develop on land, either within hard-shelled eggs or inside the mother's uterus. In most mammals, some of these membranes have evolved into the placenta, a highly specialised organ that connects directly mother and foetus (Ferner & Mess 2011). The importance of these tissues for development, therefore, cannot be overemphasised. In eutherian mammals the founders of most of these tissues are the trophoblast (TE) and the primitive endoderm (PrE). The TE is responsible for the implantation of the embryo into the uterus and contributes to the embryonic part of the placenta. The PrE develops into the visceral endoderm (VE) and the parietal endoderm (ParE) after implantation, which form the majority of the yolk sac (Gardner & Johnson 1972, Gardner *et al.* 1973, Copp 1979, Gardner & Rossant 1979, Papaioannou 1982, Gardner 1983, Nagy *et al.* 2003). Besides this embryonic nutritive role, the VE also contributes cells to the gut endoderm of the embryo (Kwon *et al.* 2008). Moreover, it provides the signals that lay down the antero-posterior axis of the foetus during gastrulation (reviewed in Beddington & Robertson (1999), Nagy *et al.* (2003), and Stern & Downs (2012)).

TE and PrE are established during the preimplantation stages of development, before the embryo attaches to the uterus. Mammalian preimplantation development is entirely dedicated to the formation of these two lineages and their segregation from the epiblast, the tissue that produces the foetus as well as the rest of extraembryonic cell types. In the mouse embryo, the most extensively studied model, the formation of these three lineages occurs in two successive cell differentiation events, which take place over ~3 days. These are the first and, arguably, most critical cell fate decisions during mammalian development. Therefore, they have been the subject of extensive research. Moreover, the progress in embryonic stem (ES) cell research and the generation of *in vitro* differentiation models have fuelled a renewed interest in understanding cell differentiation in embryo. The current evidence indicates that these lineage decisions are very much the result of cell–cell interactions, which delineate the expression pattern of master regulatory genes. Here we review the latest findings in the field and propose a comprehensive model of the mechanisms behind these early cell fate decisions.

Morphogenesis of the mouse preimplantation embryo

The mouse embryo spends nearly 5 days preparing for implantation (Fig. 1). Over this period, the blastomeres produced by the cleavage of the zygote differentiate and

Figure 1 Stages of mouse preimplantation development. Zygote (fertilized egg). The polar body is the by-product of the second meiotic division of the oocyte and degenerates during preimplantation development. E4.5 (peri-implantation blastocyst) note the altered morphology of the mural trophectoderm cells at this stage as they transform into trophoblast giant cells. Arrows denote the migration of the PrE over the TE to form parietal endoderm (ParE). The descendant of the PrE covering the epiblast at later stages is the visceral endoderm (VE). The timeline indicates the time elapsed since fertilisation in embryonic days (E). ICM, inner cell mass. Timeline not to scale. Scale bars = 20 μ m.

arrange to form the blastocyst, characterised by the presence of a fluid-filled cavity and an inner cell mass (ICM), both surrounded by the TE (Fig. 1G). In the expanded blastocyst (embryonic days, E4–E4.5), the PrE can be distinguished as an epithelium at the surface of the ICM in contact with the cavity. The rest of the ICM represents the epiblast, enclosed between the TE and the PrE (Fig. 1H).

Two morphogenetic events during this period, compaction and cavitation, create asymmetries in the embryo that affect lineage formation. Compaction of the eight-cell embryo through the development of intercellular junctions produces the morula, where individual cells are no longer evident (Fig. 1E). Cells located on the inside of the morula after subsequent cell divisions become the ICM of the blastocyst, whereas cells positioned on the surface develop into the TE (Tarkowski & Wróblewska 1967). Through cavitation at around the 30-cell stage (Smith & McLaren 1977), the morula becomes the blastocyst (Fig. 1F). The presence of the cavity eventually determines the position of the PrE in the late blastocyst (Fig. 1H). The consequences of these asymmetries for cell differentiation will be discussed further below.

Developmental potential vs development

Cell differentiation results in a concomitant loss of developmental potential. The zygote is totipotent by

definition, as it is the mother for all cells in the body and the extraembryonic membranes. However in order to build an organism, totipotency has to be forsaken; this developmental potential needs to be realised into specialised tissues with defined functions. In mammals this high developmental potential is maintained for a remarkable amount of time. In the mouse not only the zygote but all the blastomeres through the morula and even the ICM of cavitating blastocysts are totipotent, as they are able to give rise to all embryonic and extraembryonic lineages (Hillman *et al.* 1972, Garner & McLaren 1974, Kelly 1977, Handyside 1978, Hogan & Tilly 1978, Spindle 1978, Rossant & Lis 1979, Rossant & Vijn 1980, Suwińska *et al.* 2008, Tarkowski *et al.* 2010, Grabarek *et al.* 2012). The epiblast, in turn, is pluripotent, as it can produce all tissues except those derived from the TE and PrE (Gardner & Rossant 1979). The persistence of totipotent/pluripotent populations is likely to be a consequence of the particularities of mammalian development, where proper embryogenesis is delayed, sometimes until the post-implantation stages. Such a delay creates the need to maintain a population of pluripotent cells, the epiblast, capable of producing the foetus even when implantation is temporarily postponed – as it is in mammals that undergo diapause (Nichols *et al.* 2001). From an applied perspective, these extended pluripotent states have allowed the generation of ES cells from the epiblast (Evans & Kaufman 1981,

Martin 1981, Brook & Gardner 1997), therefore allowing us to capture this pluripotency *in vitro* (Smith 2001). Subsequently, trophoblast stem (TS) cells (Tanaka *et al.* 1998) and extraembryonic endoderm cells (Kunath *et al.* 2005) have also been established from the TE and the PrE respectively, thus making stem cells available from all three blastocyst lineages.

Pluripotency is lost as cells differentiate into specialised tissues. In the mouse embryo organising centres do not appear until post-implantation stages, and no maternal determinants have been described to date that can drive cell differentiation at preimplantation stages. In fact, the majority of maternal mRNAs are degraded by the two-cell stage and replaced by products of the zygotic genome (Schultz 2002). Instead, these early lineage decisions are taken based on the position of the cells within the embryo and on intercellular communication. This strategy endows mouse embryos with outstanding regulative abilities. Numerous embryological studies have shown that mouse embryos can withstand rather drastic manipulations, such as rearrangement, addition or elimination of cells (or parts of them), and still develop to term (Tarkowski 1959, 1961, Tarkowski & Wróblewska 1967, Gardner 1968, Kelly 1977, Ziomek & Johnson 1980, Zernicka-Goetz 1998, Suwińska *et al.* 2008). In the past decades, a vast amount of work using blastocyst-derived stem cells, together with experimental embryology, has helped us gain further insight into the genetic and cellular mechanisms responsible for the maintenance of these pluripotent stages and for taking these early cell fate decisions. In the next sections we will discuss these mechanisms and integrate them into a unified model of our current understanding of early lineage specification.

The specification of TE and ICM

As mentioned earlier, the first differentiation event during mammalian development is the formation of the TE and the ICM. Tarkowski & Wróblewska (1967) proposed that the exposure to different microenvironments of cells located on the periphery or inside of the morula results in differential cell fates, an idea that has come to be known as the 'inside–outside model'. Later work built onto this concept and showed that blastomeres at the eight-cell stage (E2.5) acquire a polarised apical surface upon compaction, which becomes isolated from the basolateral surfaces with the development of intercellular junctions (Ducibella & Anderson 1975, Handyside 1980, Ziomek & Johnson 1980, Johnson *et al.* 1981). This 'polarity model' postulates that it is the differential inheritance of this polarised surface, after division from 8 to 16 and from 16 to 32 cells, which determines a TE or ICM fate (Johnson & Ziomek 1981). Thus, cell divisions immediately after compaction can produce either two outer cells that inherit the polarised domain and therefore become TE (symmetric or conservative

division) or one polar and one apolar, internal cell, which becomes part of the ICM (asymmetric or differentiative division). ICM cells are produced in two successive rounds of asymmetric division, from the 8- to 16-cell stage and from the 16- to 32-cell stage (Pedersen *et al.* 1986, Fleming 1987; Fig. 1D, E and F). The number of cells internalised in each round varies widely, although they are inversely proportional, resulting in a relatively consistent number of ICM cells in the 32-cell blastocyst ($\approx$ E3.25) and suggesting that mechanisms are in place to ensure an ICM of the adequate size is produced (Fleming 1987, Bischoff *et al.* 2008).

The TE is the first epithelium to arise during mammalian development. As such, its cells are bound through an extensive network of intercellular junctions that ensure selective permeability and epithelial cohesion. These junctions develop sequentially during TE development (Calarco & Epstein 1973, Ducibella *et al.* 1975). The compaction of the eight-cell embryo is a Ca^{2+} -dependent process mediated by changes in the cytoskeleton and by the formation of adherens junctions, composed of E-cadherin (or cadherin 1; Ducibella & Anderson 1975, Hyafil *et al.* 1980, Vestweber & Kemler 1984). E-cadherin is essential for compaction and for the subsequent formation of an organised TE epithelium (Hyafil *et al.* 1980, Vestweber & Kemler 1984, Larue *et al.* 1994). The formation of adherens junctions is followed by the development of tight junctions and desmosomes, which provide the cell cohesion necessary to allow the expansion of the blastocyst cavity (Calarco & Brown 1969, Nadijcka & Hillman 1974, Ducibella *et al.* 1975, Fleming *et al.* 1989, 1991, Thomas *et al.* 2004). These intercellular junctions demarcate an apical domain containing microvilli and the actin-associated protein ezrin, as well as the atypical protein kinase C p(aPKC)–partitioning defective 6 (PAR6) complex (Calarco & Epstein 1973, Reeve & Ziomek 1981, Louvet *et al.* 1996, Pauken & Capco 2000, Plusa *et al.* 2005, Vinot *et al.* 2005). Some components of this apical domain seem to be able to affect cell position in the morula, resulting in allocation to either the TE or the ICM. Disruption of aPKC activity in a subpopulation of blastomeres before compaction reduces the size of their apical domain and causes a higher contribution of these cells to the ICM lineage through asymmetrical divisions and internalisation of cells (Plusa *et al.* 2005, Dard *et al.* 2009). Therefore, the apico-basal asymmetry established in the compacted morula seems to provide the first cue for the segregation of the TE from the ICM (Johnson & Ziomek 1981, Johnson *et al.* 1986).

The development of the apical domain in the morula precedes the action of TE-specific transcription factors, such as caudal-type homeobox-2 (CDX2). CDX2, in turn, is not needed for the polarisation of these cells (Ralston & Rossant 2008, Stephenson *et al.* 2010). CDX2 is one of the first markers for TE cells. It is genetically upstream of other trophectodermal genes, such as the

T-box gene eomesodermin (*Eomes*), integrin $\alpha 7$ (*Itga7*), or cadherin 3 (*Cdh3*) and its expression in ES cells is sufficient to induce TE differentiation (Niwa *et al.* 2005, Strumpf *et al.* 2005). *Cdx2*^{-/-} embryos show peri-implantation lethality due to a failure in the formation of a mature TE epithelium and are unable to produce TS cells in culture (Chawengsaksohak *et al.* 1997, Strumpf *et al.* 2005, Wu *et al.* 2010, Blij *et al.* 2012). CDX2 is not required for compaction or the initiation of cavitation in the morula; however, *Cdx2* null embryos fail to expand the cavity (Strumpf *et al.* 2005, Blij *et al.* 2012), indicating that epithelialisation initiates independently of CDX2, although CDX2 is necessary for maintaining the epithelial integrity and for maturation of the tissue. *Cdx2* is expressed from the eight-cell stage onwards and CDX2 is present at heterogeneous levels across all blastomeres in the morula (≥ 16 cells), where it overlaps with ICM and epiblast markers, such as octamer-binding protein 4 (OCT4/POU domain, class 5 transcription factor 1 (POU5F1)) and NANOG respectively (Fig. 2B and C). It becomes gradually restricted to TE cells only in the mid and late blastocyst and to the TE-derived extraembryonic

ectoderm at post-implantation stages (Beck *et al.* 1995, Strumpf *et al.* 2005, Dietrich & Hiiragi 2007).

Cdx2 expression is induced by the transcriptional enhancer activator (TEA) domain transcription factor (TEAD4; Yagi *et al.* 2007, Nishioka *et al.* 2008), in cooperation with either of the transcriptional co-activators Yes-associated protein 1 (YAP1 or YAP) or WW domain containing transcriptional regulator 1 (WWTR1), both repressed by the Hippo signalling pathway (Kanai *et al.* 2000, Zhao *et al.* 2007, Nishioka *et al.* 2009; Fig. 2C). TEAD4 is found in all cells at the early blastocyst stage (Nishioka *et al.* 2008). TEAD4 has also been placed upstream of another TE transcription factor, the zinc-finger, GATA-binding protein 3 (GATA3; Ralston *et al.* 2010). GATA3 co-localises with CDX2 from the eight-cell stage and can promote *Cdx2* expression (Home *et al.* 2009, Ralston *et al.* 2010). Their regulation and transcriptional targets suggest that CDX2 and GATA3 act in parallel to promote TE fate downstream of TEAD4 (Ralston *et al.* 2010; Fig. 2C). Intriguingly, it has also been recently reported that TEAD4 is excluded from the nuclei of ICM cells in later blastocysts and suggested that

Figure 2 Molecular mechanism for the specification of TE and ICM fates. POU5F1, CDX2, NANOG and GATA6 co-localise in all blastomeres at the eight-cell stage, although the interactions taking place between them are unclear. In outer blastomeres of the morula (polarised), the Hippo pathway is inactive, therefore allowing the presence of YAP in the nucleus. TEAD4 binds YAP to promote TE gene expression. In inner cells, the Hippo pathway is active and phosphorylates YAP. p-YAP is sequestered in the cytoplasm by proteins of the 14-3-3 family and can no longer interact with TEAD4, what prevents the activation of the TE genetic program.

its localisation plays a role in the specification of TE and ICM cells (Home *et al.* 2012). However, the functional relevance of this observation is still unclear.

On the other hand OCT4 is necessary for the maintenance of both the ICM and the ES cells (Schöler *et al.* 1990b, Nichols *et al.* 1998, Niwa *et al.* 2000). Zygotic *Pou5f1* is expressed in all blastomeres from the two-cell stage through the mid blastocyst. It subsequently becomes restricted to the ICM (late blastocysts), the epiblast (peri-implantation blastocysts (E4.5)) and the primitive ectoderm (post-implantation), being only expressed in primordial germ cells from mid gestation (Schöler *et al.* 1989, 1990a, Rosner *et al.* 1990). OCT4 displays a similar pattern, except that its levels have been reported to peak in the PrE before becoming completely restricted to the epiblast (Palmieri *et al.* 1994). In line with this observation, high levels of POU5F1 promote differentiation of ES cells (Niwa *et al.* 2000). In contrast, more recent reports show no such increase in OCT4 levels (Nichols *et al.* 2009, Frankenberg *et al.* 2011), or even a reduction in PrE cells, which correlates with lineage commitment (Grabarek *et al.* 2012). OCT4 is, nevertheless, necessary for maintaining pluripotency. Its absence in either the ICM or the ES cells results in them acquiring TE features (Nichols *et al.* 1998, Niwa *et al.* 2000). Its expression pattern, essentially restricted to pluripotent cells, and its requirement for the maintenance of these cells have placed OCT4 at the centre of the gene network that preserves pluripotency. Interestingly OCT4 has been observed to display different kinetics in outer and inner cells of the morula, indicating that the intracellular dynamics of transcription factors are different in these two-cell populations (Plachta *et al.* 2011). Outer cells of the morula were found to have a faster rate of OCT4 import and export to and from the nucleus (fast kinetics), whereas inner cells presented slower rates of nuclear import and export (slow kinetics). These two types of OCT4 kinetics were also found in blastomeres at the 8-cell stage and correlate with the type of division blastomeres undertake at the 8- to 16-cell transition (fast kinetics, symmetric division; slow kinetics, asymmetric division; Plachta *et al.* 2011). However, how much of a causal role these kinetics play during lineage specification and what is the relationship between the dynamics of OCT4 and the interaction of OCT4 with other transcription factors (see below) still need to be determined.

CDX2 and OCT4 eventually display complementary expression domains in the late blastocyst, where CDX2 is found only in TE cells and OCT4 only in the ICM. In fact, CDX2 and OCT4 can repress each other's activity (Niwa *et al.* 2005) and is the interplay between them and TEAD4–YAP that results in the establishment of the TE and ICM populations (Fig. 2C). In outer cells of the morula, where the Hippo pathway is inactive, TEAD4–YAP promote *Cdx2* expression, which is reinforced by CDX2 itself and GATA3 (Niwa *et al.*

2005, Home *et al.* 2009, Nishioka *et al.* 2009; Fig. 2C). CDX2 in turn activates the expression of later TE genes, alongside GATA3, and represses OCT4 activity in outer cells, which eventually leads to the restriction of OCT4 to the ICM by the late blastocyst (Niwa *et al.* 2005, Strumpf *et al.* 2005; Fig. 2C). In the absence of either *Cdx2* or *Tead4*, a mature TE fails to develop and OCT4 remains in both ICM and TE cells (Strumpf *et al.* 2005, Yagi *et al.* 2007, Nishioka *et al.* 2008). Conversely, in inner cells of the morula, activation of the Hippo pathway causes inactivation and cytoplasmic localisation of YAP via phosphorylation by the large tumour suppressor (LATS) kinase (Nishioka *et al.* 2009). In the absence of nuclear YAP, TEAD4 cannot stimulate *Cdx2* expression and CDX2 is repressed by OCT4, preventing the self-activation of *Cdx2* and TE genes (Niwa *et al.* 2005, Nishioka *et al.* 2009; Fig. 2C). Consistent with this, lack of OCT4 results in TE differentiation of the ICM (see above; Nichols *et al.* 1998), indicating that OCT4 actively suppresses TE fate in ICM cells. Moreover, OCT4 is necessary for the expression of fibroblast growth factor 4 (*Fgf4*) in the ICM (Nichols *et al.* 1998). Paracrine FGF4 is necessary for the proliferation of TE and extraembryonic ectoderm cells at late blastocyst and post-implantation stages respectively, a mechanism that ensures the co-ordinated growth of TE and ICM (Gardner & Johnson 1972, Nichols *et al.* 1998, Tanaka *et al.* 1998, Goldin & Papaioannou 2003, Saba-El-Leil *et al.* 2003).

The current evidence therefore indicates that the differentiation of TE and ICM is initiated by the allocation of cells to the periphery or the inside of the morula. Cell position and polarisation of outer cells precede lineage-specific gene expression and seem to play an instructive role in the specification of these lineages. Further support for this model comes from the ability of inner cells to re-polarise and develop a new TE when isolated from early blastocysts (Handyside 1978, Hogan & Tilly 1978, Spindle 1978, Rossant & Lis 1979, Stephenson *et al.* 2010). Thus, inheritance of apical components by outer cells and differential contacts between cells result in the patterning of lineage-specific genes, which are initially ubiquitously expressed (Fig. 2B). A recent report also argues that differential contacts are necessary for the establishment of the TE- and ICM-specific gene expression patterns (Lorthongpanich *et al.* 2012). Restriction of these regulatory transcription factors to each TE and ICM cells in turn allows the activation of the genetic programmes necessary for the maturation and further development of each lineage. The subsequent addition of epigenetic marks allows the long-term stabilisation of gene expression in each cell type (for a review on the role of epigenetic modifications in early lineage decisions, see Albert & Peters (2009) and Saitou *et al.* (2012)). In other words, the gradual loss of plasticity observed in these cells (Handyside 1978, Hogan & Tilly 1978, Spindle 1978, Rossant & Lis 1979,

Suwińska *et al.* 2008) seems to be a reflection of the gradual commitment to each lineage as a result of the stabilisation of gene expression, induced and likely reinforced, by the position of the cells within the embryo.

In this context, aPKC is a particularly interesting molecule, as it has been shown to direct the allocation of cells to TE or ICM (Plusa *et al.* 2005, Dard *et al.* 2009). Interestingly, it has been observed that CDX2 binding sites are present in the promoter of the gene(s) for aPKC (Jedrusik *et al.* 2008), indicating the possibility of a feedback loop. Jedrusik *et al.* (2008) found that the premature expression of *Cdx2* (from the two-cell to four-cell) phenocopies aPKC overexpression, allocating cells preferentially to the TE, and causes an increase in the extent of the apical aPKC domain at the 4/8-cell stage. Jedrusik *et al.* (2008) thus propose that high expression of *Cdx2* is the factor driving the allocation of cells to the TE lineage. However, from their results, elevated levels of CDX2 are not obvious. It seems more likely that forcing *Cdx2* expression prematurely, rather than increasing the levels of CDX2, results in an increase in aPKC in the injected blastomeres. These blastomeres in turn would develop, prematurely, a more extensive aPKC apical domain, which would increase their chances of becoming TE. This interpretation would agree with the reports indicating that *Cdx2* lies downstream of cell polarity in outer cells (Ralston & Rossant 2008, Stephenson *et al.* 2010). In normal conditions, polarisation of aPKC would indirectly promote *Cdx2* expression, which would in turn reinforce cell polarity via *Prkc* expression in outer cells. Moreover, aPKC has been shown to phosphorylate KIBRA/WWC1 (Büther *et al.* 2004), an upstream member of the Hippo pathway (Baumgartner *et al.* 2010; reviewed in Genevet *et al.* (2010), Yu *et al.* (2010), and Genevet & Tapon (2011)), suggesting that aPKC could provide the link between polarity and the regulation of *Cdx2* and TE specification by the Hippo pathway. This possibility certainly deserves future investigation.

The segregation of PrE and epiblast

The second differentiation event during preimplantation development results in the formation of the PrE and the epiblast. These two lineages arise in the ICM through mechanisms similar to but also remarkably different from those driving the differentiation of TE and ICM cells. The precise moment when these two lineages first emerge (if such a moment exists) is still unclear, although we now know that ICM cells begin to show differences in gene expression and cell plasticity already at the early blastocyst stage ($\approx$ E3.25), which becomes more obvious as the blastocyst expands further (E3.5) (Rossant *et al.* 2003, Chazaud *et al.* 2006, Kurimoto *et al.* 2006, Plusa *et al.* 2008, Guo *et al.* 2010, Morris *et al.* 2010, Niakan *et al.* 2010, Grabarek *et al.* 2012). However, because of

the dynamic nature of this process, it is an issue that still remains controversial. Early work proposed that the cells located on the surface of the ICM in contact with the cavity become the PrE by virtue of their position, in a process analogous to the induction of the TE (Rossant 1975). However, the ICM has been suggested to be a heterogeneous population before the appearance of a PrE layer on the surface of the ICM (Koutsourakis *et al.* 1999). Indeed, more recent studies have shown that PrE and epiblast precursors are found intermingled in the ICM of the E3.5 blastocyst, in a 'salt and pepper' pattern (Rossant *et al.* 2003, Chazaud *et al.* 2006, Kurimoto *et al.* 2006) and that these two populations become subsequently resolved into two defined layers by a number of mechanisms (Plusa *et al.* 2008, Meilhac *et al.* 2009). Thus, in the morula, cell positioning precedes the establishment of the TE- and ICM-specific gene expression patterns, whereas PrE and epiblast precursors arise intermingled within the ICM, with their spatial organisation being a rather late event during their differentiation.

Cell fate regulators in the ICM

A number of lineage-specific transcription factors have been identified in PrE and epiblast cells. Epiblast cells are marked by the homeodomain transcription factor NANOG (Chambers *et al.* 2003, Mitsui *et al.* 2003) and in late stages by OCT4 and the SRY-related HMG box-containing transcription factor SOX2 (Rosner *et al.* 1990, Schöler *et al.* 1990a, Avilion *et al.* 2003). PrE cells, on the other hand, are marked by a number of endodermal proteins, such as GATA6, platelet-derived growth factor receptor alpha (PDGFRA), SOX17, GATA4, SOX7, hepatic nuclear factor 4 (HNF4), HNF1b, or hematopoietically expressed homeobox (HEX), which appear sequentially during PrE development (Arcesi *et al.* 1993, Chen *et al.* 1994, Duncan *et al.* 1994, Thomas *et al.* 1998, Barbacci *et al.* 1999, Koutsourakis *et al.* 1999, Rossant *et al.* 2003, Kurimoto *et al.* 2006, Plusa *et al.* 2008, Artus *et al.* 2010, 2011, Morris *et al.* 2010, Niakan *et al.* 2010). These regulatory transcription factors sit at the top of the hierarchy regulating the genetic programmes responsible for the identity of these cells. *Nanog* and *Sox2* are necessary for maintaining the epiblast (Avilion *et al.* 2003, Mitsui *et al.* 2003), and have been thus placed, alongside OCT4, at the core of the pluripotency network. On the other hand, *Gata6* mutants show defects in the formation of the VE, the immediate derivative of the PrE (Morrisey *et al.* 1998, Koutsourakis *et al.* 1999). In *Gata4* mutants, the VE and PrE develop normally in most cases (Molkentin *et al.* 1997), but yolk sac closure is affected (Kuo *et al.* 1997, Molkentin *et al.* 1997). Moreover, expression of either *Gata6* or *Gata4* in ES cells is sufficient to induce differentiation towards extraembryonic endoderm (Fujikura *et al.* 2002). However, some of these genes

display an overlapping pattern at early stages. *Gata6* is the earliest PrE gene to be expressed and GATA6 is present in all blastomeres from the eight-cell stage, alongside NANOG, OCT4 and CDX2 (Fig. 2B; Dietrich & Hiiragi 2007, Plusa *et al.* 2008, Guo *et al.* 2010). *Gata6* is followed by *Pdgfra* expression in the morula (8–16 cells) and *Sox17* from the late morula/early blastocyst (16–32 cells). On the other hand *Sox2* is exclusively expressed in ICM cells from the early blastocyst stage (Guo *et al.* 2010). Consequently, GATA6, PDGFRA and SOX17 co-localise with NANOG, SOX2 and OCT4 in ICM cells of early blastocysts (≈ 32 cells; Dietrich & Hiiragi 2007, Plusa *et al.* 2008, Guo *et al.* 2010, Niakan *et al.* 2010). Interestingly, during the transition from early to mid blastocysts (≈ 64 cells), the expression of these genes becomes restricted, so that they define two different populations: *Nanog* is now expressed only in epiblast precursors, whereas *Gata6* and *Sox17* are only

expressed in PrE precursors (Rossant *et al.* 2003, Chazaud *et al.* 2006, Kurimoto *et al.* 2006, Plusa *et al.* 2008, Guo *et al.* 2010, Niakan *et al.* 2010). These two cell types, which can now be identified by their gene expression profile, appear scattered throughout the ICM, in a 'salt and pepper' fashion (Fig. 3A; Rossant *et al.* 2003, Chazaud *et al.* 2006). At this stage, PrE precursors up-regulate *Gata4* expression, thus making GATA4 the first exclusive marker of PrE cells (Kurimoto *et al.* 2006, Plusa *et al.* 2008). This shift in gene expression, from an overlapping to an exclusive pattern, raises some fundamental questions: i) what are the mechanisms responsible for this change? and ii) is this exclusive gene expression pattern a reflection of lineage commitment by PrE and epiblast precursors?

This transition from a homogeneous to a heterogeneous, 'salt and pepper' population suggests that some non-cell autonomous mechanism could act as a

Figure 3 Molecular mechanism for the specification of PrE and epiblast precursors. Heterogeneous expression of the ligand/receptor pair fibroblast growth factor 4 (FGF4)/FGF receptor 2 (FGFR2) leads to the random specification of PrE and epiblast precursors, which are scattered throughout the ICM in a 'salt and pepper' manner. Cell fate specification is followed by cell sorting (green arrows) so that PrE cells become arranged in a monolayer at the surface of the ICM in the late blastocyst. Stimulation of FGFR2 by FGF4 leads to inhibition of NANOG via ERK1/2, resulting in *Gata6* expression and promotion of PrE identity. Low expression of *Fgf2* and active repression of *Gata6* by NANOG prevents endodermal differentiation and maintains epiblast precursors in a naïve state. PrE precursors maintain OCT4 and SOX2 until very late stages, although these cells down-regulate the expression of *Fgf4*. FGF4 produced by the epiblast also stimulates cells in the polar TE (D) to promote their proliferation.

symmetry breaker in the ICM. Fibroblast growth factor (FGF) signalling has long been known to be involved in PrE formation. *Fgf4* is expressed in epiblast cells (Niswander & Martin 1992, Rappolee *et al.* 1994, Frankenberg *et al.* 2011). Its expression is activated by OCT4 and SOX2 throughout the ICM (Yuan *et al.* 1995, Nichols *et al.* 1998) and is up-regulated only in epiblast precursors during the early to mid blastocyst transition (Fig. 3B and C), whereas its receptor, *Fgfr2*, is down-regulated in these cells (Guo *et al.* 2010). PrE precursors, on the other hand, maintain *Fgfr2* expression and down-regulate *Fgf4* (Guo *et al.* 2010; Fig. 3C). Mutations in either *Fgf4*, *Fgfr2*, or members of their intracellular pathway (such as the adaptor molecule growth factor receptor-bound protein 2 (*Grb2*)) prevent PrE differentiation and the expression of endodermal genes (Feldman *et al.* 1995, Arman *et al.* 1998, Cheng *et al.* 1998, Chazaud *et al.* 2006), as does pharmacological inhibition of FGF signalling (Nichols *et al.* 2009, Yamanaka *et al.* 2010). Ablation of *Fgfr2* produces a somewhat earlier phenotype than elimination of mutants show defects already at the peri-implantation stage and display defective implantation (Arman *et al.* 1998), whereas *Fgf4* or *Gata6* null embryos look normal at blastocyst stage, but also die at early post-implantation stages due to the absence of a VE (Feldman *et al.* 1995, Morrissey *et al.* 1998, Koutsourakis *et al.* 1999). Importantly, it has been recently reported that *Fgf4* mutant blastocysts fail to maintain GATA6 past the early blastocyst (Kang *et al.* 2013). Moreover, expression of *Gata6* is able to rescue endoderm formation in *Grb2* mutants, which have defects in the FGF/ERK transduction pathway (Wang *et al.* 2011). This body of evidence suggests that, although the expression of some lineage regulators precedes cell differentiation, FGF signalling lies upstream of these genes during lineage specification.

In ES cells blocking differentiation signals mediated by the FGFR–ERK pathway is necessary to maintain them in a pluripotent, ‘ground’ state (Silva & Smith 2008, Ying *et al.* 2008). This is achieved either by inhibition of FGF signalling or through transcriptional inhibition of endodermal genes by NANOG (Chambers *et al.* 2003, Mitsui *et al.* 2003, Singh *et al.* 2007, Ying *et al.* 2008). In the embryo epiblast precursors expressing *Nanog*, *Pou5f1* and *Sox2* but not *Gata6* would represent this ground state, maintained by low FGF4 stimulation and active NANOG (Nichols *et al.* 2009, Silva *et al.* 2009, Messerschmidt & Kemler 2010; Fig. 3C). In the complete absence of maternal and zygotic FGF4, embryos express *Gata6* at the eight-cell and morula stages but fail to maintain it past the early blastocyst, and all ICM cells contain NANOG thereafter (Kang *et al.* 2013). Conversely stimulation of the FGFR–ERK pathway maintains *Gata6* expression throughout the ICM via the inhibition of NANOG, thus promoting PrE fate (Yamanaka *et al.* 2010, Frankenberg *et al.* 2011, Kang *et al.* 2013; Fig. 3C and D).

Incidentally, *Nanog* also seems to be essential for the transition from a totipotent, presumably unstable, state, represented by the ICM of the early blastocyst (containing NANOG, OCT4 and GATA6; Fig. 3B), to an ICM comprising epiblast and PrE precursors (NANOG and GATA6 mutually exclusive, in a ‘salt and pepper’ fashion; Fig. 3C). Intriguingly, during this transition, *Nanog* expression switches from being monoallelic to biallelic (Miyanari & Torres-Padilla 2012). Moreover, *Nanog* is essential for the reactivation of the paternal X chromosome (Xp) in epiblast cells at this stage (Mak *et al.* 2004, Okamoto *et al.* 2004, Silva *et al.* 2009). *Nanog* null embryos maintain the Xp inactive throughout the ICM and fail to develop an epiblast. Furthermore, *Nanog*^{−/−} embryos display a non-cell autonomous defect in PrE formation – although all ICM cells in these embryos have GATA6, they show increased apoptosis and no FGF4 production (Silva *et al.* 2009, Messerschmidt & Kemler 2010, Frankenberg *et al.* 2011). Therefore, in the absence of *Nanog*, OCT4 and SOX2 would be unable to repress *Gata6* and promote the epigenetic changes necessary to produce epiblast cells. The presence of GATA6 in these cells and the lack of FGF4 prevent the formation of neither PrE nor pluripotent epiblast cells and result in ICM degeneration (Frankenberg *et al.* 2011). Therefore, paracrine FGF4 and NANOG provide a mechanism for the restriction of gene expression and for promoting the divergence of the early ICM into the two distinct populations (epiblast and PrE precursors) that make up the ICM of the mid blastocyst.

From a functional standpoint, could this transition from an overlapping to a restricted gene expression pattern in ICM cells reflect the transition from an uncommitted, undifferentiated state to a lineage-restricted state? This hypothesis has been recently tested using chimaera assays (Grabarek *et al.* 2012). Single presumptive PrE and epiblast precursors were isolated from blastocysts at different stages and transplanted to recipient 8- or 8- to 16-cell stage embryos, which were allowed to develop to late blastocysts. Unexpectedly, it was found that both PrE and epiblast precursors could contribute to several lineages in the chimaera, even when isolated from late blastocysts, after they display restricted gene expression (Grabarek *et al.* 2012). Therefore a restricted transcription factor profile does not preclude cell fate switch when challenged by the new environment. Strikingly epiblast precursors from early blastocysts very rarely contributed cells to the PrE of the chimaeric blastocysts, whereas PrE precursors were much more plastic and were normally able to contribute to all three lineages (Grabarek *et al.* 2012). These findings indicate that epiblast cells are somewhat lineage restricted from very early stages. The lower amount of FGFR2 in epiblast cells (Guo *et al.* 2010) should result in a lower sensitivity to FGF4, which could account for their inability to produce PrE in chimaeras. In agreement with this hypothesis, addition of FGF4

restored the ability of epiblast cells to form PrE, whereas inhibition of FGF signalling restricted the plasticity of PrE precursors, which mainly produced epiblast cells (Grabarek *et al.* 2012). This lower sensitivity of epiblast precursors (or a subpopulation of them) to differentiation signals could be the means to ensure the presence of the foetal lineage in the embryo (Grabarek *et al.* 2012).

On the origin of the PrE and epiblast

Despite the evidence presented earlier, the timing and mechanism for the origin of these two populations have been a matter of debate. Early work suggested that epiblast and PrE could originate from ICM cells produced in the first and second wave of asymmetric division respectively during the morula to blastocyst transition, due to differential inheritance of cellular components, such as cytokeratin (Chisholm & Houlston 1987). This is an interesting hypothesis that has been addressed recently with opposing results (Morris *et al.* 2010, Yamanaka *et al.* 2010). Morris *et al.* (2010) found that ICM cells produced in the first round of asymmetrical division had a strong tendency to contribute to the epiblast, whereas cells produced in the second round mainly formed the PrE. On the other hand, Yamanaka *et al.* (2010) found no such correlation. These discrepancies have been attributed to the different mouse strains used and the methodological differences between the two studies (Morris 2011, Yamanaka 2011). Embryos from different strains produce different numbers of ICM cells in the first round of asymmetric division (Fleming 1987), which is also the case here (2.8 in Morris's experiments vs 4.8 in Yamanaka's experiments). When these data are analysed in the light of more recent work (Guo *et al.* 2010, Frankenberg *et al.* 2011, Grabarek *et al.* 2012), these differences in cell numbers become relevant. When few cells become internalised in the first round the overall amount of FGF4 they produce would be insufficient to stimulate their few FGFR2 molecules and thus inhibit NANOG. As a consequence they would acquire epiblast identity, down-regulating *Fgf2* and thus becoming refractory to FGF4. Interestingly these cells could represent the epiblast cells that we have found are unable to form PrE in chimaeras (Grabarek *et al.* 2012). These cells in turn would produce FGF4 that would induce PrE identity in the cells internalised in the second round. Conversely when a larger number of cells become internalised in the first round, the net amount of FGF4 they produce could then reach the threshold necessary to stimulate each other and therefore induce PrE identity in some of them.

Sorting the PrE and epiblast populations

Whatever their origin PrE and epiblast precursors become specified independently of their position. These two populations have a 'salt and pepper'

distribution in the ICM of the mid blastocyst. However, their spatial arrangement by the time of implantation is very different: PrE cells form an absorbent epithelium at the interface between the ICM and the blastocyst cavity and thus leave the epiblast enclosed between the TE and PrE (Fig. 3A). The PrE in peri-implantation blastocysts (E4.5) is an epithelium with a polarised apical surface facing the cavity and a basement membrane at the interface with the epiblast. The apical surface has microvilli, endocytic receptors such as cubilin (CUB) and LDL receptor-related protein 2 (LRP2), the endocytic adaptor disabled 2 (DAB2) and aPKC (Morrissey *et al.* 1998, 2000, Yang *et al.* 2002, Gerbe *et al.* 2008, Saiz & Plusa (unpublished observations)). The basement membrane contains laminin and collagen IV (Adamson & Ayers 1979, Dziadek & Timpl 1985, Smyth *et al.* 1999). This change in cell distribution implies that there must be a mechanism for PrE and epiblast precursors to become sorted into two separate compartments. Unless these cells are to change their gene expression depending on their position, one would expect these precursors to become rearranged into their final position. It has been described that cells within the ICM can indeed migrate in a non-directional, actin-dependent manner (Meilhac *et al.* 2009). Interestingly live imaging of embryos where PrE precursors express a reporter for platelet-derived growth factor receptor alpha (*Pdgfra*) expression (*Pdgfra*H2B-GFP; Hamilton *et al.* 2003) revealed that PrE cells migrate until they reach the surface of the ICM exposed to the cavity (Plusa *et al.* 2008). Cells that reach this surface either by active migration or by passive intercalation retain their new position and up-regulate *Pdgfra*. Conversely, PrE cells that remain deeper within the ICM eventually lose *Pdgfra* expression or die, indicating that cell position is a critical factor during PrE formation (Plusa *et al.* 2008). These cell behaviours constitute an elegant mechanism to resolve a mixed cell population with no need for directional cell migration. It has been proposed that the segregation of these two populations is due to differential adhesion properties between epiblast and PrE cells (Chazaud *et al.* 2006, Gerbe *et al.* 2008, Plusa *et al.* 2008). However, whether differential adhesion is sufficient to drive cell sorting is unclear (reviewed in Krens & Heisenberg (2011)) and work using embryoid bodies (a model for PrE differentiation) suggests endogenous properties of PrE cells, such as their ability to polarise, are the driving force of this sorting process (Rula *et al.* 2007, Moore *et al.* 2009). Further support for the importance of positional cues in the segregation of PrE and epiblast comes from the observation that PrE precursors produce surface proteins, such as LRP2 or DAB2, which localise to the apical surface of these cells when they come into contact with the blastocyst cavity (Gerbe *et al.* 2008). The polarisation of PrE cells at the surface of the ICM would be the first step in their epithelialisation and may be accompanied

by the development of cell–cell contacts with other PrE precursors that would help them maintain their position.

In summary, the evidence available indicates that PrE and epiblast cells arise in the ICM as a result of the interplay between regulatory transcription factors and FGF signalling (Fig. 3). FGF4 produced during the morula to blastocyst transition maintains Gata6 expression in some ICM cells, therefore inducing PrE fate, whereas NANOG counteracts this to promote epiblast formation. This interaction leads to the formation of two populations, epiblast and PrE precursors, with distinct gene expression profiles and epigenetic features, which are scattered through the ICM in a ‘salt and pepper’ manner (Chazaud *et al.* 2006). This restricted gene expression, however, does not result in loss of cell plasticity (Yamanaka *et al.* 2010, Grabarek *et al.* 2012), which is largely dependent on the environment surrounding the cell. Continued presence of FGF4 reinforces PrE fate and consequently SOX17 and GATA4 (Frankenberg *et al.* 2011). PrE and epiblast precursors become sorted into two separate compartments by a number of mechanisms, including cell migration and anchorage at the ICM surface to form a coherent epithelium (Gerbe *et al.* 2008, Plusa *et al.*

2008, Meilhac *et al.* 2009). Their localisation at the surface induces later endodermal markers, such as SOX7 (Artus *et al.* 2011), suggesting that correct positioning means a step forward in the maturation of the PrE. Finally, the complete separation of PrE and epiblast and the restriction of OCT4 to the epiblast seem to indicate irreversible lineage commitment at the time of implantation (Grabarek *et al.* 2012).

Variations in the lineage specification mechanisms across mammals

Preimplantation development has been best studied in mice, for evident technical and ethical reasons. Many of the molecular players involved in the specification of the blastocyst lineages in mice are known (see above and Table 1) and the regulatory networks they constitute are starting to be unveiled. However, as our knowledge advances, differences between mouse and other mammals’ preimplantation development are beginning to emerge. For instance, implantation in rodents (mice and rats) occurs on the fifth day of development. By contrast, in humans, implantation takes place around the seventh day and in cattle and sheep over two weeks into

Table 1 Summary of the main proteins involved in blastocyst formation and early cell fate decisions mentioned in the text.

Gene	Protein name	Relevant references
Epiblast		
<i>Nanog</i>	NANOG	Chambers <i>et al.</i> (2003), Mitsui <i>et al.</i> (2003), Silva <i>et al.</i> (2009), and Messerschmidt & Kemler (2010)
<i>Pou5f1</i>	Octamer-binding protein 4 (OCT4)/POU domain, class 5 transcription factor 1 (POU5F1)	Rosner <i>et al.</i> (1990), Schöler <i>et al.</i> (1990a, 1990b), Palmieri <i>et al.</i> (1994), and Nichols <i>et al.</i> (1998)
<i>Sox2</i>	SRY-related HMG box-containing transcription factor 2 (SOX2)	Avilion <i>et al.</i> (2003)
<i>Fgf4</i>	Fibroblast growth factor 4 (FGF4)	Niswander & Martin (1992), Rappolee <i>et al.</i> (1994), Feldman <i>et al.</i> (1995), Nichols <i>et al.</i> (1998), and Goldin & Papaioannou (2003)
Trophectoderm		
<i>Cdx2</i>	Caudal-type homeobox transcription factor 2 (CDX2)	Chawengsaksophak <i>et al.</i> (1997), Niwa <i>et al.</i> (2005), Strumpf <i>et al.</i> (2005), and Wu <i>et al.</i> (2010)
<i>Gata3</i>	GATA-binding protein 3 (GATA3)	Home <i>et al.</i> (2009) and Ralston <i>et al.</i> (2010)
<i>Eomes</i>	Eomesodermin	Ciruna & Rossant (1999) and Hancock <i>et al.</i> (1999)
<i>Tead4</i>	TEA domain protein family member 4 (TEAD4)	Yagi <i>et al.</i> (2007) and Nishioka <i>et al.</i> (2008)
Primitive endoderm		
<i>Gata6</i>	GATA6	Morrissey <i>et al.</i> (1996, 1998), Koutsourakis <i>et al.</i> (1999), and Fujikura <i>et al.</i> (2002)
<i>Gata4</i>	GATA4	Arceci <i>et al.</i> (1993), Soudais <i>et al.</i> (1995), Morrissey <i>et al.</i> (1996), Kuo <i>et al.</i> (1997), and Molkentin <i>et al.</i> (1997)
<i>Sox17</i>	SOX17	Kanai-Azuma <i>et al.</i> (2002), Shimoda <i>et al.</i> (2007), Morris <i>et al.</i> (2010), Niakan <i>et al.</i> (2010), and Artus <i>et al.</i> (2011)
<i>Sox7</i>	SOX7	Shimoda <i>et al.</i> (2007) and Artus <i>et al.</i> (2011)
<i>Pdgfra</i>	Platelet-derived growth factor receptor alpha (PDGFRA)	Plusa <i>et al.</i> (2008) and Artus <i>et al.</i> (2010)
<i>Fgfr2</i>	FGF receptor 2 (FGFR2)	Arman <i>et al.</i> (1998) and Haffner-Krausz <i>et al.</i> (1999)
<i>Grb2</i>	Growth factor receptor bound protein 2 (GRB2)	Cheng <i>et al.</i> (1998) and Chazaud <i>et al.</i> (2006)
<i>Dab2</i>	Disabled 2 (DAB2)	Morrissey <i>et al.</i> (2000), Morris <i>et al.</i> (2002), Yang <i>et al.</i> (2002), and Gerbe <i>et al.</i> (2008)
Others		
<i>Cdh1</i>	Cadherin 1/E-cadherin	Hyafil <i>et al.</i> (1980), Vestweber & Kemler (1984), and Larue <i>et al.</i> (1994)
<i>Prkci</i>	Protein kinase C, iota (aPKC)	Leitges <i>et al.</i> (2001), Soloff <i>et al.</i> (2004), Plusa <i>et al.</i> (2005), and Dard <i>et al.</i> (2009)
<i>Prkcz</i>	Protein kinase C, zeta (aPKC)	
<i>Yap1</i>	Yes-associated protein 1 (YAP1/YAP)	Zhao <i>et al.</i> (2007) and Nishioka <i>et al.</i> (2009)

development (Rossant 2011). Recent work suggests that these differences in timing may have an impact on the molecular mechanisms regulating early lineage decisions across mammals. It has been observed that in bovine, porcine, rabbit and human blastocysts *Pou5f1* down-regulation in the TE occurs much later in blastocyst development than it does in mice (Kirchhof *et al.* 2000, Cauffman *et al.* 2005, Kuijk *et al.* 2008, Kobolak *et al.* 2009, Berg *et al.* 2011, Puy Du *et al.* 2011). *Pou5f1* down-regulation in TE cells has been shown to mark commitment to the TE fate in mouse and bovine blastocysts (Niwa *et al.* 2005, Berg *et al.* 2011). Interestingly Berg *et al.* (2011) found that differences in the regulatory region of *Pou5f1* between mice and cattle are responsible for the early down-regulation of *Pou5f1* observed in the TE of the mouse compared with the bovine blastocyst (van Eijk *et al.* 1999, Berg *et al.* 2011). The results of this elegant work suggest that the early down-regulation of *Pou5f1* has evolved in rodents to allow a rapid commitment to the TE fate, thus catering for their shorter preimplantation development (Berg *et al.* 2011). On the other hand, in cows and other mammals where preimplantation development is longer, there may not be a need to switch off *Pou5f1* and commit to the TE fate so early.

Although the transcription factors found in PrE and epiblast cells seem to be mostly conserved among mammalian species, differences have also been found between rodents and other mammals in the specification of these two lineages. Inhibition of the FGF/MAPK signalling pathway in mouse embryos and mouse and rat ES cells prevents differentiation of the PrE lineage (Buehr *et al.* 2008, Li *et al.* 2008, Ying *et al.* 2008, Nichols *et al.* 2009). In contrast, two recent reports show that inhibition of the FGF/MAPK axis in human embryos does not prevent hypoblast (PrE) development (Kuijk *et al.* 2012, Roode *et al.* 2012). Similarly in bovine blastocysts inhibition of either FGF or MAPK signalling failed to prevent hypoblast formation, indicating that *Gata6* expression is maintained independently of FGF signalling in these embryos (Kuijk *et al.* 2012). On the other hand, stimulation with FGF4 eliminated NANOG from the ICM and caused pan-ICM GATA6 distribution, suggesting that GATA6 expression could be repressed by NANOG in these embryos, as it has been shown for mouse blastocysts (Frankenberg *et al.* 2011, Kuijk *et al.* 2012). These results show that, although the molecules and parts of the mechanism regulating PrE and epiblast specification seem to be conserved, species-specific divergence can also be observed among mammals. Considering the hypotheses put forward to explain such divergence in the TE, one could speculate as to whether differences in developmental timing might also underlie the differences observed in the formation of the PrE and epiblast. These observations, without undermining the mouse blastocyst as a model, indicate that thorough comparative studies are necessary for us to gain a better

evolutionary understanding of the mechanisms driving early lineage specification across mammals.

Conclusions

Work in the last decade has allowed the identification of some of the molecular players and mechanisms orchestrating the earliest cell fate decisions in mammalian development. Perhaps not surprisingly, it has also become clear that cell interaction and cell communication are just as important as the expression of master regulatory genes for lineage differentiation. The embryonic nuclei take command of their own fate early in development and start producing some of the transcription factors that regulate the first cell differentiation events. However, their co-expression in all the blastomeres of the morula and early blastocyst calls into question how much of an inductive role these transcription factors play at the earliest stages. Instead they rather seem to prime cells for differentiation upon the reception of the appropriate stimuli, these being cell position during the formation of the TE or paracrine FGF4 and cell position during the formation of the PrE. Cell differentiation then proceeds in a gradual manner and plasticity is retained until TE and PrE have become stable epithelia where the gene regulatory networks have become stabilised and de-differentiation is no longer possible. These mechanisms result in remarkable regulatory abilities, which allow the accommodation into the particular context of the embryo of any biases early gene expression might impose on cells. This robust mode of development, therefore, ensures the correct formation of these first lineages, critical for any further progression of mammalian development.

Despite the progress in our understanding of the mechanisms governing early cell fate decisions, many important questions still remain unanswered. We do not yet fully comprehend how the initial heterogeneities among cells arise and how are they translated into divergent patterns of gene expression (Chambers *et al.* 2007, Singh *et al.* 2007, Plusa *et al.* 2008, Kalmar *et al.* 2009, Canham *et al.* 2010). What is the relevance of these heterogeneities in the cell differentiation process and what is their importance for the maintenance of pluripotency are currently exciting research questions (Loh & Lim 2011, Martinez Arias & Brickman 2011). Finally, although we know a good deal about the gene networks that regulate lineage commitment and pluripotency, the mechanisms responsible for the remarkable plasticity of mammalian cells remain largely unknown. The challenge now remains to establish precisely how these gene networks maintain particular cellular states or make cells progress (or revert) from one state to another. Only through the deep understanding of these processes will we be able to control them to our advantage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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