The Bispecific SDF1-GPVI Fusion Protein Preserves Myocardial Function After Transient Ischemia in Mice

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Background—CXCR4-positive bone marrow cells (BMCs) are critically involved in cardiac repair mechanisms contributing to preserved cardiac function. Stromal cell–derived factor-1 (SDF-1) is the most prominent BMC homing factor known to augment BMC engraftment, which is a limiting step of stem cell–based therapy. After myocardial infarction, SDF-1 expression is rapidly upregulated and promotes myocardial repair.

Methods and Results—We have established a bifunctional protein consisting of an SDF-1 domain and a glycoprotein VI (GPVI) domain with high binding affinity to the SDF-1 receptor CXCR4 and extracellular matrix proteins that become exposed after tissue injury. SDF1-GPVI triggers chemotaxis of CXCR4-positive cells, preserves cell survival, enhances endothelial differentiation of BMCs in vitro, and reveals proangiogenic effects in ovo. In a mouse model of myocardial infarction, administration of the bifunctional protein leads to enhanced recruitment of BMCs, increases capillary density, reduces infarct size, and preserves cardiac function.

Conclusions—These results indicate that administration of SDF1-GPVI may be a promising strategy to treat myocardial infarction to promote myocardial repair and to preserve cardiac function. (Circulation. 2012;125:685-696.)

Key Words: GPVI ■ myocardial infarction ■ cells ■ SDF-1 ■ tissue repair

In recent years, extensive efforts have been made to identify mechanisms to promote myocardial repair and regeneration.1,2 It has been increasingly recognized that bone marrow (BM)–derived progenitor cells (BMCs) participate in cardiac repair mechanisms and regeneration and contribute to function recovery after myocardial infarction (MI). Elevated levels of BMCs in patients with cardiovascular disease are associated with a reduced risk of death from cardiovascular complications.3,4

The isolation of putative BMCs was first described in the late 1990s,5 and it has become evident that circulating BMCs significantly contribute to the regeneration of vascular integrity and support locally induced repair mechanisms of resident endothelial cells.6 In vivo studies in mice and rats provide evidence that vascular homing of BMCs improves endothelial regeneration and preserves myocardial function after transient coronary ischemia.7-9 Myocardial BMC transplantation into immunodeficient mice induces sustained humoral effects and leads to increased mobilization of endogenous BM-derived or progenitor cells that are incorporated into sites of neovascularization and myocardial repair.10

Clinical Perspective on p 696

Progressive adverse left ventricular (LV) remodeling after MI is the pathomorphological substrate of postinfarction heart failure and reduced survival.11 Cell therapy for patients with acute MI has been evaluated in the past by various clinical studies. In some studies, intracoronary administration of BMCs has been found to improve recovery of LV contractile function in patients with acute MI.12-14 Other clinical studies did not show a beneficial effect of intracoronary administration of BMCs on LV ejection fraction in patients after ST-segment elevation MI.15,16

Regulation of homing of BMCs plays an important role in mobilization of BMCs from the BM to the ischemic myocardium. The most prominent BMC homing factor is the chemokine stromal cell–derived factor-1 (SDF-1)/CXCL12.17 SDF-1 has been shown to be upregulated in...
experimental MI. Platelet-derived SDF-1 regulates BMC function and differentiation. Moreover, platelet-derived SDF-1 is enhanced in MI in humans and correlates with the number of circulating BMCs. Experimental data provide evidence that SDF-1 augments BMC engraftment in ischemic myocardium and preserves myocardial function. In vivo administration of recombinant SDF-1 protects mice against myocardial ischemia and leads to reduced infarct size after coronary occlusion.

Augmentation of myocardial repair mechanisms through recombinant SDF-1 is limited by its short half-life and lack of lesion-directed accumulation, leading to low enrichment in the injured myocardium. The purpose of the present study was to develop a bispecific, recombinant SDF-1 molecule that preferentially binds to tissue lesions with destroyed vascular integrity. We constructed a recombinant, bispecific molecule consisting of SDF-1 and a recombinant form of the soluble platelet collagen receptor glycoprotein VI (GPVI) serving as an anchor structure to direct BMCs to sites of vascular and myocardial damage to preserve cardiac function after MI.

Methods

Generation of SDF1-GPVI
SDF1-GPVI is a fusion protein consisting of the human chemokine SDF-1, the platelet receptor GPVI, and the Fc part of human IgG2. This fragment was produced by gene synthesis and cloned into the expression vector pcDNA5-FRT (Généart, Regensburg, Germany). The Flip-In CHO cell line (Invitrogen, Carlsbad, CA) was stably transfected, produced, and purified as described previously.

Binding and Adhesion Studies
Binding capacity of SDF1-GPVI to collagen was tested by a collagen-binding enzyme-linked immunosorbent assay. The plate was coated with collagen, incubated with SDF1-GPVI, GPVI-SDF1, GPVI, or FcIgG2, detected with 3,3’5,5’-tetramethylbenzidine, and measured with the use of the enzyme-linked immunosorbent assay 550 plate reader as described previously.

CXCR4-binding capacity of SDF1-GPVI was analyzed by a competition assay. Human monocytes (2×10^6) were freshly isolated from bone marrow, and measured with the use of the enzyme-linked immunosorbent assay 550 plate reader as described previously.

Adhesion experiments under flow conditions (flow rate 15 mL/h and shear rate 2000/s) were performed with 1.5×10^5 cells per mL. CD34^+ cells diluted in Iscove’s modified Dulbecco’s medium. Rectangular coverslips were coated with collagen type I and SDF1-GPVI, GPVI, or human SDF-1 as described previously.

Colony-Forming Unit Assay
CD34^+ cells (5×10^3/cm^2) were seeded in precoated 96-well plates and cultured at 37°C and 5% CO2 in VascuLife medium (CELL-Systems, Trois-rivières, Germany). Endothelial colony-forming units were counted at days 1, 3, 5, 7, 10, and 14 with the use of light microscopy.

 Colony-Forming Unit Assay
CD34^+ cells (5×10^3/cm^2) were seeded in precoated 96-well plates and cultured at 37°C and 5% CO2 in VascuLife medium (CELL-Systems, Trois-rivières, Germany). Endothelial colony-forming units were counted at days 1, 3, 5, 7, 10, and 14 with the use of light microscopy (Axiovert, Carl Zeiss, Oberkochen, Germany).

Additional characterization experiments such as chemotaxis, survival assay, and chorioallantoic membrane assay are described in the online-only Data Supplement.

Myocardial Ischemia and Reperfusion in Mice
All animal studies were approved by the district government of Tübingen. Myocardial ischemia was induced in 10- to 12-week-old C57BL/6J mice by ligation of the left anterior descending artery (LAD) for 45 minutes. Mice were randomly divided into the SDF1-GPVI group (n=7) or the FcIgG2 control group (n=6). SDF1-GPVI (10 μg/g body wt) and FcIgG2 (4.2 μg/g body wt) control protein was injected intravenously directly after LAD ligation and repeated 48 hours later. Both groups were treated intraperitoneally with granulocyte colony-stimulating factor (G-CSF) (100 μg/kg body wt) after the surgical procedure for 3 consecutive days. Furthermore, mice were treated after LAD ligation with SDF1-GPVI (n=4) or FcIgG2 (control group; n=6) in the absence of G-CSF, 28 days after MI echocardiography was performed, as described previously.

Preparation of Murine BMCs and Specific Detection of Injected BMCs in the Injured Myocardium
BMCs were freshly isolated from the tibias and femurs of transgenic green fluorescent protein (GFP)-positive mice (kindly provided by Dr Bischof). The BM was injected with the use of a 26-gauge needle and filtered through a 70-μm cell strainer (BD Bioscience, CA). BM cells (1×10^6) were resuspended in 250 μL phosphate-buffered saline and incubated for 30 minutes with 20 μg/mL SDF1-GPVI or 8.4 μg/mL FcIgG2 control protein. Preparated BMCs were injected intravenously directly after LAD ligation. Twenty-four hours after ischemia and reperfusion, hearts were removed, cut in cryosections, stained with 4’,6-diamidino-2-phenylindole, and analyzed by immunofluorescence. The numbers of GFP-positive BMCs were quantified from 10 random cryosections of each heart sample.

Statistical Analysis
All experiments were performed at least 3 times in duplicates. Results are depicted throughout as mean±SEM. Statistical analysis was performed with the use of SPSS statistics software for Windows version 19.0 (IBM SPSS Inc, Chicago, IL). Statistical significance was set at P<0.05. Statistical comparison of >2 experimental conditions was performed with the Kruskal-Wallis test. To compare specific pairs of outcome, 1-way ANOVA with adjustment for multiple testing (Bonferroni) was performed in case the global test was significant. Measurements taken at different times from the same experimental units were compared with repeated-measures ANOVA.

Results

Soluble SDF1-GPVI Fusion Protein
We designed, constructed, and cloned 2 proteins, the bifunctional protein GPVI-SDF1-FcIgG1 (termed GPVI-SDF1) and SDF1-GPVI-FcIgG2 (termed SDF1-GPVI). We followed these 2 concepts to provide different strategies because it is well known that the direction of different domains of the fusion protein is important for proper protein function. Both proteins (Figure 1A) contain the platelet collagen receptor GPVI, which is able to bind to collagen at the exposed extracellular matrix of disrupted endothelium or myocardium or to activated endothelium via fibronectin or vitronectin. Furthermore, the proteins exhibit a human SDF-1 domain to attract and bind CXCR4-positive BMCs for enhanced accumulation of these cells at sites of vascular and/or myocardial injury. Only SDF1-GPVI offers an amino acid linker [G(GS)_4] to separate the N-terminal SDF-1 domain from other domains, whereas the SDF-1 domain of the GPVI-SDF1 is located at the C-terminal site without any additional linker sequence (Figure 1A). Furthermore, we generated 2 corresponding control proteins, GPVI-FcIgG2 (termed GPVI), lacking SDF-1 domain, and FcIgG2, lacking SDF-1 and GPVI domains.
A predicted molecular 3-dimensional model of both proteins demonstrates a free and separate SDF-1 domain by the SDF1-GPVI molecule in contrast to the GPVI-SDF1 protein (Figure 1A). A predicted binding model of the SDF-1 domain to its CXCR4 receptor revealed a high calculated binding affinity with a dissociation constant ($K_D$) of 105 nmol/L in contrast to the low binding affinity of the GPVI-SDF1 construct with a $K_D$ of 188 mol/L, reflecting a high-affinity interaction only between the SDF1-GPVI protein and CXCR4.

Stable cell lines of all proteins were prepared and expressed as secreted soluble proteins with the use of the Flp-In CHO cell line.24 The proteins were produced and purified from cell supernatants. Using silver staining (Figure 1B), we showed that SDF1-GPVI was isolated with high purity. The molecular mass of SDF1-GPVI was confirmed by Western blotting at 82 kDa under reducing conditions (Figure 1C). An $\approx$130-kDa protein and an $\approx$250-kDa protein were identified under nonreducing conditions, which confirms that SDF1-GPVI is present as a homodimer as well as an oligomer.

Figure 1. Generation of the bispecific SDF1-GPVI. A, Schematic illustration of the construct. Structures of the 2 constructed proteins GPVI-SDF1 and SDF1-GPVI are illustrated (top). Predicted 3-dimensional models of both proteins and a predicted binding model of SDF1-GPVI to CXCR4 receptor (bottom) are shown. SDF1-GPVI shows a theoretical high binding affinity to CXCR4 receptor with a $K_D$ of 105 nmol/L in contrast to the low binding affinity from GPVI-SDF1 to CXCR4 with a $K_D$ of 188 μmol/L. B, Purity of the fusion protein SDF1-GPVI (1 μg) and control glycoprotein VI (GPVI) (1 μg) was verified by silver staining under reducing (left) and nonreducing (right) conditions. C, Purified SDF1-GPVI (600 ng) and the corresponding control proteins were immunoblotted under reducing (left) and nonreducing (right) conditions with the use of stromal cell–derived factor-1 (SDF-1), GPVI, and IgG antibodies.
SDF1-GPVI Binding to Collagen and CXCR4

Next we determined the binding activity of both proteins to their binding partners collagen and CXCR4. The specific binding of the GPVI domain to collagen was verified by a collagen/GPVI-binding enzyme-linked immunosorbent assay (Figure 2A, left) with the use of different concentrations of the proteins. Both proteins, GPVI-SDF1 and SDF1-GPVI, display a similar dose-dependent binding capacity to immobilized collagen, whereas the control FcIgG2 protein without GPVI domain showed no collagen binding, as expected. A competition experiment with solubilized collagen demonstrated that a concentration of 200 µg/mL collagen was sufficient to completely inhibit GPVI binding to immobilized collagen (Figure 2A, right). Taken together, these data demonstrated high specific binding of the GPVI domain of both proteins (GPVI-SDF1 and SDF1-GPVI) to its ligand collagen.

To explore sensitivity and specificity of the SDF-1 binding site, we used a CXCR4 competition–based binding assay. Isolated monocytes were incubated with different concentrations of the bispecific proteins to compete with phycocerythrin-labeled anti-CXCR4 antibody that was determined by flow cytometry analysis. Incubation of monocytes with the SDF1-GPVI protein displayed a clear concentration-dependent decrease of detectable CXCR4 (Figure 2B). In contrast, the GPVI-SDF1 protein revealed only low binding activity (Figure 2B). No binding to CXCR4 was detected with the control proteins GPVI or FcIgG2 (Figure 2B). These results demonstrate that the SDF-1 domain of the SDF1-GPVI protein binds its receptor CXCR4 with high...
sensitivity and specificity in contrast to the GPVI-SDF1 protein, which shows only low binding affinity. These results were in accord with the calculated $K_D$, as shown in Figure 1A.

The bispecific properties of the SDF1-GPVI construct were also shown by a dynamic adhesion assay. Collagen-coated coverslips were incubated with SDF1-GPVI, native SDF-1, or control protein. CXCR4-positive BMCs were perfused through the flow chamber under high shear rates (2000/s). A strong increase in firm adhesion of BMCs was detected when collagen-coated surfaces were preincubated with SDF1-GPVI protein before perfusion (Figure 2C). In contrast, native SDF-1 or control protein showed marginal adhesion of BMCs. Taken together, these results demonstrate high sensitivity and specificity of SDF1-GPVI binding to collagen and CXCR4 and demonstrate that SDF1-GPVI augments cell adhesion under high shear rates.

SDF1-GPVI Triggered Chemotaxis and Preserved Cell Survival

To study the functional consequences of SDF1-GPVI binding to CXCR4, we used a transwell system to test whether both proteins, GPVI-SDF1 and SDF1-GPVI, are able to trigger chemotactic activity of CXCR4-positive cells (Figure 3A). SDF1-GPVI was able to induce strong chemotactic responses of BMCs in a concentration-dependent manner. In contrast, the GPVI-SDF1 protein displayed only low chemotactic activity, as shown by the low number of migrated cells (SDF1-GPVI versus GPVI-SDF1, 4170/1100675 versus 248/1100623; n = 3; $P < 0.001$). Furthermore, we tested the ability of a neutralizing CXCR4 antibody to block the chemotactic effects. A concentration of 10 ng/mL of CXCR4 antibody was sufficient to decrease the amount of migrated cells by 60% (anti-CXCR4 antibody versus IgG2b control, 1635/11006354 versus 4065/11006867; $P < 0.04$), confirming that the specific interaction of SDF-1 and CXCR4 is responsible for the chemotactic effects of the SDF1-GPVI protein. To validate that the human SDF-1 fusion protein also triggers chemotaxis of murine CXCR4-positive cells, we performed comparable experiments confirming that SDF1-GPVI stimulates migration of mouse-derived CXCR4-positive cells as well (data not shown).
SDF-1 regulates hematopoiesis by suppressing apoptosis.\textsuperscript{27} Because of this, we performed an apoptosis assay using BMCs to investigate the potential of SDF1-GPVI as a survival factor. As shown in Figure 3B, the presence of SDF1-GPVI (177 pM) enhanced survival of living cells compared with FcIgG2 control (48.3\% versus 23.5\% live cells; \( P < 0.001 \)) to an extent similar to that of SDF-1 (56.6\% versus 2.1\%, \( P < 0.001 \)).

**SDF1-GPVI Enhanced Endothelial Differentiation**

After homing, BMCs must undergo integration into the endothelial monolayer before they can contribute to restoration of vascular integrity.\textsuperscript{28} We performed a colony-forming unit assay to test the potential of SDF1-GPVI to induce maturation of CD34\(^+\) cells into endothelial-like cells (Figure 4A). Cultivation with SDF1-GPVI propagated and accelerated the colony-forming unit of CD34\(^+\) cells (Figure 4). Within days in vitro 1 to 5 (DIV1–5), we detected 3 times more colonies on SDF1-GPVI–coated surfaces than on uncoated or control GPVI-coated surfaces (22\%\pm5.6 versus 8\%\pm2.3 versus 9\%\pm2.8 colonies per 10\(^6\) CD34\(^+\) cells, respectively). This positive effect on colony-forming unit efficiency was consistent up to DIV14 when progenitor cells cultivated on SDF1-GPVI were completely differentiated into a confluent mature endothelial monolayer, whereas cells cultivated on control coating surfaces still displayed individual colonies (control, 9.7\%\pm3.5 colonies per 10\(^6\) CD34\(^+\) cells; GPVI, 20\%\pm6 colonies per 10\(^6\) CD34\(^+\) cells), indicating a clear benefit for progenitor cells cultivated on SDF1-GPVI.

These findings were supported by the analysis of \(1,1'\)-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanin perchlorate (DiI)-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) uptake (Figure 4B, red fluorescence). CD34\(^+\) cells cultivated on SDF1-GPVI–coated surfaces displayed enhanced attachment of living cells (green Calcein staining; Figure 4B, d compared with a through c) and more rapid and more efficient uptake of \(1,1'\)-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanin perchlorate (DiI)-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) (Figure 4B, d compared with a through c). Moreover, these cells develop an endothelial-like morphology (Figure 4B, t compared with q through s).

The endothelium is essential to provide an intact and selective barrier between the vessel lumen and surrounding tissue. Integrity of intercellular junctions is a major determinant of permeability of the endothelium and essential in endothelial differentiation.\textsuperscript{29} Therefore, we examined the
formation of the adherens junction molecule vascular endothelial cadherin (red fluorescence, Figure 4C), which is known to be important for the formation of a restrictive endothelial barrier. We were able to detect the first intercellular junction formation within DIV5 when BMCs were cultured in the presence of SDF1-GPVI (arrow, Figure 4C, l). These cells displayed strong and early cell proliferation and differentiation with early intercellular vascular endothelial cadherin expression between adjacent cells (arrow, Figure 4C, f and l), resulting in a confluent endothelial monolayer at DIV14 (Figure 4C, x). In contrast, BMCs cultivated on control coating surfaces displayed the first intercellular junction formation at DIV14 (Figure 4C, u through w), indicating a retarded endothelial differentiation potential. These results indicate that SDF1-GPVI augments and accelerates endothelial differentiation of BMCs.

Consequently, we examined typical endothelial marker expression at DIV14 when confluent monolayers were formed. Within DIV14, monolayers on SDF1-GPVI displayed an expression pattern of typical endothelial markers comparable to naive human coronary artery endothelial cells (Figure 4D). We were able to detect the characteristic von Willebrand factor staining (compare Figure 4D, a and g), the adherens junction proteins vascular endothelial cadherin (compare Figure 4D, d and j) and platelet/endothelial cell adhesion molecule-1 (compare Figure 4D, f and l), as well as the occludens junction protein claudin 5 (compare Figure 4D, c and i), which were exclusively located at the intercellular borders, as is typical for endothelial cells.29 Moreover, differentiated progenitor cells showed vascular endothelial growth factor receptor 2 (compare Figure 4D, b and h) and angiopoietin receptor Tie-2 (compare Figure 4D, e and k) expression, demonstrating distinct endothelial differentiation of cultured progenitor cells after SDF1-GPVI administration.

SDF1-GPVI Mediated Proangiogenic Effects
For testing angiogenic characteristics of implants or substances, the chorioallantoic membrane assay is a well-accepted and widely used in ovo model (Figure 5A). Application of SDF1-GPVI (10 μg) resulted in a 200% a/b ratio (blood vessels growing toward the test solution [a] and those growing centrifugally away from the test solution [b] were counted, and a/b ratio was determined to evaluate the degree to which the test substance induced proangiogenic or antiangiogenic effects. Application of phosphate-buffered saline (PBS) or the control protein glycoprotein VI (GPVI) resulted in equal angiogenesis without any chemotacttractant impact. Addition of SDF1-GPVI and human stromal cell–derived factor-1 (hSDF-1) resulted in a comparably strong doubling of proangiogenic effects. When vascular endothelial growth factor (VEGF) was applied, proangiogenic events were further enhanced (n ≥3; general significance P<0.006; significance between SDF1-GPVI and phosphate-buffered saline P<0.001). ***P<0.001.

**Figure 5.** SDF1-GPVI revealed proangiogenic effects in ovo. A, Schematic illustration of the experiment. B, Chorio allantois membrane (CAM) was explanted at day 13, and blood vessel orientation was examined. Blood vessels growing toward the test solution (a) and those growing centrifugally away from the test solution (b) were counted, and a/b ratio was determined to evaluate the degree to which the test substance induced proangiogenic or antiangiogenic effects. Application of phosphate-buffered saline (PBS) or the control protein glycoprotein VI (GPVI) resulted in equal angiogenesis without any chemotacttractant impact. Addition of SDF1-GPVI and human stromal cell–derived factor-1 (hSDF-1) resulted in a comparably strong doubling of proangiogenic effects. When vascular endothelial growth factor (VEGF) was applied, proangiogenic events were further enhanced (n ≥3; general significance P<0.006; significance between SDF1-GPVI and phosphate-buffered saline P<0.001). ***P<0.001.

SDF1-GPVI Improved Cardiac Function After MI
To assess the effects of SDF1-GPVI administration on myocardial function in vivo, we used a mouse model of myocardial ischemia.25,26 In this model, myocardial damage is assessed after LAD ligation for 45 minutes followed by reperfusion. C57BL/6J mice were treated with administration (×2) of SDF1-GPVI as well as G-CSF for 3 days (Figure 6A). The pharmacokinetics of SDF1-GPVI in mouse serum was determined by enzyme-linked immunosorbert assay and Western blot analysis (Figure 6B). Results demonstrate a slow and time-dependent elimination of SDF1-GPVI with an estimated half-life of >48 hours, as shown for other Fc immunoadhesins.30,31
Figure 6. SDF1-GPVI treatment reduced infarct size and preserved cardiac systolic function in a mouse model of myocardial ischemia. 

A, In C57BL/6J mice, SDF1-GPVI (n=7) or control FcIgG2 (n=6) was injected intravenously after left anterior descending coronary artery (LAD) ligation and 48 hours later (administration ×2). All mice were treated with granulocyte colony-stimulating factor (G-CSF) (100 µg/kg body wt) for 3 days. Mice were analyzed 28 days after reperfusion. LV indicates left ventricular. B, Pharmacokinetics of SDF1-GPVI in mouse serum samples. Serum levels of SDF1-GPVI before and 6, 12, 24, and 48 hours after intravenous injection were analyzed by enzyme-linked immunosorbent assay and Western blot (n=4). C, Representative transverse cardiac sections show reduced infarct size in SDF1-GPVI–treated mice (left, pale area). Quantitative analysis of the infarct size in percentage of area at risk (%I/AaR) is shown (right; n=6; P=0.02). D, Cardiac sections from mice 24 hours after myocardial infarction. Representative images of recruited green fluorescent protein–positive bone marrow cells (BMCs) (green) into the heart (left; bar=25 µm; magnification ×200) are shown. Quantification of recruited BMCs is shown (right; n=3; P=0.081). hSDF-1 indicates human stromal cell–derived factor-1. E through G, Cardiac sections from mice 7 and 28 days after myocardial infarction were stained with hematoxylin and eosin (HE) and immunostained for FcIgG2 (hIgG), SDF-1, CXCR4, and platelet/endothelial cell adhesion molecule-1. Control (ctrl) group at day 7 was treated with glycoprotein 692 Circulation February 7, 2012
In SDF1-GPVI–treated mice, infarct areas 28 days after reperfusion, as assessed by 2,3,5-triphenyltetrazolium chloride staining to differentiate between metabolically active and inactive tissue, were reduced by 24.4% in comparison to mice treated with FcIgG2 control protein (34.3±4.2% versus 58.7±8.3% [infarct size as a percentage of area at risk]; Figure 6C). Next we determined whether treatment with SDF1-GPVI shows an enhanced recruitment of CXCR4-positive BMCs to the infarcted myocardium. Freshly isolated GFP-positive BMCs from GFP mice, incubated with SDF1-GPVI, control FcIgG2, or human SDF-1, were specifically detected in the myocardium after 24 hours. We found enhanced accumulation of GFP-positive BMCs in the presence of SDF1-GPVI (57.3±0.66 BMCs per 10 cryosections; Figure 6D) compared with control FcIgG2 (13.0±2.0 BMCs per 10 cryosections), human SDF-1 (11.67±1.2 BMCs per 10 cryosections), or control hearts (13.3±6.36 BMCs per 10 cryosections). Specific accumulation of SDF1-GPVI in the injured myocardium was proven by immunohistochemistry. SDF1-GPVI accumulation 7 days after transient ischemia was substantially enhanced in the infarcted myocardium compared with control-treated animals. Twenty-eight days after transient ischemia, a positive SDF1-GPVI signal was almost absent (Figure 6E). In parallel, detection of CXCR4-positive cells was significantly increased in infarcted myocardium of SDF1-GPVI–treated mice compared to the control group (61.77±1.88% versus 35.82±3.45% CXCR4-positive cells; Figure 6F). Twenty-eight days after LAD ligation, the percentage of CXCR4-positive cells within the infarcted myocardium was not different between SDF1-GPVI– and control-treated animals (24.04±2.98% versus 23.59±4.36% CXCR4-positive cells; Figure 6F). Capillary density in the infarct border zone was detected with a platelet/endothelial cell adhesion molecule-1 antibody. In the SDF1-GPVI–treated group, the average capillary density was enhanced at day 7 compared with the GPVI control group (991 ± 80 versus 476 ± 113 capillaries per square millimeter) as well as at day 28 compared with the FcIgG2 control group (528 ± 19 versus 282 ± 41 capillaries per square millimeter; Figure 6G). Reduction of infarct size and enhanced capillary density were functionally relevant because echocardiography 28 days after LAD ligation showed significantly improved cardiac LV function by ≈69% in mice treated with SDF1-GPVI compared with FcIgG2-treated mice (36.1±1.8% versus 21.3±1.3% fractional area change; Figure 6H). Echocardiography 7 days after LAD ligation showed no significant difference in regard to FAC between both groups. Next we analyzed the effect of SDF1-GPVI on LV function in infarcted mice in the absence of G-CSF. We found that in the absence of G-CSF, SDF1-GPVI preserved myocardial function 28 days after transient ischemia (15.7±1.1% versus 11.7±1.9% fractional area change) by ≈34% by trend without reaching statistical significance (P=0.15; Figure 6I).

Discussion
The major findings of the present study are as follows: (1) We successfully generated a bispecific protein with SDF-1 and GPVI domains that binds with high affinity to the CXCR4 receptor and extracellular matrix proteins such as collagen. (2) SDF1-GPVI triggers chemotaxis of CXCR4-positive cells and preserves cell survival in vitro. (3) SDF1-GPVI enhances endothelial differentiation of progenitor cells in vitro and reveals proangiogenic effects. (4) SDF1-GPVI administration reduces infarct size, recruits BMCs, enhances capillary density, and preserves cardiac systolic function after MI in vivo.

These findings strongly imply that the bifunctional molecule SDF1-GPVI recruits and modulates function of BMCs at sites of myocardial injury after transient ischemia. Systemic administration of SDF1-GPVI preserves myocardial function and might be an attractive tool to promote myocardial repair/regeneration after ischemic injury.

At sites of vascular or tissue injury, discontinuity of the endothelial barrier and exposure of subendothelial matrix within the (micro-)vasculature leads to platelet adhesion and activation. Platelet adhesion at the injured vessel wall is mediated by adhesion receptors, including GPVI, that recognize proteins of the subendothelial matrix such as collagen and fibronectin, leading to platelet activation and secretion of granule constituents. We found that platelets secrete SDF-1 upon activation in mice and humans. Platelet-derived SDF-1 recruits CXCR4-positive progenitor cells to the microenvironment of activated platelets and favors migration and endothelial differentiation.

SDF-1 and its receptor CXCR4 regulate trafficking and homing of BM-derived cells to BM or peripheral organs. SDF-1 is the most potent chemokine of stem and progenitor cells, as well as of monocytes, lymphocytes, and platelets. SDF-1 gene expression is regulated by the transcription factor hypoxia-inducible factor-1 in endothelial cells, resulting in selective expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tension in vivo. Hypoxia-inducible factor-1–induced SDF-1 expression increases the adhesion, migration, and homing of circulating CXCR4-positive progenitor cells to ischemic tissue. Blockade of SDF-1 in ischemic tissue or CXCR4 on circulating cells prevents progenitor cell recruitment to sites of injury. Adenoviral overexpression of SDF-1 in the heart after MI significantly enhanced BM-derived cell recruitment in injured myocardium. SDF-1 has been shown to be cardioprotective after hypoxemia and stimulates recruitment of circulating progenitor cells that promote angiogenesis and support myocyte survival. Administration of a protease-resistant...
SDF-1 mutant limits MI in mice and promotes angiogenesis in a hindlimb ischemia model.

We constructed a bifunctional fusion protein that combines binding activity to the extracellular matrix proteins like collagen and binding to the SDF-1 receptor CXCR4 (SDF1-GPVI). We hypothesized that the “anchor” molecule GPVI binds preferentially to the injured vasculature of the microcirculation and thereby increases the concentration of SDF-1 within the injured myocardium, resulting in enhanced accumulation of “repair” cells. Indeed, we found enhanced cell accumulation within the injured myocardium in mice treated with the SDF1-GPVI fusion molecule (Figure 6D). Furthermore, we provide in vitro and in vivo evidence that our fusion molecule SDF1-GPVI promotes angiogenesis (Figures 5 and 6G) and sustains cell survival (Figure 3B). After transient myocardial ischemia (LAD ligation), systemic administration of SDF1-GPVI significantly reduces infarct size and preserves myocardial function in mice (Figure 6C and 6H).

After MI, SDF-1 expression in the heart is rapidly upregulated. However, SDF-1 levels decline between days 4 to 7 after MI, and therefore CXCR4-positive BMCs are mobilized at a time when myocardial SDF-1 expression is not optimal for their recruitment. Furthermore, cardiac myocytes of the infarct border zone as well as cardiac stem cells also start to express CXCR4 after MI at a time of declining myocardial SDF-1 expression. Therefore, it is believed that sustained expression of SDF-1 is required to decrease cardiac myocyte death and improve cardiac function. Several studies followed this strategy to enhance myocardial SDF-1 levels by invasive means. These invasive strategies include adenoviral SDF-1 gene delivery, transplantation of SDF-1–expressing fibroblasts, intracardial injection of SDF-1, and intramyocardial injection of a modified protease-resistant SDF-1 protein. They together preserved cardiac function and reduced heart remodeling but are limited by the fact that only a single administration of exogenous SDF-1 can be applied to the patient directly after MI. Therefore, our aim was to provide a noninvasive pharmacological tool to enrich CXCR4-positive cells by a bispecific molecule targeting a component of the subendothelial matrix (collagen) that is exposed at injured vessels and providing SDF-1 to bind and recruit CXCR4-positive cells to damaged tissue. The molecule allows individual therapeutic application to the patient and might overcome low grafting efficiency of stem cell–based regenerative therapy.

In addition, we combined our bispecific molecule with G-CSF to increase recruitment of BMCs according to a recent report by Zaruba and colleagues that demonstrates increased mobilization of progenitor cells from the BM after transient ischemia by pharmaceutical administration of G-CSF. They verified that endogenous SDF-1 concentration after myocardial ischemia and reperfusion is not sufficient to mobilize a number of BMCs that achieve a positive effect on myocardial regeneration. This therapeutic strategy leads to systemic mobilization of progenitor cells that might be accompanied by cardiovascular risk, as shown in apolipoprotein E–/– mice with plaque destabilization after widespread endothelial progenitor cell mobilization. In contrast, binding of the bifunctional protein to injured and inflamed tissue via the anchor molecule GPVI leads to increased concentration of SDF-1 and consequently to local stimulation of BMC recruitment after tissue damage that may effectively support endothelial and myocardial healing, as shown in our MI model.

In conclusion, we provide evidence that the bispecific protein SDF1-GPVI is highly effective in preserving myocardial function after transient ischemia. Administration of SDF1-GPVI during reperfusion thereafter might be a promising strategy to treat MI to promote myocardial repair and limit heart failure.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Heart failure is the major serious complication of myocardial infarction. Circulating bone-marrow–derived stem cells play a critical role in repair mechanisms of infarcted myocardium and limit disease progression and heart failure. Clinical trials suggest that regenerative mechanisms of the diseased myocardium can be supported by administration of autologous bone-marrow–derived stem cells. One major limitation of the stem cell–based treatment option is low and undirected accumulation of cells with high regenerative potential at the site of injured myocardium. Bifunctional recombinant molecules that target both structures of the diseased myocardial microcirculation and distinct circulating bone-marrow–derived stem cells such as SDF1–GPV1 have been shown to be of great potential to augment peripheral recruitment of stem cells toward diseased myocardium. These molecules have been proven to limit heart failure and preserve myocardium in disease-related mouse models. These molecules may be a promising strategy to promote myocardial repair and to preserve cardiac function after myocardial infarction.


The Bispecific SDF1-GPVI Fusion Protein Preserves Myocardial Function After Transient Ischemia in Mice
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Supplemental Methods

Animals
Specific pathogen-free C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). Animal studies were approved by the district government of Tübingen.

Chemicals and antibodies
Human collagen I was purchased from Becton-Dickinson (BD, Heidelberg, Germany). Acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (Biomedical Technologies, USA), calcein (Molecular Probes, Leiden/Netherlands) and vascular endothelial growth factor (recombinant human VEGF165, PeproTech, London, UK) were used. 4',6-diamidino-2-phenylindole (DAPI, 500 ng/ml in PBS) was obtained from Sigma-Aldrich (Steinheim, Germany), phalloidin from Invitrogen (Carlsbad, CA, USA). The antibodies against vWF and VE-cadherin were from Santa Cruz (CA, USA). Antibodies against VEGFR-2 and PECAM-1 were obtained from Cell Signaling (Frankfurt, Germany), antibodies against claudin 5 and Tie-2 were purchased from abcam (Cambridge, UK).

Human cells
Monocytes were freshly isolated as described previously\(^1\). CD34\(^+\) cells were isolated from remnants of leukapheresis transplants as described previously\(^2\). All experiments were approved by the local ethic committee of the University of Tuebingen.

Cloning and protein production for SDF1-GPVI
The cDNA sequence coding for SDF1-GPVI includes a C-terminal FcIgG2-tag and a N-terminal IgK leader\(^3\). This fragment was produced by gene synthesis and cloned into the mammalian expression vector pcDNA5-FRT using HindIII and BamHI restriction sites.
(GENEART, Regensburg, Germany). The Flp-In™ CHO cell line (Invitrogen, Carlsbad, CA, U.S.A.) was stably transfected with either pcDNA5-SDF1-GPVI-FclgG2-FRT, pcDNA5-GPVI-SDF1-FclgG1-FRT, pcDNA5-GPVI-FclgG2-FRT or pcDNA5-FclgG2-FRT using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer’s protocol to generate a stable SDF1-GPVI-FclgG2-Flp-In™-CHO, GPVI-SDF1-FclgG1-Flp-In™-CHO, GPVI-FclgG2-Flp-In™-CHO and FclgG2-Flp-In™-CHO cell line as described previously. The stable cell lines were cultivated using Hams F12 medium supplemented with 10% FCS, 1% Penicillin/Streptomycin and 250 µg/ml hygromycin B. For protein expression, cells were grown on T-160 cell culture flasks in serum free CHO III (A) medium (Gibco, Paisley, Scotland), supplemented with 4 mM Glutamine, 4.5 g/l D-Glucose and 1% Penicillin/Streptomycin. Cell culture supernatants were harvested 8 days after subculturing and purified using Protein G Agarose Beads (Pierce, Rockford, IL, U.S.A.) according to the manufacturer’s protocol.

Molecular modeling
Computational docking and scoring studies of the interaction of SDF1-GPVI with CXCR4 were performed with Molegro Virtual Docker 2008. The original parameters of blind docking were used in combination with evaluation scheme based on binding free energy (ΔG). The structure of CXCR4 was obtained from the OPM Data Bank (3OE6).

Western blot analysis
Purified supernatant from SDF1-GPVI producing CHO cells was detected via Western blot. Supernatant was prepared with reducing or non reducing sample buffer, separated on SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Subsequently, the membrane was blocked using 5% powdered skim milk in PBST (PBS with 0.1% Tween 20) and probed with anti-hSDF1 (R&DSystems, Wiesbaden, Germany) in a dilution of 1:500, anti-human IgG-HRP (Jackson Immuno Research, PA, USA) in a dilution of 1:5000 and anti-GPVI in a dilution of 1:7500. Anti-GPVI was purified by the supernatant of hybridoma 5C4. Afterwards the membrane was incubated
with fluorescently labeled secondary antibodies in darkness. The membrane was detected by the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany).

**Silver staining**

Purified protein was prepared with reducing or non reducing sample buffer and separated on 8% SDS-polyacrylamide gel. Afterwards the gel was fixed with 20% ethanol and 5% acetic acid for 10 min, followed by washing two times in 25% ethanol. Gel was then incubated in 0.02% sodium thiosulfate for 1 min, washed in deionized water and impregnated in 0.2% silver nitrate for 15 min. After washing in deionized water the gel was immersed in developer (6% sodium carbonate, 37.5 µl formaldehyde (37%)). After silver reduction the staining was stopped with 20% ethanol and 5% acetic acid.

**Collagen binding ELISA**

An Immuno HB 96-well plate (Thermo Fisher, Schwerte, Germany) was coated with 10 µg/ml bovine collagen I in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 3 mM NaN₃ pH 9.6) o/n at 4°C. Control wells were coated with 10 µg/ml murine Laminin in PBS for one hour at RT. Wells were blocked with blocking solution (Candor Bioscience GmbH, Weißensberg, Germany) o/n at 4°C. The four proteins SDF1-GPVI, GPVI-SDF1, GPVI and FcIgG2 were prepared with Low Cross Mild buffer (Candor Bioscience GmbH, Weißensberg, Germany) in the following concentrations: 2.5, 5, 10, 20 µg/ml. For inhibition of the functional binding, proteins (10 µg/ml) were preincubated with 200 µg/ml solubilized collagen for 10 min. Wells were washed two times with PBS and were incubated with 100 µl of the proteins for one hour at RT. Afterwards, the plate was washed five times with PBS and incubated with 100 µl anti-human IgG-HRP (Jackson Immuno Research Europe, Newmarket, UK) 1:10 000 in Low Cross Mild buffer for 1h at RT. After washing 5 times with PBST, 0.05% Tween-20, wells were incubated with 100 µl TMB detection reagent (Serva, Heidelberg, Germany) for 10-15 min. Reaction was stopped by addition of 100 µl H₂SO₄, and the absorbance was measured.
at 450 nm against reference wavelength 570 nm using the ELISA 550 plate reader (Bio-Rad, München, Germany).

**Competition assay**

Every FACS tube was prepared with $2 \times 10^5$ human monocytes and washed with 1 ml ice-cold FACS buffer (PBS with 0.05% acide and 0.5% BSA).

The four proteins SDF1-GPVI, GPVI-SDF1, GPVI and FcIgG2 were prepared in the following concentrations: 200, 100, 60 and 30 µg/ml and were mixed with 10% Intratect (Biotest, Dreieich, Germany). Cells were incubated with these proteins for 10 min at 4°C. Afterwards, cells were labeled with human anti-CXCR4 PE-conjugated antibody (BD Biosciences, CA, USA) or isotype IgG2a control PE mouse antibody (BD Biosciences, CA, USA) and incubated for 30 min at 4°C. After incubation, cells were washed two times with ice-cold FACS buffer, resuspended in 500 µl FACS buffer and gently mixed. Finally, cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, CA, USA).

**Chemotaxis**

Chemotaxis was performed using 5.0 µm pore size Transwell migration chambers (Costar Corning, NY, USA). SDF1-GPVI in three different concentrations (155 pM, 77 pM and 15 pM), GPVI-SDF1 (155 pM), hSDF-1 (100 ng/ml) or only medium were included in the lower compartments. 1.5 x $10^5$ CD34+ cells were added to the upper chambers in StemSpan SFEM medium (Stem Cell, Grenoble, France). For inhibition of chemotaxis anti-CXCR4 (10 µg/ml) or control isotype IgG2b (10 µg/ml) antibodies (R&DSystems, Wiesbaden, Germany) were mixed with cells 30 min before the addition of the cells to chambers. The cultures were incubated for 6 hrs at 37°C. Finally, cells in the lower chamber were mugged and counted using flow cytometry.
Survival assay
Freshly isolated CD34+ cells were cultured in IMDM medium supplemented with 1% vitamins, 5% FCS, 1% P/S, and 1% NEAA (Gibco, Paisley, Scotland) overnight. Cell survival assay was performed using a 24-well plate. 1 x 10^5 CD34+ cells/well were incubated for 6 hrs at 37°C in serum- and cytokine-free medium (IMDM + GlutaMAX, Gibco, Paisley, Scotland) in the presence of SDF1-GPVI, FcIgG2 (each 177 pM), 500 ng/ml hSDF-1, 50 ng/ml hTGFβ1 or medium alone. The percentages of apoptotic and healthy cells were determined using Annexin V-FITC and propidium iodide (BD Biosciences, CA, USA) co-staining according to the manufacturer’s protocol and flow cytometry.

Coating procedure of culture plates
Culture plates and cover slips were coated in four different modes under sterile conditions. Coatings were performed in two steps (1st: collagen, 2nd: protein). Between these two steps wells were washed 3 times with PBS and blocked with 3 % BSA in PBS for 1 h at RT. Subsequently wells were washed with PBS and cells were immediately added to pretreated wells.

Dynamic adhesion assay
Adhesion experiments under flow conditions (flow rate 15ml/h and shear rate 2000 s⁻¹ corresponding to the native flow rate of blood in human arterioles) were performed with 1.5x10^5 cells/ml CD34+ cells diluted in IMDM medium. Rectangular coverslips were coated with collagen type I as described and fixed in the flow chamber. Cells were perfused through the flow chamber with a 50 ml syringe and a pulse-free pump for 10 min. After perfusion, all experiments were recorded in real time and evaluated offline.

Colony forming unit assay (CFU)
CD34+ cells (5 x 10⁶/cm²) were seeded in precoated 96-well plates and cultivated at 37°C and 5% CO₂ in VascuLife medium (CELLSystems®, Troisorf, Germany). After 24 hrs non-
adherent cells were removed and medium was changed every 3 days. Additionally, endothelial colony-forming units were counted at day 1, 3, 5, 7, 10 and 14 using light microscopy (Axiovert, Carl Zeiss, Oberkochen, Germany)\(^9\).

**Uptake of low-density lipoprotein Dil-Ac-LDL**

CD34\(^+\) cells (5 x 10\(^4\)/cm\(^2\)) were seeded as described in the CFU-assay. The uptake of Dil-Ac-LDL (Biomedical Technologies, USA) of seeded CD34\(^+\)-cells was analyzed at day 1, 3, 5, 7, 10 and 14 as described previously\(^4\).

**Immunocytochemistry**

CD34\(^+\) cells (5 x 10\(^4\)/cm\(^2\)) were seeded and cultivated as described in the CFU-assay. Immunohistochemistry was performed as described previously\(^4\).

**Chorioallantoic membrane assay (CAM assay)**

Fertilized Hisex brown chicken eggs were incubated at 37\(^\circ\)C and 60% humidity. After two days of incubation, 2 ml albumen was aspirated from the eggs. A window cut into the egg shell was then encircled with silicone paste and the opening was covered with a Petri dish lid. Afterwards, eggs were immediately returned to the incubator\(^10\). At day nine of incubation, a silicon ring (diameter of 9 mm, thickness of 3 mm) was placed onto the CAM (Figure 1A). 50 µl of test substance (diluted in PBS) was poured into the silicone ring, which was subsequently covered with a cover slip and the eggs were returned to the incubator for another 4 days. At day 13, CAMs were fixed in 4% PFA for 6 hrs at RT and collected. Photographs of the CAM were used to determine the orientation of blood vessels in the CAM. Followed by counting the number of blood vessels orientated towards (Figure 1B, red arrow [a]) and backwards (Figure 1B, green arrow [b]) to the implanted test substance charged silicon ring (Figure 1B). Calculations of the a/b-ratio were evaluated and revealed a pro- or anti-angiogenic effect of applied test substances.
Pharmacokinetics

C57BL/6J mice received SDF1-GPVI (10 µg/g body weight) by intravenous injection. At indicated time intervals (before, 6, 12, 24 and 48 hrs after administration), 100 µL blood was collected in a tube taken from the retro-orbital plexus under isoflurane anaesthesia. SDF1-GPVI levels were determined in the serum using a human IgG ELISA (IgG ELISA Kit, Immunotek, ZMC) according to manufacturer’s instruction and by Western Blot analysis detected with human IgG-HRP Ab (Jackson Immuno Research, PA, USA) as described recently11-13.

Myocardial ischemia and reperfusion in mice

10 to 12 weeks old C57BL/6J mice were anesthetized by intraperitoneal injection of a solution of midazolame (5 mg/kg body weight; Ratiopharm, Ulm, Germany), medetomidine (0,5 mg/kg body weight; Albrecht GmbH, Aulendorf, Germany) and fentanyl (0,05 mg/kg body weight; CuraMed Pharma, Karlsruhe, Germany). Anaesthesia was maintained with isoflurane (Albrecht GmbH, Aulendorf, Germany). Myocardial ischemia was induced by ligation of the left anterior descending artery (LAD) for 45 min. Successful performance of the occlusion was confirmed by visual inspection of color in the apex. After 28 days of reperfusion the ischemic area (area at risk, AaR) was defined by negative staining with 4% Evan’s Blue (after re-ligation of the LAD at the level marked by the suture left in place) and the infarcted area (infarct size, IS) was detected by triphenyltetrazolium staining (Sigma Aldrich, St Louis, Mo, USA)1. The ratio of IS/AaR is an accurate measure to analyze IS within ischemic myocardium and is the primary end point, which determines the effect of the treatment strategy. Areas were digitally quantified by video planimetry.

Administration of G-CSF and SDF1-GPVI

Experimental design is shown in Figure 6A. Mice were randomly divided into the SDF1-GPVI group (n=7) or the FcIgG2 control group (n=6). SDF1-GPVI (10 µg/g body weight) and FcIgG2 (4,2 µg/g body weight) control protein was injected intravenously directly after LAD
ligation and repeated 48 hrs later. Both groups were treated intraperitoneally with G-CSF (100 µg/kg body weight) for three consecutive days. G-CSF treatment was initiated immediately after surgical procedure. Further, mice were treated after LAD ligation with SDF1-GPVI (n=4) group or FclgG2 control group (n=6) in the absence of G-CSF.

**Immunohistochemistry**

Paraffin-embedded cardiac sections of day 7 and 28 were stained with an avidin-biotin-immunoperoxidase method (LSAB + System HRP, Dako, Heverlee, Belgium) using anti-PECAM-1 mAb (Santa Cruz Biotechnology, CA, USA), anti-CXCR4 mAb (RnDSystems, Wiesbaden, Germany), anti-SDF1 mAb (abcam, Cambridge, UK) or anti-hIgG Ab (Jackson Immuno Research, PA, USA) and isotype control Ab according to standard protocol. Corresponding secondary antibodies (Dako, Heverlee, Belgium) were used. The numbers of PECAM-1⁺ capillaries were quantified from six random areas from the border zone of each heart sample (n=3) and were referred to a certain tissue area (mm²). The percentage of CXCR4-positive cells were counted from six random areas in the infarcted myocardium of each heart sample (n=3) in a blinded manner.

**Echocardiography**

Echocardiography was performed using a VEVO 770 ultrasound machine and a 30 Mhz linear transducer as described recently\(^{14,15}\).
References


