Original Article

Stem Cell INVESTIGATION

An *ex vivo* human placental vessel perfusion method to study mesenchymal stem/stromal cell migration

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Background: To initiate tissue repair, mesenchymal stem/stromal cells (MSCs) must enter the blood stream, migrate to the targeted area, cross the endothelial barrier and home to the damaged tissue. This process is not yet fully understood in humans and thus, the aim of this study was to develop an *ex vivo* placental vessel perfusion method to examine human MSC movement from a blood vessel into human tissue. This will provide a better understanding of MSC migration, movement through the endothelial barrier and engraftment into target tissue, in a setting that more closely represents the *in vivo* state, compared with conventional *in vitro* human cell culture models. Moreover, important similarities and differences to animal experimental model systems may be revealed by this method.

Methods: Human placental hTERT transformed MSC lines were labelled with live-cell fluorescence dyes, and then perfused into term human placental blood vessel. After labelled MSCs were perfused into the vessel, the vessel was dissected from the placenta and incubated at cell growth conditions. Following incubation, the vessel was washed thoroughly to remove unattached, labelled MSCs and then snap frozen for sectioning. After sectioning, immunofluorescence staining of the endothelium was carried out to detect if labelled MSCs crossed the endothelial barrier.

Results: Twelve placental vessel perfusions were successfully completed. In eight of the twelve perfused vessels, qualitative assessment of immunofluorescence in sections (n=20, 5 µm sections/vessel) revealed labelled MSCs had crossed the endothelial barrier.

Conclusions: The human placental *ex vivo* vessel perfusion method could be used to assess human MSC migration into human tissue. Cells of the MSC lines were able to adhere and transmigrate through the endothelial barrier in a manner similar to that of leukocytes. Notably, cells that transmigrated remained in close proximity to the endothelium, which is consistent with the reported MSC vascular niche in placental blood vessels.

Keywords: Cell migration; mesenchymal stem/stromal cell (MSC); perfusion; placenta

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Introduction

Mesenchymal stem/stromal cells (MSCs) are well-established, important players in tissue repair and regeneration. The conventional concept of MSC-based tissue repair is that MSCs are mobilised to their target by chemoattractant signals released by the damaged tissue. Regardless of their source, MSCs respond to the inflammatory signal(s) by entering the blood stream, migrating to the target area, crossing the endothelial barrier and homing in to the damaged tissue (1,2). MSCs then initiate repair by differentiating into the appropriate cell type to replace the damaged tissue, or by releasing paracrine factors that act on resident cells, or by modulating inflammatory processes (3-6). Therefore, MSCs must be able to efficiently migrate to, and interact with, the microenvironment at the site of injury. This process is not fully understood in humans. The aim of this study therefore, was to develop an *ex vivo* human placental vessel perfusion method to examine MSC migration from the circulation and into tissue. This will provide a better understanding of MSC transendothelial migration and engraftment into target tissue, in a setting that more closely represents an *in vivo* state.

Perfusion is a technique where a fluid is injected into a blood vessel in order to reach an organ or a tissue. The placental perfusion system was first developed and described by Panigel in 1962 (7), then later modified by Schneider and Huch in 1985 (8), and other research groups, including our own (9). Perfusion of the human placenta is one of the most useful methods to examine the transplacental transfer of chemical substances and drugs through the maternal-fetal interface (10-13). The placental perfusion model has also been used to investigate the transplacental transfer of antigens, such as the blood stage malaria antigen (14), or to study the transfer of immune cells from the mother to the fetus (15), and the invasion of leukaemia cells into fetal tissues (16).

Stem cell researchers have utilized the perfusion technique to study MSC transmigration and homing in various organs. For example, Nazarov *et al.* [2012] perfused MSCs into an *ex vivo* human acute lung injury model to assess their therapeutic effect (17). Yet the majority of other studies involve perfusion of human MSCs into animal organs, most commonly murine hearts (18,19). This is because the ability to perfuse human organs, *ex vivo*, is limited by the availability of organs, and the difficulty in maintaining the physiology of the perfused organ in an *ex vivo* sitting for the duration of the experiment. The term human placenta remains largely unexploited for investigating MSC transendothelial migration and engraftment. Unlike other human organs, the term human placenta is abundant and readily available but more importantly, the *ex vivo* perfused placenta can be maintained in its physiological state for up to several days.

Here, we employ a term human placenta *ex vivo* perfusion method, where placental vessels are perfused *ex vivo* with a medium containing MSCs that have been stained with live-cell fluorescent dyes. We employed placental CMSC29 and DMSC23 cell lines, which were derived by hTERT transformation of primary, term, chorionic and *decidua basalis* MSCs respectively (see below). Both cell lines were verified to maintain the MSC phenotype and functions, including the ability to migrate. With this method, MSCs were observed to migrate from the vessel lumen into the vessel wall and cross the endothelial cell barrier.

Methods

Tissue collection

Placentae for this project were collected with approval from the Royal Women's Hospital (RWH) Human Research and Ethics Committees. All placentae were obtained following informed, written patient consent at the RWH, Parkville, Australia. The collected placentae were from clinically uncomplicated, healthy pregnancies and had no obvious macroscopic defects.

Cell culture

The CMSC29 and DMSC23 cell lines were used in this study as representatives of chorionic and decidual MSC types (CMSCs and DMSCs). CMSC29 and DMSC23 were created at the Pregnancy Research Centre (PRC)-RWH laboratory from primary, term, chorionic and *decidua basalis* MSCs respectively, by hTERT transformation (20). Both cell lines have been verified to maintain MSC characteristics and functions, including migration capacity (20). CMSC29 cells were cultured in AmnioMAX™ C-100 Basal Medium supplemented with AmnioMAX™ C-100 Supplement [(10:1, v/v), Life Technologies, Carlsbad, CA, USA] (21). DMSC23 cells were cultured in MesenCult™ Proliferation Kit (Human) [MesenCult™ MSC Basal Medium and Mesenchymal Stem Cell Stimulatory Supplements (10:1, v/v), STEMCELL Technologies,

Vancouver, Canada]. Cells were kept in a humidified incubator at 37 ℃, 5% CO_2 and 95% room air.

Optimizing live-cell fluorescent labelling for CMSC29 and DMSC23 cells

Prior to perfusing, CMSC29 and DMSC23 cells were labelled with live-cell fluorescent dyes. This was done to detect the CMSC29 or DMSC23 cells in the perfused vessel sections. Cell tracker™ Orange (CMTMR) and Cell tracker™ Green (CMFDA) live-cell florescence dyes (Life Technologies), were used to label CMSC29 and DMSC23 cells respectively. According to the manufacturer's instructions, the recommended concentration for long-term staining (i.e., >3 days) of rapidly dividing cells was 5–25 μM dye, incubated for 15–45 minutes. However, a report noted that increasing the CMTMR loading time for up to 2 hours leads to better dye retention by the cells with no impact on viability (22). Therefore, to optimise the CMTMR and CMFDA concentration and incubation time for CMSC29 and DMSC23 labelling, five dye concentrations and five incubation times were tested. Cells suspended in supplemented medium were seeded into 6-well plates and left to adhere and grow overnight. Five wells per plate were seeded, one for each concentration tested (15, 20, 25, 30 and 35 μM). Five plates in total were used, one for each incubation time tested (0.5, 1, 1.5, 2 and 2.5 hours).

To label the cells, cell culture medium was removed from the wells and pre-warmed dye working solution was added at 15, 20, 25, 30 or 35 μM. The cells were incubated for 0.5, 1, 1.5, 2 or 2.5 hours, under cell growth conditions. The dye working solution was then replaced with supplemented medium and cells were incubated for another 30 minutes under cell growth conditions. Finally, the medium was removed, and the cells were washed with phosphate buffer saline (PBS) before fresh supplemented medium was added. At day 1 and day 3 after cell labelling, the fluorescence intensity was examined by measuring the pixel intensities with an Olympus IX2 inverted microscope (Olympus Shinjuku, Tokyo, Japan) equipped with Cell^R imaging software (Olympus). To assess whether the used cell tracker concentrations affected cell viability, 3×10^5 cells were seeded per tube and stained using the manufacturer's "cells in suspension" staining protocol. Then, a viability count was carried out on the stained cell suspension and compared to the control.

Establishment of the ex vivo placental vessel perfusion method

Following delivery at term, the placenta was kept at 4 ℃ and transported to the laboratory within 20 minutes. The placenta was then assessed for any macroscopic abnormalities and perfused within two hours. The placenta was placed in the upper compartment of a dual compartment perfusion bath. The lower compartment was pre-filled with water and maintained at 37 ℃ (*Figure 1*). The blood vessel (vein or artery) chosen for perfusion was 2–4 mm in diameter and located in the central area of the chorionic plate, in a close proximity to the umbilical cord. The perfused vessel was isolated from the placental circulation by applying two tight stiches approximately 4 cm apart and the isolated area of the vessel was cannulated. The cannulas were stabilized by applying another two stiches leaving around 1–1.5 cm of vessel length between the inflow cannula and the outflow cannula. Following cannulation, the placenta was submerged in warm saline and the cannulated vessel was washed for 1 hour. Washing was done with warm Krebs-Henseleit buffer balanced salt solution (23), saturated with 5% O_2 and 5% CO_2 in N_2. Perfusion was at a constant pressure of 5 ml/min using a peristaltic pump (Thermo Fisher Scientific, catalogue No. 7554-20).

After the first wash was completed, fluorescently labelled MSCs were suspended in cell culture medium (Av.1.5×10^6 cells/ml in a volume of 3 mL) and perfused into the vessel. The labelled MSCs were injected into the perfused vessel by disconnecting the inflow cannula from the peristaltic pump circuit, and connecting the cannula to a syringe containing the cell suspension. After the cells were perfused into the vessel, the vessel was dissected from the placenta with the stabilized cannulas still attached. Then, the dissected vessel was placed in a Petri dish filled with saline and incubated at cell growth conditions (37 ℃, 5% CO_2 and 95% room air) for 3 hours up to 4 days. Following incubation, the vessel was taken out of the incubator and the perfusate was drained and the lumen of the vessel was washed thoroughly with cell culture medium using the attached cannulas and a syringe.

Finally, the perfused vessel was transferred into a foil cylinder filled with Jung Tissue Freezing Medium™ Jung (Leica Biosystems, Nuloch, Germany), and snap frozen in liquid nitrogen. The frozen vessel was either sectioned immediately or stored at –80 ℃ until sectioning. For each

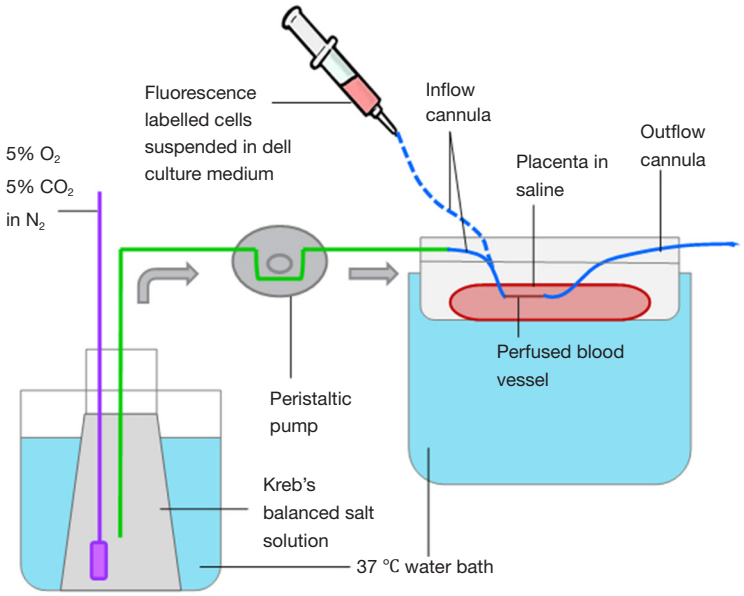

Figure 1 Schematic drawing of the main elements of the placental vessel perfusion experiment setting. The drawing shows Krebs-Henseleit buffer balanced salt solution being pumped into the perfused placental vessel through the inflow cannula. The inflow cannula is then disconnected from the pump tube and connected to a syringe containing fluorescence labelled cells suspended in cell culture medium.

perfused vessel, the block was sectioned into 20 µm × 5 µm cross sections using a cryostat (Leica CM1850 UV, Leica Bioscience). The 20 sections collected were obtained from different segments across the approximately 1 cm perfused vessel. Prior to examination, the sections were fixed with 70% ice cold ethanol [(v/v) in Milli-Q water] and immunofluorescence detection of the endothelium was carried out. The endothelium was detected with a conjugated anti-vWF antibody as described below. Cell nuclei were detected by using mounting medium for fluorescence containing DAPI nuclear stain (VECTASHIELD®, Vector Labs, Burlingame, CA, USA). Images were taken using an Olympus Provis AX-70 fluorescence microscope (Olympus) equipped with AxioVision 40 version 4.8.2.0 software (Carl Zeiss AG, Oberkochen, Germany). The criterion for migration was the detection of labelled CMSC29 or DMSC23 that had crossed the endothelial barrier.

Endothelium labelling with anti-von Willebrand factor (vWF) antibody

The endothelium of the perfused sectioned vessels was labelled with fluorescein-conjugated anti-vWF antibodies. This was done to identify the endothelial barrier and determine whether the fluorescently labelled cells had crossed this barrier. Two anti-vWF antibodies were used in this study. Antibody concentrations and incubation times were optimized for 5 µm placental vessel sections, based on qualitative assessment of fluorescence signal intensity under a microscope. The first was a FITC-conjugated sheep antibody to human vWF (Meridian Bioscience, Cincinnati, OH, USA). Sections were fixed with 70% ethanol [(v/v) in Milli-Q water] before blocking with 5% skim milk [(w/v) in PBS] for 1 hour at room temperature. After blocking, the sections were washed with PBS. Then, the anti-vWF antibody was added at 1:50 dilution [(v/v) in PBS], and incubated for 3 hours at 4 °C in a humidified chamber. The second vWF-antibody used in this study was a rabbit antibody to human vWF (Thermo Fisher Scientific, Waltham, Ma, USA), together with a Cy5-conjugated goat antibody to rabbit IgG (Abcam PLC, Cambridge, UK). The sections were first fixed, blocked and washed as described. Then, the rabbit antibody to human vWF was added at 1:50 dilution [(v/v) in PBS], and incubated overnight (at least 18 hours) at 4 °C in a humidified chamber. Next, the tissue sections were washed with PBS and conjugated goat antibody to rabbit IgG diluted at 1:100 [(v/v) in PBS], was added to the sections and incubated for 3 hours at 4 °C in a humidified chamber.

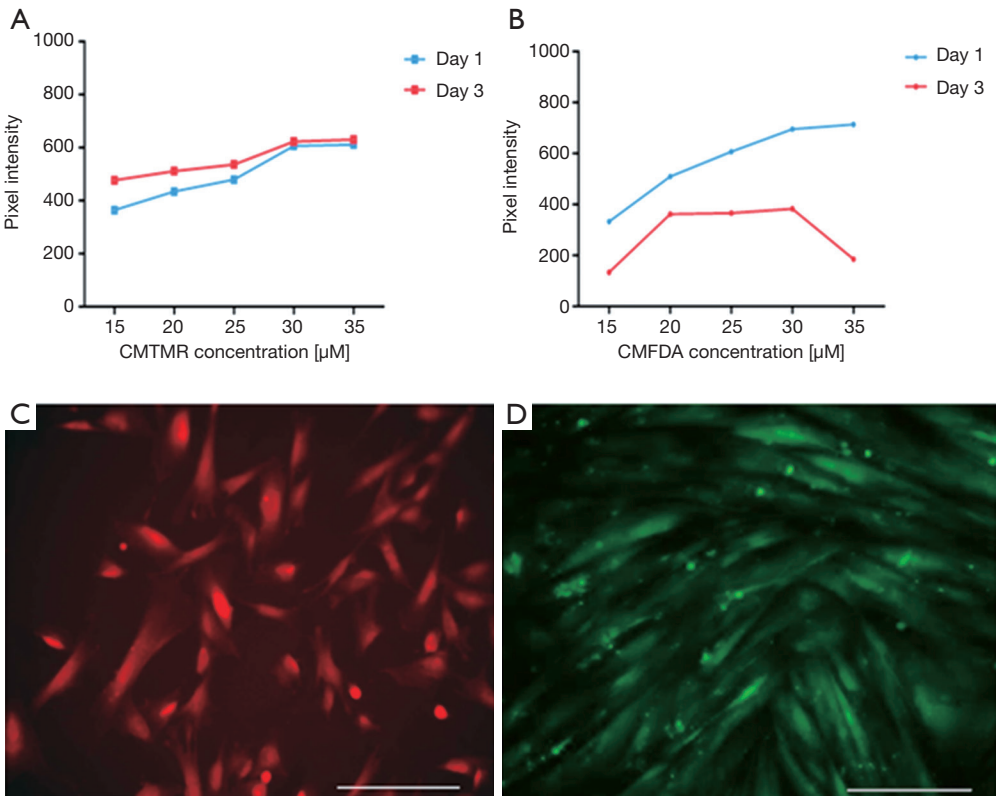

Figure 2 Fluorescence intensity of CMTMR labelled CMSC29 and CMFDA labelled DMSC23. Fluorescence intensity presented as mean pixel intensity values for (A) CMTMR labelled CMSC29 cells, and (B) CMFDA labelled DMSC23 cells, following one day and three days of staining (n=2). (C) A representative image of CMSC29 cells labelled with CMTMR (30 μM incubated for 2 hours). (D) A representative image of DMSC23 cells labelled with CMFDA (30 μM incubated for 1.5 hours), 200×. Scale bar =100 μm.

Results

Optimizing live-cell fluorescent labelling for CMSC29 and DMSC23 cells

The fluorescence intensity of CMTMR labelled CMSC29 cells and CMFDA labelled DMSC23 cells continued to increase up to 30 μM of dye concentration, before reaching a plateau (*Figure 2A,B*). For CMTMR, most of the tested concentrations gave the highest pixel intensity values following 2 hours of incubation. Whereas for CMFDA, most of the tested concentrations gave the highest pixel intensity values following 1.5 hours of incubation (data not shown). Comparing fluorescence intensity following one day and three days of labelling, it appeared that the fluorescence intensity was qualitatively higher at day three than it was at day one for CMTMR labelled CMSC29 cells, and qualitatively lower at day three than it was at day one for CMFDA labelled DMSC23 cells (*Figure 2A,B*). At all tested concentrations, there was no obvious change in the characteristic spindle-shaped MSC morphology and both CMSC29- and DMSC23-labelled cells were able to proliferate and achieve confluency (*Figure 2C,D*). The mean percentages of viable, labelled CMSC29 at 30 μM CMTMR for 2 hours was 88.3%±5.8% (SEM). The mean percentages of viable, unlabelled control CMSC29 cells was 81.7%±1.7% (SEM) (n=3). This indicates that using the Cell tracker™ Probes at a concentration of 30 μM, incubated for up to 2 hours, give high fluorescence intensity without affecting cell viability.

Establishment of the ex vivo placental vessel perfusion method

Twelve placental vessel perfusions were successfully completed. In eight of the twelve perfused vessels, qualitative assessment of immunofluorescence in cross

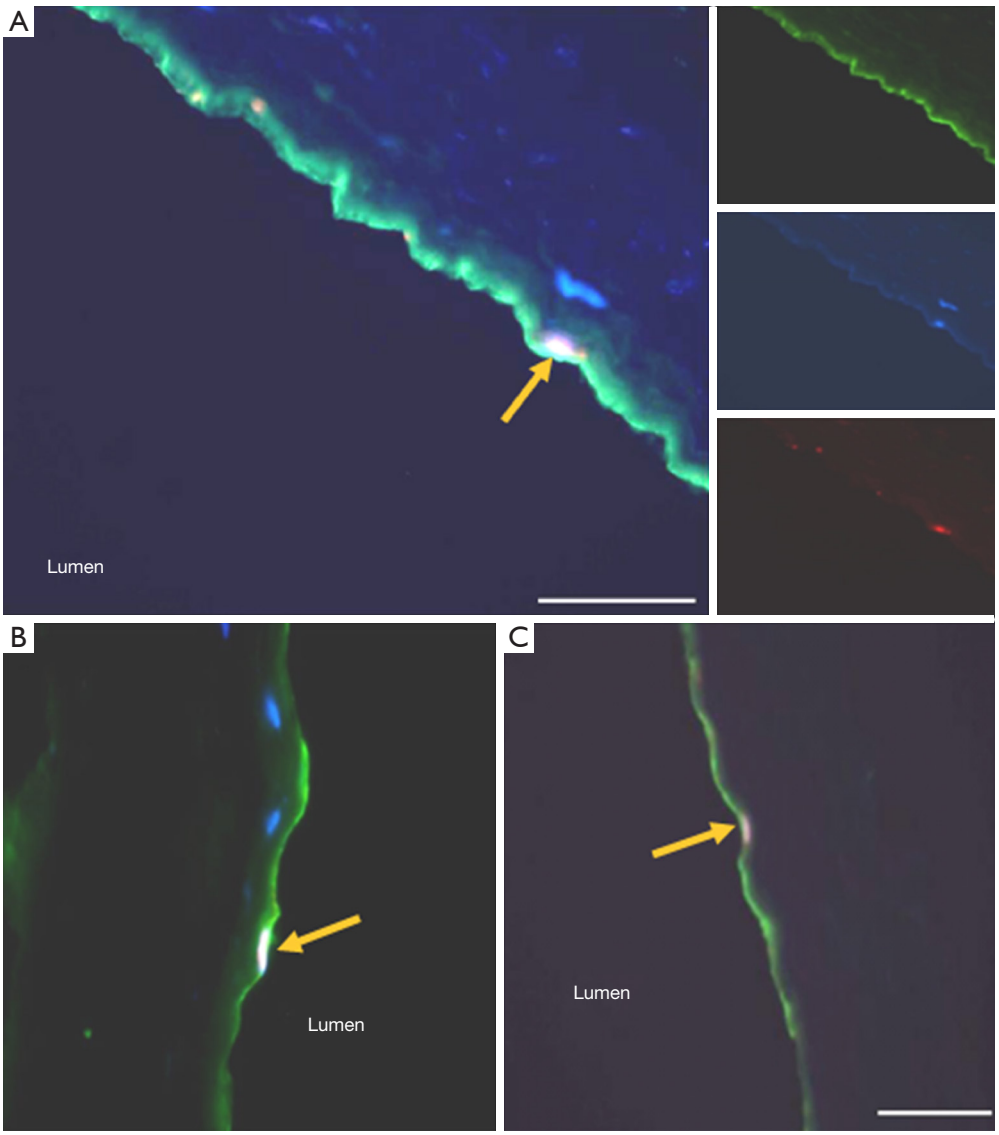

Figure 3 CMSC29 cell crossing the endothelial cell barrier in an *ex vivo* placenta vessel perfusion model. (A) Composite image of a labelled CMSC29 cell (orange) crossing the endothelial barrier, the cell appears purple due to counterstaining of the nuclei (DAPI-blue) (arrow). On the right Individual image of anti-vWF FITC staining of the endothelium (top), DAPI staining of the nuclei (middle) and CMTMR-labelled CMSC29 cell (bottom); (B,C) more composite images of CMSC29 cell (arrows) crossing the endothelial barrier, 400×. Scale bar =100 µm.

sections (n=20, 5 µm sections/vessel) revealed labelled CMSC29 cells crossed the endothelium barrier (*Figure 3*). Preliminary results from one of the vessels (n=20, 5 µm sections) showed an average of 1.05 cells/5 µm section had crossed the endothelial barrier (i.e., 210 CMSC29 cells/1 cm of vessel, constituting around 0.2% of perfused cells). Fluorescent cells were also seen protruding between the endothelial cells (*Figure 4A,B*). Other fluorescent cells were seen aggregated or attached to the luminal side of the endothelial lining (*Figure 4C,D*). These fluorescent cells are believed to be adhered to the endothelium, since these cells were not removed from the luminal wall despite extensive washing of the vessel prior to sectioning. A negative control was included, which consisted of non-labelled CMSC29 cells perfused into a placental vessel and then incubated. After washing to remove unattached cells, sections were prepared, stained with DAPI and examined for any fluorescence. In all 20 sections examined, no red-orange

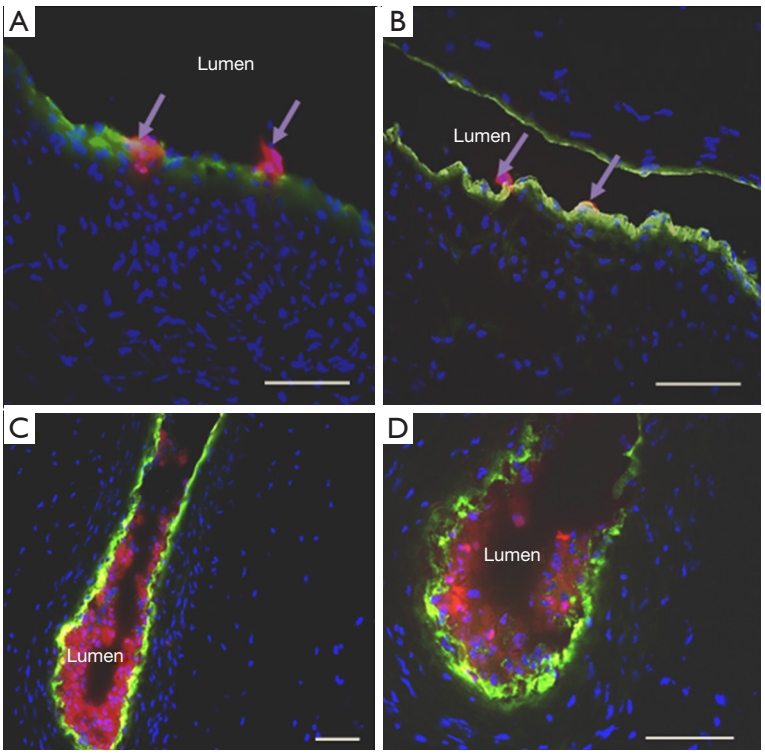

Figure 4 CMSC29 cell adherence and transmigration in *ex vivo* perfused placental vessels. Two images (A,B) each showing labelled CMSC29 cells (arrows). The cell on the right in (A,B) appears to be adhering to the endothelium. In (A,B), the cell on the left appears to be penetrating through the endothelial layer. (C,D) Labelled CMSC29 cells aggregating on the perfused vessel endothelium. (A,B,D) 400×, (C) 200× mag. Scale bar =100 µm.

fluorescence was detected (data not shown).

Perfusion of a mixture of labelled DMSC23 and CMSC29 cells into vessels

Labelled CMSC29 (CMTMR-orange) cells and labelled DMSC23 (CMFDA-green) cells were mixed in equal numbers and then perfused into placental vessels. This was done to determine whether diverse placenta-derived MSCs would behave differently in the same perfused vessel. For example, one MSC type may cross the endothelial barrier more efficiently in the same microenvironment within the perfusion vessel. Qualitative assessment of immunofluorescence in sections from two repeats revealed that both labelled CMSC29 and DMSC23 cells crossed the endothelial barrier (*Figure 5*) but there was no qualitative difference in the number of MSCs of each type that transmigrated across the endothelial barrier.

Discussion

During the development and optimization of this *ex vivo* perfusion system, transformed MSC cell lines were used to remove the confounding variable of patient-to-patient variation in primary MSC preparations. Nevertheless, other variables were observed between experiments; which included the type of the vessel perfused i.e. vein or artery, and the incubation time of cells inside the perfused vessel, which ranged between 3 hours up to 4 days. The alteration in the incubation time in different repeats was to assess the effect of different incubation periods on the efficiency of cell migration. Regardless of the type of vessel and incubation time, in eight out of twelve completed perfusions, there was evidence of successful cell migration through the perfused vessel endothelium. With regard to the 4 vessels where no fluorescent cells were observed, it could be that the 20 µm × 5 µm sections obtained from these vessels were not

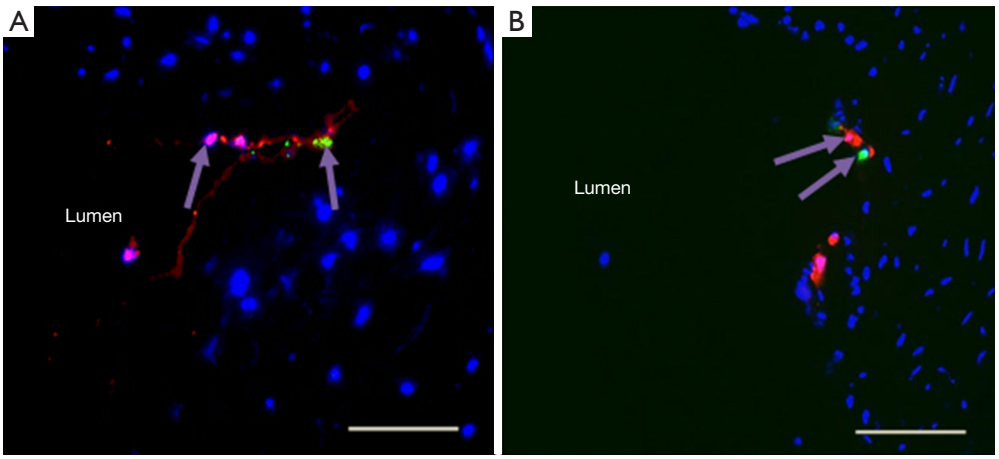

Figure 5 Mixtures of fluorescently-labelled CMSC29 and DMSC23 cells migrating in an *ex vivo* placenta vessel perfusion model. Labelled CMSC29 (orange) cell and labelled DMSC23 cells were mixed in equal numbers and then perfused into a placental vessel. Nuclei were counterstained (DAPI-blue). Cells were observed crossing the vessel endothelial barrier (A) endothelium is red due to anti-vWF Cy5-red staining. (B) No endothelial staining was performed on this section to make orange labelled CMSC29 easier to visualize, 400×. Scale bar =100 μm.

sufficient to detect any of the fluorescent cells since they represent only 1% per cm of perfused vessel area.

The mechanisms involved in this trans-endothelial movement are not yet fully understood. MSCs are assumed to utilize the same key mechanism employed by leukocyte recruitment to inflamed or injured areas (24). Indeed, in some of the examined sections, perfused cells were observed adhering to the luminal side of the endothelium, or protruding in between endothelial cells, which resembled the behaviour of leukocytes in blood vessels (*Figure 4*). Another interesting finding was that MSCs that had completely crossed the endothelial barrier, were always found to be in close proximity to the endothelial cells and were not found anywhere in the stroma or within the smooth muscle layer (*Figure 3*). The close proximity of migrated cells to the endothelium coincides with the reported MSC niche in the placenta. Both CMSCs in chorionic villous tissues, and DMSCs in the decidua, reside in a vascular niche (25,26). In this perfusion system, migrating CMSC29 and DMSC23 cells crossed the endothelial barrier within as little as 3 hours and remained in a location consistent with the MSC niche adjacent to endothelial cells even after 4 days of incubation. This contrasts with the results seen with other cells, for example, when labelled maternal lymphocytes were perfused into the maternal-side of the placenta, the lymphocytes crossed the endothelial barrier, migrated through the stroma and infiltrated the fetal villous structures (15). A possible explanation for the migration pattern of cells in this perfusion system is that there was no damage to the perfused tissue, and thus, the MSCs did not receive the appropriate damage signal to promote their movement into the tissue stroma, and instead remained in their niche. Since MSCs are reported to have a similar migration pattern to leukocytes, we expect that induction of damage to the endothelial layer will increase MSC migration in this perfusion model. Endothelial cell damage triggers the release of proinflammatory cytokines, which in turn elevate the expression of molecules involved in the transendothelial movement across the targeted endothelial site (27-29). These molecules include selectins and integrins, which are key players in the leukocyte adhesion cascade, and these factors are also involved in MSC transendothelial movement (19,30). The endothelial layer could be activated in this model using several different methods. For example, lipopolysaccharide (LPS) is a commonly used agent to activate endothelial cells to mimic the inflamed microenvironment (31). The perfused vessels can be perfused with LPS prior to cell introduction to determine whether cells preferentially migrate through the activated endothelium compared with the intact endothelium. Another approach is to perfuse the MSCs into vessels under hypoxic conditions to mimic the tissue microenvironment found in various pathologies.

Finally, both CMSC29 and DMSC23 labelled cells were perfused to determine whether diverse placenta-derived MSCs would behave differently in the same perfused vessel. Both cell types were detected crossing the endothelial cell barrier with no qualitative difference. Other combinations of MSC types (e.g., placental MSC versus adipose tissue MSCs or bone marrow MSCs), or dissimilar stem cells such as embryonic stem cells or induced pluripotent stem cells, could be investigated in the future.

In conclusion, the data from this study indicate that the human placental *ex vivo* vessel perfusion method could be used to assess human MSC migration into human blood vessels. Moreover, the method can be used to assess the migration of MSCs in an environment that could mimic different pathological conditions, or it could be used to examine and compare the migration of more than one MSC sub-type in the same microenvironment within the blood vessel. Further studies are needed to determine why MSCs that migrate across the vessel wall remain in close proximity to the endothelium, within the vascular niche of MSCs, and do not migrate into the surrounding tissue.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the Human Research Ethics Committee of the Royal Women's Hospital, Parkville, Australia (No. 05/07). All placentae were obtained following informed, written patient consent at the RWH, Parkville, Australia.

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