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

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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.

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 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* 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-Boulant 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 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 hair-pin 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 angiomin (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).

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 (trophoblast) 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

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

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 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 μ m

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”