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Inhibition of RHO–ROCK signaling enhances ICM and suppresses TE characteristics through activation of Hippo signaling in the mouse blastocyst

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ABSTRACT

Specification of the trophectoderm (TE) and inner cell mass (ICM) lineages in the mouse blastocyst correlates with cell position, as TE derives from outer cells whereas ICM from inner cells. Differences in position are reflected by cell polarization and Hippo signaling. Only in outer cells, the apical–basal cell polarity is established, and Hippo signaling is inhibited in such a manner that LATS1 and 2 (LATS1/2) kinases are prevented from phosphorylating YAP, a key transcriptional co-activator of the TE-specifying gene *Cdx2*. However, the molecular mechanisms that regulate these events are not fully understood. Here, we showed that inhibition of RHO–ROCK signaling enhances ICM and suppresses TE characteristics through activation of Hippo signaling and disruption of apical–basal polarity. Embryos treated with ROCK inhibitor Y-27632 exhibited elevated expression of ICM marker NANOG and reduced expression of CDX2 at the blastocyst stage. Y-27632-treated embryos failed to accumulate YAP in the nucleus, although it was rescued by concomitant inhibition of LATS1/2. Segregation between apical and basal polarity regulators, namely PARD6B, PRKCZ, SCRIB, and LLGL1, was dampened by Y-27632 treatment, whereas some of the polarization events at the late 8-cell stage such as compaction and apical localization of p-ERM and tyrosinated tubulin occurred normally. Similar abnormalities of Hippo signaling and apical–basal polarization were also observed in embryos that were treated with RHO GTPases inhibitor. These results suggest that RHO–ROCK signaling plays an essential role in regulating Hippo signaling and cell polarization to enable proper specification of the ICM and TE lineages.

Introduction

The first cell differentiation in mammalian development is the establishment of the two cell lineages, inner cell mass (ICM) and trophectoderm (TE). ICM is a population of pluripotent stem cells that give rise to all cell types in the body, whereas TE contributes only to extraembryonic tissues, namely trophoblasts of the placenta. When the embryo reaches the blastocyst stage, ICM and TE are distinguishable by their morphology as well as gene expression patterns (Fujimori, 2010; Marikawa and Alarcon, 2012; Stephenson et al., 2012; Zernicka-Goetz et al., 2009). TE forms a monolayer of epithelium surrounding a fluid-filled cavity, and ICM forms a single, ovoid-shaped cell aggregate that is situated within the cavity. TE expresses homeodomain-containing CDX2 and GATA family member GATA3, whereas ICM expresses POU-domain-containing POU5F1 and homeodomain-containing NANOG. These transcription factors play essential roles in maintaining the characteristics of each lineage, as demonstrated by gene knockout and knockdown studies (Chambers et al., 2003; Home et al., 2009; Mitsui et al., 2003; Nichols et al., 1998; Strumpf et al., 2005). While the nature of the initial signals that specify the two cell lineages during early development is somewhat controversial (Blij et al., 2012; Hiiragi et al., 2006; Takaoka and Hamada, 2012; Zernicka-Goetz, 2013), it is evident that the position of cells within an embryo is critical to direct lineage specification toward TE or ICM. At around 32-cell stage, cells located in the outer layer of the embryo are committed to differentiating into TE, whereas those positioned inside are destined to become ICM, as demonstrated by cell dissociation and cell repositioning experiments (Marikawa and Alarcon, 2012; Sasaki, 2010; Stephenson et al., 2012; Suwinska et al., 2008; Zernicka-Goetz et al., 2009). Thus, elucidation of the molecular mechanisms of how a cell interprets positional information to execute lineage-specific gene expression programs as well

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as to adopt lineage-specific morphology should help in understanding how the mammalian embryo regulates its first cell differentiation event.

Hippo signaling controls organ size through cell proliferation and apoptosis, and many components of the signaling pathway are conserved in various animals, ranging from fruitfly to mammals (Johnson and Halder, 2014; Yu and Guan, 2013). Key components of the Hippo signaling pathway play pivotal roles in cell-lineage specification in the mouse blastocyst. TEA-domain transcription factor TEAD4 is a downstream effector of Hippo signaling and is essential for TE formation (Nishioka et al., 2008; Yagi et al., 2007). While ubiquitously expressed in the embryo, TEAD4 activates *Cdx2* expression specifically in TE, because its transcriptional co-activator Yes-associated protein (YAP) accumulates in the nucleus only in the outer cells (Hirate et al., 2012; Nishioka et al., 2009). YAP is retained in the cytoplasm in inner cells, owing to its phosphorylation by kinases LATS1 and 2 (LATS1/2) (Hao et al., 2008). Loss of function of LATS1/2 in mouse embryos leads to ubiquitous nuclear YAP localization and *Cdx2* expression throughout the embryo, including inner cells, while they form the blastocyst cavity (Lorthongpanich et al., 2013; Nishioka et al., 2009). Recent studies have also revealed other regulators of Hippo signaling that act upstream of LATS, namely AMOT and NF2, and play critical roles in lineage formation in the mouse blastocyst (Cockburn et al., 2013; Hirate et al., 2013; Leung and Zernicka-Goetz, 2013). Thus, differential control of Hippo signaling between inner and outer cells is a crucial element for the specification of ICM and TE in the mouse blastocyst, i.e., its inhibition induces TE lineage whereas its activation promotes ICM lineage.

Another key element for linking the positional information (i.e., inside versus outside) to lineage specification (i.e., ICM versus TE) is the establishment of apical–basal cell polarity. By the end of the 8-cell stage, the event known as compaction occurs, in which the overall appearance of the embryo becomes smooth due to enhanced cell-cell adhesion. During compaction, all eight cells start to exhibit polarity along the apical and basal axis. However, the subsequent cleavages to 16- to 32-cell stages generate inner and outer cell populations, and only outer cells further establish distinct apical and basal polarity, while inner cells remain non-polarized (Eckert and Fleming, 2008; Stephenson et al., 2012). Various molecules have been identified that are localized to the apical or basal membrane in the outer cells, many of which are homologs of evolutionary conserved cell polarity regulators. For example, PARD3 (a par-3 homolog), PARD6B (a par-6 homolog) and PRKCI/PRKCZ (atypical protein kinase C or aPKC) are localized to the apical membrane, whereas SCRIB (a scribble homolog), LLGL1 (a lethal giant larva homolog) and MARK2 (a par-1 homolog) are confined to the basal membrane (Alarcon, 2010; Dard et al., 2009; Plusa et al., 2005; Tao et al., 2012; Vinot et al., 2005). Knockdown of PARD6B causes cavitation failure due to defective tight junction formation. Also, the expression of CDX2 is diminished while NANOG expression is elevated in PARD6B-knockdown embryos, indicating that PARD6B is essential for TE specification (Alarcon, 2010). Furthermore, a recent study has shown that knockdown of PARD6B impairs nuclear localization of YAP in outer cells (Hirate et al., 2013), suggesting that the activity of Hippo signaling is controlled by cell polarity regulators. Thus, delineating the molecular players that impact Hippo signaling as well as the apical–basal polarity is the key to understand the mechanisms of cell-lineage specification in the mouse blastocyst.

In the present study, we investigated the role of RHO–ROCK (Rho-associated kinase) signaling in lineage specification, specifically focusing on its link to Hippo signaling and apical–basal polarization. ROCK is a serine-threonine kinase and is activated by its association with RHO small GTPases (Amano et al., 2010; Amin et al., 2013; Nishioka et al., 2012; Thumkeo et al., 2013). ROCK phosphorylates a number of protein targets and regulates various cellular processes, such as cell migration, cytokinesis, and neurite elongation. It has been shown previously that inhibition of ROCK during mouse preimplantation development using a specific inhibitor, Y-27632, interferes with blastocyst cavity formation (Kawagishi et al., 2004), raising the possibility that RHO–ROCK signaling is required for TE lineage formation. Nonetheless, the impact of RHO–ROCK signaling inhibition on cell-lineage specification has not been explored. Moreover, recent studies with cultured cells showing that inhibition of RHO alters LATS1/2 activity and YAP localization (Mo et al., 2012; Yu et al., 2012; Zhao et al., 2012) warrant further investigations on the relationship between RHO–ROCK and Hippo signaling in mouse preimplantation embryos. Here, we report that inhibition of RHO–ROCK signaling enhances the ICM lineage and suppresses the TE lineage formation by impacting Hippo signaling and proper apical–basal cell polarization.

Materials and methods

Animals and collection of preimplantation embryos and oocytes

F1 (C57BL/6 × DBA/2) mice from the National Cancer Institute were used. Female mice were injected with pregnant mare serum gonadotropin and human chorionic gonadotropin (hCG) (EMD Millipore) at 48 h apart. For collection of preimplantation embryos, female mice were mated with males after injection with hCG, and one-cell (pronuclear) and two-cell stage embryos were collected from oviducts, using standard protocols (Nagy et al., 2003). For collection of oocytes, female mice were sacrificed about 18 h after hCG injection and oocyte-cumulus cell complex was recovered from oviducts. Cumulus cells were removed by treatment with 75 U/mL hyaluronidase in FHM HEPES-buffered medium (EMD Millipore), and zona pellucida was digested with proteases (0.5% Pronase; Roche) in FHM. Oocytes were washed in FHM several times and carefully examined to confirm no cumulus cells remained. The protocol for animal handling and use was reviewed and approved by the University of Hawaii Institutional Animal Care and Use Committee.

Embryo treatment with pharmacological inhibitors

Stocks of ROCK inhibitor Y-27632 (20 mM, EMD Millipore) and RHO inhibitor I (100 μg/mL, Cytoskeleton) were dissolved in dimethyl sulfoxide and water, respectively, and stored at −20 °C until ready for use. Embryos were treated in 20 μL droplets of Y-27632 (20 μM) and RHO inhibitor I (1 μg/mL) that were freshly diluted in KSOM-AA culture medium (MR-121-D, EMD Millipore), covered with mineral oil, and pre-equilibrated at 37 °C with 5% CO_2 in air. Control treatment was in 20 μL droplets that were prepared by adding dimethyl sulfoxide or water to KSOM-AA at a volume equal to that of the inhibitor.

Time-lapse cinematography

Embryo development was recorded in real time, as previously described (Alarcon, 2010). Briefly, embryos were cultured in a heated stage of an Axiovert 200 inverted microscope (Carl Zeiss), which was enclosed in an incubation chamber (PeCon). Temperature and CO_2 concentration were regulated by Tempcontrol 37-2 and CO2-Controller (PeCon). Images were captured every 15 minutes, using AxioCam MRm digital camera, which was controlled by AxioVision software (Carl Zeiss). The incubation chamber was covered with a black plastic sheet during time-lapse recording.