

Published in final edited form as:

Am J Obstet Gynecol. 2011 August ; 205(2): 156.e1–156.14. doi:10.1016/j.ajog.2011.03.023.

Placental protein 13 (PP13/galectin-13) undergoes lipid raft-associated subcellular redistribution in the syncytiotrophoblast in preterm preeclampsia and HELLP syndrome

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Abstract

Objective—To investigate placental protein 13 (PP13) localization in relation to cytoskeleton and lipid rafts in preeclampsia and HELLP syndrome.

Study Design—Placental cryosections from patients with preeclampsia and HELLP, and controls were stained for PP13, actin, PLAP (lipid raft marker), and CD71 (nonraft marker). BeWo cells exposed to stress conditions were stained for PP13 and actin. Protein localization were investigated by confocal microscopy, PP13 concentrations by ELISA.

Results—PP13-actin colocalization was increased in syncytiotrophoblast juxtamembrane regions in term/preterm preeclampsia and HELLP. PP13-CD71 colocalization was decreased and PP13-PLAP proximity was increased in preterm but not term preeclampsia and HELLP. PP13-release from BeWo cells was inhibited by cytoskeleton disruption, and augmented by Ca²⁺-influx and ischemic stress.

Conclusion—The actin cytoskeleton, probably in connection with lipid rafts, controls trophoblastic “nonclassical” PP13 export. PP13 is released from the syncytiotrophoblast in preterm preeclampsia and HELLP, mimicked in BeWo cells by ischemic stress, suggesting PP13 is a placental alarmin.

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Author contributions: AB, JM, NGT designed the study; TV, MS, JR, HM, RR, ZP, NGT contributed with materials or clinical specimens; AB, JP, NGT performed research; AB, JP, JM, ZD, NGT analyzed and interpreted data; AB, JM, CJK, HM, RR, ZP, NGT wrote the paper.

Keywords

alarmin; brush border membrane; damage-associated molecular pattern (DAMP); danger signal; galectin; “nonclassical” secretory pathway

INTRODUCTION

Preeclampsia, one of the “great obstetric syndromes”,^{1,2} affects approximately 3–5% of pregnant women and is a major cause of maternal and perinatal morbidity and mortality. It is characterized by the new onset of hypertension and proteinuria after 20 weeks of gestation. In the most severe cases, seizures (eclampsia), multiorgan damage including cerebrovascular, hepatic or renal failure, and placental abruption and HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome may develop.^{3–5} HELLP syndrome, which affects 0.5–0.9% of all pregnancies and 2–12% of pregnancies complicated by preeclampsia, is thought to be more severe form of preeclampsia although approximately 15–20% of the patients do not develop hypertension and/or proteinuria.^{6–11}

Both preeclampsia and HELLP syndrome are heterogeneous. The involvement of the placenta in the different pathogenic pathways is well-established because the only definitive treatment of these syndromes is delivery of the fetus and placenta. Preterm preeclampsia and HELLP syndrome are associated with abnormal placentation, impaired trophoblast invasion, and remodeling of the uterine spiral arteries.^{12–20} Failure of physiologic transformation of the spiral arteries in these cases is thought to result in intermittent, turbulent flow, damage of the placental architecture, fluctuations in placental oxygenation, and placental endoplasmic reticulum-, oxidative-, and nitrate stress.^{21–24} Placental ischemia-reperfusion injury triggers an increased release of apoptotic-necrotic syncytiotrophoblast microparticles (MPs), proinflammatory cytokines, and antiangiogenic factors, which in turn, lead to generalized maternal endothelial cell dysfunction and an exaggerated maternal systemic inflammatory response.^{3–5,22–23,25–40}

In normal pregnancy, MPs continuously shed from the syncytiotrophoblast, the outermost fused cell layer of the human placenta, to the maternal circulation as part of the natural renewal process of placental microvilli. The amount of circulating MPs increases with advancing gestational age in normal pregnancies and is significantly elevated in the blood of women with preterm preeclampsia.^{34,41} Of note, we have previously shown that syncytiotrophoblast cytoplasm protrusions, membrane blebs, and MPs shed from the syncytiotrophoblast apical membrane have intense placental protein 13 (PP13) immunostaining in preterm preeclampsia and HELLP syndrome.⁴² This is consistent with the findings that maternal serum PP13 concentration increases during normal pregnancy with a peak in the third trimester,^{43–45} and it is higher in patients presenting with preterm preeclampsia and HELLP syndrome than in preterm controls. Surprisingly, we did not find such difference in placental PP13 staining and maternal serum PP13 concentrations in patients with term preeclampsia when compared with normal term control patients.⁴²

In the context of these observations, it is important to note that PP13 is specifically expressed by the placenta in anthropoid primates, primarily in the syncytiotrophoblast.^{42,46–50} PP13 is a member of the evolutionarily conserved family of galectins (galectin-13), which are key regulatory proteins of immune-homeostasis and inflammation.^{49–53} Galectins are synthesized in the cytosol and are alternatively transported to the plasma membrane, avoiding the endoplasmic reticulum (ER) and Golgi vesicles,⁵⁴ through a so-called “nonclassical” secretory pathway.^{55,56} On the cell surface and in the extracellular matrix, galectins can initiate leukocyte signaling and modify cell fate on

binding to their cell-surface ligands, leading to either apoptosis or cell survival. Galectins can also have various effects on cell growth, cytokine secretion, cell-cell and cell-extracellular matrix interactions.^{50,57}

A previous study showed that, similar to other galectins, PP13 accumulates in the cytoplasmic side of the plasma membrane, leading us to hypothesize that it is secreted to the syncytiotrophoblast surface by the actin filamental network.⁴⁹ It has also been demonstrated that PP13 specifically binds to and colocalizes with annexin-II at the apical membrane of the syncytiotrophoblast.⁴⁹ Of note, similar to placental alkaline phosphatase (PLAP), a protein highly expressed in the syncytiotrophoblast apical membrane, annexin-II is also associated with lipid rafts,⁵⁸ which are highly dynamic, cholesterol- and sphingolipid-rich 10–200 nm microdomains in the mammalian cell membrane. Lipid rafts can grow to 1 μm and provide a stable platform for lipid-lipid and protein-lipid interactions on various stimuli, such as receptor cross-linking during signaling or endocytosis.⁵⁹

Based on these findings, we hypothesized that: (1) the actin filamental network and lipid rafts in the syncytiotrophoblast apical membrane may play a role in the normal secretory and shedding mechanisms of PP13, and (2) the pathophysiologic processes affecting the syncytiotrophoblast in various phenotypes of preeclampsia and HELLP syndrome may lead to the increased secretion and/or shedding of PP13 from the villous tree. Therefore, this study was designed to investigate whether changes in the placental localization of PP13 and its spatial relationship to lipid rafts and the actin cytoskeleton could be detected in the placentas obtained from women with preeclampsia or HELLP syndrome and gestational age-matched controls using confocal imaging. To get insight into the possible cellular mechanisms of PP13-release from the trophoblast, the role of the actin cytoskeleton and different stress factors on PP13-release was studied in vitro in BeWo cells.

MATERIALS AND METHODS

Sample collection and patient groups

The study was approved by the Health Science Board of Hungary (ad.22—164/2007-1018EKU) and the Human Investigation Committee of Wayne State University (036410M1X). Written informed consent was obtained from women before sample collection; specimens were coded and data were stored anonymously. Placental villous tissues were collected at the First Department of Obstetrics and Gynecology, Semmelweis University (Budapest, Hungary, Federalwide Assurance: FWA00002527) in the following gestational age-matched groups (n = 5 in each): (1) preterm preeclampsia (< 35 weeks), (2) preterm HELLP syndrome (< 35 weeks), (3) term preeclampsia (> 37 weeks), (4) preterm (< 35 weeks), and (5) term (> 37 weeks) controls. Patients with multiple pregnancies or with fetuses having congenital or chromosomal abnormalities were excluded. Demographic and clinical characteristics of patient groups are shown in the Table.

Term and preterm controls had no medical complications or clinical or histologic signs of chorioamnionitis, and delivered neonates of appropriate weight for gestational age.⁶⁰ Preeclampsia was defined as hypertension that developed after 20 weeks of gestation (systolic or diastolic blood pressure ≥ 140 or ≥ 90 mm Hg, respectively, measured at 2 different time points, 4 points, 4 points to 1 week apart) coupled with proteinuria (≥ 300 mg in a 24-hour urine collection, or 2 random urine specimens obtained 4 hours to 1 week apart containing $\geq 1+$ by dipstick or 1 dipstick of $\geq 2+$ protein).^{5,61} HELLP syndrome was defined as hemolysis (serum LDH >600 IU/L; bilirubin >1.2 mg/dL; presence of schistocytes in peripheral blood), elevated liver enzymes (serum ALT and/or AST >70 IU/L), and thrombocytopenia (platelet count $<100,000/\text{mm}^3$).¹⁰

None of the patients had regular uterine contractions; cesarean delivery was performed in all cases because of previous cesarean section (term controls), or preterm premature rupture of the membranes and breech presentation (preterm controls), or severe clinical symptoms (patients with preeclampsia or HELLP syndrome).

Fluorescence immunohistochemistry

Fresh placental specimens were frozen in in isopentane (Sigma-Aldrich, St. Louis, MO); subsequently, 10 μm thick cryosections (2–4 cryosections/sample) were cut and placed on Superfrost Plus slides (Thermo Scientific, Waltham, MA), fixed with 4% paraformaldehyde and then kept at 4°C in phosphate-buffered saline (PBS) until staining. Nonspecific antibody was blocked with 5% bovine serum albumin (BSA) in PBS (30 minutes at room temperature). Samples were incubated with monoclonal anti-PP13 antibody (clone: 27-2-3; 5 $\mu\text{m}/\text{mL}$; Diagnostic Technologies Ltd, Yokneam, Israel) alone or in combination with anti-PLAP (1 $\mu\text{m}/\text{mL}$; Sigma-Aldrich), anti-CD71 (5 $\mu\text{m}/\text{mL}$; Biolegend, San Diego, CA), or antiactin (8 $\mu\text{m}/\text{mL}$; Sigma-Aldrich) antibodies in PBS containing 1% BSA. Samples were stained with anti-PP13 overnight, whereas in the case of other primary antibodies, the incubation time was 45 minutes. Washing was followed by incubation (45 minutes at room temperature) with Alexa Fluor488-conjugated antimouse IgG1 (2 $\mu\text{m}/\text{mL}$; Invitrogen-Molecular Probes, Eugene, OR) for anti-PP13, Alexa Fluor633-conjugated antimouse IgG2a (2 $\mu\text{m}/\text{mL}$; Invitrogen-Molecular Probes) for anti-PLAP and anti-CD71, or Alexa Fluor647-conjugated antirabbit IgG (2 $\mu\text{m}/\text{mL}$; Invitrogen-Molecular Probes) for antiactin. Isotype control antibodies were used as negative controls; mouse IgG1 (clone MOPC-21; BD Pharmingen, San Jose, CA) and mouse IgG2a (clone HOPC-1; Southern Biotech, Birmingham, AL). Nuclei were counterstained with propidium iodide (0.2 $\mu\text{m}/\text{mL}$; Sigma-Aldrich). Washed samples were assayed with an Olympus Fluoview 500 confocal microscope (Hamburg, Germany) equipped with 3 lasers and 4 optical channels, using a 60x (N.A.: 1.1) oil-immersion objective.

BeWo cell transfection

BeWo cells (American Type Culture Collection, Manassas, VA) were incubated in 2 T-75 flasks with F12 medium (Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) until reaching 80% confluency. PP13 vector DNA (9 μm ; OriGene, Rockville, MD) was diluted in 450 μL of serum-free Opti-MEM I medium (Invitrogen-Gibco). Then, 36 μL FuGENE HD transfection reagent (Promega, Madison, WI) was added to the mix, which was incubated for 15 minutes at room temperature. Ten milliliters of fresh Opti-MEM I medium were added to 1 of the T-75 flasks after the cells were washed once with PBS. The transfection complex was then added to this flask and incubated for 6 hours before replacing the medium to F12 (supplemented with 10% FBS and 1% P/S). BeWo cells without PP13 vector transfection were used as control.

BeWo cell treatments

After 24 hours in culture, transfected and nontransfected BeWo cells were trypsinized and plated onto 6-well plates (2×10^5 cells/well) and 4-well Lab-Tek chamber slides (5×10^4 cells/well; Thermo Scientific), and were cultured for 24 hours in F12 medium supplemented with 10% FBS and 1% P/S. After medium change, 60-hour treatments started in the following regimes: (1) to inhibit actin polymerization, cells were treated with Latrunculin B (0.125 and 0.25 μM ; EMD Chemicals, Gibbstown, NJ) between hours 48–60; (2) to stimulate nonclassical protein secretion by elevated cytoplasmic Ca^{2+} -level, cells were treated with ionophore A23187 (1 μM ; Sigma-Aldrich) between hours 48–60; and (3) to induce cellular stress, cells were treated with either human recombinant TNF α (4ng/mL; R&D Systems, Minneapolis, MN) between hours 0–60, or kept under hypoxic (1% O_2) or

ischemic conditions (1% and 20% O₂ in alternating, 12 hour cycles) between hours 0–60. Control cells were treated with vehicle (DMSO; Sigma-Aldrich).

RNA isolation and quantitative real-time reverse transcribe-polymerase chain reaction

After collecting supernatants, BeWo cells were harvested from 6-well plates and total RNA was isolated using Quick-Gene-Mini80 (Fujifilm, Allendale, NJ) according to the manufacturer's protocol. Five hundred nanograms of total RNA was reverse transcribed with TaqMan Revers Transcription Reagent kit using random hexamers (Applied Biosystems, Foster City, CA). Quantitative reverse transcribe-polymerase chain reaction (qRT-PCR) was run in triplicates with *LGALS13* TaqMan assay (Hs00848811_m1) and *RPLPO* TaqMan Endogenous Control (4326314E) on a 7500 Fast Real-Time PCR System (Applied Biosystems) Ct values for *LGALS13* and *RPLPO* were averaged over 3 technical replicates. $-DCt$ value, a surrogate of log₂ mRNA concentration, was obtained for each sample as: $-DCt_{(LGALS13)} = Ct_{(RPLPO)} - Ct_{(LGALS13)}$.

Protein isolation

After collecting supernatants, BeWo cells were harvested from 6-well plates, homogenized with 120 μ L lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP40; 5 mM EDTA) and incubated for 1 hour on ice. Homogenates were centrifuged (15 minutes at 10,000 rpm) to pellet nonsoluble or solubilized proteins were collected, and their total protein contents were measured by BCA assay (Thermo Scientific).

PP13 immunoassay

PP13 content of BeWo cell lysates and supernatants was measured using a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) (Diagnostic Technologies Ltd) with a pair of PP13-specific monoclonal antibodies, marked with amplified biotin-extravidin-horseradish-peroxidase complex, and developed with tetramethylbenzidine substrate, as described previously.⁴³ The optical density was measured at 450 nm against a 650 nm background. PP13 concentrations were determined by extrapolation from a standard curve constructed using recombinant PP13 standards (0–500 pg/mL), and were normalized to total protein in cell lysates. The sensitivity of the assay was 9.6 pg/mL. The laboratory staff performing the assays was blinded to sample information.

Fluorescence immunocytochemistry

PP13-transfected, treated or untreated BeWo cells on chamber slides were simultaneously fixed and permeabilized in PBS supplemented with 4% paraformaldehyde and 0.1% Trimeton X-100 (Sigma-Aldrich), followed by washing with PBS supplemented with 1% FBS and 0.1% sodium-azide. Nonspecific antibody binding was blocked with 5% BSA in PBS (30 minutes at room temperature). Samples were incubated overnight with monoclonal anti-PP13 antibody (clone:27-2-3; 20 μ g/mL) alone or in combination with antiactin (7 μ g/mL) antibody in PBS containing 5% BSA. Washing was followed by incubation (2 hours at room temperature) with Alexa Fluor488-conjugated antimouse IgG1 (2 μ g/mL) for anti-PP13 and Alexa Fluor555-conjugated antirabbit IgG (2 μ g/mL) for antiactin. Isotype control antibodies were used as negative controls. Alternatively, cells were stained with Alexa Fluor555-conjugated phalloidin (2 U/mL; Invitrogen-Molecular probes) to visualize filamentous actin. Nuclei were counterstained with DRAQ5 (1 μ M; Biostatus, Leicestershire, UK). Washed samples were assayed with an Olympus Fluoview 500 confocal microscope equipped with 4 lasers and 4 optical channels, using 20X (N.A.: 0.7) of 40X (N.A.: 0.85) objectives.

Data analysis

Colocalization indices were determined from approximately 100 regions of interest (ROIs) each group by the ImageJ software (<http://rsbweb.nih.gov/ij>. Accessed May 2, 2011.) using the Red Green Correlator plug-in (http://www.uhnresearch.ca/facilities/wcif/imagej/colour_analysis.htm. Accessed May 2, 2011.). The Pearson's colocalization index (CI) provides a reliable estimate on the extent of protein colocalization. A CI value of -1 means the exclusive appearance of the 2 labeled specimens, its value close to zero indicates a random appearance, whereas CI values between 0 and +1 reflect a continuously increasing degree of colocalization, while CI = 1 corresponds to a full overlap between the 2 colors in each pixels of the image.^{62,63}

Line scan analysis was carried out using the Fluoview software. Lines (approximately 200/group) were laid across placental microvilli stained for PP13 and actin or PLAP. Intensity values were sorted into membrane/juxtamembrane (3 μ m at opposite sides of the villi) and cytoplasmic (internal) regions. Based on the staining pattern, 3 groups were formed for quantifying these intensity distributions: (1) PP13 and actin (or PLAP) were localized in the same region; (2) only PP13 was localized in the selected region; and (3) only actin (or PLAP) was localized in the selected region (based on the actual intensity exceeding the threshold level). The relative frequency of each staining pattern was then calculated from approximately 300 ROIs. Intensity distributions were also demonstrated on line scan histograms. The interactive 3-dimensional (3D) surface plot plug-in of ImageJ was also used to create another representation platform of the data on the different samples.

Statistical analysis

Demographic and clinical data were analyzed by Statistica8 (StatSoft Inc, Tulsa, OK). Comparisons among the groups were performed by χ^2 test and Fisher's exact test for proportions and Kruskal-Wallis test, followed by Mann-Whitney test for continuous variables. GraphPad Prism 4 (GraphPad Software, La Jolla, CA) was used to analyze the differences in the colocalization, gene expression, and protein concentration values between the groups using Mann-Whitney test or *t* test. A *P* value of < .05 was considered statistically significant.

RESULTS

Localization of PP13 in the syncytiotrophoblast in preeclampsia and HELLP syndrome

The localization of PP13 in placental villous tissues from patients with preeclampsia or HELLP syndrome and gestational age-matched controls was determined with confocal microscopy using immunohistochemical staining. Similar to earlier data,^{42,49,50} PP13 was present in the endothelium of fetal vessels, and it was found in the membrane and the cytoplasm of the syncytiotrophoblast (Figure 1). There was a shift in the localization of PP13; stronger staining was observed in the plasma membrane rather than the cytoplasm in patients with term and preterm preeclampsia and HELLP syndrome in comparison to gestational age-matched controls (Figure 1). This finding is consistent with our previous report.⁴²

Visualization of the interaction patterns of PP13 with cortical actin cytoskeleton and the raft marker PLAP or the nonraft protein CD71 in placental cryosections

Next, we analyzed the interaction patterns of PP13 with the cortical actin cytoskeleton and with 2 membrane proteins, PLAP and CD71. A relatively high colocalization was found between PP13 and actin in term and preterm controls (CIs; mean \pm standard error of the mean [SEM]: 0.357 ± 0.009 and 0.357 ± 0.008 , respectively). The colocalization of these 2 proteins (PP13 and actin) was significantly increased in all patient groups (CIs, 0.459 ± 0.008 , *P* < .05 for

term preeclampsia; 0.509 ± 0.009 , $P < .05$; and 0.520 ± 0.010 , $P < .05$ for preterm preeclampsia and HELLP syndrome, respectively) when compared with gestational age-matched controls (Figures 2, A and 3, A). The membrane localization of PP13 was also altered in preeclampsia and HELLP syndrome. Although PP13 highly colocalized with the lipid raft-associated membrane protein PLAP in both control groups (CIs, 0.468 ± 0.007 , 0.481 ± 0.012), no significant changes in the colocalization of PP13 and PLAP could be seen in term and preterm preeclampsia or HELLP syndrome (CIs, 0.468 ± 0.009 , 0.440 ± 0.009 , and 0.485 ± 0.010 , respectively) (Figures 2, B and 3, B). In contrast, PP13 highly colocalized with CD71 in term and preterm controls (CIs, 0.505 ± 0.009 and 0.466 ± 0.009 , respectively) as well as in term preeclampsia (CI, 0.490 ± 0.010), but this colocalization was significantly reduced in preterm preeclampsia (CI, 0.327 ± 0.009 , $P < .05$) and HELLP syndrome (CI, 0.350 ± 0.013 , $P < .05$) (Figures 2, C and 3, C).

Spatial coexistence of cortical filamental actin and PP13: a statistical analysis of line scanned intensity distributions

Because one of the major questions of the current study was whether any redistribution of PP13 could be observed toward the cortical actin network, the relative frequency of the spatial coexistence of PP13 and actin (or the plasma membrane PLAP) in the membrane/juxtamembrane region and in the cytoplasm was also determined. This analysis was performed by laying a line across the apical microvillous membranes of the syncytiotrophoblast on the opposing sides of the villi. The intensity distribution profile of PP13 and actin was then analyzed along this line (Figure 4, A). As shown in Figure 4, B, in all pathologic samples the coexistence of PP13 and actin showed a dramatically increased membrane/juxtamembrane: cytoplasmic distribution ratio. Such a relationship between distribution profiles of actin or PP13 alone in either of these disease groups could not be observed (Figure 4, C and D).

Although no significant difference was detectable in PP13 and PLAP colocalization, analyzed in the plasma membrane region, the altered colocalization indices between PP13 and CD71 in preterm preeclampsia and HELLP syndrome reflect a shift in the membrane localization of PP13. Therefore, we also analyzed the spatial proximity between PP13 and PLAP by line scan and 3D surface plot analysis of the images. As the representative histograms and images show (Figure 5), PP13 is relocalized near lipid rafts in the juxtamembrane regions. Collectively, the data indicate redistribution of the PP13 in the syncytiotrophoblast of patients with preterm but not in term preeclampsia and HELLP syndrome, in comparison to the respective gestational age-matched control samples.

Disruption of cytoskeletal actin inhibits PP13-release from BeWo cells

Our confocal data suggested that cortical actin network may have a role in PP13 export from the trophoblast. To investigate this phenomenon in vitro, we treated BeWo trophoblast-like cells with Latrunculin B, a toxin that disrupts actin network by inhibiting actin polymerization. The treatment with Latrunculin B resulted in the disruption of actin filaments in BeWo cells visualized by phalloidin staining of F-actin (Figure 6, A). In parallel, intracellular accumulation and increased colocalization with actin were detected for PP13 in the juxtamembrane region of Latrunculin B-treated cells, in a concentration-dependent manner (Figure 6,B). Consistently, PP13 content of the cell culture supernatants gradually decreased with increasing Latrunculin B concentrations (mean \pm SEM in pg/mL; untreated: 137.3 ± 13.5 ; $0.125 \mu\text{M}$: 106.6 ± 9.5 , $P < .05$ compared with untreated). There was no detectable PP13-release from nontransfected cells (Figure 6,C).

PP13-release from BeWo cells is facilitated by Ca²⁺ influx or ischemic stress

Next, we attempted to explore the possible mechanisms promoting PP13-release from the trophoblast under normal and stress conditions. Calcium ionophore treatment led to decreased PP13 immunostaining compared with untreated cells (Figure 7, A and B), indicating that BeWo cells released their PP13 contents when cytoplasmic Ca²⁺ levels were elevated. From the applied stress conditions, TNF α and hypoxic stress did not reduce PP13 immunostaining; however, ischemic stress did markedly decrease it (Figure 7, C–E).

To quantify PP13-release from BeWo cells under calcium ionophore treatment and ischemic stress, we determined PP13 contents of BeWo cells and their culture supernatants by ELISA. PP13 mRNA expression was negligible in nontransfected cells, but in transfected cells it reached the level previously detected in normal placentas at term⁵⁰ (Figure 8, A). PP13 content (in pg, normalized to mg total protein; mean \pm SEM) was 38.8 ± 2.6 pm/mg protein in nontransfected cells (Figure 8, C), whereas it was $851. \pm 66.8$ pg/mg protein in PP13-transfected, untreated cells. In transfected cells, ischemic stress or calcium ionophore treatment decreased PP13 content (215.5 ± 23.1 pg/mg protein, $P < .001$ or 134.1 ± 7.0 pg/mg protein, $P < .001$ respectively). In accord, PP13 concentration (mean \pm SEM) of the supernatants of nontransfected cells was under the detection limit, whereas it was 130.9 ± 10.7 pg/mL in PP13-transfected, untreated cells. In transfected cells, ischemic stress or calcium ionophore treatment increased PP13 concentrations in supernatants (160.0 ± 13.4 , $P = .06$ or 185.9 ± 6.1 , $P < .001$, respectively) (Figure 8, B).

COMMENT

Principal findings of the study: (1) PP13 was localized in the cytoplasm and in the brush border membrane of the syncytiotrophoblast in normal placentas (term and preterm); (2) similarly, PP13 was colocalized with actin in the cortical cytoskeleton of the syncytiotrophoblast, as well as with placental alkaline phosphatase in the lipid raft regions of the syncytiotrophoblast and with CD71 in the nonlipid raft regions of the brush border membranes of this structure; (3) in term and preterm preeclampsia and HELLP syndrome, there was a stronger PP13 staining in the apical plasma membrane rather than in the cytoplasm, indicating a shift in the localization of PP13 in the syncytiotrophoblast; (4) similarly, a higher degree of colocalization of PP13 and actin was found in the apical juxtamembrane area of the syncytiotrophoblast in term and preterm preeclampsia and HELLP syndrome; (5) in contrast, a lower colocalization of PP13 and CD71 in nonlipid raft membrane regions was found in the syncytiotrophoblast; in addition, accumulation of PP13 in the vicinity of PLAP containing lipid rafts was noted in preterm but not in term preeclampsia and HELLP syndrome; (6) the inhibition of actin polymerization in trophoblast-like BeWo cells led to the intracellular accumulation of PP13 along with its increased colocalization with actin in the juxtamembrane region, and to a simultaneous decrease in PP13-release to culture supernatants; (7) Ca²⁺ influx mediated by calcium ionophore treatment increased “nonclassical” PP13-release from BeWo cells; and (8) ischemic stress resulted in increased PP13-release from BeWo cells. These data support the concept that the different pattern of localization of PP13 in the syncytiotrophoblast is related to distinct placental pathology in preterm and term preeclampsia, and that the cortical actin cytoskeleton, through lipid raft domains, has an active role in the increased secretion and/or shedding of PP13 on ischemic stress in preterm preeclampsia and HELLP syndrome.

Meaning of the study

Preeclampsia and HELLP syndrome are life-threatening pregnancy complications,^{1–11} previously attributed to defective hemochorial placentation^{12–18,20,22} placental stress and antiangiogenic state,^{21–24,29,30,32,33,35} generalized maternal endothelial dysfunction,^{3–5,38,39}

and an excessive maternal systemic inflammatory response.^{4,25,26,64} Emerging evidence shows that the excessive placental secretion of antiangiogenic^{29,30,32,33,35} and proinflammatory molecules,^{27,28,37} as well as the augmented shedding of microparticles and cellular debris from the syncytiotrophoblast surface could play a key role in triggering the exaggerated maternal systemic inflammatory response in preeclampsia and HELLP syndrome, especially in early-onset disease.^{31,34,37,39–41} It is noteworthy that patients with preterm (but not term) preeclampsia and HELLP syndrome have a higher concentration of PP13, a placenta-specific innate immune lectin, in the maternal circulation than women with normal pregnancy at the onset of disease.⁴² We have provided evidence that this change can partly be attributed to an excessive shedding of PP13 from the syncytiotrophoblast into the maternal circulation; however the mechanism responsible for this phenomenon remains to be determined.⁴²

Therefore, this study was designed to investigate the changes in the localization of PP13 in the syncytiotrophoblast, focusing on its spatial relationship to the actin cytoskeleton and lipid rafts in the apical brush border membrane. We also aimed to reveal the potential mechanisms mediating PP13-release from the trophoblast in normal conditions and in preeclampsia. Because of (1) placental villous explants may disintegrate and their PP13 production is decreasing after 24–48 hours^{65,66}; (2) primary trophoblasts can only be obtained from a single placenta in less quantity⁶⁷ than needed for our parallel experiments; and (3) PP13 mRNA/protein expression is very low in trophoblastic cell lines,⁶⁸ we used PP13 DNA-transfected BeWo cells for our in vitro studies. In these cells, PP13 mRNA expression was up-regulated by approximately 45,000-fold, much higher than in BeWo cells induced to fuse and differentiate (approximately 7-fold).⁶⁸ reached the physiologic level of PP13 mRNA expression detected in term placentas.⁵⁰

Actin network and the syncytiotrophoblast secretion/shedding of PP13

The findings reported herein about the cytoplasmic and brush border membrane localization of PP13 in the syncytiotrophoblast in normal term placentas are consistent with those reported with confocal microscopy and immunohistochemistry.^{42,45,49} The colocalization of PP13 and actin in the cortical cytoskeleton of the syncytiotrophoblast is consistent with data we have previously reported, demonstrating the specific binding of PP13 to β/γ -actin in placental tissue lysates.⁴⁹ Importantly, PP13 also binds to annexin-II,⁴⁹ which is a lipid raft-associated protein that interacts with the submembraneous actin network functions as an interface between lipid rafts and the actin cytoskeleton,^{69,70} and is involved in membrane trafficking events.⁷¹ As the colocalization of PP13 with annexin II on the apical membrane of the syncytiotrophoblast has also been reported,^{49,72} our past and present results suggest that actin, in conjunction with annexin-II, has a potentially important role in the membrane trafficking of PP13 on the apical surface of the syncytiotrophoblast. This concept is strongly supported by our in vitro data showing that the disruption of the actin filamental network leads to the intracellular accumulation and decreased release of PP13 from transfected BeWo cells.

Consistent with our previous observations with immunohistochemistry,⁴² we found a stronger apical membrane staining and a weaker cytoplasmic staining for PP13 in the syncytiotrophoblast in term and preterm preeclampsia and HELLP syndrome. This shift in the localization of PP13 in the syncytiotrophoblast in these cases was also observed as a higher colocalization of PP13 with the cortical actin cytoskeleton. As PP13 is released from the syncytiotrophoblast into the maternal circulation in significantly larger amounts in preterm but not in term preeclampsia and HELLP syndrome when compared with gestational age-matched controls,⁴² the shift in the cellular localization of PP13 itself is not sufficient to explain the increased apical membrane trafficking of this galectin in preterm cases. Therefore, we asked whether the altered membrane trafficking of PP13 in preterm

preeclampsia and HELLP syndrome is related to lipid raft domains, which exist in epithelial brush borders⁷⁰ and control and apical trafficking of some galectins and their glycoconjugates.⁷³

The relationship of PP13 and lipid rafts in the syncytiotrophoblast apical membrane

Lipid rafts are highly dynamic, cholesterol-, and sphingolipid-rich micro-domains, which function in apical trafficking through interacting with the actin cytoskeleton.^{74,75} Of interest, galectin-4, an intestine-specific member of the galectin family, is a stabilizer of lipid rafts in the intestinal microvillar membranes through its interactions with lipid-raft associated proteins, such as alkaline phosphatase.^{76,77} The apical membrane of the syncytiotrophoblast is also rich in lipid rafts and raft-associated molecules, such as annexin-II, gangliosides, and the GPI-anchored PLAP.⁵⁸ In addition to our previous data on the colocalization of PP13 with annexin II,⁴⁹ in this study, we report a high colocalization of PP13 with PLAP and CD71 in the syncytiotrophoblast in control placentas, suggesting an equal distribution of PP13 in lipid raft and nonlipid raft regions of the syncytiotrophoblast brush border membrane in these cases.

Consistent with previous reports on the differences in placental pathology and global gene expression^{15,17,78} as well as in placental *LGALS13* expression and PP13 immunostaining in preterm versus term preeclampsia,^{42,79} we observed a lower colocalization of PP13 with CD71 and the accumulation of PP13 in the vicinity of PLAP containing lipid rafts in preterm but not in term preeclampsia and HELLP syndrome. These findings are also consistent with reports showing similar changes in placental pathology and global transcriptome in preterm preeclampsia and HELLP syndrome.^{16,42,80} These data suggest that the apical, nonlipid raft localization of PP13 in the syncytiotrophoblast brush border is reduced, and the association of PP13 with the juxtamembrane cortical actin network and lipid raft domains is enhanced in preterm preeclampsia and HELLP syndrome by yet unknown mechanism, possibly facilitating the secretion and/or shedding of PP13 through lipid rafts and leading to the elevated PP13 concentrations in the maternal circulation.

Preeclampsia, galectins and “danger signals”

Because PP13 lacks a signal peptide required for the classical protein secretion pathway, it was hypothesized^{42,49} that PP13, similar to other galectins,^{55,56,81,82} is transported to the syncytiotrophoblast apical membrane and into the extracellular milieu through “nonclassical” secretory mechanism (e.g. via exovesicles or by the extrusion of membrane blebs), which circumvents the endoplasmic reticulum and the Golgi apparatus. Calcium ionophores induce the formation and membrane shedding of microvesicles, which contain actin, annexin II, and galectins, enhancing their “nonclassical” membrane transport.⁵⁴ In this study, we tested the ionophore-effect on PP13-release from BeWo cells. Indeed, exposing BeWo cells to calcium ionophore treatment in vitro resulted in a marked release of PP13, suggesting microvesicle-mediated secretion of PP13 from the juxtamembrane region, where these vesicles are formed.⁵⁴ These results are consistent with observations on the first trimester villous explant cultures in which calcium treatment increased PP13-release.⁶⁶ Furthermore, our observations on clinical specimens suggest that PP13 uses carbohydrate structure in lipid raft domains similar to other galectins,^{73,83–85} rather than CD71-marked compartments, for this apical membrane trafficking.

Our earlier⁴² and current clinical data indicate that there is an increased membrane trafficking of PP13 in preterm preeclampsia and HELLP syndrome, leading to increased PP13 concentrations in maternal circulation at the onset of clinical symptoms. This phenomenon could be mimicked in BeWo cells by ischemic stress, which is in accord with increasing evidence showing that not hypoxia but oxidative stress results in an increased

trophoblastic release of antiangiogenic factors, proinflammatory debris and microparticles, triggering the exaggerated activation of the maternal immune system in preterm preeclampsia.^{3–5,21–39}

Of importance, galectins can be increasingly secreted from inflamed tissues or after cellular stress and/or damage, and they have been implicated to act as “cell-stress sensors,”^{86,87} “danger signals,” or “alarmins.”^{88–91} “Alarmins” are endogenous danger signals secreted via “nonclassical” pathways by activated cells or passively released by dying cells undergoing necrosis. “Alarmins” signal tissue/cell damage to the immune system, eliciting effector responses from innate and adaptive immune cells, and contributing to the activation and resolution of immune responses.^{88–93} Indeed, because of their cytokine-like properties, galectins are powerful regulators of both arms of the immune system.^{57,82}

“Danger signals” at the maternal-fetal interface have recently been proposed to create an abnormal placental cytokine milieu and link the activation of the innate immune system in preeclampsia, where there is considerable stress and damage to the interstitial trophoblast and syncytiotrophoblast.^{23,37,94–98} Because PP13 has proinflammatory properties on macrophages⁹⁹ and can induce T cell death in vitro,⁵⁰ we propose that PP13 may function as an endogenous danger signal or alarmin of the syncytiotrophoblast, and its increased release from the aponecrotic trophoblast upon ischemic stress may contribute to the exaggerated activation of the maternal immune system in preterm preeclampsia and HELLP syndrome.

Acknowledgments

We are grateful to Dr. Sándor Paku, Dr. Tibor Füle, and Nicolett Klucsik for their help with placental cryosections preparations (Semmelweis University), to Rona Wang, Lorri McLuckie, Hong Meng, and Sivasakthy Sivalogan for technical assistance with ELISA and cell transfection, to Adi Tarca for statistical advices, and to Sara Tipton for critical reading of the manuscript (Wayne State University).

This research was funded in part by grants from the European Union FP6 (Pregenesys/037244 to HM and NGT), Hungarian National Science Fund (OTKA CK 80935 to JM and AB) and by TÁMOP 4.2.1/B-09/1/KMR-2010-0003 grant supported partly by EU Social Fund (to JM and AB).

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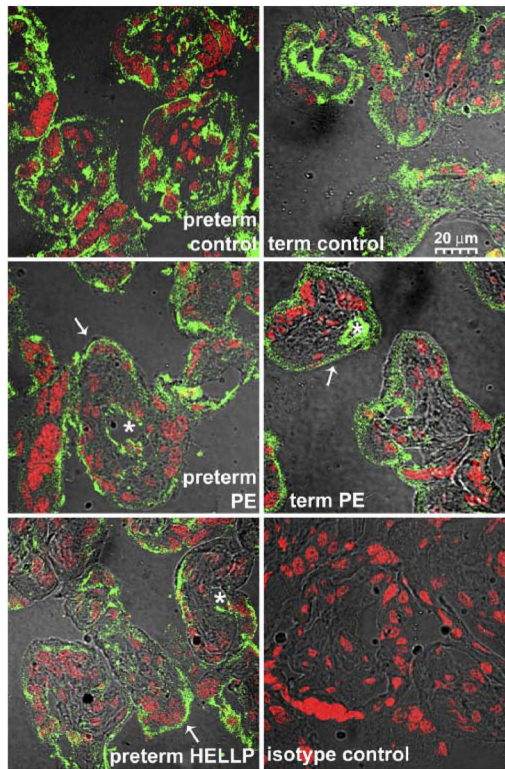


Figure 1. The syncytiotrophoblastic localization of PP13 is altered in patients with preeclampsia and HELLP syndrome

Representative confocal fluorescence-DIC composite images of cryosections of placentas from women with preterm preeclampsia, preterm HELLP syndrome, term preeclampsia, and gestational age-matched controls. Cryosections were stained with monoclonal anti-PP13 antibody (*green*) and PI for nuclei (*red*). There was a weaker cytoplasmic staining of the syncytiotrophoblast in term and preterm preeclampsia and HELLP syndrome when compared with gestational age-matched controls. *Stars* indicate PP13 staining of the endothelium of fetal vessels, *arrows* indicate the strong apical membrane staining of terminal villi in disease cases.

DIC, differential interface contrast; *HELLP*, hemolysis elevated enzymes and low platelets; *PI*, propidium iodide; *PP13*, placental protein 13.

Balogh. Subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

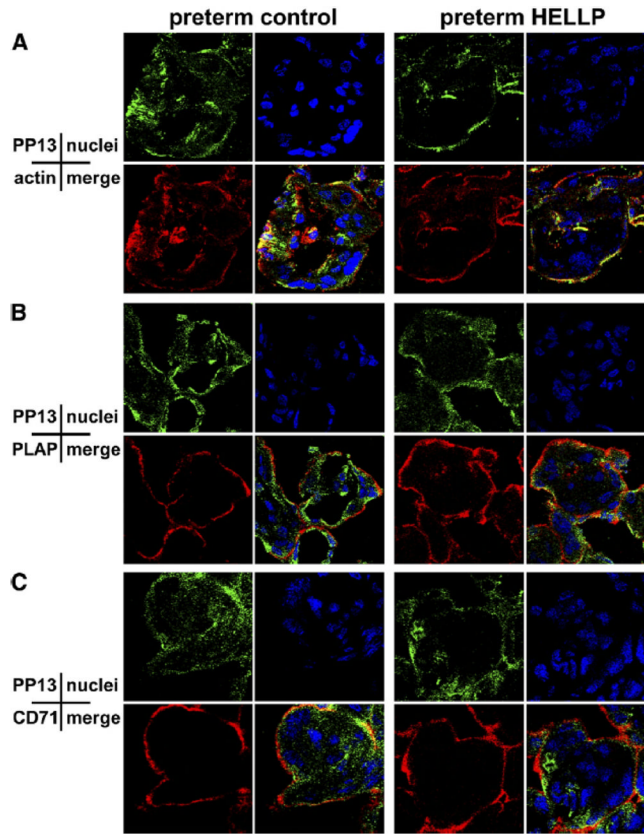


Figure 2. Colocalization of PP13 with actin, CD71 or PLAP in preeclampsia and HELLP syndrome compared to controls

Cryosections of placentas taken from women with HELLP syndrome and controls were stained with **A–C**, anti-PP13 (*green*) in combination with **A**, anti-actin, **B**, anti-PLAP, or **C**, anti-CD71 (all in *red*), and analyzed by confocal microscopy. Cell nuclei were counterstained with PI (*blue*) on all representative confocal images.

HELLP, hemolysis elevated liver enzymes and low platelets; *PI*, propidium iodide; *PLAP*, placental alkaline phosphatase; *PP13*, placental protein 13.

Balogh, subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

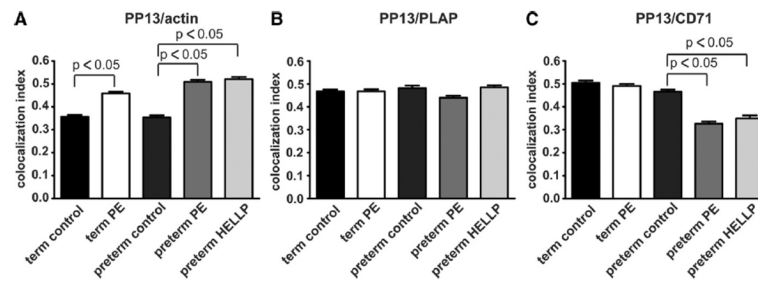


Figure 3. PP13 is redistributed in the placenta of patients with preeclampsia and HELLP syndrome

Cryosections of placentas taken from women with preterm preeclampsia or HELLP syndrome, term preeclampsia, and gestational age-matched controls were stained with anti-PP13 in combination with antiactin, anti-PLAP or anti-CD71, and analyzed by confocal microscopy. Colocalization indices for PP13 (derived from approximately 100 ROIs/group) are shown as mean \pm SEM with **A**, actin, **B**, PLAP, or **C**, CD71.

HELLP, hemolysis elevated liver enzymes and low platelets; *PLAP*, placental alkaline phosphatase; *PP13*, placental protein 13; *ROI*, regions of interest.

Balogh. Subcellular redistribution of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

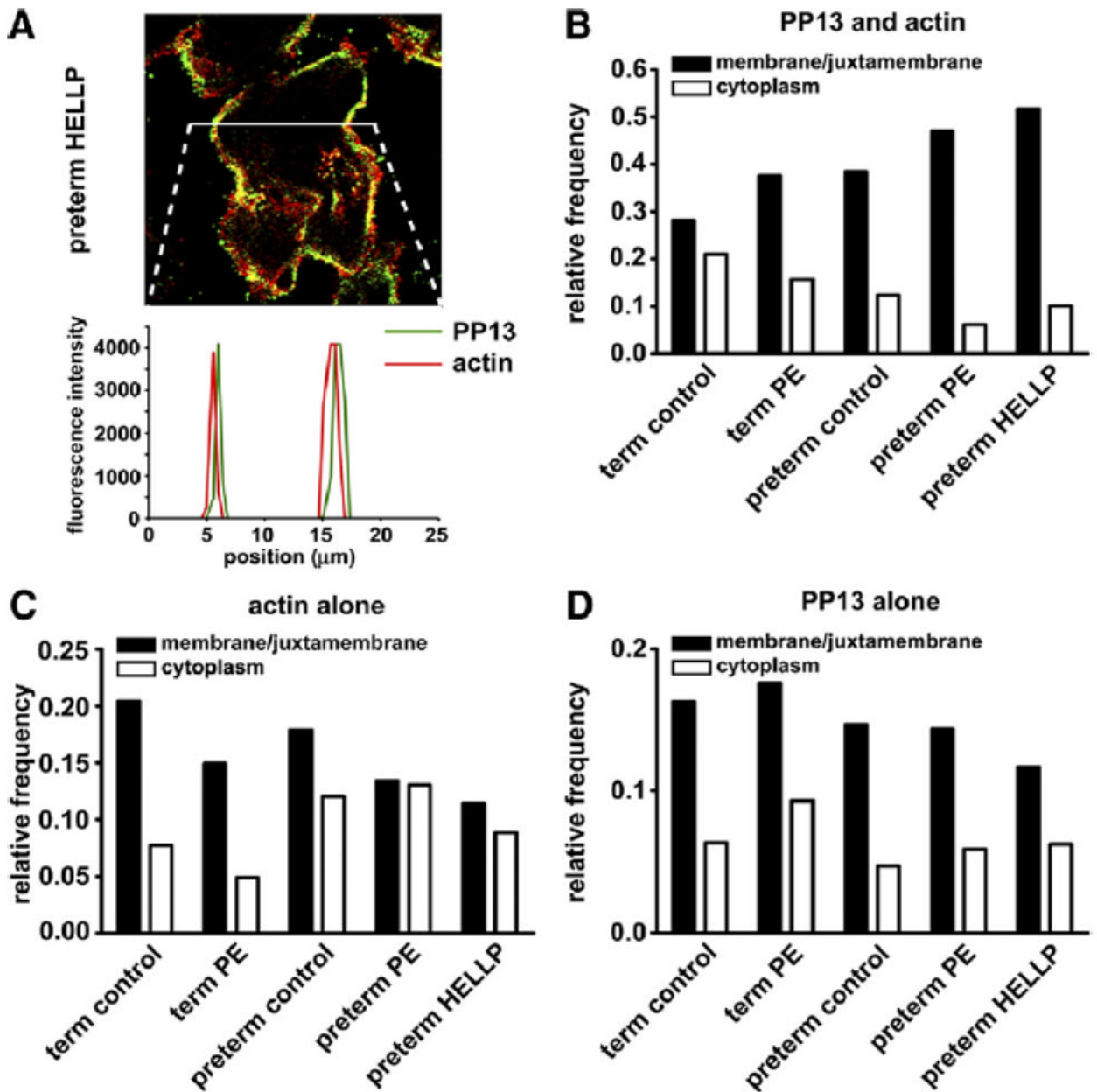


Figure 4. Coexistence of PP13 and actin in the juxtamembrane area of the syncytiotrophoblast is increased in preeclampsia and HELLP syndrome

Cryosections of placenta taken from women with preeclampsia or HELLP syndrome and gestational age-matched controls were stained for PP13 and actin. **A**, Representative image and line scan intensity distribution are shown. Relative frequency of **B**, PP13-actin coexistence and of **C**, actin, or **D**, PP13 existence alone in membrane/juxtamembrane and in cytoplasmic regions, respectively, was calculated from approximately 200 ROIs in each group.

HELLP, hemolysis elevated liver enzymes and low platelets; *PP13*, placental protein 13; *ROI*, regions of interest.

Balogh. Subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

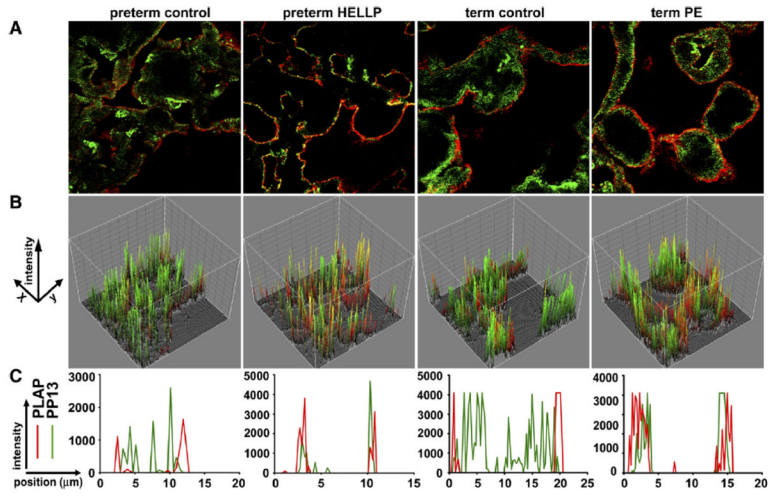


Figure 5. PP13 accumulates in the vicinity of PLAP-containing lipid rafts in preeclampsia and HELLP syndrome

Samples stained for PP13 (*green*) and PLAP (*red*) were analyzed with confocal microscopy. Representative **A**, confocal images, **B**, 3D surface plots and, **C**, line scan intensity distributions of samples derived from preterm or term patients and controls.

HELLP, hemolysis elevated liver enzymes and low platelets; *PLAP*, placental alkaline phosphatase; *PP13*, placental protein 13.

Balogh, subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

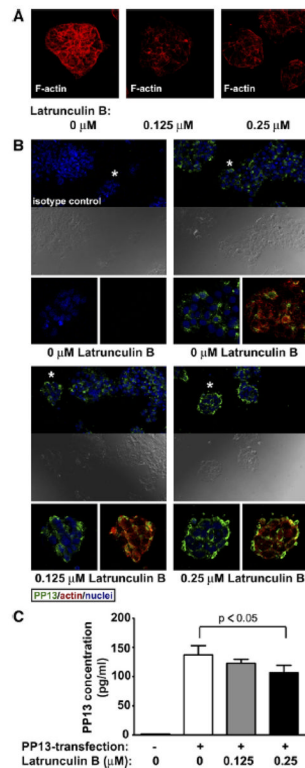


Figure 6. Disruption of the actin cytoskeleton results in intracellular accumulation of Pp13 in BeWo cells

PP13-transfected, untreated or Latrunculin B-treated cells were stained with **A**, phalloidin or with **B**, anti-PP13 (*green*), and antiactin (*red*), followed by confocal microscopic analysis (40x or 20x magnifications, respectively). Cell nuclei were counterstained with DRAQ5 (Biostatus, Leicestershire, UK) (*blue*). *Asterisks* denote enlarged areas. *Yellow color* reflects colocalization of PP13 and actin. **C**, PP13 content of cell culture supernatants of nontransfected controls, as well as PP13-transfected, untreated or Latrunculin B-treated cells were measured by ELISA (data are displayed as mean \pm SEM of 3 independent experiments).

ELISA, enzyme-linked immunosorbent assay; *PP13*, placental protein 13.

Balogh. Subcellular relocation of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

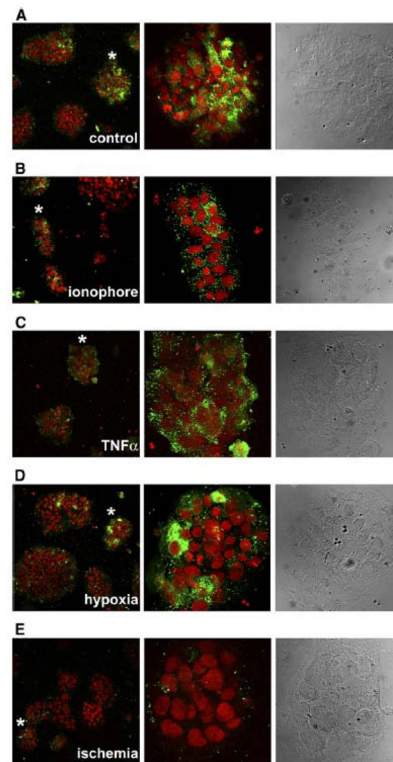


Figure 7. Calcium-influx or ischemic stress decreases PP13 immunostaining in BeWo cells
 PP13-transfected BeWo cells were treated with either **A**, calcium ionophore to increase **B**, intracellular Ca^{2+} - level, or kept under stress, such as **C**, $\text{TNF}\alpha$ treatment, **D**, hypoxia, or **E**, ischemia to mimic placental milieu in preterm preeclampsia. **B–E**, Treated or **A**, untreated control cells were stained with anti-PP13 (*green*), nuclei were counterstained with DRAQ5 (Biostatus, Leicestershire, UK) (*red*). Representative confocal images of 3 independent experiments are displayed (20x magnifications). *Asterisks* denote enlarged areas.

PP13, placental protein 13.

Balogh. Subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

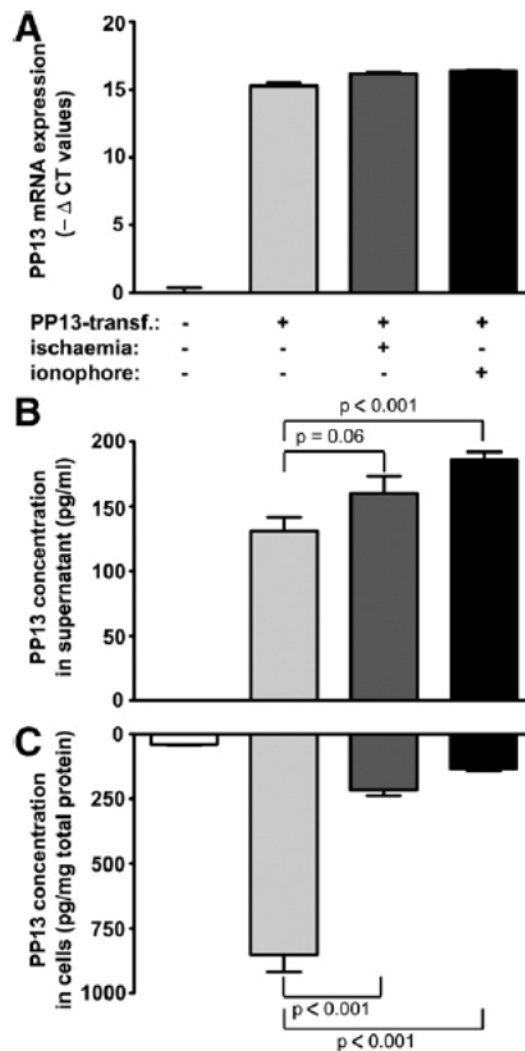


Figure 8. Calcium-influx or ischemic stress promotes PP13-release from BeWo cells
 PP13-transfected cells were treated with calcium ionophore (*black bars*), kept under ischemic stress (*dark gray bars*), or left untreated (*light gray bars*). Nontransfected cells were used as negative controls (*white bars*). **A**, PP13 mRNA expression or **B**, PP13 protein concentration in culture supernatants and **C**, in cells were measured by qRT-PCR or ELISA, respectively. Data are displayed as mean \pm SEM of 3 independent experiments. *ELISA*, enzyme-linked immunosorbent assay; *PP13*, placental protein 13; *qRT-PCR*, quantitative reverse transcribe-polymerase chain reaction.
 Balogh. Subcellular relocalization of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.

Table 1

Demographic and clinical characteristics of the study groups

Groups	Term controls	Term preeclampsia	Preterm controls	Preterm preeclampsia	Preterm HELLP syndrome
Number of cases ^a	5	5	5	5	5
Maternal age, y ^b	30.8 (30.6–31.5)	32.2 (30.3–33.6)	31.6 (31.5–34.3)	34.0 (30.2–34.5)	28.1 (24.1–29.2)
Gestational age at delivery, wk ^b	38.9 (38.7–39.0)	38.4 (37.7–39.9)	31.0 (30.9–34.0)	32.6 (30.3–34.9)	32.0 (29.3–33.1)
Primiparity ^c	40	60	40	80	60
Smoking ^c	0	0	20	0	0
Systolic blood pressure, mm Hg ^b	130 (120–135)	160 (153–170) ^d	120 (120–120)	160 (155–163) ^e	170 (170–170) ^f
Diastolic blood pressure, mm Hg ^b	80 (78–89)	90 (90–100)	80 (70–80)	100 (98–101) ^e	100 (90–110) ^f
Proteinuria ^c	0	100	0	100	100
Maternal BMI, kg/m ² ^b	26.7 (25.2–28.0)	22.0 (20.0–23.3)	23.4 (21.6–24.6)	24.2 (22.6–24.9)	21.4 (21.2–26.0)
Neonatal birthweight, g ^b	3440 (3400–4030)	2810 (2620–3200) ^d	1990 (1640–2210)	1100 (990–1200)	1480 (990–1610)
Cesarean section ^c	100	100	100	100	100
Placental weight, g ^b	518 (458–665)	458 (431–476)	301 (294–322)	217 (211–224)	302 (255–305)
Thrombocytes, M/mm ³ ^b	178 (173–238)	206 (191–250)	227 (170–298)	233 (187–360)	80 (73–93) ^e
Hgb, g/100mL	10 (10–11)	12 (12–12)	11 (11–11)	13 (13–13) ^f	13 (12–14)
SGOT, U/L ^{b,g}		25 (22–32)		31 (25–80)	333 (318–439)
SGPT, U/L ^{b,g}		17 (13–19)		33 (23–80)	337 (315–448)
LDH, U/L ^{b,g}		246 (196–332)		293 (217–350)	572 (510–646)
Bilirubin, μmol/L ^{b,g}		15 (12–15)		5 (4–12)	19 (17–21)

All women were white.

BMI, body mass index; HELLP, hemolysis elevated liver enzymes and low platelets; LDH, lactate dehydrogenase; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase.

^aValues are presented as number;

^bValues are presented as median (interquartile [IQR] range);

^cValues are presented as percentage;

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^d $P < .05$ compared with gestational age matched, term controls;

^e $P < .05$ compared with gestational age matched, preterm controls;

^f $P < .01$;

^gRoutinely not examined in normal pregnant women.

Balogh. Subcellular relocation of PP13 in preeclampsia and HELLP syndrome. *Am J Obstet Gynecol* 2011.