# MECHANISMS OF EMBRYONIC AND EXTRA-EMBRYONIC TISSUE SEGREGATION IN PRE-IMPLANTATION EMBRYOS

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## DEDICATION

I dedicate this first and foremost to my mother, Merly, thank you for instilling the concept of hard work and value of education in me, my 7th grade English teacher, Ms. Kong, I will always remember how you told me if I believe in myself, I could get far in life… and to my high school/life counselor, Mr. Fia, for reminding me college is just like anything else in life, it is attainable if your determination reflects your passion.

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## ABSTRACT

The pre-implantation embryonic stage called blastocyst comprises of inner cell mass (ICM) tissue, which is a mosaic of epiblast (EPI) and primitive endoderm (PE) cells. Prior to uterine implantation, EPI and PE segregate into distinct tissue layers, which will give rise to the fetal body and extra-embryonic structures, respectively. Rho-associated coiled-coil containing protein kinase (ROCK) is an enzyme responsible for a plethora of cellular functions, including cell migration and cell-to-cell adhesion. To investigate ROCK's role in EPI and PE segregation, blastocysts, prior to tissue segregation, were treated with ROCK inhibitor Y-27632 and immunostained for cell lineage-specific markers. Results showed EPI and PE cells remained intermingled, and ICM cells became loosely associated. Blastocysts treated with Fasudil, another ROCK inhibitor, showed similar results. This suggests that ROCK is required for segregation, possibly by mediating intercellular adhesion to maintain ICM morphology and differential cell adhesion to promote EPI and PE cell sorting.

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# CHAPTER 1. INTRODUCTION

The mouse is an ideal model system of human pre-implantation development because it is convenient to study given its short lifespan and the morphological resemblance of its pre-implantation stages to human. After fertilization, which usually happens in the ampulla, the two-cell stage embryo undergoes several cell divisions to produce a ball of cells or morula. At embryonic day 3.0 (E3.0), the embryo consists of fluid filled cavities that fuse to become one, resulting in the blastocyst. A blastocyst, which arrives in the uterus, consists of two important cell types: the inner cell mass (ICM) and the trophectoderm (TE). The ICM is comprised of pluripotent cells that can give rise to any cell type of the body as well as extra-embryonic tissues, whereas the TE gives rise to the trophoblasts which are essential for implantation and placenta formation.

Before implantation into the mother's uterine lining, the ICM of the early blastocyst (E3.5) undergoes the second cell fate decision, resulting in the emergence of two distinct cell types. They are the epiblast (EPI) and primitive endoderm (PE) which express cell lineage-specific transcription factors in a mosaic "salt and pepper" pattern. NANOG is expressed in EPI cells, and GATA4 and GATA6 are expressed in PE cells (Chazaud et al., 2006). Thus, the ICM is heterogeneous. By the late blastocyst (E4.5), EPI forms the internal layer of cells in the ICM, whereas PE forms the superficial layer of cells that faces the blastocyst cavity. The EPI gives rise to the fetus, and the PE gives rise to extra-embryonic structures, namely the yolk sac. Additionally, PE has an important role in helping to determine the body axis by defining the rostral end of the mouse embryo after implantation (Thomas & Beddington, 1996).

Currently, the question of the origin of the EPI and PE cells prior to the blastocyst stage is still controversial. Yamanaka et al. (2010) traced the generation of inner cells and their cell fate in the ICM, by co-injecting mRNA for nuclear-RFP (red fluorescent protein), membrane-RFP, and Cre recombinase into a single cell at the 8-cell stage of LacZ/Enhanced GFP (green fluorescent protein) mouse line. The Cre recombinase protein excised LacZ to induce GFP expression, which enabled long-term lineage tracing. They observed that labeled cells contributed to inner cells at the16-cell stage (early) and at the 32-cell stage (later). In both instances, outer cells divided asymmetrically to produce one outer and one inner daughter cell. Thus, at the blastocyst stage, the ICM consisted of a mixture of early and later inner cells. When the cell fate of the early and later inner cells was examined at the post-implantation stage, they observed that the EPI and PE development is independent of the timing of cell internalization in the ICM. Thus, cell origin, that is, the timing of cell internalization, does not determine whether a cell becomes EPI or PE. In contrast, Morris et al. (2010) utilized transgenic embryos that express GPI (glycosylphosphatidyl-inositol)-GFP, which is a cell surface marker, to track every cell during the live imaging of embryos. They observed that the early inner cells from the asymmetric division are biased to give rise to EPI cells.

Despite the mosaic position of the PE and EPI cells in the E3.5 ICM, there may be regulatory interactions between the two cell types. Interestingly, Yamanaka et al. (2010) showed that inhibition of the FGF signaling pathway after the early blastocyst stage prevents formation of PE precursor cells, whereas activation of the FGF signaling pathway promotes formation of PE precursor cells. Perhaps EPI cells interact with PE cells through the FGF pathway to specify cell identity.

Once the blastocyst has formed, the question is how do the EPI and PE cells segregate from one another to form two distinct tissue layers. Insight was given by Plusa et al. (2008), who combined live imaging of embryos that express GFP under the control of a PE-promoter with immunohistochemical staining of cell lineage-specific markers. They showed that GFP-expressing cells migrate to the outer layer, whereas cells that move towards the inner layer down-regulate GFP expression or undergo apoptosis. After localizing in the surface layer, cells show up-regulation of GFP. Thus, PE segregation is achieved via cell migration, regulation of gene expression that is position-dependent, and apoptosis to eliminate PE cells that are still positioned inside the ICM after the PE layer has been established.

Based on the studies of Plusa et al. (2008), there are three rationales or models to explain how EPI and PE cells form distinct tissue layers in the ICM: cell migration, cell fate based on cell position, or even a combination of both (Fig. 1). Cell migration is the possibility that cells expressing NANOG will remain in the inner part of the ICM, whereas cells expressing GATA4 or GATA6 will migrate to the surface of the ICM. Cell fate based on cell position is the possibility that cells differentiate according to their position within the ICM. After the two tissues have segregated, cells that are in the wrong position undergo apoptosis, thereby restricting NANOG-expressing cells in the EPI layer and GATA4/6-expressing cells in the PE layer.

The possible mechanisms underlying the segregation of EPI and PE cells may be through the regulation of cytoskeletal organization during cell migration and by differential cell adhesion. The Rho family GTPases and the major RhoA downstream effector Rho-associated coiled-coil containing protein kinase (ROCK) play a role in the

organization of F-actin cytoskeleton and is a regulator of cell migration. Studies done by Leong et al. (2011) revealed that inhibition of ROCK activity using Y-27632 promotes cell body elongation, protrusion, and migration of adult neural precursor cells (NPC) in the subventricular zone of the brain. NPC-derived neurospheres treated with Y-27632 results in an elongated morphology, single cell migration, and reduced membrane contact. Additionally, Y-27632 treatment modifies the migration course, such that instead of NPC being located to the rostral migratory stream (RMS), they were in the anterior olfactory nucleus and anterior cortex. In contrast, untreated neurospheres migrate as sheets of interconnected cells to the RMS.

Furthermore, a study done by Hopkins et al. (2007) showed that ROCK activity regulates the migration of epithelial cells as a cohesive unit, which is distinct from studies that have been done on single cells (e.g., fibroblasts). Cell lines derived from colonic tumors T84 and Caco-2 were allowed to migrate into wound space in the presence or absence of Y-27632. Results showed that Y-27632 treatment enhanced the migration of cells into the wound space, but they did not completely fill it because there were gaps between cells. Additionally, cells exposed to Y-27632 possessed jagged shapes in the protruding leading edge, and were dispersed from one another. In contrast, control cells showed smooth edges and migrated as a cohesive unit into the wound space with no gaps between the cells. F-actin organization was also analyzed in these cell lines by staining with Alexa-568-phalloidin. Cells exposed to Y-27632 showed no organized stress fiber networks, and F-actin aggregates were observed behind the leading edge. In contrast, control cells showed F-actin in the leading edge and tail end with organized stress fibers and lacked F-actin aggregates.

The regulation of differential cell adhesion may be another possible mechanism that segregates EPI cells from PE cells. Steinberg and Takeichi (1994) demonstrated that expression of different amounts of the homophilic adhesion molecule, P-cadherin, by two groups of cells that were intermixed allowed cell sorting because they were able to differentiate amongst each other. Taken together, cytoskeleton-mediated cell migration and differential cell adhesion may function in sorting EPI and PE cells into separate tissue layers.

ROCK is an enzyme that is responsible for a plethora of cellular functions, such as cellular migration, cell-to-cell adhesion, and apoptosis. There are two ROCK isoforms: ROCK1/ROKβ and ROCK2/ROKα with 64% homology in amino acids (Amano et al., 2010). Previous studies revealed findings of the phenotype when each isoform was knocked out. Thumkeo et al. (2003) focused on ROCK2 null mutants, and showed that most do not survive long after birth. Those that do survive are born as runts (40-50% size of control litter) with thrombosis occurring in the placenta, which results in loss of trophoblasts. Other than these problems, these runts are healthy and fertile. Shimizu et al. (2005) revealed the phenotype of ROCK1 null mutants. Most mutants are born with omphalocele, in which intestines and other internal organs are herniated through the umbilical cord. Because of these defects, most mutant pups are cannibalized by their mother, and thus, very few survive beyond week 4. ROCK1 mutants display varying degrees of open eyes at birth: 50% of embryos had fully open eyelids, and 50% of embryos had almost fully open eyelids. With the exception of the eye lesions, a small portion of ROCK1 mutants make it to full term, and are fertile and healthy. Taken together, ROCK1 and ROCK2 null mutants result in the successful birth of offspring

because the individual isoform knockout does not affect pre-implantation development. However, it may be necessary to inhibit both isoforms to reveal the role of ROCK in pre-implantation development, and in particular, during the period of development when EPI and PE segregation is occurring.

To investigate the role of ROCK in EPI and PE segregation into distinct tissue layers in the ICM, two pharmacological inhibitors were used in our experiments: Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] and Fasudil [1-(5-isoquinolinesulfonyl)homopiperazine dihydrochloride]. Y-27632 and Fasudil have been widely used in studies regarding vascular problems pertaining to smooth muscle contraction, hypertension and tumor invasion, as well as asthma (Ishizaki et al., 2000; Breitenlechner et al., 2003; Ichikawa et al., 2008). Both drugs have been used or tested in cardiovascular therapies (Hu & Lee, 2005). However, Fasudil was the first widely used drug, approved in 1995, in the treatment of cerebral vasospasm (Breitenlechner et al., 2003). More recently, these ROCK inhibitors have been discovered to prevent apoptosis and to promote survival of dissociated human embryonic stem cells, thereby contributing to the technological advancement of their successful propagation (Olson et al., 2008; Ichikawa et al., 2008).

Y-27632 and Fasudil can inhibit both ROCK1 and ROCK2, by competing for the ATP binding site and changing the conformation of the catalytic site of the kinase (Ishizaki et al., 2000; Jacobs et al., 2006; Yamaguchi et al., 2006; Ichikawa et al., 2008). Previous studies revealed that the ability of ROCK to bind to Y-27632 and Fasudil differs. Binding properties of ROCK against Y-27632 and Fasudil have been compared with homologous kinase, cAMP-dependent kinase (PKA) (Breitenlechner et al., 2003;

Jacobs et al., 2006). ROCK and PKA share highly conserved binding interactions at the catalytic site. Whereas Y-27632 binds to ROCK at a 30-fold rate over PKA, Fasudil binds equally to ROCK and PKA. Thus, Y-27632 has more specificity in binding ROCK than Fasudil. Understanding the difference of ligand selectivity between these two ROCK inhibitors may help us to understand how different ROCK inhibitors can influence different cellular events.

The goal of my Master Thesis is to understand the mechanisms behind the formation of the EPI and PE tissue layers in the ICM of the blastocyst. To this end, my research was guided by the following three Specific Aims: (1) to test the hypothesis that ROCK plays a role in the segregation of the EPI and PE cells; (2) to determine whether the effects of ROCK inhibition are reversible; and (3) to confirm that ROCK plays a role in EPI and PE segregation. For Specific Aim 1, I exposed blastocysts to Y-27632 during the period of development when EPI and PE cells normally undergo sorting. I observed the collapse and re-emergence of varying sizes of the blastocyst cavity, the loosening of cells in the ICM, and the disruption in the segregation of EPI and PE cells. These findings suggest that intercellular adhesion plays a role in the proper sorting of EPI and PE into two separate layers. Furthermore, they tell us that the ROCK pathway plays a significant role in regulating the segregation of EPI and PE by influencing cell adhesion. For Specific Aim 2, I cultured Y-27632-treated blastocysts in a drug-free medium. I observed that the subsequent exposure to a drug-free medium was not able to revive the proper segregation of the EPI and PE, as well as the ICM morphology. This indicates that cell sorting occurs within a restricted period of blastocyst development, and interferences within that time frame leads to irreversible abnormalities. For Specific Aim

3, I exposed blastocysts during the period of EPI and PE cell sorting to another ROCK inhibitor, Fasudil. I found that disruption in normal segregation was also evident, as well as varying sizes of the blastocyst cavity. These findings are reminiscent of what was examined in Y-27632-treated blastocysts. Thus, I am able to confirm that the ROCK pathway does play a role in the normal segregation of EPI and PE, and in normal ICM morphology.

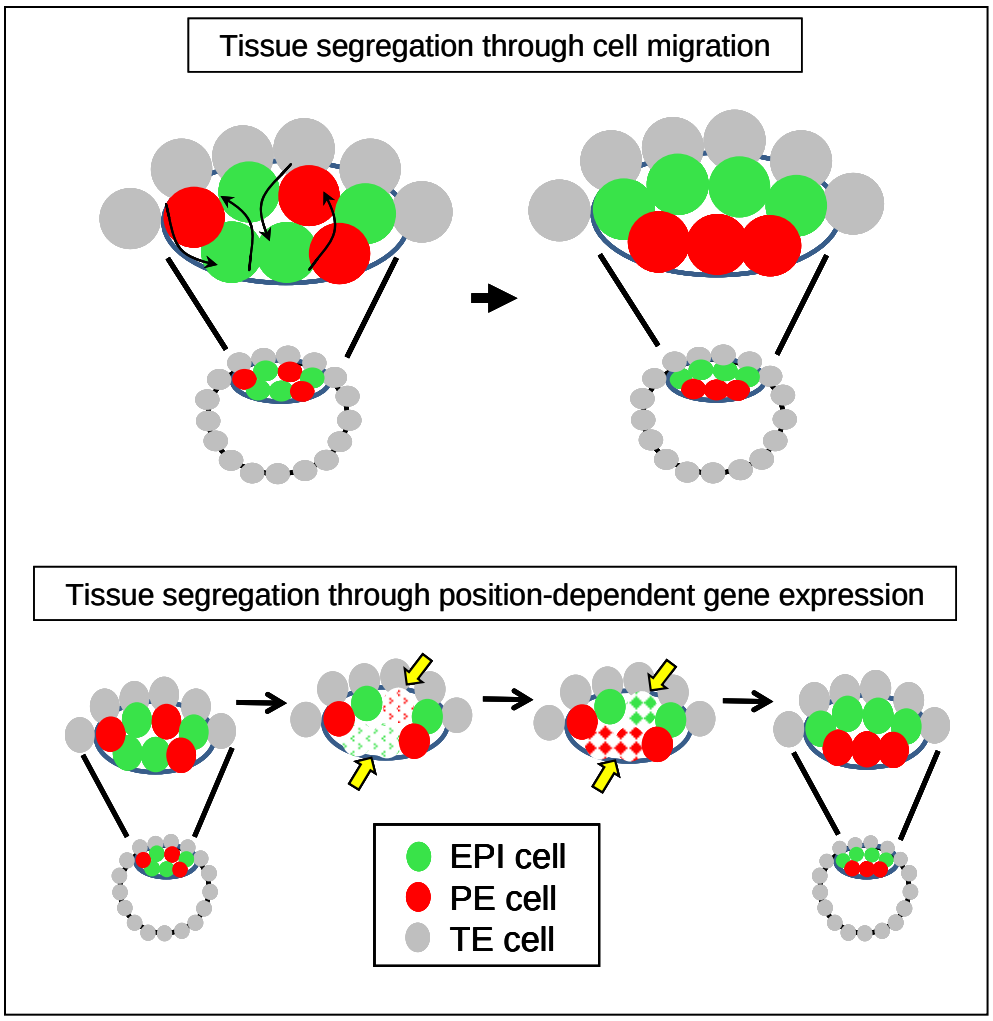


**Fig. 1. Possible mechanisms of how the EPI and PE segregate from each other. A.** Cells that express EPI marker, NANOG (green), will remain in the inner part of the ICM or migrate to the inner part of the ICM, whereas cells that express PE marker, GATA4 (red), will remain in the outer layer of the ICM or migrate to the outer layer of the ICM. **B.** Cells that are located in the inner part of ICM will continue to express NANOG, or will turn off GATA4 expression and turn on NANOG expression. In contrast, cells that are located in the outer layer of the ICM will continue to express GATA4, or will turn off NANOG expression and turn on GATA4 expression. It is possible that a combination of the scenarios depicted in **A** and **B** may also function in EPI and PE segregation.

# CHAPTER 2. MATERIALS AND METHODS

## Animals and Embryos

Female $F_1$ mice (C57BL/6 x DBA/2; National Cancer Institute) were injected with equine chorionic gonadotropin and human chorionic gonadotropin (hCG; Calbiochem) at 48 h apart, and mated with male $F_1$ mice. At 44 h after hCG injection, two cell stage embryos were flushed from the oviducts with FHM HEPES-buffered medium (MR-024-D; Millipore), and cultured up to early blastocyst stage (E3.5) in KSOM-AA (MR-121-D; Millipore) at 37°C with 5% $CO_2$ humidified air. Mice were maintained and handled according to the regulations and guidelines of the Institutional Animal Care and Use Committee.

## Pharmacological Treatment of Embryos with ROCK Inhibitors

Stocks of ROCK inhibitors Y-27632 and Fasudil (Calbiochem) were prepared in dimethyl sulfoxide (DMSO) and water, respectively, and were stored at -20ºC until ready for use. E3.5 blastocysts whose cavity volume was at least half the embryo's size were selected and split into two groups: the ROCK inhibitor-treated group which was cultured in KSOM-AA containing 20 μM Y-27632; and the control group which was cultured in KSOM-AA containing DMSO. Both groups were cultured for 24 h up to E4.5 blastocyst stage. To test the reversibility of the Y-27632 effect, E3.5 blastocysts were cultured in Y-27632 for 24 h and then cultured in KSOM-AA medium for 24 h up to E5.5.

Fasudil treatment was done similarly as the procedure above, in which E3.5 blastocysts whose cavity volume was at least half the embryo's size were selected and

split into four groups: three ROCK inhibitor-treated groups which were cultured in KSOM-AA containing 5 μM, 10 μM, and 20 μM Fasudil; and the control group which was cultured in KSOM-AA. These four groups were cultured for 24 h up to E4.5 blastocyst stage. To test the reversibility of the Fasudil effect, E3.5 blastocysts were cultured in 5 μM, 10 μM, and 20 μM Fasudil for 24 h and then cultured in KSOM-AA medium for 24 h up to E5.5.

**Immunohistochemistry**

Embryos were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS) for 30 min, and permeabilized in PBS containing 0.5% Triton X-100 for 15 min. After blocking with 5% bovine serum albumin in PBS containing 0.1% Tween-20 (PBSw), embryos were incubated in the primary antibody overnight at 4ºC, and incubated in secondary antibody for 2-3 h at 25ºC. Primary antibodies used were mouse anti-CDX2 (1:200; CDX2-88; BioGenex), goat anti-GATA4 (1:400; C-20, sc-1237; Santa Cruz Biotechnology), mouse anti-histones (1:2000; MAB052; Millipore), rabbit anti-NANOG (1:800; RCAB0002P-F; Cosmo Bio), goat anti-POU5F1 (1:200; N-19, sc-8628; Santa Cruz Biotechnology), and mouse anti-POU5F1 (1:100; C-10, sc-5279; Santa Cruz Biotechnology). Secondary antibodies used were donkey anti-rabbit, goat anti-mouse, goat anti-rabbit and rabbit anti-mouse conjugated with Alexa Fluor 488 (1:1000; Invitrogen), or donkey anti-goat, rabbit anti-goat and rabbit-anti mouse conjugated with Alexa Fluor 546 (1:1000; Invitrogen). To stain for actin filaments, phalloidin conjugated with Alexa Fluor 546 was added at a final concentration of 33 nM

in the secondary antibody solution. To visualize nuclei, embryos were stained with 4',6'-diamidino-2-phenylindole (DAPI; Invitrogen) and mounted onto slides in PBSw.

**Microscopy and Image Analysis**

Embryos were observed using Axiovert 200 fluorescence microscope (Carl Zeiss), LSM 5 PASCAL confocal laser scanning microscope (Carl Zeiss), and FV1000 confocal laser scanning microscope (Olympus). For fluorescence microcopy, embryos were imaged at different planes of focus. For confocal microscopy, serial optical sections were obtained at 2 μm intervals under a 40x objective lens with oil. The same settings (i.e., exposure time, pinhole size, detection gain, voltage) were used to record images of embryos from the same experiment. Z-axis projections of confocal images were performed with Zeiss LSM Image Browser or FV Viewer program. To visualize the ICM in three-dimension, projected images of blastocysts were rotated to observe the spatial distribution of the ICM nuclei. LSM Image Browser and FV Viewer programs were also used to examine every optical section of embryos to count nuclei.

**Statistical Analysis**

Data were analyzed by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. Data were presented as mean ± standard error of the mean (SEM).

# CHAPTER 3. RESULTS

## Y-27632 Treatment Inhibits Segregation of EPI and PE Cells in the ICM

To determine whether ROCK activity is necessary for the segregation of EPI and PE cells, blastocysts were treated with Y-27632 from E3.5 to E4.5, which is the period when the ICM normally gives rise to the EPI and PE tissue layers (Chazaud et al., 2006). In the control blastocysts, EPI and PE cells separated into distinct layers (n = 18, Fig. 2A). NANOG-positive EPI cells were positioned in the inner layer of the ICM, whereas GATA4-positive PE cells were positioned in the surface of the ICM, adjacent to the blastocyst cavity. In contrast, Y-27632-treated blastocysts did not show segregation because NANOG-positive cells were mixed with GATA4-positive cells (n = 21, Fig. 2B-D). These results show that Y-27632 treatment prevents the sorting of the EPI and PE cells from one another. This suggests that ROCK activity is required for the segregation of the EPI and PE cells into two layers of tissues in the ICM.

## Y-27632 Treatment Disrupts the Tight Aggregation of the ICM Cells

When blastocysts were initially exposed to Y-27632, the blastocyst cavity dramatically decreased in volume or collapsed. After 24 h of treatment, the cavities re-emerged, although they were smaller in volume relative to the control blastocysts whose cavities were fully expanded. Furthermore, Y-27632-treated blastocysts exhibited varying volumes of the cavity with 43% having a cavity volume at least half the embryo's size (Fig. 2B), 24% having a cavity that is less than half the embryo's size (Fig. 2C), and 33% having a small, nascent-looking cavity (Fig. 2D). The ICM morphology

appeared to be disturbed by Y-27632 treatment (compare Fig. 2A with 2B-D). To examine the ICM morphology more closely, blastocysts were immunostained for the pluripotency marker POU5F1 protein, and the distribution of the ICM nuclei was observed. Control blastocysts had a cohesive ICM (n = 32, Fig. 3A), whereas Y-27632-treated blastocysts had loosely associated ICM cells (n = 35, Fig. 3B). These results show that Y-27632 treatment disrupts the tight aggregation of the ICM cells. This suggests that ROCK activity is required for the intercellular adhesion of the ICM cells.

**Y-27632 Treatment Promotes Cell Division in the ICM Lineage and Decreases Cell Number in the TE Lineage**

To determine whether Y-27632 treatment influences cell division in the blastocyst, embryos were assessed for their total cell number, and ICM and TE cell number. Embryos were immunostained for histone proteins as a general nuclear marker and POU5F1 protein as an ICM marker, so that the number of TE nuclei is the difference between the number of total nuclei and the number of ICM nuclei (Fig. 4). There was significant reduction in total cell number in Y-27632-treated blastocysts (73.5 ± 2.3, mean ± SE; n = 25) when compared to control blastocysts (94.5 ± 2.9; n = 23) (Fig. 4). Interestingly, Y-27632-treated blastocysts have significantly more ICM cells (25.1 ± 1.5, $p < 0.002$) and significantly less TE cells (48.4 ± 2.8, $p < 0.001$) when compared to control blastocysts (19.0 ± 1.0 ICM cells, 75.5 ± 2.5 TE cells). These results show that Y-27632 treatment delays cell division in the blastocyst, which affects the TE lineage more than the ICM lineage. This suggests that ROCK inhibition allows the selective

proliferation of ICM cells, and the selective delay of cell division or selective cell death of TE cells.

To further assess whether Y-27632 treatment disturbs the differentiation of the TE lineage, embryos were immunostained for CDX2 and POU5F1 proteins, which are TE and ICM lineage markers, respectively. The results show that the TE lineage is present in Y-27632-treated embryos (n = 8), as in the control embryos (n = 8) (Fig. 5). Thus, TE lineage formation is independent of ROCK, although ROCK inhibition decreases the number of TE cells.

**The Inhibitory Effect of Y-27632 on Blastocyst Cavity Formation is Reversible**

Previously, it was shown that treatment with Y-27632 starting at the early cleavage stage blocks blastocyst cavity formation (Kawagishi et al., 2004). To determine whether Y-27632 treatment irreversibly impairs embryo development and viability, 8-cell (E2.5) embryos were treated for 24 h up to the early blastocyst (E3.5) stage and were returned to medium without Y-27632 for 24 h of culture up to the late blastocyst (E4.5) stage. The results show that Y-27632-treated embryos (n = 11) remained as a ball of cells at E3.5, which formed blastocysts that were beginning to hatch at E4.5 when cultured in a drug-free medium (Fig. 6). On the other hand, control embryos (n = 11) developed into blastocysts by E3.5, several of which were hatching by E4.5. Thus, the inhibitory effect of Y-27632 on cavitation is reversible and does not destroy embryo development and viability.

**Y-27632-Induced Scattering of the ICM Cells is Irreversible**

To determine whether the tightly aggregated morphology of the ICM can be restored, E3.5 blastocysts were cultured in medium containing Y-27632 for 24 h followed by culture in a drug-free medium for another 24 h up to E5.5. The blastocysts were double immunostained for NANOG and POU5F1 to distinguish the subset of EPI cells in the ICM. The control blastocysts (n = 8) showed POU5F1-positive ICM that appeared as a compacted disc and its inner layer consisted of NANOG-positive EPI cells (Fig. 7Aa). In contrast, scattered ICM cells persisted in all Y-27632-treated embryos and were POU5F1 positive (n = 9, Fig. 7Ab,Ac). In the scattered cells, NANOG expression was highly variable in which 34% of the blastocysts lacked expression as would be expected of the PE lineage, whereas 33% had expression in one cell and 33% had expression in a few cells as would be expected of the EPI lineage.

In a separate set of experiments, blastocysts were double immunostained for GATA4 and POU5F1 to distinguish the subset of PE cells in the ICM. The control blastocysts (n = 8) showed POU5F1-positive ICM that appeared as a compacted disc, and its surface layer consisted of GATA4-positive PE cells (Fig. 7Ba). However, in many of the Y-27632-treated blastocysts (73%, n = 11), ICM cells remained scattered, most of which expressed GATA4 (Fig. 7Bb). A small proportion of Y-27632-treated blastocysts (27%) possessed an ICM disc, although the PE cells were intermingled with POU5F1-positive cells. These results show that the Y-27632-induced scattering of the ICM cells is irreversible. This suggests that the intercellular adhesion of the ICM cells has been disrupted by ROCK inhibition, and that ROCK is essential for the aggregated ICM

morphology. However, in spite of the loss of normal ICM morphology, EPI and PE lineages are present in the dispersed ICM cells.

**Fasudil Treatment Also Inhibits Segregation of EPI and PE Cells in the ICM**

To verify that the phenotypes caused by the Y-27632 treatment is due to the inhibition of ROCK activity, the effect of another type of ROCK inhibitor called Fasudil was examined with the expectation that it would also yield the same phenotypes. Blastocysts were treated with different concentrations of Fasudil at 5 μM, 10 μM, and 20 μM from E3.5 to E4.5. In the control blastocysts, EPI and PE cells separated into two different layers (n = 37, Fig. 8A). NANOG-positive EPI cells were positioned in the inner layer of the ICM, whereas GATA4-positive PE cells were positioned in the surface of the ICM, which is adjacent to the blastocyst cavity. In contrast, Fasudil-treated blastocysts showed varying extent of disturbances in the segregation of the EPI and PE cells (Fig. 8B-D). EPI cells were intermingled with PE cells in 48% of 5 μM-treated embryos (n = 31), 12% of 10 μM-treated embryos (n = 34), and 38% of 20 μM-treated embryos (n = 39). These results show that Fasudil, like Y-27632, can inhibit tissue segregation in the ICM. However, Fasudil appears to be less potent than Y-27632, which disrupts segregation in all treated blastocysts.

Total cell number was determined by counting DAPI-stained nuclei, the number of ICM cells was determined by counting NANOG- and GATA4-positive nuclei, and the number of TE cells was the difference between total cell number and ICM cell number. The results were that total cell number decreased with increasing concentrations of Fasudil (Fig. 9A): there was 50.4 ± 4.5 (mean ± SE) cells in 5 μM-treated blastocysts (n

= 11), 44.6 ± 6.1 cells in 10 μM-treated blastocysts (n = 12), and 30.5 ± 3.4 cells in 20 μM-treated blastocysts (n = 10), which is significantly reduced when compared to control blastocysts (60.6 ± 5.0 cells, $p < 0.001$, n = 9). Similarly, the number of TE cells was significantly reduced by Fasudil treatment at the higher concentrations (Fig. 9A): there was 35.6 ± 4.9 cells at 5 μM, 27.0 ± 5.7 cells ($p < 0.05$) at 10 μM, and 13.0 ± 2.9 cells ($p < 0.001$) at 20 μM, compared to control blastocysts which had 46.7 ± 3.8 cells. On the other hand, blastocysts exhibited modest increases in the number of ICM cells with increasing concentrations of Fasudil, although the increases were not statistically significant (Fig. 9A): 14.7 ± 1.5 cells at 5 μM, 17.6 ± 1.5 cells at 10 μM, and 17.5 ± 2.0 cells at 20 μM, compared to control blastocysts which had 13.9 ± 2.0 cells. Thus, Fasudil selectively decreases the number of TE cells, which is similar to the effect of Y-27632, but it does not influence the number of ICM cells. The number of NANOG-positive EPI cells and GATA4-positive PE cells was compared between control and Fasudil-treated blastocysts (Fig. 9B). The results were that the number of EPI and PE cells was unchanged by Fasudil treatment. Specifically, there were 7.4 ± 0.9 EPI cells in control blastocysts, compared to 7.5 ± 0.9 cells at 5 μM, 8.3 ± 0.8 cells at 10 μM, and 8.6 ± 1.2 cells at 20 μM. On the other hand, there were 6.4 ± 1.2 PE cells in control blastocysts, compared to 7.2 ± 1.1 cells at 5 μM, 9.3 ± 1.1 cells at 10 μM, and 8.9 ± 1.2 cells at 20 μM. Thus, ICM cell proliferation, as well as EPI and PE cell differentiation, are not affected by Fasudil.

Additionally, the ICM morphology appeared to be disturbed by Fasudil treatment in a dose-dependent manner. Control blastocysts have an ICM composed of tightly aggregated cells (n = 37, Fig. 8A). In contrast, as Fasudil concentration increased, so did

the occurrence of loosely associated ICM cells (Fig. 8B-D). This occurred in 48% of embryos treated with 5 μM (n = 31), 76% of embryos treated with 10 μM (n = 34), and 87% of embryos treated with 20 μM (n = 39). However, unlike Y-27632-treated embryos, Fasudil-treated embryos did not yield ICM cells that were separated from the main mass. Taken together, these results confirm that ROCK activity is required for the segregation of the EPI and PE cells, the adhesion of ICM cells, and the normal number of TE cells.

**Fasudil-Induced Loosening of the ICM Cells is Irreversible**

To determine whether the loosening of the ICM disc morphology caused by Fasudil treatment can be reversed, treated E4.5 blastocysts were cultured in a drug-free medium for 24 h up to E5.5. The blastocysts were double immunostained for GATA4 and NANOG to distinguish the PE and EPI cells in the ICM, respectively. The results illustrate that control blastocysts maintained a tightly compacted ICM disc. EPI cells were in the most inner set of ICM cells, closest to the TE, and PE cells were in the periphery of the ICM, adjacent to the blastocyst cavity (n=7, Fig. 10A). However, Fasudil-treated blastocysts still showed a dose-dependent increase in the occurrence of loosely aggregated ICM cells (Fig. 10B-D): 57% of embryos treated at 5 μM (n = 7), 86% of embryos treated at 10 μM (n = 7), and 100% of embryos treated at 20 μM (n = 6). These results show that although the Fasudil-treated blastocysts were cultured subsequently in a drug-free medium, the tightly aggregated ICM morphology was not retrievable. This is consistent with the findings on blastocysts treated with Y-27632 followed by culture in a drug-free medium.

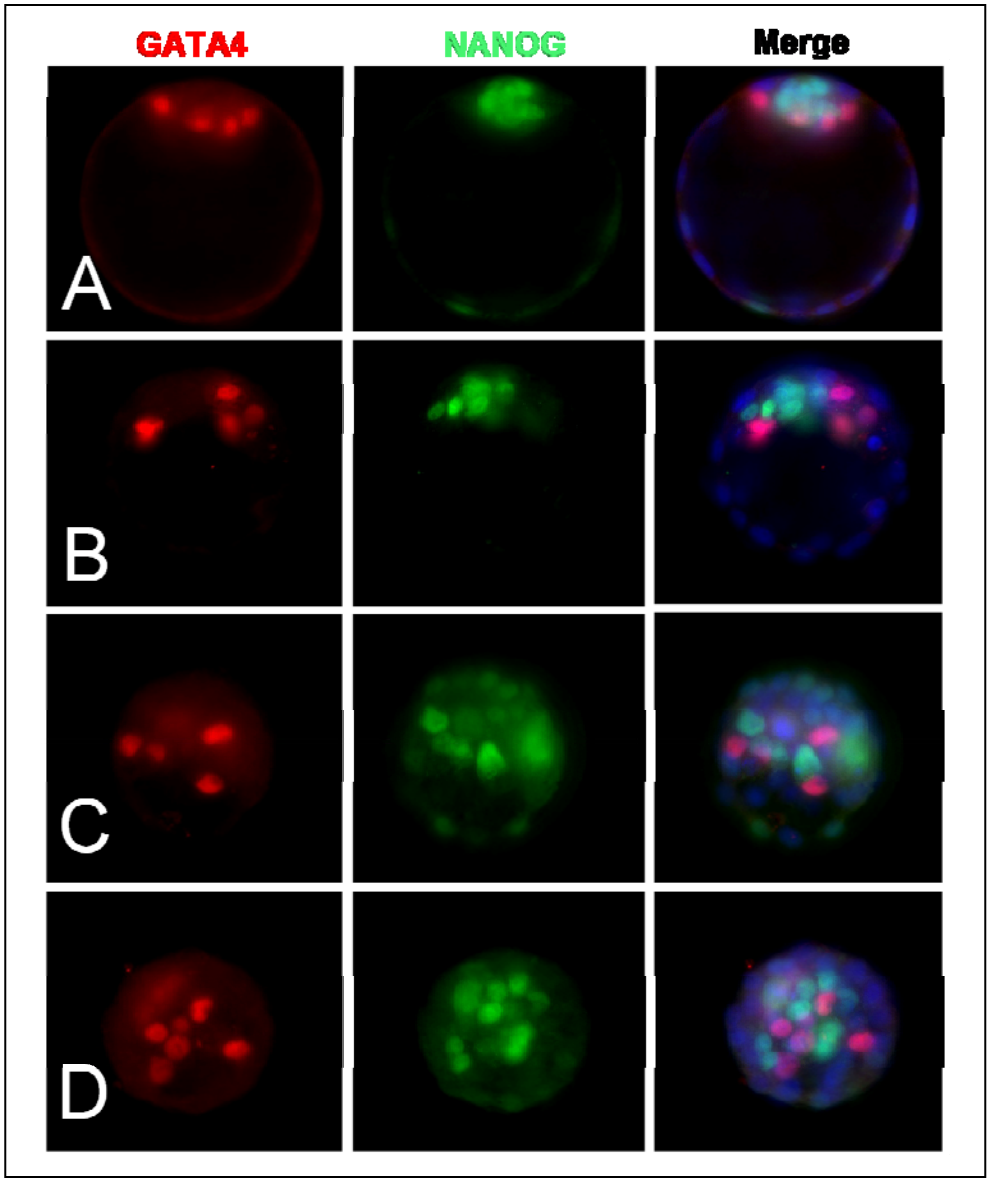


**Fig. 2. Y-27632 treatment inhibits segregation of EPI and PE cells in the ICM.** Blastocysts (E4.5) are double immunostained for PE marker GATA4 (red) and EPI marker NANOG (green), whereas nuclei are stained with DAPI. Images are obtained by fluorescence microscopy. **A.** Control blastocyst (n = 32) shows evident segregation between PE and EPI. **B-D.** Three examples of Y-27632-treated blastocysts (n = 35) show loosely aggregated ICM cells, in which GATA4-positive PE and NANOG-positive EPI cells have not sorted into separate tissues. Note that blastocysts show different sizes of cavity, see Results for details.

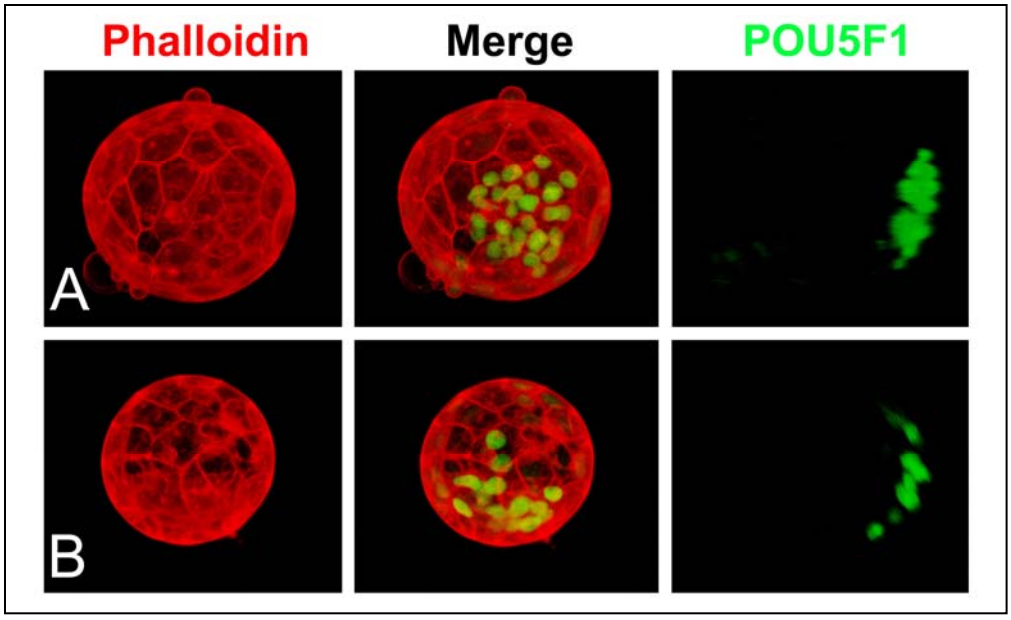


**Fig. 3. ICM cells become loosely aggregated with Y-27632 treatment.** Actin filaments are stained with phalloidin (red) to show cell boundary, whereas ICM cells are immunostained for pluripotency marker POU5F1 (green). Blastocysts (E4.5) are positioned to show the entire ICM disc (merge), and rotated to show the ICM from the side (right). Imaging is carried out using confocal microscopy. **A.** In the control blastocyst (n = 32), ICM nuclei are aggregated. **B.** In the Y-27632-treated blastocyst (n = 35), ICM nuclei appear to be scattered and less cohesive.

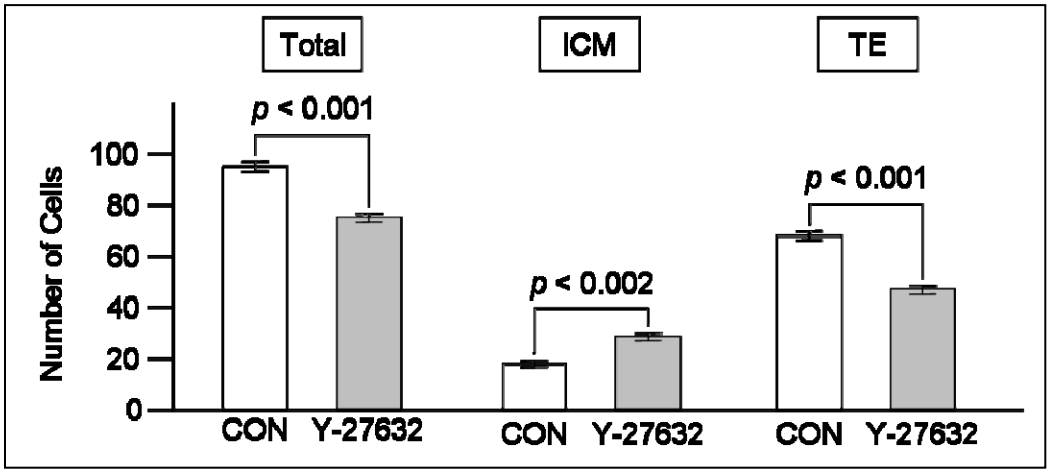


**Fig. 4. Cell number in Y-27632-treated blastocysts.** Blastocysts (E4.5) are immunostained for POU5F1 and histones to aid in counting ICM nuclei and total nuclei as an indicator of cell number, respectively. Blastocysts are imaged with confocal microscopy at 2 μm thickness, and each optical section is examined for number of nuclei. For total cell number, Y-27632-treated blastocysts at 20 μM (n = 25) show a significant decrease as compared to control blastocysts (n = 23). For ICM cell number, Y-27632-treated blastocysts show a significant increase as compared to control blastocysts. For TE cell number (total nuclei minus ICM nuclei), Y-27632-treated blastocysts show a significant decrease as compared to control blastocysts.

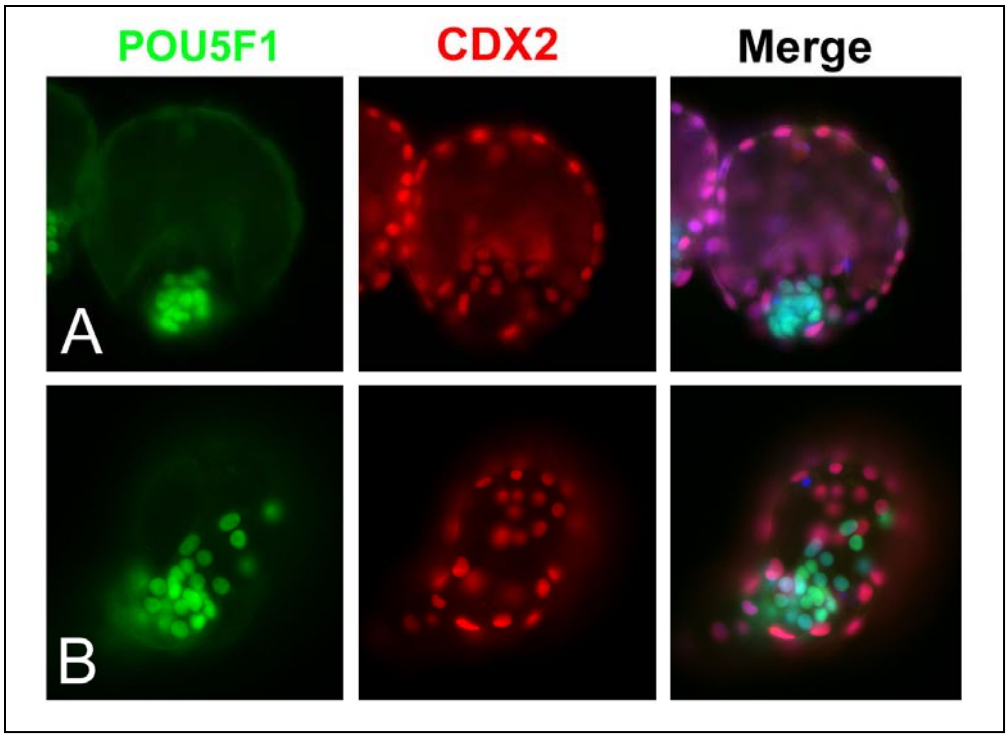


**Fig. 5. TE differentiation is apparently unaffected by Y-27632 treatment.** Blastocysts (E4.5) are double immunostained for pluripotency marker POU5F1 (green) and TE marker CDX2 (red), whereas nuclei are stained with DAPI. Images are obtained by fluorescence microscopy. **A.** In the control blastocyst (n = 8), POU5F1 localized to the ICM and CDX2 to TE. **B.** In the Y-27632-treated blastocyst (n = 8), ICM cells are loosely aggregated and several cells are scattered (50%).

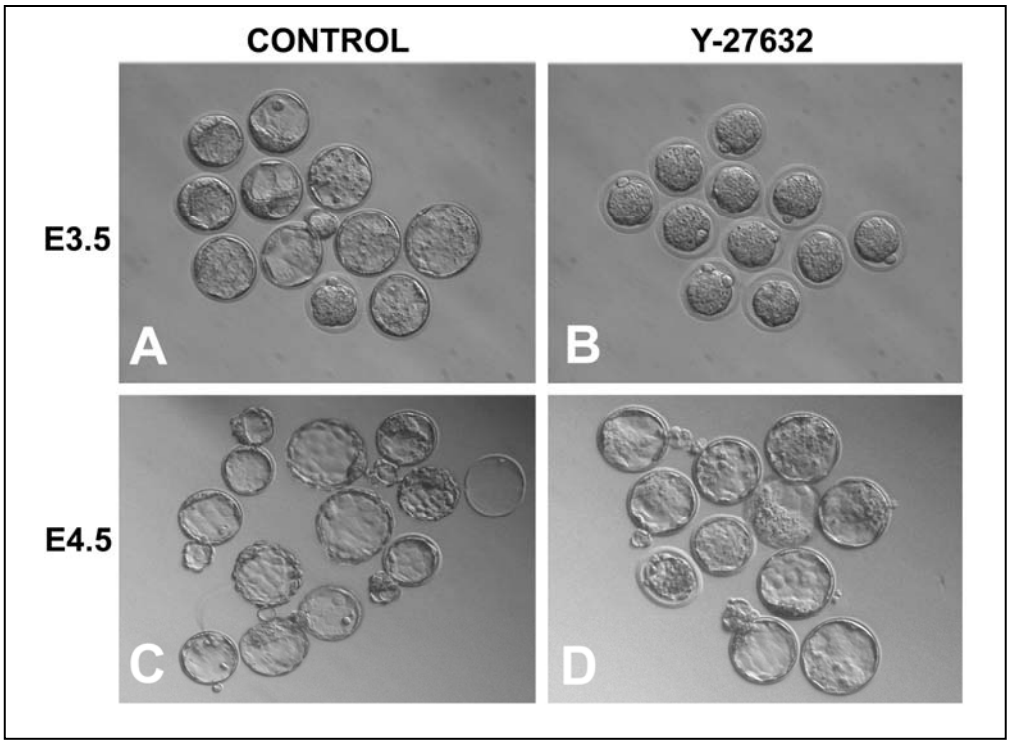


**Fig. 6. The effect of Y-27632 is reversible with respect to cavity formation. A-B.** Y-27632-treatment from 8-cell to 32-cell stage (E2.5-E3.5, 24 h) inhibits blastocyst formation, resulting in embryos appearing as a solid mass of cells. **C-D.** Subsequent culture of embryos in **(B)** under Y-27632-free condition (E3.5-E4.5, 24 h) rescues blastocyst formation. Thus, the effect of Y-27632 is reversible. Control n = 11, Y-27632-treated n = 11.

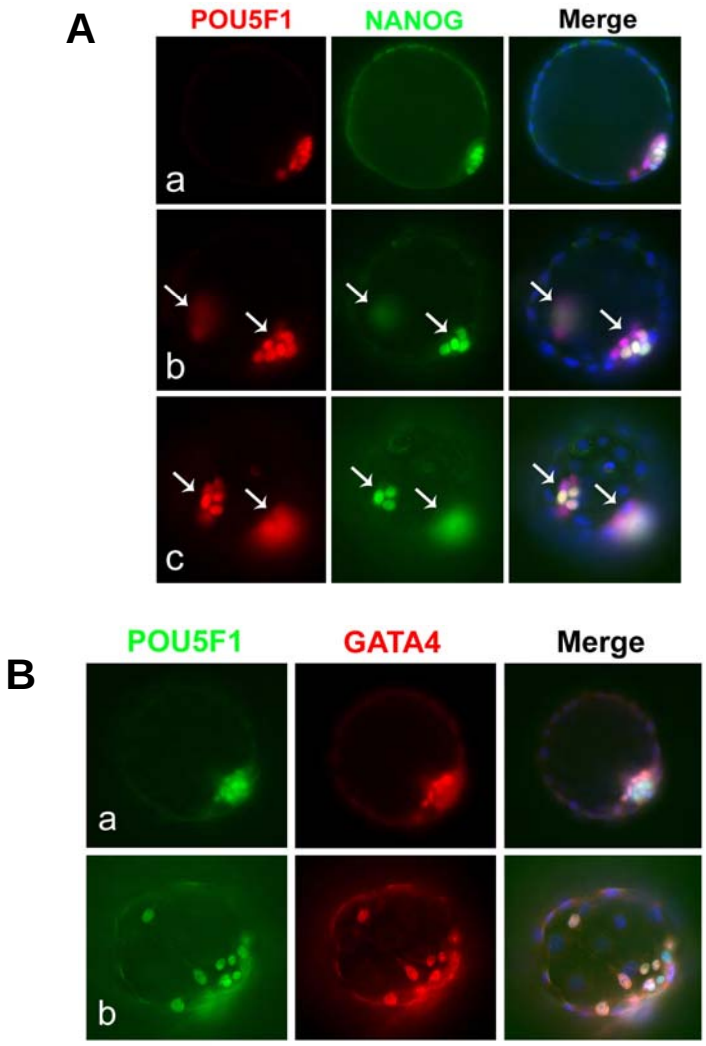


**Fig. 7. Y-27632 treatment induces irreversible scattering of ICM cells.** Blastocysts (E3.5) are treated with Y-27632 for 24 h, followed by 24 h of drug-free culture, and processed for immunohistochemistry at E5.5. Images are obtained by fluorescence microscopy. **A.** Blastocysts are immunostained for pluripotency factors POU5F1 (red) and NANOG (green), whereas nuclei are stained with DAPI. Control blastocyst (**Aa**, n = 8) shows one ICM disc. Different planes of focus of Y-27632-treated blastocyst (**Ab,Ac**, n = 9) show ICM cells which separated into two clusters (arrows). POU5F1 is expressed in all ICM cells, whereas NANOG expression is variable (see Results for details). **B.** Blastocysts are immunostained for POU5F1 (green) and PE marker GATA4 (red), whereas nuclei are stained with DAPI. Control blastocyst (**Ba**, n = 8) has an ICM disc in which all cells express POU5F1, and PE has segregated as a superficial cell layer in the ICM. In the Y-27632-treated blastocyst (**Bb**, n = 11), ICM cells are scattered, most of which express GATA4. A small proportion of Y-27632-treated blastocysts retained an ICM disc with PE cells intermingled with EPI cells (see Results for details).

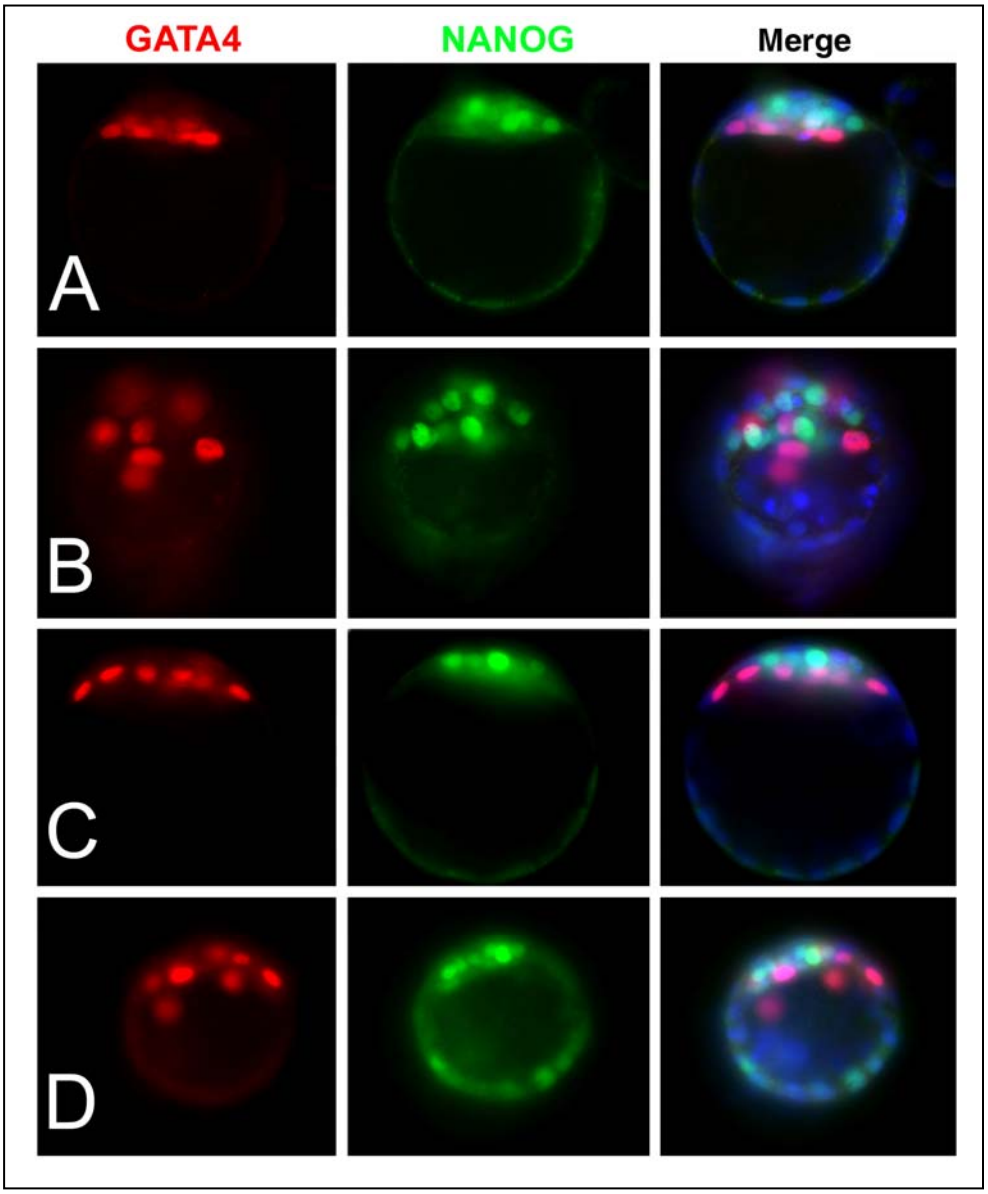


**Fig. 8. Fasudil treatment inhibits segregation of PE and EPI cells in the ICM.** Blastocysts are treated with various concentrations of Fasudil (5 μM, 10 μM, 20 μM) for 24 h from E3.5 to E4.5. Blastocysts are immunostained for PE marker GATA4 (red) and EPI marker NANOG (green), whereas nuclei are stained with DAPI. Images are obtained by fluorescence microscopy. **A.** Control blastocyst shows segregation of PE and EPI layers (n = 37). **B-D.** A proportion of blastocysts treated with 5 μM (**B**, 48%, n = 31), 10 μM (**C**, 12%, n = 34), and 20 μM (**D**,38%, n = 39) show disrupted segregation of PE and EPI cells, as well as cells scattered away from the main ICM mass.

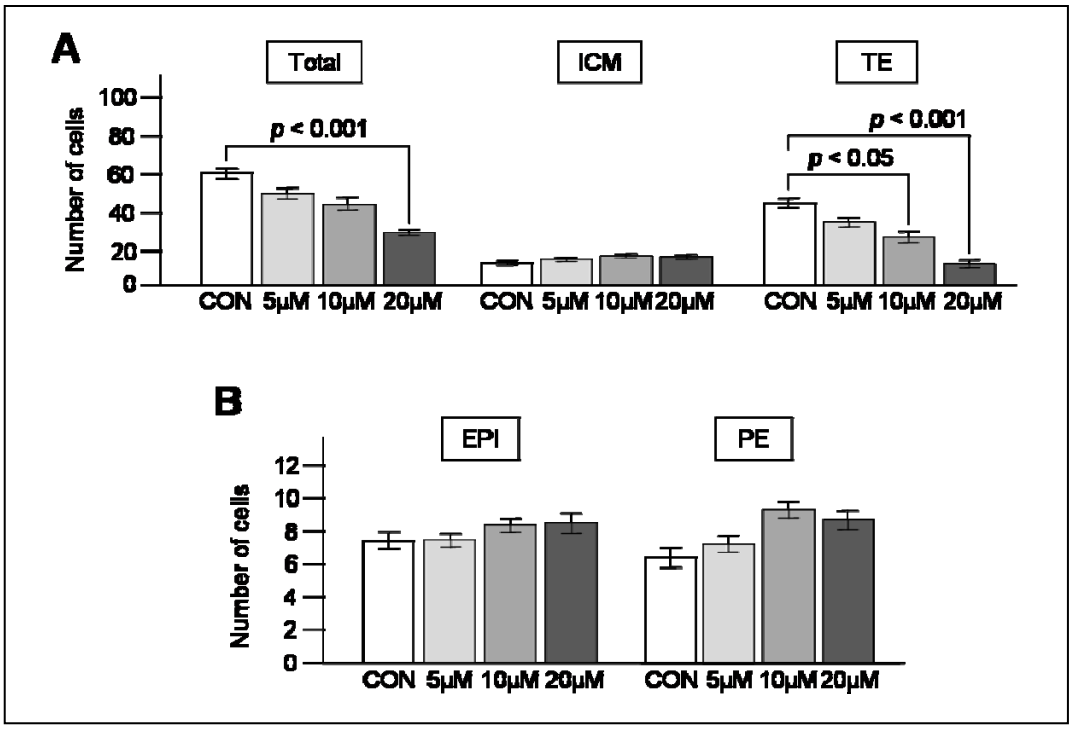


**Fig. 9. Cell number in Fasudil-treated blastocysts.** Blastocysts are treated with various concentrations of Fasudil (5 μM, 10 μM, 20 μM) for 24 h from E3.5 to E4.5, and immunostained for NANOG (EPI), GATA4 (PE), and stained with DAPI (nucleus). Blastocysts are imaged using confocal microscopy with a thickness of 2.0 μm, and each optical section is examined for number of nuclei. **A.** Total cell number in blastocysts decreases with increasing concentrations of Fasudil, and is significantly reduced at the highest concentration. ICM cell number is not significantly changed at all concentrations of Fasudil. TE cell number (total nuclei minus EPI/PE nuclei) decreases significantly at higher concentrations of Fasudil. **B.** As Fasudil concentration increases, the number of EPI and PE cells is not significantly changed. Control embryos (n = 9), 5 μM-treated embryos (n = 11), 10 μM-treated embryos (n = 12), and 20 μM-treated (n = 10).

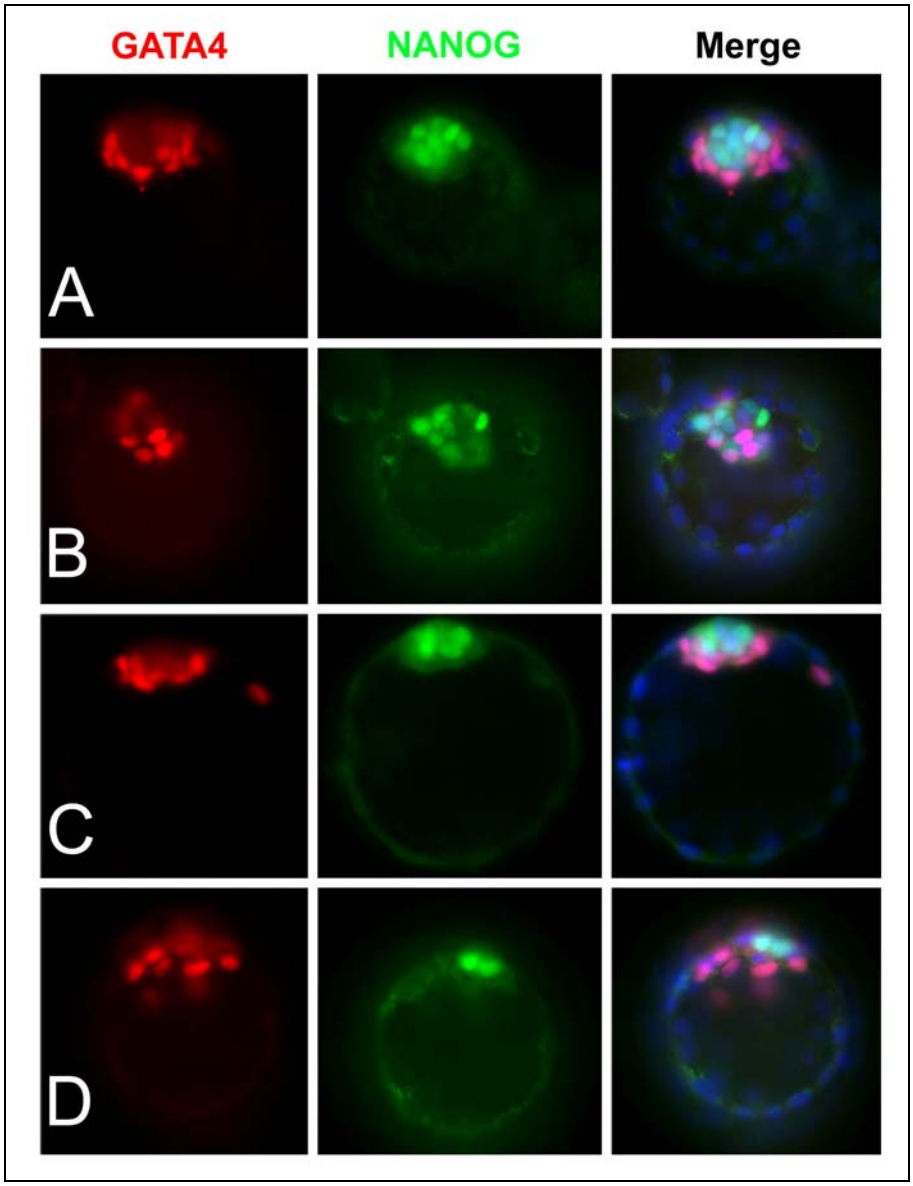


**Fig. 10. Fasudil treatment induces irreversible scattering of PE and EPI cells.** Blastocysts (E3.5) are treated with Fasudil (5 μM, 10 μM, 20 μM) for 24 h, followed by 24 h of drug-free culture, and processed for immunohistochemistry at E5.5. Blastocysts are immunostained for PE marker GATA4 (red) and EPI marker NANOG (green), whereas nuclei are stained with DAPI. Images are obtained by fluorescence microscopy. **A.** Control blastocyst (n = 7) has tightly aggregated ICM disc with an inner layer of EPI cells that segregated from an outer layer of PE cells. **B-D.** Blastocysts that are treated with 5 μM (**B**, 57%, n=7) of Fasudil, 10 μM (**C**, 86%, n = 7), and 20 μM (**D**, 100%, n = 6) show increasing incidence of scattered cells, apparently mostly the PE cells.

# CHAPTER 4. DISCUSSION

I have investigated the role of ROCK in the segregation of the two cell lineages, EPI and PE, in the blastocyst stage of the mouse embryo. During the developmental period that EPI and PE segregate into two distinct tissue layers, embryos were exposed to ROCK inhibitor, Y-27632. I observed disturbance of proper sorting of EPI and PE cells. Another ROCK inhibitor, Fasudil, revealed similar disturbances in cell sorting. Thus, EPI and PE segregation is ROCK-dependent. Inhibition of ROCK also caused disturbance in the ICM morphology, such that the ICM was not maintained as a tightly aggregated unit. Additionally, these disturbances were not reversible when Y-27632- and Fasudil-treated blastocysts were cultured additionally in a drug-free medium. Taken together, these observations show that cell sorting and maintenance of the ICM morphology are restricted to occur at the blastocyst stage prior to uterine implantation (E3.5 to E4.5). Interestingly, although the ROCK inhibitors reduced cell numbers in the blastocysts, Y-27632 in particular promoted ICM cell proliferation, which is consistent with its capability of enhancing the survival of dissociated human embryonic stem cells (Olson et al., 2008).

## Early and Later Effects of ROCK Inhibition on Cell Lineage Segregation During Pre-implantation Development

In the mouse embryo, the first two cell lineages to develop are the ICM and TE. The zygote undergoes a series of mitosis, yielding the morula stage (about 16-cell stage). At the morula stage, the inner blastomeres become the pluripotent ICM population and

express POU5F1, and the outer blastomeres become differentiated into TE and express CDX2. However, at 16-cell stage, the inner and outer blastomeres are not restricted to become either ICM or TE according to studies done by Suwinska et al. (2008). They showed that when only inner or only outer blastomeres were re-aggregated together, such recombinant embryos expressed CDX2 correctly in the outer cell layer. Furthermore, both types of recombinant embryos gave rise to healthy, fertile offspring, although the recombinants derived from only inner blastomeres had lower implantation rates. When the same experiment was repeated using 32-cell stage embryos, the inner-blastomere recombinants successfully developed into blastocysts, expressing CDX2 in the outer cell layer, but they did not implant. In contrast, the outer-blastomere recombinants became trophoblastic vesicles. These observations suggest that by 32-cell stage, TE and ICM cell fate becomes specified. Thus, Suwinska et al. (2008) was able to provide evidence that TE and ICM cell fate becomes cell position-dependent at the 32-cell stage.

The second cell lineage formation occurs in the ICM from which the EPI and PE cells emerge. Previous studies showed that there is a combination of mechanisms that segregate EPI and PE cells into two distinct tissue layers. Plusa et al. (2008) immunostained embryos for EPI and PE markers, as well as performed live imaging of embryos with GFP-expressing PE cells, using total cell number to stage embryos. They observed that in embryos with up to 33 cells (early blastocysts), EPI and PE markers exhibited overlapping expression. At less than 80 cells, most blastocysts still did not show EPI and PE segregation. However, at 80-100 cells, almost half of the blastocysts showed segregation, and it was at over 100 cells when most blastocysts possessed separate EPI and PE tissue layers. Furthermore, live imaging revealed cell behaviors

which suggested possible mechanisms by which PE separated from EPI, and they are: cell migration to the superficial layer, changes in levels of GFP expression according to cell position, and apoptosis which was seen as nuclear fragmentation by cells that are positioned in the wrong tissue layer.

Prior research by Kawagishi et al. (2004) focused on the earlier effect of ROCK inhibition on pre-implantation development. They exposed embryos, starting from 2-cell stage, to various concentrations of Y-27632 (1 μM, 10 μM, and 100 μM). As the concentration of Y-27632 increased, the amount of embryos that remained as morulae increased and blastocyst development became limited, preventing TE formation. Thus, the effect of Y-27632 is only significant after the 8-cell stage. Inhibition of blastocyst cavity formation was reversible when Y-27632-treated embryos were further cultured in drug-free medium for 24 hours. Observations revealed that as the Y-27632 dose increased, the amount of embryos whose cavity was restored also decreased. Thus, the effect of Y-27632 is dose dependent.

By comparison, my research is the first to focus on the later effect of ROCK inhibition in pre-implantation development. Exposure to Y-27632 during E3.5 initially collapsed the blastocyst cavity, which later re-emerged, and the embryos still have EPI and PE marker expression present. When blastocysts were cultured in a drug-free medium for 24 hours from E4.5, there were no apparent effects on TE cell differentiation, but rather only ICM cells showed disturbance with respect to morphology and segregation. This suggests that the ROCK pathway does not likely interface with FGF signaling that is essential for EPI and PE cell differentiation (Takaoka & Hamada, 2012). Previous studies showed that exogenous FGF in the culture medium of mouse embryonic

stem cells inhibited NANOG expression (Hamazaki et al., 2006), whereas inhibition of FGF signaling stabilized NANOG expression (Lanner et al., 2010), indicating that cell-cell interactions are necessary for EPI and PE cell differentiation.

**The Role of Cell Adhesion in Tissue Segregation**

Differential cell adhesion plays a role in helping to sort mixtures of cells into separate tissues. Steinberg and Takeichi (1994) demonstrated the role of differential cell adhesion by testing two populations of cell lines that differ only in amount of homophilic adhesion molecules on their surface with the aid of the following constructs: 1) PLβ2, an efficiently expressed P-cadherin inserted downstream of the β-actin promoter, and 2) PLs5, a less efficiently expressed P-cadherin inserted downstream of simian virus 40 thymidine kinase promoter. These two cells lines were mixed and cultured together with PLβ2 being labeled with DiI. Cell sorting was evident with PLβ2 cells forming clusters and collecting within PLs5 cells. Another experiment demonstrated cell sorting by allowing fragments of PLβ2- and PLs5-expressing aggregates to adhere together. Results were similar to the previous experiment such that PLβ2 aggregates remained clustered within PLs5 aggregates, forming a spheroid core of PLβ2 enveloped by a PLs5 outer shell. The authors discussed that PLβ2 cells expressed about more than 20 times of P-cadherin than PLs5 cells.

For my experiments, I observed that blastocyst exposure to Y-27632 led to ICM cells becoming loosely associated. This suggests a problem or disturbance of cell adhesion amongst the EPI and PE cells. These results were also witnessed when another ROCK inhibitor, Fasudil, was used in replacement of Y-27632. This suggests that the

role of ROCK does not interfere with cell differentiation of EPI and PE, but instead may play an important role in differential cell adhesion that affects the segregation of tissues in the ICM.

The mechanisms responsible for sorting out EPI and PE into two distinct layers in the blastocyst remain unclear. However, Yang et al. (2002) gave insight into the role of DAB2 (disabled homolog 2) protein in cell sorting because embryos lacking DAB2 possessed PE cells that were not able to migrate to the ICM surface. Morris et al. (2002) supplemented this observation by hypothesizing that DAB2 is an adaptor protein, which attaches actin filament-associated myosin VI to endocytic vesicles to impact polarization of cells. This possibility illustrates that DAB2 facilitates transfer of cell adhesion proteins via transport of endocytic vesicles, which may be controlling the amount of cell adhesion proteins by removing them from the apical side of a cell and localizing them to the basal side or for degradation. ROCK inhibition possibly disrupts the actin filaments, resulting in disturbance or aberrant cell adhesion interactions between cells. Thus, regulation of differential cell adhesion may be a possible mechanism behind cell sorting into different tissue layers (Takaoka & Hamada, 2012).

In my ROCK experiments, blastocysts were inhibited from E3.5 to E4.5, the stage in which EPI and PE are supposed to be segregating from one another. Perhaps, DAB2 gets localized asymmetrically in the PE cells at E3.5, resulting in the asymmetric localization of cell adhesion proteins. During this time period, inhibition of ROCK activity may have disrupted the DAB2 localization in the PE precursor cells, resulting in failure to separate from EPI cells.

**Comparison of ROCK Inhibitors and Knockout Mouse Technology as Tools to Investigate the Mechanisms of Pre-implantation Development**

ROCK inhibitors Y-27632 and Fasudil were used in the present study to investigate the role of ROCK in pre-implantation development, specifically in tissue segregation in the ICM of the mouse blastocyst. These pharmaceutical drugs are widely used in research and are very convenient because after blastocysts are treated with a ROCK inhibitor, it is possible to observe effects on cellular behaviors in about a week. However, the use of ROCK inhibitors can have off target effects, because the drugs may interact with other proteins in addition to ROCK1 and ROCK2. Thus, it will be informative to use knockout mouse embryos to analyze the role of the ROCK isoforms. However, obtaining knockout embryos involves a much longer process, such that it may take up to a year or more to get sufficient numbers of homozygous knockout embryos for each ROCK isoform because they occur at such low frequency.

Previous studies revealed that the single knockout of each ROCK isoform had no effect on pre-implantation development (Thumkeo et al., 2003; Shimizu et al., 2005) because of the possibility of one isoform compensating by functioning in place of the other. Thus, it will be necessary to generate ROCK1/ROCK2 double knockout embryos. However, it was shown previously that there is maternal expression of ROCK1 and ROCK2 (Kawagishi et al., 2004; Kono, 2009). Maternal ROCK isoforms may function during pre-implantation development so that the double knockout embryos may not show any phenotype. If this were the case, then the knockout embryos to generate would be those that do not express maternal and zygotic ROCK1/ROCK2 to reveal ROCK function in pre-implantation development.

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