Reproductive Biology 25 (2025) 100994

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.journals.elsevier.com/reproductive-biology

Original article

Trophoblast-derived factors drive human mesenchymal stem cell differentiation along an endothelial lineage: A model of early placental vasculogenesis

Claire V. Harper [a], Leah Eccles [a], James Henstock [b], Jayne C. Charnock [a,*]

[a] Department of Biology, Edge Hill University, L39 4QP, UK
[b] Faculty of Health & Life Sciences, Northumbria University, Newcastle-upon-Tyne NE1 8SU, UK

ARTICLE INFO

Keywords:
Endothelial differentiation
Vasculogenesis
Stem cells
Trophoblast
Growth factors

ABSTRACT

Mechanisms controlling the process and patterning of blood vessel development in the placenta remain largely unknown. The close physical proximity of early blood vessels observed in the placenta and the cytotrophoblast, as well as the reported production of vasculogenic growth factors by the latter, suggests that signalling between these two niches may be important. Here, we have developed an *in vitro* model to address the hypothesis that the cytotrophoblast, by the secretion of soluble factors, drives differentiation of resident sub-trophoblastic mesenchymal stem cells (MSCs) along a vascular lineage, thereby establishing feto-placental circulation. BM-MSCs (a readily available model for placental stem cells) were treated with conditioned medium containing the secretome from human BeWo trophoblast cells, or endothelial growth medium (EGM2) supplemented with exogenous growth factors (VEGF, IGF1 and EGF) for 10–12 days. Trophoblast-conditioned media, found to contain detectable concentrations of cytokines including VEGF, uPAR, TIMP-1, TIMP-2, IL6 and placental growth factor, induced the expression of the endothelial genes *CD31*, *von Willibrand factor* (*vWF*), *FLT-1*, *VEGFR2* and *VE-Cadherin*. Upregulation of vWF protein was also detected following growth in trophoblast-conditioned media, using immunocytochemistry. Wound healing (migration assay) and Matrigel-tube formation assays confirmed that the BM-MSCs cultured in trophoblast-conditioned media exhibited functional measures of endothelial cells in addition to expressing relevant markers. Identification of key trophoblast-secreted factors and their promotion of endothelial differentiation in BM-MSCs helps advance our theories regarding the close relationship of the mesenchymal stem cell-cytotrophoblast niche in coordinating the complex angiogenic events that occur in the placenta. The *in vitro* model presented here provides an accessible and reproducible tool for further investigations into placental development.

1. Introduction

Establishment of placental blood vessels early in gestation is essential for a successful pregnancy since it allows the fetus access to maternal oxygen and nutrients. Dysregulation in the development of placental vasculature can cause severe complications for the fetus. Imperfect vascularisation leads to a reduction in the level of oxygen and nutrients available, factors essential for a healthy baby to develop [1]. Failure of vascular processes to occur as normal can lead to several different placental disorders including fetal growth restriction (FGR) [2,3]. FGR occurs in around 3–7 % of pregnancies [4] and is generally described as a failure of a fetus to attain its genetic growth potential, with severe consequences for the infant including increased risk of perinatal morbidity, mortality and sudden infant death as well as impacting future disease risk [5,6].

Rather than expansion of blood vessels from the fetus into the placenta, it is believed that fetal blood vessels in the placenta originate via the process of vasculogenesis around the periphery of the villous stroma [7]. The earliest vascular structures can be observed about 21 days after conception [8]. At this early stage the placental villous trees are in their secondary phase and are made up of three layers; the central core of mesoderm surrounded by two trophoblast cell layers: the cytotrophoblast cell layer followed by the syncytiotrophoblast cell layer [9]. Various origins for the intricate array of cellular differentiation events

* Corresponding author.
E-mail address: charnocj@edgehill.ac.uk (J.C. Charnock).

https://doi.org/10.1016/j.repbio.2025.100994
Received 8 October 2024; Received in revised form 19 December 2024; Accepted 4 January 2025
Available online 16 January 2025

which lead to placental vascular development within the villous core have been proposed [10], including, though not limited to, the formation of primitive blood vessels from mesenchymal stem cells underlying the cytotrophoblast [11]. From 32 days post conception vasculogenesis continues while angiogenesis, both branching and non-branching allows for the expansion of the new vascular network [12].

These vasculogenic and angiogenic processes are complex and rely on the coordination of many different vasculogenic, angiogenic and antiangiogenic factors and cytokines. It is thought that trophoblast cells are key producers of these factors, regulating vessel formation via autocrine and paracrine systems involving receptors on angiogenic precursor cells [13]. Several teams have reported an increase in the endothelial phenotype of MSCs and endothelial precursor cells in response to treatment with one or more of these isolated factors [14–16]. In addition to growth factors, the trophoblast basement membrane is also thought to play a role in early vessel formation after [13] showed that haemangiogenic cells accumulate close to the basement membrane and formed contact by cytoplasmic processes. Immunohistochemistry and electron micrograph investigations of 1st trimester placental villus have previously illustrated the close spatial proximity of placental endothelial and cytotrophoblast cells, in which endothelial cells have been observed to be in direct contact with the trophoblast basement membrane where potential exposure to vasculogenic factors would be maximised [17–19].

An expanding body of *in vitro* evidence also points towards the trophoblast playing a significant role in the development of the placental vasculature. Troja and colleagues [20] used human placental microvascular endothelial cells (HPMVECs) harvested from human placentas and the choriocarcinoma BeWo cell line (as a model for human trophoblast cells) in co-culture to determine that factors produced by trophoblasts transcriptionally enhance the angiogenic gene expression of HPMVECs. Additionally, Ma and colleagues [21] determined that primary trophoblast conditioned medium enhanced migration and tube formation by human umbilical vein endothelial cells (HUVECs) and contains various pro-angiogenic factors.

It is currently unknown how the placenta initiates vasculogenesis. Furthermore, it is unclear how these primitive sub-trophoblastic vascular niches become incorporated into the expanding vessel network by angiogenesis. Owing to the close spatial proximity between the cytotrophoblast cell layer and very early endothelial cells plus other primitive vascular structures, and the production of vasculogenic factors by the cytotrophoblast itself, as well as the role of cellular basement membranes (such as that which underlies the cytotrophoblast layer) in stem cell differentiation [22–25], one hypothesis is that the cytotrophoblast cell layer drives the differentiation of resident subtrophoblastic MSCs along a vascular lineage, thereby establishing fetoplacental circulation. Confirmation here of vasculogenic factor production by trophoblast and the resultant differentiative effects on the phenotype and functionality of BM-MSCs along an endothelial lineage both phenotypically and functionally, provides addition evidence for this theory. These cells possess comparable antigen expression, multilineage differentiation potential, morphology and self-renewal properties to placental-derived MSCs [26–28]. The objective of the present study was to assess the vasculogenic potential of mesenchymal stem cells following exposure to trophoblast-derived factors.

2. Materials and methods

2.1. Materials

DMEM, DMEM/F-12 and fetal bovine serum were obtained from ThermoFisher Scientific, UK. VEGF, IGF and EGF were from Peprotech, UK. All other reagents were from Sigma Aldrich, UK. BeWo choriocarcinoma cells were gifted from University of Manchester. Bone-marrow derived mesenchymal stem cells (BM-MSCs) and Endothelial Growth Media-2 were from Lonza, UK.

2.2. Cell culture

BM-MSCs were cultured in low glucose DMEM supplemented with 10 % (v/v) fetal bovine serum, 2 % antibiotic-antimycotic, 1 % nonessential amino acids, 1 % L-glutamine and ascorbic acid-2-phosphate. BeWo cells were cultured in DMEM/F-12 media supplemented with 10 % (v/v) fetal bovine serum and 1 % L-glutamine. Both the cell lines were cultured in T75 flasks at 37C in a humidified atmosphere containing 5 % CO_2 to proliferate. BM-MSCs between passage 1 and 5 were used in this study.

2.3. Collection and analysis of trophoblast-conditioned media (TCM)

BeWo cells (1×10^6) were seeded into a T75 flask with 15 mL media and cultured for 4 days at 37C, 5 % CO_2 and 6 % O_2. Media was removed, centrifuged to remove cell debris, and the supernatant was stored in 15 mL aliquots at −80°C. 60 % concentrated trophoblast-conditioned media (cTCM) was generated (Concentrator Plus, Eppendorf) for 1.5 h at 45 °C (V-aqueous mode). The presence of angiogenic factors and cytokines in the TCM and cTCM was measured using a human angiogenesis antibody array kit (Abcam) and compared to control media (50:50 DMEM:DMEM/F-12 containing 10 % serum). The protocol was carried out according to the manufacturer's instructions. Briefly, membranes were washed with 2 mL blocking buffer for 30 minutes at room temperature followed by 1 mL of TCM, cTCM or control media overnight at 4°C. Membranes were washed as instructed followed by 1 mL of Biotinylated Antibody Cocktail for 2 h. Wash steps were repeated then 2 mL HRP-Conjugated Streptavidin was then added to each membrane for 2 h before repeating the wash steps. Chemiluminescent signal was detected with detection buffers using a Bio-Rad ChemiDoc Touch imaging system. Images were processed using ImageJ and quantitative measurements of integrated optical density were obtained following manufacturer's instructions.

2.4. Endothelial differentiation

BM-MSCs (1×10^5) were plated into T25 flasks. After 24 h, media was replaced with fresh control media (as described above), 50:50 TCM: control media, or EGM-2 supplemented with 50 ng/mL VEGF, 5 ng/mL EGF, 20 ng/mL IGF (EGM2-GF) for 10–12 days. Media was replaced every 2–3 days and cells were passaged during the experiment.

2.5. Real-time PCR

Cells were lysed and total RNA was extracted using RNeasy Plus mini kit (Qiagen), according to manufacturer's instructions. RNA was reverse-transcribed and amplified using the High-Capacity RNA to cDNA synthesis kit (Applied Biosystems). For RT-qPCR, cDNA was diluted with RNase-free water, and amplified using PowerUp SYBR green MasterMix (Applied Biosystems). Relative quantification of gene expression was conducted using B-actin expression level as an endogenous control. Primer sequences were, *CD31* F: agtcattcttgccccatgga, R: ggccgcaatgatcaagagag; *vWF* F: ctttgtggccaggatcgatg, R: ttaaagttgccacacagccc; *FLT-1* F: cccagtttctgccattccag, R: cgcgattttcctttccagct; *VEGFR2* F: ctcacagtcctagagcgtgt, R: agacttcgatgctttcccca (taken from [29]) and *VE-Cadherin* F: caactttaccctcacggataatcac, R: acttggcatcccattgtctgag; *B-actin* F: catccgcaaagacctgtacg, R: tctccttctgcatcctgtcg.

2.6. Immunocytochemistry

Cells were grown as described above in control media, 50 % TCM or EGM2-GF. After the final passage, cells (4×10^4) were seeded onto coverslips for the remainder of the experiment. Cells were fixed using 4 % paraformaldehyde, permeabilized in 0.1 % Triton X-100 and then blocked in 1 % BSA/PBS. The cells were then incubated with Alexa488-congugated vWF antibody (1:100, Abcam ab195028). Nuclear DNA was

labelled with DAPI (Vectashield) and coverslips were imaged at 20x magnification on a Leica DMI8 inverted microscope. A negative staining control, without directly conjugated antibody, was used to set the level of background cellular autofluorescence. Mean fluorescence intensity from each individual cell was quantified using ImageJ.

2.7. Wound healing migration assay

Silicone inserts (Sigma Aldrich) were added to a 6-well plate and marks made on the bottom of the plate at the top and bottom of the wound. 70 µL media containing 2.5×10^3 BM-MSCs was then added to each well of the inserts before incubating at 37°C, 5 % CO2 for 24 hours. Inserts were removed and cells washed with PBS before adding 50 % TCM or control media to the wells. The plate was then incubated at 37°C, 5 % CO2. After 24 hours a Leica DMi8 microscope was used to take three images at x5 magnification along the wound in each well. Change in percentage cover was analysed using ImageJ software.

2.8. Tube forming assay

Wells of a 24-well plate were coated with Matrigel™ for 30 minutes following manufacturers' instructions. BM-MSCs were seeded at a density of 5×10^4 cells per well grown in 50 % TCM or control media to the wells. After 24 hours, four representative images of each well were taken at 5x magnification on a Leica DMi8 microscope. Images of tube forming assays were assessed using the Angiogenesis Analyzer software for Image J [30].

2.9. Data analysis

Data were collated and analysed using Microsoft Excel and GraphPad Prism. Each experiment was performed at least in triplicate. Statistical tests between different treatments were performed by the non-parametric Kruskal–Wallis test with post-hoc Dunn's multiple comparisons test, where significance was determined to be $p < 0.05$. Comparison of two samples was performed using a Mann-Whitney test. ImageJ was used to determine cell size by measurement of individual cell area and to quantify mean fluorescence intensity.

3. Results

3.1. Trophoblast cells secrete angiogenic factors

A human angiogenesis antibody array was used for detection of human angiogenic proteins in TCM compared to control (serum-containing) medium. Of the 43 proteins on the array, 6 proteins showed > 2-fold increase in TCM compared to control medium levels (Fig. 1A). These BeWo-secreted proteins were the growth factors vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), the inflammatory cytokine Interleukin 6 (IL-6), tissue inhibitor of metalloproteinase proteins 1 and 2 (TIMP-1/2) and urokinase receptor (uPAR). Concentrating the TCM (by 50 % evaporation; cTCM) failed to enrich the proteins detected on the array (Fig. 1A).

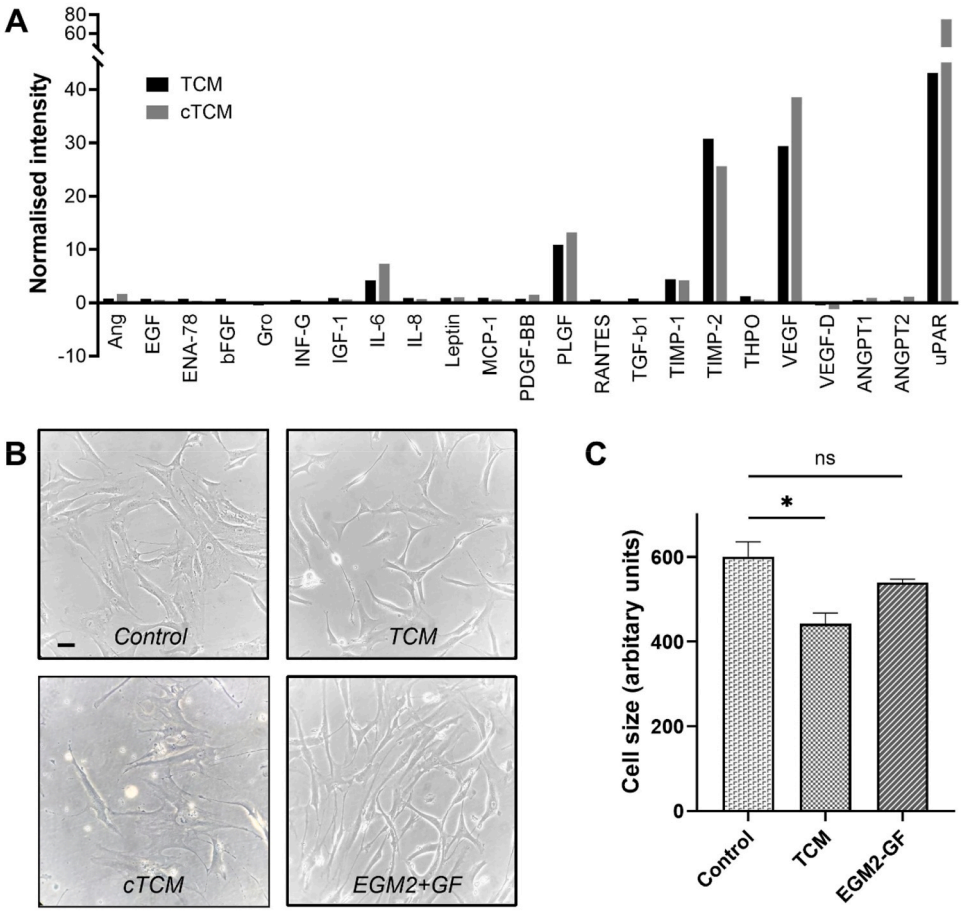

Fig. 1. Angiogenic factors secreted by BeWo trophoblast cells. (A) TCM and cTCM were analysed using an angiogenesis array normalised to control standards. (B) Images of BM-MSCs treated with control media, 50 % TCM, 50 % cTCM or EGM-2-GF. Bar represents 50 µm. (C) Cell size following the treatments in (B). At least 150 cells per treatment. * represents $p < 0.05$, ANOVA with post-hoc Friedman test.

3.2. Morphological changes in BM-MSCs

BM-MSCs were propagated for 10–12 days in control media, 50 % TCM, 50 % cTCM or EGM2-GF media. BM-MSCs maintained in 50 % TCM showed a smaller 'cobblestone' appearance compared to the classic fibroblast-like (flattened and elongated) morphology evident of BM-MSCs maintained in control media (Fig. 1B,C) and also showed a reduction in cell size (Fig. 1C; >150 cells per treatment). BM-MSCs grown in EGM2-GF media maintained a similar appearance to those cells remaining in control media (Fig. 1B,C). Growth was inhibited in cells treated with 50 % cTCM and therefore this treatment was excluded from further study.

3.3. Trophoblast-conditioned medium induces expression of markers of endothelial differentiation

Expression of the endothelial cell markers *CD31*, *vWF*, *FLT-1*, *VEGFR2* and *VE-Cadherin* and the stem cell/endothelial cell marker *CD105* was assessed by RT-qPCR in BM-MSCs maintained for 10–12 days in 50 % TCM, EGM2-GF or media control (Fig. 2). 50 % TCM stimulated a significant increase in the expression of *CD31* ($p = 0.004$; Fig. 2A), *vWF* ($p = 0.03$; Fig. 2B), *VE-Cadherin* ($p = 0.002$; Fig. 2D) and *VEGFR2* ($p = 0.045$; Fig. 2E), when compared to media alone. Although TCM induced an increase in FLT-1 expression across the experiments, this was not significant ($p = 0.41$; Fig. 2C). Variability between endothelial marker expression was detected with EGM2-GF treatment, with a significant increase in *CD31* ($p = 0.004$; Fig. 2A), *FLT-1* ($p = 0.008$; Fig. 2C) and *VEGFR2* expression ($p = 0.004$; Fig. 2E) but no significant change was measured in *vWF* ($p = 0.7$; Fig. 2B) or *VE-Cadherin* expression ($p = 0.56$; Fig. 2D). Expression of CD105 did not significantly change following 50 % TCM or EGM2-GF treatments ($p = 0.1$ and 0.07 respectively; Fig. 2F).

To further analyse the extent of trophoblast cell-mediated endothelial differentiation of BM-MSCs, the expression of vWF protein was quantified using immunocytochemistry (Fig. 3). A significant increase in expression intensity of vWF was detected in BM-MSCs treated with 50 % TCM ($p < 0.001$) and EGM-2 +GF ($p < 0.001$; $n = 3$, >150 cells per treatment) compared to control medium (Fig. 3B).

3.4. Trophoblast-conditioned media enhances functional aspects of endothelial function in BM-MSCs including wound healing and endothelial tube formation

The effect of 50 % TCM on cell migration potential was assessed using a wound closure assay. Following 48 h treatment, wound closure significantly increased compared to cells maintained in control media (15.6 ± 1.1 % closure in control media compared to 38.3 ± 7.8 % closure in 50 % TCM; $p = 0.016$; $n = 5$) (Fig. 4A,B). To further characterise endothelial potential, tube-formation was measured in BM-MSCs treated with 50 % TCM for 24 hours (Fig. 4C). A significant increase in the number of tubes ($p = 0.032$), number of junctions ($p = 0.036$) and number of nodes ($p = 0.0301$) was measured, with tube length remaining unchanged ($p = 0.486$; $n = 5$ experiments; Fig. 4D).

4. Discussion

The present study confirms the production of vasculogenic factors by the choriocarcinogenic BeWo cell line as well as the capacity of such trophoblast-derived vasculogenic factors to drive differentiation of BM-MSCs along an endothelial lineage, with respect to RNA expression, protein production and functional capabilities of the cells. These findings are supportive of the hypothesis that cytotrophoblasts drive differentiation of multipotent cells within the stroma along an endothelial lineage to establish the placental vascular network.

Verification of vasculogenic factor production by BeWo cells within this study was important to determine the origins of potential differentiation effects. Previous studies have identified a number of secreted factors, including VEGF and PlGF by trophoblast in culture (both primary and immortalised cell lines) [9,20,21,31–39]. Limitations exist in the use of BeWo choriocarcinoma cells as a model for cytotrophoblasts. Whilst they meet the four criteria that are defining of trophoblasts, are a useful tool in the investigation of placental function *in vitro* ([40]) and have been previously shown to promote angiogenesis through the secretion of angiogenic growth factors ([39,41–43]), genetic differences compared to normal trophoblast may limit the impact of findings using these cells [44]. Additional work will further focus on comparisons between this cell line and other in vitro options such as trophoblast stem cells and primary villous cytotrophoblast and regards to soluble factor secretion.

VEGF, found here at ~30-fold increased levels in conditioned media,

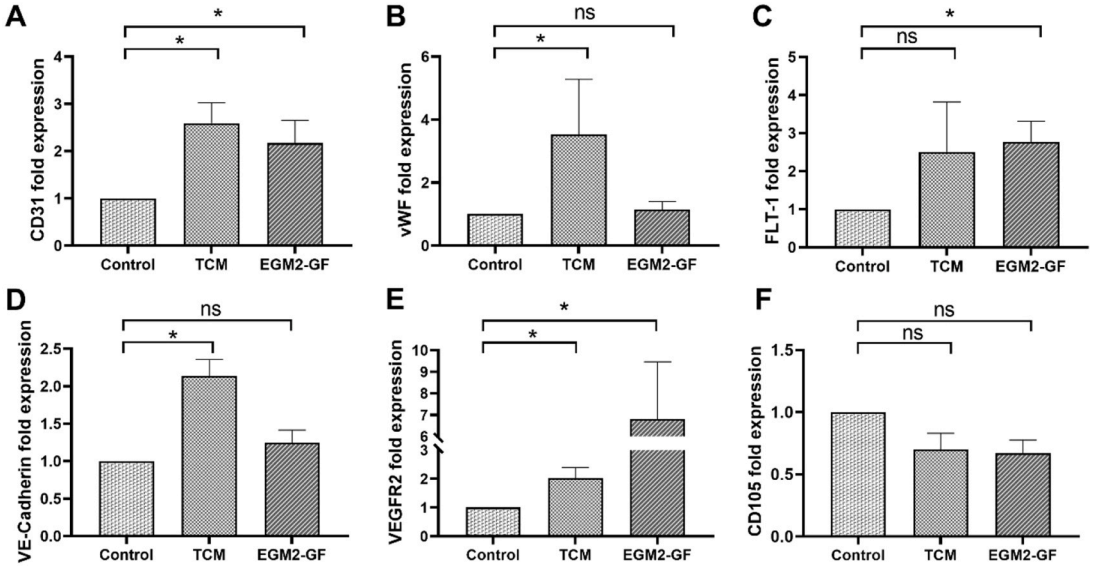

Fig. 2. Gene expression of the endothelial markers (A) *CD31*, (B) *vWF*, (C) *FLT-1*, (D) *VE-Cadherin*, (E) *VEGFR2* and (F) *CD105* in BM-MSCs maintained for 10–12 days in control media, 50 % TCM or EGM-2-GF. Graphs show mean + /-SEM for 8 experiments. ns = not significant. * represents $p < 0.05$, Mann-Whitney test.

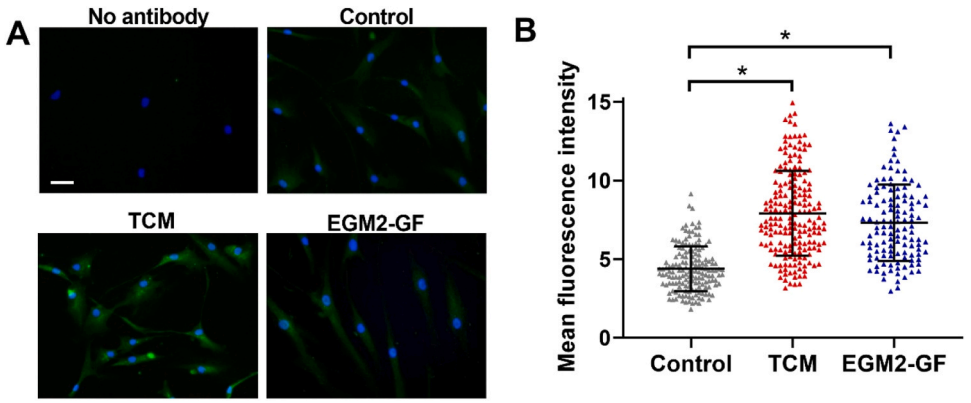

Fig. 3. Expression of von Willibrand Factor (vWF) protein in BM-MSCs following 12 days treatment with control media, 50 % TCM or EGM-2-GF (A). No primary antibody control included for comparison. Bar represents 50 μm. (B) Scatter plot showing quantification of mean fluorescence intensity per cell in the 3 treatments described in (A). Bars show mean + /-SD for each condition. * represents $p < 0.05$, paired *t*-test (n = 3 experiments, >150 cells per treatment).

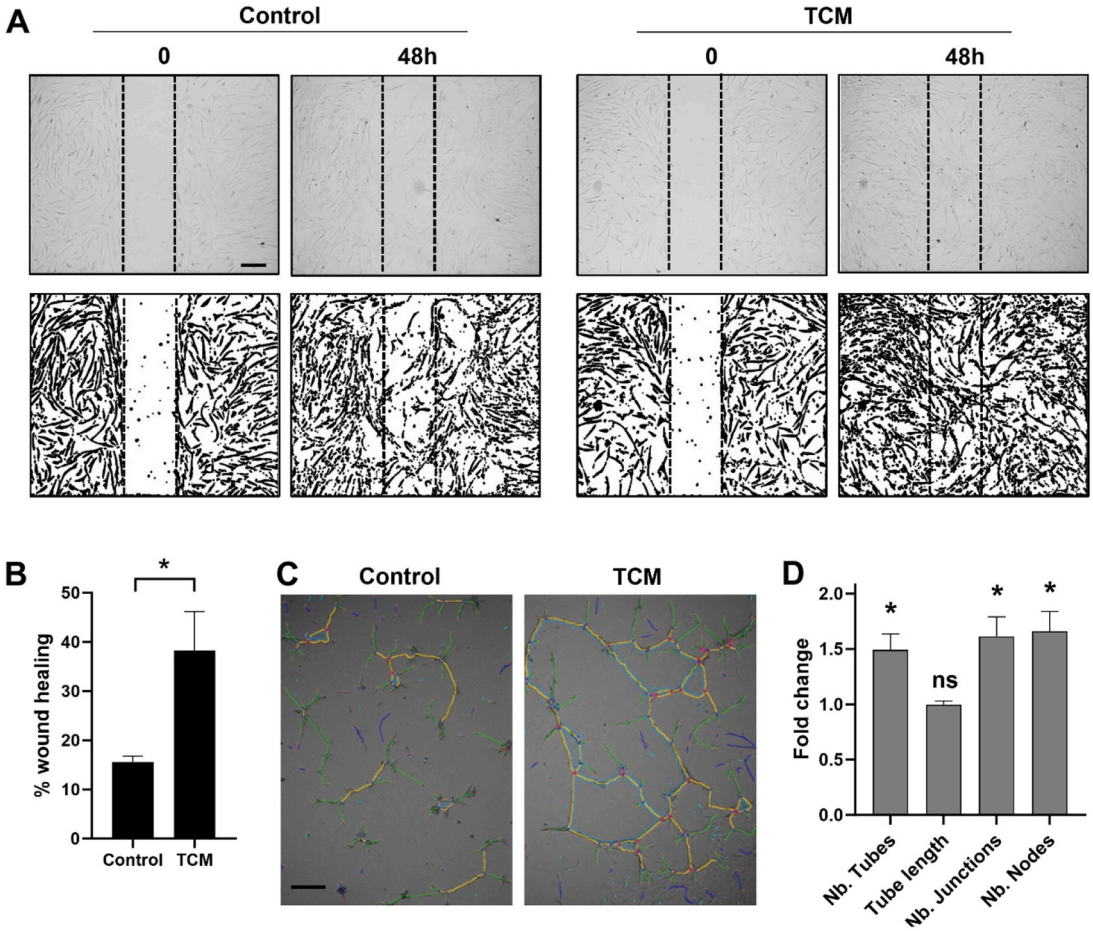

Fig. 4. The effect of trophoblast-secreted media on cell migration and angiogenic tube formation. (A) Representative images of BM-MSCs grown in control media or 50 % TCM for 48 h in a wound healing assay. Quantification of % wound closure after 48 h treatment (n = 5; p = 0.016). (C) Representative images of tube forming assay showing cells grown on Matrigel™ in control media or 50 % TCM for 24 h. Colour key as follows: Orange = master segments, green = branches, dark blue = isolated elements, light blue = mesh, blue circle = junction, red around blue circle = master junction. (D) Angiogenic potential of 50 % TCM treatment on BM-MSCs for 24 h. Fold change in number of tubes, tube length, number of junctions and number of nodes compared to BM-MSCs growth in control media (n = 4; * represents $p < 0.05$, ns=not significant). Scale bar = 250μm. Bars represent mean ± SEM.

is a known potent mitogen, morphogen and chemo-attractant for endothelial cells and is well established as an important factor for their survival and proliferation [36,45]. It is known to trigger the initial stages of vascular tube formation, haemangiogenic precursor differentiation, and fusion of early endothelial cell cords. PlGF has a significant effect on angiogenesis both directly via binding with VEGFR2 to induce vessel growth and maturation and indirectly by the recruitment of progenitor cells to growing sprouts [46,47]. In addition, levels of uPAR, IL-6, TIMP-1 and 2 were also increased in the present study, all of which have previously been identified as contributing to placental vascular

development, through promotion of matrix remodelling, endothelial cell migration and angiogenic regulation, respectively [48–53]. The correct balance of growth factors and cytokines working in unison is essential to a healthy pregnancy, and together these findings highlight the importance of the trophoblast in producing the variety of factors needed to drive the vascular development of the placenta. The trophoblast is a highly polarised cell layer which *in vivo* influences the spatial distribution and concentration of secreted factors. Whilst the release of soluble factors from the apical surface is important in adaptation of the maternal vasculature [8], the very close spatial proximity between the cytotrophoblast basal membrane and primitive placental vascular niche could maximise exposure of the former to trophoblast-derived vasculogenic factors, as well as suggesting some role for the basement membrane itself in MSC-endothelial differentiation [17–19]. In this model, total secretions are collected from BeWo monoculture, and future work will explore how the positioning of MSCs in relation to the trophoblast basement membrane could potentially modulate exposure to the factors, thereby affecting MSC behaviour and function.

Characterisation of 50 % TCM-treated BM-MSCs in this study confirmed increased expression of *CD31*, *vWF*, *VE-cadherin*, *FLT-1* and *VEGFR2*. Previous studies have similarly undertaken analysis of endothelial-lineage antigens and increased production of vWF has been commonly chosen for the basal characterisation of endothelial-like cells [27,54,55]. Increased *CD31*, *FLT-1* and *VEGFR2* expression following endothelial differentiation of other MSCs has also been described [56]. Raised placental mesenchymal stem cell expression of *VE-cadherin* and *vWF* has also been reported [54]. The work presented here builds upon the work of Troja *et al.* [20] which utilised Human Placental Microvascular Endothelial Cells (HPMVECs) harvested from human placentas and BeWo cells in co-culture to show that factors produced by trophoblasts transcriptionally promote the angiogenic gene expression of HPMVECs. Studies of morphological changes to placental stem cells have been varied in their findings however Miao et al. [57] found, as in this study, that differentiated cells exhibited a transition from a spindle-shaped, fibroblastoid morphology, to a more cobble-stoned endothelial-like one.

In addition to changes in morphology and antigen expression, 50 % TCM was found to enhance functional measures of endothelial phenotype- namely migratory and tube formation capacity. BM-MSCs treated with 50 % TCM in wound healing assays had significantly increased motility, covering a greater percentage of the wound at 48 h than BM-MSCs in control media,. This increase in motility supports the hypothesis that factors produced by trophoblast cells play a role in angiogenesis via an increase in migration- an essential process for vascular development in the placenta and other organs [58]. These results are in agreement with recent work by Ma and colleagues [21] who reported that BeWo-conditioned media increases migration in HUVECs, and Liu and colleagues [56] who found that BM-MSCs from rats that were previously treated with growth factors in EGM media showed increased migration in wound healing assays compared to those treated with control media. Additionally, Portalska *et al.* [59] reported increased BM-MSC migration following treatment with EGM-2). Of particular interest is the work of Latifi-Pupovci and colleagues [46] who showed that individual factors including IL-6, identified within TCM in the present study, increase MSC migration in wound healing assays.

Tube forming assays in this study showed a consistent pattern of increased number of tubes, nodes and junctions formed by BM-MSCs treated with 50 % TCM when compared to control media. MSC response to the matrix culture substrate involves various steps comparable to those observed during *in vivo* vessel formation, including cell adhesion, migration, alignment, protease secretion and tubule formation [60]. These stages rapidly lead to the establishment of tubular networks that resemble those of vascular beds formed *in vivo*. Again, these results are also in agreement with Ma et al. [21] who reported that conditioned media from BeWo cells increased the tube forming ability of HUVECs plated on Matrigel. Differentiated MSCs from other sources have been shown to form such capillary structures, and cultivation in the presence of VEGF appeared to enhance this process [55,61]. Indeed the tube-forming assay substrate Matrigel™ may itself promote differentiation as a proxy for the trophoblast basement membrane since key constituents such as collagen IV and laminin are shared and known to contribute to endothelial differentiation [62–64]. The average tube length remained relatively consistent across the experiments, the presence of 50 % TCM inducing no significant difference. This may potentially be due to tube forming assays being models of vasculogenesis or early angiogenesis, whereas tube elongation is part of non-branching angiogenesis and is not prioritised in early vascularisation of the placenta [11]. The variability in the number of tubes formed across each assay points to the fact that BM-MSCs may be a (strongly) heterogeneous population and predetermined as to their differentiation/angiogenic potential. This was also seen by Canosa et al. [65] who found that in culturing MSCs with HUVECs a subpopulation of about 10 % were induced to form tubes on Matrigel. It has been suggested that MSCs have a propensity to differentiate along the line of the tissue type from which they were procured [66]. This difference in predisposition may not only be between different types of MSCs but between individuals.

In this study, trophoblast-derived vasculogenic factors were capable of driving BM-MSC differentiation towards an endothelial phenotype, including altered expression of *CD31*, *vWF*, *VE-cadherin*, *FLT-1* and *VEGFR2*. These secreted factors were also observed to enhance BM-MSC tube-formation as well as migration, important events in the process of establishing a mature vascular network within the villus. These findings are strongly suggestive of the role of the cytotrophoblast in establishing the placental circulation and compliments observations of the close spatial relationship between early vascular lineages and cytotrophoblasts described previously within first trimester tissue [17,18]. Placenta-derived MSCs are morphologically indistinguishable from bone marrow-derived MSCs and share common surface antigens, adhesion molecules and multipotent differentiation capacity [57,67–69]. Whilst the findings presented here are encouraging regarding trophoblast-directed differentiation towards an endothelial lineage by utilising bone marrow-derived MSCs, and this work provides a relevant and accessible model for the study of placental developmental processes in vitro, the progression of the model to incorporate primary chorionic MSCs is logical. Doing so would augment the clinical significance of the research by also facilitating investigation of the impact of pregnancy complications such as fetal growth restriction and preclampsia on the differentiation potential of MSCs from these pathologies. Similarly, an evaluation of trophoblast-derived vasculogenic factors from such placental disorders would be extremely insightful. Such further investigations are required, alongside additional mechanisms for the induction of endothelial differentiation including the application of shear stress or flow within the culture system, approaches which have proved successful in MSC-endothelial differentiation in a previous study of placental MSCs [70]. Improved understanding of the mechanisms involved in placental vascular development will help identify therapeutic targets for diseases of pregnancy, such as FGR, in which vascular development is compromised.

Funding

This study was funded by the Edge Hill University Research Investment Fund to JCC (No. 1CHARN18) and a Society for Endocrinology equipment grant to CVH.

CRediT authorship contribution statement

C.V. Harper: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, reviewing and editing, Visualization, Supervision. **L. Eccles:** Investigation, Formal analysis, Writing – original draft. **J. Henstock:** Resources, Writing – reviewing and editing. **J.C. Charnock:** Conceptualization, Methodology, Validation, Writing –

original draft, reviewing and editing, Supervision, Funding acquisition.

Declaration of Competing Interest

None.

Acknowledgements

The authors thank Professors John Aplin and Melissa Westwood (University of Manchester) for their guidance in formulating the hypothesis and providing the inspiration for this project, and Dr Carolyn Jones (University of Manchester) for her support and discussion of her previous observations made using transmission electron microscopy. We also thank Dr Karen Forbes (University of Leeds) and Dr David Turner (University of Liverpool) for their valuable discussions regarding the experiments.

References

[1] Kingdom J, et al. Development of the placental villous tree and its consequences for fetal growth. Eur J Obstet Gynecol Reprod Biol 2000;92(1):35–43.
[2] Brouillet S, et al. Revisiting the role of hCG: new regulation of the angiogenic factor EG-VEGF and its receptors. Cell Mol Life Sci 2012;69(9):1537–50.
[3] Ahmed A, Perkins J. Angiogenesis and intrauterine growth restriction. Best Pract Res Clin Obstet Gynaecol 2000;14(6):981–98.
[4] Romo A, Carceller R, Tobajas J. Intrauterine growth retardation (IUGR): epidemiology and etiology. Pedia Endocrinol Rev 2009;6(3):332–6.
[5] Bernstein IM, et al. Morbidity and mortality among very-low-birth-weight neonates with intrauterine growth restriction. Am J Obstet Gynecol 2000;182(1):198–206.
[6] Barker D, et al. Fetal and placental size and risk of hypertension in adult life. Br Med J 1990;301(6746):259–62.
[7] Charnock-Jones DS, Burton GJ. Placental vascular morphogenesis. Best Pract Res Clin Obstet Gynaecol 2000;14(6):953–68.
[8] Gude NM, et al. Growth and function of the normal human placenta. Thromb Res 2004;114(5-6):397–407.
[9] Demir R, Seval Y, Huppertz B. Vasculogenesis and angiogenesis in the early human placenta. Acta Histochem 2007;109(4):257–65.
[10] Boss AL, Chamley LW, James JL. Placental formation in early pregnancy: how is the centre of the placenta made? Hum Reprod Update 2018;24(6):750–60.
[11] Charnock-Jones D, Kaufmann P, Mayhew TM. Aspects of human fetoplacental vasculogenesis and angiogenesis. I. Molecular regulation. Placenta 2004;25(2-3):103–13.
[12] Wang Y. Vascular biology of the placenta. in *Colloquium Series on integrated systems physiology: from molecule to function*. Morgan & Claypool Life Sciences; 2010.
[13] Demir R, et al. Fetal vasculogenesis and angiogenesis in human placental villi. Cells Tissues Organs 1989;136(3):190–203.
[14] Chen M-Y, et al. Endothelial differentiation of Wharton's jelly–derived mesenchymal stem cells in comparison with bone marrow–derived mesenchymal stem cells. Exp Hematol 2009;37(5):629–40.
[15] Ge Q, et al. VEGF secreted by mesenchymal stem cells mediates the differentiation of endothelial progenitor cells into endothelial cells via paracrine mechanisms. Mol Med Rep 2018;17(1):1667–75.
[16] Oswald J, et al. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 2004;22(3):377–84.
[17] Aplin JD, et al. Hemangioblastic foci in human first trimester placenta: distribution and gestational profile. Placenta 2015;36(10):1069–77.
[18] Jones CJ, Fox H. Ultrastructure of the normal human placenta. Electron Microsc Rev 1991;4(1):129–78.
[19] Jones CJP. An Ultrastructural and Ultrahistochemical Study of the Human Placenta in Normal and Abnormal Pregnancy. ProQuest Dissertations & Theses. Ann Arbor: The University of Manchester; 1976.
[20] Troja W, et al. Interaction between human placental microvascular endothelial cells and a model of human trophoblasts: effects on growth cycle and angiogenic profile. Physiol Rep 2014;2(3):e00244.
[21] Ma H, et al. Conditioned medium from primary cytotrophoblasts, primary placenta-derived mesenchymal stem cells, or sub-cultured placental tissue promoted HUVEC angiogenesis in vitro. Stem Cell Res Ther 2021;12(1):1–24.
[22] Kleinman HK, et al. Role of basement membranes in cell differentiation. Ann N Y Acad Sci 1987;513:134–45.
[23] Czyz J, Wobus A. Embryonic stem cell differentiation: the role of extracellular factors. Differentiation 2001;68(4-5):167–74.
[24] Solursh M. Extracellular matrix and cell surface as determinants of connective tissue differentiation. Am J Med Genet 1989;34(1):30–4.
[25] Dye J, et al. Distinct patterns of microvascular endothelial cell morphology are determined by extracellular matrix composition. Endothelium 2004;11(3-4):151–67.
[26] Chen CPL, Huang SH, Lee JP, Chen MY, Hu PC, Wu CS, Ko YH, Yang CC. Engraftment potential of human placental-derived mesenchymal stem cells after in utero transplantation in rats. Reprod Sci 2007;14(1 (iment). Abstract 803.
[27] Miao Z, et al. Isolation of mesenchymal stem cells from human placenta: Comparison with human bone marrow mesenchymal stem cells. Cell Biol Int 2006;30(9):681–7.
[28] Poloni A, et al. Characterization and expansion of mesenchymal progenitor cells from first-trimester chorionic villi of human placenta. Cytotherapy 2008;10(7):690–7.
[29] Inglis S, et al. Human endothelial and foetal femur-derived stem cell co-cultures modulate osteogenesis and angiogenesis. Stem Cell Res Ther 2016;7(1):1–16.
[30] Carpentier G, et al. Angiogenesis Analyzer for ImageJ—a comparative morphometric analysis of "Endothelial Tube Formation Assay" and "Fibrin Bead Assay". Sci Rep 2020;10(1):1–13.
[31] Amemiya K, et al. Involvement of epidermal growth factor (EGF)/EGF receptor autocrine and paracrine mechanism in human trophoblast cells: functional differentiation in vitro. J Endocrinol 1994;143(2):291–301.
[32] Shore VH, et al. Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. Placenta 1997;18(8):657–65.
[33] Arany E, Hill DJ. Fibroblast growth factor-2 and fibroblast growth factor receptor-1 mRNA expression and peptide localization in placentae from normal and diabetic pregnancies. Placenta 1998;19(2-3):133–42.
[34] Demir R, et al. Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: differences between placental vasculogenesis and angiogenesis. Placenta 2004;25(6):560–72.
[35] Demir R, et al. Sequential steps during vasculogenesis and angiogenesis in the very early human placenta. Placenta 2006;27(6-7):535–9.
[36] Ruhrberg C. Growing and shaping the vascular tree: multiple roles for VEGF. Bioessays 2003;25(11):1052–60.
[37] Zhang EG, et al. The regulation and localization of angiopoietin-1, -2, and their receptor Tie2 in normal and pathologic human placentae. Mol Med 2001;7(9):624–35.
[38] Arroyo J, Torry RJ, Torry DS. Deferential regulation of placenta growth factor (PlGF)-mediated signal transduction in human primary term trophoblast and endothelial cells. Placenta 2004;25(5):379–86.
[39] Taylor CM, et al. Influence of hypoxia on vascular endothelial growth factor and chorionic gonadotrophin production in the trophoblast-derived cell lines: JEG, JAr and BeWo. Placenta 1997;18(5-6):451–8.
[40] Orendi K, et al. Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. Placenta 2011;32:S49–54 (Suppl).
[41] Depoix C, Tee MK, Taylor RN. Molecular Regulation of Human Placental Growth Factor (PlGF) Gene Expression in Placental Villi and Trophoblast Cells is Mediated via the Protein Kinase A Pathway. Reprod Sci 2011;18(3):219–28.
[42] Eisele N, et al. Placental expression of the angiogenic placental growth factor is stimulated by both aldosterone and simulated starvation. Placenta 2016;40:18–24.
[43] Tanaka K, et al. Tumor necrosis factor-alpha regulates angiogenesis of BeWo cells via synergy of PlGF/VEGFR1 and VEGF-A/VEGFR2 axes. Placenta 2018;74:20–7.
[44] Turco MY, Moffett A. Development of the human placenta. Development 2019;146(22).
[45] Sherer DM, Abulafia O. Angiogenesis during implantation, and placental and early embryonic development. Placenta 2001;22(1):1–13.
[46] Latifi-Pupovci H, et al. In vitro migration and proliferation ("wound healing") potential of mesenchymal stromal cells generated from human CD271(+) bone marrow mononuclear cells. J Transl Med 2015;13:315.
[47] Zhou Y, et al. Human cytotrophoblasts promote endothelial survival and vascular remodeling through secretion of Ang2, PlGF, and VEGF-C. Dev Biol 2003;263(1):114–25.
[48] *The effect of bisphosphonates on the endothelial differentiation of mesenchymal stem cells.*
[49] Hung SC, et al. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem Cells 2007;25(9):2363–70.
[50] Yew TL, et al. Enhancement of wound healing by human multipotent stromal cell conditioned medium: the paracrine factors and p38 MAPK activation. Cell Transpl 2011;20(5):693–706.
[51] Ries C, et al. MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. Blood 2007;109(9):4055–63.
[52] Seo DW, et al. TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. Cell 2003;114(2):171–80.
[53] Lala PK, Chakraborty C. Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. Placenta 2003;24(6):575–87.
[54] Chen CP, et al. Engraftment potential of human placenta-derived mesenchymal stem cells after in utero transplantation in rats. Hum Reprod 2009;24(1):154–65.
[55] Oswald J, et al. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. Stem Cells 2004;22(3):377–84.
[56] Liu C, et al. Endothelial differentiation of bone marrow mesenchyme stem cells applicable to hypoxia and increased migration through Akt and NFkappaB signals. Stem Cell Res Ther 2017;8(1):29.
[57] Miao Z, et al. Isolation of mesenchymal stem cells from human placenta: comparison with human bone marrow mesenchymal stem cells. Cell Biol Int 2006;30(9):681–7.
[58] Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. Circ Res 2007;100(6):782–94.
[59] Janeczek Portalska K, et al. Endothelial differentiation of mesenchymal stromal cells. PLoS One 2012;7(10):e46842.

[60] Arnaoutova I, et al. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. Angiogenesis 2009;12(3):267–74.
[61] Liu JW, et al. Characterization of endothelial-like cells derived from human mesenchymal stem cells. J Thromb Haemost 2007;5(4):826–34.
[62] Amenta PS, et al. The extracellular matrix is an integrated unit: ultrastructural localization of collagen types I, III, IV, V, VI, fibronectin, and laminin in human term placenta. Coll Relat Res 1986;6(2):125–52.
[63] Chen CP, Aplin JD. Placental extracellular matrix: gene expression, deposition by placental fibroblasts and the effect of oxygen. Placenta 2003;24(4):316–25.
[64] Gerecht-Nir S, et al. Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. Lab Invest 2003;83(12):1811–20.
[65] Canosa S, et al. Angiogenic properties of endometrial mesenchymal stromal cells in endothelial co-culture: an in vitro model of endometriosis. Mol Hum Reprod 2017;23(3):187–98.
[66] Reinisch A, et al. Epigenetic and in vivo comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. Blood 2015;125(2):249–60.
[67] Fukuchi Y, et al. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. Stem Cells 2004;22(5):649–58.
[68] Barlow S, et al. Comparison of human placenta-and bone marrow–derived multipotent mesenchymal stem cells. Stem Cells Dev 2008;17(6):1095–108.
[69] Zhang X, et al. Successful immortalization of mesenchymal progenitor cells derived from human placenta and the differentiation abilities of immortalized cells. Biochem Biophys Res Commun 2006;351(4):853–9.
[70] Wu CC, et al. Synergism of biochemical and mechanical stimuli in the differentiation of human placenta-derived multipotent cells into endothelial cells. J Biomech 2008;41(4):813–21.