Angiogenic potential of human mesenchymal stromal cell and circulating mononuclear cell co-cultures is reflected in the expression profiles of proangiogenic factors leading to endothelial cell and pericyte differentiation

Running title Angiogenic potential of human MSC-MNC co-cultures

Katriina Joensuu[*, ☺], Liina Uusitalo-Kylmälä[☺], Teuvo A Hentunen, Terhi J Heino

Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku, Turku, Finland
[☺]These authors contributed equal work

***Corresponding Author:**

Katriina Joensuu, M.D., Ph.D.
Department of Cell Biology and Anatomy
Kiinamyllynkatu 10 C3
20520 Turku, Finland
E-mail kvjoen@utu.fi
Fax: +358 2 231 0320

Abstract

Endothelial progenitors found among the peripheral blood (PB) mononuclear cells (MNCs) are interesting cells for their angiogenic properties. Mesenchymal stromal cells (MSCs) in turn can produce proangiogenic factors as well as differentiate into mural pericytes, making MSCs and MNCs an attractive co-culture setup for regenerative medicine. In this study, we co-cultured human bone marrow-derived MSCs and PB-derived MNCs in basal or osteoblastic medium without exogenously supplied growth factors to demonstrate endothelial cell, pericyte and osteoblastic differentiation. The expression levels of various proangiogenic factors, as well as endothelial cell, pericyte and osteoblast markers in co-cultures were determined by qPCR. Immunocytochemistry for vascular endothelial growth factor receptor 1 (VEGFR1) and α smooth muscle actin as well as staining for alkaline phosphatase were performed after 10 and 14 days. Messenger RNA expression of endothelial cell markers was highly upregulated in both basal and osteoblastic conditions after 5 days of co-culture, indicating an endothelial cell differentiation, which was supported by immunocytochemistry for VEGFR1. Stromal derived factor 1 and vascular endothelial growth factor were highly expressed in MSC-MNC co-culture in basal medium but not in osteoblastic medium. On the contrary, the expression levels of bone morphogenetic protein 2 and angiopoietin 1 were significantly higher in osteoblastic medium. Pericyte markers were highly expressed in both co-cultures after 5 days. In conclusion, we demonstrated endothelial cell and pericyte differentiation in MSC-MNC co-cultures both in basal and osteoblastic medium indicating a potential for neovascularization for tissue engineering applications.

Keywords: Angiogenesis, endothelial cells, mesenchymal stromal cells, mononuclear cells, osteoblastic differentiation, pericytes, tissue engineering

1. Introduction

Angiogenesis is central in bone development and fracture healing, as well as in graft survival after implantation (Muschler *et al.,* 2004). Despite recent advances in bone tissue engineering, efficient bone formation and vascularization remains a challenge for clinical applications. Bone marrow (BM) derived mesenchymal stromal cells (MSCs) are an attractive cell source for osteoprogenitors (Muschler *et al.,* 2004, Asatrian *et al.,* 2015), and therefore the synergistic crosstalk between MSCs and endothelial cells, influencing both angiogenesis and osteogenesis, has gained interest as a potential solution for improved tissue engineered bone constructs.

Endothelial cells are inappropriate for clinical applications as they are not easily accessible and cannot be attained in sufficient quantities. Peripheral blood (PB) mononuclear cells (MNCs) include endothelial progenitor cells (EPCs), which are easy to isolate and can differentiate into blood vessel endothelial cells, and have therefore gained much interest as a potential cell source in tissue engineering (Asahara *et al.,* 1997). EPCs are found among the CD34-positive fraction of PB-MNCs and they have recently been shown to be important for angiogenesis and bone formation during clinical fracture healing (Kuroda *et al.,* 2014).

MSCs stimulate the survival and growth of endothelial cells through production of paracrine signals, including vascular endothelial growth factor (VEGF) (Kasper *et al.,* 2007, Nombela-Arrieta *et al.,* 2011, Guo *et al.,* 2012). MSCs further support blood vessel formation by differentiating into perivascular cells *in vitro* (Goerke *et al.,* 2012) and *in vivo* (Au *et al.,* 2008). Pericytes display a very similar gene expression profile and have similar characteristics as MSCs (da Silva Meirelles *et al.,* 2016) but they have also been reported to express markers such as platelet-derived growth factor receptor (PDGFR) and α smooth muscle actin (α-SMA), which are not considered characteristic for

MSCs (Wong *et al.,* 2015). Pericytes are an interesting cell population for regenerative medicine, as they are reported to support endothelial cells and vascular assembly as well as to differentiate into a variety of mesodermal cell types such as osteoblasts, myocytes and adipocytes having thereby a central role in the regulation of angiogenesis and tissue regeneration (Crisan *et al.,* 2008, Armulik *et al.,* 2011).

Aguirre and co-workers (2010) demonstrated that EPCs and MSCs interact both directly through gap junctions and indirectly through paracrine signalling. Increased expression of proangiogenic factors, such as VEGF and angiopoietin-1 (Ang-1) has recently been shown in the co-cultures of MSCs and EPCs (Aguirre *et al.,* 2010, Rahbarghazi *et al.,* 2013, Li and Wang 2013). It has also been shown that MSCs differentiate into smooth muscle cell/pericyte lineage when co-cultured with EPCs or HUVECs serving as a mural, stabilizing cells (Goerke *et al.,* 2012).

We have previously shown that PB-MNCs can differentiate into endothelial cells in MSC-MNC co-cultures without exogenously supplied growth factors (Joensuu *et al.,* 2011) and data by others has demonstrated that MSCs produce proangiogenic factors, such as VEGF and fibroblast growth factor 2 (FGF-2) in a differentiation-dependent manner (Hoch *et al.,* 2012). On the other hand, our recent results show that in MSC-MNC co-cultures the osteoblastic differentiation of human BM-MSCs is enhanced and further potentiated by exogenous VEGF (Joensuu *et al.,* 2015). Taken together, all this data suggests that MSCs and endothelial progenitors interact in a paracrine manner and can thereby regulate each other's differentiation. The aim of the current study was to evaluate the *in vitro* angiogenic potential of human MSC-MNC co-cultures both in basal conditions and during osteoblastic differentiation. This was performed by assessing the differentiation of endothelial cells and pericytes in the co-culture set-up.

2. Materials and Methods

2.1. MSC culture

Permissions for the use of human MSCs and human blood MNCs were approved by the Ethical Committees of the Hospital District of Southwest Finland and Finnish Red Cross. Human MSCs were isolated from a 5-ml aspirate of iliac bone marrow from a healthy donor. Bone marrow aspirate was mixed with α-minimum essential medium (α-MEM; Gibco) containing 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Gibco) and 20 IU/ml of heparin (Heparin Leo; LEO Pharma A/S) and cells were isolated by density gradient centrifugation (Ficoll Paque Plus, Amersham Pharmacia). Cells were seeded at 1×10^6 cells in a 25 cm^2 tissue culture flasks (Gibco) and cultured in α-MEM containing 10% fetal bovine serum (FBS) and antibiotics, hereafter referred to as basal medium. After culturing for 48 hours, non-adherent cells were discarded and medium was thereafter changed every 3-4 days. Upon confluency, cells were harvested using trypsin/EDTA (Gibco), counted and re-plated at 1000 cells/cm^2. Criteria for identifying MSCs included the phenotype analysis (CD105+, CD73+, CD90+, CD45-, CD14-), proliferation through several passages as well as osteogenic, chondrogenic and adipogenic differentiation capacity as described earlier (Alm *et al.*, 2010). Passage 2 cells were used for the experiments.

2.2. MSC-MNC co-cultures

For MSC-MNC co-cultures, MSCs were plated into 24-well tissue culture plates (2 500 cells/cm^2) and T25-cell culture flasks (1000 cells/cm^2). After 3 days of culturing MSCs in basal medium, peripheral blood samples (average volume 80 ml) were drawn from a healthy volunteer and MNCs were isolated by Ficoll density gradient centrifugation and MNCs were added to the attached MSCs for co-culturing. The experimental setup included four different groups: 1) MSCs in basal medium;

2) MSCs and MNCs in basal medium; 3) MSCs in osteogenic (OB) medium; 4) MSCs and MNCs in OB medium. Each group consisted of three (flasks) or four (wells) parallel samples. MNCs (1 x $10^5/cm^2$) were added to the groups 2 and 4 and OB medium was simultaneously added to the groups 3 and 4. OB medium was basal medium supplemented with 10 mM Na-β-glycerophosphate (Merck) and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich). Dexamethasone (10^{-7} M) was included in the OB medium for the first week. Half of the medium was changed every 4-5 days.

2.3. Real-Time Cell Imaging System (IncuCyte)

MSC-MNC co-cultures were prepared similarly as above and cultured in 24- and 48-well plates for 14 days. Cultures were visualized using a real-time cell imaging system (IncuCyte ZOOM, ESSEN BioScience Inc) to study morphological changes, which reflect cell proliferation, differentiation, migration and tube formation. Images were automatically acquired and registered by the IncuCyte software system and updates were recorded at 2 h intervals.

2.4. Osteoblastic differentiation

Osteoblastic differentiation was analyzed after 2 weeks of culture by fixing the cells in 3% paraformaldehyde and staining for alkaline phosphatase (ALP) according to manufacturer`s instructions (Alkaline Phosphatase kit, 86-R, Sigma-Aldrich). ALP stained areas were quantified using an automated image analysis. The plates were scanned using a flatbed scanner with a transparency adaptor (HP ScanJet 5370C) at 600 dpi resolution and saved as 24-bit color images in TIFF format. Transparency exposure adjustments were maintained constant to create images of equal intensity.

2.5. cDNA synthesis and quantitative real time RT-PCR

For the analysis of mRNA expression levels of various factors and markers, total RNA was isolated at 5 and 10 days of culture according to manufacturer's instructions (GenElute TM Mammalian Total RNA, Sigma-Aldrich). RNA quality was checked by Nanodrop Spectrophotometer (Thermo Fisher Scientific). 200 ng of total RNA was subjected to first-strand cDNA synthesis using MuMLV-H(-) reverse transcriptase and random 8-mers and oligo dT-16 primers (High-Capacity RNA-to-cDNA™ Kit, Thermo Fisher Scientific).

PCR reaction was performed in four parallel samples, where at least three of them were taken into expression analysis depending on the diffraction of Ct-values and the efficiencies of thermal cycling. Specific primers and probes were purchased from ITD (Integrated DNA Technologies Inc) (Supplementary Table 1). Primer concentrations in each PCR-reaction were 0.5 µM and for probes 0.25 µM. PCR was carried out in a solution containing 300 mM of primers and 200 mM of 5′ 6-FAM-labeled probe in 7 µl of Kaba Probe Fast qPCR master mix (Kaba Biosystems), and 3 µl of template cDNA in a final volume of 10 µl. Thermal cycling was performed with Master Mix QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Cycling was initiated with 3 min at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C.

Accumulation of the specific PCR products was detected real-time as an increase in fluorescence. Observed fluorescence was plotted against cycle number to generate amplification plots and to determine CT values, i.e. the cycle numbers at which the fluorescence signal exceeded a CT value of 0.05 relative fluorescence units. Each determination of a CT value was normalized with the CT values of simultaneous measurements of GAPDH expression from the same samples. Relative expression of the gene analyzed (target gene) was estimated using the formula: relative expression = $2^{-\Delta CT}$, where

ΔCT = CT(target gene) − CT(GAPDH). The quantity mRNAs was expressed as percentage of the quantity of GAPDH mRNA after multiplying relative target gene expression by a factor of 100.

2.6. Immunocytochemistry

After 10 and 14 days of culture, cells were fixed as described above. For immunocytochemistry of anti-human α-SMA, the cells were permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA) in PBS for 1h at room temperature (RT) and incubated for overnight at +4 °C with mouse anti-human α-SMA antibody (1:100) (Abcam, #ab7817). Cultures for immunocytochemistry of vascular endothelial cell receptor 1 (VEGFR1) were first treated with 3% H_2O_2 in methanol 10 min RT, washed three times with dH_2O and once with 0.05% Tween-20 in PBS, then blocked with 3% BSA for 30 min at RT, washed with PBS and incubated for overnight at +4 °C with rabbit anti-human VEGFR1 antibody (1:200) (Abcam, #32152). Biotin-conjugated secondary antibodies included goat anti-rabbit antibody (Vector Laboratories) and polyclonal rabbit anti-mouse antibody (DakoCytomation) (1:200 for both). Samples with omitted primary antibodies were used as negative controls. Bound antibodies were detected by Vectastain ABC kit and DAB (diaminobenzidine, 0.52 mg/ml) (both from Vector Laboratories). Samples were examined and photographed under light microscope (Leitz Aristoplan). Positively stained areas were quantified from the images using an automated image analysis, and the stained areas (cm^2) were converted to a percentage of the total area.

2.7. Influence of exogenous VEGF on cell sprouting capacity

Human MSCs and MNCs were prepared and co-cultured and observed using IncuCyte as previously described, but recombinant human VEGF-A at 5 ng/ml (R&D Systems) was added to the co-cultures. Half of the medium supplemented with fresh VEGF-A was changed every 4-5 days. Co-cultures of MSCs and MNCs in basal medium as well as cultures of plain MNCs in OB medium were included as controls. Sprouting cells were quantified from IncuCyte images using ImageJ. Cell counting procedure is explained in more detail in Supplementary Figure 1 (Fig. S1).

2.8. Statistical analyses

In the statistical analyses, paired Student´s t-test was used for pairwise comparisons between different groups. Each culture condition (group) consisted of four replicate samples (wells). Data is presented as mean ± SD and a p-value ≤ 0.05 is considered statistically significant.

3. Results

3.1. Morphological changes reflecting endothelial differentiation are observed in MSC-MNC co-cultures

MSC-MNC co-cultures in basal or OB medium were visualized by IncuCyte imaging system during a culture period of 14 days (Fig. 1A, B). During the first 2-3 days of culture, MNCs adhered on top of the MSC layer and at the same time, they started to sprout and form tube-like structures when cultured in basal medium (Fig. 1A). In contrast, their morphology remained rounded much longer and tube-forming cells were not observed until at the end of the culture period in OB medium (Fig. 1B). Sprouting spindle-shaped cells were quantified with Cell Counter plugin of Image Processing and Analysis Java (ImageJ) after 3, 5, 7, 10 and 14 days of co-culture in 48-well plates and significantly more sprouting tube-forming cells were observed in basal than in OB medium at each time point ($p \leq 0.01$) (Fig. 1C).

3.2. MSCs are differentiated into osteoblasts in MSC-MNC co-cultures

To study osteoblastic differentiation in MSC-MNC co-cultures, ALP mRNA expression was measured by qPCR after 5 and 10 days of culture (Fig. 2A) and cells were stained for ALP after 14 days (Fig. 2B) and positively stained areas were quantified (Fig. 2C). A strong osteoblastic differentiation of MSCs in co-cultures was observed at mRNA level on days 5 and 10 ($p \leq 0.01$ and $p \leq 0.001$, respectively) and at protein level on day 14 ($p \leq 0.001$), when comparing against non-differentiated MSCs. However, due to high level of variation, no significant differences were observed when comparing co-cultures to differentiated MSCs. More detailed study of osteoblastic differentiation in MSC-MNC co-cultures has been previously performed (Joensuu et al., 2015).

3.3. Differentiation of endothelial cells is observed in the MSC-MNC co-cultures both in basal and osteoblastic medium

The expression of VEGFR1 was significantly higher in the co-cultures in basal medium ($p \leq 0.001$) when compared to plain MSC cultures, while for vWF this did not reach the statistical significance (Fig. 3A). However, in OB medium the expression levels for both VEGFR1 and vWF were significantly higher in co-cultures than in MSC cultures ($p \leq 0.01$ for VEGFR1 and $p \leq 0.05$ for vWF, respectively) (Fig. 3A). Immunostaining for VEGFR1 after 10 days of culture (Fig. 3B) also indicated increased expression but no statistically significant differences could be observed in the quantification of VEGFR1-positively stained cells (Fig. 3C), probably due to background staining of MSCs and differentiated osteoblasts.

3.4. Expression profiles of proangiogenic factors differs between the culture conditions

To study proangiogenic factors, mRNA expression of VEGF, Ang-1, stromal-derived factor 1 (SDF-1), placental-like growth factor (PIGF), pleiotrophin (PTN), FGF-2 and bone morphogenetic protein 2 (BMP-2) were studied by qPCR after 5 and 10 days of culture. Interestingly, while endothelial cell differentiation was demonstrated in the co-cultures of both studied culture conditions (Fig. 3A, B), the expression profiles of individual proangiogenic factors seem to be different between the two culture conditions (Fig. 4A, B). Higher expression of SDF-1 and VEGF was observed in the co-cultures in basal medium when compared to co-cultures in OB medium (Figure 4A, B). SDF-1 was expressed significantly higher in MSC-MNC co-cultures in basal medium when compared to MSC-monocultures ($p \leq 0.01$ at day 5 and $p \leq 0.05$ at day 10), while VEGF expression showed no significant difference when compared to MSC-monocultures. However, Ang-1 was expressed at significantly higher levels in osteogenic induction of MSC-MNC co-cultures ($p \leq 0.01$ at day 5 and $p \leq 0.001$ at day 10) while in co-cultures in basal medium no statistically significant difference was observed when

compared to monocultures. PTN expression in the co-cultures was significantly lower in OB medium than in basal medium at day 5 ($p \leq 0.001$, Fig. 4A), while at day 10, the expression level of PTN was higher in the co-cultures in OB medium ($p \leq 0.05$, Fig. 4B) compared to co-cultures in basal medium (no statistical significance). BMP-2 was highly expressed in both co-culture setups, although the expression level was significantly higher in the co-cultures in OB medium ($p \leq 0.001$ at day 5 and $p \leq 0,05$ at day 10) compared to co-cultures in basal medium at both time points ($p \leq 0,05$ at day 5 and n. s. at day 10) (Fig. 4A, B).

3.5. Differentiation of pericytes is observed in the MSC-MNC co-cultures in both culture conditions

Pericyte inducer TGF-β1 and pericyte marker PDGFRβ were highly upregulated in both co-culture setups ($p \leq 0.01$) but no expression was observed plain MSC-cultures in basal medium (Fig. 5A), demonstrating pericyte differentiation of MSCs in the presence of PB-derived MNCs. Immunostaining for α-SMA demonstrated increased expression in co-cultures performed in osteoblastic medium ($p \leq 0.05$) (Fig. 5B and C) but α-SMA expression was also observed in MSC monocultures and no difference was observed in the presence of MNCs.

3.6. Exogenous VEGF enhances cell sprouting capacity in OB medium

We decided to study if exogenous VEGF increases cell sprouting in MSC-MNC co-cultures in osteoblastic conditions. MSCs and MNCs were co-cultured in OB medium supplemented with exogenous VEGF, monitored using IncuCyte, and the quantities of sprouting cells were quantified. As a result, after 14 days of co-culture, there were more sprouting cells in the co-cultures in OB medium with exogenous VEGF, even though this did not reach statistical significance (Fig. 6). Our observation that VEGF and SDF-1 were highly expressed in co-cultures in basal medium, while the

expression levels of BMP-2 and Ang-1 were the highest in the co-cultures in OB medium (Fig. 4A, B) could partially explain these differences in tube-formation and cell sprouting.

4. Discussion

In the current study, we demonstrated simultaneous endothelial cell, pericyte and osteoblastic differentiation in the MSC-MNC co-cultures. The expression profiles of proangiogenic factors differed between the co-cultures in basal and OB medium, even though both led to endothelial cell and pericyte differentiation. The co-culture assay could thus have a potential in tissue engineering when aiming into enhanced angio- and osteogenesis.

VEGF is a key factor in the crosstalk between ECs and MSCs/osteoblasts (Table 1). It is produced by osteoblast-lineage cells and stimulates EC proliferation, migration and formation of capillary-like structures (Gerhardt *et al.*, 2003, Grellier *et al.*, 2009). Also SDF-1 is known to be an important factor in endothelial tube formation and maturation (Stratman *et al.*, 2010). Both of these factors were expressed in the co-cultures in basal medium, but not in OB medium probably explaining the poorer tube-forming capacity of OB-supplemented co-cultures.

Pericytes are an important cell type in stabilizing the blood vessel structures (Table 1), and these cells were observed in the co-cultures in basal as well as in OB medium. MSCs are often referred to as a single cell type, but it has been suggested that they comprise a heterogeneous population of cells with a diverse differentiation capacity (Nombela-Arrieta *et al.*, 2011). Mural pericytes are suggested to be an MSC-subpopulation, although not all MSCs possess pericyte characteristics (Blocki *et al.*, 2013). In our study, immunostaining for α-SMA demonstrated that also MSC monocultures expressed this pericyte marker characteristic to mural cells, indicating the heterogeneity of BM-derived MSCs.

PDGFRβ is another pericyte marker (Table 1), which was expressed in both co-culture conditions. It is important in pericyte recruitment by endothelial cells in angiogenesis (Melchiorri *et al.,* 2014). It has also been shown that MSCs differentiate into smooth muscle cell/pericyte lineage when they are co-cultured with EPCs or HUVECs serving as a mural, stabilizing cells in the tubular structures of ECs (Goerke *et al.*, 2012), thus supporting our data on pericyte differentiation in co-cultures. This is further supported by our observation of a very low level of PDGFRβ and TGF-β mRNA expression in plain MSC cultures.

TGF- β is the main inducer of pericyte differentiation and proliferation (Melchiorri *et al.*, 2014) (Table 1) and it guides the differentiation of MSCs into mural cells (Hirschi *et al.*, 1998). TGF-β was strongly expressed in both co-culture setups, but not in MSC-monocultures, indicating a pericyte induction in the presence of MNC-derived cells. Ang-1 (Table 1) was also strongly expressed in the co-cultures in OB medium, which is an interesting observation, since Ang-1 is known to recruit MSCs to the site of neovascularization (Hirschi *et al.,* 1998). Perivascular CD146-positive cells are reported to express Ang-1, and gene knockdown of Ang-1 in these cells limited their ability to regulate microvessel assembly (Sacchetti *et al.*, 2007).

In addition to MNC-derived EPCs, MSCs are also shown to differentiate into endothelial cells (Table 1). In our previous study, MSCs cultured with exogenous VEGF did not differentiate into endothelial cells (Joensuu *et al.*, 2015) and the sprouting tube forming cells in the co-cultures were positive for TRACP, an enzyme, which is expressed by the cells of monocyte-lineage (Joensuu *et al.*, 2011) indicating that endothelial cells in the MSC-MNC co-cultures probably differentiated from MNC-derived EPCs instead of MSCs. However, we have not fully demonstrated the origin of each mature cell type in the current study. Nevertheless, we believe that these kinds of co-cultures can be performed by using patient specific stem/progenitor cells requiring no exogenous growth factors. This

makes it as a potential set-up for tissue engineering purposes, even without the detailed knowledge of specific cell communication mechanisms.

5. Acknowledgements

Professor Hannu Aro (Orthopedic Research Unit, University of Turku) as well as Professor Hannu Järveläinen (Department of Medical Biochemistry and Genetics, University of Turku) are kindly acknowledged for their valuable comments on this manuscript. The Finnish Microarray and Sequencing Centre is acknowledged for their excellent RT-qPCR analysis service. Permissions for the use of human MSCs and human blood MNCs were approved by the Ethical Committees of the Hospital District of Southwest Finland. This study has been supported by grants from the Turku University Foundation, the Finnish Cultural Foundation (Varsinais-Suomi Regional Fund) and Finska Läkaresällskapet (Einar och Karin Stroems Stiftelse). KJ is a Ph.D. student of the National Doctoral Program of Musculoskeletal Disorders and Biomaterials (TBDP). KJ and LU-K are supported Ph.D. students from Turku Doctoral Program for Molecular Medicine (TuDMM). The funding sources had no involvement in the study.

Conflict of interest. The authors declare that they have no conflicts of interest concerning this article.

6. References

Aguirre A, Planell JA, Engel E. 2010, Dynamics of bone marrow-derived endothelial progenitor cell/mesenchymal stem cell interaction in co-culture and its implications in angiogenesis. *Biochem Biophys Res Commun*, 400: 284-291

Alm JJ, Koivu HM, Heino TJ, Hentunen TA, Laitinen S, Aro HT. 2010, Circulating plastic adherent mesenchymal stem cells in aged hip fracture patients. *J Orthop Res*, 28:1634–42

Armulik A, Genové G, Betsholz C. 2011, Pericytes: developmental, physiological and pathological perspective, problems and promises. *Dev Cell*, 21: 193-215

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. 1997, Isolation of putative progenitor endothelial cells for angiogenesis. *Science*, 275: 964-967

Asatrian G, Pham D, Hardy WR, James AW, Peault B. 2015, Stem cell technology for bone regeneration: current status and potential applications. *Stem Cells Cloning*, 8: 39-48

Au P, Tam J, Fukumura D, Jain RK. 2008, Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood*, 111: 4551–4558

Blocki A, Wang Y, Koch M, Peh P, Beyer S, Law P, Hui J, Raghunath M. 2013, Not all MSCs can act as pericytes: functional in vitro assays to distinguish pericytes from other mesenchymal stem cells in angiogenesis. *Stem Cells Dev*, 22: 2347-2355

Crisan M, Yap S, Casteilla L, Chen C-W, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Péault B. 2008, A perivascular origin for mesenchymal stem cell in multiple human organs. *Cell Stem Cell*, 3: 301-313

da Silva Meirelles L, Malta TM, de Deus Wagatsuma VM, Palma PVB, Araujo AG, Malmegrim KCR, de Oliveira FM, Panepucci RA, Silva WA Jr, Haddad SK, Covas DT. 2015, Cultured human adipose tissue pericytes and mesenchymal stromal cells display a very similar gene expression profile. *Stem Cells Dev*, 24: 2822-2840

Gerhardt H, Golding M, Fruttinger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C. 2003, VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol*, 161: 1163-1177

Goerke SM, Plaha J, Hager S, Strassburg S, Torio-Padron N, Stark B, Finkenzeller G. 2012, Human endothelial progenitor cells induce extracellular signal-regulated kinase-dependent differentiation of mesenchymal stem cells into smooth muscle cells upon cocultivation. *Tissue Eng Part A*, 18: 2395-2405

Grellier M, Bordenave L, Amédée J. 2009, Cell-to-cell communication between osteogenic and endothelial lineages: implications for tissue engineering. Trends Biotechnol, 27: 562–571

Guillotin B, Bourget C, Remy-Zolgadri M, Bareille R, Fernandez P, Conrad V, Amedee-Vilamitjana J. 2004, Human primary endothelial cells stimulate human osteoprogenitor cell differentiation. Cell Physiol Biochem 14: 325-332.

Guo F, Lv S, Lou Y, Tu W, Liao W, Wang Y, Deng Z. 2012, Bone marrow stromal cells enhance the angiogenesis in ischemic cortex after stroke： involvement of Notch signaling. *Cell Biol Int*, 36: 997-1004

Hirschi KK, Rohovsky S, D`Amore PA. 1998, PDGF, TGF-beta and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation into smooth muscle fate. *J Cell Biol*, 141: 805-814

Hoch AI, Binder BY, Genetos DC, Leach JK. 2012, Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. *PloS One*, 7:e35579

Joensuu K, Paatero I, Alm JJ, Elenius K, Aro HT, Heino TJ, Hentunen TA. 2011, Interaction between marrow-derived human mesenchymal stem cells and peripheral blood mononuclear cells in endothelial cell differentiation. *Scand J Surg*, 100: 216-222

Joensuu K, Uusitalo L, Alm JJ, Aro HT, Hentunen TA, Heino TJ. 2015, Enhanced osteoblastic differentiation and bone formation in co-culture of human bone marrow mesenchymal stromal cells and peripheral blood mononuclear cells with exogenous VEGF. *Orthop Traumatol Surg Res*, 101: 381-386

Kaigler D, Krebsbach PH, West ER, Horger K, Huang YC, Mooney DJ. 2005, Endothelial cell modulation of bone marrow stromal cell osteogenic potential. *Faseb*, 19: 665-667

Kasper G, Dankert N, Tuischer J, Hoeft M, Gaber T, Glaeser JD, Zander D, Tschirschmann M, Thompson M, Matziolis G, Duda GN. 2007, Mesenchymal stem cells regulate angiogenesis according to their mechanical environment. *Stem Cells*, 25: 903-910

Keramaris NC, Calori GM, Nikolaou VS, Schemitsch EH, Giannoudis PV. 2008, Fracture vascularity and bone healing: A systematic review of the role of VEGF. *Injury*, S45-S57

Kuroda R, Matsumoto T, Kawakami Y, Fukui, T, Mifune Y, Kurosaka M. 2014, Clinical Impact of Circulating CD34-Positive Cells on Bone Regeneration and Healing. *Tissue Eng Part B Rev*, 20: 190-199

Li Q, Wang Z. 2013, Influence of mesenchymal stem cells with endothelial progenitor cells in co-culture on osteogenesis and angiogenesis: an in vitro study. *Arch Med Res*, 44: 504-513

Melchiorri AJ, Nguyen B-NB, Fischer JP. 2014, Mesenchymal stem cells: Roles and Relationships in vascularization. *Tissue Eng Part B*, 20: 218-228

Mercado- Pimentel ME, Hubbard AD, Runyan RB. 2007, Endoglin and Alk5 regulate epithelial – mesenchymal transformation during cardiac valve formation. Develop Biol 304: 420-432

Muschler GF, Nakamoto C, Griffith LG. 2004, Engineering principles of clinical cell-based tissue engineering. *J Bone Joint Surg Am*, 86: 1541-1558

Nombela-Arrieta C, Ritz J, Silberstein LE. 2011, The elusive nature and function of mesenchymal stem cells. *Nature Rev Mol Cell Biol*, 21: 126-131

Rahbarghazi R, Nassiri B, Khamzraiinia P, Kajbafzadeh A-M, Ahmadi SH, Mohammadi E, Molazem M, Zamani-Ahmadmahmudi M. 2013, Juxtacrine and paracrine interactions of rat marrow-derived mesenchymal stem cells, muscle-derived satellite cells and neonatal cardiomyocytes with endothelial cells in angiogenesis dynamics. *Stem Cells Dev*, 22: 855-865

Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P. 2007, Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell*, 131: 324-336

Stratman AN, Schwindt AE, Malotte KM, Davis GE. 2010, Endothelial-derived PDGF-BB and HB-EGF coordinately regulate pericyte recruitment during vasculogenic tube assembly and stabilization. *Blood*, 116: 4720-4730

Wong S-P, Rowley JE, Redpath AN, Tilman JD, Fellous TG, Johnson JR. 2015, Pericytes, mesenchymal stem cells and they contributions to tissue repair. *Pharmacology & Therapeutics*, 151: 107-120