Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo

Short title: PARD6B is essential for trophectoderm formation

Summary sentence: PARD6B regulates formation of trophectoderm epithelium, and is involved in the up-regulation of trophectoderm-lineage transcription factor CDX2.

Keywords: blastocyst, cavity formation, epithelium, paracellular permeability seal, TJP1 (ZO-1), PRKCZ (aPKCzeta), Cdx2, Tead4, Nanog

Vernadeth B. Alarcon
Institute for Biogenesis Research, Department of Anatomy, Biochemistry and Physiology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii

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2Correspondence: V.B. Alarcón, Institute for Biogenesis Research, Department of Anatomy, Biochemistry and Physiology, John A. Burns School of Medicine, University of Hawaii, 651 Ilalo Street, BSB 163, Honolulu, Hawaii 96813. FAX: 808-692-1962; email address: vernadet@hawaii.edu

ABSTRACT
In preimplantation mouse development, the first cell lineages to be established are the trophectoderm (TE) and inner cell mass. TE possesses epithelial features, including apical-basal cell polarity and intercellular junctions, which are crucial to generate a fluid-filled cavity in the blastocyst. Homologs of the partitioning defective (par) genes in Caenorhabditis elegans are critical regulators of cell polarity. However, their roles in regulating TE differentiation and blastocyst formation are still unclear. Here, the role of mouse Pard6b, a homolog of par-6 gene and a component of the PAR-aPKC complex, was investigated. Pard6b expression was knocked down by microinjecting RNA interference construct into zygotes. Pard6b-knockdown embryos cleaved and compacted normally but failed to form the blastocyst cavity. The cavitation failure is likely due to defective intercellular junctions, as Pard6b knockdown caused abnormal distribution of actin filaments and TJP1 (ZO-1) tight junction (TJ) protein and interfered with cavitation in chimeras containing cells from normal embryo. Defective TJ formation may be due to abnormal cell polarization, as the apical localization of PRKCZ (aPKCzeta) was absent in Pard6b-knockdown embryos. Pard6b knockdown also diminished the expression of CDX2, a TE-lineage transcription factor, in the outer cells. TEAD4, a transcriptional activator that is required for Cdx2 expression and cavity formation, was not essential for the transcription of Pard6b. Taken together, Pard6b is necessary for blastocyst morphogenesis, particularly the development of TE-specific features, namely the apical-basal cell polarity, formation of TJ, paracellular permeability sealing, and up-regulated expression of Cdx2.
INTRODUCTION
In mouse development, the first cell lineages to form are the trophectoderm (TE) and inner cell mass (ICM) at the blastocyst stage. TE is a layer of cells that surround the fluid-filled blastocyst cavity, and is the progenitor of trophoblast cells which facilitate implantation and generate the placenta. ICM is situated in the cavity as a clump of cells, and is the progenitor of all fetal tissues and extraembryonic mesoderm and endoderm. ICM cells are undifferentiated pluripotent cells from which embryonic stem (ES) cell lines can be derived. By contrast, TE cells are differentiated as epithelium. Like other types of epithelial cells in the animal body, the TE cell possesses apical-basal polarity and intercellular junctional complexes, such as tight junctions (TJs) and adherens junctions (AJs) which maintain the blastocyst cavity [1]. TE also harbors distinct sodium ion carriers, such as Na+/H+ exchanger and Na+/K+-ATPase which drive fluid flow across the epithelial layer and into the blastocyst cavity [2-5].

Epithelialization begins at the late 8-cell stage as compaction when blastomeres polarize and flatten, and is completed in the next two cell divisions at around the 32-cell stage when a nascent blastocyst cavity forms [1]. While epithelial features start to emerge at the late 8-cell stage, cell lineage formation towards either TE or ICM is not determined until around 32-cell stage. The blastomeres before the 32-cell stage are capable of contributing to both TE and ICM [6-8]. The commitment towards TE or ICM is most likely linked to the spatial location of the blastomeres within an embryo at around 32-cell stage, as external blastomeres develop to TE and internal ones to ICM [6]. Although the molecular nature of the spatial location of blastomeres is beginning to be elucidated, how it controls the specification of the two cell lineages are still elusive [9, 10].

While the molecular mechanisms that regulate the initial step of TE and ICM lineage specification are not clear, gene-knockout studies have revealed several transcription factors that play critical roles in the formation and maintenance of the two cell lineages. POU5F1 (also known as OCT4) is a POU-domain transcription factor, and is essential for the maintenance of the ICM lineage [11]. It is initially expressed ubiquitously, but becomes enriched in ICM by the late blastocyst stage [12, 13]. Although the POU5F1-null embryo gives rise to a morphologically normal blastocyst with a TE layer surrounding a cavity, an internal clump of cells expresses a TE marker, indicative of defective ICM [11]. CDX2 is a caudal-type homeodomain transcription factor, and is critical for the maintenance of the TE lineage [14, 15]. Like POU5F1, CDX2 is also expressed in most blastomeres at early stages, but later becomes restricted to TE by the blastocyst stage [16-18]. In the CDX2-null embryo, the initial phase of epithelialization and cavity formation take place normally, but by the late blastocyst stage the integrity of the epithelium is lost, resulting in the collapse of the cavity [15]. TEAD4, a TEA-domain transcription factor, has been identified as a key regulator of TE formation. In the TEAD4-null embryo, the expression of CDX2 is markedly diminished, and the blastocyst cavity does not form [19, 20]. While TEAD4 is expressed ubiquitously in the blastocyst [20], a recent study has shown that the TE-specific activity of TEAD4 to up-regulate CDX2 is mediated by YAP1 and WWTR1, which bind to TEAD4 and act as transcriptional co-activators [9]. How TEAD4 regulates the formation of the blastocyst cavity and TE epithelialization is yet to be elucidated.
TE epithelialization is accompanied by apical-basal cell polarization. The polarity manifests in all blastomeres at the late 8-cell stage and is maintained in subsequent stages in the outer cells of the embryo. During cell polarization, several PAR (partitioning defective) proteins, including components of the PAR-aPKC complex, are distinctly localized along the apical-basal axis [21, 22]. par genes were originally identified in the nematode *Caenorhabditis elegans* as genes that regulate the anterior-posterior axis and pattern of asymmetric cleavages in the zygote [23]. The critical components of the PAR-aPKC complex, namely PAR3, PAR6, and atypical protein kinase C (aPKC), are localized to the anterior cortex of the *C. elegans* zygote [24-26]. The homologs of the PAR-aPKC complex are found in a wide range of systems, and play a broader role in cell and tissue morphogenesis, including the establishment of the apical-basal polarity in various epithelial cells [27-29]. PAR3 and PAR6 are PDZ domain-containing proteins that act as scaffolds to bind and regulate aPKC, a serine/threonine kinase. The localized activation of aPKC is crucial for the action of the PAR-aPKC complex, although the phosphorylation targets of aPKC that are responsible for the apical-basal polarization are not fully elucidated. In the mouse embryo, PAR3 and aPKC homologs regulate the orientation of cell cleavage planes, and cell polarity and adhesion, which altogether can influence the allocation of blastomeres to an outer or inner position in the blastocyst [21, 30]. However, whether the PAR-aPKC complex is essential for the lineage specification and epithelialization of TE is not clear. Also, the functional role of PAR6 homologs, another component of the PAR-aPKC complex, in mouse blastocyst formation has not been investigated.

In the present study, the role of a PAR6 homolog, specifically PARD6B, in TE formation was investigated. There are three PAR6 homologs in mouse, namely PARD6A, PARD6B, and PARD6G, which are encoded by different genes [31]. Among these, PARD6B is the major gene that is expressed during preimplantation development, and neither PARD6A nor PARD6G is expressed at a detectable level [22, 32]. Here, the specific knockdown of *Pard6b* during early development using RNA interference construct revealed that PARD6B plays essential roles in the development of critical features of TE, such as the formation of the blastocyst cavity and junctional complexes, the apical localization of PRKCZ (also known as aPKCζ), and the up-regulation of *Cdx2* expression.

**MATERIALS AND METHODS**

*Cell Culture and Plasmid Transfection*

P19 mouse embryonal carcinoma cells (American Type Culture Collection, Manassas, VA) were cultured in MEM Alpha Medium containing 2.5% fetal bovine serum and 7.5% calf serum (Invitrogen, Carlsbad, CA). A day before transfection, 2 × 10^4 cells were plated per well in a 24-well plate. Transfection of plasmid DNA was performed using Lipofectamine2000 (Invitrogen), according to the manufacturer’s instruction. The full-length cDNA encoding mouse *Pard6b* was isolated by RT-PCR from P19 cells using the primers (forward: 5′-CCA TGG TTG TGT GTG CAG CGG CAG CTG TCC GG-3′ and reverse: 5′-ATT TGC GGC CGC GTG TCT CTG GCA GGT GTG GAG CCT AGA A-3′), and subcloned into the *NcoI*/*NotI* sites of pCS2+MT vector to generate the fusion of MYC-epitope tag to the N-terminus of PARD6B protein. All the shRNA plasmids used in this study were obtained commercially (Sigma-Aldrich, St. Louis, MO). *Pard6b* shRNA #1, #2, #3, #4 and #5 plasmids correspond to TRCN0000054684, TRCN0000054686, TRCN0000054687, TRCN0000054683 and TRCN0000054685, respectively. *Tead4* shRNA plasmid corresponds to TRCN0000015875. Non-target shRNA and
enhanced green fluorescent protein (Egfp) shRNA plasmids, which correspond to SHC002 and SHC005, respectively, were used interchangeably as control shRNA plasmids. For stable transfection, P19 cells were transfected with shRNA plasmid that was linearized with SfI, and cultured in the presence of 10 μg/ml puromycin for at least 10 days.

Quantitative Reverse Transcription and Polymerase Chain Reaction (qRT-PCR)
Total RNA was extracted using TRI Reagent (Sigma-Aldrich), according to the manufacturer’s instruction. For P19 cells, cDNA was synthesized from 1 μg of total RNA, using oligo dT(18) primer and M-MLV Reverse Transcriptase (Promega, Madison, WI) in a 25 μl reaction volume. For embryos, the entire total RNA extracted from each sample (containing 15-25 embryos) was used to synthesize cDNA in a 12.5 μl reaction volume. Quantitative PCR was performed using iCycler Thermal Cycler with MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). 0.5 μl of cDNA was amplified using iQ SYBR Green Supermix (Bio-Rad) in a 20 μl reaction volume with the following condition: the initial denaturation at 94°C (5 min) followed by up to 45 cycles of 94°C (15 sec), 60°C (20 sec) and 72°C (40 sec). The sequences of PCR primers are found in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org). Gapdh and Actb (also known as β-actin) were used to normalize the expression levels of all other genes for P19 cell and embryo samples, respectively. Each experiment was carried out using at least three independent sets of samples, and the results were presented as mean ± standard deviation.

Animals, Embryos, and Chimeras
The protocol for animal handling and treatment was reviewed and approved by the Institutional Animal Care and Use Committee. F1 (C57BL/6 × DBA/2) female mice (National Cancer Institute, Frederick, MD) were superovulated by intraperitoneal injections of equine chorionic gonadotropin and human chorionic gonadotropin (hCG, Calbiochem, La Jolla, CA), and were mated with F1 (C57BL/6 × DBA/2) males or with the homozygous transgenic males that ubiquitously express the Egfp transgene [33] under the CD1 (Charles River Laboratories, Wilmington, MA) background. At 20 hours (h) after the hCG injection, fertilized eggs were flushed with EmbryoMax FHM HEPES Buffered Medium (MR-024-D, Millipore, Billerica, MA) from the oviducts, and dissociated from cumulus cells using hyaluronidase [34]. Fertilized eggs were cultured in EmbryoMax KSOM with 1/2 amino acids, glucose and phenol red (MR-121-D, Millipore) at 37°C with 5% CO2 humidified air. ESGRO Complete Serum-Free Clonal Grade Medium (Millipore) was used as Embryonic Stem Cell Medium (ESCM) to culture embryos in a specific experiment. Chimeric embryos were generated as described previously [34]. Briefly, the zona pellucida was removed from embryos at the 8-cell stage with 0.5% Pronase (Roche Applied Science, Indianapolis, IN) in FHM, and two denuded embryos were placed together in a depression well, which was made with the aid of the DN-09 aggregation needle (Biological Laboratory Equipments Maintenance and Service, Budapest, Hungary).

Microinjection of shRNA Plasmid
Knockdown of specific gene expression during preimplantation development by means of the microinjection of shRNA plasmid into mouse fertilized eggs has been documented previously [35]. In the present study, shRNA plasmids were purified, using HiSpeed Plasmid Midi Kit (Qiagen, Los Angeles, CA), GenElute HP Plasmid Midiprep Kit (Sigma-Aldrich) or PureYield Plasmid Midiprep System (Promega) according to the manufacturers’ instructions. Prior to
microinjection, shRNA plasmids were diluted to 10 ng/µl in the Injection Buffer (5 mM Tris [pH 7.4], 0.1 mM EDTA, and 0.1 mg/ml Fast Green FCF [Sigma-Aldrich]), and passed through a 0.22 µm filter (Millex-GV, Millipore). Microinjection needles were made from glass capillaries with a filament (BF100-78-10, Sutter Instruments, Novato, CA), backfilled with shRNA plasmid solution, and attached to a FemtoJet air pump (Eppendorf, Westbury, NY). With the aid of NK-2 micromanipulators (Eppendorf), pronuclear microinjection [34, 35] was carried out on fertilized eggs in an FHM drop in a Petri dish under Axiovert 200 inverted microscope with Hoffman modulation contrast optics (Carl Zeiss, Thornwood, NY). At 23-25 h after hCG administration, one of the pronuclei was injected with shRNA plasmid solution that was expelled from the tip of a microinjection needle with constant pressure until the pronucleus expanded by about 20-30% in diameter by visual estimation. Accordingly, the amount of injected shRNA plasmid was estimated to be approximately 0.1-0.2 femtogram per egg. Injected eggs were transferred into KSOM drops for further culture.

Time-lapse Video Microscopy
Time-lapse recording of embryo development was performed as described previously [36]. Briefly, a Petri dish, in which a 20 µl drop of the KSOM medium was placed on a poly-D-lysine-coated coverglass (BD Biosciences, Bedford, MA) and overlaid with mineral oil, was positioned in Heating Insert P (PeCon, Erbach, Germany), whose temperature and CO2 concentration were regulated by Tempcontrol 37-2 and CO2-Controller (PeCon), respectively. The Heating Insert P was enclosed in Incubator XL-3 (PeCon), which was attached to Axiovert 200. Eight-cell stage embryos were placed in the KSOM drop, and re-positioned with the aid of a fine glass needle attached to Model MWO-202 micromanipulator (Narishige, Tokyo, Japan). Snap-shot images were captured every 20 min using the AxioCam MRm digital camera, controlled by the AxioVision software (Carl Zeiss). The Incubator XL-3 was covered with a black plastic sheet during recordings.

Immunocytochemistry and Other Fluorescence Labeling
P19 cells and embryos were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS) for 20-30 min. Samples were washed in PBS containing 0.1% Tween-20 (PBSw) and permeabilized in 0.5% TritonX-100 in PBS for 15 min. After blocking with 5% bovine serum albumin in PBSw, they were incubated in the primary antibody overnight at 4°C, and incubated in the secondary antibody for 1-2 h. The primary antibodies used were mouse anti-MYC (1/50, 9E10, Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-histones (1/2000, MAB052, Millipore) [37], rabbit anti- PARD6B (1/100, sc-67393, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-CDH1 (1/50, #33-4000, Invitrogen), rabbit anti-CLDN4 (1/800, #36-4800, Invitrogen), rabbit anti-TJP1 (1/200, #40-2200, Invitrogen), mouse anti-PRKCZ (1/200, sc-17781, Santa Cruz Biotechnology), mouse anti-CDX2 (1/800, CDX2-88, BioGenex, San Ramon, CA), goat anti-POU5F1 (1/200, sc-8628, Santa Cruz Biotechnology), and rabbit anti-NANOG (1/200, Cosmo Bio, Tokyo, Japan). Secondary antibodies used were goat antimouse, rabbit anti-goat and goat anti-rabbit conjugated with Alexa Fluor 488, or rabbit anti-mouse and goat anti-rabbit conjugated with Alexa Fluor 546 (1/1000, Invitrogen). To localize actin filaments, phalloidin conjugated with Alexa Fluor 546 (Invitrogen) was used at a final concentration of 33 nM. To visualize apoptotic nuclei by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) was used according to the manufacturer’s instruction. Stained embryos were
mounted on slides in ProLong Gold antifade reagent with DAPI (Invitrogen) or Vectashield with propidium iodide (Vector Laboratories, Burlingame, CA).

**Microscopy and Image Analysis**

Specimens were observed with Axiovert 200 fluorescence microscope and LSM 5 PASCAL laser scanning confocal microscope (Carl Zeiss). The same settings (e.g., exposure time, pinhole size, and detection gain) were used to record images of embryos to allow comparison of fluorescence intensities among embryos that were stained in the same batch. For confocal microscopy, serial optical sections were imaged at 1-2 µm intervals under a 40× objective lens. Z-axis projection of confocal images was performed using the LSM Image Browser program (Carl Zeiss) with the following setting: 10% threshold, 70% ramp, 30% maximum opacity and 1.0 brightness for histone, CDX2, POU5F1, NANOG, and propidium iodide staining; 0% threshold, 70% ramp, 30% maximum opacity and 1.0 brightness for TJP1 and actin filament staining; 0% threshold, 100% ramp, 10% maximum opacity and 3.0 brightness for TUNEL. The fluorescence intensity of PARD6B staining was measured by the AxioVision program using the images captured with AxioCam MRm. To determine the ratio of CDX2 to POU5F1 staining intensities and the ratio of NANOG to nuclear staining intensities, images of individual optical sections were opened with the ImageJ program (http://rsb.info.nih.gov/ij), the area that encompasses each nucleus was selected using the elliptical tool, and the intensities of red color (for CDX2 or nuclei) and green color (for POU5F1 or NANOG) were measured using the Measure RGB Plugin function. To measure the intensity of TUNEL, Z-axis projection images were converted to binary images, and the size of white area was measured as TUNEL-positive regions using ImageJ.

**RESULTS**

**Pard6b Down-regulation Interferes with Blastocyst Formation**

The effectiveness of commercial shRNA plasmids to knockdown Pard6b expression was first tested using mouse P19 embryonal carcinoma cell lines. qRT-PCR analysis of transiently transfected cells showed that three out of the five Pard6b-specific shRNA sequences examined reduced Pard6b mRNA level by 70-80% of the control level (Fig. 1A). To determine the efficacy of knockdown on protein expression, P19 cells were co-transfected with one of the shRNA plasmids (designated #2) and the plasmid which robustly express MYC-tagged PARD6B. This approach was taken because endogenous PARD6B protein was only weakly detectable by immunostaining in P19 cells in spite of robust mRNA expression. The result was that MYC-tagged PARD6B protein from the co-transfected plasmid was diminished by shRNA #2 plasmid (Fig. 1B). In addition, when P19 cells were stably transfected with the Pard6b shRNA #2 plasmid, Pard6b mRNA level was reduced down to 15 ± 6% (mean ± standard deviation, n = 3) of that in unmanipulated P19 cells, further demonstrating the effectiveness of the shRNA plasmid. Importantly, P19 cells that were stably transfected with the Pard6b shRNA #2 plasmid appeared healthy, as their growth efficiency was similar to unmanipulated P19 cells, i.e., doubling time of the former is 9.3 h on average whereas that of the latter is 9.4 h on average. These results suggest that the Pard6b shRNA #2 plasmid is effective in suppressing PARD6B expression without causing detrimental effects on cell growth. Thus, the Pard6b shRNA #2 plasmid was selected for use in all subsequent experiments, unless specifically stated otherwise.
To determine whether Pard6b is necessary for preimplantation development, zygotes at the pronuclear stage were injected with Pard6b shRNA plasmid or control plasmid (encoding Egfp shRNA or non-target shRNA). Injected zygotes were cultured and allowed to develop until the late blastocyst stage. When the rate of the initial cleavages by the blastomeres was compared between the two injection groups (Table 1), similar numbers of embryos reached the 2-cell stage by 41-44 h post-hCG, and the 4-8 cell stage by 65-68 h post-hCG. These results indicate that the Pard6b shRNA plasmid does not interfere with the early blastomere cleavages in the embryo.

To closely monitor development from the 8-cell to late blastocyst stage, shRNA plasmid-injected embryos were observed with the aid of time-lapse video microscopy. Both Pard6b shRNA plasmid-injected and control-injected embryos compacted at the late 8-cell stage at 68-76 h post-hCG (Fig. 1C). Subsequently, about 95% of control-injected embryos cavitated and formed blastocysts between 88 and 104 h post-hCG. By contrast, most of Pard6b shRNA plasmid-injected embryos did not cavitate or were delayed in cavitation, while they exhibited a highly compacted, smooth appearance. Specifically, only about 10% of the Pard6b shRNA plasmid-injected embryos had cavitated by 104 h post-hCG (Fig. 1D). When cultured further up to the late blastocyst stage, some Pard6b shRNA plasmid-injected embryos started to exhibit small cell fragments on the surface (117 h snap shot in Supplemental Figure S1) which was likely due to apoptosis (see Fig. 5, E and F).

To determine whether the inability of Pard6b shRNA plasmid-injected embryos to cavitate was due to developmental delay, embryos were immunostained for histone protein and nuclei were counted as the indicator of cell number. Analysis was carried out when most of the control-injected embryos reached the early blastocyst stage (100 h post-hCG) which was before the Pard6b shRNA plasmid-injected embryos underwent cell fragmentation. While nuclei in the Pard6b shRNA plasmid-injected embryos were packed more closely together than in the control-injected embryos, the two groups did not display a significant difference in cell numbers (Fig. 2, A and B), indicating that they underwent similar rates of cell division. Thus, the failure of blastocyst formation in Pard6b shRNA plasmid-injected embryos was not due to cleavage arrest or developmental delay.

To confirm the effectiveness of the Pard6b shRNA plasmid in suppressing Pard6b expression in embryos, the level of Pard6b mRNA was analyzed by qRT-PCR at approximately 100 h post-hCG, the equivalent of early blastocyst stage (E3.5). Pard6b mRNA was significantly down-regulated by approximately 70% in the Pard6b shRNA plasmid-injected embryos compared to the control-injected embryos (Fig. 2C), showing the effectiveness of the Pard6b shRNA plasmid. Consistent with the reduction in Pard6b mRNA level, PARD6B protein was also diminished in the Pard6b shRNA plasmid-injected embryos (Fig. 2D). In control-injected embryos at 16-32 cell stage, PARD6B protein had a distinctly polarized localization, being enriched in the apical domain and also moderately distributed in cell-cell boundaries and cytoplasm, consistent with the previous observation in unmanipulated embryos [22]. By contrast, immunostaining was considerably weaker in the Pard6b shRNA plasmid-injected embryos with little indication of the apical PARD6B distribution. Densitometry analysis of immunostaining intensity further corroborated that the Pard6b shRNA plasmid significantly down-regulated the level of PARD6B protein (Fig. 2D).
Pard6b is Necessary for Epithelium Integrity

The formation and maintenance of the blastocyst cavity depends on the paracellular sealing of TE cells [38]. This raises the possibility that Pard6b-knockdown embryos are unable to cavitate because they have defective paracellular junctions, namely AJ and TJ. CDH1 (also known as E-cadherin) and actin filaments are components of AJ. CDH1 is the major transmembrane protein constituent of AJ, and is essential for compaction and blastocyst cavity formation [1, 7]. When expression was examined by immunostaining, CDH1 protein was localized at the cell-cell boundaries in all the control-injected (n = 6) and Pard6b-knockdown (n = 8) embryos (Fig. 3, A and B). This showed that the intercellular distribution of CDH1 was not disturbed by Pard6b knockdown. This is consistent with the morphological observation that the down-regulation of Pard6b did not interfere with compaction (Fig. 1C). By contrast, actin filament distribution was impaired by Pard6b knockdown. Actin filaments are typically distributed in the cell cortex and are associated with the AJ through CDH1 [39]. Additionally, disturbance of actin filaments by a pharmacological inhibitor causes the collapse of the blastocyst cavity [40, 41]. In all of the control-injected embryos examined at the early blastocyst stage (n = 37), actin filaments were detected by phallodin staining as continuous lines at the apical edge of cell-cell boundaries (Fig. 3C). In Pard6b-knockdown embryos, phalloidin staining was detectable at the apical edge of cell-cell boundaries in only about half (56.1%, n = 41) the embryos at the equivalent stage of the control-injected embryos. The rest (43.9%) of the Pard6b-knockdown embryos exhibited abnormal distribution of actin filaments, i.e., phalloidin staining was indistinct at the apical edge of cell-cell boundaries, and instead it appeared to be more enriched throughout the apical cortex of outer cells compared to the control-injected embryos (Fig. 3D). Thus, with respect to AJ, the distribution of CDH1 at the cell-cell boundary was independent of Pard6b, while that of the actin filaments depended on Pard6b.

Next, the components of TJ, namely CLDN4 (claudin4) and TJP1 (also known as ZO-1), were examined by immunostaining in Pard6b-knockdown embryos. CLDN4, which is a transmembrane protein of the TJ complex [42], was distributed as continuous lines at the apical edge of cell-cell boundaries in the majority of Pard6b-knockdown (80%, n = 30) embryos, as seen in control-injected embryos (92.6%, n = 27) (Fig. 3, E and F). By contrast, the distribution of TJP1, a cytoplasmic component of TJ [43], was severely impaired by Pard6b knockdown. In the majority of the control-injected embryos (88.1%, n = 42) examined at the early blastocyst stage, TJP1 localized as continuous lines at the apical edge of cell-cell boundaries (Fig. 3G). However, in Pard6b-knockdown embryos examined at the equivalent stage, a similar TJP1 staining pattern was observed in only a small fraction (15.0%, n = 40). Many (85.0%) of the Pard6b-knockdown embryos displayed an abnormal TJP1 staining pattern, i.e., the staining was discontinuous and indistinct (Fig. 3H). In some embryos (38.2% of the abnormal cases), aberrant thick rings of TJP1 staining were observed in the apical surface of the outer cells (see arrows in Supplemental Figure S2), although the nature of these ring-like structures is currently unclear. Thus, with respect to TJ, CLDN4 distribution was independent of Pard6b, while TJP1 distribution depended on Pard6b.

To confirm that the defect in TJP1 formation is caused specifically by the knockdown of Pard6b and not by an unknown off-target effect of shRNA #2 plasmid, embryos were injected with shRNA #4 plasmid whose sequence differs from #2 but is as effective in knocking down Pard6b.
in P19 cells (Fig. 1A). The results were that shRNA #4 plasmid interfered with cavitation as observed by time-lapse video microscopy, and the TJP1 distribution was also disturbed (see Supplemental Figure S3). This further supports that the TJP1 defects are specifically caused by the knockdown of Pard6b.

**Pard6b is Essential for Paracellular Sealing to Maintain the Blastocyst Cavity**

The abnormal distributions of actin filaments and TJP1 suggest that paracellular sealing between the outer cells is defective which may be the reason for the failure of Pard6b-knockdown embryos to cavitate. Alternatively, it is possible that paracellular sealing is intact in Pard6b-knockdown embryos, but ion transport-mediated fluid movement is impaired which may account for cavitation failure. To distinguish between these possibilities, Pard6b-knockdown embryos were chimerized with normal embryos and assayed for blastocyst formation at E3.5 (Fig. 4, A and B). If Pard6b knockdown impairs paracellular sealing, chimeras would not be able to retain a cavity because any inward movement of fluid across the epithelium of normal cells would be canceled by the leakage of fluid through impaired sealing between Pard6b-knockdown cells. On the other hand, if paracellular sealing is intact between Pard6b-knockdown cells, fluid would still be retained and a cavity would form. Pard6b shRNA plasmid or non-target shRNA plasmid was injected into Egfp-transgenic one-cell embryos, which were allowed to develop to the 8-cell stage. Many of the control chimeras formed a blastocyst cavity (80%, n = 15) (Fig. 4, B and C). By contrast, only a small fraction of the Pard6b-knockdown chimeras cavitated (15%, n = 21), and the rest did not. This indicates that PARD6B is essential to establish the functional paracellular permeability seal in TE.

**Apical Localization of PRKCZ is Dependent on Pard6b**

Defective epithelium development, as shown in the experiments above, is indicative of abnormal TE formation in Pard6b-knockdown embryos. Formation of functional epithelium is dependent on apical-basal cell polarization [44]. In mouse embryos, apical-basal cell polarity develops after compaction in the outer blastomeres, which is evidenced by the apical localization of PRKCZ [21, 22]. To determine whether PARD6B is necessary for specifying the apical cell domain, morulae were immunostained for PRKCZ. The distinct apical localization of PRKCZ was observed in all control-injected embryos (n = 10, Fig. 3I) at 90 h post-hCG. Also, PRKCZ localized at cell-cell boundaries and in the cytoplasm at a lower level, as described previously [21, 22]. On the other hand, the apical localization of PRKCZ was absent in the majority (84.6%, n = 13, Fig. 3J) of Pard6b-knockdown embryos and there was an increase in cytoplasmic PRKCZ. This suggests that PARD6B is necessary to establish the normal apical cell domain.

**TE-lineage Transcription Factor Cdx2 is Diminished by Pard6b Knockdown**

Defective paracellular junction formation and apical-basal cell polarization suggest that TE formation is abnormal in Pard6b-knockdown embryos. To further assess the extent of abnormality in TE formation, expression levels of transcription factors that are involved in lineage specification were analyzed in knockdown embryos by qRT-PCR (Fig. 2C). Analysis was carried out at approximately 100 h post-hCG when the control embryos were at the early
blastocyst stage (E3.5) while the knockdown embryos have not yet undergone cell fragmentation. The expression level of TE-lineage gene Cdx2 was significantly reduced, which is consistent with abnormal TE epithelialization as previously described in Cdx2-null embryos [15]. However, the expression level of Tead4, which acts upstream of CDX2 [9], was unaffected in the knockdown embryos. The expression level of ICM-lineage gene Pou5f1 [11] was apparently unaffected by Pard6b knockdown. However, Nanog, another ICM-specific gene which encodes a homeodomain transcription factor and is required for sustaining pluripotency of ICM [45, 46], was significantly up-regulated. These results suggest that Pard6b knockdown interferes with TE lineage formation and that the embryo retains a more pluripotent feature.

CDX2 protein is initially ubiquitously expressed and eventually localizes in the outer cells of the embryo, while POU5F1 protein is expressed throughout at the early blastocyst stage [16, 18]. To gain insight into their spatial expression patterns, embryos were double-immunostained for CDX2 and POU5F1 protein. In control-injected embryos, CDX2 localized in the nuclei and was more intense in the outer cells, although it was also detectable in the inner cell nuclei (n = 13, Fig. 5A). In the Pard6b-knockdown embryos (n = 14), CDX2 appeared less intense throughout the embryo. The relative expression levels between CDX2 and POU5F1 was examined by measuring their fluorescence intensity in the outer and inner cells. In control-injected embryos, the ratio of CDX2 to POU5F1 was higher in the outer cell nuclei (Fig. 5B). By contrast, in Pard6b-knockdown embryos, the ratio was significantly lower in the outer cell nuclei than in the control, but the ratio in the inner cell nuclei was similar. Furthermore, in many of the knockdown embryos (62%, n = 34), strong expression of NANOG protein was found in most of the nuclei including those in the outer cell layer (Fig. 5, C and D). In some knockdown embryos (38%), NANOG protein expression was distinct in, at most, half of the nuclei. In many control embryos (75%, n = 28), equal to or less than half of the nuclei had distinct NANOG protein expression, and most outer cell nuclei had decreased or no NANOG staining. Taken together with the qRT-PCR data, these results show that CDX2 expression in the outer cells is not as elevated in Pard6b-knockdown embryos as in the control embryos. These results suggest that PARD6B acts upstream of CDX2 and is necessary to up-regulate CDX2 expression in TE.

Cell Death in Pard6b-knockdown Embryos
One of the characteristics of the Cdx2-knockout embryos is the increased incidence of apoptosis at the late blastocyst stage [15]. In connection with this, Pard6b-knockdown embryos, in which CDX2 level was significantly reduced, exhibited cell fragments on the surface after 110 h post-hCG, as observed by time-lapse video microscopy (see Supplemental Figure S1). To examine apoptosis in Pard6b-knockdown embryos, TUNEL labeling was carried out at the blastocyst stage. At the early blastocyst (E3.5) stage, TUNEL signal was not evident in control-injected (n = 8) and Pard6b-knockdown (n = 8) embryos. However, by the late blastocyst (E4.5) stage, there was an increase in TUNEL signal on the surface of Pard6b-knockdown embryos (Fig. 5, E and F). Interestingly, apoptosis was significantly reduced by culturing Pard6b-knockdown embryos in embryonic stem cell medium (ESCM, Fig. 5F) which is used for sustaining embryonic stem cells (i.e., ICM-like cells). These results further support the possibility that Pard6b-knockdown embryos display features of ICM, which is consistent with the Cdx2-knockout embryos [15].
**Pard6b Expression is Independent of Tead4**

Recent studies showed that Tead4 is important for TE formation [19, 20]. TEAD4, in cooperation with YAP1, acts as a transcription activator to up-regulate Cdx2 expression in the outer cells of the embryo [9]. Tead4 is also essential for blastocyst cavity formation through yet unknown mechanisms. During normal development, Pard6b mRNA level is up-regulated between 4-cell to morula stages, which is around the time of TEAD4 expression [19, 20, 32], raising the possibility that the transcriptional activation of Pard6b gene is regulated by TEAD4. To test whether the expression of Pard6b gene is dependent on TEAD4, Tead4-knockdown embryos were produced by injecting specific shRNA plasmid into zygotes at the pronuclear stage. Time-lapse video microscopy showed that Tead4 shRNA plasmid-injection interfered with cavity formation (see Supplemental Figure S4, A and B), which is reminiscent of the morphological phenotype of Tead4-null embryos [19, 20]. On the other hand, about 85% of control-injected embryos cavitated and formed blastocysts at 83-104 h post-hCG. qRT-PCR analysis showed that the Tead4 shRNA plasmid was effective, as Tead4 mRNA level was significantly down-regulated compared to control-injected embryos (Fig. 2E). Consistent with the Tead4-null embryo phenotype, Cdx2 mRNA level was significantly reduced by Tead4 knockdown, while ICM markers Pou5f1 and Nanog were expressed with the latter being significantly up-regulated. Pard6b mRNA level was not affected by Tead4 knockdown, indicating that transcription of Pard6b is not dependent on TEAD4.

**DISCUSSION**

This study shows that PARD6B is essential for normal blastocyst formation in the mouse embryo. Pard6b knockdown resulted in cavitation failure without compromising blastomere cleavage or compaction. A cause for the cavitation failure is likely to be abnormal epithelial junctions, which results in defective paracellular permeability sealing in the outer cells of the embryo. Defective epithelial junctions may be due to abnormal apical-basal cell polarization, as demonstrated by the lack of apical localization of PRKCZ. Furthermore, the outer cells in the Pard6b-knockdown embryo fail to up-regulate CDX2 normally, leading to aberrant TE differentiation.

TJ is essential for paracellular sealing of the outer cells as they undergo epithelialization to generate the blastocyst cavity [42, 43, 47, 48]. The involvement of PAR6 homologs in TJ formation has been investigated in mammalian cultured epithelial cells, in which epithelium formation is experimentally induced after the disruption of cell-cell contacts. During epithelialization in Madin-Darby canine kidney (MDCK) cells, overexpression of wild-type PAR6 or mutant PAR6 lacking its aPKC-binding site interfered with TJ formation [49, 50]. These studies suggest that the mutant PAR6 acts in a constitutively active manner and that PAR6 is a negative regulator of TJ formation. By apparent contrast, the present study suggests that PARD6B is a positive regulator of TJ formation, as it was essential for normal TJP1 distribution and paracellular sealing. Possibly, the right amount of PAR6 activity is critical so that either excessive or insufficient PAR6 is detrimental to normal TJ formation. Another possibility is that TJ formation is regulated differently by PAR6 in the two experimental systems, that is, the epithelialization in MDCK cells and TE formation in the mouse embryo. Indeed, the epithelial cell line is stimulated to reform TJ whereas the embryo establishes TJ in TE for the first time during development. TE development in the mouse blastocyst serves as a unique model for studying TJ formation within a developmentally relevant context, and would be an informative
system to further elucidate the molecular mechanisms of how Par genes as well as other cell polarity regulators contribute to TJ formation.

PAR6 forms a tripartite complex with PAR3 and aPKC, and serves as a scaffold for the action of aPKC [27-29]. Members of the complex localize to the apical side of cells in the mouse embryo [21-22], suggesting they function together at the apical domain of the plasma membrane. Indeed, the apical localization of PRKCZ was lost with Pard6b knockdown, indicating that PARD6B is required for the normal localization of PRKCZ. Previously, it has been shown that the suppression of Pard3 or Prkci (also known as aPKCiota or aPKClambda) correlates with increase in asymmetric cleavages which yield one outer and one inner daughter cell [21, 30]. This suggests that the PAR-aPKC complex plays roles in the regulation of cleavage orientation. Interestingly, Pard3 or Prkci-suppressed embryos still form blastocyst cavity. With Pard6b knockdown, the cells located in the surface of the embryo failed to form functional TE, and such embryos did not form blastocyst cavity. The difference in cavity formation between the Pard3/Prkci-suppressed embryos and Pard6b-knockdown embryos may be due to PARD6B playing additional roles essential for paracellular sealing of TE. Alternatively, the other isoforms of PAR3 and aPKC which are expressed during preimplantation development [22, 30] may compensate for the suppression of Pard3/Prkci. Another possibility is the difference in methodology. In the Pard3/Prkci studies, the suppression is achieved either by siRNA injection or overexpression of dominant-negative form through synthetic mRNA injection. By contrast in the present study, Pard6b was knocked down by injection of shRNA-encoding plasmid DNA. Pard6b shRNA might be continuously expressed from the plasmid which provided long-lasting knockdown to disrupt paracellular sealing.

In the present study, the microinjection of specific shRNA plasmid was used to knock down endogenous Pard6b gene products in mouse embryos, which resulted in the down-regulation of Cdx2. It is speculated that the knockdown of Pard6b impairs apical-basal polarity and epithelial integrity in the outer cell, which indirectly led to the failure of up-regulation in Cdx2 expression. It is unlikely that Pard6b-specific shRNA directly caused degradation of Cdx2 mRNA as an off-target effect of the shRNA, because the same shRNA plasmid did not reduce the level of Cdx2 mRNA in P19 cells (Supplemental Figure S5). Interestingly, while the level of Nanog mRNA was increased in Pard6b-knockdown embryos (Fig. 2C), it was decreased in Pard6b-knockdown P19 cells (Supplemental Figure S5). This suggests that how the transcription of this pluripotency regulator gene is controlled by Pard6b is different depending on cellular context.

The incidence of apoptosis was significantly elevated in Pard6b-knockdown embryos by the late blastocyst stage. PAR6 may play a general role in the regulation of apoptosis in epithelial cells [51, 52]. For example, overexpression of a mutant PAR6 lacking its aPKC-binding site promotes the incidence of apoptosis in MDCK cell line [52]. Importantly, in the mouse blastocyst, CDX2 deficiency also increases the number of apoptotic cells [15]. Given that the expression level of CDX2 was significantly lower in Pard6b-knockdown embryos, it is possible that the effect of PARD6B deficiency on apoptosis is through the impaired expression of CDX2. The mechanism that links PARD6B or CDX2 to the regulation of apoptosis is unclear. However, the incidence of apoptosis was significantly reduced when Pard6b-knockdown embryos are cultured in ESCM, indicating that extrinsic factors also contribute to the regulation of apoptosis in the blastocyst. Because the outer cells in Pard6b-knockdown embryos are unable
to differentiate as functional TE and are possibly adopting certain features of ICM, such as a reduced CDX2 level, elevated NANOG level, and non-epithelial morphology, they may be more stable in the culture medium that supports the survival and proliferation of ES cells. In this respect, it is of interest whether ES cell lines can be established in the complete absence of PAR6B, although their differentiation into epithelial tissues, including the primitive ectoderm and primitive endoderm, may still depend on PAR6B.

TEAD4, a transcription factor, was recently shown to be essential for cavity formation, and one of its target genes may be Cdx2 [19, 20]. However, Cdx2-null embryos can cavitate initially, so there are likely to be other target genes of TEAD4 that play critical roles in cavitation. Pard6b knockdown causes cavitation failure, reminiscent of the Tead4-knockout phenotype. Nonetheless, Pard6b is unlikely to be a transcriptional target of TEAD4, as its expression level was not diminished in Tead4-knockdown embryos. Furthermore, a significant difference between Pard6b-knockdown and Tead4-knockout embryos is that the former impairs PRKCZ localization and the latter does not [20]. This suggests that TEAD4 regulates cavity formation either downstream or independently from PAR6B. Whether Tead4-knockout embryos can form TJ is currently not known. Whether the cavitation failure in Tead4-knockout embryos is due to aberrant sealing (i.e., paracellular junction) or aberrant fluid pumping (i.e., vectorial transport of sodium ions) needs to be clarified in future studies.

While TEAD4 is expressed ubiquitously [20], its function is specific to TE. Such TE-specific action, namely the activation of Cdx2 expression, is controlled by TEAD4-binding protein YAP1, which acts as a transcriptional co-activator [9]. YAP1 translocates to the nucleus only in the outer cells of the embryo. This spatial regulation of YAP1 is controlled by kinase LATS1 and LATS2, which phosphorylate YAP1 only in the inner cells and prevent YAP1 from going into the nucleus. How LATS action is restricted to the inner cells is currently unknown. It is suggested that cell position or cell polarity plays critical roles [9]. This notion is consistent with the present study, as the disturbance in apical-basal cell polarization by Pard6b knockdown resulted in the down-regulation of Cdx2, a putative transcriptional target of TEAD4. It is of particular interest to investigate how YAP1 localization is affected by the Pard6b knockdown in future studies.

It has been shown that RAS-MAPK signaling promotes TE development [53], although its relationship to PAR6 remains to be demonstrated. Interestingly, MAPK signaling antagonizes PAR-1 signaling during embryonic polarization in C. elegans [54]. It is possible that MAPK signaling is linked to cell polarization because MARK2 (also known as EMK), the mouse homolog of PAR-1, localizes in the basolateral domain, complementary to the apical localization of PARD6B and PRKCZ at the 8- and 16-cell stage [22].

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REFERENCES


FIGURE LEGENDS

Fig. 1. Pard6b knockdown suppresses formation of the blastocyst cavity. (A) Selection of effective Pard6b shRNA plasmids in P19 mouse embryonal carcinoma cells. qRT-PCR analysis of Pard6b expression in P19 cells that are transiently transfected with shRNA plasmids for 24 h (control encoding a non-target sequence and five types of Pard6b shRNA). Pard6b mRNA levels were measured using primers for the protein-coding region (gray bars) and for the 3’UTR (white bars), and normalized by Gapdh mRNA level. Bars indicate mean ± SD. (B) Immunostaining for MYC-tagged PARD6B protein in P19 cells that are co-transfected with control or Pard6b shRNA #2 plasmid. The signal for MYC-tagged PARD6B protein is markedly reduced by Pard6b shRNA plasmid. Nuclei are stained with DAPI. (C) Images taken by time-lapse video microscopy at three different time points in one set of experiments, corresponding to 8-cell during compaction, morula and blastocyst stage. Eight embryos on the left side of the images have been injected with control shRNA plasmid, whereas eight embryos on the right side have been injected with Pard6b shRNA plasmid. Time elapsed after the administration of hCG is indicated in hours (h). (D) Comparison of the timing of blastocyst cavity formation between control shRNA plasmid-injected and Pard6b shRNA plasmid-injected embryos. The data are a compilation of three independent sets of experiments, and the total number of embryos examined is indicated (n). (B, C) Scale bar: 100 µm.

Fig. 2. Gene expression in Pard6b-knockdown and Tead4-knockdown embryos. (A) Z-series projections of confocal images of embryos at the early blastocyst stage (100 h post-hCG) which were immunostained for histone protein. Arrows point to mitotic nuclei. Embryos had been injected with control shRNA plasmid and Pard6b shRNA plasmid. (B) Bar graph summarizing the total number of nuclei per embryo (mean ± SD) as determined by histone staining. Mean number of mitotic nuclei per embryo are represented by the shaded portion of the bar. Total number of embryos examined per group is indicated (n). (C) qRT-PCR analysis of shRNA plasmid-injected embryos at the early blastocyst stage (100 h post-hCG). Relative expression levels of Gapdh, Pard6b (3’ UTR), Cdx2, Tead4, Pou5f1, and Nanog are shown as percentages of their expression levels in Pard6b shRNA plasmid-injected embryos relative to those in control shRNA plasmid-injected embryos. In each set of experiment, the expression level of each gene is normalized by that of Actb. Bars indicate mean ± SD. p values for Pard6b, Cdx2, and Nanog are based on Student’s t-test against Gapdh, indicating that the change in these genes by the Pard6b shRNA plasmid is statistically significant. (D) Immunostaining for PARD6B protein in shRNA plasmid-injected embryos at the morula stage (90 h post-hCG). A single confocal optical section near the equator of a representative embryo is shown for each group. Graph shows comparison of fluorescence intensity of PARD6B immunostaining between control shRNA plasmid-injected and Pard6b shRNA plasmid-injected embryos. Circles represent the intensity in individual embryos, and the horizontal bars represent means (n = 12 for control, n =
13 for Pard6b shRNA plasmid). p value is based on Student’s t-test between the two groups, indicating that the reduction in PARD6B protein level by Pard6b shRNA plasmid is statistically significant. (E) Knockdown of Tead4 reduces TE-lineage transcription factors and elevates pluripotent transcription factors. qRT-PCR analysis of shRNA plasmid-injected embryos at the early blastocyst stage (100 h post-hCG). Relative expression levels of Gapdh, Tead4, Cdx2, Pou5f1, Nanog, and Pard6b (3’ UTR) are shown as percentages of their expression levels in Tead4 shRNA plasmid-injected embryos relative to those in control shRNA plasmid-injected embryos. In each set of experiment, the expression levels of each gene is normalized by that of Actb. Bars indicate mean ± SD. p values for Tead4, Cdx2, and Nanog are based on Student’s t-test against Gapdh, indicating that the change in these genes by Tead4 shRNA plasmid is statistically significant. (A, D) Scale bar: 100 µm.

**Fig. 3.** Knockdown of Pard6b impairs the localization of cytoplasmic actin filaments, TJP1 and PRKCZ, but not intercellular CDH1 and CLDN4, in the outer cells of the embryo. (A, B) Immunostaining for CDH1 protein in shRNA-injected embryos (90 h post-hCG). Images are average intensity projections of three confocal optical sections near the equator. The distinct staining at cell-cell boundaries are observed in both control shRNA plasmid-injected and Pard6b shRNA plasmid-injected embryos, suggesting that the knockdown of Pard6b does not impair the expression and localization of CDH1 at cell-cell boundaries. (C, D) Phalloidin-staining for actin filaments in shRNA-injected embryos at the early blastocyst stage (100 h post-hCG). Images are Z-series projections of confocal sections of embryos. In the control shRNA plasmid-injected embryo, actin filaments are enriched at the cell-cell boundaries, particularly near the apical edge, reflecting their association with the AJ. In the Pard6b shRNA plasmid-injected embryo, the localization of actin filaments along the apical edge of cell-cell boundaries is indistinct, and actin filaments appear to be more enriched throughout the apical cortex of outer cells. (E, F) Immunostaining for CLDN4 protein in shRNA-injected embryos at the early blastocyst stage (100 h post-hCG). Embryos were imaged via fluorescence microscopy. Pard6b knockdown apparently does not disturb CLDN4 distribution, as it is localized similarly at the apical edge of cell-cell boundaries to form the TJ in control shRNA plasmid-injected and Pard6b shRNA plasmid-injected embryos. (G, H) Immunostaining for TJP1 protein in shRNA-injected embryos at the early blastocyst stage (100 h post-hCG). Images are Z-series projections of confocal sections of embryos. In the control shRNA plasmid-injected embryo, TJP1 is localized at the apical edge of cell-cell boundaries. In the Pard6b shRNA plasmid-injected embryo, TJP1 is localized at the apical edge of cell-cell boundaries. In the Pard6b shRNA plasmid-injected embryo, the localization of TJP1 is severely impaired such that the staining is weak or discontinuous along cell-cell boundaries. (I, J) Immunostaining for PRKCZ protein in shRNA-injected embryos (90 h post-hCG). Single confocal optical sections near the equator are shown. In the control shRNA plasmid-injected embryo, the PRKCZ staining is enriched at the apical cortex of outer cells but weakly at the cell-cell boundaries. In the Pard6b shRNA plasmid-injected embryo, the apical staining of PRKCZ is indistinct while cytoplasmic staining is increased. (A-D, G-J) and (E-F) Scale bars: 100 µm.

**Fig. 4.** Knockdown of Pard6b interferes with paracellular permeability sealing in the outer cells and impairs the blastocyst cavity formation. (A) A schematic overview of the chimera experiment. Egfp-transgenic fertilized eggs are injected with control (non-target) shRNA plasmid or Pard6b shRNA plasmid. At the 8-cell stage, the injected embryos are combined with non-transgenic uninjected embryos, and allowed to develop as chimeras up to the blastocyst
stage. As a comparison, non-transgenic uninjected embryos are cultured by themselves as non-chimeras up to the same stage. (B) Bright field and fluorescence images of two representative chimeras for each shRNA plasmid injection group at 96 h post-hCG. Note that both control shRNA chimeras possess a large cavity (occupying more than 50% of the embryo in volume) and that EGFP-positive cells are found in both epithelial and non-epithelial portions. By contrast, Pard6b shRNA chimeras possess only small cavities (top: occupying less than 50% of the embryo in volume) or no cavity (bottom). Scale bar: 100 µm. (C) Comparison of the efficiency of blastocyst cavity formation among two types of shRNA chimeras and non-chimeras. The definitions for large and small cavities are as described in (B). Total numbers of embryos examined are indicated (n).

Fig. 5. (A) Double immunostaining for CDX2 (red) and POU5F1 (green) proteins in shRNA plasmid-injected embryos (100 h post-hCG). Z-series projections of confocal images of embryos are shown for each group. In the control shRNA plasmid-injected embryo, CDX2 is stained more intensely in the nuclei of outer cells than in inner cells, whereas POU5F1 is stained ubiquitously among all the nuclei. As a result, outer cell nuclei exhibit the appearance of orange color in the merge image (right column). In the Pard6b shRNA plasmid-injected embryo, CDX2 is stained less intensely in all the nuclei, which as a result exhibit the appearance of green color in the merge image. Images were collected during the same confocal session using the same settings. (B) Quantification of relative intensity of CDX2 to POU5F1 immunostaining in shRNA plasmid-injected embryos. The data are a compilation of two independent experiments, and a total of 13 and 14 embryos were examined for the control and Pard6b shRNA groups, respectively. For each embryo, nuclei of 5 inner cells and 5 outer cells were arbitrarily chosen for fluorescence intensity measurement, and mean ± SD from all the measurements for each group are presented in the graph. p value is based on Student’s t-test, indicating that the relative intensity of CDX2 to POU5F1 in outer cells is significantly reduced by the knockdown of Pard6b. Outer cell nucleus was identified as lacking adjacent nucleus at one side, as images of the embryos were scanned in the z-axis via confocal microscopy. A nucleus was considered to be internal when it is surrounded by other nuclei, as observed when images of the embryos were scanned in the z-axis. (C) Immunostaining for NANOG (green) and propidium iodide (PI)-staining for nuclei (red) in shRNA plasmid-injected embryos (100 h post-hCG). Z-series projections of confocal images of embryos are shown for the control and Pard6b shRNA groups. Images were collected during the same confocal session using the same settings. (D) Levels of NANOG protein in shRNA plasmid-injected embryos. NANOG fluorescence levels were normalized by PI fluorescence levels for each nucleus. The data are a compilation of two independent experiments, and a total of 6 embryos were examined for each group. For each embryo, nuclei of 5 inner cells and 5 outer cells were arbitrarily chosen for measurement, and mean ± SD from all the measurements for each group are presented in the graph. p value is based on Student’s t-test, indicating that the relative intensity of NANOG to PI in outer cells is significantly elevated by the knockdown of Pard6b. (E) Increased incidence of apoptosis at the late blastocyst stage by the knockdown of Pard6b. shRNA plasmid-injected embryos were analyzed at 124 h post-hCG for apoptosis by TUNEL (left column) and stained with phalloidin for actin filaments (middle column). Numbers at the right-bottom corner in the TUNEL images correspond to TUNEL-positive area in the arbitrary unit that is depicted in the graph in (F). The representative samples show that TUNEL-positive area is larger in the Pard6b shRNA plasmid-injected embryos than in the control shRNA plasmid-injected embryos, both of which have been
cultured in KSOM. However, the incidence of apoptosis due to the knockdown of \textit{Pard6b} is reduced when the injected embryos have been cultured in ESCM from the 8-cell stage. (F) The graph shows mean ± SD of TUNEL-positive area in control shRNA plasmid-injected embryos cultured in KSOM, \textit{Pard6b} shRNA plasmid-injected embryos cultured in KSOM, and \textit{Pard6b} shRNA plasmid-injected embryos cultured in ESCM. Numbers of embryos examined are indicated \((n)\). Scale bar: (A, C) 50 µm, (E) 100 µm.
Table 1. Early cleavage in *Pard6b*-knockdown and control embryos.

<table>
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<th></th>
<th>Number of zygotes injected</th>
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<td>223 (93.3%)</td>
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<td><em>Pard6b</em> shRNA #2</td>
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<td>280 (88.1%)</td>
<td>266 (95%)</td>
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Figure 1  Alarcon
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Figure 4  Alarcon
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