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ORIGINAL ARTICLE

Establishment of an Early Vascular Network Promotes the Formation of Ectopic Bone

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Vascularization is crucial for the induction of bone formation. In this study, we investigated the application of two subtypes of peripheral blood-derived endothelial progenitor cells (EPCs) to stimulate vessel formation in ectopic bone constructs. Early and late outgrowth EPCs (E-EPC and L-EPC, respectively) were characterized for their ability to form network structures *in vitro* and perfused vessels subcutaneously in mice. Only L-EPCs showed the formation of fully connected networks on Matrigel two-dimensional (2D) angiogenesis assays. The presence of multipotent stromal cells (MSCs) inhibited network formation in 2D assays, but stimulated network formation in three-dimensional plugs. *In vivo* studies revealed that at 2 weeks, the highest incidence of formed perfused vessels was reached by implanted E-EPC/MSC constructs and this could be attributed to the presence of E-EPCs. L-EPCs displayed a significantly lower frequency of blood vessel formation than E-EPCs and this was accompanied by a lowering of total luminal area densities. Nevertheless, combined E-EPC/L-EPC application somewhat increased the percentage incidence of perfused vessels. After 6 weeks, differences in vascularization were still obvious as all three EPC-based constructs contained higher numbers of perfused vessels than constructs containing MSCs alone. Bone was formed in all constructs at an incidence that coincided with high density of perfused vessels after 2 weeks. Altogether, our findings suggest the differential establishment of vascular networks by E-EPCs and L-EPCs and suggest the importance of early vasculogenesis in ectopic bone formation.

Introduction

Prevascularization strategies have been widely addressed in the design of bone replacement constructs as timely vascularization of the constructs is crucial for bone formation. The application of endothelial progenitor cells (EPCs) is one of the favored components of such a graft as they can, directly or indirectly, contribute to vessel formation. Since EPCs were first described,[1] their possible role in stimulation of a vascular bed in and around grafts has been one of the main research focuses in the field of tissue engineering.[2–4] EPCs with vasculogenic potential have been isolated from bone marrow,[5–8] umbilical cord blood,[9–11] and peripheral blood[12,13] and may constitute different cell populations.[14–16] Several EPC subtypes contribute to the formation of blood vessels, either in a direct or indirect manner, but are difficult to characterize as there are no unique markers to identify them with. Expression of endothelial markers, uptake of LDL, lectin binding, and CD34/CD133/VEGFR2 expression, as well as angiogenic network formation *in vitro*, and population doubling capacity may be used to phenotypically distinguish EPCs from mature endothelial cells or from hematopoietic cells.[5,9,12,13,17,18]

Based on *in vitro* growth characteristics, two subtypes of EPCs can be distinguished after isolation; the so-called early outgrowth EPC (E-EPC) and late outgrowth EPC [L-EPC; also known as endothelial colony-forming cell (ECFC)].[9,18,19] E-EPC colonies typically arise within 3–5 days of culture after isolation as a heterogeneous population[9,13,18] and show a spindle-shaped morphology.[1,13,18] Population doublings of E-EPCs are limited depending on the species.[9] In contrast, L-EPC colonies appear around 2–4 weeks after isolation as a more homogeneous population of cells with a cobblestone morphology and high proliferative capacity.[13,17,18] It has been suggested that E-EPCs arise from the myeloid lineage as they express some well-known hematopoietic markers[1,20–23] such as CD14. In addition, the cells express several endothelial markers,[12,23] although CD31 expression subsides upon prolonged culturing.[13] This EPC subtype is able to bind isolectin B4 and take up acetylated LDL.[18,23] Although the primary origin of the L-EPC subtype is still under debate, it has been adopted that the L-EPCs arise from a more distinct endothelial lineage and thus do not express hematopoietic markers,[20,22] whereas they are positive for CD31, which remains present during culturing.[13,17] Furthermore, the E-EPCs show poor/no

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tubule formation *in vitro*,[13,24] whereas L-EPCs show very efficient tubule formation when cultured on Matrigel.[5,13,17] In addition, it is known that E-EPCs secrete high levels of cytokines,[15,18] whereas L-EPCs do not.[12] Despite their limited capacities *in vitro*, E-EPCs have been found to improve neovascularization upon implantation[12,18] through their trophic effects on the more mature endothelial cells.[9,12,25,26] In addition, it has been shown that E-EPCs contribute to the formation of vascular networks synergizing with L-EPCs or with multipotent stromal cells (MSCs).[12,26]

As shown by a number of cell-tracking studies, it has become clear that L-EPCs directly contribute to *in vivo* vascularization[6,13,27] that subsequently leads to long-term engraftment. The use of EPCs in treatment of several vascular problems has increased in the past years,[1,28] including their contribution to bone tissue-engineered constructs.[3] Recent research focused on the engineering of vascularized bone grafts has shown the added value of cell-based constructs to treat bone defects using animal models.[23,29–31] In this, the use of a single EPC subtype in combination with MSCs has shown promising results in terms of bone formation.[23,30] However, differential timing of a vascular network formed by both EPC subtypes separately or combined has not yet been addressed properly in cell prevascularization strategies.[23,29,31] In addition, the subsequent effect of an early induced vascular network on bone formation is not clear. Since sufficient vascularity is essential for bone graft survival, we investigated the application of both E-EPCs and L-EPCs in combination with MSCs to induce vessel formation and subsequent bone formation in ectopic constructs. Due to the differential contribution in the formation of vascular networks by E-EPCs and L-EPCS, we hypothesized that a mixture of E-EPC and L-EPC outperforms single application of both subtypes in terms of vascular network formation and stabilization at an early time point and its effect on bone formation at a later time point.

Materials and Methods

Cell culture

Bone marrow samples were taken from the iliac crest of adult Dutch milk goats and the suspension was filtered through a 70-μm filter mesh and cultured in complete minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA), supplemented with 10% (v/v) fetal calf serum (Cambrex, Charles City, IA), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 0.2 mM L-ascorbic acid-2-phosphate (AsAP) (Sigma-Aldrich, St. Louis, MO). MSCs were obtained by their adhesion to the tissue culture plastic and culture expanded. Passage 1 cells were characterized for their osteogenic differentiation capacity *in vitro* and cryopreserved according to established protocol.[32] Cells were thawed and used at passages 4–6 for all *in vitro* assays and *in vivo* implantation. For this, the medium was refreshed twice a week and cell cultures were maintained in a humidified incubator at 5% CO_2 and 37°C.

E-EPCs and L-EPCs were isolated from peripheral blood samples from adult Dutch milk goats and cryopreserved. Characterization of the cells with respect to marker expression and growth characteristics was described in a previous study[13] and used in view of future translation toward implantation in the goat model.[33,34] In short, mononuclear cells were isolated by Ficoll (Ficoll Paque™ Plus; GE Healthcare Biosciences AB, Diegem, Belgium) density gradient centrifugation and plated in fibronectin-coated [2.5 μg/mL in phosphate-buffered saline (PBS); Harbor Bioproducts, Norwood, MA] flasks in EBM-2 medium supplemented with SingleQuots (Lonza, Basel, Switzerland) and 20% (v/v) fetal calf serum (Cambrex). E-EPCs were obtained within 1 week after isolation and replated in fresh medium twice a week. L-EPCs were obtained within 2–3 weeks after isolation and cultured similarly. For the current study, both EPC subtypes were thawed and expanded for at least six passages in complete EBM-2 medium (Lonza) with 20% (v/v) fetal calf serum (Cambrex) before further use in *in vitro* and *in vivo* experiments.

2D Matrigel network formation assay

To assess network formation by cultured EPCs, angiogenesis assays were performed. For this, growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) was thawed overnight on ice and 50 μL aliquots were transferred to a 96-well plate with a flat bottom and incubated for 1 h at 37°C; 10^4 cells in total were seeded on the surface of Matrigel discs and incubated in complete EBM-2 medium (Lonza) supplemented with 20% (v/v) fetal calf serum (Cambrex). Cells were cultured in a humidified incubator at 5% CO_2 and 37°C. Pictures were taken after 23 h of incubation to evaluate the formation of networks.

3D Matrigel tubular network formation assay

To evaluate tubular network formation in three-dimensional (3D) Matrigel plugs *in vitro*, 10^6 cells in total were encapsulated in 80 μL growth factor-reduced Matrigel (BD Biosciences) and incubated in complete EBM-2 medium (Lonza) containing 20% (v/v) fetal calf serum (Cambrex) for 2 weeks in a humidified incubator at 5% CO_2 and 37°C, refreshing media twice a week. Plugs consisted of (1) L-EPC; (2) E-EPC; (3) L-EPC/E-EPC (ratio 1:1); (4) L-EPC/MSC (ratio 1:1); (5) E-EPC/MSC (ratio 1:1); and (6) L-EPC/E-EPC/MSC (ratio 0.5:0.5:1). Samples that also contained MSCs were incubated in complete EBM-2 mixed with complete α-MEM at a ratio 1:1. After incubation, plugs were embedded through alcohol dehydration series in paraffin and 5-μm-thick sections were stained with hematoxylin.

Preparation of constructs for in vivo *implantation*

E-EPCs, L-EPCs, and MSCs were evaluated for their effect on vessel formation and bone formation after 2 and 6 weeks. To this end, five groups were formed, consisting of 125,000 MSCs (MSC1), 250,000 MSCs (MSC2), a combination of 125,000 MSCs with either 125,000 E-EPCs (E-EPC/MSC) or 125,000 L-EPCs (L-EPC/MSC), and 125,000 MSCs with a total of 67,500 early and 67,500 late EPCs (E-EPC/L-EPC/MSC). MSCs were included in all constructs as they are necessary to induce bone formation, one of the primary outcome parameters. All constructs, for week 2 and week 6 analysis, consisted of cells embedded in 200 μL growth factor-reduced Matrigel (BD Biosciences), supplemented with 20% (w/v) of biphasic calcium phosphate (BCP) particles (0.5–1 mm Ø, BCP-1150; Xpand, Bilthoven, The Netherlands). Constructs were prepared and kept in a humidified incubator at 5% CO_2 and 37°C overnight before subcutaneous implantation.

Subcutaneous construct implantation in nude mice

Twenty-three female nude mice (Hsd-cpb:NMRI-nu; Harlan, Boxmeer, The Netherlands) were anesthetized with 2.0–2.5% isoflurane, after which the implants were placed in five separate subcutaneous pockets in the shoulder and hind limb areas and one on the back, according to a randomized block design. Each animal received all constructs. The animals were postoperatively treated with the analgesic, buprenorphine (0.05 mg/kg, sc; Temgesic; Schering-Plough/Merck, Whitehouse Station, NJ), and housed together at the Central Laboratory Animal Institute, Utrecht University. Experiments were conducted with the permission of the local Ethics Committee for Animal Experimentation and in compliance with the Institutional Guidelines on the use of laboratory animals.

Implant retrieval and embedding

During surgery, one of the mice included for analysis of vascularization (week 2) died unexpectedly. Four animals died later in the experiment due to nonexperiment-related infections. At 2 weeks ($n = 14$ animals) and 6 weeks ($n = 5$ animals) after implantation, the constructs were retrieved to analyze the formation of vessel networks and bone. Week 2 explants were retrieved, fixed overnight in 4% (v/v) formalin, and subsequently processed through alcohol dehydration series for 5-μm-thick paraffin sections, without prior decalcification. All week 6 samples were processed for polymethylmethacrylate (MMA) embedding and sectioned to analyze vessel formation and bone formation.

Evaluation of vessel formation after 2 weeks in vivo

Goldner's trichrome staining was performed to analyze vessel formation in the week 2 constructs. Samples were blinded and two independent observers identified three different categories of formed structures as follows: grade 1) individual cells and/or the presence of microluminal structures, grade 2) partially connected networks, or grade 3) erythrocyte-perfused vessels. Observed morphologies were scored in three randomly chosen fields of view per section and two sections per sample were evaluated. Scorings were expressed as category incidence per group ($n = 14$) and results given as mean ± standard deviation (SD).

Immunohistochemistry of CD31/PECAM-1

To evaluate the contribution of the host and donor cells to the formed vessels in the constructs after 2 weeks, 5-μm-thick paraffin sections were processed for antigen retrieval as follows: rehydrated sections were incubated in 0.1 M sodium citrate solution (pH 6.0) for 20 min at 95°C. Sections were then blocked in 3% (v/v) H_2O_2 in PBS for 15 min and 5% (w/v) bovine serum albumin (BSA) for 1 h at room temperature. Thereafter, the sections were stained for mouse-specific CD31/PECAM-1, which does not cross-react with goat endothelium in goat tissue control stainings. Primary rabbit-anti-CD31 (LifeSpan Biosciences, Seattle, WA) was incubated at 2 μg/mL in 5% (w/v) BSA overnight at 4°C. After three washes with PBS-Tween20 (0.1% v/v), secondary goat-anti-rabbit biotinylated antibody (0.6 μg/mL; DakoCytomation, Glostrup, Denmark) and streptavidin-peroxidase (1.4 μg/mL; DakoCytomation) were incubated, each for 30 min at room temperature. The staining was developed with diaminobenzidine (DAB), and Mayer's hematoxylin was used for counterstaining. Negative controls were treated similarly, except for exclusion of the primary antibody. Since a goat-specific CD31 antibody is not available at present for immunohistochemical analysis on paraffin, and all mouse blood vessels in adjacent tissue could be identified by the rabbit-anti-mouse CD31 mentioned above, unstained vessels and microluminal structures (based on morphology and the presence of erythrocytes) in the same sections were supposed to be goat derived.

Quantification of luminal area densities

Luminal areas of the formed vessel structures, erythrocyte perfused or not, were measured in four randomly chosen fields per construct of Goldner's trichrome-stained sections. All constructs, regardless of their scoring grade, were subjected to this quantification. Using Adobe Photoshop CS5, areas could be calculated as [vessel area/(total area − BCP scaffold area)] ×100% and expressed as a percentage of the total area per region of interest for each group. Data are expressed as mean ± SD ($n = 14$).

Evaluation of vessel formation and bone formation after 6 weeks

MMA-embedded sections of 10 μm were cut (Leica, Nussloch, Germany) and stained by basic fuchsin/methylene blue to evaluate vessel formation and bone formation using a light microscope (Olympus-BX50; Olympus, Zoeterwoude, The Netherlands). All vessels were counted based on their morphology, erythrocyte perfused or not, in four randomly chosen fields/construct. Data are expressed as mean ± SD ($n = 5$ per group).

Bone apposition could be observed based on morphology and bright pink staining lining the borders of the BCP particles in the constructs. Bone incidence was scored as the percentage of constructs that showed any bone formation calculated from all constructs within one group.

Statistical analysis

Statistical analysis was performed with SPSS 20.0 software. A randomized one-way ANOVA with a Tukey correction was applied to the data shown in Figures 3, 5, and 6. For Figure 7, Fisher's exact test with Bonferroni correction was applied to test significant differences in bone incidence between groups. *p*-Values less than 0.05 were considered statistically significant.

Results

In vitro *2D network formation by EPCs*

When E-EPCs were seeded in two-dimensional (2D) Matrigel angiogenesis assays, single cells were observed and no networks were formed after 23 h of incubation (Fig. 1a). The seeding of L-EPCs showed interconnected networks after 23 h (Fig. 1b). Combining E-EPCs with L-EPCs in a 1:1 ratio abrogated the effect of seeding L-EPCs alone, leading to clusters of cells being formed after 23 h of incubation (Fig. 1c). The addition of MSCs to E-EPCs resulted in cluster formation with some observed sprouting from the clusters (Fig. 1d). A similar phenomenon was observed when MSCs were added to L-EPCs (Fig. 1e) or to the combination of E-EPCs/L-EPCs (Fig. 1f).

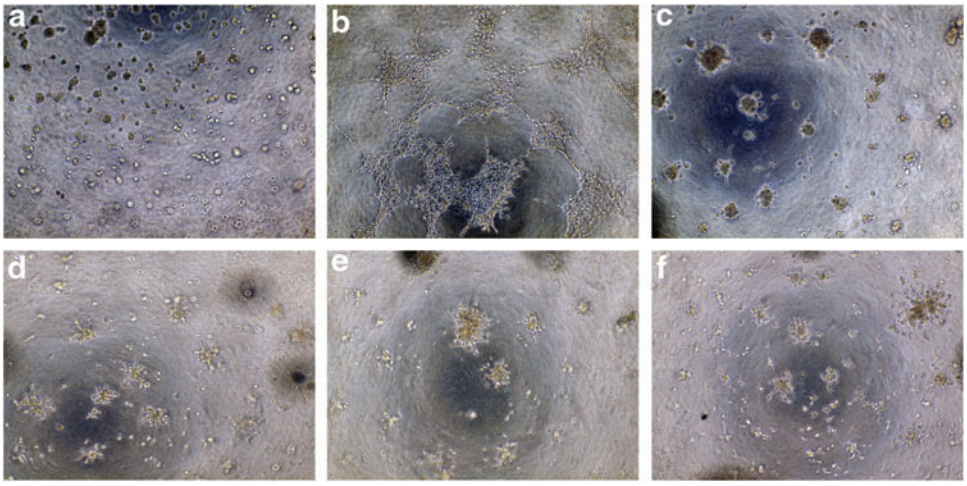


FIG. 1. *In vitro* 2D Matrigel assay. Goat E-EPCs showed no formation of networks in a 2D angiogenesis assay **(a)** in contrast to goat L-EPCs **(b)**. Combination of both EPC subtypes resulted in clusters of cells **(c)** rather than network formation. The addition of goat MSCs to either early EPCs **(d)** or late EPCs **(e)** showed cell clusters with some sprouting. Combining all three cell types showed a similar effect **(f)**. 2D, two-dimensional; EPC, endothelial progenitor cell; E-EPC, early outgrowth EPC; L-EPC, late outgrowth EPC; MSC, multipotent stromal cell. Color images available online at www.liebertpub.com/tea

3D network formation in Matrigel

To assess the effect of a 3D environment on network formation, cells were embedded in Matrigel and assessed for network formation after 2 weeks of incubation. In this study, mostly single cells were observed when E-EPCs (Fig. 2a) or L-EPCs (Fig. 2b) were embedded in the gel. Coculture at a 1/1 ratio of both EPC subtypes resulted in groups of cells spread throughout the gel, although no connections were found (Fig. 2c). Addition of MSCs to E-EPCs (Fig. 2d) or L-EPCs (Fig. 2e) showed a similar phenomenon compared with E-EPC/L-EPC, with groups of cells scattered throughout the gel. When all three cell types were combined, partially connected networks could be observed (Fig. 2f). This indicates that in contrast to what was seen in the 2D network formation assays, MSCs have a beneficial effect on network formation in 3D settings.

Vessel formation after 2 weeks in vivo

According to established protocols,[35] formed vessel networks were scored based on morphology after 2 weeks of implantation. Three distinct morphology structures could be observed (Fig. 3a–c), and the mean percentage of constructs that showed mature erythrocyte-perfused vessels (grade 3) per group was determined. The number of constructs containing both E-EPCs and MSCs that showed mature erythrocyte-perfused vessels was significantly higher when compared with MSC1, L-EPC/MSC, and E-EPC/L-EPC/MSC groups (Fig. 3d), this at the expense of grade 1 structures (data not

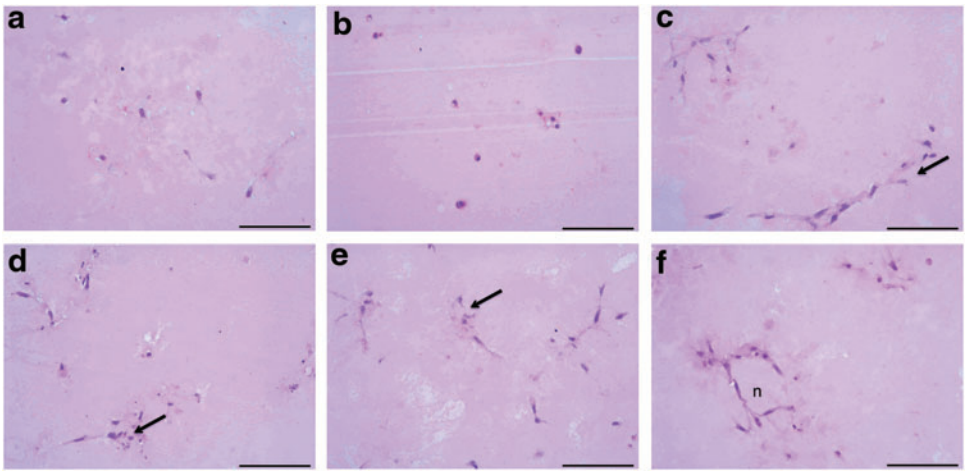


FIG. 2. *In vitro* 3D tubule formation assay. Embedding early EPCs **(a)** or late EPCs **(b)** in Matrigel plugs resulted in the presence of individual cells, whereas a combination of both EPC subtypes showed more groups of cells **(c)**. In addition, combined seeding of early EPCs with MSCs **(d)** or late EPCs with MSCs **(e)** showed the presence of groups of cells, indicated by the *arrow*. In contrast to 2D assays, combining both EPC subtypes with MSCs showed more partially connected networks, indicated with ''n'' **(f)**. Scale bars represent 100 μm. 3D, three-dimensional. Color images available online at www.liebertpub.com/tea

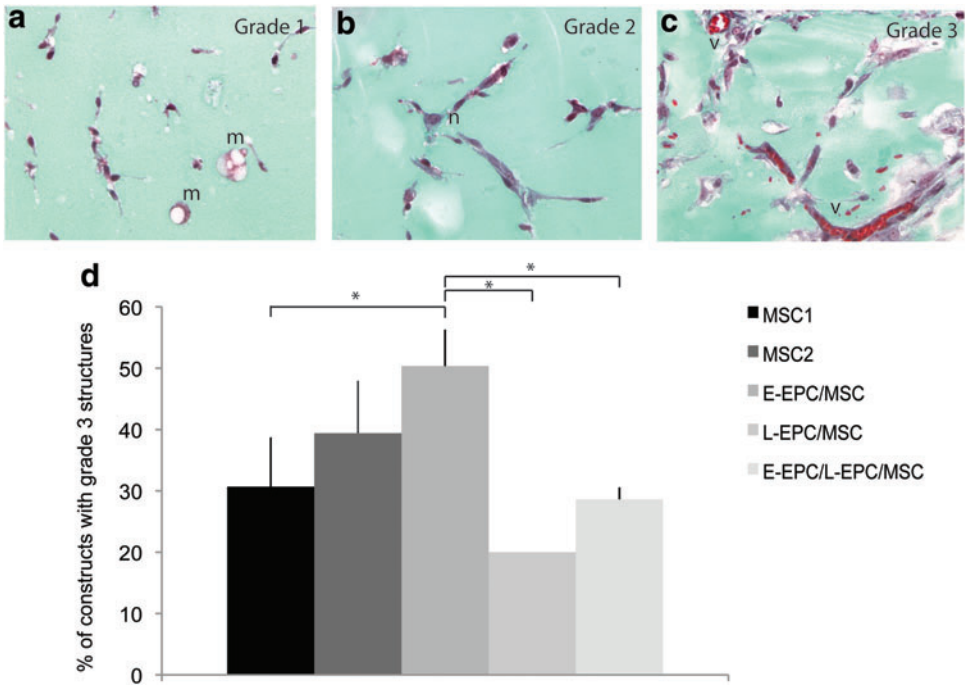


FIG. 3. Evaluation and scoring of vessel network formation after 2 weeks of implantation. In Goldner's trichrome-stained sections, three grades of vessel network formation could be observed **(a–c)**. Grade 1 showed the presence of individual cells and/or the formation of microluminal structures **(a)**. Grade 2 structures showed more partially connected networks **(b)**. Erythrocyte-perfused vessels were shown by grade 3 structures **(c)**. The mean percentage of constructs that showed perfused vessels is represented in **(d)** (of 14 constructs per group). Significantly highest percentages were observed in the E-EPC/MSC group when compared with MSC1, L-EPC/MSC, and E-EPC/L-EPC/MSC groups. No differences were observed between MSC2 and E-EPC/MSC groups. m, microluminal structure; n, partially connected network; v, erythrocyte-perfused vessel; $*p < 0.05$. Color images available online at www.liebertpub.com/tea

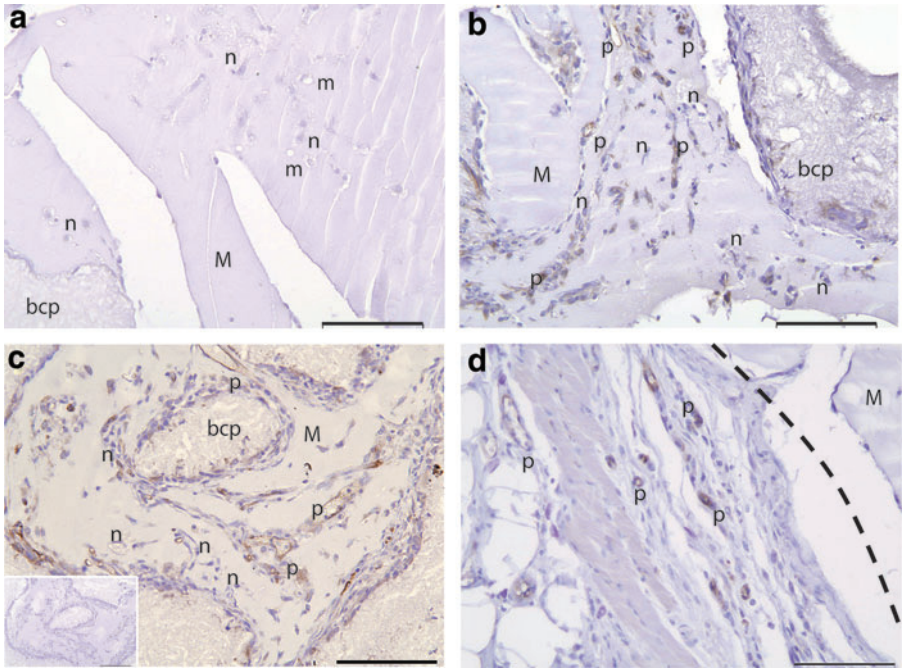


FIG. 4. Evaluation of the endothelial identity of formed vessel structures. In constructs showing the presence of individual cells and/or the formation of microluminal structures, no mouse-specific CD31/PECAM-1 staining was observed **(a)**. Constructs that showed more partially connected networks **(b)** or erythrocyte-perfused vessels **(c)** showed abundant positive CD31/PECAM-1 expression (p) throughout the constructs, as well as some donor-derived unstained structures (n). A representative negative control staining is shown by the inset of **(c)**. As a positive control, vessels in the host mouse skin (p), separated by the *dashed line*, were positive for CD31/PECAM-1 **(d)**. m, microluminal structure; M, Matrigel; bcp, biphasic calcium phosphate. Scale bars represent 100 µm. Color images available online at www.liebertpub.com/tea

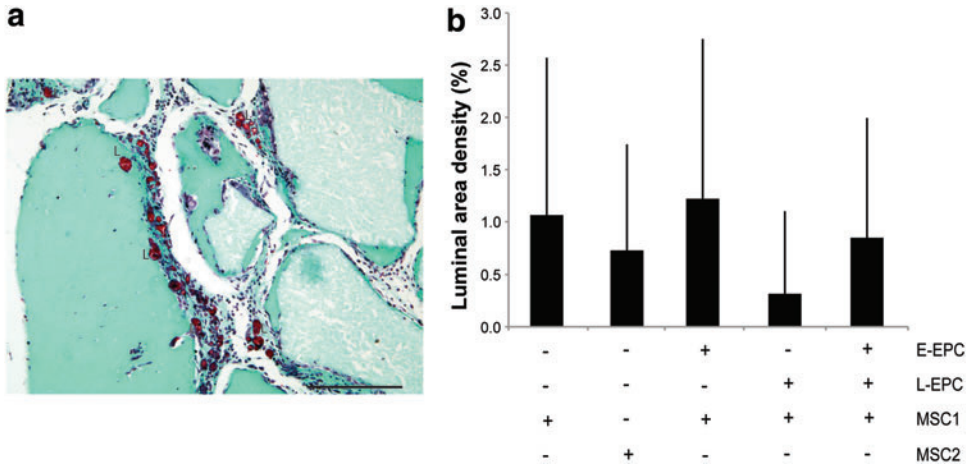


FIG. 5. Quantification of luminal area densities formed after 2 weeks of implantation. The luminal area densities found in the constructs regardless of their grade scoring, represented by L **(a)**, were quantified and showed no significant differences between all five groups **(b)**. L-EPC/MSC-containing groups showed a trend of lower luminal areas compared with all groups. L, luminal area. Scale bars represent 200 μm. Data are expressed as mean ± SD. SD, standard deviation. Color images available online at www.liebertpub.com/tea

shown). There were no significant differences observed between MSC2 and E-EPC/MSC groups (Fig. 3d). In addition, there was no additive effect in terms of the formation of mature erythrocyte-perfused vessels through application of L-EPCs or both E-EPCs and L-EPCs to MSC1 groups as the mean percentage of constructs that showed grade 3 structures was similar in all three groups (Fig. 3d). Application of a combination of E-EPCs, L-EPCs, and MSCs showed a trend of increase of the incidence percentage of grade 3 structures when compared with the L-EPC/MSC group, which may suggest an earlier induction of vascular networks through the addition of E-EPC rather than L-EPC to MSC-based constructs. The latter is supported by the significant highest incidence percentage of grade 3 structures found in the E-EPC/MSC group. Furthermore, the contribution of both donor cells of goat origin and mouse host cells to vessel formation was evaluated by mouse-specific CD31 expression throughout the constructs. In general, we could not observe any positive CD31 expression in grade 1 structures (Fig. 4a), which indicates that the goat donor cells formed the observed microluminal structures. However, when grade 2 or grade 3 structures were present (Fig. 3b, c, respectively), both CD31-positive (i.e., mouse) and -negative vessels (donor derived) could be observed (Fig. 4b, c). This indicates ongoing angiogenesis by host cells as well as vasculogenesis by donor cells in the constructs, the latter concluded from vessels that were not stained by the mouse-specific CD31 antibody since all vessels in mouse host skin were stained positive using this CD31 antibody (Fig. 4d).

Quantification of luminal area densities after 2 weeks of implantation

The total luminal area in the constructs was calculated, including vessels with or without visible blood perfusion, because erythrocytes are frequently lost during processing of the

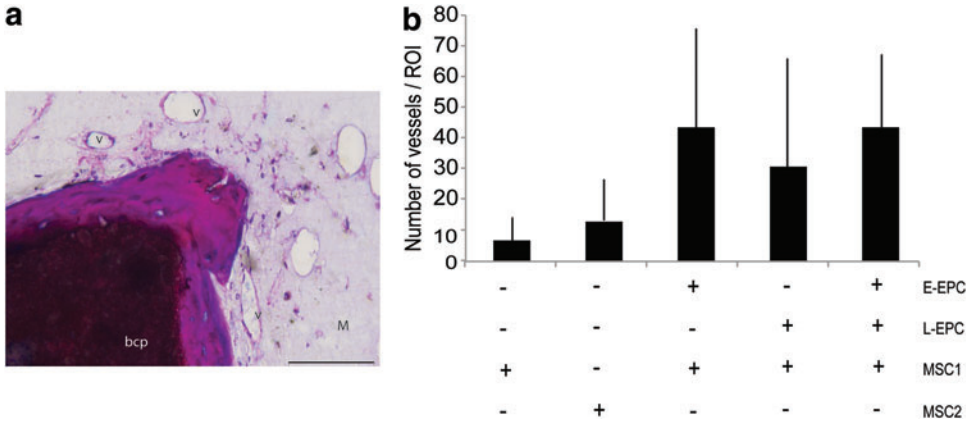


FIG. 6. Quantification of the number of formed vessels after 6 weeks of implantation. After 6 weeks of implantation, formed vessels could be observed in the constructs based on morphology **(a)**. Quantification of the number of formed vessels showed a trend of higher numbers of vessels in all combination groups compared with MSC only groups **(b)**. v, vessel; M, Matrigel; bcp, biphasic calcium phosphate. Scale bar represents 100 μm. Data are expressed as mean ± SD. Color images available online at www.liebertpub.com/tea

samples. The L-EPC/MSC group showed a trend of lower luminal area densities than the E-EPC/MSC group (Fig. 5b), which is in accordance with the lower percentage of vessels found in these constructs (Fig. 3c). E-EPC/L-EPC/MSC constructs revealed increased luminal area densities when compared with the L-EPC/MSC group (Fig. 5b), which supports the increased observed grade 3 structures in the E-EPC/L-EPC/MSC group compared with L-EPC/MSC group (Fig. 3c).

Quantification of the number of vessels after 6 weeks

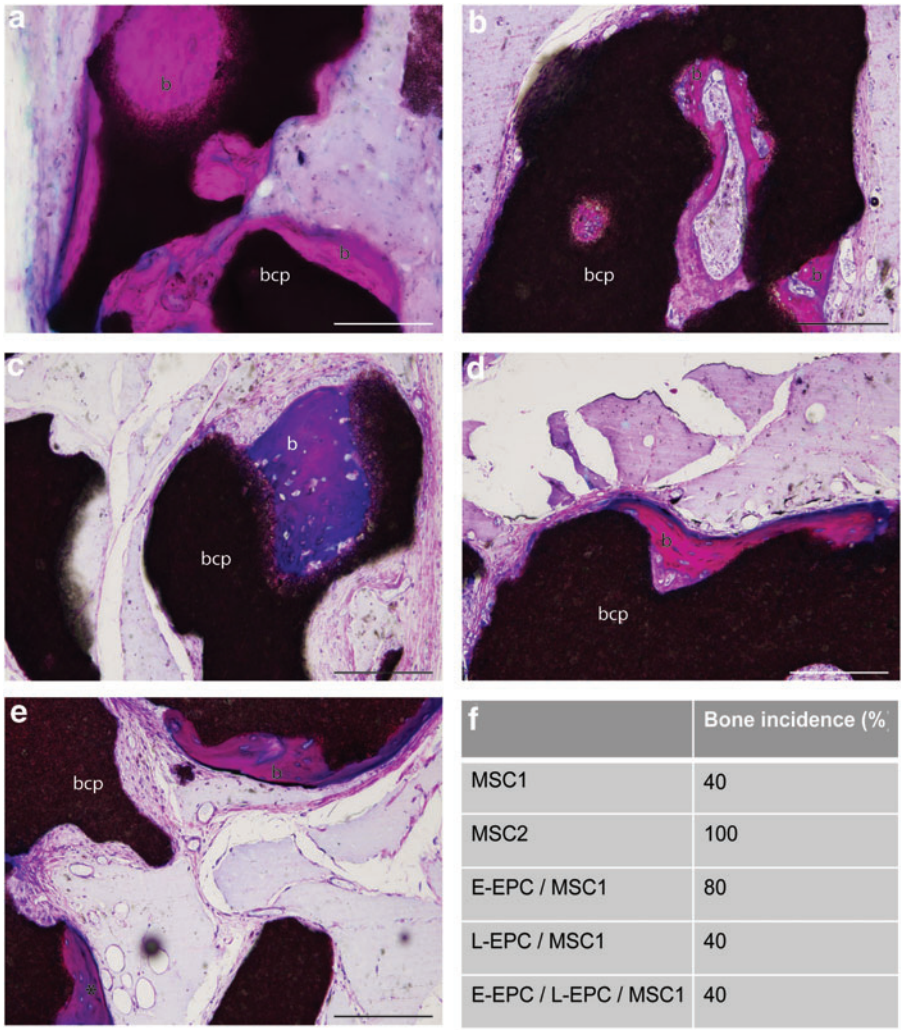
To address vascularization at a later time point, the number of vessels that were present in the constructs after 6 weeks were quantified based on morphology (Fig. 6a). Whereas groups containing MSCs alone showed induction of vasculogenesis after 2 weeks of implantation, the number of vessels after 6 weeks in MSC1 and MSC2 groups appeared lower than in the combination groups (Fig. 6b). Again, high numbers of vessels were observed in the E-EPC/MSC group (Fig. 6b). Moreover, whereas combination constructs that included L-EPCs hardly showed vessel formation after 2 weeks of implantation (Fig. 3d), the number of vessels found in the L-EPC/MSC group after 6 weeks was clearly enhanced at this time point (Fig. 6b). The E-EPC/L-EPC/MSC group tended to show a slight increase in the number of vessels after 6 weeks when compared with the L-EPC/MSC constructs (Fig. 6b), which indicates a possible differential timing of vasculogenesis induction by E-EPCs and L-EPCs.

Bone formation after 6 weeks

To obtain more knowledge of the effect of early and late vascularization on ectopic bone formation, constructs were evaluated for the deposition of bone after 6 weeks. In all five different groups, bone formation was detected at this time point (Fig. 7a–e). In groups that showed a high density of perfused vessels after 2 weeks, the MSC2 and E-EPC/MSC groups, bone formation was observed in 100% and 80% of the animals, respectively (Figs. 3 and 7f), whereas the other groups showed lower bone incidences (Fig. 7f).

Discussion

In this study, we investigated the separate and combined effects of the induction of early and late vascularization on ectopic bone formation. Using two subtypes of peripheral blood-derived EPCs, E-EPCs and L-EPCs, we have shown their network-forming capacity *in vitro* in both 2D and 3D settings. We showed that E-EPCs were considerably less efficient in the formation of networks when seeded on Matrigel discs, whereas L-EPCs showed good network formation,



f	Bone incidence (%
MSC1	40
MSC2	100
E-EPC / MSC1	80
L-EPC / MSC1	40
E-EPC / L-EPC / MSC1	40

FIG. 7. Evaluation of bone formation after 6 weeks. Basic fuchsin/methylene blue staining revealed bone formation in *pink/purple* in MSC1 **(a)**, MSC2 **(b)**, E-EPC/MSC1 **(c)**, L-EPC/MSC1 **(d)**, and E-EPC/L-EPC/MSCs **(e)** groups. Bone incidence is represented in the table **(f)**. bcp, biphasic calcium phosphate; b, bone. Scale bars represent 200 μm. Color images available online at www.liebertpub.com/tea

which is in accordance with other studies.[9,12,13,18] Combining MSCs with L-EPCs abrogated network formation when cultured on Matrigel discs, likely due to physical interference. In contrast, in 3D settings, combined MSC/EPC seeding outperformed single EPC treatment in terms of network formation; an observation that we have reported previously.[13] In addition, other studies have shown that combining MSCs with L-EPCs[5,6,13] reinforces the cells' network-forming capacities and enhances osteogenic differentiation by MSCs *in vitro*. In addition, numerous groups have also shown the reinforcement of the vasculogenic potential of EPCs by MSCs *in vivo*.[6,11,23,30] The additive effect of combined EPC/MSC treatment versus the single addition of MSCs on the induction of vascularized bone *in vivo* has been shown,[6,23,31] although no discriminations between E-EPCs and L-EPCs have been made. A few studies have used single administration of E-EPCs[1,8,12,18] or L-EPCs[12,18,27] to effectively stimulate network formation *in vitro* and/or vascularization *in vivo*. To our knowledge, only one study[12] has shown the formation of luminal networks by combining both EPC subtypes *in vivo*, although the subsequent effect on bone formation was not addressed. Therefore, we performed *in vivo* subcutaneous implantations in mice using two EPC subtypes in combination with MSCs to induce vascularized ectopic bone formation. Whereas Yoon *et al.* showed an additive vasculogenic effect by combining E-EPCs with L-EPCs compared with either cell type alone, in our study, the E-EPC/MSC group outperformed both the L-EPC/MSC group and E-EPC/L-EPC/MSC group in terms of the formation of erythrocyte-perfused vessels at 2 weeks *in vivo*. A recent study by Shi *et al.* showed a similar effect *in vitro* when comparing cocultures of MSC/E-EPC with L-EPC/MSC or E-EPC/L-EPC/MSC cultures.[25] In addition, we showed that E-EPCs are effective in the induction of vessel formation at an early time point when combined with MSCs, whereas L-EPCs rather induce vascularization at a later time point in similar constructs. The observed phenomenon may indicate that vessels formed by E-EPCs after 2 weeks of implantation *in vivo* are stable and do not dissociate in time or that vessel formation is still ongoing after 6 weeks. It has become clear that these two EPC subtypes are involved in a differential timing of vasculogenesis. Discrepancies between *in vivo* studies may be explained by our use of combined EPC subtypes with MSCs instead of single administration of EPCs since different cell types affect each other's functions.[9,12,25,26]

In addition to the scoring system based on morphology, data from quantified luminal area densities showed a similar trend of identification of the E-EPC/MSC group as the best performing and the L-EPC/MSC group as the worst performing group of the five different groups examined. Although other reports used similar methods to describe *in vivo* neovessel formation,[12,35,36] we rather emphasize the scoring of vessel structure grades as a more sensitive parameter of neovascularization. Furthermore, an increased perfused vessel density at unchanged luminal area could indicate a more intricate branched network of microvessels, which is of relevance for newly formed tissue such as bone.

We also addressed the contribution of both donor cells and host cells, hereby evaluating the effect of combining MSCs with both EPC subtypes on ongoing vasculogenesis. We observed both host vessel ingrowth as well as vessel formation by the goat donor cells in constructs showing grade 2 or grade 3 structures, but not in constructs showing grade 1 structures. Contribution of both donor and host cells to induce vascularization through cell-seeding techniques has been shown before[6,11,27] and our results correspond to these studies.

Most studies that have addressed the application of EPC/MSC constructs to favor vascularization and subsequent bone formation/mineralization *in vivo* have focused on the use of L-EPCs.[6,11,13] We are the first to address the E-EPCs as a good alternative for L-EPCs in EPC/MSC treatment to favor vascularized ectopic bone formation since ectopic bone was formed in most E-EPC/MSC groups. Moreover, groups that showed induced early vasculogenesis also showed a higher bone incidence compared with groups lacking early vascularization. These results indicate a possible higher importance of induced vascularization at an early time point, rather than at a later time point, to induce bone formation. Therefore, whereas most studies focus on EPC/MSC treatment to induce vascularized bone formation, we rather emphasize the importance of early vasculogenesis to favor bone formation *in vivo*, a suggestion also made by others.[23]

Conclusions

Altogether, we presented evidence that E-EPCs and L-EPCs perform differently in terms of *in vitro* network formation in a 2D setting, but do equally well in a 3D environment. Whereas E-EPCs enhance vascularization in MSC-based constructs *in vivo* at 2 weeks, at a later time point, both EPC subtypes show similar numbers of formed vessels when implanted subcutaneously. Prominent early vascularization at 2 weeks coincided with a higher incidence of ectopic bone formation. Therefore, these results emphasize the potential importance of differential timing of vasculogenesis by the use of two EPC subtypes, which are both obtained from peripheral blood, and which are highly relevant in the synthesis of vascularized new bone.

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Disclosure statement

No competing financial interests exist.

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