CXADR is required for AJ and TJ assembly during porcine blastocyst formation

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Abstract

Coxsackie virus and adenovirus receptor (CXADR) is a member of the immunoglobulin superfamily as well as a member of the junctional adhesion molecule family of adhesion receptor. In human pre-implantation embryos, CXADR was detected and co-localized with tight junction (TJ) proteins on the membrane of the trophectoderm. However, its physiological roles were not elucidated in terms of blastocyst formation. Here, we reported expression patterns and biological functions of CXADR in porcine pre-implantation embryos.

The transcripts of CXADR were detected at all stages of pre-implantation. Particularly, its expression dramatically increased and preferentially localized at the edge of cell–cell contacts, rather than in the nucleus from the eight-cell stage onwards. CXADR expression was knocked down (KD) by microinjecting double-stranded RNA into one-cell parthenotes. The vast majority of CXADR KD embryos failed to develop to the blastocyst stage, and a few developed KD blastocysts did not expand fully. Analysis of adherens junction (AJ)- and TJ-associated genes/proteins using qRT-PCR, immunocytochemistry and assessment of TJ permeability using FITC-dextran uptake assay revealed that the developmental failure and relatively small cavities are attributed to the defects of TJ assembly. In summary, CXADR is necessary for the AJ and TJ assembly/biogenesis during pre-implantation development.

Introduction

Once fertilized, a zygote undergoes a series of mitotic division without significant cell growth. As mitosis proceeds, the divided cells called blastomeres are tightly attached to each other in a process named compaction that is mediated by adherens junction (AJ) components such as CDH1 and CTNNB1 to form the compacted morula (Fleming et al. 1989, 2000a,b, Eckert & Fleming 2008). The outer cells of blastomeres after further division become flattened and form tight junctions (TJ) with neighboring cells together. Moreover, water influx by ion gradient (Na/K-ATPase) and water channels (AQP) across the membrane of outer cells (trophectoderm (TE)) results in a fluid-filled cavity called blastocoel. At this stage, the embryo is known as a blastocyst (Watson et al. 2004). Thus, the cavity of blastocyst is expanded by paracellular sealing via TJ at the TE and continuous fluid influx into the blastocyst. Furthermore, blastocyst development and stage status can be classified according to the morphological diameter (expansion) (Gardner et al. 2000, Ulloa Ullo et al. 2008).

In the last decade, extensive studies have identified genes involved in TJ biogenesis and cavity formation during mammalian pre-implantation development, and revealed their function by using various methods including specific inhibitor treatment, knockout and RNAi (summarized in Choi et al. 2012)). Recently, transcription factor AP 2 γ (TFAP2C) has been reported to regulate the expression of genes important for TJs in both murine and porcine embryos (Choi et al. 2012, Lee et al. 2015). Nonetheless, the mechanism underlying regulation in TJ biogenesis, constituent components and their crosstalk remains uncertain.

The coxsackie virus and adenovirus receptor (CXADR) is a member of junctional adhesion molecules, which are glycosylated transmembrane that belong to the Ig superfamily (Chiba et al. 2008). It is first identified as a cellular receptor for coxsackie B viruses and type C adenoviruses (Bergelson et al. 1997, Tomko et al. 1997). Many studies have demonstrated that CXADR is co-localized with junctional adhesion molecules including occludin (OCLN) and cytoplasmic plaque proteins such as TJ protein 1 (TJP1 also known as zonula occludens; ZO1) (Cohen et al. 2001, Coyne et al. 2004). A recent study reported that human CXADR is expressed during pre-implantation development and concentrated near the region of outer cell–cell contact

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ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-15-0397
Online version via www.reproduction-online.org
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at morula and blastocyst stages, and the subcellular localization of CXADR depends on splice variant and timing of their expression, suggesting that it may be closely associated with TJ at the early blastocyst (Krivega et al. 2014). However, biological roles of CXADR are not well defined in pre-implantation embryo development although a number of studies have showed expression and roles of CXADR as a TJ component in testes and other epithelial tissues (Raschperger et al. 2006, Wang et al. 2007, Mirza et al. 2012, Sultana et al. 2014). In the present study, we examined the spatial and temporal patterns of porcine CXADR gene expression, explored its physiological functions employing RNAi approach and investigated stability or integrity of TJs in porcine pre-implantation embryos. Here, we revealed that CXADR plays essential roles in the blastocyst formation including cavitation and proper subcellular localization of TJ proteins into the apical edge of the outer blastomeres.

Materials and methods

All chemicals were purchased from Sigma–Aldrich unless stated otherwise.

Porcine oocytes collection, embryo culture and collection

Porcine oocyte collection and embryo culture were performed as previously described (Lee et al. 2015). Briefly, ovaries were obtained from a local abattoir and transported to the laboratory in DPBS at 37 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles between 3 and 6 mm and then were washed three times with Hepes-buffered Tyrode’s medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). Groups of about 50 COCs were matured in in vitro maturation (IVM) medium at 39 °C for 44 h. After IVM, COCs were denuded by pipetting in the presence of 1 mg hyaluronidase/ml for 2–3 min. The denuded oocytes were activated using 50 μM calcium ionophore A23187 for 5 min and exposed 7.5 mg cytochalasin B/ml for 3 h in porcine zygote medium 3 (PZM3). The activated embryos were washed three times, transferred to PZM3 supplemented with 0.4% (w/v) BSA and cultured until use at 39 °C in a humidified atmosphere of 5% CO2 and 95% air.

Quantification of transcript levels

To analyze the expression levels of the genes, mRNA was isolated from denuded MI oocytes, one-cell, two-cell, four-cell parthenotes, morula and blastocyst using the Dynabeads mRNA Direct Kit (Dynal ASA, Oslo, Norway). Ten oocytes/embryos per biological replicate were used for the analysis. First-strand cDNA was synthesized by using Superscript Reverse Transcriptase enzyme (Invitrogen). qRT-PCRs were conducted using DNA Engine Opticon 2 Fluorescence Detection System (MJ Research, Waltham, MA, USA). DyNaMo SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland) was used to provide real-time quantification of targeted genes that were amplified with specific primer pairs (Table 1). The PCR was performed as follows: denaturation at 95 °C for 10 min, 40 cycles of amplification and quantification at 94 °C for 10 s, 55 or 60 °C for 30 s and 72 °C for 30 s with a single fluorescence measurement, melting at 65–95 °C with a heating rate of 0.2 °C/s and continuous fluorescence measurement and cooling to 12 °C. The relative quantification of gene expression was determined by the 2⁻ΔΔCT method (Livak & Schmittgen 2001) from three technical and biological replicates for both control and CXADR knockdown (KD) group. GAPDH was used as an internal control in all experiments.

Immunocytochemistry

The procedure was performed as described previously (Lee et al. 2015). Porcine pre-implantation embryos washed in DPBS containing polyvinyl alcohol (PVA; 1 mg/ml) were fixed for 20 min in 3.7% formaldehyde in DPBS, followed by permeabilization and blocking in DPBS containing 0.5% (v/v) Triton X-100 and

Table 1 Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence (5’–3’)</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXADR</td>
<td>GAAACCCTGGACTGGACGAG</td>
<td>GTAGCACCCACTTGGCAGAG</td>
</tr>
<tr>
<td>CDH1</td>
<td>ACTGGGTATATCCTCCCACATC</td>
<td>AAAGGGGCTTCTCTATTTT</td>
</tr>
<tr>
<td>TJP1</td>
<td>ACCACACAAAAACCCACCAA</td>
<td>CCATCTCTTGGCTGCAAACTATC</td>
</tr>
<tr>
<td>OCLN</td>
<td>CCRGGAGGAGAAGCTGGAT</td>
<td>ATCCGGCAGATCTCCACCAC</td>
</tr>
<tr>
<td>CLDN6</td>
<td>CCTGAAGAGAGGACAGGAG</td>
<td>GTCGCCGGTGAGGAAGAAG</td>
</tr>
<tr>
<td>PARDE6B</td>
<td>GATGAGAGAGGAGGAGGAG</td>
<td>GCCAGGCTGACAGGACAG</td>
</tr>
<tr>
<td>TFA2PC</td>
<td>CCTGCCAGCTGGAGTGAA</td>
<td>CCTCCGCCGAGTGATCTCCTTAC</td>
</tr>
<tr>
<td>SOX2</td>
<td>CTCGAGGCGCTGGAGTGA</td>
<td>GGGCATGATGGATGCTGCGTCT</td>
</tr>
<tr>
<td>POU5F1</td>
<td>CGGCGATGACCTATAGTGCGACACACA</td>
<td>AAACGGGATGATGCTGCGTGCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCAGGACGATGAGAAGA</td>
<td>TATACGCGAATAGTGCGATGAG</td>
</tr>
<tr>
<td>CXADR dsRNA</td>
<td>TAATACGACTCACTATAGGGGAGGACCACA-TTTCCTGGCAGAATTTCCT</td>
<td>CAGACGTCTGCAATTTGCTC</td>
</tr>
</tbody>
</table>
5% donkey serum at room temperature for 1 h. Embryos were then incubated with primary antibodies such as a rabbit polyclonal CXADR antibody (Sigma), a goat polyclonal OCT4 antibody (Santa Cruz Biotechnology), a mouse monoclonal TJP1 antibody (ZO1; ZYMED, San Francisco, CA, USA), a mouse monoclonal OCLN antibody (ZYMED), CDH1 (BD Biosciences, San Jose, CA, USA) and CTNNB1 (β-catenine; Santa Cruz) in blocking solution overnight at 4 °C, followed by incubation with Alexa Fluor 488 and 594 (Molecular Probes, Eugene, OR, USA) secondary antibodies. Embryos were then mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA) and images were obtained as z-series with 18 μm intervals with a moderately closed pinhole 56.3 μm, 2% laser power, 500–700 master gain and 1.58 μs pixel time. The acquired images were processed using Zen (Zeiss). Further image processing and analysis was performed using the freely available Image J software (ImageJ 1.48v; http://imagej.nih.gov/ij/) as described previously (Choi et al. 2013).

Double-strand RNA injection and embryo collection
After 6 h of chemical activation, 50 parthenotes per group were transferred to 10 μl drops of manipulation medium (TCM-199 supplemented with 0.6 mM NaCO3, 3 mM Hepes, 30 mM NaCl and 0.1% (w/v) BSA). Then, 100 μM CXADR-specific double-strand RNA (dsRNA) and negative control dsRNA were injected into the cytoplasm of one-cell parthenotes using an inverted microscope (Nikon, TE2000U, Japan) equipped with a micromanipulator (Narishige, Tokyo, Japan) until obvious swelling was observed. The injected embryos were incubated in PZM5 until use. Embryos at the one-cell, two-cell, four-cell, eight-cell, morula, and early blastocyst and expanded blastocyst stages were collected at 6, 30, 48, 72, 96, 120, and 148 h respectively after parthenogenetic activation.

Blastocyst expansion
To assess blastocyst expansion and predict Tj integrity, diameter of each CXADR KD and control blastocyst was measured under an inverted microscope (Nikon, TE2000U) using NIS-Elements D 2.30 Computer Software (Nikon). The diameters measured in the KD blastocyst were compared with those of the control, and the relative expansion was calculated from the relative diameter. Total 135 and 158 embryos (>40 per biological replicate) were used for the analysis.

**TJ permeability assay by FITC-dextran uptake**
To assess the effects of CXADR depletion on Tj permeability, control and the KD blastocyst were incubated in the culture medium containing 1 mg/ml of 40 kDa FITC-dextran for 30 min 39 °C. Following this incubation, the blastocysts were immediately washed in three 50 μl droplets of PZM5, placed in a fourth clean droplet of PZM5 and visualized under an inverted fluorescence microscope.

Statistical analysis
Data were analyzed by ANOVA using Statistical Analysis System Software (Statistical Analysis System, Inc., Cary, NC, USA). Data are presented as mean ± S.E.M. P values <0.05 were considered statistically significant unless otherwise stated.

Results
Expression patterns of CXADR during porcine pre-implantation development
We first examined temporal and spatial expression of porcine CXADR during pre-implantation development. CXADR transcripts were detected at all stages of pre-implantation from oocyte to blastocyst using qRT-PCR. Transcripts of CXADR were slightly reduced until two-cell stage and significantly increased at four-cell stage. Notably, a sharp increase in CXADR expression was observed from the eight-cell stage onwards (P<0.05) (Fig. 1A). We examined CXADR localization by immunocytochemistry (ICC) using confocal microscopy. Likewise, CXADR protein was present at all stages from the one-cell embryo to the blastocyst, but the abundance and the intracellular distribution of CXADR varied between early cleaving embryos and compacted/blastocyst embryos. CXADR protein was detected in both the nuclei and the perinuclear region and evenly distributed throughout the cytoplasm in the early cleaving embryos (Fig. 1B and Supplementary Fig. 1, see section on supplementary data given at the end of this article; one-cell to two-cell), but at the compacted eight-cell stage, CXADR immunostaining was localized to the perinuclei and along the entire cell membrane, preferentially apical sides (Fig. 1B and Supplementary Fig. 1, see section on supplementary data given at the end of this article). Most evidently at the morula and blastocyst stage, continuous lines of CXADR immunofluorescent staining were seen throughout the apical-most regions of outer cell–cell contacts (Fig. 1B).

In the ICC assay during pre-implantation development and another study (Krivega et al. 2014), CXADR proteins were detected by ICC staining as continuous lines at the apical edge of cell–cell boundaries at morula and blastocyst stages (Fig. 1B and C). To identify possible co-localization of adhesion and Tj complexes, we
Figure 1 Developmental expression of porcine CXADR in pre-implantation embryos. (A) qRT-PCR analysis of CXADR transcripts in porcine MII oocytes and pre-implantation embryos. 1C, one-cell parthenotes; 2C, two-cell parthenotes; 4C, four-cell parthenotes; 8C, eight-cell parthenotes; Mo, morula; BL, blastocyst. Different letters indicate statistically significant differences (\(P < 0.05\)). Data are expressed as mean \(\pm\) S.E.M.; RQ, relative quantification. (B) ICC analysis of CXADR in porcine pre-implantation development. CXADR is concentrated in the nucleus and is approximately equally distributed in the cytoplasm before compaction; this protein is predominantly found at cell–cell contact areas at the compacted eight-cell stage and onwards. Scale bars: 50 \(\mu\)m. (C) Immunofluorescent double staining revealed that CXADR is localized in TJs (TJP1 and OCLN) and AJs (CTNNB1 and CDH1), particularly a dot-like pattern was observed in mid-sectioned images. 3D projection images supported apical-lateral localization of the target proteins. Scale bars: 50 \(\mu\)m. (D) Co-localization of POU5F1 and CXADR in the nuclei of the expanding blastocyst. Enlarged images and 3D projection demonstrated the overlapped of green and red fluorescent signals. Negative control (without first antibody, CXADR) confirmed that the signal observed above is not a crosstalk artifact. Scale bars: 50 \(\mu\)m.
performed double ICC using two sets of antibodies: against CXADR and adhesion CTNNB1 also known as (a.k.a.) β-catenin (CDH1 a.k.a. E-cadherin) or against TJP1 (OCLN) respectively. In blastocyst embryos, these TJ proteins were co-localized with CXADR proteins that were distributed as continuous lines at the apical edge of cell–cell boundaries from Z-stack images and a dot-like pattern from mid-plane images while adhesion proteins were distributed as continuous lines at the edge of cell–cell boundaries without a dot-like fashion (Fig. 1C). This is supported by the 3D projection images in which the staining signals were more densely observed in apical-lateral areas (Fig. 1C).

We also detected CXADR in the nuclei of blastomeres and in the apical cortex of outer cells at the blastocyst stage. In line with one study on human embryos (Krivega et al. 2014), POU5F1 (a.k.a. OCT4), a pluripotency marker, was co-localized with CXADR in some blastocyst embryos (Fig. 1D). Co-localization between POU5F1 and CXADR was confirmed by enlarged and 3D projection images showing merged nuclei (purple) and thin continuous line (green) (Fig. 1D).

**CXADR is required for blastocyst formation**

We carried out a loss-of-function experiment using dsRNA injections to determine functions of porcine CXADR during pre-implantation development. Porcine CXADR mRNA and protein were successfully depleted by the dsRNA-mediated posttranscriptional gene silencing. We examined efficiency of the dsRNA-mediated KD of mRNA and protein expression, in comparison with non-specific dsRNA-injected control embryos.

A total of 90.4, 86.4, 55.1% of CXADR transcripts were depleted in four-cell, morula stage embryos and expanded blastocyst respectively when the abundance of CXADR was analyzed by qRT-PCR (Fig. 2A). Likewise, 73.3 and 51.4% of CXADR protein were abolished in morula and expanded blastocyst respectively when the average levels of pixel intensities of fluorescent signals were measured (Fig. 2A). Particularly, the CXADR protein was barely detectable at the apical regions of cell–cell boundaries in the CXADR KD blastocysts (Fig. 2B).

We found that CXADR down-regulation interfered with blastocyst development. In vitro development of embryos following dsRNA injection into one-cell parthenotes was examined at early cleaving, morula and blastocyst stages. There were no significant differences in initial cleavage and development to morula between control (77.5 and 52.9% respectively) and CXADR KD groups (67.5 and 43.6% respectively) although the developmental rates appeared to be lower in the KD embryos (Fig. 2C). On the other hand, the majority of the KD embryos failed to develop beyond the morula stage, and the blastocyst formation rates were found to be significantly lower in the KD group compared with those in the control at the early blastocyst (122 h post-activation; 11.2% vs 38.3%; P<0.05; Fig. 2C), and when both the embryos were further cultured for 24 h, control embryos were expanded but the KD embryos were not (Fig. 2D).

**CXADR is involved TJ integrity**

The impaired blastocyst formation in the KD embryos led us to hypothesize that genes/proteins involved in
adhesion, TJ complexes and/or cell lineage determination are affected by the morula-to-blastocyst transition. Thus, we examined the genes and proteins by qRT-PCR and ICC and subsequently, compared the relative abundance of transcripts and protein in the KD embryos with those in the control by turning ddCts into fold changes and measuring the fluorescence intensity using Image J Software (Fig. 3). CLDN4 and PARD6B coding for a cell adhesion and cell polarity regulator molecule respectively, essential in TJ on TE (Moriwaki et al. 2007, Alarcon 2010), were not affected by the CXADR KD at the morula stage, but adhesion and TJ-associated or -regulating genes including CDH1, OCLN, TJP1 and TFAP2C were significantly down-regulated in the KD embryos. In contrast, transcript levels of POU5F1 and SOX2, which are essential for normal development during mouse pre-implantation (Foygel et al. 2008, Keramari et al. 2010), were not significantly changed (Fig. 3A). According to the changes in the relative abundance of transcripts in the KD embryos, we first selected CDH1, OCLN and TJP1, which were significantly down-regulated (at least two-fold), and then examined abundance and subcellular localization by ICC. All the examined proteins were evidently distributed as continuous lines at the cell–cell boundaries in the control morula. In contrast, in the KD embryos, the immunostaining signals were relatively weak (CDH1) and indistinct (OCLN and TJP1), pointing to AJ–TJ defects in the KD blastocysts.

At 144 h after activation, we measured the diameters of control and KD blastocysts and compared the relative expansion on the basis of the measured values in each blastocyst because establishment and maintenance of cavitation largely depends on TJ function (Bell and Watson 2013). As shown in Fig. 4, the diameter of the CXADR KD blastocyst was smaller, and the relative ratio was 65 ±5% in comparison with the control. This finding was indicative of increased TJ permeability caused by disruption of the TJ complex in the KD embryos. Therefore, we examined TJ integrity with the help of 40 kDa FITC-dextran to confirm paracellular sealing and observed a significant difference in the number of embryos showing FITC signals between the control and KD groups (8.3 ±1.2% vs 27.2 ±1.7% respectively). This result was indicative of the defects of TJ integrity caused by mislocalization of TJ protein constituents.

**Discussion**

In this study, we describe the patterns of CXADR expression during porcine pre-implantation development. A loss-of-function experiment involving an RNAi-mediated KD reveals the biological function of CXADR in the blastocyst development. CXADR proteins are a part of the TJ complex, where they are associated with a variety of TJ plaque proteins, including TJP1 and OCLN, and function as an outer barrier of the TE during blastocyst formation.

Dramatic up-regulation of the CXADR transcript and changes in subcellular localization at the eight-cell stage...
onwards (Fig. 1) indicate that porcine CXADR is involved in the regulation of epithelial cell adhesion through CDH1 (Hussain et al. 2011, Morton et al. 2013) and is incorporated into the network of TJ networks during compaction/morula to blastocyst transition (Chiba et al. 2008, Krivega et al. 2014). In accordance with other studies, wherein a loss of CXADR resulted in mislocalization of OCLN and TJP1, and affected epithelial permeability (Cohen et al. 2001, Raschperger et al. 2006, Su et al. 2012), we observed compromised developmental competence of CXADR KD embryos from the morula to blastocyst stages and disruption or abnormal distribution of TJ proteins OCLN and TJP1 in the KD morula. These results suggest that CXADR directly interacts with AJ and TJ proteins and is essential for completion of TJ assembly/biogenesis.

These findings have led us to hypothesize that TJ disassembly that is caused by the CXADR KD directly affects paracellular sealing of the TE epithelium, and consequently, the KD blastocysts have relatively smaller diameters and cavities because of the leakage of blastocoel fluid in the KD embryos (Fig. 4). We wanted to find out whether the effect of the CXADR KD on blastocyst expansion could be attributed to disintegration of the TJ barrier or to the delayed blastocyst development. The increased permeability to FITC-dextran in the CXADR KD blastocyst strongly supports our supposition that CXADR does play an important role in TJ integrity.

Human CXADR has been reported to be ubiquitously expressed during pre-implantation development, and the soluble form CXADR protein localized to the nucleus was detected in undifferentiated blastomeres and in the TE nuclei after hatching while transmembrane CXADR was predominantly found at outer cell–cell boundaries (Krivega et al. 2014). We observed that CXADR accumulates in the nuclei and is co-localized with POU5F1 in the expanded porcine blastocysts (Fig. 1C). To determine whether CXADR affects the cell lineage-specific and pluripotency-specific or the related gene expression, we quantified the transcript levels of POU5F1, SOX2 and TFP2C. The TE-specific gene, TFP2C, was down-regulated, but the pluripotency genes were not affected in the CXADR KD embryos, suggesting that CXADR may not directly regulate POU5F1 and SOX2, at least at the level of transcription. In spite of the presence of nuclear CXADR in undifferentiated blastomeres and expanded/hatching blastocysts, the biological function of CXADR is still largely unknown.

In summary, our data provide strong evidence that the transmembrane protein CXADR is crucial for the correct localization and function of TJs during the morula-to-blastocyst transition. Except for the compacted eight-cell, morula and early blastocyst stages, maternally derived CXADR is mostly localized in the cytosol and nucleus; in expanded blastocysts, CXADR is co-localized with POU5F1 in the nucleus. Nevertheless, the functions of nuclear CXADR are still unknown. Further studies are needed to elucidate the mechanisms regulating CXADR expression, including its variants and its translocation into the nucleus.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0397.
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.

Funding

This was supported by Next-Generation BioGreen21 Program (CABX, Project No. PJ011213 to IC and PJ011126 to NK), Rural Development Administration (RDA), Republic of Korea.

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Accepted 4 January 2016

Received 27 August 2015
First decision 16 September 2015

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