Published in final edited form as:

Histochem Cell Biol. 2013 March ; 139(3): 487–500. doi:10.1007/s00418-012-1040-2.

Expression of flotillins in the human placenta: potential implications for placental transcytosis

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Abstract

A proteomics survey of human placental syncytiotrophoblast (ST) apical plasma membranes revealed peptides corresponding with flotillin-1 (FLOT1) and flotillin-2 (FLOT2). The flotillins belong to a class of lipid microdomain-associated integral membrane proteins that have been implicated in clathrin- and caveolar-independent endocytosis. In the present study, we characterized the expression of the flotillin proteins within the human placenta. FLOT1 and FLOT2 were coexpressed in placental lysates and BeWo human trophoblast cells. Immunofluorescence microscopy of first-trimester and term placentas revealed that both proteins were more prominent in villous endothelial cells and cytotrophoblasts (CTs) than the ST. Correspondingly, forskolin-induced fusion in BeWo cells resulted in a decrease in FLOT1 and FLOT2, suggesting that flotillin protein expression is reduced following trophoblast syncytialization. The flotillin proteins co-localized with a marker of fluid-phase pinocytosis, and knockdown of FLOT1 and/or FLOT2 expression resulted in decreased endocytosis of cholera toxin B subunit. We conclude that FLOT1 and FLOT2 are abundantly coexpressed in term villous placental CTs and endothelial cells, and in comparison, expression of these proteins in the ST is reduced. These findings suggest that flotillin-dependent endocytosis is unlikely to be a major pathway in the ST, but may be important in the CT and endothelium.

Keywords

flotillin-1; flotillin-2; placenta; trophoblast; endothelium; endocytosis

Introduction

The human placenta is a complex and vital organ that mediates the transfer of solutes, biomolecules, and gases between mother and fetus. This exchange is facilitated by a branching villous system, which invests fetal capillaries and brings them into close proximity with the maternal circulation. Intervening between these circulatory systems is the specialized syncytiotrophoblast (ST) epithelium, which serves to govern trafficking between the mother and fetus. This tightly-coordinated process is crucial for placental function in support of normal fetal development.

In contrast to gases, which passively diffuse across the trophoblast barrier, and essential small molecules (e.g., amino acids, sugars and ions), which traverse the ST through protein pumps and channels, larger macromolecules (such as maternal antibodies) must be carried into the cell via membrane-bound, endocytotic vesicles (Fuchs and Ellinger, 2004). These organelles form compartmentalized micro-environments to facilitate biochemical reactions, regulate hormonal signaling events, participate in immune surveillance, and coordinate the trafficking of proteins and solutes (Conner and Schmid, 2003). While electron microscopy reveals that the ST is crowded with various membrane-delimited compartments, the molecular composition and function of these structures has not been fully characterized.

In general, endocytosis is a highly-regulated process that encompasses both phagocytosis (in which large particles are taken up) and pinocytosis (which involves the uptake of fluid and smaller solutes). While phagocytosis is typically restricted to specialized cells, pinocytosis occurs ubiquitously through three major mechanisms: (1) clathrin-mediated endocytosis; (2) caveolar-mediated endocytosis; and (3) clathrin-/caveolar-independent endocytosis (Conner and Schmid, 2003). Of these, clathrin-dependent endocytosis has received the most attention, owing largely to the well-defined, receptor-mediated mechanisms through which it proceeds. Clathrin-coated pits have been identified in the ST (Ockleford and Whyte, 1977), and this pathway is important for the uptake and transport of numerous cargos, such as immunoglobulin G, holo-transferrin and lipoproteins (Fuchs and Ellinger, 2004; Mongan and Ockleford, 1996; Pearse, 1982). In contrast, the research to date suggests that the ST expresses little, if any, of the caveolin proteins (Byrne et al., 2001; Byrne et al., 2007; Dye et al., 2001; Kittel et al., 2004; Lambot et al., 2006; Lyden et al., 2002; Vandre et al., 2007), and the morphological entities known as caveolae have not been identified at the ultrastructural level despite efforts to detect them (Linton et al., 2003; Lyden et al., 2002). Given either the absence or extremely low levels of the proteins necessary for caveolar-mediated endocytosis in ST, the portals through which transplacental macromolecular transport can proceed are reduced, thereby raising the relative importance of clathrin-/caveolar-independent endocytosis in this specialized epithelium.

In a proteomics screen of the apical plasma membrane of human placental ST, we recently identified several proteins previously not known to reside in this layer (Robinson et al., 2008; Robinson et al., 2009c; Vandre et al., 2012). Among these were flotillin-1 (FLOT1) (flotillin 2 [FLOT2] was also identified but only a single peptide was detected and thus was not included in the list of proteins presented in that study). The flotillins belong to a class of lipid microdomain-associated integral membrane proteins (Morrow and Parton, 2005) that have recently been implicated in clathrin-/caveolar-independent endocytosis (Babuke et al., 2009; Frick et al., 2007; Glebov et al., 2006; Neumann-Giesen et al., 2007; Riento et al.,

2009). In cell culture models, these proteins appear to contribute to the uptake of certain cargos, such as the lipid-raft associated, glycosylphosphatidylinositol (GPI)-linked CD59 protein, and cholera toxin B (Glebov et al., 2006; Saslowsky et al., 2010). The current study was undertaken to examine the expression and localization of flotillins within the human placenta and the BeWo trophoblastic cell model. We hypothesized that flotillin-dependent endocytosis may serve as an important non-clathrin/non-caveolar route of endocytosis in the placenta.

Materials and methods

Reagents and antibodies

The following antibodies were used for immunoblotting and immunolabeling experiments, as indicated in the text and figure legends: mouse anti-FLOT1 (610820, BD Biosciences), mouse anti-FLOT2 (610383, BD Biosciences), rabbit anti-FLOT1 (HPA001393, Sigma-Aldrich), rabbit anti-FLOT2 (F1680, Sigma-Aldrich), rabbit anti-FLOT2 (F1805, Sigma-Aldrich), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Chemicon International), mouse anti-dysferlin (DYSF; Ham1/7B6, Vector Laboratories), mouse monoclonal anti-E-cadherin (E-cad, 610181, BD Biosciences), and mouse anti-serine peptidase inhibitor, Kunitz type 1 (SPINT1, clone C76/18) (Kataoka et al., 2000b; Kataoka et al., 2000a; Shimomura et al., 1999). Lucifer yellow carbohydrazide (LY-CH), Alexa Fluor 594-conjugated cholera toxin B subunit (CTB-594), Alexa Fluor-conjugated secondary antibodies, and Prolong antifade mounting reagent were obtained from Molecular Probes/Life Technologies. Horseradish peroxidase-labeled goat secondary antibodies were obtained from Jackson ImmunoResearch. Forskolin and MISSION Lentiviral Transduction Particles were obtained from Sigma-Aldrich. DMEM/F-12, fetal calf serum, and NuPAGE Novex Bis-Tris precast acrylamide gels were obtained from Invitrogen/Life Technologies. SuperSignal chemiluminescent kits were from Pierce Biotechnology/Thermo Scientific. Bradford assay dye was from Bio-Rad Laboratories. Tissue freezing medium for cryosectioning were obtained from Fisher Scientific. All other reagents unless otherwise specified also were obtained from Sigma-Aldrich.

Tissue collection

Human term (39–41 wk of gestation) placentas were obtained with informed consent according to a protocol approved by the Biomedical Sciences IRB at Ohio State University, Columbus, OH. Tissue samples from uncomplicated cesarean deliveries were used. In addition, first-trimester (8–12 wk) and term (39–41 wk) placental tissues were obtained according to a protocol approved by the Hospital Ethics Committee at Nippon Medical School, Tokyo, Japan. Placental tissue was either flash frozen in liquid N₂ or fixed in 4% PFA (1 h at 22°C) within 20 min of delivery. A total of 10 placentas (3 first-trimester, 7 term) were used for the studies described herein.

Cells and cell culture

Human BeWo choriocarcinoma cells were obtained from the American Type Culture Collection and were cultured in DMEM/F12 (1:1) supplemented with 10% fetal calf serum, 200 U/ml penicillin, and 200 µg/ml streptomycin. Cells were cultured in tissue culture flasks and maintained at 37°C in a humidified incubator with a 5% CO₂/95% air atmosphere. In experiments requiring cell-cell fusion, cells were incubated in the presence of 20 µM forskolin or solvent control (0.2% dimethyl sulfoxide) for 0–3 days *in vitro* as previously described (Robinson et al., 2009a). For microscopy, cells were cultured on 12-mm round glass coverslips in 24-well culture plates or 22-mm square coverslips in 35-mm Petri dishes.

Lentiviral Transduction

MISSION short hairpin RNA (shRNA) lentiviral transduction particles were designed and developed by The RNAi Consortium (Broad Institute, Cambridge, MA) using an algorithm to select and rank candidate hairpin sequences, each comprised of a 21 base stem and a 6 base loop, from reference sequence transcripts reported from the NCBI gene database. The following hairpin shRNA sequences targeting human FLOT1 mRNA were used: 5'-CCG GCC AGG ACT ATT TGC ACT CTT TCT CGA GAA AGA GTG CAA ATA GTC CTG GTT TTT (#29309); 5'-CCG GCC AGG TGA ATC ACA AGC CTT TCT CGA GAA AGG CTT GTG ATT CAC CTG GTT TTT (#29310); 5'-CCG GCC CTC AAT GTC AAG AGT GAA ACT CGA GTT TCA CTC TTG ACA TTG AGG GTT TTT (#29311); 5'-CCG GGC AGA GAA GTC CCA ACT AAT TCT CGA GAA TTA GTT GGG ACT TCT CTG CTT TTT (#29312); and 5'-CCG GAC AGA GAG ATT ACG AAC TGA ACT CGA GTT CAG TTC GTA ATC TCT CTG TTT TTT (#29313). The shRNA sequences targeting human FLOT2 RNA were: 5'-CCG GCG TGT ATG ACA AAG TGG ACT ACT CGA GTA GTC CAC TTT GTC ATA CAC GTT TTT TG (#148873) and 5'-CCG GGA GCA GTT TCT GGG TAA GAA TCT CGA GAT TCT TAC CCA GAA ACT GCT CTT TTT TG (#149396). The FLOT2 sequences were cloned into the pLKO.1-hPGK-Neo vector; all other sequences were cloned into the pLKO.1-Puro vector.

For single target knockdowns, BeWo cells were plated at 10^5 cells/well in a 24-well tissue culture dish and incubated overnight. Transduction was carried out by adding concentrated lentiviral particles to the cells at a multiplicity of infection (MOI) of 1 (10^5 transducing units/well) in the presence of 8 μ g/ml of hexadimethrine bromide. In control preparations, BeWo cells were incubated with MISSION Non-Target shRNA Control Transduction Particles (SHC002V, Sigma-Aldrich). Following overnight incubation, the media were replaced with normal growth media. On the second day following transduction, the cells were incubated in the presence of 8 μ g/ml puromycin to select for puromycin-resistant cells, or 800 μ g/ml Geneticin to select for neomycin resistant cells. Using this method, stable FLOT1- and FLOT2-deficient cell lines were produced. For double knockdowns, the same procedure was carried out starting with FLOT1-deficient cell lines and using lentiviral constructs targeting FLOT2 mRNA, in which the neomycin resistance gene was substituted for the puromycin-resistance gene.

Protein extraction and immunoblotting

Placental tissue (~60–120 mg) was pulverized under liquid N₂ using a mortar and pestle and subsequently incubated for 20 min in ice-cold lysis buffer (150 mM Na₂PO₄, 60 mM *n*-octyl β -D-glucopyranoside, 10 mM D-gluconic acid lactone, and 1 mM EDTA) containing protease inhibitor cocktail (Lang et al., 2009). BeWo extracts were prepared using the same lysis buffer. Proteins from placental and cell lysates were resolved by SDS-PAGE using 10% acrylamide gels. Immunoblotting was performed as described previously (Lang et al., 2009; Robinson et al., 2009a; Vandre et al., 2007).

Immunofluorescence microscopy (IFM) and analysis

Cryostat sections (5–6 μ m) of fixed placental tissue were prepared and labeled with anti-FLOT1 and anti-FLOT2 antibodies using published protocols (Lang et al., 2009; Robinson et al., 2009a; Vandre et al., 2007). Briefly, the cryosections were washed with PBS and incubated with 0.5% SDS for antigen retrieval (Robinson and Vandre, 2001), then 1% non-fat milk/5% normal goat serum/PBS for 1 h to reduce non-specific binding. The sections were then incubated overnight with anti-FLOT1, anti-FLOT2, and/or anti-SPINT1 antibodies at 4°C, as indicated in the text and figure legends. The sections were subsequently washed and incubated with fluorochrome-labeled secondary antibodies for 1 h at room temperature. After washing with PBS, the nuclei were stained with DAPI (4',6-

diamidino-2-phenylindole) for 10 min before mounting the slide using an anti-photobleaching reagent. In control preparations, either the primary antibodies were omitted, or normal mouse or rabbit sera was substituted prior to application of the secondary antibodies.

IFM was also performed using ultrathin cryosections as described previously (Takizawa et al., 2005; Takizawa and Robinson, 2003; Takizawa and Robinson, 2006). Briefly, placental tissues were fixed as described above, solidified with gelatin, infiltrated with 2.3 M sucrose, and mounted on specimen pins designed to fit a cryo-ultramicrotome. The samples were then stored in liquid N₂ prior to sectioning. Ultrathin cryosections (70–100 nm in thickness) were cut with a Leica ultramicrotome EM UC6b equipped with an FC6 cryounit. Ultrathin cryosections were incubated with primary and secondary antibodies and DAPI as described for conventional cryostat sections. Control incubations were also as described above.

BeWo cells cultured on glass coverslips were fixed in 4% PFA/PBS for 1 h. The cells were then washed six times in PBS and permeabilized with 0.5% SDS in PBS for 5 min prior to the application of antibodies. Immunolabeling was then performed as described for the cryosections.

Conventional epifluorescence and differential interference contrast (DIC) images were collected with a Nikon Eclipse 90i microscope equipped with a Photometrics CoolSNAP HQ2 CCD camera (Roper Scientific) or an Olympus BX60 microscope equipped with a Spot RT SE6 CCD (Diagnostic Instruments). Images were captured using either the NIS-Elements AR software package (version 3.1, Nikon Instruments, Melville, NY) or the MetaMorph image analysis system (Universal Imaging). All images were collected within the linear response range of the CCD camera. In some cases, a Zeiss 510 META laser scanning confocal microscope (Carl Zeiss, Inc.) was used to examine labeled specimens, as indicated in the figure legends.

Fluorescence intensity measurements comparing flotillin labeling in different regions of the villous tree were made using the intensity profile tool of the NIS-Elements AR software package (version 3.1, Nikon Instruments). For each antigen (FLOT1 and FLOT2), six randomly selected, non-overlapping images from three term placentas, each containing sectioned profiles of multiple villi, were imaged. A total of 35 images were used in this analysis (one image contained a processing artifact, and was eliminated from consideration). The acquisition parameters were held constant throughout imaging, and measurements for each comparison group were done together to minimize variation in excitation illumination.

Endocytosis assays

Fluid phase pinocytosis was measured using lucifer yellow carbohydrazide (LY-CH, a small, membrane-impermeable fluorescent dye that is taken up non-specifically by endocytosis). BeWo cells cultured on glass coverslips were incubated in the presence of 1 mg/ml of LY-CH dissolved in sterile PBS for 0–15 min at 37°C. Following treatments, the cells were quickly rinsed 4 times each with 2 ml of pre-warmed PBS, fixed for 1 h in 4% PFA, and then immunolabeled as described previously using anti-FLOT1 and anti-FLOT2 antibodies.

To examine the roles of FLOT1 and FLOT2 on endocytosis, we performed additional endocytosis assays in BeWo cells deficient in flotillin isoforms. Uptake of the cholera toxin B subunit was assessed, since this was shown previously to enter HeLa and COS-7 cells in a flotillin-dependent manner (Glebov et al., 2006). Following the procedures described by Glebov et al. (Glebov et al., 2006), BeWo cells (5×10^4 /well) stably transduced with non-target shRNA control transduction particles or lentivirus-based shRNAs targeting FLOT1,

FLOT2, or both, were seeded onto glass coverslips. Following overnight incubations, each of the cell lines was incubated with 20 µg/ml CTB-594 on ice for 30 min. Next, the cells were warmed to 37°C by exchange with pre-warmed medium without labeled ligand, and endocytosis was allowed to proceed for 15 min. The cells were then washed four times with ice-cold acid wash buffer (150 mM glycine, pH 2.0) to remove CTB-594 attached to the cell surface but not internalized), followed by two rinses in PBS and fixation in 4% PFA/PBS for 1 h. To quantify CTB-594 uptake, the fixed slides were mounted for IFM, and 20 non-overlapping images per condition were acquired using the 20X objective of Nikon Eclipse 90i epifluorescence microscope. All images were collected within the linear response range of the CCD camera, and exposure times were held constant throughout imaging. Average fluorescence intensity measurements for individual cells were evaluated using the NIS-Elements AR software, and at least 100 cells per cell type were analyzed.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software). A Shapiro–Wilk test for normality was initially performed on fluorescence intensity data to confirm the validity of parametric statistical testing. Comparisons of fluorescence intensity measurements were performed using one-way analysis of variance, followed by the Bonferroni multiple comparisons *post hoc* test. A *P* value <0.05 was considered significant.

Results

Flotillin proteins are expressed in the human placenta

We performed a proteomics analysis of fractions derived from the microvillous (MV) surface of term placental syncytiotrophoblast (ST) (Robinson et al., 2008; Robinson et al., 2009c; Vandre et al., 2012). Among approximately 6000 unique peptides identified by tandem mass spectrometry, our initial database query revealed eight peptides that mapped to the human FLOT1 amino acid sequence, and one unique peptide corresponding to the human FLOT2 protein. Based on these preliminary data, we examined the expression of the flotillins by immunoblotting using homogenates of fractionated proteins prepared using the small cationic colloidal silica fractionation method described previously (Robinson et al., 2009b). Both proteins were present in the crude tissue homogenate (CTH) as well as in other fractions collected during the isolation procedure (Fig. 1A), including the pelleted plasma membrane (PPM) fraction that was enriched for proteins in the apical MV surface of the ST; however, neither FLOT1 nor FLOT2 were enriched in this fraction. Coexpression of FLOT1 and FLOT2 was confirmed using whole tissue lysates prepared from the villous portion of three additional term placentas (Fig. 1B).

Flotillin expression is abundant in villous cytotrophoblasts and endothelial cells with lesser amounts in the syncytiotrophoblast

To characterize the cellular distribution of FLOT1 and FLOT2 in the human placenta, IFM was performed using conventional cryostat sections (5–6 µm thickness). In term specimens, we found that FLOT1 and FLOT2 exhibited similar patterns of distribution: both proteins were prominently expressed at the CT surface and in endothelial cells surrounding fetal vessels, but lesser amounts were evident in the ST (Fig. 2). Control specimens in which the primary antibodies were omitted, or pre-immune sera were substituted for the primary antibodies, exhibited only faint background staining in all cases. To confirm immunolabeling specificity, we repeated these experiments using additional antibodies. While the rabbit anti-FLOT1 antibody worked well, the mouse anti-FLOT1 antibody was not useful for IFM; however, the two additional anti-FLOT2 antibodies showed an immunolabeling pattern identical to that presented in Fig. 2 (data not shown).

To assess whether flotillin labeling exhibited regional variability among villous subdivisions, we performed quantitative IFM analysis. These measurements revealed that the labeling of both FLOT1 and FLOT2 was most intense in endothelial cells lining fetal vessels (Fig. 2, E and F). For either of these proteins, we noted no significant changes in endothelial labeling between intermediate and terminal villi. Within the trophoblast layers of the villous tree, terminal villi exhibited significantly greater flotillin labeling intensity than intermediate villi, and intermediate villi possessed significantly more labeling intensity than stem villi ($P < 0.05$, ANOVA with Bonferroni multiple comparison test).

In as much as these labeling results indicated that most of the flotillin was localized at or near the basal ST border (single arrows in Fig. 2, A and C), we performed additional analyses to determine the extent to which this labeling was associated with CTs. In term placenta specimens sectioned at 5–6 μm thickness, it was difficult to distinguish between CT and ST labeling due to changes in the CT layer that accompany advancing gestation (including discontinuity and an attenuation in the height of individual cells). Therefore, we examined flotillin labeling in first trimester placental specimens (in which CTs are prominent and easy to discern), in combination with a marker protein, serine peptidase inhibitor Kunitz type 1 (SPINT1) (Mori et al., 2007), which is expressed exclusively in CTs (Kataoka et al., 2000a; Potgens et al., 2003). In addition, we employed high-resolution IFM of ultrathin cryosections (70–100 nm in thickness), since this approach minimizes the possibility of false co-localization between fluorescent labels in complex tissue specimens (Takizawa et al., 2005). In ultrathin cryosections of first-trimester tissues, SPINT1 labeling was confined to the cell borders of CTs (Fig. 3B, Fig. S1). A majority of the FLOT1 (Fig. 3, A and C) as well as the FLOT2 (Fig. S1) labeling was observed within SPINT1-positive CTs. Much less punctate flotillin staining was observed in the ST. In ultrathin cryosections of term placental specimens, the SPINT1-labeled CTs exhibited marked lateral spreading (Fig. 4, Fig. S2), such that the confinement of FLOT1 and FLOT2 to CTs (as opposed to localization at the basal ST surface) was difficult to discern. Although we could not exclude the possibility that flotillin expression was present at the basal border of the ST by IFM, these results nevertheless demonstrate that flotillin expression was reduced in the ST cytoplasm and apical regions. As with the conventional sections, flotillins were highly expressed in fetal endothelial cells.

Flotillins are downregulated in BeWo cells following forskolin-induced fusion and contribute to endocytosis

Given that the expression of FLOT1 and FLOT2 was prominent in CTs but reduced in ST, we next examined the expression of these proteins in BeWo cells. Importantly, BeWo cells can be induced to fuse into syncytial structures, a process that models the fusion of CTs with the ST *in situ*. Commonly, this is achieved via a protein kinase A-dependent pathway following treatment with forskolin or cAMP analogs (Chang et al., 2005; Wice et al., 1990).

Immunoblot analysis revealed that FLOT1 and FLOT2 were coexpressed in mononuclear BeWo cells (Fig. 5A). The expression of both proteins decreased following forskolin-induced cell-cell fusion (Fig. 5A), mirroring the expression pattern observed *in situ* in which flotillin was abundant in unfused CTs, but attenuated in the ST. Reciprocally, the expression of dysferlin (DYSF, used here as a marker for cell-cell fusion) in BeWo cells increased following forskolin treatment, consistent with our previous findings (Robinson et al., 2009a).

We next examined the distribution of the flotillin proteins by IFM, which revealed that FLOT1 and FLOT2 appeared in rows of puncta along intercellular boundaries in mononuclear BeWo cells (double arrows in Fig. 5, B and C), similar to the labeling pattern in CTs within first trimester placenta sections. Both flotillins additionally localized to

perinuclear regions within (or near) the Golgi/trans-Golgi network (wide arrows in Fig. 5, B and C), and flotillin-labeled punctate structures were also prominent. Similar labeling at a lower magnification (to show context) is presented in Fig. S3.

Since the expression of both flotillins decreased concomitantly with forskolin treatment, we predicted that fused BeWo cells would exhibit diminished flotillin labeling. While the cell surface labeling was essentially absent, we observed that both FLOT1 and FLOT2 were present within fused cells, typically in crescent-shaped areas surrounding nuclear clusters (wide arrows in Fig. 5, D and E). Whereas flotillins colocalized with E-cadherin (E-cad) at cellular boundaries in mononuclear BeWo cells (Fig. S3, A and C), following fusion, the flotillins were no longer found in association with areas of residual E-cad staining (arrowhead in Fig. 5E, and Fig. S3, B and D).

To assess whether the flotillin-labeled punctate structures could represent intracellular vesicles involved in endocytosis, we performed endocytosis assays. Following incubation for 15 min in the presence of LY-CH, a fluorescent marker of fluid-phase pinocytosis, we found that internalized LY-CH could be discerned within structures that co-labeled with FLOT2 (Fig. 6A). Identical experiments were performed using anti-FLOT1 labeling, which yielded similar results (data not shown). These data suggest that a subset of the flotillin-labeled puncta represent intracellular transport vesicles, and point to a role for these proteins in endocytosis in BeWo cells.

To determine the role of flotillins in endocytosis in the BeWo model, FLOT1 and FLOT2 were knocked-down using lentivirus-mediated RNA interference and subjected to endocytosis assays using a cargo (cholera toxin B subunit) that is imported, in part, through a flotillin-dependent mechanism (Glebov et al., 2006). To generate individual knockdowns, multiple shRNA targeting vectors were screened (see “Materials and methods”), and the lines bearing constructs with superior single-target knockdown efficiencies (#29309 for FLOT1 and #149396 for FLOT2) were used for subsequent experiments. Although FLOT1 knockdown had little effect on FLOT2 expression, knockdown of FLOT2 also resulted in downregulation of FLOT1, which is consistent with the results of prior studies (Babuke et al., 2009; Solis et al., 2007; Tomasovic et al., 2012) (Fig. 6B). Indeed, in cells expressing both the #29309 and #149396 shRNA constructs, the effects of combinatorial knockdown on FLOT1 and FLOT2 expression were similar to that using the #149396 construct alone (Fig. 6B). As shown in Fig. 6C, knockdown of FLOT1 resulted in a significant decrease in intracellular CTB-594 following 15 min of uptake, with further decreases noted when FLOT1 and FLOT2 were downregulated. Collectively, these results suggest that flotillins contribute to endocytosis in BeWo trophoblast cells.

Discussion

Ultrastructural studies spanning the past five decades have demonstrated that the ST is rich in membrane-delimited cytoplasmic organelles (Fox and Sebire, 2007). However, the complement of proteins within these vesicular profiles has not been fully deciphered, and as such, information about the molecular functions of these organelles is incomplete. The vesicular profiles associated with the plasma membrane in the ST are, on average, larger (132.16 ± 52.45 nm) than would be typical of caveolae (~70 nm) (Lyden et al., 2002), and frequently lack the electron-dense material that is characteristic of clathrin-coated pits. We have been unable to demonstrate the expression of any of the caveolin proteins in human ST (Lyden et al., 2002; Vandre et al., 2007), which has prompted us to consider that a portion of the vesicles in the ST may be involved in transport processes independent of clathrin and caveolins.

The flotillins (FLOT1 and FLOT2) were initially discovered as proteins upregulated in regenerating neurons following optic nerve lesions in the goldfish (for which they were named “reggies”, for “regeneration”) (Schulte et al., 1997). Independently, the flotillins were identified as resident components of caveolae in mammalian cells (Bickel et al., 1997). The two flotillin proteins contain an evolutionarily-conserved prohibitin homology domain (PHB), which is common among members of the SPHF (stomatin/prohibitin/HflK/C/flotillin) family (Morrow and Parton, 2005). Despite being of separate protein families, the flotillins share a striking number of structural and functional similarities with caveolins. For example, like caveolins, the flotillins contain short spans of centrally-located hydrophobic amino acid residues which form hairpin-like insertions into the cytoplasmic leaflet of cell membranes (Morrow and Parton, 2005; Stuermer, 2011; Williams and Lisanti, 2004). It is thought that this non-conventional morphology, coupled with a propensity to form higher-order oligomeric structures, enables these proteins to contribute to the formation of areas of high positive membrane curvature (e.g., invaginations and budding endocytotic vesicles) (Frick et al., 2007; McMahon and Gallop, 2005). Consistently, both flotillin and caveolin paralogs have been found in association with distinct types of plasma membrane invaginations (Frick et al., 2007; Glebov et al., 2006; Volonte et al., 1999), and both have been implicated in endocytosis and other membrane trafficking events (Babuke et al., 2009; Doherty and McMahon, 2009; Glebov et al., 2006; Morrow and Parton, 2005; Stuermer, 2011; Williams and Lisanti, 2004). In addition, flotillins and caveolins both serve as scaffolding proteins within lipid microdomains (i.e., cholesterol-enriched, lipid-ordered “rafts” within otherwise fluid membranes), and through intimate association with other microdomain component proteins (such as GPI-linked membrane receptors, Src family tyrosine kinases, and G proteins), proteins of each class have been linked to a diverse variety of receptor-mediated signaling cascades, cytoskeletal and plasma membrane dynamic events, and cell motility (Morrow and Parton, 2005; Stuermer, 2011; Williams and Lisanti, 2004). Given such similarities, the identification of flotillins in our proteomics screen led us to consider that these proteins might functionally compensate for the caveolins in the ST.

Our proteomics screen suggested that, while present, FLOT1 and FLOT2 were not highly enriched in the apical membrane fraction (see Fig. 1). This is distinctly different from other proteins such as DYSF, which is highly enriched in the apical membrane (Robinson et al., 2009b; Vandre et al., 2012). Consistent with this biochemical analysis, we found that while FLOT1 and FLOT2 appeared abundant in CTs and endothelium by immunofluorescence localization, the expression of both was less in the ST. Thus we conclude that the ST is not the major site for localization of the flotillins in the human placenta. Interestingly, this observation was reinforced by our finding that FLOT1 and FLOT2 were downregulated following forskolin-induced fusion in the BeWo model system, suggesting that the process of trophoblast syncytialization is accompanied by a decrease in flotillin expression. It is noteworthy that Rashid-Doubell and colleagues reported that BeWo cells fused using dibutyryl cyclic AMP exhibited a similar decrease in the expression of caveolin-1 (Rashid-Doubell et al., 2007). These observations bolster the notion that there among numerous biochemical changes accompanying cell-cell fusion, there are also dramatic changes in cellular trafficking.

Since it is generally thought that at least one of the flotillin proteins is present (and presumably required) in virtually all cells (Babuke and Tikkanen, 2007; Volonte et al., 1999), it was unexpected that the ST would express both of these proteins at comparatively low levels. These data suggest that abundant flotillin expression is not essential for normal ST function, thus raising interesting questions regarding mechanisms of clathrin-/caveolar-independent endocytosis in this specialized epithelium. In the human placenta, the ST comprises the outermost layer of the vasculo-syncytial exchange barrier, where it serves as a transporting epithelium, with some features in common with other polarized transport

epithelial cells (e.g., proximal renal tubular cells, enterocytes, etc.) (Sibley, 2009). In the absence of placental lesions (e.g., epithelial denudations), it is presumed that large biomolecules must cross through the ST via highly-regulated endocytic and transcytotic processes, and indeed, the villous ST is acknowledged as having high endocytotic activity (Fuchs and Ellinger, 2004). However, these present results indicate that the ST appears to have limited expression of flotillin proteins. When coupled with the near-absence of caveolin proteins, the ST appears to be somewhat limited in its repertoire of endocytotic routes. As such, at least in the human placenta, the ST barrier may in part be established by limiting the complement of regulated portals of entry. Since numerous pathogens, including viruses, bacteria, and toxins, have been shown to enter cells either through endocytosis (Miyauchi et al., 2009; Vidricaire et al., 2004) or interaction with specific lipid microdomains (Campbell et al., 2001), we speculate that the ST may be developmentally specialized so as to minimize the transfer of harmful cargos without compromising the transfer of necessary macromolecules (e.g., maternal IgG, lipoproteins). Although the present results do not further identify components of the abundant clathrin-deficient membrane invaginations and vesicular profiles observed in the ST, they eliminate flotillins as the primary candidate. Recent proteomics screens of the ST have identified additional proteins associated with clathrin-dependent and -independent endocytosis, as well as the exocyst complex, suggesting that these are targets that should be examined in greater detail for their potential contribution to endocytosis in the ST in future studies (Vandre et al., 2012; Zhang et al., 2010). In addition, further work is required to determine what other routes of endocytosis (e.g., macropinocytosis, RhoA-regulated endocytosis, Arf6-dependent endocytosis, and the clathrin-independent carriers/GPI-enriched early endosomal compartment pathway [CLIC/GEEC] (Conner and Schmid, 2003; Doherty and McMahon, 2009), are available and functional in the ST.

Based on their localization patterns within other placental cell types, FLOT1 and FLOT2 are likely to contribute to the endo- and transcytotic molecular trafficking in these cells. In support of this postulate, we found that internalized LY-CH, a fluorescent pinocytotic marker, could be detected with flotillin-containing vesicles after 15 min of continuous uptake in BeWo cells. In addition, knockdown of flotillin expression decreased uptake of CTB-594, a cargo internalized in part through flotillin-dependent routes (Glebov et al., 2006). Interestingly, in term placental specimens, we found that the highest levels of trophoblastic flotillin expression occurred in the CTs of terminal villi; the terminal villi are thought to be responsible for the bulk of maternal–fetal exchange processes (Fuchs and Ellinger, 2004). It is possible that flotillins are enriched in the CTs and terminal villi to facilitate certain transcytotic events. Higher still were the levels of flotillin expression within endothelial cells of fetal vessels. In these cells, it is possible that the flotillins might be involved in the caveolar-independent trafficking of placental IgG via the Fc γ RIIb2 receptor (Takizawa et al., 2005).

In addition to trafficking, it is probable that flotillins act as scaffolding proteins within liquid-ordered microdomains of the plasma membrane, and may thus participate in a variety of structural and signaling events that are important to placental function. In other systems, flotillins have been implicated in diverse processes such as growth factor signaling, insulin receptor signaling, the coordination of axon/neurite growth in neurons, and T lymphocyte capping and activation (Morrow and Parton, 2005; Stuermer, 2011). The flotillins might participate in analogous processes in those cells where it is highly expressed in the placenta. A recent study has used E-cad labeling to distinguish the CT from the ST (Longtine et al., 2012). The situation with regard to FLOT1 and FLOT2 is very similar to that of E-cad, namely that the CT expresses high levels of these proteins while the ST expresses low levels. Thus, FLOT1 and FLOT2 can be added to the short list of proteins that mark the CT plasma membrane.

In summary, we have provided an initial characterization of flotillin protein expression in the human placenta. Although FLOT1 and FLOT2 are coexpressed, at a relatively high level, in villous placental CTs and endothelial cells, the expression of these proteins in ST is limited. These results have implications for placental endocytosis and transcytosis, as our findings suggest that the routes available for cargo transport are restricted at the hemochorial interface. With research now indicating little (if any) caveolin proteins or caveolae (Linton et al., 2003; Lyden et al., 2002; Takizawa et al., 2005; Vandre et al., 2007) and low expression of flotillins in the ST, further studies into the mechanistic basis for macromolecular transport across the vasculo-syncytial exchange barrier are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the staff at the Pathology Core Facility at The Ohio State University (Columbus, OH) for technical assistance with cryosectioning of placental biopsy specimens. We are likewise grateful to the Campus Microscopy and Imaging Facility at The Ohio State University. This work was performed while Dr. Janelle R. Walton was a Fellow in Maternal-Fetal Medicine at The Ohio State University. Portions of this work were presented in abstract form at the Annual Meeting of the Society for Gynecologic Investigation, March 24–27, 2010, Orlando, FL. The current work was supported by a grant in aid from Perinatal Resources, Inc. (Hilliard, OH) and The Ohio State University Perinatal Research and Development Fund. Additional support was provided by grants K08 HD49628 (W.E.A.) and R01 HD058084 (J.M.R.) from the National Institutes of Health.

Abbreviations

CPS	Crude placental supernatant
CT	Cytotrophoblast
CTB-594	Cholera toxin B subunit/Alexa Fluor-594
CTH	Crude tissue homogenate
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential interference contrast
DYSF	Dysferlin
E-cad	E-cadherin
FCTH	Filtered crude tissue homogenate
FLOT1	Flotillin-1
FLOT2	Flotillin-2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
IFM	Immunofluorescence microscopy
LY-CH	Lucifer yellow carbohydrazide
MV	Microvillous
PPM	Pelleted plasma membrane
SPINT1	Serine peptidase inhibitor, Kunitz type 1
ST	Syncytiotrophoblast

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Fig. 1.

FLOT1 and FLOT2 are expressed in human placental homogenates. (A) Equal amounts of protein from fractions generated during the preparation of small cationic colloidal silica-coated ST microvillous fractions (Robinson et al., 2009b) were resolved by gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies directed against either FLOT1 or FLOT2. These fractions were: crude tissue homogenate (CTH), filtered crude tissue homogenate (FCTH), crude placental supernatant (CPS), crude placental pellet (CPP), Histodenz fractions (0–50%, 50–55%), and the pelleted plasma membrane (65% PPM). Note that neither FLOT1 nor FLOT2 was enriched in the PPM fraction. (B) Immunoblotting was used to confirm the expression of both flotillins in three additional term human placenta extracts prepared following lysis in buffer containing *n*-octyl β -D-glucopyranoside (see “Materials and methods”).

Fig. 2. Distribution of FLOT1 and FLOT2 proteins in term human placental villi. (A–D) Representative photomicrographs of conventional cryostat sections (5–6 μm) of placental villi labeled with anti-FLOT1 (HPA001393; red in A) and anti-FLOT2 (1608; red C) antibodies and imaged using laser scanning confocal microscopy. The intensities of these images were adjusted to provide maximal contrast. Panels B and D are corresponding non-confocal differential interference contrast (DIC) micrographs on which DAPI staining (blue) is superimposed. Prominent flotillin labeling (red) is observed in cytotrophoblasts (CTs) and/or basal regions of syncytiotrophoblast (ST), and in fetal capillary endothelial cells (*, lumen of fetal capillary). Arrowheads indicate surface of ST; double arrows indicate flotillin labeling in ST; white single arrows indicate labeling along the basal surface of the ST and/or in CTs; black arrows indicate labeling in endothelial cells. (E, F) Fluorescence intensity measurements for FLOT1 (E) and FLOT2 (F) labeling taken within fetal capillary endothelial cells (EC) of intermediate and terminal villi, and at three levels of the villous tree: terminal villi (TV), intermediate villi (IV), and stem villi (SV). In our analysis, we found that the intensity measurements for FLOT1 EC labeling in intermediate and terminal villi were equivalent, so the data were pooled. This was also the case for FLOT2 EC labeling. Note that the differences in the absolute intensity measurements between FLOT1 and FLOT2 may represent differences in antigen-antibody interactions, and should not be interpreted as differences in relative abundance between these two proteins. Data are mean $\pm$ SD from 3 separate term placental specimens; *, $P < 0.05$ vs. SV; **, $P < 0.01$ vs. SV (ANOVA with Bonferroni multiple comparisons). Scale bar = 20 μm

Fig. 3.

FLOT1 is highly expressed in endothelial cells and CTs, but exhibits lower levels of expression in ST of first trimester placenta. (A, B) Ultrathin cryosection prepared from a first trimester placenta co-labeled using antibodies against FLOT1 (HPA001393; green in A) and SPINT1 (red in B). The specimen was counterstained with DAPI (blue in C–F). (C) A merged image of the green FLOT1 signal, the red SPINT1 signal, and the blue DAPI nuclear staining. (D) The same section with the DIC image merged with the fluorescence image of DAPI-stained nuclei. Panels E and F are the same as those presented in C and D, respectively, on which annotations have been made and white lines drawn to show the approximate demarcations of the CT and ST layers. These micrographs demonstrate that the expression of FLOT1 is higher in the CT relative to the ST. Scale bar = 20 μ m. VS, villous stroma; #, intervillous space

Fig. 4. FLOT1 is highly expressed in endothelial cells and CTs, but exhibits lower levels of expression in ST of term placenta. (A, B) Ultrathin cryosection prepared from a term placenta co-labeled using antibodies against FLOT1 (HPA001393; green in A) and SPINT1 (red in B). The specimen was counterstained with DAPI (blue in C–F). (C, E) A merged image of the green FLOT1 signal, the red SPINT1 signal, and the blue DAPI nuclear staining. (D, F) The same section with DAPI-stained nuclei superimposed on the DIC image. Panels E and F are annotated to identify the approximate locations of the ST and CT layers (indicated by the white lines). The outline of endothelial cells (EC) surrounding a fetal capillary is also demonstrated. Scale bar = 20 μ m. VS, villous stroma; #, intervillous space

Fig. 5.

Flotillin expression is downregulated and changes distribution in BeWo cells following forskolin-induced fusion. (A) Representative immunoblots of BeWo cells treated with 20 μ M forskolin from 0–72 h. Note that the immunoreactivity of FLOT1 and FLOT2 decreases with forskolin treatment, whereas that of dysferlin (DYSF) increases. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Similar results were obtained in three separate experiments. (B, C) Mononuclear BeWo cells immunolabeled with antibodies against FLOT1 (HPA001393; red in B) and FLOT2 (1608; red in C), respectively. Double arrows indicate areas of flotillin labeling along cellular boundaries; wide arrows denote areas of perinuclear staining in individual cells. (D, E) BeWo cells treated with forskolin (20 μ M for 72 h) and co-immunolabeled using antibodies generated against E-cadherin (E-cad, green) and either FLOT1 (red in D) or FLOT2 (red in E), as above. Wide arrows indicate flotillin-like immunoreactivity in crescent-shaped structures in close proximity to clusters of nuclei; arrowhead in E denotes an area of Ecad labeling that has become irregular following cell-cell fusion. (F) Cryosection (5–6 μ m) of term placental villus co-immunolabeled using antibodies generated against dysferlin (DYSF, green) and FLOT2 (1608; red) and imaged using confocal microscopy. Single arrows indicate DYSF labeling at the apical regions of the ST; double arrows indicate FLOT2 labeling. (G) BeWo cells treated with forskolin (20 μ M for 72 h) and co-immunolabeled using anti-DYSF (green) and anti-FLOT2 (red) antibodies. Arrowheads denote co-labeling in crescent-shaped structures around clusters of nuclei; the asterisk indicates DYSF immunoreactivity in fused cells. Note that non-fused cells lack DYSF labeling. All scale bars = 20 μ m

Fig. 6.

Flotillins contribute to endocytosis in BeWo cells. (A) Flotillins co-localize with a fluorescent marker of fluid phase-pinocytosis in endocytosis assays. Mononuclear BeWo cells were incubated continuously in the presence of 1 mg/ml of lucifer yellow (LY-CH, green) for 15 min prior to fixation. Following permeabilization, the cells were immunolabeled with anti-FLOT2 antibodies (red) and imaged using confocal microscopy. In the representative micrograph taken from a single optical section, areas of co-localization between the LY-CH cargo and FLOT2 (yellow in the merged images, indicated by arrows) are observed. Similar results were obtained when the cells were labeled using antibodies generated against FLOT1 (not shown). Scale bar = 10 μ m. (B) Immunoblots of BeWo cell lines stably transduced with shRNA targeting FLOT1, FLOT2, or both. Control cells were transduced with Non-Target Control Transduction Particles (LV CTRL) as described in “Materials and methods”. (C) Flotillin knockdown decreases uptake of fluorescently-labeled cholera toxin B subunit (CTB-594). BeWo cells varying in flotillin expression levels were incubated with 20 μ g/ml CTB-594 at 4°C for 30 min, then warmed to 37°C and endocytosis was allowed to proceed in media without labeled ligand for 15 min. On average, cells deficient in flotillin expression exhibited decreased CTB-594 based on average fluorescence intensity measurements. Data are mean $\pm$ SD from two separate experiments. Different letters indicate significant differences at $P < 0.05$ (ANOVA with Bonferroni multiple comparisons)