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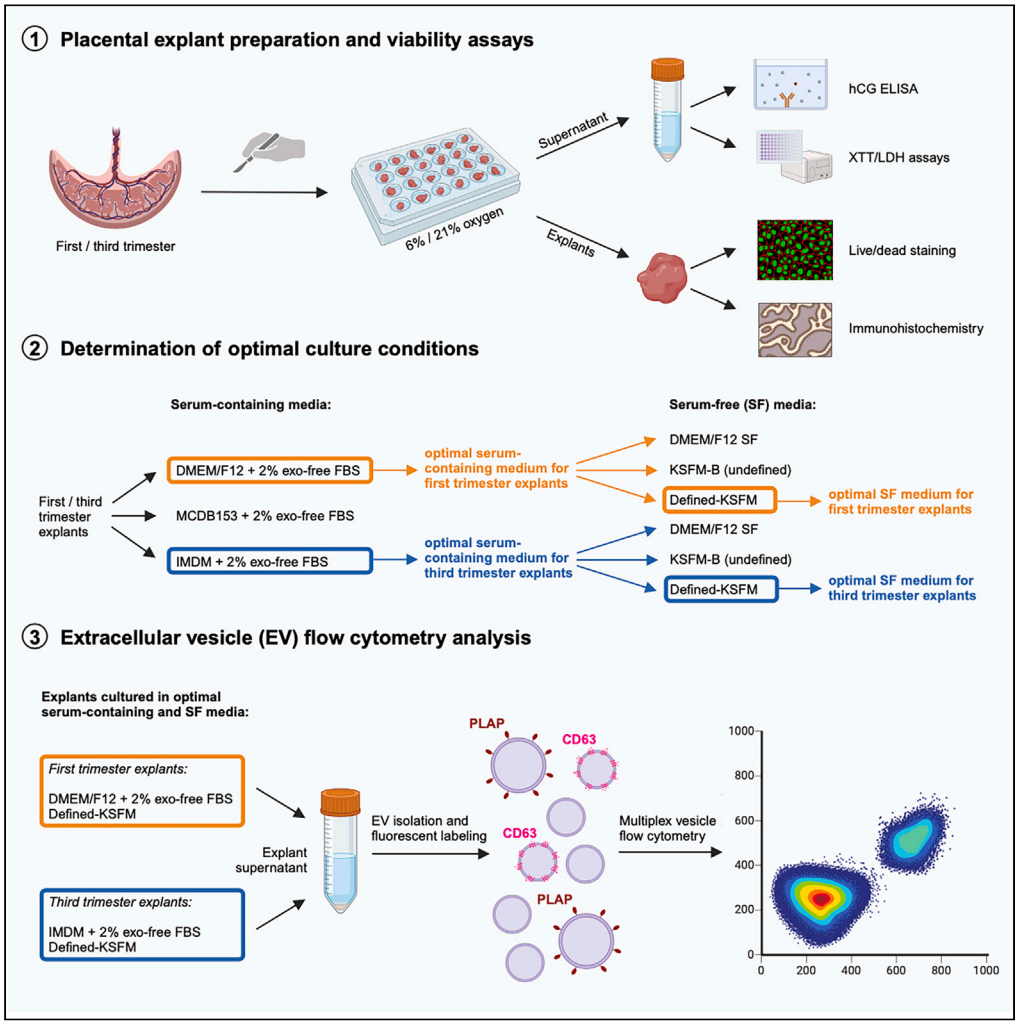

Chandana Tekkatte, Scott A. Lindsay, Erika Duggan, ..., John Nolan, Mana M. Parast, Louise C. Laurent

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Highlights

Identified optimal conditions for 1st and 3rd trimester placental explant culture

Syncytial regeneration not observed, even at placental normoxia (6% O_2)

A multiplex vesicle flow cytometry assay for placental EVs was developed

1st and 3rd trimester placental explants have distinct EV surface marker profiles

Tekkatte et al., iScience 26, 108046
October 20, 2023
https://doi.org/10.1016/j.isci.2023.108046

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Identification of optimal conditions for human placental explant culture and extracellular vesicle release

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SUMMARY

Extracellular vesicles (EVs) can mediate intercellular communication, including signaling between the placenta and maternal tissues. Human placental explant culture is a versatile *in vitro* model system to investigate placental function. We performed systematic studies in different tissue culture media types and oxygen tensions to identify a defined serum-free culture condition that supports high trophoblast viability and metabolism, as well as the release of similar populations of EVs, compared to traditional undefined conditions that contain media additives potentially contaminated with exogenous EVs. We also determined the time frame in which trophoblast viability and functionality remain optimal. Multiplex vesicle flow cytometry with classical EV and placenta-specific markers revealed three separate populations of explant-derived EVs: small CD63^{+} EVs; large PLAP+ EVs; and CD63-/PLAP- EVs. These culture and analytical approaches will enable *in vitro* modeling of short-term effects of environmental perturbations associated with pregnancy complications on placental function and EV release.

INTRODUCTION

There is increasing evidence that extracellular vesicles (EVs) play important roles as mediators of intercellular communication.[1–3] Classified based on their biogenesis pathways, size distribution, molecular cargo, morphology and functionality, EV subtypes include exosomes, microvesicles, apoptotic bodies, and oncosomes, with sizes ranging from 30 nm to 5000 nm.[2,4] EVs relay information between different cell types by facilitating the exchange of biomolecular cargo, such as extracellular RNAs (exRNAs), DNA fragments, cytosolic proteins and lipids.[5] The increased research focus on these nano-sized membrane-bound particles has revealed potential uses as clinical biomarkers and in therapeutics for various physiological and pathological disease conditions.[6,7]

EVs are of particular interest during pregnancy, a complex physiological state that is characterized by bidirectional signaling between the mother and fetus, which is in large part mediated by a sophisticated and transient organ – the placenta.[8] At the materno-fetal interface, the placental villus is comprised of a multinucleated syncytiotrophoblast (STB) layer responsible for nutrient and gas exchange, waste disposal, and production of signaling hormones and proteins that trigger maternal immunogenic adaptations to the pregnancy.[9,10] Apart from these hormone and protein signals, there is increasing evidence that the STB also produces EVs that mediate communication with the maternal immune system.[10] Diverse subtypes of EVs of placental origin have been found in abundance in maternal biofluids, starting as early as six weeks of gestation.[8,11] It has been reported that plasma concentrations of exosomes are 50-fold higher in pregnant than in non-pregnant women and are continually increasing over gestational age (GA).[12] Furthermore, the placenta is highly responsive to microenvironmental cues, such as changes in oxygen tension or glucose concentration; this is clearly reflected by higher plasma EV concentrations and unique exRNA signatures for different pregnancy complications such as preeclampsia and gestational diabetes mellitus.[7,13–15]

To investigate such disease-specific effects on the release, cargo, and function of human placental EVs, it would be desirable to establish a model system in which we can control the exposure of placental cells to disease-mimicking microenvironments and study the released EVs and exRNA cargo. *Ex vivo* whole placenta-perfusion has been used for studying placental EV release in placental dysfunction.[16–20] However,

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https://doi.org/10.1016/j.isci.2023.108046

this technique is less versatile and more labor-intensive, due to the complex experimental set-up, resulting in high technical variability and low reproducibility,[21] as well as the infeasibility of conducting adequately replicated experiments. Human trophoblast stem cell-based model systems, such as self-organizing 3D organoids, hold promise for studies of *in vitro* placental function in a three dimensional fashion,[9,22–25] but come with certain inherent limitations, such as the imperfect recreation/representation of the villus micro-architecture; specifically, organoids form as "inside-out" structures, with the apical aspects of the STB oriented inwards.

For many years, placental villus explants have been considered to be a model system that can closely mimic the *in vivo* milieu, including the tissue 3D microarchitecture, and are useful for studying placental function, metabolism, and EV release *in vitro*.[11,26–33] However, prior studies on the effect of culture media, culture duration, and oxygen tension on placental explants have not included the simultaneous evaluation of viability, function (by measuring hCG secretion from explants), morphology, and EV characteristics. Moreover, explant cultures have been traditionally prepared using media composed of undefined components (most commonly, fetal bovine serum [FBS]), which carry endogenous EVs and exRNAs that can confound studies aimed at determining the very roles of these entities in cell-cell communication.[30,34–36]

Here, we systematically compare human placental explants cultured in serum-based and chemically defined growth media at placental normoxia (6% oxygen) and ambient oxygen concentration (21% oxygen) using a comprehensive set of assays, including widely used functional parameters, as well as morphological and immunohistochemical characterization, to determine the optimal defined culture conditions and time frame where STB viability and functionality are maintained. We have also performed, for the first time, a detailed and rigorous characterization of both first and third trimester placental explant EVs, evaluating their size, count, and surface marker expression using a multiplex vesicle flow cytometry-based assay. Through this detailed protocol and comprehensive analysis, we provide a reproducible foundation on which future studies with these explants can be compared across different treatments to model placental disorders.

RESULTS

Identification of an optimal serum-containing medium for first-trimester placental explant culture

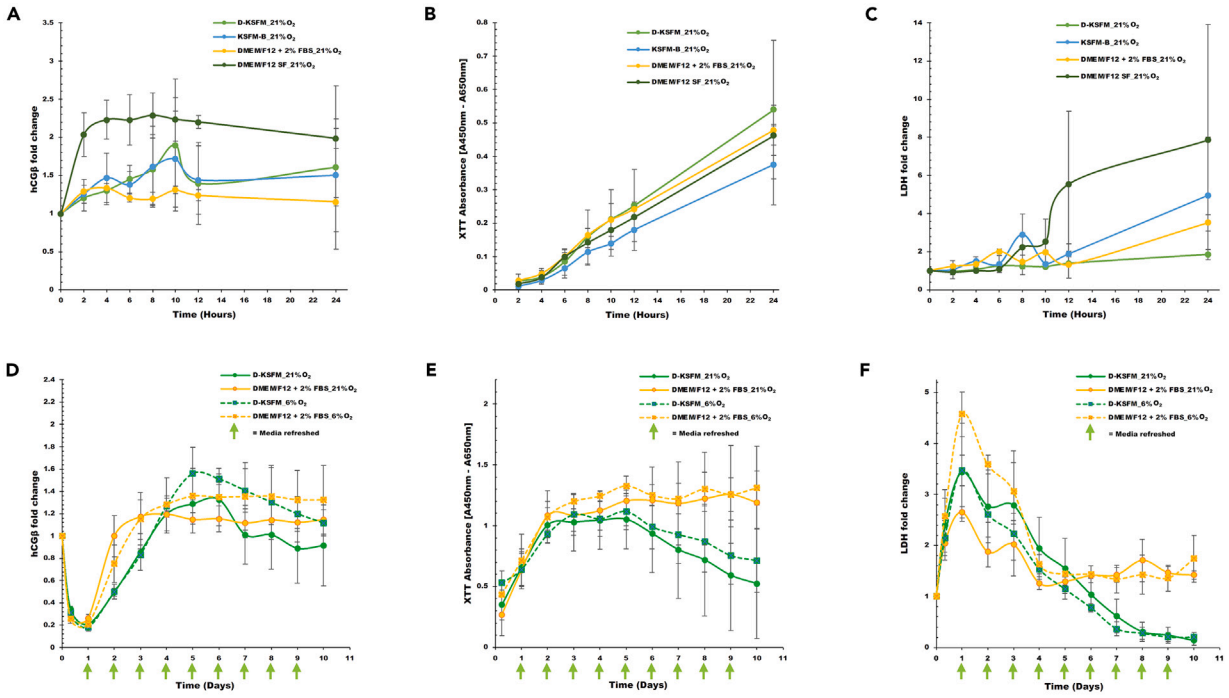
Because serum-containing media have been traditionally used for placental explant culture, but systematic studies comparing their relative performance were lacking, we first prepared placental explant cultures from first trimester [11 to 12 weeks GA] placentae (n = 2), at standard oxygen concentration, in three serum-based media types that have been previously used for human primary epithelial-type (trophoblast) cell culture: DMEM/F12, IMDM, and MCDB153. To minimize artifacts due to bovine EVs present in FBS, all serum used in the study was Gibco's "exosome-depleted" FBS (Cat # A2720801). Throughout this article, for each tested placenta, triplicate cultured wells of explants were prepared for each media type.

Active release of the human chorionic gonadotropin-beta (hCGβ) hormone is a direct functional indicator of a healthy villous STB layer. hCGβ ELISA performed on explant-conditioned media collected every 2 h of *in vitro* culture demonstrated an increasing concentration of the hormone for up to 8 h in DMEM/F12 + 2% FBS (Figure S1A). This indicates a steady secretion rate of hCGβ hormone over this time frame (Figure S1A). However, no such continuous hCGβ release was seen in IMDM +2% FBS or MCDB153 + 2% FBS conditions, where the hormone levels peaked at about 2 h of explant culture, plateauing and declining thereafter (Figure S1A). The XTT cell metabolism assay revealed that placental explants continued to have a steady rate of metabolism in all three media types during the full culture period up to 24 h after explant dissection. Notably, DMEM/F12 + 2% FBS media had a significantly higher metabolic rate ($p < 0.01$) compared to the other two media types (Figure S1B). The lactate dehydrogenase (LDH) assay was used to assess viability based on cell membrane integrity and permeability, and indicated a low level of placental cell death at the start of explant culture in DMEM/F12 + 2% FBS and IMDM +2% FBS, which did not increase during the 24 h after explant dissection (Figure S1C). In contrast, LDH levels in MCDB153 + 2% FBS were significantly higher than the other two media types ($p < 0.01$) (Figure S1C). Therefore, we concluded that first trimester placental explants cultured in DMEM/F12 + 2% FBS showed the best combination of high metabolic rate, low cell death, and continuous release of hCGβ, compared to IMDM +2% FBS and MCDB153 + 2% FBS.

Serum-free, defined media supports healthy first trimester placental explant culture, with the highest metabolic activity observed during the first 10 h

Next, we performed the same battery of assays described above on first trimester [10 to 12 weeks GA] placentae (n = 2) to determine whether the commercially available, chemically defined keratinocyte serum-free media (D-KSFM) (a low calcium medium used for primary trophoblast cell maintenance)[37] could perform as well as the best standard serum-containing medium for first trimester placental explants (DMEM/F12 + 2% FBS). We also tested the corresponding "undefined" version of KSFM (KSFM-B), which is supplemented with bovine pituitary extract (not FBS). Serum-free DMEM/F12 was also included as a "defined" serum-free standard media condition.

The hCGβ ELISA showed a positive linear slope for D-KSFM and KSFM-B, with both peaking at 10 h, (Figure 1A). hCGβ secretion in DMEM/F12 + 2% FBS remained mostly flat, indicating a halt in new secretion 2 to 4 h into explant culture (Figure 1A). hCGβ secretion was highest in serum-free DMEM/F12, but it peaked at 4 h into explant culture, suggesting a shorter period of viability (Figure 1A). The XTT assay showed that explants in all four tested media types had similar and steady metabolic rates through the 24 h after dissection (Figure 1B). The amount of LDH released was low in the first 10 h for explants in all four media types. By the 24-h timepoint, LDH detected in the spent media increased many folds in serum-free DMEM/F12 and KSFM-B, while the D-KSFM and DMEM/F12 + 2% FBS conditions showed the lowest levels of LDH on a consistent basis in comparison to the other 2 media conditions (Figure 1C). The results of this assay suggest that the apparent rapid secretion of hCGβ in serum-free DMEM/F12 (Figure 1A) may actually be due to release from dying STB. Viewed together, results from all three


A
hCGβ fold change
Time (Hours)
D-KSFM_21%O2
KSFM-B_21%O2
DMEM/F12 + 2% FBS_21%O2
DMEM/F12 SF_21%O2
B
XTT Absorbance [A450nm - A650nm]
Time (Hours)
C
LDH fold change
Time (Hours)
D
hCGβ fold change
Time (Days)
D-KSFM_6%O2
DMEM/F12 + 2% FBS_6%O2
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E
XTT Absorbance [A450nm - A650nm]
Time (Days)
F
LDH fold change
Time (Days)

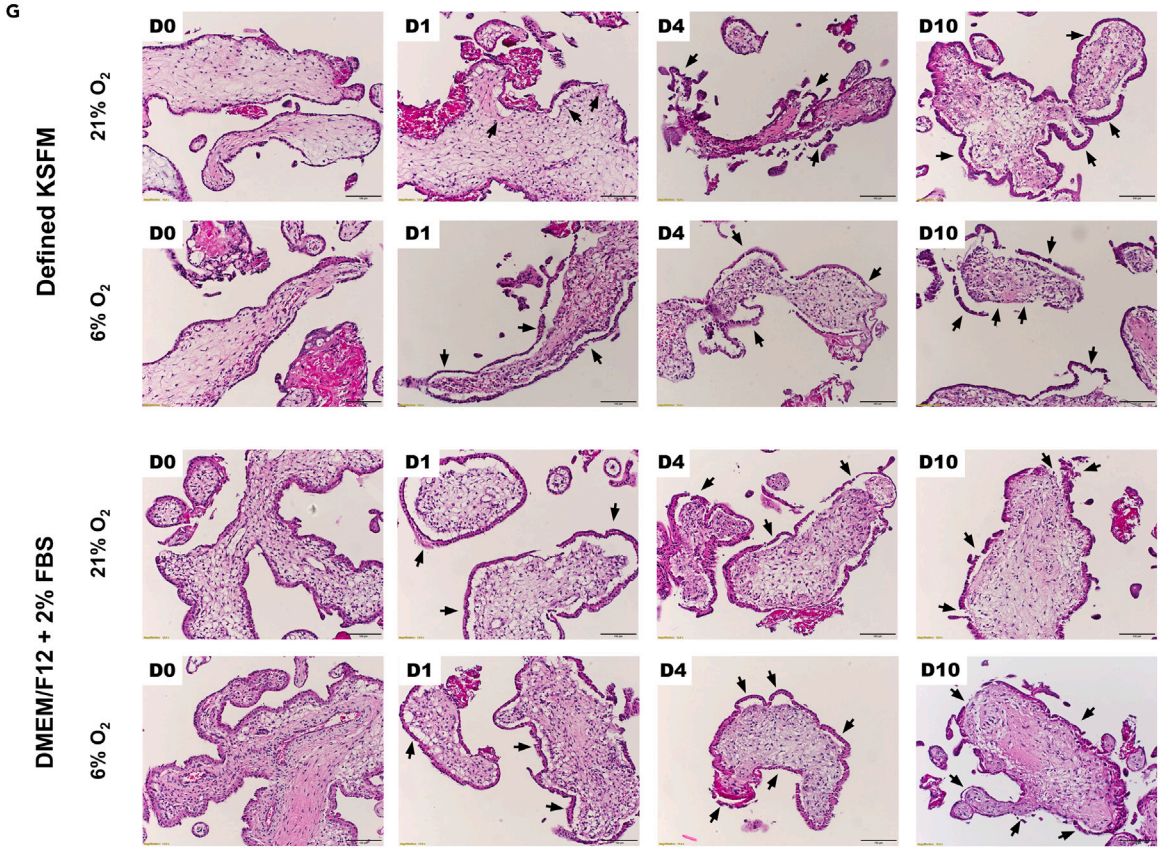

G
Defined KSFM
DMEM/F12 + 2% FBS
21% O2
6% O2
D0
D1
D4
D10

Figure 1. Serum-free, defined media supports healthy first trimester placental explant culture, with the highest metabolic activity observed during the first 10 hours

(A) D-KSFM supports a steady release of hCGβ from placental explants during the first 10 h of culture at 21% oxygen tension (data represented as mean ± SEM (n = 2)).

(B) XTT assay shows stable metabolic activity in explants for 24 h in culture (data represented as mean ± SEM (n = 2)).

(C) LDH levels in the explant media remain relatively low in D-KSFM (data represented as mean ± SEM (n = 2)).

(D) hCGβ hormone secretion shows a steady increase from D1 to D4 and then declines from D5 to D10 at 6% oxygen tension for both media types. At ambient oxygen concentration, hCGβ secretion plateaus at D3 in DMEM/F12 + 2% FBS, and at D6 in D-KSFM before declining (data represented as mean ± SEM (n = 3)).

(E) Cell metabolic activity peaks at D2 to D5 as shown in the XTT assay (data represented as mean ± SEM (n = 3)).

(F) LDH molecules released into the spent media were highest at 24 h (data represented as mean ± SEM (n = 3)).

(G) H&E staining of first trimester placental explants shows the degradation of syncytium (arrows) by D1 of culture in both media and oxygen conditions. By D4, the syncytium is partially or completely detached from the underlying villus stroma (Scale bar - 100 μm).

assays suggest that D-KSFM and DMEM/F12 + 2% FBS are the best media to support healthy first trimester placental explant culture. Interestingly, additional villus explant culture experiments using the two best-performing media showed two distinct patterns of hCGβ secretion during the first 24 h of culture among first trimester placentae in the range 10–12 weeks gestation. hCGβ secretion from explants cultured in both media steadily decreased over the first 24 h of culture in some placentae (n = 6) but remained mostly steady for other placentae of similar gestational age (n = 6) (Figure S1D).

Previous articles have reported the initial loss of STB integrity, followed by STB regeneration between days 5–7 of explant culture.[26,30,38] Because the culture media used in these studies are no longer available, we cultured 10 to 12-week placental explants (n = 3 placentae) for 10 days in the two best-performing media from the above experiments (D-KSFM and DMEM/F12 + 2% FBS) in either placental normoxia (6% oxygen) to replicate physiologic conditions of post 10-week GA placentae (*in vivo* villus oxygen of ~6–8%),[39–41] or at standard oxygen tension (21% oxygen). Under 6% oxygen tension, the amount of hCGβ released per day (relative to the starting timepoint) in both D-KSFM and DMEM/F12 + 2% FBS cultured explants was marginally higher (compared to the 21% oxygen condition) ($p < 0.01$) showing a steady increase from D1 to D4 and then steadily declining through D5 to D10 (Figure 1D). In contrast, placental explants cultured in DMEM/F12 + 2% FBS at 21% oxygen showed a steady increase in hCGβ secretion up to D3, after which hCGβ release plateaued through D10. Placental explants cultured in D-KSFM at 21% oxygen showed a steady increase in hCGβ secretion up to D6, after which hCGβ release declined through D10. Cell metabolic activity as measured by the XTT assay peaked at D2 of culture in both media and at 6% oxygen and 21% oxygen conditions and then fell gradually in D-KSFM, while the absorbance in DMEM/F12 serum media plateaued (Figure 1E). LDH in both types of explant-conditioned media, and at both oxygen tensions, peaked by the first 24 h of culture and steadily declined thereafter (Figure 1F). Interestingly, up to D3 for the DMEM/F12 + 2% FBS cultures, LDH levels in 6% oxygen were nearly 2-fold higher than in 21% oxygen, whereas LDH release in D-KSFM at both oxygen tensions remained similar throughout (Figure 1F).

Cell viability in 11-week placental explants cultured for 10 days in 21% and 6% oxygen tension was assessed each day using the "LIVE/DEAD Viability" Kit (Thermo Scientific, Cat #L3224). Explants in defined and serum-based conditions showed the highest calcein-AM staining (indicating live cells with high intracellular esterase activity) and lowest propidium iodide (PI) staining (indicating dead cells) on D0 (Figure S1E). By D1, more PI staining began to appear in the syncytial layer (denoting loss of cell membrane integrity) and increased through the week in D-KSFM in both oxygen concentrations (Figure S1E). From D1 to D7, in DMEM/F12 + 2% FBS, weak calcein-AM staining remained only in the inner stromal regions of the explants, and by D10, only bright PI-stained dead cells bordering the villus structure were observed (Figure S1E).

Dramatic decline in TP63 stained nuclei in first trimester placental explants after one day of *in vitro* culture

To investigate the effect of long-term culture under 6% and 21% oxygen conditions on tissue morphology and cellular viability, hematoxylin and eosin (H&E) staining was performed on PFA-fixed explants. Progressive loss of structural integrity of the explants starting at 24 h in culture was evident from signs of thinning and partial/complete detachment of the trophoblast from the villous stroma (arrows) (Figure 1G). Arrows highlight regions where the trophoblast that normally forms a continuous layer that closely abuts the underlying stroma is completely missing by D4 (Figure 1G).

To further evaluate tissue integrity and regeneration within the first trimester explants, we performed immunohistochemistry (IHC) using antibodies against TP63 (specific to cytotrophoblast/CTB), Cytokeratin 7 (CK7) (a pan-trophoblast marker), and Ki-67 (marker of proliferation). CK7 staining remained in explants throughout the 10 days of *in vitro* culture; however, the trophoblast layer began to appear highly vacuolated after D1, with no difference between either media or oxygen tensions (Figures 2A and 2B). There was a significant loss of TP63+ nuclei (arrows) after the first day of culture in both media and oxygen conditions (Figures 2A and 2B).

Besides trophoblast cells, proliferative stromal and endothelial cells may also contribute to explant metabolic activity. To visualize non-CTB proliferative cells, Ki-67 was used for immunostaining. Ki-67+ and TP63+ nuclei in the trophoblast layer (arrows) dramatically decreased over the first 24 h of culture, indicating loss of proliferative CTB (Figures 2A and 2B). However, some nuclei in the stromal and endothelial regions of the villi remained positive for Ki-67 (arrowheads) even by D10 of culture (Figures 2A and 2B). Overall, the long-term culture of placental explants in 6% oxygen tension did not appear to improve tissue morphology, trophoblast viability, or CTB proliferation compared to 21% oxygen; in none of the tested conditions did we observe evidence of syncytial regeneration.

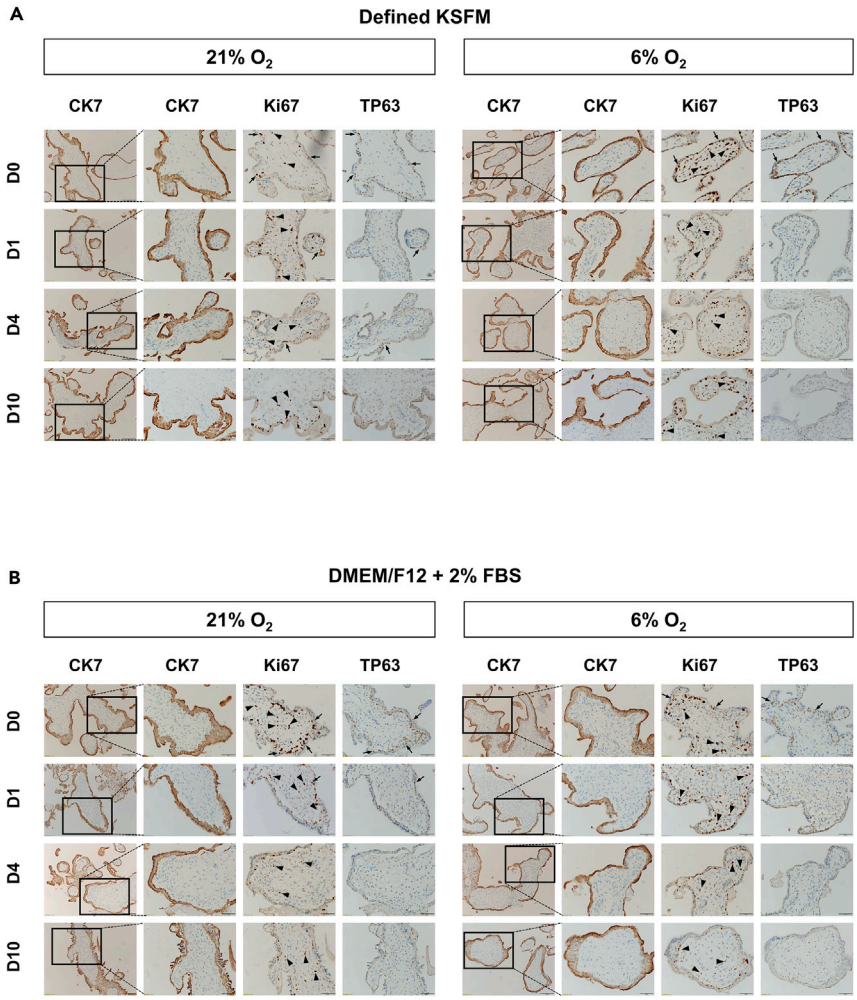


Figure 2. Dramatic decline in TP63 stained nuclei in first trimester placental explants after one day of *in vitro* culture

(A. B) Pan-trophoblast marker CK7 is expressed in explants throughout the ten-day culture period. Magnified insets of CK7, proliferative marker Ki-67, and cytotrophoblast-specific marker TP63 indicate that Ki-67+ and TP63+ nuclei in the trophoblast layer (arrows) completely disappear after 24 h of culture. In contrast, non-trophoblast Ki-67+ nuclei (arrowheads) decrease in number with culture duration but remain present in the stromal regions indicating the survival of stromal and endothelial cells even at D10 (Scale bar - 100 μm, Scale bar (insets) - 50 μm).

Third trimester placental explants were most active and viable over 8 h of culture in both serum-containing and serum-free media types

Similar to the first trimester explant studies, DMEM/F12 + 2% FBS, IMDM +2% FBS, and MCDB153 + 2% FBS were tested on placental explant cultures from third trimester [36–42 weeks GA] placentae (n = 2 placentae) at ambient atmospheric oxygen tension (21%). Third trimester placental explant cultures were similarly tested for cell metabolism, viability, and function in the three serum-containing media formulations. The hCGβ ELISA displayed a linear increase of the hormone for 8 to 10 h in IMDM +2% FBS and MCDB153 + 2% FBS media types (Figure S2A). Such a sustained hCGβ release was not observed in DMEM/F12 + 2% FBS (Figure S2A). The XTT assay showed that placental explants had similar continuous metabolic activity in all three culture conditions throughout the 24 h post-dissection (Figure S2B). The LDH assay indicated a low level of cell death in all three media types over the 24-h culture duration, with MCDB153 + 2% FBS condition showing relatively higher rates of LDH release ($p < 0.01$) (Figure S2C). Overall, for third trimester placental explants, we concluded that IMDM +2% FBS was the best serum-containing culture media, with an ideal combination of high hCGβ levels and cell metabolism, and low cell death.

We performed the same series of experiments on third trimester placentae (n = 2 placentae) comparing D-KSFM against IMDM (with and without 2% FBS), and KSFM-B. The hCGβ assay showed similar release trends of the hormone for up to 12 h in all four media types (Figure 3A). The XTT assay showed that explants maintain similar levels of metabolism in all four media types through 24 h, with highest metabolic rate in D-KSFM ($p < 0.05$) (Figure 3B). LDH release data revealed lowest numbers in IMDM +2% FBS media ($p < 0.05$) over the course of 24 h, while D-KSFM cultures had elevated LDH in comparison ($p < 0.05$) (Figure 3C).

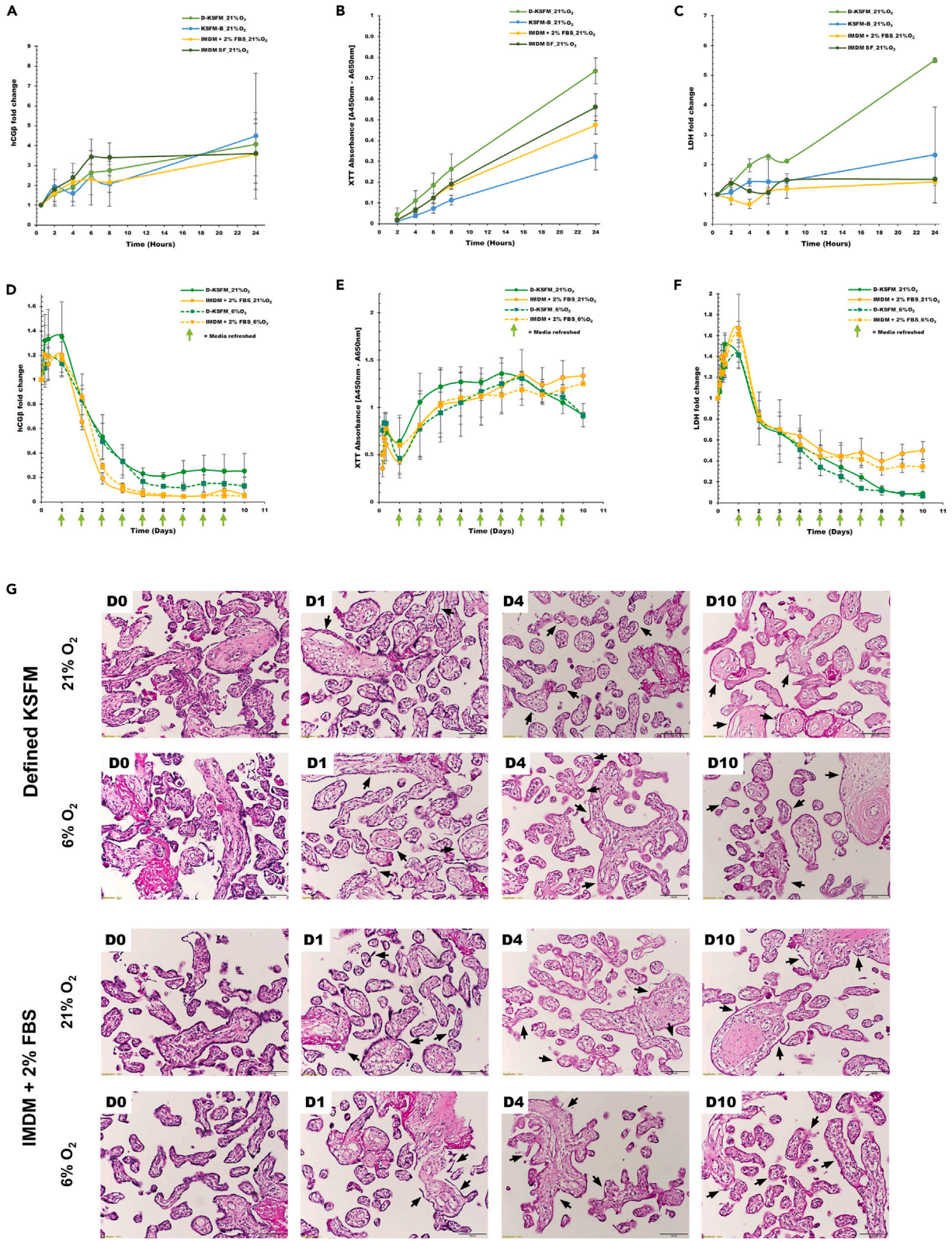

A
B
C
D
E
F
G
hCGβ fold change
XTT Absorbance [A450nm - A650nm]
LDH fold change
Time (Hours)
Time (Days)
D-KSFM_21%O2
KSFM-B_21%O2
IMDM + 2% FBS_21%O2
IMDM SF_21%O2
D-KSFM_6%O2
IMDM + 2% FBS_6%O2
= Media refreshed
Defined KSFM
IMDM + 2% FBS
21% O2
6% O2
D0
D1
D4
D10

Figure 3. Third trimester placental explants were most active and viable over 8 h of culture in both serum and serum-free media types

(A) Rate of hCGβ secretion is seen decreasing after 8 h in D-KSFM (data represented as mean ± SEM (n = 2)).

(B) XTT assay shows steady metabolism over 24 h in all four media conditions with D-KSFM having highest metabolic rate (data represented as mean ± SEM (n = 2)).

(C) Explants in IMDM +2% FBS have lowest LDH molecules released, while D-KSFM has much higher numbers (data represented as mean ± SEM (n = 2)).

(D) Third trimester explants display rapidly declining hCGβ secretion after their first day of *in vitro* culture in both oxygen concentrations indicating possible degradation of the STB layer (data represented as mean ± SEM (n = 3)).

(E) XTT assay shows a slowdown in cellular metabolism over the course of ten-day culture (data represented as mean ± SEM (n = 3)).

(F) LDH release peaks 24 h into the culture and then remained low for 10 days (data represented as mean ± SEM (n = 3)).

(G) Third trimester explants stained with H&E reveal unhealthy placental villus morphology worsening with increased time in culture, regardless of serum or serum-free conditions (Scale bar - 100 μm).

Next, we chose the best-performing medium from the above 24-h experiments (IMDM +2% FBS), as well as our defined serum-free medium (D-KSFM), and cultured third trimester placental explants in each of these media for 10 days at placental normoxia (6% oxygen) and at ambient oxygen concentration (21% oxygen) (n = 3 placentae) to observe if villus syncytial regeneration could occur in third trimester explants over a longer culture period. The hCGβ ELISA showed that hormone levels reached their peak during the first 24 h of culture followed by declining secretion throughout the ten days of culture in both media (Figure 3D) and oxygen tensions. Cell metabolic activity measured by the XTT assay exhibited a slowdown in metabolism over ten days in both media (Figure 3E). LDH release from the explants was highest at 24 h and then declined in both media, and at both oxygen tensions, over the remaining culture period (Figure 3F).

Cell viability in cultured explants was assessed each day using the LIVE/DEAD Viability Kit. Explants displayed highest green-fluorescent staining (calcein-AM) and lowest red-fluorescent staining (PI) on D0, indicating abundant live cells with high intracellular esterase activity (Figure S2D). Additional days in culture showed increased PI and decreased calcein-AM staining intensity which was particularly pronounced in the D-KSFM condition, whereby D7 there were hardly any live cells left in the explants (Figure S2D). As with the first trimester explants, loss of cell viability was evident earlier on the surfaces of the villi compared to the core, indicating earlier loss of trophoblast viability compared to stromal/endothelial cell viability.

The majority of trophoblast-specific and proliferative markers were lost in first 24 h of third trimester placental explant culture

We next fixed the third trimester explants and stained with H&E to observe for morphological alterations occurring over time in culture under 6% and 21% oxygen conditions. The explants displayed structural damage to their villi, especially the syncytial layer (arrows) by D1 (Figure 3G). Thinning of the syncytium and complete detachment of the STB monolayer from the villi (arrows) was observed by D4 in both media conditions (Figure 3G); no observable differences in morphological characteristics were seen between explants cultured in either oxygen tension.

Third trimester placental explants have vastly different cellular compositions in comparison to first trimester placentae, with significantly fewer TP63+ CTB in the trophoblast compartment,[42] and fewer proliferative cells in stromal compartments of the villus. Immunostaining showed dramatic loss of TP63+ CTB within the first 24 h of culture in both media and oxygen tensions (Figures 4A and 4B). The TP63+ population disappeared entirely by D4 (Figures 4A and 4B) in both 6% and 21% oxygen conditions. Though CK7 expression in third trimester placental explants remained present throughout the 10-day culture period, there was a reduction in the intensity of CK7 staining by D4 (Figures 4A and 4B).

Ki-67 staining was performed on third trimester placental explants to distinguish between CTB and other proliferative cell types and their expression patterns during the 10-day culture window. The Ki-67+ nuclei overlapping with TP63+ trophoblasts (arrows) disappeared quickly (within one day of culture) in third trimester placental villi, indicating unfavorable conditions for trophoblast cell proliferation in both media and oxygen concentrations (Figures 4A and 4B). The number of Ki-67+ nuclei in the villus stroma (arrowheads) remained mostly constant during the 10-day culture period (Figures 4A and 4B). Hence, the immunostaining data in third trimester placental explants highlight the absence of any functional regeneration of the STB layer even in favorable placental oxygen conditions, consistent with the hCGβ hormone release data shown in Figure 3D. Similar to first trimester explant cultures, the low oxygen/placental normoxia culture condition did not seem to induce syncytial regeneration in third trimester placental explants cultured for 10 days *in vitro*.

Vesicle flow cytometry analysis reveals that placental explants release at least three classes of extracellular vesicles

Next, we investigated placental EV production from first and third trimester placental explants cultured for 8 h in D-KSFM (Figure 5), DMEM/F12 supplemented with exosome-depleted FBS (first trimester explants) (Figure 6), and IMDM supplemented with exosome-depleted FBS (third trimester explants) (Figure 6). We conducted these experiments at 21% oxygen, as previous experiments did not reveal a significant difference between placental cultures at either 21% or 6% oxygen concentration. Additionally, this experimental design enabled us to conduct large, highly replicated experiments for EV studies in widely available culture conditions, as not all laboratories have ready access to a hypoxia incubator chamber.

For each media condition, placental explant supernatants (PES) were pooled from explant preparations from 5 placentae (of similar gestation age) and concentrated 40-fold in Amicon Ultra 4 mL Centrifugal Filters. EV characteristics, including size distribution and surface molecular phenotype were assessed by multicolor vesicle flow cytometry (vFC) assay using antibodies against EV-associated tetraspanin surface

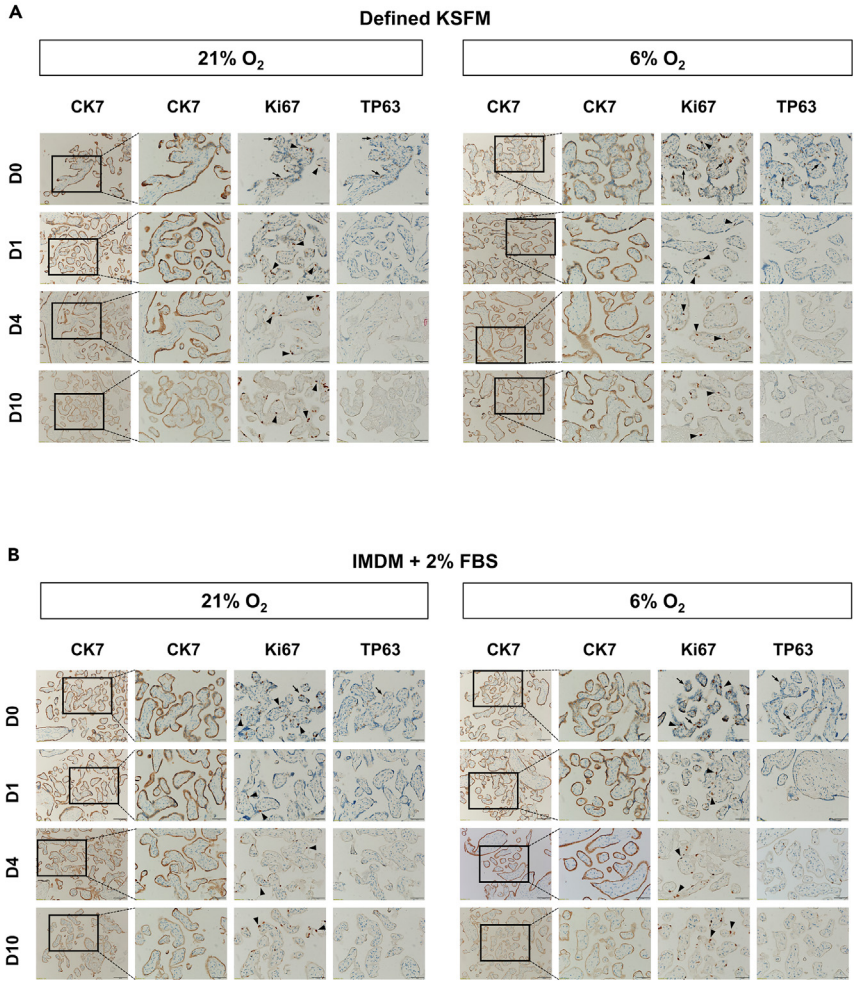


Figure 4. The majority of trophoblast-specific and proliferative markers were lost in the first 12 h of third trimester placental explant culture

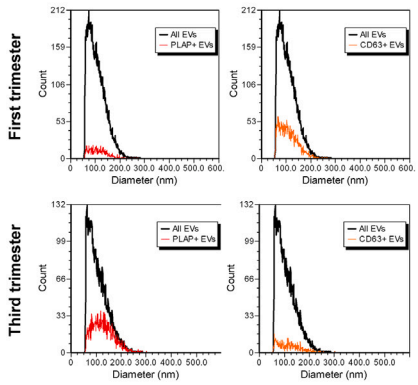
(A, B) Pan-trophoblast marker, CK7 is consistently expressed in explants throughout the ten-day culture duration. Magnified insets of CK7, proliferative marker Ki-67, and trophoblast-specific TP63 show that by D1, there are no Ki-67 and TP63 double-positive nuclei (arrows) found in the explants. However, non-CTB specific Ki-67+ nuclei (arrowheads) remain positive in the stromal and endothelial cells even at D10 (Scale bar - 100 μm, Scale bar (insets) - 50 μm).

markers (CD9, CD63, and CD81) and a placenta-specific protein biomarker, placental alkaline phosphatase (PLAP), found exclusively on the surface of trophoblast cells. We also analyzed the PES-derived EVs with antibodies against common antigens expressed on platelets (CD41), RBCs (CD235ab), immune cells (CD54), and stromal markers (CD73, CD105, CD90).

EVs in the first trimester PES ranged from ~60 nm (the estimated limit of detection for vFC on the CellStream flow cytometer) to ~250 nm in diameter (median ~99 nm, Figures 5A and 6A). EVs in third trimester PES were of a similar size, also ranging from ~60 nm to ~250 nm in diameter with a median diameter of 99 nm (Figures 5A and 6A). We then quantified the relative abundance of the PLAP epitope on PES EVs and discovered that both first and third trimester explants released EVs that showed low levels of PLAP antibody binding (1–10's of molecules/vesicle) as seen in Figures 5B and 6B. First trimester PES had ~8% of total EVs as "positive" for PLAP (median diameter ~105 nm) and is slightly above the limit of detection (at ~3 molecules/vesicle) which is defined by the gate set on background autofluorescence of unstained EVs. In contrast, EVs from third trimester placenta contained a greater fraction of PLAP positive vesicles (~35% of all EVs) and these particles appeared to be larger on average (median diameter ~124 nm) compared to all EVs or PLAP+ EVs from first trimester explants (Figures 5B and 6B). Both first and third trimester PES EVs showed a lack of expression of cell-surface markers specific to platelets (CD41), RBCs (CD235ab), immune cells (CD54), and common stromal cell markers (CD73, CD105, CD90), ruling out circulating fetal and maternal cell EV contamination, and vesicles originating from the placental stromal compartment (Figure S3).

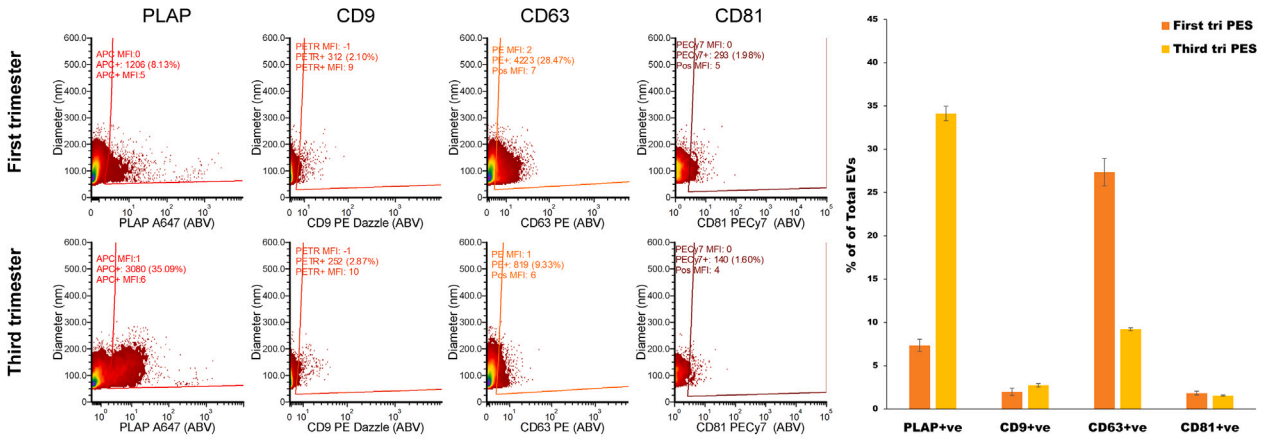
Among the EV-related tetraspanins, CD9 expression was almost undetectable in first trimester PES EVs, while CD63 and CD81 showed low levels of expression (1–10's of molecules per EV) with ~30% and ~4% appearing positive, respectively (above the background autofluorescence) (Figures 5B and 6B). Tetraspanin expression was also low in third trimester explant-derived EVs, with relatively smaller fractions appearing as $CD63^{+}$ (~9%) compared to first trimester vesicles.

A



	Median diameter (nm)		
	Total EVs	PLAP+	CD63+
First trimester	98.85	106.93	103.54
Third trimester	98.97	123.49	112.52

B



C

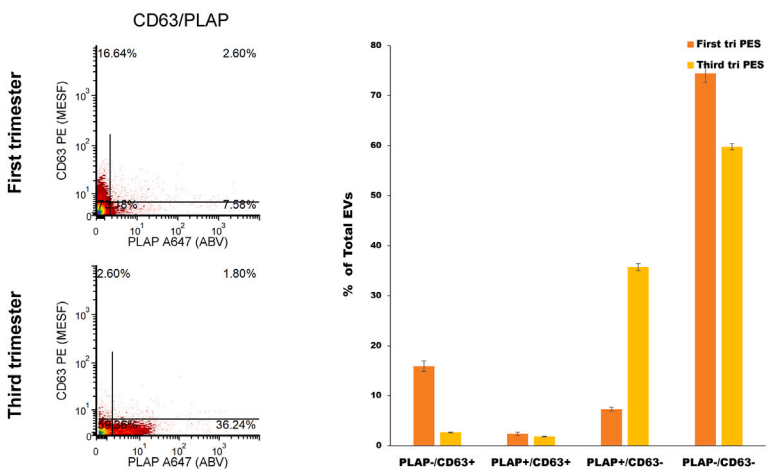


Figure 5. Vesicle flow cytometry analysis reveals that placental explants cultured in D-KSFM release at least three classes of EVs

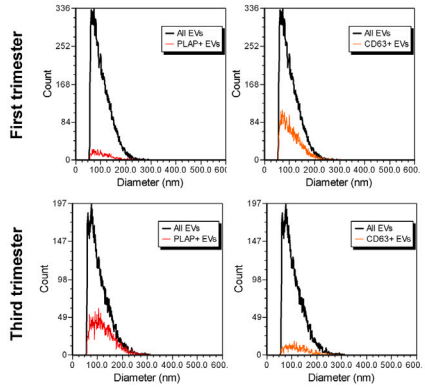
EVs from PES in defined media were analyzed by vesicle flow cytometry to measure EV size, number, and surface antigen expression.

(A) EV population size distributions show that first trimester vesicles are smaller in diameter.

(B) Expression patterns of PLAP and CD63 differ significantly between first and third trimester EVs (data represented as mean ± SD (n = 3)).

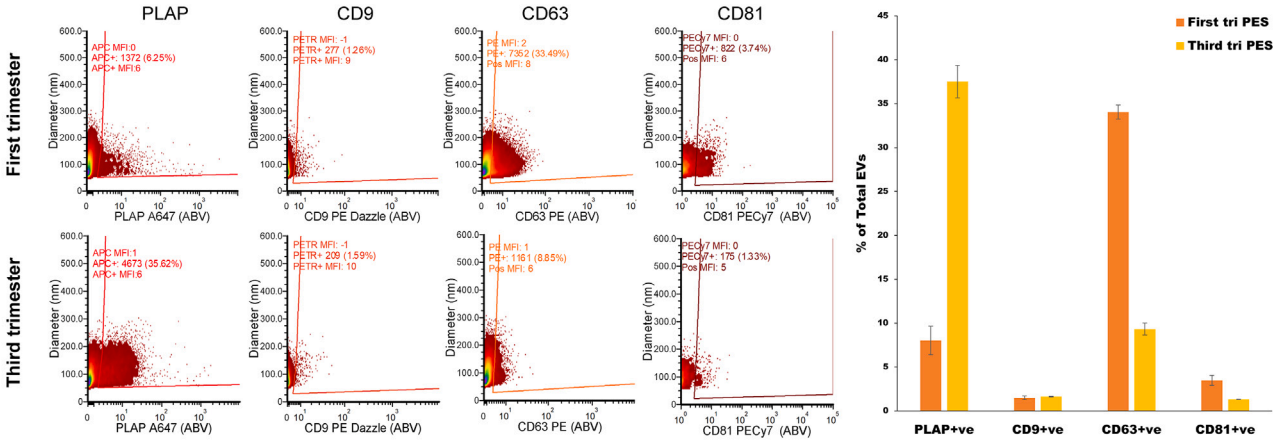
(C) PLAP and CD63 double-positive EVs are very low in number in both first and third trimester placenta (data represented as mean ± SD (n = 3)).

A



	Median diameter (nm)		
	Total EVs	PLAP+	CD63+
First trimester	95.13	99.97	100.6
Third trimester	98.01	123.9	118.17

B



C

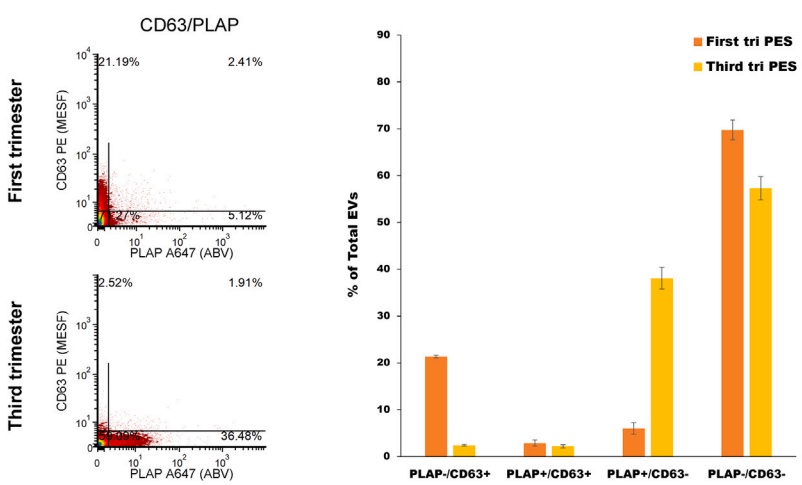


Figure 6. Vesicle flow cytometry analysis of placental explants cultured in exosome-depleted FBS media display EV profiles similar to D-KSFM

EVs from PES in serum-based media were analyzed by vesicle flow cytometry to measure EV size, number, and surface antigen expression.

(A) EV population size distributions show that third trimester vesicles are larger in diameter on average.

(B) Just as in D-KSFM, PLAP and CD63 expression is considerably different between first and third trimester EVs in exosome-depleted FBS media (data represented as mean ± SD (n = 3)).

(C) Co-expression of PLAP and CD63 is very low in vesicles from both trimester placenta samples (data represented as mean ± SD (n = 3)).

As for multiparameter analysis of EVs with the tetraspanins and PLAP, first trimester PES EVs showed little co-expression of CD63 and PLAP (Figures 5C and 6C) with less than 3% particles carrying both the surface proteins. Similarly, for third trimester PES EV's, bivariate analysis of CD63 vs. PLAP staining indicated very low co-expression of CD63 on PLAP+ EVs, in range of 1.5% double-positive vesicles (Figures 5C and 6C).

Interestingly, when we analyzed EVs derived from the BeWo cell line (a human choriocarcinoma cell line commonly used as a model for STB formation *in vitro*), the EVs showed a pattern that was markedly different from both first and third trimester placental explant-derived EVs (Figure S4). BeWo cells release a moderate level of canonical EVs, with approximately 30% $CD9^+$, 20% $CD63^+$ and 10% $CD81^+$. Around 25% of BeWo EVs also express PLAP protein (Figure S4), and these particles had a diameter ranging from 60 to 180 nm (median ~100 nm) (Figure S4), closer to the size profile of PLAP+ EVs in first trimester PES.

All of the above experiments were performed in parallel with positive and negative assay controls, including as plain buffer (Figure S5), Lipo100 vesicle standard (Figure S6), and the respective blank media controls (Figure S7) that show no particles and minimal background. It should be noted that our experimental estimates of antigen numbers are subject to several sources of uncertainty, including the bivalent nature of the antibodies, Poisson-dominated counting statistics, and related uncertainties in the brightness of the calibrators and standards used. However, because these measurements were run on a calibrated instrument, using well-characterized standards and the current best practices for quantitative flow cytometry, the results will be interpretable and comparable to results obtained on future generations of more sensitive instruments and using brighter dyes.

In conclusion, whether the medium used to culture human placental explants was chemically defined or serum-based, it did not appear to have a major effect on EV number, size, or surface marker expression (Figures 5 and 6). Overall, these vFC characterization data suggest that placental explants release at least three classes of EVs: smaller EVs that express CD63, larger EVs that express PLAP, and EVs that express neither of these markers above the limits of detection of our methods.

DISCUSSION

Human placental explants are a versatile and commonly accepted "benchmark" *ex vivo* model system for primary placental tissue. Given the lack of standardized experimental practices for culture of placental explants, we set out to rigorously validate conditions for first and third trimester villus explant preparation and culture, which we could then use to study EVs of placental origin. Our study undertook a systematic approach to identify optimal culture conditions by comparing multiple serum-containing and defined media conditions across multiple longitudinal time-points (sampling every 2 h for first 24 h and daily for 10 days). A panel of assays, consisting of ELISA for hCGβ secretion, XTT for metabolic activity, and LDH for cell viability were conducted to identify the most favorable culture system. In addition to biochemical assays, we used multiple complementary techniques to evaluate explant function and morphology, using LIVE/DEAD fluorescent viability staining and immunohistochemical analysis. To ensure the robustness of our results, we conducted a well-replicated study on both first and third trimester placentae, in which each experiment was performed using multiple placentae, and for each placenta, triplicate explants were cultured in each media type.

Identification of optimal culture conditions for first and third trimester placental explants

We found that the best serum-containing basal medium is DMEM/F12 for first trimester and IMDM for third trimester placenta using a variety of viability and functional parameters described above. This difference in the optimal basal media type for explants from placentae at different gestational ages is also true for primary human cytotrophoblast culture protocols.[43,44] Such a difference could be attributed to the dramatic changes in the cellular composition of placental tissue across gestation, especially in the trophoblast compartment[42] where significant changes in STB membrane structure and integrity have been observed.[45,46] We then used the same panel of assays to compare the favored standard serum-containing media to defined serum-free medium (D-KSFM) and found similar performance for DMEM/F12 + 2% FBS and D-KSFM for first trimester and IMDM +2% FBS and D-KSFM for third trimester placentae. This clearly demonstrates that we can achieve comparable performance with a defined serum-free medium compared to the best standard serum-containing medium. This is important, as the usage of defined media simplifies EV and exRNA studies by avoiding contamination with exogenous EVs and exRNAs present in FBS and other undefined supplements.

Interestingly, two distinct hCGβ secretion patterns were observed among first trimester placentae (10-week to 12-week). This was consistently seen across multiple placentae, and could be caused by developmental changes in trophoblast structure and function that occur around 12-week gestation, when significant vascular remodeling by extravillous trophoblast leads to increasing levels of tissue oxygenation.[47] This further illustrates the importance of documentation of precise gestational age of the placentae from which explants are derived, particularly for first trimester samples.

Villus syncytiotrophoblast regeneration does not occur with the extended culture of placental explants

A highly debated aspect of *in vitro* villus explant culture has been the "correct" post-culture time window during which the biochemical, morphological and villus microarchitecture of the villi are preserved. A few publications over the last two decades have asserted that villus STB from third trimester placental explants display signs of degeneration in the first two days of culture, only to recover and regenerate to form a new syncytium with fresh hormonal secretion by day 5–7 of explant culture.[26,30,38] In our study, we performed 10-day cultures of first and third trimester placental explants in serum-based media and chemically defined media at both 6% and 21% oxygen, and observed no evidence of STB regeneration. We only observed thinning and detachment of the trophoblast from the villus stroma indicative of tissue breakdown, which is consistent with the results of hCGβ secretion assay, where new hormone secretion declines after only a few days in culture, confirming a lack of STB regeneration. Immunohistochemical analysis of fixed and sectioned first trimester villi showed substantial loss of TP63 positive CTB after only D1 of culture in both media types. Still, Ki-67 positive nuclei continued to be observed in the villus stromal regions and these could be contributing to sustained low levels of metabolic activity seen in the XTT assay and LIVE/DEAD cell viability staining from D1 of culture onwards.

In third trimester placental explants, which differ markedly in cellular composition compared to first trimester explants, both serum-containing and serum-free media continued to support steady metabolic activity (XTT) throughout the first 24 h of culture, but we observed rapid loss of trophoblast function as indicated by the rapid decline in hCGβ secretion after 24 h. In third trimester explants, the few TP63 nuclei within the CK7+ trophoblast layer seen at D0 disappear entirely by D4. Thus, no signs of STB regeneration were observed in both serum and serum-free culture of first or third trimester placental explants, either in placental normoxia or ambient oxygen tension.

Primary placenta-derived extracellular vesicle characteristics vary widely by gestational age

It is evident that EV release can be affected by culture conditions,[48,49] and thus it is extremely important to conduct experiments using rigorously defined and reproducible culture conditions. Here, we first used a battery of biochemical and morphological criteria to identify optimal culture conditions for first and third trimester placental explants, and then used the two best conditions for each gestational age window to collect EVs for detailed characterization using cutting-edge multiplex vesicle flow cytometry.

To our knowledge, the multiplex vesicle flow cytometry assay used in this study is the first of its kind to characterize placental EV size and use canonical EV markers and a tissue-specific placental EV-associated surface marker for EV characterization from primary placental explant cultures of various gestational ages. We observed clear differences in the patterns of EV expression between first and third trimester PES, the most important of which is a higher fraction of large CD63-/PLAP+ EVs in the third trimester PES, and a higher fraction of small CD63+/PLAP- EVs in the first trimester PES. The fraction of total EVs expressing STB-specific PLAP was only ~8% in first trimester PES and ~35% in third trimester. There are two major reasons for this somewhat unexpectedly low percent of PLAP+ EVs, which are not mutually exclusive. First, the PLAP- EVs could be originating from the non-trophoblast compartment, such as placental stromal cells, macrophages (Hofbauer cells), or endothelial cells of the microvillus, and/or circulating fetal and maternal cells. However, our observations in Figure S3 ruled out circulating fetal and maternal cell EV contamination, and vesicles originating from the placental stromal compartment; so, to identify origins of the PLAP-negative EVs, vesicle flow cytometry assay with additional markers and other modes of molecular profiling will be required in future studies. Second, it is possible that a subset of trophoblast-derived EVs do not carry detectable levels of PLAP, either due to stochastic loss of PLAP during the sampling of the membrane or because PLAP is selectively excluded from these EVs. The fact that the majority of PES EVs also lack detectable expression of CD63 (the most abundant tetraspanin in this sample type), and that the majority of BeWo EVs are also PLAP-, suggest that at least the second possibility is true. We have also observed that BeWo-derived EVs showed a pattern distinct from both first and third trimester PES suggesting that this commonly used cell type has potentially important limitations for the modeling of placental EV biogenesis and function.

"Exosome-free" FBS is often recommended for use in EV experimental assays; however, a number of recent reports have shown that serum depleted of exosomal particles had adverse effects on cellular health and maintenance, indicating that exosome-depleted FBS may have a reduced capacity to support cell proliferation and survival.[49–51] Although we did not observe significant differences in the EV profiles of PES cultured in defined media compared to media containing exosome-depleted FBS, it is important to note that this study did not include comprehensive molecular profiling, and thus we cannot exclude changes at the molecular level. Nevertheless, defined media shows less batch-to-batch variability compared to those containing serum or other complex undefined components. Thus, the fact that the defined conditions were comparable to the serum-containing conditions by all of the other measures included in this study suggests that we have identified an excellent defined media condition for future human placental explant studies.

Future directions

In conclusion, our comprehensive approach has allowed us to: (a) identify robust and reproducible culture conditions for first and third trimester placental explants; (b) define the time frame during which the trophoblast layer of the explant is intact, thus establishing the optimal window for future functional experiments; and (c) quantitatively describe three distinct populations of EVs in placental explant supernatants and demonstrate differences in these populations between first and third trimester PES.

Future explant preparations using this culture system may be applied to *in vitro* placental disease modeling by manipulating glucose levels in culture media to represent gestational diabetes mellitus, or subjecting explant cultures to altered oxygen tension(s) to model the ischemic-reperfusion injury experienced by pre-eclamptic placentae. This would help us better understand EV-related responses to specific disease states and their downstream effects on exRNA expression profiles. Another potential enhancement for placental explant model system is to adapt static explant culture to sophisticated flow-based modeling, which is a more physiologically relevant representation of the *in vivo*

placenta microvillus.[31] A fluidics-based placental villus explant culture could be used in conjunction with a "placenta-on-chip," microfluidics-based co-culture system to study the pathophysiology of the human placenta and its EV characteristics in a dish.

Limitations of the study

We acknowledge that the placental explant model has limitations, as all models do. *In vitro* placental explant cultures serve as a model system at the level of a functional tissue unit, containing multiple interacting cell types, and producing a heterogeneous population of EVs and other exRNA carriers. While we have demonstrated that placental explants release EVs that likely originate from the trophoblast compartment (which carry PLAP on their surfaces), there are also PLAP-negative EVs that do not carry typical surface markers of platelets, red blood cells, immune cells, stromal cells, and endothelial cells (which are major non-trophoblast cell types present in explants). Further studies are needed to identify the cells of origin of PLAP-negative vesicles through vesicle flow cytometry analysis and other modes of molecular profiling.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108046.

ACKNOWLEDGMENTS

Graphical abstract created with BioRender.com. This work was supported by funds from the National Institutes of Health: U01HL126494 to LCL; UG3CA241687 to LCL; and R01HD089537 to MMP.

AUTHOR CONTRIBUTIONS

Conceptualization, C.T. and L.C.L., methodology, C.T. and E.D., investigation, C.T., S.L., A.C., A.H., I.S., E.D., Y.Z., J.Z., R.S., Y.L., D.S.P., M.M., and T.N.L., writing – original draft, C.T., writing – review and editing, S.L., J.N., M.M.P., and L.C.L., supervision, D.P.P., J.N., M.M.P., and L.C.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: November 8, 2021
Revised: May 17, 2023
Accepted: September 21, 2023
Published: September 26, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human anti-TP63 (p40)	Biocare, Concord, CA	Cat# ACI 3066; RRID:AB_2858274
Human anti-Ki-67	Ventana Medical Systems	Cat# 790-4286; RRID:AB_2631262
Human anti- Cytokeratin-7	Abcam	Cat# ab181598; RRID:AB_2783822
Human anti-PLAP [H17E2]	BioRad	Cat# MCA2091; RRID:AB_2226283
Human anti-CD9 PE Dazzle	Cellarcus Biosciences	Cat# CBS10-PED-100T
Human anti-CD63 PE	Cellarcus Biosciences	Cat# CBS11-PE-100T
Human anti-CD81 PE-Cy7	Cellarcus Biosciences	Cat# CBS12-PC7-100T
Human anti-CD41 PE	Cellarcus Biosciences	Cat# CBS71-PE-100T
Human anti-CD235ab PE	Cellarcus Biosciences	Cat# CBS56-PE-100T
Human anti-CD54 PE	Cellarcus Biosciences	Cat# CBS72-PE-100T
Human anti-CD73 PE	Cellarcus Biosciences	Cat# CBS81-PE-100T
Human anti-CD90 PE	Cellarcus Biosciences	Cat# CBS83-PE-100T
Human anti-CD105 PE	Cellarcus Biosciences	Cat# CBS51-PE-100T
Biological samples		
Human first trimester placental tissue [10 to 12 weeks gestation] from D&C procedures	Not disclosed, as per agreement between UCSD and source of tissue	N/A
Human third trimester placental tissue [36 to 42 weeks gestation] from planned C/S section following uncomplicated pregnancy	UCSD Health Systems	N/A
Chemicals, peptides, and recombinant proteins		
MCDB153	Sigma-Aldrich	Cat# F8105-BC
DMEM/F-12	Gibco	Cat# 10565018
IMDM	Gibco	Cat# 12440061
Fetal Bovine Serum, exosome-depleted	Gibco	Cat# A2720801
Insulin-Transferrin-Selenium-Ethanolamine	Gibco	Cat# 51500056
Penicillin-Streptomycin	Gibco	Cat# 15140122
Critical commercial assays		
Defined Keratinocyte SFM kit	Gibco	10744019
Keratinocyte SFM kit	Gibco	17005042
HCG enzyme-linked immunosorbent assay	Calbiotech	HC251F
XTT Cell Proliferation assay	Millipore Sigma	11465015001
LDH Cytotoxicity assay	Thermo Scientific	88953
vFC™ EV Analysis Assay kit	Cellarcus Biosciences	CBS4
LIVE/DEAD™ Viability Kit	Thermo Scientific	L3224
Software and algorithms		
FCS Express	*De Novo* Software	https://denovosoftware.com/
Other		
Amicon® Ultra 4 mL Centrifugal Filters	Millipore Sigma	Cat# UFC800324
384-well PP flat-bottom assay plate	Greiner Bio-One	Cat# 781207

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources sharing should be directed to and will be fulfilled by the lead contact, Prof. Louise C. Laurent (llaurent@health.ucsd.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human placenta samples were obtained following written informed consent under protocols approved by the Human Research Protections Program at UCSD Institutional Review Board (IRB numbers: 172111 and 181917). In total for this study, 24 donors were consented. Twelve third trimester placentae were collected from 12 women aged between 23 and 39 years (median age 33.5 years) who delivered at UCSD Health. Of these participants, 5 (41.7%) were non-Hispanic and White, 2 (16.7%) were non-Hispanic and Black, 2 (16.7%) were non-Hispanic and Asian, 1 (8.3%) was Hispanic and White, 1 (8.3%) was Hispanic and Other/Mixed, and 1 (8.3%) was of unknown ethnicity and race. In addition, 12 first trimester placentae were collected from 12 women undergoing elective termination procedures (age and ethnicity/race information was not collected from these donors).

To establish placental explant cultures, placentae were collected immediately after elective termination procedures (9-12 weeks GA) [experienced clinical research staff trained by a perinatal pathologist separate placental and fetal tissues post D&C], or planned C/S delivery (36-42 weeks) (following uncomplicated pregnancies) and transported to lab within an hour on ice. The first trimester placentae collected for this study were obtained from surgical cases that did not receive these medications prior to the procedure.

METHOD DETAILS

Placenta preparation and microvillus explant dissection

Under aseptic conditions, the whole placenta was placed in a sterile container. First, the chorionic and basal plates were cut out using small surgical scissors and discarded. Then, lobes of placental villi (approximately 10 g each) were excised from the placenta (avoiding large blood vessels) and rinsed in DPBS (Gibco, 14190250) until all maternal blood was washed off and the wash solution became clear. Each villus section was placed in a petri dish of warm culture media and micro-dissected into small 'tufts' of villus tissue ~ 3 mm^3-sized explants under a dissection microscope. To ensure explant tissue heterogeneity, harvest sites were frequently switched. Fifteen such microvillus explants (approximately 30 – 50 mg total) were added into each well of a 24-well plate containing 1 mL of growth media and incubated in a humidified incubator at 37°C, 5% CO_2 for ambient oxygen cultures for 10 days (growth medium was replenished once every 24 hours). For the culture of placental explants in 6% oxygen tension (to mimic physiological placental normoxia), culture plates were prepared as described above and incubated in a hypoxia incubator chamber (BioSpherix XVIVO System ® Model X3); supernatant collections and culture media replacements every 24 hours were performed in hypoxia (i.e., without exposing the plate to atmospheric oxygen concentrations). All experiments were conducted using biological triplicates or quintuplicates depending on size of placental villus tissue available for dissection (early GA placenta has lesser villus tissue). The various culture media formulations are:

1. MCDB153 Basal Medium (Sigma-Aldrich, F8105-BC) (containing 1.08 g/L D-Glucose) + 2% Fetal Bovine Serum, exosome-depleted (Gibco, A2720801) + 1 U/mL Insulin-Transferrin-Selenium-Ethanolamine (Gibco, 51500056) + 100 U/mL Penicillin-Streptomycin (Gibco, 15140122).
2. DMEM/F-12, (Gibco, 10565018) (containing 4.5 g/L D-Glucose) + 2% Fetal Bovine Serum, exosome-depleted + 1 U/mL Insulin-Transferrin-Selenium-Ethanolamine + 100 U/mL Penicillin-Streptomycin.
3. IMDM (Gibco, 12440061) (containing 4.5 g/L D-Glucose) + 2% Fetal Bovine Serum, exosome-depleted + 1 U/mL Insulin-Transferrin-Selenium-Ethanolamine + 100 U/mL Penicillin-Streptomycin.
4. Defined Keratinocyte SFM containing 4.5 g/L D-Glucose, supplemented with 1X Defined Keratinocyte SFM Growth Supplement (Gibco, 10744019) + 100 U/mL Penicillin-Streptomycin.
5. Keratinocyte SFM (containing 1.5 g/L D-Glucose) + 30μg/mL Bovine Pituitary Extract + 5 ng/mL rEGF (Gibco, 17005042) + 100 U/mL Penicillin-Streptomycin.

For the first 24 hours of explant culture, a small volume of cell-free placental explant supernatant (PES) was serially collected at 2-hour time intervals (2, 4, 6, 8, 10, 12, 18, 20, 22 and 24 hours) [at each timepoint, we ensured no physical disturbance of explants was caused during collection]. To minimize risk of any detrimental effect of frequent sampling on the cultured explants, we ensured the plates were kept for a minimal

time outside the incubator. For the explants cultured at 6% oxygen, the use of a fully contained hypoxia chamber enabled us to perform the sampling without removing the cultures from 6% oxygen. The sampled supernatants were centrifuged at 2500g for 5 minutes and used for hCGβ enzyme-linked immunosorbent assay (ELISA) (Calbiotech, HC251F) and LDH Cytotoxicity assay (Thermo Scientific, 88953). XTT Cell Proliferation assay (Millipore Sigma, 11465015001) was also performed at the same timepoints. For 10 day-long explant cultures, PES was collected for above assays before the daily media change. Placental explants were fixed at various timepoints in 4% paraformaldehyde for 1 hour at RT for immunohistochemistry for TP63, Cytokeratin 7 (CK7) and Ki-67 and H&E staining.

XTT cell viability assay

Explant cell viability was measured by XTT assay in accordance with the manufacturer's instructions (Millipore Sigma, 11465015001). Briefly, freshly prepared XTT solution was added to the explants and incubated at 37°C 5% CO_2. After 2 hours of incubation, 25 μL of the cell-free supernatant was transferred to a Greiner 384-well polypropylene white flat-bottom assay plate (Greiner Bio-One, 781207) and absorbance measured at 450 nm on a Tecan Infinite 200 plate reader. This measurement was repeated every couple of hours up to 12 hours and then once every 24 hours thereafter. Culture media and XTT solution were replaced daily and measurements were continued for up to 10 days.

hCGβ ELISA

Amount of hCGβ hormone released into culture media by the explant's STB layer was measured by enzyme-linked immunosorbent assay (Calbiotech, HC251F) according to instructions provided with the kit. In short, 25 μL of hCG standards and PES samples were pipetted into the coated well strips. 100 μL of conjugate reagent was added to all wells and incubated for 1 hour at RT. Then, all liquid was removed from the wells and washed thrice with 300 μL of 1X wash buffer. Any excess liquid was blotted on absorbent paper towels and 100 μL of TMB substrate was pipetted into all wells. After a 15-minute incubation at RT, 50 μL of stop solution was added to all wells. The plate was gently shaken to mix the reagents and absorbance measured on Tecan Infinite 200 plate reader at 450 nm within 15 minutes of adding the stop solution.

LDH release assay

Lactate dehydrogenase leakage from explants into the culture media was monitored as an indicator of cell membrane integrity. LDH assay (Thermo Scientific, 88953) was performed as per manufacturer's instructions. Briefly, 25 μL of LDH standards (provided with the kit) and explant-conditioned supernatants were added to a Greiner 384-well polypropylene, white flat-bottom assay plate (Greiner Bio-One, 781207). 25 μL of reaction mixture was added to the plate and incubated at RT for 30 minutes. Then, 25 μL of stop solution was added and absorbance measured on Tecan Infinite 200 plate reader at 490 nm and 680 nm. To determine LDH activity, 680 nm absorbance values (background signal from instrument) were subtracted from the 490 nm absorbance.

Histology and immunohistochemistry

Placental explants were fixed at various timepoints in 4% paraformaldehyde for 1 hour at RT and stored at 4°C in PBS until processed for IHC staining. To facilitate sectioning, tissue was briefly immersed in eosin and then rinsed to remove excess eosin. Explants were then immersed in 1% agarose at 65°C, rapidly chilled to solidify the agarose, then processed to dehydrate the tissue and embedded into wax. Explant sections were cut serially at 5 μm across 7 slides (after 7 slides, if any tissue available, a second piece of tissue placed on each slide so that it has two pieces of tissue roughly 40 μm apart). 5-micron tissue sections were stained with antibodies to TP63 (1:300) from Biocare Medical (Concord, CA), Ki-67 (prediluted) from Ventana Medical Systems (Tucson, AZ) and CK7 (1:1500) from Abcam (Burlingame, California). Slides were stained on a Ventana Discovery Ultra (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was performed using CC1 for 40 minutes at 95°C. The primary antibodies were incubated on the sections for 32 minutes at 37°C. Primary antibodies were visualized with DAB as a chromagen using the UltraMap (Ki-67, TP63) or OmniMap (CK7) detection systems (Ventana Medical Systems) followed by hematoxylin as a counterstain. Slides were rinsed, dehydrated through alcohol and xylene and cover slipped.

Vesicle flow cytometry

EV concentration, size, and surface marker expression were measured by single vesicle flow cytometry,[52,53] using a commercial kit (vFC™ Assay kit, Cellarcus Biosciences, La Jolla, CA) and flow cytometer (CellStream™, Luminex Corp). For EV characterization studies, PES was collected from placental cultures performed at ambient oxygen concentration (21%) at t = 8h for each media condition and centrifuged at 2500 g for 15 minutes at 4°C. The cell-free supernatant was carefully removed and transferred to a fresh tube for storage at -80°C. Similar collection methods were repeated for a total of 5 placental replicates for first trimester [10 to 12-week GA] and 5 placentae for third trimester [36 to 42-week GA]. After the completion of all replicate experiments, the cell-free explant supernatants from each replicate was thawed on ice and pooled. The pooled PES for first trimester and pooled PES for third trimester were separately processed for 40-fold concentration in Amicon® Ultra 4 mL Centrifugal Filters (Millipore Sigma, UFC800324) and proceeded to perform vFC™ EV Analysis Assay (Cellarcus Biosciences, CBS4). Briefly, samples were stained with the fluorogenic membrane stain vFRed™ and one or more fluorescent antibodies targeting canonical EVs such as tetraspanins, placenta-associated EVs using PLAP, and antibodies for common antigens expressed on platelet EVs (CD41), RBCs-derived EVs (CD235ab), immune cell-origin EVs (CD54), and stromal cell-derived EVs (CD73, CD105, CD90) (see under 'Antibodies' in key resources table), for 1h at RT and analyzed using the CellStream's Small Particle detection mode. Controls included 'buffer

only', 'media only' and positive and negative controls for antigen expression (Figures S5–S7, S11, and S12). Spectral compensation was performed using antibody-stained antibody capture beads and validated using single stained controls (Figures S13 and S14). Data were analyzed using FCS Express (*De Novo* Software) and included calibration using vesicle size and fluorescence intensity standards (Figures S13 and S14). The analysis included a pre-stain dilution series to determine the optimal initial sample dilution and multiple positive and negative controls, per guidelines of the International Society for Extracellular Vesicles (ISEV).[54] A detailed description of vFC™ methods and controls, as well as the MIFlowCyt and MIFlowCyt-EV checklists, as requested by the guidelines are provided in supplemental information (Methods S1, Figures S8–S14; Tables S1 and S2).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical comparisons were made using paired Student's t-test. Differences were considered statistically significant at $p < 0.05$.