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Endothelial progenitor cells are integrated in newly formed capillaries and alter adjacent fibrovascular tissue after subcutaneous implantation in a fibrin matrix

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Abstract

Vascularization of bioartificial matrices is crucial for successful tissue engineering. Endothelial progenitor cells (EPC) have shown vascularization potential in ischemic conditions and may also support blood vessel formation in tissue-engineered matrices. The aim of our study was to investigate the impact of a well-characterized murine embryonal EPC line (T17b-EPC) on vascularization and fibrovascular granulation tissue formation after suspension in a fibrine matrix followed by subcutaneous implantation in a separation chamber in rats. EPC were fluorescently labelled *in vitro* prior to implantation. After 3, 7 or 14 days, animals were killed followed by explantation and histological analysis of the constructs. Before the end of the experiment, Bandeirea Simplicifolia lectin was intravenously injected to mark the vascular ingrowth into the implanted constructs. The transplanted cells were histologically detected at all time-points and located almost exclusively within the fibrin matrix at day 3 but the number of cells in the clot continuously decreased over day 7 to day 14. Conversely, cells were detected within the newly formed granulation tissue in increasing numbers from day 3 over day 7 to day 14. Transplanted cells were also found in the intermuscular septa. Cell viability was confirmed by use of an EPC clone expressing β-galactosidase. Fluorescence microscopy demonstrated integration of the transplanted cells in newly formed blood vessels within the fibrovascular granulation tissue adjacent to the fibrin clot. Presence of cells in the fibrin clot lead to thicker granulation tissue and an increased blood vessel diameter compared to cell-free controls. Organ standard controls showed presence of the transplanted cells in spleens at day 14 after transplantation. In summary, EPC exhibited biological activity after subcutaneous implantation in a fibrin matrix by migration from the fibrin clot into the granulation tissue and along intermuscular septae, undergoing differentiation into mature endothelial cells and integration into newly formed blood vessels and altering fibrovascular granulation tissue development. EPC may hold promise to modulate blood vessel formation in bioartificial matrices.

Keywords: endothelial progenitor cells • fibrin matrix • angiogenesis • tissue engineering

Introduction

Over the past two decades, tissue engineering has been the focus of extensive research resulting in significant advances and clinically available products. A major challenge, however, lies in vascularization of large three-dimensional bioartificial constructs [1, 2]. Delivery of proangiogenic growth factors as proteins and *via* gene therapy as a means to stimulate angiogenesis in tissue-engineered constructs *in vitro* and *in vivo* have been extensively studied in the past with a certain success [3, 4]. A problem of the growth factor delivery approach, however, lies in the fact that the number of endothelial cells that could be locally stimulated by angiogenic molecules is limited [5]. An alternative may thus be to supply appropriate cell populations towards the formation of new blood vessels [6].

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Endothelial progenitor cells (EPC) have been shown to play a key role in different disease states where (neo)-angiogenesis and -vasculogenesis occur in adulthood. This finding implies that formation of new blood vessels does not only occur by sprouting from pre-existing vessels (angiogenesis), but also under participation of these progenitor cells that circulate in the bloodstream and differentiate *in situ*, *i.e.* vasculogenesis [5]. Indeed, experimental studies have demonstrated that EPC are recruited from bone marrow and the bloodstream to areas of new blood vessel formation where they contribute to (neo)-vascularization after myocardial infarction, stroke, lower extremity ischemia as well as in tumours [7]. Based on studies like these, a new concept of 'therapeutic vasculogenesis' was developed that implies transplantation of EPC to enhance (neo)-vascularization of ischemic tissues [6].

Although transplantation of EPC induced angiogenesis and vasculogenesis in a therapeutically efficient manner in a number of experimental and clinical studies aiming for blood vessel formation in ischemic tissues [8], knowledge about their usefulness in generating blood vessels in bioartificial tissues *in vivo* is limited [9].

Thus far, application of EPC has mostly been restricted to seeding on various three-dimensional matrices resulting in formation of capillary-like tubes *in vitro* [10]. We recently suspended the murine embryonal EPC line T17b (T17b EPC) [11] in a commercially available fibrin matrix and demonstrated that these cells could be induced towards proliferation as well as differentiation and vascular endothelial growth factor (VEGF) secretion after an incubation period of 8 days [3]. Moreover, morphological analysis showed formation of lumen-containing circular structures. The T17b EPC line is derived from murine embryos at Day E 7.5 and has been initially characterized by Hatzopoulos *et al.* [11]. T17b EPC display robust growth properties and stability of phenotype *in vitro* and have been extensively characterized *in vitro* [11, 12] and *in vivo* in several different animal models [12–14]. They displayed unlimited growth potential typical for stem-cell populations and stability of the phenotype in culture. Expression of the endothelial-specific genes tie-2 and thrombomodulin as well as the early mesodermal marker fibroblast growth factor (fgf)-3 was revealed during RNA analysis, as well as specific binding of the GSL I-B4 isolectin, a marker of early endothelial cells, to the isolated T17b cells. They can be differentiated to mature endothelial cells and were shown to form vascular structures *in vitro* [11, 12] and *in vivo* [12–14]. T17b differentiation was associated with a highly significant increase of von Willebrand Factor and VEGF receptor-2 which can serve as differentiation markers for T17b EPC. *In vivo* studies demonstrated that T17b EPC home to ischemic tumour areas where VEGF concentrations were particularly high [12], indicating a potential chemotactic recruitment of EPC *via* increased VEGF production or – in concordance with our *in vitro* studies – EPC themselves being the source of increased VEGF production in states and areas of hypoxia. Moreover, T17b EPC are a rich source of proteins that modulate angiogenesis and tissue regeneration and promote blood vessel formation in tissue ischemia [14].

In the light of these findings, we suggested that T17b EPC may also play a role in blood vessel formation in tissue-engineered matrices. The aim of this study was to use a rat model where cells are suspended in a fibrin matrix and implanted in a subcutaneous chamber as a screening model to evaluate cell survival and quantitatively analyse distribution kinetics and their effect on granulation tissue and blood vessel formation.

Materials and methods

Cell culture

T17b embryonal murine EPC were cultured in DMEM Glutamax (Invitrogen, Carlsbad, CA, USA) containing 20% foetal bovine serum 100 U/ml Penicillin, 100 μg/ml Streptomycin, 0.1 mM β-Mercaptoethanol, 1 mM non-essential amino acids and 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.5 (all purchased from Invitrogen). To track the fate of transplanted EPC, cells were marked with the fluorescent carbocyanine DiI dye (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) as previously described [15]. In brief, before cellular transplantation, EPCs in suspension were washed with phosphate-buffered saline (PBS) and incubated with DiI at a concentration of 2.5 μg/ml PBS for 5 min. at 37°C and 15 min. at 4°C. After two washing steps in PBS, the cells were resuspended in DMEM Glutamax medium.

Transfection and evaluation of β-galactosidase activity

Transfection

The day prior to transfection, cells were plated at a density of 1,000,000 cells per well of a six-well plate. For transfection 8 μg of pcDNA3.1-lacZ-Hygro (Invitrogen) plasmid DNA were diluted in 250 μl OptiMEM (Invitrogen) media per well. LipofectAMINE 2000 (Invitrogen) was diluted 1:50 in OptiMEM, and equal volumes of diluted LipofectAMINE 2000 and DNA were combined and gently mixed by inversion. After 20 min. incubation at room temperature, a volume of 500 μl OptiMEM was added and applied to the cells. After 4 hrs of incubation medium cells were washed twice with PBS and cell type specific medium was applied.

For the generation of single cell clones, transfected cells were seeded at low density of 20% to 30% confluence in 10 cm dishes 24 hrs after transfection. Cells were selected by supplementing the respective culture medium with 200 μg/ml hygromycin (Invitrogen). Resistent cell clones were harvested and expanded.

β-galactosidase assay

Stable cell clones were analysed for lacZ expression by a β-Gal Assay Kit (Invitrogen). For this purpose protein was extracted from the cells with the corresponding 1× lysis buffer. For normalization protein concentrations were determined using a modified Lowry method (Bio-Rad DC protein assay, Bio-Rad Laboratories GmbH, Vienna, Austria) with bovine serum albumin (Promega GmbH, Mannheim, Germany) as a reference standard. β-galactosidase activity was measured in a TECAN microtitre plate reader (TECAN Genios, TECAN Austria GmbH, Vienna, Austria), expressed as optical density (OD) units at 405 nm.

β-galactosidase staining of cells

The day prior staining 1,000,000 cells were seeded per well of a six-well plate. For visualization of β-galactosidase activity cells were washed twice

with ice-cold PBS, then fixed with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 20 min. After washing the cells three times with PBS, staining solution consisting of the X-GAL mixer (20 mM K3Fe(CN)6, 20 mM K4Fe(CN)6.3H2O and 2 mM MgCl2 (all reagents from Sigma) solved in PBS and 1/40 X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [Sigma] at a concentration of 40 mg/ml in dimethylformamide [Sigma]) was added to the cells and incubated at 37°C in obscurata for 2 hrs. Cells were examined by light microscopy (Zeiss Axiovert 200M, Carl Zeiss GmbH, Vienna, Austria).

T17b EPC fibrin matrix

A total of 5×10^6 EPC fluorescently labelled with DiI were re-suspended in 600 μl fibrin gel (10 mg/ml Fibrinogen, 2 IU/ml thrombin, Baxter Healthcare, Vienna, Austria, approved for clinical application) and immediately placed inside the Teflon chamber which was subsequently implanted subcutaneously on the dorsum of rats.

Animals

Male Lewis rats (Charles River Laboratories, Sulzfeld, Germany) were used and German regulations for the care and use of laboratory animals were observed throughout the entire study. All experiments were approved by the animal care committee of the University of Erlangen and the Government of Mittelfranken, Germany. Animals were housed in the veterinary care facility of the University of Erlangen Medical Center and submitted to a 12 hr dark–light cycle with free access to rodent standard diet (Altromin, Hamburg, Germany) and tap water.

Surgical procedures

All operations were performed by the same surgeon on rats under general inhalation anesthesia using isoflurane (Baxter, Unterschleißheim, Germany). In both groups, a subcutaneous pocket was created on the dorsum of the animals. The chamber was filled with 600 μl fibrin gel containing EPC. Thereafter the lid was closed and the chamber fixed on the dorsum muscle using Prolene 3–0 (Ethicon, Norderstedt, Germany) sutures. Haemostasis was achieved followed by wound closure using Vicryl 5–0 (Ethicon). After surgery, analgesia was assured by subcutaneous application of 0.2 ml Benzylpenicillin-Benzathin (Tardomycel Comp, Bayer, Leverkusen, Germany) and buprenorphin (0.3 mg/kg rat weight, Temgesic, Essex Chemie AG, Luzern, Switzerland). Thirty minutes before animals were killed, they received an intravenous injection of 500 μg Bandeirea Simplicifolia lectin (BS-1) conjugated with FITC (Vector Laboratories Inc., Burlingame, CA, USA). Six constructs per group and each time-point were examined, resulting in a total of 36 animals for the entire study.

Histological and statistical analysis

Six constructs per group and time-point were explanted *in toto* with the surrounding tissue. After fixation in 3.5% formalin solution, constructs were dehydrated in graded ethanol and embedded in paraffin. A total of 3 μm cross-sections were obtained using a Leica microtome (Leica Microsystems, Bensheim, Germany) where two standardized planes were defined (1 mm proximal and 1 mm distal of the central plane). Haematoxylin and eosin staining was performed according to standard protocols. Immunohistochemical staining was performed with the fluorescent BS-1, lectin and ED-1 for macrophage detection. Detection of the transgenic T17b-lacZ EPC was performed with β-galactosidase staining as described above. Fluorescence microscopy was carried out to detect EPC labelled with Di-I *in vitro* prior to subcutaneous implantation and in growing blood vessels decorated with BS-1 lectin which was intravenously injected immediately prior to killing of the animals. DAPI fluorescent staining was done directly on histological slides.

The images were evaluated by an independent and blinded observer. All images of haematoxylin and eosin stained cross-sections were generated with a Leica microscope and digital camera using 25× magnification. Apart from morphologic evaluation, height and volume of the matrix as well as height and volume of the fibrovascular tissue adjacent to the matrix were quantified for both groups (with or without EPC) and each time-point. Morphometric quantification of the height of both the matrix as well as the fibrovascular tissue was previously established by our group for this animal model and a detailed description can be found in [4]. Briefly, the fibrovascular tissue adjacent to the matrix of all cross-sections stained with lectin was divided into three regions of equal size, one at the central part of the section and two at either periphery. Images were generated with a Leica microscope using 200× magnification. Images were rendered bimodal for quantification (standardized threshold) (WinQ, Leica Microsystems).

Number of blood vessels, average size of blood vessels and standard deviation of blood vessel size was calculated for both groups and each time-point. Results are presented as mean ± S.D. Statistical analysis was carried out GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Two-tailed unpaired Student's *t*-test was applied for statistical analysis. A *P*-value <0.05 was determined statistically significant.

Immunohistochemistry

Lectin: The lectin BS-1 was used for immunohistochemical detection of rat endothelial cells [4]. Paraffinated sections were submitted to a xylol/ethanol sequence, rinsed in PBS followed by blocking of endogenous peroxidise activity in H_2O_2 for 10 min. Blocking was completed by incubation with avidin and biotin 15 min. each (Vector Laboratories) as well as 5% goat serum for 1 hr. Overnight incubation was performed with biotinylated lectin (BS-1, Sigma-Aldrich) in a 1:100 dilution in PBS overnight at 4°C. Slides were rinsed in PBS between incubation steps. Detection was achieved by incubation with streptavidin antibody complex/horseradish peroxidase (HRP) (Dako GmbH, Hamburg, Germany) for 30 min. followed by development with DAB + Chromogen (Dako GmbH). Cardiac muscle sections were used as positive controls, whereas absence of lectin served as negative control.

ED-1: Macrophages were detected immunohistochemically using ED-1 stainings. Sections were deparaffinated and incubated for 30 min. with 1:300 anti-ED-1 primary antibody (Serotec, Raleigh, NC, USA). A goat anti-mouse secondary antibody (Dako GmbH, Hamburg, Germany) was used at 1:20 for 30 min. After that sections were incubated with 1:50 APAAP complex (alkaline phosphatase–anti-alkaline phosphatase) for 30 min. Enhancement was achieved by repeating incubation with secondary antibody and with the APAAP complex for 10 min. each. The enzyme was revealed with 0.2 mg/ml naphthol AS.MX (Sigma Chemical Co., St. Louis, MO, USA), 0.002 mg/ml dimethyl formamide (Merck, Darmstadt, Germany) in 0.1 M Tris pH 8.2, 1 mg/ml fast red TR salt (Sigma Chemical Co.) and 0.33 mg/ml levamisole (Sigma Chemical Co.). Sections were counterstained with haematoxylin (Merck).

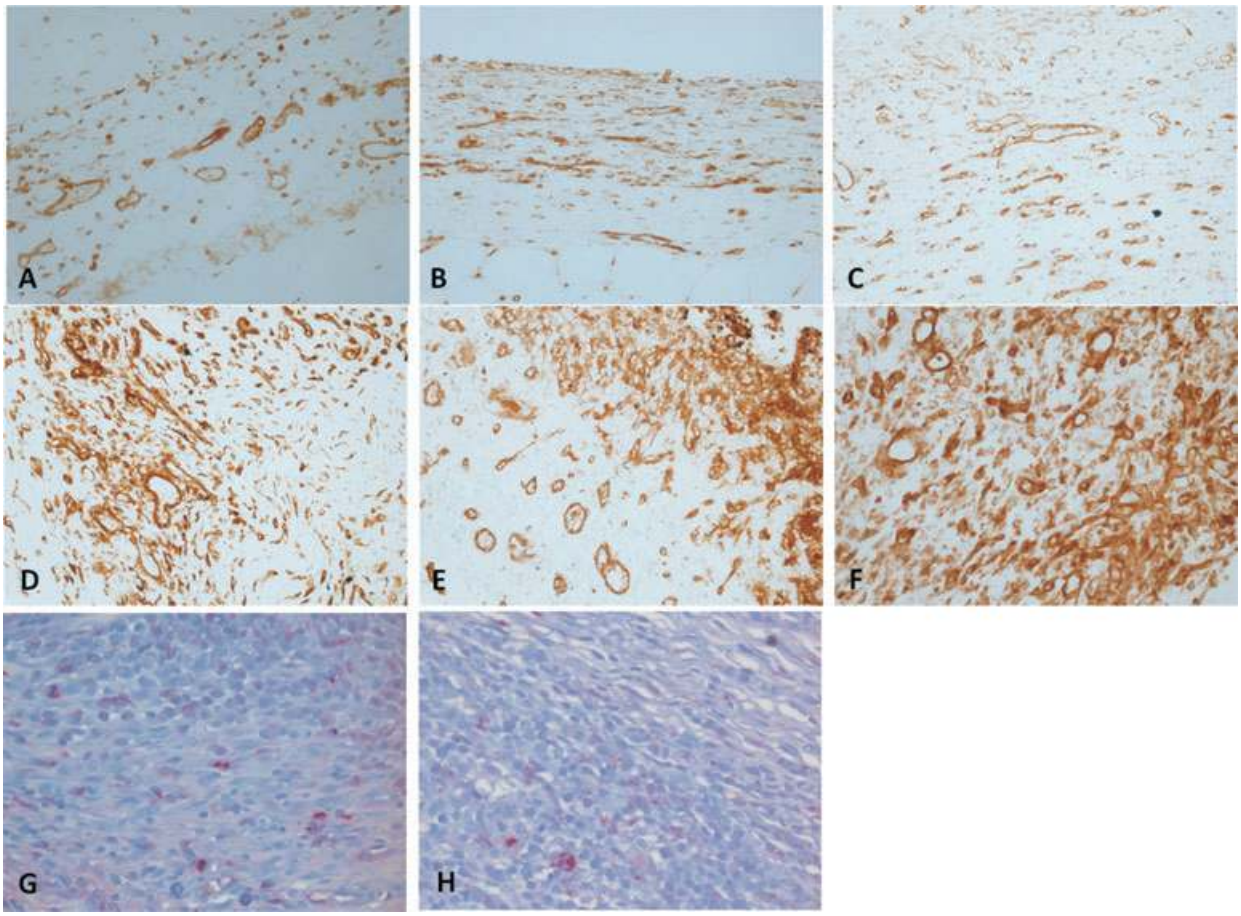

Fig. 1 Representative micrographs of fibrovascular tissue adjacent to fibrin matrices and detection of blood vessel formation by immunohistochemical analysis. Endothelial cell lectin staining of cell-free constructs (**A**–**C**) and T17b EPC-containing constructs (**D**–**F**) at day 3 (**A**, **D**), day 7 (**B**, **E**) and 14 (**C**, **F**). Representative photographs of ED-1 staining of macrophage invasion at day 14 in cell-free constructs (**G**) compared to cell-containing constructs (**H**). Numbers of blood vessels were increased at day 3 (**D** *versus* **A**) and 7 (**E** *versus* **B**) when T17b EPC were present within the construct. Magnification ×200 (**A**–**F**) and ×400 (**G**, **H**). $N = 6$ constructs per group and time-point.

Fluorescence microscopy

Images were generated with a Leica microscope using 200× magnification. Appropriate filter systems for detection of the triple fluorescence labelling was used (red – Di-I; green – BS-1 lectin; blue – DAPI).

Results

Surgery and animals

All rats tolerated the surgical procedure well. No major postoperative complications such as haematoma, infection or wound dehiscence were observed. No extrusion or perforation of the implants was noticed throughout the entire experiment.

Upon explantation, all chambers in all groups were surrounded by a connective tissue capsule. At day 3, integrity of fibrin clots was still completely preserved without signs of fibrinolysis or degradation. At 1 week, there was a significant volume reduction in both EPC-containing fibrin clots as well as cell free controls. Volume reduction was more pronounced in EPC-free clots. After 14 days, the major part of the fibrin matrix was resorbed in both groups; the average volume of EPC-containing clots being significantly reduced compared to EPC-free matrices (data not shown).

There was no histological evidence of blood vessel invasion into the fibrin matrix throughout the entire experiment. Significant foreign body reaction involving multinucleated giant cells could not be detected within fibrin clots or adjacent fibrovascular granulation tissue in either of the two groups (data not shown). Presence of blood vessels in granulation tissue was confirmed by specific immunohistochemical staining for BS-1 (Fig. 1). ED-1 immunohistochemical

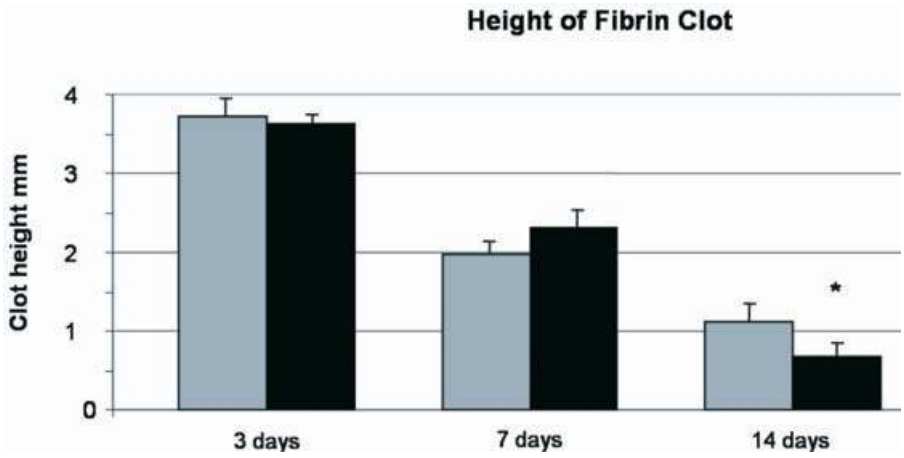

Fig. 2 Morphometric analysis of fibrin clot height. Grey bar = fibrin; black bar = fibrin + EPC. $*P < 0.05$ *versus* Fibrin at day 14. $N = 6$ constructs per group and time-point.

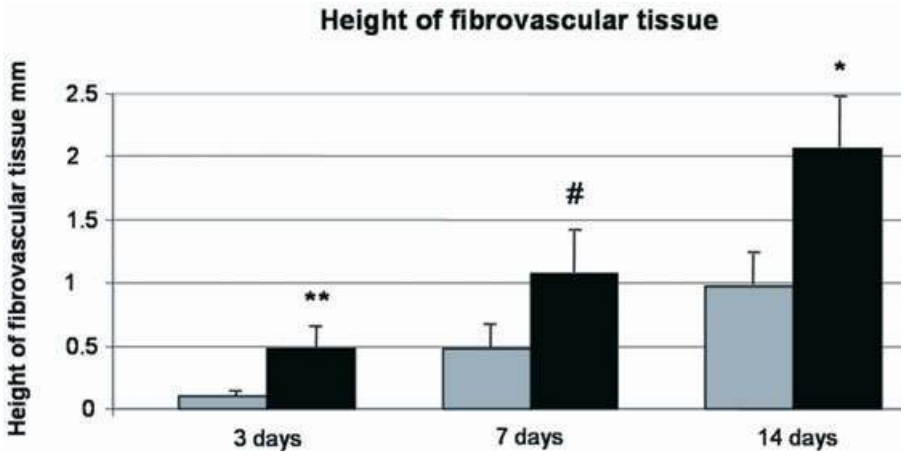

Fig. 3 Morphometric analysis of fibrovascular tissue thickness. Grey bar = fibrin; black bar = fibrin + EPC. $*P < 0.05$ *versus* Fibrin at day 14; $\#P < 0.05$ *versus* Fibrin at day 7; $**P < 0.05$ *versus* Fibrin at day 3. $N = 6$ constructs per group and time-point.

staining was performed to assess if macrophage infiltration is increased after xenogenic transplantation of murine EPC into the rat host. At 14 days after implantation, representative micrographs failed to detect significantly altered levels of macrophage invasion into the newly formed fibrovascular tissue (Fig. 1). Lectin-stained cross-sections were used for morphometric analysis in terms of quantification of blood vessel formation within the fibrovascular granulation tissue adjacent to the fibrin clots (Fig. 1).

Morphometric analysis

Addition of T17b EPC resulted in a significantly increased degradation with consecutive reduction in fibrin clot height at 14 days after implantation (Fig. 2). There was no significant influence on fibrin clot height at earlier explantation time-points days 3 and 7 (Fig. 2).

The use of EPC significantly increased height of fibrovascular tissue at 3 days, 7 days as well as 14 days compared to EPC-free controls (Fig. 3).

The total number of blood vessels counted in fibrovascular tissue adjacent to the matrices of lectin stained cross-sections was significantly increased after 3 and 7 days when T17b were present in the fibrin clot. At day 14, there was no significant difference in number of blood vessels (Fig. 4).

Conversely, the average size, *i.e.* the diameter of blood vessels in the fibrovascular tissue was significantly increased at all time-points in EPC-containing clots (Fig. 5).

Fluorescence microscopy

Fluorescence microscopy showed that number of EPC within the fibrin clot decreased over the experiment. At day 3, Di-I^+ EPC appear numerous within the clot, whereas their number decreases at day 7. At day 14 after implantation, only a small number of EPC is present within the fibrin clot (Fig. 6A-C).

The distribution kinetics of Di-I^+ EPC in the fibrovascular tissue adjacent to the fibrin clot follows a pattern exactly opposite,

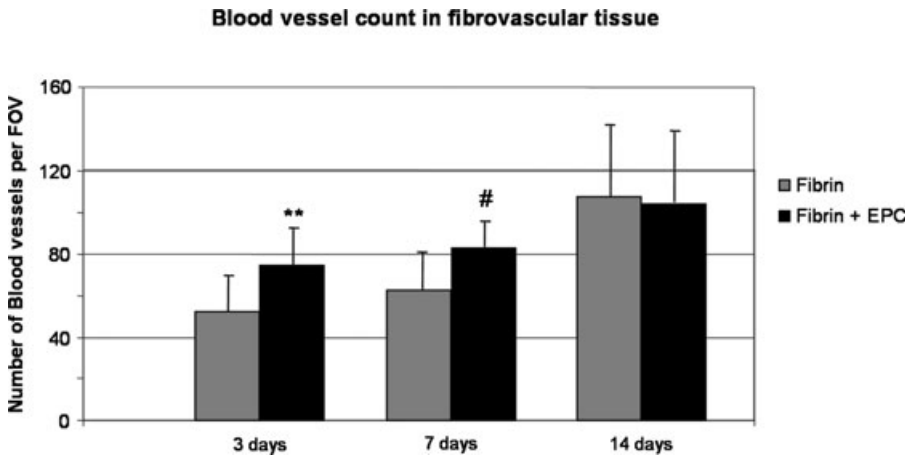

Fig. 4 Morphometric analysis of number of blood vessels. Grey bar = fibrin; black bar = fibrin + EPC. $\#P < 0.05$ *versus* Fibrin at day 7; $**P < 0.05$ *versus* Fibrin at day 3. $N = 6$ constructs per group and time-point.

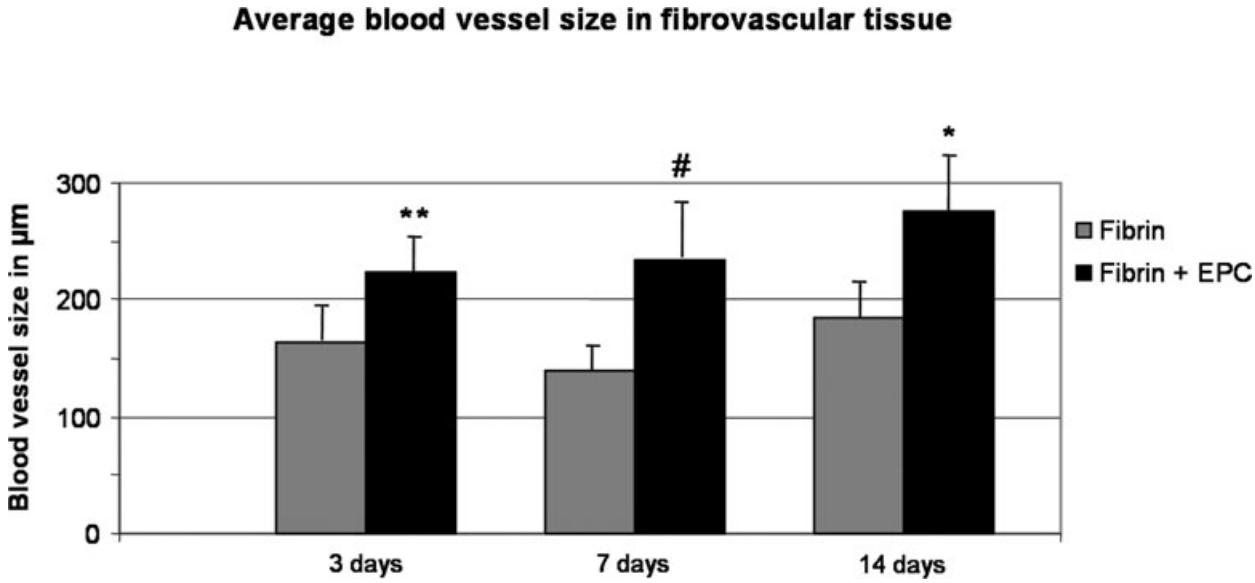

Fig. 5 Morphometric analysis of size (diameter) of blood vessels. Grey bar = fibrin; black bar = fibrin + EPC. $*P < 0.05$ *versus* Fibrin at day 14; $\#P < 0.05$ *versus* Fibrin at day 7; $**P < 0.05$ *versus* Fibrin at day 3. $N = 6$ constructs per group and time-point.

with very few cells present at day 3. The number of DI-I^+ cells in the fibrovascular tissue is increased at day 7 and even higher at day 14 (Fig. 6D-F).

Triple fluorescence microscopy for BS-1 lectin (labelling host rat endothelium and transplanted murine T17b EPC in green), Di-I (corresponding to pre-labelled murine T17b in red) and DAPI (marking all nucleated cells in blue) demonstrated that EPC take part in blood vessel formation within the fibrovascular tissue where they form part of the capillary lumina (Fig. 7A-C).

Presence of EPC within intermuscular septae was confirmed in an analogous fashion (Fig. 7D-F).

Abundant amounts of EPC could be detected in the spleen at 2 weeks after transplantation (Fig. 8A-C), but in none of the other harvested organs such as liver, lungs, heart and kidneys (data not shown).

Transplantation of the X-GAL expressing transgenic EPC clone T17b-lacZ was performed in the same fashion as for fluorescence-stained T17b EPC to confirm vitality and biologic activity of transplanted EPC. Indeed, 2 weeks after implantation, a large amounts of blue X-GAL expressing EPC was present in the granulation tissue and intermuscular septae, indicating the transplanted cells are still alive and biologically active (Fig. 9A and B). Distribution pattern and impact of T17b-lacZ on fibrin clot behaviour and fibrovascular tissue formation as well as blood vessel number and diameter were comparable to fluorescently labelled T17b EPC (data not shown).

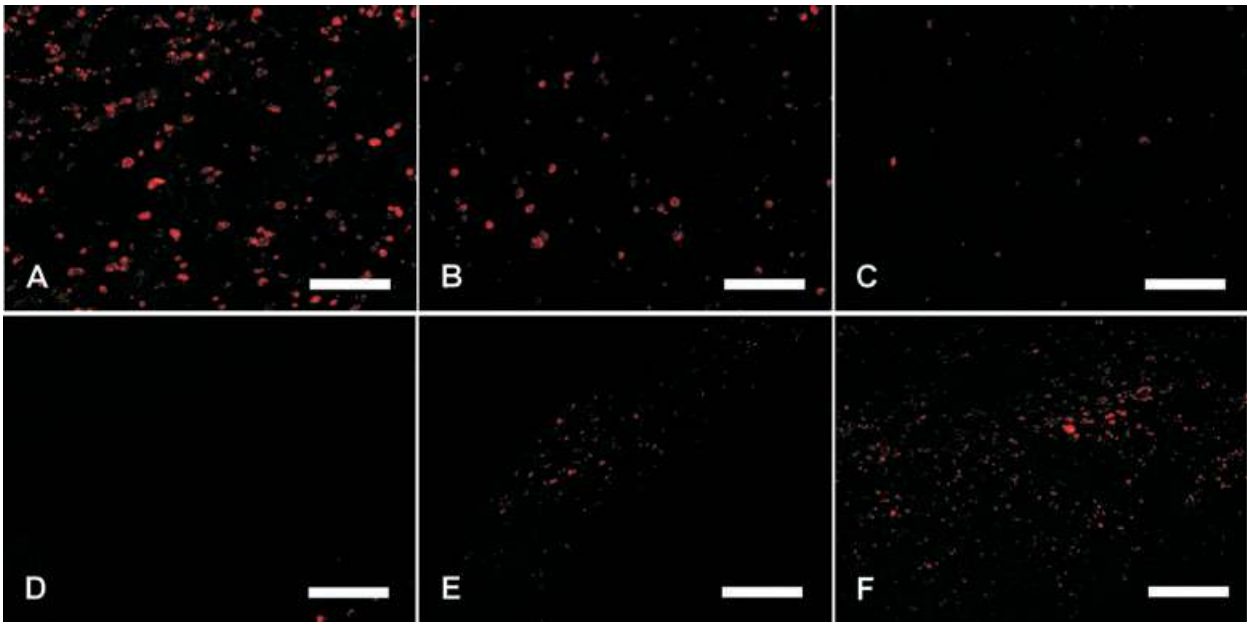

Fig. 6 Localization of DiI labelled T17b EPC in fibrin clot (**A**–**C**) and fibrovascular granulation tissue (**D**–**F**) at day 3 (**A**), (**D**), day 7 (**B**), (**E**) and day 14 (**C**), (**F**) by Fluorescence Microscopy. With increasing duration of the experiment, there was a shift of T17b cells from within the fibrin clot into the adjacent layers of tissue, *i.e.* to the granulation tissue and the adjacent intermuscular septae. At day 3, Di-I^{+} EPC appeared numerous and homogenously distributed within the clot, with only occasional cells within the newly formed granulation tissue (**A** *versus* **D**). The number of T17 EPC within the fibrin clot was decreased whereas there was an increase of cells within the granulation tissue at day 7 (**B** *versus* **E**). At day 14 after implantation, the vast majority of T17b EPC was located in the granulation tissue, whereas only a small number of cells were still present within the fibrin clot (**C** *versus* **F**). Scale bar = 200 μm. $N = 6$ constructs per group and time-point.

Discussion

In the present study we demonstrate that transplantation of murine embryonal EPC suspended in a fibrin matrix efficiently modulates fibrovascular tissue formation and is involved in the formation of neo-capillaries within fibrovascular in the subcutaneous model in the rat.

The addition of EPC did not lead to a significant decrease of construct size at earlier time-points but at day 14 compared to cell-free control matrices. The possible reason may be two-fold. On the one hand, ingrowth of fibrovascular tissue may occur with subsequent resorption of the matrix [4]. A similar mechanism could be enabled by the transplanted EPC themselves through the release of fibrinolytic enzymes, particularly matrix metalloproteinases [16], leading to enhanced degradation of the matrix while the cells migrate through the matrix towards the fibrovascular tissue that is formed adjacent to the fibrin construct. This has previously been shown to be essential during the neovascularization process supported by cells derived from the endothelial lineage [16, 17].

Generation of fibrovascular tissue was efficiently stimulated by the addition of T17b EPC immobilized within the fibrin matrix, resulting in a significant increase in volume and height of fibrovascular tissue compared to cell-free clots at all observed time-points. In addition, quantification of blood vessels staining positive for lectin revealed that the angiogenic response was accelerated at early observation times: At days 3 and 7 after implantation, numbers of blood vessels were significantly increased in cell-containing fibrovascular tissue of the constructs, whereas the size of blood vessels was increased at all observation points. Altman *et al.* recently demonstrated that fibrovascular tissue formation can be efficiently stimulated by cell therapy as they delivered human adipose-derived stem cells to a murine soft tissue injury model, resulting in increased wound healing, microvessel density and adipose-derived stem cell differentiation into fibrovascular, endothelial and epithelial components of restored tissue [18]. In the past, platelet-rich plasma has been used to enhance wound healing, and increase in fibrovascular tissue formation has been attributed to the rich source of supportive growth factors contained within the platelets [19]. In our previous study using the same subcutaneous chamber implantation model we demonstrated enhanced fibrovascular tissue formation when recombinant growth factors VEGF and bFGF were suspended in the adjacent fibrin matrix [4]. EPC differentiate after *in vivo* transplantation under the influence of multiple local growth factors and cytokines and have been shown to be a rich source of growth factors supporting angiogenesis and proliferation themselves, potentially stimulating fibrovascular tissue formation in the present study.

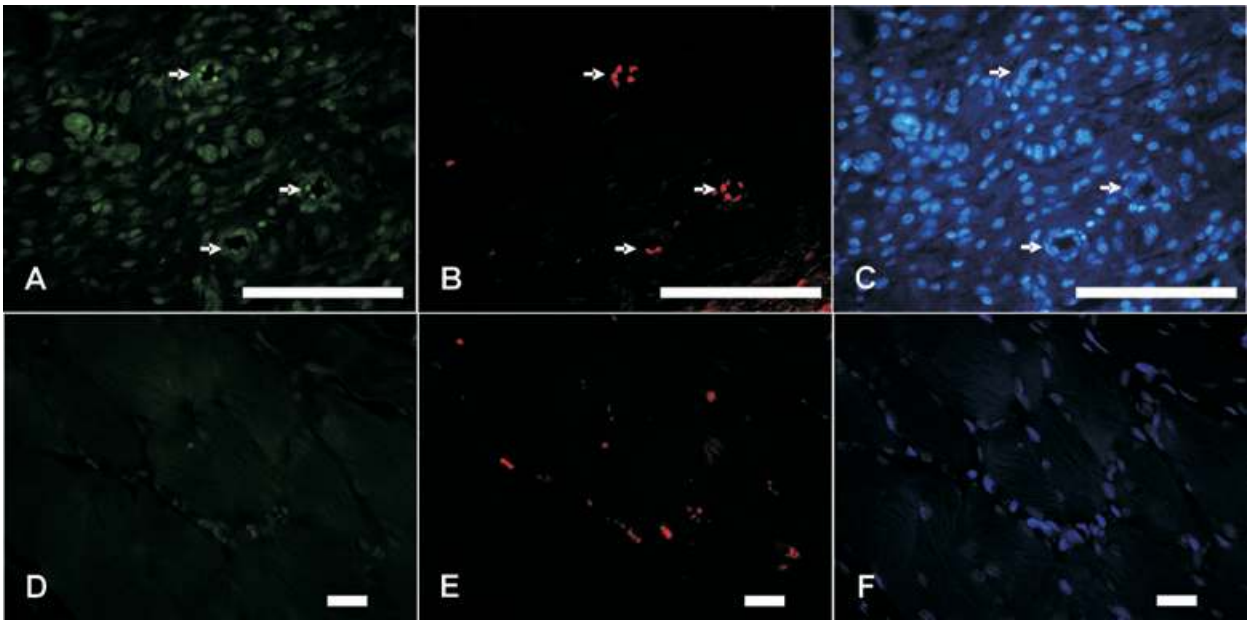

Fig. 7 Detection of EPC incorporation in fibrovascular tissue capillaries by Fluorescence Microscopy. Triple fluorescence microscopy enabled visualization of all blood vascular endothelial cells (host rat + transplanted murine T17b EPC) in green by BS-1 lectin, xenogenically transplanted murine T17b EPC in red (pre-labelled by Di-I) and all nucleated cells stained by DAPI in blue. This permitted identification of T17b EPC as well as their separation from other nucleus-positive cells, *i.e.* cells derived from the host, and demonstrated localization of T17b cells in relation to the vascular network within the granulation tissue (**A**–**C**) and the underlying muscle (**D**–**F**). Integration of the transplanted cells into newly formed capillaries within the granulation tissue is demonstrated by co-localization of murine T17b EPC (red fluorescence) and blood vascular endothelium (green fluorescence) (**A**, **B**). T17b were not only present within fibrin clot and granulation tissue but also within the underlying intermuscular septae (**D**, **E**). Scale Bar = 200 μm in all micrographs. $N = 6$ constructs per group and time-point.

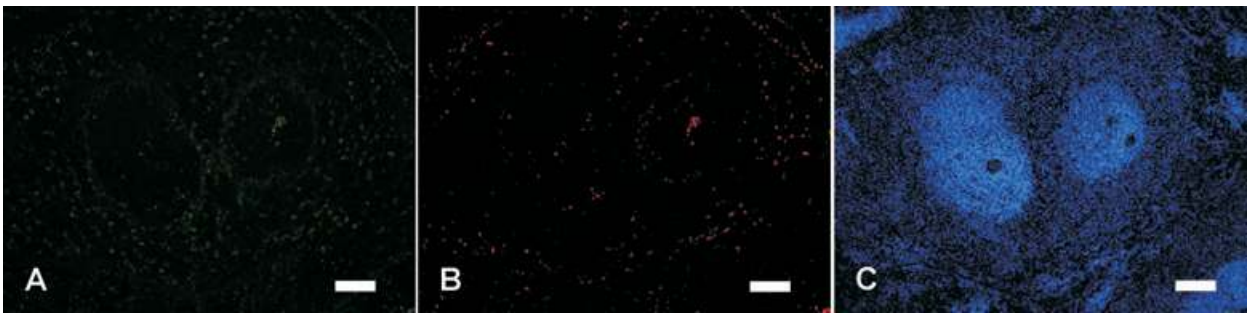

Fig. 8 Detection of EPC access to systemic circulation by Fluorescence Microscopy. Abundant amounts of EPC could be detected in the spleen at 2 weeks after transplantation (**A**–**C**), but in none of the other harvested organs such as liver, lungs, heart and kidneys (data not shown). Scale bar = 200 μm. $N = 6$ constructs per group and time-point.

In contrast to our above mentioned previous study where a combination of recombinant growth factors VEGF and bFGF enhanced blood vessel formation but did not change blood vessel size [4], the present study only showed an increased number of blood vessels at early observation times whereas the blood vessel size significantly differed from the cell- free control group at all time-points. A possible explanation is that the growth factor levels released from the now mature endothelial cells after differentiation *in vivo* differ from the concentration of the recombinant growth factors suspended in the fibrin matrix. In addition, EPC also contain an additional subset of different growth factors and cytokines that additionally modulate formation of blood vessels. Growth factor release kinetics from the murine endothelial cells may also be different compared to the suspended recombinant growth factors. Unfortunately, a diligent and reliable molecular analysis of growth factor expression by the EPC after *in vivo* transplantation is not feasible due to a high degree of homology between murine and rat. Taken together, the

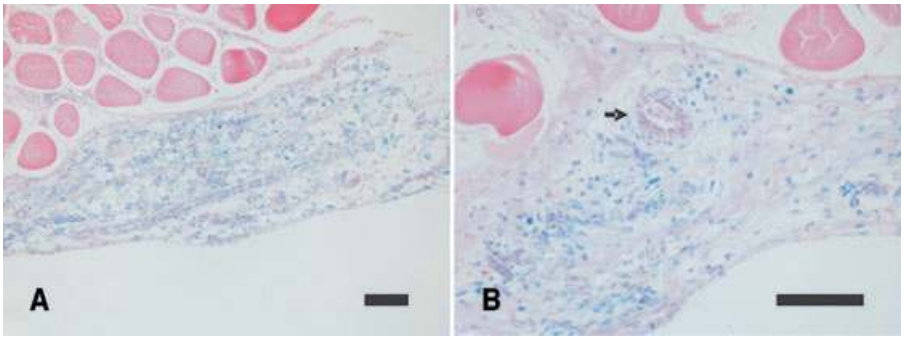

Fig. 9 Demonstration of EPC vitality and migration using the β-galactosidase expressing clone T17b-lacZ. At day 14 after implantation, large numbers of β-galactosidase-expressing EPC were present in the granulation tissue and intermuscular septae, identified by their blue staining, indicating vitality and biological activity of the transplanted cells (**A**, **B**). Some of them were located in close proximity to blood vessels (note arrow in **B**). Scale Bar = 200 μm in all micrographs. *N* = 6 constructs per group and time-point.

differences in composition, release kinetics and concentrations of the growth factors may have a significant impact on maturation of the newly formed vascular network within the fibrovascular tissue, possibly indicated by the differing size and calibre of blood vessel and their variation. This hypothesis is supported by previous work from our laboratory where remodelling was quantitatively analysed in a neovascular network in the arteriovenous loop model and variance of blood vessel calibre identified as a parameter of vascular maturation state [20].

The subcutaneous implantation model was shown to be an efficient *in vivo* screening model for the effect of EPC immobilized in a fibrin matrix on blood vessel formation. Using triple fluorescence labelling for transplanted murine embryonal EPC host vasculature and cell vitality, we demonstrated that EPC do not persist within the fibrin construct into which they were initially suspended, but gradually migrate towards the newly forming granulation tissue where they can be found in increasing numbers after differentiation with later observation times after implantation. At day 14, almost no cell can be detected within the fibrin clot but numerous cells are found within the fibrovascular tissue. The mechanisms triggering EPC migration are not yet defined, but previous experimental studies by other groups revealed that chemotaxis and oxygen gradients play a crucial role in EPC recruitment to the zone of injury [14, 21, 22]. Investigating one identical single region of interest by triple fluorescence staining, we could show co-localization of EPC with the vessel wall of blood vessels that were newly formed within the fibrovascular tissue. This is in concordance with previous studies using the same murine EPC cell line where EPC were either associated with or even integrated in newly formed blood vessels after myocardial ischemia [14] and in tumours [12, 13] after differentiation and maturation, thereby contributing to vasculogenesis. Cells were also shown to migrate further towards intermuscular septa, and even gaining access to systemic circulation, as proven by their detection within the spleen.

A drawback of fluorescence staining lies in the fact that conclusions regarding cell vitality and biologic activity are limited in their reliability because fluorescence may even persist after phagocytosis of the cell. Therefore, we used a transgenic EPC clone expressing the reporter gene β-galactosidase for analogous implantation within the fibrin matrix. Indeed, we could demonstrate that EPC remain viable after 14 days, showing abundant presence in the fibrovascular tissue and within the intermuscular septae. Despite these advantages, genetic alteration of the EPC by lacZ incorporation may, however, result in altered growth potential *in vivo* and a reduced overall vitality. We did notice a discretely reduced *in vitro* growth potential of T17b-lacZ compared to wild-type T17b EPC after fluorescence labelling but did not observe differences in their effect *in vivo* (data not shown).

Conclusion

In conclusion, we demonstrated that murine embryonal EPC can be immobilized in a fibrin matrix and can be transplanted in the subcutaneous rat chamber model where they exhibit biological activity. This was manifested by an increase in fibrovascular tissue formation and an accelerated formation of blood vessels as well as an increased size of these newly formed blood vessels. In addition to their previously documented indirect effect on vessel growth through release of proangiogenic substances, fluorescence labelling also demonstrated integration of EPC in the new blood vessels, indicating that these cells may support vasculogenesis.

These data identify EPC as a promising vehicle to enhance vasculogenesis in tissue-engineered matrices.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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