Distinct roles of *ROCK1* and *ROCK2* during development of porcine preimplantation embryos

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Abstract

Cell-to-cell contact mediated by cell adhesion is fundamental to the compaction process that ensures blastocyst quality during embryonic development. In this study, we first showed that Rho-associated coiled-coil protein kinases (ROCK1 and ROCK2) were expressed both in porcine oocytes and IVF preimplantation embryos, playing different roles in oocytes maturation and embryo development. The amount of mRNA encoding ROCK1 and the protein concentration clearly increased between the eight-cell and morula stages, but decreased significantly when blastocysts were formed. Conversely, ROCK2 was more abundant in the blastocyst compared with other embryonic stages. Moreover, immunostaining showed that ROCK1 protein distribution changed as the embryo progressed through cleavage and compaction to the morula stage. Initially, the protein was predominantly associated with the plasma membrane but later became cytoplasmic. By contrast, ROCK2 protein was localized in both the cytoplasm and the spindle rotation region during oocyte meiosis, but in the cytoplasm and nucleus as the embryo developed. In addition, ROCK2 was present in the trophectoderm cells of the blastocyst. Treatment with 15 μ M Y27632, a specific inhibitor of ROCKs, completely blocked further development of early four-cell stage embryos. Moreover, we did not detect the expression of *ROCK1* but did detect *ROCK2* expression in blastocysts. Moreover, lysophosphatidic acid an activator of ROCKs significantly improved the rates of blastocyst formation. These data demonstrate that ROCKs are required for embryo development to the blastocyst stage. Together, our results indicate that ROCK1 and ROCK2 may exert different biological functions during the regulation of compaction and in ensuring development of porcine preimplantation embryos to the blastocyst stage.

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Introduction

Rho-associated coiled-coil protein kinases (ROCKs) are downstream targets of RhoA small GTP-binding proteins, and regulate cell adhesion, cytokinesis, cell proliferation, and apoptosis (Takai *et al.* 1995, Narumiya *et al.* 1997, Etienne-Manneville & Hall 2002, Riento & Ridley 2003, Zohrabian *et al.* 2009). To date, two isoforms of ROCK, ROCK1 and ROCK2, have been described in mammalian cells (Ishizaki *et al.* 1996, Matsui *et al.* 1996). In the mouse, the ROCKs have been reported to function primarily via regulation of actin cytoskeleton dynamics (Takai *et al.* 1995, Narumiya *et al.* 1997). Recently, functional differences between the two ROCK isoforms have been identified. *In vivo* studies showed that, despite the proteins being structurally similar, expression of neither *ROCK1* nor *ROCK2* can compensate for loss of the other isoform during murine embryonic development. Moreover, *Rock1*^{−/−} is embryonic lethal because of cardiac fibrosis, and *Rock2*^{−/−} mice are embryonic lethal because of placental dysfunction (Narumiya 2003, Shimizu *et al.* 2005, Zhu *et al.* 2011). *In vitro* studies

using ROCK isoform-specific RNAi-mediated knockdown in fibroblasts showed that ROCK1 regulates fiber stress and cell adhesion, whereas ROCK2 regulates the formation of the microfilament bundle and focal adhesion. These reports suggest that ROCK1 and ROCK2 may play distinct, and sometimes opposing, roles in such cells (Yoneda *et al.* 2005, 2007). In mammals, several reports have shown that the compaction process, involving cytoskeletal rearrangement and development of intercellular adhesion, is critical for the development of viable embryos (Lu *et al.* 2002, Kawagishi *et al.* 2004). Rho family signaling is considered to be the principal mechanism whereby oocyte maturation and embryo development are controlled. The mRNAs encoding both Rac1 and Cdc42 are present throughout all preimplantation development stages in mice (Natale & Watson 2002). RhoA is also reported to regulate ooplasmic segregation, cytokinesis, and spindle rotation (Yoshida *et al.* 2003, Zhong *et al.* 2005). In addition, some studies have found that Rho-kinase is involved in mouse blastocyst cavity formation (Kawagishi *et al.* 2004). Rho-associated proteins are involved in the determination of

the polarity of intercellular adhesion, and all such proteins are important in terms of embryonic compaction (Clayton *et al.* 1999). Although the mechanisms by which adherent junctions (mediating cell–cell adhesion) are formed during the development of mouse preimplantation embryos have been studied in detail (Clayton *et al.* 1999, Pauken & Capco 1999, 2000, Fleming *et al.* 2001), the mechanisms by which the compaction process is regulated, and formation of a blastocyst with adequate cell number ensured, in early-stage *in vitro* porcine embryos have not been clearly defined.

ROCK activity can be induced in mammalian cells by lysophosphatidic acid (LPA; Hashimoto *et al.* 2003). Addition of LPA has been reported to improve the development of mouse preimplantation embryos (Kobayashi *et al.* 1994). Thus, we proposed the hypothesis that LPA enhanced embryo development by activating ROCKs. Y27632, a specific inhibitor of Rho-kinases (Ishizaki *et al.* 2000, Hahmann & Schroeter 2010), has been widely used to study enzyme function. Therefore, employing either Y27632, a specific inhibitor of ROCKs, or LPA, an activator of ROCKs, we explored the distribution and function of ROCK1 and ROCK2 during porcine oocyte maturation and embryonic cleavage. Next, we identified a specific role for ROCK2 in increasing the number of trophectoderm (TE) cells to regulate the quality of a blastocyst produced *in vitro*. Our results indicate that *ROCK1* and *ROCK2* are both present in porcine oocytes and preimplantation embryos. We found that *ROCK1* is mostly expressed in compaction stages except for blastocysts, but *ROCK2* expression is increased in blastocyst stages. Therefore, the two proteins seem to play different roles in oocyte maturation and embryo development dependent on their different localizations. Moreover, we demonstrated that Y27632 inhibits embryos from reaching the blastocyst stage, but LPA attenuates this inhibition allowing the embryos to develop to blastocysts as in normal conditions. Thus, *ROCK1* may principally regulate the compaction process, rather than blastocyst formation. On the other hand, *ROCK2* may control processes ensuring normal blastocyst formation.

Materials and methods

Chemicals

All chemicals used in the present study were purchased from Sigma unless otherwise noted.

In vitro maturation

Pig ovaries were collected from a local slaughterhouse (NH Livestock Cooperation Association, Nonsan City, Chungnam Province, Korea) where we had acquired permission to use porcine ovaries, and transported to the laboratory within 2 h in 0.9% (w/v) saline supplemented with 75 µg/ml potassium

penicillin G and 50 mg/ml streptomycin sulfate at 35 °C. Cumulus–oocyte complexes were recovered from follicles (3–6 mm in diameter) by aspiration through 18-gauge needles and placed in disposable 10-ml syringes. After washing three times in TL-HEPES medium, groups of ~50 oocytes were allowed to mature in 500 µl amounts of *in vitro* maturation (IVM) medium in four-well multi-dishes (Nunc, Roskilde, Denmark) at 38.5 °C under 5% (v/v) CO₂ in air. The medium used for oocyte maturation was NCSU-23 supplemented with 10% (v/v) follicular fluid, 0.57 mM cysteine, 10 ng/ml β-mercaptoethanol, 10 ng/ml epidermal growth factor, 10 IU/ml pregnant mare serum gonadotropin, and 10 IU/ml human chorionic gonadotropin. After culture for 22 h, the oocytes were washed three times with maturation medium and next further cultured for another 22 h in maturation medium without hormones (Petters & Wells 1993).

IVF

After completion of IVM, oocytes were subjected to IVF using the method of Abeydeera & Day (1997). The IVF medium was a modified Tris-buffered medium (mTBM) with 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris (crystallized free base; Fisher Scientific, Fair Lawn, NJ, USA), 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics. Ejaculated fresh semen was washed three times by centrifugation in Dulbecco's PBS (Gibco-BRL) supplemented with 1 mg/ml BSA (fatty acid free), 100 µg/ml penicillin G, and 75 µg/ml streptomycin sulfate. After washing, the spermatozoa were suspended in mTBM at pH 7.8 to give a final concentration of 1.5×10^5 sperm/ml. The oocytes were washed three times in mTBM containing 2.5 mM caffeine sodium benzoate and 4 mg/ml BSA and next placed into 50 µl amounts of mTBM (containing 15–25 oocytes). The oocytes were co-incubated with spermatozoa for 6 h at 38.5 °C in an atmosphere of 5% (v/v) CO₂ in air. Next, the fertilized embryos were washed three times and transferred to PZM-3 *in vitro* culture medium at 38.5 °C in an atmosphere of 5% (v/v) CO₂ in air.

For the analysis of Y27632 and LPA effects on embryo development, four-cell, eight-cell, or morula stage embryos were treated with different concentrations (0, 1, 5, 10, and 15 µM) of Y27632 or LPA for 24 h and cultured in PZM-3 medium until they reach to blastocyst development. And to evaluate the effect of Y27632 and LPA treatments on the embryo quality, one-cell stage embryos were exposed to 10 µM Y27632 or both 10 µM Y27632 and 10 µM LPA in the culture medium for 144 h. The cleavage and blastocyst formation rates were examined followed by enumeration of blastocyst nuclei.

Real-time quantitative PCR

To analyze the expression of the genes encoding ROCK1 and ROCK2, oocytes at the germinal vesicle (GV), GVBD, metaphase I (MI), and MII stages were collected at 0, 6, 22, and 44 h, respectively, of culture in maturation medium. One-cell, two-cell, four-cell, eight-cell, morula, and blastocyst-stage embryos were obtained at 48, 72, 96, and 144 h after fertilization respectively (30 samples of each stage). For analyzing anti-apoptotic and pro-apoptotic gene expressions, blastocysts were recovered after culture of one-cell stage embryos with 10 µM Y27632 or both 10 µM Y27632 and

10 μ M LPA for 144 h. Total mRNA was extracted using the Dynabeads mRNA Direct Kit (DynaL Asa, Oslo, Norway) according to the manufacturer's instructions. For RT, total mRNA from a single blastocyst was incubated in a final volume of 20 μ l with 0.5 mg oligo-dT, RT buffer (1 $\times$), 10 mM dithiothreitol, 10 mM dNTP mixture, and ten units of Moloney murine leukemia virus reverse transcriptase. The reaction proceeded at 37 °C for 50 min, followed by 70 °C for 15 min, and products were stored at 4 °C until real-time PCR analysis. Real-time PCR amplification was conducted using a RotorGene 2000 Real-Time PCR System (Applied Biosystems). The QuantiTect SYBER Green PCR Kit (Finnzymes, Espoo, Finland) was used to provide real-time quantification of desired PCR products. Each real-time PCR mixture contained 4 μ l cDNA and 10 pmol/ μ l of each of the appropriate forward and reverse primers (Table 1) in a total volume of 20 μ l. All tests were conducted in triplicate and the mRNA levels were normalized to that of mRNA encoding β -actin, which was shown to be stably expressed across all of the stages of development examined.

Immunofluorescence

Oocytes at the GV, MI, and MII stages, and porcine embryos at various stages of development, were fixed in 4% (v/v) paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% (v/v) Triton X-100 for 30 min. Oocytes and embryos were blocked overnight with 3% (w/v) BSA in PBS at 4 °C and then incubated with either rabbit polyclonal anti-ROCK1 antibody (Santa Cruz, sc5560) or rabbit polyclonal anti-ROCK2 antibody (Santa Cruz, sc5561), both diluted 1:100 in a blocking solution, for 1 h at 37 °C. After washing with 0.5% (v/v) Tween-20 in PBS, samples were reacted with anti-rabbit FITC-conjugated secondary antibody (Santa Cruz, sc2365) in a blocking solution at 1:200 for 60 min at 37 °C. Then, all samples were mounted using VECTASHIELD Mounting Medium containing DAPI. Images were obtained using a Zeiss scanning laser confocal microscope (LSM 510). The confocal microscopy was used to capture pictures through 3.5 μ m single-optical sections at all embryonic stages. At the blastocyst stage, the image was a projection of multiple 3.5 μ m thick optical slices. Serial optical sections (the Z-series) were collected at 3.5 μ m intervals; all nuclear and cytoplasmic regions were covered. The Z-series were stacked and images depicting staining patterns and intensities of all nuclear and cytoplasmic entities were

generated. All samples of oocytes and embryos were prepared and processed simultaneously before fluorescence intensity measurements. The laser power was adjusted to ensure that signal intensity was below saturation for the developmental stage that displayed the highest intensity and all images were next scanned at that laser power. All images in any particular developmental series were acquired using the same laser power output. The photographs were analyzed employing LSM Image Browser Software (Zeiss, Gottingen, Germany).

Western blotting

Porcine blastocysts (50 per sample) were washed three times in PVA–PBS, and then resuspended in extraction buffer (PRO-PREP; Intron Biotechnology, Seoung, Korea). The extracted proteins were separated by 10% (w/v) SDS–PAGE using a Bio-Rad apparatus (Bio-Rad) and then electrophoretically transferred to PVDF membranes, employing a Bio-Rad Mini Trans-Blot Cell. The membranes were blocked with 5% (w/v) skim milk and 0.5% (v/v) Tween-20 in Tris-buffered saline and subsequently exposed to primary antibodies directed against ROCK1 (Santa Cruz, sc5560), ROCK2 (Santa Cruz, sc5561) at 1:1000 dilution, β -actin (Santa Cruz, sc47778) at 1:5000 dilution, phospho-MYPT1 (Thr696-MYPT1, Upstate Cell Signalling, 5163, Lake Placid, NY, USA) or phospho-MLC (Thr18/Ser19-Myosin Light Chain 2, Cell signaling Technology, 3674S, Lake Placid, NY, USA) at 1:1000 dilution. The antibody solutions were prepared in Tris-buffered saline, containing 5% (w/v) nonfat dry milk powder and 0.1% (v/v) Tween-20. The membranes were next washed in Tris-buffered saline with 0.5% (v/v) Tween-20 for 15 min and antibody–antigen complexes were detected using anti-mouse IgG (Santa Cruz, sc2301) or anti-rabbit IgG peroxidase conjugates (Abcam, ab6721, Cambridge, MA, USA), followed by employment of an ECL detection kit (Amersham Bioscience). All experiments were carried out in triplicate. The intensities of bands on blots were measured densitometrically (Bio-Rad); the β -actin band served as a standard.

Dual differential staining

Differential staining of ICM and TE cells of blastocysts (day 6) was carried out using a previously described technique (Machaty *et al.* 1998). Briefly, the zona pellucida of blastocysts was removed by incubation in 0.5% (w/v) pronase solution.

Table 1 Primers used in this work.

Gene	Primers	Sequence	Accession number	Annealing temperature (°C)	Product size (bp)
ROCK1	Forward	TGCTGCTGGATAAATCTGGA	D89493	60	152
	Reverse	ATAACCATCGCCACCTTGAG			
ROCK2	Forward	TGCTTTGGATGCCATACACTC	XM003125386	59	162
	Reverse	TGCCGTATCACAATGCACCAT			
BAX	Forward	ACACCTCATAGCCATGAAAC	YA55048	56	232
	Reverse	ATGGCTGACATCAAGATACC			
BCL-XL	Forward	AGAGCTTTGAGCAGGTATTG	NM214285	56	253
	Reverse	GCATTGTTCCGTAGAGTTC			
CASP3	Forward	CCTCACCATCATCACACTGG	AF098067	60	279
	Reverse	AGCTCTCGGAACATCTCGAA			
β -actin	Forward	GTGGACATCAGGAAGGACCTCTA	U07786	58	137
	Reverse	ATGATCTTGATCTTCATGGTGCT

Figure 1 Detection of mRNAs encoding ROCK1 and ROCK2 during porcine oocyte maturation and preimplantation development. Real-time PCR was used to analyze the expression levels of *ROCK1* and *ROCK2* mRNA in porcine oocytes and embryos. RNA was isolated from 30 oocytes and 30 embryos at each tested developmental stage, and reverse-transcribed. One microliter of cDNA were used as templates for PCR. The *ROCK1* and *ROCK2* amplicons were separated on 1.0% (w/v) agarose gels. GV, germinal vesicle; MI, metaphase I; MII, metaphase II; 1-C, one-cell; 2-C, two-cell; 4-C, four-cell; 8-C, eight-cell; Bla, blastocyst. * $P < 0.05$ and ** $P < 0.01$, a statistically significant difference from any another group.

After rinsing in TL-HEPES medium containing 1 mg/ml PVA, zona pellucida-free embryos were exposed to a 1:5 (v/v) dilution of rabbit anti-pig whole serum for 1 h. Next, the embryos were rinsed three times for 5 min each time in TL-HEPES and transferred to a solution consisting of a 1:10 dilution of guinea pig complement containing 10 mg/ml propidium iodide and 10 mg/ml bisbenzimidazole (Hoechst 33342), for 1 h. After washing, each embryo was mounted on a slide under a cover glass and observed under u.v. light using an epifluorescence microscope (Olympus). Blue and red colors were considered to be the characteristic of ICM and TE cells respectively.

Statistical analysis

All data were analyzed by one-way ANOVA and the protected least significant difference test using general linear models of the Statistical Analysis System (SAS, Cary, NC, USA) to determine differences among experimental groups. A treatment difference was considered significant when the P value was < 0.05 .

Results

Detection of *ROCK1* and *ROCK2* in porcine oocytes and embryos

To investigate whether *ROCK1* and *ROCK2* were present in porcine oocytes and preimplantation embryos, we first examined mRNA expression from the relevant genes via quantitative real-time PCR (Fig. 1). No significant difference in the expression levels of the two genes was evident from the GV stage to the eight-cell stage. However, the *ROCK1* mRNA level increased notably at the eight-cell stage, became abundant when the morula formed, and decreased sharply at the blastocyst stage.

There was a fourfold difference between *ROCK1* and *ROCK2* mRNA abundance at the blastocyst stage, and with *ROCK2* mRNA being highly expressed at all embryonic stages.

Distribution of *ROCK1* and *ROCK2* in oocytes and embryos

ROCK1 was predominantly located in the ooplasm of oocytes, and the cytoplasm of all embryonic stages from the two-cell stage to the blastocyst, but was also present in regions adjacent to the cell membrane (Fig. 2). As the embryo progressed from the four-cell stage to become a morula, *ROCK1* fluorescence became cytoplasmic and was particularly intense in regions adjacent to the cell borders and margins. *ROCK2* was detected in oocytes from the GV to the MII stage. *ROCK2* fluorescence was present throughout the cytoplasm at the GV stage, and became localized to the meiotic spindle apparatus around the maternal chromosomes in the MI and MII stages (Fig. 3A). In porcine preimplantation stage embryos, *ROCK2* was detected in both the cytoplasm and the nucleus; staining intensity increased during development from the two-cell stage to the blastocyst stage, especially increased in the nuclei (Fig. 3B).

Detection of *ROCK1* and *ROCK2* proteins by western blotting

To confirm the expression pattern for *ROCK1* and *ROCK2* during compaction and blastocyst formation, we explored protein expression levels by western blotting. *ROCK1* levels increased significantly during development from the eight-cell stage to the morula, and fell markedly extent at the blastocyst stage. By contrast, *ROCK2* was only weakly expressed at the eight-cell and morula stages but highly expressed in blastocysts (Fig. 4).

Figure 2 Localization of *ROCK1* protein in porcine oocytes and IVF preimplantation embryos. *ROCK1* protein was detected using a specific anti-*ROCK1* antibody (green). *ROCK1* was predominantly located in the cytoplasm of MI oocytes, and all embryonic stages from the two-cell stage to the blastocyst. MI, metaphase I; 1-C, one-cell; 2-C, two-cell; 4-C, four-cell; 8-C, eight-cell; Bla, blastocyst. Single optical sections from confocal Z-series were shown at every stage of embryo development. Negative control embryos were probed directly with the secondary antibody. Nuclei were stained with DAPI (blue); Scale bar, 50 μ m.

Figure 3 Localization of ROCK2 protein in porcine oocytes and IVF preimplantation embryos. (A) ROCK2 in oocytes of different stages. (B) ROCK2 in different stages of IVF preimplantation embryos. The images from GV to eight-cell stages were single optical sections from confocal Z-series. The images of blastocysts were projected of multiple optical sections. ROCK2 was detected using a specific anti-ROCK2 antibody (green). ROCK2 was commonly localized in the cytoplasm at the GV stage, and localized to the meiotic spindle apparatus around the maternal chromosomes in MI and MII stages. GV oocytes designated as nonsurrounded nucleolus (NSN) exhibited a roughly dispersed chromatin configuration throughout the nucleoplasm. All oocytes were stained to visualize chromatin (DNA) with DAPI (blue). During embryo stages, ROCK2 was detected in both the cytoplasm and the nucleus, especially increased in the nuclei from one cell to blastocyst. GV, germinal vesicle; MI, metaphase I; MII, metaphase II; 1-C, one-cell; 2-C, two-cell; 4-C, four-cell; 8-C, eight-cell; Bla, blastocyst. Negative control embryos were probed directly with the secondary antibody. Nuclei were stained with DAPI (blue). Scale bar, 50 μ m.

These differential expression patterns of ROCK1 and ROCK2 suggest that they may have different functions, with involvement in events linked to compaction and blastocyst development, respectively, in porcine preimplantation embryos.

Functions of ROCKs during development of preimplantation embryos

To investigate the functions of ROCK1 and ROCK2 during compaction and embryo development, we examined the effect of different concentrations of Y27632 on the blastocyst formation rate. Porcine embryos at various stages of growth, including four-cell, the eight-cell, and the morula stages, were cultured for 24 h in PZM-3 medium containing various concentrations of Y27632. Figure 5A shows that blastocyst

formation was not detected when embryos at the four-cell stage were exposed to 15 μ M Y27632. Exposure of eight-cell and morula stage embryos to Y27632 reduced blastocyst formation rates in a dose-dependent fashion. In addition, when four-cell stage embryos were treated with LPA, an activator of ROCKs, the blastocyst formation rate was significantly improved at a 10 μ M concentration (Fig. 5B).

Regulation of ROCKs on blastocyst development

To further investigate the role played by ROCK2 in ensuring blastocyst formation, embryos at the one-cell

Figure 4 Semi-quantitative ROCK1 and ROCK2 protein levels in porcine embryos from the eight-cell stage to the blastocyst stage. β -actin served as a standard control. The bands appearing upon western blotting (A), and mean $\pm$ S.E.M.s of relative band intensity values (B and C), are shown. * P <0.05, ** P <0.01 and a statistically significant difference.

Figure 5 ROCK expression during the development of IVF porcine embryos. (A) The percentages of embryos treated with Y27632 (at the four-cell, eight-cell, and morula stages) that developed into blastocysts. (B) The percentage of four-cell stage embryos treated with LPA that developed into blastocysts. X-axis shows different concentrations of Y27632 (A) and LPA (B). ** $P < 0.01$ and * $P < 0.05$, a statistically significant difference.

stage were exposed to 10 μM Y27632 or both 10 μM Y27632 and 10 μM LPA in the culture medium. Rates of cleavage and blastocyst formation observed in the presence of Y27632 alone were significantly lower than those of the control group (Table 2). However, treatment with both Y27632 and LPA significantly improved developmental rates compared with the other groups. Using dual differential staining, we evaluated blastocyst quality by counting the number of ICM, TE, and all cells. The numbers of TE cells and total cells in blastocysts treated with Y27632 alone decreased significantly (21.4 ± 1.6 and 26.0 ± 2.8 respectively) compared with those of the control group (28.7 ± 2.6 and 34.1 ± 3.3 respectively). However, the group treated with both Y27632 and LPA had the highest numbers of TE and total

cells (35.2 ± 3.0 and 40.3 ± 2.0 respectively). ICM cell numbers in blastocysts did not vary among groups. Furthermore, by checking the levels of phosphorylated MYPT1 and MLC (p-MYPT1 and p-MLC) of MYPT1 and MLC, which are ROCK2-specific substrates, by western blotting, we showed that ROCK2 activity decreased significantly in blastocysts treated with Y27632 alone compared with the control group and the group treated with both Y27632 and LPA (Fig. 6).

Effects of ROCKs on apoptosis in blastocysts

We used quantitative real-time PCR to measure the expression levels of anti- and pro-apoptotic genes, including *Bcl-xl*, *Caspase 3*, and *Bax*, in blastocysts cultured with or without Y27632 and with both Y27632 and LPA (Fig. 7). A significant difference was evident between the control and Y27632-alone groups. No significant difference was apparent when the control and Y27632 + LPA groups were compared. This suggests that Y27632 induces expression of pro-apoptotic genes including, *CASP3* and *BAX*, and inhibits the expression of the anti-apoptotic gene *BCL-XL*. However, LPA, an activator of ROCKs, increased the expression of *BCL-XL* and inhibited the expression of the pro-apoptotic genes *CASP3* and *BAX* (Fig. 7).

Discussion

The distribution and function of RhoA during mouse oocyte maturation and embryo development have been explored (Cheon *et al.* 2000, Zhong *et al.* 2005). However, the roles played by the RhoA effectors, ROCK1 and ROCK2, during embryonic development have not been elucidated. In the present work, we first showed that mRNAs encoding ROCK1 and ROCK2 were present in porcine oocytes and preimplantation developmental stage embryos. Also, we showed that the distribution patterns of ROCK1 and ROCK2 differed in both porcine oocytes and embryos by using immunostaining employing specific antibodies. Although ROCK1 and ROCK2 share structural similarities, being 65% homologous in terms of amino acid sequence (92% homologous in the kinase domains), the two proteins may play distinct roles in cell differentiation and adhesion (Lock & Hotchin 2009, Hahmann & Schroeter 2010). In a previous study in mitotic cells,

Table 2 Effect of ROCKs on the quality of IVF porcine blastocysts.

Group	No. of embryos	Cleaved (%)	Blastocysts (%) (n)	Mean ($\pm$ S.E.M.) no. of nuclei		
				ICM	TE	Total
Control	159	$67.9 \pm 1.2^*$	$25.1 \pm 1.0^*$ (40)	5.4 ± 1.2	$28.7 \pm 2.6^*$	$34.1 \pm 3.3^*$
Y27632	164	$48.7 \pm 2.4^\dagger$	$12.8 \pm 1.7^\dagger$ (21)	4.6 ± 1.0	$21.4 \pm 1.6^\dagger$	$26.0 \pm 2.8^\dagger$
LPA + Y27632	156	$75.0 \pm 0.9^\ddagger$	$32.0 \pm 1.3^\ddagger$ (50)	5.1 ± 2.3	$35.2 \pm 3.0^\ddagger$	$40.3 \pm 2.0^\ddagger$

Values with different superscripts (*, †, ‡) indicate that the numbers are significantly different ($P < 0.05$). The concentration of Y27632 is 10 μM . The concentration of LPA is 10 μM .

Figure 6 The influence of ROCK2 on blastocyst quality. Embryos at the one-cell stage were treated with Y27632 (or not) or with both Y27632 and LPA, and the numbers of embryos developing into blastocysts were counted. (A and B) p-MYPT1 protein levels in blastocysts were quantitated by western blotting. β -actin served as a control. (C and D) p-MLC protein levels in blastocysts were quantitated by western blotting. β -actin served as a control. * $P<0.05$ and ** $P<0.01$, a statistically significant difference from control.

Rho was found to be important in terms of reactive oxygen species (ROS) production (Adachi *et al.* 2001). In the present work, we found that ROCK1 was localized to the cortical region of the oocyte at the MI stage (Fig. 2). This suggests that ROCK1 may play a role in the regulation of oocyte cytoplasmic maturation. At the two-cell stage, ROCK1 fluorescence was localized to the cytoplasm. At later stages, from the four-cell stage to the blastocyst, ROCK1 fluorescence was observed in the cytoplasmic region of the blastomere and adjacent to cell membrane encircling each blastomere (Fig. 2). These results imply that ROCK1 is expressed during the course of preimplantation development and that its expression may relate to the occurrence of blastomere contact and embryonic compaction.

Compared with that of ROCK1, the ROCK2 expression pattern differed at both the mRNA and protein levels; ROCK2 expression was especially high at the blastocyst stage. ROCK2 fluorescence was initially localized in the cytoplasm, but increased in intensity in the nucleus during development from the two-cell to the blastocyst stages. Notably, ROCK2 fluorescence in blastocysts seemed to be located principally in the outer cells of blastocysts, mainly TE cells (Fig. 3B). Our western blotting data analyzed the expression levels of ROCK1 and ROCK2 during development; expression from the eight-cell to the blastocyst stage was similar to the protein expression patterns seen by immunochemical staining data (Fig. 4), further supporting our speculations that ROCK2 plays an important role during porcine embryonic development, but plays a role distinct from that of ROCK1. Our findings are consistent with those of a previous report, to the effect that Rho-kinase is

involved in mouse blastocyst cavity formation (Kawagishi *et al.* 2004).

In previous study, the functions of ROCK1 and ROCK2 were not distinguished. Therefore, the specific roles played by the individual ROCK isoforms, either *in vitro* or *in vivo*, remain largely unknown. However, our results clearly demonstrated that ROCK1 and ROCK2 differ in terms of expression pattern. Although several studies have shown that two ROCK isoforms exist, and that they play distinct roles in mammalian cells (Nakagawa *et al.* 1996, Lock & Hotchin 2009, Williams *et al.* 2011), we are the first to show, in the present work, both ROCK1 and ROCK2 function in porcine oocyte and embryo development. To understand the roles played by the ROCKs in the regulation of embryo development, we used an inhibitor (Y27632) and an activator (LPA) of ROCKs during embryo culture. Y27632 has been widely used as a ROCK inhibitor in work seeking to identify and define the roles played by ROCK kinases in a variety of systems (Klages *et al.* 1999, Ishizaki *et al.* 2000, Roberts 2004, Takehara *et al.* 2008, Zhang *et al.* 2011). In addition, some studies have shown that regulation of cell proliferation and adhesion by LPA are likely achieved by the activation of ROCKs (Kobayashi *et al.* 1994, Cheng *et al.* 2011). However, the exposure of developing embryos to LPA can potentially have adverse effects through the activation of the ROCKs, which is reported to inhibit neuronal differentiation of human embryonic stem cells (Dottori *et al.* 2008). In the porcine system, Y27632 was demonstrated to specifically inhibit ROCK activity and affected ROCK-related signaling in porcine primary cells (Roberts 2004). Herein, we evaluated the effect of treating embryos with different concentrations of Y27632 or LPA on the rate of blastocyst formation and found that Y27632 and LPA indeed affected porcine preimplantation embryo development. Treatment of porcine embryos with the Y27632 inhibitor resulted in reduced development to the blastocyst stage, whereas treatment with the LPA activator improved the blastocyst rate (Table 2). Next, the expression of phosphorylated forms of substrates of ROCK2, p-MYPT1, and p-MLC in blastocysts treated with Y27632 or Y27632 + LPA (Fig. 6).

Figure 7 The relative expression levels of porcine BCL-XL, BAX, and CASP3 genes in blastocysts cultured with or without Y27632 and with both Y27632 and LPA were analyzed by real-time PCR. * $P<0.05$, significantly different from control. Each experiment was repeated three times.

The level of p-MYPT1 has been reported to be related to ROCK2 activity alone (Amano *et al.* 2010). ROCKs have been shown to directly phosphorylate the regulatory myosin light chain (p-MLC). Increased p-MLC is consistent with increased ROCK1 and ROCK2 and elevated ROCK activity (Riento & Ridley 2003). In this study, Y27632 inhibited ROCK2 activity and LPA activated the inhibition caused by Y27632 in blastocysts. It is concluded that ROCK2 activity could play an important role in blastocyst development. ROCK2 is localized in the nucleus, associates with and phosphorylates p300, and increases histone acetyltransferase activity *in vitro*, which means that it regulates transcription (Tanaka *et al.* 2006). Thus, we believe that Y27632 treatment arrests embryo development at the cleavage stage because embryonic transcription may be disturbed by the reduced activation of ROCK2. Y27632 is also known to be a potent teratogenic agent; by inhibiting Rho kinase it can block brain and somite formation and migration of precardiac mesoderm in early stages of chick and mouse embryogenesis (Wei *et al.* 2001, Fukuda & Hosoi 2008). In addition, ROCK2 has been shown to be expressed in the placenta and to facilitate normal placental development. Loss of ROCK2 causes placental dysfunction and fetal death (Thumkeo *et al.* 2003, 2005). The fact that most blastocyst ROCK2 was present in TE cells in our present work indicates that ROCK2 may play a role not only in pre-implantation embryo development but also in fetal development.

ROCK signaling is known to be involved in regulation of apoptosis, as emphasized by experiments conducted with ROCK-deficient mice (Potin *et al.* 2007, Shi & Wei 2007). Therefore, we used quantitative real-time PCR to analyze the effects of ROCK inhibition and activation on the activities of apoptotic genes in blastocysts. We found that activation of ROCKs significantly increased expression of the anti-apoptotic gene *BCL-XL* and significantly reduced the expression of the pro-apoptotic genes *BAX* and *CASP3* (Fig. 7). These data indicate that ROCK activation increases the extent of apoptosis modulated by cleaved caspase 3. Thus, ROCK expression enhances the development of porcine preimplantation embryos.

Together, our results show that ROCK1 and ROCK2 play roles in porcine oocyte maturation and preimplantation embryo development. The expression patterns of the two isoforms differ, and the two proteins exert distinct functions during development of preimplantation embryos. ROCK1 may principally regulate the compaction process evident between the four-cell and morula stages, whereas ROCK2 may play a role in positively influencing blastocyst formation.

Declaration of interest

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

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