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Acquisition of the pluripotent and trophectoderm states in the embryo and during somatic nuclear reprogramming

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How the first cell fate decision of an embryo occurs is one of the most fascinating biological questions examined over the last few decades, with numerous *in vivo* models proposed and many factors tested for their role in the process. In this review, we will primarily focus on the mouse model and discuss the role that transcription factors play during establishment and maintenance of the first lineage segregation in the embryo, towards inner cell mass or trophectoderm. We will also overview recent developments in somatic nuclear reprogramming into induced pluripotent stem cells, the inner cell mass (epiblast) equivalent, and into induced trophoblast stem cells, the trophectoderm equivalent, and discuss potential correspondences between the *in vivo* and *in vitro* models.

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Introduction

Following fertilization of an egg by a sperm, an active and rapid demethylation process of the paternal genome is initiated, by contrast to the passive and replication-dependent demethylation of the maternal genome [1]. This nuclear reprogramming process yields a totipotent cell, the zygote, which holds the capacity to give rise to all embryonic and extraembryonic tissues [2]. In the mouse, just before the zygote divides into a two-cell embryo and until a full division occurs, changes in histone marks such as H3K4me3 take place in promoter regions. These changes lead to an active transcription, termed zygotic genome activation (ZGA) [3–5], which is one of the hallmarks of totipotency.

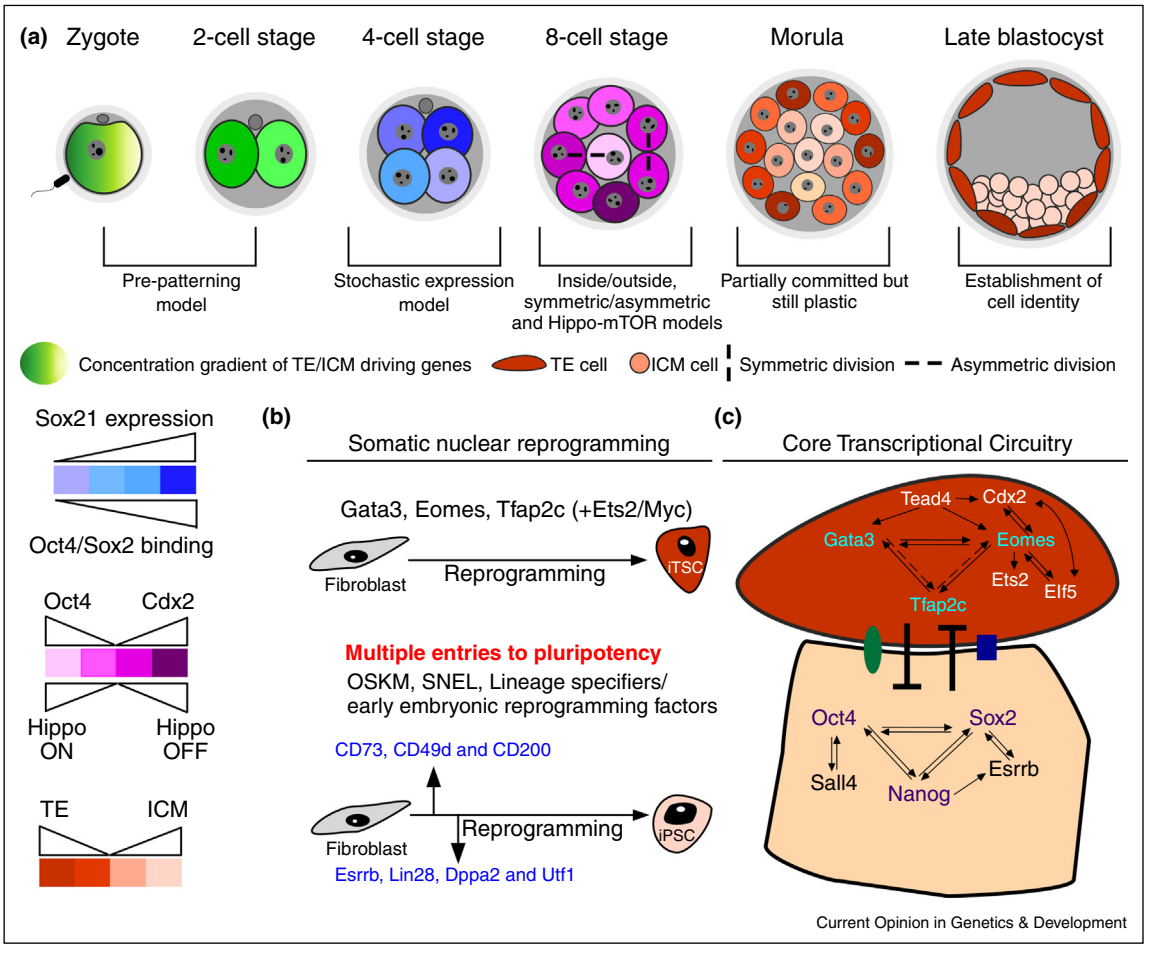
As the embryo continues to cleavage, its totipotent capacity gradually decreases. While the blastomeres of a two-cell embryo have equal developmental potential, the blastomeres of an eight-cell embryo represent the beginning of compaction, polarization, and asymmetric cell division [6]. At this stage, each of the embryo's cells has the tendency to differentiate into one of two cell fates: inner cell mass (ICM) that will give rise to the embryo proper (epiblast) and primitive endoderm (PrE), and trophectoderm (TE) that will produce extraembryonic tissues such as the placenta. By the next cell division, in the sixteen-cell compact morula, a more pronounced lineage restriction is observed, where the outer cells are more committed to the TE lineage and the inner cells to the ICM [6]. However, despite this initial specification, it is believed that most if not all cells at this developmental stage still retain some of the capacity to differentiate into both lineages [7,8].

Understanding the counteracting forces that orchestrate the first cell fate decision in the embryo is particularly intriguing, as only a couple of cell divisions later, at the late blastocyst stage, full morphological and molecular segregation take place and a strong cellular identity is established.

Establishment of the pluripotent and trophectoderm lineages in the embryo

It has been suggested that in the mouse embryo, the two first lineages are established as a consequence of reciprocal inverse expression of *Oct4* and *Cdx2* in early compact morula cells, that will give rise to ICM and TE, respectively [9]. Strikingly, despite this clear counter relation, it has been shown that depletion of the maternal and the zygotic *Oct4* gene does not affect the formation of ICM in the blastocyst [10]. Similarly, depletion of the maternal or zygotic *Cdx2* gene has no effect on the establishment of TE lineage [11,12], although a depletion of both maternal and zygotic *Cdx2* leads to TE specification defects at the late morula stage [13]. These findings suggest that at early stages, the counteracting forces between Oct4 and Cdx2 are not essential for driving cell specification in the embryo, and that most likely, their importance is in maintaining each of the lineages at later stages of specification. Another possibility is that there are robust gene redundancies within the embryo that compensate for the loss of *Oct4* or *Cdx2*. How, then, is the first cell fate decision determined? Several models have been proposed

Figure 1



Establishment of the pluripotent and trophectoderm states in the embryo and during somatic nuclear reprogramming. A scheme describing the current models for the first lineage segregation in the embryo and during nuclear reprogramming. **(a)** Three major models for lineage segregation during embryonic development are depicted: the pre-patterning model, the stochastic expression model and the inside/outside model. Color legends are presented on the bottom left. **(b)** A scheme showing the various factors (in black) and early markers (in blue) responsible for the generation of induced pluripotent stem cells (iPSCs) and induced trophoblast stem cells (iTSCs). **(c)** An illustration of two cells (upper cell: TE/iTSC, lower cell: ICM/iPSC) and the core transcriptional circuitries that safeguard their identity. Pointed arrows represent activation, blunted arrows represent inhibition, dashed arrows represent hypothetical activation, green oval and blue square represent tight junctions.

for addressing this question, and reviewed in great detail here ([14] and Figure 1). Briefly, one model is the inside/outside model, which proposes that segregation is a direct consequence of the cell's location in the early embryo. While the inner cells will form the ICM, the outer ones will form the TE, a dissection created due to the asymmetric cell division between the outer and inner cells, with each cell population being subjected to separate signaling and growth factor stimulation, dependent on its location [14]. This model further proposes that correct lineage specification is established regardless of the initial transcriptome of the cells. Supporting this is the observation that *Cdx2*-expressing cells can migrate from the outer layer towards the inner layer and as a result lose *Cdx2* expression and contribute to the ICM [15••].

Another model that points to cellular location as the basis for cell specification is the 'Hippo-mTOR pathway model', suggesting that tight junctions, presented solely at outer cells of the embryo, negatively regulate the Hippo pathway, which in turn inhibits the phosphorylation of the downstream effector YAP. YAP's inhibition causes its nuclear localization, resulting in the activation of *Tead4* and its inverse effect on the relation between Cdx2 and Oct4 during embryonic development [6,14]. Interestingly, suppression of the mTOR pathway leads to a diapause-like phase of pluripotent cells and blastocysts, that can retain this stage for a long time [16•]. A recent study claims that cell polarity, rather than cell position, is linked to the Hippo pathway activity, and that outer a-polar cells are eventually internalized by the surrounding polar cells

[17]. However, several studies have implicated distinct gene expression earlier than the morula stage, before 'inner' and 'outer' cell populations are formed. For example, using single cell RNA-sequencing throughout mouse pre-implantation development, Goolam and co-authors identified targets of the master pluripotency regulators Oct4 and Sox2 as being highly heterogeneously expressed within 4-cell stage embryos, with *Sox21* showing one of the most heterogeneous expression profiles, thus driving cell fate commitment [18••]. Blastomeres with decreased levels of *Sox21* exhibit pre-mature upregulation of *Cdx2* and a higher capacity to become TE [18••].

A third model is the 'pre-patterning model', suggesting that the axis of the mouse blastocyst is determined as early as at the oocyte, and that the balance between TE-specific and ICM-specific gene expression will determine the identity of the blastomeres already at the 2-cell stage [14,19].

Lastly, in the 'stochastic model', researchers proposed that a stochastic expression of *Oct4*, *Nanog* and *Cdx2* in the embryo's blastomeres causes a positional change of the blastomeres, depending on these genes expression [20]. This proposal was further supported by a study that used time lapse live video imaging technology, and found that blastomeres move very extensively during cell division [21]. To date, it is still unclear which of the models is the most accurate one and whether an expression of a single factor or several factors at early stages can fully predict cellular outcome. By contrast, it is well accepted that once the core transcriptional circuitry of a cell is established (i.e., *Nanog*, *Oct4* and *Sox2* for ICM and *Gata3*, *Cdx2*, *Eomes* and *Tfap2c* for TE), it safeguards cellular identity (Figure 1).

Induction of the pluripotent state by somatic nuclear reprogramming

In 2006, Takahashi and Yamanaka showed that forced expression of four transcription factors (TFs): Oct4, Sox2, Klf4 and Myc (OSKM), can reprogram mouse somatic cells into embryonic stem (ES)-like cells, termed induced pluripotent stem cells (iPSCs) [22]. Although iPSCs closely resemble ESCs in many examined aspects, often the reprogramming process yields iPSC colonies of poor quality [23], indicating that the current protocol to induce pluripotency by defined factors still requires optimization. The ability to switch the identity of somatic cells into pluripotency has added to our understanding of how pluripotency is established and which gene circuitries are involved. In the past decade, multiple TFs and many small molecules have been shown to facilitate the reprogramming process or to replace some of Yamanaka's factors [24]. Specifically, much effort has been put into identifying factors that can replace the Oct4 master regulator. Similarly to recent findings regarding its role in embryogenesis, Oct4 was eventually found to be dispensable for the induction of pluripotency, as proteins such as Nr5a1/2, Tet1, Sall4 and the Gata family proteins were able to replace it and induce pluripotency, albeit to a much lesser extent [25–27,28••]. A single cell analysis study that monitored the transcriptional changes of 48 genes during reprogramming, demonstrated that all of Yamanaka's factors can be replaced by alternative TFs, producing yet better quality iPSCs, suggesting that pluripotency can be attained via distinct entries [27,29,30••]. In accordance with this notion, Shu and co-authors in the mouse system, and Montserrat and co-authors in human cells, suggested that rival lineage specifiers can induce pluripotency [31,32]. These groups showed that mesendodermal (e.g., Gata3, Gata6, Sox7, Gata4, Cebpa, and Grb2) and ectodermal (e.g., OTX2, ZNF52, PAX6, Sox1, Sox3, Rcor2, and Gmnn) specifiers can replace Oct4 and Sox2, respectively, in inducing pluripotency [32]. The authors propose that competition between mesendodermal and ectodermal specifiers promotes successful reprogramming and pluripotency establishment, and therefore bypasses the need of the so-called critical pluripotency reprogramming factors. These innovative findings led to the proposal of the 'see-saw model', wherein cell fate is dependent on the balance between counteracting lineage specifiers [31,32]. However, careful examination of the selected factors in these studies shows a clear bias towards genes that are activated in pre/post implantation embryo or direct activators of key master pluripotency genes. The Gata family of proteins (e.g. Gata4/2/6/3) that can replace Oct4 [28••] are expressed and play a significant role in pre-implantation embryo before specification and at the blastocyst stage (TE and primitive endoderm (PrE)) [33–35]. *Sox7* and *Grab2* are expressed in the PrE [36,37], *Gmnn* is activated in the TE [38], *Rcor2* is predominantly expressed in pluripotent cells [39], Otx2 is required for ESC transition into EpiSCs and interacts with Oct4 [40,41], the Sox family of genes holds strong functional redundancy [42] and lastly, Cebpa and ZNF521 are direct activators of *Klf4*, and PAX6 activates *SOX2* [43,44]. This raises the question of whether the selected factors really induce pluripotency in somatic cells by creating counteracting forces of different lineages, or could it be that a unique set of factors that are expressed early on during embryonic development (i.e. zygote to epiblast of post-implantation embryo) have a certain capacity to induce pluripotency genes, either directly or indirectly, and thus induce pluripotency. This alternative viewpoint is supported by several observations. Firstly, TE and ICM share the expression of many pluripotency genes, such as *Sall4*, *Rex1*, *Lin28*, *Sox2*, *Utf1*, and *Esrrb* [45••], and ICM (epiblast) and PrE share genes like *Sall4* and *Oct4* (although the levels of *Oct4* are lower in PrE) [46]. Secondly, both iTS-like cells (TE equivalent) and XEN-like cells (PrE equivalent) are produced occasionally during reprogramming with OSKM [47•], and XEN-like cell state is achieved just before pluripotency in

chemical reprogramming [48•], suggesting a close core transcriptional circuitry and reprogramming capability between the three lineages. Thirdly, cells that reside in the outer layer of the early blastocyst and express TE genes, can migrate to the inner part and become pluripotent cells, suggesting a hyperdynamic chromatin state that allows a fast and robust cell fate changes [15••]. Lastly, direct conversion of cells from one lineage to another using late development-specific key master regulators often yields incompletely reprogrammed, functionally compromised cells, suggesting unique properties of nuclear reprogramming of embryonic reprogramming factors [23]. Taken together, these observations lead us to propose a model where early embryonic key master regulators harbor a unique capacity to reshape the epigenome, which is partially mediated by a direct or indirect activation of pluripotency genes. We believe that this unique feature is absent in late developmental factors, which might explain the low quality of the reprogramming process observed in direct lineage conversion [23]. To validate thoroughly the 'see-saw model', one should demonstrate induction of pluripotency using only late development-specific key master regulators, such as MyoD for a mesodermal specifier.

It has been proposed that the reprogramming process is divided at large into two major phases: an early stochastic and a later deterministic, with the later phase instigated following the activation of the endogenous *Sox2* locus, leading to a chain of gene activations, resulting in a stable pluripotency state [27,29]. In agreement with that, a recent study employed a unique methylation genomic reporter, showing that the activation of the *Sox2* enhancer is a late step in reprogramming, even later than the activation of Nanog [49]. Together, these results can explain why some of the Nanog-positive iPSC clones collapse following transgene removal. In accordance with its role as a major player in the core pluripotency circuitry, full reprogramming has been achieved only four days following downregulation of CAF-1, which allows increased binding and activation of Sox2 to pluripotency-specific targets [50••].

One of the most challenging questions in the reprogramming field is whether it is possible to detect as early as in the stochastic phase, which cells will become reprogrammed. The Jaenisch group suggested that stochastic expression of four pluripotency genes, *Esrrb*, *Utf1*, *Dppa2* and *Lin28* precedes the activation of *Sox2* and thus might have a better capability to predict reprogrammable cells [27,29]. The Wernig group expanded this model and suggested three cell surface markers, CD73, CD49d and CD200, that are absent in both fibroblasts and iPSCs, and can mark cells with higher capability to produce iPSCs [51]. However, it remains to be elucidated whether a specific gene signature can truly and fully identify reprogrammable cells at early stages of reprogramming.

Induction of the trophoblast stem cell state by somatic nuclear reprogramming

Several key TE master regulators and signaling pathways (i.e., Tead4, Cdx2, Elf5, Tfap2c, Gata3, Eomes, Ras and knockout of Oct4) have been proposed to establish and maintain the trophoblast stem cell (TSC, TE equivalent) state in transdifferentiation models, where ESCs are forced to become trophoblast stem-like cells by genetic manipulations [9,52–56]. However, a recent study demonstrated that all examined transdifferentiation models exhibit a partially reprogrammed state, as assessed by the methylation status of several gatekeepers such as *Elf5*, *Hand1*, *Tead4*, *Ezr* and *Plet1* [57]. This study raised the question of whether it is possible to cross the barrier between embryonic cells and extraembryonic cells by reprogramming factors and to produce fully reprogrammed cells (Figure 1). Recently, two independent studies from the Schorle [58••] and Buganim [45••] labs have succeeded in identifying key master regulators of the TSC state that can drive the reprogramming of mouse fibroblasts into fully reprogrammed induced trophoblast stem cells (iTSCs). Both groups attained high-quality and stable iTSCs, by implying the Yamanaka approach, using a screen of twelve transcription factors that were previously shown to have major roles in trophoblast specification or maintenance. Strikingly, they came up with the same minimal TF cocktails: Gata3, Eomes, and Tfap2c (GET). However, Kubaczka *et al.* also used Ets2, while Benchetrit *et al.* noted that Myc could boost the efficiency of iTSC formation, similarly to its role in iPSC reprogramming [29]. Of note, *Ets2* and *Tead4* did integrate into the genome of four out of five iTSC colonies analyzed by Benchetrit *et al.*, suggesting that Ets2 and Tead4 may promote successful reprogramming. The presence of Ets2 in the Schorle cocktail may provide an explanation to the shortened induction time of ten verses twenty days used by Buganim's group. Ets2 is implicated in cell proliferation and in TSC self-renewal and thus facilitates reprogramming [59]. Importantly, all 'gatekeepers' were unmethylated and expressed in iTSC colonies [45••,58••]. The most striking observation was the absence of trophoblast key master regulators such as Tead4, Cdx2 and Elf5 from the minimal reprogramming cocktail, even though these TFs are potent inducers of the trophoblast lineage in ESCs and during lineage segregation in the embryo, as discussed above. One explanation is that these TFs have a role in overcoming, or counteracting, the pluripotent state during embryonic development, but are not essential for the induction of TE, as was shown for Cdx2 in the embryo. This distinction was further highlighted by the Buganim group, which showed that GET/M can induce iTSC formation in Oct4-knockout cells, thus indicating for the first time, that pluripotency is not a requirement for the establishment of fully reprogrammed cells, or for achieving a TSC fate. A different viewpoint will argue that these are the differences between native embryonic developmental process

and the artificial process induced in differentiated cells. Gata3, Eomes, and Tfap2c were previously shown to regulate trophoblast genes, with Eomes and Tfap2c positively regulating the *Tead4*, *Gata3* and *Elf5* genes [60]. Furthermore, many genes related to TSC proliferation and potency are enriched with binding sites for Gata3, Eomes, and Tfap2c, including these three genes [61]. Gata3 positively regulates *Cdx2* gene expression and also induces the expression of *Eomes*, independently of Cdx2 regulation [55], and *Ets2* is located downstream of Eomes and directly regulates the transcription of *Cdx2* [59]. It is reasonable to conclude that GET functionally activate together the core circuitry of the TE state, by positively regulating each other's expression as well as the expression of additional genes that are implicated in TE specification (Figure 1).

Outlook

A variety of experimental approaches has provided exciting perceptions on how early lineage segregation is determined at early stages of embryogenesis (Figure 1). While much effort is put into understanding the first cell fate decision in the embryo, it remains unclear how totipotency is induced and established and which of the transcription factors are responsible for sustaining totipotency.

Very recently, attempts to generate totipotent-like state (i.e. bi-directionality) *in vitro* were made, focusing mostly on the activation of the retro transposon MERVL [62], which is considered as a totipotency marker gene owing to its specific expression at the two-cell stage. Pfaff and colleagues generated a MERVL reporter cell line, enabling them to capture and characterize a rare and transient cell population within mouse ESCs and iPSCs that expresses high levels of transcripts found in two-cell (2C) embryos [63]. A more recent article reported the creation of bi-directional cells *in vitro* by knocking out miR-34a in ESCs. miR-34a knocked-out ESCs were shown to differentiate both into functional embryonic and extraembryonic lineages, implying the role of microRNA in restricting the developmental potential of ESCs/iPSCs [64]. Other groups used over-expression of genes that are involved in ZGA, such as Dux4, to generate ESCs with chromatin structures and transcriptional profiles that are similar to those of 2C embryos [65,66]. Efforts for generating bi-directional cells were also put into modifying culture conditions, by adding inhibitors that were able to expand the developmental potential of ESCs [67].

As discussed in this review, many of the molecular mechanisms and core transcriptional circuitries are shared among the processes of somatic nuclear reprogramming and early embryonic development. It is thus tempting to speculate that future insights into the fascinating state of totipotency will emerge from somatic nuclear reprogramming approaches [68].

Conflict of interest statement

Nothing declared.

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