Trophectoderm formation: regulation of morphogenesis and gene expressions by RHO, ROCK, cell polarity, and HIPPO signaling

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Abstract

**In brief:** Trophectoderm is the first tissue to differentiate in the early mammalian embryo and is essential for hatching, implantation, and placentation. This review article discusses the roles of Ras homolog family members (RHO) and RHO-associated coiled-coil containing protein kinases (ROCK) in the molecular and cellular regulation of trophectoderm formation.

**Abstract:** The trophectoderm (TE) is the first tissue to differentiate during the preimplantation development of placental mammals. It constitutes the outer epithelial layer of the blastocyst and is responsible for hatching, uterine attachment, and placentation. Thus, its formation is the key initial step that enables the viviparity of mammals. Here, we first describe the general features of TE formation at the morphological and molecular levels. Prospective TE cells form an epithelial layer enclosing an expanding fluid-filled cavity by establishing the apical-basal cell polarity, intercellular junctions, microlumen, and osmotic gradient. A unique set of genes is expressed in TE that encode the transcription factors essential for the development of trophoblasts of the placenta upon implantation. TE-specific gene expressions are driven by the inhibition of HIPPO signaling, which is dependent on the prior establishment of the apical-basal polarity. We then discuss the specific roles of RHO and ROCK as essential regulators of TE formation. RHO and ROCK modulate the actomyosin cytoskeleton, apical-basal polarity, intercellular junctions, and HIPPO signaling, thereby orchestrating the epithelialization and gene expressions in TE. Knowledge of the molecular mechanisms underlying TE formation is crucial for assisted reproductive technologies in human and farm animals, as it provides foundation to help improve procedures for embryo handling and selection to achieve better reproductive outcomes.

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Introduction

Preimplantation development is an embryonic process that is unique to the placental mammals. During this process, the fertilized egg transforms into the blastocyst to prepare for implantation, i.e. the attachment to the uterine epithelium and the invasion into the endometrium. Implantation and the following generation of the placenta are essential for further development of the embryo, as nutrients and oxygen need to be provided by the mother through the placenta. The blastocyst is composed of two types of tissues: the trophectoderm (TE) and the inner cell mass (ICM) (Fig. 1A). TE, which is positioned at the periphery of the blastocyst, plays crucial roles in implantation by contacting the uterine epithelium and by giving rise to the trophoblasts of the placenta. By contrast, ICM is a group of undifferentiated pluripotent cells, which later undergo germ layer formation and gastrulation to generate the fetal body. Thus, the formation of TE precedes the embryonic body patterning and therefore is the top priority in mammalian development to enable the viviparous mode of reproduction.

Here, we first describe the overall process of TE formation, focusing on its morphogenesis and gene expression regulation. We then discuss the roles of the Ras homolog family members (RHO) and RHO-associated coiled-coil containing protein kinases (ROCK) pathway as one of the essential regulators of TE formation. Discussions are in large part based on studies using the laboratory mouse, which is the most widely used experimental model for mammalian development. However, when the information is available, studies using other mammalian species, such as human, cattle, and pig, are also included. Understanding of the mechanisms of TE formation is important from the clinical and agricultural points of view. In vitro fertilization and preimplantation embryo culture are routinely performed as assisted reproductive technologies (ART) for human and farm animals. Mechanistic knowledge of TE
formation should help improve procedures pertinent to embryo culture and selection for optimal reproductive outcomes.

**Key features of trophectoderm formation**

During preimplantation development, TE forms an epithelial layer that encloses the ICM and the blastocyst cavity (Fig. 1A). Because the blastocyst cavity is created by the action of TE, its presence is often regarded as the morphological hallmark of TE formation. The cavity first emerges as a small lumen, which increases in volume over time, driven by the osmotic pressure built by the action of TE. The expansion of the cavity is crucial for preimplantation development. As the oocyte is encapsulated by the protective shell called the zona pellucida (ZP), the embryo needs to break it and hatch out of it before the attachment to the uterine epithelium (Fig. 1B and C). The breaking of the ZP is driven by the physical force generated by the expansion of the cavity (Wang & Dey 2006, Li et al. 2016, Leonavicius et al. 2018). Trypsin-like digestive enzymes produced by TE may also contribute to the weakening of the ZP to facilitate hatching (Perona & Wassarman 1986, Yamazaki et al. 1994, Sharma et al. 2006). In human in vitro fertilization (IVF), the hardening of the ZP may be caused by in vitro culture, impeding efficient hatching and implantation (De Vos & Van Steirteghem 2000). To help embryos escape from their ZP during blastocyst expansion, a procedure known as ‘assisted hatching’ is performed, in which a small hole is made in the ZP using a laser beam or chemical method (Hammadeh et al. 2011, Li et al. 2016). In addition to hatching, the cavity expansion may also be important for efficient attachment to the uterine epithelium, by increasing the surface area of the blastocyst. Furthermore, a recent study, in which the cavity volume of the mouse blastocyst is experimentally reduced, suggests that cavity expansion is essential for the proper differentiation of the ICM into the epiblast and primitive endoderm (Ryan et al. 2019). Thus, the formation and expansion of the blastocyst cavity by the action of TE are crucial for successful embryo development.

Note that the epithelial morphology of TE is a temporary characteristic, which is essentially to prepare the embryo for implantation, as described above. Upon attachment to the uterine epithelium, TE cells undergo a dramatic transformation, known as the epithelial-mesenchymal transition (EMT), to enable the invasion of the embryo into the endometrial matrix (Oghbail et al. 2022). After EMT, the descendants of TE are referred to as trophoblasts, which play the major role in the construction of the placenta (Turco & Moffett 2019).

Below, we briefly describe the essential cellular and molecular features of TE formation as the foundation for later discussions with respect to the roles of RHO and ROCK. For more details, including references to the original studies, we refer the readers to some excellent reviews (Saini & Yamanaka 2018, Posfai et al. 2019, Plusa & Piliszek 2020).

**Establishment of the apical-basal cell polarity and intercellular junctions**

To form an epithelium, prospective TE cells first acquire the apical-basal polarity. In mouse, the apical-basal polarity emerges in all cells at the late eight-cell stage. Subsequent cell divisions generate outside and inside cell populations, and only outside cells retain the apical-basal polarity, which are about to become TE (Fig. 2A). Essential roles of various polarity proteins in TE formation have been largely studied using mouse embryos. However, the polarity proteins are likely to play evolutionary conserved roles, as loss of function of atypical protein kinase C (aPKC), one of the major apical proteins, impairs TE formation not only in mouse but also in human and cattle (Gerri et al. 2020).
Notably, not all apical proteins, such as EZR, may be required for the TE epithelialization or cavitation, as suggested by a recent study using cattle embryos (dos Anjos et al. 2021).

The initiation of the polarity protein assembly in mouse is dependent on the transcriptional activation of the zygotic genome. Transcription factors TFAP2C and TEAD4 are needed for the initial localization of the apical proteins, partly through the transcriptional activation of the genes encoding the polarity proteins and cytoskeleton regulators (Zhu et al. 2020). Although the apical-basal polarization normally starts at the eight-cell stage, it can also occur at later stages when non-polar inside cells are brought to the surface (Korotkevich et al. 2017, Wigger et al. 2017). The ability of inside cells to polarize upon external exposure is also shown in a study using cattle blastocysts, in which isolated ICM can regenerate TE and support full-term development (Kohri et al. 2019).

Following the apical-basal polarization, prospective TE cells form cell–cell junctions, namely the adherens junction, the desmosome, and the tight junction (Fig. 2B) (Fleming et al. 2000). In mouse, formation of the intercellular junctions starts at the late eight-cell stage through the process called compaction. Compaction transforms a loose cluster of round cells into a tightly packed mass. This morphological change is due to the increased cell–cell adhesion and cortical tension that involves the transmembrane cell adhesion protein CDH1, a key component of the adherens junction (Maitre et al. 2015, White et al. 2016). The timing of compaction is later in other species, namely 8- to 16-cell stage in human and around 32-cell stage in pig and cattle, which correlates with their later onset of cavitation compared to the mouse (Plusa & Piliszek 2020). Regardless, the assembly of the cell–cell junction components continues to attain functional maturity, which mechanically couple TE cells together to form an epithelial layer that can withstand the stretching force of an expanding cavity (Zenker et al. 2018, Chan et al. 2019).

**Formation and expansion of the blastocyst cavity**

The blastocyst cavity first emerges as a small lumen, or microlumen (Dumortier et al. 2019, Ryan et al. 2019), which in mouse occurs between 16- and 32-cell stages or about 3 days after fertilization. The timing of the cavity formation is later in other species, namely about 5 days in human, 6–7 days in pig, and 7–8 days in cattle (Plusa & Piliszek 2020). Initially, multiple microscopic lumens appear between cells throughout the embryo, which coalesce to form a single lumen (Fig. 2C). These microscopic lumens are derived from intracellular vesicles (or vacuoles) that are released from both outside and inside cells (Ryan et al. 2019). Interestingly, the formation of the intracellular vesicles can occur in a cell-autonomous manner without neighboring cells (Schliffka et al. 2021). In ‘single-celled’ embryos that are experimentally generated by cell fusion or inhibition of cytokinesis, multiple intracytoplasmic vesicles appear around the same time as in normal development and coalesce to form a single vesicle in the cytoplasm. However, no exocytosis occurs, possibly because of the lack of the basal membrane without cell–cell contact.

Further expansion of a microlumen is promoted by water influx from the external environment, driven by an osmotic gradient created by the accumulation of sodium ions (Na⁺) in the lumen (Fig. 2D) (Watson et al. 2004). Na⁺ and water molecules are retained in the lumen.
owing to the tight junction, as its experimental disruption using Clostridium perfringens enterotoxin (CPE), which specifically targets the tight junction proteins CLDN, diminishes cavity expansion (Chan et al. 2019).

While the osmotic gradient of Na⁺ persists, water molecules continuously enter the cavity and cause its expansion, which is pivotal for hatching. Nonetheless, during the in vitro culture of preimplantation embryos, temporary collapse of the cavity occurs periodically (Niimura 2003). It is still unclear whether there is a physiological role for such periodic blastocyst contractions. Experimental suppression of mitosis with DNA replication inhibitor aphidicolin abrogates periodic cavity contraction (Chan et al. 2019), suggesting that it may be caused by a transient breakage of the tight junction during the mitosis of TE cells. Notably, the pattern of contraction has been used as a morphokinetic parameter to predict the quality of blastocysts in human ART (Gazzo et al. 2020, Huang et al. 2021). It is possible that some aspects of the periodic contraction, such as the cyclic release and replenishment of cavity fluid components, may play positive roles in the further development of blastocysts.

**Transcriptional regulation of lineage-specific genes**

Differences in the gene expression profiles between TE and ICM have been studied extensively at the single-cell level in mouse (Guo et al. 2010, Yan et al. 2013, Posfai et al. 2017, Fan et al. 2020), human (Blakeley et al. 2015, Petropoulos et al. 2016, Meistermann et al. 2021), cattle (Negrón-Pérez et al. 2017, Wei et al. 2017), and pig (Ramos-Ibeas et al. 2019, Kong et al. 2020, Liu et al. 2021, Zhi et al. 2022). Some genes are preferentially expressed in TE in all these species (e.g. GATA3 and Cdx2), while other genes appear to be TE-specific only in certain species (e.g. Eomes and Elf5 in mouse TE). Differential activity of HIPPO signaling plays a key role in lineage-specific gene expressions (Karasek et al. 2020, Yildirim et al. 2021). In outside cells (i.e. prospective TE cells), HIPPO signaling is inhibited, leading to the nuclear accumulation of YAP (Fig. 3). In the nucleus, YAP functions with TEAD4 as a transcriptional co-activator to up-regulate TE-specific genes, such as Cdx2 (Rayon et al. 2014). By contrast, HIPPO signaling is active in inside cells, causing the phosphorylation of YAP for cytoplasmic retention and degradation. Functional significance of various HIPPO signaling components (e.g. LATS, AMOT, and NF2) in lineage specification has been studied mainly in mouse. However, some of these components may have similar roles in other species, as they are expressed in human (Gerri et al. 2020), cattle (Sakurai et al. 2017, Gerri et al. 2020, Sharma & Madan 2020, 2022, dos Anjos et al. 2021, Saito et al. 2021), and pig (Cao et al. 2019, Emura et al. 2019, 2020) preimplantation embryos.

In the mouse, the inhibition of HIPPO signaling in the outside cells is mediated by the apical-basal polarity. When the apical-basal polarization is experimentally disturbed, outside cells have cytoplasmic YAP and adopt gene expression profiles similar to ICM (Alarcon 2010, Hirate et al. 2013). One of the proposed mechanisms that link the cell polarity with HIPPO signaling entails the subcellular distribution of AMOT (Hirate & Sasaki 2014). In non-polar cells, AMOT is distributed throughout the plasma membrane, where it interacts with the cell–cell adhesion complex to activate HIPPO signaling (Fig. 3) (Hirate et al. 2013). By contrast, in polarized cells, the apical domain sequesters AMOT away from the site of cell–cell adhesion to prevent AMOT from activating HIPPO signaling.

The apical-basal polarity also affects the cell contractility or cortical tension, which in turn may influence HIPPO signaling. The presence of the apical domain reduces actomyosin contractility in the outside cells, which promotes polar and non-polar cells to assume the outside and inside position, respectively (Maître et al. 2016). As YAP acts as a mechanotransducer to relay cytoskeletal tension to the nucleus (Panciera et al. 2017), the polarity-dependent alteration in the cell contractility may influence HIPPO signaling. However, the mechanoregulation of YAP is highly dependent on...
cellular context (Cai et al. 2021), and it is unclear to what extent the reduced contractility in the outside cells contributes to the TE-specific gene expressions.

The transcriptional outcome of HIPPO signaling also regulates TE morphogenesis, as Tead4-null mouse embryos fail to form the blastocyst cavity (Yagi et al. 2007, Nishioka et al. 2008). However, the identity of the YAP/TEAD4 targets that are essential for TE morphogenesis is unclear. The apical-basal polarity appears intact in Tead4-deficient embryos, based on the localization of the apical proteins (Nishioka et al. 2008, Mihajlović et al. 2015). Likewise, the formation of intercellular junctions may be unaffected by the loss of TEAD4. The components of the adherens junction, CDH1 and CTNNB1, are localized at the site of cell–cell contact in Tead4-deficient embryos in a manner similar to normal embryos (Nishioka et al. 2008, Mihajlović et al. 2015). Unpublished data from our labs suggest that the tight junction may also be unaffected by Tead4 deficiency. Knockdown of Tead4 by RNAi prevents blastocyst cavity formation (Alarcon 2010, Mihajlović et al. 2015). In Tead4-knockdown embryos, tight junction component TJP1 was distributed as continuous lines at the apical edge of cell–cell boundaries, as seen in control embryos (Fig. 4A). Transmission electron microscopy also showed the presence of tight junctions in Tead4-knockdown embryos (Fig. 4A). When Tead4-knockdown embryos were chimerized with normal embryos, a blastocyst cavity formed and expanded (Fig. 4B). This was in stark contrast to chimeras between Pard6b-knockdown and normal embryos, the former of which has defective tight junction and prevents cavity formation (Alarcon 2010) (Fig. 4B). These results suggest that the structure and function of the tight junction are not compromised by the lack of Tead4. Further investigations are required to examine whether the formation of the microlumen or osmotic gradient is impaired in Tead4-deficient embryos.

Roles of RHO and ROCK in the trophectoderm formation

The remainder of the article discusses the roles of RHO and ROCK in TE formation with respect to the regulation of morphogenesis and gene expressions that are described above.

General molecular characteristics of RHO and ROCK

The RHO subfamily of small GTPases consists of three isoforms: RHOA, RHOB, and RHOC (Narumiya &...
Thumkeo 2018). These isoforms share a high degree of structural similarities with over 88% amino acid sequence identity and are collectively referred to as RHO in this article. RHO cycles between a GTP-bound (active) state and a GDP-bound (inactive) state (Fig. 5A). Guanine nucleotide exchange factor (GEF) replaces GDP with GTP, thereby activating RHO. By contrast, GTPase-activating protein (GAP) inactivates RHO by promoting the hydrolysis of GTP into GDP. Guanine nucleotide dissociation inhibitor (GDI) inactivates RHO by keeping the GDP-bound form away from the membrane, where an exchange with GTP takes place. Hence, the membrane association of RHO is prerequisite for its activation, which is enabled by the covalent attachment of a lipid moiety, namely a geranylgeranyl group. A geranylgeralyl group is provided by geranylgeranyl pyrophosphate (GGPP), a product of the mevalonate pathway, and its attachment to RHO is catalyzed by geranylgeranyltransferase (Fig. 5A; Hodge & Ridley 2016). Activated (GTP-bound) RHO binds and regulates various effector proteins, one of which is ROCK. ROCK, which collectively refers to the two isoforms ROCK1 and ROCK2 in this article, belongs to the family of serine/threonine kinases. Upon binding of active RHO, the kinase domain of ROCK becomes functional and phosphorylates various target proteins, such as myosin-binding subunit of myosin phosphatases (MYPT) and LIM motif-containing protein kinases (LIMK) (Amin et al. 2013) (Fig. 5A).

Generally, RHO and ROCK are best known for their roles in the rearrangements of cytoskeletons, particularly the actomyosin system. Through the regulation of the cytoskeleton, RHO and ROCK can influence a variety of cellular processes, including cell shape change, polarization, migration, cytokinesis, and intracellular vesicle trafficking (Narumiya & Thumkeo 2018). Understanding of the functional roles of RHO and ROCK owes greatly to experimental studies in cultured cells using RHO-specific and ROCK-specific inhibitors. The C3 exoenzyme isolated from the Clostridium botulinum bacterium is an ADP-ribosyltransferase, and it inactivates RHO but not other small GTPase subfamilies, such as CDC42 and RAC1 (Wilde et al. 2000). Y-27632 is a small molecule that is used as a selective ATP-competitive inhibitor of ROCK (Uehata et al. 1997). Exposure of cultured cells to these inhibitors causes distinct morphological changes that are largely due to the rearrangement of the actomyosin cytoskeleton (Narumiya & Thumkeo 2018). Many of the phosphorylation targets of ROCK are modifiers of the actomyosin architecture. For example, phosphorylation of MYPT by ROCK causes the activation of myosin II and enhancement of the actomyosin-driven contractility. ROCK also phosphorylates LIMK, which in turn phosphorylates the F-actin-severing protein CFL1, leading to the stabilization of actin filaments.

Roles for RHO and ROCK in TE morphogenesis

Studies in mouse and other mammalian species have shown that the inhibition of RHO or ROCK interferes with the formation of the blastocyst cavity, suggesting their essential roles in TE formation. Exposure of preimplantation embryos to C3 exoenzyme impairs cavity formation in mouse (Kono et al. 2014) and in cattle (Kohri et al. 2020). Likewise, treatment with Y-27632 inhibits cavity formation in mouse (Kawagishi et al. 2004, Kono et al. 2014, Cao et al. 2015, Mihajlović & Bruce 2016) and in pig (Son et al. 2010, Zhang et al. 2014, Kwon et al. 2016, Guo et al. 2019) and delays blastocyst formation in human (Huang et al. 2016). Cavity formation is also prevented by exposure to statins, the inhibitors of the mevalonate pathway, which provides GGPP for RHO geranylgeranylation (Surani et al. 1983, Alarcon & Figure 5 Regulation of the trophectoderm morphogenesis and gene expressions by RHO and ROCK. (A) General molecular characteristics of RHO and ROCK. See the text for details. (B) Impact of RHO or ROCK inhibition on the distributions of the apical and basal domain proteins and YAP protein in outside cells. (C) Models depicting the molecular mechanisms by which RHO and ROCK regulate two aspects of trophectoderm formation: morphogenesis and gene expression. See the text for details.
Marikawa 2016), further supporting the essential role of RHO activation in cavity formation. However, a study using cattle embryos has shown that Y-27632 does not impair the formation of the blastocyst cavity, raising the possibility that ROCK regulates the development of the cattle embryo differently from other species (Negrón-Pérez et al. 2018). Further investigations are required to determine whether a lack of adverse effects of Y-27632 on the cavity formation is due to species-dependent roles of ROCK or due to the differences in other experimental settings, such as the drug concentrations and the timing of exposures.

How mechanistically are RHO and ROCK involved in the regulation of the blastocyst formation? In cultured cells, RHO and ROCK control various cellular activities through the modification of the actomyosin assembly (Narumiya & Thumkeo 2018). Actin filaments are connected to the intercellular junctions, namely the adherens junction and tight junction, to mechanically couple epithelial cells. In the mouse blastocyst, the disruption of the actin filament with cytochalasin or latrunculin causes the collapse of the cavity, suggesting that the paracellular sealing of TE is dependent on the integrity of actin filaments (Kawagishi et al. 2004a, Marikawa & Alarcon 2019). Thus, a modulation of the actomyosin cytoskeleton by the inhibition of RHO or ROCK may impair the intercellular junctions to prevent cavity formation. In mouse, Y-27632-treated embryos exhibit a discontinuous distribution of TJP1 and TJP2 along the boundaries between the outside cells, indicative of defective tight junction (Kono et al. 2014, Mihajlović & Bruce 2016). Y-27632 also disrupts the tight junction in pig embryos, as evidenced by the impaired distribution and reduced expression of TJP1 and OCLN and by the defective paracellular sealing of TE (Kwon et al. 2016). On the other hand, the integrity of the adherens junction is unclear. In Y-27632-treated mouse embryos, CDH1 is localized throughout the cell–cell contact sites and compaction occurs normally (Kono et al. 2014, Mihajlović & Bruce 2016). By contrast, Y-27632-treated pig embryos exhibit a disrupted localization of CDH1 and a reduced level of the Cdh1 transcript (Kwon et al. 2016).

Another mechanism by which RHO and ROCK can regulate TE morphogenesis is through the control of the apical-basal polarization. In mouse, exposure of preimplantation embryos to C3 exoenzyme or Y-27632 causes aberrant localizations of the polarity proteins (Kono et al. 2014, Mihajlović & Bruce 2016). In the inhibitor-treated embryos, the apical proteins PARD6B and PRKCCZ are distributed to both apical and basal sides, and the basal proteins SCRIB and LLGL1 are also mislocalized to both sides (Fig. 5B). RHO and ROCK may directly modulate the activities of the polarity proteins, as ROCK has been shown in cultured cells to phosphorylate PARD3 to prevent its binding to aPKC (Nakayama et al. 2008). Also, a disruption of the tight junction by RHO or ROCK inhibition may cause mislocalization of the polarity proteins, as the tight junction acts as a fence to segregate the apical domain from the basal domain (Jou et al. 1998).

Whether RHO and ROCK regulate the other aspects of TE morphogenesis, namely the formation of the microlumen and osmotic gradient, is unclear. It is difficult to experimentally determine the specific roles of RHO or ROCK in these processes, as they are dependent on the prior establishment of the apical-basal polarity. However, in cultured cells, RHO and ROCK are implicated in the regulation of intracellular vesicle formation and trafficking (Olayioye et al. 2019). It may be possible that RHO and ROCK can directly influence the formation of the microlumen and osmotic gradient through modulation of the directed transport of membrane vesicles and channel proteins.

Regulation of lineage-specific gene expression and HIPPO signaling by RHO and ROCK

Inhibition of RHO and ROCK alters cell lineage-specific gene expression profiles, namely the reduced expression of the TE-specific genes. In the mouse, exposure to C3 exoenzyme or Y-27632 downregulates Cdx2 while up-regulating ICM-specific genes Nanog and Sox2 (Kono et al. 2014, Cao et al. 2015, Frum et al. 2018). The CDX2 expression is also reduced in C3 exoenzyme-treated cattle embryos (Kohri et al. 2020) and in Y-27632-treated pig embryos (Guo et al. 2019). Reduction in the TE-specific gene expression is largely due to the ectopic activation of HIPPO signaling, i.e. the cytoplasmic retention of YAP in the outside cells (Fig. 5B). In the mouse, nuclear accumulation of YAP is absent or markedly diminished in C3 exoenzyme- or Y-27632-treated embryos (Kono et al. 2014, Cao et al. 2015, Mihajlović & Bruce 2016, Shi et al. 2017). A reduction in the nuclear YAP is also observed in C3 exoenzyme-treated cattle embryos (Kohri et al. 2020). Similar alterations in gene expressions and HIPPO signaling are also observed in statin-treated mouse embryos, likely due to deficiency in RHO geranylgeranylation (Alarcon & Marikawa 2016). When LATS or AMOT is experimentally knocked down, YAP accumulates in the nucleus even when RHO or ROCK is inhibited (Kono et al. 2014, Mihajlović & Bruce 2016). These studies suggest that RHO and ROCK act upstream of LATS and AMOT to inhibit HIPPO signaling in the outside cells to enable TE-specific gene expression.

RHO and ROCK may control HIPPO signaling through the modulation of the actomyosin cytoskeleton. Studies with cultured cells have revealed that YAP acts as a mechanotransducer and responds to the state of actomyosin to regulate gene expressions. Generally, an experimental increase in actin polymerization promotes YAP nuclear localization, whereas a decrease in actin polymerization causes cytoplasmic retention of YAP.

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(Dasgupta & McCollum 2019). AMOT, which directly binds F-actin, has been implicated as a sensor of the actin status. Binding to F-actin diminishes AMOT interaction with YAP, resulting in a reduction in YAP phosphorylation by LATS (Hirate et al. 2013, Mana-Capelli et al. 2014) (Fig. 5C). However, in preimplantation embryos, it is unclear whether the modulation of the actin cytoskeleton is the major mechanism by which RHO and ROCK inhibits HIPPO signaling.

Alternatively, inhibitory actions of RHO and ROCK on HIPPO signaling may be executed through the apical-basal polarity. As described above, the proper localizations of the apical and basal proteins are dependent on RHO and ROCK and are essential for the nuclear accumulation of YAP in the outside cells. Inhibition of RHO or ROCK disturbs the apical-basal polarization, which in turn may cause cytoplasmic retention of YAP (Fig. 5C). In particular, when the mouse embryos are exposed to C3 exoenzyme or Y-27632, AMOT is mislocalized to the basolateral side in the outside cells (Mihajlović & Bruce 2016, Shi et al. 2017), which may be sufficient to activate HIPPO signaling and phosphorylate YAP.

RHO may also exert a more direct action on HIPPO signaling through its interaction with AMOT (Shi et al. 2017). Co-immunoprecipitation experiments have shown that RHO binds to AMOT and blocks the interaction between AMOT and NF2. Interaction with NF2 is required for the membrane localization of AMOT and subsequent activation of HIPPO signaling. Thus, RHO may inhibit HIPPO signaling by directly interfering with the interaction of AMOT and NF2 (Shi et al. 2017) (Fig. 5C). In this scenario, RHO affects HIPPO signaling independently of ROCK, which may explain why the inhibition of RHO causes a much severer impact than the inhibition of ROCK on HIPPO signaling in preimplantation embryos. In the mouse, Y-27632 treatment reduces the number of YAP-positive nuclei by about 60% of the control, whereas C3 exoenzyme reduces it by about 95% (Kono et al. 2014). In addition, treatment of the expanding mouse blastocyst with C3 exoenzyme, but not with Y-27632, diminishes the nuclear accumulation of YAP in TE, even though both inhibitors can collapse the blastocyst cavity (Marikawa & Alarcon 2019).

**Upstream regulators of RHO and ROCK**

In cultured cells, various extracellular signals have been identified that regulate the activity of RHO. These include cytokines, growth factors, and extracellular matrix proteins, which act through cell surface receptors, such as G-protein coupled receptors, receptor tyrosine kinases, and integrins. These receptors in turn control the actions of GEF, GAP, and GDI to alter the state of RHO between the GTP-bound active form and the GDP-bound inactive form (Bros et al. 2019). In preimplantation embryos, however, it is unclear whether such extracellular signals play a role in the control of RHO activity during TE formation. Preimplantation embryos can develop normally from fertilization to the late blastocyst stage in a simple culture medium that lacks any of the extracellular signals mentioned above (Biggers 1998). This is in stark contrast to most cultured cells, which grow in a medium containing serum, the major source of cytokines and growth factors. However, it is possible that embryos may synthesize and secrete such extracellular signals, which in turn act on themselves through an autocrine fashion.

While the presence of extracellular regulators is unclear, RHO appears to be activated preferentially in presumptive TE cells. Studies using probes that specifically detect the GTP-bound form have shown that active RHO is localized to the apical side in outside cells and is nearly absent in inside cells (Shi et al. 2017). This observation raises the possibility that the apical components may be involved in the activation of RHO. In mouse keratinocytes, loss of PARD3 leads to a reduction in GTP-bound RHOA with a concomitant increase in ARHGAP35, a RHO-specific GAP (Dias Gomes et al. 2019). It is yet to be determined in preimplantation embryos whether apical enrichment of active RHO is dependent on PARD3 or other apical components. However, as the inhibition of RHO causes mislocalizations of the polarity proteins (Kono et al. 2014), there may be a feed-forward regulatory loop between RHO and the apical components to ensure the robust maintenance of the apical-basal polarity.

While the kinase activity of ROCK is dependent on active RHO, the total amount of ROCK is regulated at the transcriptional level, which may contribute to the overall action of ROCK. TFAP2C is a transcription factor that is essential for blastocyst formation (Choi et al. 2012). An experimental knockdown of TFAP2C results in a marked reduction in the level of the transcripts encoding ROCK (Rock1 and Rock2) and also those encoding LIMK (Limk1 and Limk2), the major phosphorylation target of ROCK (Cao et al. 2015). A chromatin-immunoprecipitation study has shown that TFAP2C binds to a putative regulatory sequence of the Rock2 gene (Kidder & Palmer 2010, Choi et al. 2012), suggesting that TFAP2C directly controls the expression of ROCK, thereby promoting the formation of TE. However, it is unclear whether the levels of ROCK1 and ROCK2 are indeed higher in outside cells than in inside cells.

**Future perspectives**

The key aspect of TE formation is the creation of an epithelial layer that surrounds an expanding cavity, which is essential for hatching. Also, a distinct gene expression profile is established during TE formation, which is critical for trophoblast differentiation upon implantation. Although all these processes occur concurrently during
normal development, distinct mechanisms may regulate individual processes independently to some extent. For example, cells may be able to adopt the TE-specific gene expression profiles without epithelialization. Conversely, cells may be epithelialized while expressing ICM-specific, but not TE-specific, genes. Importantly, both processes are dependent on the activity of RHO and ROCK, as discussed in this article (Fig. 5C). RHO and ROCK regulate epithelialization and gene expressions through the modulation of the actomyosin cytoskeleton, the apical-basal polarity, and HIPPO signaling. Understanding of the mechanisms by which RHO and ROCK orchestrate these processes may provide insights into the quality assessment of embryos and improvement of culture conditions in human and farm animal ART. It may also shed light on the evolutionary mechanisms that enabled the acquisition of a unique embryonic process to generate TE at the beginning of mammalian development.

Even though referred to as RHO and ROCK collectively in this article, all isoforms (RHOA, RHOB, RHOC, ROCK1, and ROCK2) are expressed during preimplantation mouse development, at least at the transcript level (Marikawa et al. 2021). Whether individual isoforms play distinct roles in TE formation is currently unclear, partly because most of the investigative tools employed to study the functions of RHO and ROCK (e.g. inhibitors and dominant-negative construct) affect all isoforms without discrimination. However, there are studies in other experimental systems, mainly in cultured cells, implicating different functions for individual isoforms. For example, each of RHOA, RHOB, and RHOC appears to play a specific role in cell migration in cancer cells (Ridley 2013) and in the regulation of permeability barrier in endothelial cells (Pronk et al. 2019). It would be of interest to elucidate how each isoform is involved in TE formation. In the pig embryo, distinct roles are suggested for ROCK1 and ROCK2, largely based on differential temporal and spatial expression patterns (Zhang et al. 2014). Namely, during the morula-to-blastocyst transition, the transcript level of Rock1 is markedly reduced, while that of Rock2 is elevated. The localization of ROCK1 protein shifts from the cytoplasm to the plasma membrane during development, while ROCK2 becomes enriched in the nucleus. Additional functional tests are needed, possibly through experimental depletion of individual isoforms, to determine whether they regulate specific aspects of TE development.

In recent years, blastocyst-like structures, termed ‘blastoids’, have been constructed from cultured stem cells, such as embryonic stem cells and trophoblast stem cells (Rivron et al. 2018, Li et al. 2019, Sozen et al. 2019, 2021, Fan et al. 2021, Yanagida et al. 2021, Yu et al. 2021, Kagawa et al. 2022). Blastoids morphologically resemble blastocysts, as they consist of an epithelial layer surrounding a fluid-filled cavity with a small mass of cell aggregate inside. Transcriptomic analyses at the single-cell level have shown that the gene expression profiles of blastoids are similar to those of the blastocyst. Blastoids may be explored as in vitro models of blastocysts to further dissect the molecular mechanisms of TE formation, including the roles of RHO and ROCK, especially for the individual isoforms. At the same time, great caution is needed in extrapolating the experimental results using blastoids, as there may be significant differences in the molecular regulations to generate the morphology and gene expression profiles that appear to be similar. More comparative studies, particularly with functional tests, are required to reveal similarities and differences between blastoids and blastocysts, so that these novel tools are effectively used to expand our understanding of TE formation.

Conclusion

TE is the first lineage to emerge as an epithelial layer during preimplantation development and plays pivotal roles in blastocyst hatching, implantation, and placentation. RHO and ROCK are essential regulators of TE formation, as they modulate the actin cytoskeleton, apical-basal polarity, cell–cell junctions, and HIPPO signaling. The conserved functions of RHO and ROCK in TE formation have been suggested for mouse, human, cattle, and pig embryos, although some species-dependent differences may also exist. The current knowledge on RHO and ROCK is largely based on experiments using pharmacological inhibitors, and more specific molecular interrogations are needed to elucidate their upstream regulators, downstream effectors, and isoform-specific roles. While the experimental use of embryos, particularly of human, may be restricted for practical and ethical reasons, non-animal alternatives, such as stem cell-derived blastoids, may help dissect the mechanisms by which RHO and ROCK control distinct processes of TE formation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

V B A and Y M performed literature search, conceived the framework of the review, conducted experiments presented as unpublished data, and wrote the manuscript.
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