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ORIGINAL ARTICLE *Early pregnancy*

Placental Nano-vesicles Target to Specific Organs and Modulate Vascular Tone *In Vivo*

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STUDY QUESTION: How do nano-vesicles extruded from normal first trimester human placentae affect maternal vascular function?

SUMMARY ANSWER: Placental nano-vesicles affect the ability of systemic mesenteric arteries to undergo endothelium- and nitric oxide-(NO-) dependent vasodilation *in vivo* in pregnant mice.

WHAT IS KNOWN ALREADY: Dramatic cardiovascular adaptations occur during human pregnancy, including a substantial decrease in total peripheral resistance in the first trimester. The human placenta constantly extrudes extracellular vesicles that can enter the maternal circulation and these vesicles may play an important role in feto-maternal communication.

STUDY DESIGN, SIZE, DURATION: Human placental nano-vesicles were administered into CD1 mice via a tail vein and their localization and vascular effects at 30 min and 24 h post-injection were investigated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Nano-vesicles from normal first trimester human placentae were collected and administered into pregnant (D12.5) or non-pregnant female mice. After either 30 min or 24 h of exposure, all major organs were dissected for imaging ($n = 7$ at each time point) while uterine and mesenteric arteries were dissected for wire myography ($n = 6$ at each time point). Additional *in vitro* studies using HMEC-1 endothelial cells were also conducted to investigate the kinetics of interaction between placental nano-vesicles and endothelial cells.

MAIN RESULTS AND THE ROLE OF CHANCE: Nano-vesicles from first trimester human placentae localized to the lungs, liver and kidneys 24 h after injection into pregnant mice ($n = 7$). Exposure of pregnant mice to placental nano-vesicles for 30 min *in vivo* increased the vasodilatory response of mesenteric arteries to acetylcholine, while exposure for 24 h had the opposite effect ($P < 0.05$, $n = 6$). These responses were prevented by L-NAME, an NO synthase inhibitor. Placental nano-vesicles did not affect the function of uterine arteries or mesenteric arteries from non-pregnant mice. Placental nano-vesicles rapidly interacted with endothelial cells via a combination of phagocytosis, endocytosis and cell surface binding *in vitro*.

LARGE SCALE DATA: N/A.

LIMITATIONS REASONS FOR CAUTION: As it is not ethical to administer labelled placental nano-vesicles to pregnant women, pregnant CD1 mice were used as a model of pregnancy.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study to report the localization of placental nano-vesicles and their vascular effects *in vivo*. This work provides new insight into how the dramatic maternal cardiovascular adaptations to pregnancy may occur and indicates that placental extracellular vesicles may be important mediators of feto-maternal communication in a healthy pregnancy.

STUDY FUNDING/COMPETING INTEREST(S): This research was supported by the Faculty of Medical and Health Science (FMHS) School of Medicine PBRF research fund to L.W.C. M.T. is a recipient of a University of Auckland Health Research Doctoral Scholarship and the Freemasons Postgraduate Scholarship. No authors have any competing interests to disclose.

Key words: microparticle / nano-vesicle / nanoparticle / trophoblastic debris / exosome / vessel

Introduction

During normal human pregnancy, the mother undergoes dramatic physiological cardiovascular adaptations to support the requirements of the developing foetus. These changes include major increases in both plasma volume and cardiac output (Rovinsky and Jaffin, 1966; Hytten, 1985; Robson *et al.*, 1989), which are balanced by a reduction in total peripheral resistance. As such, at term, pregnant women have a slightly lower mean blood pressure than non-pregnant women (Moutquin *et al.*, 1985; Rovinsky and Jaffin, 1966). Interestingly, while the maximal increase in plasma volume occurs relatively late in gestation, the increase in cardiac output and associated decrease in total peripheral resistance is greatest in the first 20 weeks of gestation and is not driven by the increased plasma volume (Poppas *et al.*, 1997; Robson *et al.*, 1989). Currently, these cardiovascular adaptations are reported to be induced by reduced maternal pressor responses, increased signalling via the renin-angiotensin-aldosterone system, and increased oestrogen, progesterone and relaxin levels during pregnancy (Conrad, 2011; Gant *et al.*, 1973; Kristiansson and Wang, 2001; Leduc *et al.*, 1991; Walters and Lim, 1969). However, these changes cannot fully explain the dramatic cardiovascular adaptations that the mother undergoes, and other placental factors are likely to play a role.

The human placenta extrudes a large range of extracellular vesicles (EVs) throughout gestation. During the first 10–12 weeks of pregnancy, trophoblast plugs in the maternal spiral arteries prevent the passage of maternal red blood cells to the placenta, but these plugs are not solid and maternal plasma perfuses the placenta from very early in gestation. Thus, placental EVs can be carried away from the placenta via the uterine veins into the maternal circulation (Covone *et al.*, 1984). Extracellular vesicles are membrane-encapsulated packages of proteins, lipids and nucleic acids that represent a novel mode of cell-to-cell/endocrine-like communication (Raposo and Stoorvogel, 2013). The syncytiotrophoblast, which forms the outermost layer of the human placenta, produces a large range of EVs including multinucleated syncytial nuclear aggregates (SNAs, 20–100 μm), as well as smaller micro- (100–1000 nm) and nano-vesicles (<100 nm) (Tong and Chamley, 2015). Most of these EVs are thought to be formed by budding from the apical plasma membrane of the syncytiotrophoblast. In contrast, exosomes are a sub-population of nano-vesicles that have an intracellular origin (Mincheva-Nilsson and Baranov, 2014).

Once released from the syncytiotrophoblast, placental EVs are deported via the uterine veins into the maternal circulation. Placental extracellular vesicles have been reported in the maternal circulation from as early as 6 weeks of gestation (Sarker *et al.*, 2014). It was reported over 120 years ago that SNAs are entrapped in the first capillary bed they encounter after leaving the uterus, in the maternal lungs (Lapaire *et al.*, 2007; Schmorl, 1893). This was presumed to be due to the large size of the SNAs compared with the capillaries. In contrast, placental nano-vesicles are much smaller than SNAs and it is assumed that they can pass freely through the maternal pulmonary capillaries to disperse to the maternal peripheral circulation. Despite many publications that report the interactions of placental nano-vesicles with various cell types *in vitro* (Holder *et al.*, 2012; Pap *et al.*, 2008; Tannetta *et al.*, 2015; Tong and Chamley, 2015), at present, there are no reports demonstrating the distribution of human placental nano-vesicles *in vivo*.

Since placental nano-vesicles are carried in the blood, it is highly likely that they will interact with the endothelial cells that line all blood vessels. Indeed, the larger placental EVs (macro- and micro- vesicles) can interact with endothelial cells *in vitro*, affecting their viability and function (Chen *et al.*, 2012; Cockell *et al.*, 1997; Hoegh *et al.*, 2006; Smarason *et al.*, 1993; Wei *et al.*, 2016). Thus, in this study, we firstly investigated the *in vivo* localization of nano-vesicles derived from normal first trimester human placentae in pregnant mice. Then, we assessed the effects of placental nano-vesicles on maternal vascular function *in vivo*. Finally, we determined the rate and means of interaction between placental nano-vesicles and endothelial cells *in vitro* in order to gain a better understanding of how placental EVs may contribute to maternal vascular adaptations during pregnancy.

Materials and Methods

Ethical approvals

Human first trimester placentae were obtained from Epsom Day Unit, Greenlane Hospital (Auckland, NZ) following elective surgical termination of pregnancies with informed written consent. All work performed on human tissue was approved by the Auckland Regional Health and Disabilities Ethics Committee and conform to the declaration of Helsinki. The manipulation of mice used in this study was approved by the Auckland Animals Ethics Committee and performed under isoflurane anaesthesia.

Reagents

All cell culture reagents including Advanced Dulbecco's modified eagle medium/Nutrient mixture F-12 (DMEM/F12), MCDB-131 medium, L-glutamine, Penicillin/Streptomycin, foetal bovine serum (FBS), Trypsin/EDTA, fluorescent dyes and CD45+ magnetic beads were purchased from Invitrogen. Throughout the study, the same batch of FBS was used. Unless otherwise specified, all chemicals used for wire myography were purchased from Sigma Aldrich.

Collection of placental and control nano-vesicles

Placental nano- vesicles were collected from cultured first trimester placentae (8–12 weeks of gestation) as previously described (Abumaree *et al.*, 2006). Briefly, placental explants of around 400 mg were dissected from first trimester placentae and cultured in Netwell™ inserts (Corning) in Advanced DMEM/F12 medium supplemented with 2% FBS and 1%

Penicillin/Streptomycin. For some experiments, the fluorescent dye, CellTracker™ Red CMTPX, was also added (1 μg/mL, Invitrogen). Explants were cultured at 37°C in 5% CO_2/95% air for 16 h. The culture medium was then aspirated and centrifuged twice at low speed (2000 *g* for 5 min, 20 000 *g* for 1 h) to remove cellular debris and larger EVs (Avanti J30I Ultracentrifuge, JA 30.50 Ti fixed angle rotor, Beckman Coulter). The supernatant was then centrifuged at 100 000 g for 1 h at 4°C to collect placental nano-vesicles. The size and morphology of nano-vesicles collected from first trimester human placentae using this method has previously been extensively characterized and reported (Tong *et al.*, 2016). Control nano-vesicles were harvested from culture medium containing 2% FBS, but which had not been exposed to placental explants. Thus, control nano-vesicles were predominantly derived from the FBS in the medium.

Preparation of fluorescent placental nano-vesicles for administration into mice

Placental nano-vesicles were resuspended in 0.2 μm filtered phosphate buffered saline (PBS) to 3 mg/mL, as measured by the Nanodrop™ Lite spectrophotometer (Thermo Fisher), and labelled with CellTrace™ Far Red DDAO-SE (2 μg/mL) for 30 min in the dark at 20°C (Invitrogen). Excess dye was removed by diluting the samples 1:1 in PBS and centrifuging at 100 000 *g* for 1 h. Labelled placental nano-vesicles were resuspended in 0.2 μm filtered PBS to 1 mg/mL and 100 μL was administered into each mouse within 3 h of fluorescent labelling. In parallel, nano-vesicles from an equivalent volume of unused culture medium (6 mL) were collected and labelled as above to use as the study control.

Administration of placental nano-vesicles *in vivo*

Time-mated pregnant CD1 mice (12.5 ± 1 days post-coitus) between 7 and 12 weeks of age were used for this study. Placental or control nano-vesicles (from an equal volume of culture medium), freshly labelled with CellTrace™ Far Red DDAO-SE, were administered into mice via a tail vein. After 30 min or 24 h, cardiac puncture was performed to withdraw 1 mL of blood. Euthanasia was then performed by cervical dislocation and solid organs/tissues were dissected for imaging. Meanwhile, mesenteric arteries, and uterine arteries in pregnant animals, were dissected for wire myography.

Visualization on an IVIS kinetic imager

Within 1 h of euthanasia, the brain, thymus, lungs, heart, liver, spleen, pancreas, kidneys, feto-placental units and skeletal muscle (from the left forelimb and right hindlimb) were dissected from study animals in the dark and placed next to the corresponding organs from mice injected with control nano-vesicles. Organs were imaged on an IVIS Kinetic Imager (Caliper Life Sciences) at 605/640 nm using a 3 s exposure at 20°C and default settings (medium binning, F/Stop 2, EM gain off). The fluorescence level of each organ was adjusted to levels above the background level from mice that had been administered control nano-vesicles and the average radiance of each organ was recorded.

Wire myography

Within 30 min of euthanasia, second order mesenteric arteries and the main uterine arteries from pregnant mice were dissected and cleared of the surrounding adipose and connective tissues.

Three segments of each vessel were mounted on two 25 μm tungsten wires and held in place in a DMT 610 m wire myography apparatus. Vessels were submerged in a tissue bath containing physiological salt solution (PSS; 10 mM HEPES, 1.56 mM $CaCl_2$, 142 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose, pH 7.4) warmed to 37°C, and equilibrated with dry air. Vessels were normalized to a physiological luminal pressure of 90 mmHg, as determined by the LabChart software (ADInstruments) and rested for 30 min prior to assessing the integrity of the vessels, using phenylephrine, an α1-adrenergic receptor agonist (final bath concentration 10^{-5}M), and acetylcholine, an endothelium-dependent vasodilator (final bath concentration 10^{-5}M). Vessels that show a significant response to both chemicals were used for subsequent vessel function studies. The ability of the vessels to constrict and dilate in response to external stimulants was measured as previously described (Stanley *et al.*, 2011).

Assessing vasoconstriction

After initial assessment, vessels were washed twice with PSS and allowed to rest for 30 min before initiating function experiments. For mesenteric arteries, U46619, a thromboxane A2 mimetic, was chosen as the agonist to induce vasoconstriction, while for uterine arteries, phenylephrine was used. Previous work has demonstrated that these two agents produce reproducible, sustained constriction in these particular vascular beds (Stanley *et al.*, 2011). Concentration–response curves to U46619 (10^{-10}M – 3×10^{-5}M) or phenylephrine (10^{-10}M – 3×10^{-5}M) were constructed and the EC_{80} was calculated for individual vessel segments, which was subsequently used to pre-constrict arteries for vasodilatory response curves.

Assessing vasodilation

In order to determine the contribution of nitric oxide (NO) to endothelium-dependent vasodilation, one segment of the three segments of either mesenteric or uterine arteries was randomly chosen and incubated with a NO synthase inhibitor that prevents the production of NO (N-nitro-l-arginine methyl ester [L-NAME], 10^{-4}M). Other segments were incubated in PSS only and served as controls. After 30 min, all vessels were pre-constricted with U46619 or phenylephrine at the pre-determined concentrations to reach EC_{80}, and concentration–response curves to acetylcholine (10^{-10}M – 10^{-5}M) were constructed.

In order to investigate endothelium-independent relaxation, all vessels were washed and again pre-constricted with U46619 or phenylephrine at the pre-determined concentrations to reach EC_{80}. Dose-response curves for vasodilation in response to sodium nitroprusside (10^{-10}M – 10^{-5}M), an NO donor that directly stimulates smooth muscle relaxation (and thus endothelium-independent vasodilation), were constructed.

Data presentation

Finally, a 124 mM potassium solution (10 mM HEPES, 4.9 mM $CaCl_2$, 24 mM NaCl, 124 mM KCl, 2.4 mM $MgSO_4$, 1.18 mM KH_2PO_4, 5.5 mM glucose, pH 7.4) was added to maximally constrict the vessels. For vasoconstriction dose-response curves, measured values were expressed as percentages of maximal constriction induced by the 120 mM potassium solution; for vasodilation dose-response curves for both acetylcholine and sodium nitroprusside, measured values were presented relative to maximum pre-constriction achieved with either U46619 or phenylephrine. Sigmoidal dose-response curve fitting was performed and analysed on GraphPad PRISM 6.01 (GraphPad Software Inc).

Cell culture

The human microvascular endothelial cell line (HMEC-1 cells) was purchased from ATCC (CRL3243) and cultured in MCDB-131 medium supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin, at 37°C in 5% CO_2/95% air. When 90% confluent, cells were subcultured using 0.025% Trypsin/EDTA at a ratio of 1:4.

Determination of the interaction between placental nano-vesicles and endothelial cells

Visualization by confocal microscopy

In order to visualize the interaction between placental nano-vesicles and endothelial cells, HMEC-1 cells were cultured on glass coverslips until 90% confluent before labelling with fluorescent CellTracker™ Green CMFDA (1 μg/mL). CellTracker™ Red CMTPX-labelled placental nano-vesicles were then added for 24 h. Coverslips were washed with PBS and Hoechst was added (10 μg/mL) for 10 min at 20°C to stain the nuclei. Coverslips were mounted with Citifluor™ mounting medium (Citifluor Ltd) before viewing on a FluoView™ FV1000 Confocal Microscope (Olympus).

Mechanisms of placental nano-vesicles internalization by endothelial cells

In order to determine the mechanism by which placental nano-vesicles were internalized by endothelial cells, CellTracker™ Red CMTPX-labelled nano-vesicles (0.5 mg/mL) were added to 6×10^3 of HMEC-1 cells, in quadruplicates, and co-cultured for 16 h in the presence of (i) cytochalasin D (10 μg/mL), an inhibitor of phagocytosis, (ii) chloroquine (1 μg/mL), an inhibitor of endocytosis, or (iii) both inhibitors. After co-culture, the cells were washed thrice in PBS to remove unbound nano-vesicles prior to measuring fluorescence at 530/590 nm (Synergy 2 fluorescent microplate reader, Biotek). Fluorescence levels between experiments were normalized to the background fluorescence of HMEC-1 cells alone.

Time-course of interaction between placental nano-vesicles and endothelial cells

CellTracker™ Red CMTPX-labelled nano-vesicles (0.5 mg/mL) were co-cultured with 6×10^3 of HMEC-1 cells, in quadruplicates, for 30 min, 2, 6, 18, 24 or 48 h. After washing thrice, fluorescence was measured at 530/590 nm. Fluorescence readings between experiments were normalized to untreated HMEC-1 cells and maximal fluorescence was considered to be the fluorescence reading at 48 h.

Time-course of clearance of placental nano-vesicles by endothelial cells

In order to determine the rate of clearance of placental nano-vesicles by endothelial cells, 6×10^3 of HMEC-1 cells were co-cultured with CellTracker™ Red CMTPX-labelled nano-vesicles (0.5 mg/mL) in quadruplicates for 18 h. Unbound nano-vesicles were removed by washing thrice with PBS before quantifying fluorescence at 530/590 nm and this was taken to be time = 0. Then, fresh MCDB-131 medium was added and the cells were returned to the incubator for 30 min. After this, the medium was removed and the fluorescence was measured again (time = 30 min). This was repeated at 2, 24, 48 and 72 h after removal of the placental nano-vesicles. The drop in fluorescence over time was plotted relative to the fluorescence level at time = 0.

Statistical analysis

Statistical differences in the *in vivo* and *in vitro* observations were examined by the Kruskal–Wallis test with Dunn's multiple comparisons test or Mann–Whitney *U* test, as appropriate. For vascular function studies, repeated measures ANOVA (dose-response curves) or two-way ANOVA (EC_{50} and contribution of NO) were performed, followed by Bonferroni *post-hoc* test. All statistical analyses were performed on GraphPad PRISM 6.01 (GraphPad Software Inc) with an adjusted P value < 0.05 being considered statistically significant.

Results

Placental nano-vesicles were localized to the lungs, liver and kidneys of pregnant mice

In order to determine the *in vivo* localization of placental nano-vesicles, fluorescently labelled nano-vesicles from normal first trimester human placentae were administered into pregnant CD1 mice at gestational day 12.5 ± 1. After 30 min, placental nano-vesicles were localized to the lungs and liver of the pregnant mice (Fig. 1, $n = 7$) while after 24 h, nano-vesicles were localized to the lungs, liver and kidneys of the pregnant mice (Fig. 1, $n = 7$). We did not observe localization of exogenous placental EVs to the brain, thymus, heart, spleen, pancreas, skeletal muscle (of the left forelimb and right hindlimb) or foetal-placental units of the pregnant mice (Fig. 1, $n = 7$).

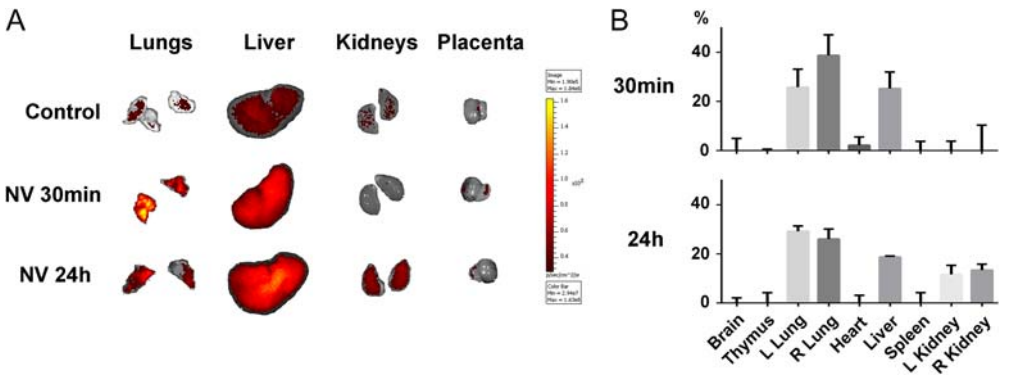

Figure 1 Distribution of placental nano-vesicles in pregnant CD1 mice. Nano-vesicles (NV) were collected from first trimester human placentae, fluorescently labelled and administered into pregnant CD1 mice at gestational day 12.5 ± 1 via a tail vein (100 μg). After 30 min or 24 h of exposure, cardiac puncture was performed and 10 major organs/tissues (brain, thymus, lungs, heart, liver, spleen, pancreas, kidneys, feto/placental units, skeletal muscle) were dissected for imaging on an IVIS Kinetic Imager (**A**). Fluorescence intensities were normalized to background fluorescence of organs from control mice that had been exposed to nano-vesicles derived from an equivalent volume of unconditioned placental culture medium (6 mL). The distribution of placental nano-vesicles to each organ (% total fluorescence, mean ± SEM) was semi-quantified ($n = 7$ mice at each time point, **B**).

Placental nano-vesicles did not affect uterine artery function in pregnant mice

In order to investigate whether placental nano-vesicles can affect vascular function *in vivo*, placental nano-vesicles were administered into pregnant mice at gestation day 12.5 ± 1 and after 30 min or 24 h, uterine arteries were dissected and wire myography was performed to assess vascular function *ex vivo*. Exposure to placental nano-vesicles did not affect the ability of uterine arteries to constrict in response to phenylephrine (Fig. 2A, $n = 6$); or to undergo endothelium-dependent vasodilation in response to acetylcholine (Fig. 2B, $n = 6$). Exposure to nano-vesicles also did not affect the ability of uterine arteries to undergo endothelium-independent vasodilation in response to sodium nitroprusside (Fig. 2C, $n = 6$).

Placental nano-vesicles affected the ability of mesenteric arteries to undergo endothelium-dependent vasodilation in pregnant mice

Exposure of mesenteric arteries from pregnant mice to placental nano-vesicles *in vivo* did not affect their ability to undergo vasoconstriction in response to U46619 ($P > 0.05$, $n = 6$, Fig. 3A) or endothelium-independent vasodilation in response to sodium nitroprusside ($P > 0.05$, $n = 6$, Fig. 3B).

In contrast, mesenteric arteries from pregnant mice that had been exposed to placental nano-vesicles for 30 min *in vivo* showed significantly increased endothelium-dependent vasodilation in response to acetylcholine, compared to mesenteric arteries that had been exposed to control nano-vesicles ($P < 0.05$, $n = 6$, Fig. 3C and D). Conversely, mesenteric arteries from pregnant mice that had been exposed to placental nano-vesicles for 24 h *in vivo* showed reduced endothelium-dependent vasodilation in response to acetylcholine compared to control mesenteric arteries ($P < 0.05$, $n = 6$, Fig. 3C and D).

The ability of placental nano-vesicles to affect endothelium-dependent vasodilation of mesenteric arteries was partially mediated by nitric oxide

When mesenteric arteries from pregnant mice were incubated with L-NAME, an inhibitor of NO synthase, prior to the addition of acetylcholine, the ability of the vessels to undergo endothelium-dependent vasodilation was reduced, regardless of whether they had been exposed to placental nano-vesicles or not (Fig. 4A and B). In mesenteric arteries that had been exposed to placental nano-vesicles for 30 min *in vivo* prior to myography, the contribution of NO to maximal endothelium-dependent vasodilation was significantly increased compared to control arteries (69.8 ± 6.5% vs. 41.5 ± 5.9%; mean ± SEM, $P < 0.05$, $n = 6$, Fig. 4C). However, the contribution of NO to maximal endothelium-dependent vasodilation was significantly reduced in mesenteric arteries that have been exposed to placental nano-vesicles for 24 h compared to control arteries (37.7 ± 11.8% vs. 69.8 ± 6.5%; $P < 0.05$, $n = 6$, Fig. 4C).

Placental nano-vesicles did not affect mesenteric artery function in non-pregnant mice

In order to investigate whether placental nano-vesicles alone can affect vascular function in the absence of a pregnant state, mesenteric arteries from non-pregnant female CD1 mice that had been exposed to placental nano-vesicles for either 30 min or 24 h were dissected and mounted for wire myography. Exposure to placental nano-vesicles *in vivo* did not affect the ability of mesenteric arteries of non-pregnant mice to constrict in response to U46619 (Fig. 5A, $n = 6$), to undergo endothelium-dependent vasodilation in response to acetylcholine (Fig. 5B), or to undergo endothelium-independent vasodilation in response to sodium nitroprusside (Fig. 5C, $n = 6$).

Placental nano-vesicles interacted rapidly with endothelial cells *in vitro*

In order to determine the rate and mechanisms of interaction between placental nano-vesicles and endothelial cells, *in vitro* co-culture experiments were employed. Firstly, confocal microscopy showed that fluorescently labelled placental nano-vesicles can be internalized by HMEC-1 endothelial cells ($n = 3$, Fig. 6A and B). The interaction between placental nano-vesicles and HMEC-1 cells was rapid, with 96.3% (89.2–100.4) [median (25–75 percentiles)] of maximal interaction being achieved by 30 min, the shortest time point studied ($n = 6$, Fig. 6C). Both cytochalasin D, an inhibitor of phagocytosis, and chloroquine, an inhibitor of endocytosis, significantly reduced the interaction between placental nano-vesicles and endothelial cells by 4% (0–12%)

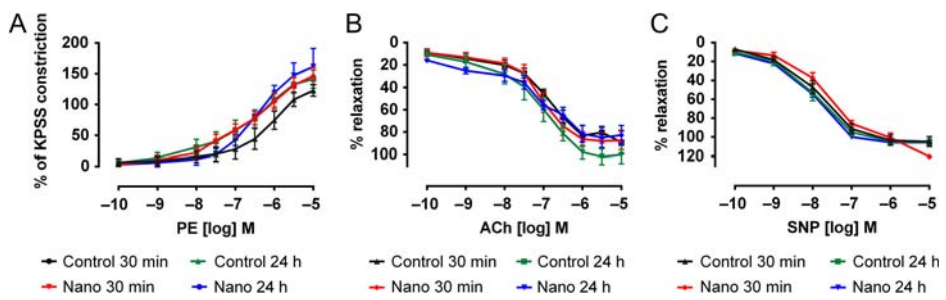

Figure 2 The effect of placental nano-vesicles on uterine artery function in pregnant mice. The effect of placental nano-vesicles on the ability of uterine arteries to constrict in the presence of phenylephrine (**A**), or to undergo endothelium-dependent vasodilation in response to acetylcholine (**B**), or to undergo endothelium-independent vasodilation in response to SNP (**C**), was investigated by wire myography. Data presented as mean ± SEM ($n = 6$ at each time point).

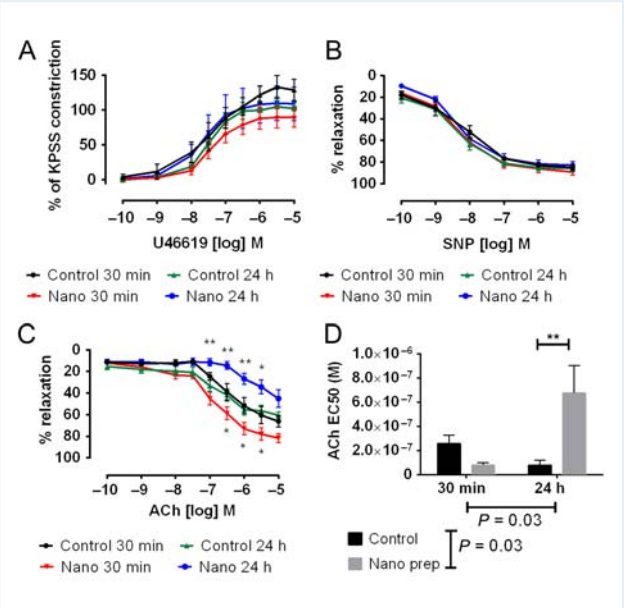

Figure 3 The effect of placental nano-vesicles on mesenteric artery function in pregnant mice. The effect of placental nano-vesicles on the ability of mesenteric arteries to constrict in the presence of a thromboxane A2 mimetic U46619 (**A**), or to undergo endothelium-independent vasodilation in response to SNP (**B**), or to undergo endothelium-dependent vasodilation in response to acetylcholine (**C**) was investigated by wire myography. Exposure to placental nano-vesicles *in vivo* significantly affected the response of mesenteric arteries to acetylcholine depending on the length of exposure (*$P < 0.05$, **$P < 0.001$). There was a significant effect of both nano-vesicle exposure ($P = 0.03$) and length of exposure ($P = 0.03$) on the sensitivity of mesenteric artery response to acetylcholine. Sensitivity to acetylcholine was significantly reduced (higher EC50) in arteries from animals exposed to exogenous nano-vesicles for 24 h in comparison to time-control animals (**$P < 0.001$, **D**). Data presented as mean ± SEM ($n = 6$ at each time point).

and 8% (1–21%), respectively ($P < 0.0003$, $n = 10$, Fig. 6D), however they did not have an additive effect (Fig. 6D). Neither cytochalasin D (10 μM) nor chloroquine (1 μM) affected the viability of HMEC-1 cells as assessed by the Alamar blue viability assay (data not shown). Placental nano-vesicles were cleared by HMEC-1 cells relatively rapidly in the first 30 min after the removal of unbound nano-vesicles ($n = 6$, Fig. 6E).

Discussion

During the first 20 weeks of pregnancy, the mother undergoes dramatic cardiovascular adaptations, including a 30–50% increase in cardiac output balanced by a reduction in peripheral vascular resistance (Christianson, 1976; Poppas *et al.*, 1997; Robson *et al.*, 1989). The reduction in total peripheral resistance can be mainly attributed to widespread vasodilatation, beginning early in the first trimester and peaking at 20–24 weeks of gestation (Poppas *et al.*, 1997; Robson *et al.*, 1989). Hormonal and neural factors are known to play a role in mediating these changes; however, other factors produced by the placenta are also likely to play a part. This study revealed that nano-vesicles extruded from first trimester human placentae localized to specific maternal organs *in vivo* and can affect endothelium-dependent vasodilation of mesenteric arteries in pregnant mice through a nitric oxide (NO)-mediated pathway. This work also showed that placental nano-vesicles can rapidly interact with endothelial cells through phagocytic and endocytic mechanisms *in vitro*. Thus, this study supports the concept that placental extracellular vesicles (EVs) may play an important endocrine role in mediating maternal physiological cardiovascular adaptations during early human pregnancy.

From as early as 6 weeks of gestation, the human placenta begins to extrude EVs into the maternal circulation (Salomon *et al.*, 2014; Sarker *et al.*, 2014). It was over 120 years ago that placental macro-vesicles were first reported in the lungs of pregnant women, presumably trapped due to their large size relative to that of the pulmonary capillaries (Lapaire *et al.*, 2007; Schmorl, 1893). However, the *in vivo* distribution patterns of the smaller placental EVs remained unclear. In this study, we have shown that after 30 min of exposure, placental nano-vesicles localized to the lungs and liver of pregnant mice *in vivo*, while after 24 h, placental nano-vesicles were localized to the lungs, liver and kidneys of pregnant mice.

That placental nano-vesicles were localized to the lungs *in vivo* regardless of time of exposure suggests that placental EVs are specifically targeted to this organ, rather than simply being entrapped as they attempt to pass through the lungs. In a previous description of the proteome of placental EVs, we reported that there are a number of adhesion molecules present on the EVs, including integrins α6 and β1 (Tong *et al.*, 2016). The α6β1 integrin has recently been shown to be responsible for targetting breast cancer cell exosomes to the lungs (Hoshino *et al.*, 2015) and it is likely that this integrin is at least in part responsible for the targeting of placental nano-vesicles to the maternal lungs. The maternal lungs undergo significant anatomical and physiological changes during pregnancy, such as changes to the composition of the extracellular matrix and a reduction in the production of angiotensin converting enzyme (Elkus and Popovich, 1992; Langer *et al.*, 1998; Merrill *et al.*, 2002), and it is possible that placental nano-vesicles are involved in regulating these changes.

Placental nano-vesicles were also localized to the liver of pregnant mice. The significance of this observation is currently unclear, as most other localization studies of vesicles from different sources also report the presence of vesicles/liposomes/nanoparticles in the liver, and the spleen (Lai *et al.*, 2014; Peinado *et al.*, 2012). Indeed, in our previous study, 200 nm carboxylate beads were also localized to the liver and spleen of pregnant mice (Tong *et al.*, 2017). Both the liver and spleen have reticuloendothelial systems with patrolling mononuclear phagocytes that can readily phagocytose administered liposomes or vesicles (Wiklander *et al.*, 2015). It seems likely that placental nano-vesicles are cleared from the maternal blood via the liver and kidneys but this remains to be confirmed. While it is perhaps not suprising that placental nano-vesicles are localized to the liver, their absence from the well-vascularized spleen is remarkable. As the spleen carries out both innate and adaptive immune functions (Mebius and Kraal, 2005), it is perhaps reasonable that placental nano-vesicles may lack signals targeting them to this organ or, alternatively, possess signals to evade this organ.

During pregnancy, the pattern of blood flow to different organs is also significantly altered (Buelke-Sam *et al.*, 1982; Hytten, 1985), including increased renal blood flow and glomerular filtration rates

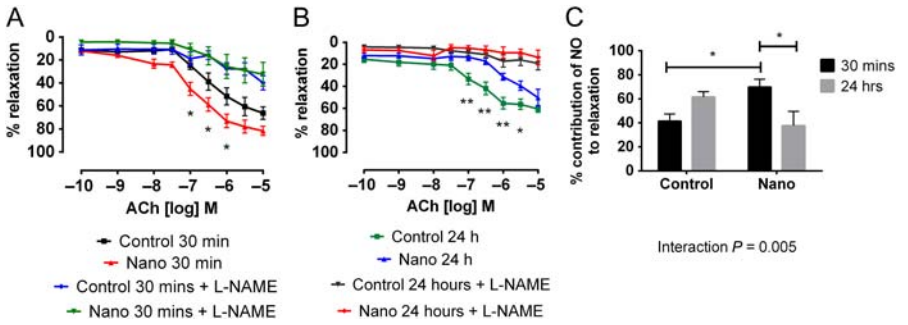

Figure 4 The effects of placental nano-vesicles on mesenteric artery function in the presence of L-NAME. When incubated with the nitric oxide synthase inhibitor L-NAME, mesenteric arteries from pregnant mice that have been exposed to control or nano-vesicle preparations for 30 min had their response to acetylcholine significantly reduced (**A**, $^{*}P < 0.05$). A similar effect of L-NAME was also observed for mesenteric arteries from mice exposed to control or nano-vesicle preparations for 24 h (**B**, $^{*}P < 0.05$, $^{**}P < 0.01$). The percent contribution of nitric oxide (NO) to the maximal response to acetylcholine was determined (**C**) and a significant interaction between nano-vesicle treatment and length of exposure was observed ($P = 0.005$). Following 30 min of exposure to nano-vesicles *in vivo*, there was a significant increase in the contribution of NO to the maximal endothelium-dependent relaxation ($^{*}P < 0.05$; **C**). Following 24 h of exposure to nano-vesicle *in vivo*, there was a significant decrease in the contribution of NO in comparison to 30 min exposure ($^{*}P < 0.05$; **C**). Data presented as mean ± SEM ($n = 6$ at each time point).

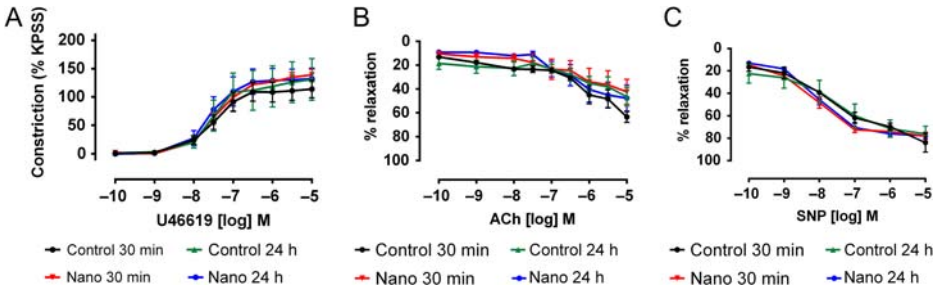

Figure 5 The effect of placental nano-vesicles on mesenteric artery function in non-pregnant mice. The effect of placental nano-vesicles on the ability of mesenteric arteries to constrict in the presence of a thromboxane A2 mimetic U46619 (**A**), or to undergo endothelium-dependent vasodilation in response to acetylcholine (**B**), or to undergo endothelium-independent vasodilation in response to SNP (**C**) was investigated by wire myography. Data presented as mean ± SEM ($n = 6$ at each time point).

(Anderson, 2005; Cheung and Lafayette, 2013; Davison and Dunlop, Pavek *et al.*, 2009). That placental nano-vesicles are found in the kidneys of pregnant mice only at the later time point suggests that the kidneys may be a mechanism for clearance of placental nano-vesicles *in vivo*, with the possibility that these EVs could contribute to the blood flow changes in the kidneys during pregnancy.

In addition to the organ-specific targeting of placental nano-vesicles *in vivo*, this work has also shown that placental nano-vesicles can remain in the maternal body for relatively long periods of time (over 24 h). This finding, combined with the fact that the human placenta is constantly extruding EVs into the maternal circulation, supports the hypothesis that placental EVs can potentially contribute significantly to the maternal physiological adaptations that occur during pregnancy. Indeed, in a wide range of other fields, EVs have increasingly been recognized as important mediators of cell-to-cell communication (Nilsson *et al.*, 2009; Peinado *et al.*, 2012; Raposo and Stoorvogel, 2013; Record *et al.*, 2014; Zitvogel *et al.*, 1998). While there is substantial evidence demonstrating that placental EVs play a role in mediating feto-maternal communication *in vitro*, this has not previously been demonstrated *in vivo*. Thus, in order to determine whether placental EVs can mediate some of the early maternal physiological adaptations to pregnancy, we specifically investigated whether placental nano-vesicles can affect the function of mesenteric and uterine arteries *in vivo*.

Firstly, uterine arteries were investigated since these arteries control the flow of blood to the uterus and therefore the growing feto-placental units. Uterine artery function has also previously been reported to be impaired in preeclampsia and intrauterine growth restriction (IUGR), two common obstetric complications (Fayyad and Harrington, 2005; Khaw *et al.*, 2008; Verlohren *et al.*, 2008). Placental nano-vesicles did not affect the ability of uterine arteries to constrict or dilate in pregnant mice. Conversely, exposure of pregnant mice to placental nano-vesicles affected the ability of mesenteric arteries to

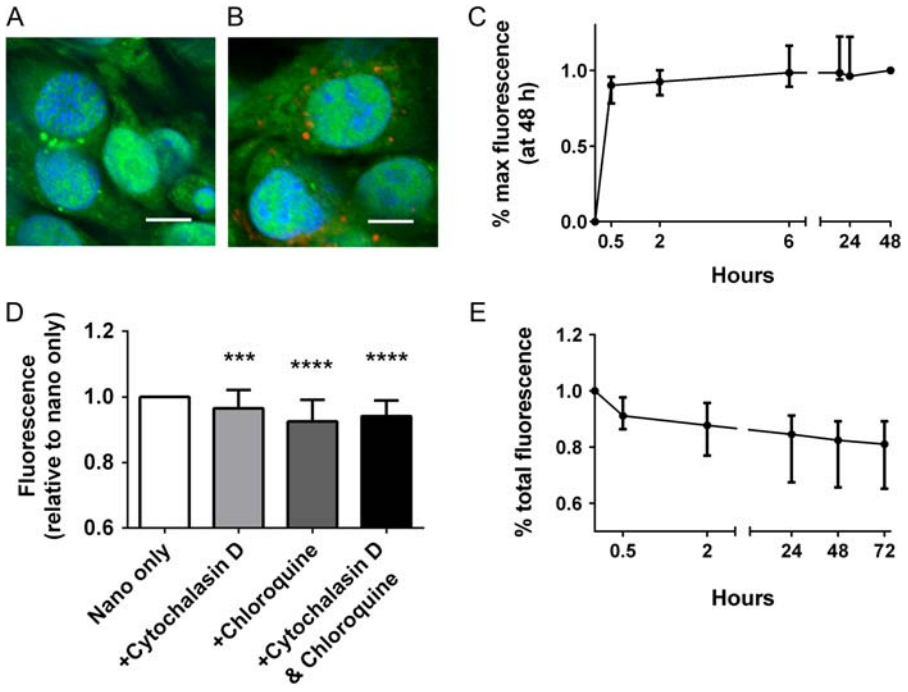

Figure 6 Interaction between placental nano-vesicles and endothelial cells *in vitro*. Representative confocal microscopy images showing cultured HMEC-1 endothelial cells (cytoplasm = green; nucleus = blue; **A**) and their internalization of placental nano-vesicles after 24 h of co-culture (red; **B**; $n = 3$, scale bar = 100 μm). The interaction between placental nano-vesicles and HMEC-1 cells was measured by quantifying the increase in fluorescence between 30 min to 48 h of co-culture (**C**, $n = 6$, median ± range). The mechanism of internalization of placental nano-vesicles by HMEC-1 cells was investigated by the addition of cytochalasin D (10 μM), chloroquine (1 μM) or both inhibitors (**D**, $n = 10$, ***$P < 0.001$, ****$P < 0.0001$, median ± IQR). The rate of clearance of placental nano-vesicles by HMEC-1 cells was measured by culturing HMEC-1 cells with placental nano-vesicles for 18 h before washing and quantifying the decrease in fluorescence from 0 min to 72 h (E, $n = 6$, median ± range).

undergo endothelium-dependent vasodilation. Mesenteric arteries are part of a major class of resistance vessels that contribute substantially to the control of total peripheral resistance in the systemic circulation. These observations support the idea that under normal physiological conditions, placental nano-vesicles may play an endocrine role in signalling to cells/organs distant to the placenta, rather than directly affecting the placenta's paracrine environment.

In this work, both the short term (30 min) and longer term (24 h) effects of placental nano-vesicle exposure were studied. After 30 min of exposure, placental nano-vesicles augmented endothelium-dependent relaxation of mesenteric arteries induced by acetylcholine, while after 24 h, placental nano-vesicles reduced endothelium-dependent vasodilation. In both cases, placental nano-vesicles signalled through NO, a major vasodilatory factor produced by the endothelium in both mice and humans (Durand and Gutterman, 2013). Nitric oxide has previously been reported to play a key role in the enhanced vasodilatory response observed in arteries from pregnant subjects (Cooke and Davidge, 2003; Kublickiene *et al.*, 1997). Providing further mechanistic insight to how placental EVs affect maternal vascular reactivity, others have recently reported the presence of bioactive endothelial nitric oxide synthase (eNOS) on EVs from normal term placentae (Motta-Mejia *et al.*, 2017). This observation, combined with our findings in this study, suggests that placental EVs can participate in the regulation of maternal vascular adaptations in pregnancy. This is further supported by our recent publication showing that EVs from first trimester human placentae carry other vasoactive factors including vascular endothelial growth factor (VEGF) and fms-like tyrosine kinase-1 (Flt-1), the receptor for VEGF (Tong *et al.*, 2017).

As the placenta is constantly extruding EVs into the maternal circulation, it remains unclear whether the observed increased (30 min post-injection) or decreased (24 h post-injection) vascular responsiveness following a single bolus of nano-vesicles in our model more closely resembles what might happen in pregnant women *in vivo*, but what is clear is that these normal placental EVs affected maternal vascular responses in key resistance vessels in an NO-dependent manner. It is possible that the differences observed between the two time-points are due to the breakdown of placental nano-vesicles *in vivo* or to the depletion of vasoactive factors present in the vesicles with time. This requires further investigation. While the exposure of pregnant mice to placental nano-vesicles affected the ability of mesenteric arteries to undergo endothelium-dependent vasodilation, exposure of virgin female mice to placental nano-vesicles did not affect vascular function. This suggests that the vasculature may be altered or primed/sensitized to placental EVs during pregnancy, or that placental EVs may be synergizing with other factors present in the blood of pregnant mice to augment the effects of the vesicles. Similar observations have been made with other factors, such as hypoxia inducible-factor 1α and sFlt-1, where overexpression was reported to induce hypertension and

proteinuria only in pregnant mice and not non-pregnant mice (Lu et al., 2007; Tal et al., 2010). This demonstrates that pregnancy can significantly alter an animal's physiology and often, pregnancy may present as a 'sensitized state' to exogenous factors, allowing these factors to affect normal physiological responses.

A caveat in this work is that we studied the location of human placental nano-vesicles in mice. The use of the mouse model may result in some differences due to intrinsic disparities between women and mice. However, it is unlikely that the findings we observed here are simply due to xenogenic responses as it has been reported by many studies that the administration of human extracellular vesicles into mice is well-tolerated (Doeppner et al., 2015; Wiklander et al., 2015). Furthermore, while the structure of the rodent placenta is dramatically different to that of the human, rodents undergo similar cardiovascular adaptations to those seen in women during pregnancy, making mice a useful model for studying maternal cardiovascular adaptations during early pregnancy (Kulandavelu et al., 2006; Wong et al., 2002). Of particular interest and relevance to our findings, the NO pathway has also been reported to be partially responsible for the pregnancy-induced cardiovascular adaptations observed in pregnant mice (Kulandavelu et al., 2006).

In a previous proteomic study, we reported that placental nano-vesicles carry some eat-me signals (calreticulin and Annexin V) as part of their protein cargo, but these were not balanced by the presence of don't-eat-me signals, such as CD31 and CD47 (Tong et al., 2016). We hypothesized that this would allow placental nano-vesicles to rapidly interact with target cells. Indeed, the *in vitro* observations in this study showed rapid interaction between placental nano-vesicles and endothelial cells (with over 90% of maximal interaction occurring by 30 min). However, phagocytosis and clathrin-mediated endocytosis accounted for less than 5% and 10%, respectively, of the total interaction between endothelial cells and placental nano-vesicles *in vitro*. This is in contrast to placental micro-vesicles where inhibiting phagocytosis and clathrin-dependent endocytosis of endothelial cells reduced the interaction between placental micro-vesicles and endothelial cells by 12% and 10%, respectively (Tong et al., 2017). It is possible that surface binding/interaction with endothelial cells is sufficient for placental nano-vesicles to affect endothelial cell function or, alternatively, that other mechanisms of interaction between placental nano-vesicles and endothelial cells exist, such as caveolin 1-mediated uptake and lipid raft-mediated internalization. The mechanisms of interaction between endothelial cells and placental nano-vesicles require further investigation.

In summary, this work has shown for the first time that nano-vesicles from first trimester human placentae target to specific organs *in vivo* and that these nano-vesicles can rapidly interact with endothelial cells. In addition, placental nano-vesicles affected endothelium-dependent vasodilation of mesenteric arteries in pregnant mice via an NO-dependent pathway. Thus, this work suggests a novel mechanism by which physiological maternal cardiovascular adaptations may be effected during the first trimester of pregnancy and suggests that placental EVs are important mediators of feto-maternal communication in a healthy pregnancy.

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Authors' role

All authors contributed to study conception and design. P.R.S. provided the placental samples. M.T., J.L.S. and Q.C. conducted the experiments. M.T., J.L.S., J.L.J., P.R.S. and L.W.C. analysed the results. M.T., J.L.S. and L.W.C. drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that no conflict of interest exists.

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