Cell Tissue Bank (2017) 18:27–43
DOI 10.1007/s10561-016-9606-1

In-vivo quantification of the revascularization of a human acellular dermis seeded with EPCs and MSCs in co-culture with fibroblasts and pericytes in the dorsal chamber model in pre-irradiated tissue

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Received: 16 September 2016 / Accepted: 8 December 2016 / Published online: 21 December 2016
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Abstract
Introduction Transplantation of a cell-seeded graft may improve wound healing after radiotherapy. However, the survival of the seeded cells depends on a rapid vascularization of the graft. Co-culturing of adult stem cells may be a promising strategy to accelerate the vessel formation inside the graft. Thus, we compared the in vivo angiogenic potency of mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC) using dorsal skinfold chambers and intravital microscopy.
Materials and methods Cells were isolated from rat bone marrow and adipose tissue and characterized by immunostaining and flow cytometry. Forty-eight rats received a dorsal skinfold chamber and were divided into 2 main groups, irradiated and non-irradiated. Each of these 2 groups were further subdivided into 4 groups: unseeded matrices, matrices + fibroblasts + pericytes, matrices + fibroblasts + pericytes + MSCs and matrices + fibroblasts + pericytes + EPCs. Vessel densities were quantified semi-automatically using FIJI.
Results Fibroblasts + pericytes − seeded matrices showed a significantly higher vascular density in all groups with an exception of non-irradiated rats at day 12 compared to unseeded matrices. Co-seeding of MSCs increased vessel densities in both, irradiated and non-irradiated groups. Co-seeding with EPCs did not result in an increase of vascularization in none of the groups.
Discussion We demonstrated that the pre-radiation treatment led to a significant decreased vascularization of the implanted grafts. The augmentation of the matrices with fibroblasts and pericytes in co-culture increased the vascularization compared to the non-seeded matrices. A further significant enhancement of vessel ingrowth into the matrices could be achieved by the co-seeding with MSCs in both, irradiated and non-irradiated groups.

Keywords Graft vascularization · In vivo vascularization · Human acellular dermis · Angiogenic potential adult stem cells

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Introduction

Soft tissue defects caused by trauma or tumor resection provide a challenging problem to reconstructive surgery and tissue engineering. Tissue engineering

techniques offer the development of bioactive tissue constructs that can regenerate soft tissue in both structure and function (Choi et al. 2010). Cell-seeded biodegradable natural materials like acellular dermis may be used for soft tissue augmentation (Krauss 1999). Nonetheless, the absence of a vascular network within such a graft may have a negative effect on the viability of the seeded autologous cells in the graft due to an inadequate supply with nutrients and gas exchange after implantation (Laschke et al. 2006; Phelps and Garcia 2010). Thus, a rapid vascularization of the biomaterial plays a pivotal role in the survival and proliferation of the seeded cells to gain a successful integration of the graft into the host tissue. In addition, a neoadjuvant irradiation treatment has deleterious effects on neoangiogenesis and can delay the vascularization of the biomaterial (Kesler et al. 2013; Mao 2006; Martin 2013; Reinhold et al. 1990).

The advantage of in vivo vascularized tissue engineered grafts is the formation of a native complex branched vascular network with physiologically functional vessels within the engineered implant. However, a major drawback of this method is that the neovascularization of the implanted matrix needs several days to weeks, depending on the size of the implant and the implantation site (Rouwkema et al. 2008). Hypoxic areas within an implant may have negative impact on the cell survival and proliferation after implantation (Vitacolonna et al. 2015b). Several strategies have been investigated in order to enhance the in vivo vascularization of an engineered graft (Phelps and Garcia 2010). A promising approach is the co-cultivation of adult stem cells with stromal and perivascular cells inside a matrix (Baiguera and Ribatti 2013; Baldwin et al. 2014; Kirkpatrick et al. 2011; Phelps and Garcia 2010; Schumann et al. 2014; Shepherd et al. 2006). Numerous wound healing studies demonstrated that adult stem cells are capable to improve significantly chronic and acute wound healing (Parekkadan and Milwid 2010). The currently most widely studied adult stem cells for the reconstruction of musculoskeletal structures are mesenchymal stem cells (MSC) (Bianco et al. 2001; Maxson et al. 2012). MSCs can differentiate into a variety of different cells of the mesoderm (Farini et al. 2014; Vater et al. 2011) and are able to induce indirect trophic effects by secretion of a large number of different growth factors and cytokines (Phinney and Prockop 2007). Endothelial progenitor cells (EPC) participate in vivo in the renewal of the vasculature and promote de-novo vessel formation (adult vasculogenesis), their therapeutic potential for revascularization of ischemic tissue or tissue-engineered implants have been intensively studied (Asahara et al. 1997; Zammaretti and Zisch 2005). Perivascular or mural cells like pericytes are an additional essential cell type in vascular formation. They regulate the maturation and stabilization of new formed vessels (Abramsson et al. 2002; Alajati et al. 2008; Bryan and D'Amore 2008; Hegen et al. 2011; Rouwkema et al. 2006). Stromal cells like fibroblasts play also an important role in wound healing by autocrine and paracrine secretion of growth factors and cytokines such as e.g. TGF-ß, FGF and VEGF (Costa-Almeida et al. 2015; Novaes et al. 2007; Wenger et al. 2005).

To investigate whether MSC and EPC co-seeded with autologous pericytes and fibroblasts are capable to accelerate the vascularization of a human acellular dermis in radiated and non-irradiated tissues in vivo, we established a dorsal skinfold chamber model in rats. The vessel ingrowth was examined in vivo using intravital microscopy at day 0, 3, 9 and 12. Unseeded matrices and matrices seeded with fibroblasts and pericytes served as control groups.

Materials and methods

Animals

Forty-eight male Fisher-344 rats (150 g, Charles-River, Germany) were used for this study. All animals were held in the vivarium of the University Medical Centre Mannheim (Study approval by state authorities: Regierungspräsidium Karlsruhe, Germany: AZ 35-9185.81/G-187/09).

Study design

The study comprised a total of 6 treatment groups and 2 control groups. The rats were divided initially into 2 main groups (n = 24 rats) of equal size. One main group received neoadjuvant irradiation prior to the implantation of the skinfold chamber, the other does not. Each of the two main groups were subdivided into four groups each with n = 6 rats. Group 1 and 2 (control groups 1) received dermis without cells. Animals in group 3 and 4 (control groups 2) received a

fibroblast/pericytes augmented-dermis to assess the effects of the fibroblasts/pericyte co-culture on the neovascularization. Rats in group 5 and 6 received dermis seeded with fibroblasts, pericytes and MSCs in co-culture. Rats in group 7 and 8 received dermis with EPCs in co-culture with fibroblasts and pericytes. The matrices were microscopically examined at day 3, 6, 9 and 12 after transplantation. The rats were sacrificed thereafter. Table 1 details the study design.

Human acellular dermis

Epiflex® (German Institute for Cell and Tissue Replacement (DIZG), Berlin, Germany) was used as human acellular dermis (hAD). The mechanical processing, decellularization, sterilization, preservation methods (Rossner et al. 2011; Vitacolonna et al. 2014) and composition (Roessner et al. 2012) are described in detail elsewhere.

Isolation and culture conditions of fibroblasts from rat subcutaneous fat

Autologous fibroblasts were obtained from the subcutaneous fat as described previously (Vitacolonna et al. 2015b). Briefly, the adipose tissue was digested using 2 mg/ml collagenase type 2 at 37 °C for 2 h. The suspension was washed twice with Dulbecco's modified Eagle medium (DMEM). After digestion, the suspension was centrifuged at 400 g for 5 min. The resultant cell pellet was plated onto 100 mm^2 tissue culture plates (Greiner Bio One, Germany) supplemented with DMEM (with 10% FBS and 1% penicillin/streptavidin solution (PAA, Germany)) and maintained at 37 °C in an incubator with 5% CO_2.

Isolation and culture conditions of primary endothelial progenitor cells (EPC) from rat bone marrow

EPCs were isolated from tibia and femur of fischer-344 rats as described modified elsewhere (Kahler et al. 2007). Bone marrow was flushed out with DMEM supplemented with 10% FBS, 1000 U/ml Heparin (Sigma-Aldrich, USA) and 100U/ml penicillin G and 100 mg/ml streptomycin using a 20G needle (Becton–Dickinson, USA). To remove bone fragments and to obtain a single cell suspension, bone marrow was filtered through a 100 µm cell strainer (Becton–Dickinson, USA). The mononuclear cell fraction was obtained by a density gradient centrifugation using HSITOPAQUE®-1083 (Sigma-Aldrich, USA) for 30 min at 400 g. The mononuclear cell fraction was transferred into a new 50 ml tube, washed 3× with 30 ml DMEM and centrifuged at 250 g for 10 min. The resultant cell pellet was then suspended in EBM2-MV Medium (Lonza, USA) supplemented with 5% FBS and 100 U/ml penicillin G and 100 mg/ml streptomycin. Cells were plated on 6-well plates coated with 5 µg/cm^2 rat-derived fibronectin (Sigma-Aldrich, USA), 10 µg/cm^2 collagen I (Sigma-Aldrich, USA) and 2 µg/cm^2 laminin (Sigma-Aldrich, USA). After 24 h the non-adherent cell population was transferred to new coated wells. The cells were cultured for further 24 h. This procedure was repeated to remove rapidly adherent cells such as mature endothelial cells or MSCs. The resultant fraction was cultured in EBM-2-MV medium containing vascular endothelial growth factor (VEGF), human fibroblast growth factor-B (hFGF-B), insulin like growth factor (IGF-1), human epidermal growth factor (hEGF), stem cell growth factor (SCGF), ascorbic acid, hydrocortisone, gentamycin and amphotericin B (MV-Kit,

Table 1 Tabular summary of the treatment groups

Group	Treatment	Radiation
1 (Control group 1)	hAD unseeded	−
2 (Control group 1)	hAD unseeded	+
3 (Control group 2)	hAD + fibroblasts + pericytes	−
4 (Control group 2)	hAD + fibroblasts + pericytes	+
5 (MSC group)	hAD + fibroblasts + pericytes + MSC	−
6 (MSC group)	hAD + fibroblasts + pericytes + MSC	+
7 (EPC group)	hAD + fibroblasts + pericytes + EPC	−
8 (EPC group)	hAD + fibroblasts + pericytes + EPC	+

Each group consisted of n = 6 animals

hAD human acellular dermis, *MSC* mesenchymal stem cells, *EPC* endothelial progenitor cells

Lonza, USA). In addition, rat-specific rFGF and rVEGF (both R&D Systems, USA) were supplemented at a concentration of 100 ng/ml each. Medium was changed every 3 days. After 20 days of cultivation, cells were characterized by immunofluorescence staining and flow cytometry.

Characterization of EPCs from rat bone marrow

EPCs were characterized by immunofluorescence, flow cytometry and the incorporation of acetylated low density lipoprotein (acLDL; Biomedical Technologies, USA). Immunofluorescence staining was performed using antibodies against rat CD11b-biotin (AbD Serotec, USA), CD31 (Genetex, USA), CD34-AlexaFluor488 (Santa Cruz Biotechnology, Inc., USA), CD45-biotin (Cederlane, USA), CD106 (Becton–Dickinson, USA), CD133 (Abnova, USA), FLK-1 (Santa Cruz, USA) and Von Willebrand factor (vWF) (Santa Cruz, USA). Secondary FITC-labeled antibodies goat anti-mouse-IgG, goat anti-rabbit-IgG and streptavidin were purchased from Rockland (Rockland Immunochemicals, USA). Cells were fixed with ice cold acetone for 10 min. Stainings were performed in 8-well culture slides (BD Falcon^{TM}, Becton–Dickinson, USA) using manufacturers protocol. After blocking for 20 min with PBS containing 1% BSA (PAA, Germany), cells were incubated with primary antibodies for 2 h at room temperature (RT). After incubation, cells were washed 3 times with PBS for 5 min and incubated with secondary antibodies for 1 h. After 3 subsequent washing steps with PBS for 5 min VECTASHIELD mounting medium with DAPI (Vector Labs, USA) was used to prevent fading. Staining with acLDL was performed using manufacturer's protocol. For additional characterization, flow cytometry (FACS) analyses were performed using standard protocol. Antibodies against rat CD11b-biotin (AbD Serotec, USA), CD31 (Genetex, USA), CD34-AlexaFluor488 (Santa Cruz, USA), CD44-Fitc (Immunotools, USA), CD45-biotin (Cederlane, USA), CD54-Biotin (Cederlane, USA), CD73 (Becton–Dickinson, USA), CD106 (Becton–Dickinson, USA), CD133 (Abnova, USA) and FLK-1 (Santa Cruz, USA) were used. Secondary FITC-labeled antibodies goat anti-mouse IgG, goat anti-rabbit IgG and streptavidin were purchased from Rockland (Rockland Immunochemicals, USA). Briefly, $1x10^6$ cells/ml were incubated for 30 min at 4 °C with the primary antibodies. After three washing steps with PBS supplemented with 1% FBS, cells were incubated for further 30 min at 4 °C with the secondary antibodies. Measurements were done with a FACScalibur cytometer equipped with a 488 nm argon laser (Becton–Dickinson, USA) using BD CellQuest software. At least 10,000 events were collected and WinMDI 2.8 software was used to create the histograms.

Isolation and culture conditions of primary mesenchymal stem cells (MSC) from rat bone marrow

MSC were isolated from tibia and femur of fischer-344 rats based on the plastic adherence of these cells (Schumann et al. 2009). Subsequently, an enrichment using the surface marker CD90 as described elsewhere (Zhang and Chan 2010) was performed in order to increase the purity of the MSCs. Bone marrow was isolated and processed as described in the EPC section. After density gradient centrifugation, the mononuclear cells were plated in 10 cm dishes and cultured overnight in DMEM supplemented with 10% FBS, 100U/ml penicillin G, 100 mg/ml streptomycin and 50 ng/ml rat fibroblast growth factor (bFGF) (R&D Systems, Germany). After 24 h, the supernatant was aspirated and 10 ml of fresh medium was added. The cells were incubated for 1 week at 37 °C in a 5% CO_2 incubator and the medium was changed every 3 days. Once the plates were approximately 80% confluent, the purity of the MSCs was increased by a positive isolation using CD90-biotin (Cederlane, USA) and streptavidin-coupled Dynabeads (Dynabeads® system; Invitrogen-Dynal, Sweden). The purification was performed according to the manufacturer's instructions. Briefly, 1×10^7 cells/ml were incubated with CD90-biotin for 10 min at 4 °C. After 3 washing steps, cells were incubated for 20 min at 4 °C with the streptavidin-beads and washed afterwards three times. After the magnetic isolation procedure, cells were resuspended in DMEM supplemented with 10% FBS and 50 ng/ml rbFGF and plated at a density of 500cells/cm^2 in 10 cm dishes. The cells were incubated at 37 °C in a 5% CO_2 incubator and the medium was changed every 3 days. Once the cells were approximately 80% confluent, they were trypsinized and diluted to a concentration of 500cells/cm^2 in 10 cm dishes.

Characterization of MSC from rat bone marrow

For immunofluorescence characterization, cells were stained with rat anti CD29-biotin (Becton–Dickinson, USA), CD90-biotin (Cederlane, USA), CD73 (Becton–Dickinson, USA), CD44-Fitc (Immunotools, USA), CD34-AlexaFluor-488 (Santa Cruz, USA), CD45-biotin (Cederlane, USA), CD31 (Genetex, USA), CD106 (Becton–Dickinson, USA), vWF (Santa Cruz, USA), fibronectin (Abcam, USA) and collagen I (Rockland, USA). Secondary FITC-labeled antibodies goat anti-mouse-IgG, goat anti-rabbit-IgG and streptavidin were purchased from Rockland (Rockland Immunochemicals, USA). Flow cytometry analyses were performed as described above. Antibodies against rat anti CD29-biotin, CD90-biotin, CD73, CD44-Fitc, CD34-AlexaFluor-488, CD45-biotin, CD31, CD106, fibronectin and collagen I were used.

To proof the preservation of multi-potency, two different differentiation assays were performed following the manufacturers protocol. For the differentiation of MSC into osteoblasts, cells were cultured 21 days in OsteoPrime® Induction Medium (Invitrogen-Gibco, USA) supplemented with ascorbic acid 2-phosphate, ß-glycerophosphate and dexamethasone. To show calcium deposition, cultures were washed once with PBS and stained for 5 min at RT with Alizarin Red S (Sigma-Aldrich, USA). To induce adipogenic differentiation, MSCs were cultured for 21 days in StemPro® Adipogenesis Differentiation Kit (Invitrogen-Gibco, USA). To stain the adipocytes, cells were fixed in 10% paraformaldehyde (Merck, Germany) for 1 h at RT and subsequently stained 20 min at RT with Oil-Red-O (3 volumes of 0.5% Oil-Red-O in isopropanol added to 2 volumes of aqua dest) (Sigma-Aldrich, USA).

Isolation and culture conditions of primary pericytes from rat bone marrow

Pericytes were isolated from tibia and femur of fischer-344 rats as described above in the EPC section. After density gradient centrifugation, depletion of MSC by negative magnetic separation with the MSC marker CD73-biotin (Becton–Dickinson, USA) was carried out as described above. The resultant cell pellet was plated in 10 cm dishes and cultured in EBM2-MV medium supplemented with 5% FBS, VEGF, hFGF-B, IGF-1, hEGF, SCGF, ascorbic acid, hydrocortisone, gentamycin and amphotericin B (MV-Kit, Lonza, USA). In addition, rat-specific rFGF and rVEGF (both R&D Systems, USA) were supplemented at a concentration of 100 ng/ml each. After about 10–15 days, isolated colonies were identified with different morphologies, containing presumably mature endothelial cells, EPC and MSC. Pericytes were identified based on their typical round nucleolus and numerous cell extensions as described in the literature (Bryan and D'Amore 2008; Dore-Duffy and Cleary 2011; Tigges et al. 2012). To purify the pericytes, isolated colonies were trypsinized using cloning rings (Sigma-Aldrich, USA). The detached cells were pooled and transferred into a collagen I, fibronectin, and laminin-coated 6-well plate. This procedure was repeated 2-4 times in order to obtain a homogeneous pericyte culture. EBM2-MV medium was changed every 3 days. After 20 days of cultivation, cells were characterized by immunofluorescence staining.

Characterization of primary pericytes from rat bone marrow

For immunofluorescence characterization, cells were stained with different markers typically expressed by pericytes (Farrington-Rock et al. 2004; Lamagna and Bergers 2006). Since there exists no specific marker for pericytes, antibodies against rat anti CD54 (Cederlane, USA), desmin (DAKO, Germany), smooth muscle actin (α-SMA) (DAKO, Germany) and platelet-derived growth factor receptor-ß (PDGFR-ß) (Becton–Dickinson, USA) were used. Secondary FITC-labeled antibody goat anti-mouse-IgG and streptavidin were purchased from Rockland (Rockland Immunochemicals, USA).

Irradiation

Twenty-four animals were assigned to the irradiation group. The radiation has been described previously (Vitacolonna et al. 2015b). Dorsal skin of the anesthetized animals was positioned under the irradiation apparatus and fixed using surgical clips. Twenty gray were administered topically 14 days prior to the implantation of the chamber in a single dose with an Intrabeam® device (PEC Photoelectronic Corporation PRS400; Voltage 50 kV, Current 40 µA; Run Time 6 min).

Implantation of the dorsal skinfold chamber

The surgical implantation of the skinfold chamber has been described previously in detail (Vitacolonna et al. 2015b). Briefly, after anesthesia the back skin was shaved, depilated and disinfected. Using an operation microscope (Zeiss OPMI 9-FC, Zeiss, Germany) an area (15 mm diameter) of the top layer of the dorsal was circularly excised and the underlying fascial layers were removed carefully from the thin skin muscle layer (panniculus carnosus). The remaining layer of the striated muscle, the subcutaneous tissue, the dermis and the epidermis, were covered with the counterpart of the dorsal skinfold chamber and screwed together. The observation window was filled with Ringer's solution and covered with a detachable cover glass (Menzel, Germany). After preparation, the animals were allowed to recover from anesthesia and surgery for 48 h before the implantation of the scaffolds. During the period of study, the chambers and the implanted biomaterial underwent qualitative daily assessment. Animals with pathological findings or chamber defects were excluded from the experiment. Inflammation, hemorrhage, edema and persisting restriction of movement with consequent impairment of nourishment were termination criteria.

Cell seeded acellular dermis

Human acellular dermis (Epiflex®, DIZG, Berlin, Germany) with a thickness of 0.5–0.8 mm were cut into 5 mm × 5 mm pieces and rehydrated in DMEM for 2 h at 37 °C in 48-well plates. The rehydrated hADs were initially degassed with a chamber evacuation method to remove air trapped within the matrix (Hasegawa et al. 2010; Vitacolonna et al. 2013) and subsequently seeded with fibroblasts, pericytes and MSCs or EPCs in co-culture dynamically at a concentration of each $5x10^5$ cells/hAD. Dynamic seeding (centrifugal cell immobilization) is described modified in a previous work (Vitacolonna et al. 2015a). Briefly, scaffolds were placed in a 48-well plate and a cell suspension with the above mentioned concentration was pipetted onto the scaffolds. The plate was then centrifuged at 300 g for 5 × 1 min in a common plate centrifuge, in order to allow a deeper penetration of the cells inside the dermis. Culture plates were incubated after the seeding procedure at 37 °C with 5% CO_2 for 2 h to enable cell attachment. Immediately thereafter, the matrices were implanted into the skinfold chamber.

Implantation of the seeded dermis

The animals were anesthetized as described before. The cover slip was removed, the seeded graft was attached to the striated muscle in the centre of each chamber window with an 8/0 suture (Ethicon, USA). The window was closed with a sterile coverslip avoiding air bubbles.

In-situ fluorescence microscopy

Microscope examination was performed under anesthesia (as described above) at days 0, 3, 6, 9 and 12 using an Axiotech® Vario 100 intravital microscope (Zeiss, Germany). The 4 borders of the implanted dermis were defined as regions of interest (ROIs). Five pictures were taken from each ROI using a 5× objective (each ROI corresponded an area of 5 mm × 1 mm). The images were captured with a digital camera (AxioCam ICm1, Zeiss, Germany). Images were acquired with Axiovision LE V4.8.2 software (Zeiss, Germany). Images were stitched together using ICE (Image Composite Editor, V1.4.4.0, Microsoft, USA) to an edge-to-edge view of the full width of each ROI.

Quantification of vessel density

To quantify the vascularization of the implanted matrices, vessel density per ROI was analyzed. Whole photographs of each ROI (composed of each 5 single pictures) were binarized and segmented using FIJI and the advanced WIKA segmentation plug-in (Schindelin et al. 2012). Vessel density in the observed area was calculated automatically and expressed as % area occupied by blood vessels per ROI. A total of $n = 24$ ROIs per group were evaluated.

Statistics

All statistical tests were conducted with GraphPad Prism V6 (GraphPad Software Inc, USA). The data were analyzed by two-way ANOVA and a subsequent Bonferroni's multiple comparisons test (<0.05 at a confidence level of 95%. $p < 0.05$ was considered significant).

Results

Characterization of EPC from rat bone marrow

Figure 1a1–2 shows representative colonies of EPCs typically growing between 10 and 15 days in culture. To confirm the endothelial phenotype, the cells were stained after 20 days in culture for immunofluorescence characterization using specific antibodies and Dil-Ac-LDL. The cells were positive for Dil-Ac-LDL, CD11b, CD31, CD106, CD133, VEGFR2, vWF and CD34 (Fig. 1a3–10). The cells showed no expression of CD29, CD73 and the hematopoietic markers CD45 (data not shown). The cytometry analysis demonstrated the expression of CD11, CD31, VEGFR2, CD133, CD34 and CD54, but they were negative for CD29, CD73 und CD45 (Fig. 1b). The absence of CD29, CD45 and CD73 indicates that neither

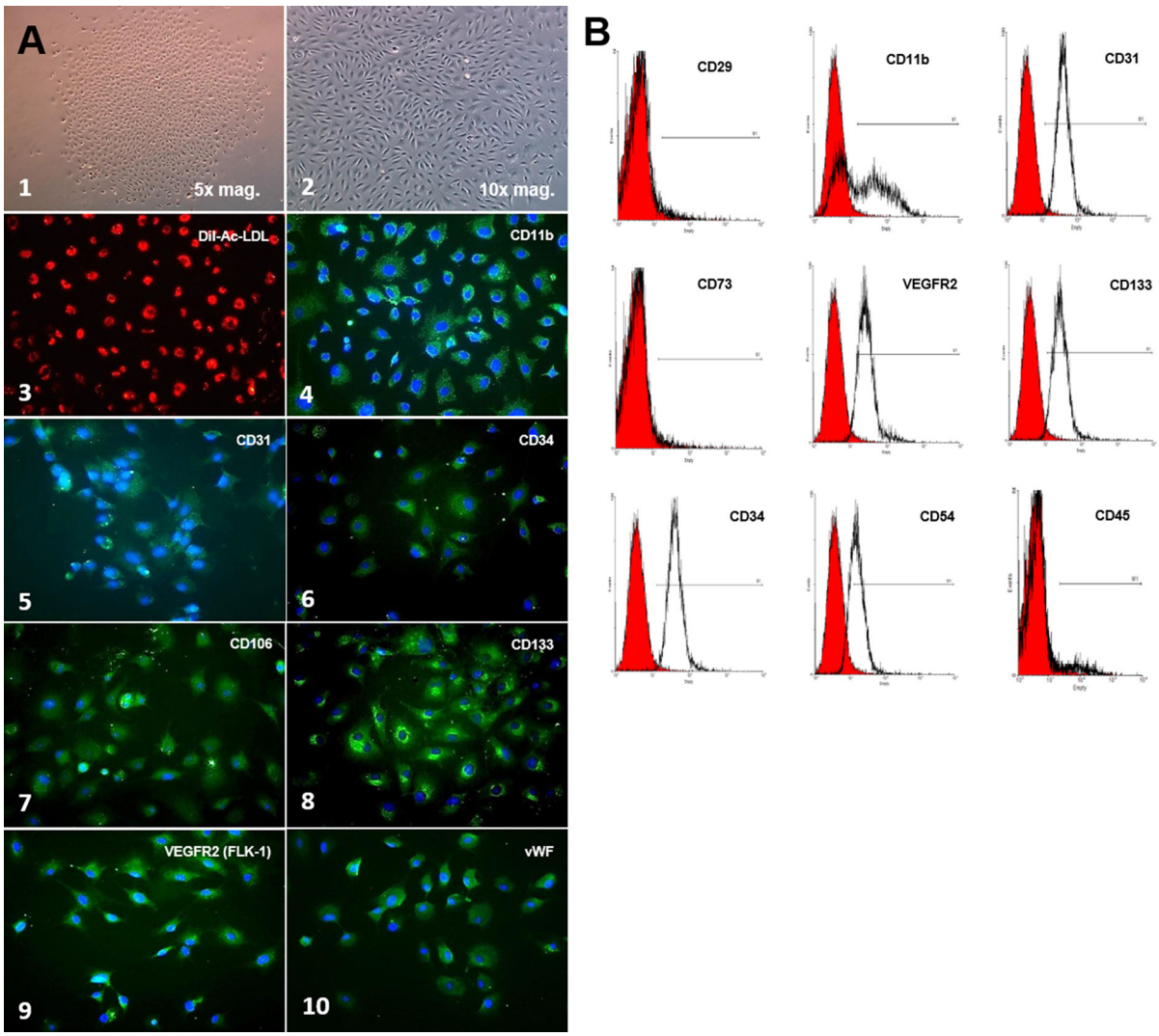

Fig. 1 **a1–2** EPC from bone marrow 10 and 20 days after isolation, cultured in EBM-2 medium; a3–10 Immunofluorescent characterization of the isolated EPC. Cells were positiv for Dil-AC-LDL, CD11b, CD31, CD34, CD106, CD133, FLK-1 and vWF. Cell nuclei were stained *blue* with DAPI. **b** The FACS analysis of the EPC illustrates the different degrees of expressions of the endothelial marker CD11b, CD31, VEGFR2, CD133, CD34 and CD54. The absence of CD29, CD73 and CD45 shows the high purity of the culture. *Red* represents the respective isotype control. (Color figure online)

hematopoietic cells nor MSC or fibroblasts were present in the culture.

Characterization of MSC from rat bone marrow

Adherent MSCs were present after 5–7 days in culture with spindle-shaped fibroblastoid morphology. The MSCs grew in colonies clearly defined from each other (Fig. 2a1–2). Immunofluorescence characterizations demonstrated expression of MSCs marker CD29, CD73, CD90, CD44, fibronectin and collagen-I (Fig. 2a3–8). Staining with CD14, CD45, CD31 and CD34 were negative (data not shown). Using flow cytometry, the cells expressed the stromal antibodies CD29, fibronectin, CD44, CD90 and CD73, whereas neither the macrophage marker CD14 nor CD31 and CD34 were expressed. The hematopoietic stem cell marker CD45 was weakly detected (<5%) (Fig. 2b). The majority of the MSCs cultured in osteogenic differentiation medium exhibited significant morphological changes including the production of a mineralized matrix, typically formed by osteoblasts (stained red with Alizarin Red) (Fig. 2c1). Cells incubated in adipogenic medium expressed lipid vacuoles

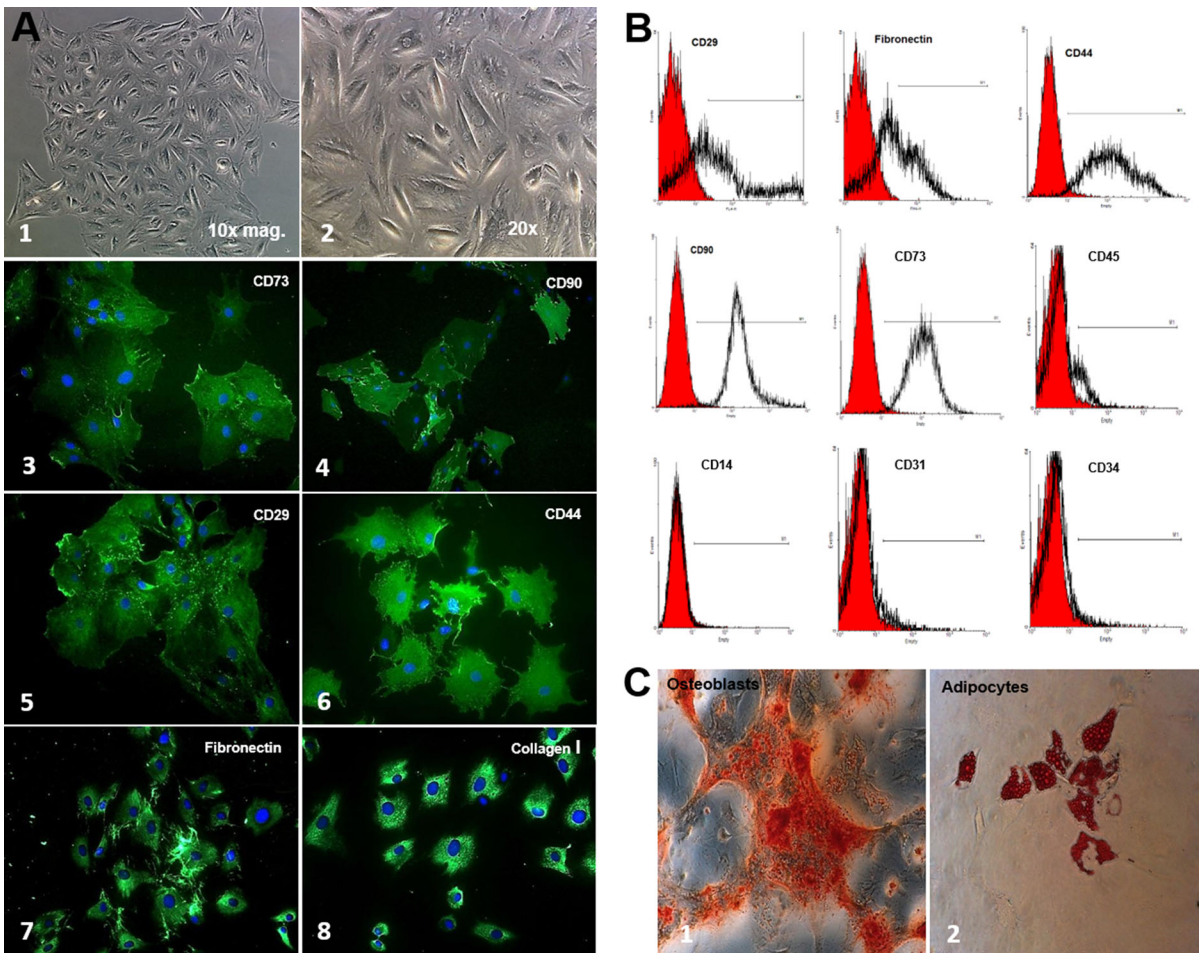

Fig. 2 **a1–2** Phase contrast microscopic images of MSC colonies after 20 days of culture. **a3–8** Immunofluorescence staining of MSC with antibodies against CD73, CD90, CD29, CD44, fibronectin and collagen-1. Cell nuclei were stained *blue* with DAPI. **b** FACS analysis of the MSC. The cells were positive for CD29, fibronectin, CD44, CD90, CD73 and negative for CD45, CD14, CD31 and CD34. *Red* represents the respective isotype control. **c1** Cell culture in osteogenic differentiation medium led to cell differentiation into osteoblasts. Cells were stained with *alizarin red* to visualize the formed mineralized matrix. **c2** Culture in adipogenic differentiation medium led to cell differentiation into adipocytes. The lipid droplets were stained by the lipid dye Oil-Red-O. (Color figure online)

formations of different sizes. The vacuoles were stained with the lipid dye Oil-Red-O to prove the differentiation into adipocytes (Fig. 2c2). Thus, accordingly to the International Society of Cellular Therapy (ISCT) the common set of minimal criteria for defining MSCs were confirmed in our study (Dominici et al. 2006).

Characterization of pericytes from rat bone marrow

After isolation of the pericytes using cloning rings, a relatively homogeneous population could be cultured for numerous passages (Fig. 3a, b). The characteristic prominent nucleus, irregular triangular cell bodies and long extensions of pericytes (Hirschi and D'Amore 1996; Liu et al. 2014) were present in all cells used in this experiment (Fig. 3b). All cells expressed desmin, α-SMA, CD54 and PDGFR-ß (Fig. 3c–f).

Quantification of vessel densities

The vessel densities were analyzed semi-automatic using the WEKA segmentation plugin and FIJI and expressed as % vessel density per observed region. Group 1 and 2 (unseeded hAD; ±radiation) were compared with group 3 and 4 (hAD seeded with fibroblasts and pericytes; ±radiation) to assess the influence of the co-cultured fibroblasts and pericytes on dermis vascularization. The control groups 3 and 4 (hAD seeded with fibroblasts +pericytes; ±radiation) were compared with the stem cell-augmented matrices (group 5–8) to evaluate the influence of EPCs and MSCs on the vascularization.

Figure 4 shows representative intravital microscopic pictures of an observed ROI between day 6–12 (group 5: +fibroblasts +pericytes +MSC −radiation). Perfused macro-vessels were clearly distinguishable from the high intrinsic fluorescence of the dermis without fluorescent staining. In all groups first vessel ingrowth were observable at day 6 with an exception of group 5 (+fibroblasts +pericytes +MSC −radiation). In group 5 first vessel ingrowth became visible at day 3 within the border zones.

At any time point the macrovascular density of cell seeded scaffolds was significantly higher compared to the unseeded control groups with an exception at day 12 in the non-irradiated groups. In addition, we found, that the irradiation caused a significant decrease of vessel density in all groups compared to the non-irradiated groups during the entire observation.

Figure 5 displays the calculated vessel densities of the non-irradiated groups (group 1, 3, 5 and 7).

The comparison between the control group 1 (group 1; −cells −rad) and control group 2 (group 3; +fibroblasts +pericytes −rad) showed no differences at day 3 (both groups 0%). At day 6 the vessel density in group 3 was significant higher than in group 1 (group 1: 3.6 ± 0.4% and group 3: 16.2 ± 1.3%; $p = 0.0217$). At day 9 a significant higher vessel ingrowth was detectable in the seeded hAD (group 3: 35.6 ± 4.6%) compared to the unseeded hAD (group 1: 14.7 ± 5.3%) ($p < 0.0001$). At day 12 there were no differences between both non-irradiated control groups (group 1: 28.4 ± 5.4% and group 3: 39.2 ± 19.5%).

Co-seeding with MSCs (group 5) leads to an earlier detectable vessel ingrowth starting at day 3 (3.1 ± 2.2%) compared to the other groups. The EPC group (group 7) achieved none visible vascularization at day 3. At day 6 vessel density in group 5 (23.9 ± 1.9%) was significant higher than in group 3 ($p = 0.0324$). Group 7 (+EPC) showed no differences (14.2 ± 2.2%) compared to the control group 2. At day 9 vessel density in group 5 (+MSC) was significant higher (47.6 ± 2.1%) than in group 3 ($p = 0.0327$) while group 7 (+EPC) showed no significant difference (38.0 ± 4.5%). At day 12 neither MSCs nor EPCs increased the vessel density compared to group 3 (group 5: 46.6 ± 13.9% and group 7: 41.1 ± 24.1%).

Figure 6 depicts the vessel densities from the irradiated groups 2, 4, 6 and 8.

The comparison at day 3 between control group 1 (group 2; −cells +rad) and control group 2 (group 4; +fibroblasts +pericytes +rad) showed in both groups none visible vessel ingrowth. At day 6 vessel ingrowth was not detectable in the unseeded matrices (group 2) while matrices of control group 2 (group 4; +fibroblasts +pericytes +rad) achieved a vessel density of 3.1 ± 0.4% per ROI ($p = 0.0004$). At day 9 vessel density in group 2 (2.2 ± 1.3%) was significant lower than in group 4 (8.4 ± 0.7%) ($p = 0.0008$). At day 12 vascular density in group 4 (12.9 ± 5.2%) increase compared to the non-seeded matrices (group 2; 4.3 ± 3.1%) ($p < 0.0001$).

In none of the stem cell-augmented groups (group 6 and group 8) and control group 2 (group 4) were

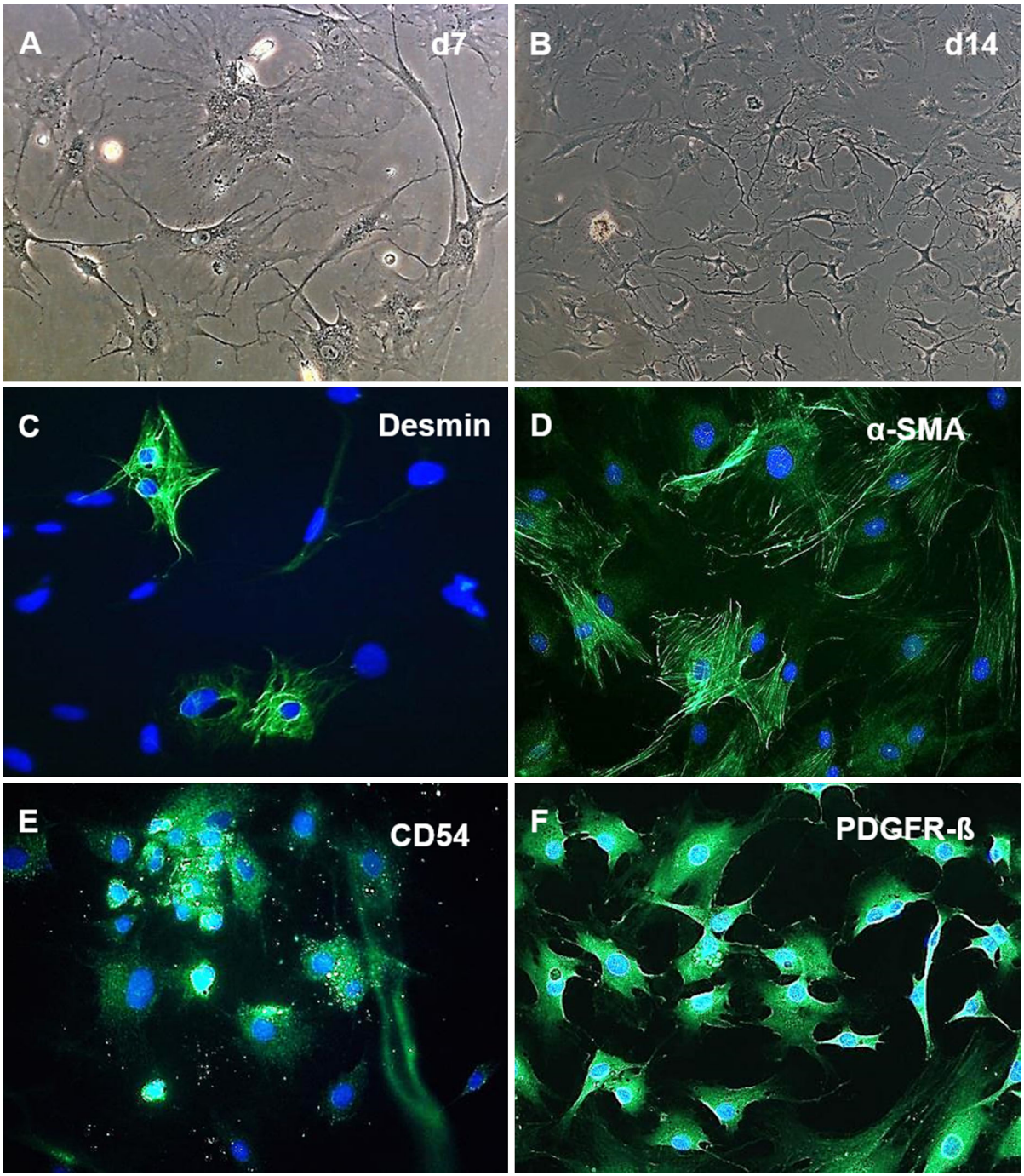

Fig. 3 Phase contrast microscopic images of pericytes after **a** 7 and **b** 14 days in culture. **c–f** Immunofluorescent staining of pericytes were positiv for Desmin, α-SMA, CD54 and PDGFR-beta. Cell nuclei were stained *blue* with DAPI. (Color figure online)

vessels detectable at day 3. At day 6 matrices co-seeded with MSCs (group 6) achieved a higher vessel density (9.6 ± 1.2%) than control group 2 (group 4; $p = 0.0004$). EPCs (group 8; 2.5 ± 0.8%) had no beneficial effect on dermis vascularization compared to the fibroblasts +pericytes-seeded control group. MSCs showed also at day 9 a superior vessel growth (21.8 ± 1.2%) in comparison to group 4

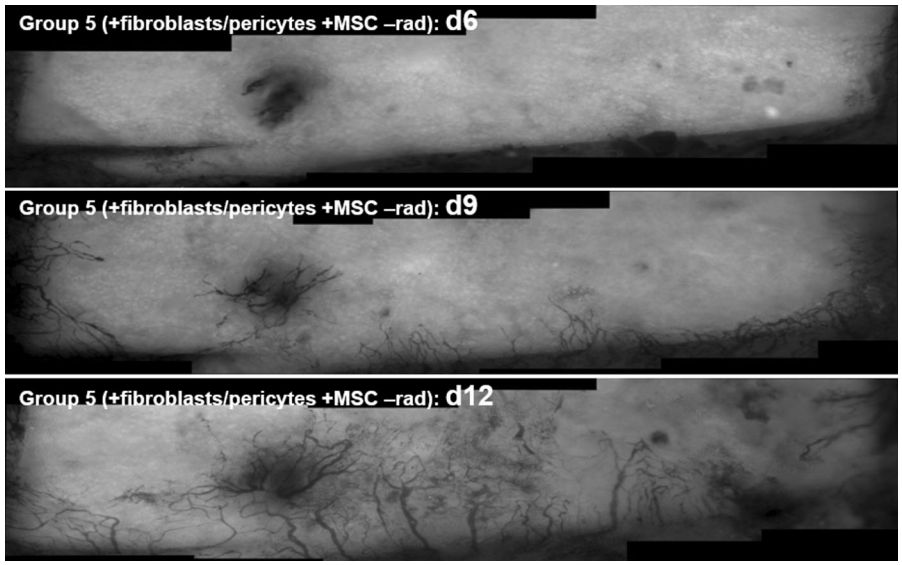

Fig. 4 Representative intravital microscopic picture of the observed ROI between day 6 to 12 (group 5: hAD +fibroblasts/pericytes +MSC −radiation). Perfused macro-vessels were clearly distinguishable from the high intrinsic fluorescence of the dermis. Vessel ingrowth started to be visible at the borders of the hAD. Newly formed macro-vessels interconnect with each other and form a dense microvascular network within the border zones until day 12

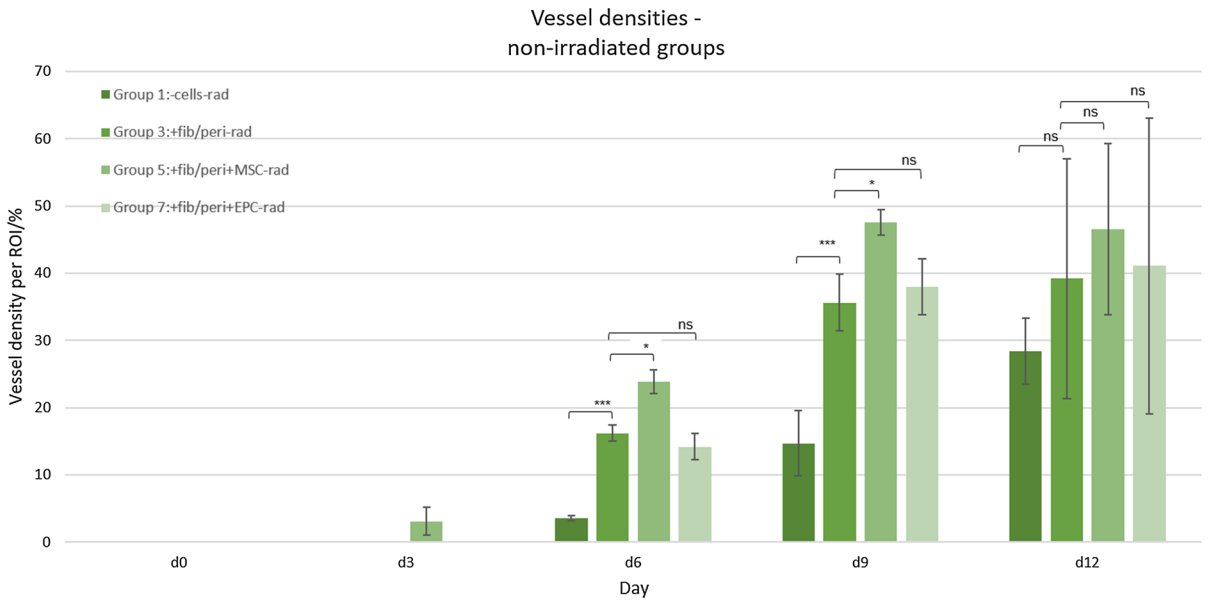

Fig. 5 Vessel densities in %/ROI of the non-irradiated groups at day 3–12

($p < 0.0001$). Even at day 9 EPCs were not able to increase vascular density significantly (group 8; 6.3 ± 1.5%). At day 12 the co-seeding of MSCs lead to a higher vessel density (group 6; 22.4 ± 10.0%) ($p < 0.0001$). As well as seen at earlier time points no improvement of vascularization could be detected in the EPC group 8 (11.0 ± 3.7%).

Discussion

In the present study we compared the vascularization potential of EPC and MSC in co-culture with fibroblasts and pericytes seeded on an acellular human dermis. We were further simulating a multi-modal neoadjuvant therapeutic approach by pre-implantation

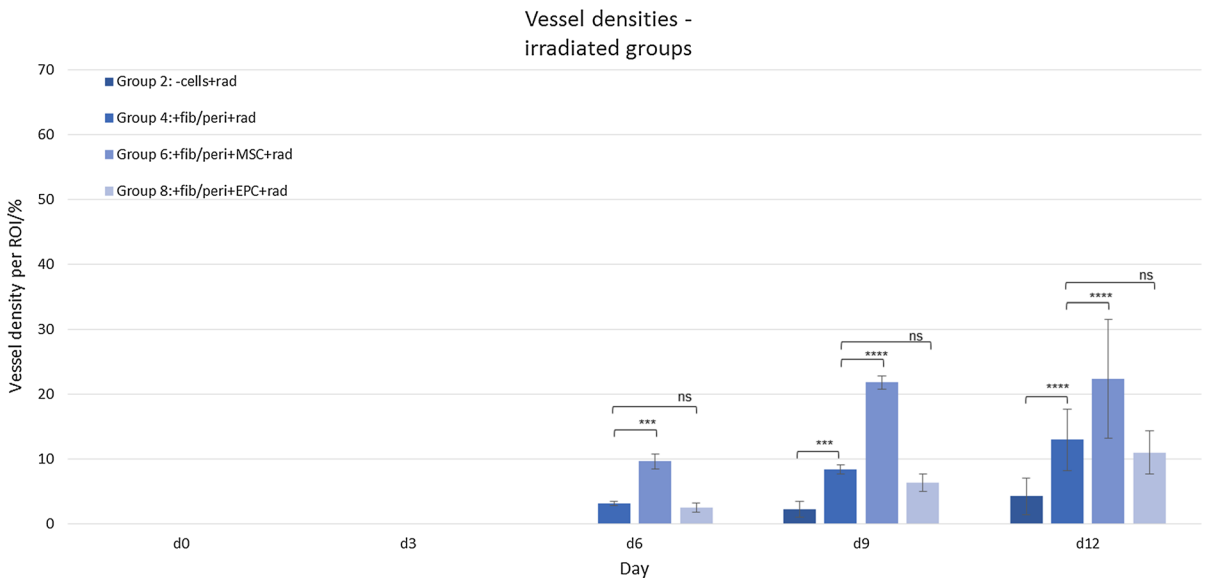

Fig. 6 Vessel densities in %/ROI of the irradiated groups at day 3–12

irradiation to investigate the effect of radiation on the vessel ingrowth.

Our study demonstrates that the pre-irradiation treatment results in a significant weaker graft vascularization in all groups. The augmentation with fibroblasts and pericytes in co-culture significantly enhanced the vascularization at the border zones of the matrices compared to the unseeded matrices in both, irradiated and non-irradiated groups. These results are consistent with previous findings showing that vitalization of scaffolds with cells causes a significant improvement in blood vessel formation in vivo (Kampmann et al. 2013; Laschke et al. 2013; McFadden et al. 2013; Schumann et al. 2014). In many wound healing studies the transplantation of vital, non-irradiated fibroblasts resulted in normal wound healing or an improvement of wound breaking strength after radiation (Chen et al. 2010; Dantzer et al. 2003; Ferguson et al. 1999; Roessner et al. 2013, 2011). Fibroblasts act as important indirect supporting cells during angiogenesis by constitutively expressing angiogenic factors, which regulate endothelial tube formation (Baldwin et al. 2014). As well, perivascular cells like pericytes provide direct support to endothelial cells and are essential to stabilizing and maturing new vessels and capillaries in tissue engineered implants (Golas et al. 2013; Jain 2003; Stratman and Davis 2012; Yamamoto et al. 2010; Yang et al. 2012).

The co-seeding of MSCs leads into a further significant increase of vessel density. Their regenerative effects seem to induce the vascular growth in hypoxic tissue in both non-irradiated and irradiated tissues. MSCs demonstrated the capability to improve wound healing in both animal models and humans in the treatment of acute and chronic wounds (Falanga et al. 2007; Kwon et al. 2008; Liu et al. 2006; McFarlin et al. 2006; Shin and Peterson 2013; Wu et al. 2007; Yoshikawa et al. 2008). The reparative and angiogenic properties of MSCs were also intensively studied in the recent years (Au et al. 2008; Hegen et al. 2011; Sanz et al. 2008). For example, MSC were used to engraft and ameliorate limb ischemia or to enhance angiogenesis in different stroke models (da Cunha et al. 2013; Hoffmann et al. 2010; Honmou et al. 2012; Madonna et al. 2013; Martins et al. 2014; Watt et al. 2013; Wu et al. 2007). Recent studies indicate that the involvement of MSCs in wound healing is not limited by engraftment and differentiation, but also by broad immunoregulatory effects, possessing immunosuppressive and anti-inflammatory properties (Baiguera et al. 2012; Bernardo and Fibbe 2013; English 2013; Salem and Thiemermann 2010; Yagi et al. 2010). In addition, MSCs secret a variety of bioactive factors that have angiogenic and antiapoptotic properties and serve to limit tissue

damages at the injured sites, regenerating blood supply, and recruiting progenitor cells (Murray et al. 2014). MSCs have as well been shown to be an effective therapy option in the treatment of acute radiation injuries by enhancing the recovery of hematopoiesis and reducing apoptosis (Chang et al. 2013; Hu et al. 2010; Lange et al. 2011; Semont et al. 2006). Although we have demonstrated a significant increase in the vascularization in the MSC groups, the exact mechanism behind is still unclear. However, we hypothesize that a multifactorial interaction of their varied properties may be responsible for this effect. To elucidate the exact mechanism, further studies are needed.

The co-seeding with EPCs had no effect on the vessel density, although they were used in numerous studies to improve vascularization of wounds after transplantation or injection in vivo (Hess et al. 2002; Kocher et al. 2001; Takahashi et al. 1999). In contrast to these results, there are some studies, in which transplanted EPCs caused an aggravation of the disease or injury (Pearson 2010). EPCs consist of at least two subtypes, the "early"—and "late"-outgrowth EPCs with different morphologies and growth patterns. Late-EPCs exhibit a high proliferative capacity and typical endothelial antigens (Mukai et al. 2008). It was reported, that late-EPCs augment the angiogenesis by direct incorporation into the neovasculature and the secretion of angiogenic growth factors, while early-EPCs are very short-lived in culture and not able to form tubular structures (Hur et al. 2004; Suh et al. 2005). Instead, it was postulated that these cells are involved rather in inflammatory processes than in angiogenesis (Fadini et al. 2008). The EPCs used in this study were derived from the non-adherent fraction of mononuclear bone marrow cells, which grew after 48 h in fibronectin-coated cell culture plates to angioblast-shaped cells as reported before (Salazar et al. 2001). The cells presented within the first 5 days in culture an elongated, thin spindle-shaped morphology, as described in the literature as "early-outgrowth" EPCs. Between day 8–15 the proliferation rate increased and the cells showed an endothelial cell-like shape, which may be typical for "late outgrowth" EPCs. FACS analysis of the transplanted EPCs demonstrated a weak expression of the monocyte marker CD14 after 15 days in culture. After more than 2 weeks in culture, the "late"—subtype dominated. These cells expressed typical antigens of endothelial origin, such as CD34, CD31, or vWF. Although we have seeded late-subtype EPCs onto the hADs, they did not increase the vessel density compared to the groups with fibroblast/pericyte-seeded matrices. The results suggest that the in vitro expansion decreased the migratory capacity of the EPCs and led to a maturation of the cells into endothelial cells which resulted into a partial loss of their vasculogenic ability, as reported elsewhere (Ingram et al. 2004; Khan et al. 2006; Melero-Martin et al. 2007; Singh et al. 2011).

Limitation of the study

The unequivocal verification of purity of the pericyte culture with antibodies against desmin, SMA, PDGFR-ß and CD54 may be critical due to the lack of unique pericyte-specific markers. So far, known pericyte markers are also expressed in various other cell types (Kloc et al. 2015). Therefore, the identification may rely on multiple criteria including morphology and co-expression of several different pericyte markers.

Conclusion

We demonstrated that the co-seeding of fibroblasts, pericytes and MSCs have the potential to accelerate the vascularization of an implanted matrix. Since MSCs are easy to isolate, possess an extraordinary plasticity and retain their ability to differentiate even after massive in vitro expansion, their co-seeding with target cells into a graft prior the surgical intervention could represent a promising vascularization strategy to increase the vessel number within the construct in vivo after implantation.

Acknowledgements The authors thank Dr. Jan C. Brune and Dr. Mark D. Smith from the German Institute for Cell and Tissue Replacement (DIZG) for advice relating to use of Epiflex® and for assistance with data analysis and editing of the final manuscript.

Compliance with ethical standards

Conflict of interest Dr. Mario Vitacolonna, Dr. Djeda Belharazem, Prof. Dr. Peter Hohenberger and Dr. Eric Roessner declare that they have no conflict of interest.

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