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REVIEW

Lineage specification in the mouse preimplantation embryo

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ABSTRACT

During mouse preimplantation embryo development, totipotent blastomeres generate the first three cell lineages of the embryo: trophectoderm, epiblast and primitive endoderm. In recent years, studies have shown that this process appears to be regulated by differences in cell-cell interactions, gene expression and the microenvironment of individual cells, rather than the active partitioning of maternal determinants. Precisely how these differences first emerge and how they dictate subsequent molecular and cellular behaviours are key questions in the field. As we review here, recent advances in live imaging, computational modelling and single-cell transcriptome analyses are providing new insights into these questions.

KEY WORDS: Cell heterogeneity, Lineage differentiation, Morphogenesis, Mouse, Preimplantation

Introduction

How a zygote develops from a single cell into a complex multicellular organism is a fundamental question in developmental biology. Mammalian preimplantation development is unique because the mammalian zygote contains no apparent polarity that may be linked to later developmental events. In particular, the mouse preimplantation embryo is highly tolerant of various experimental perturbations, displaying a high level of plasticity and self-organization. Although the general outline of how the mammalian embryo develops throughout preimplantation embryogenesis has long been known, the mechanisms that underlie this plasticity and self-organization are only just beginning to be understood.

Preimplantation development in mice involves several distinct cellular events (Fig. 1). After fertilization, three rounds of cleavage give rise to an 8-cell embryo. Polarization and compaction then take place at the 8-cell stage, followed by asymmetric division rounds that generate two distinct cell types: trophectoderm (TE), which contributes to the placenta, and inner cell mass (ICM) cells. Subsequently, as the embryo develops into a hollow sphere of cells known as a blastocyst, the ICM differentiates into epiblast (EPI), which produces all fetal cells, and primitive endoderm (PE), which contributes mostly to the extra-embryonic yolk sac. Although all embryos develop through the same processes, a wide variation in the frequency and timing of events exists among embryos. In addition, many molecular and cellular events are not fully synchronized, either between blastomeres within an embryo or among littermate embryos, yet all zygotes form fairly similar looking blastocysts. This raises the questions of how equal early blastomeres are and whether early embryos can be considered a homogenous population. Given the recent recognition of various pluripotent states in stem cells *in vitro* (Wu and Izpisua Belmonte, 2015), a comprehensive understanding of preimplantation development and self-organization in the mouse embryo is important for furthering cell reprogramming approaches. Such studies also have important implications for understanding early human development and for understanding the pluripotent state in humans, as there are clear parallels between preimplantation development in mice and humans, although some differences do exist (see Box 1).

In recent years, advances in live imaging, computational modelling and single-cell transcriptomic approaches have allowed researchers to probe the mechanisms of blastocyst formation in mice. Here, we review these recent advances, describing the molecular events and cellular behaviours taking place in the mouse embryo that lead to the differentiation of the first three embryonic cell lineages.

Early blastomeres: are they equivalent in fate, potency and gene expression?

It is generally considered that until the 8-cell stage each blastomere retains totipotency – the ability to generate all three lineages (TE, EPI and PE). By aggregating one or two donor blastomeres with a genetically distinct host embryo, a classical study showed that early blastomeres are totipotent (Kelly, 1977). In this study, it was shown that many donor blastomeres from 4- and 8-cell embryos contributed to all three lineage derivatives in chimeric embryos and pups, suggesting that they are totipotent, although some showed skewed contributions. The development of long-term cell labelling strategies with Cre-loxP recombination technology made it possible to examine the contribution of early blastomeres in intact preimplantation embryos and through to mid-gestation (Fujimori et al., 2003). Using this approach, it was shown that whereas the progeny of single 2-cell blastomeres almost always contribute to both TE and ICM derivatives, the progeny of single 4-cell blastomeres exclusively contribute to TE derivatives in 16 out of 54 labelled embryos (30%). More recently, the contributions of all blastomeres in single 4-cell embryos was assessed using Rainbow reporter mice, in which individual cells can be uniquely labelled with different colours (Tabansky et al., 2013). In this study, 30% of blastocysts labelled at the 4-cell stage displayed a statistically significant TE/ICM bias. Although it is difficult to control the precise timing of Cre-mediated DNA excision, and hence the exact developmental time point at which cells are labelled (as reflected in the uneven size of labelled progeny), these studies suggest that early blastomeres do not always contribute equally to all three lineages, but instead exhibit a skewed lineage contribution. This raises an interesting question: is the skewed lineage contribution due to non-equivalent potency? Alternatively, is the skewed pattern created by chance in blastomeres that otherwise exhibit equal potency?

A number of studies have aimed to determine if all early blastomeres are identical. Initial studies examined whether there is a link between the polarities of the oocyte and those in the blastocyst, aiming to determine if any of the blastomere progeny preferentially

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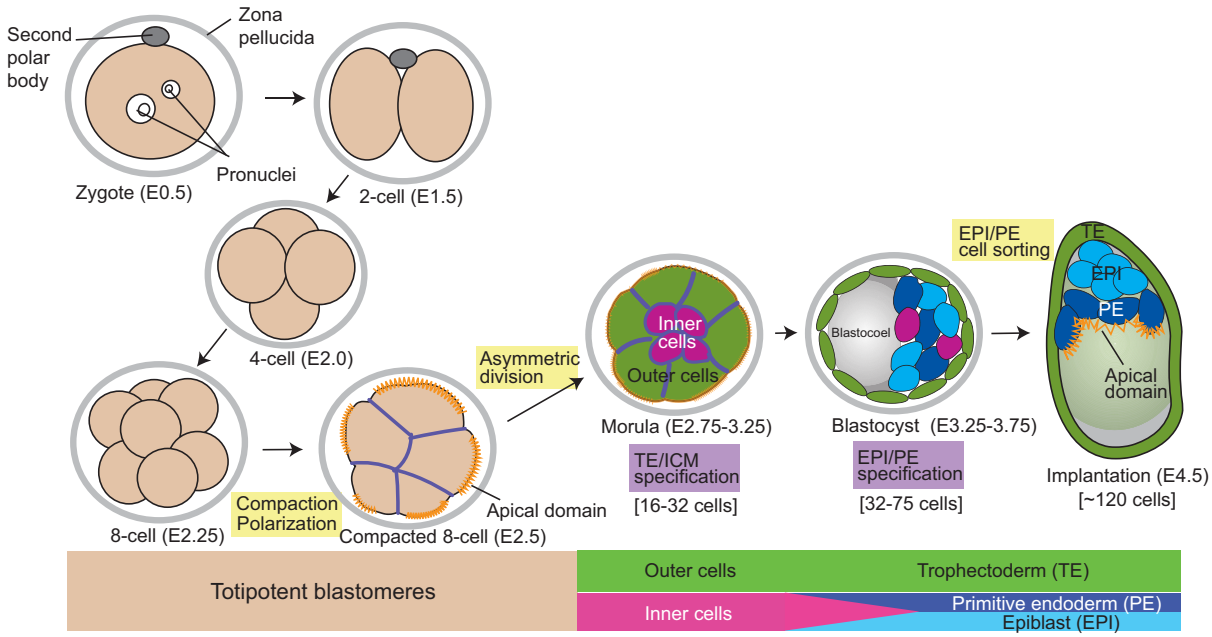

Fig. 1. Preimplantation mouse development. Schematic of the morphological changes and lineage differentiation steps of mouse preimplantation embryo development. The coloured bars show the sequential lineage progression from totipotent blastomere to the first three lineages: trophectoderm (green), epiblast (light blue) and primitive endoderm (dark blue). Important morphogenetic events are highlighted in yellow. Lineage specification events are highlighted in purple. Orange lines indicate the apical domains of cells. Figure modified from Rossant and Tam (2009).

take up a specific position in a blastocyst (Gardner, 2001; Piotrowska-Nitsche and Zernicka-Goetz, 2005). However, these results were controversial due to their reliance on using the second polar body as a reference point (Hiiragi and Solter, 2004) and to unsuccessful reproducibility in different laboratories using different mouse strains (Motosugi et al., 2005; Hiiragi et al., 2006; Alarcon, 2010). Although the controversy is not fully settled yet, some of these studies demonstrated heterogeneities in developmental potency and histone methylation patterns of blastomeres in a subset (~40%) of 4-cell embryos generated by a unique divisional pattern after the 2-cell stage (Fig. 2A) (Piotrowska-Nitsche and Zernicka-Goetz, 2005; Torres-Padilla et al., 2007). In this subset of embryos, one of the blastomeres only inherits the vegetal portion of the oocyte. Interestingly, despite this blastomere being totipotent (as it contributes to all three lineages), when chimeric embryos are generated with only this type of blastomere by aggregation they cannot develop beyond gastrulation, presumably because they contain a smaller number of EPI cells at the blastocyst stage (Piotrowska-Nitsche et al., 2005; Morris et al., 2012). This 'potency-limited' blastomere has the lowest level of methylation at the arginine 26 residue of histone H3 (H3R26me) compared with the other three blastomeres of the same 4-cell embryo (Torres-Padilla et al., 2007), and this molecular heterogeneity is only observed in this subset of embryos. Furthermore, increasing the level of H3R26me, by overexpressing the maternally expressed H3 arginine methyltransferase CARM1 (PRMT4), induces precocious expression of the pluripotency factors NANOG and SOX2, and ultimately increases the contribution of the blastomere to the ICM (Torres-Padilla et al., 2007). This biased ICM contribution is due to an increase in the frequency of asymmetric divisions and enhanced inward cell movements after divisions associated with the modulation of polarity protein expression levels, although the mechanism by which the level of H3R26me regulates specific gene expression is unknown (Parfitt and Zernicka-Goetz, 2010).

Heterogeneity in PRDM14 expression is also observed (Fig. 2B). PRDM14 is an epigenetic modifier, later enriched in the ICM, that can directly interact with CARM1 (Burton et al., 2013). *Prdm14* mRNA expression is highest at the 2-cell stage and gradually declines by the 8-cell stage. By the late 4-cell stage, heterogeneity in its mRNA and protein levels emerge: two blastomeres of 4-cell embryos exhibit PRDM14 mRNA/protein expression, whereas the remaining two show no or very low expression; unfortunately, whether these two sets of cells are sister pairs was not examined. The overexpression of PRDM14 by microinjection of *Prdm14* mRNA increases the level of H3R26me in injected blastomeres, and their progeny more frequently contribute to the ICM, similar to the situation observed following

Box 1. From mouse to human: species-specific differences in preimplantation embryo development

The recent analyses of human embryos, and embryos from other mammals, have highlighted similarities and possible species-dependent differences in preimplantation development. Although the lineage-specific transcriptional networks appear to be generally conserved, the signalling pathways regulating lineage specification might be diversified. During EPI/PE specification, for example, the salt-and-pepper expression of NANOG and GATA6 is probably common to all mammals, as it is found even in marsupials (Frankenberg et al., 2013). However, the importance of FGF signalling during EPI/PE development appears to be less prominent in human and bovine species. Indeed, activating or blocking the pathway in these embryos does not impact ICM composition as strongly as it does in the mouse (Kuijk et al., 2012; Roode et al., 2012). Nonetheless, it should be noted that the *in vitro* derivation of 'naïve' human ESCs that resemble early *in vivo* EPI cells often involves MEK1 (MAP2K1) inhibition (Manor et al., 2015), and thus the role of ERK signalling *in vitro* and *in vivo* is still unresolved. Interestingly, cooperation between FGF and Wnt pathways is required for PE formation in marmoset embryos (Boroviak et al., 2015). Therefore, the relative importance of these various signalling pathways could have evolved differently in diverse mammalian species to induce the same core lineage-specific transcriptional networks.

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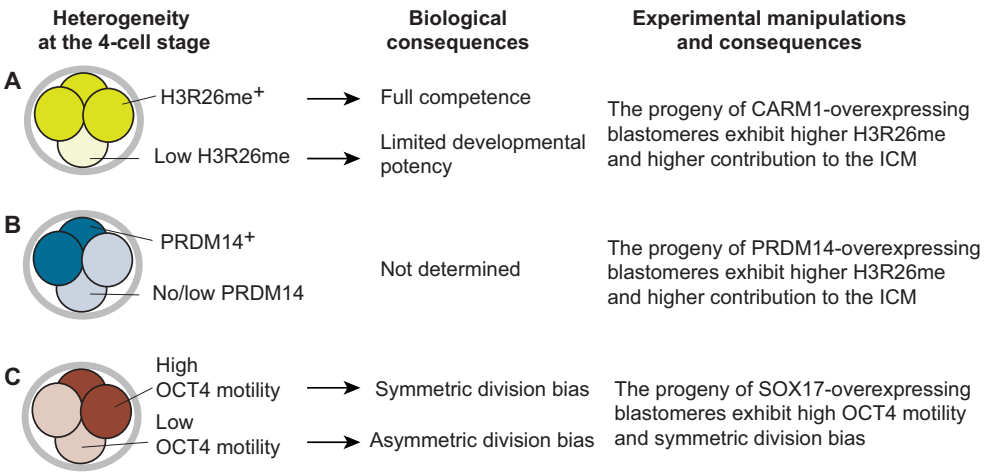

Fig. 2. Reported heterogeneities in early blastomeres. (A) Heterogeneity in H3R26me levels in a subset of 4-cell embryos. One blastomere with limited developmental potency has low levels of H3R26me (light yellow). (B) Heterogeneity in PRDM14 expression. Two of the blastomeres in a 4-cell embryo express PRDM14 (dark blue), whereas the remaining two exhibit low/no PRDM14 expression (light blue) at the late 4-cell stage. (C) Heterogeneity in OCT4 motility. About half of the blastomeres in 4- and 8-cell embryos exhibit high nuclear OCT4 motility (dark brown), whereas the others exhibit low nuclear OCT4 motility (light brown). It is not known whether these heterogeneities are linked to each other.

CARM1 overexpression. However, whether no/low PRDM14 expression is associated with low H3R26me in intact embryos, and whether these heterogeneities are transient at the 4-cell stage or maintained in the 8-cell stage and later, is not yet clear.

Heterogeneity in the dynamics of OCT4 (POU5F1) – a key transcription factor involved in early development and pluripotency – has also been reported (Fig. 2C). Although OCT4 is uniformly expressed in early blastomeres, its motility in nuclei is not the same in all blastomeres (Plachta et al., 2011). Thus, outer cells at the 16-cell stage have higher OCT4 motility than inner cells. The low OCT4 motility is likely to be due to OCT4 binding to DNA; a truncated mutant form of OCT4 that lacks the DNA-binding homeodomain shows only high motility (Plachta et al., 2011; Kaur et al., 2013). Surprisingly, high and low OCT4 motility can be already distinguished in the blastomeres of 4- and 8-cell embryos (Plachta et al., 2011). In 8-cell embryos, blastomeres with low OCT4 motility tend to divide asymmetrically during the 8- to 16-cell division (71% asymmetric division), whereas those with high OCT4 motility tend to divide symmetrically (18% asymmetric division). Thus, the skewed lineage contribution of early blastomeres in the lineage tracing studies mentioned above might be a consequence of the biased frequency of symmetric division in some blastomeres, which limits their chance to contribute to the ICM. Whether OCT4 motility correlates with the expression of PRDM14 or the level of H3R26me remains a fascinating question.

Recent single-cell transcriptomic studies have also highlighted the presence of heterogeneities in gene expression in early blastomeres (Biase et al., 2014; Deng et al., 2014; Piras et al., 2014; Shi et al., 2015). Gene expression heterogeneity is observed as early as the 2-cell stage. Because zygotic transcription begins after the late 2-cell stage in mouse embryos, this heterogeneity is thought to be due to 'partitioning errors' that occur during early cell divisions, i.e. the unequal distribution of mRNAs during division. It was further shown that the genes expressed at lower levels are more vulnerable to such partitioning errors (Biase et al., 2014). In addition, although dozens of protein-coding genes show reproducible bimodal patterns in the level of their expression in sister blastomeres (i.e. one has high expression, the other has low), some bimodally expressed genes show correlation or anti-correlation with other genes, suggesting that the heterogeneity is not due to random transcriptional noise. For example, most blastomeres in 8-cell embryos are dominated either by *Carm1* or by *Cdx2* (Shi et al., 2015). Whether this heterogeneity is sufficient to control subsequent cellular behaviour needs to be tested in the future.

Although the studies described above indicate that early blastomeres are molecularly heterogeneous, it is important to note that most of these studies have been descriptive, and only a few tested the importance of heterogeneities in correlative overexpression experiments (Plachta et al., 2011; Torres-Padilla et al., 2007; Burton et al., 2013). In addition, these heterogeneities have not yet been independently confirmed by other laboratories, probably owing to technical difficulties or potential differences in mouse strains. Furthermore, whether the patterns of heterogeneity are reproducible in all embryos or only in a subset of embryos has not been fully explored. It is possible that individual embryos are unique in the degree, pattern and variety of heterogeneity that they exhibit. Directly monitoring this heterogeneity in live embryos (Plusa et al., 2005; Dietrich et al., 2015; Xenopoulos et al., 2015) and the development of more sophisticated gene manipulation strategies will be important for gaining a full understanding of the functional consequences of heterogeneity in early blastomeres.

The generation of outer and inner cells: compaction, polarization and asymmetric division

At the 8-cell stage, two key morphogenetic processes take place: compaction and polarization. Early 8-cell blastomeres are spherical and morphologically distinguishable before compaction. Through compaction, they flatten against each other and minimize the total surface area of the embryo, making each blastomere morphologically indistinguishable (Ducibella and Anderson, 1975). Studies have shown that E-cadherin and β-catenin are essential for compaction and blastocyst formation (Ducibella, 1980; Hyafil et al., 1980; Shirayoshi et al., 1983; Larue et al., 1994; de Vries et al., 2004). Embryos completely lacking the E-cadherin-encoding gene *Cdh1* can specify the TE lineage but cannot form a TE epithelium or the ICM (de Vries et al., 2004; Stephenson et al., 2010). Given that E-cadherin plays a role in cell adhesion, it was classically thought that compaction is initiated by changes in cell adhesion to increase cell-cell contact (Fig. 3A). Although the exact cellular mechanism by which this occurs remains unknown, two recent studies have begun to shed light on our view of the compaction process (Fig. 3A). Using live imaging techniques in 8-cell embryos, one study identified CDH1-dependent filopodia formation in tight coordination with cell flattening (Fierro-González et al., 2013). This study also showed that the overexpression of myosin X is sufficient to trigger the formation of the CDH1-dependent filopodia to induce precocious compaction at the 4-cell stage. Laser ablation of these filopodia prevents blastomere

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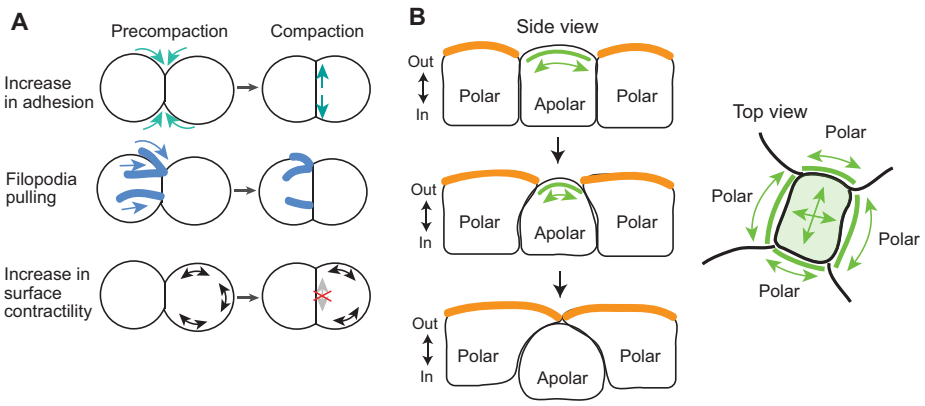

Fig. 3. Morphogenetic processes controlling compaction and cell allocation. (A) Models for compaction at the 8-cell stage. In the classical model (top), an increase in cell adhesion triggers an increase in cell-cell contacts (green arrows). In a second model (middle), CDH1-dependent filopodia (blue) create the pulling force (blue arrows) for cell flattening. In the final model (bottom), an increase in surface contractility at the medium-cell interface (double-headed arrows) controls compaction. In this model, CDH1-mediated cell-cell contacts prevent an increase in contractility at the contacts. (B) The apical constriction of cells can result in the outer/inner positioning of polar/apolar cells. After asymmetric divisions, some apolar cells are present in an outer position (side view, top). The surface of such apolar cells exhibits a higher level of actomyosin contractility to initiate apical constriction (green double-headed arrows). This increases the intracellular pressure of the apolar cell to initiate curvature formation at cell-cell contacts with surrounding polar cells (side view, middle). The size of the non-contacting surface gradually becomes smaller, both by apical constriction and by shrinkage of the gap between the surrounding polar cells through an increase in actomyosin contractility at the edge of the polar cells (top view). The apolar cell then becomes fully internalized and the gap between the polar cells is closed (side view, bottom). Apical domains of polar cells are shown in orange.

flattening and thus blocks compaction, suggesting that the filopodia generate the mechanical force to change cell shape during compaction. By contrast, a separate study showed that compaction is driven primarily by an increase in tension at the cell-medium interface, i.e. at the embryo surface (Maître et al., 2015), differing from a previous model that hypothesized that adhesion molecules generate the initial forces driving compaction. This study instead suggests that CDH1-mediated cell-cell contact acts to prevent an increase in surface contractility at cell-cell contacts (Fig. 3A) to facilitate compaction (Maître et al., 2015).

At the 8-cell stage, blastomeres also undergo polarization, whereby their apical domains are established. Blastomeres from 8-cell embryos can polarize without cell-cell contacts, which is in clear contrast to tissue culture cells (Johnson and Ziomek, 1981b; Stephenson et al., 2010; Anani et al., 2014). The apical domain is enriched for microvilli, F-actin and an evolutionarily conserved apical protein complex containing PAR3 (PARD3), PAR6 (PARD6) and atypical protein kinase C (aPKC) (Yamanaka et al., 2006). This apical protein complex plays important roles in the formation of the blastomere apical domain (Plusa et al., 2005; Alarcon, 2010). Rho GTPase activity and microtubule dynamics are also required for initiating apical domain formation, but how they are linked to the apical protein complex is unknown (Clayton et al., 1999; Houliston et al., 1989; Kono et al., 2014).

After polarization and compaction, each blastomere in the 8-cell embryo divides either symmetrically (such that both daughter cells inherit part of the apical domain, resulting in two polar cells) or asymmetrically (such that only one daughter cell inherits the apical domain, resulting in one polar and one apolar cell). Although original studies in isolated 8-cell blastomeres defined the division types as inheritance of the apical domain (Johnson and Ziomek, 1981a,b), this was not directly examined in intact embryos because of technical limitations at that time to the identification of sister pairs after divisions. As the apical domain forms at the surface of the embryo, it was thus simply proposed that the division angle regulates the inheritance of the apical domain and hence the fate of daughter cells, i.e. if the division is planar, aligned along the embryo surface, then it will be symmetric, whereas if it is orthogonal then the division will be asymmetric (Johnson and McConnell, 2004; Yamanaka et al., 2006). Using live imaging microscopy techniques, several recent studies have examined the relationship between division angles and cell position (Sutherland et al., 1990; Watanabe et al., 2014; McDole et al., 2011; Yamanaka et al., 2010). The overall conclusion of these studies is that the division angles in intact embryos are not only planar or orthogonal but are also oblique with respect to the embryo surface, and are thus not a simple predictor of symmetric versus asymmetric divisions or final cell position.

Interestingly, some cells placed on the surface of the embryo after the 8-cell division can internalize to take an inner position (Sutherland et al., 1990; Watanabe et al., 2014; McDole et al., 2011; Yamanaka et al., 2010). More recently, two studies demonstrated that this internalization is mediated by active cellular behaviours (Anani et al., 2014; Samarage et al., 2015). One study focused on inheritance of the apical domain after the 8-cell division and demonstrated that outer apolar cells are actually internalized instead of adopting polarity in intact embryos or in isolated blastomeres (Anani et al., 2014). Enrichment of phospho-myosin was observed at the non-contact surface of the outer apolar cells, suggesting that the internalization process is regulated by increased cortical actomyosin contractility. The second study directly observed the internalization process in intact embryos and demonstrated that apical constriction is a driving force of this internalization, although the identity of internalizing cells was not specified (Samarage et al., 2015). Together, these studies suggest that symmetric/asymmetric divisions in this context should be defined by inheritance of the apical domain rather than by division angles, and that apical constriction at the non-contact surface of outer apolar cells initiates apolar cell internalization to establish the outer/inner configuration of polar/apolar cells (Fig. 3B).

Finally, although it is evident that the frequency of asymmetric divisions defines the proportion of TE/ICM cells in the embryo, how exactly this frequency is regulated is unclear. There is wide variation in the frequency of asymmetric divisions during the 8- to

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16-cell transition (Fleming, 1987; Anani et al., 2014). In some 8-cell embryos, all blastomeres divide asymmetrically, generating 8 polar and 8 apolar cells at the 16-cell stage (Anani et al., 2014), whereas other embryos may only exhibit one or two asymmetric divisions. The frequency can be changed in isolated or paired 8-cell blastomeres (Johnson and Ziomek, 1981a; Johnson et al., 1986; Pickering et al., 1988), suggesting context-dependent regulation of the choice between asymmetric and symmetric divisions (e.g. via cell-cell contact) rather than being predetermined. Various interdependent factors, such as the size of the apical domain, nuclear position and cell-cell contact, also appear to affect the frequency of asymmetric division (Johnson and Ziomek, 1983; Johnson et al., 1986; Anani et al., 2014; Ajduk et al., 2014). Interestingly, it is known that the embryo has compensation mechanisms: if the number of asymmetric divisions is low during the 8- to 16-cell transition, more outer cells at the 16-cell stage divide asymmetrically in the next division to compensate the number of inner cells (Fleming, 1987). The regulation of the frequency of asymmetric division, and the subsequent adjustment of the number of inner cells in an embryo, are thus open questions for future studies.

The regulation of lineage-specific gene expression in the TE and ICM

CDX2 is a key transcription factor that is expressed specifically in the TE and is essential for functional TE formation (Strumpf et al., 2005). In *Cdx2* zygotic mutants, which form morphologically normal early blastocysts, ICM-specific genes such as *Oct4* and *Nanog* are ectopically expressed in the TE, suggesting CDX2-dependent transcriptional repression of *Oct4* and *Nanog*. Additionally, CDX2 directly interacts with OCT4 to suppress its transcriptional activity during early TE formation (Niwa et al., 2005). Since CDX2 overexpression in embryonic stem cells (ESCs) is sufficient to convert them to trophoblast stem cells, a number of studies have aimed to identify what controls CDX2 expression.

The presence of the apical domain is tightly correlated with enhanced CDX2 expression (Ralston and Rossant, 2008; Stephenson et al., 2010). The apical localization of *Cdx2* mRNA and its asymmetric inheritance by polar cells during asymmetric division is proposed to be involved in establishing TE-specific CDX2 expression (Jedrusik et al., 2010; Jedrusik et al., 2008; Skamagki et al., 2013). Precisely when CDX2 acts, however, remains unclear: some studies suggest that maternal CDX2 is required for proper cell cycle progression and cell survival (Jedrusik et al., 2015); however, a separate study has shown that there is no clear contribution of maternal CDX2 to lineage specification and that maternal CDX2 is dispensable for preimplantation development (Blij et al., 2012). What causes the discrepancy in these two studies is not known, but differences in mouse strains and/or *in vitro* embryo culture conditions might have affected their experimental outcomes.

Data also suggest that the Hippo/YAP signalling cascade plays a central role in TE/ICM lineage specification, particularly for TE-specific gene expression (Fig. 4). The Hippo/YAP signalling cascade was originally identified in *Drosophila* as a growth control pathway, and it is now recognized as a well-conserved pathway in mammals (Pan, 2010; Yu et al., 2015). This signalling cascade is used to sense cellular environments such as cellular density, position and the stiffness of extracellular matrices. The involvement of this signalling cascade in TE/ICM specification was first identified in embryos lacking *Tead4*, which encodes a TEAD family DNA-binding protein. In *Tead4* null embryos, a functional

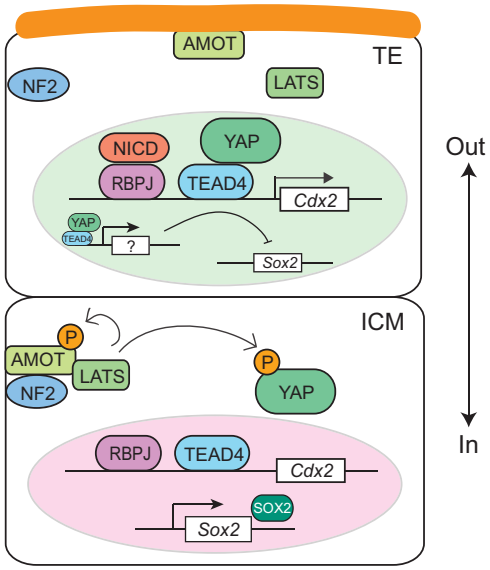

Fig. 4. Signalling cascades controlling TE and ICM segregation. In a TE cell, non-phosphorylated AMOT localizes to the apical domain (orange) and does not form an active complex with NF2 and LATS kinases. Non-phosphorylated YAP translocates into the nucleus to bind TEAD4 and activate target genes such as *Cdx2*. In parallel, Notch signalling (NICD and RBPJκ) is activated to contribute to the activation of *Cdx2* expression, while *Sox2* expression is suppressed by unknown Hippo signalling-dependent mechanisms. In an ICM cell, however, AMOT is phosphorylated and forms an active complex with NF2 and LATS kinases to phosphorylate YAP. Phosphorylated YAP is sequestered in the cytoplasm, preventing *Cdx2* expression in the ICM.

TE is not formed and CDX2 expression is severely downregulated (Yagi et al., 2007; Nishioka et al., 2008). A subsequent study demonstrated that YAP (YAP1) acts as the transcriptional activator of TEAD4 for upregulating TE-specific genes (Nishioka et al., 2009). YAP can shuttle between the nucleus and cytoplasm in a phosphorylation-dependent manner. In the TE, YAP translocates into the nucleus to activate TE-specific genes such as *Cdx2* and *Gata3* (Nishioka et al., 2009; Ralston et al., 2010), whereas in the ICM YAP is phosphorylated by LATS kinases leading to its cytoplasmic sequestration and extrusion from the nucleus. This differential activity of YAP/Hippo signalling is only required for a short period of time for establishing the stable, distinct gene expression patterns in the TE and ICM (Lorthongpanich et al., 2013). Furthermore, although modulation of the YAP/Hippo signalling cascade is sufficient to disengage CDX2 expression from outer TE cells, it does not change cell position and the status of cell polarity.

More recent studies have identified a role for NF2 (Merlin) – another component of the Hippo pathway – in establishing TE fate. NF2 is a FERM domain protein that acts upstream of LATS kinases, as indicated by genetic analyses in *Drosophila* (Maitra et al., 2006; Hamalatoglu et al., 2006). In maternal zygotic *Nf2* mutant embryos, nuclear YAP localization and CDX2 expression are not restricted to outer/TE cells but are also evident in inner/ICM cells (Cockburn et al., 2013). Although NF2 is evenly distributed at the cell cortex/membrane of all cells, it is only necessary in inner/ICM cells to activate Hippo signalling, i.e. for YAP phosphorylation.

Similarly, junction-associated scaffolding Angiomotin (AMOT) family proteins are also required for the activation of Hippo signalling in inner/ICM cells but show differential subcellular localizations in outer/TE and inner/ICM cells (Hirate et al., 2013;

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Leung and Zernicka-Goetz, 2013). In outer/TE cells, AMOT is localized at the apical domain but excluded from the basolateral domain, whereas in inner cells the serine 176 (S176) of AMOT is phosphorylated and AMOT is distributed throughout the plasma membrane (Hirate et al., 2013). The phosphorylation of AMOT on S176 prevents its association with F-actin in *in vitro* biochemical assays and in tissue culture cells (Mana-Capelli et al., 2014; Chan et al., 2013). It was also shown that a phosphomimetic form of AMOT, which acts as a constitutively active form, is distributed in the cytoplasm with strong punctate signals, whereas a non-phosphorylated form of AMOT colocalizes with enriched F-actin and has a very weak ability to activate Hippo signalling (Hirate et al., 2013). S176 phosphorylation enhances AMOT interactions with LATS kinases to facilitate YAP phosphorylation. These results suggest that in outer cells AMOT is sequestered at the apical domain, whereas S176-phosphorylated AMOT in inner cells can form an active complex with LATS kinases (and likely with NF2) at cell-cell junctions.

Interestingly, the YAP/Hippo signalling cascade regulates not only TE-specific genes but also the ICM-restricted expression of SOX2, which is one of earliest markers of inner cells. Indeed, preventing YAP nuclear localization in outer TE cells is sufficient to induce ectopic SOX2 expression (Wicklow et al., 2014). The suppression of SOX2 expression in the TE is CDX2 independent and thus differs from NANOG and OCT4 regulation (Strumpf et al., 2005). This indicates that YAP activates a distinct repression mechanism in outer cells for the regulation of SOX2 expression (Wicklow et al., 2014).

Although the Hippo/YAP signalling cascade appears to play a central role in TE/ICM specification, the requirement for TEAD4 during TE formation can be bypassed when *Tead4* null embryos are cultured in low O_2 conditions, which reduces oxidative stress and mimics the *in vivo* situation (Kaneko and DePamphilis, 2013). This suggests that YAP can have different DNA-binding partners to activate target genes (Imajo et al., 2015) or that parallel signalling cascades could compensate for the loss of TEAD4 in low O_2 conditions. The analysis of TE-specific enhancers of *Cdx2* reveals that Notch signalling cooperates with Hippo/YAP signalling (Rayon et al., 2014) for full activation of *Cdx2* expression. It will be interesting to examine whether Notch signalling can compensate for TEAD4 function in low O_2 conditions or whether other mechanisms are involved.

Tipping the balance between EPI and PE fates

Shortly after their internalization, ICM cells differentiate into EPI and PE lineages. This is a multistep process that starts with binary EPI/PE specification and is followed by lineage maturation (i.e. the establishment of specific gene networks and differentiation within each lineage) and cell sorting to form two distinct compartments – an EPI cluster and a PE epithelium.

The transcription factors NANOG and GATA6 are the earliest markers for the EPI and PE, respectively (Chazaud et al., 2006; Kurimoto et al., 2006). Both are present in all blastomeres at the 8-cell stage but, from the ∼32-cell stage, ICM cells choose to express either NANOG, leading to an EPI fate, or GATA6, leading to a PE fate (Plusa et al., 2008; Guo et al., 2010). This gives rise to a mutually exclusive 'salt and pepper' expression pattern of NANOG and GATA6 by embryonic day (E) 3.75. Cell lineage tracing and live cell tracking analyses (Chazaud et al., 2006; Kurimoto et al., 2006; Plusa et al., 2008; Meilhac et al., 2009; Xenopoulos et al., 2015) show that this process occurs in individual ICM cells asynchronously, spanning from ∼E3.0-E3.75 (Gerbe et al., 2008; Plusa et al., 2008; Bessonnard et al., 2014). Therefore, a few cells coexpressing both transcription factors can still be identified at ∼E3.75 in some embryos, and the emergence of $NANOG^{+}$/$GATA6^{-}$ EPI cells is currently the first known sign of the specification process.

Since *Nanog* and *Gata6* expression levels are relatively high from the 8-cell stage (Guo et al., 2010), the mechanism controlling the specification process is likely to act through selective mRNA decay, rather than via a selective increase in transcription. Indeed, at the protein level, EPI/PE specification can be visualized by a combination of the increased expression of one of the transcription factors and the fading of the other (Guo et al., 2010; Bessonnard et al., 2014), revealing slightly different dynamics between the RNAs and proteins. However, mutual repression between the two transcription factors also appears to exist: in *Gata6* mutants, all ICM cells uniformly express NANOG without any PE markers, indicating the acquisition of an EPI fate (Bessonnard et al., 2014; Schrode et al., 2014), and, conversely, in *Nanog* mutants all ICM cells express GATA6 (Frankenberg et al., 2011). NANOG and GATA6 might directly repress each other's transcription, as suggested by their binding sites identified in chromatin immunoprecipitation studies of ESCs (Singh et al., 2007) and of induced extra-embryonic endoderm (iXEN) cells (Wamaitha et al., 2015).

A series of studies has revealed that FGF signalling plays an essential role during PE lineage formation (Fig. 5A). *Fgf4* specifically labels EPI cells during blastocyst formation (Kurimoto et al., 2006; Guo et al., 2010; Frankenberg et al., 2011; Ohnishi et al., 2014) and is not expressed in *Nanog* mutants (Frankenberg et al., 2011). *Fgfr2* is expressed in all early ICM cells at E3.25 before its restriction to PE cells by E3.5, suggesting that all early ICM cells are capable of responding to FGF ligands (Ohnishi et al., 2014; Boroviak et al., 2015). Blocking FGF signalling is sufficient for all ICM cells to adopt an EPI fate (Chazaud et al., 2006; Nichols et al., 2009; Yamanaka et al., 2010; Kang et al., 2013; Krawchuk et al., 2013), whereas the addition of excess FGF4 is sufficient to differentiate all ICM cells into PE (Yamanaka et al., 2010). Interestingly, GATA6 is expressed until the early blastocyst stage in *Fgf4* mutants (Kang et al., 2013; Krawchuk et al., 2013), meaning that FGF4 is required for the salt-and-pepper patterning but that other factors regulate the initial expression of GATA6. In addition, FGF4 administration can repress NANOG expression without GATA6 (Bessonnard et al., 2014), while blocking FGF signalling can repress GATA6 expression in the absence of NANOG (Frankenberg et al., 2011). However, these inhibitions must be initiated before NANOG and GATA6 start to be expressed, i.e. around compaction stage, as their expression becomes insensitive at later stages (Frankenberg et al., 2011; Bessonnard et al., 2014; Schrode et al., 2014). Together, these findings highlight that FGF signalling, NANOG and GATA6 form a regulatory network that drives the specification process. The proportion of EPI/PE cells within the ICM is therefore regulated by the relative levels of the network components in individual cells (Yamanaka et al., 2010; Kang et al., 2013; Krawchuk et al., 2013; Bessonnard et al., 2014; Schrode et al., 2014; Schröter et al., 2015). For example, reducing the levels of *Fgf4* expression increases the number of NANOG-expressing EPI cells (Kang et al., 2013; Krawchuk et al., 2013). In *Gata6* heterozygous embryos, the number of PE cells specified is reduced and compensated by an equivalent number of EPI cells (Bessonnard et al., 2014; Schrode et al., 2014). *Gata6* heterozygous embryos also show precocious specification of EPI cells, which is likely to be due to reduced inhibition of *Nanog*

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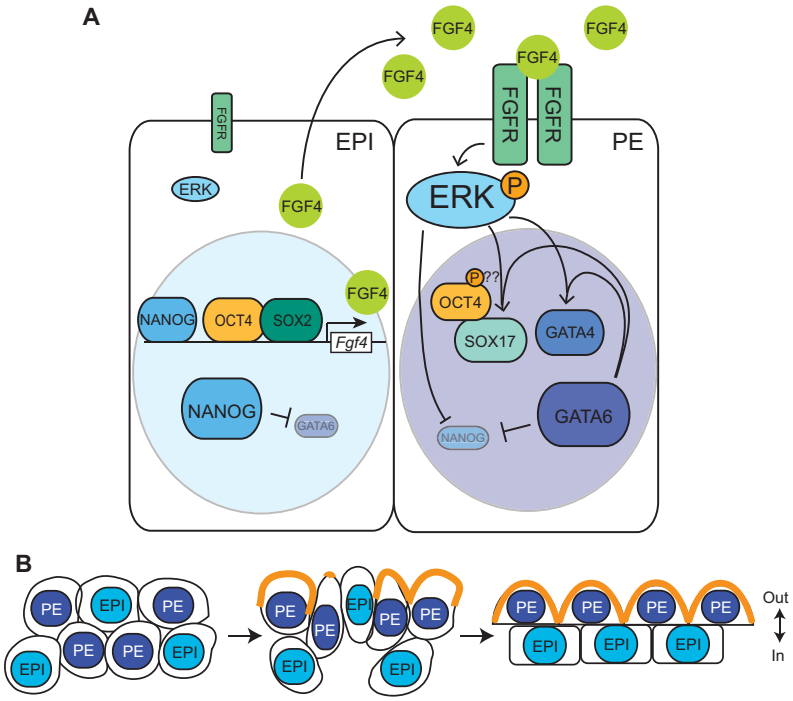

Fig. 5. The regulation of EPI/PE formation. (A) In EPI cells (left), a higher level of NANOG in collaboration with OCT4 and SOX2 upregulates *Fgf4* expression and represses GATA6 expression as well as the level of FGF signalling, as indicated by the low level of phosphorylated ERK. In PE cells (right), FGF4 secreted from EPI cells activates FGF signalling. This higher FGF signalling upregulates GATA6 expression and represses NANOG expression. FGF signalling and GATA6 cooperatively activate downstream genes, such as *Sox17* and *Gata4*. (B) Following their specification, EPI (light blue) and PE (dark blue) cells segregate and the PE undergoes epithelization. Once PE progenitors hit the ICM surface, they initiate formation of the apical domain (orange) to prevent further cell mixing and a PE epithelium forms around the clustered EPI cells.

expression (Bessonnard et al., 2014; Schrode et al., 2014), although removing just one allele of *Nanog* does not impact PE/EPI specification (Frankenberg et al., 2011; Miyanari and Torres-Padilla, 2012). More recently, a study using an *in vitro* tuneable GATA expression system also revealed GATA dose-dependent binary selection of PE fate (Schröter et al., 2015).

The EPI/PE gene regulation network has recently been transposed into a mathematical model (Bessonnard et al., 2014) to examine how the mutually exclusive salt-and-pepper pattern is established in the ICM. This modelling takes into consideration the mutual repression of NANOG and GATA6 coupled to self-activation, and FGF signalling positively regulating GATA6 while inhibiting NANOG. With these initial setup conditions, the model is sufficient to recapitulate the *in vivo* developmental process. Simulations show that one or a few cells in the early ICM promote NANOG expression leading to an increase in FGF4 secretion. This higher local concentration of extracellular FGF4 induces PE fate in neighbouring cells, suggesting that individual ICM cells can asynchronously adopt an EPI or PE fate based on their local FGF4 concentration. The asynchronous EPI/PE specification events observed in embryos can thus be explained by the heterogeneous propagation of FGF4 paracrine activity. In addition, ICM cells express FGFR2 homogeneously at E3.25 (Ohnishi et al., 2014), suggesting that differences in ERK (MAPK) activity are initiated by differences in local FGF4 availability and are later amplified by NANOG-mediated downregulation of FGFR2 in EPI cells. Another refined model recapitulating the binary choice has been proposed based on an *in vitro* GATA ectopic expression system in ESCs (Schröter et al., 2015). This model considers the mutual repression of NANOG and GATA6, but without the positive-feedback enforcement loops, and the sole inhibition by FGF signalling of NANOG expression. Indeed, in this ESC system, a *Gata6* transcriptional reporter is insensitive to ERK inhibition upon GATA4 induction (Schröter et al., 2015). It will thus be interesting to establish whether FGF4 acts on NANOG only or also on GATA6 expression at both the RNA and protein levels *in vivo*.

Both mathematical models predict that, once they have made their choice, ICM cells do not change their fate in intact embryos (Bessonnard et al., 2014; Schröter et al., 2015). This was previously suggested by cell lineage tracing experiments (Chazaud et al., 2006; Meilhac et al., 2009) and confirmed by live cell tracking with a *Nanog-GFP* reporter (Xenopoulos et al., 2015). Interestingly, however, it was shown (Xenopoulos et al., 2015) that a few cells expressing low levels of *Nanog-GFP* until a relatively late blastocyst stage can strongly upregulate *Nanog-GFP* expression eventually, which is suggestive of fate reversion. The relative levels of NANOG and GATA6 were not examined in this study and it might be the balance between the two transcription factors that is important rather than their absolute amounts. It thus remains uncertain whether these cells truly undergo a fate reversion or correspond to late specifying cells that exhibit low levels of NANOG until ∼E3.75 (Bessonnard et al., 2014). It should also be noted that, although most ICM cells are specified by E3.75, they still retain the plasticity to change into an alternative fate, either through the modulation of FGF signalling (Nichols et al., 2009; Yamanaka et al., 2010) or by changing their neighbours through cell transplantation (Grabarek et al., 2012). This plasticity asynchronously disappears at ∼E4.0 (Yamanaka et al., 2010; Grabarek et al., 2012) and is first lost in EPI cells (Grabarek et al., 2012), probably reflecting their earlier specification (Bessonnard et al., 2014).

Despite these insights into the FGF-NANOG-GATA6 network, the events that trigger the early differences in ICM cells are not known. The earliest heterogeneity observed in ICM cells is bimodal *Fgf4* expression at E3.25 (Kurimoto et al., 2006; Guo et al., 2010; Ohnishi et al., 2014). Several hypotheses have been proposed to explain this induction of the salt-and-pepper pattern (reviewed by Hermitte and Chazaud, 2014): (1) stochastic activation of gene expression reinforced by cell-to-cell differences in FGF secretion and signalling (Dietrich and Hiiragi, 2007; Ohnishi et al., 2014); (2) reduced phosphorylated ERK, or the presence/absence of another factor, in a few cells promotes NANOG

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expression that facilitates FGF4 secretion (Bessonnard et al., 2014); (3) cell division history bias, based either on *Fgfr2* expression (Morris et al., 2010, 2013; Mihajlović et al., 2015) or the accumulation of inner cells expressing FGF4 (Krupa et al., 2013). None of these hypotheses has yet been discarded or favoured; however, mathematical simulations mimicking high transcriptional noise of all the core factors indicate that this internal noise is unlikely to be the initiating mechanism (Bessonnard et al., 2014; DeMot et al., 2016). Thus, although the initial step is known to be the commitment of one or a few ICM cells to an EPI fate at ∼E3.0, always preceding PE specification in other cells (Bessonnard et al., 2014; Xenopoulos et al., 2015), we still do not know why FGF4 and/or NANOG expression increases in just a few ICM cells.

The regulation of gene expression during PE maturation

After specification, PE cells turn on mature PE markers such as SOX17, GATA4, DAB2 and PDGFRα. At this stage, any experimental condition or manipulation that decreases PE cell numbers is not compensated by an increase in EPI cells, and vice versa, because both EPI and PE cells are already specified, i.e. their fate is already 'locked-in'. A number of studies have revealed that, although GATA6 plays a key role in PE specification, it appears to act in concert with other signalling factors and pathways during the subsequent stages of PE maturation (Fig. 5A). For example, although GATA6 is expressed in all ICM cells of *Nanog* mutant embryos, the expression of SOX17 and GATA4 is severely downregulated (Mitsui et al., 2003; Silva et al., 2009; Messerschmidt and Kemler, 2010), demonstrating that GATA6 expression alone is not sufficient to initiate PE maturation. This downregulation of SOX17 and GATA4 is likely to be due to lower availability of FGF4 (Messerschmidt and Kemler, 2010; Frankenberg et al., 2011). Indeed, *Fgf4* expression is severely downregulated in *Nanog* mutant EPI cells, and FGF4 supplementation can rescue SOX17 and GATA4 expression (Frankenberg et al., 2011). Thus, FGF signalling is required for both the initial specification of the PE, to tilt the balance between NANOG and GATA6, and for its further maturation. Are these two steps concomitant or sequential? There is no clear answer at this point, but this raises the question of whether the ICM cells of *Nanog* null embryos should be considered as PE cells or as progenitor cells lacking NANOG expression and expressing GATA6 only. In addition, FGF4 administration cannot induce SOX17 and GATA4 expression in *Gata6* mutants (Bessonnard et al., 2014; Schrode et al., 2014), highlighting that both the activation of FGF signalling and GATA6 expression are required for the expression of mature PE markers.

Another EPI-specific marker, SOX2, is also necessary for PE maturation but is not required for EPI/PE specification. Indeed, *Sox2* mutant embryos are able to generate NANOG-expressing and GATA6-expressing cells in the correct proportions; however, the level of GATA6 expression is lower and there are fewer cells expressing SOX17 at E3.75 (Wicklow et al., 2014). By E4.25, the number of SOX17-expressing cells is restored, suggesting that the expression of SOX17 is simply delayed in PE cells in these mutants. FGF4 administration can rescue this phenotype, implying reduced expression of FGF4 in *Sox2* mutants. The delayed SOX17 expression in these mutants is probably the consequence of a slower accumulation of FGF4, which finally reaches the required levels to induce SOX17. It could also be due to the low levels of GATA6, the expression of which requires FGF4 to be maintained at high levels. *Sox2* null embryos also illustrate the sequential steps of PE differentiation, starting with PE specification (downregulation of NANOG) and followed by PE maturation (the induction of PE-specific genes such as *Sox17*).

The pluripotency factor OCT4 is homogeneously expressed in ICM cells but is then downregulated in PE cells at ∼E4.5, while being maintained in EPI cells until their differentiation during gastrulation. OCT4 is required to maintain ESC pluripotency and prevents their differentiation into TE, while high levels of OCT4 can promote ESC differentiation into PE (Niwa et al., 2000). In *Oct4* mutant embryos, NANOG-expressing and GATA6-expressing cells emerge in the correct proportions but GATA6 expression is not maintained and very few cells express SOX17, partly owing to impaired FGF4 production (Frum et al., 2013; Le Bin et al., 2014). SOX2-OCT4 complexes can directly activate *Fgf4* expression *in vitro* (Ambrosetti et al., 2000), underlining the cooperation between SOX2, OCT4 and NANOG to generate enough FGF4 for the correct production of mature PE cells, with regard to both time and numbers. In addition, chimera experiments demonstrate that OCT4 is required cell-autonomously for the maintenance of PE cells by sustaining SOX17 and SOX7 expression. Surprisingly, the administration of either FGF signalling inhibitors or FGF4 to *Oct4* mutants fails to inhibit GATA6 or NANOG expression, respectively (Frum et al., 2013). As OCT4 can be phosphorylated by ERK (Brumbaugh et al., 2012), it is tempting to speculate that phosphorylated OCT4 prevents NANOG expression in PE cells, while unphosphorylated OCT4 inhibits GATA6 in EPI cells. Thus, OCT4 would cell-autonomously reinforce each lineage identity in cooperation with FGF signalling (Fig. 5A) (Frum et al., 2013). This is also evidenced by the partnership of OCT4 with either SOX2 or SOX17, targeting specific genes in each lineage (Aksoy et al., 2013).

BMP is another signalling pathway involved in early lineage differentiation. BMP4 is expressed in the EPI of the early blastocyst (Coucouvanis and Martin, 1999; Guo et al., 2010; Graham et al., 2014; Boroviak et al., 2015). The overexpression of a dominant-negative form of BMPR2 in one blastomere at the 2-cell stage reduces the PE contribution of the progeny of this blastomere. Blocking BMP signalling with specific inhibitors, such as NOGGIN and Dorsomorphin, severely reduces the number of PE cells via cell death (Graham et al., 2014). Since the number of NANOG-expressing cells is not changed, EPI/PE specification is not affected, revealing a defect in the survival of the specified PE cells in this context. Cell survival in the PE also appears to be controlled by PDGF signalling. A recent single-cell transcriptome analysis reveals that, like FGF4 and BMP4, PDGFA is specifically produced by EPI cells (Boroviak et al., 2015). By contrast, PDGFRα is expressed specifically in PE cells and its loss induces PE cell death (Artus et al., 2013).

Together, these studies highlight that several signalling pathways are involved in PE maturation and survival, and seem to act concomitantly, possibly to provide robustness. In addition, although several of these pathways influence cell survival, the extent to which cell death actively contributes to lineage differentiation remains unclear. Apoptosis rarely occurs during preimplantation, except during a short time window at ∼E3.75-E4.0. During this time, when cell sorting occurs (discussed below), ∼10% of ICM cells are eliminated (El-Shershaby and Hinchliffe, 1974; Copp, 1978). Live imaging movies following EPI or PE cells show that both cell types can undergo apoptosis during this time window (Plusa et al., 2008; Xenopoulos et al., 2015). Currently, EPI and PE cells cannot be examined simultaneously in single embryos owing to incompatibility between reporter lines, but it would be interesting to examine the timing and proportion of apoptosis in each lineage

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within single embryos. Dying cells could be non-specified precursors expressing NANOG and GATA6 at equal levels, as proposed in one mathematical model (Bessonnard et al., 2014). Alternatively, apoptosis could occur to eliminate misplaced cells following the sorting/segregation of PE and EPI (Plusa et al., 2008).

Cell sorting and epithelium formation

Owing to their specification mechanism (the salt-and-pepper patterning), EPI and PE cells must undergo cell sorting to form two distinct tissues (Fig. 5B). This process is not yet well understood, but studies are beginning to provide insights into the factors that regulate sorting. For example, lineage-specific transcription factors have been shown to modulate cell type-specific mechanical characteristics, including adhesion properties, as illustrated by the specific expression of laminin 1 and collagen IV in PE cells (Gerbe et al., 2008; Niakan et al., 2010). The acquisition of polarity may also influence cell position and sorting. The apical localization of DAB2 and LRP2 is the first sign of cell polarization at the surface of PE cells (Gerbe et al., 2008). Subsequently, apical aPKC localization is observed (Saiz et al., 2013). The establishment of cell polarity, through aPKC and DAB2 pathways, is required for epithelium formation but also to prevent cell remixing (Yang et al., 2002; Saiz et al., 2013). Thus, a polarity-dependent anchoring mechanism could lead to the separation of PE cells from the EPI cluster (Moore et al., 2009). However, since some PE cells need to cross two or three cell layers to reach the ICM surface, it is possible that other mechanisms, such as directional movement, are necessary to facilitate the sorting (Meilhac et al., 2009). Indeed, experiments using a mix of undifferentiated and differentiated ESCs have shown that differential adhesion is probably not the driving force for cell sorting (Moore et al., 2009). Mathematical modelling also shows that differential adhesion alone is not sufficient to sort cells in this context (Krupinski et al., 2011).

Although inhibiting microtubule activity does not prevent sorting, cytochalasin D treatment, which affects actin polymerization, does perturb cell movements (Meilhac et al., 2009). The actomyosin network is a major contributor to cortical tension, which could differ according to cell type and create cell sorting, as has been shown in zebrafish (Krieg et al., 2008). The directionality of sorting could be influenced by the differential pressure between the blastocoel and the polar TE, which would facilitate movement of the more elastic cells, or the less adhesive ones, toward the lowest tension pole (Krupinski et al., 2011). Thus, cell sorting due to cortical tension and/or differential adhesion, combined with epithelial anchoring through cell polarization, could provide a working model to explain cell sorting and its maintenance during blastocyst development.

Conclusions

Despite the advances described in this Review, we still do not fully understand how the first differences observed in early blastomeres emerge, whether they can control subsequent molecular and cellular events, and whether they can regulate gene expression to control lineage specification. The lack of stereotypic patterns, as well as the asynchronicity observed, complicate interpretations of some analyses and challenge our comprehension. In addition, maternal contributions cannot be ignored. However, it is becoming clear that the complexity of mouse preimplantation development cannot be analyzed with simple static analyses alone. Ideally, monitoring transcriptome dynamics in single cells in live embryos, combined with the tracking of individual cellular behaviours, would provide the most useful information for understanding the self-organizing properties of the mouse embryo. Although we are far from attaining this approach, recent developments in imaging techniques and in biophysics approaches have considerably increased our knowledge. Computational simulations have also proven to be a powerful approach, but require further adaptions to factor in the complexity of the molecular and cellular interactions that are occurring. A combination of sophisticated new technology with traditional genetic approaches and embryo manipulations will hopefully take us to a new level of understanding mammalian preimplantation development.

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Competing interests

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