

# Endothelial Differentiation of Mesenchymal Stromal Cells

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## Abstract

Human mesenchymal stromal cells (hMSCs) are increasingly used in regenerative medicine for restoring worn-out or damaged tissue. Newly engineered tissues need to be properly vascularized and current candidates for *in vitro* tissue pre-vascularization are endothelial cells and endothelial progenitor cells. However, their use in therapy is hampered by their limited expansion capacity and lack of autologous sources. Our approach to engineering large grafts is to use hMSCs both as a source of cells for regeneration of targeted tissue and at the same time as the source of endothelial cells. Here we investigate how different stimuli influence endothelial differentiation of hMSCs. Although growth supplements together with shear force were not sufficient to differentiate hMSCs with respect to expression of endothelial markers such as CD31 and KDR, these conditions did prime the cells to differentiate into cells with an endothelial gene expression profile and morphology when seeded on Matrigel. In addition, we show that endothelial-like hMSCs are able to create a capillary network in 3D culture both *in vitro* and *in vivo* conditions. The expansion phase in the presence of growth supplements was crucial for the stability of the capillaries formed *in vitro*. To conclude, we established a robust protocol for endothelial differentiation of hMSCs, including an immortalized MSC line (iMSCs) which allows for reproducible *in vitro* analysis in further studies.

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## Introduction

Human mesenchymal stromal cells (hMSCs), also referred to as colony forming unit-fibroblasts (CFU-F), mesenchymal stem cells, or mesenchymal progenitor cells were first identified as a subpopulation of bone marrow cells by Friedenstein [1]. Their abundance among other bone marrow cells was estimated to be 0.01% to 0.001% [1–3]. Later research showed that MSCs can be isolated from other types of tissues as well, including adipose tissue, placenta, periosteum, trabecular bone and femur [4–7]. MSCs can be characterized based on their fibroblast-like morphology and ability to differentiate into various cell types [8]. To induce adipogenic differentiation, stimulation with insulin, dexamethasone and IBMX is typically applied [9]. MSCs cultured in dexamethasone differentiate towards osteoblasts and can participate in new bone formation after implantation in critical size bone defects [10–12]. TGF- $\beta$  stimulation, especially when combined with BMP-2 treatment, can trigger chondrogenic differentiation of MSCs [13]. While MSC differentiation towards adipo-, osteo- and chondrogenic lineages is widely investigated, some studies have also shown that MSCs can differentiate towards muscular and neural phenotype, but those are less documented and the differentiation protocols are not yet widely applied. For instance, myoblasts can be obtained from MSCs after applying basic fibroblast growth factor (bFGF) and forskolin [14–16]. On the other hand, platelet-derived growth factor (PDGF) together with

forskolin and glial growth factor (GGF-2) stimulation results in differentiation of MSCs into cells with a Schwann cell-like phenotype [17]. Continuous trials to obtain neural cells have resulted in several studies demonstrating the possibility of obtaining MSC-derived cells that can support the regeneration of nerves and participate among others in therapy of erectile dysfunction, multiple sclerosis and spinal cord injury [18,19]. MSCs are also capable of suppressing allo-responses and appear to be non-immunogenic [20]. Therefore, hMSCs are increasingly used in regenerative medicine as a source of cells for restoring worn-out or damaged tissues such as cartilage, cardiac muscle or bone. In the field of bone tissue engineering there are a total of 9 human clinical trials performed to date [21]. Other clinical trials with MSCs are performed to improve cardiac functions after myocardial infarction [22,23] and to restore liver and kidney function after failure [24,25].

The standard approach in regenerative medicine when MSCs are used is to identify the cell type necessary for the therapy and then differentiate MSCs towards this phenotype. Differentiated cells are then used in animal models and when the therapy is successful, clinical trials are performed. There is, however, a clear need for endothelial cells (EC) in this approach. ECs are needed to line artificial vessels and to restore vascularization of ischemic tissues. This is a crucial point in therapy of peripheral vascular diseases [26] which is a growing medical problem in Western

societies and manifests itself in obstruction of large arteries. This leads to retraction of small arteries and capillaries followed by acute or chronic ischemia in surrounding tissues. Only the recovery of the vascular network in such tissues can restore blood flow and prevent limb amputation [27]. Another difficulty that may be solved by providing a good source of endothelial cells is the maintenance of cell survival after graft implantation. The key parameter in this problem is the supply of nutrients and oxygen in which diffusion is a limiting factor. Metabolically active cells must be situated within 150–200  $\mu\text{m}$  distance from blood vessels in order to maintain proper functions [28,29]. To maintain cell survival in large grafts, a vascular structure needs to be introduced within the graft which can rapidly connect to the host's blood vessels upon implantation. Without that, cell death and lack of new tissue formation in the interior of the implant will occur [30]. The proof of principle for the application of ECs in the above-mentioned conditions has been achieved using endothelial progenitor cells (EPCs) [31] and endothelial cells. EPCs are immature cells capable of differentiating into mature endothelial cells *in vitro* and *in vivo* [32]. EPCs are cells with some self-renewing capacity present both in the bone marrow and in circulation [33]. Recently, the vasculogenic potential of different EPCs was investigated and compared [34,35]. EPCs adhere to gelatin and fibronectin, take up acetylated LDL, bind lectins from *Ulex Europaeus*, and express marker proteins of the EC lineage (e.g. CD31, KDR, vWF). However, they either lose their potential after prolonged expansion or their expansion capacity is not enough to provide sufficient numbers of cells for therapeutic applications [36,37]. Mature ECs isolated from umbilical vein or aorta are considered as another cell source for graft vascularization [38]. These cells can be expanded *in vitro* and perform well in creating vascular networks *in vivo* [39,40]. Autologous isolation is however only possible by sacrificing a current vessel of the patient. Since bone marrow derived MSCs were shown to differentiate into adipogenic, osteogenic and chondrogenic lineages [41] they can also be considered as a promising source for obtaining endothelial cells that are able to create vascular networks. There are several factors reported to influence endothelial differentiation and maintain endothelial potential *in vitro* while using embryonic stem cells or EPCs. Specifically, the effects of endothelial growth supplements [42–44], shear forces [45–49] and composition of extracellular matrix [44,50] are important factors in EC culture. These factors influence the efficiency of capillary-like structure formation on Matrigel, endothelial markers expression (CD31, vWF, KDR), the ability to take up acetylated LDL and their *in vivo* performance. In the last 10 years, much effort was put into establishing protocols for endothelial differentiation of mesenchymal stromal cells. In particular, cells isolated from adipose tissue were reported to respond positively for endothelial differentiation [51,52]. In the case of MSCs isolated from bone marrow (BM-MSCs) several studies were conducted with various outcomes. The work of Oswald *et al.* shows that BM-MSCs can acquire *in vitro* phenotypic and functional features of ECs [53]. Silva *et al.* demonstrated that MSCs injected into ischemic myocardium can differentiate into smooth muscle cells and endothelial cells *in vivo*, resulting in increased vascularity and improved cardiac functions [54]. Differentiation of MSCs into endothelial cells *in vitro* did not improve their performance *in vivo* [55], which could be due to a sub-optimal differentiation protocol, similar to previous attempts to improve ectopic bone formation by hMSCs. Only after precise adjustment of several parameters a protocol was obtained that resulted in an improvement *in vivo* [56]. In this manuscript we demonstrate a robust and efficient endothelial differentiation protocol for MSCs, which describes the isolation, expansion and

differentiation of BM-MSCs and their potential in tissue engineering as endothelial-like cells.

## Materials and Methods

### Isolation and Culture

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent [57]. Aspirates were resuspended using a 20G needle and plated at a density of 0.5 million mono-nucleated cells per  $\text{cm}^2$ . Cells were grown in MSC proliferation medium which contains minimal essential medium (alpha-MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (GIBCO), 10  $\mu\text{g}/\text{ml}$  streptomycin (GIBCO), 2 mM L-glutamin (GIBCO), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma-Aldrich) and 1 ng/ml bFGF (Fisher Scientific) at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. Cells were expanded up to passage 2. For further experiments hMSCs from two different donors and one immortalized clone (iMSCs, courtesy of Ola Myklebost, University of Oslo, Norway) were cultured in basic medium (alpha-MEM supplemented with 10% FBS, 100 U/ml penicillin, 10  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM L-glutamin and 0.2 mM ASAp). Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured in endothelial growth medium (EGM-2, Lonza). Mouse skeletal myoblast cells (C2C12) and mouse embryonic fibroblasts (MEF) (Cell Essentials) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% FBS, 100 U/ml penicillin and 10  $\mu\text{g}/\text{ml}$  streptomycin.

### Endothelial Induction of MSCs

hMSCs from passage 2 and iMSCs from passage 25 were used for the endothelial induction protocol. Cells were seeded at a density of 3,000 cells/ $\text{cm}^2$  on tissue culture plastic in EGM-2 and cultured for 10 days. After one day in static culture shear force was applied using an XYZ shaker (3D shaker). Cultures were rotated at a rate of 20 rpm. Cells that were cultured according to this protocol will be referred to as pre-differentiated MSCs.

Phalloidin and DAPI stainings were used for cell size and shape analysis. Pictures were taken with a BD Pathway<sup>TM</sup> Bioimager and analyzed using Attovision software. A minimal number of 300 cells per condition were analyzed.

For induction on Matrigel, wells of 6-well plates were covered with 1 ml of growth factor reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. Cells were seeded at a density of 30,000 cells/ $\text{cm}^2$  and cultured in a humid atmosphere with 5% CO<sub>2</sub> for 24 hours. The formation of capillary-like structures (CLS) was observed over time using an inverted microscope (Nikon Eclipse TE300). Pictures were taken at different time points using a Nikon DS-L2 camera.

### Wound Healing Assay

iMSCs were cultured for 10 days in EGM-2 on an XYZ shaker and were then trypsinized and seeded in 6-well plates at a density of 15,000 cells/ $\text{cm}^2$ . iMSCs expanded in basic medium were used as a negative control. After 24 h of culture, cells reached 90% confluency. The cell monolayers were gently scratched using a pipette tip across the entire diameter of the well. Cells were washed twice with PBS to remove cellular debris and then cultured in basic medium to avoid differential growth factor stimulation during the assay. The size of the wounds directly after making the scratch and following wound closure was observed over time using an inverted microscope (Nikon Eclipse TE300). Pictures were taken at each time-point using a Nikon DS-L2 camera. The wound

size was determined using TScratch software (CSElab) as percentage of the picture area that was not occupied by cells.

### TubeCount

Custom image recognition and analysis software has been implemented in C++ programming language with the aid of dlib library (<http://dlib.net/>) used for image processing and graphical user interface, Anti-Grain Geometry library (<http://www.antigrain.com/>) as vector graphics engine and FFTW library (<http://www.fftw.org/>) for calculating Fourier transforms. The purpose of the software is to quantify tube formation efficiency which is performed in two stages. The first stage is a fully automated segmentation routine which partitions the image into background region (insignificant to further analysis) and foreground region (containing cells). Segmentation is based on the assumed characteristics of cell images obtained with phase contrast microscopy (Fig. 1A) and primarily relies on the existence of well-defined cell edges and on low pixel intensity (dark shades) within the cells as compared to image background. To extract regions which fulfill these criteria the image is first enhanced using generic image quality improvement methods such as homomorphic filtering, median-based noise removal and contrast stretching (Fig. 1B). Such enhanced images are then treated with a localized intensity thresholding algorithm extracting pixels which have lower-than-average intensity with the average intensity calculated over a window of a specified size (Fig. 1C). The algorithm is repeated for multiple window sizes and the final result is an intersection of the results obtained in all iterations. Thus, the pixels which show below-average intensity regardless of averaging window size are classified as potential foreground pixels. On the other hand, the enhanced cell image is also processed using a Sobel edge detector in order to find cell boundaries (Fig. 1D). The detected edges are processed using a fill/connect algorithm which connects neighboring edges resulting in a uniform binary bitmap containing pixels corresponding to objects with distinct edges (Fig. 1E). An intersection of this binary bitmap with the one obtained with intensity thresholding is assumed to be a correct foreground mask (Fig. 1F).

The second stage consists of manual tube identification where the software user depicts beginning and ending points of individual tubes on the processed image. As result of this process a tube topology graph is formed which, in combination with the information obtained in the segmentation stage, enables to gather valuable statistics such as total and average tube length, average tube width, number of tube branching points and total tube area (Figure 1G).

### EL-MSC Characterization

**RNA isolation and quantitative PCR.** Total RNA was isolated using TRIZOL reagent according to the manufacturer's protocol. Briefly, 1 ml of Trizol reagent was added per T25 flask (cells cultured in basic medium) or per well (cells cultured on Matrigel in 6-well plates). Samples were incubated for 5 min at room temperature to allow complete dissociation. Phase separation was performed by adding chloroform, and then samples were shaken vigorously for 15 seconds and incubated for 3 min at room temperature. After that samples were centrifuged at 12,000×g for 15 min. RNA was precipitated by mixing the aqueous phase with isopropyl alcohol followed by 10 min incubation at room temperature. Samples were centrifuged again and the remaining RNA pellet was washed with 75% ethanol. The obtained samples were dissolved in water. The quantity and quality of RNA was analyzed using spectrophotometry (ND-1000 spectrophotometer).

For first strand cDNA synthesis 500 ng of RNA was used in combination with Superscript II (Invitrogen) according to the manufacturer's protocol. One ul of 3× diluted cDNA was used for further gene amplification. PCR was performed in a Light Cycler real time PCR machine (BioRad). Data was analyzed using BioRad iQ5 software. Expression of endothelial genes was calculated relative to GAPDH levels by the comparative  $\Delta$ CT method. Primers used in the study are listed in Table 1.

### Immunostaining

Cells for immunostaining were fixed with 70% ethanol and permeabilized with 0.01% Triton-X. To block non-specific background staining, cells were incubated with 5% BSA (Sigma-Aldrich) in PBS for 30 min. Cells were then incubated with mouse anti-human CD31 antibody (Dako) or with rabbit anti-human VEGFR2 (Cell Signaling) for 2 hours. Cells were washed in PBS with 1% BSA and subsequently incubated with the secondary antibody (AlexaFluor 488 conjugated goat anti mouse or AlexaFluor 594 conjugated goat anti rabbit, Invitrogen) for 1 hour. Cells were washed and counterstained with DAPI and photographed with a BD Pathway<sup>TM</sup> Bioimager.

**Acetylated low-density lipoprotein (ac-LDL) uptake assay.** iMSCs were cultured in EGM-2 on an XYZ shaker for 10 days, then transferred to Matrigel and cultured for another 24 hours. Cells were then recovered from Matrigel by 30 min incubation in a 1:1 mixture of 0.25% trypsin and dispase (BD Bioscience). Recovered cells were seeded in 96-well plates and cultured in EGM-2 for one more day. Subsequently, cells were incubated for 4 hours in EGM-2 supplemented with 10 ug/ml Dil-labeled ac-LDL (Invitrogen) at 37°C in a humid atmosphere. Finally, cells were washed with PBS 3 times.

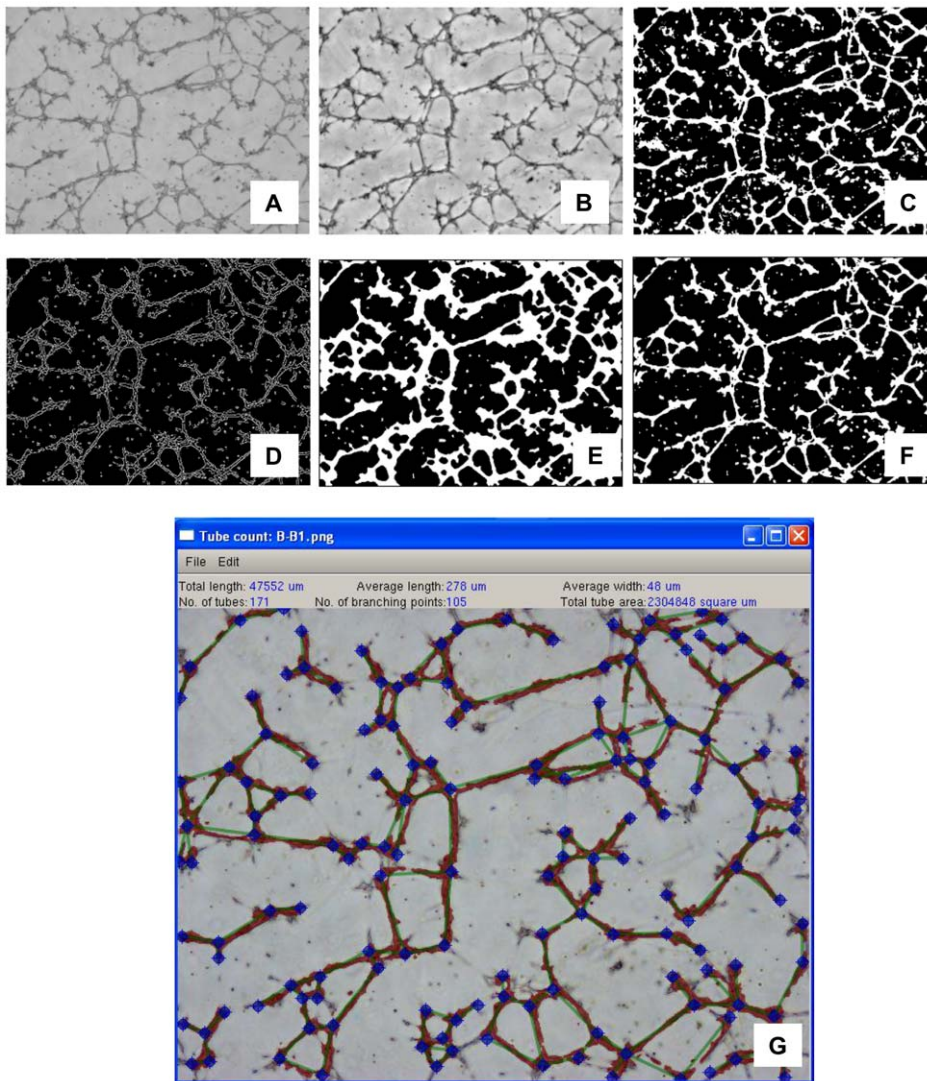
### Biodegradable Scaffold Preparation

Porous scaffolds composed of a 50/50 blend of poly(L-lactic acid) (PLLA) and polylactic-glycolic acid (PLGA) were fabricated by a salt-leaching process. PLLA (Polysciences) and PLGA (Sigma-Aldrich) were dissolved 1:1 in chloroform (Fisher Scientific) with a final concentration of 5% (wt/vol). Two ml of this solution was poured into Teflon containers (Saville) with a diameter of 50 mm and homogenized with 3.4 g of sodium chloride particles with an average grain size of 425  $\mu$ m. The solvent was evaporated overnight under nitrogen flow. To leach the salt, the obtained polymer films were immersed in distilled water for 6 hours (changed every hour). The leached films with a thickness of 500  $\mu$ m and a pore size of 200±60  $\mu$ m were lyophilized overnight and subsequently cut in circular disks with a diameter of 5 mm. Before culture the scaffolds were sterilized in 70% ethanol for 2 days, washed three times with PBS and incubated in culture medium overnight.

### PLLA/PLGL Construct Preparation and Implantation

Constructs for implantation were prepared as described before [58]. Briefly, 500,000 cells were pooled and resuspended in 20  $\mu$ l of a 1:1 mixture of EGM-2 medium without growth factors and growth factor reduced Matrigel. This suspension was applied onto the scaffold and allowed to be absorbed and solidify for 30 min at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. Culture medium (EGM-2) was then added; scaffolds were detached from the wells and cultured further on a shaker at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. Medium was changed every other day. After 10 days of culture samples were fixed in 10% formalin or implanted into mice.

Male 6-week old NMRI-nu mice (Harlan) were anesthetized with a mixture of isoflurane and oxygen after which constructs



**Figure 1. Predifferentiation of iMSCs.** Cell shape after 10 days of culture in basic medium (A) and in EGM-2 (B). Average cell area (C) was compared. Cell shape was characterized based on circularity coefficient (D). Error bars represents 95% confidence interval, \* denotes statistical significance ( $P < 0.05$ ).

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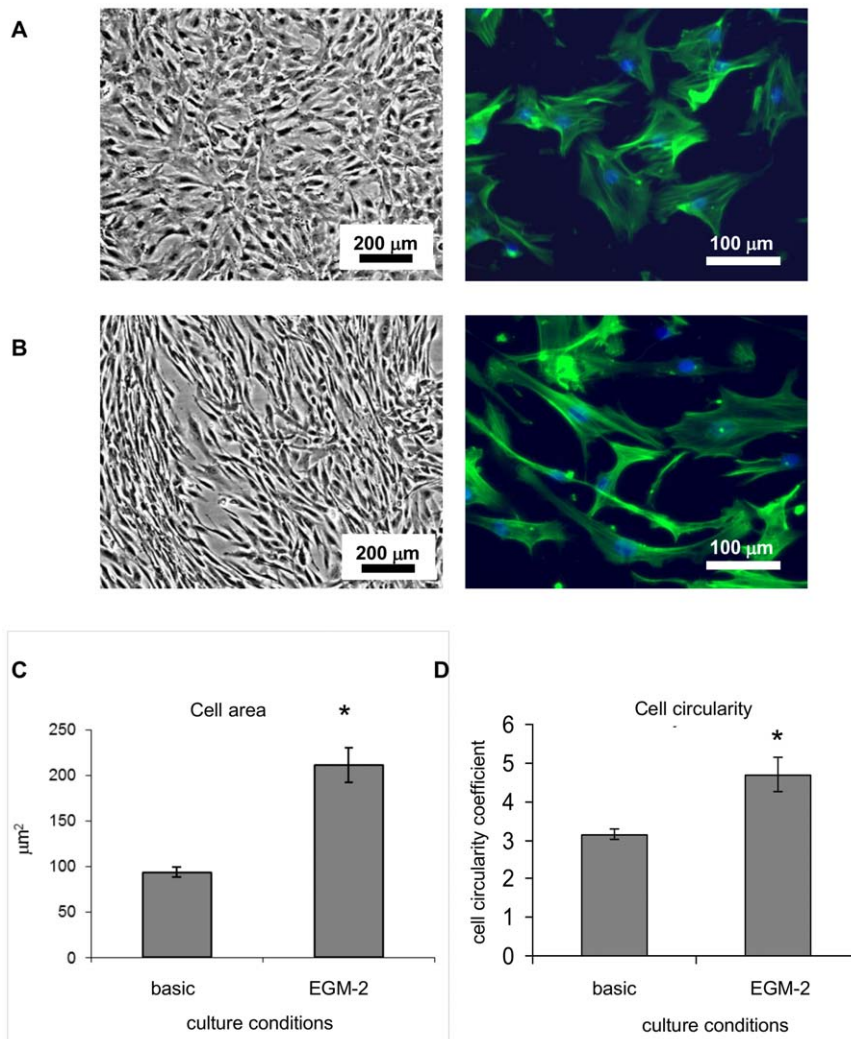
were subcutaneously implanted in four pockets. Animals were housed at the Central Laboratory Animal facility (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and use committee. Two

weeks after implantation mice were sacrificed and implants were recovered. Samples were fixed in 10% formalin, embedded in paraffin and sectioned at 5  $\mu\text{m}$  before staining.

**Table 1. Primers used for qPCR.**

CD31 (Platelet Endothelial Cell Adhesion Molecule-1)	F 5' TCTATGACCTCGCCCTCCACAAA 3' R 5' GAACGGTGTCTTCAGTTGGTATTCA 3'
KDR (VEGF receptor 2)	F 5' ACTTTGGAAGACAGAACCAAATTATCTC 3' R 5' TGGGCACCATCCACCA 3'
vWF (von Willebrand factor)	F 5' TGCTGACACCAGAAAAGTGC 3' R 5' AGTCCCAATGGACTCACAG 3'
GAPDH	F 5' CGCTCTCTGCTCCTCTGTT 3' R 5' CCATGGTGTCTGAGCGATGT 3'

doi:10.1371/journal.pone.0046842.t001



**Figure 2. Wound healing assay.** Pictures were taken directly after making the wound, 12 and 24 hours later (A). Quantification of wound recovery was performed in TScratch program and presented on the graph (B). Error bars represent standard deviation, \* denotes statistical significance ( $P < 0.05$ ).

doi:10.1371/journal.pone.0046842.g002

### Histochemical Staining and Image Analysis

Hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) staining as well as Masson's trichrome (Merck Chemicals) staining were performed according to manufacturers' protocols. Samples were photographed using a Nikon Eclipse E600 microscope. Based on Masson's trichrome stainings vessels were counted manually by 4 observers blinded to the sample composition. Three areas of each sample (10 samples per condition) were used for this quantification.

For detecting endothelium of human origin CD31 staining was performed. Antigen retrieval was achieved with IHC-Tek Epitope Retrieval Solution (IHC World). Sections were then incubated with mouse-anti-human CD31 primary antibody (Dako), which does not cross-react with mouse tissue. Following this, biotinylated horse anti-mouse secondary antibody (Antibodies-online.com) was applied. Slides were developed with Labeled Streptavidin Biotin (LSAB) with DAB Chromogen (IHC World) and weakly counterstained with Mayer's hematoxylin (Sigma-Aldrich).

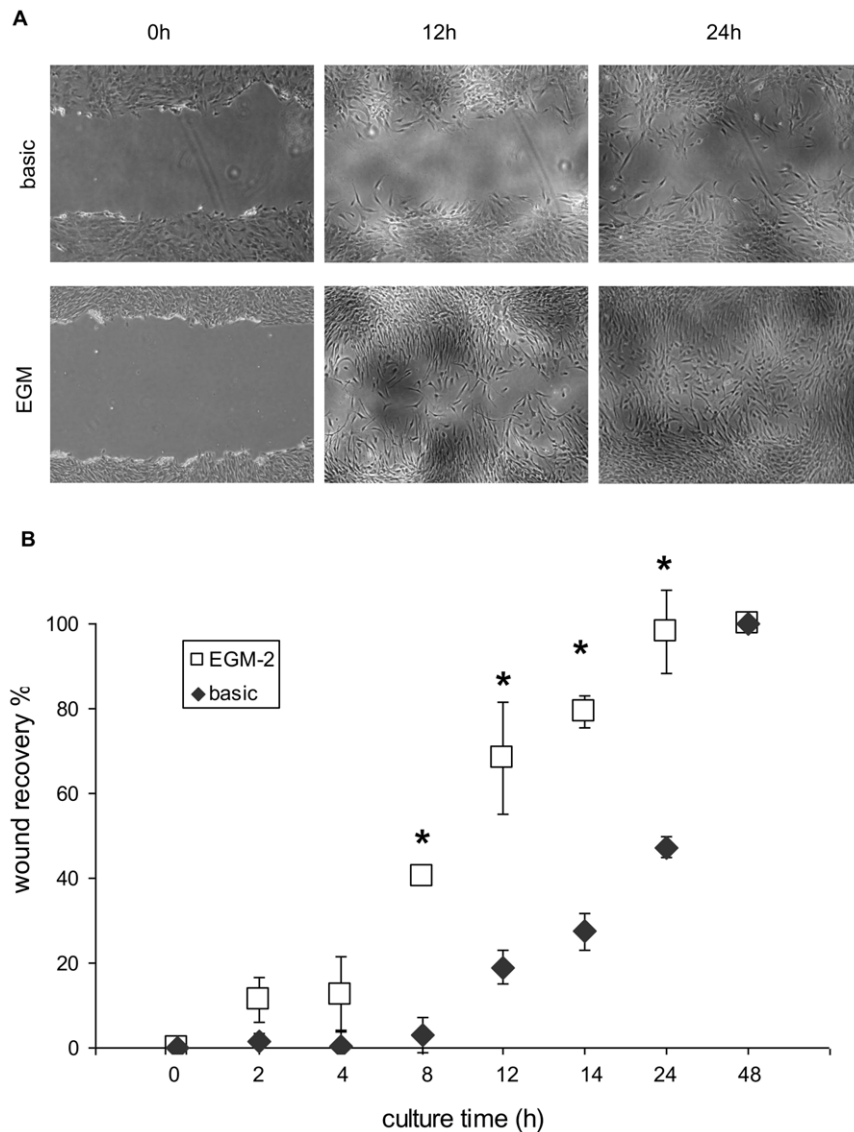
### Statistics

Each experiment was performed in triplicate. The data was analyzed using Student's t-test at  $p < 0.05$ . Data that required multiple comparison test was analyzed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ( $P < 0.05$ ). Error bars on graphs represent standard deviation or 95% confidence interval as indicated in the graph legends.

### Ethics Statement

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent. This study was carried out in strict accordance with the recommendations of Medisch Ethische ToetsingsCommissie Twente (Medical Ethical Research Committee Twente) and was approved by this Committee.

The animal study reported on in this manuscript was ethically assessed a priori by an animal ethics committee 2010-III-10-125 DEC-Utrecht. Animals were housed at the Central Laboratory Animal facility (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and use



**Figure 3. Capillary-like structures formation on Matrigel.** EL-hMSCs, EL-iMSCs and HUVECs were cultured on Matrigel for 24 h in EGM-2 medium (A). Total tube length, total tube area and number of branching points were compared (B). \* and \*\* denotes statistical significance ( $P < 0.05$ ). doi:10.1371/journal.pone.0046842.g003

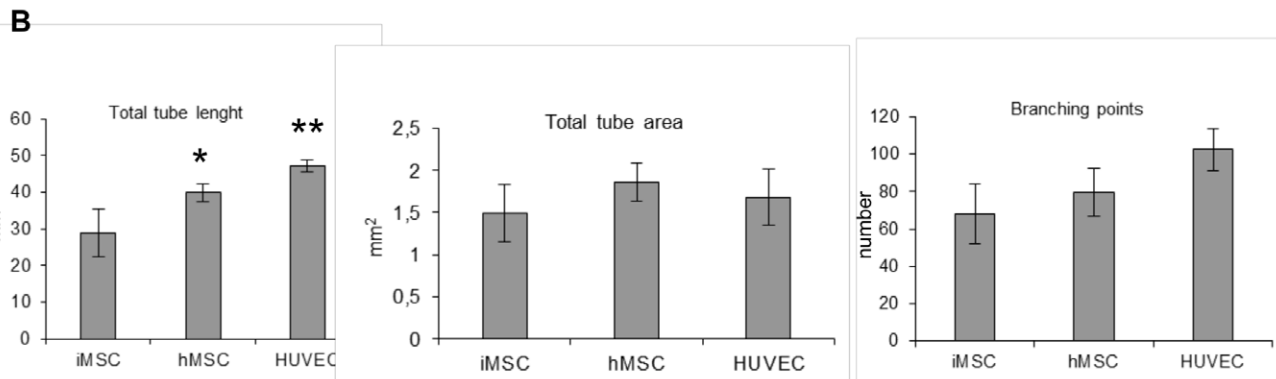
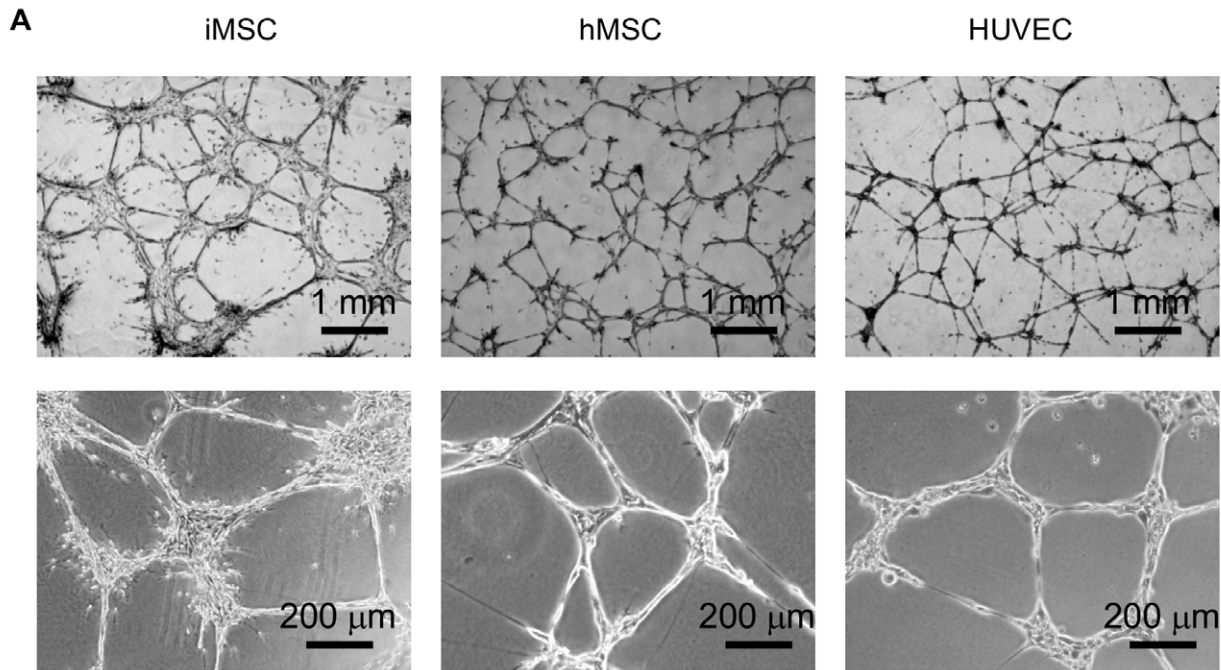
committee Dierexperimentencommissie Academisch Biomedisch Centrum (DEC-ABC). All surgery was performed under isoflurane/oxygen anesthesia, and all efforts were made to minimize suffering.

## Results

### Endothelial Differentiation Medium Effects Cell Shape

Because EGM-2 induces endothelial differentiation in human amniotic fluid-derived stem (AFS) cells, [46] we decided to use this medium to differentiate MSCs towards an endothelial-like phenotype. First, we analyzed whether MSCs cultured in EGM-2 acquired an endothelial-like phenotype. HUVEC were used as positive control for expression of endothelial markers. Cytoskeleton staining and qPCR study showed that the observed change of shape and size of pre-differentiated MSCs was not followed by expression of endothelial specific markers such as CD31, KDR or vWF (data not shown). Additionally, MSCs grown in above-mentioned conditions did not take up ac-LDL (data not shown).

We did observe a difference in cell shape between MSCs cultured in EGM-2 and basic medium. Cells cultured in EGM-2 were clearly more elongated than MSCs cultured in basic medium but exact measurements of cell shape and area were difficult due to the high cell density. To quantify this phenomenon, sub-confluent cell culture was necessary. Cells were trypsinised and seeded at lower density and further growth was allowed for 2 more days to ensure cell spreading. Morphology of naïve MSCs showed a typical fibroblast-like shape in contrast to cells grown in EGM-2 (Fig. 2A–B). The average size of MSCs cultured in basic medium was approximately  $100 \mu\text{m}^2$  and the average cell circularity was 3 (circularity of 1 represents round objects, the higher the coefficient is, the less round object it describes). The shape of pre-differentiated MSCs was significantly altered: the area increased to approximately  $200 \mu\text{m}^2$  and the circularity coefficient was close to 5, suggesting that cells grown in EGM2 were more elongated than naïve MSCs (Fig. 2C–D). The observed phenomenon was opposite to what was expected because MSCs from other sources



**Figure 4. Capillary-like structures formation on Matrigel. Time course study.** iMSCs were cultured for 10 days in basic or EGM-2 medium and then seeded on Matrigel in basic or EGM-2 medium. Dynamics of capillaries formation was observed for following 7 days. doi:10.1371/journal.pone.0046842.g004

[59] acquired a cobblestone-like morphology typical for endothelial cells.

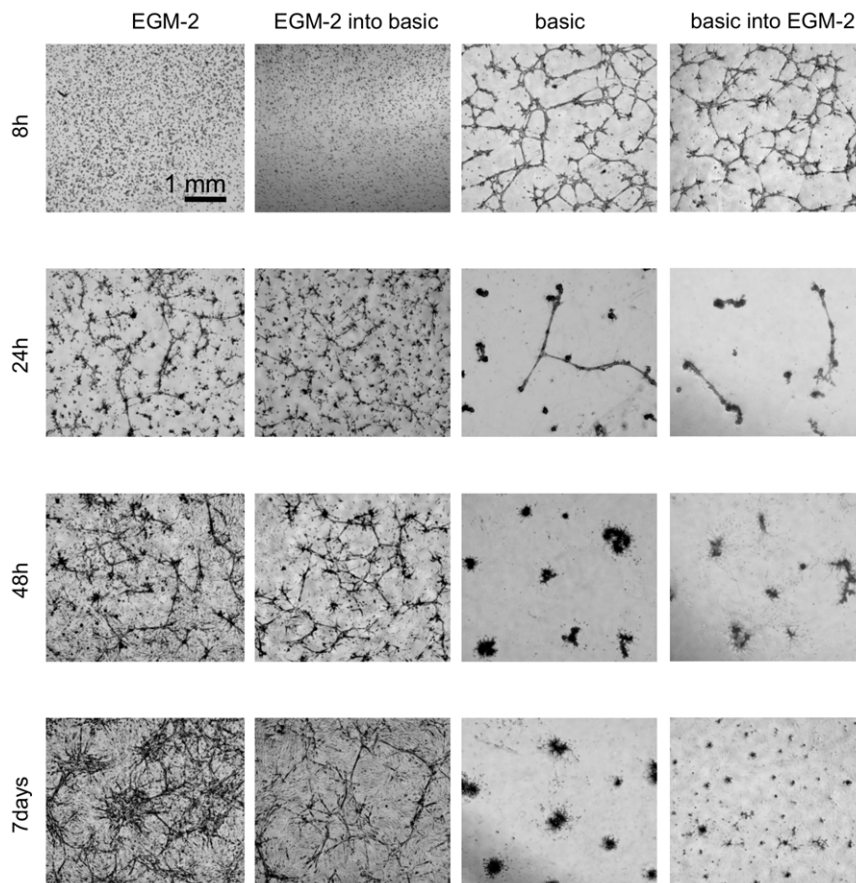
### Wound Healing Assay

The wound-healing assay is a simple method to study directional cell migration *in vitro* [60]. Migration of vascular endothelial cells plays an important role in vasculogenesis and angiogenesis [61]. We used the scratch wound healing assay of tissue-culture cell monolayers to assess the migration potential of predifferentiated MSCs. The wound recovery increased over time and the rate of this was taken as a measure of cell migration (Fig. 3A). We observed that 10 days culture in EGM-2 significantly increased the migration rate of MSCs (Fig. 3B). The size of the wound in EGM-2-cultured MSC monolayer was reduced by 40% after 8 hours and the wound closed completely after 24 hours. Naïve MSCs needed 24 hours for a 45% reduction in wound size and 48 hours to close the wound completely. This assay showed that MSCs cultured in EGM-2 migrate faster than naïve MSCs.

### Capillary-like Structures Formation

The angiogenic capability of various cell types was assessed using an *in vitro* capillary formation assay on Matrigel. Three cell types were used for this study: HUVEC which served as positive control, bone-marrow derived hMSCs and iMSCs. We decided to test whether iMSCs react in a similar way to hMSCs to serve as a cell source for future studies without problems associated with donor variation. As shown in Fig. 4A, cells from each cell type performed with similar efficiency. Measurement of total tube length (Fig. 4B) revealed significant but not critical differences between iMSCs and hMSCs and HUVECs. Total tube area was similar in all three cases and the number of branching points did not reveal significant differences between iMSCs, hMSCs and HUVECs. Comparison of all those parameters allowed us to use iMSCs as a model of MSCs for further study.

As we described above, MSCs expanded in endothelial medium undergo significant changes in shape. We hypothesized that these changes might influence the results of the Matrigel assay. In order



**Figure 5. Image segmentation steps.** Original image (A), quality enhancement (B), intensity thresholding (C), edge detection (D), edge connection/filling (E), final segmentation result (F). Print Screen of the analysis of tube topology (G). doi:10.1371/journal.pone.0046842.g005

to confirm this, we performed a Matrigel assay with naïve MSCs and MSCs expanded in EGM-2 (pre-differentiated MSCs). Both naïve MSCs as well as pre-differentiated MSCs were seeded onto Matrigel in basic medium or in EGM-2. We observed cell behavior on Matrigel in a time course study (Fig. 5) where we found that naïve MSCs seeded in basic medium start to form capillaries on Matrigel at an earlier time point than pre-differentiated MSCs but those capillaries were very unstable and disrupted after 24 hours. A similar situation was observed when naïve MSCs were seeded on Matrigel in EGM-2. In contrast, pre-differentiated MSCs started to form capillaries with a 20 hours delay compared to naïve MSCs. However, those capillaries grew thicker and created a more complex network during the next 7 days. This showed that the expansion phase in EGM-2 is crucial for the stability of capillary structures.

Quantification of the capillaries obtained in different conditions (Fig. 6) demonstrated a significant increase in the total tube length, total tube area, number of tubes and branching points when cells were expanded in EGM-2 even when the process of tube formation was performed in basic medium. This means that cells expanded in EGM-2 behave more like endothelial cells even when used in less promoting conditions (basic medium is not supplemented with growth factors as much as EGM-2). This is interesting when taking into account that endothelial cells need well supplemented medium to survive prolonged culture: HUVECs seeded in basic medium did not survive (data not shown).

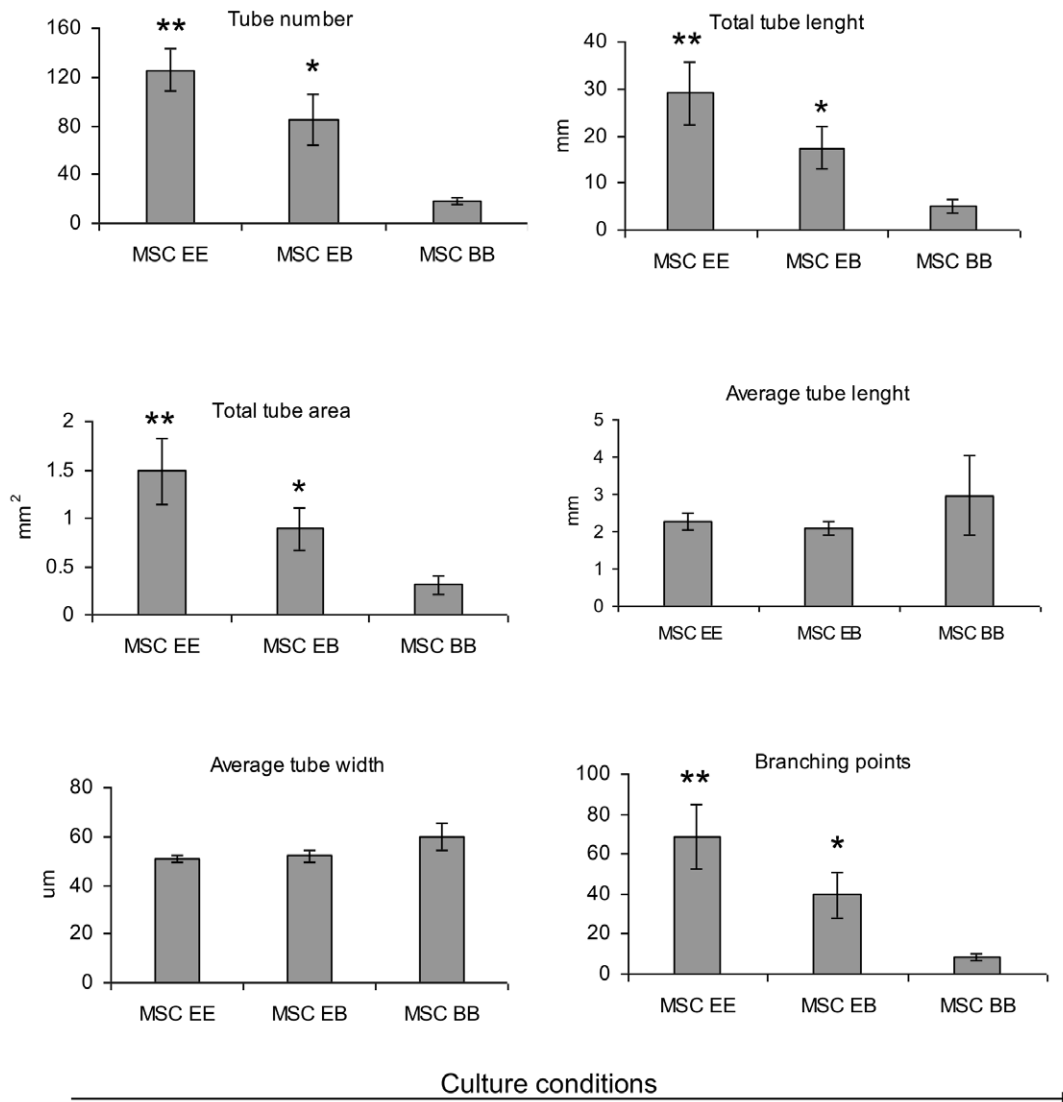
### Endothelial Induction of MSCs

Since we did not observe endothelial marker expression in MSCs when growth factors and shear stress were applied we decided to test whether introduction of extracellular matrix could trigger an endothelial phenotype in MSCs. Cells cultured for 10 days in EGM-2 on a XYZ shaker were reseeded on Matrigel for another 24 hours. This short period of Matrigel culture allows for endothelial induction without the risk of MSCs modifying the gel itself [62]. Cells obtained in this way will be called endothelial-like MSCs (EL-MSCs).

### EL-MSCs Express Endothelial Markers

To characterize the phenotype of EL-MSCs, the expression of several endothelial genes, CD31, KDR and vWF, was assessed. hMSCs from 2 donors and iMSCs were used in this study. In all three cases the expression of CD31 and KDR was significantly higher in EL-MSCs than in naïve MSCs from the same source. CD31 expression was up-regulated between 15–60 times and KDR expression between 80–1000 times (Fig. 7A). In the case of vWF the expression was 5–6 times higher in hMSCs from both donors but not in case of the immortalized clone (Fig. 7A). To confirm the qPCR data, immunostaining was performed. iMSCs expanded in EGM-2, seeded on Matrigel for 24 h and recovered with dispase/trypsin solution were stained for CD31 and KDR. Staining directly on Matrigel was not possible due to the very high background signal generated by unspecific antibody binding to Matrigel. HUVECs and naïve





**Figure 6. Quantification of capillary-like structures on Matrigel.** Quantification of tube formation by cells cultured in 3 different conditions: cells predifferentiated in EGM-2 and seeded on Matrigel in the same medium (EE), cells predifferentiated in EGM-2 and seeded on Matrigel in basic medium (EB) and naïve MSCs expanded in basic medium and seeded on Matrigel in basic medium (BB). Graphs show total tube length, average tube length and average tube width, total tube area, number of tubes and number of branching points per picture. Error bars represent standard deviation, \* and \*\* denotes statistical significance ( $P < 0.05$ ) towards all other bars. doi:10.1371/journal.pone.0046842.g006

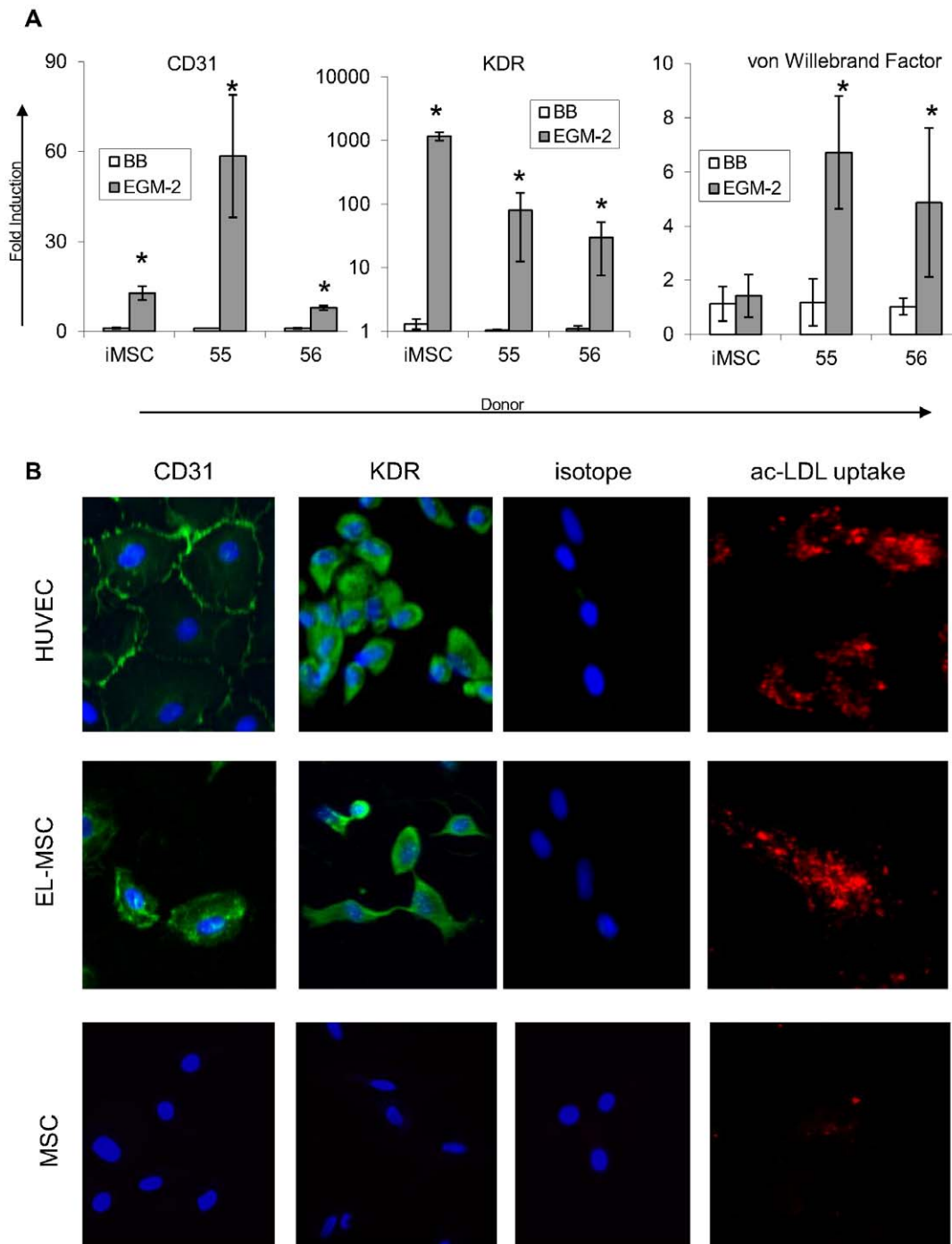
iMSCs served as positive and negative control respectively. We observed cells positive for tested markers among EL-iMSCs whereas naïve iMSCs were negative (Fig. 7B). The specificity of the antibody for both markers was confirmed by HUVECs staining – cells recovered from Matrigel maintained round shape and did not spread on tissue culture plastic, which made the observation of characteristic staining on the border of neighboring cells very difficult.

The last characteristic of endothelial cells we tested was the ability of EL-MSCs to take up ac-LDL. iMSCs that were expanded in EGM-2 for 10 days and seeded on Matrigel for 24 hours were then recovered from the gel and reseeded on tissue culture plastic. Twenty-four hours later cells were incubated with ac-LDL for 4 hours, washed and imaged. Most of the cells were lost during this process. The cells that survived returned to a fibroblast-like morphology but were still able to take up ac-LDL. This uptake was however limited in the view of the number of positive cells

when compared to HUVECs but was not observed at all in naïve MSCs (Fig. 7B).

### PLLA/PLGL Construct

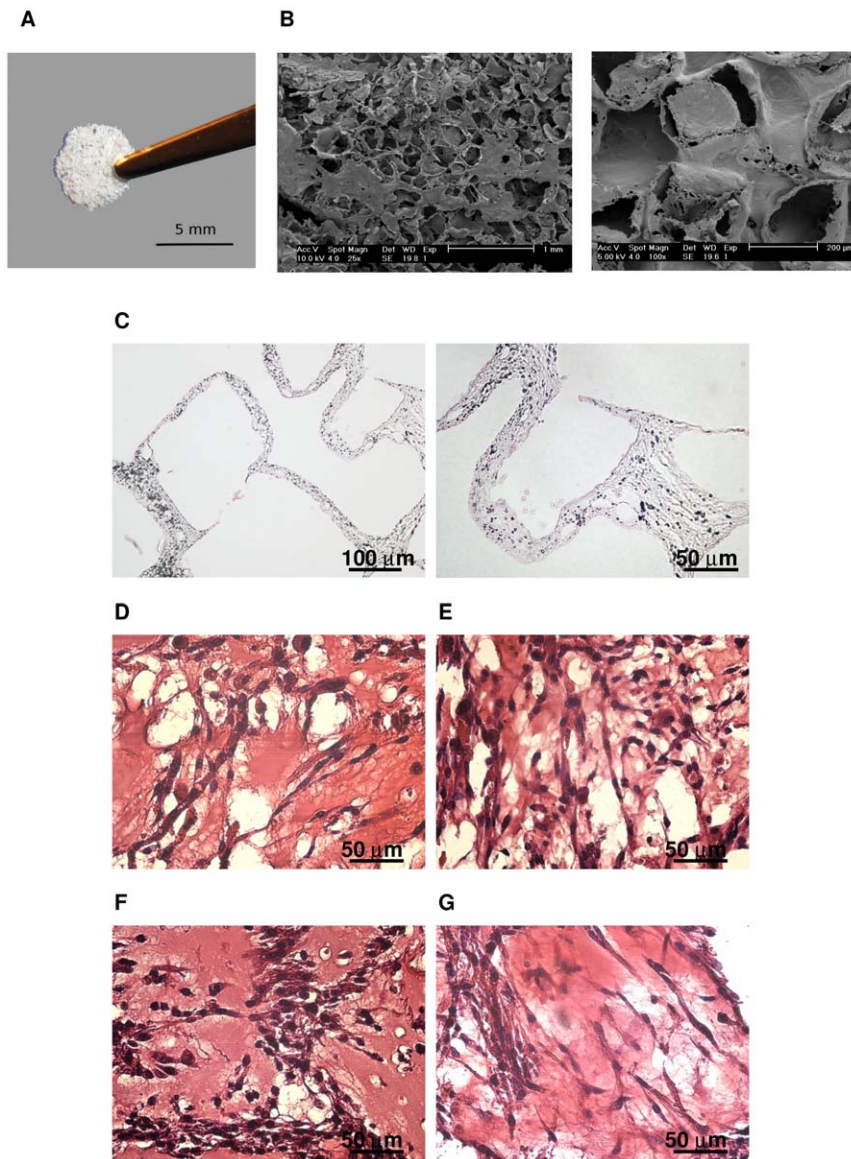
We previously reported the induction of vessel networks in engineered tissue constructs using a three-dimensional coculture system consisting of cells seeded on porous and biodegradable polymer scaffolds [58]. The scaffolds were composed of 50% PLLA and 50% PLGL, with a pore size of  $200 \pm 60 \mu\text{m}$  (Fig. 8A and 8B). We have previously vascularized muscle tissue with tissue engineered constructs combining PLLA/PLGL scaffolds, Matrigel and HUVECs [58]. To assess the angiogenic potential of EL-MSCs we have compared their ability to improve construct vascularization with that of HUVECs and naïve MSCs. C2C12 monoculture was used as negative control for angiogenic potential of constructs seeded with C2C12 with HUVECs, MSCs or EL-MSCs in 1:1 ratio.



**Figure 7. Endothelial marker expression in differentiated MSCs.** Gene expression profiles of endothelial markers in EGM-2 and Matrigel culture (EE) (A). Expression is indicated as fold induction compared to cells grown in basic medium (BB) on plastic and normalized to GAPDH RNA. hMSCs from donors 55 and 56 and iMSCs were used in this experiment. Error bars represent standard deviation, \* denotes statistical significance ( $P < 0.05$ ). Staining for endothelial markers (B).  
doi:10.1371/journal.pone.0046842.g007

We showed that scaffolds filled with Matrigel without cells and cultured for 10 days in EGM-2 kept their structure (Fig. 8C). Hematoxylin/eosin staining performed on cross sections of constructs 10 days after cell seeding indicates that cells attached to and grew on the scaffolds in all four culture systems (Fig. 8D–G), no differences in cell density were detected. Elongated cells were observed in all used schemes, indicating cell-matrix interactions.

To observe the therapeutic potential of EL-MSCs we subcutaneously implanted tissue engineered constructs in immunodeficient mice (NMRI-nu, Harlan). The constructs were permeated with host blood vessels (Fig. 9) and the number of ingrown vessels were quantified. There were no significant differences between constructs seeded with C2C12 cells alone or in coculture with HUVECs or with naïve MSCs (Fig. 10A). Only in constructs



**Figure 8. In vitro vascularization of polymeric construct combined with Matrigel and cells.** PLLA/PLGL scaffold before cell seeding (A). Scanning electron microscope (SEM) pictures of scaffold taken after gold coating (B). Eosin/hematoxylin staining of tissue sections taken from constructs after 10 days of *in vitro* culture without cells (C), with C2C12 (D), with C2C12 and MSCs (E), with C2C12 and HUVECs (F), with C2C12 and EL-MSCs (G).

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seeded with C2C12 cells in coculture with EL-MSCs the number of vessels was significantly higher compared to all other tested conditions. These *in vivo* results showed that introduction of EL-MSCs in the implant improve construct vascularization and by that can promote cell survival in large grafts.

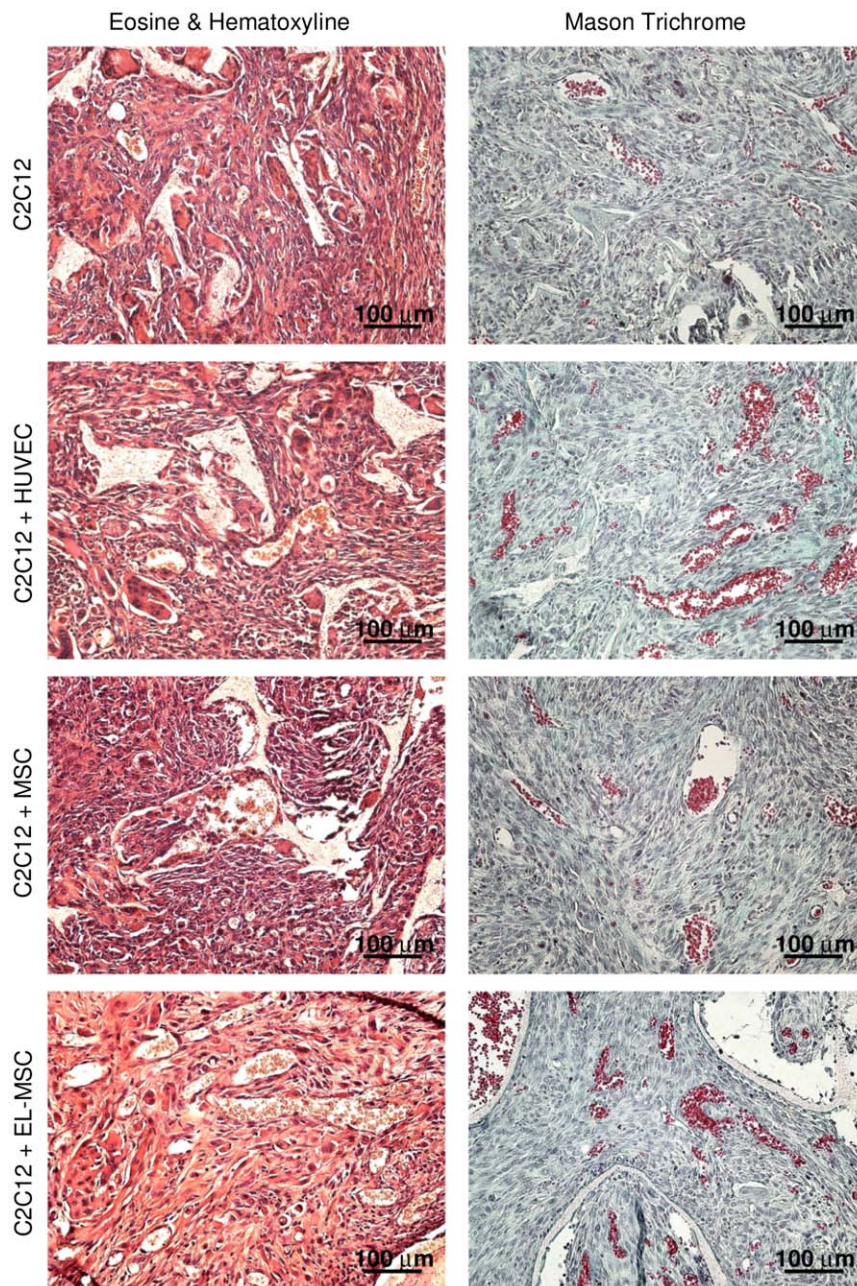
Staining of implants with anti-human specific endothelial antibody (anti-CD31) demonstrated that in the constructs seeded with EL-MSCs vessels lined with human cells were present (Fig. 10B). Moreover, these vessels contained intraluminal red blood cells suggesting that the vessels had anastomosed with the host vasculature.

## Discussion

Proper vascularization is essential for maintaining tissue well-being and functionality. It is also crucial for engineering of bone

graft constructs, liver and many other tissues used for transplantation [63–65]. To provide extensive tissue or graft vascularization, a source of endothelial cells must be found. Those cells have to be available in large quantities and be able to create a vascular network within the tissue. Furthermore, this network should be both structurally and functionally appropriate.

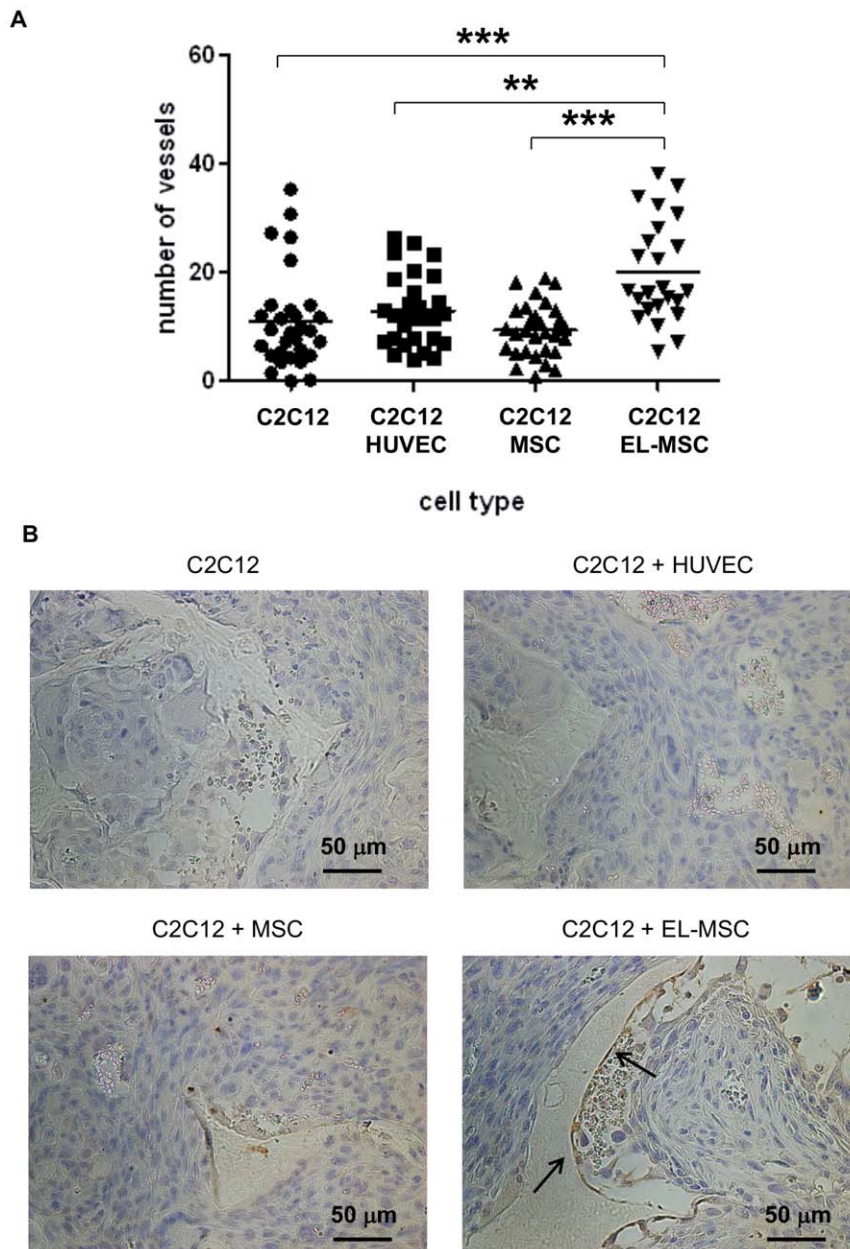
Since BM-MSCs are widely used for bone tissue engineering, we decided to investigate whether we can use the same cell source for vascular network formation. In this study we used a number of techniques including immune-fluorescent imaging, qPCR and quantitative image analysis to examine endothelial differentiation of MSCs at the cellular and protein level. This study is the first to fully describe differentiation of MSCs into endothelial-like cells that perform in an *in vivo* study better than undifferentiated (naïve) MSCs.



**Figure 9. In vivo vascularization of polymeric construct combined with Matrigel and cells.** Vessel-like network formation in vitro in 3D constructs. Tissue construct sections were stained with eosin (orange for tissue, red for erythrocytes) and counterstained with hematoxylin (brown) or with Masson's trichrome staining (blue or green for collagen, red for erythrocytes) and counterstained with hematoxylin (brown). doi:10.1371/journal.pone.0046842.g009

BM-MSCs can be obtained in large quantities and further expanded *in vitro*. For most *in vivo* applications, a minimal number of cells, in the range of  $10^8$ – $10^9$ , are necessary [66] and this can easily be obtained from MSCs after only a few passages. We did not observe any serious loss of endothelial potential in cells expanded up to such a level. In the differentiation protocol MSCs were first cultured for 10 days in EGM-2 medium on an orbital shaker. The effect of this step was limited to changes in morphology and ability to perform in the Matrigel assay. After this culture period we did not observe a change either in marker expression or in the ability to take up ac-LDL. Nevertheless, this step was crucial for further performance in functional assays and

differentiation. Functional properties of EGM-2-cultured MSCs were demonstrated using the Matrigel assay. MSCs that underwent 10 days of culture in EGM-2 medium performed with similar efficiency to endothelial cells (HUVECs) in Matrigel sprouting assay and much better than naïve MSCs. Interestingly, capillaries formed by MSCs were more stable than the ones formed by HUVECs – when applying prolonged culture, capillaries formed by HUVECs get disorganized whereas capillaries formed by MSCs remain stable. This can indicate that while forming capillaries MSCs can play both the role of endothelial cells that create vessels as well as the role of pericytes that stabilize those vessels [67]. The fact that MSCs can take the role of pericytes in



**Figure 10. Quantitative analysis of vessels in polymeric constructs.** Number of vessels per sample was quantified by four people blinded for the conditions (A). \*\* denotes statistical significance ( $P < 0.01$ ), \*\*\* denotes statistical significance ( $P < 0.001$ ). Tissue construct sections were stained with anti-human CD31 antibody (brown, indicated by black arrows) and counterstained with hematoxylin (blue) (B). doi:10.1371/journal.pone.0046842.g010

various engineered constructs has already been shown by Chamberlain *et al* [68]. They reported that in co-culture with HUVECs on collagen modules MSCs became smooth muscle actin positive and migrated to surround the EC layer of the vessel (location typical for pericytes).

Pre-differentiated MSCs were also able to acquire endothelial characteristic (endothelial markers expression and ability to take up ac-LDL) after 24 h culture on Matrigel which was not the case when using naïve MSCs. This showed that growth supplements combined with shear force play an important role in triggering endothelial differentiation of MSCs, though this effect cannot be observed based on marker expression only. Although the molecular mechanisms of angiogenesis and vasculogenesis are

currently not fully understood, there is evidence strongly supporting the crucial role of VEGF in both processes [69]. The expression of the main receptor for VEGF, KDR, did not increase in hMSCs after culture in EGM-2, but other studies showed that many key events in VEGF signaling occur inside endothelial cells and are regulated by endosomal receptor trafficking [70]. According to this model even basally expressed KDR can propagate the VEGF signaling. The VEGF-KDR complex is endocytosed directly after signal transduction and then proteolytic cleavage takes place, releasing the cytoplasmic KDR dimer and making it available to form new signaling complexes. In our work we did not check for expression of Neuropilins and Ephrins that play a crucial role in both VEGF and KDR endocytosis [71,72]

but we hypothesize that this can be the mechanism that triggers MSC differentiation towards endothelial lineages. This would be similar to the processes that occur in the embryo when the multipotent mesodermal progenitor cells differentiate *in situ* into endothelial cells during early stages of vascular development. VEGF, together with bFGF, is known to play a critical role in these events [73].

The input of Matrigel on pre-differentiated MSCs that leads to further differentiation towards endothelial cell phenotype can be explained based on the work of Lozito *et al.* [62]. They showed that change in the composition and crosslinking level of the matrix influences MSC differentiation towards endothelial and smooth muscle phenotype. Osawa *et al.* [74] showed that CD31 can be the mechanoresponsive molecule in case of endothelial cells, but we did not observe an increase in CD31 expression in MSCs before Matrigel stimulation. Therefore, two possible explanations of the effect of Matrigel on MSCs can be suggested. There can be another molecule on MSCs that acts as mechanoreceptor or there is a positive feedback loop between the ECM stimulation and CD31 expression in MSCs. Culture on Matrigel also induced KDR expression in MSCs which can further stimulate endothelial differentiation by increasing MSC sensitivity to VEGF present in culture medium. These results are similar to the ones obtained by Gu *et al.* [75] while studying murine embryonic stem cells. They showed that increased expression of endothelial markers is induced by extracellular matrix via CEACAM1, a glycoprotein involved in cell-cell adhesion. Therefore we can hypothesize that the role of Matrigel in our differentiation system is mainly to provide the environment in which MSCs can create cell-cell contact promoting endothelial differentiation. Nevertheless, those results suggest that careful examination of the MSC differentiation protocols is necessary as our knowledge concerning their signaling pathways is still limited.

CD31 staining performed on the constructs with EL-MSCs revealed the presence of human cells lining the walls of vessels. These vessels were fully functional as demonstrated by the presence of erythrocytes in the vessel lumens. This is a proof that EL-MSCs actively participated in building of these vessels. Nevertheless, there were also vessels present that were not human CD31 positive. This can suggest that the EL-MSC influence on vascularization is also due to their trophic effect on surrounding tissues. There is also the possibility that EL-MSCs create a network within the sample that is then gradually replaced by in-growing vessels. In that case the input of EL-MSCs can be limited to providing the route for host endothelial cells.

Although the primary purpose of this study was to differentiate hMSCs into endothelial-like cells, the results also serve as proof of concept for using bone marrow-derived hMSCs to create a vascularised graft. Our *in vivo* study showed that introduction of EL-MSCs in the engineered construct doubles the number of

vessels in-growing in the construct 2 weeks after implantation. The fact that HUVECs did not induce vessel in-growth when compared to C2C12 or C2C12/MSC seeded constructs indicates that these endothelial cells are not able to create stable vascular structures without the support of other cells, pericytes or smooth muscle cells, which will stabilize newly formed blood vessels. Similar results were obtained by Levenberg *et al.* [58] where they have shown that the addition of embryonic fibroblasts promotes stable vessel formation. According to our results, EL-MSCs do not require the presence of an additional cell type to improve construct vascularization. Increased level of vascularization is crucial for the implantation of large grafts and the possibility to use the patient's own cells to stimulate such an effect is a promising finding for a number of different applications. Further studies are required to verify whether differentiation of hMSCs prior to application can further improve performance of these cells in peripheral vascular disease (PVD) treatment as our data suggest.

This study also presents the opportunity of using iMSCs as model cells for studying the endothelial differentiation of MSC. This can be a useful tool for further study since it provides cells that are not affected by potential donor variation and isolation procedures.

In summary, hMSCs derived from bone marrow acquire several endothelial-like characteristics when cultured in endothelial cell growth supplement and exposed to shear force and extracellular matrix stimuli. These features include both phenotypical as well as functional characteristics. Furthermore, when applied *in vivo*, EL-MSCs show greater angiogenic potential than both naive MSCs as well as truly endothelial cells, HUVECs, that are generally used as golden standard for vascularization studies. This study presents a facile protocol for MSC preconditioning that improves *in vivo* performance of these cells with respect to attracting neovascularization. We believe that this approach has potential applications in tissue engineering and provides a tool for various clinical studies where improved vascularization is desired.

To conclude, training MSCs *in vitro* can be an efficient way to manipulate the fate of these cells *in vivo*.

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## Author Contributions

Conceived and designed the experiments: KJP AL NG HF LM CvB JdB. Performed the experiments: KJP AL NG. Analyzed the data: KJP AL NG HF JdB. Contributed reagents/materials/analysis tools: KJP AL NG HF LM CvB JdB. Wrote the paper: KJP AL NG HF LM CvB JdB.

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