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Primitive endoderm differentiation: from specification to epithelium formation

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In amniotes, primitive endoderm (PrE) plays important roles not only for nutrient support but also as an inductive tissue required for embryo patterning. PrE is an epithelial monolayer that is visible shortly before embryo implantation and is one of the first three cell lineages produced by the embryo. We review here the molecular mechanisms that have been uncovered during the past 10 years on PrE and epiblast cell lineage specification within the inner cell mass of the blastocyst and on their subsequent steps of differentiation.

1. Introduction

In mammals, extraembryonic tissues differentiate first to prepare a nutrient support as early as possible. During these steps of differentiation, subsets of cells keep the ability to give rise to all embryonic and adult tissues. These epiblast (Epi) pluripotent cells segregate from two differentiation events during blastocyst formation: first, during the trophoblast versus inner cell mass (ICM) differentiation and second, during Epi versus primitive endoderm (PrE) specification within the ICM. The trophoblasts will participate in the formation of the placenta, whereas PrE cell derivatives will be a major constituent of the yolk sac. Several reports have shown the importance of extraembryonic tissues not only for nutrient supply but also to induce the adjacent embryonic tissue, notably for the proper establishment of the anterior–posterior axis [1]. Moreover, it was shown recently that some cells of the visceral endoderm, a PrE derivative, could integrate the definitive endoderm, revealing a potential role in gut development [2].

Here, we review the recent discoveries regarding the molecular characterization of PrE differentiation.

2. The core molecular regulatory network for primitive endoderm versus epiblast cell specification

After fertilization, the cells (blastomeres) of the mouse embryo divide and reach the 8-cell stage (at embryonic day (E) 2.5), the time of compaction. During this process, adhesion increases between blastomeres that concomitantly polarize. During the following two to three rounds of division, inner, unpolarized cells are produced through asymmetric division or internalization [3–7]. Subsequently, inner and outer cells are fated to the ICM and the trophectoderm, respectively (figure 1). It has been known for a long time that the ICM gives rise to Epi and PrE cells around E4.5 [8], but the exclusive expression pattern of Nanog and Gata6 in a 'salt and pepper' organization indicated for the first time that the ICM is already heterogeneous at E3.5 [9,10]. These intermingled ICM cells were further characterized by cell lineage tracing and cell grafting, which showed that most of them are engaged towards an Epi or a PrE identity from the mid-blastocyst stage [10–12]. Altogether, these experiments revealed that Epi and PrE cells specify within the ICM in an apparent random salt and pepper pattern.

The mechanism leading to the exclusive expression of Nanog and Gata6 is not entirely clear, but such a pattern suggests that these transcription factors inhibit each other. This is the case at least for Nanog as it can directly bind to *Gata6* regulatory sequences [13] and repress its expression. However, the regulation of

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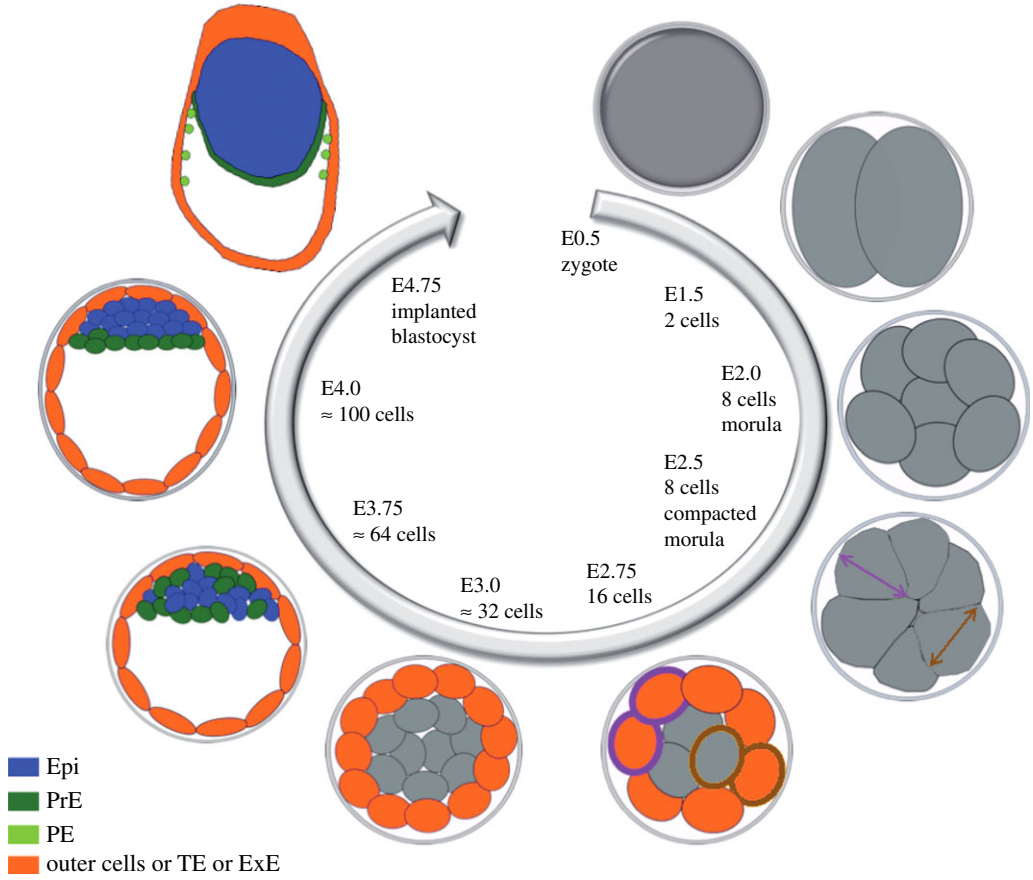

Figure 1. Schematic of cell lineages differentiation during mouse preimplantation. After fertilization, the embryo reaches the 8-cell stage and undergoes compaction. During the next two rounds of division, cells divide either symmetrically (purple arrow and cell) or asymmetrically (brown arrow and cell) to give rise to two outer cells or to one outer cell and one inner cell, respectively. Cells can also be internalized. From E3.0 to E3.25 inner cells start to adopt Epi or PrE signatures. At E3.75, a high majority of cells have specified into Epi or PrE. At E4.0, Epi and PrE cells have sorted to form two distinct tissues and have lost their plasticity. By E4.75, PrE cells have formed an epithelium and started to produce parietal endoderm cells that migrate along the trophectoderm. ExE, extraembryonic ectoderm (trophectoderm derivative); PE, parietal endoderm; TE, trophectoderm.

Nanog and Gata6 expression is more complex, because these proteins are co-expressed in all blastomeres at least from the 8-cell stage [14], and their RNAs can be detected as early as the 2-cell stage [15,16]. Thus, the early ICM progenitors co-express Nanog and Gata6 before acquiring distinct identities cell by cell. This is reminiscent of other cell specification events, as coexpression of prodifferentiation factors in progenitor cells has been described during haematopoiesis or pituitary development [17,18].

(a) High or low Erk activity drives lineage specification

Several experiments showed that the switch from a common to an exclusive expression of Nanog and Gata6 depends on FGF4 expression. It was first shown genetically and then with pharmacological inhibitors that blocking the Erk pathway inhibits PrE specification while promoting Epi identity, visualized by Nanog expression in all ICM cells [3,10,19]. On the reverse, FGF4 administration induces PrE cells, at the expense of the Epi cells [3]. Altogether, these experiments indicated that the ICM precursor has a binary fate choice, which is dependent on low or high Erk activity leading either to an Epi or to a PrE identity, respectively. Recently, expression and/or role of Nanog, Gata6 and active Erk were converted into differential equations, setting up a mathematical model reproducing *in vivo* cell behaviours [20]. Importantly, this series of embryo culture experiments also demonstrated the high plasticity of ICM cells, as cell identity can be reversed by artificially applying low or high levels of active Erk. This ability to change identity upon exogenous treatments ceases around E4.0, and cell lineages are thus considered as determined [3]. Using chimaera essays with donor cells of different stages (from the early blastocyst stage to E4.5), Grabarek *et al.* [12] showed that plasticity is lost in all ICM cells only after the late blastocyst stage. These results also suggest that the expression of Nanog and Gata6 in an exclusive manner is not sufficient to lock the cell identity [12], a hypothesis substantiated by the mathematical model [20]. While the levels of Nanog and Gata6 proteins in the donor cells would need to be quantified, this might indicate that other factors could be required for the loss of cell plasticity.

Genetic analyses demonstrated that FGF4 is the RTK ligand required to induce the PrE identity, as *Fgf4*$^{-/-}$ embryos express Nanog in all ICM cells at the late blastocyst stage [21–23], phenocopying *Grb2* mutants [10]. Strikingly, Gata6 is expressed in *Fgf4*$^{-/-}$ embryos until E3.25, the time of the salt and pepper set-up, but cannot be maintained afterwards [21–23]. This means that Gata6 pre-blastocyst expression is independent of the early 2-cell stage expression of FGF4 [15]. Thus, another signalling pathway is required to induce Gata6 expression. The factors required for Nanog onset of expression before the 8-cell stage also remain unknown.

(b) *Nanog* requirements for epiblast specification

Several groups have analysed *Nanog*$^{-/-}$ embryos [24–27], and showed that the first role of Nanog is to specify Epi. Indeed, in *Nanog*$^{-/-}$ embryos, all ICM cells express Gata6. This also confirms that Nanog represses Gata6 expression *in vivo*. The ICM marker Oct4 and the trophectoderm marker Cdx2 are correctly expressed in these mutants, demonstrating that cell specification between ICM and trophectoderm occurs properly. Through single-cell quantitative real-time PCR (RTqPCR), *Fgf4* was shown to be expressed specifically in Epi precursor cells of wild-type embryos [15,28]. Fluorescent *in situ* hybridization analyses showed that this specific Epi expression disappears in *Nanog* mutants, strongly suggesting that *Fgf4* expression is induced by Nanog [27]. This regulation is probably direct as Nanog binds to *Fgf4* regulatory sequences during chromatin immunoprecipitation (ChIP) experiments in embryonic stem (ES) cells [29]. Conversely to *Fgf4*$^{-/-}$ embryos, *Nanog*$^{-/-}$ embryos—that also lack FGF4—maintain Gata6 expression until at least E4.5 [27]. This indicates that in *Fgf4*$^{-/-}$ embryos, the decay of Gata6 expression is not directly due to the absence of FGF4, but is rather the consequence of Nanog high expression that inhibits that of Gata6.

While Gata6 induction of expression is not impaired before the 8-cell stage in the absence of FGF4, it is inhibited when FGFR and Mek activities are blocked at an early time point (before compaction) in a *Nanog* mutant context [27]. These results suggest that another RTK ligand is active early on to induce Gata6 expression, even if *Fgf4* is expressed at these early stages [15]. Thus, another RTK must be active to induce Gata6 expression. Interestingly, once Gata6 has been induced, the RTK pathway is not required anymore to maintain its expression. Indeed, in *Nanog* mutant embryos treated with FGFR and Mek inhibitors around E3.25, Gata6 expression is not downregulated [27]. These results could be interpreted in two ways: (i) during the salt and pepper set-up in wild-type embryos, FGF4 promotes Gata6 expression only through Nanog downregulation or (ii) in *Nanog* mutant embryos, ICM cells specify rapidly into PrE as Nanog is absent, and go beyond the stages of plasticity that normally occur around E4.0. In any case, there are two consecutive phases of Gata6 expression, first induced through an unknown RTK activation and then maintained independently of the direct RTK/FGF4 signalling.

(c) *Gata6* requirements for primitive endoderm specification

In vitro, *Gata6* ectopic expression in ES cells is sufficient to transdifferentiate them into PrE cells [30,31], whereas it is not the case via RNA injections into ICM cells during embryo cultures [5,11]. Moreover, *Gata6* mutant ES cells are unable to differentiate into PrE, even in the presence of retinoic acid that normally drives them towards a PrE identity [32,33].

After several reports on the requirement of Gata6 only after implantation, it is now clear that *Gata6* is necessary for PrE epithelium formation *in vivo* [20,32,34–36]. Two recent reports reveal that *Gata6* is necessary to specify PrE cells and is an important component of the binary Epi/PrE cell fate decision as all ICM cells express the Epi markers Nanog and Sox2 in *Gata6*$^{-/-}$ embryos [20,36]. Administration of FGF4 cannot rescue PrE specification [20,36], suggesting that Gata6 is downstream of the FGF4/FGFR2 pathway. However, Gata6 early expression does not depend on the presence of FGF4 as was shown with *Fgf4* and *Nanog* mutant analyses [21,22,27]. One explanation could be that *Gata6*$^{-/-}$ ICM cells do not respond to FGF4 because, becoming Epi cells, they have lost FGFR2 expression. Indeed, *Fgfr2* is repressed in Epi cells only from E3.5 in wild-type embryos as shown by single-cell RTqPCR [15,23,28]. In *Gata6* mutants, the loss could be due either to Nanog repression or to the absence of Gata6 activation. Unfortunately, the expression of FGFR2 was not examined in *Gata6* mutant ICMs.

Using complementary time-windows treatments, the two studies analysed the effect of an RTK activation on Nanog expression [20,36]. Administration of FGF4 to *Gata6* mutants at the 8-cell stage prevents Nanog expression [20]. This means that Nanog can be repressed by the RTK pathway independently of Gata6. Higher levels of Nanog expression are indeed released by Erk inhibition independently of the genotype [36]. Moreover, ectopic active FGFR2 expression in ES cells indicates that this repression is direct [37]. A later FGF4 treatment does not impair Nanog expression [20,36]. The switch into an insensitive state is very rapid as it occurs around the 8/16-cell stage [36], whereas it takes place from E3.75 in wild-type embryos [3]. The mathematical model, indeed, predicts a two-phase state of Nanog expression and explains that Nanog (or the network of pluripotency activity) levels need to become high to counteract the direct inhibitory effect FGF4/FGFR2 activation [20].

(d) Modulation of cell fate choice and timing by gene dosage

In *Gata6* heterozygous mutant embryos, less PrE cells are specified compared with their wild-type littermates. As a consequence, there are more Epi cells within the same size ICM [20,36]. Such imbalanced proportions of Epi and PrE cells can be found in *Fgf4*$^{+/-}$ embryos [21]. Under a different genetic background, this effect is unmasked by deleting maternal *Fgf4* (*MFgf4*$^{+/-}$) [22]. Altogether, these studies on heterozygous embryos show that the dosage of FGF4 and Gata6 is important to balance PrE and Epi identities.

Single-cell quantification *in situ* at different stages enabled the demonstration that Epi cells are specified earlier in *Gata6*$^{+/-}$ embryos than that in their wild-type littermates [20,36]. This result was strengthened by an earlier expression of *Fgf4* [20], used there as an Epi marker [15,27]. Thus, the relative levels of Nanog and Gata6 not only modulate Epi/PrE ratios, but also control the timing of specification. Using FGF4 treatments, it was shown that cell plasticity is also lost earlier in *Gata6*$^{+/-}$ embryos. Indeed, by E3.25, Epi cells are already insensitive to FGF4 as Nanog and PrE cell number are the same in treated and untreated *Gata6*$^{+/-}$ embryos recovered at E3.75 [20]. Conversely, PrE cells still require RTK activation as administration of FGFR2 and Erk inhibitors can push the cells towards an Epi identity as late as E4.0 [20]. Different results were found by Schrode *et al.* [36], who showed that PrE cells are insensitive to Erk inhibition as early as E3.5. This result is puzzling, as about one-third of ICM cells are not specified at the 64–128 stage in *Gata6*$^{+/-}$ ICMs [36]. It remains to be known whether an FGFR2 activity other than Erk can explain such a difference.

Interestingly, PrE cell numbers are restored in *Gata6*$^{+/-}$ and *MFgf4*$^{+/-}$ embryos at E4.5 [20,22]. As Epi cells lose their plasticity early in *Gata6*$^{+/-}$ embryos [20], it is unlikely that the recovery of PrE cell number is due to a conversion of Epi cells into PrE cells. Although it was not examined, this increase in PrE cells could be due to a higher cell proliferation.

Recently, it was shown that *Nanog* is first expressed monoallelically until the late blastocyst stage and then switches to a biallelic expression [16]. The results were disputed [38,39], but a recent single-cell allelic sequencing analysis confirms the monoallelic expression [40]. As the levels of Nanog, Gata6 and active Erk seem to balance cell fate in the ICM, it was surprising that Epi/PrE specification occurs properly in Nanog heterozygous early blastocysts [16,27]. This could be explained by an absence of difference in Nanog levels between wild-type and heterozygous embryos, as it is monoallelically expressed. This could imply that Nanog must be monoallelically expressed to maintain the proper balance with Gata6 and the FGF signalling pathway during lineage specification.

(e) Other factors implicated in epiblast/primitive endoderm specification

So far, no other transcription factors or signalling pathways have been found to clearly modulate Epi versus PrE specification. Despite Oct4 impact on ES cell pluripotency, *Oct4* mutant embryos produce Epi and PrE cells in correct proportions [41,42]. This suggests that Oct4 is dispensable for Epi versus PrE specification. However, these embryos do not respond to artificially strong variations of the Erk pathway like their wild-type littermates. Indeed, in $Oct4^{-/-}$ embryos, Gata6 expression is not downregulated by FGFR/Mek inhibitor treatment, with some cells co-expressing both Nanog and Gata6. In addition, Nanog is still present upon FGF4 administration [41]. Thus, some of the artificially high and low Erk activities seem to be transduced through Oct4, revealing an involvement of Oct4 in cell plasticity. The physiological relevance of these interesting results is not clear as yet, and additional experiments will be needed to understand the interactions between Oct4 and the RTK pathway.

The RTK pathway does not impact ICM cell specification in human embryos, whereas salt and pepper expression of Nanog and Gata6 is present [43,44]. This striking feature shows that Nanog and Gata6 are the conserved factors, while a pathway other than RTK must be regulating them. Their antagonism seems to be conserved as shown by Nanog siRNA experiments in human ES cells. Indeed, despite their mature Epi identity compared with mouse ES cells [45], these human ES cells induce PrE and trophectoderm markers upon Nanog knockdown [46]. Bovine embryos are influenced by variations in RTK pathway activity but are less sensitive than mouse or rat embryos [43,44]. Thus, other signals must reinforce the FGF pathway in the bovine embryo and replace it in human ones. For example, the Wnt and Bmp pathways are good candidates as they have been reported to be involved in mouse ES cell maintenance or differentiation [47–49]. *Bmp4* is indeed specifically expressed in Epi precursors at E3.5 [15] and plays a role in extraembryonic endoderm lineage differentiation, possibly as early as PrE formation, in embryoid bodies [50].

3. Different hypotheses for the induction of the salt and pepper pattern

Nanog and Gata6 are coexpressed in all ICM cells before the induction of the salt and pepper pattern around E3.25. Single-cell quantification *in situ* of these markers revealed that Epi cells are specified first [20] through an as yet unknown mechanism. Remarkably, the specification into either Epi or PrE is the consequence of the down- rather than upregulation of these two factors, both at the RNA and protein level [15,20,23,36,51]. Another important feature is that the prevalence of one of the factors is not synchronous in all ICM cells, but gradually extends to reach completion around E3.75 [14,23].

The FGF4/FGFR pathway is required to set up the salt and pepper pattern [21,22]. Surprisingly, the use of increasing exogenous doses of recombinant FGF4 cannot rescue a proper salt and pepper pattern in *Fgf4* mutants, the ICM behaving in a bimodal fashion with either only Epi or only PrE cells [21,22]. The need for a heterogeneous FGF4 distribution is a likely interpretation [21] that can be explained mathematically [20]. A few FGF4-expressing cells would induce a PrE identity in neighbouring cells while further away from the FGF4 source other cells would adopt an Epi identity. The asynchronous specification would be thus a consequence of the propagation of FGF4 and an important component of the cell lineage specification mechanism.

It is not known whether FGF4 is the induction trigger or whether it transmits an earlier signal. Indeed, *Fgf4* expression is under the control of Nanog and, as there is a progressive increase of Nanog protein expression, with cell-to-cell variations, from compaction to the early blastocyst stage [20,51], a defined threshold could induce *Fgf4* expression. The mathematical model predicts that only one Nanog-expressing cell would be sufficient to produce enough FGF4 to propagate the salt and pepper mechanism [20]. However, these few cells might be difficult to detect as the cell-to-cell variation of Nanog and Gata6 expressions are small at the RNA level [15,23], even if they seem more obvious at the protein level [20,51]. A slight difference between these factor levels nevertheless might be sufficient to induce a burst of FGF4 expression. Indeed, FGF4 is one of the first factors to clearly appear in a salt and pepper pattern [15,23]. Alternatively, a timely cofactor could help Nanog in inducing FGF4. It is also possible that Nanog only maintains an earlier FGF4 induction.

Altogether, the results published in the recent years suggest different scenarios for the induction of the salt and pepper pattern.

(a) Stochastic cell-to-cell expression heterogeneity

Single-cell transcriptomic analyses reveal that there is no clear RNA expression pattern in ICM cells at E3.25, as no correlation can be established between expressed genes, even for the genes that show a segregated pattern at E4.5 [15,23]. These genes are expressed at different levels, with a random distribution in the ICM population, suggesting a stochastic activity of their expression. Single-cell quantification *in situ* supports this hypothesis as no spatial correlation between specifying cells can be found [36]. During the subsequent stages, gene expression correlations can be observed depicting the beginning of an identity acquisition. At E3.5, most of the cells can be classified with Epi or PrE signatures [15,20,23,28,36]. Thus, when the cells start to specify they gradually acquire their lineage-related markers while downregulating the ones from the alternative lineage (figure 2*a*). This model proposed by Ohnishi *et al.* [23] also takes into account the action of the secreted FGF4, which reinforces the cell-to-cell segregation. These studies confirm that lineage identity is only acquired around E3.5 [14]. However, these acquired identities are the output of an earlier specification mechanism. Stochastic gene

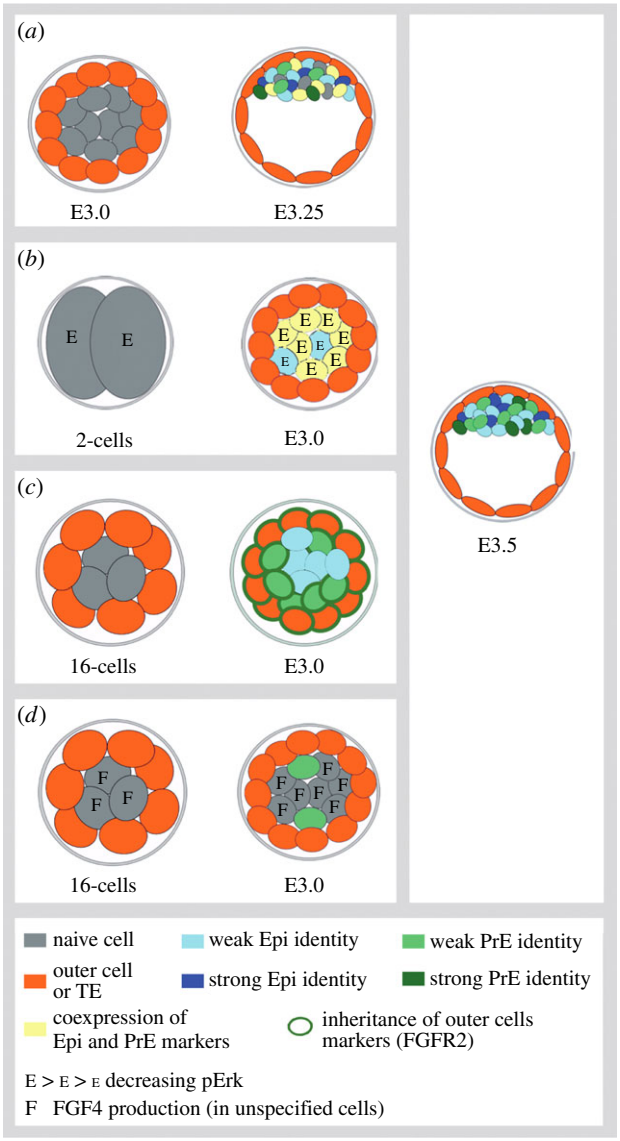

Figure 2. Proposed models for the initiation of the salt and pepper pattern. (*a*) **Stochasticity**. Before E3.25, inner cells express some Epi and PrE markers randomly. From E3.25, cells progressively acquire either an Epi or a PrE signature, owing to cell-to-cell stochastic expression reinforced by cell interactions. By E3.75, the two lineages are distinct [23]. (*b*) **Nanog expression gain through pErk decrease**. Cells express high levels of active Erk at the two cells stage, owing to high levels of FGF4. The transcription of *Fgf4* diminishes subsequently. From the 8-cell stage, Nanog and Gata6 are coexpressed in all the cells. Around E3.0–E3.25, the FGF4 protein levels are heterogeneous leading to a lower Erk activity in some cells. As a consequence, these cells promote Nanog expression, which in turn re-induces FGF4 expression at high levels. The neighbouring cells receive the FGF4 message and differentiate into PrE. The specification of the two lineages is asynchronous and leads to a progressive cell-to-cell identity acquisition [20]. (*c*) **Inherent differences between In1 and In2 cells**. At the 32-cell stage (E3.0), In2 cells inherit FGF2 from their mothers. As a consequence, In2 cells preferentially become PrE cells, whereas In1 cells favour an Epi identity [52]. In1 cells could acquire an Epi identity as soon as they are internalized or later, as a default state. (*d*) **Inside cell accumulation**. Inner cells (In1 and In2) produce FGF4. The accumulation of FGF4-expressing cells leads to high concentration of secreted FGF4 that is sufficient to induce PrE differentiation in some cells [53]. Epi identity could be acquired as an induced or default state.

activation followed by mutual reinforcement could be one explanation. Still, the early expression of *Fgf4* in a subset of cells at E3.25 [15,23] clearly designates it as the earliest identified marker for the salt and pepper set up. How FGF4 is produced in a subset of cells is not clear yet, and it remains to be known whether stochastic activation of FGF4 alone can be the switch for Epi versus PrE specification.

(b) Dilution of early FGF4 secretion promotes Nanog expression

Throughout preimplantation, the expression of *Fgf4* is very dynamic. While being high at the 2-cell stage, it then decreases until the set-up of the salt and pepper pattern and is high again in Epi cells only [15]. These dynamic changes in FGF4 levels could trigger the binary decision. During cell divisions, the early secretion of FGF4 is certainly diluted and diffuses, possibly creating the local heterogeneities needed to set up the salt and pepper pattern. As Epi cells are specified first, it would be a decrease—and not an increase—of FGF4 production that would be the trigger [20,54]. The lower Erk activity in some cells would promote Nanog and downregulate Gata6 expression (figure 2*b*). Nanog-expressing cells would then generate the salt and pepper pattern by inducing the later FGF4 expression. This hypothesis is supported by the mathematical model, but the role of early FGF4 expression, however, will be difficult to assess as well as the active Erk decrease (predictions indicate that a 15% decrease would be sufficient) [20]. Moreover, the mathematical model refers to Gata6, Nanog and the FGF4/FGFR2 pathway activities only, whereas any additional factor, such as another RTK ligand or a transcription factor, promoting Nanog expression could act to switch on the salt and pepper expression pattern.

(c) History from different rounds of asymmetric division

More than 25 years ago Chisholm & Houliston [55] observed a difference of cytokeratin expression between cells issued from the first (In1) or the second (In2) asymmetric division, and it was suggested that the round of cell internalization could influence ICM cell fate [9,55]. More recently, cell-lineage-tracing experiments through microinjection did not support this hypothesis, showing equal potential fates for In1 and In2 cells [3]. Conversely, manual cell-tracking analyses showed that a great majority of In1 cells preferentially contribute to the Epi while In2 cells rather produce PrE cells [5,52]. It was suggested that the time spent outside biases cell fate of In2 cells, as outer cells producing In2 would be less pluripotent, owing to their differentiation towards trophectoderm. This hypothesis was tested by enclosing a single 8-cell stage blastomere or an outside cell from the 16-cell stage with 8-cell stage 'host' blastomeres. While blastomere size or the number of enclosing cells were not considered, this experiment showed a different Epi/PrE ratios contribution between 8- and 16-cell stage blastomeres [52].

Higher levels of *Fgfr2* mRNAs were observed *in situ* in outside cells at the 16-cell stage. The authors suggested that the receptor could be maintained in In2 cells [52]. This differential expression, initially driven by outside cells, would then promote a PrE state in In2 cells (figure 2*c*). It was thus proposed that In1 and In2 cells are inherently different [52]. This interesting scenario would have to be sustained by more FGFR2 expression experiments at different stages to confirm and understand its dynamic pattern. Additionally,

overexpressing FGFR2 from the 2-cell stage shows that it can bias cells towards a PrE identity [52]. However, it does not indicate whether FGFR2 is required already at the 16-cell stage or only later during the propagation of the salt and pepper pattern around E3.25–E3.5, as was shown using FGFR/Mek inhibitors [3,19].

(d) Accumulation of inner FGF4-expressing cells leads to the initiation of the salt and pepper pattern

The number of In1 cells at the 16-cell stage can vary, according to the genetic background and to the method used to identify them [4–7,52,53,56]. Time-lapse microscopy revealed a correlation between In1 and Epi fate only if the In1/In2 ratio is low [52,56], reconciling the divergent cell tracing reports [3,5]. To examine the influence of the number of In1 cells on Epi/PrE specification, different ratios of inner/outer cells at the 16-cell stage were analysed to assess cell potential through blastomere reaggregation [53]. This analysis also concluded that there is a strong bias of In1 for Epi and In2 for PrE when the number of internalized cells at the 16-cell stage is low. Thus, from relatively similar results [52,53,56], two different models were proposed (figure 2*c*,*d*). One favours that the influence is driven by outside cells resulting in intrinsically molecularly different In1 and In2 cells (figure 2*c*) [5,52]. The other model privileges the number of internalized cells as the biasing factor with no impact of their origin from the first or second round of asymmetric divisions (figure 2*d*). As a differential expression of *Fgf4*, although not significant, was found between In1 and In2 cells, the authors suggested that cell specification depends on the accumulation of this ligand. During their internalization, In1 or In2 cells would have an equal potential and express low levels of FGF4. The accumulation of these *Fgf4*-expressing cells would lead to greater levels of extracellular FGF4, which, at a defined level would induce PrE differentiation (figure 2*d*) [53]. A low number of In1 cells would secrete low amounts of FGF4, unable to induce any PrE specification, thus pushing them towards an Epi identity. In this case, In1 cells would be biased towards an Epi identity. In the case that the number of In1 cells is high, the pool of available FGF4 would be sufficient to induce a PrE identity within In1 cells, erasing any correlation between inner cell generations and cell fate. Therefore, in this model (figure 2*d*), the bias would be generated by inside cells.

Thus, each of these models will require additional experiments to be confirmed. The proposed mechanisms are not exclusive, and a combination of these models might be the final solution. It will be important to clearly determine the dynamic expression patterns of FGF4 and FGFR2 and their aetiology.

4. Differentiation of the specified lineages

(a) Epiblast differentiation

Once specified, Epi and PrE lineages rapidly differentiate. Little is known about Epi lineage maturation. The changes might be minor as the cells must be kept in a pluripotent state. A downregulation of Nanog is observed from E4.5 [57], which is dependent on the presence of FGF4 [21]. Thus, like in ES cells [58], the FGF signalling might be preparing Epi cells for their differentiation into the three germ layers. In *Gata6*$^{-/-}$ embryos, Nanog expression diminishes at the same time as in wild-type littermates, meaning that this decay does not depend on the PrE [20]. Thus, despite a precocious specification, these mutant Epi cells are reset at a normal timing.

Recent reports have shown that *Oct4* and *STAT3* are necessary for the maintenance of both Epi and PrE lineages as early as E4.5 [41,42,59]. The defects in the Epi are probably owing to the reactivation of Cdx2 expression [60]. As *Oct4* is a direct target of the STAT3 pathway [59], the phenotype of the corresponding mutants might be similar.

Some markers, such as *Fgf5* [61] or the *Nodal* enhancer ASE [62], are upregulated during Epi maturation. Interestingly, the downregulation of Nanog is heterogeneous in the Epi cells at E4.5 and corresponds to the appearance of the ASE-GFP transgene. The two markers are thus expressed in a complementary and exclusive manner, suggesting a release of inhibition by Nanog on the ASE [62]. As Nodal is required to keep the Epi from neural differentiation [63,64], the results suggest that it might relay Nanog in maintaining pluripotency [62], probably with different features.

(b) Primitive endoderm differentiation

PrE cells differentiate fast, as they need to be functional as soon as possible to sustain the nutritional needs of the embryo. The analyses of *Nanog* mutant embryos revealed that Epi cells have a critical role in PrE differentiation. Indeed, while they express Gata6, *Nanog*$^{-/-}$ embryos have very few or any cells expressing Sox17, Gata4 or Pdgfra [25–27]. The lack of Sox17 or Gata4 expressing cells is rescued when wild-type Epi cells are present, either through ES cells chimaera complementation [26] or through mosaic inactivation of Nanog [27]. Because *Fgf4* is transcribed specifically in Nanog-expressing cells and is absent in E3.5 *Nanog*$^{-/-}$ embryos, it suggested that FGF4 is potentially the non-cell-autonomous factor required for PrE maturation. Strikingly, the administration of recombinant FGF4 to *Nanog*$^{-/-}$ embryos rescues the expression of Sox17, Gata4 and Pdgfra expression, confirming the hypothesis [27]. Altogether, experiments with *Nanog* mutants show that Gata6 alone cannot induce the expression of these maturation genes. They require an activation by FGF4 that is secreted from Epi cells under the control of Nanog. As well, FGF4 cannot activate Sox17 and Gata4 in the absence of *Gata6* [20,36] meaning that a cooperation between Gata6 and FGF4 is needed.

Similar experiments were carried out with *Oct4* zygotic mutant embryos. These embryos specify PrE cells but cannot maintain them, the number of Gata6 expressing cells diminishing drastically [41]. FGF4 expression is missing in *Oct4*-deleted embryos at E3.5 [42]. In these embryos, where *Oct4* is excised from the 8-cell stage, administration of recombinant FGF4 can rescue Sox17 expression, but not later aspects of PrE development. This means that either Oct4 induces another non-cell-autonomous factor from the Epi or that Oct4 is required cell-autonomously in the PrE. To this end, chimaera complementation experiments were carried out with wild-type ES cells. In embryos where *Oct4* is deleted from the 8-cell stage, wild-type ES cells can rescue a PrE phenotype when injected early (at the 8-cell stage) as opposed to late (at the blastocyst stage). This shows that in this case *Oct4* is not required within PrE cells. In addition, it suggests that the required non-cell-autonomous factor(s) needs to accumulate or to act early on to be fully efficient. Another chimaera essay with wild-type ES cells aggregated to 8-cell stage zygotic mutant embryos failed to rescue

Sox17 expression. While the role of Cdx2 re-expression in the ICM needs to be assessed, this result means that *Oct4* is required cell-autonomously in the PrE [41]. The difference between the two chimaera assays may reside in the timing of *Oct4* deletion and that in 8-cell-stage deleted embryos the cell-autonomous factor might have acted beforehand. A difference could also be due to different genetic backgrounds. It is noteworthy that Oct4 changes target DNA binding sites due to different partners, from Sox2 to Sox17, upon PrE differentiation and this reflects a cell-autonomous requirement within the PrE [65]. Altogether, the three studies are complementary and suggest that Oct4 activates first an unknown cell-autonomous factor before the 8-cell stage and then a non-cell-autonomous factor, which both allow the later differentiation of PrE-specified cells.

After maturation by FGF4, PrE cells sort from Epi cells to join the surface of the ICM, in contact with the blastocoel cavity. Before or during their migration, PrE cells acquire additional expression markers. Several of these were found at E3.5 days during the first single-cell RNA analysis of the blastocyst: *Sox17*, *Gata4*, *Pdgfra*, *Cubn*, *Lama1*, *Foxq1*, *Serpinh1*, *Col4a2* [28]. Expression of many of these and other genes was confirmed *in situ* at different stages [23,66–68]. A recent single-cell RNA analysis led to a ranking of PrE-expressed genes according to their timing of activation, their preponderance and their coexpression among PrE cells [23]. Collectively, the data present a hierarchy in the sequence of gene activation. A first group comprises genes/proteins expressed shortly after PrE specifies, before the cells have sorted: Gata6, Sox17, Serpinh1, Laminin1, followed by Gata4, Lrp2, Pdgfra, Col4a [14,23,66–68]. Some proteins such as Sox7, Lrp2 or Dab2 initiate their expression or change subcellular localization when individual cells reach the surface of the ICM [66,68].

Dab2 excepted, all these PrE differentiation genes are not absolutely required to proceed to the next steps of differentiation towards visceral or parietal endoderm. ChIP analyses reveal complex interactions between these genes [67], suggesting mutual reinforcement of their expression and possible redundant activities.

At the time of cell sorting, a high proportion of ICM cells, about 20%, undergo apoptosis [69]. The reason is unknown as it is impossible to identify dying cells on fixed embryos. There are several hypotheses that are not exclusive. Some cells could not manage to resume either an Epi or a PrE identity and would die as a consequence. This is supported by the mathematical model that also predicts undetermined cells during ICM cell specification [20]. Interestingly, in *Nanog* mutant embryos in which all ICM cells have chosen to become PrE, there is no cell death at E3.75, also reinforcing this hypothesis. Alternatively, some specified cells might die because they do not manage to reach their final position, which is inside for an Epi cell or at the surface for a PrE cell. Apoptosis would thus eliminate misplaced cells to achieve two homogeneous tissues. However, some rare PrE cells can be found deep in the ICM as late as E4.75 in wild-type embryos (C. Chazaud 2010, unpublished data) or in sorting impaired embryos [70]. It is not excluded that cells from any compartment, Epi and PrE, could die. Absence of Pdgfra increases PrE cell death [71], but this might be unrelated to the observed wild-type cell death. Indeed, a Z-VAD caspase inhibitor treatment cannot prevent cell death of wild-type embryos, whereas it can rescue the number of *Pdgfra*$^{-/-}$ PrE to wild-type levels [71], suggesting different mechanisms.

(c) Cell sorting mechanisms and epithelium formation

The cell sorting process became evident when cell lineage tracing and cell grafts revealed that most ICM cells at E3.5 do not change their identity [10]. The use of the Pdgfrα-GFP reporter enabled filming and tracking of PrE cells at the late blastocyst stage and revealed that the segregation into two layers is completed around the 100-cell stage *ex vivo* [14]. The beginning of these directional movements is difficult to define as the frequent cell divisions also cause cell displacement [11]. Although it is possible that PrE cells seek the ICM surface as soon as they have specified, thus in an asynchronous manner. This would lead to an increasing bias in finding PrE cells at the surface, which can already be detected around E3.5 [23]. The mechanism(s) involved in these cell movements are still not clear, but mathematical modelization suggests that both cell adhesion and directional cues are required [72]. Experiments recapitulating the Steinberg affinity hypothesis [73] were carried out in embryoid bodies lacking E- or N-cadherin. The authors found that the difference of adhesiveness can result in the segregation of Epi and PrE cells. But, surprisingly, removing cadherins did not prevent cell sorting when the cells are primarily treated with retinoic acid, used as a primer for PrE differentiation, suggesting that other mechanisms are involved [74,75]. Passive rearrangements after cell division participate in the sorting [11]. In addition, experiments with cytochalasin D show the involvement of actin-dependent movements [11]. Active cell movements imply polarization of the PrE cells, but so far no polarized proteins have been found during cell migration through the ICM. The first polarization cues that are detected are Lrp2 and Dab2, which are localized on the future apical side of the epithelium only once the cells have reached the surface. Interestingly, cell polarization occurs in individual cells, before all the cells have attained the surface [66]. Analyses in embryoid bodies show that this polarization, once at the surface, seems to be progressive [75], suggesting that the cells are not polarized beforehand, at least with these markers. While being involved in many early polarization events and directional cell migration [76], the atypical protein kinase C (aPKC) protein is enriched apically in the PrE epithelium only around E4.5 [77], thus after Lrp2 and Dab2 polarized expression. Impairment of aPKC function through RNAi or with a pharmacological inhibitor during four-dimensional imaging reveals that cell sorting occurs correctly in these aPKC deficient embryos, but maintenance of the PrE epithelium is affected [77]. The endocytic adaptor protein Dab2, which interacts with LDL receptor complexes such as Lrp2, is required for PrE cell sorting [70,78]. This is surprising as the protein is only apparent once the cells are at the ICM surface [66]. This could be due to undetectable expression at earlier stages or, as in aPKC-deficient embryos, owing to a failure to maintain the cells at the surface. Interestingly, the inactivation of *Oct4* in embryoid bodies leads to a cell-sorting defect as Dab2 or α-fetoprotein expressing cells remain inside the clump [79]. This phenotype remain to be checked in embryo live imaging and could provide novel cues for the cell sorting model.

Formation and maintenance of the PrE epithelium remains largely unknown with very few experiments reporting potential mechanisms. Embryoid bodies [80] resulting from ES cell aggregation have provided a lot of information, notably on the importance for the laminin/integrin pathway [81]. As mentioned above, a deficient aPKC signalling induces defects in epithelium formation. One of the consequences of this

inhibition is Gata4 localization in the cytoplasm instead of its usual expression in the nucleus [77]. Gata4 is probably not the only target of aPKC as *Gata4*$^{-/-}$ embryos survive that stage and die of later postimplantation defects [82,83], despite a lack of PrE differentiation in mutant ES cells [84]. This Gata4-mislocalized expression is also observed in integrin-β1 null embryoid bodies [85]. In these embryoid bodies, which recapitulate the embryonic phenotype [78], specified and sorted PrE cells detach because of the lack of a proper basement membrane. Thus, potential interactions between aPKC and integrin pathways could lead to the set-up or the maintenance of epithelial polarity, as was shown in other systems [86,87]. Sox17 is also implicated in the maintenance of the epithelium as Sox17-deficient embryos have PrE cells that are scattered on the mural trophectoderm cells. Whether this defect is linked to the aPKC/integrin pathway or is a distinct phenotype remains to be established.

5. Conclusion

The different novel technologies such as single-cell transcriptomics or live imaging have provided considerable help in understanding the molecular networks driving PrE differentiation. Deciphering how the epithelial structure of the PrE is set up from scattered progenitors will be of high interest. Moreover, despite considerable advances in understanding PrE specification and differentiation, some main questions remain unanswered. Finding what is triggering the salt and pepper pattern is definitively a key issue that is highly debated. A challenge will be also to find the mechanism in human embryos, as they are not sensitive to variations of the FGF/RTK pathway for Epi and PrE specification.

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