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Anatomy of a blastocyst: cell behaviors driving cell fate choice and morphogenesis in the early mouse embryo

Nadine Schrode^{1,*}, Panagiotis Xenopoulos^{1,*}, Anna Piliszek^{1,2}, Stephen Frankenberg³, Berenika Plusa⁴, and Anna-Katerina Hadjantonakis^{1,#}

¹ Developmental Biology Program, Sloan-Kettering Institute, New York, USA. ² Department of Experimental Embryology, Institute of Genetics and Animal Breeding, Polish Academy of Sciences, Jastrzebiec, Poland. ³ Department of Zoology, The University of Melbourne, Victoria 3010, Australia. ⁴ Faculty of Life Sciences, Manchester University, Manchester, UK.

Abstract

The preimplantation period of mouse early embryonic development is devoted to the specification of two extra-embryonic tissues and their spatial segregation from the pluripotent epiblast. During this period two cell fate decisions are made while cells gradually lose their totipotency. The first fate decision involves the segregation of the extra-embryonic trophoblast (TE) lineage from the inner cell mass (ICM); the second occurs within the ICM and involves the segregation of the extra-embryonic primitive endoderm (PrE) lineage from the pluripotent epiblast (EPI) lineage, which eventually gives rise to the embryo proper. Multiple determinants, such as differential cellular properties, signaling cues and the activity of transcriptional regulators, influence lineage choice in the early embryo. Here, we provide an overview of our current understanding of the mechanisms governing these cell fate decisions ensuring proper lineage allocation and segregation, while at the same time providing the embryo with an inherent flexibility to adjust when perturbed.

Keywords

mouse embryo; preimplantation; morula; blastocyst; inner cell mass; epiblast; trophoblast; primitive endoderm; cell lineage commitment

A. LINEAGE SPECIFICATION IN THE PREIMPLANTATION EMBRYO

Preimplantation development is devoted to the segregation of embryonic and extraembryonic tissues

As in all amniotes, the initial stages of mouse development involve the allocation of embryonic as well as extraembryonic tissues that have a principal role in the transfer of nutrients to the embryo (reviewed in (Arnold and Robertson, 2009; Nowotschin and Hadjantonakis, 2010; Rossant and Tam, 2009; Zernicka-Goetz *et al.*, 2009)) (**Figure 1**). The first extraembryonic lineage to be specified – the trophoblast (TE) – contributes to the fetal portion of the placenta. The second extraembryonic lineage – the primitive endoderm (PrE) – gives rise to the parietal and visceral endoderm of the yolk sac as well as a subpopulation of cells in the early gut tube of the embryo (Kwon *et al.*, 2008). The PrE lineage is segregated from the epiblast (EPI) lineage within the inner cell mass (ICM) of the

Correspondence: Kat Hadjantonakis. hadj@mskcc.org. 1-212-639-3159 (Phone). 1-646-422-2355 (FAX)..

*These two authors made an equal contribution to the preparation of this review.

preimplantation embryo; the EPI lineage then gives rise to the embryo proper. Thus, mouse embryonic stem cells (mESCs) can be derived from and represent the EPI lineage *in vitro*. Stem cells can also be derived from the extraembryonic lineages PrE and TE, these being extraembryonic endoderm (XEN) cells and trophoblast stem (TS) cells respectively (reviewed in (Artus and Hadjantonakis, 2012)). Here, we review our current understanding of the mechanisms that are employed for cell fate determination and allocation to an embryonic or extraembryonic developmental pathway during mouse preimplantation development.

Regulative development

Unlike in the majority of metazoans, zygotes of eutherian mammals lack clearly localized determinants, related in large part to the absence of yolk. Since the pioneering study of Tarkowski and Wróblewska (Tarkowski and Wróblewska, 1967), it has been recognized that fate choices in the preimplantation mouse embryo are, at least in part, determined by cells detecting their relative position (inside-*versus*-outside) within the morula. Numerous subsequent studies demonstrated that early blastomeres (from 2-cell to 8-cell stages) are totipotent, meaning they can contribute cellular descendants to all embryonic and extraembryonic lineages. Indeed, single early blastomeres are able to support development to term (Tarkowski, 1959), but also chimeras formed by combining two or more early embryos, as first shown by Tarkowski and Mintz in the early 1960s, can result in development of fully functional individuals (Mintz, 1965; Tam and Rossant, 2003; Tarkowski and Wróblewska, 1967). It is now widely accepted that mouse development is a regulative process, during which the totipotency of each blastomere is gradually lost as embryonic and extraembryonic lineages become specified. Thus cell lineage specification and spatial segregation results in two cell fate decisions, which are generally believed to be sequential, during preimplantation development. The first involves the TE-*versus*-ICM choice, followed by the second involving the EPI-*versus*-PrE allocation within the ICM (Figure 1). In the following sections we review recent advances in understanding the mechanisms governing these two cell fate decisions.

B. FIRST FATE DECISION: TE *VERSUS* ICM CHOICE

TE differentiation is driven by cell position and cell polarity

A large number of studies over the past three decades have revealed many of the properties of early blastomeres, especially with respect to allocation of TE and ICM lineages. Until the 8-cell stage, all blastomeres have exposure to the outer surface of the embryo and are essentially equivalent in their totipotency. At the 8-cell stage each of these blastomeres acquires an apical-basal polarity concomitant with compaction, a morphogenetic process in which cell-cell contacts increase (Johnson and Ziomek, 1981). Compaction requires the presence of the homophilic adhesion molecule E-cadherin and results in the formation of an apical zone of microvilli and apical localization of molecules such as atypical protein kinase C (aPKC), the PAR (PARTitioning defective) proteins PAR3 and PAR6 and the actin-associated protein Ezrin (Dard *et al.*, 2001; Louvet *et al.*, 1996; Pauken and Capco, 1999, 2000; Plusa *et al.*, 2005; Reeve and Ziomek, 1981; Vinot *et al.*, 2005). During the next two rounds of cell division (from the 8-cell to 16-cell and 16-cell to 32-cell stages), blastomeres divide in either of two modes. Symmetric (also referred to as conservative) divisions, with the division plane perpendicular to the surface of the embryo, give rise to two polar daughter cells that both maintain an outer position and inherit part of the apical cytoplasm and membranes. Asymmetric (also referred to as differentiative) divisions, with the division plane parallel to the surface of the embryo, give rise to one polar outer cell, which inherits all of the apical cytoplasm of the parental cell, and one apolar inner cell. Thus two sub-populations of cells are generated that differ in their properties and position within the

embryo as a direct result of their mechanism of generation. Interestingly, Yamanaka and colleagues have recently suggested an alternative model for the allocation of the polar/outer *versus* apolar/inner cells, by proposing an engulfment mechanism promoting the internalization of apolar cells and segregating them from polar ones (Yamanaka *et al.*, 2010). This would result in apolar cells intrinsically taking up an inner position, regardless of their initial position after blastomere division. Indeed, this study proposed that differential cortical tension of daughter cells might be a contributing factor directing cellular position within the 16-cell embryo. Though an intriguing possibility, at present, one cannot exclude the possibility that these internalization events result from experimental perturbations of cells resulting from their microinjection or due to intrinsic strain differences.

During the fifth and sixth cell division cycles, outer cells exhibit an increasing maturation of epithelial properties. The assembly of cell-cell junctional complexes culminates in functional tight junction formation by the time cavitation is initiated, when embryos have approximately 28-33 cells (Smith and McLaren, 1977). Although the allocation of ICM *versus* TE fate occurs prior to cavitation (between the 8-cell and 32-cell stages), the specification of ICM and TE cell fate in the early blastocyst does not, however, appear to reflect their actual developmental potency. This is revealed by the fact that ICMs isolated from early blastocysts (corresponding to 32-cell to 64-cell stage) by immunosurgery (Solter and Knowles, 1975) can form blastocyst-like structures, indicating that early ICM cells retain the ability to respond to positional signals, polarize, and form a functional TE epithelium (Handyside, 1978; Hogan and Tilly, 1978; Rossant and Lis, 1979; Spindle, 1978; Stephenson *et al.*, 2010). This suggests that at the early blastocyst stage, simple exposure of ICM cells to the outside environment is sufficient to divert their fate towards different lineages and that these cells are still responsive to polarity cues. However, this plasticity disappears by the fully expanded blastocyst stage, corresponding to around the 100-cell stage (Dyce *et al.*, 1987; Gardner and Nichols, 1991).

It was initially proposed that cell position alone determines the TE *versus* ICM cell fate choice, reflecting an inside *versus* outside position within the morula during the symmetric/asymmetric divisions at the 8-to-16-cell and 16-to-32-cell stage transitions. This could explain observations from experiments where spatial rearrangements have an effect on cell fate (Hillman *et al.*, 1972; Tarkowski and Wroblewska, 1967). However, we now know that acquisition of cell polarity affects cell fate perhaps earlier than the emergence of inside and outside cells, as was first shown by Johnson and Ziomek (Johnson and Ziomek, 1981). Disruption of aPKC activity before compaction promotes internalization of blastomeres and their increased contribution to the ICM (Dard *et al.*, 2009; Plusa *et al.*, 2005). Moreover, Pard6b, a component of the partitioning defective (PAR)-aPKC complex was shown to directly affect TE specification and blastocyst formation (Alarcon, 2010). According to this cell polarity model, the differential polarity status of early blastomeres at the time of compaction provides the first cue towards the choice between TE and ICM.

The role of lineage specific transcriptional factors in TE versus ICM specification

Besides their differential polarity status, the outer and inner blastomeres generated at the 8-to-16-cell and 16-to-32-cell transitions also differ in the expression levels of some lineage-specific transcription factors (Guo *et al.*, 2010). This differential activity of genes in a lineage-specific manner is equally essential for both the TE *versus* ICM and the latter EPI *versus* PrE cell fate choices (**Figure 2**). One of the earliest events taking place during the first fate choice involves the expression of Cdx2 and suppression of the ICM-specific factors Nanog and Oct4, in TE precursor cells (Niwa *et al.*, 2005; Ralston and Rossant, 2008; Strumpf *et al.*, 2005).

Nanog, a variant homeodomain protein, is first expressed at the 8-cell stage and is thereafter maintained in the ICM and EPI at the blastocyst stage; however its expression declines when the embryo is close to implantation and becomes reestablished coincident with the onset of gastrulation and in the germline (Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Plusa *et al.*, 2008). Nanog was initially identified as a key factor required for ICM/EPI maintenance, although recent data suggest that it acts primarily in construction of ICM and germ cell states rather than in the maintenance of pluripotency (Chambers *et al.*, 2007; Messerschmidt and Kemler, 2010).

Oct4 (also known as Pou5f1) is a POU domain protein expressed in the oocyte and in all blastomeres of the cleavage stage embryo (Scholer *et al.*, 1989). In the mouse, Oct4 expression becomes restricted to the ICM during the blastocyst stage and later it is maintained exclusively in the epiblast (thus for longer than Nanog). Thereafter, its expression is strictly confined to the primordial germ cells, precursors of the gametes (Palmieri *et al.*, 1994). Oct4 is the key transcriptional determinant of the pluripotent state (Chambers and Smith, 2004). *Oct4* mutant embryos do form early blastocysts however they fail to develop an ICM, while inner blastomeres acquire a trophoblast character (Nichols *et al.*, 1998). Thus, Oct4 plays a role in suppressing differentiation of embryonic cells into trophectoderm by regulating the activity of the homeodomain transcription factor Cdx2 (Niwa *et al.*, 2005). Expression of another pluripotency-associated factor, Sox2, overlaps with that of Oct4 in early blastomeres; both factors are required for the specification of ICM (and later EPI) lineages but unlike Oct4, Sox2 is present in TE derivatives at postimplantation stages even though it is not expressed in the trophoblast cells of the blastocyst (Avilion *et al.*, 2003).

The Caudal-related homeobox 2 (Cdx2) is a key factor involved in TE formation and maintenance/renewal (Niwa *et al.*, 2005; Strumpf *et al.*, 2005). Mutant embryos lacking zygotic Cdx2 form morphologically normal blastocysts, however they exhibit ectopic expression of genes characteristic to ICM and do not implant due to a failure in TE specification (Strumpf *et al.*, 2005). Several groups have reported that Cdx2 is detectable in a subset of blastomeres as early as the 8-cell stage (Dietrich and Hiiragi, 2007; Niwa *et al.*, 2005; Ralston and Rossant, 2008) and by the 16-cell stage in most if not all blastomeres at variable levels that appear largely independent of cell position (Dietrich and Hiiragi, 2007). This position-independent variability is restricted to a 10- to 20-h period post-compaction (16- to 32-cell stage) (Dietrich and Hiiragi, 2007). During subsequent stages, however, outer cells exhibit increasingly higher expression than inner cells until fully restricted to the TE soon after blastocyst formation (Dietrich and Hiiragi, 2007; Niwa *et al.*, 2005; Ralston and Rossant, 2008; Strumpf *et al.*, 2005). This restricted expression of Cdx2 in the TE is required to repress the initially ubiquitous expression of Oct4 and Nanog (Niwa *et al.*, 2005; Strumpf *et al.*, 2005; Wang *et al.*, 2010).

These data suggest that Cdx2 and Oct4 interact in a mutually repressive manner to regulate each other's expression. It is also likely that this mechanism operates from early stages to determine the ultimate TE-specific and ICM-specific expression domains of these two factors, respectively. Supporting this, blastomeres at the 8-cell stage, even though morphologically indistinguishable, have been shown to display differential Oct4 transcription factor kinetics; those with slow kinetics mostly undergo asymmetric divisions and predominantly contribute to ICM, whereas those with rapid kinetics divide symmetrically and predominantly give rise to outer TE cells (Plachta *et al.*, 2011). However, it seems that although Cdx2 begins to be restricted to outer cells during morula stages and is completely restricted soon after blastocyst formation, Oct4 continues to be expressed in Cdx2-expressing TE cells for a considerable period during blastocyst expansion and only begins to be down-regulated after the embryo comprises around 60 cells (Dietrich and

Hiiragi, 2007). Based on these observations, it has been proposed that Oct4 may be present in all blastomeres until the first lineages are irreversibly specified (Dietrich and Hiiragi, 2007; Palmieri *et al.*, 1994). Therefore, reciprocal inhibition between Oct4 and Cdx2 could function in the down-regulation of Oct4 in TE cells only after the actual patterning process has occurred. In addition to this, Cdx2 expression in the ICM of mid-blastocyst embryos is inhibited by Nanog. Therefore, it has been proposed that Nanog might play a supportive role to Oct4 during the first fate decision, ensuring proper segregation of ICM and TE cells (Chen *et al.*, 2009).

If mutual repression of Cdx2 and Oct4/Nanog does not begin to take effect until around the mid-blastocyst stage, then some other mechanism must act to ensure TE-specific Cdx2 expression in the early blastocyst. Cdx2 expression has been shown to be regulated by the transcription factor Tead4 (Nishioka *et al.*, 2008; Yagi *et al.*, 2007), however, Tead4 is expressed and localized to the nucleus in all blastomeres (Nishioka *et al.*, 2008), raising the question as to how it might differentially regulate Cdx2. A mechanism for the involvement of Tead4 in the predominantly outer cell-specific expression of Cdx2 came from studies of the Hippo pathway and the regulation of the subcellular localization of the two co-activators of Tead4 – Yap and Taz (also called Wwtr1) (Nishioka *et al.*, 2009) (**Figure 2**). The current model posits that Hippo signaling is active in inner cells leading to phosphorylation and subsequent degradation of Yap/Taz, resulting in an inability of Tead4 to direct expression of target genes. Conversely, the Hippo pathway is inactive in outer cells, leading to the nuclear localization of Yap/Taz and binding of Tead4 to target loci, such as *Cdx2* and *Gata3*, with the latter encoding an additional TE-specific factor which has been shown to promote Cdx2 expression in outer blastomeres (Ralston *et al.*, 2010) (**Figure 3**). Yet another TE- (as well as subsequently an EPI-) specific factor, Klf5, which acts upstream of Cdx2 (Lin *et al.*, 2010), might function as a transcription factor partner to Yap/Taz as has been shown in breast cell lines (Zhi *et al.*, 2012).

It is worth noting that the above model was questioned by a study from Home *et al.* proposing that rather than differential Hippo signaling, it is differential Tead4 localization that determines TE versus ICM specification; thus nuclear localization of Tead4 in the outer TE lineage induces TE-specification whereas lack of Tead4 in the nucleus of inner cells allows their maturation toward the ICM lineage (Home *et al.*, 2012). This study reported the impaired Tead4 nuclear localization within the ICM lineage to be a conserved phenomenon during mammalian preimplantation development as it was observed in blastocysts from rats, cattle, rhesus monkeys, and humans. Intriguingly, Yap was proposed to be localized to the nuclei of both TE and ICM cells, raising questions as to the role of differential Hippo signaling on TE versus ICM lineage allocation (Home *et al.*, 2012). Recently however, Hirate and colleagues repeated the immunostaining experiments with the antibodies used by Home and colleagues and noted that Tead4 is constitutively nuclear localized, whereas Yap signal is strongly nuclear in outer cells but absent in inner cells, thus providing firm support for the direct role of the Hippo signaling pathway in regulating the first fate decision (Hirate *et al.*, 2012).

Based on these observations, the prevailing model posits that transcription of TE-specific factors, such as Cdx2 and Gata3, are directly regulated by Tead4, which in turn is regulated by the differential activation of the Hippo signaling pathway. This then raises the question of whether Hippo signaling is itself regulated by cell position and the emergent apico-basal polarity within early blastomeres. Interaction of Yap/Taz and the Crumbs polarity complex has been reported to promote nuclear accumulation of Smad proteins, thereby linking Tgf- β signaling with the Hippo pathway (Varelas *et al.*, 2010). Intriguingly, a recent study reported that absence of cell-cell contact and established polarity does not prohibit activation of the Hippo/Yap signaling pathway in separated early blastomeres and their daughter cells

(Lorthongpanich *et al.*, 2012). In the absence of positional and polarity clues, these isolated blastomeres failed to exhibit normal lineage-specific gene expression and rather exhibited a unique expression profile of both ICM- and TE-specific genes, although somewhat biased towards the latter (Lorthongpanich *et al.*, 2012).

An attractive possibility is that cell position and polarization of outer blastomeres plays an instructive role in the TE versus ICM fate choice, in regulating Hippo signaling and TE-specific Cdx2 and Gata3 expression. By analyzing embryo fragments derived from individual polar or apolar 8-cell blastomeres, a recent study reported that proper TE-specific Cdx2 expression is the result of established cell polarization, which depends on the outer position of the cell (Kondratiuk *et al.*, 2012). Cdx2 was expressed exclusively in those blastomere progeny that had inherited the polarity domain, which has been shown to be crucial for TE specification through its involvement in induction of Cdx2 in TE precursor cells (Jedrusik *et al.*, 2008; Ralston and Rossant, 2008). The aforementioned results provide further support for the cell polarity model, which posits that acquisition of cell polarity precedes and is essential for Cdx2 expression in TE precursor cells. In an apparent contradiction to these studies, it was suggested that maternal Cdx2 might play a role at early stages for trophectoderm cell polarization and cell fate specification (Jedrusik *et al.*, 2008). However, it was recently shown that there is no phenotype in mutant embryos with a maternal ablation of *Cdx2*, arguing against such a role for the maternal pool of Cdx2 and instead suggesting that it is not essential for the TE versus ICM fate choice and overall blastocyst development (Blij *et al.*, 2012). *In toto*, it is now becoming widely accepted that TE specification and induction of Cdx2 lies downstream of cell polarity in outer cells. This explains observations reporting that isolation of ICMs from early blastocysts can result in the *de novo* establishment of apical-basal polarity and formation of a new, superficial layer of TE (Rossant and Lis, 1979; Spindle, 1978; Stephenson *et al.*, 2010).

C. THE SECOND FATE DECISION: CHOICE BETWEEN EPIBLAST AND PRIMITIVE ENDODERM

The mechanisms underlying the second fate decision, which results in the specification of the PrE and EPI lineages, have only recently received attention, in part because of the relative difficulty in accessing and manipulating cells of the ICM. The primitive endoderm first becomes morphologically apparent in the late blastocyst, as a layer of epithelial cells positioned on the surface of the ICM adjacent to the blastocyst cavity. It has been shown that at E4.5, PrE and EPI cells exhibit distinct fates (Gardner and Rossant, 1979). Therefore, like the ‘inside-outside’ model proposed for the TE *versus* ICM cell fate decision, an early model for the PrE *versus* EPI cell fate decision proposed that initially identical ICM cells differentiate depending on their position: cells adjacent to the blastocyst cavity would adopt a PrE fate and deeper-lying ICM cells an EPI fate (Enders *et al.*, 1978).

This model was challenged by the observation that after the mid blastocyst stage the ICM no longer comprises a homogenous population of cells, but is rather a mosaic of cells expressing transcription factors at different levels. Nanog and Gata6, markers of the EPI and PrE respectively, were shown to be expressed in a non-overlapping (salt-and-pepper) manner within the ICM prior to the appearance of a differentiated and sorted primitive endoderm tissue layer (Chazaud *et al.*, 2006; Gerbe *et al.*, 2008; Kurimoto *et al.*, 2006; Plusa *et al.*, 2008; Rossant *et al.*, 2003). These two factors are implicated in specifying PrE (Gata6) and EPI (Nanog) cell fate (Chambers *et al.*, 2003; Koutsourakis *et al.*, 1999; Mitsui *et al.*, 2003). It was suggested that precursors of EPI and PrE are specified prior to any influence of positional signals relating to the blastocyst cavity. This observation was also supported by lineage tracing studies in which individually labeled ICM cells of E3.5 blastocysts contributed in most of the cases to only one lineage (PrE or EPI), similar to

earlier observations on E4.5 cells (Chazaud *et al.*, 2006). Therefore, an alternative model has recently emerged, proposing that individual early ICM cells are already biased in fate to either EPI or PrE. In this way, ICM cells become specified earlier than the overt appearance of the PrE layer and only later become fully committed and sort into their respective layers (Chazaud *et al.*, 2006; Rossant *et al.*, 2003).

These observations led to the proposition of a three-phase model (Plusa *et al.*, 2008): (1) initial co-expression of lineage-specific transcription factors (at around the 32-cell stage), (2) subsequent mutually-exclusive expression and salt-and pepper distribution of EPI- and PrE-precursor cells (at around the 64-cell stage), and (3) dynamic cell movements leading to the final sorting and spatial segregation of the EPI and PrE cell lineages (by around the 100-cell stage).

Early blastocyst: Co-expression of lineage-specific transcription factors

It is now widely accepted that EPI *versus* PrE lineage allocation within the ICM is linked to the dynamics of gene regulatory networks driving the proper temporal and spatial expression of lineage-specific transcription factors that specify cell fate (**Figure 2**). EPI cells are marked by the pluripotency-associated factors Nanog, Sox2 and Oct4; however, Nanog is the only factor that is earlier specific to EPI-biased cells and thus is thought to be the main factor driving their cell fate decision (Chazaud *et al.*, 2006; Plusa *et al.*, 2008).

Conversely PrE cells, which until implantation also express Sox2 and Oct4, are marked by the upregulation of the zinc finger transcription factor Gata6. Notably, studies on *Nanog* mutant embryos have shown that Nanog is required not only for formation of the EPI lineage, but also for the maintenance of the PrE, suggesting that cross-talk between emerging EPI and PrE lineages is essential for proper development at this stage (Messerschmidt and Kemler, 2010; Silva *et al.*, 2009). Nevertheless, up until the 32-cell stage the ICM exhibits a homogenous appearance with cells exhibiting similar gene expression patterns and notably co-expression of Gata6 and Nanog (Chazaud *et al.*, 2006; Gerbe *et al.*, 2008; Plusa *et al.*, 2008) (**Figure 2**).

Mid blastocyst: Emergence of a salt-and-pepper distribution of lineage-biased progenitors

After the 32-cell stage, cells within the ICM begin upregulating lineage-specific transcription factors, which are further restricted until a distribution of cells exhibiting mutually exclusive transcription factor expression emerges (Chazaud *et al.*, 2006). A subset of ICM cells down-regulate Gata6 while still expressing Nanog, biasing them towards an EPI fate. The remaining ICM cells down-regulate Nanog but maintain Gata6 expression and subsequently up-regulate Sox17 (Artus *et al.*, 2011), which primes them for a PrE fate. It remains an open question if levels of the maintained factors (Nanog in EPI and Gata6 in PrE) remain stable at the expression level they exhibit early on or if these levels change during a cell's development. Oct4 and Sox2 maintain their expression in all ICM cells and function as a complex to autoregulate themselves (Avilion *et al.*, 2003; Chew *et al.*, 2005; Okumura-Nakanishi *et al.*, 2005).

Additionally Oct4 in cooperation with Sox2 are thought to promote upregulation of Nanog (Liang *et al.*, 2008; Rodda *et al.*, 2005; Wang *et al.*, 2006). Interestingly, Oct4 has also been reported to cooperate with Sox17, forming a complex that drives endodermal gene expression (Jauch *et al.*, 2011; Ng *et al.*, 2012; Stefanovic *et al.*, 2009). Sox17 has also been shown to interact with many of the same promoter sequences as the core pluripotency genes and overexpression of Sox17 in ES cells has been shown to be sufficient to displace Nanog from its binding sites (Niakan *et al.*, 2010). A possible switch of Oct4 binding partners from

Sox2 to Sox17 during lineage specification of the PrE may constitute a plausible mechanism that needs to be investigated.

As discussed earlier, it seems likely that cross-talk between Nanog- and Gata6-expressing cells is necessary for their proper specification. The missing link might be a non-cell-autonomous factor enabling this cross-talk, which is now thought to be Fgf4 (Goldin and Papaioannou, 2003; Kang *et al.*, 2012; Nichols *et al.*, 2009; Yamanaka *et al.*, 2010). Indeed, before the formation of a salt-and-pepper pattern of expression, Fgf4, whose expression is regulated by Oct4 and Sox2 (Ambrosetti *et al.*, 2000), becomes down-regulated in some ICM cells (PrE-biased) (Guo *et al.*, 2010). It is thought that Nanog could play a role in this process by reinforcing the expression of Fgf4 in EPI biased cells. Conversely, Fgf4's putative receptor Fgfr2 becomes down-regulated in EPI biased cells while being maintained in PrE-biased cells, which in turn enhances their sensitivity to Fgf4-induced signaling (Guo *et al.*, 2010). Activation of the Fgf/MAPK signaling pathway in these cells (whilst not necessary for its initial activation) reinforces upregulation of Gata6 and thus repression of Nanog (Frankenberg *et al.*, 2011) (**Figure 2**). Furthermore, the prominent role of Fgf signaling is emphasized by the fact that ablation of Fgf4, Fgfr2 or downstream mediators of the Fgf signaling cascade, such as Grb2, results in a complete failure to specify PrE (Arman *et al.*, 1998; Chazaud *et al.*, 2006; Feldman *et al.*, 1995; Kang *et al.*, 2012). Though Gata6 is activated already in the 8-cell stage embryo, in the absence of Fgf4 its expression cannot be maintained beyond the 32-cell stage, and no salt-and-pepper pattern of expression is established. Instead, all ICM cells acquire an EPI fate. This points to an indispensable role for Fgf signaling in the formation of the two ICM lineages and links lineage specification with the salt-and-pepper distribution of lineage-biased cells. Remarkably, treatment of *Fgf4* mutants with exogenous Fgf does not restore the salt-and-pepper distribution. Instead, it creates an all-or-nothing situation with the ICM either remaining all EPI or becoming all PrE (Kang *et al.*, 2012). This suggests a local effect of non-cell-autonomous Fgf signaling in the allocation of EPI- and PrE-biased cells within the ICM.

Even though cells are biased towards either an EPI or a PrE fate by the salt-and-pepper stage, they are not fully committed to their respective identities. Grabarek and colleagues reported that isolated PrE and EPI cells were able to contribute to all lineages when transplanted into 8-cell host embryos. Notably, the PrE precursors represented a heterogeneous population with respect to levels of the PrE marker *Pdgfra*, with *Pdgfra*-expressing PrE precursors exhibiting greater plasticity than EPI precursors and giving rise to a higher proportion of different lineages (Grabarek *et al.*, 2012).

One of the biggest questions that arises from the observations of these early steps of lineage commitment is how the transition from a state of Nanog and Gata6 co-expression to a mutually exclusive salt-and-pepper state is achieved. The identity of the inducing factor or event that shifts the balance towards PrE or EPI within any individual cell of the seemingly homogenous ICM is to date unknown. While the initial theory of a position-induced cell fate decision comparable to the allocation of the TE lineage has been superseded by the discovery of the salt-and-pepper state, another model has emerged that gives significance to cell positioning, though in a different context.

In this so-called “time-inside/time-outside” model, Morris and colleagues propose an influence of the wave of asymmetric cell division on the fate of the ICM cell to which it gives rise (Morris *et al.*, 2010). This model posits that internalized cells from the first wave of asymmetric divisions (between the 8- and 16-cell stage) are biased towards an EPI fate. Internalized cells from the second and third asymmetric divisions (16-to-32 and 32-64 cell stages) on the other hand would be primed to become PrE because their parental cells have

had more time to progress further in their development and to express markers of differentiation such as Gata6 (**Figure 4A**).

Though seemingly integrating a role for positional information with the salt-and-pepper distribution, the “time-inside/time-outside” model was challenged by Yamanaka and colleagues who, by tracing cells in a different way, concluded that cells generated from the first or second waves of asymmetric divisions give rise to PrE or EPI in a random, non-biased fashion (Yamanaka *et al.*, 2010). It has subsequently been suggested that the discrepancies between the two studies may be due to technical differences – for example the fact that different methods were used for lineage tracing (Bruce and Zernicka-Goetz, 2010). In the case of a more randomly initiated lineage allocation, a possible model posits that levels of the transcription factors Nanog (EPI) and Gata6/Sox17 (PrE) fluctuate slightly and diverge due to their mutual repression. This would lead to one factor prevailing over the other in some cells and vice versa in other cells, this would in turn create two populations of lineage-biased cells that would become arranged in a random distribution. The fact that the PrE-specific marker *Pdgfra* shows high and low expressing populations inside the ICM instead of fluctuations may be due to *Pdgfra* being a receptor that is active in the PrE but neither a transcription factor nor required for PrE formation (**Figure 4B**). It remains to be demonstrated whether fluctuations or differential gene expression in the two lineages exist *in vivo*.

Late blastocyst: Cell Sorting

After the 64-cell stage the salt-and-pepper distribution of EPI- and PrE-biased cells is established in the ICM. At this point EPI cells exhibit high levels of Nanog, while PrE cells express Gata6, Sox17 and Gata4 (Arceci *et al.*, 1993; Artus *et al.*, 2011; Laverriere *et al.*, 1994; Morrissey *et al.*, 1998; Plusa *et al.*, 2008). Subsequently, PrE cells become sorted to form an epithelialized layer positioned adjacent to the EPI at the interface with the blastocyst cavity. At this time cells express Sox7, the latest-expressed PrE-specific transcription known to date (Artus *et al.*, 2011).

Live imaging of the PrE-specific single-cell resolution fluorescent reporter *Pdgfra*^{H2B-GFP} revealed a variety of different cell behaviors, which resulted in PrE cells moving from a position deep within the ICM to the nascent PrE layer on its surface (Plusa *et al.*, 2008). However, sorting by changing position was not the only mechanism. Some GFP-negative cells lying in contact with the blastocyst cavity were observed to up-regulate GFP expression and subsequently contribute to the PrE, while some GFP-positive cells lying deep within the ICM failed to contribute to the PrE, instead either appearing to down-regulate GFP or undergoing apoptosis. Interestingly, GFP-positive cells already lining the cavity were rarely observed to change position. By contrast, deeper-lying GFP-positive cells tended to be more migratory yet lost this property upon reaching the cavity. These observations revealed that sorting does not simply involve active and inactive cell movements, but also that PrE precursors failing to integrate into the nascent PrE layer either undergo apoptosis or possibly revert to an EPI fate.

Once on the surface, PrE cells rarely change their position, which suggests a possible involvement of cell polarity in reinforcing lineage identity. Indeed, it has been demonstrated that artificially interfering with polarity in a subset of blastomeres in the morula drastically diminishes their ability to maintain an outside position, forcing them to be “out-competed” by blastomeres with a greater ability to polarize and flatten (Plusa *et al.*, 2005). In support of this model, Gerbe and colleagues reported expression of several markers, including *Lrp2*/*Megalin* and *Dab2*, that are apico-basally polarized in cells having contact with the cavity (Gerbe *et al.*, 2008). Moreover, position-induced signaling cues have been shown to directly affect the PrE sorting process. To this end, *Wnt9A*, which has been suggested to be

expressed on the surface of the ICM, has been reported as facilitating the re-positioning of Gata6-expressing cells when microinjected into them (Meilhac *et al.*, 2009). Thus, the combination of differences in the ability of cells to polarize and positional signals from the cavity can both contribute to effective cell sorting during PrE formation.

D. CONCLUDING REMARKS: DEVELOPMENTAL BIASES AND PLASTICITIES DURING BLASTOCYST FORMATION

The mechanisms governing early fate decisions during preimplantation development provide the embryo with the necessary flexibility to adjust in changing circumstances. Early on, the activation of the embryonic genome and random expression of lineage-specific genes provides plasticity as cells undergo differentiation (Grabarek *et al.*, 2012; Lorthongpanich *et al.*, 2012; Morris *et al.*, 2012). Therefore, the early totipotent blastomeres, or thereafter the early ICM cells, can be considered as hybrid TE/ICM or EPI/PrE cells respectively, being plastic in changing their developmental path when challenged with the appropriate stimuli. To this end, heterogeneities or ‘noise’ in gene expression could provide a mechanism permitting flexible cell fate choices during differentiation (Arias and Hayward, 2006; Eldar and Elowitz, 2010). Indeed, recent studies have demonstrated heterogeneous gene expression in the early mouse embryo. *Cdx2* levels are highly variable when expression is first initiated after the 8-cell stage, showing no relation to either *Oct4* levels or cell position (Dietrich and Hiiragi, 2007). Similarly, *Nanog*, *Gata6* and *Pdgfra* exhibit heterogeneous expression that is mutually independent (Plusa *et al.*, 2008). Therefore, such heterogeneities may provide a lineage bias rather than commitment early during the first or the second fate decision, thereby allowing the embryo to adapt to new conditions should the emergent pattern be perturbed.

However, among this early apparent randomness in the expression of key regulatory genes, a bias to a certain lineage eventually gives rise to final commitment and proper tissue specification. Cellular properties, such as cell-cell contact, position or polarity provide instructive cues for correct lineage specification during the first TE *versus* ICM decision. Accordingly, differential signaling cues, inferred by the Hippo and Fgf pathways, also play instructive roles. Recently, epigenetic marks including DNA methylation and chromatin modifications have also been implicated in the processes controlling lineage specification in the blastocyst (reviewed in (Bergsmedh *et al.*, 2011; Gasperowicz and Natale, 2011)). Therefore, we now recognized that developmental flexibility during differentiation, in the context of the heterogeneous expression of lineage-specific factors, gives rise to properly restricted tissue patterning through instruction from position and polarity signaling cues, as well as epigenetic modifications. Moreover, according to the ‘time-inside/time-outside’ model, the developmental history of a cell might influence its fate, providing an additional layer of non-random instructive regulation (Morris *et al.*, 2010) (**Figure 4**).

Additionally, the evolutionary relationships between some of the key regulatory transcription factors for lineage commitment could be informative for shedding light on the mechanisms of lineage specification in the early embryo. In particular, the second cell fate decision, between EPI and PrE, is mechanistically not fully understood. Intriguingly, many of the regulators driving these cell fate decisions during embryo development are members of the same transcription factor family. Indeed, the Gata and Sox transcription factor families are majorly involved in developmental processes (reviewed in (Bowles *et al.*, 2000; Patient and McGhee, 2002)). During preimplantation development, *Gata3* is involved in trophoblast specification (Ralston *et al.*, 2010). *Gata6* and *Gata4* are sequentially activated in the PrE lineage and are both individually sufficient to convert ES cells to XEN cells (Artus *et al.*, 2011; Cho *et al.*, 2012). It is plausible that in the embryo, *Gata6* primes cells for possible primitive endoderm specification while still allowing for plasticity. *Gata4*

then would be activated at a later stage when cells have progressed further into acquiring their developmental fate. Additionally, members of the Sox family play an important role in the early embryo. Sox2 is involved in transcription regulation of ICM cells, while Sox17 and Sox7 are sequentially activated in PrE cells (Ambrosetti *et al.*, 2000; Artus *et al.*, 2011; Avilion *et al.*, 2003; Chew *et al.*, 2005; Liang *et al.*, 2008; Okumura-Nakanishi *et al.*, 2005; Rodda *et al.*, 2005; Wang *et al.*, 2006). The close similarity of these transcription factor family members might involve a scenario in which a factor such as Oct4 could easily switch Sox binding partners, thereby facilitating a rapid responsiveness that is necessary early in development. A binding partner switch from Sox2 to Sox17 would not only disrupt the balance of the core Oct4 – Sox2 – Nanog pluripotency network, but might also initiate a PrE-specific transcription factor program that could shift cells towards a PrE fate.

One could speculate that evolution has affirmed proper development of the PrE through the sequential activation of four transcription factors that assure cells are locked in their fate. A similar process can be found during nervous system development in *Drosophila*, where neuroblasts express a sequence of four progenitor temporal transcription factors to sequentially generate neurons and glia (Jacob *et al.*, 2008). Intriguingly, examples of sequential transcriptional regulators priming and subsequently affirming cell fate can also be found in non-vertebrate, prokaryotic organisms. During *Bacillus subtilis* sporulation, a cascade of prokaryotic transcription factors (known as sigma factors) regulate the genetic programs of the two differentiated cell types formed, the spore and the mother cell (Kroos *et al.*, 1999). Nevertheless, it remains to be determined if the two transcription factor families (Sox and Gata) act independently, which would mean an additional fail-safe mechanism, or if they cooperate to accomplish PrE specification.

Parallels can be drawn between the mechanisms governing lineage specification in the preimplantation embryo and those controlling self-renewal or differentiation of embryo-derived stem cells. To this end, as in the embryo, heterogeneous gene expression has been demonstrated in mouse and human pluripotent (and induced pluripotent) stem cells (Chambers *et al.*, 2007; Enver *et al.*, 2005; Kalmar *et al.*, 2009; Lanner *et al.*, 2010; Narsinh *et al.*, 2011; Singh *et al.*, 2007; Stewart *et al.*, 2006). It has been suggested that heterogeneities represent an inherent feature of pluripotent cells, being critical for stem cell function and the regulation of cell fate decisions (Enver *et al.*, 2009; Martinez Arias and Brickman, 2011). Particularly for mouse embryonic stem cells, heterogeneities have been correlated with dynamic fluctuations in the expression of certain pluripotency-associated factors (Chambers *et al.*, 2007; Kalmar *et al.*, 2009; Toyooka *et al.*, 2008; Wray *et al.*, 2011); these studies have mostly centered on the transcription of *Nanog*, for which it was additionally revealed that there is allelic regulation both *in vitro* and *in vivo*, further promoting heterogeneities in its expression (Miyanari and Torres-Padilla, 2012). Moreover, it was recently shown that Nanog represses its own expression and this autorepressive activity is the major regulator for inducing heterogeneities in its transcriptional status (Fidalgo *et al.*, 2012; Navarro *et al.*, 2012). It is still not known whether these dynamic heterogeneities exist *in vivo*. Live cell imaging would allow for quantitative analysis of gene expression in single-cells, and if combined with mathematical modeling should provide insights into how dynamic heterogeneities can influence cell fate decisions. To achieve this goal, the construction of single-cell resolution reporters for all three blastocyst lineages that can be simultaneously imaged would facilitate the direct visualization of how the each cell lineage emerges and segregates *in situ* within the developing embryo.

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Figure 1. Overview of preimplantation development leading to blastocyst formation

Proper lineage segregation is ensured by two cell fate decisions. The first giving rise to trophectoderm and inner cell mass and the second leading to the allocation of primitive endoderm and epiblast. E: embryonic day. Of note, these cell fate decisions are schematized as being sequential but this may not strictly be the case.

Figure 2. Active gene regulatory networks throughout preimplantation development

For details, see text. Green: trophectoderm cells, blue: primitive endoderm cells, red: epiblast cells, purple: unspecified ICM cells, arrows: positive regulation, blocked arrows: negative regulation, circle arrows: autoregulation.

Figure 3. Model for trophectoderm lineage allocation

The prevailing model posits cell polarity cues and differential Hippo signaling activation. Outside cells are polarized, consequently Hippo signaling is inactive. YAP can be transported into the nucleus, where it co-activates Tead4 to drive expression of TE specific genes. Conversely, Hippo signaling in unpolarized inner cells results in phosphorylation and degradation of YAP leading to no co-activation of Tead4.

Figure 4. Working models for primitive endoderm lineage allocation

A Time inside - time outside model. Cells from the first wave of asymmetric divisions are biased to become EPI while cells from the second and third wave are biased to become PrE due to the further developed identity of their parental cells. **B** Stochastic model. Cells are unbiased by the wave of asymmetric divisions they arise from. Rather, stochastic fluctuations in gene expression direct cell identity in a random fashion. By comparison a *Pdgfra* transcriptional reporter exhibits differential expression that can be classified into two subpopulations or PrE biased cells, prior to being expressed at high levels in all PrE cells and low levels or absent in EPI cells (dashed lines).