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Sustained delivery of SDF-1α from heparin-based hydrogels to attract circulating pro-angiogenic cells

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ABSTRACT

Enrichment of progenitor cells in ischemic tissue has become a promising therapeutic strategy in the treatment of myocardial infarction. Towards this aim, we report a biology-inspired concept using sulfated glycosaminoglycans to sustainably generate chemokine gradients for the localized accumulation of early endothelial progenitor cells (eEPCs). StarPEG-heparin hydrogels, which have been previously demonstrated to support angiogenesis, were functionalized with SDF-1α, a potent chemoattractant known to act on EPCs. The gels were quantitatively shown to release the chemokine in amounts that are adjustable by the choice of loading concentrations and by matrix metalloprotease (MMP) mediated hydrogel cleavage. Transwell assays confirmed significantly enhanced migration of early EPCs towards concentration gradients of hydrogel-delivered SDF-1α *in vitro*. Subcutaneous implantation of SDF-1α-releasing gels in mice resulted in massive infiltration of early EPCs and subsequently improved vascularization. In conclusion, sustained delivery of SDF-1α from pro-angiogenic starPEG-heparin hydrogels can effectively attract early EPCs, offering a powerful means to trigger endogenous mechanisms of cardiac regeneration.

1. Introduction

The deterioration of cardiac function after myocardial infarction is a major cause of high morbidity and mortality [1]. Effective repair of ischemic tissue after myocardial infarction (MI) remains challenging as cardiomyocytes have a limited regeneration potential and current conventional treatment modalities are restricted to incomplete recovery of tissue structure and function. The success of cell therapy approaches in alleviating the consequences of MI was concluded to be influenced by the incorporation of injected cells into new capillaries and on the release of factors that promote angiogenesis or limit apoptosis in a paracrine manner [2]. While various stem and progenitor cell types, including embryonic stem cells, hematopoietic stem cells, and endothelial progenitor cells were applied and different injection schemes were employed, the retention, survival, differentiation, and integration of the transplanted cells so far remained rather low [3].

In view of the fact that regeneration is an integrated process, involving not only cells but also their supporting matrices, the activation of the organism's inherent regeneration potential by injecting biofunctional polymer matrices into ischemic tissue holds great promise for inducing regeneration [4]. ECM-derived biomaterials including fibrin, collagen or Matrigel were primarily explored for that purpose [5]. While these materials were shown to enable cell adhesion and therefore improve cell survival and reduce apoptosis *in vivo* [5], they hardly allow for the systematic or independent variation of their inherent signaling characteristics that is, however, required to elicit particular cellular responses selectively. Engineered matrix materials have to provide cells with a protective microenvironment that undergoes cell-mediated remodeling during regeneration without formation of toxic degradation products [4]. Currently, most ECM-based cardiac biomaterials degrade too fast to allow for improvement of cardiac function or are inappropriately persistent causing the formation of isolated, nonfunctional cell "islands" [6]. Therefore, materials with cell-responsive degradation are needed to maintain their structural integrity for longer time periods while locally allowing for remodeling and matrix replacement. Cell attachment and engraftment can be achieved by functionalization of polymer scaffolds with adhesion receptor ligand peptides resulting in improved cell viability and accelerated cardiac tissue regeneration [7]. Additionally, incorporation and site-specific

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delivery of regulatory molecules facilitate the recruitment of cells from the blood stream to the scaffold, improve vascularization and guide differentiation of recruited cells. In this context, immobilization and delivery of angiogenic factors such as VEGF and FGF-2 from hydrogels (fibrin, gelatin, hyaluronic acid, collagen, alginate, chitosan etc.) was found to promote neovascularization [6].

In parallel, chemotactic molecules have received increasing interest in cardiac regeneration. Stromal cell-derived factor-1α (SDF-1α), a member of the CXC chemokine family of pro-inflammatory mediators, was identified to be a potent chemoattractant for EPCs [8]. Upregulation of SDF-1α expression after MI was described to cause elevated chemokine levels in ischemic tissue [9] and to induce mobilization and migration of EPCs from the bone marrow to the ischemic site. Further insight into that process was gained by quantification of time-dependent plasma and bone marrow levels of SDF-1α in a mouse model of hindlimb ischemia that was directly related to the mobilization of progenitor cells from the bone marrow and their transmigration into the blood stream [10].

Binding of SDF-1α to sulfated glycosaminoglycans (GAG) of the ECM such as heparan sulfate (HS) and heparin (a chemically related GAG) [11] plays a crucial role by locally accumulating and protecting the protein against degradation [9]. ECM-mediated concentration gradients of SDF-1α were reported to trigger EPC homing and migration in the infarct zone [9,12]. Furthermore, incorporation of the accumulated EPCs into new capillaries was found to promote neovascularization [12,13] and tissue regeneration after MI was concluded to be augmented by EPC secreted factors that support angiogenesis and reduce apoptosis in the ischemic tissue [2,14]. However, although increased levels of circulating EPCs were observed after MI *in vivo*, this process only peaks 7 days after vascular injury, and the delayed response appears to be insufficient to prevent cardiovascular damage [15]. Moreover, the homing rates of administered progenitor cells to ischemic tissues seem to be very low [16]. This points to the need for augmenting EPC homing to ischemic tissue immediately after MI and several studies aimed at modulating SDF-1α levels to enhance cardiac regeneration. Intramyocardial injections and adenoviral or non-viral (plasmid-based) delivery of SDF-1α were shown to promote cell mobilization and neoangiogenesis improving cardiac structure and function after MI [17]. Nevertheless, direct intramyocardial delivery of SDF-1α was found to be limited due to the rapid diffusion of the chemokine and its degradation by proteolytic enzymes [18]. Protection and controlled delivery of SDF-1α for effective recruitment of EPCs is therefore an important additional aim for biomaterials to support cardiac regeneration.

Consequently, we set out to create well-defined and localized SDF-1α gradients capable of attracting early EPCs to the ischemic site. Several recent reports concern the use of polymer matrices to release SDF-1α and ultimately support angiogenesis in bone [19], skeletal muscle [20] and for wound healing [21]. Also, experimental studies have explored the SDF-1α-induced migration of hematopoietic progenitor cells [22,23], mesenchymal stem cells [24] or bone marrow stromal cells [25]. However, biomaterials affording adjustable, defined and sustained delivery of SDF-1α in ischemic tissue remain to be developed. Therefore, a modular starPEG-heparin hydrogel system [26,27], which has been previously customized to support angiogenesis [28–30], was extended here to provide ECM-like binding, protection and sustained release of SDF-1α in precisely defined quantities.

2. Materials and methods

2.1. Preparation of starPEG-heparin hydrogel networks

StarPEG-heparin hydrogel scaffolds were formed by covalently crosslinking amino-end-functionalized starPEG with EDC/s-NHS activated carboxylic acid groups of heparin [31]. All hydrogels were formed with a total polymer content of 11.6% (w/w), at a molar ratio of starPEG to heparin (γ) of 3.

Heparin (14,000 g/mol, Calbiochem (Merck), Darmstadt, Germany) and four-arm starPEG (10,000 g/mol, Polymer Source, Inc., Dorval, Canada) were each dissolved in one third of the total volume of ice-cold deionized, decarbonized water (MilliQ) by ultrasonication. In a similar manner, EDC (Sigma–Aldrich, Munich, Germany) and s-NHS (Sigma–Aldrich, Munich, Germany) were each dissolved in one sixth of the total volume of ice-cold MilliQ. A ratio of 2:1:1 EDC:s-NHS:NH_2-groups of starPEG [mol/mol] was used. Subsequently, EDC and s-NHS solutions were added to heparin, mixed well, and incubated for 15 min. The dissolved starPEG was then mixed with the activated heparin by vortexing the solution for 15 s. All solutions were kept on ice (2–4 °C) unless otherwise indicated.

To allow for cell-mediated remodeling of the matrix, hydrogel scaffolds with matched viscoelastic properties (to non-degradable gels; $\gamma = 3$) were similarly produced using a starPEG conjugated to an MMP-cleavable peptide sequence [32]. For visualization of the gels with fluorescence microscopy, 1% of the heparin was substituted by Alexa-488-labeled heparin (synthesized by M. Tsurkan, IPF Dresden).

According to the experiment planned, the gels were either prepared as free-floating clots or immobilized to the surface of glass cover slips. To allow for basic characterization of SDF-1α uptake and release, and to evaluate the chemotactic properties of the SDF-1α-loaded polymer scaffolds in cell culture experiments, surface-bound hydrogels with a final thickness of 50 μm were prepared. Additionally, 200 μm thick fluorescence-labeled, surface-bound gels were used in vertical migration studies. In detail, for 50 μm thick scaffolds 3.11 μl of the gel mixture per cm^2 and for 200 μm 13.6 μl per cm^2 were added on freshly amino-functionalized glass cover slips to allow for covalent attachment of heparin through its activated carboxylic acid groups [32]. The liquid gel mixture was covered with a second Sigmacote (Sigma–Aldrich, Munich, Germany)-treated hydrophobic glass cover slip in order to spread the solution equally. Presented results are expressed for scaffolds prepared from 5.5 μl for 50 μm thick gels and 24 μl for 200 μm thick gels.

To prepare free-floating gel clots for implantation, 50 μl of the liquid gel mixture were placed onto a 0.2 cm^2 (5 mm) hydrophobic glass cover slip. After polymerization overnight (22 °C), the hydrophobic cover slips were removed and the gels were washed in phosphate buffered saline (PBS, Sigma–Aldrich) to remove EDC/s-NHS. The PBS was exchanged five times, once per hour, and once again after storage overnight. The swollen gels were then immediately used for experiments. Furthermore, gels used for cell culture experiments and *in vivo* studies were sterilized by UV-treatment for 30 min.

Swelling, mechanical properties and pore sizes of heparin-starPEG hydrogels were determined as described elsewhere [31].

2.2. Biomodification of the hydrogel scaffolds

2.2.1. Modification of starPEG-heparin hydrogels with RGD

Biomodification with cyclo(Arg-Gly-Asp-D-Tyr-Lys) (Peptides International, Louisville, KY, USA) was performed as described in [31]. Briefly, the swollen free-floating hydrogel clots were rinsed three times with 1/15 M phosphate buffer (pH 5) at 4 °C. Subsequently, this solution was replaced with EDC/s-NHS solution (50 mM EDC, 25 mM s-NHS in 1/15 M phosphate buffer, pH 5) to activate the heparin carboxylic acid groups. After incubation for 45 min, the gel clots were washed three times in borate buffer (100 mM, pH 8, 4 °C) to remove unbound ECD/s-NHS. Next, the gels were incubated in 800 μl RGD-solution (50 μg/ml, dissolved in borate buffer) for 2 h at room temperature. Finally, all samples were washed in PBS three times. All solutions and peptides were sterile unless otherwise indicated.

2.2.2. Modification of starPEG-heparin hydrogels with SDF-1α

To immobilize SDF-1α (Miltenyi Biotech, Bergisch Gladbach, Germany) within the starPEG-heparin hydrogels, the swollen surface-bound scaffolds were incubated with 200 μl/cm^2 SDF-1α solution dissolved in PBS to the desired concentration. After 24 h, all scaffolds were rinsed twice with PBS. Similarly, SDF-1α was immobilized within free-floating clots by incubating the gels with 5 μg/ml SDF-1α in PBS. Before using the clots for *in vivo* studies, all scaffolds were washed twice in PBS.

2.3. Analysis of SDF-1α uptake and release

2.3.1. Qualitative determination of SDF-1α uptake by cLSM

To visualize protein uptake and distribution within the gel matrix, SDF-1α was labeled with tetramethylrhodamine (TAMRA) according to the FluoReporter Tetramethylrhodamine Protein Labeling Kit Manual (Molecular Probes, distributed by Invitrogen, Netherlands). SDF-1α was diluted to a final concentration of 5 μg/ml in PBS and added to starPEG-heparin hydrogels ($n = 3$, 200 μl/cm^2), immobilized in glass bottom 24-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Leica SP5 (Leica, Bensheim, Germany) confocal Laser scanning microscope with a 40× magnification objective (HCxPL APO, Leica) and aperture pinhole set at 68 μm was used for quantitative determination of SDF-1α uptake. Fluorescence intensities of the Alexa-488-labeled starPEG-heparin hydrogels and TAMRA-labeled SDF-1α were measured by exciting the gel matrix with the argon laser (excitation wavelength 488 nm, laser intensity 20%) and the SDF-1α solution with the DPSS laser (excitation wavelength of 561 nm, intensity 20%), respectively. While emission of

Alexa-488 was monitored at 500–550 nm, TAMRA emission was quantified in the 570–630 range. Time-dependent intensity profiles (XZ profiles) of TAMRA-SDF-1α were imaged for the supernatant and the gel body. For evaluation of SDF-1α immobilization, fluorescence intensities at three different X-positions were analyzed for each time point.

2.3.2. Enzyme-linked immunosorbent assay (ELISA) for quantitative analysis of SDF-1α uptake and release

Surface-bound hydrogels ($n = 3$) were placed in custom-made immobilization chambers that allowed for only minimal interaction of SDF-1α with surface not originating from the hydrogels. SDF-1α was diluted in PBS (1, 2.5, 5, 10 or 15 μg/ml) and 200 μl/cm^2 solution were added to the gels and immobilized overnight at 22 °C. After SDF-1α solution was taken out, the gels were washed twice to remove any unbound protein. To determine the release kinetics of SDF-1α, 250 μl/cm^2 endothelial basal medium (EBM) (Lonza, Walkersville, USA) was added. For determination of SDF-1α release from MMP-cleavable starPEG-heparin hydrogels ($\gamma = 1$, stiffness comparable to non-cleavable hydrogel $\gamma = 3$) 0.5 U/ml Collagenase IV (Biochrom AG, Berlin, Germany) was added to the release medium. The incubation medium was collected at defined time points (3, 6, 24, 96 and 168 h) and an equal volume of fresh medium was added back to the hydrogels. All solutions were collected and frozen at −80 °C until they were assayed in duplicate using an ELISA SDF-1α Quantikine kit (R&D Systems, Minneapolis, USA) in accordance with the manufacturer's instructions.

2.4. Analysis of chemotactic properties of starPEG-heparin hydrogels

2.4.1. Isolation of early endothelial progenitor cells (early EPCs) and cell culture

Early EPCs (myeloid EPC) were isolated from human peripheral blood buffy coats as previously described [33]. Briefly, leucocyte-rich buffy coats were diluted 1:1 with PBS, and overlaid on Biocoll Separating solution (Density 1.077, Biochrom AG, Berlin). The mononuclear cells were collected by density gradient centrifugation (800 × g for 20 min at room temperature). After centrifugation, the interface cells were carefully removed and transferred to a new conical tube. The cells were washed three times with PBS, centrifuged at 800 × g for 10 min and then suspended in endothelial basal medium (EBM) supplemented with SingleQuots (EGM-Bullet-Kit; Lonza, Walkersville, USA) and 100 ng/ml vascular endothelial growth factor (VEGF; PeproTech GmbH, Hamburg, Germany). Subsequently, the cells were counted and 2.1 × 10^6 cells/cm^2 plated on culture dishes coated with 2 μg/cm^2 fibronectin (Roche Diagnostics, Mannheim, Germany). Cells were cultured for 3 days at 37 °C and 5% CO_2 in a humidified atmosphere. After 3 days in culture, the non-adherent cells were removed by thoroughly washing the cells with PBS and adherent early EPCs (0.5–1% of the initially applied mononuclear cells) were incubated in fresh medium for another 24 h before initiation of the experiments.

2.4.2. Dil-Ac-LDL/Lectin staining of early EPC

Early EPCs were characterized by fluorescent staining with fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin (UEA)-1 (Sigma–Aldrich, Munich, Germany) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled, acetylated, low-density lipoprotein (Dil-Ac-LDL) (Cell Systems, Troisdorf, Germany). To detect dual binding of both components, cells were first incubated with 2.4 μg/ml Dil-Ac-LDL for 1 h at 37 °C. Subsequently, cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min and then counterstained with 10 μg/ml lectin for 1 h at room temperature in the dark. After staining, the samples were visualized by fluorescence microscopy (DMIRE2, Leica) using a 20× oil immersion objective (HC PL Fluotar 20× 0.5). Dil-Ac-LDL fluorescence was monitored by excitation with a helium-neon-laser (excitation wavelength 537 nm, emission wavelength 566 nm) whereas lectin-positive cells were excited with an argon laser (excitation wavelength 492 nm, emission wavelength 520 nm). Cells demonstrating double-positive fluorescence were identified as early EPCs [33]. Fluorescence images confirmed that the majority of adherent cells were positive for both acLDL uptake and UEA-1 binding (>95%, supplemental Fig. 1).

2.4.3. Transwell migration assay

To determine chemotactic properties of starPEG-heparin scaffold, surface-bound hydrogels ($n = 3$) were loaded with 2.5, 5, 10 and 15 μg/ml SDF-1α and placed in the lower compartment of a modified Boyden chamber. Scaffolds containing no SDF-1α and samples where soluble SDF-1α (concentration adapted to released SDF-1α concentration from starPEG-heparin hydrogels within 24 h) was added to the bottom well were used as controls. After flushing twice with PBS, the gels were pre-incubated in 600 μl EBM for 30 min at 37 °C. Meanwhile, early EPCs grown for 4 days were harvested and resuspended in EBM. A total of 2.5 × 10^4 cells in 200 μl was added to the upper chamber of the modified Boyden chamber (Millicell hanging cell culture insert, 8 μm pore size, 0.3 cm^2 membrane surface area, Millipore, Bedford, MA) previously coated with fibronectin (5 μg fibronectin/filter membrane) and flushed with PBS. Subsequently, the inserts were placed in the 24-well culture dish containing the pre-incubated gels. After 20 h incubation at 37 °C in 5% CO_2, non-adherent cells in the top well were washed off with 100 μl PBS and adherent cells on the top side of the filter were removed by gently swabbing with a cotton tip. Transmigrated early EPCs on the bottom side of the filter were fixed with 90% ethanol and stained using 0.25% Crystal violet (Sigma–Aldrich) diluted in MilliQ. After visualizing the cells with a light microscope (Olympus IX50, Olympus, Hamburg, Germany), the stained cells were lysed for 20 min with 10% acetic acid. Measuring the absorbance of the cell lysate at 590 nm (Genios, Tecan, Crailsheim, Germany), the percentage of migrated cells was quantified with respect to the control containing neither hydrogel nor SDF-1α. Shown results correspond to data analysis of at least 4 independent experiments.

2.4.4. Invasion assay: vertical migration of early EPCs into MMP-cleavable hydrogels

Vertical migration of early EPCs into starPEG-heparin hydrogels was visualized in a sandwich system consisting of two separate gels layers. A surface-bound Alexa-488-labeled MMP-cleavable starPEG-heparin gel with a thickness of 200 μm formed the bottom layer. The layer was functionalized with 2.5, 5, 10 and 15 μg/ml SDF-1α and starPEG-heparin hydrogels without SDF-1α were similarly used as control. The top layer of the gel sandwich was formed from a collagen gel that was prepared as previously described [34]. Briefly, to prepare the collagen gel layer, bovine dermal collagen I (purified and pepsin-solubilized in 0.012 N HCl, PureCol, Inamed, Milmont Drive, USA) was brought to physiological pH by mixing eight parts acidified collagen solution (3.0 mg/ml) with one part 10-fold concentrated phosphate buffered saline (PBS, Sigma, Steinheim, Germany) and one part 0.1 M NaOH. All components were kept on ice before and after mixing. Subsequently, 5 × 10^5 early EPCs were resuspended in 200 μl of the collagen gel and the mixture was added to the flushed hydrogels. After 30 min incubation at 37 °C and 5% CO_2 the whole sandwich was flushed with PBS, 1 ml EBM was added and the samples were further incubated at 37 °C and 5% CO_2 for 3 days. After that, F-actin of the cells was stained with AlexaFluor 633-labeled phalloidin as described previously [29]. Briefly, the samples were washed with PBS and fixed with 4% paraformaldehyde (Fluka, Deisenhofen, Germany) for 10 min. Thereafter the cells were permeabilized and non-specific binding sites were blocked with a solution of 0.2 vol.-% Triton X-100 (Fluka, Deisenhofen, Germany) and 1wt.-% Bovine serum Albumin (BSA, Sigma–Aldrich) (room temperature, 10 min). Afterwards the samples were washed again with PBS and subsequently stained with phalloidin-AlexaFluor633 (Invitrogen) for 1 h at room temperature in the dark and washed before imaging. A Leica SP5 confocal Laser scanning microscope equipped with a 40× immersion oil objective and aperture pinhole set at 68 μm was used for the investigation of cells. Horizontal stacks (2 μm step size) were taken to determine the cell localization within the gel scaffold. Migration depth of eEPCs in the scaffolds was analyzed with ImageJ.

2.4.5. Subcutaneous implantation of SDF-1α loaded starPEG-heparin hydrogels in mice

For *in vivo* implantation experiments, nude mice were anesthetized and the incision site marked and disinfected with 70% ethanol. A vertical incision was made down the midline of the back and the SDF-1α-loaded gel clots or unmodified control scaffolds were implanted subcutaneously. At the end of the experiments, animals were sacrificed and implants and surrounding tissue was embedded in paraffin or frozen in OCT embedding medium.

To analyze vessel growth and angiogenesis, the scaffolds were harvested after 7 days. The blood vessel/cell infiltration of the gels was quantified by hematoxylin & eosin staining (H&E stain, Sigma, St. Louis, MO) and analysis of sections stained by a Cy3-labeled anti smooth muscle actin antibody (Sigma–Aldrich, Germany) and by Lectin-FITC using a Zeiss confocal microscope (LSM 510, Carl Zeiss, Jena, Germany).

For determination of early EPC homing to the hydrogel scaffolds, 200 μl PBS solution containing 3.5 × 10^6 human early EPCs labeled with CellTracker CM-DIL (Invitrogen, Germany) were injected into the tail vein of the mice and the scaffolds were harvested after 48 h. To assess homing of human early EPCs to scaffolds, explanted starPEG-heparin hydrogels were stained after cryofixation with anti-CD31-FITC (BD, Germany). CellTracker CM-DIL positive and CD31 positive cells were visualized with a confocal microscope (LSM510, Carl Zeiss, Jena, Germany) and the number of positive cells per high power field was counted. A minimum of 10 high power fields were assessed per mouse. The data are presented as early EPC/mm^2.

2.5. Data analysis

All data are presented as mean ± standard deviation. Multiple samples were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey–Kramer multiple comparison test to evaluate the statistical significance. p values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Uptake and release of SDF-1α from starPEG-heparin hydrogels

SDF-1α plays a key role in regulating the trafficking of stem and progenitor cells and local delivery of SDF-1α was reported to induce recruitment, mobilization and homing of cells, and consequently improve neovascularization of the ischemic tissue [2,12]. The

suitability of starPEG-heparin hydrogels as an SDF-1α delivery system was first tested in binding and release studies.

3.1.1. SDF-1α immobilization

Binding of SDF-1α was qualitatively analyzed by detecting the fluorescence intensity of the hydrogel matrix at different time points after application of the TAMRA-labeled chemokine (Fig. 1A). The fluorescence images demonstrate a homogeneous distribution of SDF-1α within the scaffold indicating the absence of structural heterogeneities (Fig. 1B). Furthermore, comparable intensities of gel body and supernatant 1 min after application prove the immediate penetration of SDF-1α into the gel network (Fig. 1A: relative fluorescence intensities of gel body and supernatant 58% versus 42%). These observations reflect the high affinity of SDF-1α for heparin (K_d 27.7 nM) [11]. The association process is known to originate from electrostatic interactions between the highly negatively charged heparin and clusters of positively charged residues of SDF-1α (SDF-1α has an overall positive charge of +8), thus explaining the driving force for the fast immobilization [35,36]. Based on the fast and efficient binding process of the small molecule SDF-1α (8 kDa, ~5 nm) [37] steric restrictions of the conjugation by the polymer network (see Table 1, mesh size 6.5 nm) can be excluded. These findings are consistent with previous studies where fast immobilization of FGF-2 (17.2 kDa; ~3 nm) and VEGF (38.2 kDa; 6 nm) [28] to starPEG-heparin gels was observed. Over time, the binding led to accumulation of SDF-1α in the gel matrix and depletion from the supernatant. Thus, for this particular setting, 24 h after SDF-1α application, the relative fluorescence intensity in the supernatant was decreased to ~20% of the initial value. The significant depletion in the supernatant can be explained by the excess of heparin (large amounts in the scaffold: A single 9 kDa heparin can accommodate up to 6 molecules of SDF-1α [11]). In sum, starPEG-heparin hydrogels allow for fast and effective binding of homogenously distributed SDF-1α.

For quantification, the immobilized amount of SDF-1α after 24 h was further determined by ELISA. Administering SDF-1α loading concentrations in the range of 2.5–15 μg/ml, resulted in a linear correlation between the applied SDF-1α-concentration and the amount of SDF-1α immobilized in the matrix (Fig. 2A right); no saturation of the matrix was reached within the tested concentration range. The data corresponds to an immobilization efficiency of ~99.6% irrespective the SDF-1α concentration in the immobilization medium (data not shown; SDF-1α to heparin ratio is only 0.03 for a loading concentration of 15 μg/ml SDF-1α).

These data show that starPEG-heparin hydrogels are suitable for incorporating large amounts of SDF-1α via conjugation to heparin. Association of SDF-1α to heparin was previously reported to facilitate SDF-1α-dimer formation, which is important for triggering the biological response [11].

3.1.2. SDF-1α release

As sustained chemokine gradients are required to induce migration of EPCs [9], the ability of starPEG-heparin hydrogels to deliver SDF-1α was analyzed. Similarly to the SDF-1α binding studies, surface-bound hydrogels were pre-incubated with SDF-1α solution and after 24 h, the immobilization solution was exchanged against SDF-1α-free release medium. Using ELISA, SDF-1α concentrations in the supernatant were determined over time to obtain release profiles for different initially applied SDF-1α concentrations. The release profile was characterized by an initial burst release (after 3–6 h) and a sustained, lower release over the course of one week (Fig. 2A right). As EPC mobilization and recruitment needs to be initiated by higher amounts of SDF-1α [6], the observed release characteristics are considered particularly

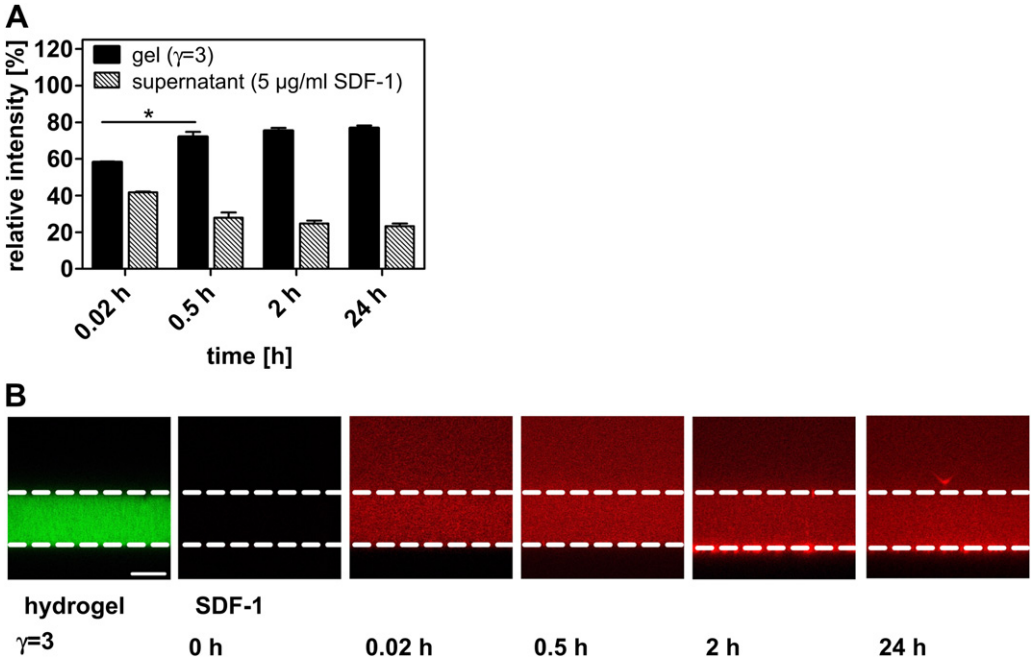

Fig. 1. Quantitative (A) and qualitative (B) SDF-1α uptake experiments. 1A: Average fluorescence intensity of TAMRA-labeled SDF-1α in starPEG-heparin gels ($\gamma = 3$) and in the corresponding supernatant at different time points. Measurements were performed using confocal laser scanning microscopy (cLSM). All data are presented as average over three Z-lines from at least two different gel samples ± root mean square deviation. * indicates statistically significant differences ($p < 0.05$; ANOVA). 1B: visualization of SDF-1α uptake and distribution within the gel matrix. Alexa-488-labeled surface-bound gel material (thickness ~ 50 μm, green, 1B left) was incubated with TAMRA-labeled SDF-1α (red, right). Pictures show X-Z-cLSM scan of the gel body at different time points (before addition of SDF-1α, 0.02 h, 0.5 h, 2 h and 24 h after addition of SDF-1α, 1B right). White dotted lines show the upper and lower gel boundary. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Key characteristics of the different heparin-starPEG hydrogel types.

starPEG/heparin ratio [mol/mol]	Heparin content [μg/μl]	Volume swelling [−]	Storage modulus [kPa]	Pore size [nm]
3 (non-cleavable)	7.8	30	2.57	11.7
1 (MMP-cleavable)	14.85	37	2.9	11

promising. To explore the dose-dependency of the cellular response, the SDF-1α-release was modulated through immobilization from solutions of different concentrations of SDF-1α (2.5–15 μg). Fig. 2B (right) illustrates that loaded and delivered amount of SDF-1α correlate well (cumulative release after one week: 51.5 ± 2.8 ng/ml for 2.5 μg/ml, 100.38 ± 1.2 ng/ml for 5 μg/ml, 203.2 ± 8.4 ng/ml for 10 μg/ml and 367.9 ± 16.8 ng/ml for 15 μg/ml). As recent studies showed that cell motility and neovascularization are induced by concentrations of 40–100 ng/ml (soluble) SDF-1α [13,20,24,38] we expected that starPEG-heparin hydrogels loaded with 2.5–10 μg/ml SDF-1α would be effective in stimulating early EPC migration.

To mimic the cell-responsive characteristic of the ECM, MMP-sensitive cleavage sites were incorporated into starPEG-heparin hydrogels [32]. Binding of SDF-1α to MMP-cleavable gels and the amount of chemokine released after degradation of the matrix with collagenase IV was analyzed as described above. As shown in Fig. 2B (left), the introduction of MMP-cleavable crosslinks did not affect the immobilization of SDF-1α (99.8 ± 1.6% non-cleavable hydrogels versus 99.4 ± 0.5% MMP-cleavable hydrogels, $n = 3$) because of the low SDF-1α to heparin ratio in the matrices. However, the amount of SDF-1α released from MMP-degradable scaffolds was found to be significantly accelerated (Fig. 2B right) with the addition of 0.5 U/ml collagenase IV (2.6 fold increase of SDF-1α release compared to non-degradable scaffolds).

StarPEG-heparin hydrogels can consequently provide engrafting cells with the appropriate mechanical integrity, but at the same time enable localized matrix degradation in temporal and spatial synchrony with the formation of new tissue. Importantly, degradation does not form any toxic products.

3.2. Chemotactic characteristics of SDF-1α loaded starPEG-heparin hydrogels

3.2.1. Early EPC migration assay

EPCs contribute to tissue neovascularization after ischemia. The neovascularization promoting capacity of EPCs is among other prerequisites (e.g., distribution, alignment and functional integration) dependent on the effective homing of the progenitor cells to ischemic tissues [16]. This homing requires a local SDF-1α concentration gradient along which EPCs can migrate toward the

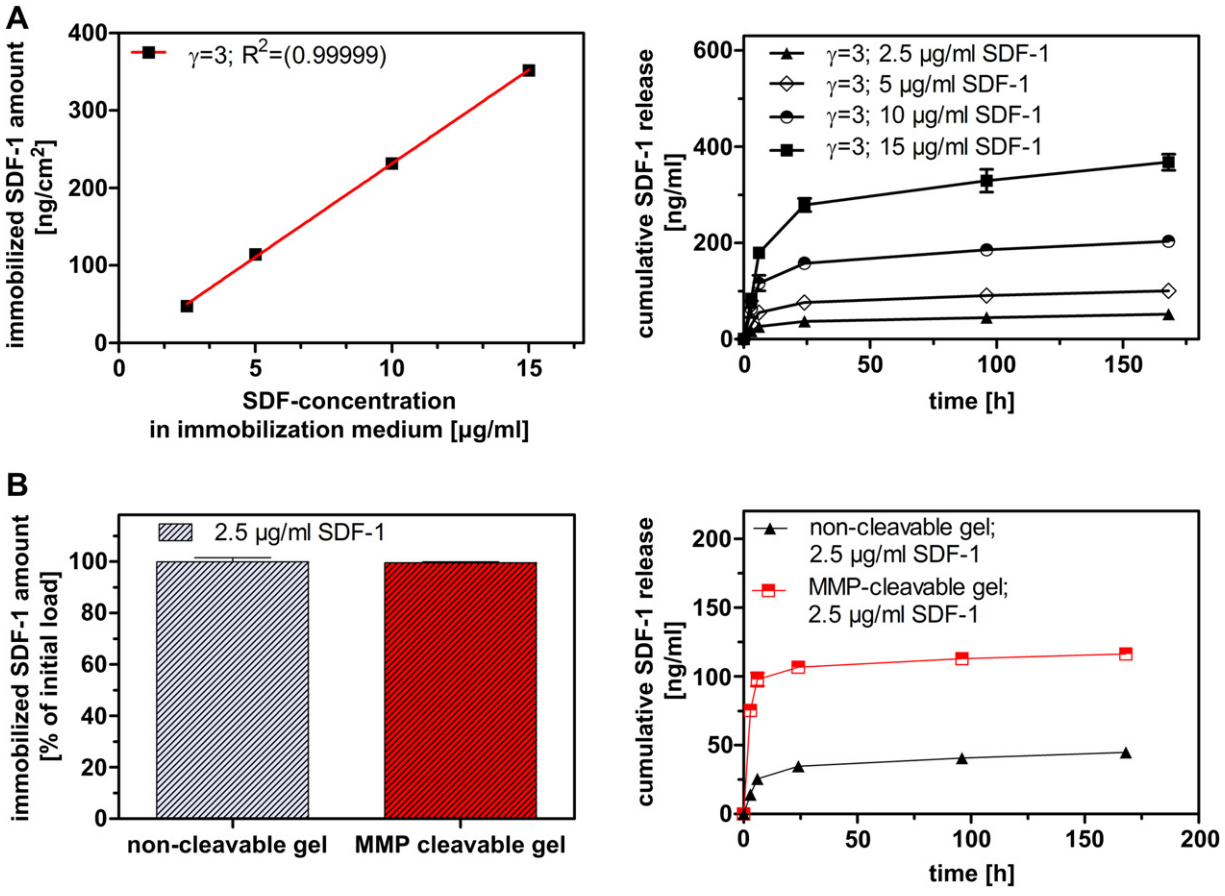

Fig. 2. SDF-1α uptake and release experiments for non-cleavable (A) and cleavable (B) starPEG-heparin hydrogels as quantified via ELISA. 2A (left): immobilized SDF-1α amount per cm² scaffold at varied SDF-1α concentration in solution, linear regression, $R^2 = 0.9999$. 2A (right): cumulative release of SDF-1α in dependence on protein concentration upon immobilization. All data are presented as mean ± root mean square deviation, $n = 3$. 2B: Modulation of SDF-1α release (B) using protease-mediated degradation of cleavable hydrogels at unaffected SDF-1α immobilization as quantified via ELISA. 2B left: immobilized SDF-1α amount per cm² scaffold comparing non-cleavable gel matrices with MMP-degradable hydrogels ($p > 0.05$; ANOVA). 2B right: cumulative release of electrostatically bound SDF-1α released by the gels with/without enzyme-mediated degradation ($p < 0.001$; ANOVA). All data are presented as mean ± root mean square deviation, $n = 3$.

site of injury [10,13]. For an ischemic limb model in mice [10], a peak in the SDF-1α concentration at day 1 and day 2 after initiation of the ischemic conditions in the peripheral blood was detected, correlating to a gradient formation of approx. 1 ng/ml SDF-1α from the peripheral blood towards the bone marrow. Therefore, a total amount of approx. 5 ng SDF-1α per day (mice) defines a minimal effective dose that was clearly exceeded in order to boost an angiogenic response in former *in vivo* applications (e.g., upon release of 100 ng/ml from matrigel, [10]).

First, the chemotactic properties of starPEG-heparin hydrogel scaffolds were investigated in a modified Boyden chamber assay *in vitro*. Early EPC migration towards the chemokine gradient established by placing the hydrogel in the bottom well was visualized by staining the early EPCs adherent to the lower side of the filter (Fig. 3A). Microscopic images of the stained early EPCs clearly show the increased chemotactic response of the cells with increasing amounts of SDF-1α immobilized within the matrix. Subsequently, early EPC migration was determined for control conditions (i.e., no gel, no chemoattractant) by measuring the absorption of the cell lysate. Untreated scaffolds (no SDF-1α) only slightly improved early EPC migration compared with the control (4.7%) (Fig. 3B). SDF-1α released from the cleavable or non-cleavable scaffolds significantly augmented early EPC migration in a dose-dependent manner (Fig. 3B) with a maximum motility at a loading concentration of 10 μg/ml SDF-1α, which corresponds to a cumulative SDF-1α release of approximately 150 ng/ml (25.4% increase of early EPC migration compared with control). In line with previous reports on a reversed cell migration at high SDF-1α concentrations [39,40], we found that higher loading concentrations of SDF-1α (15 μg/ml, which corresponds to 300 ng/ml SDF-1α released) slightly but significantly decreased cell migration.

SDF-1α loaded, matrix metalloprotease (MMP) cleavable starPEG-heparin gels (red bars) induced an even higher early EPC migration due to the cellular production of MMPs [41] resulting in elevated SDF-1α release upon MMP-driven gel degradation (Fig. 2). Furthermore, association of SDF-1α with soluble heparin produced by MMP-mediated gel degradation can be assumed to protect the chemokine against CD26/dipeptidyl peptidase IV (DPP4) cleavage [42,43] thereby maintaining its activity for prolonged time periods *in vivo* [9,42].

The presented data show that the reported hydrogel system induces chemotaxis of early EPCs by establishing a sustained and well-defined chemokine gradient. Compared to chemotaxis assays in which soluble SDF-1α is added directly to the bottom well,

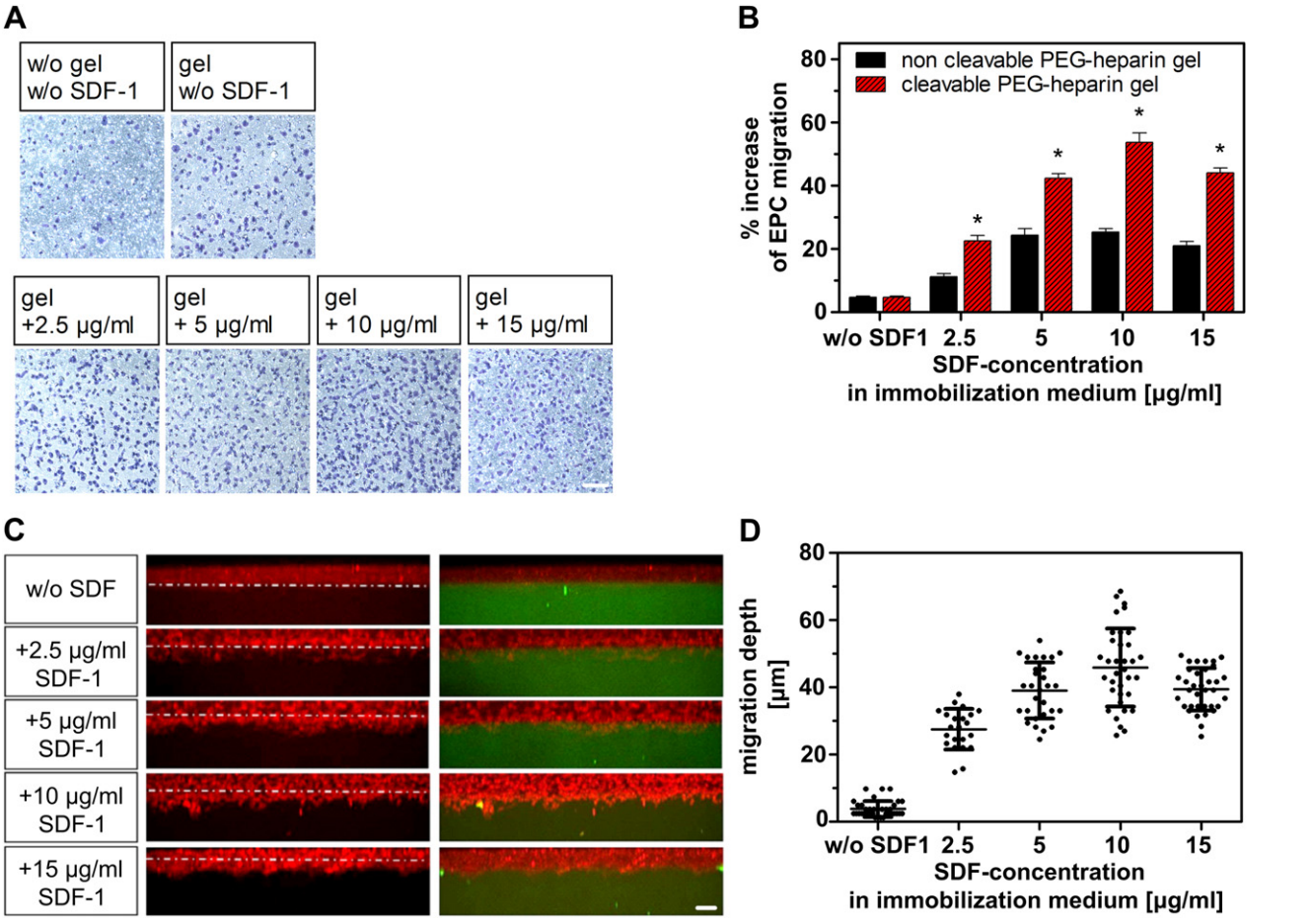

Fig. 3. Migration of eEPCs as determined by a modified Boyden chamber assay (A + B) and in vertical migration experiments (C + D). 3A Representative images of migrated eEPCs on the lower site of the transwell membrane. Migration assay was performed with starPEG-heparin hydrogels loaded with indicated concentrations of SDF-1α in the bottom well. A sample without hydrogel and without SDF-1α was used as control. 3B Quantification of relative eEPC migration on non-cleavable (black) and MMP-cleavable (red) starPEG-heparin hydrogels loaded with indicated SDF-1α concentrations. Data reflect the percentage increase of migratory activity compared to control without hydrogel and without SDF-1α in the bottom well (* indicates $p < 0.05$ versus non-cleavable hydrogel loaded with equal SDF-1α concentration; ANOVA). 3C Representative cross-sectional images showing vertical eEPC migration (red) into starPEG-heparin hydrogels (green) loaded with indicated concentrations of SDF-1α. White dotted lines show the upper gel boundary. 3D Quantification of eEPC migration depth in starPEG-heparin hydrogels. All data are presented as mean ± root mean square deviation from $n = 4–6$. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

matrix-based SDF-1α delivery resulted in significantly enhanced early EPC migration (see Fig. 1 supplemental). Soluble SDF-1α was reported to equilibrate within 5 h by diffusion processes [44]. In contrast, the starPEG-heparin hydrogel system allowed for a sustained delivery of SDF-1α that generated a constant chemokine gradient and continued early EPC migration for at least 20 h (the time period followed in this study).

3.2.2. Vertical migration

To visualize the process of migration and invasion of early EPCs, the vertical migration into cleavable starPEG-heparin scaffolds was additionally analyzed (Fig. 3C). SDF-1α was immobilized within surface-bound starPEG-heparin hydrogel layers with a thickness of 200 μm. A gradient of the chemokine was established by using a sandwich system consisting of the hydrogel as bottom layer and a collagen gel (in which the early EPCs were suspended) on top. Unmodified hydrogels were used as control and the migration process was analyzed after 3 days. Fig. 3C displays the early EPC distribution (red) within the starPEG-heparin matrix (green). The gradient of SDF-1α generated in the hydrogel led to the migration of early EPCs into the MMP-cleavable gel matrix. The migration depth of the cells increased similarly to the results of the modified Boyden chamber assay in a dose-dependent manner while in unmodified gels in the absence of SDF-1α almost no migration of cells into the hydrogel matrix was found. This indicates that a hydrogel-based chemokine gradient promoted the attraction and subsequent invasion of early EPCs. Moreover, as the cells were able to penetrate the SDF-1α loaded hydrogels, these experiments further illustrate that MMP-sensitive hydrogels do indeed allow for cell-mediated remodeling.

3.3. Vascularization of subcutaneously implanted, SDF-1α releasing starPEG-heparin hydrogels

To analyze the potential of SDF-1α loaded hydrogels to promote angiogenesis *in vivo*, materials were implanted subcutaneously into the backs of nude mice and histologically analyzed after 7 days. Based on the results of the performed *in vitro* migration experiments and published information about the minimal effective dose [10] hydrogels were adjusted for a cumulative release of ~100 ng/ml during 24 h.

Hematoxylin and eosin staining of paraffin-embedded sections show that the unmodified control scaffold was nearly intact with almost no cells residing inside the scaffold. In contrast, SDF-1α loaded hydrogels exhibited remodeling and degradation and contained a significantly greater overall number of cells within (Fig. 4A; quantification in Fig. 4B, control gels: 19.5 cells/field, SDF-1α loaded gels: 105.2 cells/field). Although both materials allowed for cell-mediated remodeling due to the incorporation of MMP-sensitive cleavage sites, the results show that SDF-1α was crucial in initiating cell infiltration. Furthermore, staining and subsequent quantification of lectin-positive cells illustrate that SDF-1α loaded implants contained a significantly greater number of endothelial cells, suggesting that the formation of the hydrogel-based chemokine gradient could initiate angiogenic processes inside the scaffold (Fig. 4A; quantification in Fig. 4B, control gels: 35.9 lectin-positive cells/field, SDF-1α loaded gels: 70.2 lectin-positive cells/field). Moreover, H&E staining showed a large number of red blood cells inside the SDF-1α containing implant in addition to a substantial increase in SMA-positive cells (images not shown, quantification in Fig. 4A; control gels: 6.2 SMA-positive cells/field, SDF-1α loaded gels: 24.7 SMA-positive cells/field) further demonstrating the ability of the SDF-1α functionalized starPEG-heparin hydrogels to efficiently promote neovascularization *in vivo*. In sum, SDF-1α modified hydrogels showed increased attraction and infiltration of endothelial and smooth muscle cells as required in angiogenic tissue engineering.

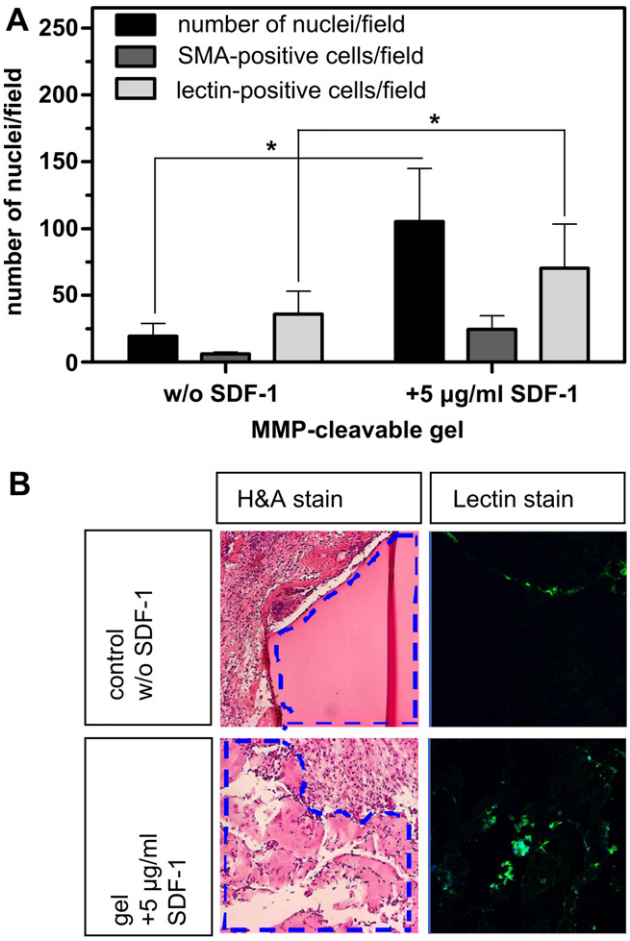

Fig. 4. Subcutaneous implantation of SDF-1α loaded starPEG-heparin hydrogels increased the number of cells infiltrating and degrading the tissue and supports vessel growth in the scaffold. 4A Quantification of overall number, SMA-positive and lectin-positive cells present in the hydrogel after 7 days of implantation. Data are presented as mean ± standard error of the mean from $n = 4–5$ (* indicates $p < 0.05$ versus control without SDF-1α; ANOVA) 4B Representative images of hematoxylin and eosin stain and lectin-stained cells in starPEG-heparin hydrogels after implantation of RGD-functionalized scaffolds loaded with SDF-1α in comparison to unloaded hydrogel.

3.4. Homing of early EPCs to subcutaneously implanted, SDF-1α-releasing starPEG-heparin hydrogels

Circulating EPCs can home to sites of ischemic tissue, participate in neovascularization and reduce cardiomyocyte apoptosis after MI [2]; however, under physiological conditions the number of circulating EPCs in the blood is rather low (EPCs represent only 0.01% of circulating mononuclear cells [45]). Accordingly, several studies have explored the therapeutic administration of EPCs in hindlimb ischemia assays and MI models [46–48]. To test the *in vivo* potential of SDF-1α-loaded starPEG-heparin hydrogels to enhance the specific recruitment of early EPCs, implantation of SDF-1α functionalized materials was combined with an intravenous injection of human early EPCs in mice. SDF-1α-loaded and unloaded control scaffolds were implanted subcutaneously in the back of nude mice and CellTracker CM-DIL-labeled human early EPCs were injected intravenously into the tail vein of nude mice. Two days after the intravenous cell injection, the homing of early EPCs to the hydrogel scaffolds was evaluated. SDF-1α functionalized scaffolds attracted

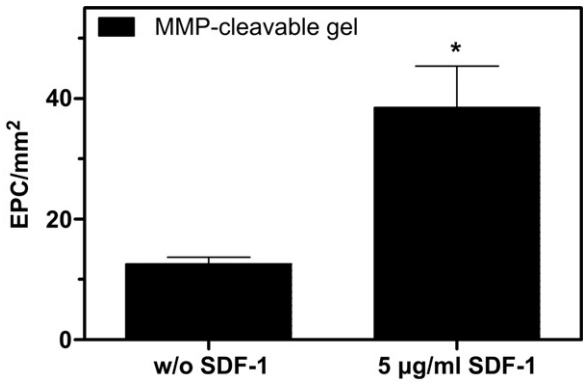

Fig. 5. Subcutaneous implantation of SDF-1α loaded starPEG-heparin hydrogels increased the number of EPCs homing to the tissue. Quantification of the number of EPCs present in the hydrogel after 48 h of implantation. Data are presented as mean ± standard error of the mean from $n = 4–5$ (* indicates $p < 0.05$ versus control without SDF-1α; ANOVA).

a significantly larger number of tail vein-supplemented human $CD31^+$ and DIL^+ cells than the unloaded control gels (Fig. 5A, control gels > 12.5 early EPC/mm^2, SDF-1α loaded gels: 38.5 early EPC/mm^2).

Thus, a combination of early EPC injection and implantation of SDF-1α-loaded starPEG-heparin hydrogels at the site of ischemia could be clinically beneficial as it could not only augment the number of circulating progenitor cells but also activate the intrinsic regeneration. As such, our approach may offer an interesting alternative to recently reported gene therapy schemes that have successfully employed overexpression of the chemokine to improve cardiac function after chronic heart failure [38].

4. Conclusion

StarPEG-heparin hydrogels allow for precisely adjusted, long term delivery of SDF-1α, thus enabling the formation of sustainable concentration gradients in tissues. The heparin-containing hydrogel stabilizes the chemokine and protects it against enzymatic degradation. Early EPC migration *in vitro* towards matrix-based SDF-1α gradients was found to be clearly superior to the administration of soluble SDF-1α. *In vivo* studies showed improved early EPC homing, cell infiltration and pro-angiogenic response to the SDF-1α releasing matrices. The design of the biohybrid materials can further support endogenous cardiac regeneration by providing adhesion sites and incorporating MMP-cleavable peptides to facilitate cell attachment and cell-mediated remodeling.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.03.039.

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