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Cocultivation of Mesenchymal Stem Cells and Endothelial Progenitor Cells Reveals Antiapoptotic and Proangiogenic Effects

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Stem cells · Regenerative medicine · Proliferation · Apoptosis · Angiogenesis

Abstract
Integrating bioartificial tissues into the host vasculature is a prerequisite for tissue engineering applications. Endothelial progenitor cells (EPCs) display a high angiogenic potential and a low donor-site morbidity, making them ideal for tissue engineering applications. In our study we used a murine EPC cell line (T17b) and rat mesenchymal stem cells (MSCs) for cocultivation experiments. MSCs were cocultured with increasing T17b EPC amounts. Furthermore, MSCs in monoculture were treated with conditioned medium (CM) from T17b EPCs and T17b EPCs were treated with CM from MSCs. Proliferation and apoptosis were quantified with a bromodeoxyuridine ELISA and a DNA fragmentation ELISA, respectively. Osteogenic differentiation was detected with an alkaline phosphatase assay and bone morphogenetic protein-2 ELISA. The production of proangiogenic molecules was measured with a matrix metalloproteinase-3 and vascular endothelial growth factor ELISA as well as nitric oxide assay. We could show that T17b EPCs stimulated MSC proliferation but not vice versa. On the other hand, MSCs promoted the cell survival of EPCs. The growth-inducing and antiapoptotic effects were dependent on heterotypic cell contacts and paracrine mediators. Moreover, proangiogenic growth factors were found in the coculture. Collectively, our results indicate that the coapplication of MSCs and T17b EPCs provides new perspectives for tissue engineering applications.

Abbreviations used in this paper

ALP	alkaline phosphatase
BMP	bone morphogenetic protein
BrdUrd	bromodeoxyuridine
CM	conditioned medium
DM	differentiation medium
EPCs	endothelial progenitor cells
MMP	matrix metalloproteinase
MSCs	mesenchymal stem cells
NO	nitric oxide
PBS	phosphate-buffered saline
VEGF	vascular endothelial growth factor

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Introduction

Despite the advantages in microsurgery, the reconstruction of large-volume tissue defects as a result of trauma or cancer still represents a therapeutic challenge for the plastic surgeon. Autologous tissue transfer is the current gold standard for the treatment of such defects. On the other hand, autologous tissue transfer is limited by the defect size and donor site morbidity. A promising approach to overcoming these problems is the emerging field of tissue engineering. However, adequate vascularization of tissue-engineered constructs is the crucial step for clinical implementation. In the initial phase of the implantation of bioartificial tissues into the host vasculature, oxygen and nutrition supply depends on diffusion. As diffusion is limited to a maximum range of 100–200 µm, various strategies have been developed to improve vascularization [Ko et al., 2007; Rouwkema and Khademhosseini, 2016]. These therapeutic approaches comprise the delivery of angiogenic growth factors [Street et al., 2002; Arkudas et al., 2007; Moimas et al., 2015], the implementation of endothelial cells [Koob et al., 2011], or the microsurgical improvement of the vascularization by the use of arteriovenous loops [Horch et al., 2014; Weigand et al., 2015]. As endothelial progenitor cells (EPCs) display a high angiogenic and vasculogenic potential as well as a low donor site morbidity they represent an interesting cell source for the prevascularization of bioartificial tissues [Asahara et al., 1997; Kupatt et al., 2005b; Yoder et al., 2007; Young et al., 2007]. It is generally accepted that EPCs are recruited from the bone marrow to hypoxic tissues or tumor parenchyma supporting neovascularization [Asahara et al., 1999; Ahn and Brown, 2009]. Based on these findings first efforts have been made to use the angiogenic and vasculogenic potential of EPCs in a therapeutic manner [Folkman, 1998; Kocher et al., 2001; Iba et al., 2002; Kupatt et al., 2005a; Wang et al., 2015]. For the recent study, we used the well-established EPC cell line T17b isolated from mice embryos on day E7.5 [Hatzopoulos et al., 1998]. Endothelial differentiation of T17b EPCs can easily be induced in vitro with retinoic acid and cAMP as previously shown by an upregulation of endothelial marker genes such as thrombomodulin, flk-1, or von Willebrand factor [Hatzopoulos et al., 1998; Bleiziffer et al., 2009]. Moreover, T17b EPCs assume the characteristic cobblestone morphology and form tube-like structures when seeded onto Matrigel as a proof of their endothelial character [Hatzopoulos et al., 1998]. In a previous study we could demonstrate that T17b EPCs proliferate, form tube-like structures, and secrete vascular endothelial growth factor (VEGF) in vitro when seeded into a fibrin matrix [Bleiziffer et al., 2009]. Furthermore, we could demonstrate that T17b EPCs display an increased proliferation rate as well as VEGF-secretion under hypoxic conditions which might be an explanation for their great vasculogenic potential, especially in ischemic tissues [Asahara et al., 1999]. As a next step, we subcutaneously implanted T17b EPCs in a fibrin matrix and found that T17b EPCs support the formation of fibrovascular tissue as indicated by an increased blood vessel quantity and diameter [Bleiziffer et al., 2011].

Mesenchymal stem cells (MSCs) have also gained much interest in the field of regenerative medicine because of their capability of differentiating into multiple cell types, including adipocytes, osteoblasts, chondrocytes, and even endothelial cells [Janeczek Portalska et al., 2012].

T17b EPCs do not express major histocompatibility complex I molecules and therefore can be used as an interesting cell source for xenogenic cell transplantation. Prior a possible in vivo application, we performed cocultures with rat MSCs and T17b EPCs and investigated whether coculturing has supportive effects on proliferation, apoptosis, osteogenic differentiation, and angiogenesis in vitro. Furthermore, we tried to figure out the ideal cell ratio of MSCs and T17b EPCs in order to improve the generation of bioartificial tissues based on both cell types.

Materials and Methods

Cell Culture

MSC Isolation and Cultivation

The isolation of MSCs was approved by the Animal Care Committee of the University of Erlangen and the Government of Mittelfranken, Germany (55.2-2532.1-53/14). MSCs were isolated and characterized by an established protocol [Rottensteiner et al., 2014; Vielreicher et al., 2015]. Briefly, male Lewis rats were sacrificed, the femoral bones were prepared, and the bone marrow was flushed with phosphate-buffered saline (PBS) and fetal calf serum (Biochrom, Berlin, Germany). The cell suspension was centrifuged for 10 min at 15,000 rpm and the cell pellet was resolved in DMEM (Gibco/Life Technologies, Carlsbad, CA, USA) containing 20% fetal bovine serum (Biochrom), 1% penicillin/streptomycin (Gibco Invitrogen) and 1% L-glutamine (Sigma-Aldrich, Schnelldorf, Germany). The cells were filtered through 100-µm cell strainers (BD™, Becton Dickinson, Heidelberg, Germany) and purified by density gradient centrifugation with Histopaque® (Sigma-Aldrich). Thereafter, the cells were seeded in culture flasks at a density of $2.0 \times 10^6/cm^2$ in a humidified atmosphere (37°C; 5% CO_2). After 48 h, the medium was changed and nonadherent cells were washed out. The remaining cells were further cultured until they reached a confluence of 80%. Then, cells were detached from the cell culture dishes with Accutase solution (PromoCell, Heidelberg, Germany) and used until passage 5.

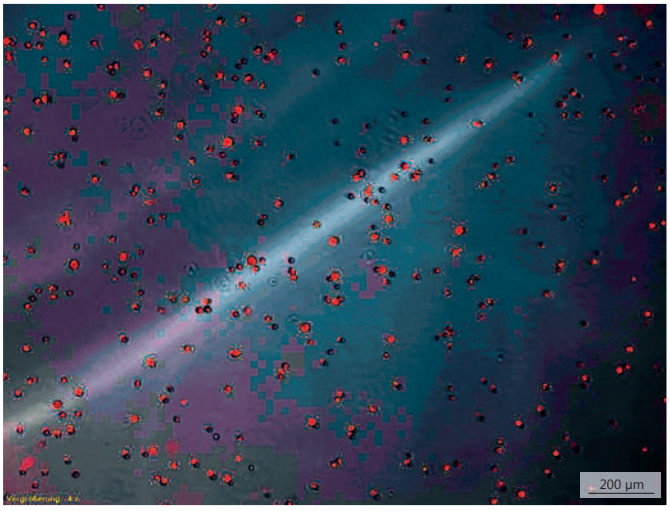

Fig. 1. After 72 h of incubation in DM, T17b EPCs stained positive for DiI-ac-LDL.

T17b EPC Cultivation and Differentiation

Cell culture flasks (Greiner Bio-One, Frickenhausen, Germany) were coated with bovine skin gelatin type B (Sigma-Aldrich, Schnelldorf, Germany) prior to seeding with T17b EPCs. Cell cultivation was performed using a basal medium (BM) containing high glucose DMEM GlutaMAX® (Gibco/Life Technologies), 20% fetal calf serum (Biochrom), 100 U/mL penicillin (Biochrom), 100 µg/mL streptomycin (Biochrom), 1 mM nonessential amino acids (Gibco), 2 mM HEPES buffer pH 7.5 (Gibco), and 0.1 mM 2-mercaptoethanol (Gibco). Endothelial differentiation was induced by supplementing 1 µM all-trans retinoic acid (Sigma-Aldrich) and 0.5 mM dibutyryl cyclic AMP (Sigma-Aldrich) for 72 h, as described by Hatzopoulos et al. [1998]. Endothelial differentiation was confirmed by positive DiI-ac-LDL staining as previously described (Fig. 1) [Brandl et al., 2014].

Experimental Design

In order to differentiate between the effects due to heterotypic cell contacts or paracrinely acting mediators, 2 dimensional cocultures of MSCs and T17b EPCs and monocultures of MSCs or T17b EPCs were performed. To determine the optimal T17b EPC/MSC cell ratio, MSCs were cocultured with 25, 50, and 75% T17b EPCs. Monocultures of MSCs received differentiation medium (DM) or conditioned medium (CM) from T17b EPCs. Vice versa, monocultures of T17b EPCs received DM or CM gained from MSCs. DM was used for mono- and cocultivation experiments. CM was gained from supernatants of T17b EPC or MSC monocultures after 48 h, and supplemented with fresh DM in a 1:1 ratio. We used 1.3×10^4 cells/cm^2 except in the bromodeoxyuridine (BrdUrd) ELISA (5×10^3/cm^2).

BrdUrd ELISA

The effect of T17b EPCs on MSC cell proliferation was investigated using a BrdUrd cell proliferation ELISA. According to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany), cell culture dishes were washed with PBS after each time point (24, 72, 120 h) and BrdUrd labeling solution was added for 24 h. Briefly, an anti-BrdUrd antibody labeled with peroxidase was added and subsequently substrate solution was added. Thereafter, absorbance was analyzed at 450 nm and a reference wavelength of 690 nm with an ELISA reader.

DNA Fragmentation ELISA

To quantify the rate of apoptotic cells a cell death detection ELISA (Roche Diagnostics, Mannheim, Germany) was carried out. Briefly, cells were detached from cell culture dishes after 72 h. Firstly, the cells were centrifuged at 12,000 rpm for 5 min including the supernatants. Thereafter, cell lysis was performed. The resulting supernatants containing the fragmented DNA were transferred onto a microtiter plate coated with an antihistone antibody. Afterwards, a second anti-DNA antibody labeled with peroxidase and the ABTS (2,20-Azino-di[3-ethylbenzthiazolin-sulfonat]) substrate solution were added. Photometric analysis was performed with an ELISA reader at 405 nm and a reference wavelength of 490 nm.

VEGF ELISA

VEGF production was measured using a mouse VEGF Quantikine® ELISA kit (R&D Systems, Wiesbaden, Germany). Briefly, the supernatants of 3 samples per group were collected after 72 h and transferred to an anti-VEGF-coated microplate. VEGF was quantified by measuring the absorbance at 450 nm and reference wavelength of 540 nm with an ELISA reader.

Matrix Metalloproteinase-3 ELISA

Corresponding to manufacturer's instructions (R&D Systems, Wiesbaden, Germany), a Quantikine® ELISA was carried out to quantify the production of matrix metalloproteinase (MMP)-3. Briefly, supernatants of 3 samples per group were gathered after 72 h and transferred to an anti-MMP-3-coated microplate. Thereafter, a second antibody specific for MMP-3 conjugated to horseradish peroxidase and the substrate solution were added. Absorbance was measured at 450 nm and a reference wavelength of 540 nm with an ELISA reader.

Nitric Oxide Assay

Nitric oxide (NO) assay was performed using the improved Griess method. Briefly, cells were detached from cell culture dishes and sonicated. After centrifugation (10,000 rpm), the supernatants were treated with ZnSO4 for deproteination. Colorimetric determination was performed following manufacturer specification (Antibodies-Online Inc., Atlanta, GA, USA). Absorbance was measured at 540 nm with an ELISA reader.

Alkaline Phosphatase Assay

The enzyme activity of the osteogenic differentiation marker alkaline phosphatase (ALP) was measured as described previously [Hager et al., 2009]. Briefly, cells were washed with PBS after 48 h of cell culturing. A lysis buffer was added containing 25 mM of Tris-HCl (pH 8.5) and 0.5% Triton X-100, and cell lysis was conducted by 3 freeze-thaw cycles. Thereafter, CSPD substrate (Applied Biosystems, Forster City, CA, USA) was added to the samples and the luminescence was measured.

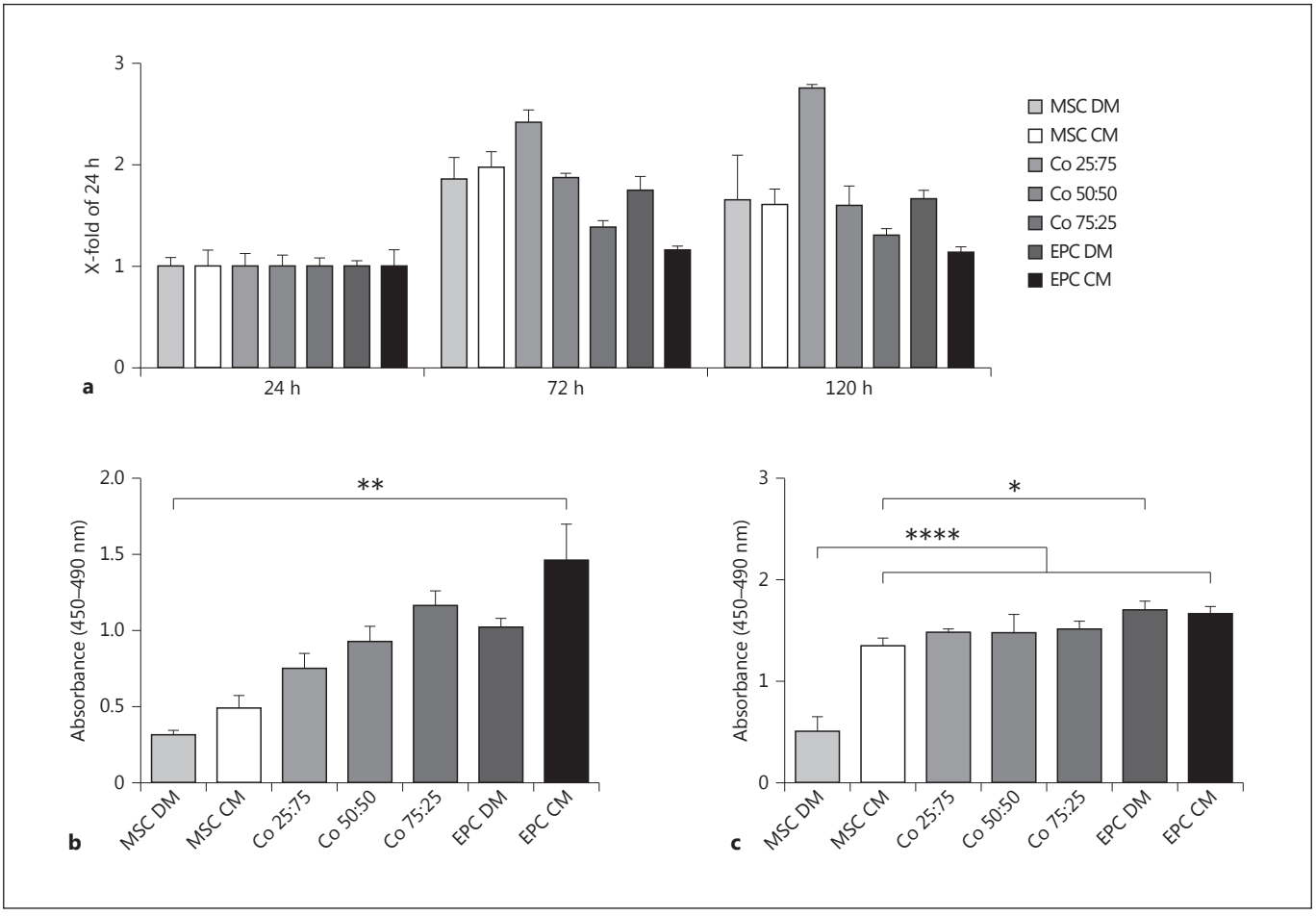

Fig. 2. Proliferation of T17b EPCs and MSCs under mono- and coculture conditions. **a** Proliferation was measured via BrdUrd incorporation and depicted as the relative increase compared to the BrdUrd amount 24 h after cell seeding. The subfigures (**b**, **c**) depict the absolute proliferation rate (shown as absorbance) 24 and 120 h after cell seeding. MSCs and T17b EPCs were grown under monoculture conditions with DM and served as the control groups. MSCs were treated with CM from T17b EPCs and T17b EPCs were treated with CM from MSCs. Cocultures were performed with an increasing T17b EPC cell ratio, beginning with 25% up to 75% (Co 25:75, Co 50:50, Co75:25). Statistically significant differences are indicated as * $p \leq 0.05$, ** $p \leq 0.01$, and **** $p < 0.0001$.

Bone Morphogenetic Protein-2 ELISA

According to the manufacturer's instructions, an immunoassay kit (R&D Systems, Wiesbaden, Germany) was used to quantify the bone morphogenetic protein (BMP)-2 production. Briefly, the supernatants were collected after 72 h of cell culturing and transferred onto a microplate coated with a BMP-2 antibody. Absorbance was measured at 450 nm and a reference wavelength of 540 nm with an ELISA reader.

Statistical Analysis

We first tested for a Gaussian distribution. If the data were normally distributed, a 1-way ANOVA and Bonferroni correction for multiple testing were performed. Otherwise, a nonparametric analysis was performed with the Kruskal-Wallis test and Dunn multiple comparisons test. p values ≤0.05 were considered statistically significant. Data are either shown as mean arbitrary units ± SD or box plots from triplicate determinations. GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis.

Results

Effects of Coculturing T17b EPCs and MSCs on Proliferation

In order to analyze the influence of coculturing T17b EPCs and MSCs on cell proliferation, a BrdUrd ELISA was performed. In general, the BrdUrd incorporation had increased in all groups in relation to the BrdUrd amount at 24 h after cell seeding (Fig. 2a). At that time point

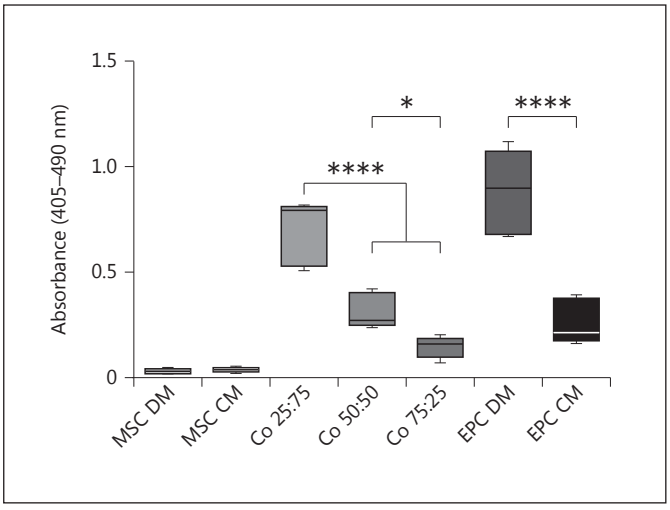

Fig. 3. Apoptosis of T17b EPCs and MSCs quantified by DNA fragmentation ELISA 72 h after cell seeding. MSCs and T17b EPCs were grown under monoculture conditions with DM and served as the control groups. MSCs (MSC CM) were treated with CM from T17b EPCs. T17b EPCs (T17b EPC CM) were treated with CM from MSCs. Cocultures were performed with an increasing T17b EPC cell ratio, beginning with 25% up to 75% (Co 25:75, Co 50:50, Co75:25). Statistically significant differences are indicated as * $p \leq 0.05$ and **** $p < 0.0001$.

(Fig. 2b), T17b EPCs treated with DM or CM, displayed higher proliferation rates compared to the corresponding MSC cultures (MSC DM and MSC CM). Furthermore, there was a statistically significant difference between T17b EPCs treated with CM gained from MSCs and MSCs cultured in DM ($p < 0.01$). In the coculture groups, we could detect higher absolute proliferation rates with an increasing MSC cell ratio after 24 h, although this trend was not statistically significant (Fig. 2b). Interestingly, MSCs displayed a statistically significant lower absolute proliferation rate under monoculture conditions compared to the other groups after 120 h ($p < 0.0001$). Moreover, CM from T17b EPCs seemed to have a strong influence on MSC proliferation but not vice versa ($p < 0.0001$; Fig. 2c).

Effects of T17b EPC and MSC Coculture on Cell Survival

A DNA-fragmentation ELISA was carried out to investigate whether MSCs have an effect on T17b EPC cell survival. For this reason, T17b EPCs and MSCs were treated with CM or grown under coculture conditions for 72 h. As shown in Figure 3, cocultures of MSCs and T17b EPCs displayed a lower apoptosis rate compared to the

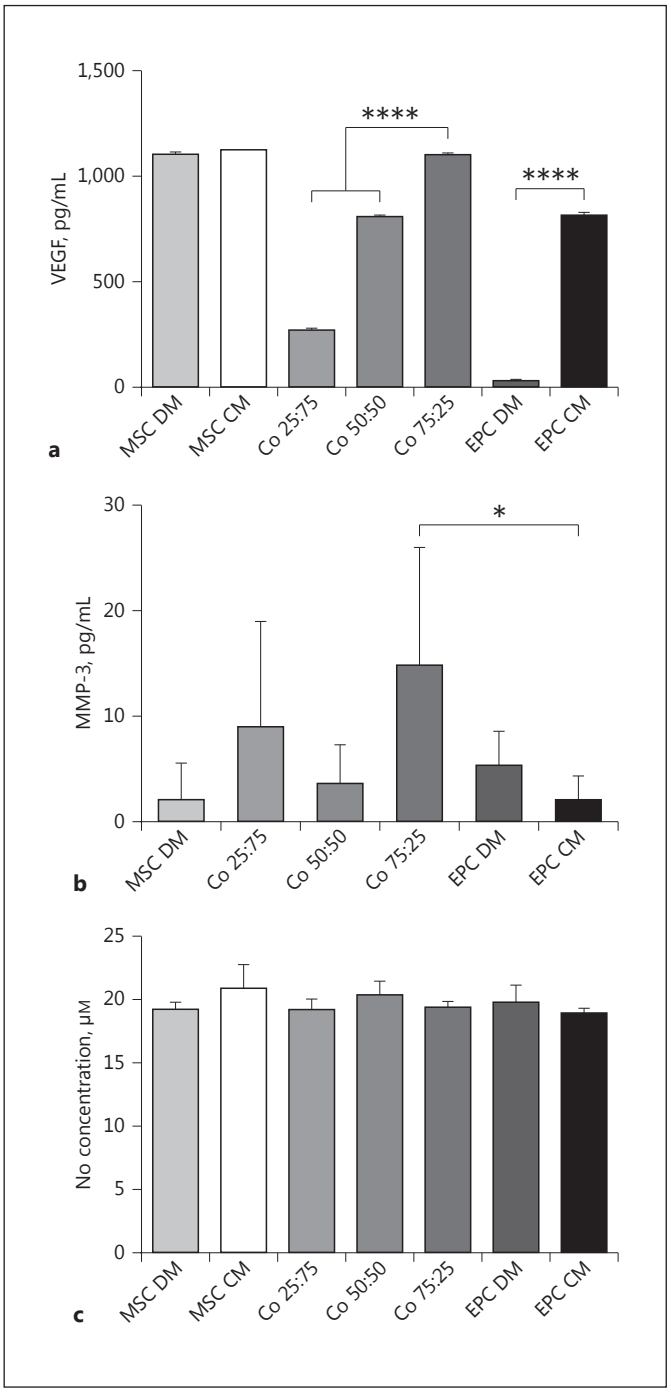

Fig. 4. Measurement of angiogenic factors produced by T17b EPCs and MSCs. Experiments were performed 72 h after cell seeding. MSCs and T17b EPCs were either grown under monoculture conditions with DM or treated with CM. Cocultures were performed with an increasing T17b EPC cell ratio, beginning with 25% up to 75% (Co 25:75, Co 50:50, Co75:25). The concentration of VEGF (**a**) and MMP-3 (**b**) are indicated as pg/mL. **c** The NO concentration was measured using the Griess method and is indicated as µM. Statistically significant differences are indicated as * $p \leq 0.05$ and **** $p < 0.0001$.

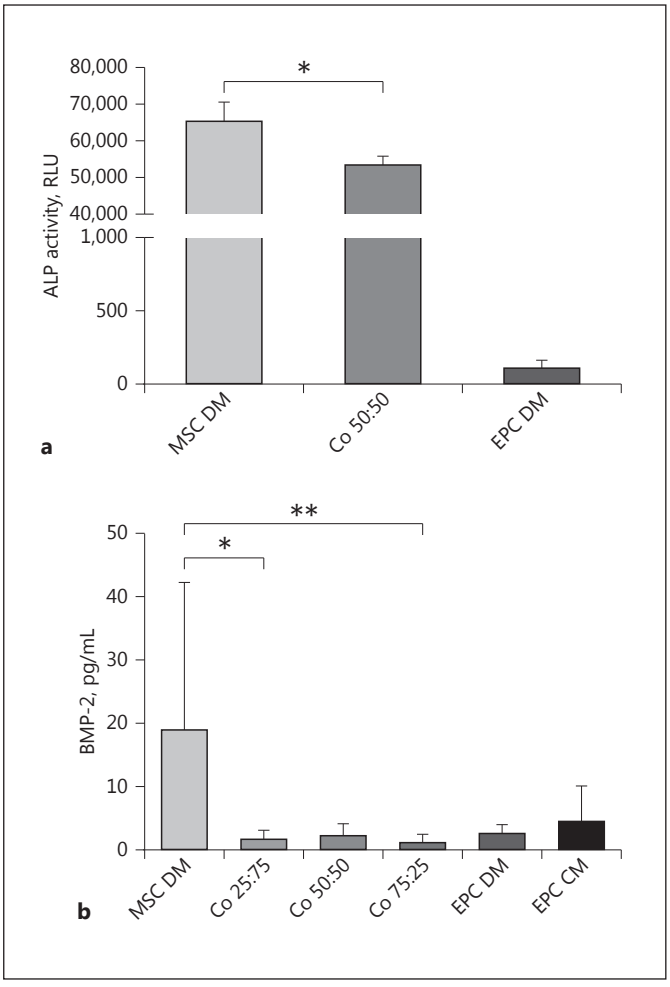

Fig. 5. Quantification of osteogenic differentiation markers of ALP in relative luminescence units (RLU) and BMP-2 (pg/mL). **a** For the ALP assay, MSCs were either grown in monoculture (MSC DM) or in coculture with T17b EPCs (Co 50:50) for 48 h. **b** For BMP-2 ELISA, MSCs were either grown under monoculture conditions (MSC DM) or cocultured with T17b EPCs with an increasing T17b EPC cell ratio, beginning with 25% up to 75% (Co 25:75, Co 50:50, Co 75:25). The BMP-2 ELISA was performed after 72 h. Statistically significant differences are indicated as * $p \leq 0.05$ and ** $p \leq 0.01$.

T17b EPC cultures (T17b EPC DM/CM). Interestingly, this effect was even more pronounced with an increasing MSC cell number in the coculture groups. By using CM from MSCs, the apoptosis rate could be significantly reduced in the T17b EPC monoculture group, whereas the apoptosis rate of MSCs treated with CM from T17b EPCs was unaffected, indicating that MSCs reduced the apoptosis of T17b EPCs through direct cell-cell contacts as well as paracrine-acting soluble mediators.

T17b EPCs and MSCs Produce and Secret Proangiogenic Molecules

As a next step, we investigated the production of factors involved in angiogenesis, namely VEGF, MMP-3, and NO. Under monoculture conditions, the amount of VEGF in MSCs was higher compared to T17b EPCs (Fig. 4a). The treatment with CM from T17b EPCs did not affect the VEGF production in MSCs. On the contrary, T17b EPCs treated with CM from MSCs displayed a higher VEGF amount compared to T17b EPCs treated with DM ($p < 0.0001$). Furthermore, we could detect a cell-dependent increase of VEGF in the coculture groups. It is well known that angiogenesis also involves the degradation of the extracellular matrix. By performing an MMP-3 ELISA, we could measure the highest MMP-3 amount in the coculture group containing 75% MSCs (Fig. 4b). Interestingly, we detected lower amounts of MMP-3 in the T17b EPC group treated with CM from MSCs compared to the untreated T17b EPCs. By using a NO assay, we could prove that all groups produced NO with no relevant differences between each treatment group (Fig. 4c).

Influence of Coculturing T17b EPCs and MSCs on Osteogenic Differentiation

In order to investigate the osteogenic differentiation, we performed an ALP assay as well as a BMP-2 ELISA. As shown in Figure 5, we could not prove a stimulation of the osteogenic differentiation by coculturing MSCs and T17b EPCs. Moreover, we could detect a statistically significant decrease of ALP activity in the coculture group ($p < 0.05$; Fig. 5a). By measuring the production of BMP-2, we could also detect a decrease of this osteoinductive factor in all coculture groups, indicating that T17b EPCs do not stimulate the osteogenic differentiation of MSCs (Fig. 5b).

Discussion

The present study aimed at investigating the interplay between the murine EPC cell line T17b and rat MSCs concerning proliferation, apoptosis, osteogenic differentiation, and angiogenesis. A BrdUrd ELISA was performed to investigate the effect of coculturing MSCs and T17b EPCs on cell proliferation. Our results display a strong effect of T17b EPCs on MSCs proliferation, which is coherent with previous studies demonstrating that endothelial cells as well as EPCs have an impact on MSC proliferation [Steiner et al., 2012; Wen et al., 2016]. This

effect seems to be stimulated by heterotypic cell-cell contacts as well as paracrine-acting mediators if CM was used. We could prove that the proliferation rate of MSCs was highly stimulated by paracrine-acting soluble mediators secreted from T17b EPCs. These results conform with other studies demonstrating that CM from endothelial cells or EPCs enhanced the proliferation of MSCs [Villars et al., 2000; Wen et al., 2016]. Conversely, CM from MSCs had no effect on T17b EPC proliferation. All coculture groups displayed higher proliferation rates compared to the MSCs in the monoculture after 72 h, but we could not detect any differences between the coculture groups and the T17b EPCs. It is alluring to speculate that the growth-supporting effect of the T17b EPCs in the coculture system might be unidirectional and restricted to the MSCs.

Next, we investigated whether MSCs might have an effect on T17b EPC survival. Our data revealed that coculturing MSCs and T17b EPCs has an antiapoptotic effect in the 2-dimensional cell culture. This effect was even more pronounced with an increasing MSC amount in the coculture groups. In this context, gap junctions might play a role in intercellular signaling concerning cell survival in the direct cocultures [Villars et al., 2002; Hager et al., 2009]. Using CM from MSCs, we could prove that MSCs also promote cell survival of T17b EPCs in a paracrine manner. Moreover, we could demonstrate that cocultures of MSCs and T17b EPCs displayed a lower apoptosis rate compared to T17b EPCs cultured in DM (T17b EPC DM). As the apoptosis rate decreases with increasing MSC amounts in the cocultures, MSCs might increase the survival of T17b EPCs.

On the other hand, cell survival of MSCs seemed to be unaffected if CM from T17b EPCs was used. Interestingly, we could prove a relatively high apoptosis rate in the T17b EPC groups. Considering the results of a previous study using MSCs and HUVECs (human umbilical vein endothelial cells), it seems to be a normal phenomenon that endothelial cells display higher apoptosis rates compared to MSCs [Steiner et al., 2012]. Considering the fact that MSCs produced VEGF under mono- or coculture conditions, these antiapoptotic effects might be explained. In our experiments, we have observed an inverse correlation between increasing VEGF amounts and decreasing apoptosis in the coculture groups and the T17b EPCs treated with CM, indicating a strong effect of VEGF on T17b EPC cell survival. Our results are consistent with the pertinent literature, reporting that VEGF is an important survival factor for endothelial cells [Gerber et al., 1998a, b]. These authors demonstrated that the addition of VEGF induced the expression of the antiapoptotic signal molecules Bcl-2 and A1 in HUVECs. Other cell types, such as osteoblasts, also secrete VEGF, thereby regulating the apoptosis by increased BCL-2 gene expression [Street and Lenehan, 2009]. In accordance with the work of Thébaud et al. [2012], we could observe a significant decline of VEGF in the coculture groups containing ≤50% MSCs compared to the MSC monoculture. On the other hand, if 75% MSCs were used in the cocultures with T17b EPCs, we could detect an equal amount of VEGF compared to the MSC monoculture. Previous studies demonstrated that endothelial cells stimulate the gene expression of VEGF in cocultured human osteoprogenitor cells [Grellier et al., 2009; Kolbe et al., 2011]. In our own study, we could not prove that coculturing MSCs and T17b EPCs stimulated the VEGF protein secretion compared to the MSC monoculture, which is in accordance with the results of Kolbe et al. [2011]. However, it is important to bear in mind that the relative gene expression does not necessarily correlate with the level of protein expression. On the other hand, the VEGF production in the MSC monoculture could also be dependent on the selected cell culture medium [Kolbe et al., 2011]. Taken together, our experiments reveal that proliferation and apoptosis seem to be regulated via direct cell-cell interactions as well as paracrine mediators.

As previously demonstrated by Brandl et al. [2014], we could confirm endothelial differentiation of T17b EPCs by DiI-ac-LDL uptake. In order to prove that coculturing MSCs and T17b EPCs stimulate the production of proangiogenic molecules, we additionally performed an MMP-3 ELISA as well as an NO assay. It is well known that angiogenesis is a well-orchestrated process involving a multitude of sequentially coordinated events. One important step is the degradation of the extracellular matrix in order to promote endothelial cell invasion. In this context, the MMPs play an important role through degrading the extracellular matrix [Chang and Werb, 2001; Chen et al., 2013]. As MMP-3 is discussed in the literature as an important protease in angiogenesis [Sage et al., 2003; Jin et al., 2006], we measured the production of MMP-3 in the 2-dimensional cell culture. In this context, we could detect the highest amount of MMP-3 in the coculture group containing 75% MSCs. Interestingly, CM from MSCs reduced the amount of MMP-3 in T17b EPCs (T17b EPC CM), suggesting that the stimulation of MMP-3 production is dependent on heterotypic cell contacts between MSCs and T17b EPCs, and not paracrine-acting mediators. It has been shown that NO is an important signal molecule regulating angiogenesis as well as vascular to-

nicity and thereby the tissue perfusion [Fukumura et al., 2006; Isenberg et al., 2009]. In our experiments, we could detect a constant NO production in all groups without any differences. These results, especially regarding the generation of bioartificial tissues based on T17b EPCs and MSCs, are very promising. Although still speculative, the coimplantation of both cell types could support vascularization through the secretion of VEGF, facilitated sprouting, as well as endothelial cell invasion by MMP-3 release and enhanced perfusion of the surrounding tissue by NO.

Knowing that endothelial cells and EPCs can induce the osteogenic differentiation of MSCs [Thébaud et al., 2012; Fu et al., 2015], we measured the early osteogenic differentiation marker ALP as well as the concentration of the osteogenic growth factor BMP-2. In the pertinent literature, ALP is described as an early osteogenic differentiation marker. In this regard, Hager et al. [2009] could demonstrate that the osteogenic differentiation of osteoblasts was stimulated by coculturing with endothelial cells 48 h after cell seeding. Interestingly, we could detect a statistically significant downregulation of ALP activity in the coculture group. BMP-2 is a well-known osteogenic growth factor that supports osteogenic differentiation as well as bone formation [Betz et al. 2010; Hosogane et al., 2010]. However, concordantly with the results from the ALP assay, the BMP-2 levels have been reduced in the coculture groups suggesting that T17b EPCs inhibit the osteogenic differentiation of MSCs. These results are surprising given the fact that the coimplantation of MSCs and EPCs leads to enhanced vascularization and bone formation in vivo [Usami et al., 2009; Zigdon-Giladi et al., 2015]. Taking into consideration that we chose an EPC cell line, these results might be partially explained. Although described in the literature, the 48- and 72-h time points might be too early for measuring the osteogenic differentiation in our coculture system [Hager et al., 2009]. Furthermore, it could be possible that T17b EPCs induce the differentiation of MSCs into another cell type. In this context, Goerke et al. [2012] demonstrated that MSCs can be differentiated into smooth muscle cells upon cocultivation with EPCs.

Conclusion

Our data reveal that T17b EPCs increase the proliferation rate of MSCs. Furthermore, MSCs reduced the apoptosis of T17b EPCs. These effects seemed to be regulated through heterotypic cell contacts and paracrine-acting factors. In this context, it might be intriguing to investigate whether VEGF secreted from MSCs is the antiapoptotic reagent. As we could prove that T17b EPC/MSC cocultures also produce angiogenic molecules, this coculture system is a promising tool for further in vivo experiments. Taking into account that T17b EPCs inhibited the osteogenic differentiation of MSCs, further studies are required. Our study shows that the ideal coculture contains 25% T17b EPCs and 75% MSCs. This cell ratio displayed a robust proliferation rate, the highest VEGF and MMP-3 secretion, as well as the lowest apoptosis rate.

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Disclosure Statement

The authors declare no conflicts of interest.

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