Involvement of nephrin in human placental trophoblast syncytialization

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Abstract

The placenta has numerous functions, such as transporting oxygen and nutrients and building the immune tolerance of the fetus. Cell fusion is an essential process for placental development and maturation. In human placental development, mononucleated cytotrophoblast (CTB) cells can fuse to form a multinucleated syncytiotrophoblast (STB), which is the outermost layer of the placenta. Nephrin is a transmembrane protein that belongs to the Ig superfamily. Previous studies have shown that nephrin contributes to the fusion of myoblasts into myotubes in zebrafish and mice, presenting a functional conservation with its *Drosophila* ortholog sticks and stones. However, whether nephrin is involved in trophoblast syncytialization remains unclear. In this study, we report that nephrin was localized predominantly in the CTB cells and STB of human placenta villi from first trimester to term pregnancy. Using a spontaneous fusion model of primary CTB cells, the expression of nephrin was found to be increased during trophoblast cell fusion. Moreover, the spontaneous syncytialization and the expression of syncytin 2, connexin 43, and human chorionic gonadotropin beta were significantly inhibited by nephrin-specific siRNAs. The above results demonstrate that nephrin plays an important role in trophoblast syncytialization.

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Introduction

The syncytialization of trophoblast cells is a key event in human placental morphogenesis. Syncytialization occurs around the time of embryo implantation and continues until delivery. Primary syncytialization is a process in which trophoblast cells at the embryonic pole of the blastocyst fuse intercellularly and form a multinucleated syncytium that adheres to and penetrates the maternal uterine epithelium; this event is crucial for a successful embryo implantation (Red-Horse *et al.* 2004). After the blastocyst is fully covered by the maternal uterine epithelium and the placental villi are formed, the syncytiotrophoblast (STB) changes its function and stops invasion. Instead, it develops into the outermost layer of the placental villi, where it directly contacts maternal blood. During the remaining stages of pregnancy, the STB plays important functions in immune tolerance, the production of hormones such as human chorionic gonadotropin beta (β -hCG) and the exchange of nutrients and waste products (Malassine & Cronier 2002, Leisser *et al.* 2006, Huppertz & Borges 2008). In the so-called

secondary syncytialization, mononucleated cytotrophoblast (CTB) cells lining the placental villi fuse with the multinucleated STB. Fusion and communication between the CTB cells and STB is fundamental for placentalation and for a successful pregnancy (Gauster *et al.* 2010).

Cell fusion appears in a wide variety of organisms and in many cellular processes, such as yeast mating, *Caenorhabditis elegans* epidermal cell fusion, myotube formation, fertilization, macrophage fusion, tumorigenesis, and placentalation (del Campo *et al.* 2005, Chen & Olson 2005). A number of proteins have been implicated in the fusion process. Syncytin proteins function as fusogens during trophoblast cell fusion through binding with their receptors (Blond *et al.* 2000, Mi *et al.* 2000, Blaise *et al.* 2003, Esnault *et al.* 2008). Connexin 43, ADAM12, CD98, and cadherin 11, all have been demonstrated to play the important roles in trophoblast cell–cell membrane merging (Getsios & MacCalman 2003, Mori *et al.* 2004, Dunk *et al.* 2012). Other factors which are involved in trophoblast cell fusion include proteases (caspases 8, 10, and 14), cytokines (LIF, MIC1, and TNF α), growth factors (VEGF, CSF, and EGF), and transcription factors (GCM1, Mash-2, and Twist)

(Yusuf *et al.* 2002, Baczyk *et al.* 2009, Butler *et al.* 2009, Huppertz & Gauster 2011). Disturbances in syncytial trophoblast differentiation or maintenance may lead to pregnancy disorders such as intrauterine growth restriction and pre-eclampsia. However, the mechanisms governing syncytial fusion remain poorly understood.

Nephrin is the protein product of the *NPHS1* gene, which belongs to the Ig superfamily. Nephrin has been found to be highly expressed in the kidney glomerulus (Lenkkeri *et al.* 1999). As a transmembrane protein, nephrin has a short intracellular domain and a large extracellular domain with eight distal Ig-like motifs (Kestila *et al.* 1998). Nephrin is a central component of the slit diaphragm in glomerular podocytes (Ruotsalainen *et al.* 1999). The cell adhesion activities of nephrin have been confirmed in kidney podocytes and HEK-293 cells (Khoshnoodi *et al.* 2003, George *et al.* 2012). It is well established that nephrin plays a critical role in the renal ultrafilter. However, little is known about the potential roles of nephrin in extra-renal tissues. Sticks and stones (Sns), the *Drosophila* ortholog of nephrin, functions as a positive regulator of myoblast fusion and is also required for the adhesion and fusion of insect nephrocytes (Zhuang *et al.* 2009). Nephrin also has a conserved function in the fusion of zebrafish and mouse myoblasts. Indeed, it has been shown that nephrin mRNA is upregulated in nascent myotubes, and the total amount of myoblast fusion is decreased in nephrin-knockout mice compared with the WTone (Sohn *et al.* 2009). Therefore, nephrin is critical for the fusion of myoblasts into nascent myotubes (Sohn *et al.* 2009). So far as we know, there is no report on the expression of nephrin during the fusion of primary human CTB cells and BeWo cells, which are well-characterized models of human placental trophoblast syncytialization. Given its conservative and critical role in myoblast fusion, we hypothesized that nephrin may also play a role in human trophoblast syncytialization.

In this study, we first investigated the expression of nephrin in different human trophoblast cells from first, second, and third trimester placentas. Using a spontaneous syncytialization model of primary CTB cells, we observed increased expression in both nephrin mRNA and protein during the process of syncytialization. Furthermore, the spontaneous syncytialization of primary CTB cells and the expression of syncytin 2, connexin 43, and β -hCG were significantly inhibited when nephrin was silenced. And the expression of β -hCG was significantly elevated in nephrin stably overexpressed BeWo cells. The above evidence strongly supports a role for nephrin in human trophoblast syncytialization.

Materials and methods

Tissue collection

Human placental villi and term tissues from healthy 27- to 31-year-old women were collected from 301th Hospital of

People's Liberation Army with written consent. Approval to obtain and use these samples for this study was in accordance with the Ethics Committee of the 301th Hospital of People's Liberation Army and the Institute of Zoology at the Chinese Academy of Sciences. For immunohistochemistry and western blotting, nine normal placental tissues at different gestational stages (three from the first trimester, three from the second trimester, and three at term) were used from women who underwent legal abortion or normal delivery. After being washed three times in PBS, the villi were fixed in 4% paraformaldehyde (PFA) for 24 h at 4 °C and kept in 70% ethyl alcohol until processing.

Isolation of primary CTB cells

The isolation of CTB cells from human term placentas was performed as described previously (Zhou *et al.* 2013). The placentas were cut into pieces and digested in DMEM (HyClone, Thermo Scientific, Dubuque, IA, USA) with 0.125% trypsin (Sigma-Aldrich, Inc.) and 0.02% DNase I (Sigma) four times. The supernatant was centrifuged for 15 min at 1200 *g* to obtain the cell pellets, which were separated by Percoll (GE Healthcare BioSciences AB, Uppsala, Sweden) density gradient centrifugation. The discontinuous 5–65% Percoll gradients were made from 3 ml of 90% Percoll and 1 $\times$ HBSS mixtures. CTB cells were purified in a 30–50% Percoll density gradient. CTB cells were seeded in 35 mm dishes (2×10^6 /dish) in IMDM (HyClone) with 10% fetal bovine serum (FBS; Gibco BRL), 100 units/ml penicillin, and 100 units/ml streptomycin and cultured in a 5% CO₂ air incubator at 37 °C.

BeWo cell culture and fusion assay

The human choriocarcinoma cell line BeWo was maintained in Ham's F-12K (Kaighn's; Gibco BRL)/DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin in 5% CO₂/95% air at 37 °C. Cell fusion was induced by treating BeWo cells with forskolin (FSK, Sigma-Aldrich, Inc.) for 48 h. The achievement of cell fusion was assessed by two methods. First, immunofluorescence using anti-human E-cadherin antibody (sc-71008; Santa Cruz Biotechnology) showed the loss of cell membrane during the fusion process. The number of multinucleated cells was counted in five randomly selected areas under the microscope. In addition, whole cell lysates of BeWo cells subjected to cell fusion induction exhibited increased levels of β -hCG, as shown by western blotting analysis.

Total RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Two micrograms of RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) with oligo (dT) as primers. Real-time PCR was performed by using a SYBR Premix Ex Taq PCR Kit (Takara Biotechnology Co. Ltd, Dalian, China) on an ABI Prism 7500 Real-Time PCR System. Specific primers used were as follows: GAPDH, forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-TGGACTCCACGACGTACTC-3'; β -hCG,

forward, 5'-GAGCTACCCCCAGCATCCTATCACC-3' and reverse, 5'-TTGATGGGGCGGCACCGTGG-3'; and nephrin, forward, 5'-TAGGCAGTTGCTGGTCTGTG-3' and reverse, 5'-GCACTAGGGGGAAAGTGAC-3'.

Western blotting analysis

The cells were washed three times with cold PBS and mixed with whole-cell lysis buffer (4 mM EGTA, 3 mM EDTA pH 8.0, 125 mM NaF, 0.5 mM Na_3VO_4 , 2.5 $\mu\text{g}/\text{ml}$ aprotinin, 25 $\mu\text{g}/\text{ml}$ trypsin inhibitor, 12.5 mM HEPES pH 7.4, 1% Triton X-100, and 25 μM phenylmethylsulphonyl fluoride) as described previously (Zhang *et al.* 2013). A BCA Protein Assay Kit (Pierce Biotechnology, Thermo Scientific, Dubuque, IA, USA) was used to determine the protein concentration by spectrophotometry at 562 nm (Beckman DU530, Fullerton, CA, USA). Twenty-five micrograms of each protein sample was subjected to western blotting analysis with the following primary antibodies: rabbit polyclonal anti-nephrin (1:500; ab58968, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-human β -hCG (1:1000; ab54410, Abcam), mouse monoclonal anti-E-cadherin (1:1000; sc71008, Santa Cruz Biotechnology), mouse monoclonal anti- β -actin (1:2000; TA-09, Zhongshan Golden Bridge Crop., Beijing, China), rabbit polyclonal anti-syncytin 2 (1:500; AP13018A, Abgent, San Diego, CA, USA), mouse monoclonal anti-connexin 43 (1:1000; 610061, Transduction Laboratories, Lexington, KY, USA), and HRP-conjugated secondary antibodies. Signals were detected by the GeneGnome Imaging System (Syngene Bio-imaging, Cambridge, UK).

Nephrin stable overexpression in BeWo cells

A full-length human nephrin coding sequence was generated by PCR from a pCMV-SC-CF plasmid (a kind gift from Puneet Garg at University of Michigan School of Medicine, Ann Arbor, MI, USA). Nephrin was cloned into a pGEM-T vector (Promega) and subcloned into a CSII-CMV-MCS-IRES2-Venus vector using the NheI and NotI sites. The lentivirus vectors were kindly provided by Dr Hiroyuki Miyoshi (Riken BioResource Center, Tsukuba, Japan). All of the lentiviral vectors were generated by transient transfection of 293T cells using the calcium phosphate precipitation method. Briefly, 293T cells were seeded into 100 mm dishes with DMEM medium containing 10% FBS. The mixture of the main plasmid and the packaging plasmids was transfected into each dish by calcium phosphate precipitation. The supernatants collected at 48 and 72 h were concentrated and purified by ultracentrifugation with filter units (Millipore, Schwalbach, Germany), and then the lentivirus was titrated onto HT1080 cells. BeWo cells were infected with nephrin or empty lentivirus and passaged three times to purity by flow cytometry to ensure a transfection rate of more than 95%.

Immunohistochemistry

Placental tissues from different stages of pregnancy were embedded in paraffin after they were fixed in 4% PFA, and 5 μm sections were cut with a conventional microtome (Leica, Nussloch, Germany). Immunodetection was performed in accordance with the instructions from the biotin-streptavidin-peroxidase and

diaminobenzidine kits (Zhongshan Golden Bridge Crop.), as previously reported (Fu *et al.* 2009). Briefly, slides were boiled in citrate buffer (10 mM citrate sodium, 10 mM citric acid, and pH 6.0) for 15 min to retrieve antigen after dewaxing and then were rehydrated. Hydrogen peroxide (3%) was used to quench endogenous peroxidase activity. After blocking with normal goat serum for 20 min, the slides were incubated overnight at 4 °C with primary antibodies against nephrin (1:150, ab58968, Abcam), cytokeratin 7 (1:200, ZM-0071, Zhongshan Golden Bridge Crop.) or β -hCG (1:200, ZM-0134, Zhongshan Golden Bridge Crop.). Purified Igs (IgG) from rabbit or mouse were used as negative controls.

Immunofluorescence

Primary CTB cells were rinsed in PBS and fixed. After blocking with 5% donkey serum for 20 min, the slides were incubated with monoclonal anti-E-cadherin (1:100; sc71008, Santa Cruz Biotechnology) overnight at 4 °C and rinsed in PBS three times for 5 min each. The cells were then incubated with anti-mouse Alexa Fluor 555 (Invitrogen) secondary antibody for 1 h at room temperature. The nuclei of the CTB cells were stained with 6'-diamidino-2-phenylindole (DAPI). To quantify primary CTB cells syncytialization without bias, three different nonoverlapping fields were randomly selected for each time point under the microscope. The fusion index was calculated using the format of $(N - S)/T$, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei (Matsuura *et al.* 2011). All experiments were performed in triplicate and the results are presented as means $\pm$ S.E.M.

RNA interference

The cells were seeded at 2×10^6 cells into 35 mm dishes. One-hundred nanomolar nephrin-specific siRNA (Invitrogen; 5'-UGG CUC GGA CCA AAC CAA CAU UCA A-3' and 5'-CUG GGC ACU UGU AUG AUG ATT-3') or negative control siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3') was diluted in OPTI-MEM and transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendations for 6 h. The effect of siRNAs on nephrin levels at 72 h after transfection was detected by real-time PCR and western blotting.

Statistical analysis

Each experiment was repeated three times. Data are presented as the mean $\pm$ S.E.M. One-way ANOVA and *t*-tests were used for statistical analyses with $P < 0.05$ being considered as significant.

Results

Expression of nephrin in human placentas at different stages of pregnancy

To examine the expression of nephrin during human placentation, we carried out immunohistochemistry on placental tissues from the first, second, and third trimesters. CK7, an epithelial marker, was used to

immunolabel CTB cells, extravillous trophoblast cells in the trophoblast cell column or cells which invaded into the maternal decidua (d and d'), while β -hCG specifically immunostained the STB layer (c and c'). As shown in Fig. 1A, B and C, nephrin was highly expressed in both the CTB cells and STB from first to third trimesters (b and b'). Control sections using normal IgG instead of a primary antibody exhibited no specific staining, as is representatively shown in a and a'.

Validation of spontaneous syncytialization of primary CTB cells

To elucidate the function of nephrin in placentation under physiological conditions, we first set up a model

Figure 1 Immunohistochemical localization of nephrin in human placentas at different stages of pregnancy. (A) First-trimester placenta (6 W; $n=3$), (B) second-trimester placenta (19 W; $n=3$), and (C) term placenta (39 W; $n=3$). (a and a') Negative control images in which normal IgG is used to replace the primary antibody. (b and b') In the placentas of the first and second trimesters (A and B), nephrin is stained in the CTB cells and STB; in the term placenta (C), nephrin is expressed in the presented STB. (c and c') β -hCG immunostaining shows specific signals in the STB. (d and d') Cytokeratin 7 (CK7, an epithelial marker) staining shows CTB cells. CTB, cytotrophoblast cells; STB, syncytiotrophoblast; bar = 100 μ m.

for spontaneous syncytialization of primary CTB cells. We isolated fresh CTB cells from human term placenta and obtained a population of cells, more than 95% of which were CK7-positive (data not shown). We cultured the cells *in vitro* for 72 h. During this period of time, mononucleated CTB cells spontaneously fused and formed multinucleated syncytia. As shown in Fig. 2A, most cells were mononucleated at 0 h and showed clear E-cadherin immunostaining on their cell membranes. Some syncytia appeared at a 24-h culture with multiple nuclei in a single cell boundary (arrow). At 48 and 72 h, large syncytia with more than ten nuclei appeared abundantly (arrow). The fusion index was also increased with the process of spontaneous syncytialization (Fig. 2B; $P<0.05$); at 72 h of culture, ~76% of the cells fused and ended up as multinucleated syncytia. Message levels of β -hCG were significantly increased (over 1000- and 2000-fold at 48 and 72 h respectively) during spontaneous fusion of primary cells (Fig. 2C; $P<0.05$ as compared with 0 h). Western blotting analysis showed that protein levels of syncytin 2, connexin 43, and β -hCG were also elevated (Fig. 2D).

Nephrin expression was increased during the spontaneous syncytialization of primary CTB cells

Next, the expression of nephrin mRNA and protein levels during the syncytialization of primary CTB cells was examined by real-time PCR and western blotting using RNA and protein samples harvested at 0, 24, 48, and 72 h of syncytialization. As shown in Fig. 3A, the level of nephrin mRNA was low at 0 and 24 h, but was markedly increased at 48 h and then slightly decreased at 72 h of culture ($P<0.05$ as compared to 0 h). Furthermore, nephrin protein levels showed a similar expression profile (Fig. 3B).

Requirement of nephrin in the fusion of primary CTB cells

To further assess the function of nephrin in the fusion of primary CTB cells, we used two different siRNAs specifically targeting nephrin to silence the expression in CTB cells. As shown in the Fig. 4A, the level of β -hCG mRNA was decreased by ~40% ($P<0.05$) upon nephrin siRNA1 knockdown. Western blotting analysis showed that the expression levels of syncytin 2, connexin 43, and β -hCG were decreased when silencing nephrin (Fig. 4B). Immunofluorescence showed that E-cadherin staining disappeared in the negative-control siRNA group (Fig. 4C, left panel), but in the nephrin-silenced group, E-cadherin staining was clearly visible on mononucleated trophoblast cell membranes (Fig. 4C, right panel), and more mononucleated cells were present compared with cells treated with the negative control siRNA. Statistical analysis of the fusion index after nephrin silencing showed that the efficiency of

Figure 2 Primary CTB cells from term placenta spontaneously fuse during *in vitro* culture. (A) Immunofluorescence images show that human primary CTB cells fuse into syncytia *in vitro*. The nuclei are stained with DAPI. E-cadherin staining (red) shows the cell membrane boundary at 0, 24, 48, and 72 h of *in vitro* culture. Arrows show syncytium. Bar=100 μ m. (B) Fusion index of human primary CTB cells syncytialization (* P <0.05). (C) The levels of β -hCG mRNA are increased during syncytialization of primary CTB cells (* P <0.05). (D) Expression of syncytin 2, connexin 43, and β -hCG is increased during primary human CTB cells syncytialization (n =3).

syncytialization in the nephrin siRNA group was less than that of the negative control siRNA group (Fig. 4D, P <0.05).

Nephrin overexpression promoted β -hCG expression in BeWo cells

To further prove whether there might be a positive relationship between nephrin expression and syncytialization, we constitutively overexpressed nephrin in BeWo cells. Western blotting analysis of the nephrin-overexpressed BeWo cells showed that levels of β -hCG were increased, as indicated in Fig. 5A and B (P <0.05). Moreover, the fusion index of the nephrin-overexpressed cells was higher than that of the cells transfected with an empty vector (Fig. 5C).

Discussion

This study suggests that nephrin is involved in trophoblast syncytialization based on the following observations. First, nephrin was expressed in human CTB cells and STB in placentas from the first, second, and third trimesters. Both nephrin mRNA and protein levels were dramatically increased during the spontaneous syncytialization of primary human CTB cells.

Moreover, knockdown of nephrin by siRNA not only reduced the number of syncytia, but also decreased the expression of syncytin 2, connexin 43, and β -hCG.

Membrane merging is critical for cell–cell fusion. Indeed, the discovery of syncytins is a milestone in the understanding of human syncytialization. The syncytin family includes syncytin 1 and 2 in humans and syncytin A and B in mice (Dupressoir *et al.* 2005, Lavielle *et al.* 2013), which are one of the two groups of proteins that are called fusogens in the cell–cell fusion process. The other family of fusogens includes EFF1 and AFF1, which have been discovered in *C. elegans* (Mohler *et al.* 2002, del Campo *et al.* 2005, Sapir *et al.* 2007). In addition to the above-mentioned fusogens, the expression of connexin 43, a hexamer from which gap junctions are comprised, is increased during the differentiation of CTB cells (Dunk *et al.* 2012), while the expression of zona occludens-1, an adherens junction protein that ‘zips’ cells together, is predominantly present during CTB cells aggregation but is significantly decreased upon cell fusion (Pidoux *et al.* 2010). Calponin 3 (CNN3), a CNN family member, is also a negative regulator of trophoblast fusion (Shibukawa *et al.* 2010). CD98 and its proposed ligand, galectin-3, may play a positive role in promotion of syncytialization (Mori *et al.* 2004). Our previous studies have shown that the proprotein convertase furin is required for trophoblast

Figure 3 Expression of nephrin during spontaneous syncytialization of primary human CTB cells. (A) Nephrin mRNA is increased in primary human CTB cells spontaneous syncytialization, especially at 48 h (n =3; * P <0.05). (B) Expression of nephrin protein is increased during syncytialization of primary human CTB cells (n =3).

Figure 4 Silencing nephrin inhibits syncytialization in primary CTB cells. Primary human CTB cells were transfected with negative control siRNA or nephrin siRNA1 or 2 ($n=3$). (A) Approximately 60% of nephrin mRNA is knocked down by siRNA1 ($n=3$; $*P<0.05$). (B) Expression of syncytin 2, connexin 43, and β-hCG is significantly downregulated after nephrin knockdown. (C) Immunofluorescence images of primary human CTB cells transfected with nephrin siRNA (right) or negative control siRNA (left). More mononucleated cells appear in the nephrin siRNA-transfected group compared with the negative control siRNA group (*mononucleated cell). (D) Bar graph of the fusion index after silencing nephrin ($*P<0.05$).

syncytialization, and the expression of furin in the STB is significantly lower in placentas from pre-eclampsia patients (Zhou *et al.* 2013). In addition, the processing of insulin-like growth factor 1 receptor (IGF1R) by furin is necessary for trophoblast syncytialization (Zhou *et al.* 2013). E-cadherin levels are significantly increased during earlier stages of syncytialization (Coutifaris *et al.* 1991). However, as syncytialization progresses, for example, ~24 h later in culture in the spontaneous syncytialization system, E-cadherin levels are decreased (Coutifaris *et al.* 1991). In contrast, cadherin-11 expression increases during the formation of multinucleated syncytia, and it promotes the morphological and functional differentiation of human CTB cells (Getsios & MacCalman 2003).

The expression of nephrin has been found in the kidney, pancreas, brain, and lymphoid tissues (Putala *et al.* 2001, Astrom *et al.* 2006). Verma *et al.* (2006) have observed that mutation or deletion of nephrin protein in the slit diaphragm in the kidney leads to a defect in podocyte foot process morphogenesis, and that proteinuria is the clinical symptom. Sohn *et al.* (2009) have confirmed that nephrin knockout myoblasts fuse less efficiently into nascent human myotubes, and down-regulation of nephrin expression results in muscle defects in zebrafish and mice. Interestingly, nephrin interacts with SH2–SH3 domain-containing adapter Nck

to regulate actin cytoskeletal dynamics in podocytes (Verma *et al.* 2006), and in myoblast fusion, the appearance of actin foci is obligatory for fusion (Onel & Renkawitz-Pohl 2009, Haralalka *et al.* 2011). Coexpression of Sns and EFF1 can induce highly efficient cell fusion in *Drosophila* S2R+ cells (Shilagardi *et al.* 2013). Our study adds a new function for nephrin during primary trophoblast syncytialization, which is accompanied by the increase in important fusion-related molecules, including syncytin 2, connexin 43, and β-hCG. When the expression of nephrin was knocked down, trophoblast syncytialization as well as the expression of syncytin 2 and connexin 43 was decreased. In the placenta, syncytin is located on the cell membrane and mediates placental CTB cell fusion by binding to the syncytin receptor (Mi *et al.* 2000, Esnault *et al.* 2008, Huppertz & Borges 2008, Vargas *et al.* 2009). As a gap junctional protein, connexin 43 is required for CTB cell fusion into syncytia (Dunk *et al.* 2012, Pidoux *et al.* 2014). We therefore speculate that nephrin may be involved in the syncytialization machinery via enhancing the expression of syncytin and connexin 43, and the underlying mechanisms await further study.

Aberrant fusion of the CTB cells with the STB will impair the maintenance and integrity of the placental barrier, which may thus be involved in pregnancy disorders. Interestingly, it has been reported that serum and urine concentrations of nephrin are associated with pre-eclampsia and small for gestational age infants (Yang *et al.* 2013). Therefore, nephrin may play a role in the pathogenesis of proteinuria in pre-eclampsia. Further investigation is necessary for elucidating the clinical

Figure 5 Nephrin overexpression promotes β-hCG expression in BeWo cells. Nephrin stably overexpressed BeWo cells and control BeWo cells were incubated in a medium with or without 50 μM FSK for 48 h. (A) Western blotting analysis shows that levels of β-hCG protein are increased in nephrin overexpressed cells compared with control cells. (B) Bar graph of β-hCG band density ($*P<0.05$). (C) Bar graph of the fusion index after nephrin overexpression in BeWo cells.

significance of placental nephrin in pregnancy-related diseases. Taken together, we herein demonstrate a new function for nephrin in promoting the syncytialization of human trophoblast cells.

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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