Placenta-derived extracellular vesicles induce preeclampsia in mouse models

Cha Han,[1,2*] Chenyu Wang,[3*] Yuanyuan Chen,[1] Jiwei Wang,[4] Xin Xu,[2] Tristan Hilton,[2] Wei Cai,[3] Zilong Zhao,[5] Yingang Wu,[6] Ke Li,[1] Katie Houck,[2] Li Liu,[5] Anil K. Sood,[7] Xiaoping Wu,[2] Fengxia Xue,[1] Min Li,[3*] Jing-fei Dong[2,8] and Jianning Zhang[5]

[1]Department of Obstetrics and Gynecology, Tianjin Medical University General Hospital, Tianjin, China; [2]Bloodworks Research Institute, Seattle, WA, USA; [3] Institute of Pathology, School of Medical Sciences and Gansu Provincial Key Laboratory of Preclinical Study for New Drug Development, Lanzhou University, Lanzhou, China; [4]Department of Neurosurgery, Tianjin Key Laboratory of Cerebral Vascular and Neurodegenerative Diseases, Tianjin Huanhu Hospital, Tianjin, China; [5]Department of Neurosurgery, Tianjin Medical University General Hospital and Tianjin Neurological Institute, Tianjin, China; [6]Department of Neurosurgery, the First Affiliated Hospital, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China; [7]Department of Gynecologic Oncology and Reproductive Medicine, Division of Surgery, MD Anderson Cancer Center, University of Texas, Houston, TX, USA and [8]Division of Hematology, Department of Medicine, University of Washington, School of Medicine, Seattle, WA, USA.

**CH and CW contributed equally as co-first author.*

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Correspondence: *MIN LI* - limin@lzu.edu.cn

JIANNING ZHANG - jianningzhang@hotmail.com

JING-FEI DONG - jfdong@bloodworksnw.org

SUPPLEMENTAL METHODS AND RESULTS

Patient study

In addition to samples of patients with PE, blood samples from 7 age- and gestation-matched pregnant women were identically analyzed as pregnancy control. Five non-pregnant and age-matched female volunteers were analyzed as baseline controls. This study was approved by the Ethic Committee of the Tianjin Medical University General Hospital.

Mouse models

For the first model, C57BL/6J female mice (10-16 wks and 20-25g, Jackson Laboratory, Bar Harbor, ME) were bred under a regular diet and normal light cycles to pregnancy. For this study, we defined a mouse PE phenotype by hypertension, proteinuria, and kidney injury.[1] Blood pressure (BP) was measured at baseline, 17-18 *days post-coitum* (dpc), and 7-10 days postpartum using a noninvasive mouse tail-cuff BP analyzer (CODA; Kent Scientific Co., Torrington, CT).[2] Urinary Albumin and creatinine in a pooled urine sample collected over a 24hr period were measured and their ratio was calculated to define proteinuria (supplemental method).

This pregnancy model has a limited ability to detect the specific activity of pcEVs because EVs found in the peripheral blood of these pregnant mice are heterogeneous, including not only pcEVs but also those from blood and endothelial cells. We addressed this pitfall in a second model, wherein non-pregnant C57BL/6J female mice were infused with 1×10^7/mouse of purified pcEVs from normal placenta subjected to freeze-thawing injury (supplemental method) or an equal volume of the vehicle PBS. This dosage of pcEVs was selected through a pilot dose-titration experiments. BP was measured 30 min after the infusion and 24hr urine samples were analyzed for proteinuria.

The third model was used to specifically investigate the role of EV clearance. C57BL/6J female mice were infused with pcEVs along with either 400 μg/kg of the apoptotic cell- and microvesicle-scavenging factor lactadherin[3,4] (Haematologic Technologies, Inc., Essex Junction, VT) or an equal volume of PBS. BP was measured 30 min after pcEV infusion. Blood and 24hr urine samples were examined for plasma levels of pcEVs and proteinuria, respectively. After blood and urine collections, the mice were sacrificed by cervical translocation under anesthesia to dissect the lungs, liver, and kidneys for tissue

histology. In reciprocal experiments, mice deficient in lactadherin and their wild-type littermates (supplemental method) were bred to pregnancy. They were then examined for plasma pcEVs, BP, and proteinuria. In a subset of experiments, the kidneys and placenta were collected at 17-18 dpc for tissue histology. To specifically measure the rate of microvesicle clearance, purified pcEVs from injured placenta were biotinylated (supplemental method) and infused into non-pregnant C57BL/6J female mice with 400 μg/kg of lactadherin or PBS.[4] Blood samples were collected over time to measure plasma pcEVs by APC-conjugated streptavidin (ThermoFisher Scientific) using flow cytometry.[4] The mice were sacrificed 6 hrs after infusion to collect the liver for tissue histology.

For these mouse models, whole blood-cell counts and hematocrit were also measured using a VetScan HM5 hematology analyzer (Abaxis, Inc., Union City, CA).

Flow cytometry

Levels of pcEVs in plasma samples from women with normal pregnancy or PE patients were measured using an FITC-antibody against placental alkaline phosphatase (PLAP, LifeSpan Biosciences, Inc., Seattle, WA).[5-8] For the mouse study, we used syncytin as the marker for pcEVs because PLAP was not expressed in mouse placenta.[9] Syncytin expresses on mouse syncytiotrophoblasts,[10] induces trophoblast fusion during placental development,[11,12] and contributes to PE pathogenesis.[13,14] Blood samples collected at the baseline, 17-18 dpc, and postpartum using 3.8% of sodium citrate as an anticoagulant were centrifuged at 1500×g for 20 min at 25°C to collect platelet-poor plasma (PPP). PPP was incubated with a FITC-conjugated polyclonal syncytin antibody (FabGennix International Inc., Frisco, TX) for 30 min at room temperature and analyzed using flow cytometry (Beckon Dickinson, San Jose, CA). These pcEVs were first identified by particle size (≤ 1 μm) using standard microbeads (Biocytex, Marseille, France) and then by syncytin positivity.

Vascular leakage

We used Evans blue extravasation test to measure pcEV-induced vascular leakage in vivo (Supplemental methods).[4] female C57BL/6J mice were infused with pcEVs through the tail vein (1×10^7/mouse in 100 μl) and, after 30min, with 100 μl of 2% Evans blue (Sigma Aldrich, St. Louis, MO). They were sacrificed 2 hrs after the infusion to collect the kidneys. For each mouse, one kidney was collected, washed extensively to remove

blood, snap-frozen in liquid nitrogen, homogenized in formamide (1:20 w/v), and incubated at 60°C overnight (The other kidney was processed for tissue histology). The kidney homogenates were centrifuged at 16,000×g for 30 min, and Evans blue in the supernatant was quantified at OD 620 nm (Molecular Devices, Sunnyvale CA). We also measured the ability of pcEVs to disrupt the integrity of cultured cells from the mouse endothelial line bEnd.3 (ATCC, Manassas, VA), as we previously described (supplemental method).[15,16]

Tissue histology

The lungs, kidneys, liver and placenta were extensively washed with PBS, fixed in 5% paraformaldehyde, embedded, and processed into 3-5 μm tissue sections. The lung, liver and placenta tissues were stained with Hemotoxylin and Eosin (H&E) to detect vascular leakage and tissue necrosis, respectively. The kidney and liver sections were also stained with phosphotungstic acid hematoxylin (PTAH) to detect intravascular fibrin deposition,[4,15] which is a hallmark of systemic hypercoagulation. The livers from mice infused with biotinylated pcEVs were identically processed and stained with HRP-streptavidin to detect microvesicle accumulation.[4]

Microvesicle-induced vasoconstriction

We measured vascular wall tension using a protocol modified from a previous study.[17] Briefly, a 2 mm segment was gently dissected from the carotid artery of a C57BL/6J female mouse immediately after euthanasia. The segment was mounted on two stainless steel wire probes in the Multi Myograph System DMT 610 M (Danish Myo Technology, Denmark) and submerged in 5 ml of PSS buffer (130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.5 mM glucose, 0.026 mM EDTA, 1.6 mM $CaCl_2$, pH 7. 4). After 30 min incubation at 37°C to set the baseline at 4 mN, the PSS buffer was replaced with the oxygenated (95% O_2) KPSS buffer (74.7 mM NaCl, 60 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.5 mM glucose, 0.026 mM EDTA, 1.6 mM $CaCl_2$, pH 7. 4) to trigger vasoconstriction at 37°C in order to ensure the contractility of the vessel segment. After washing with PSS to reset the baseline, the vessel segment was incubated with pcEVs and continuously monitored for changes in the vascular wall tension for 60 min at 37°C. The segment was then washed to remove pcEVs, incubated with PSS for 50 min at 37°C, and incubated with pcEVs again. Changes in isometric force were recorded by the Power Lab System

(ADInstruments, Sydney, Australia). We also measured pcEV-induced calcium influx in cultured mouse smooth muscle cells (supplemental method) using flow cytometry.[15]

Laser speckle contrast analysis (LASCA)

LASCA uses spatiotemporal properties of the laser speckle pattern to visualize microvascular blood flow in real-time.[18,19] We used this non-invasive technique to measure the effect of pcEVs on blood flow in the cerebral cortical vessels because LASCA can not accurately measure the blood flow of internal organs such as the kidney without surgery, which would be a confounding injury that is difficult to stratify. An anesthetized female mouse was fixed on a surgery platform with the head constrained. The hairs were removed from an area of 2 cm×3 cm to expose the scalp in the frontoparietal region. A laser beam from the PeriCam PSI System (Perimed AB, Järfälla-Stockholm, Sweden) was aimed at the shaved area 15 cm away from the scalp. After 5 min to stabilize the baseline blood flow, the mouse was infused with 1×10^7 of pcEVs through the tail vein and continuously monitored for 15 min. The data were quantified for the time period of interest (TOI).

Generation of mouse pcEVs in vitro

For this study, we tested EVs from whole placenta that was subjected to freeze-thawing injury in order to evaluate the collective effect of microvesicles from all placental cells. This technique has been previously used to generate EVs from platelets[20] and the brain.[15] Testing EVs from whole placenta is more physiologically relevant for two reasons. First, while widely used as a marker, there is no evidence that syncytiotrophoblast EVs are the type or only type that causes PE. In fact, placental injury found in PE involves not only syncytiotrophoblast cells, but cells from connective tissues and vascular cells. Second, we generated pcEVs from injured placenta, instead of non-injured syncytiotrophoblast cells in culture, consistent with placental injuries as a cause of PE. Testing pcEVs made in vitro also overcomes a significant technique huddle in studying specific types of microvesicles in mouse models because microvesicles found in circulating blood of pregnant mice are highly heterogeneous and originated from placenta, endothelial cells and blood cells. Purifying pcEVs from this plasma pool of diverse microvesicles require multi-step purifications that would inevitably damage the structure and function of pcEVs.

To obtain pcEVs, placentas were dissected from C57BL/6 female mice at 17-18 dpc, washed with ice cold sterile PBS to remove blood, cut into small pieces, and snap frozen in liquid nitrogen as we previously described.[15] They were then gently homogenized in 1ml of PBS at 4℃. The placenta homogenates were centrifuged at 1500×g for 20 min at 4℃to remove intact cells. The supernatant was centrifuged first at 13000×g for 5 min at 4℃ to remove large cell debris and then at 100,000×g at 4℃ for 60 min (twice) to collect pcEV pellets, which were re-suspended in PBS. We made pcEVs immediately before experiments. The pcEVs generated using this protocol were similar to those detected in the peripheral blood of pregnant mice in terms of size and syncytin expression,[12] but they expressed a higher level of anionic phospholipids detected by annexin V (**Supplemental Figure S3**).

Genotyping lactadherin null mice

Lactadherin$^{-/-}$ mice (B6;129-Mfge8<tm1Osa>/OsaRbrc Mice, No.RBRC01726)[3,21] were provided by the RIKEN BioSource Center (Ibaraki, Japan). Lactadherin$^{-/-}$ mice and their wild-type littermates were genotyped as previously described.[4] Briefly, the genomic DNA was extracted from mouse tails using a commercial MyTaq Extract-PCR kit (Bioline, Taunton, MA) per the manufacturer's instructions. A specific sequence flanking the junction of the lactadherin gene was amplified by polymer chain reaction (PCR) using the following primers: sense-GTGAACCTTCTGCGGAAGAT, antisense-GGGCATAAACTCCAGCTCAC and Nor R- CGTGGGATCATTGTTTTTCT (Integrated DNA Technologies, Coralville, Iowa). For PCR amplification, the genomic DNA was first denatured at 94^{o}C for 120 sec followed by 30 cycles of amplification: 10 sec denaturing at 95^{o}C, 30 sec annealing at 65^{o}C, and 60 sec extension at 68^{o}C. The PCR produced a 570 kb DNA fragment from lactadherin$^{+/+}$ mice and a 310 kb fragment from lactadherin$^{-/-}$ mice (**Supplemental Figure S6**). Lactadherin$^{-/-}$ and lactadherin$^{+/+}$ mice, not heterozygous mice were studied.

Immunoprecipitation and Immunoblots

We used a polyclonal antibody against endoglin (CD105, ThermoFisher Scientific) to cross-validate pcEVs detected by the syncytin antibody in pregnant mice. Endoglin is expressed on syncytiotrophoblasts[22] and plays a role in the PE pathogenesis.[23,24] Because endoglin is also expressed on endothelial cells, pcEVs were first immunoprecipitated from plasma of pregnant mice using a monoclonal syncytin antibody coupled to magnetic microbeads. The immunoprecipitated syncytin^{+} microvesicles were

solubilized in 1% SDS, separated on 4-7% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and probed first with a polyclonal rabbit endoglin antibody (60 min at room temperature) and then with a HRP-conjugated rat anti-rabbit IgG (60 min at room temperature).

PS-dependent clotting assay

We used a modified clotting assay[20] that specifically measured PS- and EV-dependent coagulation, as we previously described.[4] Briefly, purified microvesicles from mouse plasma were re-suspended in 25 μl of PBS. They were mixed with phospholipid-deficient porcine plasma (IMVS, Adelaide, Australia) and bovine factor Xa (0.02 U/ml in a buffer containing 15 mM $CaCl_2$, 100 mM NaCl, 20 mM HEPES, and 0.001% polybrene [pH 7.0]). Time to clot formation induced by pcEVs at 37°C was recorded on a CoaScreener coagulation analyzer (American Labor Corp., Durham, NC). Purified phosphatidylserine (PS) and phosphatidylcholine (PC, both from Avanti Polar Lipids, Inc. Alabaster, AL) were tested as positive and negative controls, respectively.

Transwell assay

Purified pcEVs were labeled with PKH26 (Sigma-Aldrich Corp., St. Louis, MO), which is a membrane lipid dye,[25] as previously described.[15] After washing with PBS, the PKH26-labeled pcEVs were collected by centrifugation at 100,000×g for 1 h at 4°C and re-suspended in PBS for immediate experiments.

Cells from the mouse brain vascular endothelial line bEnd.3 cells (ATCC, Manassas, VA) were grown on a collagen-coated PTFE membrane (0.4 μm pore) in a transwell culture insert (Corning, Tewksbury, MA) in DMEM medium (VWR, Radnor, PA). Confluent cells were incubated with PKH26-labeled pcEVs in the presence and absence of platelets (50,000/μl) for 3 hrs at 37°C. The medium in the bottom chamber was collected for detecting PKH26-labeled pcEVs using flow cytometry.

Quantitative detection of proteinuria

Mice subjected to various experiment conditions were kept in individual cage for 24 hrs during which total urine was collected. The 24hr urine samples were centrifuged at 2,000×g for 10 min at room temperature to collect the supernatant, which was diluted 3,000-fold with PBS and examined for albumin detected by a commercial ELISA kit (ThermoFisher Scientific). To control for volume variations of urine samples and to be consistent with reports in the literature, we also measured urinary creatinine (ELISA,

Exocell Inc., Philadelphia, PA). Proteinuria was defined by the ratio of albumin to creatinine.

Biotinylation of pcEVs

The purified pcEVs were biotinylated using an EZ-link sulfo-NHS-biotin kit according to the manufacturer's instructions (Thermo Fisher Scientific).[4] Briefly, pcEVs at 1×10^{10}/ml × 1 ml were incubated with 224 μl of the biotinylation buffer that contained 10 mM of biotin for 30 min at room temperature. They were then washed three times with PBS that contained 100 mM of glycine to remove excess biotin and suspended in PBS. Flow cytometry detected more than 50% of pcEVs were biotinylated as they bound FITC-conjugated streptavidin (ThermoFisher Scientific) (**Supplemental Figure S9**).

PcEV-induced calcium influx

Calcium influx induced by pcEVs was measured using a protocol modified from our previous study of brain-derived microvesicles.[15] Briefly, mouse smooth muscle cells (ATCC, Manassas, VA) were grown in DMEM medium (VWR, Radnor, PA) until confluent and then washed with Ca^{++} and Mg^{++} Hank's phenol red-free Balanced Salt Solution (HBSS, ThermoFisher Scientific). The cells were incubated with pcEVs (2×10^4/μl or 2×10^5/μl) for 30 min at 37℃. Control cells were incubated with an equal volume of PBS. At the end of incubation, the cells were washed with HBSS to remove free pcEVs and then incubated with 3μM of Fluo 3-AM (Dojindo Molecular Technologies, Inc.). After 30 min incubation at 37°C, the cells were washed to remove the dye that was nonspecifically associated with the cell surface and incubated with HBSS for 30 min to allow complete de-esterification of intracellular AM esters. They were then detached with 0.25% of trypsin, washed with HBSS, and analyzed for intracellular fluorescence using flow cytometry.

SUPPLEMENTAL FIGURES AND LEGENDS

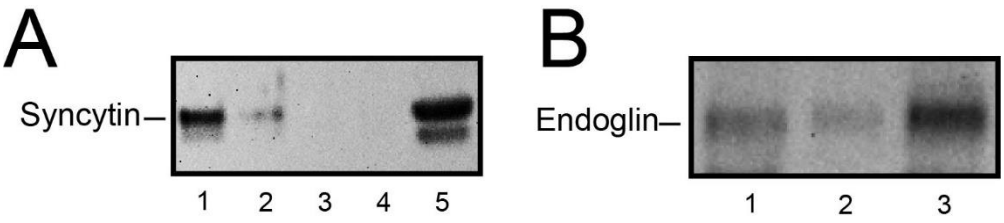

Figure S1: Detection of pcEVs using immunoblots. (A) Syncytin expressed on total EVs purified by ultracentrifugation from plasma of pregnant mice was detected by a monoclonal syncytin antibody (lanes 1 & 2: undiluted and 10 time diluted plasma; lane 3: plasma from non-pregnant mice; lane 4: platelet lysate; and lane 5: mouse placental homogenates). **(B)** pcEVs immunoprecipitated by the monoclonal syncytin antibody were probed with a polyclonal endoglin antibody (Lane 1: whole plasma from a non-pregnant mouse, Lanes 2 and 3: syncytin antibody-precipitated plasma from a non-pregnant mouse and a pregnant mouse at 17 dpc, respectively. The data are representatives from 3-5 separate experiments.

Note: *Because endoglin is also expressed in endothelial cells, directly probing plasma from mice would introduce false positivity, as indicated in lane 1. To avoid this background stain, we immunoprecipiated pcEVs with a monoclonal mouse syncytin antibody and then probed the immunoprecipitates with a polyclonal rabbit endoglin antibody.*

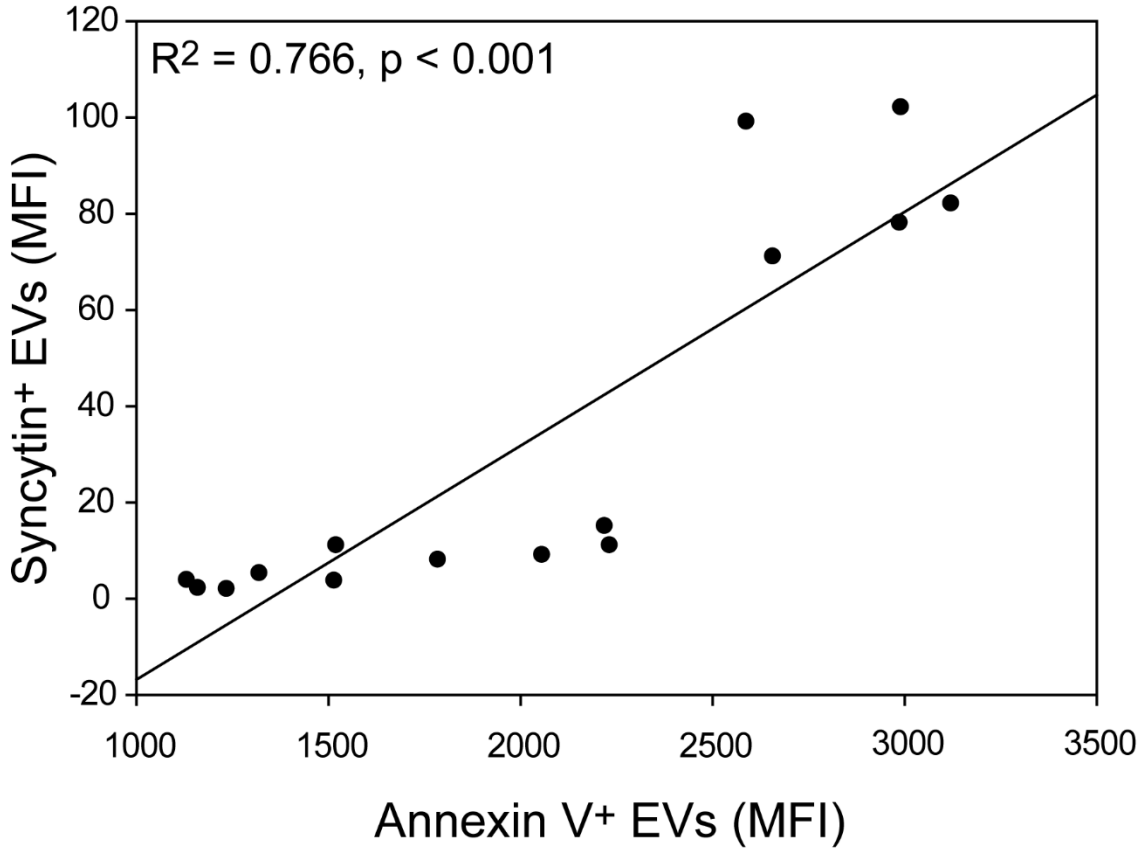

Figure S2: A correlation plot of syncytin$^+$ and annexin V$^+$ EVs found in plasma samples collected from C57BL/6J pregnant mice at 17-18 dpc (n = 15).

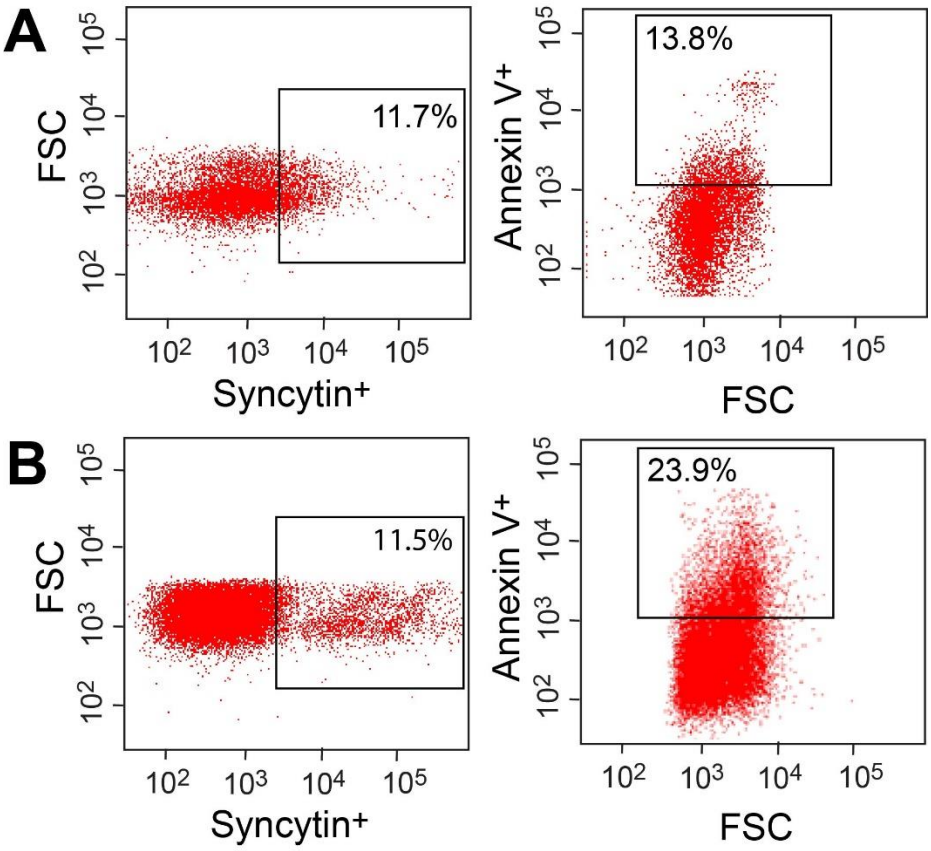

Figure S3: Comparison of pcEVs found in pregnant mice (panel A) and those made in vitro (panel B) for their sizes, levels of syncytin expression, and annexin V binding (representative of 12-56 separate measurements).

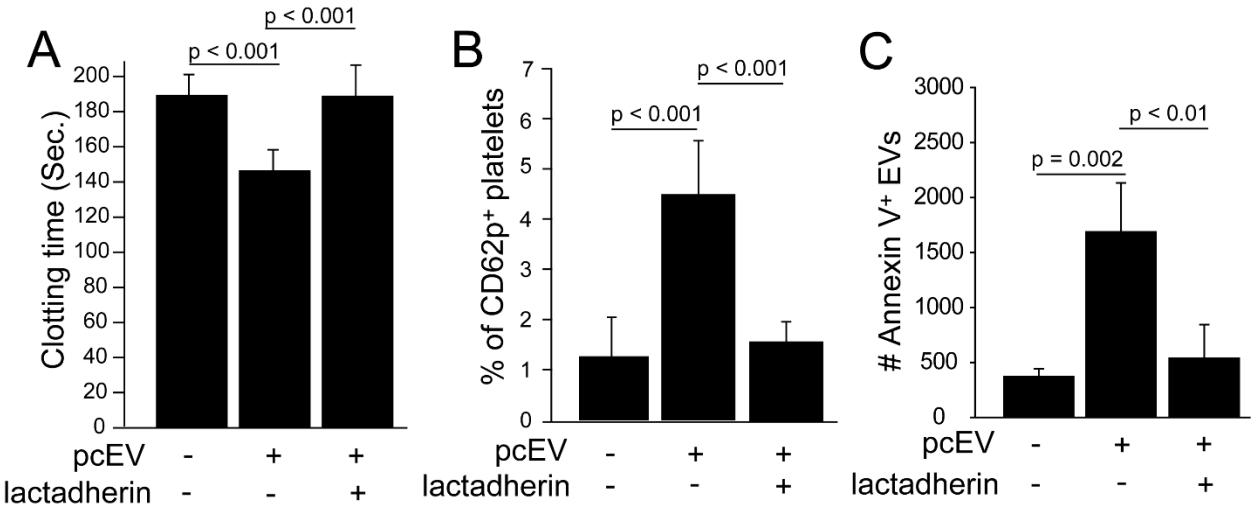

Figure S4: Non-pregnant C57BL/6J mice were infused with 1×10^7/mouse of purified pcEVs and either 400 μg/kg of lactadherin or an equal volume of PBS. Blood samples were collected 60 min after infusion to measure PS-dependent clotting time (**A**), CD62p expression on platelet surface (**B**), and annexin V-bound EVs (**C**). Data were generated from 18 mice and analyzed with one-way ANOVA.

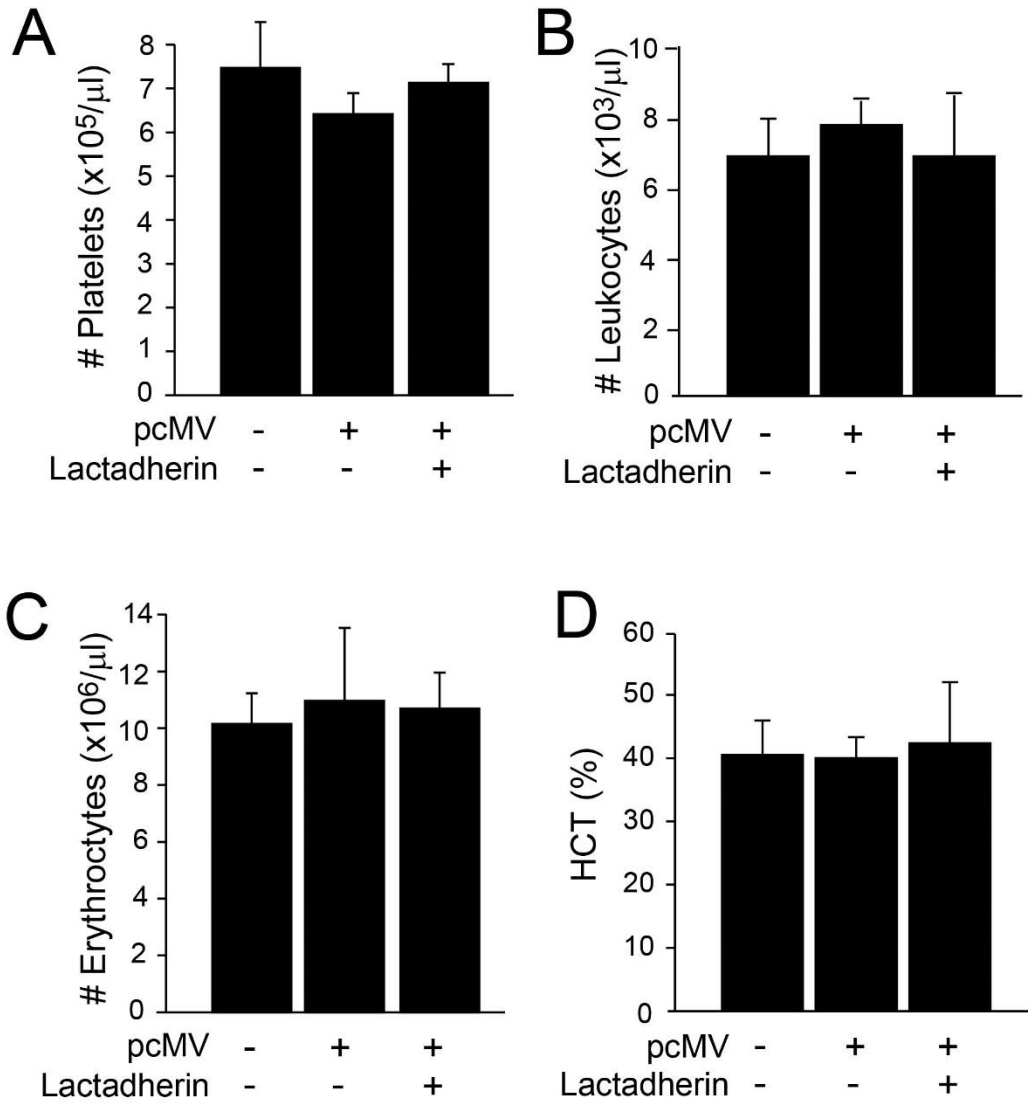

Figure S5: Non-pregnant mice infused with $1x10^7$/mouse of pcEVs with and without 400μg/kg of lactadherin. Blood samples were collected 30 min after infusion to measure platelet counts (**A**), leukocyte counts (**B**), RBC counts (**C**) and hematocrit (**D**) using an automated mouse CBC device (Abaxis, Inc., Union City, CA). The data are summary from 9 groups of mouse experiments and analyzed with one-way ANOVA.

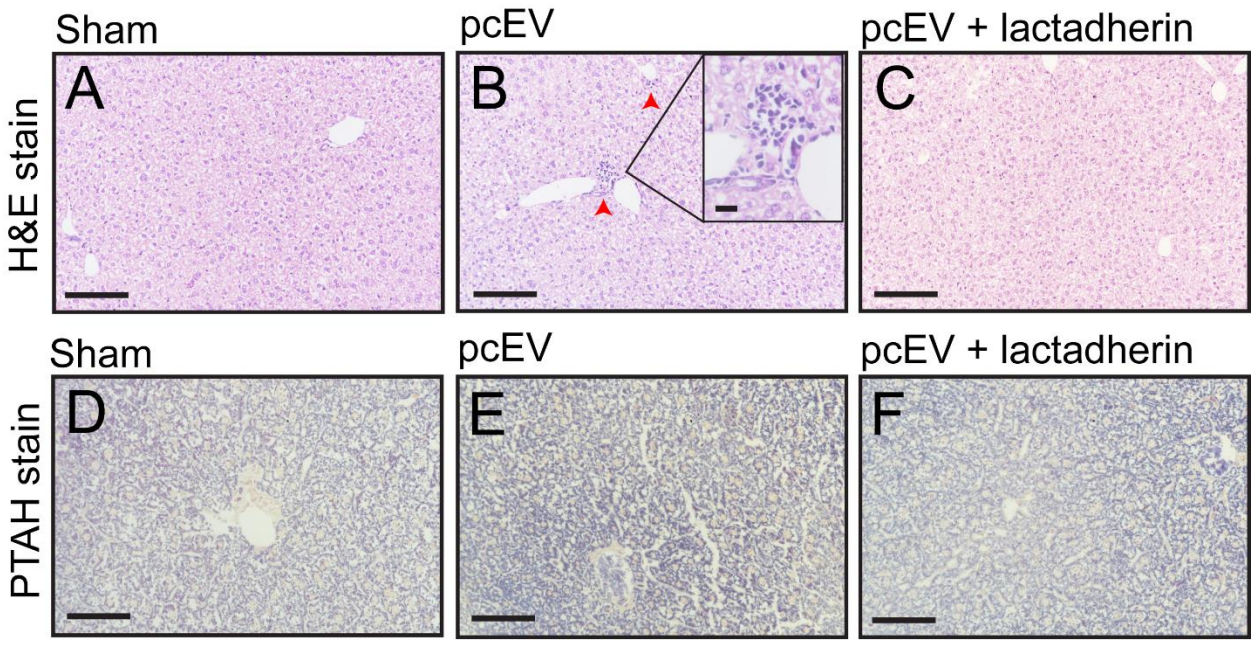

Figure S6: Tissue histology of the liver. A-C: H&E stain of liver tissue from non-pregnant sham mice and mice infused with either pcEVs or pcEVs combined with lactadherin. The enlarged insert in B indicates focal necrosis from the original image. D-F: PTAH stain (which specifically detects intravascular fibrin depsotion) of liver section from the same groups of mice. Bar = 100 μm except the insert in B (bar = 5 um). The images are representative of tissues from 21 mice.

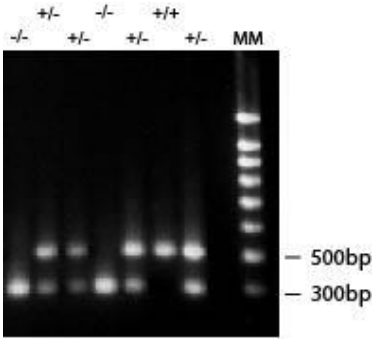

Figure S7: Genotyping lactadherin$^{-/-}$ and lactadherin$^{+/+}$ mice. Genomic DNA was extracted from lactadherin null, heterozygous, and wild-type mice. They were amplified with specific primers,[4] which amplified a 570 kp DNA fragment from the wild-type mice and a 310 kp fragment from the lactadherin null mice.

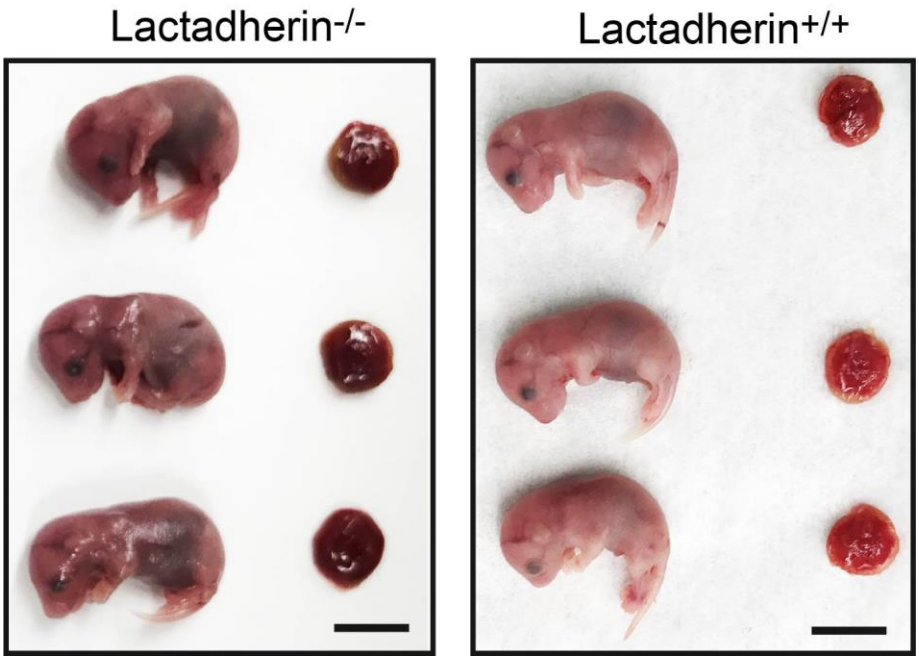

Figure S8: Representative placentas and foetuses of lactadherin$^{-/-}$ and lactadherin$^{+/+}$ mice collected at 17-18 dpc of pregnancy (bar = 10 mm).

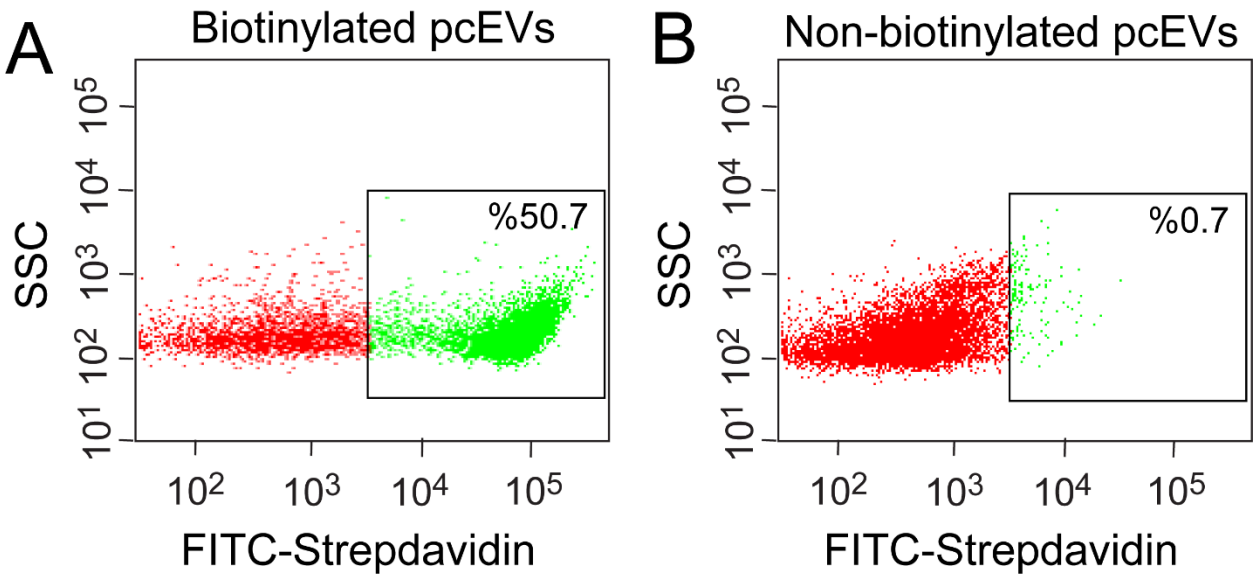

Figure S9: Purified pcEVs were biotinylated using an EZ-link sulfo-NHS-biotin kit. The successful biotinylation was determined by binding of FITC-streptavidin to biotinylated pcEVs using flow cytometry.

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