Differential Expression of Ezrin and CLP36 in the Two Layers of Syncytiotrophoblast in Rats

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The syncytiotrophoblast, which regulates maternal–fetal transfer of drugs, consists of a single layer in humans, but two layers, *i.e.*, *SynI* and *SynII*, in rodents. Polar distribution of transporters in the apical and basal plasma membranes of syncytiotrophoblast is important for placental function in terms of vectorial transport of substrates, but the mechanisms that control protein distribution in the syncytiotrophoblast remain unclear. We have previously established rat syncytiotrophoblast cell lines, TR-TBT 18d-1 and TR-TBT 18d-2, which retain characteristics of *SynI* and *SynII*, respectively. In this study, we aimed to characterize the gene expression profiles in the two layers by using these cell lines. DNA microarray analysis indicated that more than 25 mRNAs, including cytoskeleton binding proteins, ezrin and CLP36, are differentially expressed between TR-TBT 18d-1 and TR-TBT 18d-2. Quantitative real time-polymerase chain reaction (PCR) analysis indicated that mRNA expression of ezrin, CLP36, CCN1, and CCN2 is higher in TR-TBT 18d-1 and mRNA expression of elf-1a, hsc70 and flot2 is higher in TR-TBT 18d-2, compared with their counterparts. Immunohistochemical analysis indicated that ezrin is expressed in rat placental villi *in vivo*, and is located on the apical membranes of TR-TBT 18d-1, while CLP36 is located in the apical and basal sides of TR-TBT 18d-1. The expression of ezrin was highest at gestational days 14 and 18 and was highest among the ezrin/radixin/moesin (ERM) family members. These results may help to clarify the mechanisms controlling polarization of the syncytiotrophoblast and the significance of the double epithelial layers in rat and mouse.

Key words *SynI*; *SynII*; trophoblast; ezrin; blood–placenta barrier; CLP36

The syncytiotrophoblast in the placenta serves as an interface for efficient maternal–fetal exchange of nutrients, metabolites and xenobiotic compounds, and membrane transport processes play pivotal roles in efficient uptake of certain compounds and in exclusion of others; this is referred to as the blood–placenta barrier.[1,2] In humans, the syncytiotrophoblast is derived from cell fusion of the progenitor cytotrophoblast and consists a polar single epithelium. The apical and basal plasma membranes of the syncytiotrophoblast face the maternal blood and the endothelial cells of the fetal blood vessels, respectively. Specific localization of transporters in the apical and the basal plasma membranes of the syncytiotrophoblast is important for plancental function in terms of vectorial transport of substrates. For example, ATP-binding cassette transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein, are localized in the apical membrane of the syncytiotrophoblast, where they restrict the entry of xenobiotics, including digoxin, saquinavir, vinblastine, topotecan and mitoxantrone.[3] On the other hand, organic anion transporting polypeptide 2B1 is localized in the basal membrane of the syncytiotrophoblast and mediates uptake of dehydroepiandrosterone sulphate from fetal blood to placenta.[4]

The postsynaptic density 95 (PSD-95)/Discs-large/zonula occludens-1 (ZO-1) (PDZ) domain-containing scaffold proteins, such as PDZ domain containing 1 (PDZK1) and Na^+/H^+ exchanger regulatory factor 1 (NHERF1) , play a role in the association and functional regulation of organic anion transporters in the apical membranes of the renal proximal epithelial cells.[5] It has also been reported that PDZK1 functions as a regulator for oligopeptide transporter (PEPT) 2 and organic cation/carnitine transporter 2, through direct interaction with their C-termini.[6,7] However, the mechanisms that control the polar distribution of proteins in the syncytiotrophoblast are still unclear.

In mouse and rat, however, the labyrinthine part of the placenta is the principal site of maternal–fetal exchange. The labyrinthine wall, which lies between the maternal blood space and fetal vessels, is composed of three trophoblast layers,[1,8—10] *i.e.*, the porous cytotrophoblast layer, and the maternal-side and fetal-side syncytiotrophoblast layers (also called *SynI* and *SynII,* respectively). It is suggested that connexin 26 gap-junctional channels and transporters allow diffusion of nutrients between the two syncytiotrophoblast layers.[11—13] Therefore, it is considered that the apical membrane of *SynI* and the basal membrane of *SynII* correspond to the apical and basal membranes of human syncytiotrophoblast, respectively, although the physiological role of the double epithelial layers, in contrast to the single epithelial cell layer in humans, remains unclear.

We established conditionally immortalized rat syncytiotrophoblast cell lines, TR-TBT 18d-1 and TR-TBT 18d-2 from

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a pregnant transgenic rat harboring temperature-sensitive simian virus 40 (SV40) large T-antigen gene.[14,15] TR-TBTs express various transporters that are expressed in rat placenta.[16—18] In rat placenta, a facilitative glucose transporter (GLUT) 1, is localized on the apical (maternal-blood side) membrane of *SynI* and the basal membrane of *SynII*.[11,14,19] On the other hand, GLUT3 is localized on the apical membranes of both *SynI* and *SynII*.[11,14,19] Immunocytochemical studies showed that localization of GLUT1 and GLUT3 in TR-TBT 18d-1 and TR-TBT 18d-2 cells coincide well with those of rat *SynI* and *SynII in vivo*, respectively.[14,15] Therefore, we chose to use these cell lines to clarify the mechanism, physiological role and pharmacological significance of the polar distribution of membrane proteins in the syncytiotrophoblast.

The aim of the present study was to identify genes involved in the polarization of syncytiotrophoblast and the specific localization of proteins in the syncytiotrophoblast. We examined the mRNA expression profiles of various genes in TR-TBT 18d-1 and TR-TBT 18d-2 by using DNA microarrays. We focused on the ezrin-radixin-moesin (ERM) family protein ezrin and carboxyl terminal lin-11/isl-1/mec-3 (LIM) domain protein of 36 kDa, CLP36 as candidates, and confirmed that these proteins are expressed in TR-TBT cells and rat placenta.

MATERIALS AND METHODS

Animals Pregnant Wistar rats were purchased from SLC Inc. (Shizuoka, Japan). All animal experiments were approved by the Institutional Animal Care Committee, and complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in Keio University Faculty of Pharmacy.

Cell Culture TR-TBT 18d-1 and TR-TBT 18d-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 100 units/ml of benzylpenicillin, 100 μg/ml of streptomycin sulfate (both from GIBCO^{TM} Invitrogen Co., Grand Island, NY, U.S.A.) and 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, U.S.A.) at 33 °C in a humidified atmosphere of 5% CO_2/air as described previously.[14] To isolate total RNA from the TR-TBTs, cultures were incubated at 37 °C on type I collagen-coated dishes (Asahi Techno Glass Co., Tokyo, Japan).

DNA Microarray Analysis of TR-TBT 18d-1 and TR-TBT 18d-2 Cells Total cellular RNA was prepared from Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS(−))-washed cells using an RNeasy Mini Kit (QIAGEN Sciences, Maryland, MD, U.S.A.) according to the manufacturer's instructions. After treatment with DNase I (Takara Shuzo Co., Ltd., Shiga, Japan) to remove contaminating genomic DNA, mRNA was extracted using an mRNA Purification Kit (Amersham Biosciences Co., Piscataway, NJ, U.S.A.). Each sample was reverse-transcribed in the presence of Cy5-labeled deoxyuridine 5′-triphosphate (dUTP) (TR-TBT 18d-1) or Cy3-labeled dUTP (TR-TBT 18d-2) (Amersham Biosciences). DNA microarray slides bearing 4854 rat genes (Sigma Genosys Co., Hokkaido, Japan) were incubated with prehybridization buffer (5×SSC, 0.1% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA); filtered through a 0.2 μm filter) and washed with 2-propanol. Samples were mixed together and hybridized to the DNA microarray in ArrayHyb^{TM} Low Temp Hybridization buffer (Sigma-Aldrich Co., St. Louis, MO, U.S.A.). Hybridized slides were scanned, and the fluorescence intensities of Cy5 and Cy3 for each gene spot were quantified by using a GenePix4000B (Molecular Devices Co., Union City, CA,

Table 1. DNA Microarray Analysis of Genes Differentially Expressed between TR-TBT 18d-1 and TR-TBT 18d-2 Cells

Gene name	Biological functions	Array ratio
U3A snRNA	snRNA	2.825
Fat1 (FAT tumor suppressor homolog 1)	Cell adhesion molecule	2.681
Per2	Circadian clock gene	2.591
U4 snRNA	snRNA	2.469
CCN2 (CTGF)	ECM-associated signaling molecule	2.513
Ezrin	Apical anchoring protein	2.421
CLP36	LIM-PDZ protein	2.364
Cadherin-10	Cell adhesion molecule	2.320
p65 (Synaptotagmin I, SytI)	Synaptic vesicle specific protein	2.165
Uncoupling protein 2, mitochondrial (ucp2)	Regulator of insulin secretion	2.141
Calpactin I light chain	Membrane trafficking protein	2.137
CCN1 (cyr61)	ECM-associated signaling molecule	2.062
PKCq-interacting protein (PICOT)	PKCtheta-interacting protein	2.053
Very low density lipoprotein receptor (vldlr)	Syncytiotrophoblast marker	2.045
Reticulon 4 (rtn4)	Inhibitor of cell spreading	2.028
DD6A4-4 (epithelium specific gene)	Expressed in fusiform cell	2.024
Elongation factor-1 alpha (elf-1α)	Transcriptional factor	0.337
70 kD heat-shock cognative protein (hsc70)	Chaperone protein	0.375
Alpha-prothymosin	c-Myc targeted gene	0.395
Cathepsin L (ctsl)	Lysosomal proteinase	0.461
Thyroxine-binding globulin (TBG)	Hormone binding protein	0.464
Ribosomal protein S2	Ribosomal protein	0.465
Flotillin 2 (flot2)	Raft associated protein	0.477
Fibronectin 1 (fn1)	Extracellular matrix	0.484
Adenylate cyclase 8 (adcy8)	ATP related enzyme	0.486

Only genes showing changes of more than 2-fold or less than 0.5-fold are listed.

U.S.A.) with an Array-Pro Analyzer (Media Cybernetics Inc., Silver Spring, MD, U.S.A.). Each slide contained 66 housekeeping genes to normalize the signal intensities of the fluorescent dyes. The intensities of Cy5 and Cy3 were adjusted so that the mean Cy5 and Cy3 intensities of the probe cDNA binding to the housekeeping genes were equivalent. The average difference measurement computed in the Array-Pro Analyzer served as a relative indicator of the level of expression.

Quantitative Real-Time-Polymerase Chain Reaction (PCR) Analysis Expression of rat ezrin (Accession number: NM_091357.1), CLP36 (NM_017365), CCN1 (NM_031327.2), CCN2 (NM_022266.2), DD6A4-4 (AF034242), elf-1α (NM_175838.1), hsc70 (NM_024351.2), flot2 (NM_031830.1) and G3PDH (NM_017008) in TR-TBT cells and rat placenta was evaluated by quantitative real-time PCR analysis. Total cellular RNA was prepared from PBS(−)-washed cells or tissues using an RNeasy Mini Kit (QIAGEN Sciences) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using oligo $(dT)_{15}$ as a primer, with molony murine leukemia virus (M-MLV) reverse transcriptase (RT) (ReverTraAce, Toyobo Co., Ltd., Osaka, Japan) for TR-TBT cells and Superscript III (Invitrogen) for rat placenta. Real-time PCR analysis was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, U.S.A.) with 2X SYBR Green PCR Master Mix (PE Applied Biosystems) according to the manufacturer's protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using pT7Blue-2 Vector (EMD Biosciences, Inc., Darmstadt, Germany) containing each gene. A control lacking the RT enzyme was assayed in parallel to monitor any possible genomic contamination. PCR was performed through 40 cycles of 95 °C for 30 s, 55—58 °C for 1 min, and 72 °C for 1 min after pre-incubation at 95 °C for 10 min, using specific primers. There was no difference in the mRNA levels of G3PDH between TR-TBT 18d-1 and 18d-2 (data not shown). The sequences of sense and anti-sense primers are shown in Table 2.

Table 2. Primer Sequence of Quantitative Real-Time PCR Analysis

Gene		Sequence (5′ to 3′)
Ezrin	Sense primer	CCGTTGACTCTGAGCAATGA
	Anti-sense primer	TTGGTGTTGCCCTGCCTGAT
CLP36	Sense primer	GACCACCCAGCAGATTGTTC
	Anti-sense primer	CCCATCAATGGCTGTGATCA
CCN1	Sense primer	GAAGTGCGTCCTTGTGGACA
	Anti-sense primer	ACACTGGAGCATCCTGCATA
CCN2	Sense primer	GCCCTGACCCAACTATGATG
	Anti-sense primer	CAGAGACGACTCTGCTTCTC
DD6A4-4	Sense primer	GCTGTTTTCGAACTGATTTC
	Anti-sense primer	CGGTGAACTTCTTTTGCTTC
Elf-1α	Sense primer	GAACCATCCAGGCCAGATCA
	Anti-sense primer	CGGCATCACCAGACTTCAAG
Hsc70	Sense primer	TTGCCAATGACCAGGGTAAC
	Anti-sense primer	ATCGAACCTACGTCCGATCA
Flot2	Sense primer	TCGCCCAGGTGAAGATCATG
	Anti-sense primer	CCAAGGATGGAGCGTAGATG
G3PDH	Sense primer	GCATGGCCTTCCGTGTTCCTA
	Anti-sense primer	GTCCACCACCCTGTTGCTGTA
Radixin	Sense primer	TCACGCACAGTAGATTCAACTTG
	Anti-sense primer	CCACTGTACAACAGTCACTTCAA
Moesin	Sense primer	CTGCTGACTCTGAGCAATGAG
	Anti-sense primer	GGCTCCAGCAGGGTGTTA

Immunohistochemical Analysis Pregnant Wistar rats at gestational day 18 were anesthetized with an intraperitoneal injection of pentobarbital (NEMBUTAL Injection, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and injected with 200 U of heparin sulfate (Wako Pure Chemical Ind., Osaka, Japan) from the cervical vein. Then, the rats were perfused with cold PBS(−) and 3% paraformaldehyde in 0.1 M phosphate buffer from the uterine artery. Following perfusion, the placenta was dissected out, kept for 2 h in 0.1 M phosphate buffer containing 3% paraformaldehyde at 4 °C, and then stored in 0.1 M phosphate buffer containing 30% sucrose at 4 °C.

For immunofluorescence analysis, placenta sections (10 μm) were fixed with 10% methanol/TBS-Ca (50 mM Tris, 1 mM $CaCl_2$, 150 mM NaCl) for 30 min at −20 °C or methanol for 10 min at 4 °C. After incubation in DAKO protein blocking reagent (DakoCytomation Inc., Glostrup, Denmark) or blocking buffer (0.02% NaN_3/0.2% skim milk/2% FBS/0.1% glycine/1% BSA/0.01% Triton in PBS) for 2 h at room temperature, the sections were reacted overnight with anti-ezrin antibody (H-276, rabbit polyclonal immunoglobulin G (IgG), Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) or anti-P-gp antibody (C219, mouse monoclonal IgG, DakoCytomation Inc.) at 4 °C, and then with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes., Inc., Eugene, OR, U.S.A.) or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) for 2 h at room temperature. Finally, sections were incubated with 4′-6-diamidino-2-phenylindole (DAPI) (Molecular Probes) to stain nuclei. The sections were sealed with VECTASHIELD (Vector Laboratories, Burlingame, CA, U.S.A.). For immunoperoxidase analysis, placenta sections were pretreated with 10% methanol/PBS for 30 min at 4 °C and then pretreated with 0.3% H_2O_2/methanol for 10 min at room temperature. After incubation in DAKO protein blocking reagent (DakoCytomation Inc.) for 2 h at room temperature, the sections were reacted overnight with anti-ezrin antibody (H-276) at 4 °C, and then with anti-rabbit IgG conjugated to horseradish peroxidase (NA934, GE Healthcare, Buckinghamshire, U.K.) for 30 min at room temperature. The sections were incubated in 0.08% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in Tris 0.05 M with 0.02% H_2O_2 and sealed with CRYSTAL/MOUNT (Biomeda Corp, Foster, CA, U.S.A.). These sections were viewed with a fluorescence microscope (Nikon, Tokyo, Japan).

TR-TBT 18d-1 and TR-TBT 18d-2 cells were cultured on glass coverslips, then fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 30 min at 4 °C. After incubation in DAKO protein blocking reagent for 1 h at room temperature, these were reacted with anti-ezrin antibody or anti-CLP36 (51, mouse monoclonal IgG1, BD Biosciences Co., San Jose, CA, U.S.A.) antibody for 1 h at room temperature, and then with Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-rabbit IgG or Alexa Fluor 594 goat anti-mouse IgG for 30 min at room temperature. To stain *F*-actin, some slides were stained with Alexa Fluor 488-labeled phalloidin (Molecular Probes). Finally, the cells were incubated with SYTOX (SYTOX Green Nucleic Acid Stain, Molecular Probes) or DAPI to stain nuclei. The glass was sealed with VECTASHIELD Hard Set Mounting Medium (Vector Labo-

ratories) using a fluorescence microscope or a Leica confocal laser scanning microscope (TCS NT, Heidelberg, Germany).

RESULTS

Differential mRNA Expression in TR-TBTs Using rat DNA microarrays, we analyzed genes that are differentially expressed between TR-TBT 18d-1 and TR-TBT 18d-2, using the criteria that significant differences should be more than 2.0-fold or less than 0.5-fold. According to this analysis, 25 genes were differentially expressed between the two cell lines. Sixteeen genes were higher in TR-TBT 18d-1 compared with TR-TBT 18d-2 (Table 1), including genes encoding membrane anchor proteins, such as ezrin and CLP36. On the other hand, 9 genes were lower in TR-TBT 18d-1 compared with TR-TBT 18d-2 (Table 1). Based on their reported biological functions, we selected 8 that may be related to cell polarity and/or localization of transporters, and quantitatively analyzed their mRNA expression levels by means of real-time PCR.

The mRNA expression levels of ezrin, CLP36, nephroblastoma overexpressed gene (CCN1) and CCN2 were approximately 2.0-, 35-, 5.8- and 4.5-fold higher in TBT 18d-1 than in TR-TBT 18d-2, respectively (Fig. 1). On the other hand, the mRNA expression levels of elongation factor (elf-1α), heat shock cognate protein (hsc70), flotillin (flot2) and DD6A4-4 were approximately 2.0-, 17-, 2.0- and 9.0-fold higher in TR-TBT 18d-2 than in TR-TBT 18d-1, respectively (Fig. 1). These results suggest that ezrin, CLP36, CCN1 and CCN2 are preferentially distributed in TR-TBT 18d-1, while hsc70, elf-1α and flot2 are preferentially distributed in TR-TBT 18d-2. We have further focused on ezrin and CLP36. These proteins are known to be related with some membrane proteins and cytoskeleton in other tissues,[20,21] although their physiological roles in the placenta are still unclear.

Localization and Expression of Ezrin and CLP36 in Conditionally Immortalized Syncytiotrophoblast Cell Lines, TR-TBTs We performed immunocytochemical analysis to examine the protein expression and intracellular localization of ezrin and CLP36 in TR-TBTs. In TR-TBT 18d-1, ezrin was predominantly expressed in the membrane protrusions, *i.e.*, filopodia and microvilli-like structures, as well as cytosol (Fig. 2A). Ezrin was colocalized in part with actin fiber structures (Fig. 2G), which is in agreement with the report that the actin binding domain is located at the C-terminus of ezrin and exists in both cytosolic and membrane-bound forms.[20] CLP36 was highly expressed in lamelipodia and the leading edge of TR-TBT 18d-1 (Fig. 2B). CLP36 was also colocated in actin fiber structures in the cell (Fig. 2H). The localizations of ezrin and CLP36 overlap in part, especially at the leading edge of the cell (Fig. 2I). Confocal microscopic analysis of TR-TBT 18d-1 indicated that ezrin was predominantly expressed underneath the apical membrane (Fig. 3C), while CLP36 was predominantly localized on the basal membrane, although a part of it was expressed in the apical membrane (Fig. 3D). Ezrin and CLP36 proteins were hardly detected in TR-TBT 18d-2 cells by immunocytochemical analysis (data not shown). These results suggested that ezrin might have a role in the polar distribution of apically localized proteins, including transporters.

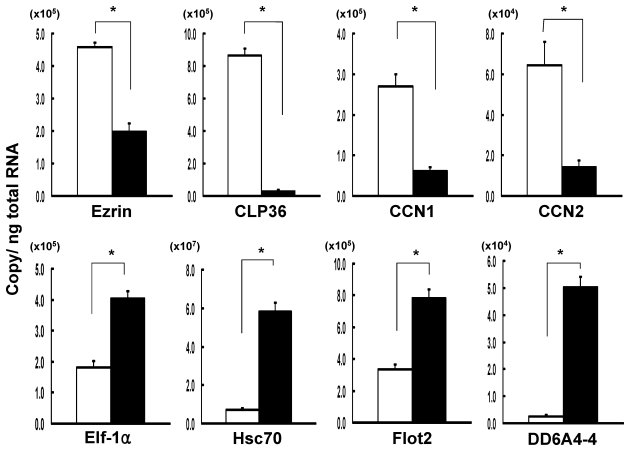

Fig. 1. Quantitative PCR Analysis of Differentially Distributed Genes in TR-TBT 18d-1 Compared with TR-TBT 18d-2

All data are given as the mean±S.E.M. of three determinations. $*p<0.01$, a significant difference between the two cell lines. Open column, TR-TBT 18d-1; closed column, TR-TBT 18d-2.

Expression of Ezrin in Placental Villi of Rats We employed immunohistochemical staining to confirm the protein expression and localization of ezrin in rat placenta. Ezrin was expressed in the labyrinthine wall at embryonic days of 14, 18 and 21 (Figs. 4B, D, E), and localized at plasma membrane mainly (Fig. 4C). At larger magnification, ezrin appeared to be localized at the maternal side of villi at embryonic days 14, 18 and 21 (Figs. 4B—D, inset). To clarify the localization of ezrin in the labyrinthine wall, we performed double immunostaining against ezrin and P-gp, which was expressed at maternal side of the syncytiotrophoblast in labyrinthine zone.[22] Ezrin was co-localized with P-gp at least in the zone where the intensive signal for P-gp was found (Fig. 4F). We also confirmed the apical localization of P-gp in rat renal proximal tubule by the same method (data not shown). These data suggested that ezrin is localized in the apical membrane of the trophoblasts in rats; this is in agreement with the finding in human placenta.[23] The expression of ezrin was not prominent in rat placenta at embryonic day 12, when the polarization of villi remains incomplete. Thus, protein expression of ezrin was low in the early phase of placentation and increased during pregnancy, concomitantly with the morphogenesis of villi.

mRNA Expression Levels of ERM Family Members in Rat Placenta As ERM family members may compensate their function each other, the mRNA expression level of ezrin in rat placenta was compared with those of other ERM family members, radixin and moesin. The mRNA expression of ezrin was highest among the ERM family members. The highest mRNA expression level of ezrin was observed at gestational day 14 and 18, and thereafter decreased gradually (Fig. 5).

DISCUSSION

Polar expression of mRNA or protein in *SynI* and *SynII* has been little investigated, except for the protein localization of glucose transporters GLUT1 and GLUT3 in mice and rats.[19] Here, we found that ezrin and CLP36 are highly expressed in TR-TBT 18d-1 compared with TR-TBT 18d-2

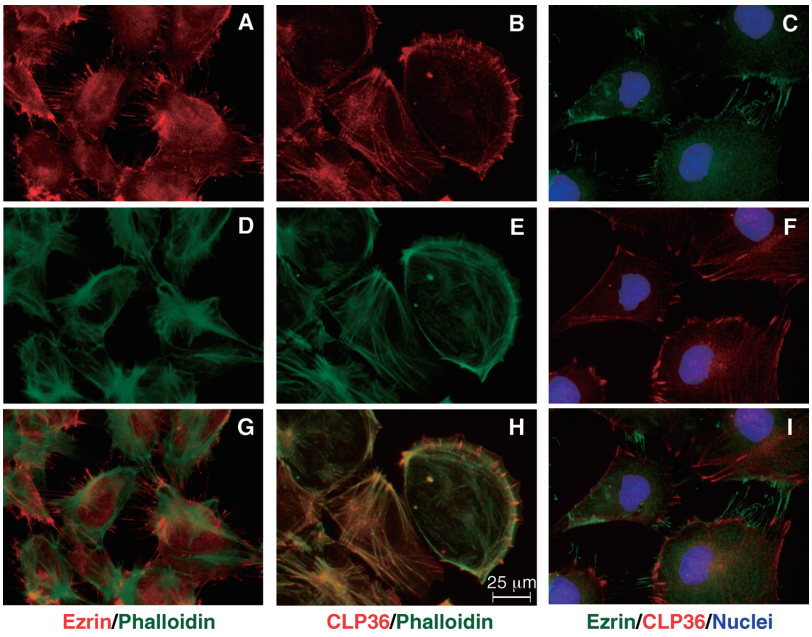

Fig. 2. Expression of Ezrin and CLP36 Proteins in Syncytiotrophoblast Cell Line TR-TBT 18d-1

TR-TBT 18d-1 was stained with anti-ezrin rabbit polyclonal antibody (A, C) and anti-CLP36 mouse monoclonal antibody (B, F). The immune reactivity was visualized by using Alexa Fluor 488 (green) or 594 (red) labeled secondary antibodies. Nuclei were stained with DAPI (C, F, I) (blue). Alexa Fluor 488-labeled phalloidin (green) was used to visualize *F*-actin (D, E) Figs. G, H and I represent merged images of A+D, B+E and C+F, respectively.

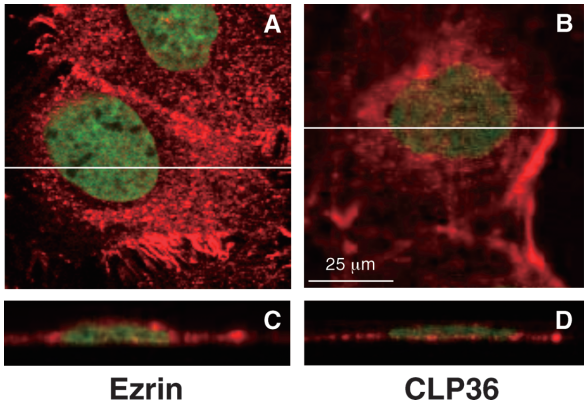

Fig. 3. Confocal Imaging of TR-TBT 18d-1 Cells Immunostained with Anti-ezrin or Anti-CLP36 Antibodies

TR-TBT18d-1 was stained with anti-ezrin antibody (A, C) or anti-CLP36 antibody (B, D). Nuclei were stained with SYTOX (green). Figures C and D represent vertical cross sections generated from Figs. A and B, respectively.

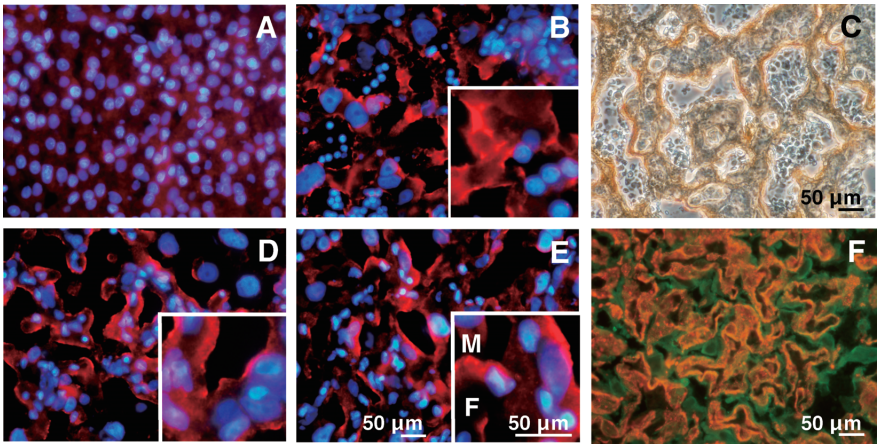

Fig. 4. Immunohistochemical Analysis of Ezrin in Rat Placenta

Rat placenta sections at embryonic day 12 (A), 14 (B, C), 18 (D) or 21 (E) were stained with anti-ezrin antibody and visualized by using Alexa Fluor 594 (red) labeled secondary antibody or by using DAB (brown). Nuclei were stained with DAPI (blue). Rat placenta section at embryonic day 18 (F) was stained with anti-ezrin antibody and anti-P-gp antibody and visualized by using Alexa Fluor 594 (red) and 488 (green), respectively. Colocalization of the red and green signals is shown in yellow. M, maternal blood side; F, fetal blood side. Insets, enlarged images.

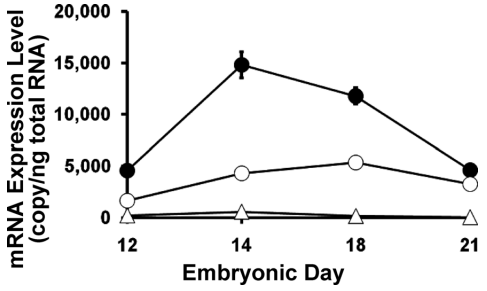

Fig. 5. Quantitative Real-Time PCR Analysis of Rat Placenta at Different Embryonic Days

Each point represents the mean±S.E.M. of three determinations. Closed circle, ezrin; open circle, radixin; open triangle, moesin.

(Table 1, Figs. 1, 2). Ezrin and CLP36 are cytoskeleton-binding proteins localized in the apical membranes of syncytiotrophoblast cell line (Figs. 2, 3) and labyrinthine villi of rat placenta (Fig. 4). These proteins are also co-localized in part with *F*-actin in TR-TBT 18d-1 (Fig. 2). Ezrin associates with actin *via* the C-terminal domain, and with membrane-associated proteins through its N-terminal domain.[20,24] The physiological role of ezrin in rat placenta is unknown, although Tamura *et al.* have shown that ezrin knockout mice were born at a submendelian segregation ratio, did not grow, and showed no increase in body weight after birth.[25] Ezrin belongs to the ERM family, and proteins of the ERM family play structural and regulatory roles in the assembly and stabilization of specialized plasma membrane domains.[23,26] Saotome *et al.* reported that ezrin controls the localization and/or function of apical membrane proteins in mouse intestine.[27] Decreased labeling of surface transporters such as GLUT1 was also observed in retinal pigment epithelium cells from ezrin knockout mice.[28] It was also reported that the localization of multidrug resistance associated protein (MRP2) on bile canalicular membranes was altered in radixin-deficient mice,[29] and that ERM family proteins mediated P-glycoprotein-actin association and regulated P-glycoprotein function in human lymphoma cells.[30]

Polarization of epithelial cells is regulated by actin-binding PDZ-related proteins, cell–cell adhesion proteins, and cell differentiation. Among the PDZ-related proteins, ezrin is localized on the maternal side of the human syncytiotrophoblast[23,24,31] and CLP36 acts as an adapter between stress fibers in nonmuscle cells.[32] Recently, Watanabe *et al.* reported that the transport activity of PEPT1 was stimulated by a PDZ protein-mediated interaction with Na^+/H^+ exchanger (NHE3).[33] As ezrin is expressed in the apical membranes of the syncytiotrophoblast, one can hypothesize that ezrin regulates the localization and function of transporters at the syncytial apical membrane in rat placenta.

The mRNA expression level of CLP36 in TR-TBT 18d-1 was approximately 35-fold higher than that in TR-TBT 18d-2 (Fig. 1). CLP36 has been reported to be expressed in several non-muscle tissues, and associates with actinin-1, -2 and -4 to act as a cellular cytoskeleton anchoring protein.[34,35] CLP36 protein is expressed in seminiferous epithelium, which is a component of the blood–testis barrier.[36] CLP36 is also expressed in trophoblast in human placenta.[37,38] A homeodomain LIM protein, hydrogen peroxide-inducible clone-5 (Hic-5), also interacts with dopamine transporter (DAT) and reduces the membrane localization of DAT in synapses.[39] Therefore, our results suggest that CLP36 may regulate the localization of apical and basal-side membrane proteins in rat *SynI*.

CCN1 and CCN2 are extracellular matrix (ECM)-associated proteins and induce cell polarization.[40] Their mRNAs were preferentially expressed in TR-TBT 18d-1 compared with TR-TBT 18d-2 (Table 1, Fig. 1). It has been reported that CCN1-deficient mice show a specific defect in vessel bifurcation at the labyrinth zone, associated with impaired vascular endothelial growth factor-C expression. Furthermore, CCN1 mediates the adhesion of human umbilical vein endothelial cells. CCN2 promotes the adhesion, proliferation, and migration of vascular endothelial cells.[40] One could therefore hypothesize that CCN family members are involved in adhesion of rat syncytiotrophoblast cells, and may contribute to the regulation of vessel formation in the placenta.

The mRNA expression levels of elf-1α, hsc70 and flot2 were higher in TR-TBT 18d-2 than in TR-TBT 18d-1 (Table 1, Fig. 1). During mammalian development, elf-1α mRNA levels remain unchanged in its sister gene S1-negative tissues, but sharply decrease in S1-positive tissues.[41] It was reported that hsc70 is a marker for progenitor B-cells in bone marrow.[42] Flot2 protein is assembled in a complex that contains activated glycosylphosphatidylinositol-linked cell adhesion molecules. Over expression of flot2 resulted in induction of filopodia.[43] Flot2 protein is associated with membrane rafts and is involved in various cellular processes such as T-cell activation, phagocytosis and insulin signalling. The expression of DD6A4-4 was down-regulated as part of the normalization process, during which IC-12 cells changed morphology from a fusiform to a cobblestone-like appearance and their anchorage-independent growth was markedly reduced (described in AF034242 from gene bank information). One possibility is that the mRNA expression level of these genes is related to the differentiation stage of epithelial cells.

In this analysis, we showed the localization of ezrin in TR-TBTs is comparable to that in rat placenta. We previously reported that the mRNA expression of various transporters and the localization of GLUT1 and GLUT3 in TR-TBTs are comparable to those in rat placenta.[14,15] These data suggested that TR-TBTs may be good model of rat syncytiotrophoblasts for analysis of the localization and the protein expression of transporters and anchoring proteins in blood–placenta barrier.

In conclusion, we have identified 8 genes that are differentially expressed between TR-TBT 18d-1 and TR-TBT 18d-2 by DNA microarray and quantitative RT-PCR. These were including genes encoding membrane anchor proteins, such as ezrin and CLP36, and 4 genes were higher in TR-TBT 18d-1 compared with TR-TBT 18d-2, while 4 genes were lower in TR-TBT 18d-1 compared with TR-TBT 18d-2. Immunohistochemical studies showed that ezrin is expressed in the rat placental villi *in vivo*, and ezrin is located on the apical side of TR-TBT 18d-1, while CLP36 is located on the apical and basal sides of TR-TBT 18d-1. Our results should help to clarify the mechanisms that control polarization of the syncytiotrophoblast and the significance of the double epithelial layers in rat and mouse.

Acknowledgement This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, a grant from the "High-Tech Research Center" and "Open Research Center" Project for Private Universities matching fund subsidy, the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan, and a grant from the Joint Research Project under the Japan–Korea Basic Scientific Cooperation Program of the Japan Society for the Promotion of Science (JSPS) and Korea Science & Engineering Foundation (KOSEF).

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