Introduction

Placentation is an oxygen sensitive process. The events that occur from the time of implantation to maternal perfusion of the placenta are influenced and directed by site-specific oxygen tensions [1]. At the time of embryo implantation, the intrauterine oxygen tension is ~3% [2] while the decidua and myometrium oxygen tension is ~8–12% [3]. This standing oxygen gradient is thought to promote and direct the invasion of extravillous trophoblast cells (EVT) into the decidua and myometrium where they engage and remodel maternal spiral arterioles [4,5]. Intraluminal EVT occludes spiral arterioles to maintain a low oxygen tension environment that is requisite for normal early placental and fetal development. Towards the end of the first trimester, low resistance, high capacity flow is restored and the placental intervillous space is perfused with the maternal blood thus establishing effective materno-fetal exchange.

Factors that compromise trophoblast cell (both cytotrophoblast (CT) and EVT) function during this critical period of development may dramatically affect subsequent fetal growth, outcome of pregnancy and the life-long disease risk profile of the newborn [6,7]. EVT migration is affected by oxygen tension [8,9]. Perturbation of intrauterine oxygen tensions, therefore, may compromise normal placentation development. The molecular mechanisms by which oxygen tension regulates EVT function and cell-to-cell communication with maternal tissues remain to be fully elucidated.

Recently, the role of exosomes in cell-to-cell communication has been established [10–13]. Exosomes are nanoparticles (40–100 nm) membrane vesicles that are released following the exocytotic fusion of multi-vesicular bodies with the cell membrane[14]. They have been identified in plasma under both normal and pathological conditions [15] and their concentration increases with disease severity and/or progression [16], and in response to oxidative stress [17].

Recently, we demonstrated that exosomes are released from first trimester placental mesenchymal stem cells (pMSC) and increases endothelial cell migration and vascular tube formation [18]. In addition, the release of exosomes from pMSC was increased under low oxygen tension. These data are consistent with the hypothesis...
that in response to changes in the environmental milieu (such as oxygen tension) placental cells release exosomes that modify the phenotype of recipient cells. The role of cytrophoblast cell-derived exosome in cell-to-cell communication and, in particular, their effect on EVT has yet to be established. Similarly, the effect of oxygen tension on the release and bioactivity of cytrophoblast exosomes is not known.

The aim of this study was to test the hypotheses that (i) exosomes released by cytrophoblast cells (CT) increase EVT proliferation and invasion; and (ii) the bioactivity and protein content of CT-derived exosomes is altered by oxygen tension. An in vitro treatment/control experimental design was used to test these hypotheses. Exosomes were isolated from primary first trimester placental villous CT. A first trimester EVT cell line (HTR-8/SVneo) was used to assess the effect of CT-derived exosomes on cell proliferation, invasion and bioactivity. The data obtained in this study are consistent with the hypothesis that exosomes from first trimester CT promote HTR-8/SVneo invasion and proliferation, and exosomal protein content is oxygen tension dependent.

Material and Methods

First trimester sample collection

Tissue collection was approved by the Human Research Ethics Committees of the Royal Brisbane and Women’s Hospital, and the University of Queensland (HREC/09/QRBW/14). Written informed consent was obtained from women for the use of placental tissue for research purposes after clinically indicated termination of pregnancy in compliance with national research guidelines. All experimental procedures were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility. All data were recorded within a 21 CFR part 11 compliant electronic laboratory notebook (Irisnote, Redwood City, CA, USA).

Isolation of cytrophoblast cells

First-trimester cytrophoblast cells (CTs) were isolated from placenta (8–12 weeks) derived by the legal termination of pregnancy (n = 6 biological samples and 2 independent duplicate cultures per placenta) as previously described [10]. Briefly, 10–20 g of chorionic villi were washed in phosphate buffer saline (PBS) and were subjected to three sequential treatments with digestion buffer (0.25% trypsin (Gibco® Trypsin, Life Technologies™, Carlsbad, CA) and 0.1 mg of DNase I (Sigma-Aldrich™, Saint Louis, USA) per ml in HBSS (1x) + HEPES (25 mM). After each 15 min step, the supernatant fluid was layered over fetal calf serum (25 ml supernatant over 5 ml FCS). The supernatant fluid was centrifuged at 400 × g for 10 min and the cell pellet was suspended in PBS and washed and re-centrifuged (100,000 × g for 75 min). Recovered exosomes pellet was resuspended in 50 μl PBS and their protein concentration determined by the dyc-binding assay [19].

Exosome protein quantification.

Exosome-containing fractions (1,146 to 1.199 g/ml) from sucrose gradients were combined in a single tube and centrifuged at 110,000 × g for 70 min. Exosome pellets were resuspended in 50 μl PBS and their proteins determined by the DC™ Protein Assay (BIO-RAD, Hercules, CA, USA). Briefly, exosome samples (5 μl) were prepared by adding RIPA buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 0.5% DOC, 1 mM EDTA, 150 mM NaCl, protease inhibitor) directly to exosomes suspended in PBS and sonicated at 37°C for 15 s three times to lyse exosome membrane and solubilize the proteins. Bovine serum albumin (BSA) diluted in RIPA buffer and PBS mixture (1:1) was prepared as protein standards (0, 200, 400, 600, 800, 1,000, 1,500 μg/ml). Standards and samples (exosomes) were transferred to 96-well plates. Alkaline copper tartrate solution (BIO-RAD, USA) and dilute Folin Reagent (BIO-RAD, USA) were added to the samples and incubated for 15 min. The absorbance was read at 750 nm with the Paradigm Detection Platform (Beckman Coulter, USA).

Western Blot.

Protein from each sucrose gradient fraction (10 in total) obtained after exosome isolation were separated by polyacrylamide gel electrophoresis, transferred to Immobilon-P® polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and probed with primary mouse monoclonal anti-CD63 (1:2000; ab18219, Abcam, Sapphire Bioscience Pty Ltd, NSW, Australia), anti-CD81 (1:1500, MAB6435, Abnova, Taipei City, Taiwan), anti-CD9 (1:1,500; ab22155, Abcam, Sapphire Bioscience) and anti-Placental Alkaline Phosphatase (PLAP; 1:1000; ab96588, Abcam, Sapphire Bioscience), as previously described [19,20] exosome specific markers. Membranes were washed in Tris buffer saline and incubated (1 h) in TBST/0.2% BSA containing horseradish peroxidase– conjugated goat anti-mouse antibody. Proteins were detected by enhanced chemiluminescence with using a SRX-101A Tablettop Processor (Konica Minolta, Ramsey, NJ, USA). Bands densitometry ratio was determined using the GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

Transmission electron microscopy.

Exosomes isolated by differential and continuous sucrose gradient centrifugation were
analyzed by transmission electron microscopy. Exosome pellets (as described above) were fixed in 3% (w/v) glutaraldehyde and 2% paraformaldehyde in cacodylate buffer, pH 7.3. 5 μl of sample was then applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The samples were examined in an FEI Tecnai 12 transmission electron microscope (FEI TM, Hillsboro, Oregon, USA).

Effect of oxygen tension on exosome release

The effects of oxygen tension on the release of exosomes from CTs were assessed by incubating cells for 48 h in exosome-free culture medium (culture media was depleted of the contaminating exosomes using the same protocol for exosome isolation described previously and exosome-free culture media was confirmed by electron microscope) under an atmosphere of 5% CO2-balanced N2 to obtain 1%, 3% or 8% O2 pO2 ~6.75, ~20.25 or ~54 mmHg, respectively, n = 6 biological replicates in duplicate) in an automated PROOX 110-scaled hypoxia chamber (BioSpherics TM, Lacona, NY, USA). The three-compartment hypoxia chamber allowed the simultaneous analysis of CT-cells from individual placentae thus reducing inter-assay variation. Cell viability was determined by Trypan Blue exclusion and Countess® Automated cell counter (Life Technologies™). In all experiments, viability remained at >95% and was not significantly different between groups (p = 0.85). Incubation media pO2 and pH were independently confirmed using a blood gas analyzer (Radiometer®, Brønshøj, Denmark) and NeoFox oxygen probe (Ocean Optics TM, Dunedin, FL, USA).

Effect of exosomes on extra-villous trophoblast cell invasion and proliferation

A first trimester cell line (HTR-8/SVneo) was used to establish the effects of CT-derived exosomes on cell invasion. HTR-8/SVneo cells were kindly donated by Dr Charles H. Graham (Queen’s University, Ontario, Canada) [21,22]. The cells were cultured in RPMI-1640 (HyClone, South Logan, USA) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (HyClone), at 37°C and 5% CO2. Before experiments, HTR-8/SVneo cells were cultured in RPMI-1640 supplemented with 0.2% FBS in 96-well culture plate (Corning Life Science, Tewksbury, MA, USA) according to the manufacturer’s instructions.
Characterization of cytotrophoblast-derived exosomes

Exosomes were isolated by ultracentrifugation after adding urea (8 M). The supernatant was collected, and proteins were precipitated with trichloroacetic acid (TCA) at 4°C for 30 min. The samples were washed with cold acetone and dried under vacuum. The dried samples were dissolved in a buffer containing 6 M urea, 50 mM ammonium bicarbonate, pH 8.5, and reduced with DTT for 1 h. The samples were alkylated in 10 mM iodoacetic acid (IAA) for 1 h. The samples were desalted by solid phase extraction and layered with 3 mg/ml collagen type I (Life Technologies, Carlsbad, CA) and incubated at 37°C for 30 min. During experiments, cells were visualized using a real-time cell imaging system (IncuCyte live-cell ESSEN BioScience Inc, Ann Arbor, Michigan, USA) and were imaged every 1–2 h to monitor treatment-induced effects on cell invasion. Time course and dose response effects were established using exosomes released from CTs incubated under 1% O2 for up to 48 h.

Time Course

To determine the effects of CT-derived exosomes on cell invasion, HTR-8/SVneo cells were incubated in the presence (treatment: 100 μg exosomal protein/ml) or absence (control) of exosomes for up to 24 h (n = 12). Cell invasion was monitored by scratch assays [10]. A scratch was made on confluent monolayers using a 96-pin WoundMaker (BioScience Inc, Ann Arbor, Michigan, USA) and layered with 3 mg/ml collagen type I (Life Technologies). During experiments, cells were visualized by the IncuCyte live-cell ESSEN BioScience Inc, Ann Arbor, Michigan, USA) and were imaged every 1–2 h to monitor treatment-induced effects on cell invasion. Time course and dose response effects were established using exosomes released from CTs incubated under 1% O2 for up to 48 h.

Dose Response

To assess the effect of exosome concentration on extra-villous trophoblast invasion and proliferation, HTR-8/SVneo cells were cultured in the presence of increasing exosomes concentration (5, 10, 20, 50 and 100 μg exosomal protein/ml) for 24 h. Data are presented as half-maximal effective concentration (EC50).

Effects of oxygen tension on CT-derived exosome bioactivity

To determine the effects of oxygen tension on exosome bioactivity, exosomes were obtained from CTs incubated under 1%, 3% and 8% O2 (as detailed above) for 24 h. The effects of exosomes on HTR-8/SVneo cell invasion and proliferation were assessed as detailed above.

Proteomic analysis of cytotrophoblast derived-exosomes by mass spectrometry (MS)

Isolated exosomes were solubilized in 8 M urea in 50 mM ammonium bicarbonate, pH 8.5, and reduced with DTT for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid (IAA) for 1 h in the dark. The sample was diluted to 1:10 with 50 mM ammonium bicarbonate and digested with trypsin (20 μg) at 37°C for 18 h. The samples were desalted by solid phase extraction using a STAGE tip protocol (Stop and go extraction tips for matrix-assisted laser desorption/ionization, nano-electrospray, and LC/MS sample pre-treatment in proteomics). The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and redissolved in Solvent A. The resulting peptide mixture was analysed by Liquid Chromatography (LC)/Mass Spectrometry (MS) LC-MS/MS on a 5600 Triple TOF mass spectrometer (AB Sciex, Framingham, U.S.A.) equipped with an Eksigent Nanoflow binary gradient HPLC system and a nanospray III ion source. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. MS/MS spectra were collected using Information Dependent Acquisition (IDA) using a survey scan (m/z 350–1500) followed by 25 data-dependent product ion scans of the 25 most intense precursor ions. All mass spectra were analysed using the Mascot and Protein Pilot search engines against the Swissprot-swissprot database with the species set as human (scores greater than 30). False discovery rate (FDR) was estimated using a reversed sequence database. Finally, proteins identified were submitted to bioinformatic pathway analysis (Ingenuity Pathway Analysis [IPA]; Ingenuity Systems, Mountain View, CA; www.ingenuity.com).

Statistical Analysis

Data are represented as mean ± SEM, with n = 6-12 different cell cultures of CTs isolated from first trimester pregnancies. Comparisons between two group means were performed by unpaired Student’s t-test. Multiple groups were compared using the analysis of variance (ANOVA) post hoc analyses were used for pair-wize comparisons (Bonferroni correction test). Statistical significance was defined as p<0.05.

Results

Characterization of cytotrophoblast-derived exosomes

Characterization included analysis of the physical properties of exosomes on a continuous sucrose gradient and protein composition by Western blot. CD63, CD9, CD81 and PLAP positive nano-particles displayed a buoyant density of 1.146 – 1.199 g/ml.
Exosomes Induces Extravillous Trophoblast Invasion

A

Initial wound  -exosomes  +exo-CT-1%

B

Relative Wound Density (%) vs Time (hours)

C

EC₅₀ [µg/ml] = 29.48 ± 0.64

% activation (EVT invasion) vs log(exosomes)
Figure 3. Cytotrophoblast cell-derived exosomes increased EVT invasion. EVT cells were grown to confluence in complete media. A wound was made using 96 well WoundMaker and then overlaid to form a 3D matrix-gel (see Methods). EVT invasion was measured in absence (white circles) or presence (black circles) of 100 µg/ml of exosomes from cytotrophoblast cells exposed to 1% O2 (exo-CTs-1%) for 24 h. (A) Top a, Wound imaged immediately after wounding; b, Graphical representation from a showing the calculation of initial wound width (black); c and e, Image at the midpoint of the experiment; d and f, Graphical representation from c and e of cell invasion (gray) at the midpoint of the experiment. (B) Time course of wound closure for HTR8/SVneo expressed as relative wound density (%). Data are presented as mean ± SEM for control (no exosomes, open circles) and treatment (100 µg/ml exosomal protein, closed circles). (C) Dose response curve for the effect of CT-derived exosomes on HTR8/SVneo invasion. Data are presented as a non-linear regression analysis (curve fit) and mean ± SEM.

doi:10.1371/journal.pone.0079636.g003

Exosomes Induces Extravillous Trophoblast Invasion

Effects of oxygen tension on exosome release

The release of exosomes (µg exosomal protein/106 cell/48 h) from CTs was simultaneously assessed at 1%, 3% and 8% O2 using a 3-compartment hypoxia chamber and presented in Figure 2. At 1%, 3% and 8% O2, exosomal protein release averaged 0.32±0.04, 0.19±0.02 and 0.11±0.01 µg protein/106 cells/48 h respectively (ANOVA, p<0.0001, n = 6 in duplicate). Post-hoc tests (Bonferroni’s multiple comparison test) identified significant differences between all group means (p<0.05). Cell viability (>95%) and incubation medium pH at 48 h displayed no significant treatment effects (p = 0.80). Incubation chamber oxygen tensions were independently monitored and verified.

CTs-derived exosomes induce EVT invasion

Time course. Representative photomicrographs of HTR-8/SVneo wound closure for treatment and control experiments are presented in Figure 3A. The effect of CT-derived exosomes on HTR-8/SVneo cell invasion is presented as relative wound density (percent) over time (Figure 3B). The rate of wound closure was significantly increased in the presence of CT-derived exosomes as measured by ST50 (9.4±0.4 versus 3.2±0.2, p<0.001) and area under the curves (1333±74 versus 2108±122, p<0.001). The effect of exosomes was concentration dependent (Figure 3C).

Dose Response. The effect of increasing concentrations of CT-derived exosomes on EVT invasion is presented in Figure 4. Exosomes significantly increased HTR-8/SVneo cell invasion and proliferation in a concentration-dependent manner. For cell invasion, EC50 = 29.4 ±1.1, 47.8±5.2 and 81.3±6.1 µg/ml for treatment in presence of exosomes isolated from 1%, 3% and 8% O2, respectively (p<0.005).

Effect of oxygen tension on CT-derived exosome bioactivity

In this study, exosome bioactivity was defined as the half maximal effective concentration (EC50) and half-maximal stimulatory time (ST50) of exosomes on EVT invasion (percent) over time (source). Values are mean ± SEM. *p<0.05 versus 8% O2.

doi:10.1371/journal.pone.0079636.g004

Figure 4. Effect of oxygen tension on exosome bioactivity. EVT cell invasion was measurement in presence of exosomes isolated from cytotrophoblast cells exposed to three different oxygen tension (1%, 3% and 8% O2). (A) The graph represents the changes of half-maximal effective concentration (EC50) and (B) half-maximal stimulatory time (ST50) exosomes on EVT invasion in response to oxygen tension (source). Values are mean ± SEM. *p<0.01 versus all conditions; †p<0.05 versus 8% O2.

CTs-derived exosomes increase EVT proliferation

A real-time imaging system (IncuCyte™) was used to measure cell proliferation using non-label cell monolayer confluence approach. The proliferation ratio (±exosomes/-exosomes for each
hour) was significantly higher (p<0.05) compared to the control in the absence of exosomes (Figure 5). At 24 hours, exo-CTs-1% increased EVT proliferation by ~1.5 fold compared to those in absence of exosomes (control).

Proteomic analysis of CTs-derived exosomes

Mass spectrometry analysis identified over 160 exosomal proteins (Table 1). We identified unique proteins for each condition (Figure 6). The biological relevance of differentially expression proteins was analyzed using Ingenuity Pathway Analysis (IPA) software. Exosomal proteins isolated from CT exposed to different oxygen tensions were associated with cellular movement and morphology, immune cell trafficking and cellular assembly and organization in accordance with IPA analysis. The canonical pathways associated with our exosomal proteins defined by IPA Core comparison analysis showed that the score (-log \( p \)-value) for proteins associated with HIF-\( \alpha \) signalling (Figure 6B) and IL-8 signalling (Figure 6C) were inversely correlated to oxygen tension. Finally, we investigated the molecular network that can be activated by the unique proteins identified in exosomes isolated from cytotrophoblast cells exposed to 1% \( O_2 \) (31 proteins) (Figure 7). We found molecules involved in cellular movement such as MMP9, TGF-\( \beta \), P38 MAPK, VEGF and others.

Discussion

Extravillous trophoblast invasion into the maternal tissue is a critical process in placentation. Hypoxia is a risk factor for complications of pregnancy and may adversely affect placentation and development of the materno-fetal vascular exchange. In particular, during early pregnancy low oxygen tension may impact on EVT migration and interactions with the maternal spiral arterioles [24–26]. Exosomes from cytotrophoblast cells may interact with EVT and modify their invasiveness, however, the effect and role of exosomes from placental cells has yet to be defined. The aim of this study was to establish the effect of oxygen tension on the release and bioactivity of CT-derived exosomes on EVT invasion and proliferation in vitro. The data obtained are consistent with the hypothesis that exosomes released from cytotrophoblast cells incubated under low oxygen tension promote HTR-8/SVneo invasion and proliferation. While the role of CT exosomes in vivo remains to be established, their release under hypoxic conditions within the placenta may be an adaptive response to promote proliferation and invasion of extravillous trophoblast.

Jauniaux et al., (2000) measured in situ oxygen tension during early pregnancy (at 60 days) within the chorionic placenta, intervillous space (IVS), and maternal endometrium underlying the placenta [31]. Oxygen tension was ~3%, 1% and 8%, respectively. Low oxygen tension within the placenta and IVS at this stage of pregnancy may promote the release of exosomes from cytotrophoblast cells and enhance cell-to-cell communication. In particular, exosomes released from CTs may induce functional changes in EVTs that promote cell invasion and proliferation.
Consistent with this hypothesis, in this study we demonstrated that CT-derived exosomes increase invasion and proliferation of the EVT cell line HTR-8/SVneo.

During the first trimester of pregnancy, EVT cells invade the decidua and myometrium and regulate the flow of maternal blood into the IVS. EVTs co-localize with maternal spiral arterioles and are present within the lumen of these vessels (where they are thought to prevent flow into the IVS). Subsequent perfusion of the IVS is associated with the transformation of these arterioles from high resistance, low capacity to low resistance, high capacity vessels. EVTs play a role in remodelling these vessels from high resistance, low capacity to low resistance, high capacity vessels. Abnormal EVT function may result in failure to transform these vessels resulting in compromised placental perfusion and hypoxia [34]. What regulates EVT invasion and/or function and their interactions with maternal vessels remains to be clearly established.

The data obtained in this study, however, establish that exosomes released from CTs increase EVT cell invasion in a concentration-dependent manner, and that the activity of exosomes is increased under low oxygen tension.

In this study, HTR-8/SVneo were cultured under low oxygen tension for at least 48 h before experimental manipulation and incubated in the presence of CT-derived exosomes for 24 h. An automated real-time imaging system was used to maintain cells in optimal conditions for quantifying cell invasion and proliferation. Exosome-treatment reduced EVT ST50 compared to control incubations (i.e. absence of exosomes). Furthermore, exosomes isolated from CTs incubated under low oxygen tension (i.e. 1%) displayed greater activity (per unit exosomal protein) than

Figure 6. Analysis of cytotrophoblast cell-derived exosome proteins. (A) The Venn diagram represents the distribution of common and unique proteins identified by nanospray LC-MS/MS (ABSciex 5600) in exosomes released from trophoblast cells exposed to 1%, 3% and 8% of oxygen tension. Comparison of canonical pathways: (B) HIFs, and (C) IL-8 signaling identified by IPA core analysis. Values are mean ± SEM. In B and C, *p<0.005 versus all condition; †p<0.05 versus 8% O2.

doi:10.1371/journal.pone.0079636.g006
exosomes obtained from cells incubated at higher oxygen tensions (i.e., 3 and 8%). Similar effects (but of lower magnitude) were observed for EVT cell proliferation.

The effect of low oxygen tension on EVT invasion remains controversial and disparate data have been reported [8,33,35,36]. Studies performed on EVT cells isolated from placental tissue (5 to 10 weeks of gestation) established that low oxygen tension reduces invasion of EVT cells through decreased MMP-2 [36] and appears to be mediated by urokinase plasminogen activator (PLAU) system [8]. In contrast, low oxygen tension (<1% O2) increased HTR-8/SVneo cell invasion when compared to cell incubated under 20% O2 [37].

The data obtained in the current study establish a role for exosomes in the intercellular communication between placental cells and in regulating EVT cell invasion in an oxygen-dependent manner. Low oxygen tension increased exosome release and

Figure 7. Ingenuity Pathway Analysis of Exosomal Proteins. Unique proteins identified in exosomes isolated from cytotrophoblast cells exposed to 1% oxygen were submitted to IPA network analysis. Green: signaling involving in cellular movement. doi:10.1371/journal.pone.0079636.g007
### Table 1 List of proteins identified in exosomes from CT exposed to different oxygen level.

| Protein ID      | Symbol | Entrez Gene Name | Location                          | Type(s)                  |
|-----------------|--------|------------------|-----------------------------------|--------------------------|
| 1% O2           |        |                  |                                   |                          |
| ABHD8_HUMAN     | ABHD8  | abhydrolase domain containing 8 | unknown                      | enzyme                   |
| ACTN1_HUMAN     | ACTN1  | actinin, alpha 1 | Cytoplasm                        | other                    |
| AGPAT1_HUMAN    | AGPAT1 | 1-acrylglycerol-3-phosphate O-acyltransferase 1 | Cytoplasm                  | enzyme                   |
| ALB_HUMAN       | ALB    | albumin          | Extracellular Space              | transporter              |
| ANXA1_HUMAN     | ANXA1  | annexin A1       | Plasma Membrane                  | other                    |
| ANXA2_HUMAN     | ANXA2  | annexin A2       | Plasma Membrane                  | other                    |
| AP0A1_HUMAN     | AP0A1  | apolipoprotein A-I | Extracellular Space             | transporter              |
| C3              | C3     | complement component 3 | Extracellular Space             | peptidease               |
| C9              | C9     | complement component 9 | Extracellular Space             | other                    |
| CO3A1_HUMAN     | CO3A1  | collagen, type IV, alpha 1 | Extracellular Space             | other                    |
| CO4A2_HUMAN     | CO4A2  | collagen, type IV, alpha 2 | Extracellular Space             | other                    |
| DHX58_HUMAN     | DHX58  | DEXH (Asp-Glu-X-His) box polypeptide 5B | Cytoplasm                    | enzyme                   |
| FBLN1_HUMAN     | FBLN1  | fibulin 1        | Extracellular Space              | other                    |
| FLT1            | FLT1   | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) | Plasma Membrane            | kinase                   |
| FN1             | FN1    | fibronectin 1    | Extracellular Space              | enzyme                   |
| FSTL3_HUMAN     | FSTL3  | follistatin-like 3 (secreted glycoprotein) | Extracellular Space            | other                    |
| GAPDH           | GAPDH  | glyceraldehyde-3-phosphate dehydrogenase | Cytoplasm                  | enzyme                   |
| GC              | GC     | group-specific component (vitamin D binding protein) | Extracellular Space          | transporter              |
| HBB_HUMAN       | HBB    | hemoglobin, beta | Cytoplasm                        | transporter              |
| HBD_HUMAN       | HBD    | hemoglobin, delta | Cytoplasm                        | transporter              |
| H2B1B_HUMAN     | H2B1B  | histone cluster 1, H2bb | Nucleus                              | other                    |
| HTRA4_HUMAN     | HTRA4  | HtrA serine peptidase 4 | unknown                               | other                    |
| INS_HUMAN       | INS    | insulin          | Extracellular Space              | other                    |
| ITIH4_HUMAN     | ITIH4  | inter-alpha-trypsin inhibitor heavy chain family, member 4 | Extracellular Space            | other                    |
| KRT1            | KRT1   | keratin 1        | Cytoplasm                        | other                    |
| KRT10           | KRT10  | keratin 10       | Cytoplasm                        | other                    |
| KRT18           | KRT18  | keratin 18       | Cytoplasm                        | other                    |
| KRT19           | KRT19  | keratin 19       | Cytoplasm                        | other                    |
| KRT7            | KRT7   | keratin 7        | Cytoplasm                        | other                    |
| KRT8            | KRT8   | keratin 8        | Cytoplasm                        | other                    |
| LGALS3BP_HUMAN  | LGALS3BP | lectin, galactoside-binding, soluble, 3 binding protein | Plasma Membrane               | transmembrane receptor   |
| MMP12_HUMAN     | MMP12  | matrix metallopeptidase 12 (macrophage elastase) | Extracellular Space            | peptidase                |
| MMP2_HUMAN      | MMP2   | matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase) | Extracellular Space         | peptidase                |
| MMP9_HUMAN      | MMP9   | matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) | Extracellular Space          | peptidase                |
| MS4A7_HUMAN     | MS4A7  | membrane-spanning 4-domains, subfamily A, member 7 | unknown                             | other                    |
| MYOH9_HUMAN     | MYOH9  | myosin, heavy chain 9, non-muscle | Cytoplasm                        | transporter              |
| NCTR1_HUMAN     | NCTR1  | natural cytotoxicity triggering receptor 1 | Plasma Membrane             | transmembrane receptor   |
| NUGGC_HUMAN     | NUGGC  | nuclear GTPase, germinal center associated | Nucleus                              | other                    |
| NXF1_HUMAN      | NXF1   | nuclear RNA export factor 1 | Nucleus                              | transporter              |
| Protein ID      | Symbol | Entrez Gene Name | Location      | Type(s)          |
|-----------------|--------|------------------|---------------|------------------|
| PLGF_HUMAN      | PGF    |                  | Extracellular | growth factor    |
| PLXNB2_HUMAN    | PLXN B2|                  | Plasma Membrane| transmembrane receptor |
| POTEE_HUMAN     | POTEE/POTEF | POTE ankyrin domain family, member F | unknown  | other |
| PRG2_HUMAN      | PRG2   | proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein) | Extracellular | other |
| PSA2_HUMAN      | PSMA2  | proteasome (prosome, macropain) subunit, alpha type, 2 | Cytoplasm | peptidase |
| PSA6_HUMAN      | PSMA6  | proteasome (prosome, macropain) subunit, alpha type, 6 | Cytoplasm | peptidase |
| PSA7_HUMAN      | PSMA7  | proteasome (prosome, macropain) subunit, alpha type, 7 | Cytoplasm | peptidase |
| PSB2_HUMAN      | PSMB2  | proteasome (prosome, macropain) subunit, beta type, 2 | Cytoplasm | peptidase |
| PSB7_HUMAN      | PSMB7  | proteasome (prosome, macropain) subunit, beta type, 7 | Cytoplasm | peptidase |
| QSOX1_HUMAN     | QSOX1  | quiescin Q6 sulphydryl oxidase 1 | Cytoplasm | enzyme |
| S100A11_HUMAN   | S100A11| S100 calcium binding protein A11 | Cytoplasm | other |
| PAI1_HUMAN      | SERPINE1| serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | Extracellular | other |
| QSOX1_HUMAN     | QSOX1  | quiescin Q6 sulphydryl oxidase 1 | Cytoplasm | enzyme |
| SMCA1_HUMAN     | SMARCA1| SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 | Nucleus | transcription regulator |
| SYCP2L_HUMAN    | SYCP2L | syntaptonemal complex protein 2-like | Nucleus | other |
| TRFE_HUMAN      | TF      | transferrin       | Extracellular | transporter |
| TPPI1_HUMAN     | TPPI   | tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) | Extracellular | other |
| TSP1_HUMAN      | THBS1  | thrombospondin 1  | Extracellular | other |
| TIMP1_HUMAN     | TIMP1  | TIMP metallopeptidase inhibitor 1 | Extracellular | other |
| TINAL_HUMAN     | TINAGL1| tubulointerstitial nephritis antigen-like 1 | Extracellular | transporter |
| TMEM5_HUMAN     | TMEM5  | transmembrane protein 5 | Plasma Membrane | other |
| TRRAP_HUMAN     | TRRAP  | transformation/transcription domain-associated protein | Nucleus | transcription regulator |
| VAT1_HUMAN      | VAT1   | vesicle amine transport protein 1 homolog (T. californica) | Plasma Membrane | transporter |
| WNT7A_HUMAN     | WNT7A  | wingless-type MMTV integration site family, member 7A | Extracellular | cytokine |
| YIPF1_HUMAN     | YIPF1  | Yip1 domain family, member 1 | Cytoplasm | other |

### 3% O2

| Protein ID      | Symbol | Entrez Gene Name | Location         | Type(s)          |
|-----------------|--------|------------------|------------------|------------------|
| ABHD8_HUMAN     | ABHD8  | abhydrolase domain containing 8 | unknown | enzyme |
| ACTB_HUMAN      | ACTB   | actin, beta      | Cytoplasm       | other |
| ACTN1_HUMAN     | ACTN1  | actinin, alpha 1 | Cytoplasm       | other |
| AGPAT1_HUMAN    | AGPAT1 | 1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, alpha) | Cytoplasm | enzyme |
| ALB_HUMAN       | ALB    | albumin          | Extracellular    | transporter |
| ASB18_HUMAN     | ASB18  | ankyrin repeat and SOCS box containing 18 | unknown | other |
| BPTF_HUMAN      | BPTF   | bromodomain PHD finger transcription factor | Nucleus | transcription regulator |
| C4B_HUMAN       | C4B    | complement component 4B (Childo blood group) | Extracellular | other |
| CCIN_HUMAN      | CCIN   | calcin           | Cytoplasm       | other |
| CGB_HUMAN       | CGB    | chorionic gonadotropin, beta polypeptide | Extracellular | other |
| COL4A1_HUMAN    | COL4A1 | collagen, type IV, alpha 1 | Extracellular | other |
| CTND1_HUMAN     | CTND1  | catenin (cadherin-associated protein), delta 1 | Nucleus | other |
| Protein ID   | Symbol | Entrez Gene Name | Location | Type(s)          |
|-------------|--------|------------------|----------|------------------|
| VGFR1_HUMAN | FLT1   | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) | Plasma Membrane | kinase           |
| FINC_HUMAN  | FN1    | fibronectin 1    | Extracellular Space | enzyme          |
| VTD8_HUMAN  | GC     | group-specific component (vitamin D binding protein) | Extracellular Space | transporter     |
| HBD_HUMAN   | HBD    | hemoglobin, delta | Cytoplasm | transporter       |
| H2B1B_HUMAN | HIST1H2BB | histone cluster 1, H2bb | Nucleus | other            |
| HTRA4_HUMAN | HTRA4  | HtrA serine peptidase 4 | unknown | other            |
| INS_HUMAN   | INS    | insulin          | Extracellular Space | other          |
| KIF3C_HUMAN | KIF3C  | kinesin family member 3C | Cytoplasm | other          |
| K1C18_HUMAN | KRT18  | keratin 18       | Cytoplasm | other          |
| K2C8_HUMAN  | KRT8   | keratin 8        | Cytoplasm | other          |
| LG3BP_HUMAN | LGALS3BP | lectin, galactoside-binding, soluble, 3 binding protein | Plasma Membrane | transmembrane receptor |
| MMP12_HUMAN | MMP12  | matrix metalloproteinase 12 (macrophage elastase) | Extracellular Space | peptidase       |
| MMP2_HUMAN  | MMP2   | matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase) | Extracellular Space | peptidase       |
| RM43_HUMAN  | MRPL43 | mitochondrial ribosomal protein L43 | Cytoplasm | translation regulator |
| NEUL_HUMAN  | NLN    | neurolysin (metalloproteinase M3 family) | Cytoplasm | peptidase       |
| NXF1_HUMAN  | NXF1   | nuclear RNA export factor 1 | Nucleus | transporter |
| PLXB2_HUMAN | PLXNB2 | plexin B2        | Plasma Membrane | transmembrane receptor |
| GDN_HUMAN   | SERPIN E2 | serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 | Extracellular Space | other          |
| SNPH_HUMAN  | SNPH   | syntaphilin      | Plasma Membrane | other          |
| SYC2L_HUMAN | SYCP2L | synaptosomal complex protein 2-like | Nucleus | other          |
| TRFE_HUMAN  | TF     | transferrin       | Extracellular Space | transporter |
| TFP1_HUMAN  | TFP1   | tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) | Extracellular Space | other          |
| TSP1_HUMAN  | TSP1   | thrombospondin 1  | Extracellular Space | other          |
| VAT1_HUMAN  | VAT1   | vesicle amine transport protein 1 homolog (T. californica) | Plasma Membrane | transporter |
| WNT7A_HUMAN | WNT7A  | wingless-type MMTV integration site family, member 7A | Extracellular Space | cytokine       |

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| Protein ID   | Symbol | Entrez Gene Name | Location | Type(s)          |
|-------------|--------|------------------|----------|------------------|
| A2MG_HUMAN  | A2M    | alpha-2-macroglobulin | Extracellular Space | transporter |
| ABHD8_HUMAN | ABHD8  | abhydrolase domain containing 8 | unknown | enzyme          |
| ADH7_HUMAN  | ADH7   | alcohol dehydrogenase 7 (class I), mu or sigma polypeptide | Cytoplasm | enzyme          |
| ALBU_HUMAN  | ALB    | albumin          | Extracellular Space | transporter |
| ANIR17_HUMAN | ANKR1D7 | ankyrin repeat domain 17 | Nucleus | other          |
| ANXA1_HUMAN | ANXA1  | annexin A1       | Plasma Membrane | other          |
| APOA1_HUMAN | APOA1  | apolipoprotein A-I | Extracellular Space | transporter |
| ASB18_HUMAN | ASB18  | ankyrin repeat and SOCS box containing 18 | unknown | other          |
| AT2B1_HUMAN | ATP2B1 | ATPase, Ca++ transporting, plasma membrane 1 | Plasma Membrane | transporter |
| C03_HUMAN   | C3     | complement component 3 | Extracellular Space | peptidase |
| CO4A_HUMAN  | C4B (includes others) | complement component 4B (Chido blood group) | Extracellular Space | other          |
| CF11B_HUMAN | C6orf118 | chromosome 6 open reading frame 118 | unknown | other          |
| CGHB_HUMAN  | CGB (includes others) | chorionic gonadotropin, beta polypeptide | Extracellular Space | other          |
| CO4A1_HUMAN | COL4A1 | collagen, type IV, alpha 1 | Extracellular Space | other          |
| CRB4A_HUMAN | CRYBA4 | crystallin, beta A4 | unknown | other          |
| Protein ID       | Symbol | Entrez Gene Name | Location       | Type                      |
|------------------|--------|------------------|----------------|---------------------------|
| EF1A1_HUMAN      | EEF1A  | eukaryotic translation elongation factor 1 alpha 1 | Cytoplasm      | translation regulator     |
| EVC_HUMAN        | EVC    | Ellis van Creveld syndrome | Cytoplasm | other                     |
| EZR1_HUMAN       | EZR1   | ezrin            | Plasma Membrane | other                     |
| FBLN1_HUMAN      | FBLN1  | fibrillin 1      | Extracellular Space | other                   |
| VGRF1_HUMAN      | VGRF1  | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) | Plasma Membrane | kinase                    |
| FMN1_HUMAN       | FMN1   | formin 1         | Plasma Membrane | other                     |
| FINC_HUMAN       | FINC   | fibronectin 1    | Extracellular Space | enzyme                   |
| GKN1_HUMAN       | GKN1   | gastronexin 1    | Extracellular Space | growth factor             |
| HBB_HUMAN        | HBB    | hemoglobin, beta | Cytoplasm      | transporter                |
| HBD_HUMAN        | HBD    | hemoglobin, delta| Cytoplasm      | transporter                |
| H2B1B_HUMAN      | HIST1H2B | histone cluster 1, H2bb | Nucleus | other                     |
| HEMO_HUMAN       | HEMO   | hemopexin        | Extracellular Space | transporter                |
| ILF3_HUMAN       | ILF3   | interleukin enhancer binding factor 3, 90kDa | Nucleus | transcription regulator    |
| INS_HUMAN        | INS    | insulin          | Extracellular Space | other                     |
| ITI_HUMAN        | ITI    | inter-alpha-trypsin inhibitor heavy chain family, member 4 | Extracellular Space | other                     |
| KIF13A_HUMAN     | KIF13A | kinesin family member 13A | Cytoplasm      | transporter                |
| KIF3C_HUMAN      | KIF3C  | kinesin family member 3C | Cytoplasm      | other                     |
| KGC1_HUMAN       | KRT1   | keratin 1        | Cytoplasm      | other                     |
| K1C18_HUMAN      | KRT18  | keratin 18       | Cytoplasm      | other                     |
| K2C8_HUMAN       | KRT8   | keratin 8        | Cytoplasm      | other                     |
| LG3BP_HUMAN      | LGALS3BP | lectin, galactoside-binding, soluble, 3 binding protein | Plasma Membrane | transmembrane receptor    |
| MMP2_HUMAN       | MMP2   | matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase) | Extracellular Space | peptidase                |
| MYH9_HUMAN       | MYH9   | myosin, heavy chain 9, non-muscle | Cytoplasm      | transporter                |
| SLIP_HUMAN       | NUGGC  | nuclear GTPase, germlinal center associated | Nucleus | other                     |
| PCLO_HUMAN       | PCLO   | piccolo (presynaptic cytomatrix protein) | Cytoplasm      | transporter                |
| PLXNB2_HUMAN     | PLXNB2 | plexin B2        | Plasma Membrane | transmembrane receptor    |
| PRG2_HUMAN       | PRG2   | proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein) | Extracellular Space | other                     |
| PSG3_HUMAN       | PSG3   | pregnancy specific beta-1-glycoprotein 3 | Extracellular Space | other                     |
| RET_HUMAN        | RBP4   | retinol binding protein 4, plasma | Extracellular Space | transporter                |
| SEM4G_HUMAN      | SEMA4G | sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G | Plasma Membrane | other                     |
| ANT3_HUMAN       | SERPINC1 | serpin peptidase inhibitor, clade C (antithrombin), member 1 | Extracellular Space | other                     |
| GDN_HUMAN        | SERPINE2 | serpin peptidase inhibitor, clade E (nexitin, plasminogen activator inhibitor type 1), member 2 | Extracellular Space | other                     |
| SNPH_HUMAN       | SNPH   | syntaphilin      | Plasma Membrane | other                     |
| SYC2L_HUMAN      | SYCP2L | synaptosomal complex protein 2-like | Nucleus | other                     |
| TFRF_HUMAN       | TF     | transferrin      | Extracellular Space | transporter                |
| TSP1_HUMAN       | THBS1  | thrombospondin 1 | Extracellular Space | other                     |
| Y016_HUMAN       | TUBB5P | tubulin, beta pseudogene 5 | unknown | other                     |
| VAT1_HUMAN       | VAT1   | vesicle amine transport protein 1 homolog (T. california) | Plasma Membrane | transporter                |
| WNT7A_HUMAN      | WNT7A  | wingless-type MMTV integration site family, member 7A | Extracellular Space | cytokine                  |

All mass spectra were analysed using the Mascot and Protein Pilot search engines against the Swissprot-swissprot database with the species set as human (score over 30). Exosomal proteins identified by mass-spectrometry were analyzed with the Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). False discovery rate (FDR) was estimated using a reversed sequence database. List of total exosomal protein from cytotrophoblast cells exposed to different oxygen level are presented as Protein ID, Symbol, Entrez Gene Name, Location and type. 

doi:10.1371/journal.pone.0079636.t001
modified their protein content. When CTs were incubated under different oxygen tensions, exosomal protein content was altered significantly. Ingenuity Pathway Analysis (IPA) of exosomal proteins identified oxygen-dependent changes in HIF-α and HIF-β signalling pathways. In addition, when CTs were incubated under low oxygen tensions, exosomal proteins identified were predominantly associated with pathways involved in the activation of MMP-9, TGF-β, MAPK, VEGF, p38MAPK, TIMP1 and ERK1/2. It remains to be established whether or not specific changes in exosomal protein content are causally related to changes in EVT invasion and proliferation. EVT cells have a crucial role in placentaation, characterized by their invasion of spiral uterine arteries to establish a low-resistance, high-capacity perfusion system. The mechanism involved in EVT invasion is not completely understood, however, there is a consensus that invading EVT cells up-regulate proteins such as MMPs, integrins (α5β1 and α1β1) and VE-cadherin which support uterine wall invasion. Our data suggest that the exosome released from cytotrophoblast cells also promote EVT invasion including the activation of MMPs, MAPK and invasiveness pathways. We are only beginning to develop an understanding of the role of placental-derived exosomes (e.g. cytotrophoblast cells) in early pregnancy events and, in particular, how they might affect the function of key cell-types (e.g. EVT) involved in the development of the placenta and its vascular communication with both mother and fetus.

In summary, the release of exosomes from primary culture of cytotrophoblast cells is oxygen tension-dependent. CT-derived exosomes increase EVT cell invasion and proliferation in a concentration and oxygen-dependent manner. Exosomal protein content is altered in response to oxygen tension, with the enhancement of signals involved in cellular invasion and migration. The release of CT-derive exosomes under hypoxic conditions within the placenta may be an adaptive response to promote proliferation and invasion of extravillous trophoblast cells.

Author Contributions

Conceived and designed the experiments: CS GER. Performed the experiments: CS MK KA LS MDM GER. Contributed reagents/materials/analysis tools: CS MK KA LS MDM GER. Wrote the paper: CS GER.

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