

Mapping the journey from totipotency to lineage specification in the mouse embryo

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Understanding the past is to understand the present. Mammalian life, with all its complexity comes from a humble beginning of a single fertilized egg cell. Achieving this requires an enormous diversification of cellular function, the majority of which is generated through a series of cellular decisions during embryogenesis. The first decisions are made as the embryo prepares for implantation, a process that will require specialization of extra-embryonic lineages while preserving an embryonic one. In this mini-review, we will focus on the mouse as a mammalian model and discuss recent advances in the decision making process of the early embryo.

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Current Opinion in Genetics & Development 2015, **34**:71–76

This review comes from a themed issue on **Cell reprogramming, regeneration and repair**

Edited by **Amander T Clark** and **Thomas P Zwaka**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 3rd September 2015

<http://dx.doi.org/10.1016/j.gde.2015.08.002>

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Introduction

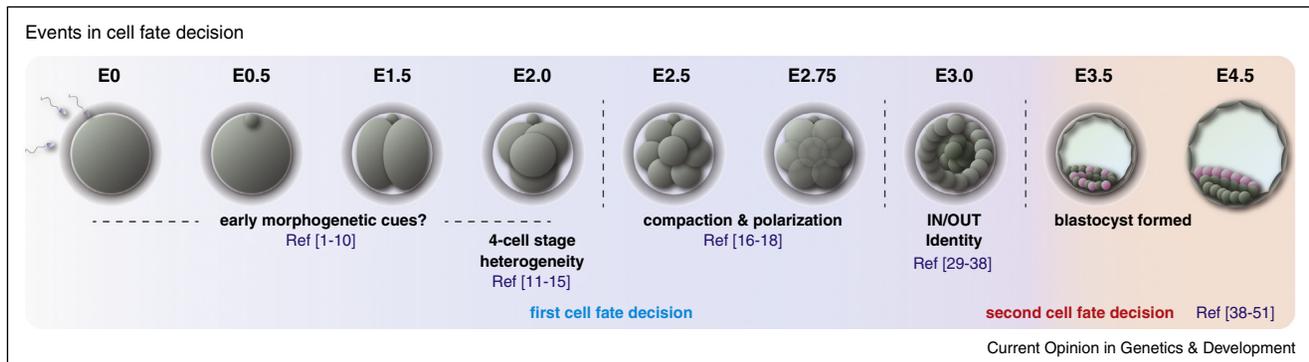
In mammals, a new generation begins when an oocyte is fertilized by a sperm to form a zygote. From this point until the embryo implants, cell fate decisions revolve around the partitioning of the two extra-embryonic lineages and the embryonic one. Cleavage divides the zygote into blastomeres without increasing its size and by E3.5 a cavity will have formed within the embryo to distinguish two cell populations: the extra-embryonic trophoblast (TE), a one-cell thick layer of epithelial-like cells surrounding the pluripotent inner cell mass (ICM) that lies to one side of the cavity. This lineage specification process is referred to as the first cell fate decision (Figure 1). A further differentiation event will occur within the ICM, setting apart the primitive endoderm (PE) and the epiblast (EPI) in the second cell fate decision. The PE is another extra-embryonic lineage that will develop into the yolk sac, and the EPI is the truly embryonic lineage that will form the embryo proper. By

E4.5 the PE cells will have moved to the cavity side of the ICM, so that the embryonic lineage is coated by the PE on one side and TE on the other. This is the final product of pre-implantation development, and the embryo is ready to implant. Thus pre-implantation cell fate decisions address whether to be or not to be an epiblast cell.

Morphogenetic cues for cell fate

Intensive research has been carried out on the underlying mechanisms of cell fate specification in the pre-implantation embryo. In many vertebrates, the zygote has a defined axis with asymmetrically distributed determinants ready to instruct cell fate. In the mammalian zygote, a polarized cell fate determinant either doesn't exist or has yet to be discovered. Earlier work was focused on determining the existence of an initial cue that would break symmetry to specify the first embryonic axis, the embryonic-abembryonic axis, as defined by the position of the ICM. Possible cues could relate to the sperm entry point and the position of the polar bodies, which are byproducts of asymmetric meiotic divisions that mark the 'animal pole' of the animal-vegetal axis (AV axis) of the zygote. Our research among others observed a bias between the polarity of the zygote and the contribution to distinct cell lineages that depends on the orientation and order of the cell divisions to the 4-cell stage [1–4]. But others have not observed any fate bias in cell fate [5–9] or proposed that the mechanical constraint of the zona pellucida leads to specification of the embryonic-abembryonic axis [9]. Supportive evidence for symmetry breaking by the 4-cell stage comes from the finding that when the same 4-cell stage blastomeres are combined together, the resulting embryos differ in developmental potential depending whether their cells originate from animal or vegetal pole [10,11]. Recent genetic tracing using the Rainbow transgenic mouse confirmed that indeed individual blastomeres of the 4-cell embryos differ and show a bias towards a particular cell fate [12*]. Such discrepancies likely arose due to the highly sensitive nature of early mouse embryos and also various technical limitations. Mouse embryos are remarkably plastic. Even within the same litter, embryos can exhibit different developmental dynamics, can compensate for experimental manipulations and are the antithesis of eutelic organisms such as nematodes that follow a deterministic developmental program. Technical limitations also hamper attempts to understand this period of development. The few number of cells in these embryos precludes use of conventional biochemical techniques applied in traditional cell biology. Furthermore these embryos essentially

Figure 1



Overview of key cell fate decision events during pre-implantation development. During the first four and a half days of mouse development the free-floating embryo undergoes cleavage and differentiates extra-embryonic lineages from the embryonic one. The fertilized egg, also known as the zygote, lacks polarization of any known cell fate determinant. Little is known about the molecular mechanism of cell fate specification in these early stages up until the 4-cell stage, where blastomeres express different levels of epigenetic regulators, and each blastomere has a bias towards a particular lineage. Compaction at the 8-cell stage allows the generation of inside/outside (IN/OUT) cells, the first time where blastomeres take on physically different positions within the embryo. Inside cells are more likely to form the ICM while outside cells is predisposed to the trophoblast. The process in which this occurs has generally been termed the first cell fate decision. After the formation of the blastocyst cavity at E3.5, some cells in the ICM will start to express markers of the primitive endoderm and sort towards the cavity side of the ICM. This process is called the second cell fate decision and produces the second extra-embryonic lineage of the embryo.

have two transcriptomes: one inherited from the oocyte and the newly combined zygotic transcriptome. This maternal-to-zygotic transition is very poorly understood and complicates various genetic approaches. To breakthrough these barriers requires a new generation of technology, and with recent progress in techniques such as single-cell sequencing and live imaging, it may become possible to shed more light on this mysterious period of development.

Molecular regulators of cell fate before compaction

The challenge now is to account for the findings about early asymmetry in molecular terms. Epigenetic differences have been found between blastomeres as early as the 4-cell stage. The epigenetic regulator Prdm14 is heterogeneously expressed between 4-cell stage blastomeres and artificially elevating levels of H3 methylation via Prdm14 or Carm1, another epigenetic modifier, directs cells to the epiblast fate [13,14]. However, it is still not clear how this early heterogeneity becomes established. The intracellular kinetics of exogenously introduced Oct4 also differentially influence cell fate already at the 4-cell stage [15] but it will take further work to understand the significance of this and of how well it represents the behavior of endogenous Oct4. Later on in development, compaction at the 8-cell stage provides mature cell-cell contacts that are crucial for polarization and cell fate decisions [16,17], but what regulates the timing of this process remains mysterious. During compaction the mRNA of the key TE determinant Cdx2 localizes to the apical domain of blastomeres, mirroring mechanisms for localization of developmentally impor-

tant transcripts in other non-mammalian vertebrate and invertebrate embryos. This localization of Cdx2 transcripts contributes to a process whereby Cdx2 expression becomes restricted to outside cells, thus biasing those cells to become TE [18*]. Cdx2 is maternally deposited as well as zygotically transcribed, and the role of maternal Cdx2 has been of debate. Although it has been suggested that maternal Cdx2 is dispensable [19,20], the elimination of both maternal and zygotic Cdx2 leads to defects in TE specification at the cleavage stages, thus earlier than when only zygotic Cdx2 is eliminated [21,22].

Molecular regulators of cell fate after compaction

Compaction eliminates intercellular space and allows blastomeres to divide asymmetrically enabling them to populate the inside of the embryo. This marks the first time where distinct, 'inside' or 'outside', populations of blastomeres emerge. Members of the Hippo signaling pathway play a crucial role in setting up the differential lineage bias of these two populations. When the Hippo pathway is activated, the kinases Lats1/2 phosphorylate the transcriptional co-activators Yap/Taz [23–25]. Yap/Taz phosphorylation results in their cytoplasmic sequestration and, consequently, their target genes are not expressed [25]. In ES cells, Yap has been reported to promote pluripotency [26,27], which is mediated through a Yap-Tead2 interaction [27]. However, in the embryo Yap has a different mode of function as instead of Tead2 [28], Tead4 is required for successful pre-implantation development [29,30]. TE genes such as Cdx2 and Eomes are not expressed in most Tead4^{-/-} embryos

leading to a developmental arrest at the morula stage [29,30], suggesting *Tead4* is an activator gene for the TE fate. Despite being a TE regulator, *Tead4* is expressed constitutively in all cells of the blastocyst [29]. However, its function is differentially controlled by the differential localization of Yap/Taz. An active Hippo pathway in inside cells sequesters Yap/Taz from the nucleus, *Tead4* is not activated and the cells take on an ICM fate. In contrast in outside cells the Hippo pathway is inactive, Yap/Taz activates *Tead4* and the TE program is switched on [31**]. The other Hippo pathway kinase *Mst1/2* does not seem to feature in YAP localization in the pre-implantation embryo [32].

Polarization of the Par complex is a known determinant for the TE fate [33], however there was no molecular pathway linking it to nuclear transcription. Recent progress identified the junction-associated protein Angiomotin (*Amot*) as the missing link. *Amot* is distributed apically in outside cells but does not show any polarity in inside cells [34**,35]. *Amot* is able to activate Lats in the inside cells as well as sequester Yap independently [34**,35]. Downregulating polarity components such as *Pard6b* or disrupting aPKC function via dominant negative constructs results in an apolar distribution of *Amot* in outside cells. Consequently these outside cells have cytoplasmic Yap and *Cdx2* is not expressed. Furthermore, *Amot* can be phosphorylated and activated by Lats, suggesting a positive feedback loop [34**]. Another upstream component of the Hippo pathway, *Nf2*, has also been identified as a cell fate regulator. *Nf2* mutant embryos fail to phosphorylate YAP in the inside cells, and the phenotype is compounded in a maternal-zygotic mutant, indicating *Nf2* is also maternally supplied. *Nf2* has been proposed to function in the adherens junction complex along with *Amot*, therefore *Nf2* and *Amot* may be functionally linked [32,34**].

Apart from the Hippo signaling pathway, Notch signaling also appears involved in the first cell fate decision. Analysis of an upstream cis-regulatory enhancer of *Cdx2* revealed that Notch signaling cooperates with *Tead4* to ensure sufficient activation of *Cdx2* in the TE, and over-expression of the Notch1 intracellular domain (NICD) is able to drive blastomeres to the TE [36]. However, Notch signaling does not affect expression of other TE genes such as *Eomes* or *Gata3* indicating that it is not required for the overall development of the TE [36].

Low oxygen bypasses *Tead4* requirement

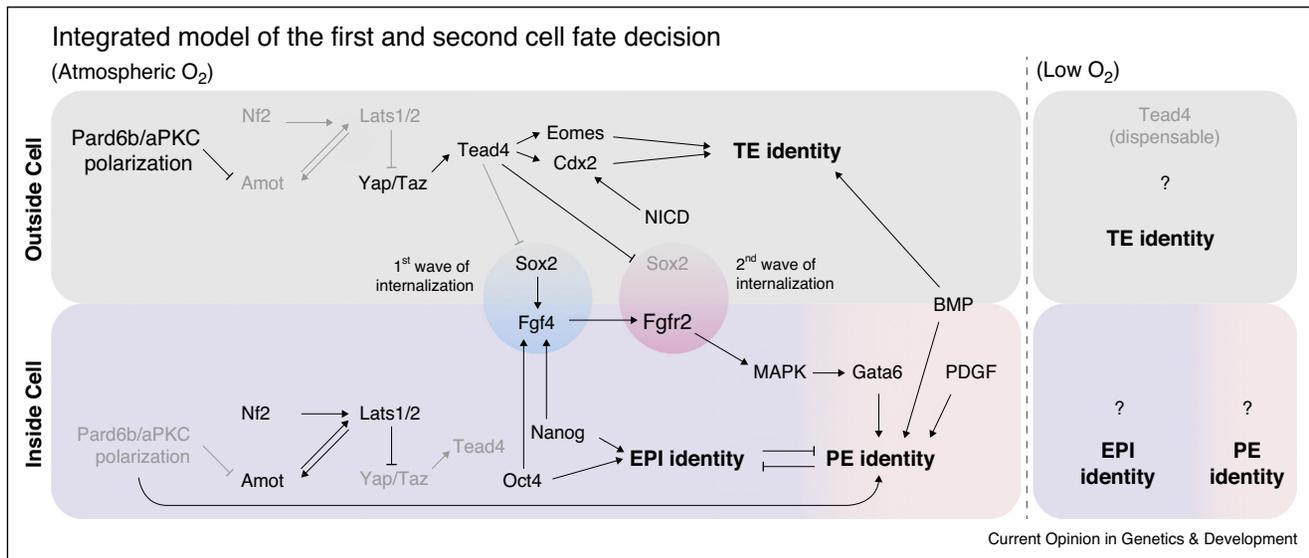
Recently, the Hippo/*Tead4*-centric viewpoint was put into question by a study that cultured embryos in low oxygen (5%), which more closely resemble the *in vivo* environment, rather than the conventionally used atmospheric level (21%) [37**]. Under 5% O₂ *Tead4*^{-/-} embryos were able to develop to the blastocyst stage as well as express TE marker genes such as *Cdx2*, *Eomes*, *Gata3*

and *Elf5*. This surprising but important finding indicates *Tead4* may actually be dispensable for TE development. However *Tead4*^{-/-} embryos were reported to arrest their development at E3.5 *in vivo*, without a well-defined TE [30,37**]. It was hypothesized that oxidative stress-inducing metabolic substrates present *in vivo* but not *in vitro* was the cause for this discrepancy. Supplementing the *in vitro* culture media with glucose, essential amino acids and glutamine caused *Tead4*^{-/-} embryos in low oxygen to arrest in development, similar to their counterparts developing *in vivo*. Furthermore, addition of an antioxidant was able to rescue *Tead4*^{-/-} embryos under otherwise non-permissive conditions. Together these results suggest *Tead4* protects the embryo from oxidative stress, and without oxidative stress *Tead4* becomes dispensable for TE development [37**]. But why should *Tead4*^{-/-} embryos have a TE-specific phenotype? This could be because the TE is much more enriched in mitochondria than the ICM [10,11], and uses oxidative phosphorylation while the ICM uses glycolysis [11,22]. The high energy consumption in the TE is likely used to drive expansion of the blastocyst cavity. Therefore it is possible that *Tead4* (and also the Hippo pathway) acts as a protective measure for the TE, rather than a true cell fate determinant. Such a finding reveals there is still a lot more to be learnt about the first cell fate decision.

Rise of the primitive endoderm

The second cell fate decision differentiates the EPI from PE within the ICM, but this process may begin much earlier, before the ICM is formed. The timing of blastomere internalization affects the probability of it becoming PE or EPI: blastomeres internalized in the first round of asymmetric division (8- to 16-cell stage) are more likely to become EPI, and those internalized in the second or third round of asymmetric division (16- to 32-cell stage and 32- to 64-cell stage) are more likely to become PE [38*]. This was not observed in another study [39], but was recently validated by a third group [40]. This bias was found to be due to differential levels of *Fgfr2* [41]. FGF signaling is a known component for PE formation [39], and blastomeres internalized in the second wave upregulate *Fgfr2*, thereby becoming more sensitive to FGF signaling essential for PE formation [41]. *Nanog*-expressing cells in the ICM produce FGF [42*] and although FGF signaling is the limiting factor for the amount of PE cells, it is not required for the initial expression of PE markers [43]. Recent work suggests a role for *Oct4* in PE formation, in agreement with the previously known role of *Oct4* and *Sox2* in lineage priming [44]. Thus, in the embryo *Oct4* appears to be required for the expression of FGF, maintenance of early PE marker *Gata6* and for the expression of late PE markers *Sox17* and *Pdgfra* [45,46]. Mature PE cells acquire apical-basal polarity and the apical polarity marker aPKC is found to take part in the second cell fate decision as well as the first. aPKC is enriched in PE progenitors and after cell sorting within the ICM, aPKC

Figure 2



An integrated model of the first and second cell fate decision. Molecular determinants that can be consolidated into a connected network are shown. Lines between the first and second cell fate decisions are blurred after the discovery that Tead4, the central protein of the first cell decision, downregulates Sox2 expression, and that Sox2 can promote the PE fate. Spanning both the first and second cell fate decisions, this could provide the molecular basis for the PE bias of blastomeres internalized later in development. All of the pathways shown are elucidated through culturing embryos under atmospheric oxygen levels. Low oxygen levels relieve the Tead4 requirement for trophectoderm formation and opens up the question whether Tead4 is a true cell fate determinant.

becomes polarized at the cavity interface. Disrupting aPKC function results in failure in TE formation [33] as well as in PE sorting and maturation [47], which links first and the second cell fate decision process. PDGF signaling is also involved in PE formation. This pathway appears independent of the FGF pathway and does not affect lineage commitment or cell sorting, but rather PE cell survival [48].

Parts of a whole: integrating the two cell fate decisions

Thus far the first and second cell fate decision have been treated as separate events, but the lines have been blurred after recent discoveries that both BMP signaling [49] and also Sox2 [50^{*}] play a role in both. A deep sequencing screen of different cell populations within the embryo revealed previously anticipated, pre-implantation role of BMP signaling [49,51]. Interfering with the functions of various BMP signaling components leads to impaired development of both extra-embryonic lineages while the EPI lineage develops normally [49,51]. Tead4 downregulates Sox2 expression in the outside cells, independently of Cdx2, making Sox2 the first pluripotency factor to be restricted to the inside cells at the morula stage. After the first cell fate decision, Sox2 then specifies the PE by upregulating PE genes via FGF4, as well as maintaining EPI gene expression [50^{*}]. This falls in line with an integrated cell fate model, where prolonged exposure to

TE determinants would bias blastomeres to the PE when their progenies become positioned inside (Figure 2).

Conclusion

Despite a rapidly expanding field, we have much to learn about the mechanisms that underlie transition from totipotency to embryonic pluripotency and from embryonic pluripotency to lineage specification in the embryo. Most of our current knowledge is concentrated upon the later stages, where some molecular determinants are known. In contrast, our understanding of the earlier stages, especially before compaction, is far from complete. We have yet to piece together the growing evidence for the influence of early events upon cell fate choices into a coherent picture. Consolidation of all of these findings will give us insight into the earliest cues leading to exit from totipotency to pluripotency and finally to lineage specification.

Acknowledgements

We are grateful to the Wellcome Trust for supporting the research in our group.

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