Biochemical and Biophysical Research Communications 651 (2023) 20–29

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Placental capillary pericytes release excess extracellular vesicles under hypoxic conditions inducing a pro-angiogenic profile in term pregnancy

Neva Kandzija [a, 1], Maryam Rahbar [a, 1], Gabriel Davis Jones [a], Carolina Motta-Mejia [a, b], Wei Zhang [a], Yvonne Couch [c], Ain A. Neuhaus [c], Uday Kishore [b], Brad A. Sutherland [d], Christopher Redman [a], Manu Vatish [a, *]

[a] Nuffield Department of Women's & Reproductive Health, University of Oxford, Women's Centre, John Radcliffe Hospital, Oxford, UK
[b] Biosciences Division, College of Health and Life Sciences, Brunel University London, London, UK
[c] Acute Stroke Program, Radcliffe Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK
[d] Tasmanian School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Australia

ARTICLE INFO

Article history:
Received 18 January 2023
Received in revised form
2 February 2023
Accepted 6 February 2023
Available online 7 February 2023

Keywords:
Placental pericytes
Hypoxia
Small extracellular vesicles
Angiogenesis

ABSTRACT

Pericytes are multifunctional cells wrapped around capillary endothelia, essential for vascular health, development, and blood flow regulation, although their role in human placental chorionic villi has not been fully explored. The second half of normal pregnancy is characterized by a progressive decline in placental and fetal oxygen levels which, by term, comprises a substantial degree of hypoxia. We hypothesized this hypoxia would stimulate pericyte regulation of chorionic villous capillary function. This study's objective was to investigate the role of hypoxia on normal term placental pericytes (PLVP) and their signaling to endothelial cells. First, we confirmed fetoplacental hypoxia at term by a new analysis of umbilical arterial blood oxygen tension of 3,010 healthy singleton neonates sampled at caesarean section and before labor. We then measured the release of cytokines, chemokines, and small extracellular vesicles (PLVPsv), from PLVP cultured at 20%, 8% and 1% O_2. As O_2 levels decreased, secreted cytokines and chemokines [interleukin-6 (IL-6), interleukin-1α (IL-1α) and vascular endothelial growth factor (VEGF)], and small extracellular vesicle markers, (Alix, Syntenin and CD9) increased significantly in the culture supernatants. When primary human umbilical vein endothelial cells (HUVEC) were cultured with PLVPsv, polygon formation, number, and tube formation length was significantly increased compared to cells not treated with PLVPsv, indicating PLVPsv stimulated angiogenesis. We conclude that adding PLVPsv stimulates angiogenesis and vessel stabilization on neighboring endothelial cells in response to hypoxia in term pregnancy compared to no addition of PLVPsv. Our finding that PLVP can release angiogenic molecules via extracellular vesicles in response to hypoxia may apply to other organ systems.

1. Introduction

Placental development requires extensive angiogenesis, which is regulated throughout pregnancy. During the first and second trimester, branching angiogenesis stimulates new vessel formation through sprouting. In the third trimester, nonbranching angiogenesis predominates, forming elongated capillary loops. The process is regulated by growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and placental growth factor (PlGF) [1]. Human placental oxygenation varies in a biphasic manner during pregnancy and is maintained by partial pressure gradients between the maternal and fetal blood flow [2]. Human placental chorionic tissue initially grows at low oxygen tensions until the end of the first trimester, when uteroplacental spiral arteries open. This happens relatively quickly causing transient oxidative stress as the intra-placental oxygen tension rises [3,4]. Placental oxygen content peaks at ≈16–24 weeks before declining progressively until term [5,6].

Capillary pericytes (PC) are multipotent, contractile spherical

* Corresponding author.
E-mail address: manu.vatish@wrh.ox.ac.uk (M. Vatish).
[1] These authors share first authorship.

https://doi.org/10.1016/j.bbrc.2023.02.015

cells with long cytoplasmic processes, which wrap around capillaries [7]. PCs can communicate with the underlying endothelial cells by direct contact through gap junctions and/or paracrine signaling [8]. They are involved in vessel stabilization, angiogenesis, signaling, and blood flow regulation [9–12]. PCs are fundamental to vascular function and present in all capillary systems. PCs are also found around micro-vessels in the placenta [placental capillary pericytes (PLVP)], but their physiological role is unclear. It has been reported PLVPs are involved in placental development and homeostasis during pregnancy [13]. Although currently there is no evidence of how PLVPs respond to a stimulus such as severe hypoxia, and how this response may affect neighboring cellular function, it is likely to involve significant inter- and intra-cellular communication pathways. Here, we focused on inter-cellular communication via extracellular vesicles (EVs).

EVs are derived from the cell surfaces of all cells, being shed either after extrusion from the plasmalemma or secreted from intracellular multivesicular bodies of the endosomal pathway. They may be small, medium/large, or apoptotic bodies. This study is confined to small and medium/large EVs. They carry different cargoes (proteins, lipids, nucleic acids) and signals to target cells when they bind with various degrees of selectivity and are internalized. In this study we refer only to small and medium/large vesicles without imputing their specific biogenesis. Pericytes are designated as follows: PC (Pericyte), PLVP (Placental pericyte); their small and medium/large EVs are designated PLVPsv and PLVPmlv, respectively. Generally, after docking, they can induce phenotypic and functional changes in target cells [14]. Some studies have shown EVs release from brain PCs but there is a paucity of information on PLVP derived EVs (PLVPev) [15–17].

In this study, we first aimed to confirm the relationship between umbilical artery (UA) oxygen content and gestational length in healthy pregnancies, as an indicator of decreased placental perfusion as pregnancy progresses. We then characterized the effects of various oxygen conditions on PLVP function, especially on PLVPev release.

2. Materials & methods

2.1. Fetal oxygenation at term

Ethical approval was obtained from NRES Committee South Central Oxford with approval number 13/SC/0153. Newborn UA pO_2 measures were routinely acquired at birth. We analyzed 3,010 samples from women delivering between 36 + 0 and 41 + 0 weeks via elective caesarean section. Newborns included were congenitally normal singletons, between the 11th and 90th birthweight centiles, with UA pH > 7.05, not requiring resuscitation or admission to special care, and healthy at hospital discharge. Statistical analysis was performed using R (R Foundation, version 4.1.1). Outlier pO2 values > 2 standard deviations below the mean for each gestational week were removed as outliers. A linear regression model was fitted to the data, with gestational age at delivery (days) and UA pO_2 as independent and dependent variables. We determined the 95% confidence interval for each gestational age (days) and the mean UA pO_2 for each gestational age (weeks).

2.2. Pericyte source and culture

Pure populations of PLVP, isolated from chorionic villi microvessels from normal term human placentae, were purchased (C-12980; PromoCell). PLVP were cultured in PC growth medium (C-28040, PromoCell) for 3–4 days before passage, according to the manufacturer's instructions. PC growth medium was equilibrated for 24-h in an incubator with modified O_2 conditioned to 1% or 8% v/v O_2 at 37 °C prior to incubation with cells at these respective conditions. PLVPs were grown in 12-well plates or T75 flasks under standard tissue culture conditions (20% O_2). Once cells reached 80% confluence, PLVPs were washed with warm phosphate buffered saline (PBS) (Sigma-Aldrich, UK) and cultured in 1%, 8%, or 20% O_2 conditioned PC growth medium (C-28040; PromoCell) and then grown in incubators maintained at either 20%, 8% or 1% O_2 for 24-h. After the experiments were completed, cells were washed again with warm PBS (Sigma-Aldrich) and were lysed with radioimmunoprecipitation (RIPA) lysis buffer (ThermoFisher Scientific) or processed for RNA isolation. The culture medium (where EVs would be present) was processed by 1,500×*g* centrifugation for 10 min at room temperature to remove dead cells and debris (Beckman Coulter Avanti J-20XP centrifuge and Beckman Coulter JS-5.3 swing out rotor), 5 mL aliquots were stored at −80 °C for multiplex cytokine and chemokine array analysis, while the remaining supernatant was processed for the PLVPev isolation (see the section *Isolation of PLVP derived EV).*

2.3. Multiplex cytokine array analysis

Supernatant (processed as described above) from PLVP incubated at 20%, 8% and 1% O_2 concentration were analyzed in duplicate using a Milliplex MAP Human cytokine/chemokine magnetic bead panel kit (HCYTOMAG-60K; EMD Millipore). 25 μl of standard, controls and supernatant sample were added in a 96-well plate (NUNC; Sigma-Aldrich), followed by 25 μl of assay buffer. Next, 25 μl of magnetic beads were added and, after 2-h room-temperature incubation, the media was removed. 25 μl of detection antibodies were incubated with the beads for 1-h at room temperature. To this, 25 μl of Streptavidin-Phycoerythrin conjugate was then added to each well and incubated for 30-min in the dark at room temperature. After a washing step with assay buffer, 150 μl of sheath fluid was added to each well, and the plate was read using the Luminex MAGPIX instrument (Luminex, ThermoFisher).

2.4. Isolation of PLVP derived EV

Small and medium/large PLVP were separated by two-stage centrifugation [18]. After removing dead cells and debris (as described above), the culture supernatant was ultra-centrifuged at 10,000×*g* for 35-min at 4 °C (Beckman L80 ultracentrifuge and Sorvall TST28.39 Swing-out rotor) to collect the pellet containing PLVP derived medium/large EVs (PLVPmlv). The remaining supernatant was then passed through a 0.2 μm Stericup filter (Millipore), and ultracentrifuged at 150,000×*g* for 125-min at 4 °C (Beckman L80 ultracentrifuge and Sorvall TST28.39 swing-out rotor) to isolate the PLVP derived small vesicles (PLVPsv) pellet, which was washed and resuspended in 100 μL of filtered PBS (Sigma-Aldrich). Both PLVPmlv and PLVPsv samples were assayed for protein content using bicinchoninic acid (BCA) assay kit (Pierce, ThermoFisher) before storage at −80 °C, and particle size and concentration were analyzed using nanoparticle tracking analysis (NTA).

2.5. Nanoparticle tracking analysis

To examine the size and concentration of PLVPsv, NTA was carried out using a NanoSight NS500 (Malvern Panalytical) and NanoSight Ltd 3.1 software (Malvern Panalytical) as described in our previous publication [19] (see Supplementary).

2.6. Western blot analysis

Western blot analysis was carried out using standard protocols with cell lysate, isolated PLVPmlv and PLVPsv from all culture

conditions as previously described [20]. Reactions were visualized by using an appropriate secondary antibody conjugated to horseradish peroxidase (HRP) before incubation with HRP substrate enhanced luminescence (ThermoFisher). The antibodies used (with corresponding concentrations) are listed in Table S1 (see Supplementary).

2.7. Cell immunofluorescence with PKH26 stained EV

10 μl of PLVPsv pellet were stained using the PKH26 red fluorescent cell linker kit (MINI26; Sigma-Aldrich) according to the manufacturer's instructions (see Supplementary). Stained PLVPsv were washed thoroughly using the qEV 35 nm original size exclusion columns (Izon Science) to remove unbound dye. 1×10^9particle/mL of PLVPsv were used to treat 2.5×10^5 cells/mL of primary HUVEC for 2- and 6-h. Cells were then incubated with 5 μg/mL of wheat germ-like agglutinin (WGA) green (Vector Laboratories), and 1/5,000 Hoechst 33342, Trihydrochloride Trihydrate (Life Technologies), and fixed with 2% v/v paraformaldehyde (PFA) (Sigma-Aldrich).

2.8. HUVEC angiogenic activity

Cultured HUVECs' angiogenic activity under treatment using PLVPsv from different degrees of oxygenation was quantified. Endothelial cells can differentiate to form capillary-like tubes using a Matrigel basement membrane to attach and migrate towards each other. Tube formation can be imaged and analyzed over time. Primary HUVECs were used after 3 or 4 passages at 80% confluency. 50 μl of Matrigel basement membrane with reduced matrix growth factor (356231; Scientific Laboratory Supplies Limited) was added to a 96-well plate (NUNC; Sigma-Aldrich, UK). 1×10^4 cells/mL of HUVEC were resuspended in HUVEC media with 4% fetal calf serum (FCS) (pre-ultracentrifuged to render it EV-free) and added onto the Matrigel pre-coated plate. 1×10^9particles/mL of PLVPsv and EV-depleted supernatant from PLVP incubated at 20%, 8% and 1% O_2 conditions were subsequently added onto the plate for a 16-h incubation at 37 °C under standard tissue culture conditions (20% O_2). After incubation, the plate was washed thoroughly with cold filtered PBS, fixed with cold 4% PFA for 1-h at 4 °C, and finally washed with PBS. Images of samples from each well were captured using a Leitz Labovert FS optical microscope (Leica Microsystems) at X4 magnification and photographed using a digital camera (Q-Imaging 5.0 RTV) linked to a computer hard drive and software Q-capture PRO 7 (Q-Imaging). Images were analyzed using ImageJ software (ImageJ/Fiji, version 1.51 v9). Image files were converted from 8-bit to red, green, blue (RGB) before using 'Batch Image Treatment Tool' and 'HUVEC phase contrast' angiogenesis analyzer macro software for tube length and polygon HUVEC formation.

2.9. Statistical analysis

The data were analyzed using Prism (GraphPad Software, Version 6). Normality testing was performed using the Kolmogorov-Smirnov test and visual inspection. The data were analyzed using the unpaired *t*-test, or one-way repeated measures ANOVA. Data were presented as Mean ± Standard Error Mean (SEM).

3. Results

3.1. Relationship between gestational age and umbilical artery pO_2

We modelled the relationship between UA pO_2 (carrying fetal blood to the placenta) and increasing gestational age at delivery in 3,010 healthy babies born via elective caesarean section (Fig. 1). We focused on elective caesarean sections to exclude the contribution of labor to UA pO_2. Simple linear regression indicated a negative relationship between UA pO_2 and gestational age at delivery between 36 + 0 and 41 + 0 weeks. The y-intercept and beta-coefficient for gestational age was 28.93 ($p < 0.001$) and −0.05 ($p < 0.01$). The adjusted R-squared and F-statistic values were 0.004 and 12.24. Given these results may suggest placental hypoperfusion and consequently hypoxia in term fetuses, we next assessed hypoxia's effects on pericytes.

3.2. Pericytes increase release of immunomodulatory factors under hypoxic conditions

To confirm functional as well as morphological changes in response to hypoxia, we collected supernatants from PLVP cultured at the three O_2 concentrations and removed apoptotic cells/cellular debris using centrifugation. We assessed the supernatant by using an immunological MILLIPLEX assay ($n = 3$; Fig. 2), which assays cytokines, chemokines and growth factors [21]. From the nine cytokines tested, both IL-6 and IL-1α showed increased secretion in 1% O_2 when compared to 20% O_2 or 8% treated cells, which was significant for both cytokines ($p < 0.01$; Fig. 2A). Interestingly, both IL-6 and IL-1α exhibited a small reduction at 8% O_2 when compared to 20% O_2 (Fig. 2A). Within the five chemokines assayed, IP-10 showed a significant reduction in release as oxygen concentrations fell ($p < 0.05$; Fig. 2B). The other four chemokines were also detected (IL-8, MCP-1, GRO and Fractalkine) but showed no response to altered oxygen concentrations suggesting detected alterations were specific.

Growth factor, VEGF, exhibited a similar pattern of release seen with IL-6 and IL-1α in that 8% O_2 caused a non-significant secretion reduction, but extreme hypoxia (1% O_2) caused a robustsecretion increase ($p < 0.05$; Fig. 2C). FGF-2 was released in large quantities but showed no response to altered oxygen concentrations.

3.3. Phenotype of PLVP EVs and their increased number under hypoxia

PLVP cell lysate and PLVP derived EVs were obtained from 20%, 8% and 1% O_2 conditions (see Methods) ($n = 3$; Fig. 3). PLVPsv were found to express the well-known small EV markers Alix, Syntenin and CD9 (Fig. 3A), which showed enhanced expression compared to the PLVPmlv. Further EV characterization was performed by NTA, determining the particle size and concentration of PLVPmlv (Fig. 3B) and PLVPsv (Fig. 3C). PLVPmlv were significantly increased in particle number as oxygen levels decreased (20% O_2 vs 1% O_2, $p < 0.001$; 8% O_2 vs 1% O_2, $p < 0.001$; Fig. 3Bii), while modal size remained unchanged between samples (Fig. 3Biii). PLVPsv also significantly increased in particle number when the O_2 conditions became more hypoxic (20% O_2 vs 8% O_2: $p < 0.05$; 20% O_2 vs 1% O_2: $p < 0.0001$; Fig. 3Cii). Like PLVPmlv, PLVPsv modal size remained unchanged between samples (Fig. 3Ciii).

3.4. Internalization of PKH26 labelled EVs into HUVECs

Given the significantly higher numbers of PLVPsv (10^{10}) compared to PLVPmlv (10^8), we focused on PLVPsv for further studies. Confocal microscopy was used to assess whether PLVPsv could be internalized by HUVECs at different time points (2- and 6-h; $n = 3$; Fig. 4). PLVPsv from normal O_2 conditions (20% O_2) were pre-stained with the lipophilic dye, PKH26 (red). The PLVPsv were separated from unbound dye by use of an Izon qEV size exclusion column (see Supplementary). HUVEC cell membranes were labelled with WGA-Alexa 488 (green) and HUVEC nuclei with

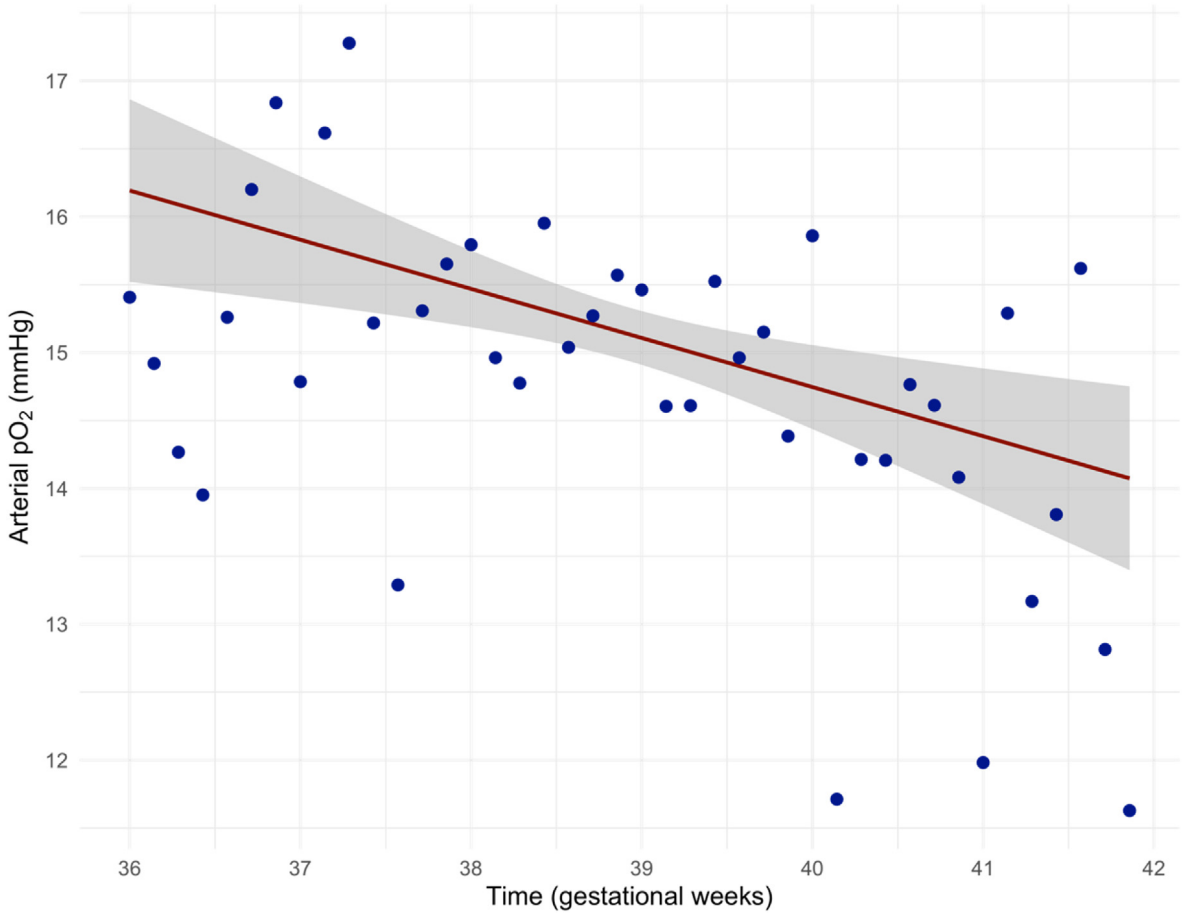

Fig. 1. Relationship between gestational age and umbilical artery pO_2. The average umbilical arterial pO_2 decreases by 0.05 mmHg with each increasing day of gestation between 36 + 0 and 41 + 0 weeks ($p < 0.001$). The red line represents the line of best fit using a linear regression model (y-intercept = 28.93, β-coefficient = −0.05, adjusted R-squared 0.004, F statistic 12.24, $p < 0.01$). The grey band represents the 95% confidence interval. Blue dots represent the mean arterial pO_2. The sample size for each gestational age (weeks) was 108 (36), 266 (37), 957 (38), 1487 (39), 175 (40) and 17 (41).

Hoechst 33342 (blue). PLVPsv were internalized by HUVECs in similar numbers at both 2- and 6-h incubation (Fig. 4). These data confirmed PLVPsv internalization.

3.5. PLVPsv stimulates HUVEC tube length and polygon formation

To investigate the effect of PLVPsv derived from normal and hypoxic conditions, we performed a tube formation assay with HUVECs (n = 3; Fig. 5). Equal numbers (1×10^9particles/mL) of PLVPsv and EV-depleted supernatant samples from 20%, 8% and 1% O_2 conditions were added to 1×10^4 cells/mL (HUVECs) for a 16-h incubation. Captured images from HUVECs incubated with PLVPsv or EV-depleted supernatant and untreated HUVECs were analyzed using ImageJ software, demonstrating the presence or lack of tube length, and polygon formation (Fig. 5A).

Averages were calculated for tube polygons total number (Fig. 5B). PLVPsv derived from all oxygen conditions (20%, 8%, 1%) stimulated significant increases in polygon number on HUVECs compared to untreated HUVECs (20% 19.15 ± 2.1; 8% 15.8 ± 1.5; 1% 17 ± 1.6; control: 5.7 ± 1.9; $p < 0.0001$ for all). EV-free supernatant from the three different O_2 conditions did not cause a change in polygon total number in comparison to controls suggesting an EV specific effect. Next, we assessed HUVEC tube formation length (Fig. 5C). PLVPsv derived from 20% O_2 conditions stimulated tube formation significantly on HUVEC (10,823 ± 552 pixels) compared to untreated HUVEC (control: 5,131 ± 845 pixels; $p < 0.0001$). This was also seen in HUVEC treated with PLVPsv derived from 8% O_2 (9,821 ± 452 pixels vs control; $p < 0.0001$) and 1% O_2 (9,129 ± 504 pixels vs control; $p < 0.0001$). PLVPsv demonstrated similar increases in tube formation across different O_2 concentrations. Notably, EV-free supernatant did not induce these increases again, suggesting an EV-specific effect. These results established PLVPsv stimulate an angiogenic role on HUVECs, not replicated by EV-free supernatant.

3.6. Validation of pro-angiogenic factors carried by PLVPsv

Given PLVPsv's previously identified pro-angiogenic roles, we decided to investigate the presence on well-known pro-angiogenic factors on PLVPsv by Western blot. Pooled PLVPsv (n = 3), were run and probed against our chosen proteins (PDGFRβ, VEGFR2, NRP1 and ENG). Our results demonstrated that PLVPsv derived from all three O_2 conditions expressed PDGFRβ (180 kDa), VEGFR2 (105 kDa) and ENG (95 kDa) at their corresponding molecular weights. Interestingly, we were only able to detect the dimer form of NRP1 (250 kDa) in PLVPsv from 20% to 1% O_2 (Fig. 6). These molecules' presence suggests a pro-angiogenic role for PLVPsv.

4. Discussion

We have demonstrated a possible negative correlation between increasing gestational age (36+0–41 + 0 gestational weeks) and UA

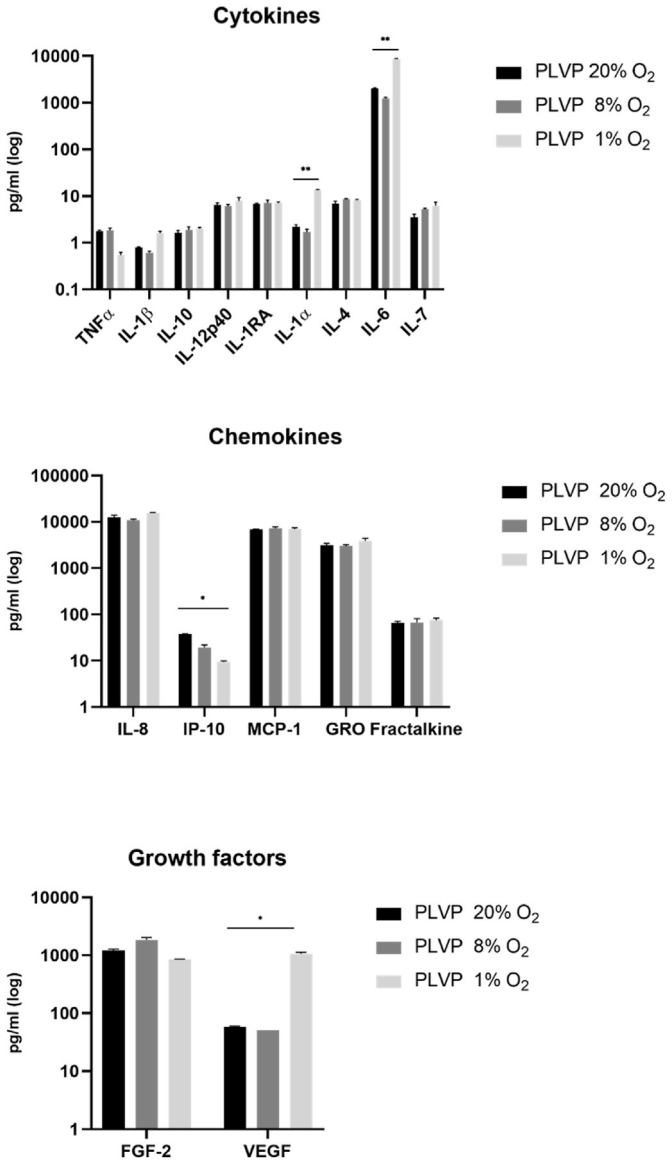

Fig. 2. MILLIPLEX assay analysis of PLVP supernatant after a 24-h incubation at 20%, 8% and 1% O_2 conditions (n = 2). (**A**) Cytokines: TNFα, IL-1β, IL-10, IL-12p40, IL-1RA, IL-1 α, IL-4, IL-6, IL-7 (**B**) Chemokines: IL-8, IP-10, MCP-1, GRO, and (**C**) Growth factor profiles: FGF-2, and VEGF from PLVP supernatant. Data presented as Mean ± SEM, one-way repeated measures ANOVA test were used for statistical analysis. Significant difference shown as $p < 0.05$ (*), and $p < 0.01$ (**).

pO_2 at delivery in healthy newborns delivered via elective caesarean section, removing biochemical confounding associated with labor (itself associated with hypoxia). This expands upon the finding that the placenta is exposed to an increasingly hypoxic environment as pregnancy proceeds in healthy babies born via vaginal delivery [5,6,22]. We wanted to explore the effects of hypoxia on PLVP given that these cells are crucial in regulating blood flow and oxygen delivery. We demonstrated that hypoxia alters the secretion of cytokines and increases the release of PLVPsv. These PLVPsv were characterized by size and expression of known small EV markers and we determined that their increased release correlated directly with decreasing oxygen concentration. Furthermore, we showed that PLVPsv were internalized by endothelial cells where they significantly stimulated both tube length and polygon formation, not seen by EV-free media. We next performed western blot analysis and validated key differentially expressed pro-angiogenic proteins.

The placenta is the primary exchange surface across which nutrients and oxygen cross to fetuses, and the functioning unit of the placenta is the chorionic villus. Nutrition, oxygen and waste products travel between maternal and fetal circulations across the syncytiotrophoblast – the villus' outermost surface with direct contact with maternal blood [23]. Fetal circulation, on the other hand, circulates in capillaries contained within the villus. These placental capillaries comprise two interacting cell types: endothelial cells which form the lumen of the vessel, and PCs which surround the vessel [13,24,25]. PCs exert significant blood flow regulation in capillaries; PC deficient capillaries become hemorrhagic and hyper dilated whilst, in the presence of hypoxia, pericytes constrict and close capillary blood flow. The latter effects have been studied extensively in the brain where hypoxia causes pericyte death in rigor (with associated morphological changes), irreversibly constricting capillaries [12]. However, little work on placental PCs has been carried out. Our observation of decreasing UA pO_2/increasing hypoxia as pregnancy progresses prompted our investigation of hypoxia's effects on PLVPev and their communication with endothelial cells. Since placental hypoxia can lead to pathological conditions, pericytes could serve the placenta to adapt to negative conditions of hypoxia by promoting vascular health and intracellular communications.

Whilst many cellular systems are assessed in 20% oxygen, it is well described that in the normal placenta, tissue oxygen level is closer to 8% and hypoxia can be represented by oxygen concentrations of 1% [26]. We therefore used these three oxygen concentrations to explore PLVP secretion of cytokines, chemokines, and growth factors.

By responding to a series of different stimuli, PCs have been shown to discharge a heterogenous secretion of pro/anti-inflammatory molecules, pleiotropic cytokines and various stem cell chemokines [27,28]. Relatively few papers have used hypoxia as a form of stimulus with PCs. Chen et al. showed that, under hypoxic conditions, microvascular pericytes express elevated levels of IL-6, LIF, COX-2, HMOX-1, and HIF-1α. This study also demonstrated that under hypoxic conditions, genes encoding pro-angiogenic factors such as, VEGFA, PDGFRβ, and TGFβ1 were upregulated significantly, whereas other pro-angiogenic factors such as bFGF/FGF2 and EGF were downregulated in hypoxic conditions [29].

We studied cytokines' release into the cell culture media from PLVPsv. Similarly to microvascular PCs, PLVPsv showed significantly higher levels of IL-6 and VEGF induced by hypoxia at 1%, alongside a non-significant reduction in FGF2. We also noted a significant increase in IL-1α levels as O_2 concentrations decreased, alongside a significant oxygen-concentration-dependent decrease of IP-10 levels. In addition, we noted a trend towards increasing levels of IL-7.

IL-6 is known to play a role in maintaining cellular homeostasis in pregnancy [30], while VEGF clearly plays a role in angiogenesis [31]. Elevated release of these molecules may be an attempt to protect vascular integrity. FGF2 is associated with vascular remodeling [32] as well as placental implantation [33] – their decreased release would negatively impact placental health.

IL-1α plays a role in immune response, specifically inflammation. It is involved in implantation, placental development, protection against infection and pregnancies' successful outcomes [33]. IL-7 is another proinflammatory cytokine. While there is limited data concerning its role in healthy pregnancy, it has been shown as significantly elevated in spontaneous and recurrent miscarriage [34]. The combination of pro-angiogenic and vascular remodeling molecules PLVPsv release suggests a protective mechanism in response to hypoxic stress.

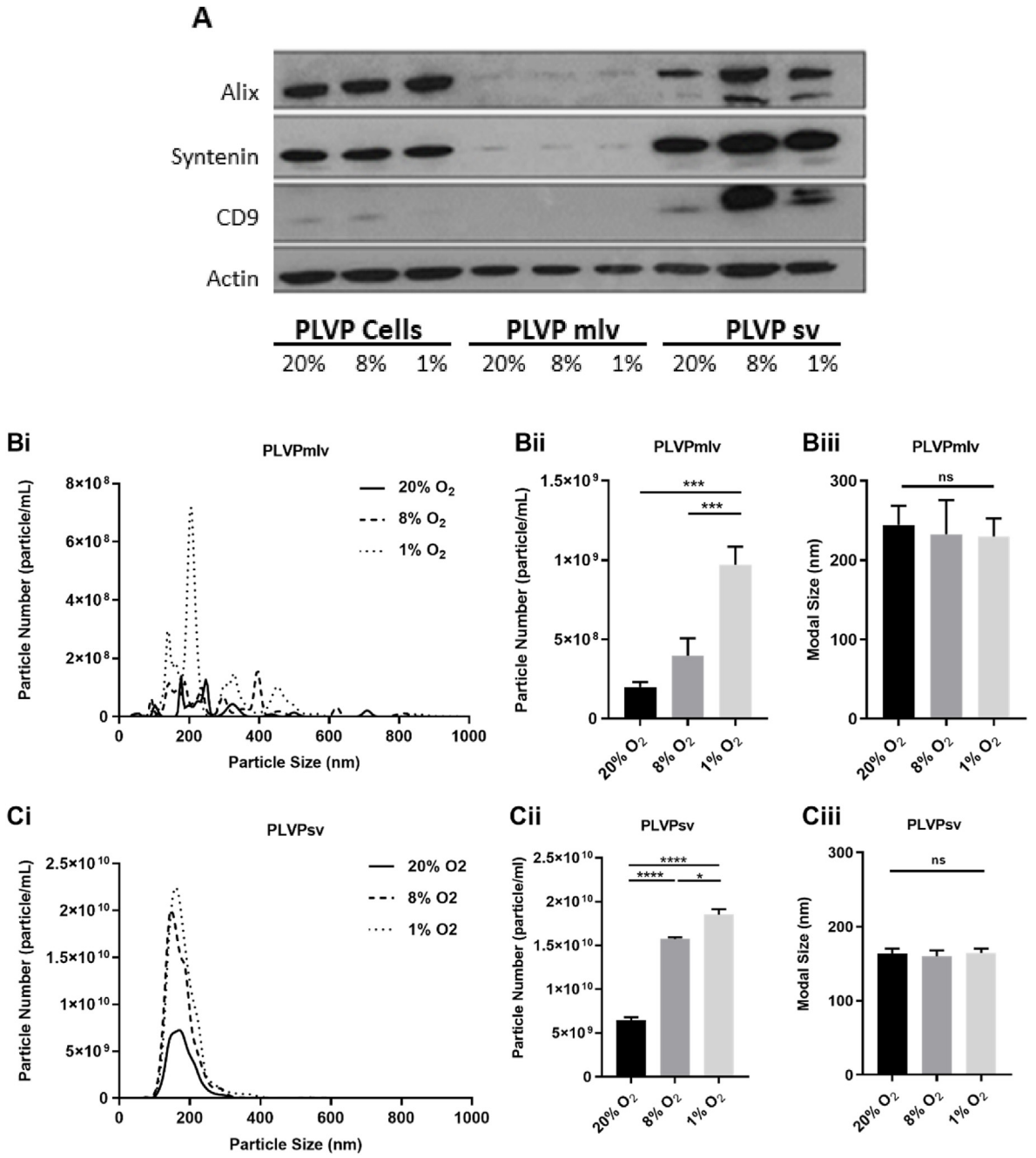

Fig. 3. Characterization of PLVPs, PLVPmlv and PLVPsv. (**A)** Representative immunoblot showing expression of Actin (42 kDa), the loading control on PLVP cell lysate, PLVPmlv and PLVPsv; and enrichment of EV markers Alix (96 kDa), Syntenin (60 kDa) and CD9 (24 kDa) on PLVPsv. (**Bi)** Representative NTA size and particle number distribution profiles of PLVPmlv; (**Bii**) particle number of PLVPmlv in response to different oxygen concentration; (**Biii**) and PLVPmlv modal size in different oxygen concentrations. (**Ci)** Representative NTA size and particle number distribution profiles of PLVPsv; (**Cii**) particle number of PLVPsv samples in response to different oxygen concentration; (**Ciii**) PLVPsv modal size in different oxygen concentrations. Data presented as Mean ± SEM, significant difference shown as $p < 0.05$ (*), $p < 0.001$ (***), $p < 0.0001$ (****) or non-significant (ns).

Our study is the first to demonstrate PLVP secrete EVs (both medium/large and small), and this release is enhanced in an oxygen dependent manner. Additionally, PLVPsv are not only internalized by primary HUVEC, but also stimulate HUVEC tube polygon and tube length formation. EV-free conditioned media do not induce a pro-angiogenic function on HUVEC, suggesting PLVPsv mediate this angiogenesis.

Our results align with those few research groups studying PC derived small EVs. Mayo and Bearden showed brain PCs perturbated with cobalt chloride promoted wound healing in cell culture, cord formation in collagen matrices, and microvascular density in spinal cord explants through the secretion of small EVs [35]. Similarly, Gaceb et al. demonstrated brain PCs secreted pro-regenerative molecules, including the release of medium/large EVs carrying growth factors, in response to stress. They also emphasized the potential importance of EVs in cell-to-cell communication between PCs and endothelial cells, especially in vessel regeneration and repair [28].

Additionally, we analyzed known pro-angiogenic factors using western blot analysis of PLVPsv. The proteins chosen for

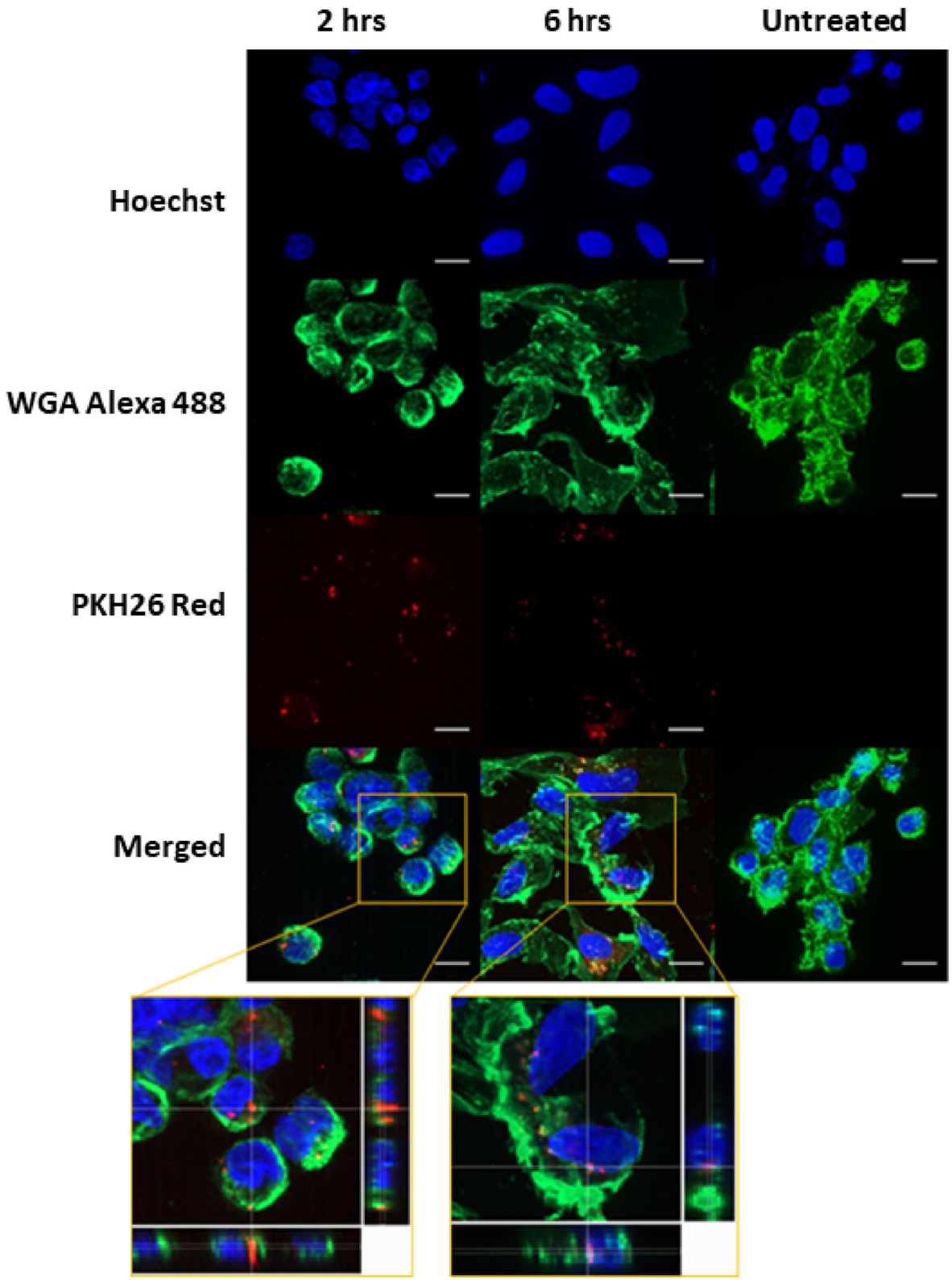

Fig. 4. Internalization of PLVPsv derived from 20% O_2 conditions into HUVEC (n = 3). HUVECs were treated with PKH26 stained PLVPsv (1×10^9particle/mL) at 2- and 6-h incubation. Cells' nuclei labelled with Hoechst dye (blue), cells' membrane labelled with WGA Alexa 488 (green), PLVPsv labelled with PKH26 dye (red) and merged channels are shown. HUVEC incubated with EV-free supernatant were used in the untreated cells. The A stack confirmed internalization of PLVPsv. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

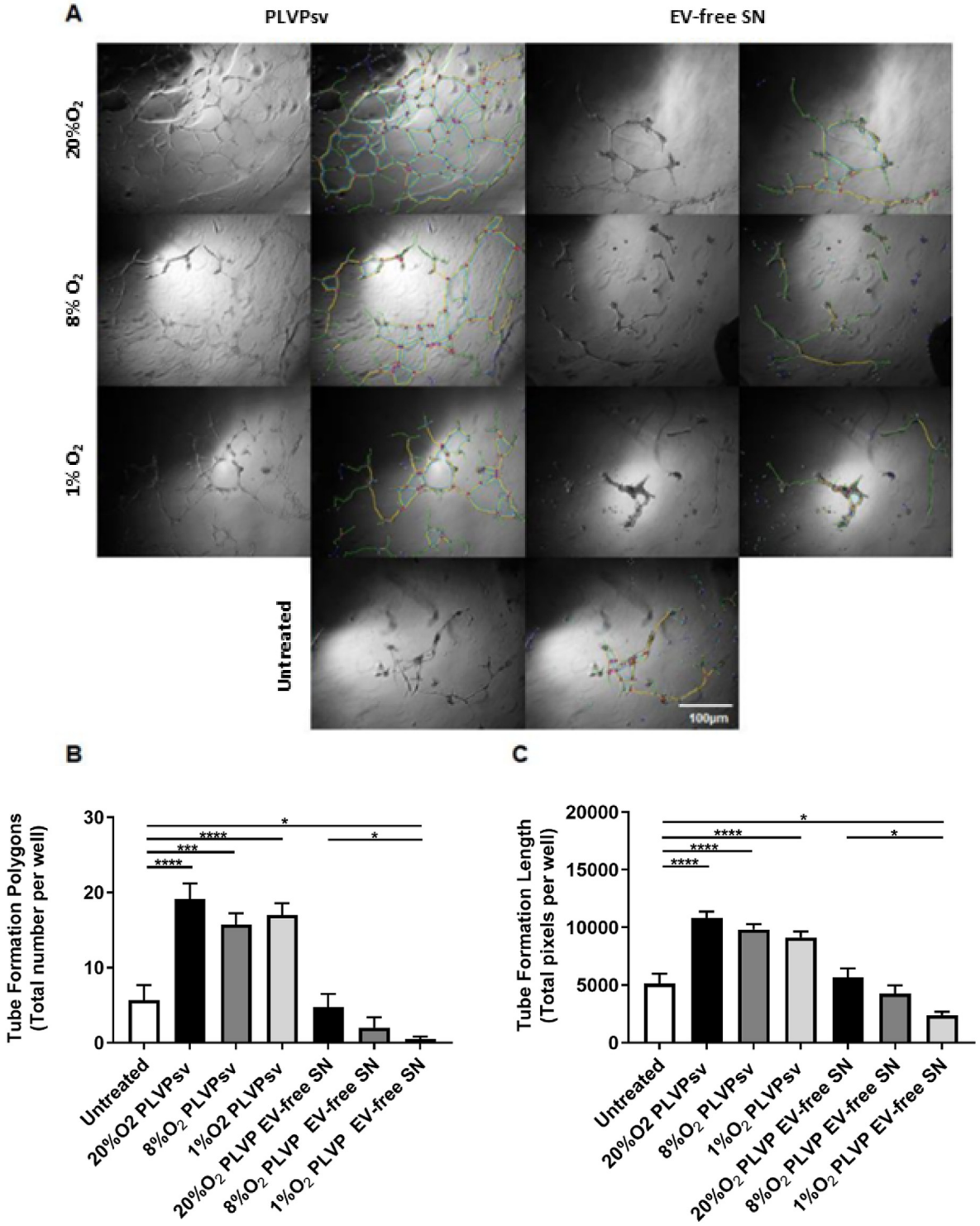

Fig. 5. Tube formation analysis of HUVEC treated with PLVPsv and EV-free supernatant from normal and hypoxic conditions (n = 3). (**A**) Images of HUVEC treated with PLVPsv and EV-free SN derived from 20%, 8% and 1% O_2 conditions and untreated HUVEC were analyzed by ImageJ. **(B)** Analysis of HUVEC tube polygon formation **(C)** and tube length formation when treated with each sample. Data presented as Mean ± SEM, significant difference shown as $p < 0.05$ (*), $p < 0.001$ (***), $p < 0.0001$ (****). Scale bar, 100 μm.

investigation were VEGFR2, neuropilin 1 (NRP1), and endoglin (ENG). Furthermore, we identified the known PC marker, platelet derived growth factor receptor β (PDGFRβ) in addition to established small EV markers CD9 and Syntenin. Importantly, the Western blots likely underestimate the quantities of these molecules released (equal protein loading) since we have demonstrated significantly more EVs are released in hypoxia.

Small EVs are known to be involved in cell-to-cell communication, and we have demonstrated PLVPsv can be internalized into endothelial cells. Thus, PLVPsv associated ENG, VEGFR2, and NRP1 could promote angiogenesis. PDGFRβ, a known PC marker, plays important roles in recruitment of vascular pericytes into ischemic

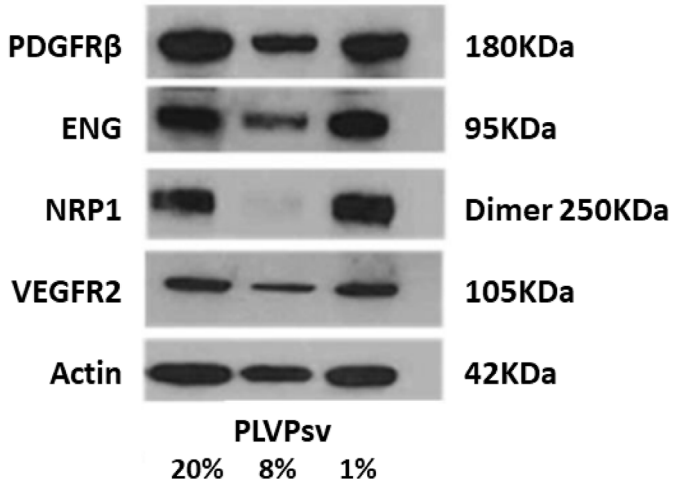

Fig. 6. Western blot validation of pro-angiogenic molecules. Representative image of PLVPsv protein from 20%, 8% and 1% O_2 conditions (n = 2) expressing PDGFRβ (180 kDa), ENG (95 kDa), as well as VEGFR2/KDR (105 kDa). The dimer form of NRP1 (250 kDa) was observed but not its monomer form (130 kDa). Actin was used as loading control.

lesions [36]. Thus, hypoxic injury in the placenta might lead to a dual reaction, an enhanced angiogenic response, coupled to recruitment of nearby vascular pericytes to mitigate the effects of hypoxic damage. Our Western blot analysis revealed a significant difference in the expression of NRP1 between placental normoxia (8%) and hypoxia (1%). NRP1 facilitates transduction of VEGF's arterial effects and has essential roles in vascular and neuronal development. Hence, the secretion of PLVPsv-bound NRP1 in high amounts under hypoxia might potentially outline a survival mechanism by which PLVP sustain fetoplacental vascular remodeling and maturation in reduced oxygen levels. The selected proteins did not show a significant difference between 20% and 1% oxygen. While not the primary focus of our work, it is interesting that hyperoxia may also drive changes in protein expression.

We are not the first to imply EVs' content might have a role in the pericyte-endothelial crosstalk communication. Studies have investigated PCs as EV recipient target cells. Caporali et al. showed the shedding of miR-503 enriched microparticles by endothelial cells in response to high glucose culturing conditions, and the integrin-mediated uptake in the recipient PCs, reducing Ephrin B2 (EFNB2) expression and VEGFA, resulting in impaired migration and proliferation [37,38]. Likewise, another group demonstrated stimulation with inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) and endotoxin induced the shedding of endothelial cell-derived EV loaded with miRNAs, such as miR-328−3p and let-7d-3p [39]. While in the above-mentioned studies, the PCs were shown to be EV target cells, we proposed PC-derived small vesicles play a role in the regulation of endothelial cell function.

Although other factors may contribute to PLVPsv's excess release, we focused here on delineating PLVP's response to different oxygen concentrations, specifically hypoxia. Hypoxia's relevance is seen throughout pregnancy as oxygen levels change and hypoxia is induced toward the end of pregnancy. We identified the pro-angiogenic factors PLVP release in response to hypoxia and showed the increase in the release of small EVs with decreasing oxygen levels. It would be interesting to isolate and study PLVP and PLVP derived EVs from different pregnancy outcomes such as pre-eclampsia or intra-uterine growth restriction where hypoxia is exacerbated. Our findings are descriptive rather than mechanistic, warranting further research to better understand the mechanisms involved in this response. Our findings of increased pro-angiogenic chemokine, cytokine, and PLVPsv release in response to hypoxia provides a rationale for similar investigation of PC derived from other hypoxia-effected organ systems (e.g., brain, heart, etc.).

For the first time, we show PLVP release EVs more so in response to hypoxia, carrying increased cargoes of pro-angiogenic factors such as VEGF. We also show PLVPsv, released under hypoxia, can induce increased tube and polygon formation in cultured HUVECs. Thus, we demonstrate a new mode of communication between PLVP and HUVECs in addition to those involving gap junctions and paracrine signaling.

Funding

This study was supported by an MRC Program Grant (MR/J003360/1).

Brad Sutherland is supported by the National Health and Medical Research Council of Australia (APP1137776).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank research midwives Fenella Roseman and Lotoyah Carty for recruiting patients for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.02.015.

Abbreviations

PCs	Pericytes
PLVP	Placental capillary pericytes
PLVPev	PLVP derived extracellular vesicles
PLVPsv	PLVP derived small extracellular vesicles
PLVPmlv	PLVP medium/large extracellular vesicles
HUVEC	Human umbilical vein endothelial cells
UA	Umbilical artery
RIPA	Radioimmunoprecipitation
DTT	Dithiothreitol

References

[1] R. Demir, U.A. Kayisli, S. Cayli, B. Huppertz, Sequential steps during vasculogenesis and angiogenesis in the very early human placenta, Placenta 27 (6–7) (2006) 535–539.
[2] G.A. Nye, E. Ingram, E.D. Johnstone, O.E. Jensen, H. Schneider, R.M. Lewis, et al., Human placental oxygenation in late gestation: experimental and theoretical approaches, J. Physiol. 596 (23) (2018) 5523–5534.
[3] J. Espinoza, R. Romero, Y.M. Kim, J.P. Kusanovic, S. Hassan, O. Erez, et al., Normal and abnormal transformation of the spiral arteries during pregnancy, J. Perinat. Med. 34 (6) (2006) 447.
[4] G.J. Burton, E. Jauniaux, A.J. Murray, Oxygen and placental development; parallels and differences with tumour biology, Placenta 56 (2017) 14–18.
[5] J. Walker, Foetal anoxia, Int J Obstet Gynaecol 61 (1954) 162–180.
[6] P.W. Soothill, K.H. Nicolaides, C.H. Rodeck, S. Campbell, Effect of gestational age on fetal and intervillous blood gas and acid-base values in human pregnancy, Fetal Ther. 1 (4) (1986) 168–175.
[7] P. Dore-Duffy, K. Cleary, Morphology and properties of pericytes, Methods Mol. Biol. 686 (2011) 49–68.
[8] G. Bergers, S. Song, The role of pericytes in blood-vessel formation and maintenance, in: Neuro-Oncology, vol. 7, Oxford University Press, 2005, pp. 452–464.
[9] M. Sweeney, S. Ayyadurai, B. Zlokovic, Pericytes of the neurovascular unit: key

functions and signaling pathways, Nat. Neurosci. 19 (6) (2016) 771–783.
[10] R. Navarro, M. Compte, L. Álvarez-Vallina, L. Sanz, Immune regulation by pericytes: modulating innate and adaptive immunity, Front. Immunol. (NOV) (2016) 480, 0.
[11] R.K. Jain, M.F. Booth, What brings pericytes to tumor vessels? J. Clin. Invest. 112 (8) (2003) 1134–1136.
[12] C.N. Hall, C. Reynell, B. Gesslein, N.B. Hamilton, A. Mishra, B.A. Sutherland, et al., Capillary pericytes regulate cerebral blood flow in health and disease, Nature 508 (1) (2014) 55–60.
[13] R.S.N. Barreto, P. Romagnolli, A.D. Cereta, L.M.C. Coimbra-Campos, A. Birbrair, M.A. Miglino, Pericytes in the placenta: role in placental development and homeostasis, Adv. Exp. Med. Biol. 1122 (2019) 125–151.
[14] D. Tannetta, R. Dragovic, Z. Alyahyaei, J. Southcombe, Extracellular vesicles and reproduction–promotion of successful pregnancy, Cell. Mol. Immunol. 11 (6) (2014) 548–563.
[15] E. Gonul, B. Duz, S. Kahraman, H. Kayali, A. Kubar, E. Timurkaynak, Early pericyte response to brain hypoxia in cats: an ultrastructural study, Microvasc. Res. 64 (1) (2002) 116–119.
[16] L.S. Brown, C.G. Foster, J.-M. Courtney, N.E. King, D.W. Howells, B.A. Sutherland, Pericytes and neurovascular function in the healthy and diseased brain, Front. Cell. Neurosci. (2019) 282, 0.
[17] J. Rustenhoven, D. Jansson, L.C. Smyth, M. Dragunow, Brain pericytes as mediators of neuroinflammation, Trends Pharmacol. Sci. 38 (3) (2017) 291–304.
[18] R.A. Dragovic, G.P. Collett, P. Hole, D.J.P. Ferguson, C.W. Redman, I.L. Sargent, et al., Isolation of syncytiotrophoblast microvesicles and exosomes and their characterisation by multicolour flow cytometry and fluorescence Nanoparticle Tracking Analysis, Methods 87 (2015) 64–74.
[19] N. Kandzija, W. Zhang, C. Motta-Mejia, V. Mhlomi, J. McGowan-Downey, T. James, et al., Placental extracellular vesicles express active dipeptidyl peptidase IV; levels are increased in gestational diabetes mellitus, J. Extracell. Vesicles 8 (1) (2019).
[20] C. Motta-Mejia, N. Kandzija, W. Zhang, V. Mhlomi, A. Cerdeira, A. Burdujan, et al., Placental vesicles carry active endothelial nitric oxide synthase and their activity is reduced in preeclampsia, Hypertension (New York) 70 (2) (2017) 372–381.
[21] K. Nakagome, Y.A. Bochkov, S. Ashraf, R.A. Brockman-Schneider, M.D. Evans, T.R. Pasic, et al., Effects of rhinovirus species on viral replication and cytokine production, J. Allergy Clin. Immunol. 134 (2) (2014) 332–341.e10.
[22] M.L. Kitlinski, K. Källén, K. Marsál, P. Olofsson, Gestational age–dependent reference values for pH in umbilical cord arterial blood at term, Obstet. Gynecol. 102 (2) (2003) 338–345.
[23] N. Gude, C. Roberts, B. Kalionis, R. King, Growth and function of the normal human placenta, Thromb. Res. 114 (5–6) (2004) 397–407.
[24] S.E. Harris, K.S. Matthews, E. Palaiologou, S.A. Tashev, E.M. Lofthouse, J. Pearson-Farr, et al., Pericytes on placental capillaries in terminal villi preferentially cover endothelial junctions in regions furthest away from the trophoblast, Placenta 104 (2021) 1–7.
[25] C.J.P. Jones, G. Desoye, A new possible function for placental pericytes, Cells Tissues Organs 194 (1) (2011) 76–84.
[26] G.J. Burton, T. Cindrova-Davies, H wa Yung, E. Jauniaux, Hypoxia and reproductive health: oxygen and development of the human placenta, Reproduction 161 (1) (2021) F53–F65.
[27] A. Gaceb, I. Özen, T. Padel, M. Barbariga, G. Paul, Pericytes Secrete Pro-regenerative Molecules in Response to Platelet-Derived Growth Factor-BB, vol. 38, 2017, pp. 45–57, 1.
[28] A. Gaceb, M. Barbariga, I. Özen, G. Paul, The pericyte secretome: potential impact on regeneration, Biochimie 155 (2018) 16–25.
[29] C.-W. Chen, M. Okada, J.D. Proto, X. Gao, N. Sekiya, S.A. Beckman, et al., Human pericytes for ischemic heart repair, Stem Cell. 31 (2) (2013) 305–316.
[30] C. Omere, L. Richardson, G.R. Saade, E.A. Bonney, T. Kechichian, R. Menon, Interleukin (IL)-6: a friend or foe of pregnancy and parturition? Evidence from functional studies in fetal membrane cells, Front. Physiol. (2020) 891, 0.
[31] D. Chen, J. Zheng, Regulation of placental angiogenesis, Microcirculation 21 (1) (2014) 15.
[32] M. Martinez-Fierro, G. Hernadez-Delgadillo, J. Flores-Mendoza, C. Alvarez-Zuñiga, M. Diaz-Lozano, I. Delgado-Enciso, et al., Fibroblast growth factor type 2 (FGF2) administration attenuated the clinical manifestations of pre-eclampsia in a murine model induced by L-NAME, Front. Pharmacol. 12 (2021).
[33] F.-Y. Tian, X.-M. Wang, C. Xie, B. Zhao, Z. Niu, L. Fan, et al., Placental surface area mediates the association between FGFR2 methylation in placenta and full-term low birth weight in girls, Clin. Epigenet. 10 (1) (2018) 1–11.
[34] T. Vilsmaier, N. Amann, S. Löb, E. Schmoeckel, C. Kuhn, A. Zati Zehni, et al., The decidual expression of Interleukin-7 is upregulated in early pregnancy loss, Am. J. Reprod. Immunol. 86 (2021), e13437.
[35] J. Mayo, S. Bearden, Driving the hypoxia-inducible pathway in human pericytes promotes vascular density in an exosome-dependent manner, Microcirculation 22 (8) (2015) 711–723.
[36] L. Cao, Y. Zhou, M. Chen, L. Li, W. Zhang, Pericytes for therapeutic approaches to ischemic stroke, Front. Neurosci. (2021) 667, 0.
[37] A. Caporali, M. Meloni, A. Nailor, T. Mitić, S. Shantikumar, F. Riu, et al., p75NTR-dependent activation of NF-κB regulates microRNA-503 transcription and pericyte–endothelial crosstalk in diabetes after limb ischaemia, Nat. Commun. 6 (1) (2015) 1–13.
[38] A. Caporali, A. Martello, V. Miscianinov, D. Maselli, R. Vono, G. Spinetti, Contribution of pericyte paracrine regulation of the endothelium to angiogenesis, Pharmacol. Ther. 171 (2017) 56–64.
[39] S. Yamamoto, S. Niida, E. Azuma, T. Yanagibashi, M. Muramatsu, T.T. Huang, et al., Inflammation-induced endothelial cell-derived extracellular vesicles modulate the cellular status of pericytes, Sci. Rep. 5 (1) (2015) 1–10.