A STROMAL CELL-DERIVED FACTOR-1 RELEASING MATRIX ENHANCES THE PROGENITOR CELL RESPONSE AND BLOOD VESSEL GROWTH IN ISCHAEMIC SKELETAL MUSCLE

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Abstract

Although many regenerative cell therapies are being developed to replace or regenerate ischaemic muscle, the lack of vasculature and poor persistence of the therapeutic cells represent major limiting factors to successful tissue restoration. In response to ischaemia, stromal cell-derived factor-1 (SDF-1) is up-regulated by the affected tissue to stimulate stem cell-mediated regenerative responses. Therefore, we encapsulated SDF-1 into alginate microspheres and further incorporated these into an injectable collagen-based matrix in order to improve local delivery. Microsphere-matrix impregnation reduced the time for matrix thermogelation, and also increased the viscosity reached. This double-incorporation prolonged the release of SDF-1, which maintained adhesive and migratory bioactivity, attributed to chemotaxis in response to SDF-1. *In vivo*, treatment of ischaemic hindlimb muscle with microsphere-matrix led to increased mobilisation of bone marrow-derived progenitor cells, and also improved recruitment of angiogenic cells expressing the SDF-1 receptor (CXCR4) from bone marrow and local tissues. Both matrix and SDF-1-releasing matrix were successful at restoring perfusion, but SDF-1 treatment appeared to play an earlier role, as evidenced by arterioles that are phenotypically older and by increased angiogenic cytokine production, stimulating the generation of a qualitative microenvironment for a rapid and therefore more efficient regeneration. These results support the release of implanted SDF-1 as a promising method for enhancing progenitor cell responses and restoring perfusion to ischaemic tissues via neovascularisation.

Keywords: Cytokines, hydrogels, injectables, neovascularisation, regenerative medicine, vascular biology

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Introduction

Myopathies, such as ischaemic heart disease and peripheral arterial disease (PAD) are significant killers in developed nations. In particular, PAD afflicts an estimated 27 million people in Europe and North America (Belch *et al*., 2003). This disease manifests when the flow of blood to extremities is acutely or chronically reduced (Selvin and Erlinger, 2004; Shamoun *et al*., 2008). Attributed to the lack of blood flow, symptoms typically include: pain, cramping, weakness and a poor ability to heal. To restore perfusion, current treatments include stent or vascular transplants with surgical intervention, or amputation; however, these options are invasive, carry risks, and amputation significantly reduces quality of life. Therefore, non-invasive options for restoring perfusion would be invaluable for PAD patients. Recent years have seen the generation of many novel approaches for developing tissue substitutes to replace or regenerate ischaemic muscle; however, the supply of sufficient and appropriate vasculature represents a major limiting parameter for a successful therapeutic approach (Ko *et al*., 2007; Kaully *et al*., 2009).

To augment reparative processes, regenerative therapies using bone marrow-, blood-, or tissue-derived progenitor cells have emerged; however, the results of these treatments are controversial and there is growing evidence to suggest that non-embryonic transplanted cells do not successfully integrate with host tissue (Murry *et al*., 2006; Suuronen *et al*., 2007). It is now believed that functional improvement results from neovascularisation and the restoration of blood flow to ischaemic muscle, and that this phenomenon is initiated, maintained, and enhanced by paracrine factors and secondary recruitment of progenitor cells (Cho *et al*., 2007; Formigli *et al*., 2007).

After ischaemic injury, an endogenous response to recruit the endogenous progenitor cells is initiated (Hofmann *et al*., 2005). Regarded as critical to this process is the up-regulation and release of the cytokine stromal cell-derived factor-1 (SDF-1) from ischaemic muscle (Askari *et al*., 2003) and the subsequent recruitment of circulating progenitor cells (CPCs), mobilised from distal tissues, such as bone marrow, into ischaemic tissue (De Falco *et al*., 2004). However, the stem cell recruitment response is short-lived and tissue accumulation is low (Wojakowski *et al*., 2004; Fazel *et al*., 2006). Recruited CPCs are observed to be pro-angiogenic (Park *et al*.,

2004; Suuronen *et al*., 2009) and are thought to augment functional recovery by promoting neovascularisation.

This study aims to use the SDF-1 signalling mechanism in an effort to amplify the endogenous response to ischaemia and the recruitment of vasculogenic progenitor cells. Herein, we report on the encapsulation of SDF-1 into microspheres, which are added to an injectable collagen-based matrix that thermogels at physiological temperature. We provide evidence that treatment with an SDF-1 releasing collagen matrix improves the vasculogenic response of ischaemic muscle, mediated by the recruitment of progenitor cells.

Methods

All reagents were obtained from Sigma-Aldrich (Oakville, Canada), unless otherwise indicated.

Generation of microspheres

Through a physical cross-linking reaction, blank (no peptide added) alginate microspheres were created with 1.25 % sodium alginate (w/v). The solution was added to a syringe and forced through a J1 Encapsulation Device (Nisco, Zürich, Switzerland) at 1 mL/min, using a syringe pump, and 9.8 L/min N_2 gas. Microspheres were allowed to fall into a 2 % $CaCl_2$(w/v) cross-linking bath, and kept stirring for 20 min before washing with phosphate-buffered saline (PBS), and snap-freezing in liquid nitrogen. SDF-1 loaded microspheres were created by adding 200 ng SDF-1 per g of alginate solution during sodium alginate solubilisation. Microspheres with both human (Cedarlane Laboratories, Hornby, Canada) and murine SDF-1 (Biovision, San Francisco, CA, USA) were generated, and stored at -80 ºC.

Microsphere morphology

Microspheres were thawed and suspended in PBS. Images were taken using an inverted phase contrast microscope (Olympus 1x81F), and microsphere size was assessed using Image-Pro Plus. Microsphere ultrastructure was observed using a Philips/FEI XL-30 scanning electron microscope (Hillsboro, OR, USA); WD = 7.0 mm and keV = 1.2. Microspheres were fixed in 3 % glutaraldehyde for 2 h, washed and subsequently dehydrated in various ethanol dilutions (30 %, 50 %, 70 %, 80 %, 90 %, 95 %, 99 %) for 5 min each before being critically point-dried. Specimens were mounted on stubs and coated with Pd/Au using a Hummer Sputter Coater (Ladd Research, Williston, VT, USA).

Matrix preparation

As described previously (Suuronen *et al*., 2009), a collagen matrix was created on ice, using a cross-linking mixture containing a 1:1 molar ratio of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS; 13 mM) in 2(-N-morpholino) ethanesulfonic acid (MES) buffer, a solution of 1 % porcine type I atelocollagen (w/v; Nippon Ham, Tskuba, Japan) and 40 % chondroitin sulphate-C (CSC) (w/v; Wako Chemicals, Osaka, Japan). The cross-linked collagen solution was diluted with PBS before adjusting the pH to 7.2 ±0.2 using 1 N NaOH or HCl. The final concentration of collagen and CSC were 0.59 % (w/v) and 2.4 % (w/v), respectively. The proportion of microspheres in the matrix was 21 % (w/v), and when included, the SDF-1 concentration was 40 ng/mL. SDF-1 microsphere-matrix was prepared using the same procedure, except 400 mg of microspheres was added in the PBS dilution step. For *in vitro* use, matrices were thermogelled for 20 min at 37 °C.

Reagent sterilisation

All liquid reagents (PBS, MES, CSC, alginate) were sterile filtered (0.45 μm). Collagen (1 %) was prepared with sterile water and the resulting solution was sterilised by exposure to ultraviolet (UV) light for a period of 15 min.

Rheology

The rheological properties of the collagen matrix were measured using a Brookfield R/S Plus Rheometer as previously described (Deng *et al*., 2010). Samples of collagen matrix or microsphere-collagen matrix (1.5 mL) were subjected to a constant shear rate of 5 s^{-1} for 20-30 min using a C50-2 spindle (spindle gap of 4 μm, according to the spindle specifications), and the temperature was maintained at 37 °C. Rheo3000 v1.2 software was used to monitor viscosity (Pa·s) and time to gelation (s). The time at which maximum viscosity was reached was considered to be the material's time to gelation. In addition to time viscosity profiles, elastic storage (G') and loss (G") modulus as a function of temperature at a frequency of 1 Hz was also measured.

SDF-1 release

Microspheres containing human SDF-1 were used to assess release kinetics. Microspheres alone or embedded in a collagen matrix were added to a 20 mL flask with 5 mL of PBS. At various time points, samples were taken and immediately frozen at -80 ºC and fresh PBS was added to replace the removed aliquot volume. SDF-1 content in the supernatant was assessed using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Data is reported as a ratio of concentration at time *t*, relative to the maximal release.

Release kinetics

To analyse SDF-1 release from microspheres (+/- matrix embedding), various release kinetic models were used to describe the observed release kinetics. Correlation coefficients were determined for data fit to zero-order release (Hadjiioannou *et al*., 1993), first-order release (Bourne *et al*., 2002), Higuchi (Higuchi, 1963), Hixson-Crowell (Hixson and Crowell, 1931) and Korsmeyer-Peppas (Korsmeyer *et al*., 1983) models.

Cell culture

Procedures for the isolation of human circulating progenitor cells (CPCs) were approved by the Human Research Ethics Board of the University of Ottawa Heart Institute.

After obtaining informed consent, total peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy human volunteers by Histopaque 1077 density-gradient centrifugation, as previously described (Ruel *et al*., 2005). Cells were cultured on fibronectin-coated plates in endothelial basal medium (EBM-2; Clonetics, Guelph, Canada) containing 5 % FBS (v/v), VEGF, R^3-IGF-1, and hEGF supplements. After 4 d in culture, supernatant and non-adherent cells were removed, and adherent populations were considered to be circulating progenitor cells (CPCs).

Adhesion assay

CPCs (2×10^4) were resuspended in 1 mL of medium and seeded in 12-well dishes with fibronectin-coated coverslips, containing medium with 50 mg of blank microspheres or medium with 50 mg of human SDF-1 loaded microspheres. After 1 h at 37 ºC, medium was aspirated and adherent cells were fixed with 4 % paraformaldehyde (PFA). Coverslips were washed with PBS and mounted on slides with 4',6-diamidino-2-phenylindol-(DAPI)-containing mounting medium (Vector Laboratories, Burlington, Canada). Six random fields-of-view were imaged using a Zeiss Z1 fluorescent microscope and $DAPI^+$ cells were counted.

Migration assay

CPCs (2×10^4) in VEGF-free medium were added to the top chamber of a Transwell tissue culture well (Corning, New York, NY, USA). The lower chamber contained VEGF-free medium with 50 mg of blank microspheres or VEGF-free medium with 50 mg of human SDF-1 loaded microspheres. The bottom of each well contained a fibronectin-coated coverslip. After 24 h of incubation, cells were fixed, DAPI-stained, visualised, and counted.

Chemokinesis *versus* chemotaxis assay

To investigate the mode of action through which SDF-1 induces CPC migration, assays, based on a previously described protocol for the evaluation of chemokinesis *versus* chemotaxis (Misiak-Tloczek and Brzezinska-Blaszczyk, 2009), were carried out. Three treatment conditions (represented as the presence of SDF-1 in the upper well/lower well) were tested: SDF-1/0 (SDF-1 only in the upper well); SDF-1/SDF-1 (SDF-1 in both upper and lower wells); 0/SDF-1 (SDF-1 in the lower well only). SDF-1 was used at a concentration of 9 ng/mL, as this is the approximate amount released from microspheres after 24 h. Quantification was performed in the same manner as described for the migration assay above.

Animal model

All procedures were performed with the approval of the University of Ottawa Animal Care Committee, in compliance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Bone marrow transplantation was performed as previously described (Whitman *et al*., 2004). Briefly, female C57BL/6J mice (8-9 weeks old, Jackson Laboratories, Bar Harbor, ME, USA) were irradiated with a total of 900 rads from a caesium source, delivered in 2 equal doses, 3 h apart. Donor bone marrow cells (7×10^6) from green fluorescent protein (GFP) transgenic mice (C57BL/6-Tg(CAG-EGFP)10sb/J, Jackson Laboratories) were injected into the tail vein of irradiated recipient mice. Six weeks after transplantation, proximal femoral arteries in left hindlimbs were ligated as described (Limbourg *et al*., 2009), using 4.0 silk thread, under 2 % isoflurane. Limbs subsequently received 80 µL injection of: 1) PBS ($n = 9$); 2) collagen matrix ($n = 8$); or 3) collagen matrix containing murine SDF-1 microspheres ($n = 8$). Treatments were delivered by 3 equivolumetric injections into the adductor muscle downstream of the ligation site, using a 27-gauge needle.

Blood perfusion of both hindlimbs was measured before and after ligation, and at days 4, 7 and 14 post-operatively using a multifibre needle probe (8 separate collecting fibres), and a laser Doppler blood flow monitor (Moor Instruments, Axminster, UK).

Blood samples (~100 µL) were procured from the right saphenous veins on days 0 (pre-operative baseline), 1, 4, 7 and 14 post-operatively. PBMCs were isolated using density-gradient centrifugation and immediately characterised using flow cytometry as described below.

Flow cytometry

Cells were labelled with antibodies against the following antigens: c-kit (Southern Biotech, Birmingham, AL, USA), CXCR4 (BD Biosciences, Mississauga, Canada), and flk-1 (eBioscience, San Diego, CA, USA), and analysed with a FACSAria flow cytometer (BD Biosciences). Isotype-matched immunoglobulin antibodies were used as controls.

Immunohistochemistry

Animals were sacrificed at day 14. Hindlimb muscle tissue was collected and fixed overnight in PFA before paraffinisation. All samples were analysed in cross-section. Samples were de-paraffinised and hydrated with sequential washes in toluene and decreasing concentrations of ethanol. Antigen retrieval was performed using boiling citrate buffer. All staining was performed in PBS containing 10 % normal horse serum (Vector Laboratories). The following antibodies were used: anti-α-smooth muscle actin (SMA; pre-diluted, Abcam, Cambridge, MA, USA), anti-GFP (1:100; Abcam), and anti-CXCR4 (1:50; Abcam). For all tissue sections, mounting medium with DAPI (Vector Laboratories) was used to visualise nuclei. All measurements and cell counts were determined from 6 random microscopic fields-of-view and averaged from 2 blinded observers.

Cytokine Antibody Arrays

Relative cytokine levels in hindlimb lysates and serum from sacrificed animals ($n = 5$ per treatment group) were analysed using RayBio Mouse Cytokine Antibody Array Kits (Raybiotech, Norcross, GA, USA), according to the manufacturer's protocol.

Statistical Analysis

Unless otherwise stated, values are expressed as means ± standard error. Statistical analyses were performed using SPSS (IBM, Somers, NY, USA). Comparisons of

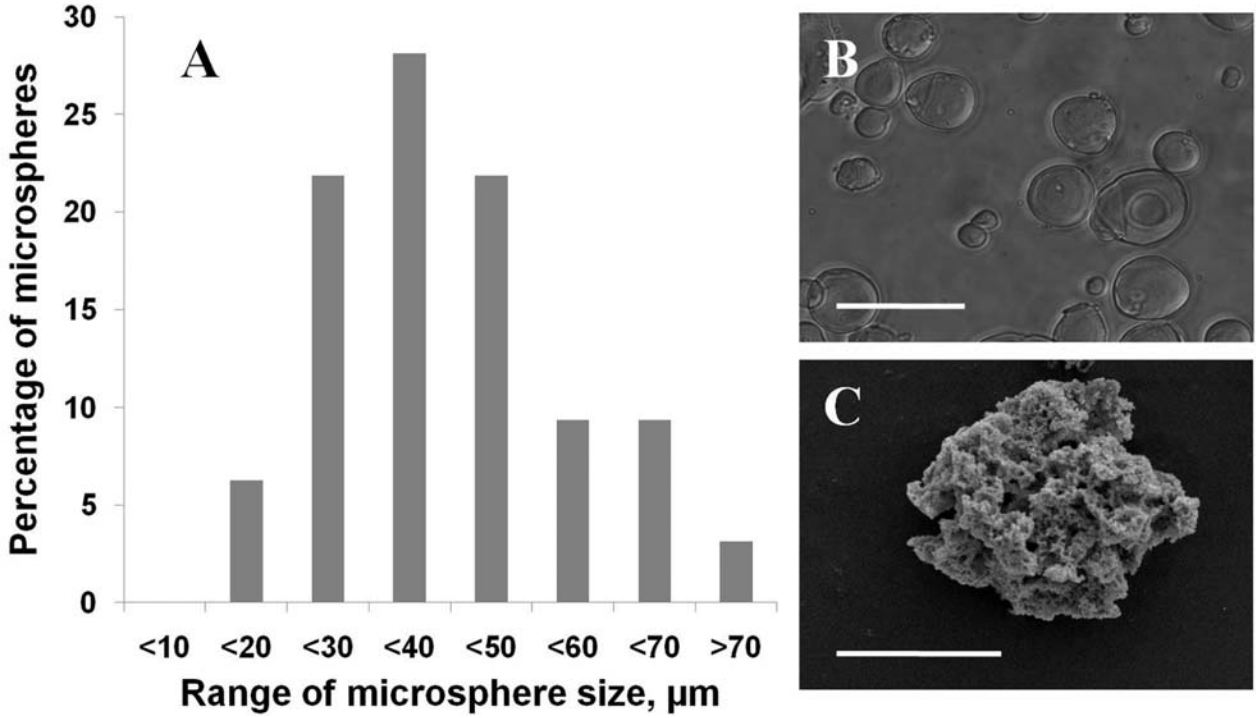

Fig. 1. Characterisation of SDF-1-containing alginate microspheres. Average size of microspheres was 38.5 µm **(A)**. Microspheres displayed a rounded morphology in saline solution (**B**; 400x), and a lattice structure after drying and imaging using scanning electron microscopy (**C**; 379x). Scale bars = 50 µm.

continuous data between groups were performed with a one-way analysis of variance and comparisons between individual groups were performed with a two-tailed Student's *t*-test. For *in vitro* CPC analysis, results were paired by donor, and subjected to a paired *t*-test. Probability values of $p < 0.05$ were considered statistically significant.

Results

Generation of SDF-1 microspheres

Microspheres had a mean diameter of 38.5 µm (±14.0 µm (SD); Fig. 1A). When hydrated, microspheres had a smooth, spherical morphology (Fig. 1B), and a rougher surface upon dehydration as visualised by scanning electron microscopy (SEM) (Fig. 1C).

Microsphere-matrix impregnation

The addition of microspheres to the collagen matrix reduced the time to gelation and caused the matrix to solidify at a greater viscosity, as early as 400 s after application ($p = 0.05$; Fig. 2A, 2B), which was maintained over time. Maximum viscosity reached was greater for the matrix containing microspheres (1.42 Pa·s), compared to matrix alone (1.18 Pa·s; $p = 0.003$). Microspheres in solution released their SDF-1 content within 1 d, but matrix impregnation prolonged the maximal release up to approximately 10 d (Fig. 2C). Analysis of SDF-1 release kinetics shows that release from microspheres best fits a first order model, indicated by the greatest correlation coefficient, but after impregnation in a matrix, release follows the Higuchi model (Table 1, Fig. 2D).

Bioactivity of SDF-1 microspheres

Blank microspheres did not confer any difference in adhesion potential with cultured CPCs, but the addition of SDF-1 loaded microspheres supported an increase in adhesive CPCs by 2.3-fold after 1 h of exposure ($p = 0.04$; Fig. 3A-C). When CPCs were given a chemotactic stimulus of blank or SDF-1 loaded microspheres, 3.2-fold more cells migrated towards the SDF-1-releasing microspheres than the blank ones after 24 h ($p = 0.004$; Fig. 3D-F).

Table 1. Correlation coefficients (R2) for SDF-1 release from microspheres alone or embedded in a collagen matrix, based on classical drug release models.

	Zero Order	First Order	Hixson-Crowell	Higuchi	Korsmeyer-Peppas
Free MS	**0.93**	**0.95**	**0.94**	**0.84**	**0.92**
Matrix-Embedded MS	**0.66**	**0.75**	**0.78**	**0.94**	**0.59**

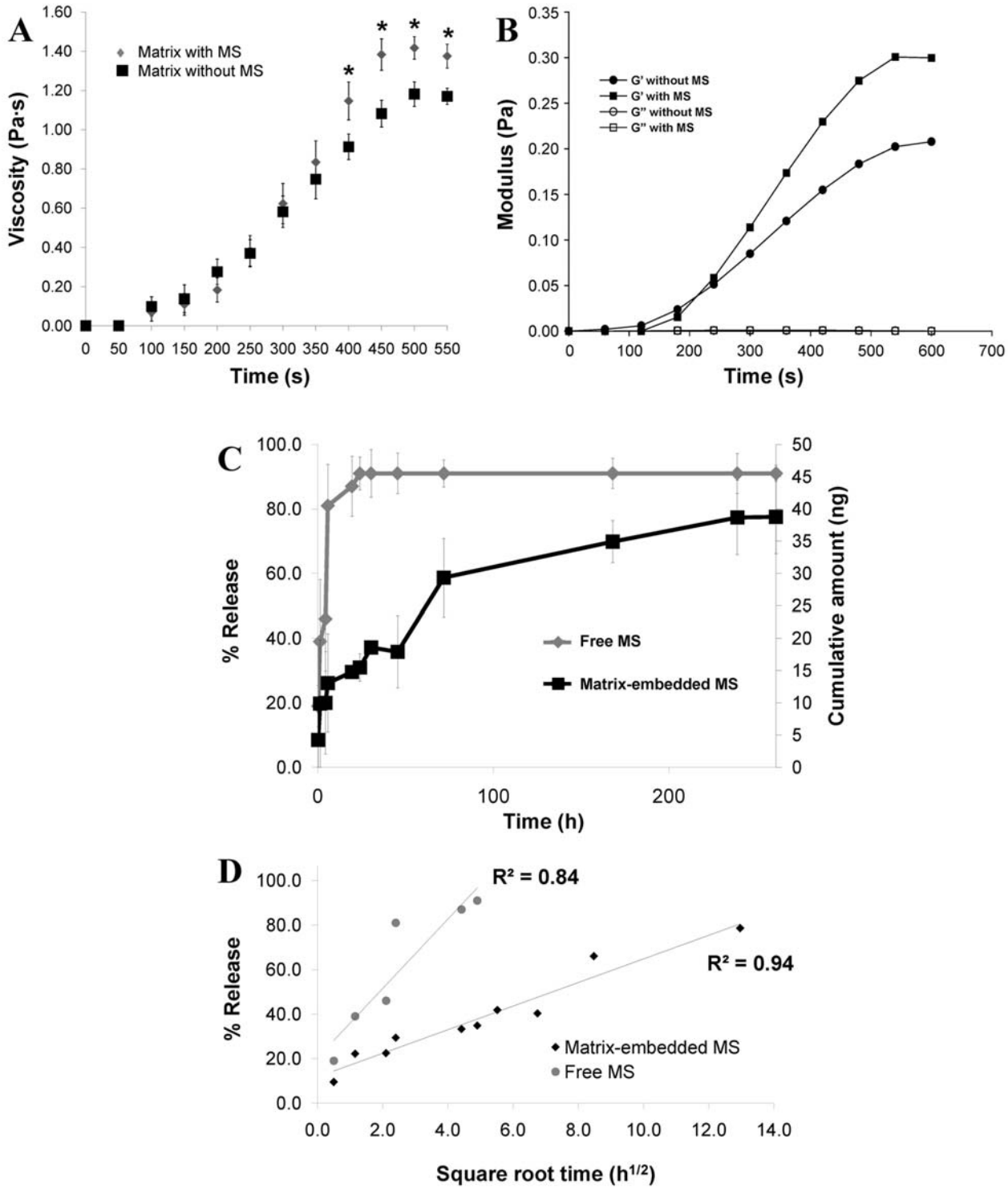

Fig. 2. Matrix-microsphere effects. After microsphere (MS) impregnation into collagen matrix, rheological properties of the matrix were altered: time to gelation was reduced by 16 %, and viscosity increased by 22 % (**A**; $n = 10$). Differences in gelation were first noted at 400 s. **B** shows representative modulus *versus* time curves, which illustrate G' (storage) and G" (loss) values for the different hydrogels. The burst-release effect of SDF-1 was reduced upon incorporation into matrix; the maximal release was delayed from approximately 1 to 10 d (**C**; $n = 3$). Analysis of release kinetics demonstrates that when microspheres are embedded in a matrix, SDF-1 release fits the Higuchi model (**D**; $R^2 = 0.94$). $*p < 0.05$ *versus* matrix without microspheres at the same time point.

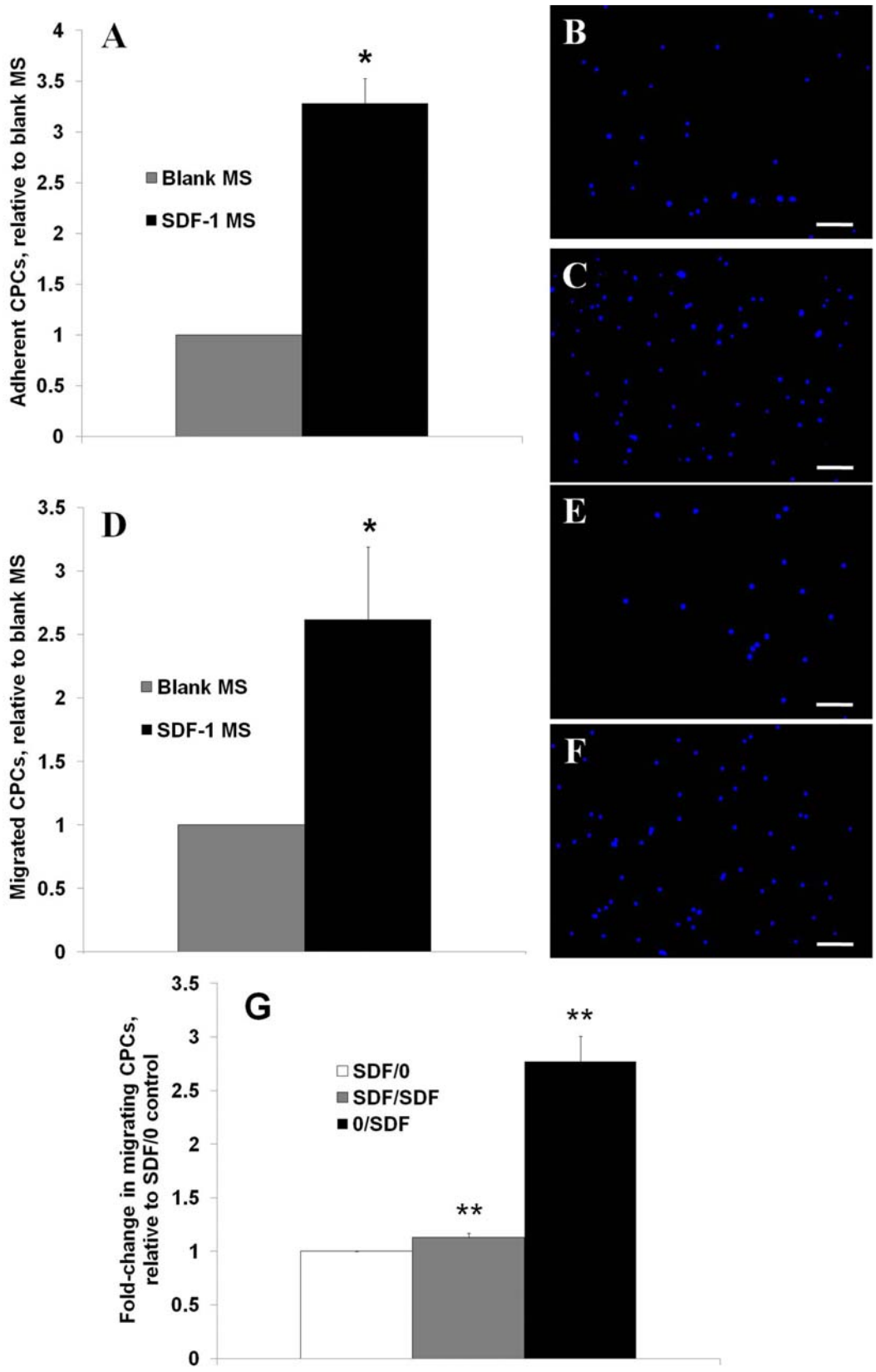

Fig. 3. Effects of blank (no SDF-1) and SDF-1 loaded microspheres on cultured primary CPCs. In the presence of SDF-1 loaded microspheres (MS), over 2-fold more CPCs were adherent to fibronectin within 1 hour of exposure (**A**; $n = 4$); **B**, **C** are representative images of adherent CPCs in the presence of blank or SDF-1 loaded microspheres, respectively. More CPCs migrated towards SDF-1 microspheres (**D**; $n = 4$); **E**, **F** are representative images of blank and SDF-1 loaded microsphere-induced migration of CPCs, respectively. The effect of SDF-1 on CPC migration was mainly chemotactic, rather than chemokinetic, as evidenced by the greater migratory effect of CPCs towards SDF-1 when it is presented as a gradient **(G)**. Scale bars = 100 μm. *$p < 0.05$; **$p < 0.05$ *vs*. all others.

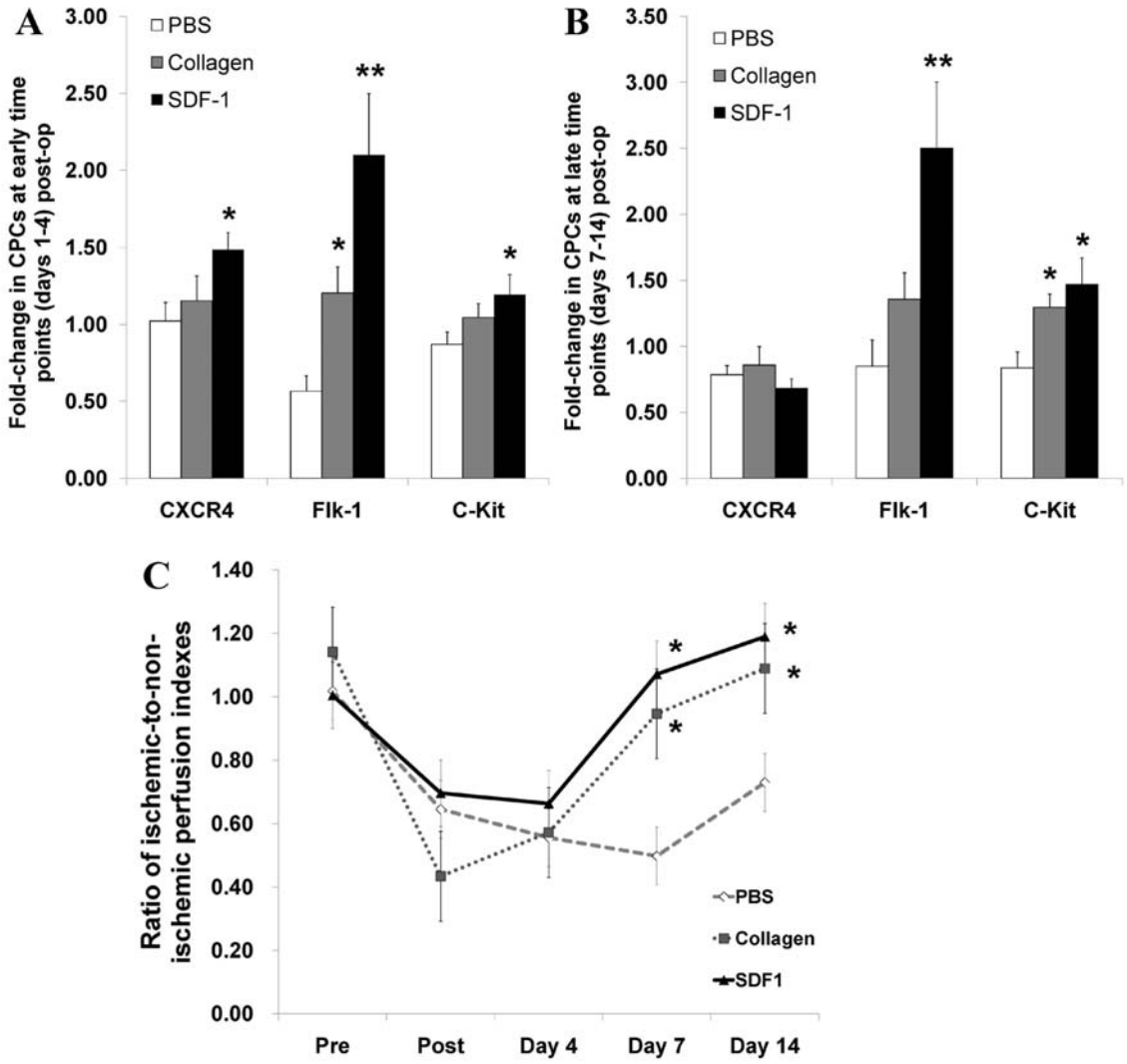

Fig. 4. Functional effects of collagen & SDF-1 microsphere matrix treatments *in vivo*. At early time points after treatment (**A**; days 1-4), animals receiving SDF-1 microsphere-matrix (SDF-1) had greater numbers of circulating CPCs. This was also observed at later time points (**B**; days 7-14). By 7 d post-op, hindlimb perfusion was restored to baseline levels with matrix treatments (**C**; n = 8-9 per group). *$p < 0.05$ *versus* PBS; **$p < 0.05$ *versus* PBS & collagen.

SDF-1 mediated-migration of CPCs is mainly chemotactic

Compared to the lack of a chemotactic stimulus (absence of SDF-1 in the lower well), chemokinesis, stimulated by equivalent amounts of SDF-1 in the upper and lower chambers, increased CPC movement to the lower chamber by 13 % ($p = 0.04$; Fig. 3G). The chemotactic stimulus with SDF-1 only in the lower chamber induced the greatest migration of CPCs, by 177 % ($p = 0.006$; Fig. 3G).

In vivo **mobilisation of CPCs by SDF-1 treatment**

Based on the *in vitro* chemotactic properties of SDF1 microsphere matrix, we defined whether the construct is able to enhance the recruitment of CPC in an *in vivo* system. GFP^+ (bone marrow-derived) CPCs in the peripheral blood were analysed over time, and compared to baseline. Interestingly, the SDF-1 microsphere matrix treatment significantly increased circulating $CXCR4^+$ cells (by 45 %; $p = 0.02$), $flk\text{-}1^+$ cells (by 105 %; $p = 0.001$), and $c\text{-}kit^+$ cells (by 18 %; $p = 0.04$) at early time point post injury (Fig. 4A) and was able to maintain elevated numbers of circulating $flk\text{-}1^+$ cells (by 149 %; $p = 0.002$) and $c\text{-}kit^+$ cells (by 48 %; $p = 0.01$) for an extended period (7-14 d) post operation (Fig. 4B). Notably, the collagen matrix alone increased circulating flk^+ cells (by 20 %; $p = 0.009$) at the earlier evaluation and $c\text{-}kit^+$ cells at both early (by 48 %; $p = 0.01$) and late time point (by 29 %; $p = 0.02$; Fig. 4A, B).

Restoration of perfusion by matrix treatments

Indeed, both matrix treatments (with or without SDF-1 microspheres) were able to restore perfusion to baseline levels by one week post-treatment, compared to PBS controls (Fig. 4C; $p < 0.04$), which was maintained at two weeks post-treatment ($p < 0.05$).

Arteriole density of ischaemic hindlimbs

Arteriole density in ischaemic hindlimbs was not different between collagen and SDF-1 collagen matrices ($p = 0.9$);

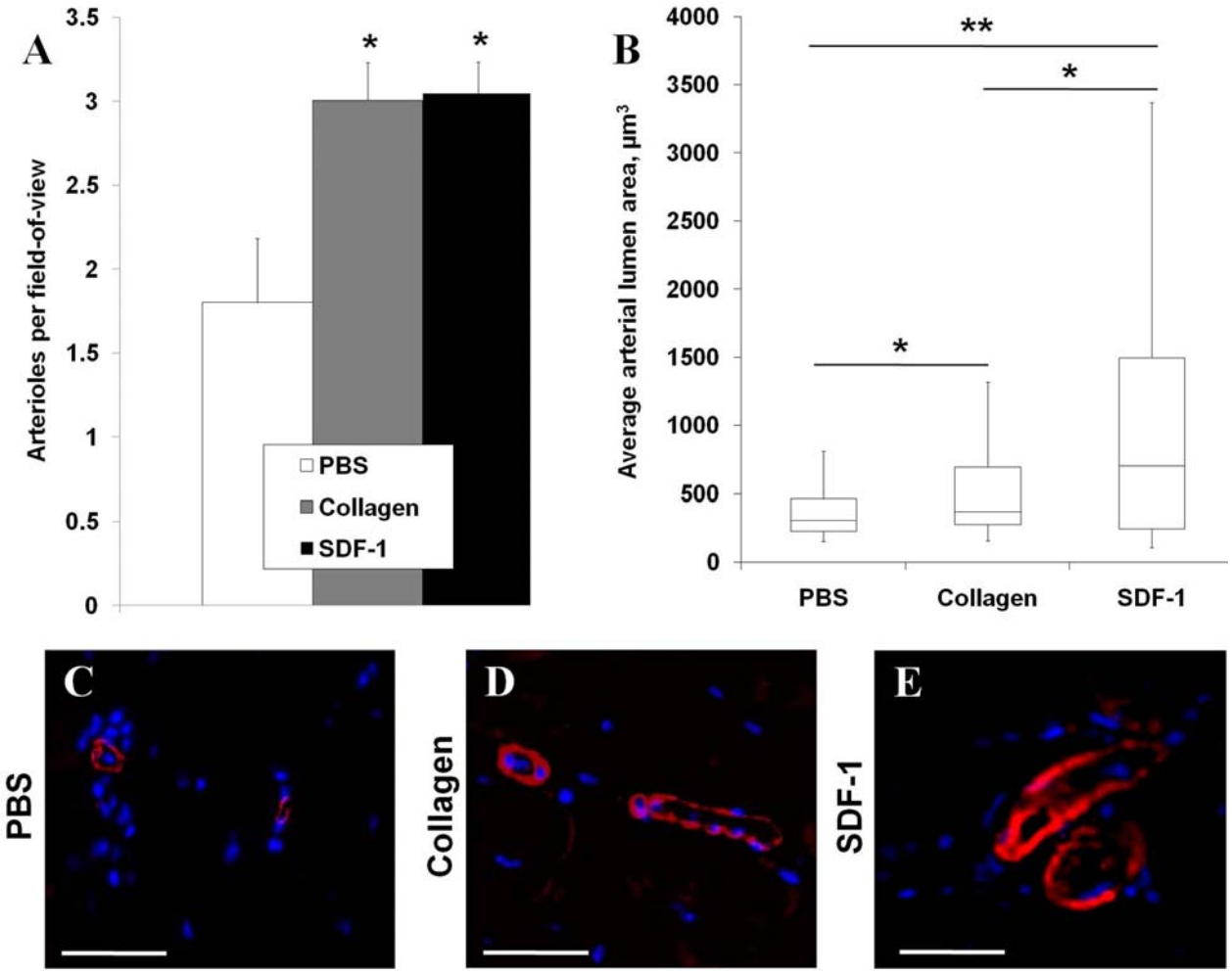

Fig. 5. Assessment of vasculature in treated hindlimbs. There was a trend for increased arteriole counts with matrix treatments **(A)**. Larger arterioles were produced in animals receiving collagen or SDF-1 microsphere-matrix (SDF-1) treatments, with the SDF-1 microsphere-matrix treatment leading to the largest arterioles **(B)**. **C**, **D**, **E** are representative images of arterioles (indicated by SMA^+, red), and counter stained with DAPI (blue). Scale bars = 50 µm. n = 6-8 per group. $^{**}p \leq 0.05$; $^{*}0.05 < p < 0.10$.

however, both matrix and SDF-1 microsphere matrix treatments increased arteriole density compared to PBS by 67 % (p = 0.01) & 69 % (p = 0.02), respectively (Fig. 5A-E). Mean arteriole size was greater in the SDF-1 microsphere matrix treatment group compared to PBS-treated mice ($p < 0.05$; Fig. 5B-E). There was also a trend for greater arteriole cross-sectional area with SDF-1 microsphere matrix treatment compared to collagen matrix (p = 0.09), and for collagen matrix treatment compared to PBS (p = 0.08; Fig. 5B).

Recruitment of bone marrow-derived cells to treated hindlimbs

SDF-1 microsphere matrix and collagen matrix treatments recruited 17.7- and 4.2-fold more GFP^+ cells to treated hindlimbs, compared to PBS (p = 0.0007 and 0.02), respectively (Fig. 6A-D). There was a trend for SDF-1 microsphere matrix treatment to recruit more GFP^+ cells than collagen matrix alone (p = 0.06; Fig. 6A).

Engraftment of $CXCR4^+$ cells in ischaemic hindlimbs

Overall, SDF-1 microsphere matrix treatment recruited 4.3- and 1.8-fold more $CXCR4^+$ cells to treated hindlimbs compared to PBS and collagen (p = 0.0004 and 0.05; Fig. 7A-D). Separating the analysis of $CXCR4^+$ cells into those recruited from the marrow versus those recruited locally, SDF-1 microsphere matrix treatment recruited 4.9-fold more $CXCR4^+GFP^+$ (bone marrow-derived) and 4.5-fold more $CXCR4^+GFP^-$ (local) cells, compared to PBS and collagen (p = 0.008 and 0.02; Fig. 7A). Collagen matrix alone also recruited 2.8-fold more $CXCR4^+GFP^+$ cells compared to PBS (p = 0.03; Fig. 7A).

Cytokine profiles

In the hindlimb (Fig. 8A), interleukin-1α (IL-1α; p = 0.04) and monocyte inhibitory protein-3α (MIP-3α; p = 0.04) were reduced, and vascular cell adhesion molecule-1 (VCAM-1; p = 0.03) and insulin-like growth factor-1 (IGF-1; p = 0.03) were increased with SDF-1 microsphere

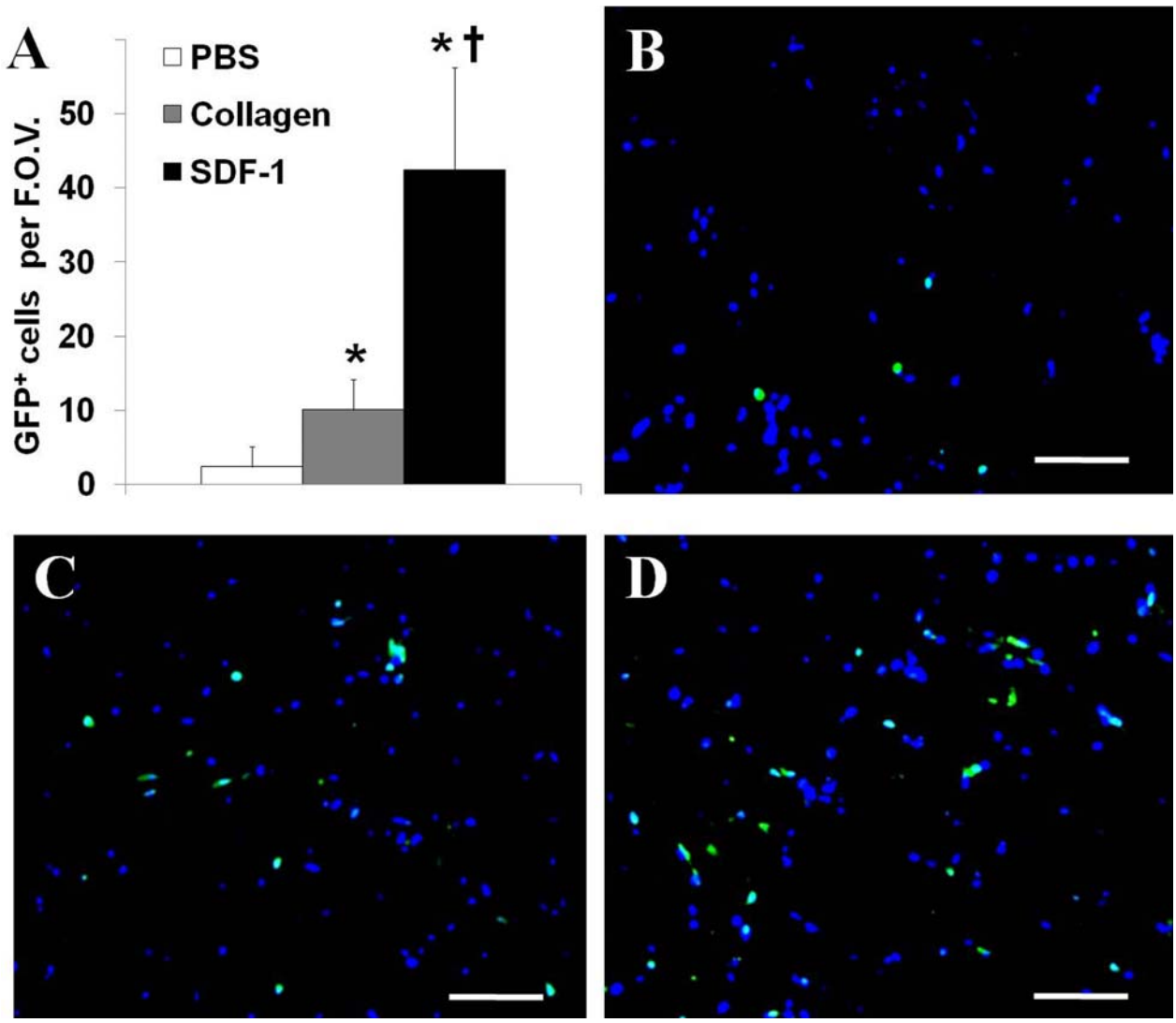

Fig. 6. Recruited bone marrow-derived cells. More GFP^+ cells were engrafted in ischaemic hindlimbs treated with collagen or SDF-1 microsphere-matrix (SDF-1), with a trend for more GFP^+ cells in the SDF-1 microsphere-matrix treatment, compared to matrix alone **(A)**. Representative images of GFP^+ cells recruited to PBS- **(B)**, collagen matrix- **(C)**, or SDF-1 microsphere-matrix- **(D)** treated hindlimbs. Scale bars = 100 μm; n = 6-8 per group. *$p \leq 0.02$ *vs*. PBS, †$p = 0.06$ *vs*. collagen.

matrix treatment. In the serum (Fig. 8B): L-selectin was increased with SDF-1 microsphere matrix treatment ($p = 0.01$) and reduced with collagen alone ($p = 0.01$); basic fibroblast growth factor (bFGF) was increased with collagen alone ($p < 0.001$) and with SDF-1 microsphere matrix treatment ($p = 0.02$); and VCAM-1 was reduced with SDF-1 microsphere matrix treatment compared to collagen matrix ($p = 0.007$) and PBS ($p < 0.001$). For both serum and hindlimb, no significant differences between any treatments were observed for the following inflammatory cytokines: GM-CSF, TNF-α, IL-1β and IL-6 (all $p > 0.4$).

Discussion

In this study, we demonstrated that the release of SDF-1 from alginate microspheres can be effectively prolonged by incorporation of the microspheres into a thermogelling collagen matrix. Released SDF-1 was bioactive, supported rapid adhesion and migration of CPCs, and also stimulated the mobilisation of CPCs from bone marrow when applied to ischaemic muscle. Both matrix alone and SDF-1-releasing matrix restored perfusion and improved arteriole density by 2 weeks, but the SDF-1 treatment best supported the growth of arterioles. SDF-1 microsphere matrix treatment also recruited more bone marrow-derived cells and local $CXCR4^+$ cells to the ischaemic muscle, and altered levels of local angiogenic factors.

Incorporation of alginate microspheres into the collagen matrix increased the hydrogel's viscosity and reduced the time to gelation. This is thought to be mediated by the cross-linking reaction, whereby functional groups on collagen ($–NH_2$) and alginate (–COOH) are covalently bound by EDC/NHS (Mitra *et al*., 2011), increasing the total number of cross-links within the material and enhancing its strength, as has been previously reported (Liu *et al*., 2008). An earlier onset of gelation is advantageous

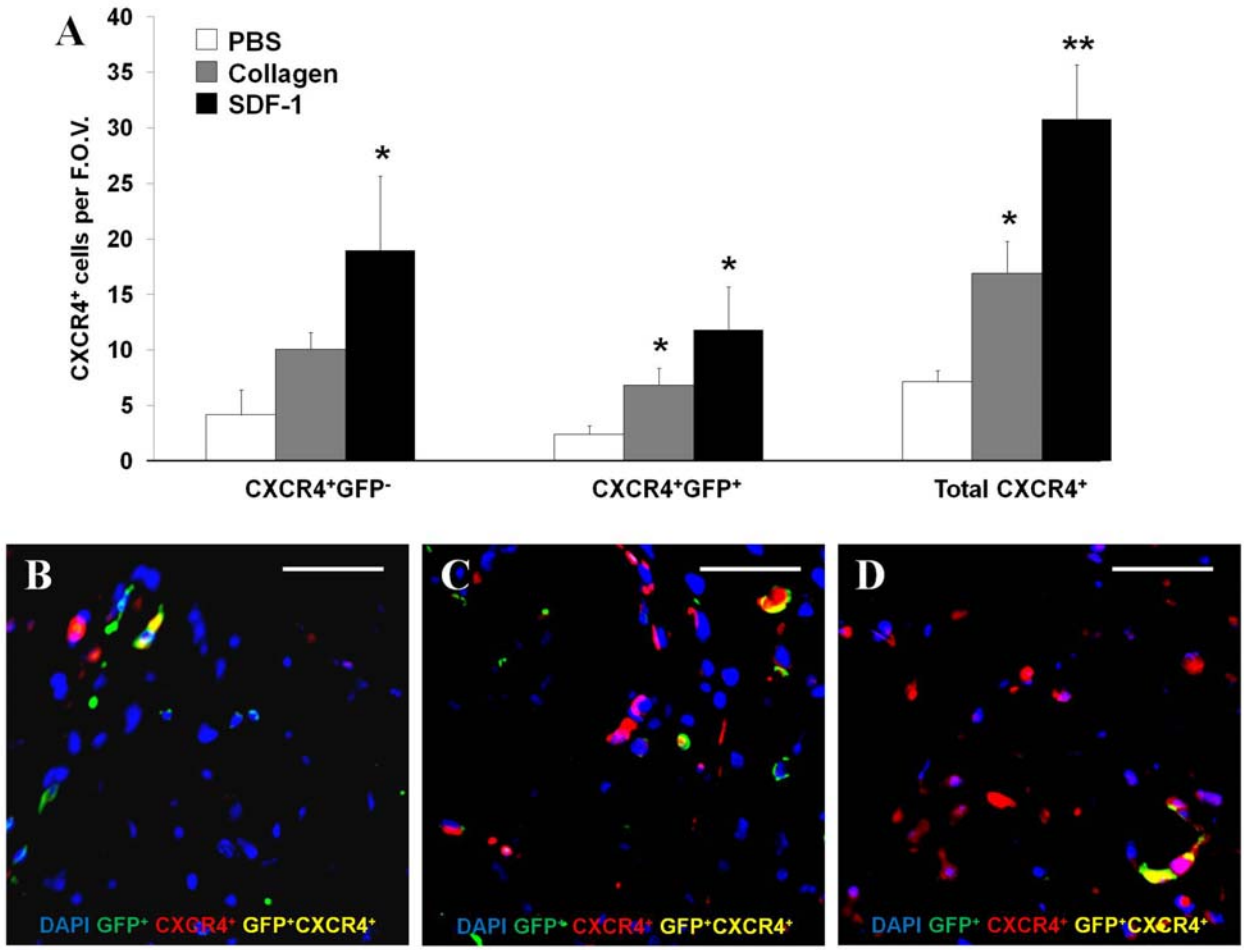

Fig. 7. Localised CXCR4$^+$ cells. SDF-1 microsphere-matrix (SDF-1) treatment recruited more CXCR4$^+$ cells, from both bone marrow (GFP$^+$) and locally (GFP$^-$). **(A)** Representative images of GFP$^+$ (green), CXCR4$^+$ (red), or GFP$^+$CXCR4$^+$ cells (yellow) in PBS- **(B)**, collagen matrix- **(C)**, or SDF-1 microsphere-matrix- **(D)** treated hindlimbs. Scale bars = 200 μm; n = 6-8 per group. *$p < 0.05$ *vs*. PBS, **$p < 0.05$ *vs*. all.

in a clinical setting, allowing for a reduction in the time required for stable material integration within host tissue. Additionally, a positive correlation between hydrogel viscosity and resistance to degradation has been noted (Yang *et al*., 2010), suggesting a better persistence *in vivo* of microsphere-containing hydrogels.

During the period of release, SDF-1 kinetics from matrix-embedded microspheres fit the Higuchi model of release whereby the initial drug concentration >> drug solubility in the matrix, swelling is negligible, perfect sink conditions are maintained and edge effects are negligible, suggesting that diffusion is the primary mechanism of SDF-1 release (Higuchi, 1963). In contrast, microspheres alone initially demonstrated a burst release followed by a first order release of SDF-1. Although a burst-release effect was observed with microspheres alone, the embedding of microspheres in matrix was able to prolong the SDF-1 release by 10-fold. This observation can be explained by the properties of the microspheres and the collagen-hydrogel system. Hydrogels have an innate ability to retain small peptides (Cadee *et al*., 2002; Ruvinov *et al*., 2010), and incorporation of peptide-containing microspheres into hydrogels has been shown previously to extend the release profile of the peptide (Kempen *et al*., 2008). SDF-1 bioactivity was maintained during the microsphere generation and cross-linking procedure; upon its release, SDF-1 augmented rapid adhesion of CPCs, as well as inducing chemotaxis of CPCs, rather than chemokinesis, indicating its ability to induce CPC homing towards a chemotactic gradient rather than the induction of random mobilisation.

In the ischaemic hindlimb study, marrow-mobilised cells expressing the SDF-1 receptor CXCR4 were increased in circulation of SDF-1 microsphere matrix-treated animals at early time points, but this effect was later lost. This can be explained by previous studies showing the eventual down-regulation of CXCR4 following cytokine-induced mobilisation from the bone marrow (Kim *et al*., 2006). More specifically, CXCR4 expression is dose-dependent on SDF-1 levels; doses of ≤ 1 μg in a mouse model have shown CXCR4 levels equivalent to controls that did not receive SDF-1 (Kimura and Tabata, 2010). Interestingly, higher doses of SDF-1 have the same effect, suggesting a potential negative feedback loop for the SDF-1/CXCR4 axis. Notably, SDF-1 microsphere matrix treatment also stimulated an early mobilisation of flk-1$^+$ and c-kit$^+$ cells from the bone marrow into circulation, and maintained this effect up to 2 weeks post-treatment.

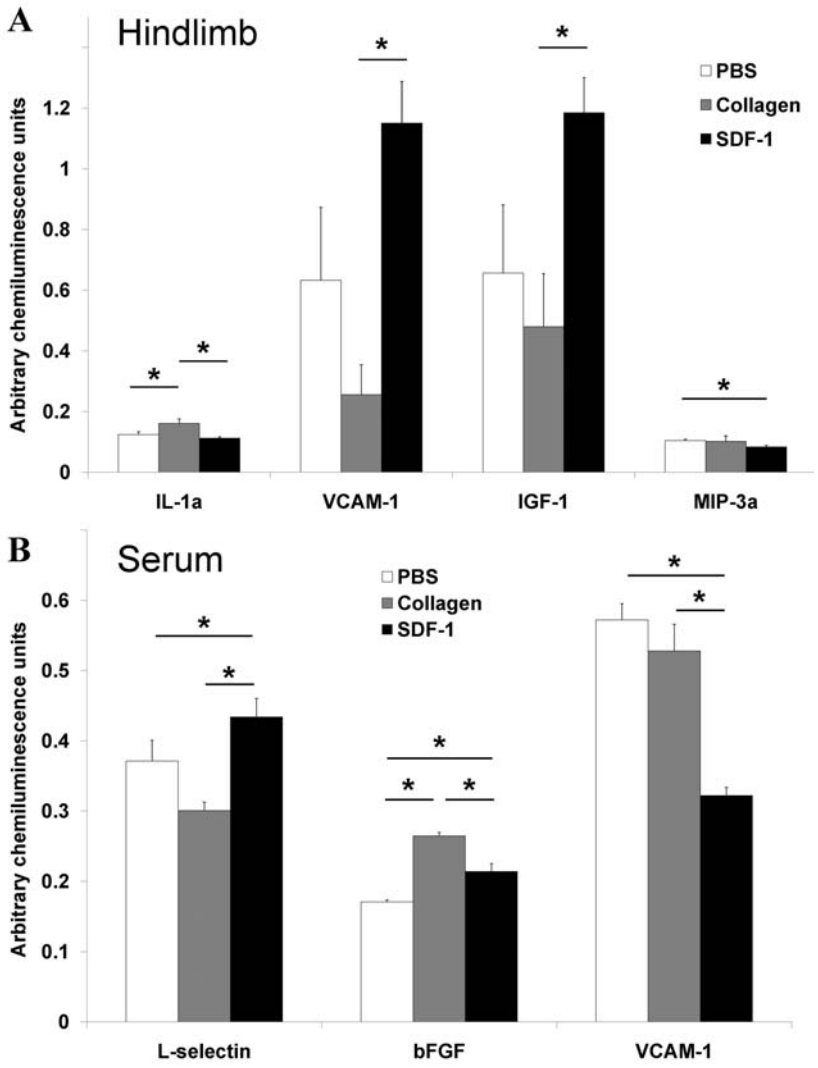

Fig. 8. Cytokine profiles. Observed differences in cytokines in hindlimb lysates **(A)** and serum **(B)**, two weeks after treatment ($n = 5$). *$p < 0.05$.

CXCR4 is SDF-1's exclusive receptor and CXCR4$^+$ CPCs are reduced over time, mechanisms other than SDF-1 release are needed to explain the increase in mobilised CPCs expressing flk-1 and c-kit. We have previously shown that systemic transplantation of CPCs can induce a potent response from the host's CPCs (Suuronen *et al*., 2009). Additionally, this effect has been documented in humans, and CPC persistence in the circulation has been observed up to 1 year after cell transplantation (Turan *et al*., 2010). Therefore, it is likely that the initiation of the SDF-1/CXCR4 mobilisation and recruitment response by SDF-1 microsphere matrix treatment activates other endogenous progenitor cell mechanisms.

Ischaemia was induced in all mice (average of 55 % of normal perfusion). By one week post-treatment, animals that received matrix treatment (with or without SDF-1 microspheres) had hindlimb perfusion restored to baseline levels, which is similar to previous reports of matrix treatment for hindlimb ischaemia (Suuronen *et al*., 2009). It has been previously demonstrated that an increase in perfusion is attributable to increased local vasculature (Kim *et al*., 2010; Suuronen *et al*., 2010), an observation that was seen in the current study; matrix treatments increased arteriole density in ischaemic hindlimbs. It was hypothesised that SDF-1 microsphere matrix treatment would confer superior restoration of perfusion, but instead, the matrix-only treatment was equally effective. Kimura and Tabata (2010) also attempted to enhance neovascularisation using an SDF-1-releasing hydrogel, and observed increased capillary density with SDF-1 treatment at 4 d post-treatment, which was abrogated by 10 d. Another SDF-1 release study did not show a difference in vascular density between SDF-1 and controls at 9 d post-treatment (Rabbany *et al*., 2010). It may be that the addition of SDF-1 accelerates the regenerative response to collagen matrix treatment. This is supported by the observation that SDF-1 treatment increased the cross-sectional size of arterioles. Cross-sectional area of new vasculature has been shown to increase over a period of 4 weeks (Ruvinov *et al*., 2011), demonstrating that vessel area is indicative of vessel maturity. Therefore, it is plausible that SDF-1 treatment had an earlier effect on neovascularisation, allowing for more rapid growth/maturation of arterioles, compared to both matrix and PBS treatments.

The SDF-1 microsphere matrix treatment recruited the most GFP^+ marrow-mobilised cells in our animal model. Other SDF-1-release studies have also shown recruitment of stem cells, positive for c-kit (Zhang *et al*. 2007; Thevenot *et al*., 2010) expression. In particular, it was expected that SDF-1 treatment would increase the recruitment and engraftment of $CXCR4^+$ cells. Both the matrix and SDF-1 microsphere matrix treatments increased homing of bone marrow-derived $CXCR4^+$ cells to the treated hindlimbs (SDF-1 microsphere matrix treatment had the most pronounced effect), but the SDF-1 microsphere matrix treatment also demonstrated an improved recruitment of $CXCR4^+$ cells of non-bone marrow origin. Recently, it was shown that local pools of progenitor cells co-expressing CXCR4 and endothelial markers reside in tissues (Sandstedt *et al*., 2010), suggesting a potential role in neovascularisation. In our experiment, SDF-1 treatment may be recruiting a similar population, as evidenced by $CXCR4^+GFP^-$ staining. Microvascular endothelial cells have also been documented to express CXCR4 (Takagi *et al*., 2009), suggesting another CXCR4-expressing population that is possibly activated in treated hindlimbs. Regardless, $CXCR4^+$ fractions have been observed to be superior to whole $CXCR4^-$ fractions with respect to potential for invasion, neovascularisation, and restoration of perfusion (Seeger *et al*., 2009).

Compared to controls, inflammatory cytokines (IL-1α and MIP-3α) were reduced in hindlimbs treated with SDF-1 microsphere matrix, a result similar to a study by Thevenot *et al*. (2010). The latter examined cytokine responses to SDF-1 delivery in a synthetic scaffold; however, our results also suggest that the SDF-1 microsphere matrix supports a favourable environment for angiogenic activity through local factors, as evidenced by increased IGF-1 and VCAM-1 in hindlimb lysates. IGF-1 is a stimulator of angiogenesis (Su *et al*., 2003) and is cytoprotective (Li *et al*., 1997), and elevated IGF-1 is ideal for recovery. Pelosi *et al.* (2007) have shown that persistent IGF-1 expression accelerates the regenerative response and restores architecture and structure soon after skeletal muscle injury. VCAM-1 is a cell adhesion molecule expressed by endothelial cells. When VCAM-1 is solubilised and detected in serum, it is used as an indicator of dysfunctional endothelium (Balciunas *et al*., 2009). Our SDF-1 microsphere matrix treatment had significantly less circulating VCAM-1, further suggesting a vasculo-protective role of this therapy. Furthermore, both matrix treatments increased systemic bFGF, which is a potent angiogenic cytokine (Hosseinkhani *et al*., 2006).

Conclusion

In this study, we demonstrated that SDF-1 can be successfully encapsulated in an alginate microsphere system and further incorporated into an injectable matrix for non-invasive delivery. The treatment of ischaemic mouse hindlimbs with SDF-1-releasing matrix enhanced progenitor cell mobilisation and recruitment. Compared to collagen-only treatment, the addition of SDF-1 appears to confer an earlier effect on neovascularisation, as suggested by greater arteriole maturity. These findings suggest that application of an SDF-1-releasing matrix constitutes a suitable therapy for prevalent myopathies with reduced perfusion, with the potential to augment progenitor cell mobilisation and homing, as well as its ability to rapidly support neovascularisation.

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Discussion with Reviewers

Reviewer II: Could the authors foresee applications for their injectable hydrogel loaded with SDF1 microspheres in damaged bone tissue with compromised vascularity?
Authors: Vascularisation is believed to play an important role in bone wound healing (Giannoudis *et al*., 2008, additional reference), yet defective neovascularisation in some patients leads to an avascular fracture site. A recent study reported that inhibiting angiogenesis in a rat tibia osteotomy model resulted in non-union of the fracture over the early healing stages, demonstrating the importance of the vasculature for proper bone wound repair (Fassbender *et al*., 2011, additional reference). Notably, the local application of SDF-1 was able to increase neo-blood vessel maturation leading to accelerated bone regeneration, characterised by increased callus formation in a distraction osteogenesis model (Fujio *et al*., 2011, additional reference). Therefore, we can envision that a strategy such as the system reported here may be applicable in bone trauma, whereby blood vessel growth is stimulated at the early stages after bone injury, possibly allowing for more effective wound healing to occur, and without delay.

Reviewer II: In the discussion, the authors suggest that the matrix is important to support the reperfusion and that SDF-1 supports early neovascularisation. Would it be possible that a burst release of SDF-1 from a hydrogel matrix would be equivalent or even better than the material solution proposed with a relatively long release profile?
Authors: After the induction of limb ischaemia, the natural *in vivo* response is that of a burst-release of SDF-1 (De Falco *et al*., 2004, text reference). This is also suggested by the observation that patients with acute limb ischaemia have greater levels of SDF-1 than those with chronic ischaemia (van Weel *et al*., 2007, additional reference). However, the data so far suggests that a prolonged release and presence of SDF-1 is superior to burst release in improving angiogenesis. For example, this has been demonstrated for subcutaneous implantation (Kimura and Tabata, 2010, text reference) and for the repair of ischaemic cardiac tissue (Segers *et al*., 2007, additional reference; Sundararaman *et al*., 2011, additional

reference). However, in the present study, we observed an increase in vessel size, but no difference in vascular density between the two matrix treatments (+/- SDF-1 release), suggesting that the SDF-1 effect was early and accelerated the regeneration response conferred by the injected collagen matrix. Therefore, it is conceivable that a burst release of SDF-1 from microspheres to augment the body's natural SDF-1 release response may be as good as, or better than, prolonged release for treatment strategies involving therapeutic matrix injections. However, we also cannot exclude the possibility that the prolonged presence of SDF-1 may have a role in other effects, such as the vessel maturation process, or the prevention of apoptosis, which have been reported previously by others (Reddy *et al*., 2008, additional reference; Ho *et al*., 2010, additional reference), but were not examined in the present study.

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