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In vivo targets of human placental micro-vesicles vary with exposure time and pregnancy

Mancy Tong[1], Qi Chen[1], Joanna L James[1], Michelle R Wise[1,2], Peter R Stone[1,2] and Lawrence W Chamley[1]

[1]*Department of Obstetrics and Gynaecology, The University of Auckland, Auckland, New Zealand and* [2]*Maternal Fetal Medicine, Auckland City Hospital, Auckland, New Zealand*

Correspondence should be addressed to M Tong; Email: mancy.tong@auckland.ac.nz

Abstract

Throughout human gestation, the placenta extrudes vast quantities of extracellular vesicles (EVs) of different sizes into the maternal circulation. Although multinucleated macro-vesicles are known to become trapped in the maternal lungs and do not enter the peripheral circulation, the maternal organs and cells that smaller placental micro-vesicles interact with *in vivo* remain unknown. This study aimed to characterise the interaction between placental micro-vesicles and endothelial cells *in vitro* and to elucidate which organs placental micro-vesicles localise to *in vivo*. Placental macro- and micro-vesicles were isolated from cultured human first trimester placental explants by sequential centrifugation and exposed to human microvascular endothelial cells for up to 72 h. *In vivo*, placental macro- and micro-vesicles were administered to both non-pregnant and pregnant CD1 mice, and after two or 30 min or 24 h, organs were imaged on an IVIS Kinetic Imager. Placental EVs rapidly interacted with endothelial cells via phagocytic and clathrin-mediated endocytic processes *in vitro*, with over 60% of maximal interaction being achieved by 30 min of exposure. *In vivo*, placental macro-vesicles were localised exclusively to the lungs regardless of time of exposure, whereas micro-vesicles were localised to the lungs, liver and kidneys, with different distribution patterns depending on the length of exposure and whether the mouse was pregnant or not. The fact that placental EVs can rapidly interact with endothelial cells and localise to different organs *in vivo* supports that different size fractions of placental EVs are likely to have different downstream effects on foeto–maternal communication.

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Introduction

During early human pregnancy, extensive maternal physiological adaptations must be effected to allow the semi-allogeneic foetus to be accommodated and adequately nourished by the mother. These maternal adaptations include a switch from a Th1 to a Th2 immune state (Wegmann *et al.* 1993, Mor *et al.* 2005), an increase in blood volume (Hytten & Paintin 1963) and a decrease in vascular resistance (Clark *et al.* 1989, Robson *et al.* 1989). Precisely, how such extensive transformations are induced during a normal human pregnancy is unclear, but endocrine factors have been shown to play a role, and it is postulated that the production of extracellular vesicles (EVs) by the placenta may also be important in mediating these adaptations (Mincheva-Nilsson & Baranov 2010, Tong & Chamley 2015).

The surface of the human placenta is covered by a single giant multinucleated cell, the syncytiotrophoblast. When areas of the syncytiotrophoblast become old or damaged, blebs on the apical surface of this layer are produced. Such membrane blebs or micro-vesicles (previously termed syncytiotrophoblast membrane microparticles (STBM)) are one type of EV produced by the human placenta. The syncytiotrophoblast also produces larger multinucleated vesicles, called syncytial nuclear aggregates (SNAs) as well as smaller nano-vesicles and exosomes (Chamley *et al.* 2014, Tong & Chamley 2015).

As the human placenta is haemochorial, the syncytiotrophoblast is bathed in maternal blood throughout most of gestation. Therefore, once extruded from the syncytiotrophoblast, EVs are carried away from the uterus into the systemic maternal circulation. Over 120 years ago, it was observed that placental macro-vesicles/SNAs become trapped in the first capillary bed they encounter, in the maternal lungs, after leaving the placenta. It is likely that the large size of macro-vesicles (20–100 µm) relative to the pulmonary capillaries (7 µm in diameter) prevents their passage beyond the lungs and very few macro-vesicles can be found in the maternal peripheral circulation (Schmorl 1893, Covone *et al.* 1984, Johansen *et al.* 1999). In contrast, the much smaller placental micro-vesicles (100–1000 nm) are able to pass through the pulmonary capillary bed and enter the maternal peripheral circulation (Goswami *et al.* 2006, Lok *et al.* 2008). To date, which maternal organs

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placental micro-vesicles interact with *in vivo* remain unknown.

Recently, EVs have increasingly been recognised as a novel mode of cell-to-cell communication, and it is likely that placental EVs play an important role in mediating foeto–maternal communication during pregnancy. For example, placental EVs may induce maternal immune tolerance and vascular adaptations to pregnancy (reviewed in Mincheva-Nilsson & Baranov 2010, Tong & Chamley 2015). Indeed, there is a growing body of evidence suggesting that EVs from normal healthy placentae can modulate the immune system (Abrahams *et al.* 2004, Frangsmyr *et al.* 2005, Abumaree *et al.* 2006*a*, 2012, Mincheva-Nilsson *et al.* 2006, Hedlund *et al.* 2009, Stenqvist *et al.* 2013). However, in comparison, the effects of placental EVs on vascular function are less clear. We have previously shown that SNAs from normal first trimester human placentae can protect endothelial cells against subsequent activation/dysfunction by endothelial damaging factors, such as IL-6 and lipopolysaccharide (Chen *et al.* 2012). In contrast, others have shown that micro-vesicles derived from healthy term placentae can inhibit the proliferation and increase apoptosis of endothelial cells (Smarason *et al.* 1993, Cockell *et al.* 1997). Clearly, more work to investigate the interaction between placental EVs and endothelial cells, and the effects of placental EVs on endothelial cell function, is required.

In order to better understand the potential targets and functions of placental EVs during a healthy human pregnancy, this study used EVs collected from normal first trimester human placentae to determine the kinetics and mechanisms of interaction between placental EVs and endothelial cells and to investigate whether there is specific localisation of placental EVs to maternal organs *in vivo*.

Methods

Ethical approvals

The collection of human placentae for this study was approved by the Auckland Regional Health and Disabilities Ethics Committee. All placentae (9–12 weeks of gestation) were obtained from Epsom Day Unit, Greenlane Hospital (Auckland, NZ) following elective surgical termination of pregnancy with informed written consent. The manipulation of mice used in this study was approved by the Auckland Animals Ethics Committee.

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, CD45+ magnetic beads and fluorescent dyes (CellTracker Red CMTPX, CellTracker Green CMFDA and CellTrace Far Red DDAO-SE) were purchased from Invitrogen. Throughout the study, the same batch of FBS was used.

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 (v/v), at 37°C with 5% CO_2/95% air.

Collection of placental EVs

Placental macro- and micro-vesicles were collected from the first trimester placentae (9–12 weeks of gestation) using a well-established explant culture model (Abumaree *et al.* 2006*b*, Tong *et al.* 2016). Briefly, placental explants of ~300 mg wet weight were dissected from the first trimester placentae and cultured in Netwell inserts (Corning, NZ) in Advanced DMEM/F12 medium supplemented with 2% FBS and 1% penicillin/streptomycin. For some experiments, fluorescent CellTracker Red CMTPX dye was added (1 µg/mL). After 16 h, the culture medium was aspirated and centrifuged sequentially at 2000 ***g*** for five minutes and then 20,000 ***g*** for one hour to collect macro- and micro-vesicle fractions respectively (Avanti J30I Ultracentrifuge, JA 30.50 Ti fixed angle rotor, Beckman Coulter, NZ). Contaminating red blood cells were removed from the macro-vesicle fraction by hypotonic lysis in ultrapure water (EMD Millipore), and contaminating leukocytes were depleted using anti-CD45 magnetic beads according to the manufacturer's instructions (Invitrogen).

Previous work using the same culture system has confirmed that the macro-vesicles collected from this culture system resemble those isolated from the uterine vein (Johansen *et al.* 1999, Abumaree *et al.* 2006*b*) and showed that the average length of macro-vesicles is 72 ± 21 µm with a mean volume of 48.32 µm^3 (Holland *et al.* 2016). In contrast, micro-vesicles have a mean diameter of 290 ± 72 nm, as measured by dynamic light scattering (Tong *et al.* 2016). Supplementary Figure 1 (see section on supplementary data given at the end of this article) shows representative images of these two vesicle types.

Visualisation of the interaction between placental EVs and endothelial cells

To visualise the interaction between placental EVs and endothelial cells, HMEC-1 cells were cultured on glass coverslips until 90% confluence before labelling with fluorescent CellTracker Green CMFDA (1 µg/mL) for two hours at 37°C. Placental macro- and micro-vesicles that have previously been labelled with CellTracker Red CMTPX were then added and co-cultured for 24 h. Coverslips were washed with PBS and Hoechst 33342 was added (10 µg/mL, Sigma-Aldrich) for ten minutes to stain the nuclei. Coverslips were washed again and mounted with Citifluor (Citifluor Ltd, UK) before viewing on the Olympus FluoView FV1000 Confocal Microscope (Olympus). Images were processed using FluoView v3.0 software and merged using Adobe Photoshop 7.0.

Investigation of the mechanism of placental EVs internalisation by endothelial cells

In order to determine the mode of internalisation of placental EVs by endothelial cells, 6×10^3 HMEC-1 cells were pretreated with cytochalasin D (10 µM), an inhibitor of phagocytosis or chloroquine (1 µM), an inhibitor of clathrin-mediated endocytosis, for 30 min. Cells were then co-cultured with CellTracker Red CMTPX-labelled macro- or micro-vesicles in quadruplicates (0.5 mg/mL), in the presence of cytochalasin D or chloroquine, as previously described (Chen *et al.* 2006). After 18 h, cells were washed and fluorescence was measured at 530/590 nm (Synergy 2 Microplate reader, BioTek). Fluorescence readings between experiments were normalised to the background fluorescence of endothelial cells alone, and all readings are presented relative to the readings from the co-culture of HMEC-1 cells with placental EVs alone.

Time-course of the interaction between placental EVs and endothelial cells

In order to establish a time-course of the interaction between placental EVs and endothelial cells, CellTracker Red CMTPX-labelled placental EVs were co-cultured with 6×10^3 HMEC-1 cells in quadruplicates for 30 min, two, six, 18, 24 or 48 h (0.5 mg/mL). At each time point, after washing thrice, the fluorescence of the cells was measured at 530/590 nm. Fluorescence readings were normalised to the reading from untreated endothelial cells, and all readings are presented relative to maximum fluorescence at 48 h.

Time-course of the clearance of placental EVs by endothelial cells

In order to establish a time-course for clearance of placental EVs by endothelial cells, CellTracker Red CMTPX-labelled placental EVs were co-cultured with HMEC-1 cells (0.5 mg/mL) for 18 h before removal of unbound EVs by washing. Fluorescence was measured at 530/590 nm, and this was taken as time = 0, at the start of the clearance curve. Fresh MCDB-131 medium was then added, and the cells are returned to the incubator for thirty minutes. After this, the medium was removed, the cells were washed again and the fluorescence was measured (time = 30 min). This was repeated at 2, 24, 48 and 72 h. The drop in fluorescence of endothelial cells over time was plotted relative to maximal fluorescence at time = 0.

Determination of the localisation of placental EVs in vivo

Labelling of placental EVs

Placental macro- and micro-vesicles were collected as described and resuspended in PBS. Placental EVs were labelled with CellTrace Far Red DDAO-SE (2 µg/mL) for 30 min at ambient temperature in the darkness. Excess dye was removed by centrifugation (2000 ***g*** × 5 min for macro-vesicles, 20,000 ***g*** × 1 h for micro-vesicles) and labelled placental EVs were resuspended in sterile PBS for administration.

Administration of placental EVs

In this study, both non-pregnant and time-mated (gestational day 12.5 ± 1) CD1 mice at week 7–12 of age were used. Mice were anaesthetised using isofluorane and 100 µL of CellTrace Far Red DDAO-SE-labelled placental micro-vesicles were administered via a tail vein (1–3 mg/mL). Control mice were injected via a tail vein with 100 µL of CellTrace Far Red DDAO-SE-labelled micro-vesicles that have been isolated from an equivalent volume of fresh culture medium that have not been exposed to placental explants (3–9 mL). In some experiments, CellTrace Far Red DDAO-SE labelled placental macro-vesicles (from 1.2 g of placenta) or 200 nm FluoSphere carboxylate beads (diluted 1:100, Thermo Fisher) were administered, with PBS being the negative control. Mice either remained anaesthetised for two minutes or were allowed to recover and after 30 min or 24 h, mice were anaesthetised again, and a cardiac puncture was performed. After one millilitre of blood was drawn, mice were killed by cervical dislocation.

Visualisation of dissected organs on an IVIS Kinetic Imager

Animals were dissected within one hour of euthanasia to remove the brain, thymus, heart, lungs, liver, spleen, pancreas, kidneys, uterus/placenta and skeletal muscle (left arm and right leg). Organs were imaged on an IVIS Kinetic Imager at 605/640 nm at 20°C. Exposure time was fixed to three seconds, with medium binning, F/Stop 2 and EM gain turned off. Background fluorescence of individual organs was adjusted to the fluorescence level of the corresponding control organ from mice injected with either CellTrace Far Red DDAO-SE-labelled micro-vesicles derived from fresh culture medium or PBS.

Statistical analysis

Statistical differences in the *in vitro* experiments were assessed either by the Kruskal–Wallis test with Dunn's multiple comparisons test or two-way ANOVA as appropriate. Observations from *in vivo* experiments were statistically examined either by the Kruskal–Wallis test with Dunn's multiple comparisons test or Mann–Whitney *U* test as appropriate. Statistical comparisons were performed on GraphPad Prism, 6.01 (GraphPad Software) with P value <0.05 being considered statistically significant.

Results

Placental macro- and micro-vesicles were internalised by endothelial cells in vitro

In order to determine whether placental EVs can interact with and be internalised by endothelial cells, placental EVs were fluorescently labelled and exposed to endothelial cells *in vitro* for 24 h ($n = 3$ placentae). Confocal microscopy showed that both placental macro- and micro-vesicles can be internalised by endothelial cells (Fig. 1A, B and C).

The mechanism of internalisation of macro- and micro-vesicles by endothelial cells differs

In order to investigate the mechanism of internalisation of placental EVs, endothelial cells were exposed to fluorescently labelled placental macro- or micro-vesicles ($n=10$ placentae) in the presence of (1) cytochalasin D (10 μM), an inhibitor of phagocytosis, or (2) chloroquine (1 μM), an inhibitor of clathrin-dependent endocytosis or (3) both inhibitors. After 24 h, free EVs were washed off and fluorescence was measured. Treatment of endothelial cells with cytochalasin D or chloroquine did not affect their viability as measured by the alamarBlue assay (data not shown).

Cytochalasin D significantly inhibited the interaction between placental macro-vesicles and endothelial cells by 10.7% (0.6–18.3) (median (25–75 percentiles), $P=0.0075$, Fig. 2A) whereas chloroquine did not affect this interaction. Similarly, cytochalasin D inhibited the interaction between placental micro-vesicles and endothelial cells by 11.5% (0–19.8) ($P<0.0001$, Fig. 2B). In contrast to macro-vesicles, chloroquine prevented 9.6% (1.7–24.8) of interactions between micro-vesicles and the endothelial cells ($P<0.0001$, Fig. 2B). There was no additive effect of combining both inhibitors on the interaction between either macro- or micro-vesicles and endothelial cells.

Placental EVs rapidly bind to endothelial cells and can be cleared by endothelial cells in vitro

In order to investigate the rates of interactions between placental EVs and endothelial cells, the time-course of binding between placental macro- or micro-vesicles and endothelial cells was studied between 30 min and 48 h (Fig. 3A, $n=4$). After 30 min of exposure of endothelial cells to macro- or micro-vesicles, 70.5% (55.3–76.6) and 60.5% (59.9–70.4) of the maximal interaction had occurred respectively (median (25–75 percentiles)).

In order to determine the rate of clearance of placental EVs by endothelial cells, fluorescently labelled placental macro- or micro-vesicles were exposed to HMEC-1 cells for 18 h (the time for maximal interaction, Fig. 3A). Clearance of placental EVs by HMEC-1 cells was quantified as a drop in fluorescence from the maximum over a time-course up to 72 h (Fig. 3B). The decline in fluorescence with time was significantly faster for micro-vesicles than that for macro-vesicles (Fig. 3B, $P<0.016$, $n=4$).

In vivo *localisation of placental micro-vesicles in non-pregnant female mice*

In order to investigate whether micro-vesicles from normal first trimester human placentae localise to particular organs *in vivo*, fluorescently labelled placental micro-vesicles (300 μg) or control micro-vesicles from an equivalent volume of culture medium that had not been exposed to placental explants (9 mL) were administered via a tail vein into non-pregnant female CD1 mice. Cardiac puncture and cervical dislocation were performed 2, 30 min or 24 h after administration, and ten major organs (brain, thymus, heart, lungs, liver, spleen, pancreas, kidneys, skeletal muscle and uterus) were dissected. The fluorescence levels of the organs dissected from mice injected with placental micro-vesicles were compared to those from mice injected with control vesicles, which was taken to be the background level of autofluorescence ($n=6$ at each time point).

After two minutes of exposure, placental micro-vesicles were localised in the lungs only whereas, by 30 min, placental micro-vesicles were localised to the lungs, liver and kidneys of mice (Fig. 4). After 24 h, placental micro-vesicles were localised to the liver and kidneys of non-pregnant mice (Fig. 4).

In vivo *localisation of placental micro-vesicles in pregnant mice*

As maternal physiology undergoes significant adaptations during pregnancy, we next investigated the *in vivo* distribution of placental micro-vesicles in time-mated pregnant mice (day 12.5 p.c.). In preliminary experiments, we observed that pregnant mice were more sensitive to placental micro-vesicles than non-pregnant animals and the dose of 300 μg (total protein) of micro-vesicles that we used in non-pregnant mice was lethal. Thus, for the following experiments, the dose of micro-vesicles was reduced to 100 μg (total protein). Pregnant mice injected with fluorescently labelled micro-vesicles

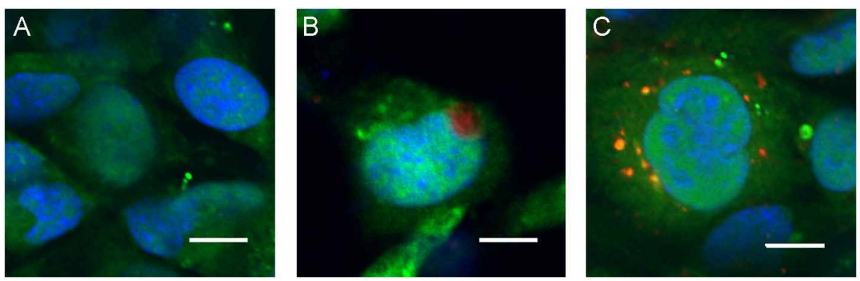

Figure 1 Representative confocal microscopy images showing the internalisation of placental EVs by HMEC-1 cells. The cytoplasm of HMEC-1 cells was labelled with CellTracker Green CMFDA (green) and the nuclei were counterstained with Hoechst (blue) (A). Macro- (B) and micro- (C) vesicles from normal first trimester human placentae were labelled with CellTracker Red CMTPX (red) and exposed to endothelial cells for 24 h ($n=3$ placentae). Representative images are taken on the FV1000 confocal microscope at 40× magnification (scale bar = 10 μm).

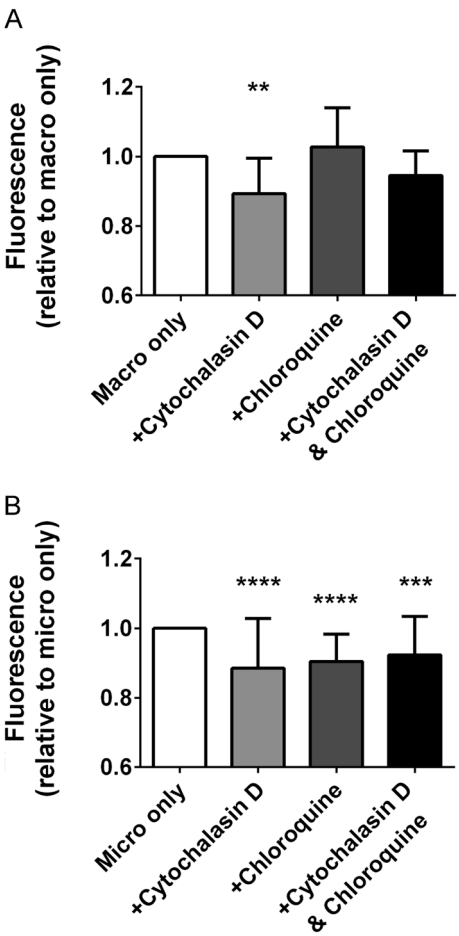

Figure 2 Mode of internalisation of placental EVs by HMEC-1 cells. CellTracker Red CMTPX-labelled macro- (A) and micro- (B) vesicles extruded from normal human first trimester placentae were exposed to endothelial cells, in quadruplicates, in the presence and absence of cytochalasin D (10 µM), chloroquine (1 µM) or both inhibitors ($n = 10$ placentae). After 24 h, unbound placental EVs were removed and fluorescence was measured at 530/590 nm (median ± IQR). The detected fluorescence was normalised to the fluorescence level of co-cultures with placental EVs only (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

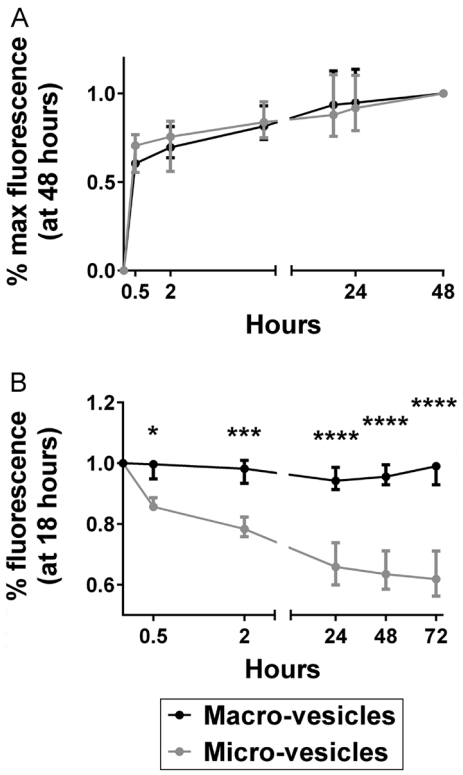

Figure 3 Time course for placental EVs to bind to and be cleared by HMEC-1 cells. CellTracker Red CMTPX-labelled macro- (black) and micro- (grey) vesicles were collected from normal first trimester human placentae and exposed to endothelial cells in quadruplicates for 30 min up to 48 h to quantitate binding/interaction ($n = 4$ placentae, (A). After washing, fluorescence at each time point was measured and fluorescence at 48 h was taken to represent 100% interaction. To quantitate clearance, CellTracker Red CMTPX-labelled placental macro- and micro- vesicles were exposed to endothelial cells in quadruplicates for 18 h ($n = 4$ placentae). Unbound vesicles were removed, and clearance was monitored by a decline in fluorescence relative to the starting fluorescence at 18 h (B). For both experiments, two-way ANOVA with Bonferroni's multiple comparisons test was performed to compare between changes in macro- and micro-vesicle fluorescence levels with changes in culture length (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$).

derived from an equivalent volume of placental culture medium that had never been exposed to explants (3 mL) were used as negative controls. Fluorescently labelled placental macro-vesicles and fluorescent 200 nm carboxylate beads were also administered to pregnant mice as additional controls. We examined the localisation of placental micro-vesicles to brain, thymus, heart, lungs, liver, spleen, pancreas, kidneys, skeletal muscle and foeto–placental units by visualisation on an IVIS Kinetic Imager.

After 30 min, placental micro-vesicles were localised only to lungs of pregnant mice, whereas, by 24 h, these vesicles were localised to lungs and liver of pregnant mice (Fig. 5, $n = 6$ at each time point). In contrast, regardless of the time of exposure, placental macro-vesicles were localised exclusively to the lungs of pregnant mice, whereas the 200 nm beads were localised to the liver and spleen of pregnant mice (Fig. 5, $n = 3$ at each time point).

In non-pregnant mice, with increasing time of exposure, there was a statistically significant reduction in the distribution of fluorescence/micro-vesicles to both the left ($P = 0.0051$) and right lungs ($P = 0.0167$), accompanied by an increase in micro-vesicle distribution to the liver and right kidney ($P < 0.05$, Fig. 6). Conversely, for pregnant mice, after 24 h of exposure, there was a statistically significant reduction in the proportion of micro-vesicles distributed to the left lung ($P = 0.0079$) and an increase in the proportion of micro-vesicles distributed to the liver ($P < 0.02$, Fig. 6).

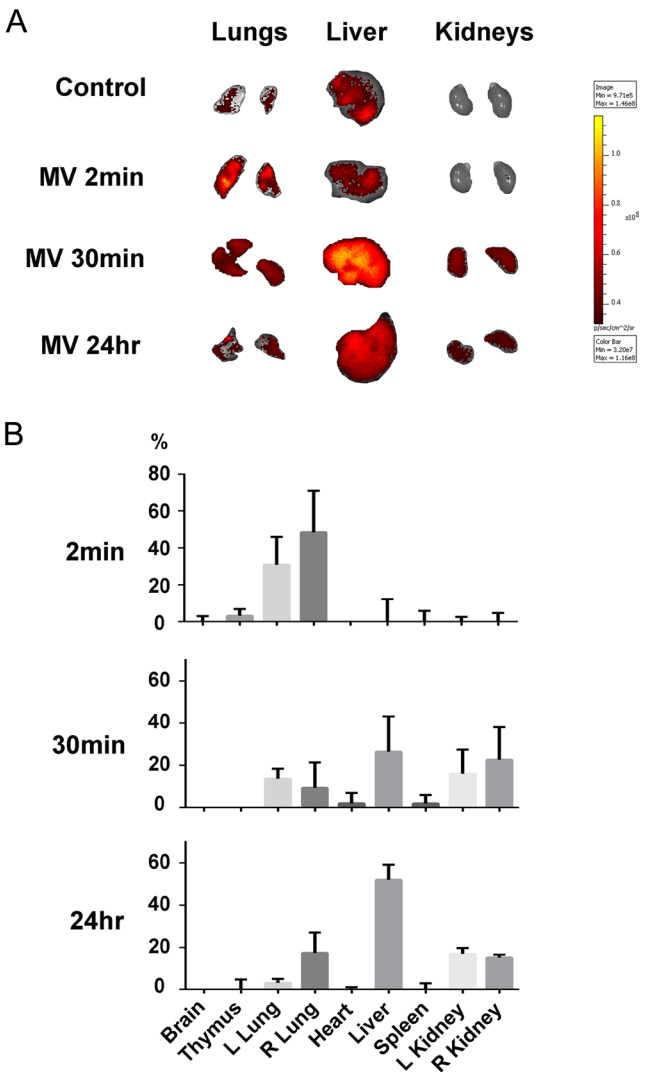

Figure 4 Organ distribution of placental micro-vesicles in female non-pregnant CD1 mice. Micro-vesicles from the first trimester human placentae were labelled with CellTrace Far Red DDAO-SE and administered to female CD1 mice through a tail vein. After 2 min, 30 min or 24 h, cardiac puncture was performed, and the fluorescence levels of ten major organs (brain, thymus, lungs, heart, liver, spleen, pancreas, kidney, skeletal muscle and uterus) were quantified using an IVIS Kinetic Imager at 605/640 nm ($n = 6$ at each time point, A). Mice injected with micro-vesicles from culture medium that had not been exposed to placental explants were used as controls to correct for background fluorescence. The distribution of fluorescence in each organ is shown in B (mean ± S.E.M.).

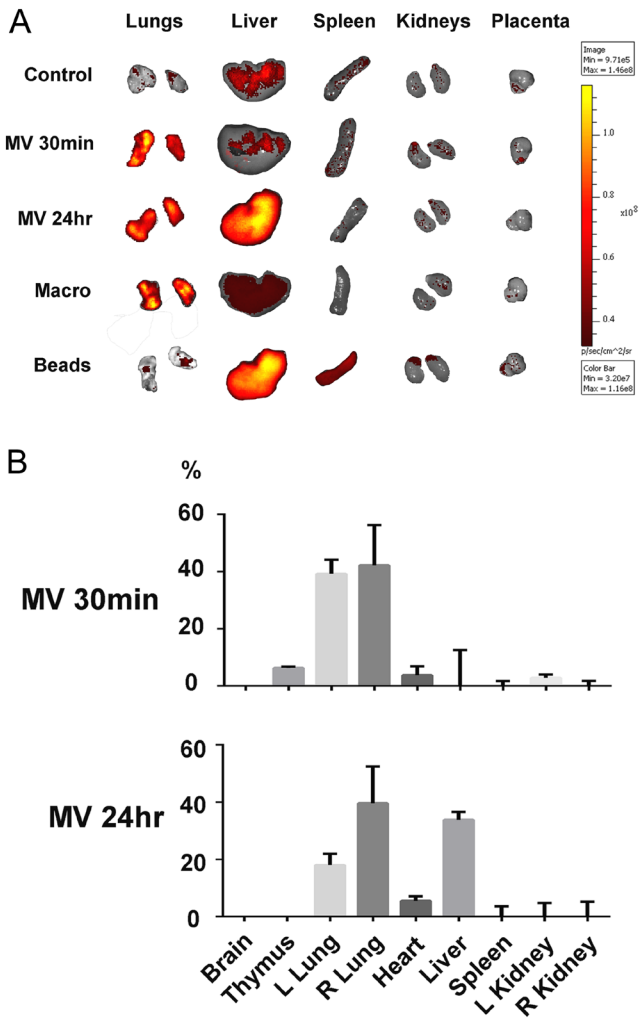

Figure 5 Organ distribution of placental EVs in pregnant CD1 mice. Placental macro- and micro-vesicles were labelled with CellTrace Far Red DDAO-SE and administered into pregnant CD1 mice through a tail vein. In other mice, 200 nm fluorescent synthetic beads were administered via a tail vein. After 30 min or 24 h, cardiac puncture was performed and the fluorescence levels of ten major organs (brain, thymus, lungs, heart, liver, spleen, pancreas, kidney, skeletal muscle and foeto-placental units) were quantified using an IVIS Kinetic Imager at 605/640 nm ($n = 6$ at each time point, A). Mice injected with micro-vesicles from culture medium that had not been exposed to placental explants were used as controls to correct for background fluorescence (A). As the distribution of placental macro-vesicles and synthetic beads did not change with time of exposure, only representative observations from the 30-min time point are depicted (A). The distribution of placental micro-vesicles to each organ is shown in B (mean ± S.E.M.).

Discussion

The human placenta is known to extrude EVs of different sizes into the maternal circulation throughout gestation, and placental macro-vesicles have been localised to the lungs of pregnant women over 120 years ago (Schmorl 1893, Lapaire *et al.* 2007). However, how placental micro-vesicles interact with endothelial cells and whether placental micro-vesicles localise to any specific organs *in vivo* remains unknown. Using a combination of *in vitro* and *in vivo* techniques, this study revealed that placental micro-vesicles can rapidly interact with and be cleared by endothelial cells *in vitro* and has also shown for the first time that micro-vesicles from first trimester human placentae are specifically localised to the lungs, liver and kidneys of mice. Interestingly, the distribution

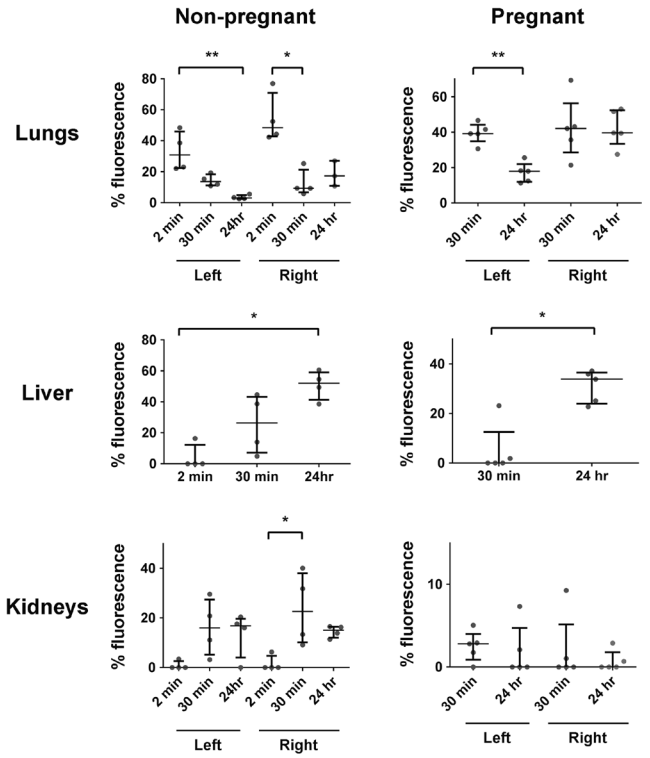

Figure 6 Organ distribution of placental micro-vesicles varied with time of exposure and pregnancy status. The changes in distribution of placental micro-vesicles to the lungs, liver and kidneys with time of exposure for both pregnant and non-pregnant mice are shown (median ± IQR). Statistical differences were examined by non-parametric tests (*$P < 0.05$, **$P < 0.01$).

of placental micro-vesicles *in vivo* varied with time of exposure and pregnancy status of the animal.

Placental EVs carry abundant proteins and nucleic acids that are likely to play an important role in foeto–maternal communication *in vivo* (Rajakumar *et al.* 2012, Delorme-Axford *et al.* 2013, Tong *et al.* 2016). Previous *in vitro* studies have suggested that interactions between placental EVs and immune cells may be important in mediating maternal immunological tolerance to the semi-allogeneic foetus during a normal pregnancy (Abrahams *et al.* 2004, Abumaree *et al.* 2006*a*, Hedlund *et al.* 2009), whereas other studies have shown the effects of placental EVs on endothelial cell function (Cockell *et al.* 1997, Chen *et al.* 2012). We have previously characterised the proteomes of different size fractions of placental EVs and showed that these EVs carry a varied array of proteins that may potentially mediate interaction(s) between the EVs and recipient cells (Tong *et al.* 2016). These include a large range of integrins (integrin α1, αIIb, α5, α6, αM, β1, β2, β3 and β5) as well as other molecules (cadherin 1, malectin, plectin and galectin 1, 3 and 7) that may be involved in targeting placental EVs to specific sites in the maternal body that express ligands for those adhesion molecules (Tong *et al.* 2016). Placental EVs also carry 'eat me' signals, such as calreticulin and Annexin V, and 'don't eat me' signals, such as CD31 and CD47, which are important in the clearance of cellular debris in other settings (Fadok *et al.* 2001, Gardai *et al.* 2005). In light of the findings from this study, further work characterising the orientation and function of these targeting molecules is imperative. We have also previously demonstrated that endothelial cells can phagocytose placental macro-vesicles and that the effects of macro-vesicles on endothelial cell function were dependent upon phagocytosis (Chen *et al.* 2006, 2012). However, those studies focused exclusively on placental macro-vesicles and did not examine the mechanism by which placental micro-vesicles interact with endothelial cells.

It is important to understand the interaction between placental EVs and endothelial cells as endothelial cells line all blood vessels and are the first adherent cell type that the placental EVs must interact with in order to leave the vasculature and enter potential target organs. Our findings in this study support our previous observation that endothelial cells can phagocytose macro-vesicles and further showed that clathrin-dependent endocytosis was not a significant mechanism for placental macro-vesicles to be internalised by endothelial cells. In contrast, placental micro-vesicles were internalised by endothelial cells through a combination of phagocytosis and clathrin-dependent endocytosis. It is important to note that while blocking, phagocytosis and clathrin-dependent endocytosis reduced the level of interaction between placental micro-vesicles and endothelial cells; this reduction was modest (10%), and there are likely to be other pathways that mediate interaction between placental EVs and target cells, such as micropinocytosis, membrane fusion and cell-surface tethering/binding. These mechanisms remain to be elucidated.

In order to accurately design future studies, it is important to also understand the kinetics of the interactions between placental EVs and endothelial cells. Here, we have shown that both placental macro- and micro-vesicles rapidly interacted with endothelial cells, achieving over 60% of maximal interaction after 30 min after exposure. Furthermore, there was a rapid initial clearance of placental micro-vesicles by endothelial cells with approximately 10% of the total fluorescence being cleared by 30 min and a slower clearance rate after that. Studying the clearance of placental EVs using the current method is complicated by the possibility that the fluorescent label from the EVs may be released into the endothelial cells after the phagocytosis/endocytosis of placental EVs. Thus, this may explain why we did not see complete clearance of either macro- or micro-vesicles from the endothelial cells even after 72 h. Nevertheless, fluorescence confocal microscopy showed that at least by 24 h, some of the dye and vesicles remained in vesicular structures inside the endothelial cells (Fig. 1). On the whole, our results suggest that in the future, *in vitro* interaction and

functional studies of placental EVs and endothelial cells should focus on shorter time points in addition to the longer (often 24 h) time points that have previously been investigated by ourselves and others (Hoegh *et al.* 2006, Chen *et al.* 2012, Tannetta *et al.* 2013).

In vivo, it is well established that placental macro-vesicles are localised exclusively to the maternal lungs in pregnant women and are not found in any other maternal organ (Schmorl 1893, Lapaire *et al.* 2007). The number of placental macro-vesicles in the maternal peripheral circulation is also very low (Attwood & Park 1961, Covone *et al.* 1984, Johansen *et al.* 1999). Our results here show that when human placental macro-vesicles were administered into pregnant mice via a tail vein, they were also localised only to the maternal lungs, mirroring the observation in pregnant women and other experimental animal models (Schmorl 1893, Lapaire *et al.* 2007, Lau *et al.* 2013). Thus, this observation endorses the use of tail vein injections to model the natural route of placental EV deportation from the placenta and also confirms the validity of using human placental EVs in mice.

In non-pregnant mice, placental micro-vesicles appeared to be localised first to the lungs after two minutes of exposure, then later to the liver and to a lesser extent, the kidneys. The rapid localisation of placental micro-vesicles to the maternal lungs with later localisation to the liver and kidneys may be explained in at least two ways: (1) there may be a strong first-pass effect and physical entrapment of the micro-vesicles in the lungs with later movement of the micro-vesicles to the liver and kidneys or (2) some micro-vesicles may be rapidly targeted to the lungs, whereas other micro-vesicles are targeted to the liver and kidneys via lower affinity interactions, and therefore, slower detection. With the current study design, we were unable to quantify the amount of EVs remaining in the maternal blood at each time point and therefore it remains unclear which scenario is the case.

As placental micro-vesicles did not fully distribute in non-pregnant mice until after 30 min of exposure, only the 30-min and 24-h time points were further studied in pregnant mice. In pregnant mice, placental micro-vesicles were localised to the lungs after 30 min of exposure and remained there until 24 h, whereas micro-vesicles were only localised to the liver by 24 h. These differences in the localisation of placental micro-vesicles between pregnant and non-pregnant animals suggest that pregnancy may be altering the expression of ligands to which these micro-vesicles home to in target organs. Alternatively, pregnancy may be altering the clearance rates of placental micro-vesicles from the lungs (decreased in pregnancy) and kidneys (increased in pregnancy). That pregnancy affected the targeting of EVs in our study raises an important caveat for similar research investigating the *in vivo* localisation of liposomes or EVs from non-placental sources as pregnancy may also alter the targeting of those particles. To further elucidate how pregnancy affects the targeting of EVs, it may be useful to include a pseudo-pregnant control group to determine whether it is the changes in hormone levels or physical changes due to pregnancy *per se* that caused the differences in distribution *in vivo*. In these studies, it would be ideal to compare the hormonal status of both the pregnant and pseudo-pregnant mice with the localisation patterns observed. Although such a control would be interesting in studies of EVs from non-placental sources, for example, EVs from cancer cells, it must be remembered that non-pregnant women (or mice) would not be exposed to placental EVs physiologically.

It was interesting to observe that the distribution of placental micro-vesicles (lungs, liver and kidneys) was quite different from that of similarly sized synthetic beads (liver and spleen) that we used as a control. This demonstrates that the localisation of placental micro-vesicles was not just a consequence of their size. The fact that placental micro-vesicles were localised to the lungs of both pregnant and non-pregnant mice while similarly sized beads were not suggests that the localisation of placental micro-vesicles to the lungs is not simply due to physical entrapment and that there are likely to be particular signals carried by micro-vesicles that target them specifically to, and retain them in, the lungs.

The tropism of placental micro-vesicles for the lungs is likely to have important physiological consequences for pregnant women. The maternal lungs are known to undergo significant anatomical and functional changes during pregnancy, such as changes to the composition of the extracellular matrix, increased oedema and increased phagocytic activity (Taylor 1961, Toppozada *et al.* 1982, Elkus & Popovich 1992, Hegewald & Crapo 2011). Pulmonary endothelial cells are also responsible for the production of angiotensin-converting enzyme whose activity may be relevant to the reduction in total peripheral resistance observed in pregnant women (Langer *et al.* 1998, Merrill *et al.* 2002). We have previously suggested that the localisation of placental macro-vesicles to the maternal lungs is likely to have important functional consequences for normal and complicated pregnancies (Chen *et al.* 2006, 2010, 2012, Lau *et al.* 2013, Chamley *et al.* 2014, Tong *et al.* 2016). The placental micro-vesicles are also specifically targeted to the maternal lungs; this suggests that in addition to endocrine factors, it is possible that placental EVs may also be involved in inducing maternal pulmonary adaptations during pregnancy.

After administration, synthetic beads were localised to the spleen of mice, whereas neither placental macro- nor micro-vesicles localised to this organ. The spleen is a highly vascularised organ that possesses a reticuloendothelial system with patrolling phagocytes (Wiklander *et al.* 2015). Therefore, logically, placental micro-vesicles should have been captured and

localised to the spleen. In addition, placental EVs have been reported to interact with various immune cells *in vitro* (Abrahams *et al.* 2004, Frangsmyr *et al.* 2005, Hedlund *et al.* 2009, Abumaree *et al.* 2012, Stenqvist *et al.* 2013). Several other studies investigating the targeting of EVs from other cellular sources (such as melanomas and hepatocytes), as well as liposomes, have also reported distribution to the spleen (Bocci *et al.* 1980, Peinado *et al.* 2012, Lai *et al.* 2014). The absence of placental EVs from the spleen suggests that placental EVs may possess mechanisms that allow them to evade this organ. In light of the immunological functions of the spleen to produce lymphocytes and antibodies, it may not be entirely surprising for EVs derived from the semi-allogeneic placenta to avoid this organ to evade maternal immune recognition/attack. The 'don't eat me' signals that we have previously identified in the proteome of placental micro-vesicles may contribute to this apparent evasion of the spleen (Tong *et al.* 2016).

It was also surprising to find that placental micro-vesicles did not target the placenta *in vivo* as it has been reported that placental EVs can interact with trophoblasts *in vitro* (Vargas *et al.* 2014). It is possible that micro-vesicles do target the placenta (and other organs) *in vivo* at levels below the sensitivity of the imager that we have used; however, this finding also raises the caveat that *in vitro* experiments in which large amounts of EVs are loaded onto single 'target' cell types may produce misleading results that do not occur *in vivo*. That placental EVs are not localised to the placenta *in vivo* may also suggest that placental EVs have a more important role in affecting maternal physiology systemically, rather than affecting the immediate uterine environment in a paracrine manner. This makes sense from an anatomical perspective as EVs deported from the placenta would have to travel around the maternal systemic circulation before returning to the uterine circulation to interact with trophoblasts and affect placental function *in vivo*.

There are several caveats to the work we report here. It is possible that cross-species differences in receptors and ligands may alter the interactions between maternal organs and the administered placental EVs. However, the gross anatomical differences in the structure of human and murine placentae mean that murine placental EVs are not derived from the syncytiotrophoblast (in rodent placentae, the syncytiotrophoblast is not exposed to maternal blood) and mice do not produce macro-vesicles that served as an important control in our localisation experiments. Thus, it is not possible to use murine placental EVs to understand the localisation of human placental EVs. It is also possible that xenogeneic differences may explain why the initial higher dose of micro-vesicles administered to pregnant mice was not well tolerated, although this seems unlikely as pregnancy is considered in some ways to be an immunosuppressed state and the non-pregnant animals tolerated the higher dose. Alternatively, pregnant mice may be more sensitive to the dose of placental EVs administered due to their endogenous load of placental EVs. Again, this seems unlikely as placental EVs make up only a small proportion of the total load of circulating EVs in women (Dragovic *et al.* 2013).

In summary, this study has confirmed that, as it occurs naturally in pregnant women, human placental macro-vesicles target exclusively to the maternal lungs when injected into pregnant mice. We have also shown that placental micro-vesicles can rapidly interact with endothelial cells *in vitro* through a combination of phagocytosis and endocytosis, as well as other as yet unidentified mechanisms, and these EVs can be cleared by endothelial cells. Furthermore, for the first time, micro-vesicles from normal human first trimester placentae have been shown to target specific organs *in vivo*, namely the lungs, the liver and the kidneys, suggesting that these organs are likely to be most affected by placental micro-vesicles during pregnancy. However, the distribution pattern of placental micro-vesicles varied with time of exposure and the pregnancy status of the study animal. A better understanding of the *in vivo* localisation and targeting mechanisms of placental EVs will allow us to better comprehend how these EVs may contribute to maternal physiological adaptations during a normal pregnancy, and in the future, to investigate whether this process is altered in obstetric complications such as preeclampsia and recurrent miscarriage.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-16-0615.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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