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# Reprogramming Bone Marrow Stem Cells to Functional Endothelial Cells in a Mini Pig Animal Model

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**Background:** The aims of this study were to compare the morphological, biochemical, and functional properties of reprogrammed bone marrow stem cell (BMSC)-derived arterial endothelial cells (AECs) and venous endothelial cells (VECs), following adenosine triphosphate (ATP)-stimulation in a mini pig animal model.


**Material/Methods:** Bone marrow aspiration was performed in six adult mini pigs. Harvested mononuclear cells were isolated, cultured, and treated with vascular endothelial growth factor (VEGF) (16 µg/ml). Transformed cells were characterized using immunofluorescence staining for CD31 and von Willebrandt factor (vWF) and expression of endothelial nitric oxide synthase (eNOS). Cell release of nitric oxide (cNO) was measured using spectrophotometry. Matrigel assays were used to investigate angiogenesis in transformed BMSCs.

**Results:** Reprogrammed BMSCs in culture showed a typical cobblestone-like pattern of growth. Immunofluorescence staining was positive for CD31 and vWF expression. Expression of eNOS, using immunofluorescence staining and Western blot, showed no difference between the reprogrammed BMSCs and VECs. Spectrophotometric examination following stimulation with 10mmol/l ATP, showed comparable cNO release for reprogrammed BMSCs (10.87±1.76 pmol/10<sup>6</sup> cells/min) and VECs (13.23±2.16 pmol/10<sup>6</sup> cells/min), but reduced cNO release for AECs (3.44±0.75 pmol/10<sup>6</sup> cells/min). Matrigel assay for angiogenesis showed vascular tube formation of differentiated BMSC endothelial cells (grade 3.25). BMSCs cultured without VEGF did not demonstrate vascular tube formation.

**Conclusions:** The findings of this study showed that eNOS expression and release of NO could be used to show that BMSCs can be reprogrammed to functional VECs and AECs.

**MeSH Keywords:** **Adult Stem Cells • Blood Vessel Prosthesis • Bone Marrow Cells • Endothelial Cells**

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## Background

Peripheral arterial disease (PAD) and coronary artery disease (CAD) are increasingly common in an aging population, requiring arterial bypass grafting; diabetes mellitus is also common with renal failure requiring vascular access for chronic hemodialysis [1,2]. When autologous venous grafts are unavailable, prosthetic grafts may be used [1,2]. If there is no monolayer of functional endothelial cells on the inside of the prosthetic graft, intimal hyperplasia may occur, which leads to graft thrombosis [3]. In 1984, an early study showed that endothelial cell seeding of vascular grafts could promote graft patency [3,4]. In small caliber vascular grafts, but also other when other prosthetic vascular or cardiac scaffolds are used, endothelial cell re-seeding improves the function of these grafts in humans and may be due to the protective functions of mature endothelial cells [4–6]. Also, an endothelial cell monolayer acts as a natural barrier to suppress leucocyte infiltration and reduces vascular smooth muscle cell (VSMC) proliferation [1].

Endothelial nitric oxide synthase (eNOS) plays a major role in vascular homeostasis and vascular protection [7–10]. Studies using eNOS knock out mice have shown an association with the development of systemic hypertension [7], platelet aggregation and thrombosis [8], atherogenesis [9], and abnormal pulmonary vascular reactivity [10]. The homodimeric enzyme eNOS is composed of an oxygenase and a reductase domain; all isoforms of nitric oxide synthase (NOS) use L-arginine as a substrate and use molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates [11]. At the hem site, the electrons are used to reduce and activate O<sub>2</sub> and to oxidize L-arginine to L-citrulline and nitric oxide (NO), which is an important vasodilator; deficiency of NO has been linked to the pathogenesis of several cardiovascular diseases [12]. Due to the anti-proliferative effects of NO on VSMCs, hyperplasia can be prevented by NO in vascular prostheses [1]. Also, changes in the activity of NADPH-diaphorase (NADPH-d) has been reported to be a marker for the activity of NOS and eNOS [13,14].

The transformation abilities between endothelial cells, mesenchymal cells, and bone marrow stem cells (BMSCs) have recently been studied [13–19]. In 1998, Wang and colleagues showed that even in early embryogenesis, the endothelial cells of arteries and veins had different properties, and these findings were confirmed by molecular studies [14,15]. By 2001, Othman-Hassan and colleagues showed that, in an avian model, the function of arterial endothelial cells (AECs) was controlled by local cues and that AECs and venous endothelial cell (VEC) phenotypes remained preserved [16]. These findings may explain the problems found in clinical studies where venous grafts are used to repair, bypass, or reconstruct femoral and coronary arteries that have been occluded by atherosclerosis and

thrombosis [17]. Venous graft failure, which occurs in between 15–40% cases, is due to intimal hyperplasia [18]. However, the use of circulating peripheral blood endothelial cells and/or endothelial progenitor cells (EPCs) for pre-seeding prosthetic grafts are being studied [1,19]. For example, in 2005, a study showed that the level of circulating CD34-positive and KDR-positive EPCs predicted the occurrence of cardiovascular events and death from cardiovascular disease [20].

Multipotent BMSCs can undergo multilineage differentiation, including differentiation into mature endothelial cells *in vitro* [21]. The typical characteristics of BMSCs include their adherence to plastic surfaces in cell culture, and their expression of typical cell surface markers, including CD90 and CD105, and their lack of expression of CD45 and CD34, characteristics that may be used in translational research studies [21,22]. In cardiac transplantation, vasculopathy is a manifestation of chronic cardiac allograft rejection that leads to graft failure [23]. In 2001, Shimizu et al. showed that host BMSCs were a source of donor intimal SMCs in murine transplant arteriopathy [24]. In 2003, Fujiyama and colleagues showed that BMSCs adhered to injured endothelium and accelerated re-endothelialization as EPCs [25]. Therefore, the use of BMSCs may be a possible pretreatment for vascular grafts to endothelialize the lining of the graft [25].

There is still a lack of clarity regarding BMSC differentiation to endothelial cells of an AEC or VEC phenotype for use in prosthetic graft cell seeding with bone marrow-derived EPCs. Therefore, the aims of this study were to compare the morphological, biochemical, and functional properties of reprogrammed BMSC-derived AECs and VECs, including the expression of eNOS and release of NO following adenosine triphosphate (ATP)-stimulation in a mini pig animal model.

## Material and Methods

### Ethical approval and animal welfare

All experiments were performed in accordance with the Principles of the Laboratory Animal Care from the National Society of Medical Research (NSMR), and the 1996 Guide for the Care and Use of Laboratory Animals developed by the US National Institute of Health (NIH) and the German laws for animal welfare. The study was approved by the Ethical Committee of the University Leipzig.

### Cell isolation

Venous endothelial cells (VECs) were harvested from the jugular vein (diameter of about 0.5 cm), and arterial endothelial cells (AECs) were harvested from the descending aorta (diameter

of about 2.0 cm) from six adult mini pigs (median weight 52 kg; range 50–60 kg). The vessels were gently rinsed with phosphate buffered saline (PBS) to remove blood cells. Endothelial cells were released from the luminal vessel side with collagenase IV (Merk Millipore, Berlin, Germany) (350 U/ml in PBS) for 15 minutes in a water bath at 37°C. The suspension was dissolved in Dulbecco's modified Eagle's Medium (DMEM) (Lonza, Hessisch Oldendorf, Germany) with 10% fetal bovine serum (FBS) (Lonza, Hessisch Oldendorf, Germany), centrifuged for 5 minutes at 700×g at 25°C, and the cells were re-suspended in DMEM containing 10% FBS, 1% penicillin/streptomycin (P/S) (Sigma Aldrich, Taufkirchen, Germany) and 16 µg/ml vascular endothelial growth factor (VEGF) supplement from bovine brain (Oberdora, Germany). Cell culture medium was changed first after 24 h and thereafter every second day. Confluent cells were passaged with accutase (Sigma Aldrich, Taufkirchen, Germany) for 3–4 minutes at 37°C until the fourth passage.

BMSCs were harvested by pelvic bone marrow aspiration, and mixed with PBS containing 2mM EDTA (1: 1) and filtered with a 100µm cell strainer (Becton-Dickinson, Heidelberg, Germany) to eliminate bone remains and tissue fragments. Bone marrow-derived mononuclear cells were isolated by sucrose gradient centrifugation adding Histopaque 1077 (Sigma Aldrich, Taufkirchen, Germany) at 500 g for 30 min at 25°C in 6 ml Histopaque and a 9 ml BMSC-PBS-2mM EDTA solution. Mononuclear cell fractions were diluted in DMEM, containing 10% FBS and 1% P/S. After 24 h, the medium was changed to remove non-adherent cells [26]. The addition of VEGF supplement (16 µg/ml) was used to differentiate BMSCs to endothelial cells up to the fourth passage.

### Cell characterization by fluorescence staining

At the third passage, endothelial cells were fixated with 4% paraformaldehyde, and permeabilized at 30 minutes in 0.1% Triton X 100 (Sigma Aldrich, Taufkirchen, Germany) and blocked with 0.1% bovine serum albumin (BSA) for 1 hour. Primary antibody was incubated overnight at 4°C. For characterization of endothelial cells, rabbit anti-eNOS polyclonal antibody (dilution 1: 250) (Abcam, Cambridge, UK), mouse anti-CD31 monoclonal antibody (dilution 1: 250) (Abcam, Cambridge, UK) and rabbit polyclonal anti-von Willebrand factor (vWF) antibody (dilution 1: 250) (Abcam, Cambridge, UK) were used. To identify mesenchymal stem cells from bone marrow, an adhesion molecule antibody to CD90 conjugated with fluorescein isothiocyanate (FITC) [27] was used as a positive marker (dilution 1: 100) (Becton-Dickinson, Heidelberg, Germany) and a mouse monoclonal antibody to CD45, a trans membrane leukocyte protein, was used as negative marker (dilution 1: 100) (Santa Cruz, Heidelberg, Germany).

The secondary antibodies (dilution 1: 250) were incubated for 1h (Alexa Fluor® 488 goat anti-mouse; Alexa Fluor® 488 goat anti-rabbit IgG; Alexa Fluor® 555 goat anti-mouse IgG) (Invitrogen, New York, USA). Staining of cell nuclei was performed with 4',6-diamidino-2-phenylindole (DAPI) 0.02 mg/ml (Roche Diagnostics, Mannheim, Germany) for 1 minute. All washing steps were performed with 0.1% BSA in PBS. Cover slips were investigated using a Zeiss Axiolab fluorescence microscope (Zeiss, Jena, Germany) and a commercial image analysis system (SigmaScan Pro 5, Erkrath, Germany). The average of BMSCs counted for each marker was 23 (magnification ×400).

### Differentiation of adipocytes from BMSCs

BMSCs were cultured to confluency without growth factors from the first passage with the StemPro® Adipogenesis Differentiation Kit (Life Technologies, New York, USA) for three weeks. As a negative control, we used BMSCs grown without VEGF. Cells were fixed with 4% paraformaldehyde for 30 minutes and fat vesicles within the cells were detected with oil-red-O (ORO) staining. Nuclear staining was performed with methylene blue. Six randomized visual fields (magnification ×80) were analyzed by the number of cells containing fat vesicles, using an Axiovert 25 inverted microscope (Zeiss, Germany).

### Angiogenesis

An *in vitro* angiogenesis assay kit was used (Merk Millipore, Berlin, Germany) using the method previously described [28]. Confluent cells were grown in 25 cm<sup>2</sup> culture flasks and were detached using Accutase® solution, centrifuged at 700×g, for 5 min, at 25°C, and seeded onto polymerized 100 µl Matrigel (Becton-Dickinson, Heidelberg, German) for three-dimensional growth in one chamber of the Lab-Tek® II Chamber Slide system, 0.8 cm<sup>2</sup> per well (Lab-Tek, Naperville, USA). After 18 h at 37°C and 5% CO<sub>2</sub> the cell angiogenic properties were evaluated under an Axiovert 25 inverted microscope (Zeiss, Germany) using the grading according to the manufacturer's instructions: Grade 0=single cells; Grade 1=cells beginning to orientate; Grade 2=cells forming tubes, with only a few branches; Grade 3=cells forming tubes, but with no branches; Grade 4=the beginning of a complex network; Grade 5=a complex network of vascular tubes. For each angiogenesis assay (AECs, VECs, reprogrammed BMSCs, BMSCs) three random fields were viewed at a magnification of ×80 and were graded at each viewing and the means were calculated (Axiovert 25, Zeiss, Germany).

### Functionality of endothelial nitric oxide synthase (eNOS)

The activity of the NADPH-diaphorase, a co-enzyme of eNOS, was histochemically investigated. This enzyme is the utcase domain of eNOS and catalyzes the following chemical reaction:  $NADPH+H^{++}+acceptor \rightarrow NADP^{++}+reduced\ acceptor$ .

Cultured cells were fixed with 4% paraformaldehyde and incubated with nitroblue tetrazolium (NBT) (0.5mM) and NADPH (1.0M) for 20 h at 37°C. Thereby active NADPH-diaphorase transferred H<sup>+</sup> ions to NBT which led to a paranuclear blue staining in eNOS expressing cells (n=6). We used the method of difference spectroscopy for NO- determination, as described previously [28]. The basic principle of investigation of NO-release is the redox reaction of Fe<sup>2+</sup> in the hem group of oxyhemoglobin with NO to Fe<sup>3+</sup> (so-called methemoglobin), which results in the  $\gamma$  Soret band shift of the absorption maximum detectable from 415 nm of oxyhemoglobin to 404 nm of methemoglobin.

To produce oxyhemoglobin 1mg/ml methemoglobin (purified from rat) (Sigma Aldrich, Taufkirchen, Germany) were dissolved in phosphate buffer (50 mmol/l Na<sub>2</sub>HPO<sub>4</sub>; 30 mmol/l NaOH; pH=7.4), aerated with vapor water saturated with carbogen (95% oxygen/5% carbon dioxide). To reduce methemoglobin to oxyhemoglobin, a spatula tip of sodium dithionite was added. After further 15 minutes aeration, oxyhemoglobin was filtrated and purified by size exclusion chromatography with a sephadex G-25 column (flow velocity 2 ml/min). Concentration (c) of oxyhemoglobin was determined at  $\gamma_{\text{max}}=542$  nm using the Beer-Lambert law:  $c = \frac{E}{\epsilon \cdot d}$  E=measured absorption;  $\epsilon$ =absorption coefficient oxyhemoglobin 14,370 l/mol/cm; d=layer thickness of the cuvette. Confluent cells growing in the petri dish (surface area: 68 cm<sup>2</sup>) were incubated for 20 min with 5 ml HEPES buffer (145 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l CaCl<sub>2</sub>, 1 mmol/l MgCl<sub>2</sub>, 10 mmol/l HEPES, 5 mmol/l glucose, pH 7.4, at 37°C.

Then, 4  $\mu$ mol/l of freshly isolated oxyhemoglobin was added, and the measurements began in the wavelength range  $\lambda=350-450$  nm for every 10 minutes, over a period of 30 minutes for determination of basal NO-liberation. Furthermore, 10 mmol/l adenosine triphosphate (ATP) was added to stimulate NO-release as a functional parameter, and methemoglobin formation as an indicator of NO-liberation was measured for further 10 minutes. Using the difference spectroscopy method we evaluated  $\Delta E_{401-411}$  (subtraction of the measured value by absorption maximum 401 nm from the isobest 411nm), which indicated methemoglobin formation. Using the Beer-Lambert law with 39.83 mM<sup>-1</sup>cm<sup>-1</sup> we calculated NO-release as pmol/10<sup>6</sup> cells/min according to previous studies [26]. As a control for spontaneous methemoglobin transformation we used HEPES buffer with 4  $\mu$ mol/l oxyhemoglobin (without cells).

### Western blot

Confluent cells were lysed with lysis buffer. After 10min incubation on ice, mechanical extraction was performed, followed by further 10 min on ice. Then, the cell lysate was centrifuged for 10 min at 16,000 x g and 4°C, with the supernatant collected and stored at -80°C. The sample of 40  $\mu$ g of protein was solubilized in sodium dodecyl sulphate (SDS) sample buffer

was performed using SDS-polyacrylamide gel electrophoresis (PAGE) with 4% stacking gel and 10% running gel, and then transferred to a nitrocellulose membrane using a wet blotting method (Towbin et al., 1979). Blocking non-specific binding sites was achieved with 5% dried milk powder in Tris-buffered saline containing Tween (TBST) for 1 hour at room temperature. Then, the membrane was incubated with rabbit monoclonal anti-eNOS antibody (dilution 1: 1000) (Abcam, Cambridge, UK) overnight at 4°C, followed by the incubation of horseradish-peroxidase (HRP)-linked secondary anti-rabbit IgG polyclonal antibody (Sigma Aldrich, Taufkirchen, Germany). Protein visualization was performed by enhanced chemiluminescence. The membranes were incubated with the mouse anti-GAPDH IgG antibody (HyTest, Turku, Finland) and appropriate polyclonal secondary antibody (anti-mouse IgG) (Sigma Aldrich, Taufkirchen, Germany).

### Statistical analysis

Continuous variables were expressed as mean  $\pm$ SEM and analyzed by univariate ANOVA. If ANOVA indicated significance, *posthoc* unpaired Student's t-test or Kruskal-Wallis test was performed, as appropriate, and corrected for multiple comparisons if necessary using the Bonferroni correction. The categorical data were compared using the chi-square test or Fisher's exact test and Kruskal-Wallis test. The level of significance was set at  $p \leq 0.05$ . For all statistical analyses, we used SPSS Statistic 17.0 (SPSS Science, Erkrath, Germany).

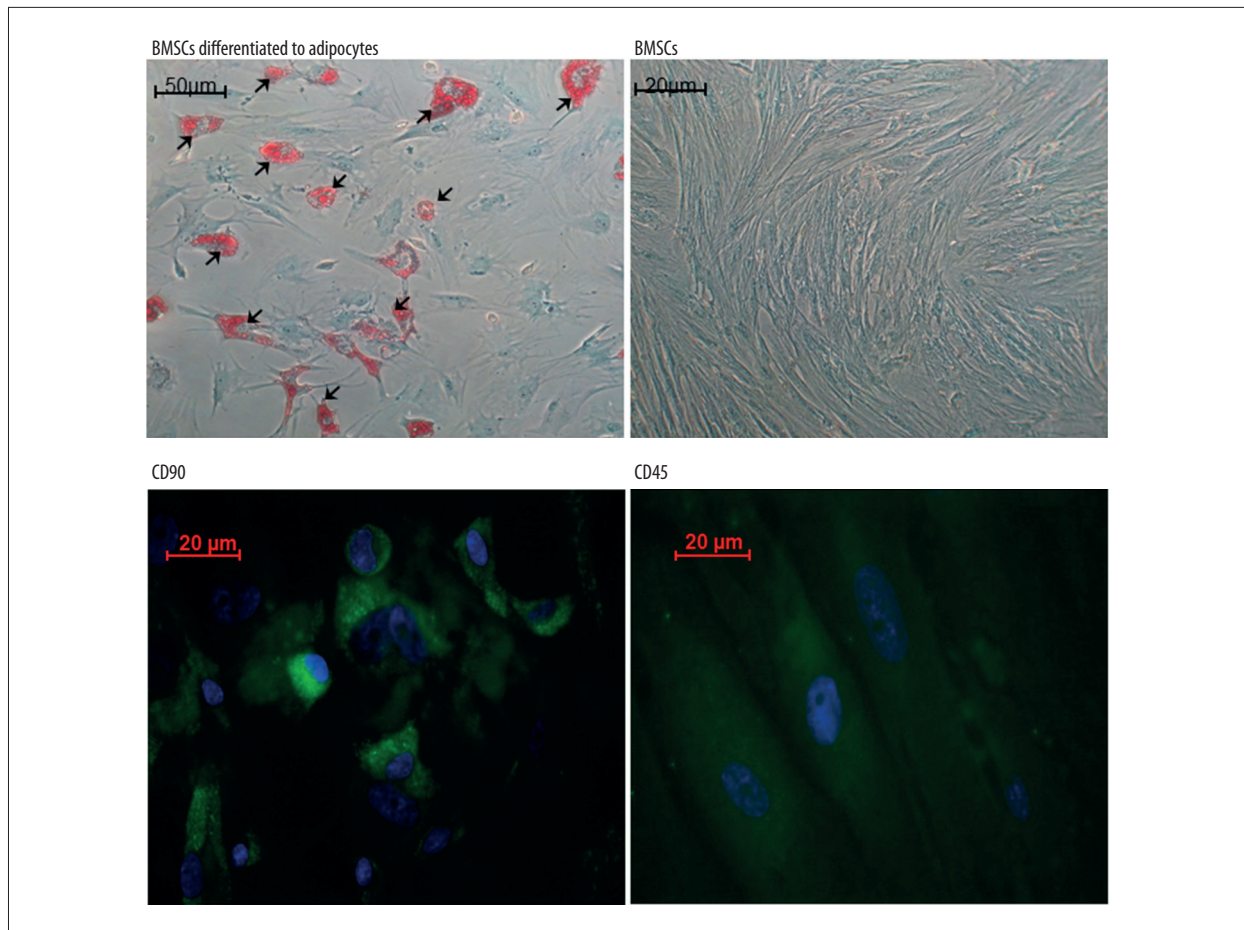
## Results

### Characterization of isolated bone marrow stem cells (BMSCs)

After the third passage, mononuclear BMSCs cultured with non-endothelial media (n=5) were characterized by immunofluorescence staining for surface antigens CD90 and CD45. Analysis of five images showed a typical pattern of stem cells with 90.67 $\pm$ 5.07% CD90-positive cells and 17.45 $\pm$ 5.44% CD45-positive stained cells (Figure 1A).

Adipogenic differentiation of cells was identified in cells cultured for three weeks with StemPro<sup>®</sup> Adipogenesis Differentiation Kit and subsequently stained with oil-red-O (ORO). We observed that 25.57 $\pm$ 4.00% of cells contained fatty vesicles, while in the BMSC control cells cultured with media without endothelial cell growth factors, they were negative ( $p \leq 0.05$ ) (Figure 1B). Therefore, isolated BMSCs had the potential to differentiate into another cell type, adipocytes, which is a characteristic good quality of stem cells.





**Figure 1.** Photomicrographs of the immunofluorescence staining of bone marrow stem cells (BMSCs) and histochemical staining for adipocyte differentiation. **(A)** Representative immunofluorescence (green) staining of isolated CD90-positive bone marrow stem cells (BMSCs) and CD45 as a negative immuno-marker. Counterstaining of cell nuclei was performed with 4',6-diamidino-2-phenylindole (DAPI) (blue). **(B)** Isolated BMSCs cultured with the StemPro® Adipogenesis Differentiation Kit for adipocytes differentiation of BMSCs (**left panel**). The right panel shows BMSCs in cultured media containing 10% FBS as control. Oil-red-O (ORO) staining (red) shows the lipid-filled cytoplasmic vesicles, with the cytoplasm counterstained by methylene blue.

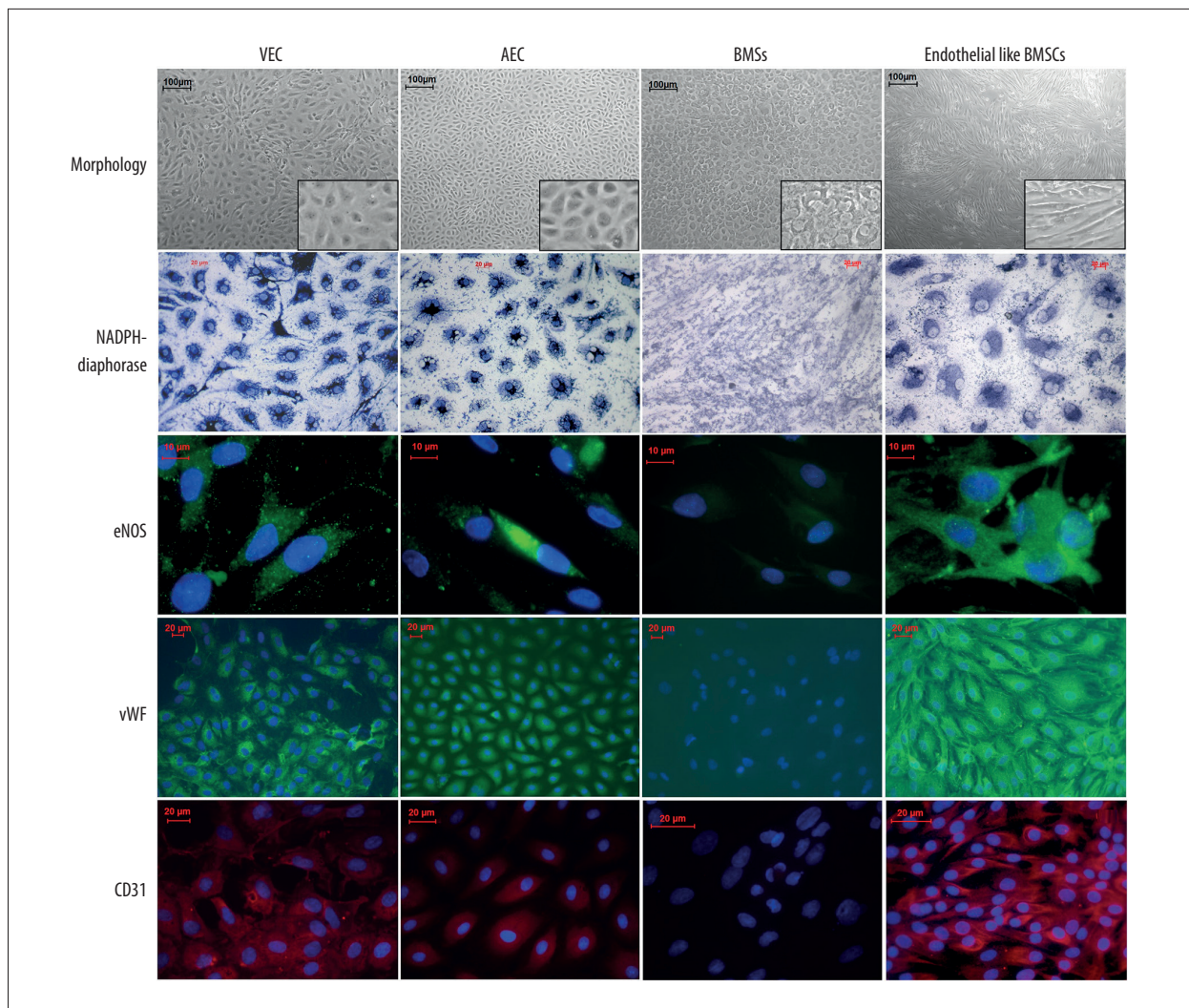
### Functional comparison of reprogrammed BMSCs with endothelial cells

By comparing cell morphology of arterial endothelial cells (AECs) and venous endothelial cells (VECs) with reprogrammed BMSCs, we saw no differences. All three cell-lines showed a cobblestone-like pattern in culture. However, reprogrammed BMSCs were more polygonal and non-homogeneous compared with VECs and AECs. As shown in the insets of Figure 2 (upper panel) reprogrammed BMSCs also exhibited a pale nucleus with two nucleoli, typical of VECs or AECs. However, the nucleus was slightly larger than in AECs or VECs. BMSC control cells exhibited a typical stem cell fibroblast-like appearance (Figure 2, upper panel).

In addition to cell morphology reprogrammed BMSCs were positive for typical endothelial cell markers, endothelial nitric

oxide synthase (eNOS), von Willebrand factor (vWF), and CD31 in the same way as VECs and AECs, as shown by immunofluorescence staining. In BMSCs cultured without VEGF, the endothelial cell markers, vWF and CD31, were not expressed, while eNOS was expressed but at a lower level of intensity when compared with other cell lines (Figure 2, last three panels).

The Western blot analysis (Figure 3) showed that reprogrammed BMSCs exhibited eNOS protein expression similar to VECs, with an eNOS/GAPDH ratio in reprogrammed BMSCs of  $0.818 \pm 0.172$  vs. VECs of  $0.809 \pm 0.549$ . However, AECs showed  $0.316 \pm 0.111$ ,  $p=0.024$  when compared with reprogrammed BMSCs. Also, in AECs, eNOS expression was significantly lower than in VECs; increased expression of eNOS was found in reprogrammed BMSCs compared with BMSCs.

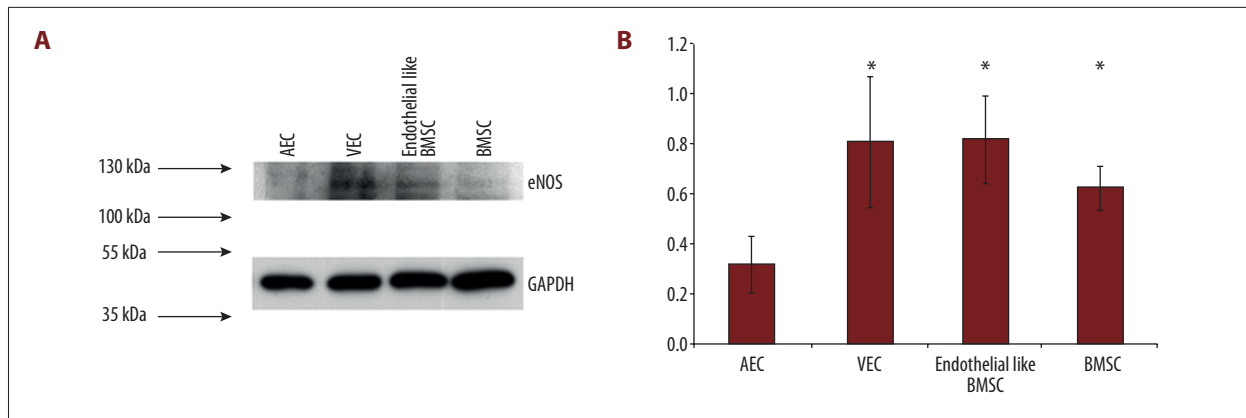


**Figure 2.** Photomicrographs of the phase-contrast, NADPH-diaphorase, and immunofluorescence staining of bone marrow stem cells (BMSCs) reprogrammed to endothelial cells. **Morphology:** Observed cell morphology in all groups. Reprogrammed bone marrow stem cells (BMSCs) showed a similar morphology, with a cobblestone appearance similar to venous endothelial cells (VECs) or arterial endothelial cells (AECs), which was not seen in isolated BMSCs cultured with media containing 10% fetal bovine serum (FBS). **NADPH-diaphorase histochemistry:** Para-nuclear (blue) staining is shown, indicating that eNOS was active in reprogrammed BMSCs, VECs and AECs, but not in normal BMSCs. **Immunofluorescence staining for eNOS:** Expression of eNOS in reprogrammed BMSCs was comparable to VECs and AECs, but to a lesser degree in normal BMSCs. **Immunofluorescence staining for von Willebrand factor (vWF):** Reprogrammed BMSCs expressed vWF, with a similar pattern to VECs and AECs, but not in normal BMSCs. **Immunofluorescence staining for CD31:** Reprogrammed BMSCs expressed CD31, which was not shown in normal BMSCs.

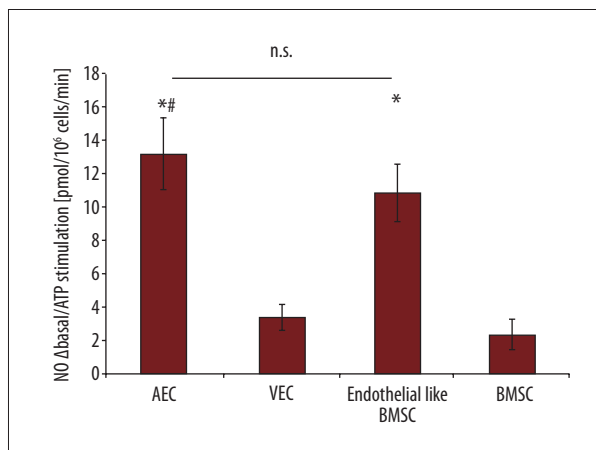
NADPH-diaphorase histochemistry was used as an indirect test for active eNOS expression and showed blue paranuclear staining. Especially in reprogrammed BMSCs, NADPH-diaphorase activity could be detected similarly in both VECs and AECs, but not in BMSCs without growth factors (Figure 2, second panel). The detection of eNOS-activity in reprogrammed BMSCs is further supported by difference-spectroscopy NO-measurements.

The original NO-measurements shown in Figure 4 represent end NO release at different time points in all three cell lines

and in the control (with only HEPES buffer with oxyhemoglobin to detect spontaneous methemoglobin transformation). The isobest was found at 411 nm as previously published [29]. We found a time-dependent and ATP-inducible increase in absorption at 401 nm. Methemoglobin formation was evaluated as  $E_{401-411}$  (i.e. relative to the isobest) and converted to NO (pmol/ $10^6$  cells/min) (see Methods section) using the molar extinction coefficient of  $39,83 \text{ mM}^{-1} \cdot \text{cm}^{-1}$  according to the Lambert-Beer Law.



**Figure 3.** Western blots for endothelial nitric oxide synthase (eNOS). **(A)** Representative Western blot of eNOS (130 kDa) in all bone marrow stem cells (BMSC) cell lines together with the reference protein GAPDH (37 kDa). **(B)** Statistical data from the Western blots (eNOS and GAPDH) show that reprogrammed BMSCs expressed eNOS to the same degree as venous endothelial cells (VECs). The eNOS expression for arterial endothelial cells (AECs) was significantly lower, compared with other cell lines. \*  $p \leq 0.05$  vs. AECs.



**Figure 4.** Nitric oxide (NO) production by cell groups. Nitric oxide (NO) production by all cell groups showed that reprogrammed bone marrow stem cells (BMSCs) released NO to the same degree as venous endothelial cells (VEC) (\*  $p \leq 0.05$  vs. BMSCs. #  $p \leq 0.05$  vs. VEC).

We found inducible NO-release in reprogrammed BMSCs ( $10.87 \pm 1.76$  pmol/10<sup>6</sup> cells/min) after ATP-stimulation in VEC-like cells ( $13.23 \pm 2.16$  pmol/10<sup>6</sup> cells/min). This finding indicated that reprogrammed BMSCs liberated NO in a similar way to functional VECs. The stimulated NO release in AECs was significantly lower than that in VECs and higher (non-significant) than in BMSCs grown without VEGF (AECs:  $3.44 \pm 0.75$  pmol/10<sup>6</sup> cells/min; BMSCs:  $2.37 \pm 0.93$  pmol/10<sup>6</sup> cells/min) (Figure 4).

Investigation of the angiogenic properties of reprogrammed BMSCs showed that they had the ability to form new capillaries and mesh-like structures in the same way as AECs and VECs; this could not be observed in BMSCs grown without VEGF ( $p \leq 0.05$ ) (Figure 5).

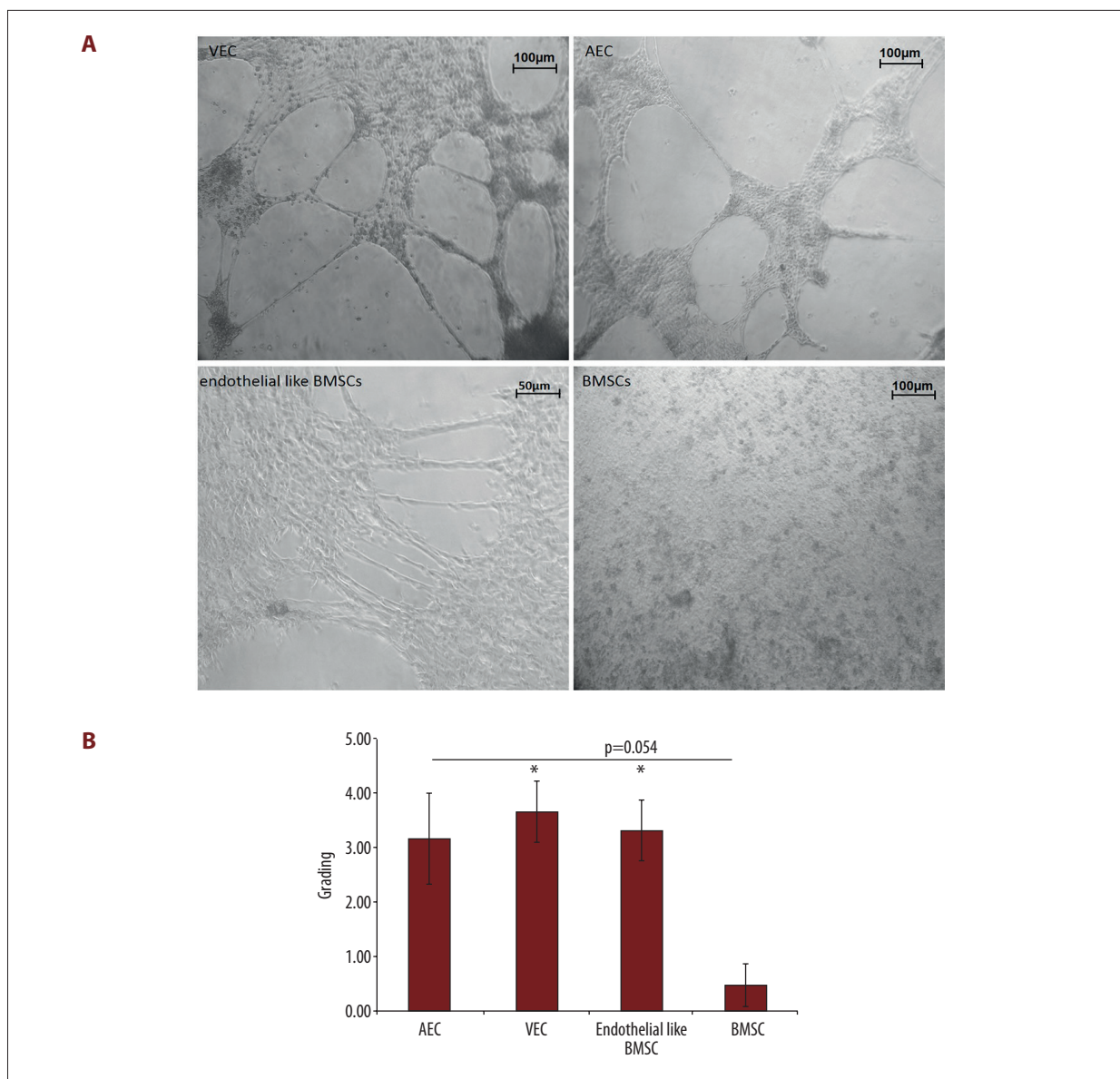
## Discussion

The findings of this study showed that mini pig-derived bone marrow stem cells (BMSCs) when grown in culture, could be reprogrammed to arterial endothelial cells (AECs) and venous endothelial cells (VECs) using treatment with vascular endothelial growth factor (VEGF) and adenosine triphosphate (ATP). In this study, we analyzed and characterized reprogrammed BMSCs to compare their functionality with purified BMSCs, VECs and AECs.

Although re-seeding of prosthetic vascular grafts with endothelial cells seems to be a possible treatment in patients with vascular dysfunction, for patients with diabetes, hyperglycemia has been shown to affect endothelial cell apoptosis, which may be an issue for endothelial cell seeded grafts [30]. However, reprogrammed BMSCs have emerged as a promising cell source for endothelial cells [21]. Because of their immunological compatibility auto-derived BMSCs can be used for the production of allogeneic cell products for transplants [31,32].

Isolated BMSCs cultured without growth factors adhere to plastics and have shown the presence of typical surface antigens like CD90, reduced CD45 expression, lack of endothelial cell marker expression and differentiation into other cell lines [22]. The findings of this study supported previous studies that have used BMSC reprogramming to endothelial cells using VEGF. Oswald and colleagues [21] observed that BMSCs could differentiate into cells that express endothelial-specific markers including von Willebrandt factor (vWF), and vascular endothelial growth factor (VEGF)-receptor 1 and 2 by adding of 50 ng/mL VEGF into the culture medium for a period of seven days [27]. However, in contrast to our results, this previous study did not observe morphological differences between





**Figure 5.** Angiogenesis and reprogrammed bone marrow stem cells (BMSCs). **(A)** Representative images of angiogenesis seen in reprogrammed bone marrow stem cells (BMSCs), arterial endothelial cells (AEC), and venous endothelial cells (VEC) but not in normal BMSCs. **(B)** Quantitative investigation of angiogenesis using a grading system: Grade 0=no angiogenesis, to Grade 5='mesh-like' branching structures. \*  $p \leq 0.05$  when compared with normal BMSCs.

differentiated and non-differentiated BMSCs. By comparing the morphology of reprogrammed BMSCs with AECs and VECs, we found similarity between the three cell lines. However, reprogrammed BMSCs differed in homogeneity, shape, and nuclear size, which were slightly larger and paler than in VECs and AECs. These findings supported those reported by Fujiyama and colleagues in 2003, who isolated bone marrow-derived CD34-/CD14+ cells of monocyte lineage, cultured them with VEGF and showed an increase in endothelial cell-specific markers, including VEGF-receptor 2 and CD34 [25].

In this study, reprogrammed BMSCs expressed typical endothelial cell markers, including vWF, CD31, and endothelial nitric oxide synthase (eNOS) after cell culture with VEGF [33,34]. Transforming growth factor  $\beta$  (TGF- $\beta$ ) is linked to neovascularization and modulates vascular development by promoting VEGF synthesis through protein kinases B and extracellular signal-regulated kinases [35,36]. These and other factors could also be included in the VEGF supplement and promote BMSC differentiation into reprogrammed cells, and changes in the microenvironment may also play an essential role in this process [37,38].



In this study, we investigated whether differentiated cells showed functional properties of endothelial cells, by detecting eNOS leading to the production of nitric oxide (NO). To our knowledge, this is the first study that has compared the release of NO between reprogrammed BMSCs and endothelial cells. NADPH-diaphorase activity was also used as evidence of indirect activity for eNOS [13]. Detection of activity of NADPH-diaphorase in reprogrammed BMSCs supported the functionality of eNOS, which was similar in VECs and AECs, but not in purified BMSCs. Also, eNOS expression and NO production in AECs were significantly reduced when compared with VECs. Previous studies have shown that stimulation of release of NO with acetylcholine acting on type 5 muscarinic receptors [39] was markedly increased in modulatory vessels with small diameter compared with large diameter vessels, indicating that eNOS expression is more marked in smaller vessels [39–41]. There were no differences found in eNOS expression in reprogrammed when BMSCs compared with BMSCs.

In the findings of NO production in this study, in bone marrow, eNOS is involved in bone development and healing, and osteogenic differentiation. In the findings of NOI it was possible to detect a time-dependent ATP-inducible production of NO in reprogrammed BMSCs, which was more comparable to that shown by the VECs than the AECs and was increased when compared with the purified BMSCs. A previous study has shown that the production of NO in rat bone marrow cells in culture was found by trapping of NO radicals (measuring  $38 \pm 6$  pmol per  $10^8$  cells) by NO-Fe  $2^+$ -diethyldithiocarbamate using electron paramagnetic resonance (EPR) spectroscopy, which supports our results (NO release by BMSC:  $2.37 \pm 0.93$  pmol/ $10^6$  cells/min) [42]. Furthermore, they showed by isoform specific inhibitors [1400W] that the majority of basal NO was produced by eNOS (66%) whilst the remaining proportions from inducible NOS and neuronal NOS.

Therefore, reprogrammed BMSCs showed VEC-like eNOS-activity and eNOS-expression, which was not shown by BMSCs. These results could be explained by different activation potentials of eNOS. In endothelial cells, eNOS becomes active by dislocation from the membrane and binds to calmodulin, and heat shock protein (HSP), 90 together with phosphorylation of serine sites (coupled form). When the substrate-level of L-arginine and the tetrahydrobiopterin-level (an essential cofactor of eNOS) is inadequate, eNOS becomes unstable

and uncoupled, leading to less NO production [43]. It could be that eNOS may be activated during the differentiation process, for instance, through VEGF, which could activate eNOS by stimulating the phosphatidylinositol 3-kinase-protein kinase B and eNOS and NO signaling pathway [44]. Furthermore, it has been reported that eNOS is essential for mobilization of stem and progenitor cells through eNOS, NO, cyclic guanosine monophosphate and matrix metalloproteinase 9 (MMP9) signaling [45]. Considering the findings of our study, the induced eNOS-activity, indicated by the increased production of NO by the reprogrammed BMSCs, could play a role in the differentiation process of BMSCs. Hypothetically, the activation of eNOS in BMSCs may not only initiate their mobilization but also may lead to differentiation of BMSCs into reprogrammed BMSCs.

The findings of this study may indicate that reprogrammed BMSCs expressed e-NOS and were able to produce NO by activation of eNOS to the same degree as VECs, but not AEC. In contrast, in an angiogenesis model, AECs, VECs, and reprogrammed BMSCs showed similar findings. Multiple pathways could regulate vessel formation, such as hypoxia, or secreted angiogenic peptides, including VEGF, through eNOS-dependent and -independent mechanisms [46].

## Conclusions

The function of endothelial cells in the vascular system is highly complex, including in vascular inflammation, in platelet aggregation, and thrombus deposition, and NO production [12,18,47]. The 'plasticity' of endothelial cells was demonstrated in 1998 by Wang and colleagues, who showed that ephrin-B2, a transmembrane ligand of the Ephrin family, is only expressed in arterial endothelial cells, but ephrin-B2 receptor tyrosine kinase Eph receptor B4 was only found in VECs [14]. Several other proteins are expressed by AECs. SS, including delta-like 4, ALKAL1 and endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. neuropilin 2 (NRP2) and COUP-transcription factor II (COUP-TFII) are known as specific VEC markers [17]. The findings of this study supported the role of the ability of BMSCs to differentiate into functional VECs, as shown by eNOS expression and the release of NO. Further studies are recommended to identify whether reprogrammed BMSCs also express specific VEC markers for their use in reseeding small caliber vascular grafts.

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