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Short Communication

Traf4 is required for tight junction complex during mouse blastocyst formation

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ABSTRACT Traf4 (Tumor necrosis factor Receptor Associated Factor 4) is a member of the tumor necrosis factor receptor (TNFR) - associated factors (TRAFs) family. TRAF4 is overexpressed in tumor cells such as breast cancer and associated with cytoskeleton and membrane fraction. Interestingly, TRAF4 was localized with tight junctions (TJs) proteins including OCLN and TJP1 in mammary epithelial cells. However, the expression patterns and biological function of Traf4 were not examined in preimplantation mouse embryos although Traf4-deficient mouse showed embryonic lethality or various dramatic malformation. In this study, we examined the temporal and spatial expression patterns of mouse Traf4 during preimplantation development by qRT-PCR and immunostaining, and its biological function by using siRNA injection. We found upregulation of Traf4 from the 8-cell stage onwards and apical region of cell – cell contact sites at morula and blastocyst embryos. Moreover, Traf4 knockdown led to defective TJs without alteration of genes associated with TJ assembly but elevated p21 expression at the KD morula. Taken together, Traf4 is required for TJs assembly and cell proliferation during morula to blastocyst transition.

Keywords: blastocyst, preimplantation, p21, tight junction, Traf4

INTRODUCTION

A fertilized one-cell zygote undergoes a series of cleavage divisions and a process known as compaction to become a morula. The compacted morula eventually develops into a blastocyst, a distinct mammalian embryo stage during the preimplantation period. This morphogenetic transition is observed in early stage mouse embryos, for example via activation of paracellular adhesion and cellular polarization at the 8-cell stage (Kidder and McLachlin, 1985; Tesarík, 1988; Zhou et al., 2020). Subsequently, external blastomeres start to take up fluid from the uterus and pump it centrally, creating the blastocoel via tight junction (TJ) assembly or biogenesis (Watson and Barcroft, 2001). Finally, the external blastomeres differentiate into the trophectoderm (TE) and the internal cells develop into the inner cell mass (ICM). The TE and ICM contribute to establishment of the placenta and fetus, respectively (Cockburn and Rossant, 2010).

Tumor necrosis factor receptor associated factor 4 (Traf4) belongs to the tumor necrosis factor receptor-associated factor (TRAF) family, which is found in the cell membrane and cytoplasm (Régnier et al., 1995; Kedinger and Rio, 2007). TRAF4 is overexpressed in breast cancer, and exists in the cytoskeleton and membrane fractions in other cancers (Xu et al., 2002; Camilleri-Broët et al.,

2007). In epithelial cells, TRAF4 is localized with TJ markers such as OCLN and TJP1 (also known as ZO-1) in the apical regions of cell-cell boundaries, and affects the stability of apical cell-cell junctions in mammary epithelial cells (Kédinger et al., 2008; Rousseau et al., 2011; Park and Kim, 2019). The Traf4-deficient mouse shows approximately 30% embryonic lethality, and causes various changes and dramatic malformations in the trachea and axial skeleton (Régnier et al., 2002). However, the expression patterns and biological function of Traf4 have not been examined in preimplantation mouse embryos.

In this study, we investigated the expression and localization of Traf4 during preimplantation development, and its biological functions, by injecting 1-cell zygotes with siRNA.

MATERIALS AND METHODS

All the animal studies were approved by Institution Animal Care and Use Committee guidelines from the Chungnam National University Animal Welfare and Ethical Review Body (License No. CNU-00702), and all chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Embryo collection and microinjection

As previously described (Jeong and Choi, 2019), five female mice (6-8 week-old C57 BL/6; KOATECH, Pyeongtaek, Republic of Korea) were superovulated by injecting 5 IU of pregnant mare's serum gonadotropin (PMSG) followed by 5 IU human chorionic gonadotropin (hCG) 48 hours later for one biological experiment. Immediately, female mice were mated with B6/n males (10-24 week-old; KOATECH) after injection of hCG. Fertilized one-cell zygotes were collected in M2 medium at 16-18 hours post hCG (hph) injection from ampulla of the mouse oviducts and then cultured in M16 medium including 0.003% EDTA, 0.06% penicilin G postassuim salt, 0.005% streptomycin sulfate under mineral oil at 37℃ in a humidified atmosphere of 5% O2, 5% CO_2, 90% N_2. For knock down (KD) experiments, 5-10 pl of 100 μM TRAF4 siRNA (siGenome; Dharmacon, CO, USA), or 100 μM control non-target siRNA (Dharmarcon) was injected into the cytoplasm of one-cell zygote embryos using microinjector at 19-21 hph, and the injected embryos were cultured for four days. Traf4 KD (n = 203), and control (n = 193) embryos were used for developmental analysis.

Real-time quantitative PCR

Total RNA was isolated from groups of at least ten embryos using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) and cDNA was synthesized by using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time qPCR analysis was performed on a StepOnePlus real-time PCR system (Applied Biosystems, Foster, CA, USA) using gene-specific designed primers (Table 1). Ubtf and GFP was used as endogenous and exogenous controls for KD and preimplantation development stage, respectively. For gene expression analysis, 60 control and 60 Traf4 KD morula embryos were used (10 embryos per biological replication and 6 times repeated).

Immunocytochemistry

Preimplantation embryos were fixed with 3.7% paraformaldehyde for 20 minutes, permeabilized with phosphate-

Table 1. Primers sequences for qRT-PCR

Gene	Forward sequences (5'-3')	Reverse sequences (5'-3')
Traf4	TGCCAGCCACATTCTACCTG	TACCTCCCACCCCCACTATG
Tjp1	TTTGGGCTGTGCATCTGA	TGCTTTATTGCTGCAGAGG
Ocln	ACGTCGTGGACCGGTATC	AAAAACAGTGGTGGGGAAC
Adam10	CCTGCCATTTCACTCTGTCA	GGGCTCCTTCCTCTACTCCA
p21	GCAGATCCACAGCGATATCC	CAACTGCTCACTGTCCACGG
p53	GGGGAGGAGCCAGGCCATCA	CCGCGCCATGGCCATCTACA
Rock1	ACCCACCATCTGGCTTTGTC	CGGTTTATCAGGTAGCATCCC
Rock2	GATGGTTGTCATTGCCTGTGC	TGCTCTTTATCTTGTTCGCTGT
Ubtf	CGCGCAGCATACAAAGAATACA	GTTTGGGCCTCGGAGCTT
GFP	AAGCTGACCCTGAAGTTCATCTGC	CTTGTAGTTGCCGTCGTCCTTGAA

buffered saline (PBS) containing 0.1% Tween 20 for 20 minutes, blocked with PBS containing 0.1% bovine serum albumin (BSA) for 1 hour at room temperature, and incubated with a primary antibody, Traf4 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or Tjp1 (also known as ZO-1, 40-2200; Invitrogen) overnight in blocking solution at 4℃. For detection, the treated embryos were incubated with AlexaFluor488- or 594-labelled secondary antibody (Molecular Probes, Eugene, OR, USA) for 30 min at room temperature, and then mounted in Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Images were captured using a laser scanning confocal microscope (C2puls; Nikon, Tokyo, Japan) and processed with NIS-Elements software (Nikon).

FITC-dextran uptake assay

52 control (17-36/assay) and 79 KD blastocyst (16-20/assay) were incubated in M16 medium containing 4kDa FITC-dextran (1mg/mL) for 10 min at 37℃ and then washed in fresh M2 medium three~ four times. Diffusion of FITC-dextran into the blastocoel was examined in a clean drop of M2 medium by an inverted fluorescence microscope (Nikon Eclipse Ti-U, Nikon).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using GraphPad Prism (Version 5.03, GraphPad Software, San Diego, CA, USA), and are presented as mean ± s.e.m. (Standard error mean). p values < 0.05 were considered statistically significant differences unless otherwise stated.

RESULTS AND DISCUSSION

We previously identified several genes involved in tight junctions of blastocyst such as Tfap2c, Cxadr, Adam 10, and Rock in pig and mouse embryos and reported that TJ biogenesis and assembly is conserved across mammalian embryos (Kwon et al., 2016a; Kwon et al., 2016b; Kwon et al., 2016c; Jeong et al., 2019). Here we reported that expression patterns of Traf4 and its biological function in terms of tight junction assembly during morula to blastocyst transition.

Expression of TRAF4 in mouse preimplantation embryos

To assess the expression of *Traf4* during mouse preimplantation development, we examined transcript levels using qRT-PCR and ICC. *Traf4* transcripts were detected at all preimplantation embryo stages, although the expression gradually decreased until the 4-cell stage. However, the zygotic expression of *Traf4* transcripts increased from the 8-cell stage onwards (Fig. 1A). Traf4 proteins were observed in the cytoplasm of early cleaving embryos, and were more densely located in cell-cell contact sites. Apical localization was detected from the 4-cell stage and was more obvious in the morula and blastocyst (Fig. 1B). Moreover, ICC assay showed that Traf4 and Tjp1 co-localized in the apical regions of outer cells (Fig. 1B).

Effects of Traf4 KD on preimplantation embryo development

To abolish maternally derived and zygotic Traf4 mRNA during early embryo development, Traf4 siRNA (100 μM) was injected into 1-cell zygote embryos. The knockdown (KD) efficiency was measured using qRT-PCR, which showed that Traf4 mRNA was successfully depleted (by > 80% in the morula).

We observed significant differences in blastocyst development between control (81.5 ± 3.0 %) and *Traf4* KD (49.2 ± 4.7 %) embryos (Fig. 2A and 2B). Interestingly, we also observed collapsed blastocyst cavities in the KD embryos (Fig. 2A), suggesting that the loss of *Traf4* affects TJ assembly during the morula to blastocyst transition.

Next, to determine whether the deletion of Traf4 causes defective TJs in blastocysts, blastocoel diffusion assay was performed using FITC-dextran uptake to assess cell-cell barrier function (Choi et al., 2012). There were significant differences in TJ permeability (FITC-positive signals) between the control (19.9 ± 4.7 %) and *Traf4* KD (43.4 ± 4.7 %) embryos (Fig. 2C and 2D), suggesting that Traf4 localized to apical regions is involved in intercellular sealing via TJ assembly rather than AJs.

Effects of Traf4 KD on the gene expression associated with TJs and the cell cycle

In agreement with previous TJ assembly studies in mouse and pig embryos (Choi et al., 2012; Kwon et al., 2016a; Kwon et al., 2016c; Jeong et al., 2019; Kwon et al., 2019), we attributed defective paracellular sealing in

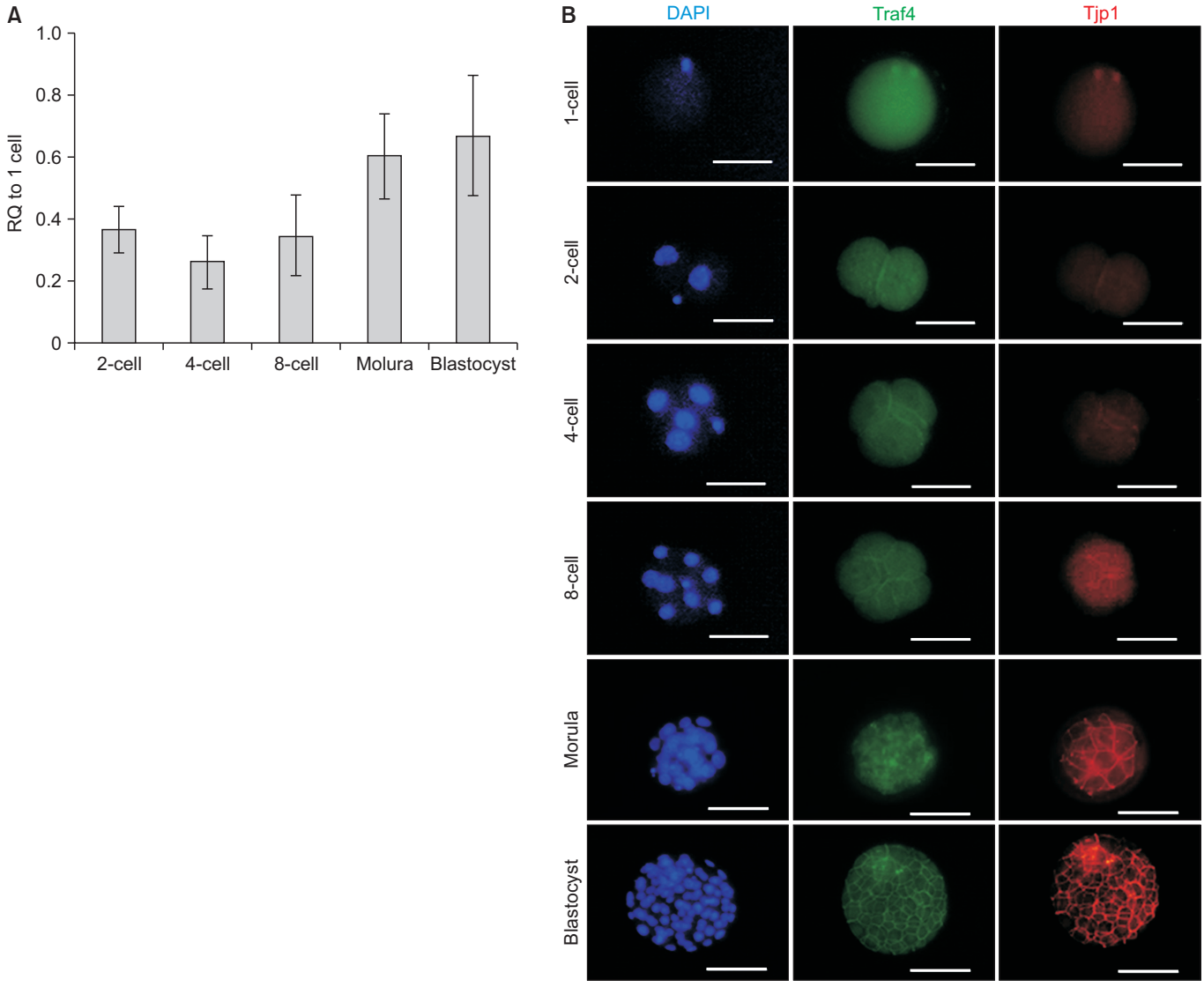

Fig. 1. Expression patterns of Traf4 during mouse preimplantation development. (A) Transcription levels of Traf4 were measured from the 1 cell to blastocyst stages using qRT-PCR. Expression levels were normalized to an exogenous control gene (Gfp). (B) Cellular localization of Traf4 were analyzed by immunocytochemistry. Scale bar (50 μm). Error bars represent mean ± standard error.

Traf4 KD blastocysts to the altered gene expression and protein localization required for establishing TJs, or to abnormal cell cycle checkpoint gene expression during cell proliferation. We examined the expression of the TJ genes *Tjp1*, *Ocln*, *Adam10*, *Rock1*, and *Rock2* (Kwon et al., 2016b; Jeong et al., 2019), and the cell proliferation genes *p21* (also known as *Cdkn1a*) and *p53* (also known as *Trp53*), in morula embryos using qRT PCR (Hoeferlin et al., 2011; Muñoz-Espín et al., 2013; Ock et al., 2020).

The expression of genes associated with TJ biogenesis was not changed in the Traf4 KD embryos. However, the KD morula embryos showed increased *p21* expression compared with control embryos (Fig. 3). In line with previous reports of the upregulation of *p21* transcripts in mouse embryos associated with embryonic arrest during the morula to blastocyst transition (Choi et al., 2012; Ock et al., 2020), the retarded development of morula embryos might be attributed to the elevated p21 expression. Although we did not find direct evidences for an inverse relationship between Traf4 and p53-p21 signaling activity (Rozan and El-Deiry, 2006; Deng et al., 2019), Traf4 suppression might affect cell proliferation in preimplantation embryos via the p21 pathway.

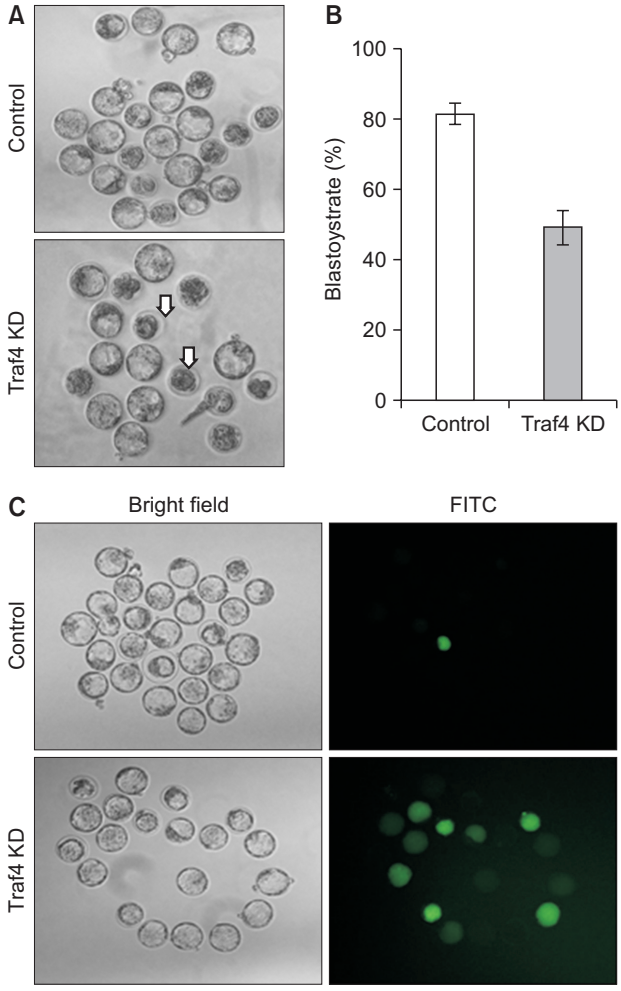

Fig. 2. Effects of Traf4 KD on embryos. (A) Representative images of blastocyst development at 120 h after hCG. (B) Blastocyst development rate. (C) Representative images of defective paracellular sealing in Traf4 KD blastocysts. (D) 4kDa FITC-dextran uptake assay was used for TJs permeability. White arrows indicate collapsed blastocoel.

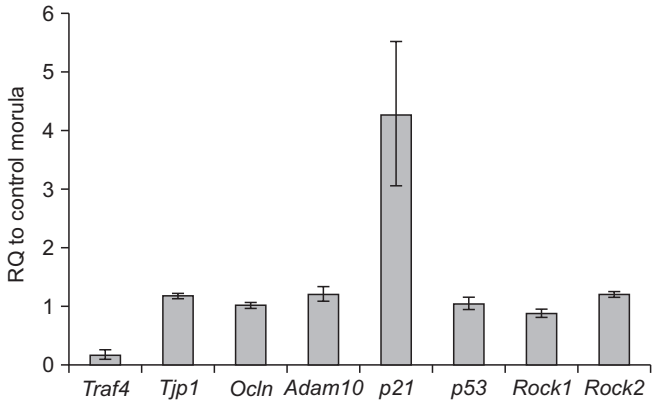

Fig. 3. Gene expression patterns of Tfa4 KD embryos. In the Traf4 KD morula, expression levels of TJs and cell cycle associated genes were not changed except for p21. RQ (Relative Quantification). Error bars represent mean ± standard error.

CONCLUSION

This study examined the spatiotemporal expression of mouse Traf4 during preimplantation development, and investigated its function using RNAi. The upregulation from the 8-cell stage onwards, apical localization, and loss of function study indicates that Traf4 is required for blastocyst development rather than morula compaction. The permeability assay confirmed the need for Traf4 for TJ assembly, and elevated p21 expression was associated with embryonic arrest at the morula stage in the KD embryos. Moreover, it is critical to evaluate and select developmentally competent embryos during preimplantation stage because the embryos produced by *in vitro* system (*in vitro* maturation, fertilization and culture) show lower blastocyst development and fail to implant.

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