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Am J Reprod Immunol. Author manuscript; available in PMC 2019 July 01.

Published in final edited form as:

Am J Reprod Immunol. 2018 July ; 80(1): e12860. doi:10.1111/aji.12860.

EXTRACELLULAR VESICLES GENERATED BY PLACENTAL TISSUES *EX VIVO:* A TRANSPORT SYSTEM FOR IMMUNE MEDIATORS AND GROWTH FACTORS

Wendy Fitzgerald[1], **Nardhy Gomez-Lopez**[2,3,4], **Offer Erez**[2,3], **Roberto Romero**[2,5,6,7,*], and **Leonid Margolis**[1]

[1]Section of Intercellular Interactions, Eunice Kennedy-Shriver National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services, Bethesda, MD, and Detroit, MI

[2]Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, Eunice Kennedy-Shriver National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services, Bethesda, MD, and Detroit, MI

[3]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan, USA

[4]Department of Immunology, Microbiology and Biochemistry, Wayne State University School of Medicine, Detroit, Michigan, USA

[5]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan, USA

[6]Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, Michigan, USA

[7]Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan, USA

Abstract

PROBLEM—To study the mechanisms of placenta function and the role of extracellular vesicles (EVs) in pregnancy, it is necessary to develop an *ex vivo* system that retains placental cytoarchitecture and the main metabolic aspects, in particular the release of EVs and soluble factors. Here, we developed such a system and investigated the pattern of secretion of cytokines, growth factors and extracellular vesicles by placental villous and amnion tissues *ex vivo*.

*Correspondence to: Roberto Romero, MD, D. Med. Sci. Perinatology Research Branch, NICHD/NIH/DHHS, Wayne State University/Hutzel Women's Hospital 3990 John R, Box 4, Detroit, MI 48201, USA, Telephone: (313) 993-2700, Fax: (313) 993-2694, prbchiefstaff@med.wayne.edu.

Author Contributions:

W.F. conceived, designed and performed experiments, analyzed and discussed data, and contributed to writing of the manuscript. N.G-L. and O.E. analyzed and discussed data and contributed to writing of the manuscript. R.R. and L.M. conceived and designed experiments, analyzed and discussed the data and contributed to writing the manuscript.

Conflict of Interests

The authors declare no conflict of interests.

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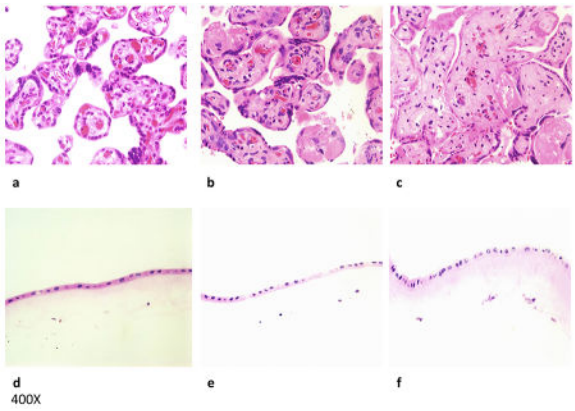
METHODS OF STUDY—Placental villous and amnion explants were cultured for two weeks at the air/liquid interface and their morphology and the released cytokines and EVs were analyzed. Cytokines were analyzed with multiplexed bead assays and individual EVs were analyzed with recently developed techniques that involved EV capture with magnetic nanoparticles coupled to anti-EV antibodies and flow cytometry.

RESULTS—*Ex vivo* tissues (i) remained viable and preserved their cytoarchitecture; (ii) maintained secretion of cytokines and growth factors; (iii) released EVs of syncytiotrophoblast and amnion epithelial cell origins that contain cytokines and growth factors.

CONCLUSION—A system of e*x vivo* placental villous and amnion tissues can be used as an adequate model to study placenta metabolic activity in normal and complicated pregnancies, in particular to characterize EVs by their surface markers and by encapsulated proteins. Establishment and bench-marking the placenta *ex vivo* system may provide new insight in the functional status of this organ in various placental disorders, particularly regarding the release of EVs and cytokines. Such EVs may have a prognostic value for pregnancy complications.

Graphical abstract



Keywords

Cytokine; pregnancy; 3D cultures; growth factors; syncytiotrophoblast; amnion; alarmins

Introduction

The placenta plays a critical role in fetal growth and development and orchestrates major maternal adaptations of pregnancy such as carbohydrate intolerance[1–5] and immune adaptations[6–30]. Placental dysfunction has been implicated in major complications of pregnancy such as preeclampsia[31–56], fetal growth restriction[57–72], fetal death[73–80], and preterm labor[81–90]. The placenta has also been considered at the center of the chronic disease universe[91, 92].

The study of human placenta *in vivo* is challenging and has significant restrictions. Animal models have been useful, although there are fundamental differences in placentation among mammals[93–97]. Many studies of human placenta utilize isolated primary cells or placenta-derived cell lines[98–101]. While major discoveries have emerged from such studies[102–104],

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isolated cells do not adequately recapitulate important aspects of tissue function related to cell-cell communications *in vivo*. This is the rationale to develop three-dimensional models which maintain the cellular relationships *ex vivo*. Such three-dimensional models have proven to be of major value in investigating cancer development[105–109], viral pathogenesis[110–113], and testing anti-cancer[114] and antiviral compounds[115] under controlled laboratory conditions.

It is now increasingly apparent that the maternal-fetal dialogue is more complex than previously recognized[116–119]. In addition to many soluble factors, such as hormones and cytokines implicated in this communication, it is now recognized that extracellular vesicles (EVs) can also mediate crosstalk between the feto-placental unit and the mother[120–131]. EVs carry lipids, proteins and miRNA that can convey information about the status of the fetus and placenta[132–134]. Moreover, EVs carry immune mediators (e.g. cytokines) that facilitate cell-to-cell communication, which are present on both the surface and inside the microvesicles[135–145].

To study the mechanisms of placenta function and the role of EVs in pregnancy, it is necessary to develop an *ex vivo* system that retains placental cytoarchitecture and continues to release EVs and soluble factors under controlled laboratory conditions. Here, we report on such a system. Using nanotechnology, we analyzed individual EVs released by placental tissues *ex vivo* and assessed EV-bound and EV-encapsulated cytokines. Establishment and bench-marking this placenta *ex vivo* system provides a basis to study the nature of various placental disorders, and in particular the release of EVs and cytokines. Their release by the syncytiotrophoblast into the maternal circulation has been proposed as a placental liquid biopsy, which can provide insight into the functional status of the organ and may be a source of biomarkers to predict pregnancy complications[146]. Herein, we report a system of e*x vivo* placental villous and amnion tissues that can be used as an adequate model to study physiological and pathological processes during normal and complicated pregnancies.

Methods

Sample preparation and storage

Placental tissues (the placenta and fetal membranes) from women who delivered at term without labor (n=10) were obtained at the Detroit Medical Center, Wayne State University, and the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, US Department of Health and Human Services (NICHD/NIH/DHHS) (Detroit, MI, USA). The collection and utilization of biological materials for research purposes were approved by the Institutional Review Boards of these institutions. All participating women provided written informed consent. Immediately after delivery, three random samples from the placental villi were collected using a metal grid and the Random Position Generator DICE software (Perinatology Research Branch, Detroit, MI, USA). Amnion was gently separated from the chorion of the fetal membranes. Samples from the placental villi and amnion were placed in 50mL tubes containing DMEM and shipped overnight to NIH on cold packs. Upon receipt, villi were sectioned into 2 mm × 6 mm strips, washed thoroughly in 1X phosphate-buffered saline (PBS) and cultured on Gelfoam absorbable collagen

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sponges (Pfizer, New York, NY) at the air-liquid interface, as has been described for other tissues[147] in 0.1 μm filtered phenol red free DMEM supplemented with 5% characterized, charcoal stripped FBS, 50 μg/ml gentamicin and 2.5 μg/ml Amphotericin B at 37°C, 5% CO_2. Amniotic membrane was sectioned into 3 × 3 mm pieces, washed thoroughly with PBS, and cultured in same medium. Equivalent masses were cultured in triplicate for each donor. Tissues were collected at day 1, 7 and 14 and fixed in 10% formalin, sent for paraffin embedding, sectioning, and H&E staining. H&E sections were evaluated by perinatal and obstetric pathologists at Wayne State University School of Medicine. Medium was collected and changed at days 1, 4, 7, 10 and 14 after initiation. Medium samples were centrifuged at 400 × g for 5 minutes to remove cells and frozen at −80°C.

Preparation of EV fractions

Medium samples were split into multiple fractions. One aliquot was kept untreated, another portion was treated with Exoquick TC (System Biosciences, Palo Alto, CA), according to manufacturer's protocols. Briefly, ExoQuick TC was added to supernatants at a ratio of 100 μl of ExoQuick TC to 500 μl of sample and refrigerated overnight at 4°C. ExoQuick/sample mixtures were centrifuged at 1500 × g for 30 minutes to pellet EVs. Supernatant was collected and saved for cytokine measurement of EV-free supernatant. The pellet was centrifuged again at 1500 × g for 5 minutes and all traces of fluid were removed resulting in an EV enriched preparation. The pellet was resuspended in 1X PBS in the original volume and cytokines were measured on intact and lysed EVs.

Cytokine measurement

We previously developed an in-house multiplexed bead-based assay for measurement of the following cytokines/growth factors: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15, IL-16, IL-18, IL-33, Calgranulin A (S100A8), Calgranulin C (S100A12), C-reactive protein (CRP), CXCL6 (granulocyte chemotactic protein 2), CXCL13 (B lymphocyte chemoattractant), Eotaxin (CCL11), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulated alpha (GRO-α or CXCL1), HMGB1 (high mobility group box 1), interferon-β (IFN-β), interferon-γ (IFN-γ), interferon-γ-induced protein (IP-10 or CXCL10), interferon-inducible T-cell alpha chemoattractant (ITAC or CXCL11), lactoferrin, macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1 or CCL2), macrophage migration inhibitory factor (MIF), monokine induced by IFN-γ (MIG or CXCL9), macrophage inflammatory protein-1α (MIP-1α or CCL3), MIP-1β (CCL4), MIP-3α (CCL20), regulated on activation normally T-cell expressed and secreted (RANTES or CCL5), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and TNF related apoptosis inducing ligand (TRAIL), as previously described with minor modifications[148–150]. All antibody pairs and protein standards were purchased from R&D Systems except those for IFN-β and lactoferrin (Abcam, Cambridge, MA). Additional in-house assays were designed for the following growth, angiogenic and anti-angiogenic factors and hormones: activin A, A disintegrin and metalloproteinase domain 12 (ADAM-12), adiponectin, angiogenin, CD40L, epidermal growth factor (EGF), endoglin, fasL, fibronectin, galectin-1, human chorionic gonadotropin (hCG), intercellular adhesion molecule 1 (ICAM-1), insulin-like growth factor-binding protein 1 (IGFBP1), interleukin-1 receptor antagonist (IL-1Ra), IL-27, leptin, matrix metalloproteinase-7 (MMP-7), MMP-9,

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pregnancy-associated plasma protein-A (PAPP-A), prostaglandin E2 (PGE2), placental growth factor (PIGF), resistin, serpin E1, tissue factor pathway inhibitor (TFPI), transforming growth factor beta 3 (TGFβ3), tyrosine-protein kinase receptor Tie-2, tissue inhibitor of matrix metalloproteinases 1 (TIMP-1), tissue factor, toll-like receptor 2 (TLR2), triggering receptor expressed on myeloid cells 1 (TREM-1), urokinase-type plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 1 (VEGFR1 or Flt-1), and vascular endothelial growth factor receptor 2 (VEGFR2 or Flk-1). Antibody pairs and proteins were purchased from R&D Systems except those for hCG and PGE2 (Abcam).

Magnetic beads (Luminex, Austin, TX) with distinct spectral signatures (regions) were coupled to cytokine specific capture antibodies according to manufacturer's recommendations and stored at 4°C. All antibody pairs were verified to be free of cross reactivity. Standards and samples were combined with bead mixtures and incubated overnight at 4°C. Intact EV samples and lysed EV samples, to which Triton X was added at final concentration of 1%, were run in separate wells. Plates were washed two times and incubated with mixtures of polyclonal biotinylated anti-cytokine antibodies for one hour at room temperature. Plates were washed two times and incubated for 25 minutes with 16 μg/ml streptavidin-phycoerythrin in PBS. Plates were washed two times and beads were resuspended in PBS and read on a Luminex 200 analyzer with acquisition of 100 beads for each region and analyzed using Bioplex Manager software (BioRad, Hercules, CA). Cytokine concentrations were determined using 5P regression algorithms.

EV labeling and capture

EVs were captured from culture supernatants via magnetic nanoparticles (MNPs) (Ocean NanoTech, San Diego, CA). MNPs were coupled to anti-PLAP (clone 8B6,Thermo Fisher, Waltham, MA and clone H17E2, BioRad), anti-CD90 (clone 5E10, Biolegend, San Diego, CA), anti-CD9 (clone H19a, Biolegend), anti-CD63 (H5C6 Biolegend), anti-HLA-ABC (W6/32 Biolegend) or mouse IgG (SouthernBiotech, Birmingham, AL) antibodies, per manufacturers' protocol and as previously described[151]. Briefly, 200 μl of 15nm MNPs are activated and then coupled with 1mg antibody overnight. Coupled MNPs are washed twice on a magnet then resuspended in 2 ml of wash/storage buffer and stored at 4°C. EVs in 100 μl of culture supernatant were labeled with 1 μM Bodipy FL Maleimide [BODIPY™ FL N-(2-Aminoethyl) Maleimide, Thermo Fisher] for 15 minutes at RT, then captured with 20 μl of MNPs. MNPs are added in huge excess to EVs, and the ratio of MNPs to EVs was optimized to allow good capture efficiency and single particle detection, as previously described[151]. Fluorescent detection antibodies were added for 30 minutes at room temperature. Detection antibodies for placental villous cultures included mouse anti-human antibodies to CD51-PE (Sony Biotechnology, Champaign, IL), CD63-BV711 (BD Biosciences, San Jose, CA), CD105-PECy7 (Biolegend), CD200 BV650 (BD Biosciences), CD274 BV605 (Biolegend), syncytin-1 (Abnova, Walnut, CA) in-house labeled with AlexaFluor 647, and HLA-ABC APC/Cy7 (Biolegend). Detection antibodies for amnion explants included mouse anti-human antibodies to CD29 APC (Thermo Fisher), CD44 PE (Thermo Fisher), CD105 PECy7 (Biolegend), CD140b BV421 (BD Biosciences), CD324 PerCP/Cy5.5 (Biolegend), CD326 BV650 (Biolegend) and HLA-DR APC/Cy7 (Biolegend).

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Control staining was also performed with mouse anti-human CD31, CD41, and CD45 APC/Cy7 (Biolegend). The captured and stained complexes were separated from unbound EVs and antibodies using MS magnetic columns (Miltenyi Biotec) in a magnetic field using OctoMACS magnet (Miltenyi Biotec), washed four times with 500 μl of PBS and eluted from the column outside the magnet with 200 μl PBS and fixed with 1.5% paraformaldehyde. 123count ebeads (Thermo Fisher) were added to tubes for EV quantification. All antibodies were tested on EV/MNP complexes singly and in combination to verify that antibodies bound with the same efficiency and spectral overlap could be compensated.

EV flow cytometry analysis

Purified complexes were acquired on low speed on an LSRII (BD Biosciences) flow cytometer equipped with 355-, 407-, 488-, 532- and 638-nm lasers by triggering on Bopidy FL fluorescence to acquire only labeled EVs. Fluorescence minus one stainings and isotype controls were used were used for setting gates, compensations, and determining background staining. Megamix SSC beads (BioCytex, Parsippany, NJ) were used to set parameters for estimated EV size; in general EV size is overestimated due to the binding of MNPs to the EVs. Data were acquired with Diva 6.3 and analyzed with FlowJo software v10.4.1 (Treestar Software, Ashland, OR).

Measurement of EV-associated cytokines

EVs were captured as above using MNPs coupled to mouse anti-human antibodies to PLAP (8B6, Thermo Fisher), CD31 (WM59, Biolegend), CD90 (5E10, Biolegend) or HLA-G (87G, Biolegend) antibodies. 20 μl of MNPs were incubated with 100 μl of culture supernatants overnight at 4°C and purified using MS magnetic columns as above. EV/MNP complexes were eluted off columns, resuspended in their original volume, split in two and analyzed by multiplexed bead assays on intact fractions and lysed (1% Triton X) fractions. Total EVs from culture supernatants were collected using ExoQuick TC as above and analyzed the same way.

Statistical Analysis

We conducted statistical analysis using JMP10 (SAS Institute, Cary, NC). Results are represented as means ± standard error of the mean (SEM). The statistical differences were evaluated with paired Student's t test. All hypothesis tests were two-tailed and a p value of ≤ 0.05 defined statistical significance.

Results

Ex vivo tissue viability and function

Histology—Samples of the villus tree and amnion were dissected and cultured as described in the methods section. Tissue samples were collected at day 1, 7 and 14 of culture, fixed, paraffin embedded, sectioned, and H&E stained (Fig. 1). At the start of culture, chorionic villi were viable and maintained normal morphology with well-preserved synctiotrophoblasts, intact blood vessels, and a lack of karyorrhexis; amnion tissue was well preserved as well. At day 7, much of the syncytiotrophoblast appeared viable and well

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preserved, with focal areas of early degenerative changes in the form of karyorrhectic debris in blood vessels and villous stromal-vascular karyorrhexis. Most of the amnion appeared well-preserved and viable at day 7. By day 14, placental villous tissue showed slightly more pronounced karyorrhexis and degeneration of syncytiotrophoblast than at day 7. Amnion tissue at day 14 also showed mild degenerative changes in the form of pyknosis.

Cytokine production—The release of cytokines by villi and amnion cultures over the entire culture period was determined using in-house designed multiplexed bead-based assays[150]. These assays revealed that cytokines are steadily produced in both placental villous and amnion cultures (Fig. 2a, c). Villous tissue produced large amounts of the pro-inflammatory cytokines IL-6, IL-8, GRO-α, IP-10 and MCP-1, as well as CRP and TRAIL (Fig. 2a). Cultures also released considerable amounts of the alarmins calgranulin A, calgranulin C, and HMGB1, and the antibacterial protein lactoferrin. IL-13, IL-16, and IL-33 were also released, as well as the chemokines ITAC, MIF, MIG, MIP-1α, MIP-1β, MIP-3α, and RANTES. Other cytokines were produced in smaller quantities (see Table S1).

Amnion explants, similar to villi explants, produced cytokines constantly over the duration of the culture period (Fig. 2c). Amnion and villus explants also produced large amounts of the pro-inflammatory cytokines IL-6, IL-8, GRO-α, IP-10 and MCP-1 as well as CRP and TRAIL. Such explants also produced the antimicrobial proteins calgranulin C and lactoferrin as well as smaller amounts of calgranulin A. Moreover, the explants produced the prototypic alarmin HMGB1 as well as IL-10, IL-13, IL-16, IL-33, MIP-1α, MIP-1β, MIP-3α, MIF, CXCL6 and smaller amounts of ITAC, RANTES, and CXCL9 (see Table S1).

Production of growth factors, angiogenic and anti-angiogenic factors—The release of other growth factors, angiogenic factors, anti-angiogenic factors and hormones was determined by multiplexed bead assays. Both villi and amnion explants also continuously produced these factors over the duration of the culture period (Fig. 2b, d). Villi explants produced large amounts of ADAM-12, adiponectin, angiogenin, fibronectin, galectin-1, ICAM-1, IGFBP1, IL-1Ra, IL-27, PAPP-A, Serpin E1, TFPI, TIMP-1, uPA, uPAR, VEGFR1 and VEGFR2, as well as hCG and PGE2 (Fig. 2b). A complete list of factors produced is available in Table S2.

Amnion explants produced large amounts of many of the same growth and angiogenic factors as villi explants including adiponectin, angiogenin, fibronectin, galectin-1, IGFBP1, IL-1Ra, IL-27, Serpin E1, TFPI, TIMP-1, VEGFR1, uPA and uPAR, and the hormones hCG and PGE2 (Fig. 2d) (See Table S2 for complete list).

Analysis of Placental Villous EVs

To analyze EVs specifically from syncytiotrophoblasts (STB) of the explants, magnetic nanoparticles (MNPs) coupled to anti-PLAP antibody, an antigen specific to STB[123, 152–155], were used. EVs were labeled with Bodipy FL as described in Methods. Among several commercially available anti-PLAP antibodies, we selected one (clone 8B6) that after coupling to MNPs was specific in capture of STB-generated EVs and captured EVs most efficiently. We analyzed the STB-generated EVs for other antigens that have been described on STBs or STB EVs.

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Selection of PLAP antibodies for capture of syncytiotrophoblast EVs—We coupled two clones of PLAP antibodies to MNPs and captured EVs from placental villous culture supernatants. MNPs coupled to two PLAP clones captured similar amounts of EVs: With MNPs coupled to clone H17E2 we captured 108.8 ± 11.6% of EVs captured with MNPs coupled to clone 8B6. However, MNPs coupled to clone H17E2 captured 3.3 ± 0.3 (n=3) times more of non-specific EVs, expressing HLA-ABC. Therefore, we selected clone 8B6 for future experiments since MNPs coupled to the antibodies of this clone seemed to be more specific to capture PLAP-positive EVs.

Specificity of EV capture—We further verified the specificity of our anti-PLAP MNPs by incubation with amnion explant supernatants which should not contain PLAP+ EVs[156] and found they captured on average 4.7 ± 0.5% of total EVs (n=3). That was not different from the amount captured with control mouse IgG isotype MNPs: With these MNPs we captured from the placental villous tissue supernatants 4.8 ± 1.1% of EVs that were captured by specific anti-PLAP MNPs (n=3).

The lack of non-STB antigens on anti-PLAP captured STB-generated EVs—To further confirm specificity of the PLAP-captured EVs, we captured EVs from villous samples pooled from multiple donors and stained for CD31, CD41, CD45, and HLA-ABC, all of which should be absent on STB EVs[157, 158]. All antibodies were labeled with the same fluorophore, and collected into a single "dump" gate. We found that they were present on only 1.6 ± 0.5% of captured EVs (n=3). For the remaining experiments, we included only HLA-ABC, and used the lack of this marker as an additional criterion for STB EVs. Single staining for HLA-ABC on EVs captured by anti-PLAP MNPs revealed 0.7 ± 0.3% of total EVs (n=3).

Syncytiotrophoblast markers on PLAP-positive EVs—Next, we evaluated the distribution of several "phenotypic" markers on the EVs captured by MNPs through PLAP. We chose markers which have been previously described in the literature as being surface markers either of STB themselves, or of the STB-generated EVs[154, 159–162] namely, CD51, CD63, CD105, CD200, CD274, and Syncytin-1. Culture supernatants were stained with BoDipy-FL to label EVs, and then captured with anti-PLAP MNPs, stained with antibodies to the above-listed markers (as well as with antibodies to HLA-ABC). The MNP/labeled EV complexes were washed on magnetic columns, eluted and acquired on a flow cytometer set to threshold on the BoDipy-FL EV label. HLA-ABC+ EVs were excluded from the analysis and the rest quantified by expression of the markers and approximate size. (See Figure S1 for gating strategy).

Flow-cytometry analysis revealed that CD200 had the highest expression on PLAP-MNPs captured EVs, being present on 67.3 ± 3.1% of vesicles at day 1, and Syncytin-1 was the lowest at 1.7 ± 0.2%. The other markers were expressed on 24 to 34% of vesicles (Fig. 3a). EV size was estimated using Megamix SSC beads. The vesicles formed a continuum rather than discreet populations, but small vesicles of the size 200nm or less were the most plentiful and over 500nm the least common (Fig. 3b).

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The distribution of the markers varied with vesicles of different sizes (Fig. 3 c-h). Only CD200 was highly expressed (58.8 ± 4.4%) at day 1 on small vesicles (of the size of 200nm or less), while all other markers were present at lower levels on these small vesicles (0.5 – 18.4%). Levels of CD51, CD63, CD105, and CD274 were highest on vesicles of the size of 250–500nm, and syncytin-1 was highest on vesicles of the size above 500nm.

We analyzed co-expression of markers on individual vesicles and found CD51, CD63, CD105, and CD274 were most often co-expressed with CD200, the most highly expressed marker on the placental villous EVs (Fig. S2). Syncytin-1 was the least co-expressed molecule, but was most often co-expressed with CD105.

Assessment of PLAP-captured vesicles over time showed that the total number of vesicles decreased throughout the culture period. Total EVs at day 1 were $1.91 \pm 3.3 \times 10^6$ EVs/ml and declined to $9.4 \pm 1.7 \times 10^4$ EVs/ml by day 14 (see Table S3 for EV counts). The distribution of EVs in different size ranges shifted slightly over time (Fig. 3b). The amount of small vesicles (≤200nm) decreased over time, starting at 52.1 ± 3.0% at day 1 and dropping to 25.5 ± 3.6% at day 14, whereas vesicles of all other size ranges increased slightly in percentage with length of culture.

The amount of PLAP-captured vesicles expressing each marker were similar at day 1 and 4, but decreased slightly by day 7 and further by day 14, except for syncytin-1 expressing EVs, which increased in over time (Fig. 3c–h). Except for syncytin-1, all markers maintained over time a similar distribution between EVs of different size. The percentage of EVs double positive for markers was stable up to day 14, except EVs double positive for syncytin-1 and all other markers which increased slightly over time (Fig. S2a).

These results demonstrate that placental villous explants produce EVs carrying typical STB markers throughout the culture period. EVs expressing each marker maintained a similar size distribution over time, but the overall percent of vesicles carrying most of these markers decreased at later days of culture.

Analysis of Amnion EVs

In parallel to the analysis of the STB-released EVs, we analyzed the EVs released by amnion explants by identification of specific cellular antigens on these EVs. EVs were labeled with Bodipy FL as described in Methods. The main cells of interest in amnion explants were amnion epithelial cells (AECs) (since they are in contact with amniotic fluid, thus likely to be involved in fetal communication), as well as the underlying amnion mesenchymal stem cells (AMSCs). We used MNPs coupled to antibodies specific to antigens that these cells carry. Since CD90 is a marker expressed by both AECs and AMSCs[163], we investigated this protein as a target for capture with MNPs using anti-CD90 antibodies.

Optimizing capture of amnion EVs

We incubated amnion explant culture supernatants with anti-CD90 MNPs to capture EVs and compared them to capture with anti-CD63, anti-CD9 and anti-HLA-ABC coupled

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MNPs. MNPs coupled to CD9, HLA-ABC and to CD63 captured 113 ± 5.3%, 75.8 ± 16.7%, and 93.7 ± 11.8% of that of coupled to CD90, respectively (n=3). Since CD90 is the most exclusive marker for our cells of interest, we used anti-CD90 MNPs for our further experiments.

Specificity of EV capture

Next, we verified whether anti-CD90 MNPs specifically capture only EVs carrying CD90. As a negative control, we used these MNPs to capture EVs from placental villous culture supernatants (which should release very few EVs carrying CD90, potentially from placental MSCs[164]). We found that these MNPs captured on average only 2.5 ± 0.8% of total EVs (n=3). We also confirmed MNP specificity by incubating amnion tissue supernatants with mouse IgG isotype MNPs, which captured 6.3 ± 1.4% of EVs compared to anti-CD90 MNPs (n=3).

Lack of irrelevant antigens on AEC-generated EVs

We captured EVs from amnion samples from multiple donors with anti-CD90 MNPs and stained captured EVs for CD31, CD41, CD45, and HLA-DR, which should not be present on EVs of this origin[165]. All antibodies were labeled with the same fluorophore, APC-Cy7, and collected into a single "dump" gate. Our staining revealed that these markers were present on only 4.8 ± 0.5% of captured EVs. Further analysis of amnion EVs included only antibodies against HLA-DR, which contributed 2.8 ± 0.3% of total EVs (n=3), and this population was excluded from flow cytometry analysis.

AEC and AMSC markers are revealed on amnion explant EVs

The distribution on EVs of several "phenotypic" markers expressed by AECs or AMSCs[166, 167], namely CD29, CD44, CD105, CD140b, CD324, and CD326, were determined. EVs were labeled with BoDipy-FL, captured with anti-CD90 MNPs and stained with antibodies to the above markers (in addition to HLA-DR). The labeled EV-MNP complexes were washed on magnetic columns, eluted and then acquired on a flow cytometer set to threshold on the BoDipy-FL label. Any vesicles positive for HLA-DR were excluded and the remainder quantified by size, estimated by Megamix SSC beads, and expression of the markers of interest (see Fig. S1 for gating strategy).

First, we evaluated EVs from amnion culture supernatants at day 1 of culture (Fig. 4a). We found that CD105 was the most highly expressed marker being present on 18.5 ± 0.7% of all captured EVs, and CD140b was the least expressed on 4.0 ±0.6% of EVs (n=10). CD44, CD326, CD324, and CD29 were on approximately on 15, 12, 10, and 9% of EVs respectively. EVs were equally distributed among most size ranges, except EVs of 200nm or less which were only 4.6 ± 0.6% of all EVs (Fig. 4b). Most markers were more likely to be on larger vesicles (Fig 4c–h). CD105 was highest on vesicles of the size of 500nm and over, CD140b was much higher on vesicles with the size over 500nm, and CD44, CD324, CD326 and CD29 were distributed more evenly between all size ranges except the smallest.

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Evaluation of marker co-expression demonstrated that CD29 and CD44 were the most commonly found together (4.2 ± 0.7% of EVs at day 1), followed by CD140b and CD326 (3.1 ± 0.7% EVs at day 1) (Fig. S2b).

Next, we investigated how the number of CD90-captured vesicles changed over time. Unlike PLAP captured EVs from placental villous explants, the amount of amnion-generated vesicles captured with CD90-MNPs did not decrease over time. The total concentration of vesicles at day 1 was $9.5 \pm 1.4 \times 10^4$/mL and at day 14 was $9.9 \pm 1.8 \times 10^4$/mL (see Table S3 for all EV counts). The amount of amnion EVs remained constant over the entire culture period in all aspects: in size ranges of vesicles (Fig. 4b), in the fractions of total EVs for each (Fig. 4c–h), and for the fractions of double positive EVs (Fig. S2b).

These results confirm that amnion explants continually produce EVs representative of AECs and AMSCs over 14 days of culture.

Analysis of EV-associated cytokines

EVs from different cells carry different cytokines

We captured EVs from culture supernatants at day 4 with MNPs coupled with specific capture antibodies to investigate whether EVs with different surface markers (i.e. generated by different cells) carry different cytokines.

Placental villous EVs

Total EVs were isolated from placental villous culture supernatants using Exoquick TC™. From this isolate we captured several types of EVs using anti-PLAP coupled MNPs to capture STB-generated EVs, anti-CD31 MNPs to capture EVs generated by endothelial cells, and HLA-G to capture EVs released by cytotrophoblasts and placental MSCs. Following MNP capture, EVs were magnetically isolated as described in Methods, and the EV-associated cytokines and growth factors were evaluated. Surface associated proteins were measured directly with multiplexed bead assays, and total EV proteins were measured after EVs were lysed. We then subtracted the surface quantity from the total to determine the internal protein concentrations.

Most cytokines were found associated with EVs, and those in the greatest amounts were IL-4, IL-8, IL-10, IL-13, IL-33, Calgranulin C, CRP, IFNγ, IP-10, MIF, MIG, MIP-3α, and TRAIL. (See Table S4 for cytokine concentrations). Overall, cytokines tended to be EV-encapsulated rather than on their surface (Fig. 5). HLA-G captured EV had slightly more cytokines on their surface compared to anti-PLAP or anti-CD31 captured EVs. PLAP captured EVs carried significantly more IL-4, IL-16, MIG, and TGF-β compared to both other types of capture ($p<0.05$, $n=5$), and were located predominantly inside EVs (Fig. 5b). CD31 captured EVs were significantly higher in MIP3α and CXCL6 compared to HLA-G captured EVs only ($p<0.05$, $n=5$), and these were encapsulated (Fig. 5c). HLA-G captured EVs were higher than both other captures in GM-CSF, IP-10 and MIF ($p<0.05$, $n=5$) and these were both on the surface and encapsulated (Fig. 5d).

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Amnion EVs

Total EVs were isolated from amnionic culture supernatants using Exoquick TC™. Amnion EVs were captured with anti-CD90 MNPs, to capture presumably EVs from both AECs and AMSCs, and HLA-G antibodies to capture EVs from selected cells, as HLA-G has been reported in various levels on AECs and only weakly on AMSCs. Also, many cytokines were associated with EV (see Table S5) especially IL-4, IL-8, IL-10, IL-13, IL-33, Calgranulin C, GRO-α, IFNγ, MIF, MIG, MIP-3α, and TRAIL. Similar to EVs from placental villous explants, for amnion explant EVs, most cytokines were predominantly inside EVs (Fig. 6). HLA-G captured EVs expressed slightly more cytokines on their surface compared to CD90 captured EVs (Fig. 6b–c). CD90 MNP-captured EVs had significantly higher amounts of IL-4, IL-10, IL-13, IL-33, CXCL6, Eotaxin, ITAC, MIG, MIP3α, and TGF-β than HLA-G captured EVs ($p<0.05$, n=5) and most were predominantly inside (Fig. 6b). HLA-G captured the highest levels of Calgranulin C, GM-CSF, MIF and MIP-1β compared to CD90 captured EVs ($p<0.05$, n=5), and most were internal to the EVs (Fig. 6c).

EVs from different cells carry different growth factors

EVs from placental villous tissue also contained several growth factors and angiogenic related factors (see Table S6). Activin A, adiponectin, endoglin, fibronectin, galectin-1, ICAM-1, IL-1RA, IL-27, MMP-9, PAPP-A, serpin E1, TFPI, TIMP-1, TREM-1, uPA, uPAR, and VEGFR2 were found in the greatest quantities, as well as hCG and PGE2. Similar to cytokines, these growth factors were predominantly encapsulated within EVs rather than on their surface (Fig. 7), although HLA-G captured EVs had more surface-associated than the other two captures. PLAP captured EVs had significantly higher amounts of EV-associated ADAM12, endoglin, and PIGF than either CD31 or HLA-G captured EVs ($p<0.05$, n=5). PIGF was mostly on the surface of EVs, whereas ADAM12 and endoglin were predominantly encapsulated (Fig. 7b–d). CD31 captured EVs carried significantly higher amounts of internal IL-27 and TREM-1 than HLA-G EVs ($p<0.05$, n=5). HLA-G captured EVs contained significantly more adiponectin, CD40L, EGF, FasL, fibronectin, galectin-1, PGE2, Resistin, TFPI, TGF-β3, Tie-2, tissue factor, TREM-1, uPA, uPAR, VEGFR1, and VEGFR2 than both PLAP and CD31 captured EVs ($p<0.05$, n=5).

Growth and angiogenic factors were also found associated with amnion EVs (see Table S7), with activin A, adiponectin, fibronectin, galectin-1, ICAM-1, IL-1Ra, PAPP-A, serpin E1, TFPI, TIMP-1, TREM-1, uPA, uPAR, and VEGFR1, as well as hCG and PGE2, secreted in the highest amounts. Amnion EVs also carried most growth factors predominantly inside EVs, and HLA-G captured EVs had slightly more surface growth factors than CD90 captured (Fig. 8). CD90 MNPs captured EVs had significantly higher amounts of PAPP-A, and TREM-1 ($p<0.05$, n=5), with PAPP-A being predominantly inside and TREM-1 being both on the surface and inside (Fig. 8b). HLA-G captured significantly higher levels of adiponectin, CD40L, EGF, endoglin, FasL, galectin-1, ICAM-1, IGFBP1, IL-1Ra, PGE2, resistin, TFPI, TGF-β3, Tie-2, tissue factor, uPA, uPAR, VEGFR1 and VEGFR2 ($p<0.05$, n=5), much the same as HLA-G captured villi EVs (Fig. 8c).

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Discussion

Previous studies of placental explants

Several techniques for maintaining placental explants have been described[168–171], with different models being useful for different purposes. Typically, placental tissues are immersed in the culture medium either free floating or supported by Matrigel™ or Millicell inserts. In these experiments, tissues remain viable up to 9 days and produce human chorionic gonadotropin (hCG) and placental lactogen [172]. Most of these models report that STB are lost in the first 1–2 days of culture but some regeneration was observed by 5–7 days[173, 174].

Following the pioneer works of Hoffman et al[175, 176], we developed cultures of *ex vivo* tissues maintained on collagen sponges at the medium/air interface to study HIV pathogenesis in human lymphoid[177–181], cervico-vaginal[182–184] and recto-sigmoid tissues[185], and to investigate the physiology of atherosclerotic plaques [186, 187] *ex vivo*. A comparable culture method was used to study cytomegalovirus infection[188]. Here, we apply a similar technique to study placental tissue secretion of EVs, cytokines and growth factors *ex vivo*.

The establishment of a three-dimensional culture to study extracellular vesicles and cytokines

The purpose of the present study was to develop a laboratory model to study soluble factors and EVs generated by placental villous tissue. This is important since both EVs and soluble factors, in particular placental cytokines[189, 190], are implicated in maternal-fetal communication. This especially concerns STB that are in direct contact with the maternal blood, and amnion epithelial cells that are surrounding the amniotic cavity containing the fetus. We found that under our protocol, explants of both placental villous tissue and amnion are viable for at least 14 days as evidenced by histological analysis. Both types of explants continue to secrete cytokines and growth factors over 14 days of culture providing further evidence of tissue viability and functionality.

Evaluating these secreted factors in tissue models allows the determination of tissue origin of these factors, which is not easily accomplished *in vivo*. Yet, we cannot rule out a minor contribution of factors derived from entrapped maternal or fetal cells in placental vessels.

A number of publications have addressed placental EVs and their potential role in pregnancy and its complications[130, 132, 133, 146, 191–225]. Several *ex vivo* (e.g. placental perfusion)[214, 226–232] and *in vivo*[233–236] systems have been used as a source of EVs. Placental perfusion is a useful method for obtaining large numbers of EVs directly from the placenta; however, this technique is suitable only for a short period of time (2–6 hours) after delivery[237]. *In vivo* studies on EVs obtained from maternal blood are difficult to interpret because of multiple potential cellular sources of these EVs. Focusing on the analysis of EVs generated by placental cells requires the ability to trace particular EVs to their cells of origin. Towards this goal, rather than “bulk” analysis of EVs, we employed a newly developed nanotechnology platform[238], which allows capture of EVs with magnetic nanoparticles (MNPs) coupled to specific antibodies against EV surface antigens and

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analyzing these EVs *individually*. The captured EVs can then be stained with additional antibodies to reveal specific antigens of interest. Here, we applied this analysis to EVs generated by placental explants.

Analysis of placental villous extracellular vesicles

We found that STB-specific EVs can be captured from placental villous culture supernatants using anti-PLAP MNPs. PLAP is a sialoglycoprotein enzyme that is present almost exclusively on STB and has been used as a marker of STB-derived EVs[123, 153, 155]. We first demonstrated specificity of capture by demonstrating that anti-PLAP MNPs capture significant amounts of EVs from placental villous explants but very few EVs from culture supernatants of amnion explants. PLAP captured EVs also do not express non-STB markers including CD31, CD41, CD45, and HLA-ABC above the background level (EVs captured by isotype control MNPs). EVs were expressed throughout the entire 14 days of culture, though their quantities declined at day 14.

We assessed the PLAP-captured EVs for other surface proteins that have previously been described to be expressed on STB or on their EVs, CD51, CD63, CD105, CD200, CD274, and syncytin-1. All these proteins were found albeit in various quantities on PLAP-MNP-captured EVs[239]. CD51, or vitronectin receptor alpha chain, is an adhesion molecule[239]. CD63 is a tetraspanin known to associate with membranes of intracellular vesicles[239]. CD105, also known as endoglin, has a crucial role in the regulation of angiogenesis[240]. CD200, also named OX-2 membrane glycoprotein, may have a role in macrophage differentiation[241]. CD274 or programmed death-ligand 1(PD-L1) is an immune checkpoint molecule that may have a role in immune suppression during pregnancy[242–244]. Syncytin-1 mediates trophoblast fusion and may have a role in tolerance to fetal antigens[245, 246]. Herein, CD200 was the most widely expressed marker and syncytin-1 the least expressed. These markers demonstrated some differences in their expression on EVs of different size ranges, for instance most markers were expressed on only a small percentage of small EVs, except for CD200. These differences may reflect differential function of these EVs. Whatever are these functions, the overall the pattern of these antigens expression on the different sizes of EVs remained constant again demonstrating viability of the *ex vivo* tissues. Also, co-expression of the various markers remains fairly constant over time.

Analysis of extracellular vesicles produced by amnion

EVs were also produced by amnion and were captured with anti-CD90 MNPs. CD90 is a cell surface glycoprotein involved in cell adhesion that is expressed on both AECs and AMSCs, as well at varying levels on fibroblasts, neurons and activated endothelial cells[163, 247–250]. We confirmed specificity of capture by showing anti-CD90 MNPs captured very few EVs generated by placental villous tissue. Also, CD90 MNP-captured EVs lacked expression of markers that should not be present on amnion-generated EVs, including CD31, CD41, CD45, and HLA-DR. EVs were generated at constant levels throughout all the 14 days of culture, and maintained the same size distributions. These EVs carried other proteins on their surface that have previously been described on AECs and AMSCs. These included CD29, CD44, CD105, CD140b, CD324, and CD326, which are involved in cell-cell and cell-matrix interactions, cell adhesion, and migration[251]. CD29 (integrin beta-1) acts as a

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fibronectin receptor[252]. CD44 is a receptor for hyaluronic acid[253]. CD140b is a tyrosine kinase receptor for members of the platelet derived growth factor family and a marker for naive AMSCs[254]. CD324 or E-cadherin is a regulator of epithelial junction formation[255]. CD326, also known as Ep-CAM, is an epithelial cell surface antigen[256]. Herein, CD105 was the most widely expressed marker and CD140b was the least expressed on amnion-derived EVs. These markers demonstrated some differences in their expression on EVs of different size ranges, but all were least prevalent on the smallest vesicles. Overall the pattern of expression on the different sizes of EVs remained constant over time. Moreover, co-expression of the various markers remains fairly constant over time.

Cytokines and other factors in EVs of different phenotype

We previously reported that various cytokines are associated with EVs[257]. Here, we demonstrate that not only cytokines, but many other growth factors, angiogenic and anti-angiogenic factors are associated with EVs from placental villous and amnion tissues. These factors can be on the EV-surface or encapsulated within the vesicles. In this study, we took this analysis one step further from the analysis of association of these factors with general EVs to their association with EVs that carry particular membrane proteins. Specifically, we captured EVs using MNPs coupled to antibodies that select for certain EV populations, and analyzing the cytokine and growth factor content of these EV fractions.

We found that placental villous EVs captured via PLAP, CD31, and HLA-G not only carry different levels of these factors, but their distributions between the EV surface and internal space were different. Some cytokines segregated completely between different EVs. For example, Eotaxin and HMGB1 were present only in HLA-G MNP-captured EVs, and ITAC was observed exclusively in CD31 MNP-captured EVs. IL-13, RANTES, and PGE2 were not present in CD31 EVs but were found in both PLAP and HLA-G captured EVs, whereas hCG was absent in HLA-G EVs. Some cytokines were carried exclusively on the EV surface, for example IL-4, IL-13, and Eotaxin in HLA-G MNP-captured EVs, whereas IL-16, IL-33 and RANTES were exclusively inside HLA-G MNP-captured EVs. Other cytokines were found on the surface in EVs captured through one membrane protein, but internally in EVs captured through another protein. For example, IL-4 and MIG were found internally in EVs captured with PLAP MNP and CD31 MNP, but on the surface in HLA-G MNP-captured.

In amnion tissue, we specifically captured EVs using anti-CD90 and anti-HLA-G MNPs, analyzed their cytokine and growth factor content, and found differences in amounts and distributions of these EV-associated proteins. For example, only EVs captured via CD90 but not via HLA-G carried IL-4, Eotaxin and ITAC. CD40L, PGE2, and uPAR were encapsulated in CD90 MNP-captured EVs but were present both inside and on the surface HLA-G MNP captured EVs.

This complex differential distribution of cytokines between EVs of different origin and phenotype suggests a fine regulation of their biogenesis and indicates different biological functions of these EVs. To identify these functions EVs should be characterized individually rather than in bulk. The ability to characterize and distinguish individual EVs generated by different cell types and carrying various cytokines and growth factors is the major advantage

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of our methods. Also, we can identify EVs that co-express different membrane proteins. For instance, CD90 and HLA-G in amnion may be co-expressed on some EVs, and CD31 and HLA-G may be co-expressed in placental villous tissue. This distinction may be the reflection of their differential biological role.

The use of the placental tissue culture described herein coupled with the newly described nanotechnology provides a novel and powerful tool for probing maternal-fetal communication through EVs that can be now traced to their cellular/tissue origin, characterized by their surface-associated and encapsulated proteins. This multifactorial characterization of EVs in an *ex vivo* tissue system will enable us to narrow the search for possible placental biomarkers in maternal blood and amniotic fluid and identify their changes in various pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported, in part, by the Perinatology Research Branch (PRB), Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services (NICHD/NIH/DHHS), and, in part, with federal funds from the NICHD/NIH/DHHS under Contract No.HHSN275201300006C. N.G-L is also supported by the Wayne State University Perinatal Initiative in Maternal, Perinatal and Child Health. We thank the physicians and nurses from the Center for Advanced Obstetrical Care and Research and the Intrapartum Unit, as well as the research assistants from the PRB Clinical Laboratory, for their help in collecting samples. In addition, we thank Suzanne M. Jacques, M.D. and Faisal Qureshi, M.D. for analysis of histological sections of tissue explants. This work was supported by NICHD Intramural Program.

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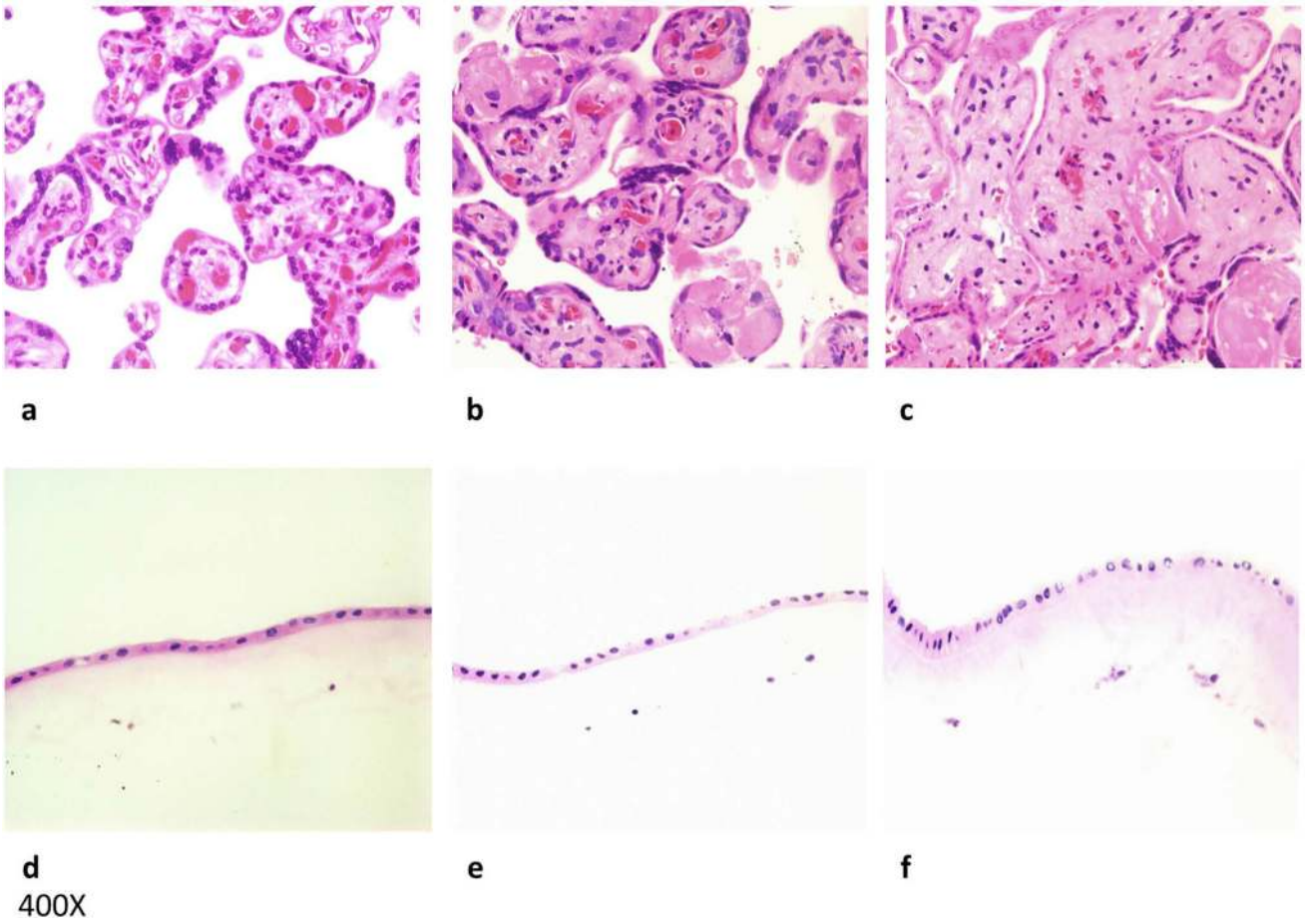

Figure 1. Placental villous and amnion tissue explants maintain their cytoarchitecture

H&E sections of placental villous explants at **(a)** day 1, **(b)** 7, and **(c)** 14 of culture (one representative tissue out of 10). Villi maintained normal morphology with well-preserved syncytiotrophoblasts and blood vessels with some focal degenerative changes. H&E sections of amnion explants at **(d)** day 1, **(e)** 7, and **(f)** 14 of culture also show well-preserved tissue with focal degenerative changes at day 14.

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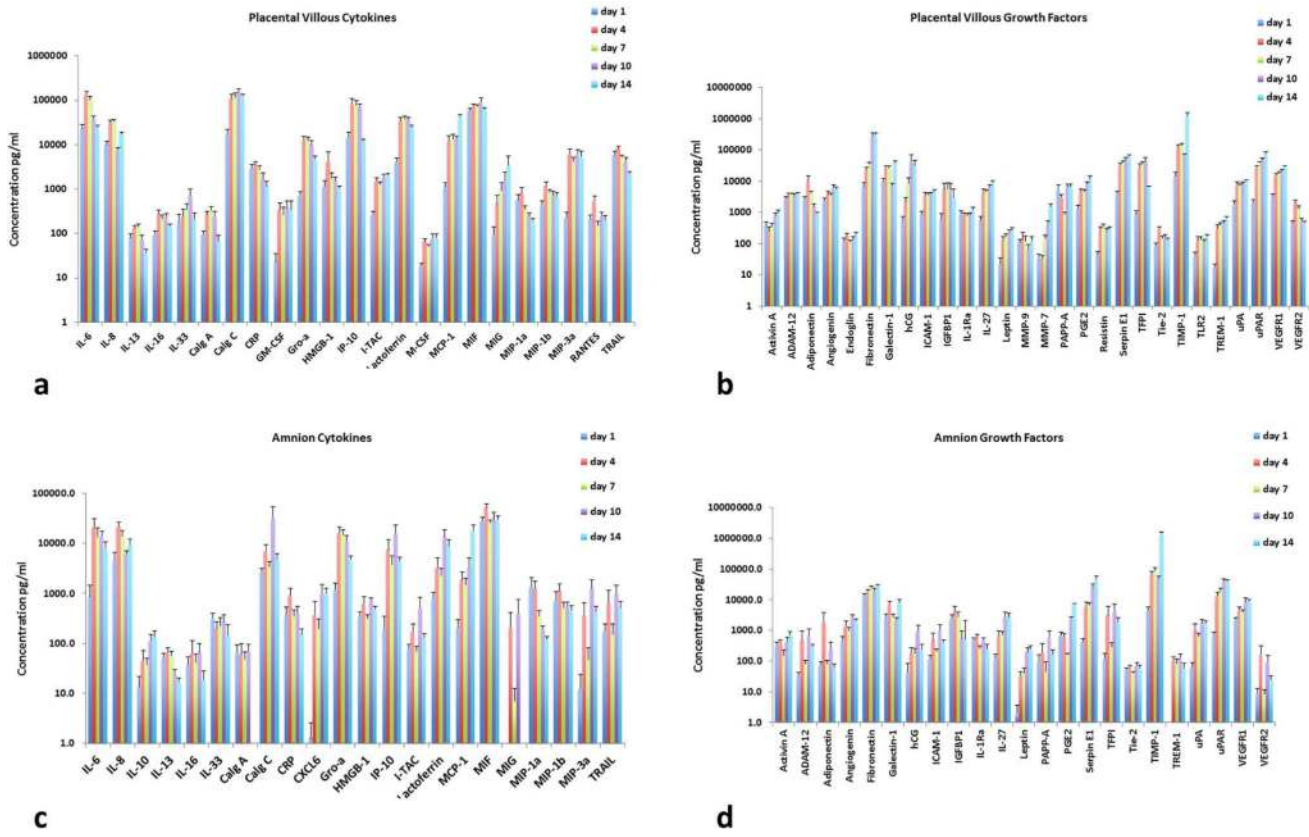

Figure 2. Placental villous and amnion tissue explants maintain cytokine and growth factor production throughout culture period

Soluble cytokines, growth factors, angiogenic and anti-angiogenic factors are produced by explants over the entire 14-day culture period (presented are average productions, mean ± SEM) as measured by multiplexed bead assays. Culture medium is replaced at each sampling time point.

(a) Placental villous explants: amounts of cytokines released at day 1, 4, 7, 10, and 14, n=10; (**b**) Placental villous explants: amounts of growth factors released at day 1, 4, 7, 10, and 14, n=10; **(c)** Amnion explants: amounts of cytokines released at day 1, 4, 7, 10, and 14, n=10; **(d)** Amnion explants: amounts of growth factors released at day 1, 4, 7, 10, and 14, n=10.

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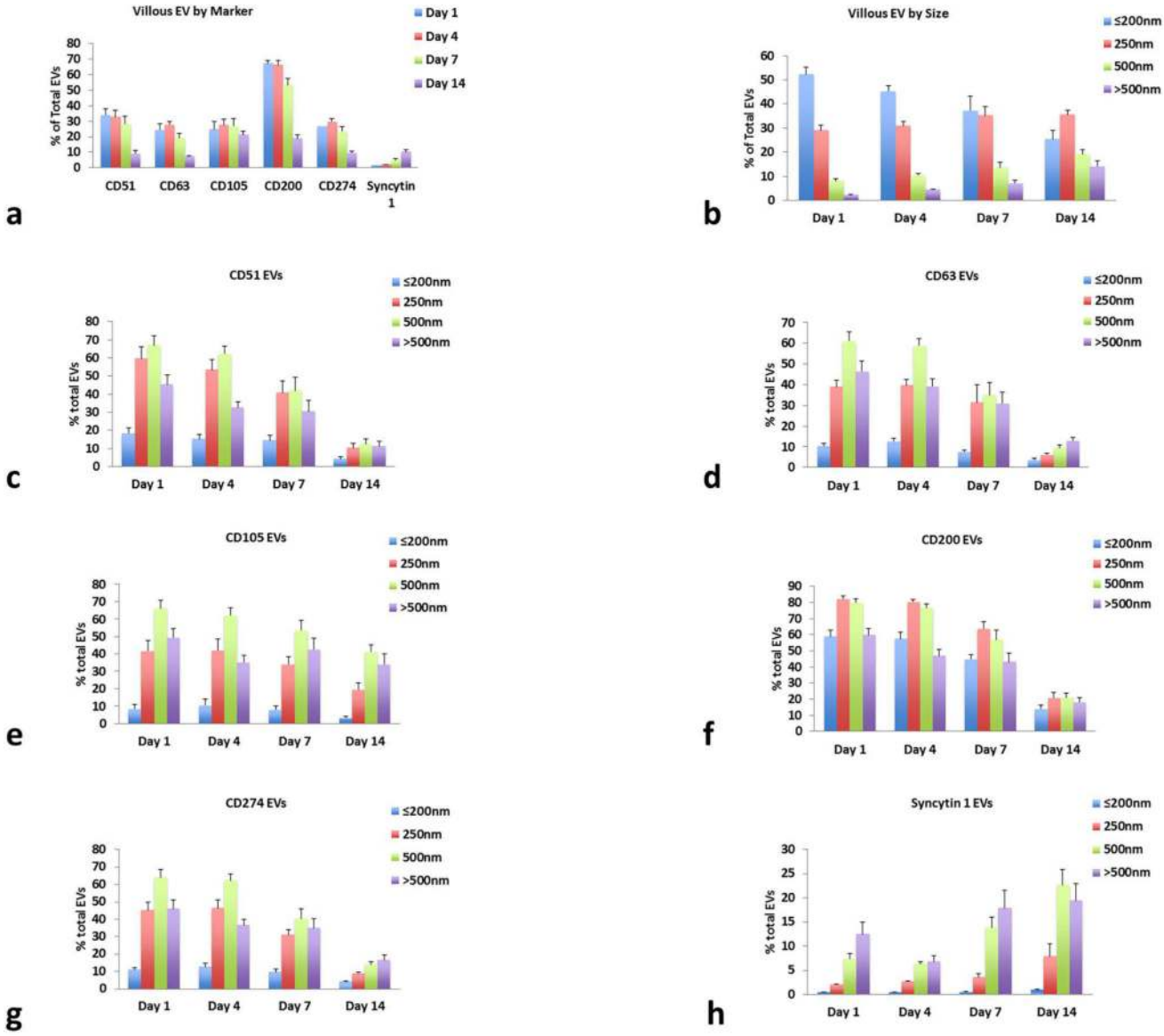

Figure 3. Placental villous tissues release a variety of EVs carrying different surface markers

Placental villous explants release EVs that **(a)** carry surface markers that are representative of syncytiotrophoblast cells throughout culture and **(b)** are of a variety of sizes (average % of total EVs for each time point ± SEM, n=10). EVs carrying (**c**) CD51, **(d)** CD63, **(e)** CD105, **(f)** CD200, **(g)** CD274, and **(h)** syncytin-1 maintain similar patterns of expression over time and some are preferentially on EVs of certain sizes (Average % of total EVs for each size range. Mean ± SEM, n=10).

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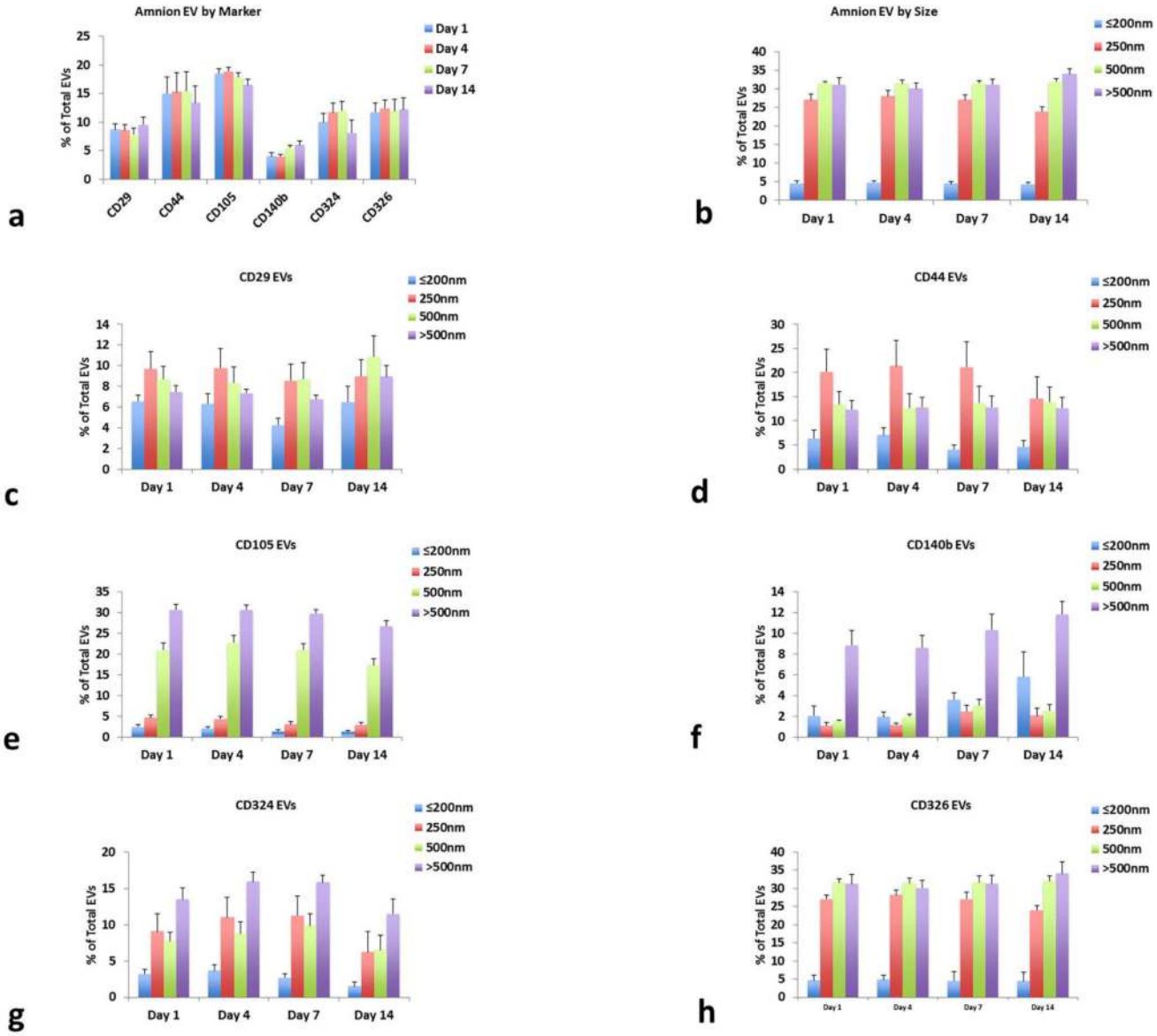

Figure 4. Amnion tissues release a variety of EVs carrying different surface markers

Amnion explants release EVs that **(a)** carry numerous surface markers that are representative of amnion epithelial and mesenchymal cells throughout culture and **(b)** are of a variety of sizes (average % of total EVs for each time point ± SEM, n=10). EVs carrying **(c)** CD29, **(d)** CD44, **(e)** CD105, **(f)** CD140b, **(g)** CD324, and **(h)** CD326 maintain similar patterns of expression over time and some are preferentially on EVs of certain sizes (average % of total EVs for each size range. Mean ± SEM, n=10).

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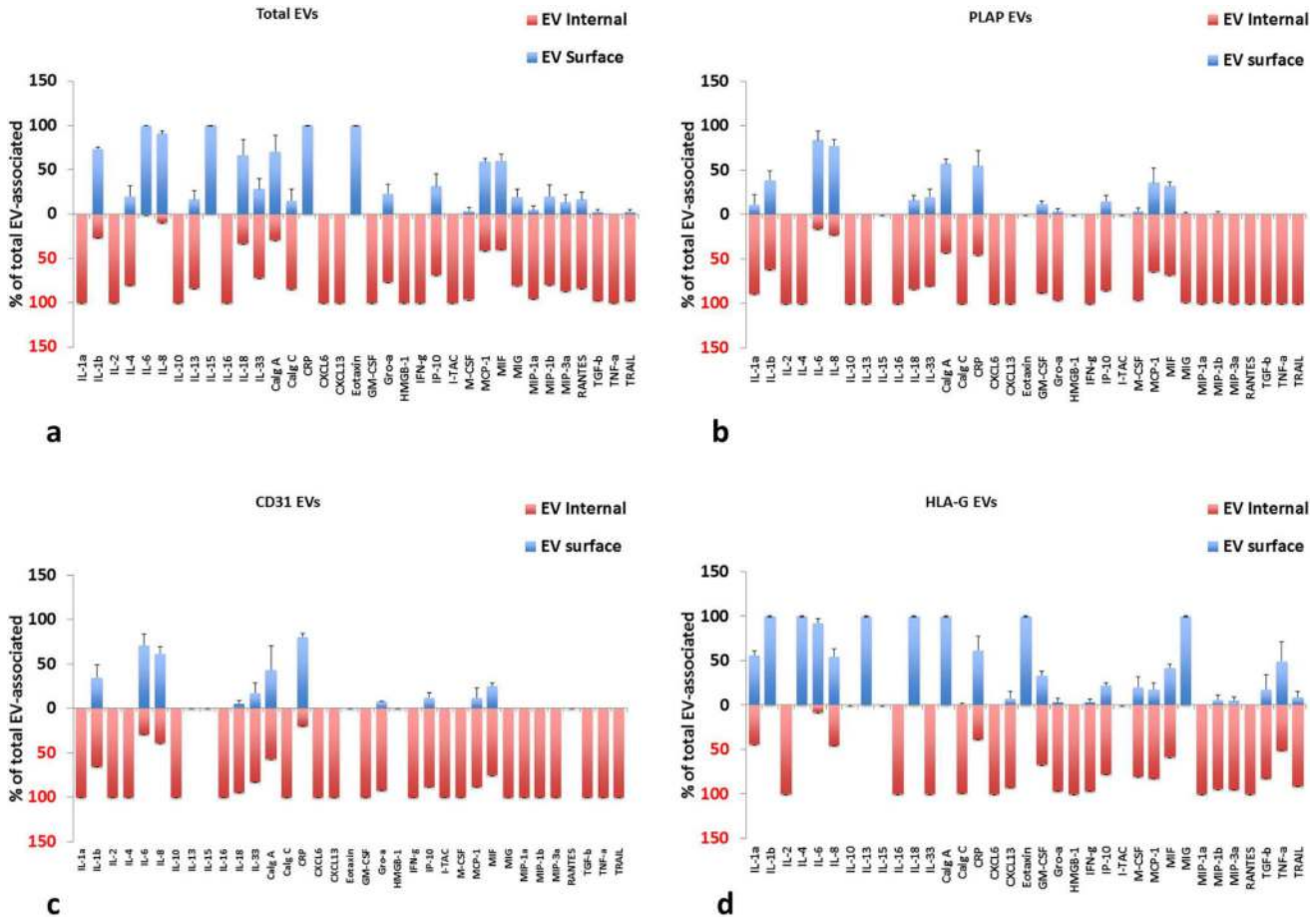

Figure 5. Distribution of cytokines between the surface and inner volume of EVs from placental villous tissues

Distribution between encapsulated and surface cytokines is shown for placental villous cultures. **(a)** Total EVs isolated by Exoquick™ **(b)** anti-PLAP MNP-captured EVs; **(c)** anti-CD31 MNP-captured EVs; **(d)** anti-HLA-G MNP-captured EVs. Free and EV-associated cytokines are expressed as percent of total (Mean ± SEM, n=5). Blue bars: surface-associated cytokines, red: EV-encapsulated. Multiplexed bead assay measurements on samples collected at day 4 (cumulative amount for days 1–4 of culture).

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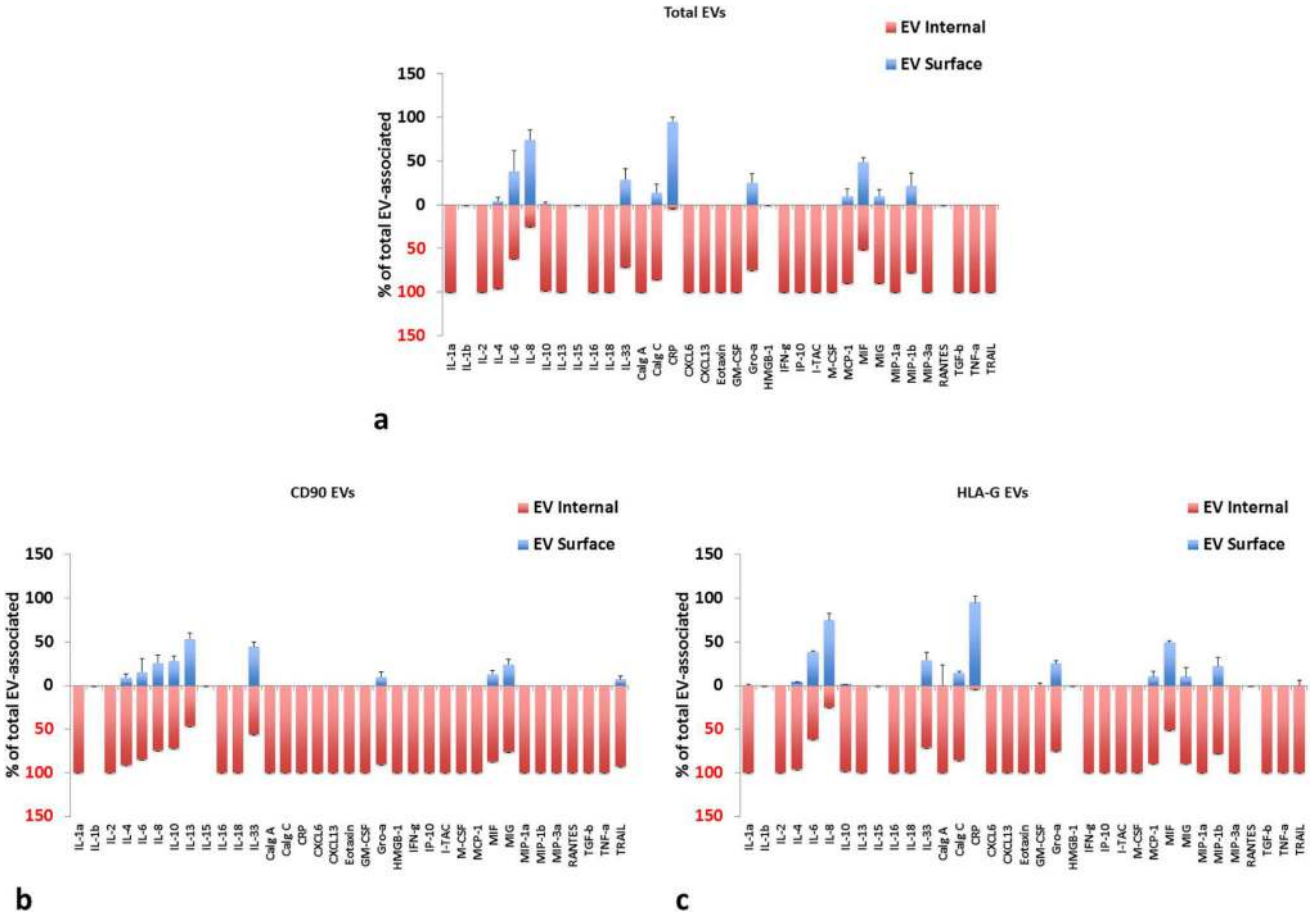

Figure 6. Distribution of cytokines between the surface and inner volume of EVs from amnion tissues

Distribution between encapsulated and surface cytokines is shown for amnion cultures **(a)** Total EVs isolated by Exoquick™; **(b)** anti-CD90 MNP-captured EVs; **(c)** anti-HLA-G MNP-captured EVs. Free and EV-associated cytokines are expressed as percent of total (Mean ± SEM, n=5). Blue bars: surface-associated cytokines, red: EV-encapsulated. Multiplexed bead assay measurements on samples collected at day 4 (cumulative amount for days 1–4 of culture).

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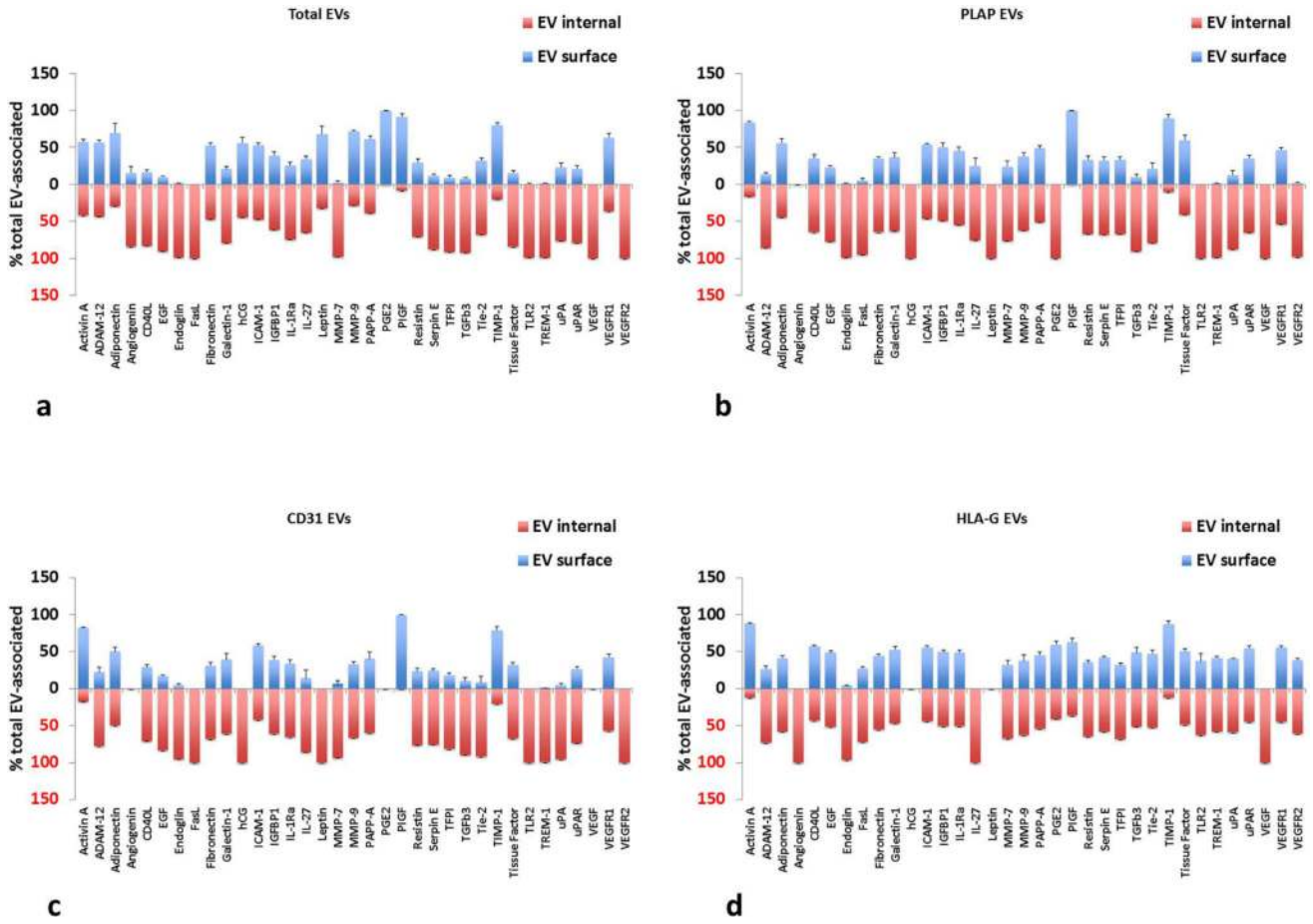

Figure 7. Distribution of growth factors between the surface and inner volume of EVs from placental villous tissues

Distribution between encapsulated and surface growth factors is shown for placental villous cultures. **(a)** Total EVs isolated by Exoquick™; **(b)** anti-PLAP MNP-captured EVs; **(c)** anti-CD31 MNP-captured EVs; **(d)** anti-HLA-G MNP- captured EVs. Free and EV-associated growth factors are expressed as percent of total (Mean ± SEM, n=5). Blue bars: surface-associated growth factors, red: EV-encapsulated. Multiplexed bead assay measurements on samples collected at day 4 (cumulative amount for days 1–4 of culture).

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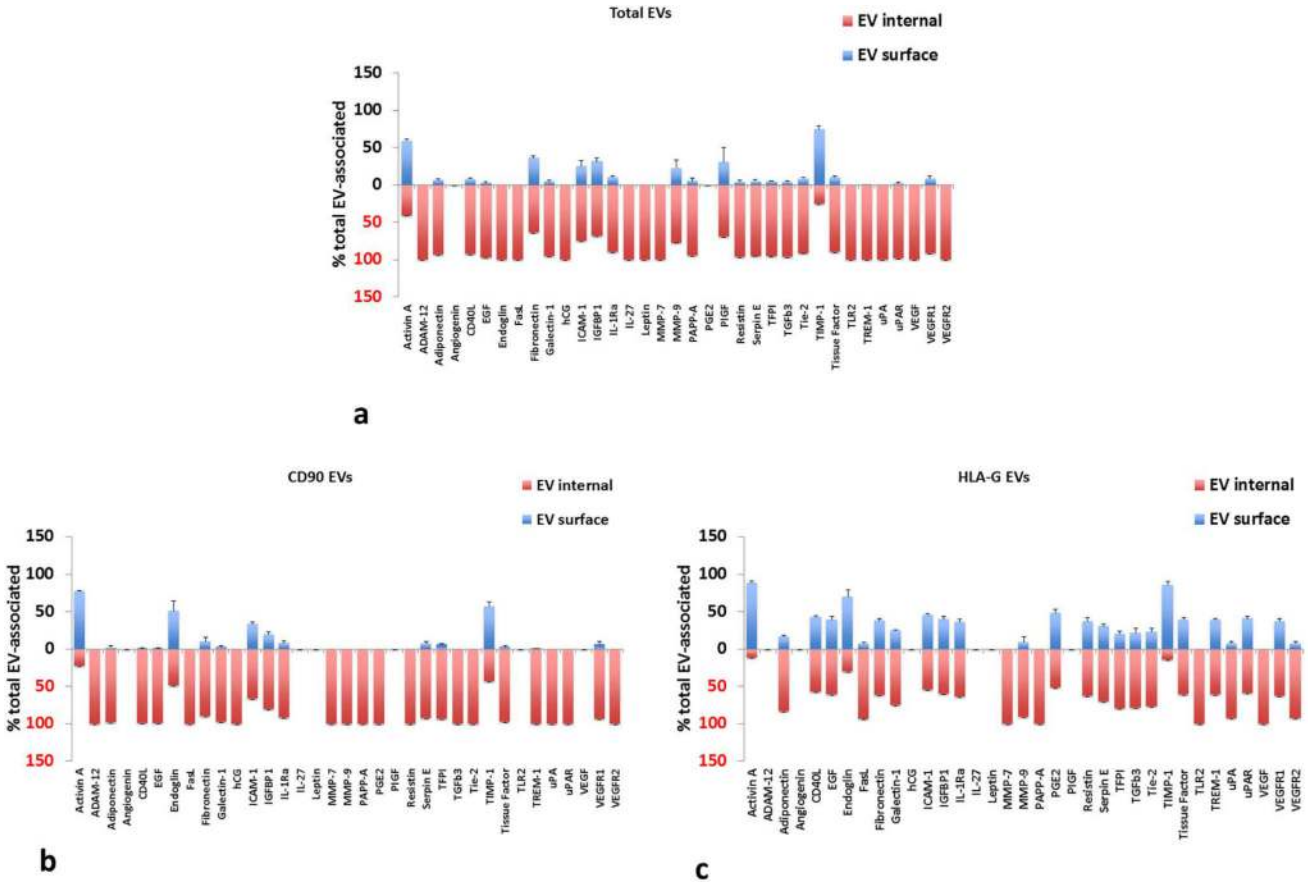

Figure 8. Distribution of growth factors between the surface and inner volume of EVs from amnion tissues

Distribution between encapsulated and surface growth factors is shown for amnion cultures. **(a)** Total EVs isolated by Exoquick™; **(b)** anti-CD90 MNP-captured EVs **(c)** anti-HLA-G MNP-captured EVs. Free and EV-associated growth factors are expressed as percent of total (Mean ± SEM, n=5). Blue bars: surface-associated growth factors, red: EV-encapsulated. Multiplexed bead assay measurements on samples collected at day 4 (cumulative amount for days 1–4 of culture).