Rho-associated Kinase is Involved in Preimplantation Development and Embryonic Compaction in Pigs

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ABSTRACT

The first morphogenetic event of preimplantation development, compaction, was required efficient production of porcine embryos *in vitro*. Compaction of the porcine embryo, which takes place at post 4-cell stage, is dependent upon the adhesion molecule E-cadherin. The E-cadherin through β-catenin contributes to stable cell-cell adhesion. Rho-associated kinase (ROCK) signaling was found to support the integrity of E-cadherin based cell contacts. In this study, we traced the effects of ROCK-1 on early embryonic development and structural integrity of blastocysts in pigs. Then, in order to gain new insights into the process of compaction, we also examined whether ROCK-1 signaling is involved in the regulation of the compaction mediated by E-cadherin of cellular adhesion molecules. As a result, real-time RT-PCR analysis showed that the expression of ROCK-1 mRNA was presented throughout porcine preimplantation stages, but not expressed as consistent levels. Thus, we investigated the blastocyst formation of porcine embryos treated with LPA and Y27632. Blastocysts formation and their qualities in LPA treated group increased significantly compared to those in the Y27632-treated group ($p<0.05$). Then, to determine whether ROCK-1 associates embryonic compaction, we explored the effect of activator and/or inhibitor of ROCK-1 on compaction of embryos in pigs. The rate of compacted morula in LPA treated group was increased compared to that in the Y27632-treated group (39.7 vs 12.0%). Furthermore, we investigated the localization and expression pattern of E-cadherin at 4-cell stage porcine embryos in both LPA- and Y27632-treated groups by immunocytochemical analysis and Western blot analysis. The expression of E-cadherin was increased in LPA-treated group compared to that in the Y27632-treated group. The localization of E-cadherin in LPA-treated group was enriched in part of blastomere contacts compared to that Y27632-treated group. ROCK-1 as a crucial mediator of embryo compaction may plays an important role in regulating compaction through E-cadherin of the cell adhesion during the porcine preimplantation embryo. We concluded that ROCK-1 gene may affect the developmental potential of porcine blastocysts through regulating embryonic compaction.

(Key words : Rho associated kinase, Y27632, compaction, preimplantation development, pig)

INTRODUCTION

In vitro production (IVP) of porcine embryos, including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) of oocytes and their subsequent *in vitro* culture (IVC), have been modified by many researchers, but are still at a low level because of that incomplete cytoplasmic maturation, polyspermy, low percentage of male pronucleus (MPN) formation and weak developmental capability of the blastocyst have not been overcome until today (Abeydeera, 2002). In preimplantation embryos, developmental events include the first cleavage, the activation of the embryonic genome, and the compaction of morula and formation of blastocyst (Butz and Larue, 1995). Among them, compaction is the process of increased cellular flattening and adhesion in 8-cell stage embryo that requires the formation of junctional complexes and results in a polarized distribution of cell surface and intracellular components (Hyafil *et al.*, 1980; Butz and Larue, 1995; Ohsugi *et al.*, 1996). Therefore, the process of compaction is essential for further embryonic development.

Compaction creates the circumstances that bring about the first differentiation in mammalian development. It is mediated by events occurring at the cell surfaces of adjacent blastomeres. In the first stage of compaction, each of the eight blastomeres interacts with neighbors to undergo membrane polarization. During compaction, blastomeres flatten upon each other,

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due to the cell adhesion molecule E-cadherin, also known as unomorulin, and many cellular components are positionally reorganized (Hyafil *et al.*, 1980; Vestweber and Kemler, 1985). The E-cadherin is the first and best characterized cell adhesion molecule of the cadherin family (Ohsugi *et al.*, 1997). The E-cadherin, the prototype and founding member of the cadherin superfamily of calcium-dependent cell adhesion molecules, plays a central role in cell adhesion and determination of cell shape (Kemler *et al.*, 1977; Yagi and Takeichi, 2000).

Recently, Rho family GTPases including RhoA, Rac1, and Cdc42 have emerged as important regulators of cadherin-mediated cell-cell adhesion. The Rho family GTPases are implicated in a variety of physiological functions associated with changes in the cytoskeletal responses to extracellular signals including lysophosphatidic acid (LPA) and certain growth factors, which form stress fibers and cause focal adhesion (Ridley and Hall, 1992; Riento and Ridley, 2003). Rho family GTPases is also implicated in other physiological functions associated with cytoskeletal rearrangements such as cell morphology (Paterson *et al.*, 1990), cell aggregation (Tominaga *et al.*, 1993), cell motility (Takaishi *et al.*, 1994) and cytokinesis (Kishi *et al.*, 1993; Tominaga *et al.*, 1993). The Rho-associated kinases (ROCK) are ubiquitously expressed, which are involved in diverse cellular functions, including cell adhesion, smooth muscle contraction, actin cytoskeleton organization and motility, and gene expression (Noma *et al.*, 2006). Furthermore, ROCKs were found to support the integrity of cadherin-based cell contacts in other study (Vaezi *et al.*, 2002).

We hypothesized that Rho-associated kinase signaling would have some role in the development of preimplantation stage porcine embryos and may promote blastocyst formation and embryonic quality. Chemical compounds such as Y-27632 and LPA were used to inhibitor or activator of Rho kinase. We investigated the effects of Y-27632 and LPA on the blastocyst formation and the embryonic compaction. Furthermore, we also examined whether ROCK-1 signaling is involved in the regulation of the compaction mediated by E-cadherin of cellular adhesion molecules.

MATERIALS AND METHODS

1. Chemicals

Unless otherwise noted, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. *In Vitro* Maturation (IVM) and *In Vitro* Fertilization (IVF)

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25~30℃ in 0.9% saline supplemented with 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate. Cumulus oocyte complexes (COC) were aspirated through an 18 gauge needle into a disposable 10 ml syringe from follicles of 3 to 6 mm in diameter. After washing three times with TL-HEPES medium, approximately 50 oocytes were matured in 500 ml of the *in vitro* maturation medium in a 4-well multidish (Nunc, Roskilde, Denmark) at 38.5℃, 5% CO_2 in air. The medium used for oocyte maturation was NCSU-23 medium supplemented with 10% porcine follicular fluid, 0.57 mM cysteine, 10 ng/ml β-mercaptoethanol, 10 ng/ml EGF, 10 IU/ml PMSG, and 10 IU/ml hCG. At this step, 1 μM dbcAMP were added to the maturation medium of the experimental samples. After 22 h of culture, the oocytes were washed three times and then further cultured in maturation medium without both hormone and dbcAMP supplementation for an additional 22 h. After the completion of IVM, the oocytes were subjected to IVF, as described by Abeydeera and Day (Abeydeera and Day, 1997). The IVF was carried out in modified Tris-buffered medium (mTBM) consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 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 with Dulbecco's phosphate buffered saline (DPBS; Gibco-BRL, Grand Island, NY, USA) containing 1 mg/ml BSA (Fraction V; Sigma), 100 μg/ml penicillin G, and 75 μg/ml streptomycin sulfate. After washing, the spermatozoa were suspended in mTBM for 15 min pH 7.8. The oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 4 mg/ml BSA (fatty acid-free) and then placed into 48 μl drop of mTBM under paraffin oil. Diluted spermatozoa (2 μl) were added to 50 μl drop of mTBM containing 15~25 oocytes to give a final concentration of 1.5×10^5 sperm/ml. The oocytes were co-incubated with the spermatozoa for 6 h at 38.5℃ in an atmosphere of 5% CO_2 in air.

3. *In Vitro* Culture (IVC)

The presumptive zygotes were cultured in 50 μl drops of PZM3 medium supplemented with 3 mg/ml BSA at 38.5℃, 5% CO_2 in air. After 24 h of culture, cleaved 2-cell embryos were cultured in PZM3 medium supplemented with or without Y- 27632 (10 μM) and/or LPA (10 μM). On day 4, embryos

were divided into two categories: compacted and non-compacted described by Tao *et al.* (2002). The category 'compacted em bryo' included the early compacting and the fully compacted. The early compacting embryo was identified by blastmeres which had begun to compact tightly, forming a clustered cell mass. Each individual cell was identifiable but not distinct. At the fully compacted stage, blastomeres compacted completely. At this stage, the cell boundary might not be visible. Embryos that did not show any sign of compaction on day 4 were considered non-compacted embryos. Sometimes compaction would occur on day 5. However, they were still considered non-compaction, and were not evaluated in detail in the present study. Then, at 144 h after IVF, blastocysts formation and their numbers of ICM and TE nuclei were observed by differential staining method.

4. RNA Extraction and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 20 each developmental stage embryos using a commercial kit (RNeasy; Qiagen, Chatsworth, CA, USA) and subjected to real-time RT-PCR using an ABI 7500 Fast Real Time PCR System (Applied Biosystems, Inc., Foster City, CA). RT-PCR was carried out in a final reaction volume of 20 μl with SYBR Green (Applied Biosystems, Inc., Foster City, CA), a fluorophore that binds to all double-stranded DNA. For each cDNA, a 1-μl aliquot of the RT reaction was used. For Housekeeping gene, all the samples were quantified simultaneously during the same run. ROCK-1 (gene bank D89493) mRNA was amplified using the gene-specific primers, sence: TGCTGCTGGATAAATCTGGA; anti-sence: ATAACCATCGCCACCTTGAG, resulting in a 152 bp fragment. Beta-actin (gene bank AB046171) mRNA was amplified using the gene-specific primers, sence: CCATTCCATTGTTTG TGCAG; anti-sence: GTTGCCACACCTTCATTCCT, resulting in a 114 bp fragment. The amplification program was as follows: pre-incubation for fast-start polymerase activation at 95℃ for 10 min, followed by 40 amplification cycles of denaturation at 95℃ for 15 sec, annealing and elongation at 60℃ for 1 min, and then acquisition of fluorescence. After the end of the last cycle, a melting curve was generated by initiating fluorescence acquisition at 72℃ and taking measurements every 0.1℃ until 95℃ was reached. After the completion of the quantitative PCR analysis, PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized by exposure to UV light. The images were obtained using a Gel Doc apparatus (BioRad, Hercules, CA, USA).

5. Differential Staining

Differential staining of ICM and TE cells of blastocysts (Day 6) was performed using the technique described by Machaty *et al.* (1998). Briefly, the zona pellucida of blastocysts was removed by 1 min incubation in 0.5% pronase solution. After rinsing in TL-HEPES medium containing 1 mg/ml PVA, zona pellucida-free embryos were exposed to a 1:5 dilution of rabbit anti-pig whole serum for 1 h. Then, they were then rinsed three times for 5 min in TL-HEPES and placed into a 1:10 dilution of guinea pig complement containing bisbenzimide for 1 h. After brief rinsing in TL-HEPES, the stained embryos were mounted on slides under cover glass and observed under UV light using an epifluorescent microscope (Olympus). Blue and red colors were counted as ICM and TE cells, respectively.

6. Immunocytochemistry

Porcine embryos were fixed in 10% formalin solution containing 4% (w/v) formaldehyde for 30 min at room temperature (RT) and washed in PBS with 0.05% Tween 20 (PBST). After permeabilization for 30 min in PBS containing 0.1% Triton X-100, the samples were blocked in PBS with 2% BSA for 1 h at RT, and incubated for 1.5 h at 37℃ with rabbit anti-E-cadherin antibody (1:300). Embryos were washed in PBST, incubated with secondary antibody, chick-anti-rabbit conjugated Alexa 594 (1:300, Molecular Probe), for 1 h at 37℃, washed in PBST, and mounted on Poly-prep slide glass with a mounting media containing DAPI (Vectashield). Samples were observed under Carl Zeiss Axiovert 200M fluorescence microscope equipped with Apotome apparatus (Zeiss, Oberkochen, Germany). Images were captured digitally using different filter sets and merged using Axiovision (v4.5) or Adobe Photoshop software (v7.0).

7. Western Blot Analysis

For analyzing E-cadherin expression, the 4-cell stage embryos were collected after culture in IVC medium for 2 days. Twenty embryos were placed into 2 μl of PBS supplemented with 0.1% polyvinyl pyrrolidone, 18 μl of lysis buffer. The protein concentration was determined with Bio-Rad protein assay solution, based on the method of Bradford. The mixture was then denatured at 100℃ for 5 min. The protein samples

were separated on a 10% polyacrylamide gel via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA) for 2 h at 60~80 V, and transferred to a nitrocellulose membrane (161-0115; Bio-Rad) overnight at 20 mA. The membrane was blocked with 1.5% BSA in Tris-buffered saline (TBS) containing 0.25% Tween 20 (TBST) for 2 h at room temperature, rinsed in TBST, and probed with monoclonal mouse anti-E-cadherin antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST containing 1.5% BSA overnight at 4℃. The membrane was washed three times for 10 min each in TBST and subsequently incubated with HRP-conjugated secondary antibody (mouse IgG: 1:3,000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. After washing four times in TBST for 15 min each, the signals were detected using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Cardiff, UK) according to the manufacturer's instructions.

8. Statistical Analysis

Each experiment was replicated at least four times. All percentage data were subjected to arcsine transformation. All percentage data obtained in the present study are presented as the mean±SD throughout the text. Embryo development, compaction rate and cell numbers in blastocysts were analysed by ANOVA and student's *t*-test. Densitometric values obtained from immunoblotting were compared using Duncan's multiple range test with the GLM procedures in the SAS package (SAS Institute, Cary, NC, USA). $p<0.05$ was considered significant.

RESULTS

1. Expression Pattern of ROCK-1 mRNA during Preimplantation Development in Pigs

The expression of ROCK-1, downstream effectors of the small GTP-binding protein RhoA, was examined by quantitative real-time RT-PCR in each developmental stage. ROCK-1 mRNA presented the different levels during preimplantation development of porcine embryos (Fig. 1). The level of ROCK-1 mRNA was decreased gradually from GV-oocytes to 2-cell stage, but increased at 4- and 8-cell stages. In morula and blastocyst stages, the levels of ROCK-1 mRNA were rapidly reduced. Especially, expression of ROCK-1 mRNA in blastocyst stage was showed the weak level, indicating the reduction of ROCK-1 may associate with low developmental competence in porcine preimplantation embryos.

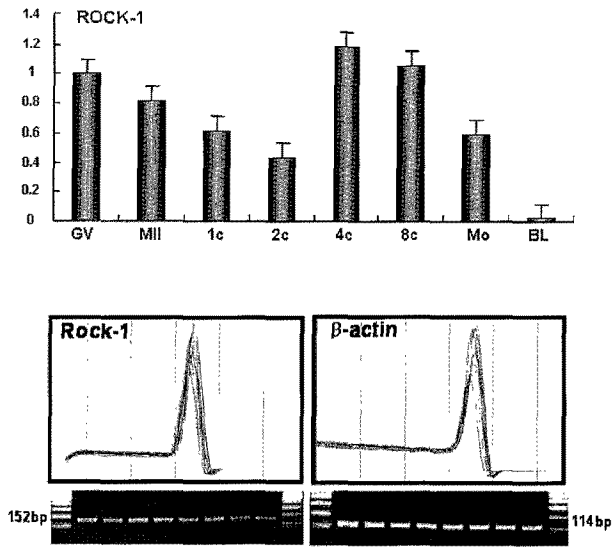

Fig. 1. Relative mRNA expression of the ROCK-1 gene in the various developmental stages of embryos by real-time RT-PCR analysis. The experiment was repeated four times, with β-actin used as the internal standard.

2. Effects of ROCK-1 Expression on Preimplantation Development of Porcine Embryos

We investigated that the expression of ROCK-1 affects on *in vitro* developmental competence and blastocyst quality by using inhibitor (Y-27632) or activator (LPA) of ROCK-1. As a result, developmental rate of LPA treated embryos was increased (54.0±5.5%, 69/132), but Y-27632 treated embryos were showed lower developmental rate (8.6±1.3%, 14/162) compared with untreated embryos (37.9±2.9%, 46/123), respectively (Fig. 2, $p<0.05$). We also checked the numbers of ICM, TE, and total cells by using differential staining. As shown in Fig. 3, total cell number of LPA treated blastocysts (43.3±12.4, n=48) was significantly different than that of Y-27632 treated blastocysts (17.4±8.6, n=11; $p<0.05$), although there was no significant difference in untreated blastocysts (36.8±11.2, n=35), respectively. The numbers of ICM and TE cell in Y-27632 treated blastocysts (1.5±1.2 and 15.8±8.0) was also significantly lower than those of LPA treated blastocysts (4.7±2.0 and 38.6±12.0) and non-treated blastocysts (4.4±1.9 and 32.4±11.2; $p<0.05$).

3. Effects of ROCK-1 on Compaction of Morula Stage Porcine Embryos

To investigate the role of ROCK-1 in compaction of porcine embryos, we analyzed compaction patterns of control, LPA-, and Y-27632-treated embryos. As indicated in Fig. 4, the percen-

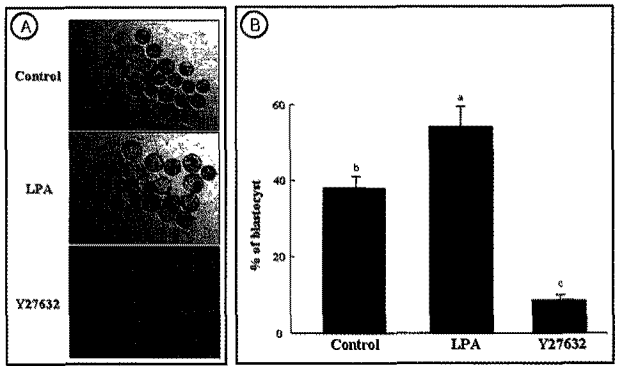

Fig. 2. Effect of LPA and Y-27632 on the blastocyst development of porcine embryos. Morphology of blastocysts derived from control, Rho associated kinase activator (LPA) treated-, and Rho associated kinase inhibitor (Y-27632) treated-embryos (A). The rate of blastocyst formation derived from 2-cell stage porcine embryos cultured in the PZM-3 medium supplemented with LPA and/or Y-27632 (B). This experiment was repeated four times. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

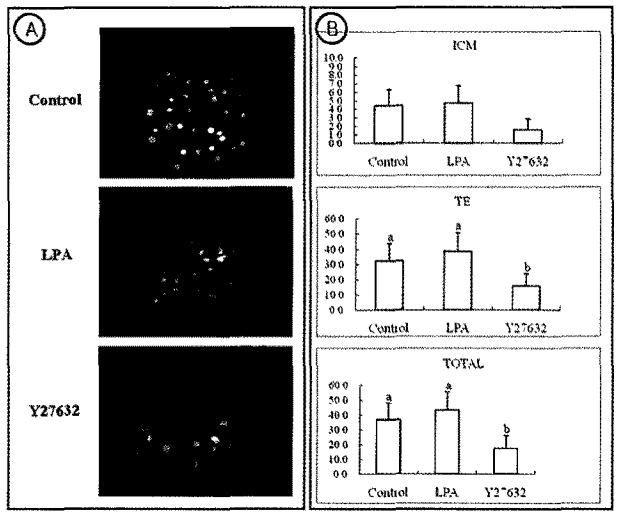

Fig. 3. Structural integrity of blastocysts derived from control, LPA treated and Y-27632 treated embryos. Differentially-stained images of porcine blastocysts (A). Blue and pink colors represent ICM and TE, respectively. Comparisons of cell numbers of blastocysts derived from each treatment group (B). This experiment was repeated three times. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

tage of compacted morula derived from LPA treated embryos (39.7±0.5%) was increased significantly compared with Y-27632

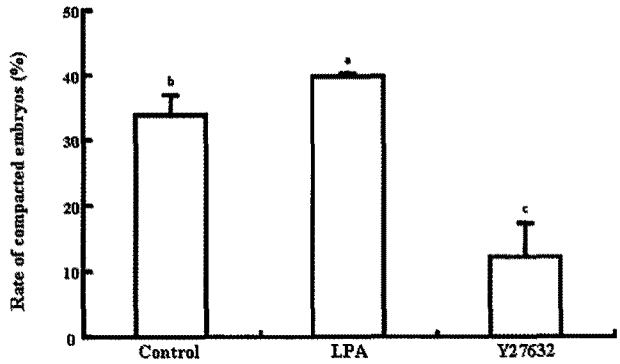

Fig. 4. Effects of LPA and Y-27632 on compaction of morula stage pig embryos. Percentage of compacted embryos on 4 days after IVF in LPA (10 μM) and Y-27632 (10 μM) treated groups. This experiment was repeated three times. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

treated embryos (12.0±5.2%) and untreated embryos (33.7±3.2%, $p<0.05$). Consequently, this result implicates that embryonic compaction is associate with Rho signaling pathway, involving ROCK-1, coincide with enhanced embryonic development.

4. Effects of ROCK-1 on the Expression of E-cadherin in 4-Cell Stage Embryos

In order to test whether the Rho signaling pathway is associated with formation of E-cadherin adherens junctions in 4-cell embryos, we analyzed the distribution and the expression level of E-cadherin in control, LPA and Y-27632 treated embryos. As a result of immunostaining, E-cadherin of LPA treated embryos enriched in adhesion region of blastomeres compared to untreated embryos (Fig. 5). However, E-cadherin at the blastomere contacts was reduced in 4-cell stage embryos of treatment with ROCK inhibitor Y-27632, as evident from the weak level of immunostaining. A comparative Western blot analysis of the LPA treated, Y-27632 treated and untreated embryos was performed to determine the relative levels of E-cadherin. Although the levels of E-cadherin in the control, LPA treated and Y-27632 treated embryos were not significantly different (Fig. 6), the intensity of immunoreactive band in the Y-27632 treated embryos was comparatively decreased.

DISCUSSION

To regulate the developmental competence of *in vitro*-produced (IVP) porcine embryos, the present study investigated

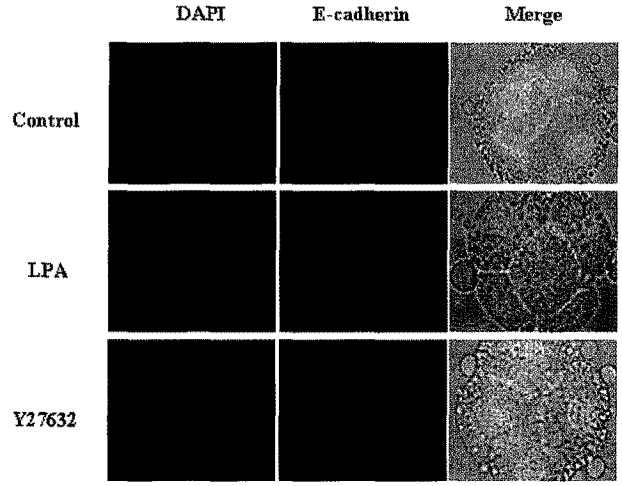

Fig. 5. Expression patterns of E-cadherin in 4-cell stage embryos treated with LPA and Y-27632 by immunocytochemical analysis. Rho kinase activator (LPA) and inhibitor (Y-27632) treated embryos were stained by anti E-cadherin (red color) in the cytoplasmic membrane. DAPI (blue) were counterstained in nucleus.

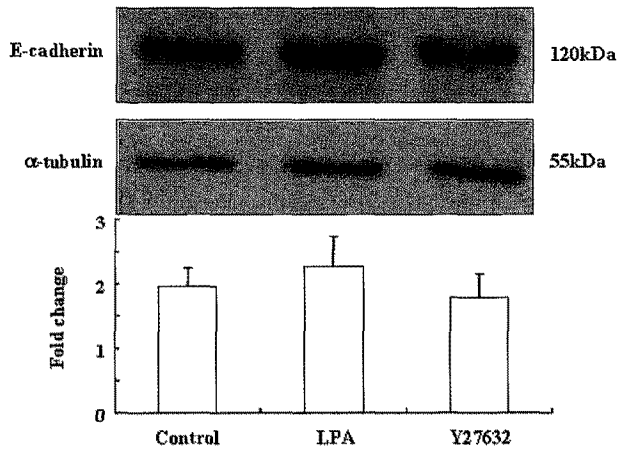

Fig. 6. Expression patterns of E-cadherin at 4-cell stage in LPA and Y-27632 treated embryos *in vitro*. Western blots were performed by using specific antibodies as anti-E-cadherin. Quantification of E-cadherin expression in control, LPA and Y-27632 treated embryos.

the effect of ROCK-1 on blastocyst development and embryonic compaction in IVP porcine embryos. In normal embryos compaction starts at the late 8-cell stage, continues in the morula. Moreover, compaction is the process of cell flattening and adhesion, and leads to a polarized distribution of cell surface and intracellular components (Hyafil *et al.*, 1980; Butz and Larue, 1995; Leung *et al.*, 1995; Ohsugi *et al.*, 1996). Specific cell surface proteins play important role in this process. One of such molecules, E-cadherin, a 120-kDa adhesive glycoprotein, is the first and best characterized cell adhesion molecule of the cadherin family and is synthesized at the 2-cell stage and is uniformly spread throughout the cell membrane. ROCK has been shown to induce adhesions and ROCK inhibition resulted in the elimination of E-cadherin-based adherens junctions at the cell-cell contacts. In the present study, we found that ROCK-1 activation was associated with increased blastocyst development by enhancing embryonic compaction, and increased the expression levels of E-cadherin through regulating Rho kinase.

Firstly, we observed that ROCK-1 mRNA was differently expressed throughout porcine preimplantation embryo development by using quantitative Real time RT-PCR. Interestingly, the expression pattern of ROCK-1 was distinct transcript levels, suggesting that the expression of ROCK-1 mRNA was degraded gradually during from oocyte to 2-cell stage, and was degraded again gradually during from 4-cell to blastocyst stage. Thus, we considered the ROCK-1 expression levels may influence the critical step compaction during preimpantation development. According to a previous report, Rho kinase is involved in mouse blastocyst formation (Kawagishi *et al.*, 2004). Thus, in the present study, we investigated the developmental competence of porcine embryos treated with Rho kinase activator LPA and inhibitor Y-27632. We confirmed that treatment of porcine embryos with Rho kinase inhibitor Y-27632 resulted in inhibition of blastocyst formation, whereas Rho kinase activator LPA was stimulated the blastocyst formation. This result clearly suggests that blastocyst formation is mediated by Rho kinase in pig, although the molecular basis for the stimulatory effect of Rho kinase on blastocoels expansion is not known. Moreover, blastocysts that developed in the LPA treated group had greater numbers of TE and total cells than that of the Y-27632 (Fig. 3). Fewer TE cells in the group that were Y-27632 treated during embryo culture period might have been due to reduced embryonic compaction. Our findings suggest that ROCK-1 activity is related with differentiation of TE cells, and may improve the developmental competence and the quality of blastocysts.

Clayton *et al.* (1999) reported that inhibition of Rho with *Clostridium botulinum* C3-transferase prevented cytocortical microfilament polarization of 8-cell blastomeres and interfered with compaction. In the present study, we examined the effect of ROCK-1 on compaction of porcine embryos treated con-

tinuously from the 2-cell stage with LPA and Y-27632 for 4 days. The rate of compacted morula in LPA treated group was significantly increased compared with Y27632 treated and untreated control groups. These results suggest that compacted morula formation might be mediated by ROCK-1. Furthermore, we investigated the relationship between ROCK-1 and expression of E-cadherin in 4-cell stage porcine embryos by immunostaining and Western blot analysis. As a result of immunostaining, E-cadherin of LPA treated embryos enriched in adhesion region of blastomeres compared to untreated embryos. However, E-cadherin at the blastomere contacts was reduced in 4-cell stage embryos of treatment with ROCK inhibitor Y-27632, as evident from the weak level of immunostaining. In Western blot analysis, although the levels of E-cadherin in the control, LPA treated and Y-27632 treated embryos were not significantly different, the intensity of immunoreactive band in the Y-27632 treated embryos was comparatively decreased. Thus, ROCK-1 signaling most likely participates in the actin filament-mediated translocation of E-cadherin to the cytoplasmic membrane (Tsukita *et al.*, 1992). Moreover, the results explained that ROCK-1 activation showed to induce E-cadherin based adherens junctions and ROCK-1 inhibition resulted in the elimination of E-cadherin based adherens junctions at the cell-cell contacts, namely the results indicated the involvement of ROCK-1 in control of E-cadherin based intercellular adhesion in porcine embryos.

In conclusion, our findings suggest the activation of ROCK-1 stimulates embryonic compaction in pig. Especially, culture conditions (i.e. the presence or absence of LPA or Y-27632) affect both the developmental potential and embryonic qualities of porcine IVF embryos. These results indicate that the use of Rho kinase activator during culture improves the *in vitro* development of porcine embryos. It will be important to investigate the mechanisms of Rho kinase signaling during the porcine preimplantation embryo development.

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