Molecular basis of the first cell fate determination in mouse embryogenesis

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Through proliferation and differentiation, a single cell, the zygote, can give rise to a complex organism composed of many types of cells. Up to the eight-cell embryo stage, the blastomeres are morphologically identical and distributed symmetrically in the mammalian embryo. Functionally, in some species, they are all totipotent. However, due to the compaction of blastomeres and the asymmetrical cell division at the late phase of the eight-cell embryo, the blastomeres of the morula are no longer identical. During the transition from morula to blastocyst, blastomeres differentiate, resulting in the first cell fate decision in embryogenesis, namely, the segregation of the inner cell mass and the trophectoderm. In this review, we will discuss the regulatory mechanisms essential for the cell fate choice during blastocyst development, including transcriptional regulation, epigenetic regulation, microRNAs, and signal transduction.

Keywords: inner cell mass; trophectoderm; embryogenesis

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Introduction

A zygote can give rise to a complex organism through cell proliferation and differentiation. The first differentiation event in embryogenesis is the segregation of the trophectoderm (TE) and the inner cell mass (ICM) in the blastocyst. During the formation of the blastocyst, the zygote gradually loses its developmental potential. The zygote is totipotent, developing into not only the fetus but also the placenta. The totipotency is maintained in the blastomeres of the two-cell stage embryo. After mechanical separation of the blastomeres of the two-cell stage embryo, each blastomere is able to give rise to an adult mouse [1]. As the embryo develops further, the developmental potential of individual blastomere decreases. No

mouse has been derived from a single blastomere at the four-cell stage or later [2, 3]. Yet, when aggregated with 'carrier' blastomeres, single four- and eight-cell stage blastomeres are able to contribute to all the tissues of the embryo, suggesting that they are pluripotent [4]. Upon the formation of the blastocyst, cells in the embryo are differentiated into two distinct lineages, the multipotent TE and the pluripotent ICM. TE cells develop into the extraembryonic tissues, while ICM cells are able to give rise to the embryo proper and another extraembryonic tissue, the primitive endoderm (PE) [5]. How TE and PE form the fetal part of the placenta is described in more detail elsewhere [6].

Regarding the formation of the blastocyst, several questions of great biological importance remain unanswered or controversial. When and how do the cells become different from each other in the embryo? When and how are the fates of cells determined? How is the developmental potential of cells regulated during the transition from the totipotent zygote to the pluripotent ICM cells and the multipotent TE cells? In this review, we will focus on these questions, summarizing the recent progress

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in the field of early mouse embryogenesis.

Developmental bias of the early blastomeres in mouse embryos

Presently, when the segregation of the TE and the ICM begins remains controversial. Blastomeres in mouse embryos are morphologically identical up to the eight-cell stage. After compaction, blastomeres in the late eight-cell embryo are polarized. The first obvious differences between blastomeres appear during the transition from the 8-cell to the 16-cell embryo, when cells are allocated to either inside or outside positions. The differentiation of ICM and TE appears to be initiated at this developmental stage. Yet, some data suggest that as early as the two-cell stage, the blastomeres are biased toward either the TE or the ICM lineage, and the orientation and the order of the second cleavages correlate with blastocyst pattern in the mouse embryo. In about 80% of embryos, one of

the second cleavages is equatorial or oblique (E), and the other is meridional (M) with respect to the second polar body (Figure 1A). These embryos can be divided into two groups, EM and ME embryos, according to the order of E and M cleavages. When the earlier of the second cleavage occurs meridionally (ME embryos), the progeny of the meridionally dividing cell populate predominantly the embryonic part of the blastocyst. In contrast, when the two-cell blastomere first divides equatorially or obliquely, the earlier equatorially dividing cell has an equal chance to develop into either the embryonic or the abembryonic part of the blastocyst. In the remaining 20% of embryos in which two-cell blastomeres undergo either EE or MM divisions, the contribution of progeny of the two-cell blastomeres is random, and developmental competence is compromised in EE and MM embryos [7–9]. The orientation and the order of the second cleavages might affect cell fate by asymmetric distribution of certain proteins. In four-cell ME embryos where pattern is predictable, the blastomeres derived from the E division tend to express more *Cdx2*, a TE-specific transcription factor [10]. Meanwhile, the M-derived blastomeres had higher levels of H3R26 methylation than the E-derived blastomeres [11]. How *Cdx2* and H3R26 affect the cell fate choice is discussed in the sections on transcriptional and epigenetic regulation, respectively.

In contrast, other data argue that no strong developmental bias exists until the eight-cell stage. When the TE and ICM cells were traced back to the four-cell stage by *in vitro* time-lapse imaging, the majority of four-cell blastomeres gave rise to both embryonic and abembryonic regions. Only 7.6% and 8.3% of four-cell blastomeres contributed exclusively to the TE or the ICM, respectively. Furthermore, lineage tracing experiments demonstrated no such tendency for one of the two-cell blastomeres to contribute mainly to either the embryonic or abembryonic part of the blastocyst [12]. Therefore, it may be that some fractions of two-cell embryos have already segregated for TE and ICM, as in ME embryos, but in most cases, a developmental bias at this stage is insufficient to direct the ICM/TE lineage commitment. It has been suggested that embryo rotation within the zona pellucida and blastomere movement during cell division might change the location of blastomeres, hence, affecting cell fate commitment [12]. Certainly, complex events yet to be discovered are required to ensure proper TE and ICM differentiation.

Cellular mechanism of allocating cells to the ICM or the TE

TE cells are polarized epithelial cells on the surface of

Figure 1 Schematic illustration for different types of cell division. **(A)** Equatorial (E) and meridional (M) cleavages in the two-cell embryo. The red circle is the second polar body. The dashed lines mark the cleavage planes. **(B)** Symmetric and asymmetric divisions in the eight-cell embryo. Only four blastomeres in the eight-cell embryo are shown. Symmetric division results in two polarized blastomeres with equal position in the embryo, while asymmetric division produces one polarized outer cell and one apolar inside cell.

the blastocyst, while ICM cells are apolar cells in the inside of the blastocyst. Both cell polarity and cell position in the embryo play critical roles in initiating TE/ICM cell fate. Polarization of blastomeres begins at the late eight-cell stage, as proteins including JAM1, aPKC, and PAR3, are localized at the apical membrane, and PAR1 becomes restricted to the basolateral regions [13, 14]. The polarity of the blastomeres results in two types of division, symmetric and asymmetric, during the transition from 8-cell to 16-cell embryos (Figure 1B). Symmetrical division results in two equally positioned and polarized cells, whereas asymmetrical division positions cells differentially. The outer cells retain polarity, and the inside cells inherit the basal pole of the parental cell, and hence lose polarity. The inside apolar cells tend to develop into the ICM, while the outside polarized cells tend to contribute to the TE [15]. In addition, cell polarity and cell position cross-regulate each other. When transplanted to outside positions, inside cells become polarized and adopt a TE fate [16–18]. Conversely, downregulation of polarity molecules, such as PAR3 and aPKC, promotes allocation of the cells to inside positions [13].

The molecular basis underlying the segregation of the TE and the ICM

As polarization of blastomeres occurs and the cells are allocated to either outside or inside positions, the specification of the TE and the ICM becomes apparent. Yet, even at the 16-cell stage, cells are not fully committed into the TE/ICM lineage. Manipulation of blastomeres at this stage may change cell fate, suggesting that 16-cell blastomeres remain plastic [19]. These blastomeres are able to sense environmental cues, which are transduced into cells through signaling pathways. Consequently, many changes within the cells, including transcriptional activity and epigenetic modifications, are directed by the signaling pathways to solidify the cell fate choice.

Cell fate determination is associated with changes in transcriptional profiles. Consequently, different sets of proteins are expressed to carry out distinct biological functions in various cells. Transcription of genes is regulated through many mechanisms, including transcription factors, epigenetic modifications, and microRNAs (miRNA). In addition, extracellular environmental cues can be transduced into intracellular compartments through signaling pathways, and regulate transcriptional activities.

Transcriptional regulation in the TE/ICM specification

During the development of the blastocyst, some transcription factors show restricted expression patterns as-

sociated with the segregation of the ICM and the TE. For example, Nanog and Oct4 are expressed in ICM cells, but not in TE cells, while Cdx2 is exclusively expressed in the TE [20–22]. These transcription factors not only serve as markers for the ICM and the TE, but also play crucial roles in cell fate determination.

The homeodomain transcription factors Oct4 and Nanog were the first proteins identified as critical for both early embryo development and maintenance of pluripotency in embryonic stem (ES) cells [20, 21, 23]. Oct4 and Nanog are expressed in all blastomeres in the early morula, and become restricted to the ICM cells of blastocysts [23–26]. Both *Oct4*-null and *Nanog*-null embryos develop to the blastocyst stage, yet, no ES cells can be derived from them. *Oct4*-null ICM cells tend to differentiate along the extraembryonic trophoblast lineage, while *Nanog*-null ICM cells are limited to TE differentiation or apoptosis [20, 27], suggesting that *Oct4*-null and *Nanog*-null ICM cells are not pluripotent. Sox2, an HMG-box transcription factor, is also necessary for ICM formation. Like *Oct4*-null ICM, *Sox2*-null ICM cells divert into the trophoblast lineage after *in vitro* culture [28]. Sox2 heterodimerizes with Oct4 to regulate numerous genes in mouse ES cells [29]. Thus, loss of Sox2 might compromise Oct4 binding to its target genes, explaining why *Sox2*-null and *Oct4*-null ICMs show similar developmental defects.

In contrast, the development of the TE requires a distinct set of transcription factors, including Cdx2, Tead4, Eomes, and Elf5. Cdx2 and Tead4 act at an early stage for initial TE commitment, while Eomes and Elf5 reinforce the initial commitment to the trophoblast lineage [22, 30–33].

Cdx2-null blastocysts fail to implant, apparently due to inappropriate development of TE cells, as aberrant expression of Oct4 and Nanog can be detected in *Cdx2*-null TE cells [22]. Moreover, overexpression of Cdx2 in ES cells leads to differentiation toward the TE lineage [34]. Cdx2 may specify cells into the TE fate through several mechanisms. First, Cdx2 activates the transcription of TE genes. Second, Cdx2 promotes symmetric divisions at the eight-cell embryo stage, thus allocating more blastomeres to the outside TE. Third, Cdx2 affects cell polarity by enhancing aPKC expression. Consequently, polarized blastomeres are associated with asymmetric distribution of Cdx2 mRNA. Therefore, Cdx2 and cellular polarity reinforce each other to make a firm commitment to the TE fate [10].

Tead4 is also necessary for TE formation. Deletion of *Tead4* results in preimplantation lethality. *Tead4*-null embryos fail to activate TE-specific genes, such as *Cdx2*, *Eomes*, and *Fgf2*, while ICM markers, Nanog and

Oct4, are expressed in all blastomeres. Moreover, no TS cells can be derived from *Tead4*-null embryos, whereas ES cell derivation is not affected [30, 31]. These data indicate that *Tead4* is essential for TE development. In addition, it has been demonstrated that the transcriptional activity of *Tead4* is modulated by the cellular localization of its coactivator Yes-associated protein 1 (Yap1). *Tead4* cooperates with unphosphorylated and nuclear-localized Yap1 to activate *Cdx2* and other TE genes [35]. Taken together, these data suggest that *Tead4* acts upstream of *Cdx2* in the specification of the TE lineage, but we cannot completely rule out the possibility that *Cdx2* also regulates the expression of *Tead4* and YAP to form a feed-forward regulatory loop.

Deletion of *Eomes* or *Elf5* does not affect blastocyst formation and implantation, suggesting that they work downstream of *Cdx2* and *Tead4* in the TE development. Loss of *Eomes* prevents TE differentiation into trophoblast, resulting in embryonic lethality soon after implantation [32]. Deletion of *Elf5* impairs the development of extraembryonic ectoderm [33].

Due to the importance of these lineage-specific transcription factors, their expression levels have to be tightly regulated. First, a feed-forward loop is formed among *Nanog*, *Sox2*, and *Oct4* to maintain pluripotency [29, 36–38]. In addition, *Oct4* and *Nanog* are regulated by many other factors, such as *Klf4*, *Zfx*, *Essrb*, *Smad1*, *STAT3*, and *Sall4* [38–40]. Second, ICM-specific and TE-specific transcription factors cross-regulate each other. *Oct4* and *Cdx2* occupy each other's promoter to suppress transcription. The antagonistic regulation between these two factors directs cells differentiating toward either the ICM or the TE lineage [34]. Similarly, cross-regulation of *Nanog* and *Cdx2* also affects the segregation of the ICM and the TE [41]. Furthermore, a pluripotency-associated factor *Sall4* not only regulates the ICM genes *Oct4* and *Nanog*, but also suppresses TE gene expression. Knockdown or knockout of *Sall4* in ES cells results in elevated *Cdx2* expression [40, 42, 43]. Moreover, under the appropriate conditions, ES cells can be re-specified into the TE lineage by reducing *Sall4* expression. Knockdown of *Sall4* by siRNA microinjection into zygotes leads to aberrant *Cdx2* expression in the ICM [40]. It has been shown that *Sall4* binds to the *Cdx2* promoter directly. Consequently, *Sall4* may recruit histone deacetylase complex to inactivate *Cdx2* transcription [43]. In addition, *Sall4* activates the expression of *Nanog* and *Oct4* by binding to their enhancer regions [39, 40]. Thus, *Sall4* might regulate *Cdx2* expression through *Nanog* and *Oct4*. Despite *Sall4*'s function in TE gene suppression, *Sall4*-null mutation does not affect lineage commitment in the blastocyst, as expression of lineage markers *Oct4*

and *Cdx2* is not altered in *Sall4*-null embryos. Rather, *Sall4* mutation impairs ICM proliferation, resulting in embryo death shortly after implantation [42].

Although many studies have focused on transcriptional regulation in ES and ICM cells, less is known about the transcription network in TE cells. Genetic studies reveal a hierarchy among the TE-specific factors: *Tead4* is the most upstream factor, followed sequentially by *Cdx2*, *Eomes*, and *Elf5* [22, 30–33]. In addition, *GATA3* is involved in trophoblast development downstream of *Tead4* and in parallel to *Cdx2* [44]. Elevated expression of any one of these factors affects others, suggesting that they are regulating each other [34, 35, 45], but there is no strong biochemical evidence that these TE factors directly bind to each other's promoters. Regulation of *Cdx2* is the best studied among these four factors. *Cdx2* binds to the promoter and the intron 1 region of its own gene locus to activate itself [41]. *GATA3* also upregulates *Cdx2* expression by directly binding to the intron 1 region of the *Cdx2* locus [46]. Other factors regulating the expression of these TE-specific factors remain to be identified.

Figure 2A illustrates the transcriptional regulation of TE/ICM differentiation, whereby lineage-specific factors positively regulate each other, and concomitantly suppress promoters of the alternate lineage, thereby reinforcing the cell fate commitment.

Epigenetic regulation in the TE/ICM differentiation

From the zygote to the formation of the blastocyst, the epigenome undergoes dramatic changes, such as DNA methylation, histone modification, and incorporation of histone variants [47, 48]. The different epigenetic marks in blastomeres at various stages orchestrate the developmental potential of these blastomeres. The segregation of the TE and the ICM is also associated with asymmetric epigenetic marks [49].

Histone modifications Histone modification, including acetylation, methylation, phosphorylation, and ubiquitylation, is an important mechanism of epigenetic regulation [50]. The first wave of epigenetic reprogramming during preimplantation development occurs in the male pronucleus (PN). Upon fertilization, protamines associated with the paternal chromatin are rapidly replaced by acetylated histones, which are not methylated at H3K4, K9, or K27 [51]. Immediately after protamine-histone exchange, mono-methylation of H3K4, K9, and K27 becomes detectable, implying that histones are rapidly deacetylated and mono-methylated [52]. Further methylation of mono-methylated histones is delayed. For example, H3K4me3 is not present until the latest pronuclear stage [52, 53]. While dynamic changes of histone

Figure 2 Molecular mechanisms for the ICM/TE segregation. **(A)** The regulatory network of key transcription factors in ICM/TE specification. *Cdx2*, *Tead4*, *Eomes*, and *Elf5* are the major TE-specific transcription factors. *Tead4* cooperates with *Yap1* to activate *Cdx2*, which in turn upregulates *Eomes* and *Elf5*. Conversely, *Elf5* is able to enhance *Cdx2* expression. In addition, *GATA3* directly binds to the intron 1 region of the *Cdx2* locus and activates *Cdx2* expression. In contrast, *Oct4*, *Nanog*, and *Sox2* form a core regulatory circuitry to promote ICM cell fate. *Sall4* not only activates *Oct4*, *Nanog*, and *Sox2* but also suppresses *Cdx2* expression, and this is critical to the differentiation of the ICM and the TE. Furthermore, cross-regulation between the ICM-specific factors and the TE-specific factors secures the appropriate cell fate in individual blastomeres. So far, no biochemical evidence demonstrates that *Cdx2*, *Tead4*, *Eomes*, and *Elf5* bind to each other's promoters. To distinguish from other interactions with direct promoter binding, the genetic interactions among these four factors are marked with orange arrows. **(B)** MicroRNAs regulate the expression of pluripotency factors, *Oct4*, *Nanog*, and *Sox2*. Only the experimentally identified regulatory interactions are shown. **(C)** Two signaling pathways, the Hippo and MAPK pathways, are involved in the ICM/TE cell fate determination. In inside cells, *Lats* in the Hippo pathway phosphorylates *Yap1*, resulting in cytoplasmic distribution of *Yap1*. Without nuclear *Yap1*, *Tead4* is unable to activate *Cdx2*. Thus, these cells develop to the ICM. In contrast, due to the inactive Hippo pathway in outer cell, *Yap1* remains unphosphorylated and is localized in the nucleus. *Yap1* cooperates with *Tead4* to activate *Cdx2*, promoting the TE fate. *Erk2* in the MAPK pathway activates *Cdx2* and suppresses *Nanog*, facilitating the TE formation. Yet, the mechanisms for *Erk2* regulating *Cdx2* and *Nanog*, and the events upstream of *Hippo* and *Erk2*, remain unknown.

modifications occur in the male PN, maternal chromatin maintains all types of histone H3 methylation throughout zygotic development [51, 53, 54]. This reprogramming event might be essential for zygotic genome activation and establishment of totipotency.

During the formation of the blastocyst, the distinct TE and ICM lineages become marked by different histone modification profiles. Mono-, di-, and tri-methylation of H3K27 are more abundant in ICM cells than in TE cells, while methylation of H3K9 shows no significant difference between the TE and the ICM [55]. In addition to the

global difference of H3K27 methylation, differential histone modifications are associated with specific loci in TE and ICM cells. For example, the active epigenetic marks, H4K8ac and H3K4me3, are more abundant at the promoters of *Pou5f1* and *Nanog* in the ICM than in the TE. The repressive epigenetic mark H3K9me2 is lacking in the promoters of *Pou5f1* and *Nanog* in the ICM relative to the TE. In contrast, less H3K9me2 and more H4K8ac and H3K4me3 at the *Cdx2* promoter are found in TE cells relative to ICM cells [56]. Moreover, in ICM cells, H4K16ac is relatively high at the promoters of *Pou5f1*

and *Nanog*, and is relatively low at the *Cdx2* promoter [57]. All these histone modifications cooperate together to ensure the appropriate spatial and temporal expression of lineage-specific genes.

DNA methylation DNA methylation is another important epigenetic modification, normally associated with inactive genes. Following protamine replacement in the zygote, the DNA of the paternal genome is demethylated (except for some imprinted loci and repeat elements). This global demethylation event occurs before DNA replication, suggesting that this demethylation process is an active enzymatic reaction [58, 59]. It has been suggested that DNA deaminases might initiate the DNA demethylation reaction by deaminating 5meC. Cytidine deamination mutates the C to T; the T:G mismatch is then repaired, resulting in replacement with an unmethylated cytidine [60]. Two recent papers have implicated the activation-induced cytidine deaminase AID in demethylation in the germ lineage, and in heterokaryons between ES cells and fibroblasts [61, 62]. The action of AID provides an appealing and plausible mechanism for demethylation, but further substantiation is required before acknowledging AID as the long-sought demethylase. Gadd45a has been shown to be involved in the repair-mediated DNA demethylation [63]. A recent study has revealed that components of the elongator complex Elp1, Elp3, and Elp4 are essential for zygotic paternal genome demethylation [64]. In the opposite direction, PGC7/Stella has been shown to protect maternal DNA from demethylation in early embryogenesis [65]. Thus, maternal DNA remains methylated while the paternal genome is rapidly demethylated at the zygote stage. In the subsequent divisions, the maternal genome is gradually demethylated [59, 66]. The major cause for this demethylation is the absence of the maintenance methyltransferase Dnmt1 during DNA replication [67]. Through active and passive demethylation, respectively, paternal and maternal DNA methylation reaches a low level in the morula [66, 68]. The global reprogramming of DNA methylation may cooperate with histone modifications to facilitate zygotic genome activation and to safeguard early embryo development.

From the morula to the blastocyst stage, global levels of methylation increase, yet unequally in the ICM and the TE. DNA methylation is greatly increased in the ICM, while TE cells remain relatively unmethylated despite an increment from the morula stage [59]. Proper DNA methylation levels in the ICM and the TE are critical for embryonic development. The loss of the maintenance methyltransferase Dnmt1 leads to severely retarded and abnormal development of the embryo proper,

whereas development of extraembryonic tissues remains largely unaffected [49]. In addition, due to inefficient reprogramming, cloned embryos have high levels of DNA methylation. Particularly, TE cells in cloned embryos display aberrantly high levels of DNA methylation [69, 70], consistent with the observation that the prevailing developmental abnormality of cloned embryos is malformation of the placenta [49]. In summary, hypomethylation predominantly affects embryonic regulation and development rather than extraembryonic tissues, while hypermethylation significantly impairs epigenetic regulation and development of extraembryonic tissues.

X chromosome inactivation Another important epigenetic event in early embryogenesis is X chromosome inactivation (XCI) in female embryos. In each female mammalian cell, one of the two X chromosomes is transcriptionally inactivated to balance X-linked gene dosage between male (XY) and female (XX). After fertilization, the silencing of the paternal X chromosome starts at zygotic gene activation (two-cell stage) [71, 72]. Yet, how the paternal X chromosome is predominantly inactivated remains elusive. At the morula stage, the paternal X chromosome is inactivated in all cells. During the formation of the blastocyst, the inactive paternal X chromosome is reactivated in the epiblast, while TE and primitive endoderm cells maintain the silent status of the paternal X chromosome [72, 73]. After implantation, as epiblast cells further differentiate, the two X chromosomes undergo random inactivation [74].

XCI involves non-coding RNAs (ncRNAs), histone modifications, and DNA methylation. The ncRNA *Xist* is pivotal in XCI [75]. Upon initiation of XCI, *Xist* is transcribed from one of the two X chromosomes, and the resulting *Xist* RNA coats the X chromosome from which it is transcribed. The coating of *Xist* recruits factors responsible for transcriptional repression to the inactive X chromosome (Xi). Through the cooperation of these factors, the Xi is epigenetically marked with histone hypoacetylation and histone hypermethylation [76]. The expression of *Xist* is regulated by other ncRNAs, such as *Tsix* and *Xite* [77-79]. *Yy1* and *Ctcf* have been characterized as *trans*-acting factors for both *Xist* and *Tsix* [80, 81]. More detailed mechanisms of XCI can be found in other review articles [76, 82].

While XCI has been studied extensively, little is known about the reactivation of the inactive paternal X chromosome in the ICM. Recently, it has been shown that the expression pattern of *Nanog*, but not *Oct4* or *Sox2*, defines the ICM cells in which the Xi is reactivated, implying that *Nanog* might be involved in X chromosome reactivation. Moreover, *Nanog*-null ICMs fail to re-

activate Xi [27]. Biochemical studies reveal that Nanog, Oct4, and Sox2 bind to the intronic region of *Xist* in ES cells [38, 83]. In addition, Oct4 and Sox2 also occupy the *Tsix* and *Xite* loci [84]. It is possible that Xi reactivation is mediated by *Xist* suppression, and that the suppression of *Xist* in turn requires the cooperation of Nanog, Oct4, and Sox2. More investigations are necessary to better understand the mechanism of Xi reactivation. Recent success in the derivation of induced pluripotent stem (iPS) cells provides us an opportunity to survey the reactivation of Xi *in vitro* [85, 86].

Epigenetic regulation and developmental potential

The differential epigenetic profiles of the ICM and the TE beg the question of which comes first: are the distinct epigenetic profiles a consequence of differentiation of the ICM and the TE, or do the differential epigenetic profiles precede lineage allocation and mediate the cell fate commitment? Intriguingly, uneven distribution of histone H3 with methylated arginine residues (R2, R17, and R26) is observed in the four-cell stage ME embryo, and the level of H3 methylation at these arginine residues (H3Rme) is correlated with cell fate and potency. Four-cell blastomeres with maximal H3Rme contribute more to the ICM and the polar TE, whereas blastomeres with minimal H3Rme contribute more to the mural TE. Moreover, overexpression of the H3-specific arginine methyltransferase CARM1 in individual blastomeres promotes their progeny to the ICM [11]. These data argue that epigenetic information precedes and dictates the ICM/TE cell fate determination, by acting on critical lineage-specific genes. For example, overexpression of CARM1 enhances the expression levels of Nanog and Sox2 in the injected blastomeres [11]. In ES cells, Oct4 recruits the histone H3K9 methyltransferase Eset to the *Cdx2* promoter. Eset in turn catalyzes the methylation of H3K9 at the *Cdx2* locus to suppress *Cdx2* expression. Knockdown of *Eset* induces ES cell differentiation into the TE lineage [87, 88]. *Eset*-depleted cells contribute to the TE of a blastocyst and, subsequently, placental tissues [88]. How epigenetic marks are first initiated remains a central question.

miRNAs in the TE/ICM commitment

miRNAs, a family of ncRNAs of 22 nucleotides, regulate gene expression through at least two distinct mechanisms: mRNA degradation and mRNA translational repression [89]. Given their important roles in gene regulation, miRNAs are also crucial for proper development, including early embryogenesis. Recent studies have revealed that miRNA expression profiles undergo dynamic changes during preimplantation embryo devel-

opment, although significantly different miRNA profiles were observed with different methodologies [90-92]. The importance of miRNA in preimplantation embryo development is further supported by knockout of *Dicer*, an RNase III-family nuclease critical for RNA interference (RNAi) and miRNA generation. Loss of *Dicer* in mice results in embryonic death before E7.5, with a lack of detectable stem cells [93]. miRNAs are also important for ES cell differentiation. *Dicer* knockout ES cells show compromised proliferation and deficient differentiation [94, 95]. Because both RNAi and miRNA generation are affected by *Dicer* knockout, it is not clear which pathway is responsible for the defect in ES cells. To assess the role of miRNAs in ES cells specifically, DGCR8, an RNA-binding protein that assists the RNase III enzyme Drosha in the processing of miRNA, was knocked out in mouse ES cells. Upon differentiation, DGCR8-deficient ES cells express some differentiation markers. However, pluripotency markers are not fully suppressed in DGCR8-deficient ES cells, confirming the crucial role of miRNAs in ES cell differentiation [96]. Altogether, these data suggest that miRNAs are essential for the differentiation of ICM and ES cells.

In addition, miRNAs might be involved in earlier stages of embryo development. To evaluate the functional importance of maternal miRNAs and *Dicer*, *Dicer* was conditionally knocked out in the growing oocyte, leading to defects in spindle organization and chromosomal alignment in the mature oocyte [90, 97]. When *Dicer* mutant oocytes were fertilized with wild-type sperm, the resulting zygotes failed to proceed through the first cell division [90]. Moreover, knockdown of Ago2, the catalytic component of the RNA-induced silencing complex, stabilizes one set of maternal RNAs and reduces zygotic transcripts of another set of genes, resulting in arrested embryos at the two-cell stage [98]. These data imply that miRNAs and/or siRNAs in the oocyte are essential for the earliest stages of embryogenesis. Further investigations are necessary to distinguish the specific effect of miRNAs and siRNAs.

Given the important role of miRNAs in embryogenesis, studies have been carried out to elucidate the function of individual miRNAs. By analyzing miRNA expression in undifferentiated and differentiated ES cells, a group of miRNAs have been found to be specifically expressed in undifferentiated ES cells, implying a role in maintenance of pluripotency. Conversely, another set of miRNAs is upregulated upon ES cell differentiation, indicating their functions in promoting differentiation [99]. Among the ES cell-specific miRNAs, the miR-290 cluster is of particular interest. The expression of the miR-290 cluster begins at the four-cell stage, and steadily in-

creases through the blastocyst stage, suggesting a critical role of the miR-290 cluster in early embryo development [92]. Mice with homozygous deletion of the miR-290 cluster die as embryos [100]. Furthermore, miR-291-3p, miR-294, and miR-295 enhance the derivation efficiency of mouse iPS cells by Oct4, Sox2, and Klf4, confirming their function in pluripotency establishment [101]. Similarly, miR-302 can reprogram human skin cancer cells into an ES-like pluripotent state [102]. In addition to the ES cell-specific miRNAs, a set of candidate miRNAs, which might be involved in TE specification, have also been identified, through comparing miRNA expression profiles in ES cell-derived TE cells and in staged murine embryos [92]. The functions of these miRNAs remain to be established experimentally.

To regulate gene expression, miRNAs bind to specific target mRNAs through imperfect match sequences. Hence, it is difficult to predict miRNA target genes. Nevertheless, several miRNAs have been demonstrated to regulate ICM-specific genes (Figure 2B). For example, miR-134 and miR470 target *Sox2* and *Oct4*, respectively. Both miR-296 and miR470 regulate *Nanog* expression. More importantly, the target sites of these miRNAs are located in the coding sequence, instead of the 3' untranslated regions [103]. In human ES cells, miR-145 represses the expression of *OCT4*, *SOX2*, and *KLF4* [104]. All these miRNAs negatively regulate pluripotency factors. It implies that these miRNAs might be involved in TE specification and ICM differentiation at a later stage. One target of the ES-specific miR-290 cluster has been identified. miRNAs of the miR-290 cluster suppress the expression of a Dnmt3 repressor Rbl2. Loss of the miR-290 cluster elevates Rbl2 expression level, and in turn reduces Dnmt3 expression, resulting in DNA hypomethylation in ES cells and defect in stable *Oct4* silencing after differentiation [105, 106]. Thus, the miR-290 cluster plays important roles not only in pluripotency maintenance but also in ES cell differentiation and likely in ICM differentiation. So far, although a group of TE-specific miRNAs have been identified, none of their targets have been characterized in this context, and no miRNA regulators of TE-specific genes have been identified. Knowledge of these miRNAs will shed light on how miRNAs promote the segregation of TE and ICM.

Signal transduction in TE/ICM segregation

Blastomeres can relocate within the embryo during cell division or can be actively manipulated experimentally. In both scenarios, the final location of blastomeres determines the cell lineage, regardless of their original position, indicating that individual blastomeres can sense environmental cues and adopt the appropriate cell fate

accordingly. To affect cell fate choice, the extracellular environmental cues must be transduced into intracellular compartments. Thus, signaling pathways play important roles in such a process.

Ras signaling appears to be essential for preimplantation embryo development. Inhibition of Ras by antisense oligonucleotides or monoclonal antibody does not affect normal oocyte maturation, but expression of the dominant-negative Ras N-17 mutant in fertilized eggs arrests the embryo at the two-cell stage. The inhibitory effect of Ras N-17 is overcome by simultaneous expression of an active *raf* oncogene. In contrast, expression of Ras N-17 starting from the late two-cell stage does not affect development of morula to blastocyst [107]. Thus, the Ras/Raf signaling pathway is specifically required at the two-cell stage.

Additionally, the MAPK pathways downstream of Ras are necessary for later stages of embryogenesis. The MAPK pathways include the extracellular signal-regulated protein kinase (ERK) pathway, the Jun N-terminal kinase (JNK) pathway, and the p38 pathway. Early studies showed that inhibition of the JNK or the p38 pathway produced defects in blastocyst formation [108, 109]. Subsequently, more studies have revealed a role for the ERK pathway in blastocyst formation. It has been demonstrated that ectopic expression of an activated HRas1^{Q61L} induced TE differentiation from ES cells. Erk2 is a major downstream effector of Ras for this cell fate switch. Interestingly, Erk2 is asymmetrically distributed in the apical membrane of the eight-cell embryo just before morula compaction. Inhibition of the ERK pathway in eight-cell embryos compromises Cdx2 expression, delays blastocyst development, and reduces TE outgrowth from embryo explants [110, 111]. All these data imply that Erk2 might be involved in cell polarization, activation of Cdx2, and, consequently, in TE fate determination (Figure 2C). Furthermore, knockout of *Erk2* results in early embryonic lethality, associated with defects in the ectoplacental cone and extraembryonic ectoderm, further confirming the crucial role of Erk2 in TE function [112].

In addition to the Ras-MAPK pathway, the recently discovered Hippo pathway is also implicated in ICM/TE differentiation [35, 113]. Yap1 is a coactivator regulated by the Hippo pathway component Lats. In the mouse embryo, the phosphorylation status of Yap1 is correlated with the position of blastomeres, and regulates the cellular localization of Yap1 protein. In outside cells, unphosphorylated Yap1 is localized in the nucleus and cooperates with Tead4 to activate *Cdx2*. In inside cells, Yap1 becomes phosphorylated and cytoplasmic, hence downregulating the transcriptional activity of Tead4/Yap1 complex [35]. Thus, through modulating the tran-

scriptional activity of Tead4, the Hippo pathway facilitates the differentiation of the TE and the ICM (Figure 2C).

Isoforms of protein kinase C (PKC) might also be involved in the TE/ICM segregation. Activation of PKC leads to earlier compaction than normal embryos. Conversely, inhibitors of PKC, but not inhibitors of other kinases, block compaction [114]. PKC α is localized at the basolateral cortex adjacent to cell contact in 8-cell embryos, while PKC μ is distributed to the outer cortex in 4-cell embryos, and to outside blastomeres in 16-cell embryos. PKC ξ also displays an asymmetric pattern in the four-cell embryo [114, 115]. These results implicate PKC isotypes in the specification of the TE and the ICM through regulating cell polarization. However, the details of how PKC isotypes affect cell polarity remain unclear.

In many other situations, signaling pathways coordinately induce cellular responses to environmental cues [116]. It remains to be clarified whether and how the Ras-MAPK pathway, the Hippo pathway, and the PKC isotypes cross-regulate each other. Another important question is how the cell senses the outside/inside position and transduces this information to intracellular pathways.

Conclusions

The very first cell fate decision made in the early mammalian embryo – the segregation of ICM and TE – remains a remarkably complex and little understood process. Changes of transcriptional profiles have pointed toward transcription factors as the key players. Oct4, Sox2, and Nanog form a regulatory circuit to promote ICM formation, whereas Tead4 and Cdx2 operate to specify the TE. The establishment of new transcriptional profiles in distinct lineages is thereafter solidified by epigenetic modification and the action of miRNAs. The developmental potential of blastomeres is also tightly regulated through epigenetic modifications. Signaling pathways transduce environmental cues into the cells and affect gene expression, thus modulating cell fate determination.

Although our knowledge on the mechanisms of the ICM/TE segregation has grown considerably, there are still many questions unanswered. How do the key transcription factors interact with epigenetic regulators (such as histone-modifying enzymes) to activate or suppress gene expression? Which miRNAs are essential for blastocyst development? What are the critical targets of these miRNAs? How do blastomeres transduce extracellular signal into the intracellular signaling pathways? How do the Ras-MAPK, Hippo, and PKC pathways coordinately regulate cell fate commitment? These central questions constitute the challenge for the future.

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