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.

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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 blastocoel. Furthermore, blastocyst development and stage status can be classified according to the morphological diameter (expansion) (Gardner *et al.* 2000, Ulloa Ulloa *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

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 TJs 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 MII 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 $2^{-\Delta\Delta C_t}$ 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% (w/v) paraformaldehyde in DPBS, followed by permeabilization and blocking in DPBS containing 0.5% (v/v) Triton X-100 and

Table 1 Primer sequences.

Gene	Forward sequence (5'–3')	Reverse primer
CXADR	GAAACCTGGACTGGACGAG	GTAGCACCCTTGCAGAGGA
CDH1	ACTGGGTATCCCTCCCATC	AAACGGGCTTTCTCATTTT
TJP1	ACCCACAAACCCACCAA	CCATCTCTTGCTGCCAACTATC
OCLN	GCRGGAGGAAGACTGGAT	ATCCGAGATCCCCTAAC
CLDN6	CCTGTAAGACGGAGCCAGAG	GTGTCCAGGGTGGAGAAAGA
PAR6B	GATGAAGACAGCGATGACGA	GCTACGGGTGCTAAGCTGAC
TFAP2C	GAAACCTGGACTGGACGAG	GTAGCACCCTTGCAGAGGA
SOX2	CTGCGAGCGCTGCAGATGAA	CCTCCGGGAGTGTGTACTTATCCT
POU5F1	CGTGGAGACTTTGCAGCCTGA	GGCGATGTAAGTGATCTGCTG
GAPDH	GGGCATGAACCATGAGAACT	AAGCAGGGATGATGTTCTGG
CXADR dsRNA	TAATACGACTCACTATAGGGAGACCACA- TTTGCCGTGCAGATTACC	TAATACGACTCACTATAGGGAGACCACA- CACAGCTCCTGCAATTGTTC

F, forward; R, reverse.

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 -canetene; 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 acquired with using a laser confocal scanning microscope (Zeiss LSM710, Jena, Germany) equipped with Zeiss AxioObserver and C-Apochromat 40 $\times$ 1.20 W Korr M27, and an argon/krypton-ion laser with 405, 488 and 568 nm excitation wavelength was used. Images were obtained as z-series with 18 μ m intervals with a moderately closed pinhole 56.3 μ m, 2% laser power, 500–700 master gain and 1.58 μ s 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 μ l 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 μ M 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 μ l 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 $\pm$ 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 μ 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 μ 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 μ m.

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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 maker, 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

Figure 2 Effects of RNAi-mediated ablation of CXADR on early porcine embryo development. (A) Validation of dsRNA-mediated KD of CXADR transcripts in four-cell embryos and morulas by qRT-PCR (filled bars), and the abundance of CXADR was semi-quantitatively analyzed by using ImageJ (open bars). (B) Representative images of CXADR protein depletion in early blastocysts by ICC. Scale bars: 50 μ m. (C) Developmental rates of pre-implantation embryos injected with control dsRNA (control; filled bars) and CXADR dsRNA (CXADR KD; hatched bars). (D) Representative images of CXADR KD and control embryos at the blastocyst stage (144 h after activation). Scale bars: 200 μ m. Different letters indicate statistically significant differences ($P < 0.05$). Data are expressed as mean $\pm$ S.E.M.; RQ, relative quantification.

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*, *OCN*, *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*, *OCN* 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 (*OCN* 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 $65 \pm 5\%$ 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 ($8.3 \pm 1.2\%$ vs $27.2 \pm 1.7\%$ 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 *OCN*,

Figure 3 Effects of RNAi-mediated ablation of *CXADR* on gene expression and TJ-associated proteins. (A) qRT-PCR analysis of putative *CXADR* target genes in *CXADR* KD and control morulas. AJ- and TJ-associated genes such as *CDH1*, *OCN* and *TJP1* and their regulating factor *TFAP2C* were significantly down-regulated in the *CXADR* KD embryos. Asterisks indicate statistically significant differences ($P < 0.05$). Data are expressed as mean $\pm$ S.E.M.; RQ, relative quantification. (B) Protein expression and subcellular localization of the proteins that were down-regulated by more than twofold in the *CXADR* KD embryos. Asterisks indicate statistically significant differences ($P < 0.05$). Data are expressed as mean $\pm$ S.E.M.; RQ, relative quantification. (C) Subcellular localization of *CDH1* was not changed, but the amount was reduced according to the transcription levels in the KD embryos. TJ proteins *OCN* and *TJP1* were significantly down-regulated and barely detectable at the apical edge of cell–cell contacts.

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

Figure 4 Effects of RNAi-mediated ablation of CXADR on the barrier function of the TE. (A) Blastocyst embryos from the KD and control groups were collected, and their diameters were measured; the diameters were compared with those in the control (control; filled bars). The ratio denotes relative expansion of the KD blastocysts (CXADR KD; hatched bars). (B) To test the permeability of TJs in the KD embryos (CXADR KD; hatched bars) and control blastocyst (control; filled bars), both groups of blastocysts were incubated in the culture medium containing 1 mg/ml 40 kDa FITC-dextran for 30 min and then the blastocysts were examined under a fluorescence microscope. A significant difference in the FITC signals was observed between the two groups when the blastocysts were visualized under an inverted fluorescence microscope. (C) Representative images of blastocysts treated with FITC-dextran. Green signals: different letters indicate statistically significant differences ($P < 0.05$). Scale bars: 200 μ m.

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 *TFAP2C*. The TE-specific gene, *TFAP2C*, 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.

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