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Jason Knott
Keith Latham *Editors*

Chromatin Regulation of Early Embryonic Lineage Specification

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229

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Preface

Remarkable strides have been made recently in understanding the mechanisms that regulate early cell fate decisions in mammalian embryos and the role of key chromatin regulators in that process. This book combines contributions from leaders in the field who have been the driving force behind these advances. The book is intended to be a key reference for anyone with an interest in early mammalian development. Readers will discover the close connections between cell polarization that accompanies the generation of inside and outside cell populations and signaling mechanisms that modify chromatin structure and establish lineage-dependent gene expression programs.

East Lansing, MI — Jason Knott
East Lansing, MI — Keith Latham

Contents

Introduction

Jason G. Knott and Keith E. Latham

Abstract In this special volume on "*Chromatin regulation of early embryonic lineage specification*," five leaders in the field of mammalian preimplantation embryo development provide their own perspectives on key molecular and cellular processes that mediate lineage formation during the first week of life. The first cell-fate decision involves the formation of the pluripotent inner cell mass (ICM) and extraembryonic trophectoderm (TE). The second cell-fate choice encompasses the transformation of ICM into extraembryonic primitive endoderm (PE) and pluripotent epiblast. The processes, which occur during the period of preimplantation development, serve as the foundation for subsequent developmental events such as implantation, placentation, and gastrulation. The mechanisms that regulate them are complex and involve many different factors operating spatially and temporally over several days to modulate embryonic chromatin structure, impose cellular polarity, and direct distinct gene expression programs in the first cell lineages.

The roles of two early acting chromatin regulators in ICM and TE formation, CHD1 and CHD4, are explored by *Suzuki and Minami* and by *Miller and Hendrich*, respectively. *Suzuki and Minami* describe a role for CHD1 in inducing early expression of *Hmgpi*, which in turn activates expression of key ICM and TE genes such as *Pou5f1* and *Nanog and Cdx2*, respectively. In doing so, CHD1 initiates a sequential chain of nuclear reprogramming and genome activation events leading to lineage commitment. *Miller and Hendrich* emphasize the opposing functions of CHD4 as both a transcriptional repressor and activator. Deficiency of CHD4 leads to a failure of cells to adopt a clear cell fate. They instead continue to express a mixed set of markers from both ICM and TE. CHD4 works at first independently of the NuRD complex during cell lineage specification and then in conjunction with the NuRD complex during epiblast formation in the ICM. Additionally, proteins expressed by translation of maternally encoded mRNAs inherited in the oocyte are required for embryo viability during cleavage, but embryonic

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genetic deficiency is not detrimental until after implantation. These observations illustrate the key early roles played by chromatin regulators, as they reprogram embryonic genomes and begin cellular specialization.

The chapter by *Cui and Mager* builds on this theme of progressive reprogramming leading to cellular specialization before implantation. Recent studies in mice indicate that transcriptional and epigenetic heterogeneities exist between cells of the 2-cell and 4-cell embryo, respectively. Early differences in mitochondrial 16S RNA between cells and then between intracellular regions are described. Because reprogramming is an ATP-dependent process, this raises the possibility that differential mitochondrial distribution may lead to differential genome reprogramming spatially within the embryo. Additionally, the impact of cellular heterogeneities in histone H3 arginine 26 methylation (H3R26me) and other epigenetic modifications on lineage commitment is reviewed. Specific histone modifications such as H3R26me may facilitate ICM commitment by potentiating SOX2–DNA interactions at pluripotency genes. Lastly, the authors highlight the results of a large-scale RNAi screen for genes required for lineage commitment in mouse preimplantation embryos. Several of the identified genes encode for transcriptional regulators. Embryos that fail to transition to blastocyst tend to have disruptions in TE lineage genes, whereas embryos that form blastocysts but fail to hatch or form outgrowths have disruptions in ICM lineage genes, arguing for distinct mechanistic relationships between specific transcriptional regulators and gene pathways/networks that drive cellular specialization.

In the chapter by *Alarcon and Marikawa*, the role of the RHOA subfamily of small GTPases and RHO-associated coiled-coil kinases (ROCK) is explored. RHO/ROCK participate in intracellular compartmentalization of HIPPO pathway effectors to promote cell polarization and TE specification. Disruption of RHO/ROCK blocks TE formation and promotes ICM characteristics. An exciting aspect developed in this article is the sensitivity of RHO/ROCK signaling to exogenous pharmacological agents that are used clinically to treat disease. Specific medications such as cholesterol-lowering drugs may directly interfere with post-translational modifications of RHO interfering with its ability to promote TE lineage development. This raises the possibility that the use of these drugs may impact early embryo viability and subsequent development and thereby affect fertility in women.

Ralston provides an overview of the transcriptional and epigenetic mechanisms that mediate PE lineage formation and extraembryonic endoderm (XEN) cell self-renewal. XEN cells are multipotent stem cells that play crucial roles in yolk sac formation, fetal patterning, and germ cell differentiation. Studies in mice have led to the discovery of specific signaling pathways and transcription factors that can be manipulated in vitro to derive XEN cells from embryos, pluripotent stem cells, and somatic cells. These include the ERK signaling pathway and cell-fate regulators such as GATA4 and GATA6. The unique epigenetic landscape of XEN cells and the impact on "stemness" are also reviewed. Lastly, *Ralston* highlights the genetic conservation between mice and humans and describes potential applications of XEN cells in research.

Enjoy the volume!

Chromatin Remodelling Proteins and Cell Fate Decisions in Mammalian Preimplantation Development

Anzy Miller and Brian Hendrich

Abstract The very first cell divisions in mammalian embryogenesis produce a ball of cells, each with the potential to form any cell in the developing embryo or placenta. At some point, the embryo produces enough cells that some are located on the outside of the embryo, while others are completely surrounded by other cells. It is at this point that cells undergo the very first lineage commitment event: outer cells form the trophectoderm and lose the potential to form embryonic lineages, while inner cells form the Inner Cell Mass, which retain embryonic potential. Cell identity is defined by gene expression patterns, and gene expression is largely controlled by how the DNA is packaged into chromatin. A number of protein complexes exist which are able to use the energy of ATP to remodel chromatin: that is, to alter the nucleosome topology of chromatin. Here, we summarise the evidence that chromatin remodellers play essential roles in the successful completion of preimplantation development in mammals and describe recent efforts to understand the molecular mechanisms through which chromatin remodellers facilitate the successful completion of the first cell fate decisions in mammalian embryogenesis.

Introduction

Within the first 5 days of mouse development, a single-celled zygote progresses, by an ordered series of cleavage divisions and successive differentiation events, to the specification of three lineages: trophectoderm (TE), epiblast and primitive

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endoderm (PrE) within the developing embryo (Rossant and Tam 2009). The TE is the first lineage to become specified and forms the outer epithelial layer which contributes to the placenta and is required for implantation into the uterus (Carson et al. 2000). The inner cells go on to segregate into epiblast or PrE. The epiblast forms the embryo proper, while TE and PrE lineages give rise to extraembryonic structures that support the development of the embryo (Rossant and Tam 2009; Chazaud and Yamanaka 2016). This developmental progression is highly reproducible, and these three lineages are defined by specific gene expression programmes which must be carefully established and controlled.

Although this descriptive information about embryo development has long been known, many questions are still unanswered as to exactly how these lineages are formed. A cell's identity is defined by the genes it expresses and those it represses, so to understand cell fate decisions we must understand how gene expression is controlled. Every cell in an organism contains the same genome, and yet there are multiple distinct cell types, all of which exhibit different dynamic gene expression profiles. Therefore, when we consider cell fate choices, we are actually witnessing the rewiring of these gene regulatory networks (GRN) to create new stable cell types. For this to occur, each cell must respond appropriately to stimuli and ensure the correct expression of specific genes. Understandably, chromatin modifiers and remodellers are key in this context: they facilitate transcription factor and RNA Polymerase II access to those genes required in the new cell state and prevent aberrant expression of those that are undesirable. Recent evidence indicates that cell signalling creates the environment for appropriate decision-making, while specific changes in gene expression cement a cell fate transition. This model prompts questions such as: How are the signals decoded by the cells at the chromatin level? How are specific genes brought 'into play' by the signalling machinery? Whether a gene is transcribed or silent is largely dependent upon its chromatin environment. In this chapter, we will focus on the role of chromatin remodellers in forming the required chromatin environment for each cell decision in mammalian preimplantation development.

Events Associated with the First Lineage Decision: TE Versus ICM

Initial rounds of cell division, from fertilisation until approximately the eight-cell stage, result in an increase in cell number without any overall increase in size of the embryo (Aiken et al. 2004). Although the exact time when the first lineage decision begins is a matter of some debate (Piotrowska-Nitsche et al. 2005; Dietrich and Hiiragi 2007; Torres-Padilla et al. 2007; Ralston and Rossant 2008; Dietrich et al. 2015), it is after the eight-cell stage that the first signs of loss of symmetry occur. At the eight-cell stage, cell-to-cell adhesion increases and the cells undergo a process called compaction (Johnson and Ziomek 1981). The cells flatten and the embryonic

surface becomes smooth. Concomitant with compaction, the cells acquire apical–basal polarity (Fig. 1). This is characterised by the reorganisation of the cytoplasm (Reeve and Kelly 1983; Fleming and Pickering 1985; Maro et al. 1985), the formation of an apical domain (Pauken and Capco 2000; Plusa et al. 2005; Vinot et al. 2005) and the presence of microvilli on the apical domain (Ducibella et al. 1977). Other polarity proteins are also localised basolaterally (Vinot et al. 2005). From this point on, outer cells retain these polarised features and go on to form TE, while inner cells (which become ICM) become apolar. During the next two rounds of cell division, blastomeres divide either symmetrically or asymmetrically, passing down either all or part of the apical cytoplasm to their daughter cells.

The Second Lineage Decision: Epiblast Versus PrE

Once the TE and ICM decisions have been made, the embryo has formed a cavitated structure known as the blastocyst, with the TE cells forming an outer layer and ICM cells forming a tight ball to one side of the inside structure (Kunath et al. 2004; Chazaud and Yamanaka 2016). The ICM cells then undergo the second lineage decision: epiblast versus PrE. Whereas cell position plays a large part in TE specification, it seems that the reverse is true for PrE: cell fate commitment both precedes and facilitates cell positioning (Rossant and Tam 2009). The PrE cells form an epithelial layer between the epiblast cells and blastocoel cavity (Fig. 1). Any presumptive PrE cell not positioned in this layer migrates towards it, switches fate to epiblast or undergoes apoptosis (Plusa et al. 2008). By E4.5, the specification of the PrE is complete, and epiblast and PrE cells show distinct fates (Gardner and Rossant 1979). Finally, following PrE specification, the embryo implants and progresses to the early egg cylinder stage (E5.5). Shortly after this point, the embryo undergoes gastrulation and forms all three primary lineages: definitive endoderm, ectoderm and mesoderm.

The Role of Chromatin Modifiers in the Formation of the Early Embryonic Lineages

ATP-dependent chromatin remodelling complexes can be divided into four distinct classes based upon the chromatin remodelling subunit, which fall into the SWI/SNF, ISWI, INO80 and CHD families (Hargreaves and Crabtree 2011). Their ATPase subunits use the energy derived from ATP hydrolysis to move, eject or slide nucleosomes along the DNA (Clapier and Cairns 2009).

Studies of these remodelling proteins in vitro have provided clear pictures of how these proteins function biochemically. Connecting these biochemical activities to the alterations in gene expression and developmental progression seen in vivo

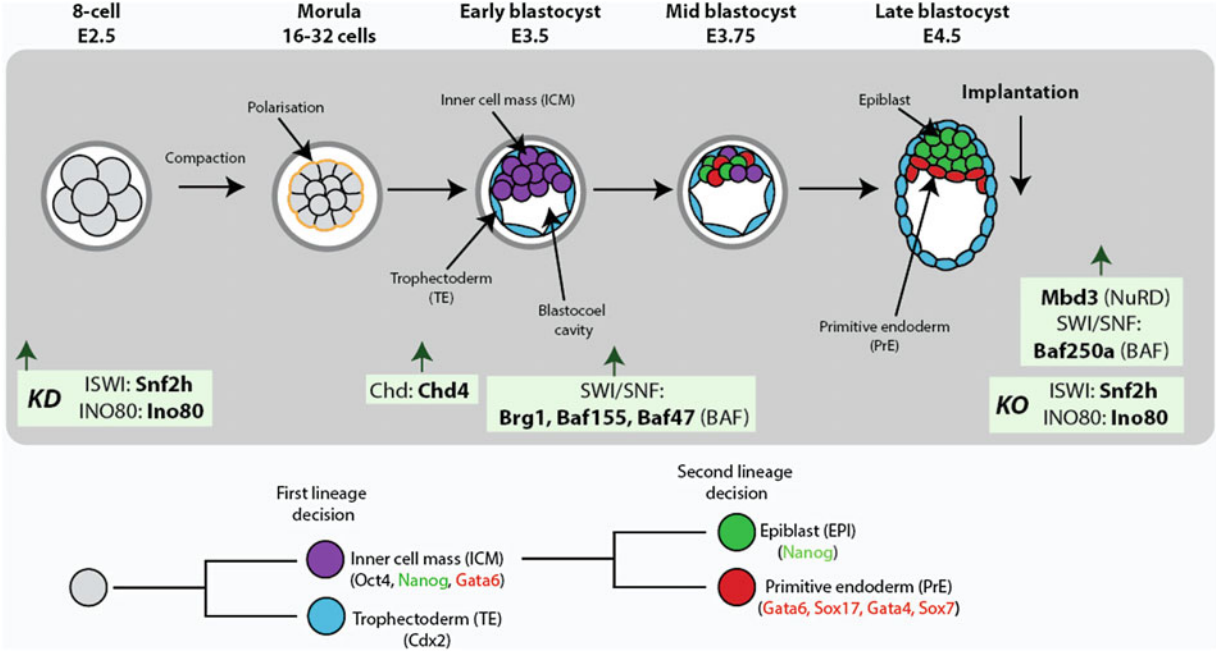

Fig. 1 Diagram to illustrate mouse preimplantation development and the stages at which specific chromatin remodellers are required. From the eight-cell stage, the blastomeres undergo polarisation and compaction to form a morula. By E3.5, the embryo has cavitated to form a blastocyst, and there is a polarised epithelium of cells on the outside of the embryo (trophectoderm) enclosing the ICM. By the mid-blastocyst stage, some cells show upregulation of epiblast or PrE factors, and this decision is completed by E4.5. Here, the ICM cells have segregated into epiblast and PrE cells, and shortly after this point, implantation occurs. *Green boxes* indicate the stage at which certain chromatin remodellers are required and the family and/or complex to which these proteins belong

using genetic mutants has often been difficult. While chromatin remodellers may all appear to possess similar biochemical activities, each complex clearly controls distinct aspects of chromatin biology.

CHD4: A Precise Subtle Regulator of Fate

CHD4 is a founding member of the NuRD (*Nu*cleosome *R*emodelling and *D*eacetylation) complex (Fig. 2). The NuRD complex regulates transcription and has long been assumed to be a transcriptional repressor, in part due to the presence of histone deacetylase (HDAC) proteins within the complex. While NuRD certainly is capable of mediating transcriptional repression (Ahringer 2000; McDonel et al. 2009), the advent of genome-wide analyses has led to the realisation that CHD4 and NuRD are just as likely to be associated with transcriptional activation (Gunther et al. 2013; Reynolds et al. 2013; Shimbo et al. 2013; Kim et al. 2014). As well as containing a chromatin remodelling protein (either CHD4 or CHD3) and HDAC proteins (HDAC1/2), NuRD contains zinc-finger proteins GATAD2A/B and SALL4 (in ES cells), SANT domain proteins MTA1/2/3, histone chaperones RBBP4/7, structural proteins MBD2/3 and the small CDK2AP1 protein (Le Guezennec et al. 2006; Allen et al. 2013). Although CHD4 is the founding member of the complex (Tong et al. 1998; Wade et al. 1998; Xue et al. 1998; Zhang et al. 1998), there is accumulating evidence that CHD4 can also function independently of the NuRD complex (O'Shaughnessy and Hendrich 2013).

CHD4 function is critical for the first lineage decision in mouse preimplantation development. Although embryos lacking CHD4 form a morphologically normal blastocyst, they are unable to form functional TE (O'Shaughnessy-Kirwan et al. 2015). Single-cell gene expression analysis was used to show that CHD4 is required for embryos to establish exclusive lineage-appropriate gene expression programmes at the 16-cell stage, before the formation of the morphological

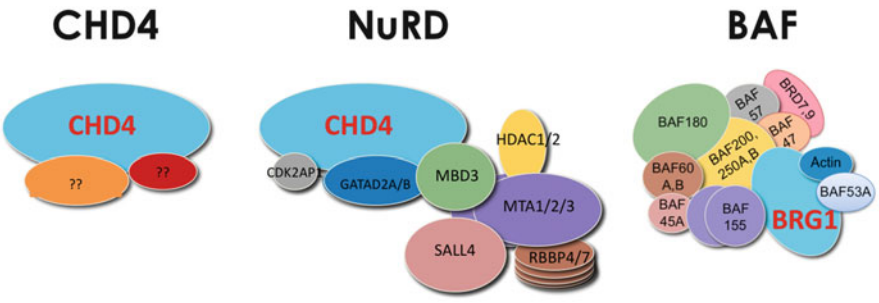

Fig. 2 CHD4, NuRD and BAF Complexes. Protein components of the NuRD and esBAF complexes. Whether CHD4 associates with other proteins when it is not part of NuRD is not known. The components of the BAF complex shown correspond to the ES cell-specific complex, esBAF

blastocyst. Cells of 16-cell embryos express markers of PrE, TE and epiblast in a stochastic manner, in a process that resembles lineage priming (Enver et al. 2009). In the absence of CHD4, these cells express lineage markers at increased frequency. The consequence of this increased transcriptional 'noise' is that cells are unable to accurately specify the TE lineage, and, despite forming an embryo that resembles a blastocyst, they lack trophectoderm function in that they are unable to maintain the integrity of the blastocoel or to implant in vivo or ex vivo (O'Shaughnessy-Kirwan et al. 2015). The activity of CHD4 in restricting gene expression probability, rather than gene expression levels per se, is thus essential for successful completion of the first lineage decision.

Although CHD4 binds broadly to active chromatin, this study did not find global dysregulation of gene expression in *Chd4*-mutant blastocysts (O'Shaughnessy-Kirwan et al. 2015). Instead, the essential function of CHD4 in the developing embryo is focused on lineage-specific genes. This work highlights two important points: that the essential targets of a chromatin remodeller may vary depending on the tissue and/or developmental stage and that, while the developing embryo is fairly robust, only small changes in gene expression can be enough to culminate in developmental failure. It remains to be seen whether this function of CHD4 is specific to the TE lineage, or whether CHD4 function similarly controls the PrE versus epiblast lineage decision.

Surprisingly, this function of CHD4 was exerted independently of the NuRD complex (O'Shaughnessy-Kirwan et al. 2015), indicating that despite the majority of chromatin remodelling proteins forming multi-protein complexes, their role isn't necessarily restricted to that within complexes. Impairment of NuRD activity through deletion of the gene encoding MBD3, an important NuRD structural component, results in developmental failure of the epiblast (Kaji et al. 2007). These observations lead to a model in which CHD4 functions on its own to facilitate the first lineage decision; it subsequently functions as part of NuRD to enable the development of epiblast cells during implantation (Kaji et al. 2007). This raises the interesting question of whether the biochemical activity of CHD4 is somehow altered when it is incorporated into NuRD or alternately whether NuRD acts to target CHD4 activity to specific sites in vivo.

BRG1/BAF Chromatin Remodellers

BAF complexes, which belong to the SWI/SNF family of remodellers, are large polymorphic complexes that contain at least 15 different subunits, the precise composition of which is cell type specific (Ho and Crabtree 2010; Kadoch and Crabtree 2015) (Fig. 2). Loss of many of the BAF complex subunits is incompatible with successful completion of early mammalian development, often resulting in pre- or peri-implantation lethality (Hota and Bruneau 2016). Loss of either the ATPase subunit (BRG1/SMARCA4) or of two other major components (BAF47 and BAF155) in embryos results in similar phenotypes: embryos develop to the

blastocyst stage but fail to progress further. Neither trophectoderm nor ICM outgrowths are viable in these mutants, indicating that BAF function is essential for the continued proliferation of both lineages ex vivo (Bultman et al. 2000; Klochendler-Yeivin et al. 2000; Guidi et al. 2001; Kim et al. 2001; Kidder et al. 2009; Panamarova et al. 2016). Loss of the BAF250A subunit results in developmental arrest later in development, shortly after implantation (Gao et al. 2008).

Although the ES cell-specific BAF complex has been extensively studied (Ho et al. 2009a, b, 2011) and the requirement for various BAF complexes in embryo viability is well known, the essential molecular mechanisms through which BAF activity sustains viability in the early mouse embryo are less well characterised. While initial work showed that zygotic deletion of *SMARCA4* resulted in a peri-implantation lethality, it was not clear how development failed in these mutants (Bultman et al. 2000). The fact that null blastocysts were morphologically normal at embryonic day 3.5 and were able to attach to plastic substrate (after removal of the zona pellucida) was an indication that the TE-ICM decision may not require zygotic BRG1 activity. Knockdown of *SMARCA4* in zygotes, which would deplete both maternally supplied and zygotic mRNA, did not prevent formation of a morphological blastocyst; however, antibody staining indicated evidence for specification defects in trophectoderm. Specifically, TE cells were found to inappropriately express OCT4 and NANOG, two proteins which should only be expressed in epiblast cells (Wang et al. 2010; Carey et al. 2015). While BRG1 and CHD4 appear to exert opposing effects on chromatin accessibility (Morris et al. 2014; de Dieuleveult et al. 2016), it appears that both activities are necessary for complete silencing of epiblast lineage genes during TE specification and/or for maintaining the silent state of these genes.

More recent work has demonstrated that the BAF155 subunit is essential for accurate lineage specification in early mouse embryos (Panamarova et al. 2016). This study found that loss of BAF155 resulted in ectopic expression of the epiblast marker NANOG in the TE, while increased *BAF155* expression resulted in upregulated expression of differentiation genes *CDX2* and *SOX17*. This study further showed that BAF155 acted to control the levels of stable BAF complex formation and that extraembryonic lineages showed increased complex formation or stability compared to embryonic lineages (Panamarova et al. 2016). This study raises the possibility that quantitative changes in the abundance or stability of chromatin remodellers could play an important role in cell fate transitions.

INO80 and ISWI Chromatin Remodellers

Two more chromatin remodelling ATPase proteins that have been implicated in early embryonic development are the INO80 DNA helicase and the SNF2H (SMARCA5) ATPase (Lazzaro and Picketts 2001; Bao and Shen 2007; Gerhold and Gasser 2014), although what their functions might be in preimplantation development is not yet clear. Knockdown of *INO80* in zygotes severely impaired

blastocyst formation after in vitro culture (Wang et al. 2014), but zygotic deletion of *INO80* had no detrimental effects until gastrulation, with zygotic *INO80*-null blastocysts appearing morphologically normal and appropriately expressing markers of epiblast and PrE (Lee et al. 2014; Qiu et al. 2016). These observations indicate either that maternally contributed INO80 plays an essential role in early cleavage stages or that the embryo is able to compensate for a genetic deficiency but cannot similarly compensate for a knockdown (Rossi et al. 2015). Similarly, a large proportion of zygotes injected with siRNA against *SMARCA5* displayed developmental failure prior to reaching the blastocyst stage (Torres-Padilla and Zernicka-Goetz 2006), whereas embryos homozygous null for *SMARCA5* showed no defects prior to implantation (Stopka and Skoultchi 2003). *SMARCA5* knockdown embryos showed reduced expression of a number of genes, indicating that the observed embryonic lethality is indeed due to changes in gene expression (Torres-Padilla and Zernicka-Goetz 2006). These studies demonstrate that in contrast to BRG1 and CHD4, neither zygotic INO80 nor zygotic SNF2H is necessary for successful completion of the first cell fate decisions or for implantation. Rather, protein derived from maternally supplied mRNA from both *INO80* and *SMARCA5* is essential for the viability of early cleavage stage embryos.

Concluding Remarks

Chromatin remodelling proteins play key roles in ensuring that cell fate decisions occur correctly throughout mammalian development. While CHD4 and BAF proteins are essential for the first cell fate decision, zygotic SNF2H and INO80 are required only after implantation. In contrast, while maternally derived BRG1 is dispensable for the viability of cleavage stage embryos, both INO80 and SNF2H made from maternally deposited mRNA play essential roles. This simple relationship illustrates the separation of function that exists between these very different chromatin remodelling proteins.

Until recently, genetics was the only concrete method of determining whether a specific chromatin remodeller was necessary during preimplantation development, but this provided little mechanistic information, if any. The recent explosion in the abundance of single-cell analysis methods allows us to better understand how cell fate decisions are made and to determine why, at the molecular level, specific proteins are necessary for cell fate decisions (Nimmo et al. 2015). Such work has demonstrated that both CHD4 and the BAF complex are indispensable for ensuring that only the appropriate gene expression programme is active in cells undergoing the ICM versus TE cell fate decision and that complex abundance or stability could be an important factor in successful completion of the first cell fate decision. As the limits of molecular analyses continue to recede, the molecular details of exactly how each remodeller exerts its functions in early embryos will come into increasingly sharper focus.

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CHD1 Controls Cell Lineage Specification Through Zygotic Genome Activation

Shinnosuke Suzuki and Naojiro Minami

Abstract In mammals, the processes spanning from fertilization to the generation of a new organism are very complex and are controlled by multiple genes. Life begins with the encounter of eggs and spermatozoa, in which gene expression is inactive prior to fertilization. After several cell divisions, cells arise that are specialized in implantation, a developmental process unique to mammals. Cells involved in the establishment and maintenance of implantation differentiate from totipotent embryos, and the remaining cells generate the embryo proper. Although this process of differentiation, termed cell lineage specification, is supported by various gene expression networks, many components have yet to be identified. Moreover, despite extensive research it remains unclear which genes are controlled by each of the factors involved. Although it has become clear that epigenetic factors regulate gene expression, elucidation of the underlying mechanisms remains challenging. In this chapter, we propose that the chromatin remodeling factor CHD1, together with epigenetic factors, is involved in a subset of gene expression networks involved in processes spanning from zygotic genome activation to cell lineage specification.

Introduction

After fertilization in mammals, the zygote, which consists of two differentiated haploid genomes, becomes a totipotent cell that initiates a developmental program resulting in production of a new organism, consisting of myriad differentiated cells, after extensive reconfiguration. This reconfiguration is dependent on transcripts and proteins stored in maternally derived oocytes, and these transcripts and proteins

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remodel the maternal and paternal genomes in preparation for the onset of the developmental program. After chromatin status is established following fertilization, zygotic genome activation (ZGA) is initiated and zygotic transcripts regulate development as maternal transcripts are degraded.

It has gradually become clear that epigenetic modification plays an important role in this remodeling and the subsequent identification of cell lineages (Ho and Crabtree 2010; Roper and Hemberger 2009). Epigenetic regulation is primarily controlled by chemical modifications, such as DNA methylation and posttranslational modifications of histones, resulting in changes in chromatin structure and nuclear organization (Bernstein et al. 2007). Although genetic information provides a blueprint for the production of RNA and proteins, chromatin structure and nuclear organization regulate gene expression, especially by controlling the binding of transcriptional machinery to DNA (Schneider and Grosschedl 2007). Shortly after initiation of the developmental program, the totipotent cells of the embryo are destined to form cells of two different types: the embryonic cell lineage (i.e., inner cell mass: ICM) and the extraembryonic cell lineage (i.e., trophectoderm: TE). This specification of cell fate, along with expression of lineage-specific marker genes, is initiated by the eight-cell stage in the mouse (Cockburn and Rossant 2010). In this chapter, we focus on trimethylated lysine 4 of histone H3 (H3K4me3), a histone modification that positively regulates gene expression. In particular, we describe the pathway of cell lineage specification by H3K4me3 at the time of ZGA and recognition of this histone modification by CHD1.

Mouse Preimplantation Development

In mammals, maternal mRNAs are actively transcribed and stored throughout oocyte growth. In fully grown oocytes, germinal vesicle breakdown (GVBD) is caused by a surge in luteinizing hormone from the pituitary, after which transcription ceases. Therefore, in the absence of transcription, oocyte maturation and fertilization occur using maternal mRNAs and proteins stored during oogenesis. After fertilization, the first step of chromatin remodeling takes place in parental genomes, and the first transcriptional event (termed minor ZGA) is initiated. This activation is followed by a large wave of transcription at the two-cell stage, major ZGA, which is required for normal development (Hamatani et al. 2004; Levey et al. 1977; Li et al. 2010; Minami et al. 2007; Schultz 1993; Wang and Dey 2006; Warner and Versteegh 1974): the development of one-cell embryos treated with α-amanitin, an RNA polymerase II inhibitor, is arrested at the two-cell stage (Levey et al. 1977; Warner and Versteegh 1974). ZGA is induced by maternal mRNA and protein stored during oogenesis, and knockout of several maternal-effect genes leads to developmental arrest at the early stages of embryogenesis (Bultman et al. 2006; Burns et al. 2003; Payer et al. 2003; Ramos et al. 2004; Tong et al. 2000; Tsukamoto et al. 2008; Wu et al. 2003). Zygotes generated from females carrying an oocyte-specific deletion of *Mll2*, an H3K4 methyltransferase, undergo

developmental arrest between the one- and four-cell stages due to impairment in ZGA (Andreu-Vieyra et al. 2010), and zygotes generated from females carrying an oocyte-specific deletion of *Brg1*, a subclass of SWItch/sucrose non-fermentable ATP-dependent chromatin remodelers, undergo developmental arrest at the two- and four-cell stage, suggesting that chromatin remodeling factors play roles in ZGA. The second transcriptional event is mid-preimplantation gene activation (MGA) (Kari et al. 2016), which occurs between the four- and eight-cell stages in the mouse. During this period, genes required for cell lineage specification, e.g., the transcription factors *Pou5f1* (also known as *Oct4*), *Nanog*, and *Cdx2*, are expressed; these genes are key regulators governing differentiation of the ICM and TE (Hamatani et al. 2004; Nichols et al. 1998; Niwa et al. 2005; Strumpf et al. 2005; Wang and Dey 2006; Yoshikawa et al. 2006). However, the mechanisms of gene regulation at ZGA and MGA have not yet been elucidated. In general, gene expression is regulated through changes in several epigenetic factors, including transcription factors, chromatin remodeling factors, and histone modification enzymes. Dynamic changes occur in chromatin structure during preimplantation development in mammals (Abdalla et al. 2009; Albert and Helin 2010; Burton and Torres-Padilla 2010; Corry et al. 2009; Morgan et al. 2005; Rasmussen and Corry 2010; Shi and Wu 2009). Histone posttranslational modifications are introduced in a variety of ways. Several enzymes contribute to histone methylation (Zhang and Reinberg 2001), acetylation (Sterner and Berger 2000), phosphorylation (Nowak and Corces 2004), and ubiquitination (Shilatifard 2006). With respect to methylation, modifications of lysines 4, 36, and 79 of histone H3 (referred to as H3K4, H3K36, and H3K79, respectively) are associated with transcriptional activation, whereas modifications of lysines 9 and 27 of histone H3 and lysine 20 of histone H4 (referred to as H3K9, H3K27, and H4K20, respectively) are associated with transcriptional repression (Lepikhov and Walter 2004; Sarmento et al. 2004). The level of H3K4me3 gradually increases throughout oocyte growth (Kageyama et al. 2007), and in fully grown and mature oocytes, H3K4me3 is distributed as broad peaks at transcription start site (TSS)-containing and non-TSS-containing domains (Dahl et al. 2016; Zhang et al. 2016a). After fertilization, H3K4me3 is maintained at a high level until ZGA in the maternal chromosomes of the embryo (Kageyama et al. 2007). Until major ZGA occurs, the amount of H3K4me3 decreases in non-TSS-containing domains, whereas that in TSS-containing domains is maintained (Dahl et al. 2016; Zhang et al. 2016a). On the other hand, in the paternal chromosomes, the loci marked with H3K4me3 are extensively reprogrammed and depleted following fertilization, and then the peaks of H3K4m3e in TSS-containing domains reappear around major ZGA (Albert and Peters 2009; Lepikhov and Walter 2004). Establishment of chromatin state after fertilization allows ZGA, and zygotic transcripts begin to control early embryogenesis. The mechanisms of regulation of ZGA by histone modifications such as H3K4me3 and H3K27me3 have been reviewed previously (Ostrup et al. 2013). In this chapter, we will focus on the mechanisms regulating cell lineage specification in early preimplantation embryos from the viewpoint of epigenetic factors during ZGA and MGA.

CHD1 (*C*hromodomain *H*elicase *D*NA-Binding Protein *1*) (Fig. 1)

CHD1 is a member of the family of ATPase-dependent chromatin remodeling factors (Woodage et al. 1997). CHD1 recognizes H3K4me3 (Sims et al. 2005) and has been implicated in transcriptional activation in yeast (Simic et al. 2003), *Drosophila* (Stokes et al. 1996), and mammalian cells (Sims et al. 2007). In cancer cells, CHD1 is required for generation of mature mRNA via stable association with the SF3a subcomplex of U2 snRNP (Sims et al. 2007), and it plays an essential role in homologous recombination-mediated DNA repair by promoting recruitment of CtIP, RPA1, and RAD51 to DNA repair sites (Kari et al. 2016). These observations suggest that CHD1 has important functions in posttranscriptional mRNA stability (Sims et al. 2007) and DNA repair mechanisms (Kari et al. 2016) in humans. In mouse, CHD1 also plays important roles in the maintenance of embryonic stem (ES) cell pluripotency by associating with euchromatic promoter regions (Gaspar-Maia et al. 2009). In *Drosophila* embryos, CHD1 is necessary at fertilization for the incorporation of the variant histone H3.3 into paternal pronuclear chromatin in the absence of transcription (Konev et al. 2007), and it is also important for incorporation of H3.3 during early preimplantation development in bovines (Zhang et al. 2016b). These observations suggest that CHD1 plays important roles after fertilization and in peri-implantation embryos. However, the function(s) of CHD1 in mouse preimplantation embryos has not yet been determined.

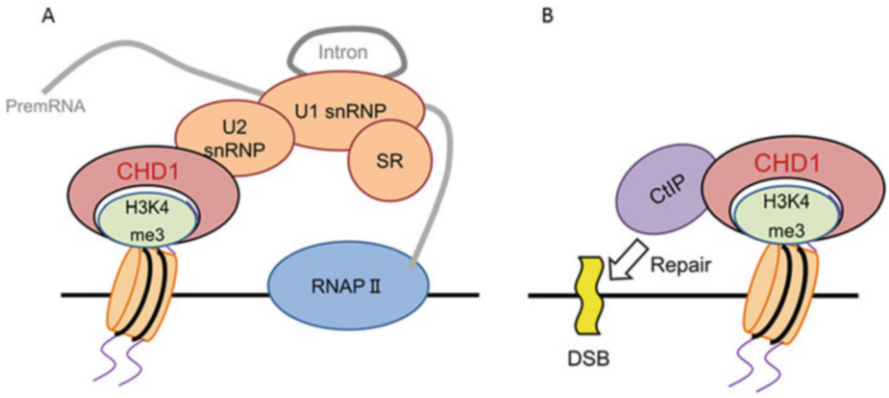

Fig. 1 Schematic illustration of CHD1 function. (**A**) During transcription, CHD1 binds to H3K4me3; recruits the splicing complex containing U2 snRNP, U1 snRNP, and SR; and participates in RNA splicing. (**B**) At DNA double-strand breaks (DSBs), CHD1 recruits CtIP (also known as RB binding protein 8; RBBP8) and repairs the DNA DSBs. *snRNP* small nuclear ribonucleoprotein, *SR* seine/arginine-rich protein, *RNAP II* RNA polymerase II, *CtIP* C-terminal binding protein interacting protein

Cell Lineage Specification

During MGA, several key regulators governing formation of the ICM and TE, e.g., the transcription factors *Pou5f1*, *Nanog*, and *Cdx2*, begin to be expressed (Hamatani et al. 2004; Nichols et al. 1998; Niwa et al. 2005; Strumpf et al. 2005; Wang and Dey 2006; Yoshikawa et al. 2006). The maintenance of pluripotency depends on the functions of OCT4 (encoded by *Pou5f1*) and NANOG during preimplantation development (Chen et al. 2009; Mitsui et al. 2003; Nichols et al. 1998; Shao et al. 2008). *Pou5f1* and *Nanog* negatively interact with *Cdx2*, and these three genes are key regulators in cell lineage specification (Chen et al. 2009; Niwa et al. 2005; Ralston et al. 2010; Strumpf et al. 2005). *Pou5f1* or *Nanog* knockout mouse embryos can develop into morphologically normal blastocysts; however, developmental arrest occurs during the postimplantation period due to a loss of pluripotency and the accompanying expression of *Cdx2* in all blastomeres (Chen et al. 2009; Ralston et al. 2010). By contrast, *Cdx2* knockout mouse embryos are arrested at the early blastocyst stage because loss of *Cdx2* fails to downregulate *Pou5f1* and *Nanog* in the outer cells of the blastocyst (Strumpf et al. 2005). Therefore, to understand cell lineage specification, the regulatory mechanisms underlying expression of lineage-specific marker genes such as *Pou5f1*, *Nanog*, and *Cdx2* at MGA must be elucidated. Several genes involved in cell lineage specification by regulating the expression of *Pou5f1*, *Nanog*, or *Cdx2* have been identified. For example, the transcription factor *Tead4*, a component of the Hippo pathway, is expressed during ZGA in mouse preimplantation embryos. A deficiency of *Tead4* leads to the failure of cell lineage specification due to suppression of *Cdx2* expression at MGA and developmental arrest at the morula stage (Yagi et al. 2007). At the beginning of cell lineage specification after the 8- to 16-cell stage, the Hippo pathway, which is involved in cell contact-mediated polarity, regulates the activity of *Tead4* (Anani et al. 2014; Cockburn et al. 2013; Hirate et al. 2012, 2013; Nishioka et al. 2009). After the 16-cell stage, the Hippo pathway is inactivated in outer cells that ultimately become TE but activated in inner cells that become ICM. In outer cells, AMOT strongly localizes to the apical domain and interacts with actin filaments. As a result, LATS cannot interact with AMOT, and YAP localizes to the nuclei and interacts with TEAD4, leading to the active regulation of gene expression. On the other hand, in inner cells, AMOT localizes to adherens junctions (AJs) and is phosphorylated by LATS. Phosphorylated AMOT, LATS, and NF2 form a complex and induce phosphorylation of YAP, and phosphorylated YAP suppresses its nuclear accumulation, leading to the inactivation of TEAD4 (Anani et al. 2014; Cockburn et al. 2013; Hirate et al. 2012, 2013; Nishioka et al. 2009). During the second cell lineage specification, which occurs between E3.5 and E4.5, the ICM gives rise to the epiblast (EPI), which predominantly expresses *Nanog*, and the primitive endoderm (PE), which predominantly expresses *Gata6*; however, these gene expression patterns in ICM at E3.5 are mosaic and random, well known as the "salt-and-pepper" distribution (Chazaud et al. 2006). The EPI will eventually give rise to the fetus, whereas the PE will develop into the visceral and

parietal endoderm of the yolk sacs, and the TE will become the fetal placenta. Additionally, there is a negative interaction between *Nanog* and *Gata6*, which are, respectively, key regulators of the establishment of EPI and PE fates (Frankenberg et al. 2011; Kang et al. 2013; Morris et al. 2010; Schrode et al. 2014). NANOG can directly downregulate the expression of *Gata6* by binding to its promoter in *Nanog*-positive cells (EPI progenitor) (Singh et al. 2007), and *Nanog*-deficient mouse embryos undergo arrest during postimplantation development due to widespread expression of *Gata6* throughout the ICM (Frankenberg et al. 2011). By contrast, *Gata6*-deficient mouse embryos are arrested during postimplantation development due to widespread expression of *Nanog* throughout the ICM (Schrode et al. 2014). Additionally, *Fgf4* is expressed in *Nanog*-positive cells, FGF4 is bound to FGFR2 in *Gata6*-positive cells, and *Gata6* expression is upregulated via the Fgf/Map kinase pathway (Yamanaka et al. 2010). Therefore, to understand the first and second cell lineage specifications, we must discern the regulatory mechanisms underlying expression of *Nanog* at MGA.

The Roles of CHD1 During Mouse Preimplantation Development

During mouse preimplantation development, expression of *Chd1* mRNA gradually increases at the two-cell stage, peaks at the eight-cell stage, and then dramatically decreases. CHD1 is localized in the nuclei of all blastomeres, and its staining intensity increases at the two-cell stage, suggesting that *Chd1* plays important roles in ZGA. *Chd1*-knockout embryos undergo developmental arrest at E6.5 due to failure to maintain the epiblast (Guzman-Ayala et al. 2015). In addition, although *Chd1*-knockdown embryos at ZGA exhibit morphologically normal growth (normal numbers of cells in both ICM and TE, relative to controls) until the blastocyst stage (E4.5), *Chd1* knockdown leads to embryonic lethality after implantation. Outgrowth experiments showed that ICM-derived colony formation and litter size after embryo transfer are significantly reduced in *Chd1*-knockdown embryos (Table 1). To determine the cause of this defect, the expression of lineage-specific markers, *Pou5f1*, *Nanog*, and *Cdx2*, was assessed at the mRNA and protein levels (Fig. 2). In *Chd1*-knockdown embryos, *Pou5f1* and *Nanog* mRNA expression is suppressed at the four- to eight-cell stages but increases dramatically at these stages in normal embryos. In normal embryos, *Cdx2* mRNA is first detected at the eight-

Table 1 Effect of *Chd1* knockdown on development of mouse embryos

Treatment	Outgrowth rates (%)	Live offspring rates (%)
siControl	81.9 ± 5.58	48.9 ± 2.22
si*Chd1*	29.0 ± 3.92*	13.3 ± 0.03*

*$p < 0.05$

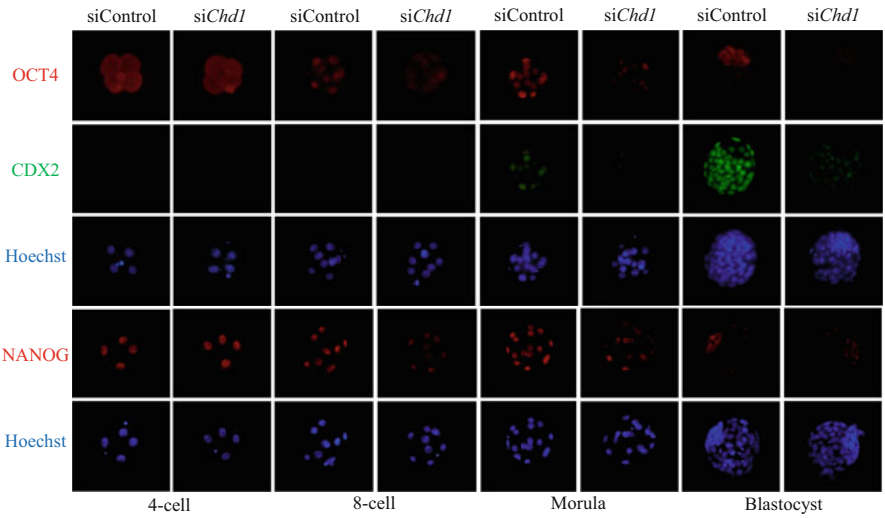

Fig. 2 Effects of *Chd1* knockdown on the expression of *Pou5f1*, *Nanog*, and *Cdx2* during mouse preimplantation development. Immunofluorescence detection of OCT4, NANOG, and CDX2 in control and *Chd1*-knockdown embryos (*red* OCT4, *red* NANOG, *green* CDX2, *blue* chromatin). The reduction of OCT4 and NANOG proteins in Chd1-knockdown embryos was observed from the eight-cell stage until the blastocyst stage. The level of CDX2 protein, first detected at the morula stage, was also reduced after the morula stage in Chd1-knockdown embryos. OCT4 and NANOG proteins detected at the four-cell stage are maternal origin

cell stage and then gradually increases in abundance, whereas its mRNA expression is suppressed during this stage in *Chd1*-knockdown embryos. Furthermore, in *Chd1*-knockdown embryos, immunofluorescence detection of OCT4, NANOG, and CDX2 reveals that maternal OCT4 and NANOG are maintained until the four-cell stage, whereas levels of newly synthesized OCT4 and NANOG are reduced starting at the eight-cell stage, and the CDX2 level is reduced starting at the morula stage. The localization of OCT4 in the ICM, NANOG in the EPI, and CDX2 in the TE does not change in *Chd1*-knockdown embryos. Expression of *Chd1* mRNA and proteins is suppressed at ZGA in *Chd1*-knockdown embryos, suggesting that CHD1 function during ZGA affects the later stages of embryogenesis. Although CHD1 is required for optimal transcriptional output in mouse ES cells (Guzman-Ayala et al. 2015), global transcriptional activity in *Chd1*-knockdown embryos at ZGA and MGA does not change compared with normal embryos. This observation suggests that CHD1 functions in selective regulation of gene expression that controls mouse preimplantation development (Suzuki et al. 2015b). At the blastocyst stage, SMYD3, an H3K4 methyltransferase, regulates the expression of ICM/EPI markers, e.g., *Oct4*, *Nanog*, and *Sox2*; PE markers, e.g., *Gata6*; and TE markers, e.g., *Cdx2* and *Eomes*. However, in *Smyd3*-knockdown embryos, global H3K4me3 levels are unchanged (Fig. 3) (Suzuki et al. 2015a), suggesting that selective regulation of gene expression in early preimplantation development is controlled by epigenetic modifications. In cancer cells, SMYD3

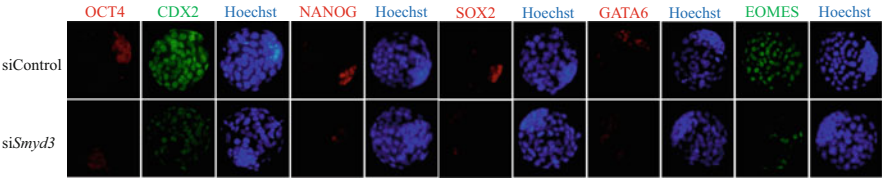

Fig. 3 Effects of *Smyd3* knockdown on the expression of lineage-specific genes in blastocysts. Immunostaining of OCT4, CDX2, NANOG, SOX2, GATA6, and EOMES in *Smyd3*-knockdown and control blastocyst embryos (OCT4 *red*, CDX2 *green*, NANOG *red*, SOX2 *red*, GATA6 *red*, EOMES *green*, chromatin *blue*). Expression of all lineage-specific proteins was downregulated in *Smyd3*-knockdown embryos

selectively modifies H3K4 methylation in oncogene promoter regions (Cock-Rada et al. 2012; Liu et al. 2013; Medjkane et al. 2012). Accordingly, it is possible that H3K4me3 levels depend on the type of methyltransferase, that SMYD3 modifies H3K4 within the promoter regions of the lineage-specific genes, and that CHD1 binds these regions and activates the expression of these genes. These observations also indicate that suppression of chromatin modifiers at ZGA affects later stages of embryogenesis, including cell lineage specification.

The Relationship Between CHD1 and HMGPI During Mouse Preimplantation Development

The mechanisms that regulate *Pou5f1*, *Nanog*, and *Cdx2* expression during mouse preimplantation development have gradually been elucidated. HMGPI, a transcription factor, regulates the expression of *Pou5f1* and *Nanog* in ICM and *Cdx2* in TE (Ema et al. 2008; Lin et al. 2010; Yamada et al. 2010). The expression of *Hmgpi* starts at the late two-cell stage, peaks at the four-cell stage, and then gradually decreases. In *Chd1*-knockdown embryos, the expression of *Hmgpi* is dramatically reduced at all preimplantation stages, and protein levels are also reduced from the four-cell stage onward (Fig. 4). In embryos lacking functional *Hmgpi*, developmental arrest occurs during peri-implantation development due to suppression of *Pou5f1*, *Nanog*, and *Cdx2* expression (Ema et al. 2008; Lin et al. 2010; Yamada et al. 2010), suggesting that *Hmgpi* regulates the initiation of cell lineage specification. These observations indicate that CHD1 regulates the expression of *Pou5f1* and *Nanog* in ICM and *Cdx2* in TE via activation of *Hmgpi* expression and is involved in lineage specification during early embryogenesis. To test this hypothesis, HMGPI expression was rescued by injection of *Hmgpi* mRNA into *Chd1*-knockdown embryos. *Chd1* mRNA levels were not restored in *Chd1*-knockdown/*Hmgpi*-rescue embryos at any stage. Nonetheless, the suppression of zygotic *Pou5f1*, *Nanog*, and *Cdx2* in *Chd1* knockdown was recovered in *Chd1*-knockdown/*Hmgpi*-rescue embryos; levels of OCT4, NANOG, and CDX2

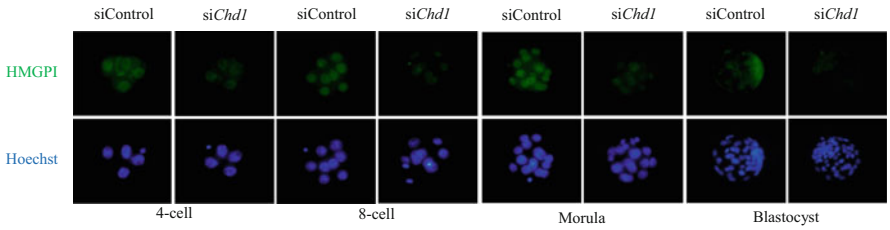

Fig. 4 Effects of *Chd1* knockdown on the expression of *Hmgpi* during mouse preimplantation development. Immunofluorescence detection of HMGPI in control and *Chd1*-knockdown embryos (*green* HMGPI, *blue* chromatin). Representative photos of embryos at each stage are shown. HMGPI protein expression was downregulated after the four-cell stage in *Chd1*-knockdown embryos

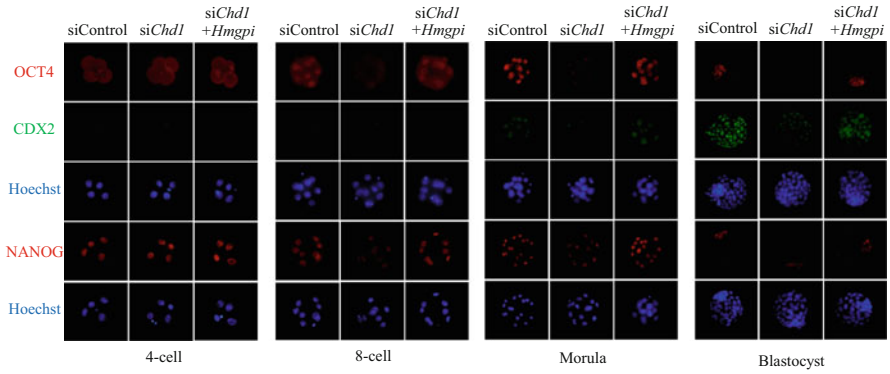

Fig. 5 Effects of *Hmgpi* rescue in *Chd1*-knockdown embryos on the expression of *Pou5f1*, *Nanog*, and *Cdx2*. Immunofluorescence detection of OCT4, NANOG, and CDX2 in control, *Chd1*-knockdown, and *Chd1*-knockdown/*Hmgpi*-rescue embryos (*red* OCT4, *red* NANOG, *green* CDX2, *blue* chromatin). The levels of lineage-specific proteins (OCT4, NANOG, and CDX2) were recovered in *Chd1*-knockdown/*Hmgpi*-rescue embryos

Table 2 Effect of *Hmgpi* rescue on development of *Chd1*-knockdown embryos

Treatment	Outgrowth rates (%)	Live offspring rates (%)
siControl	76.1 ± 5.43	44.4 ± 2.22
si*Chd1* + *Hmgpi mRNA*	71.0 ± 5.70	48.9 ± 8.01

were restored (Fig. 5); and normal ICM-derived colony formation and litter size were rescued (Table 2) (Suzuki et al. 2015b), suggesting that CHD1 plays important roles as a trigger for *Pou5f1*, *Nanog*, and *Cdx2* expression by regulating *Hmgpi* expression at ZGA.

Developmentally important genes are marked by H3K4me3 before ZGA and have a strong propensity to be activated after ZGA (Lindeman et al. 2011).

CHD1, which recognizes H3K4me3, functions in the regulation of gene expression at ZGA and affects the development of mouse pre- and postimplantation embryos. Therefore, to understand the regulatory mechanisms of early embryogenesis, it is important to elucidate the transcriptional mechanisms involving H3K4me3 at ZGA.

In conclusion, CHD1 regulates the initiation of zygotic *Oct4*, *Nanog*, and *Cdx2* expression at MGA by activating *Hmgpi* expression at ZGA. Thereafter, HMGPI, under the control of CHD1, regulates the expression of *Pou5f1*, *Nanog*, and *Cdx2* and thereby controls initiation of cell lineage specification (Fig. 6).

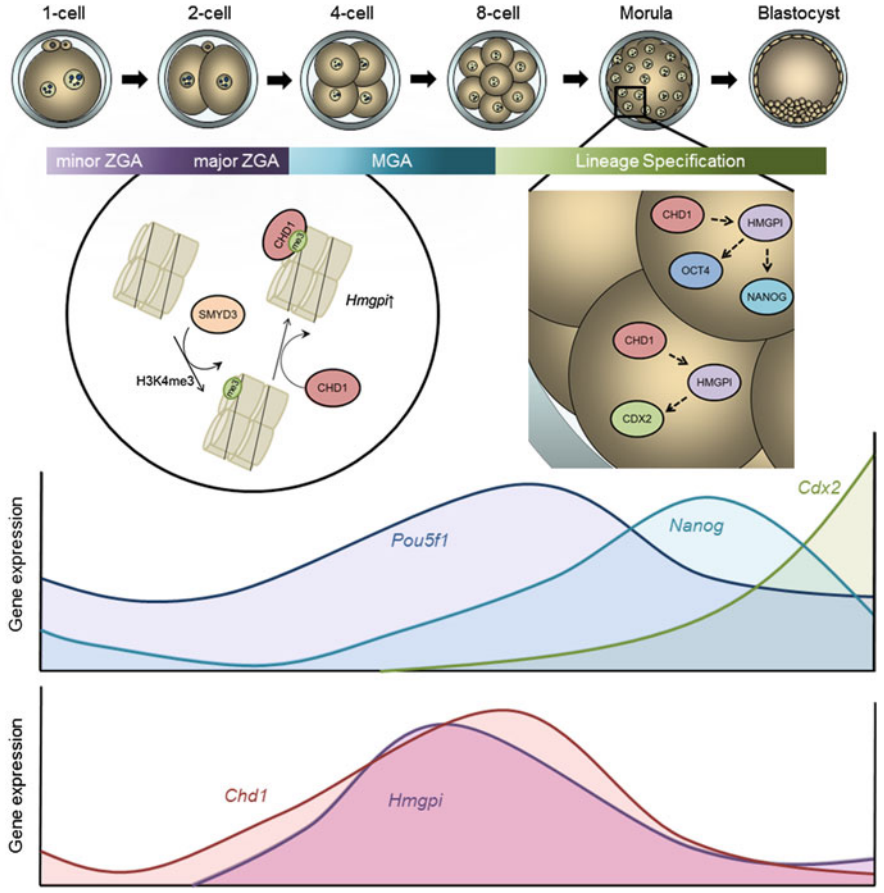

Fig. 6 Model of CHD1 function during mouse preimplantation development. After fertilization, minor ZGA, a small transcriptional wave, is initiated at the one-cell stage and followed by major ZGA, the first large wave of transcription at the two-cell stage. The second large wave of transcription (Kari et al.) occurs between the four- and eight-cell stages. During this period, the transcription factors *Pou5f1*, *Nanog*, and *Cdx2*, the key regulators of cell lineage specification, are expressed, and consequently, ICM differentiation and TE segregation occur. CHD1 begins to be actively transcribed at major ZGA and at the same time regulates the expression of downstream gene *Hmgpi*. HMGPI regulates the expression of *Pou5f1*, *Nanog*, and *Cdx2* at MGA. Thereafter, the expression of *Pou5f1*, *Nanog*, and *Cdx2* controls initiation of cell lineage specification

Conclusion and Future Directions

In this chapter, we discussed the events of mouse preimplantation development and subsequent cell lineage specification from the viewpoint of ZGA, which occurs after fertilization, and regulation of gene expression by epigenetic modifications. Following the encounter between the terminally differentiated sperm and egg, radical cell reprogramming is initiated. The sperm and egg are cells that have completely terminated gene expression, but following fertilization they form a totipotent zygote. The fertilized egg subsequently loses its totipotency shortly after major ZGA, converting instead to a pluripotent state. Then, cell lineage specification occurs before implantation and differentiation progresses, gradually leading to production of an organism. Although it has long been known that the first gene expression after fertilization is controlled by maternal factors, it remains unclear which genes are controlled by maternal factors and what kinds of mechanisms are involved in the regulation. Although it has gradually become clear that epigenetic modifications are largely involved in gene expression after fertilization, it remains to be determined which genes are under the spatiotemporal control of epigenetic modifications, and it is not yet known how the selectivity is determined. Answering this question represents a major challenge for future work. In this chapter, we also discussed the gene regulatory network that controls processes from fertilization to implantation in regard to the ZGA. Thus, the genes expressed at the time of ZGA control the subsequent MGA, and then the genes expressed at MGA control the expression of genes involved in the specification of cell lineage. However, it is still far from clear how the gene expression required for each developmental stage is controlled. Future studies will elucidate the mechanisms of control of gene expression leading to birth.

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Transcriptional Regulation and Genes Involved in First Lineage Specification During Preimplantation Development

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Abstract The successful development from a single-cell zygote into a complex multicellular organism requires precise coordination of multiple cell-fate decisions. The very first of these is lineage specification into the inner cell mass (ICM) and trophectoderm (TE) during mammalian preimplantation development. In mouse embryos, transcription factors (TFs) such as Oct4, Sox2, and Nanog are enriched in cells of ICM, which gives rise to the fetus and yolk sac. Conversely, TFs such as Cdx2 and Eomes become highly upregulated in TE, which contribute to the placenta. Here, we review the current understanding of key transcriptional control mechanisms and genes responsible for these distinct differences during the first cell lineage specification. In particular, we highlight recent insights gained through advances in genome manipulation, live imaging, single-cell transcriptomics, and loss-of-function studies.

Mammalian Preimplantation Development

Preimplantation development refers to the period from fertilization to implantation, during which the fertilized oocyte progresses through a number of cleavage divisions and three major transcriptional and morphogenetic events that lead to the first cell-fate decision and development into a blastocyst capable of implantation (Fig. 1).

Maternal-to-Zygotic Transition

The first well-characterized event is the maternal-to-zygotic transition (MZT), which includes degradation of maternal mRNAs and replacement with zygotic

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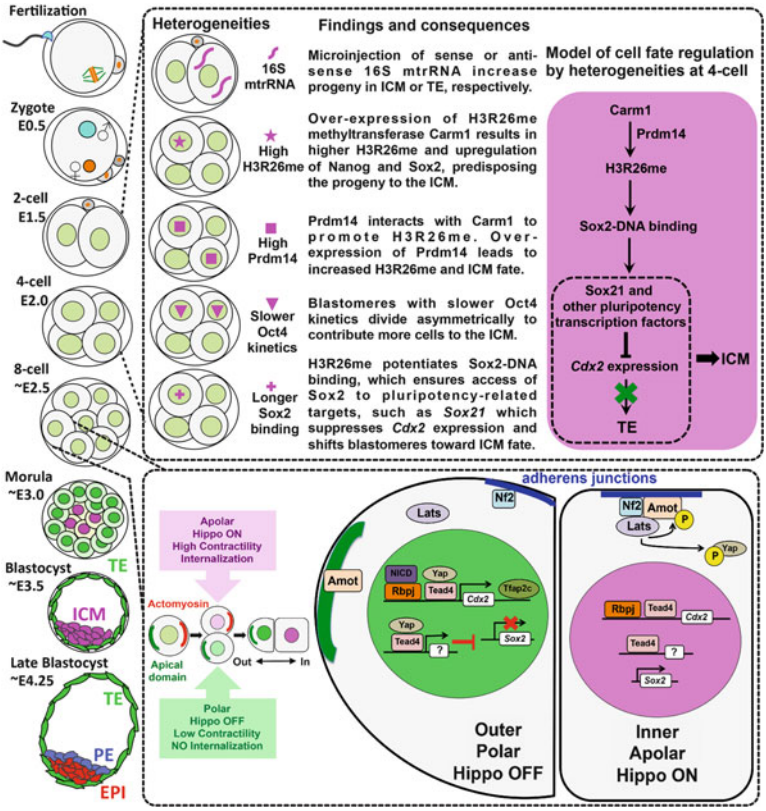

Fig. 1 Schematic representation of morphological changes and cell lineage specification during mouse preimplantation development. Cell fates and cell types are color coded. Heterogeneities detected in early blastomeres, biased lineage segregation, and proposed transcriptional control mechanisms underlying the intrinsic heterogeneities are highlighted in the *upper box*. At late eight-cell stage, blastomere compaction and polarization occur. During 8-cell to 16-cell, two kinds of daughter cells are generated: polar cells with apical domain and low actomyosin contractility and apolar cell without apical domain but high actomyosin contractility. Initiation of Hippo signaling is regulated by polarity: Hippo is off in the polar cells and active in apolar cells. Internalization of outer apolar cells is mainly driven by higher actomyosin contractility. In the outer cells, Amot, Nf2, and Lats do not form an active complex, such that Yap will not be phosphorylated. Unphosphorylated Yap translocates into the nucleus to bind Tead4 and activate target genes such as *Cdx2*. Expression of *Cdx2* is also promoted by the transcription factor Tfap2c through an intronic enhancer, as well as by Notch signaling through Notch intracellular domain (NICD) and Rbpj. Yap and Tead4 also activate an unknown component, which represses *Sox2* expression. In inner apolar cells, Amot, Nf2, and Lats form an active complex; Yap will be phosphorylated and kept out of the nucleus preventing expression of *Cdx2* and allowing expression of *Sox2*. Meanwhile, Notch signaling is not active in inner cells, resulting in no activation of *Cdx2*. Cell polarity, internalization, and key transcriptional regulation signals are highlighted in the *lower box*. *E* embryonic day, *TE* trophectoderm, *ICM* inner cell mass, *PE* primitive endoderm, *EPI* epiblast

transcripts. This dramatic reprogramming of gene expression is necessary for the establishment of totipotency and embryo development (Latham et al. 1991; Li et al. 2013). In the mouse, this process is first detectable at the one-cell stage but occurs mostly during the two-cell stage (Schultz 2002; Zhou and Dean 2015). Recent studies taking advantage of high-resolution live imaging and single-cell transcriptomic assays have discovered heterogeneities in transcription factor (TF) binding and gene expression between blastomeres as early as the two-cell stage, which may bias the first cell-fate determination (Fig. 1, upper box, reviewed below).

Embryo Compaction and Polarization

The second major event is embryo compaction and polarization, which initiate during the eight-cell stage in mouse embryos. Blastomere morphology becomes flattened, and biochemical changes to cellular metabolism, ion transport, and cell–cell contacts result in early embryonic cells first resembling somatic cells (Fleming et al. 2001). In addition to E-cadherin and β-catenin (De Vries et al. 2004), E-cadherin-dependent filopodia (Fierro-Gonzalez et al. 2013) and actomyosin cortex-derived force (Maitre et al. 2015) have been recently found essential for compaction. Proper compaction is required for the accompanying cell polarization and following cell division-dependent repositioning (Cockburn and Rossant 2010; White et al. 2016b).

During compaction, blastomeres also initiate polarization, to establish apical domains (Fig. 1, lower box), where apical and basal associated proteins first become localized [examples are, Pard3, Pard6, F-actin, and atypical protein kinase C (aPKC) (Yamanaka et al. 2006; Rossant and Tam 2009)]. In addition, other factors like microtubule (Houliston et al. 1989) and Rho-GTPase (Clayton et al. 1999; Kono et al. 2014) are also actively involved in this process. Daughter cell inheritance of polarity in subsequent divisions has been recently confirmed as critical for solidification of first cell-fate acquisition (Leung et al. 2016).

Blastomere Allocation and ICM/TE Separation

Following compaction and polarization, the third critical event is blastomere allocation into ICM and TE cell fates. Two distinct types of divisions occur during 8- to 16-cell transition: symmetric divisions that give rise to two polar cells both inheriting an apical domain versus asymmetric division that gives rise to one polar cell with an apical domain and one apolar cell (Chazaud and Yamanaka 2016). Additionally, Korotkevich et al. have recently used both in vivo and in vitro manipulated blastomeres to suggest that acquisition of an apical domain is both required and sufficient for initiation of first lineage specification (Korotkevich et al.

2017). Importantly, studies have found that some daughter cells on the outside of the embryo after 8- to 16-cell division can internalize and adopt an inner position and ICM fate (Morris et al. 2010; Yamanaka et al. 2010; Watanabe et al. 2014), suggesting that division orientation of blastomeres alone cannot predict or regulate the fate of daughter cells. Notably, recent studies revealed that this important internalization process is driven by higher actomyosin contractility within these cells induced by asymmetric segregation of the apical domain (Fig. 1, lower box) (Anani et al. 2014; Samarage et al. 2015; Maitre et al. 2016). These findings have shed new light on the link between cell polarity, cell position, and the Hippo signaling—a key pathway involved in the establishment of the first cell lineage separation (discussed below).

Apolar cell internalization and outer/inner configuration is the first sign of two-cell lineage specification: inner cell mass (ICM) and trophectoderm (TE). The apolar blastomeres located inside of the morula give rise to the ICM from which the embryo proper and yolk sac tissue are derived, whereas the outer polar blastomeres differentiate exclusively into the TE from which placenta is derived (Arnold and Robertson 2009; Zernicka-Goetz et al. 2009). Well-defined gene expression patterns occur within these two distinct lineages. For example, in the mouse embryo, transcription factors Oct4 (also known as Pou5f1), Nanog, and Sox2 are enriched in ICM and function to promote pluripotency and inhibit differentiation, while Cdx2 and Eomes become highly expressed in TE to potentiate epithelial differentiation (Marikawa and Alarcon 2009; Burton and Torres-Padilla 2014; Marcho et al. 2015). Appropriate regulation and mutually exclusive localization of these TFs is critical for successful ICM/TE lineage separation and formation of a competent blastocyst (Rossant and Tam 2009; Paul and Knott 2014).

Key Transcriptional Regulation During ICM/TE Separation

Whereas distinct localizations of transcription factors within ICM/TE lineages have been well illustrated, their functions, interactions, and upstream regulatory networks are still not fully delineated. Previous studies and recent advances have shown that Hippo signaling, Notch signaling, transcription factor AP-2γ (Tfap2c)-dependent regulation, heterogeneities in early blastomeres, epigenetic regulation, as well as many newly discovered genes are involved in the expression of these master TFs and cell lineage specification.

Hippo Signaling and TE Specific Genes

The Hippo signaling pathway is conserved in both *Drosophila* and mammals, regulating cell proliferation, differentiation, and death. Hippo signaling activity can be influenced by multiple stimuli, including cell position and cell–cell adhesion

(Yu and Guan 2013). Interestingly, recent studies indicated that in mouse preimplantation embryos, initiation of Hippo signaling is linked to blastomere polarity rather than cell position (Anani et al. 2014). Core components of Hippo pathway include the protein kinase Lats, transcriptional coactivator Yap, transcription factor Tead, and other key members such as Nf2 and angiomotin (Amot). When this pathway is activated, Yap is phosphorylated by Lats and is excluded from nucleus. Without binding to Yap, Tead cannot be activated, therefore preventing transcription of its target genes. Conversely, inactivation of the pathway induces dephosphorylation of Yap, which can then enter the nucleus and activate Tead to promote transcription of its target genes (Manzanares and Rodriguez 2013; Sasaki 2015). In sum, Hippo activation suppresses Tead gene targets, while Hippo inhibition induces gene expression downstream of Tead.

The connection between the Hippo signaling cascade and cell lineage specification was first discovered through the analysis of mutant mouse embryos lacking transcription factor TEA domain family member 4 (Tead4) (Yagi et al. 2007; Nishioka et al. 2008). Yagi et al. found that *Tead4*-null embryos failed to make blastocoel cavity and significantly reduced Cdx2, and all blastomeres were shifted into ICM with *Oct4* and *Nanog* expression. These results suggested *Tead4* is upstream of *Cdx2* and required for TE formation. Further experiments showed that *Tead4*-null embryos exhibit a more severe phenotype than *Cdx2*-null embryos (Strumpf et al. 2005) and confirmed that *Tead4* is upstream of both *Cdx2* and Gata3 (Ralston et al. 2010), another TE-specific transcription factor.

Subsequent studies focusing on other core components of the Hippo pathway have revealed the important role in regulation of TE-specific genes. Nishioka and colleagues (Nishioka et al. 2009) found that in inner cells, Yap is phosphorylated (Hippo signaling on) and excluded from nucleus, leading to Tead4 inactivation, while in outer cells, Yap is unphosphorylated (Hippo signaling off) and localized in the nucleus to activate Tead4. Studies on angiomotin (Amot) (Hirate et al. 2013) and Nf2 (Cockburn et al. 2013) demonstrated that in inner cells, Amot localizes to adherens junctions, is phosphorylated by Lats, and combines Nf2 to form an active complex to phosphorylate Yap. In outer cells, Amot is sequestered away from adherens junctions to apical domains, resulting in dephosphorylated Yap that can translocate into the nucleus to bind Tead4 and activate target genes (Fig. 1, lower box).

Hippo Signaling Promotes ICM Fate Acquisition

Cell lineage separation is controlled by expression of specific TFs in each lineage. Sox2 is the first known factor selectively located in inner cells, prior to other TFs such as Oct4 and Nanog (Guo et al. 2010). During the exploration of how Sox2 is regulated and restricted to ICM progenitors, Wicklow and colleagues (Wicklow et al. 2014) discovered an essential role of Hippo pathway in restriction of Sox2 to the inner cells that promotes ICM fate acquisition. As Cdx2 restricts expression of *Oct4* and *Nanog* to ICM by inhibiting their expressions in outer TE cell (Strumpf

et al. 2005), Cdx2 may also restrict Sox2 to ICM. However, Sox2 is still restricted to ICM in *Cdx2*-null embryos, indicating that restriction of Sox2 to ICM is Cdx2 independent (Wicklow et al. 2014) and necessitating additional unknown regulatory mechanisms.

It has been shown that Tead4 is activated in outer TE cells, and to promote transcription of its target genes (Yagi et al. 2007; Nishioka et al. 2008, 2009), these results together support a hypothesis that in outer cells, activated Ted4 upregulates an unknown factor that represses *Sox2* expression and that conversely, in inner cells, inhibition of Sox2 is not established (Fig. 1, lower box). Indeed, overexpression of *Lats2* prevents Yap nuclear localization and results in ectopic *Sox2* expression in outer cells (Wicklow et al. 2014). Thus, Hippo signaling cascade regulates not only TE-specific TFs but also ICM-restricted expression of *Sox2*, to establish complementary expression patterns and ICM/TE segregation.

Notch Signaling and TE Specific Genes

Interestingly, *Tead4*-null embryos can express *Cdx2* and form blastocoels with normal lineage commitment when cultured at 5% oxygen condition, which reduces oxidative stress (Kaneko and DePamphilis 2013). Experiments also confirmed that Tead4 regulates energy homeostasis and prevents accumulation of excess reactive oxygen species (ROS) (Kaneko and DePamphilis 2013).

Considering previous studies that demonstrated the central role of Tead4 in TE development and lineage specification (Yagi et al. 2007; Nishioka et al. 2008, 2009; Ralston et al. 2010), as well as recent findings that Tead4 can directly regulate *Cdx2* (Home et al. 2012; Rayon et al. 2014), a possible explanation is that under low oxygen conditions, other Tead proteins or other parallel signaling pathways functionally compensate for loss of *Tead4*. Indeed, an earlier study had reported that overexpression of activator-modified Tead1 also increased *Cdx2* expression (Nishioka et al. 2009). Recently, involvement of Notch signaling in TE lineage specification was also uncovered (Rayon et al. 2014). During a search for *cis*-regulatory elements responsible for TE-restricted expression of *Cdx2*, a TE-specific enhancer was identified that contains functional binding sites for both Tead and Rbpj, the transcriptional effector of the Notch signaling pathway (Tun et al. 1994; Koch et al. 2013). Experiments confirmed that Notch signaling is active at eight-cell stage and then gradually restricted to outer cells of the morula. Forced expression of Notch can drive cells to the outer position and TE cell fate (Rayon et al. 2014). Taking advantage of double mutants for *Tead4* and the Notch effector *Rbpj*, Rayon et al. demonstrated that Hippo and Notch signals converge on *Cdx2* to cooperatively promote TE lineage specification. Notably, they also reported that inhibition of Notch signaling only reduced *Cdx2* expression but had no effect on other TE-specific TF genes such as *Gata3* and *Eomes* (Rayon et al. 2014), which may explain why Notch signaling itself is not strictly required for TE development and blastocyst formation (Souilhol et al. 2006).

Transcription Factor AP-2γ (Tfap2c) Promotes TE

AP-2 family members have been demonstrated to be involved in multiple cellular events such as cell proliferation, morphogenesis, and tumor progression. In mammals, AP-2 family includes four transcription factors, Tfap2a, -b, -c, and -d (Bosher et al. 1995; Hilger-Eversheim et al. 2000). Previous studies have confirmed the essential role of *Tfap2c* in proliferation and differentiation of trophoblast cells, and *Tfap2c* mutant embryos exhibited malformed development in extraembryonic tissue (Auman et al. 2002; Werling and Schorle 2002). In addition, forced expression of *Tfap2c* in embryonic stem cells induced expression of trophoblast stem cell markers and trophectoderm cell fate (Kuckenberg et al. 2010). However, role of *Tfap2c* in preimplantation embryos was not defined.

Recently, Choi et al uncovered an essential role of *Tfap2c* in the regulation of tight junction biogenesis and cavity formation during mouse blastocyst development (Choi et al. 2012). Follow-up studies identified significant functions of *Tfap2c* in TE lineage specification during first cell-fate determination (Cao et al. 2015) and showed that Tfap2c directly regulates *Cdx2* expression through an enhancer in intron 1 during early cleavage stages (Fig. 1, lower box). Tfap2c also potentiates apical polarity via regulation of *Pard6b* expression, which is a key regulator for the establishment of cell polarity (Alarcon 2010). Importantly, these results suggest that Tfap2c also acts upstream of Rho-associated protein kinase (ROCK); thus, Tfap2c can repress position-dependent Hippo signaling in outer blastomeres through Pard6b and ROCK signaling, to promote TE formation during preimplantation development.

Heterogeneities in Early Blastomere Bias Cell Fate

Although it has been generally accepted that initiation of first cell-fate determination occurs during 8-cell to 16-cell transition in mouse embryos, many studies suggest that blastomeres are predetermined or biased at earlier stages. The link between oocyte polarities and blastocyst patterning is controversial (Hiiragi and Solter 2004; Plusa et al. 2005; Hiiragi et al. 2006), but studies have demonstrated heterogeneities in two-cell and four-cell blastomeres that may predict cell fate.

Taking advantage of single-cell RNA sequencing, different groups have confirmed the transcriptional heterogeneities between two-cell blastomeres that contribute to cell lineage separation (Biase et al. 2014; Piras et al. 2014; Shi et al. 2015). A recent study also reported differential distribution of 16S mitochondrial ribosomal RNA (mtrRNA) at two-cell stage in mouse embryos (Zheng et al. 2016). In situ hybridization results showed that while early two-cell blastomeres contain similar amount of 16S mtrRNA, late two-cell stage blastomeres exhibit apparent difference in 16s quantity. Furthermore, from the four-cell stage and onward, 16S mtrRNA is enriched in basal–lateral regions and is mainly detected in the ICM at

the blastocyst stage. Interestingly, microinjection of sense 16S mtrRNA significantly increased ICM progeny, while injection of antisense16S mtrRNA increased TE cells. These findings suggest that 16S mtrRNA may have an important role in promoting ICM lineage through an unknown mechanism (Zheng et al. 2016).

Compared with two-cell blastomeres, more heterogeneous properties have been discovered among four-cell blastomeres. Torres-Padilla and colleagues revealed arginine 26 residue of histone H3 (H3R26me), and its methyltransferase Carm1 levels vary among four-cell blastomeres. Overexpression of *Carm1* leads to higher H3R26me and a significant upregulation of Nanog and Sox2, biasing the progeny to ICM lineage (Torres-Padilla et al. 2007). Explaining these observations it has been shown that Prdm14 expression is also heterogeneous at four-cell stage and that Prdm14 can interact with Carm1 to promote H3R26me inducing ICM fate (Burton et al. 2013).

Using the fluorescence decay after photoactivation (FDAP) method, Plachta and colleagues demonstrated that blastomeres with slower Oct4 kinetics divide asymmetrically to produce more cells to ICM, while those with faster Oct4 kinetics contribute mostly to the TE through symmetric divisions (Plachta et al. 2011). Recently, two studies have shed new light on the transcriptional control mechanisms underlying the intrinsic heterogeneities that predict cell fate (Goolam et al. 2016; White et al. 2016a). They demonstrate that H3R26me potentiates long-lived Sox2-DNA binding, which ensures more access of Sox2 to its pluripotency-related targets, such as *Sox21*. Sox21 then suppresses *Cdx2* expression and biases blastomeres toward ICM (Goolam et al. 2016; White et al. 2016a). More importantly, this model may explain the early heterogeneities (Fig. 1, upper box) and strongly supports the possibility of nonrandom cell-fate determination during early mammalian development.

Epigenetic Control on Transcription During Lineage Specification

Besides key signaling pathways and crucial TFs, epigenetic control of gene transcription also plays an important role in cell differentiation and lineage specification (Paul and Knott 2014; Marcho et al. 2015). As DNA methylation has been shown to be dispensable for growth and differentiation of extraembryonic lineages (Sakaue et al. 2010), studies have focused on histone modifications during lineage specification. A link between histone modifications and gene expression patterns has also been illustrated. For example, histone lysine acetylation is normally considered as an active mark that correlates with chromatin accessibility and active transcription, whereas lysine methylation can be either active or repressive depending on the particular lysine residue which is modified (Tsukada et al. 2006; Bernstein et al. 2007).

During TE and ICM commitment, they exhibit asymmetries in specific histone modifications, and the last several years have seen a plethora of findings in this regard. For example, H4- and H2AS1P are increased in TE (Sarmento et al. 2004), while H3K27me3 is enriched in ICM (Erhardt et al. 2003). High level of H3R26me promotes DNA accessibility and biases cells to ICM fate (Torres-Padilla et al. 2007; Goolam et al. 2016; White et al. 2016a). Studies also demonstrate that H3K9me3 at *Cdx2* promoter is important for maintaining pluripotency and that loss of ESET in early embryos results in ICM failure (Yeap et al. 2009). Additionally, Suv39h methyltransferase mediates repressive H3K9me3 at ICM-specific gene promoters specifically in the TE lineage (Alder et al. 2010; Rugg-Gunn et al. 2010). H3K4me3 and H3K27me3 are enriched at promoters of genes exclusively expressed in ICM or TE in both murine and bovine embryos (Dahl et al. 2010; Herrmann et al. 2013). Loss of repressive H3K27me3 participation at TE-specific genes is essential for TE lineage development and embryo implantation (Saha et al. 2013; Paul and Knott 2014). In addition to methylation of histone H3 residues, acetylation of histone H4, such as H4K8ac and H4K12ac, also functions in early lineage specification (VerMilyea et al. 2009; Zhang et al. 2013a). It is likely that many more histone posttranslational modifications play similar roles but have yet to be defined during preimplantation lineage commitment.

Identification of Genes Essential for Lineage Specification

With readily available transcriptome-wide data, understanding the role of each expressed gene is an essential next step for elucidating developmental networks at play. Although RNAi technologies allow for genome-wide screens in cultured cells, these approaches cannot replace strategies for discovery in the embryo. Our lab has adopted a pooling strategy to allow for efficient RNAi-mediated forward genetic knockdown screen to identify genes required during preimplantation lineage specification. We recently accomplished a large-scale RNAi screen in mouse early embryos where 712 genes were screened and 53 genes were found to be required for successful lineage development and/or specification (example in Fig. 2), including *Suds3* (Zhang et al. 2013a), *Ctr9* (Zhang et al. 2013b), *Nop2* (Cui et al. 2016b), and a battery of genes without known early functions (Cui et al. 2016a). Interestingly, our results highlight that during the morula to blastocyst transition, TE lineage is more critical and/or more vulnerable as the majority of phenotypes that fail to form a blastocyst have TE defects rather than ICM defects. However, knockdown phenotypes that form a blastocyst but fail to hatch or outgrow have predominant defects in the ICM lineage. This finding suggests that while both lineages are essential during early embryo development, there are specific windows when proper function/specification of each is essential (Cui et al. 2016a).

To interpret the relationship between the genes identified in our screen and known pathways, we performed induced network module analysis (Kamburov et al. 2013) which showed ten of our genes (seeds in Fig. 3) form a network with

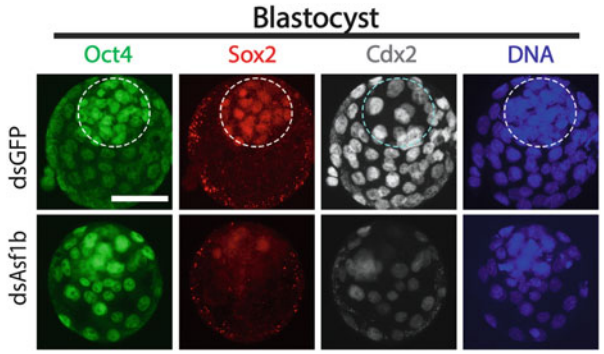

Fig. 2 Specific lineage markers of ICM (Oct4, Sox2) and TE (Cdx2) were characterized in both dsGFP control and dsAsf1b KD blastocysts by immunofluorescence. ICM cells (*circled*) in dsGFP control blastocysts are tightly arranged with robust expression of *Oct4* and *Sox2*, and TE cells are uniformly arranged with specific expression of *Cdx2*. Most dsAsf1b KD blastocysts exhibit ubiquitous Oct4 signal and with severely damaged *Sox2* and *Cdx2* expression, indicating impaired lineage specification. Oct4 (*green*), Sox2 (*red*), Cdx2 (*white*), and DAPI (*blue*). Scale bar, 50 μm

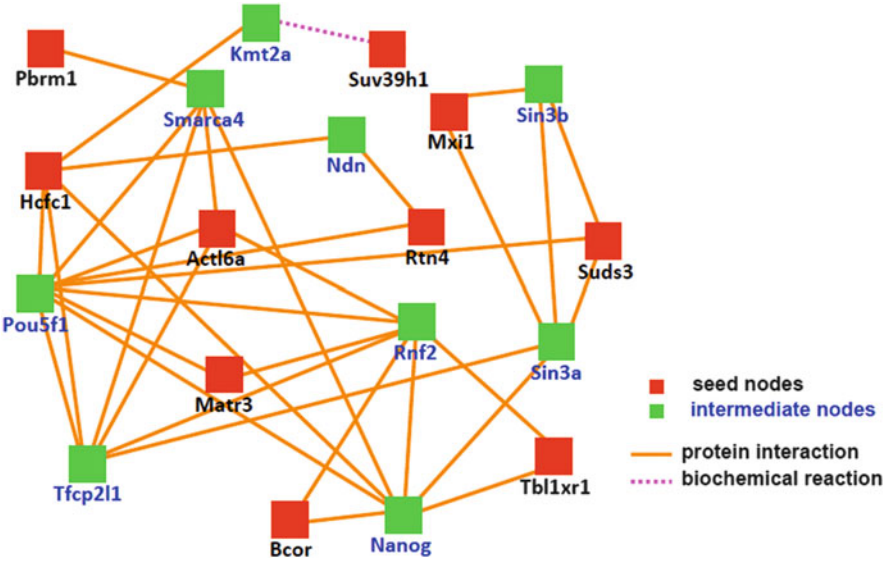

Fig. 3 In our recent screen, 712 genes were knocked down and 53 phenotypes were identified. Induced network module analysis showed 10 of our novel phenotypes (seed nodes in *red*) are connected with nine other genes (intermediate nodes in *green*), indicating that this network is essential for lineage specification and embryo development

nine other known genes (intermediates in Fig. 3). Importantly, these nine known genes already have knockout models that all confirm essential roles during early embryonic development (Cui et al. 2016a). These data suggest not only that this developmental network is essential for embryo development—but that this

screening strategy does not need to reach genome saturation to identify other/all pathways required.

Our screen selected genes to target based solely on expression during preimplantation and resulted in 7.4% of genes (53/712) with phenotypes. If there are ~11,000 genes expressed during preimplantation (Stanton and Green 2001), our results suggest that ~800 genes are required for lineage development and/or specification during preimplantation—the majority of which have yet to be discovered. We predict that screening one-fifth of all expressed genes (threefold more than we have finished) may be sufficient to identify the vast majority of networks/pathways required for early lineage events during preimplantation.

Conclusions

Here, we have reviewed recent advances in understanding transcriptional control mechanisms and crucial genes involved in first cell lineage specification, in particular, recent insights into Hippo signaling, Notch signaling, TF AP-2γ (Tfap2c) function, early heterogeneities, and epigenetic regulation. The first cell lineage decision is determined by many distinct mechanisms: some that act in parallel and some that act in networks. Although many dozen genes and pathways have been identified and—omics technologies have advanced in recent years, a comprehensive understanding of the genes required for the first lineage specification remains elusive.

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ROCK and RHO Playlist for Preimplantation Development: Streaming to HIPPO Pathway and Apicobasal Polarity in the First Cell Differentiation

Vernadeth B. Alarcon and Yusuke Marikawa

Abstract In placental mammalian development, the first cell differentiation produces two distinct lineages that emerge according to their position within the embryo: the trophectoderm (TE, placenta precursor) differentiates in the surface, while the inner cell mass (ICM, fetal body precursor) forms inside. Here, we discuss how such position-dependent lineage specifications are regulated by the RHOA subfamily of small GTPases and RHO-associated coiled-coil kinases (ROCK). Recent studies in mouse show that activities of RHO/ROCK are required to promote TE differentiation and to concomitantly suppress ICM formation. RHO/ROCK operate through the HIPPO signaling pathway, whose cell position-specific modulation is central to establishing unique gene expression profiles that confer cell fate. In particular, activities of RHO/ROCK are essential in outside cells to promote nuclear localization of transcriptional co-activators YAP/TAZ, the downstream effectors of HIPPO signaling. Nuclear localization of YAP/TAZ depends on the formation of apicobasal polarity in outside cells, which requires activities of RHO/ROCK. We propose models of how RHO/ROCK regulate lineage specification and lay out challenges for future investigations to deepen our understanding of the roles of RHO/ROCK in preimplantation development. Finally, as RHO/ROCK may be inhibited by certain pharmacological agents, we discuss their potential impact on human preimplantation development in relation to fertility preservation in women.

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Introduction

All vertebrates, including mammals, share the same body plan that is constructed during embryogenesis by evolutionarily conserved mechanisms. In placental mammals, the formation of the body plan is preceded by the unique process of preimplantation development, which transforms the fertilized egg into the blastocyst to prepare for implantation (Fig. 1). To generate the blastocyst, two distinct cell lineages, trophectoderm (TE) and inner cell mass (ICM), are established. TE is the epithelial layer that surrounds a fluid-filled cavity, attaches to the uterine endometrium, and gives rise to trophoblasts of the placenta. In contrast, ICM is the cell aggregate located inside of the blastocyst and serves as the pluripotent precursor for the entire fetal body (Fig. 1). Thus, preimplantation development presents the first cell differentiation event inherent to placental mammals, which separates the extraembryonic lineage responsible for placentation from the embryonic lineage that constructs the body plan.

How do the TE and ICM lineages differentiate? In many nonmammalian species, the first cell differentiation is coordinated by "determinants," i.e., factors in the fertilized egg that are unequally partitioned into subsets of the blastomeres during

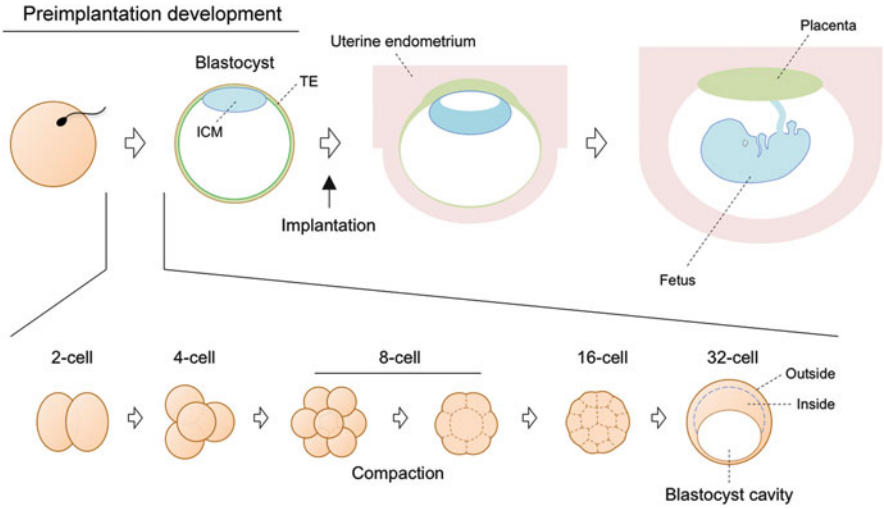

Fig. 1 Preimplantation development, an event unique to placental mammals. *Top row* Conceptual scheme depicts the emergence of embryonic and extraembryonic lineages during preimplantation development in a variety of species, including human. ICM (inner cell mass) is the embryonic lineage that gives rise to the fetus, whereas TE (trophectoderm) is the extraembryonic lineage that becomes trophoblasts of the placenta after implantation. *Bottom row* The preimplantation stages are shown for mouse, on which significant investigations have been done to elucidate the mechanisms of development and on which this review is mostly based. Cell–cell boundaries become indistinct upon compaction, which starts at the end of eight-cell stage. The blastocyst cavity, a fluid-filled space, emerges between 16- and 32-cell stages. Outside cells that are located in the surface are TE, and inside cells are ICM

early cell divisions. However, experimental studies suggest that no determinants exist to specify the TE or ICM lineages (Wennekamp et al. 2013; Solter 2016). Instead, differentiation of the two lineages takes place in accordance with the position of cells within the embryo, i.e., those in the surface give rise to TE, whereas those in the inside become ICM (Fig. 1). What kinds of mechanisms would allow each cell to interpret its position within the embryo and to execute specific differentiation programs? In this chapter, we discuss the current knowledge and models pertinent to this question. We particularly emphasize roles of the RHOA subfamily of small GTPases and RHO-associated coiled-coil kinases (ROCK) in relation to the regulation of HIPPO signaling pathway and apicobasal cell polarity, which are core aspects of cell lineage specification. While various species have been employed to study the mechanisms of preimplantation development, we focus on the mouse because a significant number of experimental investigations have been conducted using this species at the cellular and molecular levels. The knowledge obtained from mouse studies should serve as a foundation for further investigations to understand the preimplantation development of other mammalian species, especially human, which bears clinical significance for fertility preservation in women, as also discussed in this chapter.

Regulation of Lineage-Specific Gene Expressions by HIPPO Signaling and Cell Position

The genome content is equivalent among most cell types in the embryo, so that cell differentiation mainly occurs through differential gene expressions by transcriptional regulations of distinct sets of genes in a lineage-specific manner. In mouse preimplantation development, the zygote forms the blastocyst by the 32-cell stage, which takes about 3 days after fertilization (Fig. 1). By then, outside cells (TE) and inside cells (ICM) exhibit unique gene expression profiles, including those encoding transcription factors, such as *Cdx2*, *Gata3*, and *Id2* for TE and *Sox2* for ICM (Strumpf et al. 2005; Home et al. 2009; Guo et al. 2010; Wicklow et al. 2014). Some of these genes already exhibit position-specific expressions at the 16-cell stage, when inside and outside cell populations emerge for the first time (Fig. 2A) (Guo et al. 2010; Posfai et al. 2017). This highlights a tight link between cell position and lineage-specific transcriptional regulations.

Tead4 encodes a TEA-domain-containing transcription factor that plays a crucial role in the establishment of cell position-specific differential gene expressions. In homozygous *Tead4* knockout embryos ($Tead4^{-/-}$), expressions of TE-specific genes (*Cdx2*, *Gata3*) are absent, whereas ICM-specific genes (*Sox2*, *Nanog*) are ectopically expressed in outside cells (Yagi et al. 2007; Nishioka et al. 2008, 2009; Ralston et al. 2010; Wicklow et al. 2014). This suggests that *Tead4* is essential to activate TE-specific genes and to suppress ICM-specific genes in outside cells. However, it has also been reported that $Tead4^{-/-}$ embryos can form TE with *Cdx2*

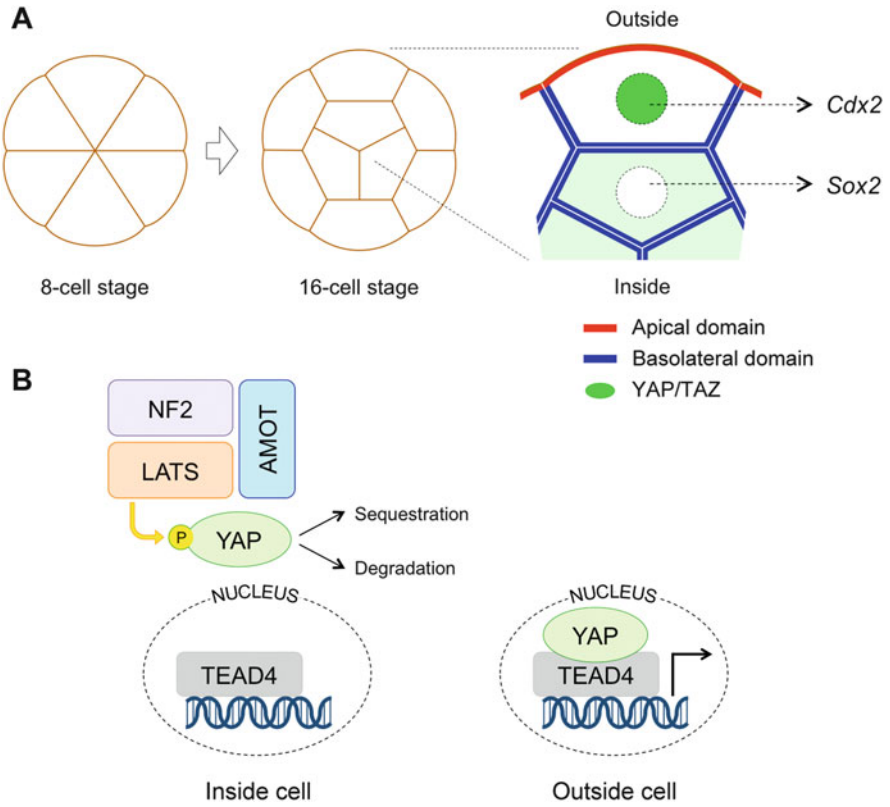

Fig. 2 Lineage-specific gene expression is linked to cell position in preimplantation development. (**A**) Two cell populations emerge at around 16-cell stage: the outside and inside cells, which are polar (i.e., apical and basolateral domains are segregated) and apolar (i.e., basolateral domain surrounds the entire cell), respectively. Transcriptional co-activators YAP/TAZ are differentially localized between outside and inside cells, resulting in the expression of transcription factors (*Cdx2*, *Sox2*) in a cell position-specific manner. (**B**) Scheme of the differential activation (nuclear localization) of YAP/TAZ and gene expression between the inside and outside cells under the regulation of the HIPPO signaling pathway. HIPPO pathway components NF2, LATS (LATS1/2), and AMOT (AMOT/AMOTL2) altogether are necessary to phosphorylate YAP, which remains in the cytoplasm of inside cells by sequestration or becomes degraded. In contrast, unphosphorylated YAP translocates to the nucleus in outside cells and binds to TEAD4 to promote TE lineage-specific gene expressions

and *Gata3* expressions when they are cultured under a low oxygen tension (Kaneko and DePamphilis 2013) or when NOTCH signaling is active in outside cells (Rayon et al. 2014). Further investigations are necessary to determine how the lack of *Tead4* can be compensated under these conditions. Notably, other TEA-domain-containing genes, specifically *Tead1* and *Tead2*, are expressed in preimplantation embryos (Nishioka et al. 2008), and overexpression of activator-modified TEAD1 can ectopically activate *Cdx2* expression in inside cells (Nishioka et al. 2009),

raising the possibility that other *Tead* genes may be involved in the compensatory mechanisms for the lack of *Tead4*.

Although *Tead4* is specifically required for TE formation, the gene products (mRNA and protein) are ubiquitously expressed in both inside and outside cells (Nishioka et al. 2008; Hirate et al. 2012). The TE-specific action of TEAD4 is due to position-dependent regulation of its binding proteins, YAP/TAZ, which act as transcriptional co-activators (Vassilev et al. 2001; Nishioka et al. 2009). YAP/TAZ are sequestered in the cytoplasm in inside cells, whereas they are localized to the nucleus in outside cells to form a complex with TEAD4 (Fig. 2B). Outside cell-specific nuclear localization of YAP/TAZ is already evident at 16-cell stage and persists up to the late blastocyst stage (Nishioka et al. 2009; Hirate et al. 2015). *Cdx2* may be a direct transcriptional target of the TEAD4–YAP/TAZ complex, as its upstream regulatory sequence contains TEAD-binding sites (Rayon et al. 2014). In contrast, it is unclear how outside cell-specific transcriptional repression of *Sox2* is achieved in a *Tead4*-dependent manner (Wicklow et al. 2014). Because the TEAD4–YAP/TAZ complex acts as a transcriptional activator, it is possible that *Sox2* is indirectly repressed by outside cell-specific factors whose transcriptions are activated by TEAD4–YAP/TAZ, although *Cdx2* is dispensable for the *Sox2* repression in outside cells (Wicklow et al. 2014).

YAP/TAZ are the key downstream effectors of HIPPO signaling, an evolutionarily conserved pathway that integrates multiple upstream signals to regulate cell cycle, tissue growth, and cell fate decisions (Meng et al. 2016; Sun and Irvine 2016). In preimplantation development, several components of HIPPO signaling are involved in cell position-dependent regulation of YAP/TAZ nuclear localization, namely, LATS1/2, NF2, and AMOT/AMOTL2 (Fig. 2B). Phosphorylation of YAP/TAZ, which is required for their cytoplasmic retention, is catalyzed by serine/threonine kinases LATS1/2. Loss of function of LATS1/2, through knockout, knockdown, or dominant-negative construct, causes ectopic YAP/TAZ nuclear localization and *Cdx2* expression in inside cells, whereas overexpression of LATS2 is sufficient to prevent YAP/TAZ nuclear localization in outside cells (Nishioka et al. 2009; Lorthongpanich et al. 2013; Kono et al. 2014). Loss of function of NF2 or AMOT/AMOTL2 also results in similar phenotypes, i.e., ectopic YAP/TAZ nuclear localization and *Cdx2* expression in inside cells (Cockburn et al. 2013; Hirate et al. 2013; Leung and Zernicka-Goetz 2013). NF2 is a membrane-associated FERM domain protein that operates upstream of LATS1/2 in the HIPPO pathway, while AMOT/AMOTL2 act as scaffolds to bind NF2, LATS1/2, and YAP/TAZ. Thus, LATS1/2, NF2, and AMOT/AMOTL2 are altogether involved in the phosphorylation and inactivation of YAP/TAZ in inside cells (Fig. 2B).

Currently, it is unclear whether other core components of the HIPPO pathway, such as MST1/2, SAV1, and MOB1A/B, all of which are to phosphorylate and activate LATS1/2, play any role in the differential regulation of YAP/TAZ in preimplantation embryos. Because the dominant-negative construct of MST1/2 has no effect on YAP/TAZ localization (Cockburn et al. 2013), it is possible that MST1/2 are dispensable for the activation of LATS1/2 in preimplantation embryos. In other cell types, however, a pathway involving multiple members of the MAP4K

family acts in parallel to MST1/2 to phosphorylate and activate LATS1/2 (Meng et al. 2015; Zheng et al. 2015). Also, YAP/TAZ are phosphorylated and inactivated by NDR1/2 independently from LATS1/2 (Zhang et al. 2015). Whether MAP4K and NDR1/2 play any role in preimplantation development is yet to be tested.

Interestingly, the inside cells in LATS1/2 or NF2 loss-of-function embryos also exhibit other TE-like features, namely, accumulations of ZO-1 (tight junction component) and ATP1B1 (ion pump involved in cavity formation) (Cockburn et al. 2013; Lorthongpanich et al. 2013). Nonetheless, these cells also express NANOG, a marker of the ICM-derived tissue, epiblast (Lorthongpanich et al. 2013), raising the possibility that other types of cell position-dependent regulations, in addition to differential HIPPO signaling, take place in inside cells to promote ICM-specific gene expressions.

Apicobasal Cell Polarity Connects Cell Position to HIPPO Signaling

Position of cells within the embryo is closely associated with establishment of the apicobasal polarity. Apicobasal polarity is a key feature of all types of epithelial cells, in which a specialized apical membrane faces one side (e.g., surface of the body, lumen of gastrointestinal tract), and a basolateral membrane is localized at the opposite side. In most epithelial cells, apical and basolateral membranes are marked with a unique set of conserved proteins, such as PAR3, PAR6, and aPKC for the apical and PAR1, SCRIB, and LGL for the basolateral membrane (Rodriguez-Boulan and Macara 2014). In preimplantation development, features of apicobasal polarity first emerge in all cells at the end of eight-cell stage. The next cell divisions toward the 16-cell stage generate the inside and outside cell populations, and apicobasal polarity is retained and fortified in outside cells but is absent in inside cells (Fig. 2A). At these stages, many of the apical and basolateral proteins described above are localized to the corresponding membrane domains (Plusa et al. 2005; Vinot et al. 2005; Dard et al. 2009; Alarcon 2010; Tao et al. 2012).

Polarity proteins play essential roles in outside cells to enable YAP/TAZ nuclear localization and TE-lineage formation. Loss of function of apical components, such as PAR6 (*Pard6b*), aPKC (*Prkci*/*Prkcz*), and CDC42, prevents TE formation with failure in YAP/TAZ nuclear localization (Alarcon 2010; Hirate et al. 2013, 2015; Cao et al. 2015; Korotkevich et al. 2017). Loss of function of basolateral components, namely, knockdown of PAR1 (*Mark2*/*Mark3*), also results in similar phenotypes (Hirate et al. 2013, 2015). Importantly, outside cells of these polarity protein-deficient embryos exhibit features of ICM, such as ectopic expression of NANOG. Thus, the acquisition of proper apicobasal polarity is essential to interpret the outside position to promote YAP/TAZ nuclear localization. A recent study, in which transplantation of a small cell fragment carrying the apical membrane is

sufficient to induce YAP/TAZ nuclear localization in apolar cells (Korotkevich et al. 2017), further highlights the critical nature of the apicobasal polarity in TE-lineage specification.

AMOT/AMOTL2 may play a pivotal role in linking apicobasal polarity to the regulation of HIPPO signaling in outside cells. AMOT/AMOTL2 are restricted to the apical membrane and excluded from the basolateral membrane, where adherens junctions are located (Hirate et al. 2013; Leung and Zernicka-Goetz 2013). Experimental disturbance of the apicobasal polarity allows AMOT/AMOTL2 to associate with AJ in the basolateral membrane, where LATS1/2 become activated to prevent YAP/TAZ nuclear localization (Hirate and Sasaki 2014). Other types of mechanistic links between the apicobasal polarity and YAP/TAZ nuclear localization are demonstrated in other systems, namely, cell lines. Apicobasal regulator DLG5 directly interacts with MST1/2, and its deficiency causes activation of MST1/2 to block YAP/TAZ nuclear localization (Kwan et al. 2016). Also, apical component PAR3 (*Pard3*) mediates dephosphorylation of LATS1/2 by protein phosphatase 1A (PP1A), and knockdown of PAR3 prevents nuclear localization of YAP/TAZ (Lv et al. 2015). Whether DLG5 and PAR3 operate in the same manner in preimplantation development is currently unknown, although PAR3 is suggested to play a role in lineage specification through the regulation of orientation of cell division planes (Plusa et al. 2005).

RHO and ROCK in Cell Lineage Specification

Recent studies suggest that the RHOA subfamily of RHO small GTPases and their major downstream effectors, RHO-associated coiled-coil kinases (ROCK), play essential roles in lineage specification, particularly of TE, by regulating apicobasal polarity and HIPPO signaling. RHO GTPases act as molecular switches that cycle between an inactive (GDP-bound) and an active (GTP-bound) state, which is controlled by various upstream regulators (Hodge and Ridley 2016). Active form of RHOA promotes serine/threonine kinases ROCK to phosphorylate various target proteins. RHOA and ROCK are involved in diverse cellular processes, including motility, morphology, polarity, cell division, and gene expression. Many of their actions are considered to be via modulation of the actomyosin cytoskeletal system, although other mechanisms may also exist (Hodge and Ridley 2016).

The RHOA subfamily consists of three members (RHOA, RHOB, RHOC) and ROCK family consists of two members (ROCK1, ROCK2), all of which are encoded by different genes. Each gene has its unique expression pattern, and each knockout causes a unique phenotype (Liu et al. 2001; Thumkeo et al. 2003; Hakem et al. 2005; Shimizu et al. 2005; Pedersen and Brakebusch 2012). However, molecular properties are similar within a family and many cell types express multiple members simultaneously, resulting in significant overlap and redundancy in function (Kamijo et al. 2011; Melendez et al. 2011; Konigs et al. 2014; Kumper

et al. 2016). Because of this, some investigations benefit from the use of agents that inhibit all members of a family. The most commonly used inhibitor of RHOA subfamily is C3 exoenzyme isolated from the bacteria *Clostridium botulinum*. C3 covalently attaches ADP-ribosyl group to RHO (A, B, C) to cause their inactivation (Vogelsgesang et al. 2007). It is often used as a specific inhibitor for RHOA subfamily members, as it does not affect other related GTPases, such as CDC42 and RAC1 (Wilde et al. 2000). Various pharmacological agents have been identified that inhibit ROCK. Of those, Y-27632 is most commonly used as a ROCK inhibitor, although it may also inhibit other kinases at high concentrations (Davies et al. 2000). All members of the RHOA subfamily and ROCK are expressed in preimplantation embryos (Hamatani et al. 2004; Zeng et al. 2004; Xie et al. 2010; Kono et al. 2014), and both maternal and zygotic gene products are possibly present. Therefore, most studies to investigate RHOA and ROCK in preimplantation development to date have been performed by taking advantage of these inhibitors, as described below.

Activities of RHO and ROCK Are Essential for TE Formation

The first indication that RHO/ROCK signaling may be involved in cell lineage specification came from observations of mouse preimplantation embryos that are cultured in the presence of ROCK inhibitor Y-27632 (Kawagishi et al. 2004). Embryos treated with Y-27632 from the two-cell stage undergo cell division and develop into morulae, but they fail to form blastocysts, hinting at problems in the formation and function of the TE lineage (Fig. 3A). Time-lapse movies of the development of untreated and Y-27632-treated embryos from the two-cell stage to the blastocyst (Kono et al. 2014) are available online for viewing (www.ncbi.nlm.nih.gov/pmc/articles/PMC4404313, under Supplementary Material; Movie 1: control, Movie 2: Y-27632 treatment). ROCK inhibition does not interfere with cleavages or compaction, whereas it prevents formation of the blastocyst cavity. ROCK-inhibited embryos exhibit aberrant ZO-1 distribution, indicative of defective tight junctions, possibly contributing to the cavitation failure (Kono et al. 2014). Likewise, embryos treated with RHOA inhibitor C3 exhibit cavitation failure. Furthermore, Y-27632-treated embryos downregulate expression of TE lineage-specific transcription factors (*Cdx2*, *Gata3*) and concomitantly upregulate the expression of ICM lineage-specific transcription factors (*Nanog*, *Sox2*). Downregulation of CDX2 expression is also observed in C3-treated embryos (Kono et al. 2014). Thus, embryos depleted of ROCK and RHO activity exhibit morphological and molecular characteristics of failed TE formation with enhanced ICM properties.

Activity of RHO and ROCK is essential in outside cells to properly regulate HIPPO signaling to allow TE specification. The nuclear localization of YAP/TAZ is diminished in Y-27632- or C3-treated embryos (Fig. 3B) (Kono et al. 2014; Cao et al. 2015; Mihajlovic and Bruce 2016). By combining genetic manipulation with

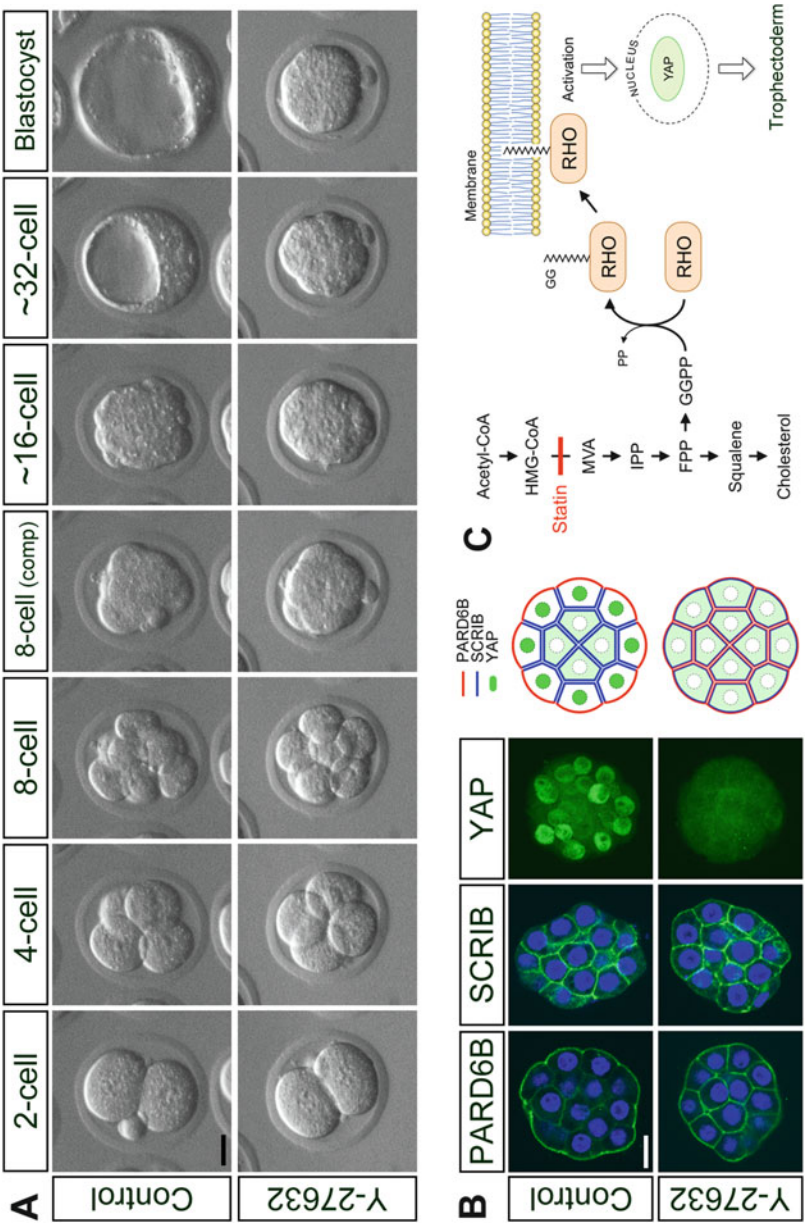

Fig. 3 Activities of ROCK and RHOA are essential for trophectoderm-lineage formation. Only images for ROCK-inhibited embryos are shown (**A**, **B**), since RHOA-inhibited embryos exhibit a similar phenotype. (**A**) Representative images of developing embryos cultured in the absence (control) and presence of ROCK inhibitor (Y-27632) from two-cell stage to the expanding blastocyst stages. The ROCK-inhibited embryo undergoes cell division and compaction

Fig. 3 (continued) (comp) but fails to transform into a blastocyst. (**B**) ROCK-inhibited embryos exhibit abnormal distributions of polarity proteins and YAP. Immunostaining shows that the apical protein PARD6B (*green*) and basolateral protein SCRIB (*green*) localize all around the cell cortex and have lost their asymmetrical distribution as in the control embryos. YAP (*green*) nuclear localization in the outside cells is abrogated in the ROCK-inhibited embryo. The last column has diagrams summarizing the distributions of polarity proteins and YAP in control and Y-27632-treated embryos. Immunostained images are optical sections (PARD6B, SCRIB) and *z*-axis projections (YAP) of serial optical sections captured by confocal microscopy. *Blue*, DAPI staining for nuclei. (**C**) Scheme of the mevalonate pathway, which produces the geranylgeranyl lipid attachment that is necessary for the membrane localization and activation of RHO. Activation of RHO is required for nuclear YAP localization to confer the trophectoderm cell fate in outside cells. Statins act by specifically inhibiting HMG-CoA reductase from converting HMG-CoA into MVA. *Acetyl-CoA* acetyl coenzyme A, *HMG-CoA* 3-hydroxy-3-methylglutaryl-coenzyme A, *MVA* mevalonic acid, *IPP* isopentenyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate. Scale bar (**A**, **B**): 20 μm

inhibitor treatment, the action of RHO and ROCK has been linked with LATS1/2-mediated regulation of YAP/TAZ (Kono et al. 2014). Specifically, when Y-27632- or C3-treated embryos express dominant-negative LATS2 or LATS1/2 short hairpin RNAs (shRNAs) for their knockdown, the nuclear accumulation of YAP is rescued. Furthermore, prevention of YAP nuclear accumulation by the ROCK inhibitor is dependent on LATS1/2-mediated phosphorylation of YAP, because a non-phosphorylatable form of YAP (S112A) can localize in the nucleus even in the presence of Y-27632 (Kono et al. 2014). Altogether, these studies show that prevention of YAP nuclear localization by interference with RHO/ROCK depends on the activity of LATS1/2 to phosphorylate YAP/TAZ (Figs. 2B and 3B). Consistently, nuclear localization of YAP/TAZ can also be restored in Y-27632-treated embryos by knockdown of AMOT, a HIPPO signaling component essential for LATS1/2 activation (Mihajlovic and Bruce 2016). Possible mechanisms by which RHO and ROCK intersect with HIPPO signaling in preimplantation embryos are discussed in a later section "Possible Mechanisms of How RHO and ROCK Regulate TE Specification."

Statins Cause Phenotypes Similar to Inhibition of RHO and ROCK

The function of most RHO GTPases, including the RHOA subfamily members, is dependent on prenylation, the posttranslational modification by covalent attachment of isoprenoid lipids, which promotes the association of RHO GTPases with the plasma and intracellular membranes to modulate their activities (Eisa-Beygi et al. 2014). Necessity of proper prenylation for lineage specification in preimplantation embryos has been demonstrated through investigations into the effects of statins, which are common cholesterol-lowering medications (Surani et al. 1983; Alarcon and Marikawa 2016). Treatment of preimplantation embryos with statins, such as lovastatin, atorvastatin, and cerivastatin, prevents blastocyst cavity formation, accompanied by reduced YAP nuclear localization and *Cdx2* expression, indicating that statins compromise the TE-lineage specification (Alarcon and Marikawa 2016).

The adverse effect of statins on TE specification is due to inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key rate-limiting enzyme in cholesterol biosynthesis of the mevalonate pathway (Fig. 3C). However, the TE defect is not caused by insufficiency of cholesterol but rather by insufficiency of isoprenoid lipids, the other metabolic products downstream of HMG-CoA (Fig. 3C). Supplementation of one of the isoprenoid products, geranylgeranyl pyrophosphate (GGPP), is sufficient to overcome the adverse effect of statins, rescuing the formation of blastocysts with correct TE and ICM marker expressions (Alarcon and Marikawa 2016). Furthermore, inhibition of geranylgeranyl transferase (GGTase), the enzyme responsible for the attachment of the geranylgeranyl moiety of GGPP to RHO GTPases, also results in similar TE-defect phenotypes, suggesting that protein prenylation, specifically geranylgeranylation, is essential for TE specification

(Alarcon and Marikawa 2016). Because such phenotypes are similar to the effect of C3 treatment, it is possible that the adverse effect of statins is mainly through inactivation of RHOA subfamily members. However, geranylgeranylation occurs not only to RHOA subfamily members but also to other GTPases, including CDC42 (Yeganeh et al. 2014). In preimplantation embryos, CDC42 is localized apically, and its maternal-zygotic knockout results in failure in TE formation (Korotkevich et al. 2017). Thus, the targets of statins and GGTase inhibitor may be more diverse than those of C3. Nonetheless, studies in cell lines show that the inhibition of YAP nuclear localization by GGTase treatment is reversed by modified RHOA that can be activated without geranylgeranylation, suggesting that activation of RHOA is mainly responsible for the regulation of HIPPO signaling (Sorrentino et al. 2014). Similar investigations are essential in future studies using preimplantation embryos to determine whether the RHOA subfamily members are the major players in geranylgeranylation-dependent TE specification.

Effect of RHO/ROCK Inhibition on Apicobasal Cell Polarity

RHO and ROCK are involved not only in the proper regulation of HIPPO signaling but also in the establishment of proper apicobasal polarity in preimplantation embryos. Apicobasal polarity of outside cells at the 16- to 32-cell stages is disturbed in RHO- or ROCK-inhibited embryos in a distinct manner. Components of the PAR3–PAR6–aPKC system and the PAR1–SCRIB–LGL system are normally localized to the apical and basolateral membranes, respectively, in a mutually exclusive manner. However, in RHO- and ROCK-inhibited embryos, the polarity proteins lose confinement from their plasma membrane domains and become distributed all around the cell cortex (Fig. 3B) (Kono et al. 2014; Cao et al. 2015; Mihajlovic and Bruce 2016). In ROCK-inhibited embryos, components of the adherens junctions, namely, E-cadherin (CDH1) and angiomotin (AMOT), also become localized all around the cortex of the outside cells, similar to the pattern in inside cells (Kono et al. 2014; Mihajlovic and Bruce 2016). Disturbance in apicobasal polarity by inhibition of RHO/ROCK may occur as early as the eight-cell stage, as apical enrichment of pericentriolar material 1 (PCM1) is absent in Y-27632-treated embryos (Kono et al. 2014), and aPKC is mislocalized to the basolateral membrane in C3-treated embryos (Liu et al. 2013).

As demonstrated in various epithelial cell types, strict confinement of apical and basolateral membrane domains may depend on mutual phosphorylation between apical and basolateral components. For example, aPKC phosphorylates basolateral components PAR1 and LGL to prevent their association with the apical membrane (Yamanaka et al. 2003; Hurov et al. 2004; Suzuki et al. 2004), while PAR1 phosphorylates PAR3 to interfere with its interaction with aPKC (Benton and St. Johnston 2003). RHO and ROCK may be involved in these mutual exclusion mechanisms, as ROCK phosphorylates PAR3 to regulate its association with the PAR3–PAR6–aPKC system (Nakayama et al. 2008). Conversely, cell polarity may

regulate activities of RHO and ROCK, because aPKC can phosphorylate ROCK in a PAR3/PAR6-dependent manner to modulate the integrity of apical junctional complexes (Ishiuchi and Takeichi 2011). Whether these mechanisms also operate in preimplantation embryos is still unknown.

Possible Mechanisms of How RHO and ROCK Regulate TE Specification

What are the mechanisms that connect the RHO/ROCK activities to TE specification, namely, nuclear localization of YAP/TAZ? One possible model is that RHO/ROCK signaling enables establishment of the apical domain, which in turn serves as a platform to regulate HIPPO signaling to promote YAP/TAZ nuclear localization (Fig. 4). This is a plausible model because: (1) inhibition of RHO or ROCK disturbs the localization of key apical components, such as PAR6 and aPKC, and (2) loss of function of these apical components blocks TE specification and YAP/TAZ nuclear localization (Alarcon 2010; Hirate et al. 2013, 2015; Kono et al. 2014; Cao et al. 2015; Mihajlovic and Bruce 2016). Nonetheless, it is not clear whether apical components are dysfunctional in RHO/ROCK-inhibited embryos, because they are still expressed in the outside cells, albeit not confined to the apical domain (Fig. 3B). It is possible that their colocalization with the basolateral components, such as PAR1, SCRIB, and LGL, may render the apical components inactive, although whether such mechanisms operate in preimplantation embryos is unknown. While the apical domain is clearly responsible for TE specification (Korotkevich et al. 2017), the mechanistic details on how it promotes YAP/TAZ nuclear localization await further investigations.

Another possible mechanism, which could operate in parallel to the above model, is that RHO and ROCK influence HIPPO signaling independently from their impact on the apicobasal polarity (Fig. 4). In various cell types, RHO and ROCK are well-known regulators of the cytoskeleton, exerting their actions partly through phosphorylation of actomyosin modulators, such as myosin light chain (MLC) and myosin phosphatase target subunit 1 (MYPT1) (Amano et al. 2010; Amin et al. 2013; Thumkeo et al. 2013). HIPPO signaling is modulated in an actomyosin-dependent manner in various cell types, playing key roles in sensing of physical properties, such as substrate stiffness and mechanical tension, even though the exact mechanisms that connect actomyosin to HIPPO signaling are still elusive (Dupont 2016; Sun and Irvine 2016). Thus, inhibition of RHO or ROCK in preimplantation embryos may disrupt actomyosin, which in turn regulates HIPPO signaling (Fig. 4). This is largely consistent with the situation in many other cell types, in which nuclear localization of YAP/TAZ, induced by external stimuli, is abrogated by disruption of actomyosin with pharmacological inhibitors, such as latrunculin and cytochalasin (Sansores-Garcia et al. 2011; Wada et al. 2011).

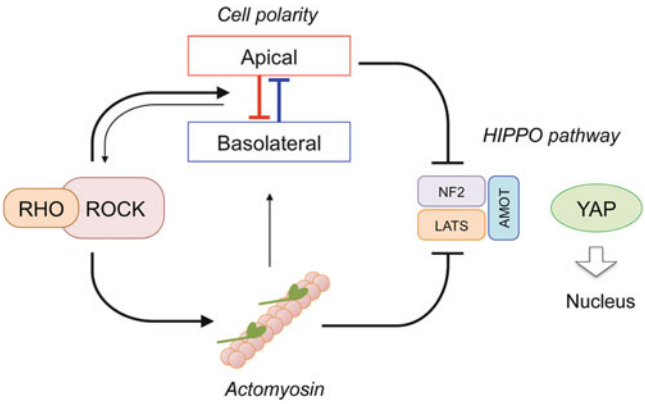

Fig. 4 Model for RHO/ROCK-dependent regulation of trophectoderm-lineage specification. RHO/ROCK signaling promotes the trophectoderm cell fate via two major inputs integrated by the HIPPO pathway to activate YAP. The first involves RHO/ROCK establishing the apical domain, whereas the second involves RHO/ROCK regulating the actomyosin cytoskeletal system. Both converge toward and modulate the HIPPO pathway, resulting in LATS (LATS1/2) suppression in the outside cells where unphosphorylated YAP promotes expression of trophectoderm lineage-specific genes. It is possible that RHO/ROCK and cell polarity mutually regulate each other and that RHO/ROCK also acts through actomyosin to modulate cell polarity. See text for details, sections "Possible Mechanisms of How RHO and ROCK Regulate TE Specification" and "Questions to Be Answered"

Interestingly, ROCK inhibition does not seem to impair other actomyosin-dependent events in preimplantation development, namely, cell division (cytokinesis) and compaction (Fig. 3A). During compaction, E-cadherin-mediated cell–cell adhesion is enhanced by the actomyosin-dependent mechanisms that involve changes in cortical tension and formation of filopodia (Stephenson et al. 2010; Fierro-Gonzalez et al. 2013; Maitre et al. 2015). Because global disruption of actin polymerization dynamics by cytochalasin or latrunculin prevents compaction (Clayton et al. 1999), the impact of ROCK inhibition on actomyosin may be more restricted or more specific to regulation of HIPPO signaling.

Some studies implicate that actomyosin-dependent cell contractility is involved in cell lineage specification in preimplantation embryos. Differences in contractility influence cell positioning within the embryo, such that cells with high contractility become surrounded by those with low contractility, resulting in inside and outside populations, respectively (Anani et al. 2014; Samarage et al. 2015; Maitre et al. 2016). In this case, differential contractility regulates lineage specification indirectly through positioning of cells rather than through direct action on HIPPO signaling. Nonetheless, pharmacological inhibition of myosin activity by blebbistatin prevents nuclear localization of YAP in cells that are not surrounded by other cells (Maitre et al. 2016), implicating that actomyosin may modulate HIPPO signaling independently of cell positioning.

Questions to Be Answered

While it is evident that activities of RHO/ROCK are required for outside cells to execute TE differentiation programs (i.e., apicobasal polarization, YAP/TAZ nuclear localization), it is unclear whether their activities are differentially regulated between the outside and inside cells. Namely, are RHO/ROCK inactive in inside cells, or are they active throughout the embryo regardless of cell position? The former case would implicate more instructive roles for RHO/ROCK in TE specification, whereas the latter would suggest more permissive roles. To monitor spatial and temporal regulations of RHO GTPases within a cell, various fluorescence probes have been engineered that specifically detect GTP-bound (active) forms (Pertz 2010). Such tools may help assess where and when RHO/ROCK signaling is activated within the embryo. Additionally, one can test whether ectopic activation of RHO/ROCK would compromise ICM formation. Use of constitutively active constructs of RHOA (Sauzeau et al. 2010) or ROCK (Wong et al. 2015) may help assess this possibility, although such constructs may exert pleiotropic effects to compromise other cellular activities independently of lineage specification.

If RHO/ROCK are to be spatially regulated in a cell position-dependent manner, what are the upstream factors that regulate them? An obvious candidate is the apicobasal cell polarity, which may act as an activator of RHO/ROCK signaling in outside cells. Interestingly, in embryos that are deficient in cell polarity regulator PRICKLE2, localization of apicobasal proteins is diminished, and the amount of active form of RHOA appears to be reduced at the eight-cell stage (Tao et al. 2012). Although further investigations are required to determine the cascade of events, this raises the possibility that cell polarity and RHO/ROCK activation may mutually regulate each other (Fig. 4).

Another question is: do RHO and ROCK act along the same pathway? Up to this point, our discussion is mostly based on this notion, because of the similarities in phenotypes between ROCK-inhibited and RHO-inhibited embryos. But there are some differences, such as the more severe impact by RHO inhibitor (Kono et al. 2014). Indeed, in our unpublished studies, we find distinct differences between the effects of ROCK inhibition and RHO inhibition on YAP/TAZ nuclear localization when treatments are initiated at later stages. Even though ROCK is generally considered to be a direct downstream effector of RHOA, there is speculation that ROCK may be active constitutively even without RHO binding (Truebestein et al. 2015). Interestingly, in human embryonic stem cells, impact of RHO inhibition by C3 and that of ROCK inhibition by Y-27632 are totally different, as the former causes cell death through action on YAP/TAZ, whereas the latter enhances cell survival (Ohgushi et al. 2015).

Concluding Remarks

While the main topic of this review is the role of RHO/ROCK system and cell lineage specification, studies suggest that RHO/ROCK are also involved in other cellular events during preimplantation development. For example, C3 treatment at around eight-cell stage interferes with cell division and compaction (Clayton et al. 1999; Liu et al. 2013). Also, Y-27632 treatment during the expanding blastocyst stage affects morphogenesis of the ICM, which impairs postimplantation development (Laeno et al. 2013). As upstream and downstream regulators of RHO/ROCK may be different for each event, elucidation of the mechanisms would be challenging. Investigations would surely benefit from cutting-edge technologies, such as real-time monitoring of RHO/ROCK activities, and molecular engineering of signaling components. One can also take advantage of the use of inhibitors that may be applied at different time points of development, so that stage-specific aspects of RHO/ROCK actions can be studied.

Although this review focuses on mouse studies, RHO and ROCK may also play essential roles in the preimplantation development of other placental mammals, including humans (Xie et al. 2010; Yan et al. 2013; Huang et al. 2016). The human situation is of significant concern with respect to fertility in women, as embryo loss may result from exposure to environmental agents that interfere with RHO or ROCK during the first week of conception (i.e., preimplantation stages). There are various natural and synthetic agents that inhibit RHO or ROCK, which may come in contact with women of reproductive age. RHO GTPases are specifically targeted by various bacterial toxins, including C3, through covalent modifications, such as ADP-ribosylation, glycosylation, adenylation, proteolytic cleavage, and deamidation (Aktories 2015). Numerous pharmacological inhibitors of ROCK have been explored for therapeutic purposes to treat cerebral vasospasm, ocular hypertension, glaucoma, cancer, and other clinical conditions (Feng et al. 2016).

Of particular concern are statins, which are widely prescribed for people with hypercholesterolemia, a prevalent condition that includes women of reproductive age. Statins prevent activation of RHO GTPases by reducing the production of isoprenoid lipids, as discussed in an earlier section "Statins Cause Phenotypes Similar to Inhibition of RHO and ROCK." Does statin intake impair preimplantation embryos in women? The serum statin levels that are found in patients (Arnett et al. 2005; Lewis et al. 2005; Hamidi et al. 2009) appear much lower than the lowest concentrations that completely block mouse blastocyst formation (Alarcon and Marikawa 2016). However, mouse studies are based on the hybrid strain (C57BL/6 × DBA/2) F1, which generally yields developmentally robust embryos, with nearly 100% of fertilized eggs consistently giving rise to fully expanded blastocysts. In contrast, developmental competence of human embryos is highly variable and generally less robust, as many fertilized eggs fail to develop into expanded blastocysts (Gardner and Lane 1998). Thus, human embryos may be more vulnerable to environmental insults than mouse embryos and may be susceptible to lower concentrations of statins. In addition, the serum statin levels in certain

patients may be significantly higher depending on their genetic background (e.g., poor metabolizers who carry variants of metabolizing enzyme genes), health condition (e.g., liver or kidney dysfunction to slow down drug elimination), and concomitant intake of other medications that inhibit metabolizing enzymes (Lynch and Price 2007; Neuvonen 2010; Hirota and Ieiri 2015).

Further investigations are crucial to determine whether statins as well as other RHO- or ROCK-interfering agents can pose a significant threat to preimplantation development in human. Importantly, human epidemiology is practically ineffective to identify environmental insults on preimplantation development. Women become aware of being pregnant only after embryo implantation through a rise in the chorionic gonadotropin level. When embryos are destroyed before implantation, women cannot even recognize that they produced an embryo and lost it. Therefore, in vitro studies with both human and model animal embryos are essential for a comprehensive understanding of the mechanisms underlying preimplantation development, so agents that interfere with critical molecular events may be suspected to be detrimental to preimplantation development.

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XEN and the Art of Stem Cell Maintenance: Molecular Mechanisms Maintaining Cell Fate and Self-Renewal in Extraembryonic Endoderm Stem (XEN) Cell Lines

Amy Ralston

Abstract The extraembryonic endoderm is one of the first cell types specified during mammalian development. This extraembryonic lineage is known to play multiple important roles throughout mammalian development, including guiding axial patterning and inducing formation of the first blood cells during embryogenesis. Moreover, recent studies have uncovered striking conservation between mouse and human embryos during the stages when extraembryonic endoderm cells are first specified, in terms of both gene expression and morphology. Therefore, mouse embryos serve as an excellent model for understanding the pathways that maintain extraembryonic endoderm cell fate. In addition, self-renewing multipotent stem cell lines, called XEN cells, have been derived from the extraembryonic endoderm of mouse embryos. Mouse XEN cell lines provide an additional tool for understanding the basic mechanisms that contribute to maintaining lineage potential, a resource for identifying how extraembryonic ectoderm specifies fetal cell types, and serve as a paradigm for efforts to establish human equivalents. Given the potential conservation of essential extraembryonic endoderm roles, human XEN cells would provide a considerable advance. However, XEN cell lines have not yet been successfully derived from human embryos. Given the potential utility of human XEN cell lines, this chapter focuses on reviewing the mechanisms known to govern the stem cell properties of mouse XEN, in hopes of facilitating new ways to establish human XEN cell lines.

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Stem Cell, Defined

While many kinds of stem cells exist, each with unique function, what makes stem cells different from differentiated, somatic, or even cancerous cells is that only stem cells are capable of both self-renewal and differentiation. One of the most intensively studied kinds of stem cell is the pluripotent stem cell, derived from early mammalian embryos. Cell lines such as embryonic stem (ES) cells and epiblast stem cells (EpiSCs) are pluripotent because they can give rise to any cell type of the body but also proliferate to an unlimited degree (Brons et al. 2007; Evans and Kaufman 1981; Martin 1981; Tesar et al. 2007). One important reason that ES cells and EpiSCs are pluripotent is because they are derived from embryos at such an early stage that their progenitors are not yet committed to specific fetal cell fates. Pluripotent cells are, however, no longer capable of efficiently producing extraembryonic cell types, such as placenta and yolk sac. Interestingly, during early developmental stages, embryos are not only able to give rise to pluripotent stem cell lines, but they are also able to give rise to multipotent stem cell lines, derived from the extraembryonic tissues. Thus, the early mammalian embryo is a rich resource for studying stem cell neogenesis.

The discovery of stem cell lines from the extraembryonic lineages, called trophoblast stem (TS) and extraembryonic endoderm stem (XEN) cells (Kunath et al. 2005; Tanaka et al. 1998), opened several opportunities not possible before their discovery. First, the existence of TS and XEN cell lines provides an opportunity to learn how extraembryonic lineages influence normal fetal development and how defects in the extraembryonic tissues might contribute to birth defects. Second, the existence of TS and XEN cell lines provides an opportunity to discover how stem cell progenitors are specified during embryogenesis—which could lead to the discovery of novel approaches to reprogram somatic cells to specific endpoints. Finally, the existence of TS and XEN cells provides an opportunity to understand how cell fate and "stemness" are maintained in a cell population. These exciting topics will be explored in this chapter, with an emphasis on XEN cells as a stem cell model of extraembryonic tissues.

What Are the Extraembryonic Tissues?

During the first week of mammalian embryonic development, multiple extraembryonic lineages are specified, including placenta, amnion, allantois, and yolk sac (Fig. 1a). These extraembryonic lineages are important because they will give rise to tissues that play multiple essential roles during development is gestation. Some of the most widely appreciated roles of the extraembryonic tissues are to surround, nourish, and protect the fetus. However, several extraembryonic tissues also actively guide embryonic development, cell fate specification, and patterning. While species-specific differences do exist among mammals, the functions of the

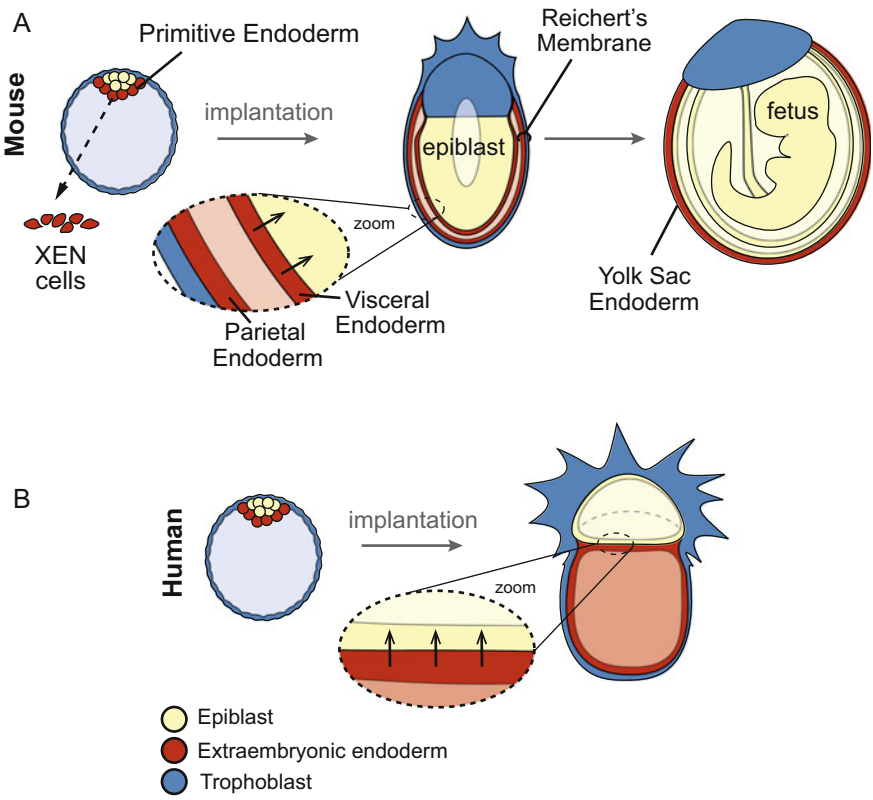

Fig. 1 Origins and roles of extraembryonic endoderm during mammalian embryonic development. (**a**) In the mouse, primitive endoderm is the progenitor of XEN cells and will give rise to both visceral and parietal endoderm after implantation. The zoomed region illustrates signals transmitted from the visceral endoderm to overlying epiblast that influence epiblast patterning and differentiation. Meanwhile, the parietal endoderm comprises Reichert's membrane, a transient extraembryonic tissue. Later in development, extraembryonic endoderm gives rise to yolk sac endoderm. *Yellow* epiblast lineage, *red* extraembryonic endoderm lineage, and *blue* trophectoderm/trophoblast/placenta lineage. (**b**) In the human blastocyst, the primitive endoderm (also known as hypoblast) underlies the epiblast as in mouse blastocysts. After implantation, the human epiblast exhibits a flattened rather than *bowl*-shaped morphology, yet visceral endoderm remains closely apposed to the epiblast, suggesting that visceral endoderm-induced epiblast patterning and differentiation is conserved between mice and humans

extraembryonic tissues are remarkably conserved between humans and mice (Rossant 2015). Notably, recent studies of cultured human embryos have permitted visualization of the extraembryonic endoderm after implantation (Deglincerti et al. 2016; Shahbazi et al. 2016). These studies have revealed that extraembryonic endoderm maintains contact with the epiblast in mouse and human embryos alike (Fig. 1b), strongly suggesting that extraembryonic endoderm plays an active role in fetal patterning in both species. Therefore, mice provide an excellent experimental

model for understanding the molecular mechanisms underlying the specification of extraembryonic lineages and the molecular mechanisms by which extraembryonic lineages influence fetal development.

The developmental roles of the extraembryonic endoderm are progressive, meaning that the roles of the extraembryonic endoderm lineage change over the course of development, with diverse, stage-specific roles. This is important because this means that there are multiple developmental processes that could be studied in vitro using stem cell models of extraembryonic endoderm. The extraembryonic endoderm is first established at the blastocyst stage as primitive endoderm, and serves as a pool of stem cell progenitors (Kunath et al. 2005). After implantation, the primitive endoderm differentiates into parietal and visceral endoderm (Gardner 1982), the functions of which are to mediate implantation and instruct fetal patterning starting around the time of gastrulation, respectively. The visceral endoderm influences the patterning of numerous fetal tissues, including neural, cardiac, intestinal, and blood, and germ cells (Belaoussoff et al. 1998; Madabhushi and Lacy 2011; Thomas and Beddington 1996). Additionally, visceral endoderm plays a role in guiding germ cell differentiation (Brons et al. 2007; de Sousa Lopes et al. 2004). Finally, the visceral endoderm differentiates into yolk sac endoderm, and both may help ensure proper neural tube closure (Mao et al. 2010). Thus, derivatives of the extraembryonic endoderm lineage influence fetal development in different ways at different times in gestation. Given the diverse functions of the extraembryonic endoderm, much excitement has surrounded the identification and study of XEN cell lines from the mouse embryo.

Stem Cell Lines from the Extraembryonic Endoderm

XEN cells were first derived from mouse embryos at the blastocyst stage (Kunath et al. 2005). Subsequently, it was shown that XEN cells can be derived from a variety of sources, but regardless of their origins, XEN cells are considered *bona fide* stem cells if they are capable of self-renewal and differentiation. For example, XEN cells can be derived from ES cells following overexpression of endodermal transcription factors, such as GATA6, GATA4, and SOX17 (McDonald et al. 2014; Shimosato et al. 2007; Wamaitha et al. 2015). XEN cells can also be derived from ES cells following treatment with Retinoic Acid and Activin (Cho et al. 2012). XEN cells can be derived from fibroblasts by reprogramming with the Yamanaka factors *Oct4*, *Sox2*, *Klf4*, and *Myc* (OSKM) (Parenti et al. 2016). Additionally, during chemical reprogramming, a XEN-like intermediate cell type has been observed in cells that are in the process of acquiring pluripotent properties (Zhao et al. 2015). However, this intermediate XEN-like cell could not be isolated or expanded as a stable cell line. Finally, XEN cell lines can also be derived from postimplantation embryos (Lin et al. 2016), and possess expanded in vivo developmental potential, discussed further below.

Several lines of evidence have shown that XEN cell lines are multipotent, meaning that XEN cells can differentiate into more than one mature cell type. For example, when injected into host blastocysts, XEN cell lines can contribute to development of the parietal endoderm. For reasons that are not clear, however, XEN cell lines do not contribute efficiently to visceral endoderm. Interestingly, XEN cell lines isolated from postimplantation embryos have been shown to contribute to visceral endoderm more efficiently than do XEN cell lines derived from the blastocyst (Lin et al. 2016). This observation strongly suggests that there are fundamental, stage-dependent differences in XEN cell developmental potential. However, the mechanisms defining these differences are still unclear.

Although most XEN cell lines do not contribute efficiently to visceral endoderm in vivo, several groups have employed in vitro protocols to produce visceral endoderm-like tissue from XEN cells. XEN cell lines can be differentiated to visceral endoderm in vitro by addition of Bone Morphogenetic Protein 4 (BMP4) (Artus et al. 2012; Paca et al. 2012). In addition, XEN cell lines that arise created during reprogramming are also capable of differentiating to visceral endoderm in vitro (Parenti et al. 2016). The ability to produce visceral endoderm-like cells from XEN cells provides several opportunities in the fields of regenerative medicine and birth defects research. First, in vitro models of visceral endoderm provide a way to study and identify essential patterning cues transmitted by the visceral endoderm to the epiblast. Second, in vitro models of visceral endoderm could provide a way to harness these essential cues to direct the differentiation pluripotent stem cells and, for example, pattern the organogenesis in pluripotent stem cell-derived organoids.

Maintaining the XEN Cell State

One exciting and unsolved topic in stem cell research is how stem cell lines maintain their fate during the course of unlimited expansion. Cell fate can be defined in terms of unique morphology or gene expression and also in terms of developmental potential. Several assays are available for examining the developmental potential of stem cell lines, including in vitro differentiation assays, and in vivo differentiation assays, such as teratomas and chimeras. Using these assays, it is apparent that XEN cells maintain the capacity to differentiate into differentiated cell types of the extraembryonic endoderm lineages, as described above. However, many researchers have attempted to understand how XEN cell lines remember what they are and what they are supposed to do. The following sections will examine the roles of extrinsic and intrinsic cues known to promote XEN cell proliferation and to repress XEN cell differentiation.

Extrinsic Cues Maintaining the XEN Cell State

One approach to understanding the balance between self-renewal and differentiation in XEN cells has been to identify pathways that promote the self-renewal of XEN cells. A variety of culture media can maintain XEN cells in the proliferative state (Niakan et al. 2013), and most XEN cell culture media include serum. Therefore, the nature of the specific cytokines or growth factors that maintain XEN cell proliferation is still somewhat obscure. Identifying factors that promote stem cell self-renewal and limit differentiation is important because this is the first step toward developing protocols to efficiently differentiate stem cell lines to desired endpoints.

Studies to identify signaling pathways that regulate XEN cell proliferation and differentiation showed that ERK signaling plays an important role in maintaining XEN cells in vitro, consistent with the role of ERK signaling in maintaining development of the XEN progenitor in the blastocyst (Nichols et al. 2009). In XEN cell lines, inhibitors of MEK activity slow XEN cell proliferation (Artus et al. 2010) and lead to upregulation of markers of visceral endoderm (Spruce et al. 2010). In blastocysts, ERK signaling in XEN progenitors depends on Fibroblast Growth Factor 4 (FGF4) (Kang et al. 2013; Nichols et al. 2009; Yamanaka et al. 2010). However, *Fgf4* is dispensable for the proliferation of established XEN cell lines (Kang et al. 2013). Similarly, inhibitors of FGF receptors fail to interfere with XEN cell proliferation (Spruce et al. 2010). These observations indicate that a non-FGF family receptor tyrosine kinase (RTK) stimulates ERK signaling in XEN cell lines. The RTK inhibitor Gleevec decreased proliferation of XEN cell lines in a dose-dependent manner (Artus et al. 2010), although the effect of Gleevec on XEN cell differentiation was not examined. These observations suggest that RTK signaling stimulates ERK, which promotes XEN cell proliferation and limits XEN cell differentiation.

One RTK that is important for maintaining XEN cell stemness has been identified. XEN cell proliferation has been shown to require a Platelet-Derived Growth Factor (PDGF) family RTK, since XEN cells lacking a PDGF receptor encoded by *Pdgfra* exhibited reduced proliferation (Artus et al. 2010). However, *Pdgfra* is probably not the only RTK regulating XEN cell stemness, because the proliferation of *Pdgfra*-null XEN cells was further reduced by the RTK inhibitor Gleevec. Moreover, no role for *Pdgfra* in limiting XEN cell differentiation was described (ibid.). These observations point to the existence of additional, as-yet unidentified RTKs regulating ERK-dependent XEN cell self-renewal and limiting their differentiation.

Intrinsic Cues Maintaining the XEN Cell State

Intrinsic XEN cell-regulating cues that have been investigated include transcription factors and epigenetic mechanisms. Several transcription factors have been shown to be important regulators of extraembryonic endoderm cell fate because they are

essential for establishing XEN cell progenitors in the blastocyst or sufficient to induce formation of XEN cells in ES cell lines. These potent cell fate regulators include GATA4, GATA6, and SOX17 (McDonald et al. 2014; Shimosato et al. 2007; Wamaitha et al. 2015). Several of these transcription factors are known to function as pioneer factors—able to bind and open closed chromatin, consistent with important roles in initiating cellular commitment to extraembryonic endoderm cell fate. Whether these factors are also important for maintaining extraembryonic endoderm cell fate is less clear, because loss of these factors from existing XEN cell lines has not been examined. Moreover, levels of *Gata4*, *Gata6*, and *Sox17* are reportedly uniform during XEN cell differentiation in vitro to visceral endoderm (Artus et al. 2012). Therefore, the levels of these factors is unlikely to regulate the transition between stem cell and differentiated states.

Besides transcription factors, epigenetic mechanisms, including DNA methylation, histone modifications, and microRNAs, are another way that XEN cells could remember their fate. Interestingly, a unique pattern of genome-wide DNA methylation was observed in XEN cell lines compared with other embryo-derived stem cell lines (Senner et al. 2012). For example, genes that are primed for expression in pluripotent stem cell lines were observed to be hypermethylated in XEN cell lines, whereas XEN cell genes were observed to be less methylated in XEN cell than in pluripotent stem cell lines. These observations indicate that DNA methylation patterns generally correlate with gene expression in embryo-derived stem cell lines. Future studies may focus on comparing DNA methylation patterns in XEN cells and their differentiated derivatives to understand how DNA methylation could reinforce not just cell fate but also stemness.

In addition to exhibiting a unique pattern of DNA methylation, XEN cell lines have been shown to possess unique chromatin signatures, when compared with other stem cell lines. For example, the repressive histone modification H3K27me is detected within very few gene loci, compared with the level at which this mark is detected in ES cells (Rugg-Gunn et al. 2010). The mechanism limiting H3K27 methylation in XEN cells is thought to be the lower levels of expression of the machinery that deposits this mark, including lower levels of *Jarid2,* as well as several members of the Polycomb Repressive Complex 2 (PRC2). XEN cells are proposed to rely instead on H3K9 methylation as a mechanism for limiting inappropriate gene expression in XEN cells (ibid.). However, this mark has not yet been surveyed on a genome-wide level.

XEN Cell Applications

Since the extraembryonic endoderm plays such a critical and important role in supervising differentiation of fetal cell types, one obvious application of XEN cells is to use XEN cells or XEN cell conditioned medium to influence and guide the differentiation of pluripotent stem cells. Although studies of this kind are still in

their infancy, a few groups have successfully used XEN cells to influence differentiation of blood and cardiac cells (Artus et al. 2012; Verzi et al. 2010).

Human XEN cell lines would enable exciting studies of the inductive interactions between embryonic and extraembryonic tissues that are not otherwise possible. However, XEN cells have not been successfully derived from the human blastocyst. This could be due to the fact that the growth factors that support specification or proliferation of XEN cell progenitors differ between humans and mice (Kuijk et al. 2012; Roode et al. 2012). Some studies have examined whether overexpression of endodermal transcription factors is sufficient to induce formation of human XEN cell lines (Séguin et al. 2008; Wamaitha et al. 2015). These studies reported that overexpression of *Sox7*, *Sox17*, and *Gata6* is sufficient to differentiate human pluripotent stem cell lines and induce expression of many endodermal markers. However, since XEN cells from the human blastocyst do not exist, it is unclear what the molecular, proliferative, and developmental hallmarks of XEN cells are. For these reasons, the mouse will continue to serve as the superlative model for stem cell and birth defects research.

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