Transplantation of Human Pericyte Progenitor Cells Improves the Repair of Infarcted Heart Through Activation of an Angiogenic Program Involving Micro-RNA-132

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Rationale: Pericytes are key regulators of vascular maturation, but their value for cardiac repair remains unknown. Objective: We investigated the therapeutic activity and mechanistic targets of saphenous vein-derived pericyte progenitor cells (SVPs) in a mouse myocardial infarction (MI) model. Methods and Results: SVPs have a low immunogenic profile and are resistant to hypoxia/starvation (H/S). Transplantation of SVPs into the peri-infarct zone of immunodeficient CD1/Foxn-1−/− mice ameliorated left ventricular dilatation and improved ejection fraction compared to vehicle. Moreover, SVPs reduced myocardial scar, cardiomyocyte apoptosis and interstitial fibrosis, improved myocardial blood flow and neovascularization, and attenuated vascular permeability. SVPs secrete vascular endothelial growth factor A, angiopoietin-1, and chemokines and induce an endogenous angiocrine response by the host, through recruitment of vascular endothelial growth factor B expressing monocytes. The association of donor- and recipient-derived stimuli activates the proangiogenic and prosurvival Akt/eNOS/Bcl-2 signaling pathway. Moreover, microRNA-132 (miR-132) was constitutively expressed and secreted by SVPs and remarkably upregulated, together with its transcriptional activator cyclic AMP response element-binding protein, on stimulation by H/S or vascular endothelial growth factor B. We next investigated if SVP-secreted miR-132 acts as a paracrine activator of cardiac healing. In vitro studies showed that SVP conditioned medium stimulates endothelial tube formation and reduces myofibroblast differentiation, through inhibition of Ras-GTPase activating protein and methyl-CpG-binding protein 2, which are validated miR-132 targets. Furthermore, miR-132 inhibition by antimiR-132 decreased SVP capacity to improve contractility, reparative angiogenesis, and interstitial fibrosis in infarcted hearts. Conclusion: SVP transplantation produces long-term improvement of cardiac function through a novel paracrine mechanism involving the secretion of miR-132 and inhibition of its target genes. (Circ Res. 2011;109:894-906.)

Key Words: pericytes-based cell therapy myocardial infarction angiogenesis VEGF-B microRNA-132

With myocardial infarction (MI) remaining a major cause of morbidity and mortality worldwide, cell therapy now aims to offer a novel option for cardiac repair. Clinical trials showed that administration of bone marrow-derived progenitor cells (PCs) improves left ventricular (LV) function in patients with coronary artery disease. However, more specialized cells are warranted to fulfill specific regenerative needs of the ischemic myocardium.

Pericytes provide the physical strength and nurturing signals that instruct new vessels to organize in a stable and efficient tubular network. On the other hand, ischemic disease and associated risk factors may impair pericyte recruitment. Therefore, a supply-side approach with fresh pericytes from exogenous sources could be helpful therapeutically. However, difficulties in isolating and expanding bona-fide pericytes from accessible human tissues have so far precluded clinical applications.

Two main mural cell populations, probably originating from a common embryonic ancestor, have been described in adult tissues based on stringent topographical, clonogenic, antigenic, and functional criteria. A population of CD34+ cells, typically located around capillaries and microvessels in multiple human organs and hence acknowledged as pericytes, give rise to mesenchymal cells with multilineage differentiation capacity and promote reparative myogenesis on trans-
plantation in models of muscular dystrophy. Another population of CD34+ cells, located around the vasa vasorum in the adventitia of arteries and veins, also express typical pericyte markers (NG2, PDGFRβ, and RGS5) together with stemness antigens (Oct-4 and Sox-2). This adventitial subset contains PCs that may contribute to angiogenesis as well as athero-antigens (Oct-4 and Sox-2). This adventitial subset contains PCs that may contribute to angiogenesis as well as athero-antigens (Oct-4 and Sox-2). This adventitial subset contains PCs that may contribute to angiogenesis as well as athero-antigens (Oct-4 and Sox-2). This adventitial subset contains PCs that may contribute to angiogenesis as well as athero-antigens (Oct-4 and Sox-2).

Recently, we have shown that CD34+ PCs from human fetal aorta adventitia possess a robust regenerative capacity in mouse models of peripheral limb ischemia and ischemic below-knee ulcers in diabetic mice. Aiming to find a similar regenerative population for autologous cell therapy, we succeeded in isolating and expanding a clonogenic population of CD34+/CD31- pericyte progenitors from saphenous vein leftovers of patients undergoing coronary artery bypass graft surgery. On transplantation into mouse ischemic limbs, saphenous vein-derived pericyte progenitors (SVPs) proved to be superior to an equal dosage of circulating proangiogenic cells (previously identified as EPCs) in supporting blood flow recovery. The benefit was attributed to potentiation of reparative angiogenesis by as yet incompletely understood interactions between SVPs and endothelial cells (ECs).

The present study demonstrates that SVP transplantation induces long-term improvements in a mouse model of MI, through angiocrine activities of donor and recipient cells. Moreover, we show that SVPs produce and release the microRNA-132 (miR-132), which exerts proangiogenic, pro-survival, and antifibrotic activity via inhibition of its targets Ras-GTPase activating protein (RasGAP, also named RASA1) and methyl-CpG-binding protein 2 (MeCP2).

Methods

Expanded methods are provided as an online supplement, available at http://circres.ahajournals.org.

Induction of Infarct and Cell Therapy

MI was induced in 8-week-old male immunodeficient CD1-FOX-O−/−/O−/− (Charles River) or immunocompetent CD1 mice (Harlan) by occlusion of the left anterior descending coronary artery, followed by injection of Dil-stained SVPs (3×10^5 or 1×10^6 cells per heart), human bone marrow mesenchymal stem cells (MSCs; 1×10^6 per heart) or PBS at 3 different sites along the infarct border zone. To investigate the importance of miR-132 in post-MI recovery, cells were transplanted with anti-miR-132 or scrambled sequence (Applied Biosystems) before transplantation.

Hemodynamic Measurements

Cardiac function was monitored using a high-resolution echocardiograph (Vevo 770, Visual Sonics, n=14 per group). LV pressures were assessed using a high-fidelity 1.4F transducer tipped catheter inserted into the LV chamber. Myocardial blood flow was measured using intraventricularly-injected fluorescent microspheres. In vivo vascular permeability was determined by assessing the distribution of fluorescent-conjugated dextran (see Expanded Methods).

Analyses on Explanted Hearts

LV samples were collected at 5, 14 and 42 days post-MI for histological, immunohistochemical and molecular analyses. Cell apoptosis and scar size were determined using terminal deoxynucleotidyl transferase dUTP nick end labeling and Azan Mallory staining, respectively.

Cell Culture for In Vitro Experiments

SVPs and human umbilical vein ECs (HUVECs, Lonza) were cultured on fibronectin and in endothelial growth medium containing 2% FBS (Lonza). MSCs were grown using mesenchymal cell medium (Lonza). In selected experiments, the effects of hypoxia (1% O2) and serum deprivation (H/S) on cell proliferation (BrdU Incorporation Assay, Roche), apoptosis (caspase-3/7, Promega), and expression profile (Western blot and RT-PCR) were studied.

Adult rat cardiomyocytes were isolated using collagenase perfusion and used immediately for experiments. Mouse cardiac fibroblasts were isolated and cultured as described.

Role of miR-132 on SVP-Associated Functions

SVPs were transplanted with scrambled oligonucleotide or anti-miR-132 (50 nmol/L) and tested for their capacity to promote network formation by HUVECs on Matrigel (BD Biosciences). Similar assays were repeated using HUVECs exposed to conditioned medium from SVPs (SVP-CM) or MSCs (MSC-CM) or unconditioned medium (UCM).

To verify SVP paracrine influence on cardiac cells, rat cardiomyocytes were exposed to hypoxia for 18 hours in the presence of SVP-CM and then assayed for caspase-3/7 activity. Similarly, we determined the effect of SVP-CM on mouse cardiac fibroblast proliferation and differentiation into myofibroblasts, as induced by angiotensin II (AngII, 100 nmol/L). Myofibroblasts were distinguished by double immunostaining for α-smooth muscle actin and MeCP2.

Molecular Analyses

Total RNA was isolated from flash-frozen LV myocardium, SVPs, SVP-CM, and MSC-CM using TRIzol (Invitrogen) and reverse-transcribed using Qiagen reverse transcriptase kit, followed by cDNA amplification using Quantitect predefined primers (Qiagen).
miR-132 was measured by TaqMan PCR. Data were normalized to the small RNA RNU6B (Applied Biosystems).

Protein extracts from LV myocardium or SVPs were used for western blotting and ELISA. AngII and norepinephrine levels in plasma and LV myocardium were measured using commercial ELISA.

**Statistical Analysis**

Comparison of multiple groups was performed by ANOVA. Two-group analysis was performed by Student t test. Values were expressed as means±SEM. Probability values (P) less than 0.05 were considered significant.

**Results**

**Verification of SVP Antigenic Profile**

By flow cytometry and immunocytochemistry, we confirmed the typical SVP antigenic phenotype described earlier (Online Figure I, A and B) and verified that it is not modified by Dil-labeling prior to transplantation or culture under H/S. Following in vitro exposure to an enriched medium that supports cardiomyocyte differentiation, SVPs acquired the typical cardiomyocyte markers αSA, GATA4, and connexin-43, while retaining the NG2 marker (Online Figure I, C). Furthermore, we found that SVPs express intermediate levels of major histocompatibility complex class I human leukocyte antigens A, B, and C and are negative for class II human leukocyte antigen HLA-DR. SVPs were also negative for CD80 and Fas ligand as assessed by RT-PCR (Online Figure I, D and IE).

**SVP Transplantation Benefits Early Post-MI Recovery in Both Immunodeficient and Immunocompetent Mice**

As shown in Online Table I, transplantation of SVPs (1×10⁶) in the heart of CD1−FOXO1−/−immunodeficient mice attenuated the LV anterior wall thinning (P<0.05 versus vehicle) and the decrease of LV ejection fraction (LVEF; P<0.05) and cardiac output (P<0.05) at 14 days post-MI. Furthermore, cell therapy improved pressure indexes (P<0.05).

Next, we tested SVPs (at 3×10⁵ or 1×10⁶) in the infarcted heart of immunocompetent mice and followed up the recovery for 2 weeks. Both cell doses improved volumetric and functional parameters (Online Figure II, A−H) and pressure indexes (Online Figure II, I−K) and induced a leftward shift of the LV pressure-volume curve compared to vehicle (Online Figure II, L).

**SVPs Exert Long-Term Improvements in Immunocompetent Mice**

Next, we tested the long-term effect of SVP transplantation in immunocompetent mice with MI. Human bone marrow-derived MSCs were used as a cellular control. As shown in Online Figure III, A, the SVP-treated group had an improved survival. The experiment was stopped at 42 days because the pre-established mortality end point (50%) was reached in vehicle-injected mice. HR increased following MI, with no difference between groups (data not shown).

Echocardiographic parameters did not differ among groups at 2 days post-MI, thus confirming the consistency of procedure for MI induction but were remarkably improved by SVPs at subsequent measurements (Figure 1A−D and Online Figure III, B and C). Specifically, SVP transplantation blunts the progression of adverse cardiac remodeling (Figure 1A and 1B and Online Figure III, B and C) and decline of LVEF (Figure 1C). At 42 days, cardiac output was reduced by 36% in vehicle-injected mice, whereas it had completely recovered in SVP-transplanted mice (Figure 1D). The frequency of the composite end point, consisting of mortality plus an LVEF of less than 35%, was 40% in SVP-treated and 100% in vehicle-treated mice. Furthermore, LV pressure indexes and pressure/volume relationship were improved in SVP-transplanted mice at 42 days post-MI (Figure 1E&F). SVP-transplanted mice showed reduced neurohormonal activation especially at the myocardial level (P<0.05 versus vehicle-injected, Online Figure III, D and E).

Human MSCs, used as a cellular control, showed similar improvements in survival (Online Figure III, A) and contractility (Figure 1C−1F) but were inferior to SVPs in blunting LV anterior wall thinning (P<0.05, Online Figure III, C) and LV chamber dilatation (P<0.05, Figure 1A and 1B).

**SVPs Maintain Their Original Phenotype After Engraftment in the Infarcted Myocardium**

Next, we checked the engraftment of transplanted Dil-labeled SVPs using confocal microscopy. At 5 days post-transplantation, Dil-labeled SVPs formed patches in the border zone (Figure 2Ai), while, from 14 days on, they were fewer and prevalently located in the infarct zone (Figure 2Aii). SVP engraftment was confirmed by staining for human mitochondrial antigen (data not shown).

Multicolor fluorescence microscopy shows that Dil-labeled SVPs are juxtaposed to isoelectin-positive capillary ECs and express typical pericyte markers NG2 and PDGFRβ (Figure 2B), with some being positive for the proliferation marker proliferating cell nuclear antigen (Figure 1C). Tridimensional confocal microscopy confirms the association of SVPs with perfused vessels, stained by intracardially injected Isolectin (Online Movie). Altogether, these data indicate that SVPs retain their original antigenic and peri-vascular phenotype on transplantation in the infarcted heart.

**SVP Transplantation Improves Myocardial Perfusion and Reparative Neovascularization**

Using fluorescent microspheres, we documented that SVP transplantation increases LV blood flow at 14 days post-MI (Figure 2D). Furthermore, SVPs attenuate vascular permeability (Figure 2E), which reportedly contributes to myocyte damage and interstitial fibrosis.

Immunohistochemistry analysis of peri-infarct microvasculature from early to late phase of recovery showed persistently higher levels of capillaries and arterioles in SVP-transplanted hearts (P<0.01 versus vehicle, Figure 2F). This was associated with increased EC proliferation (P<0.05, Online Figure IV, A) and reduced EC apoptosis (P<0.05, Online Figure IV, B). MSC-transplanted hearts showed a similar improvement in capillarization but not in arteriogenesis (Online Figure V, A and B).

**Angiocrine Response Activated by SVP Transplantation**

SVPs secrete large amounts of vascular endothelial growth factor (VEGF)-A and angiopoietin-1 (Ang-1) and modest...
quantity of VEGF-B_{186}, as assessed by ELISA of CM. Secretion of VEGF-A and Ang-1 is further augmented by H/S, which simulates in vitro the ischemic environment encountered by SVPs on transplantation (Online Figure VI). Moreover, SVP transplantation increased the myocardial levels of immunoreactive VEGF-A, VEGF-B_{186} and Ang-1 (Figure 3A).

As shown in Figure 3B, SVP transplantation also increased the mRNA levels of murine VEGF-B and Ang-1 in infarcted myocardium. Recruited CD45 and Mac-3 monocytes represent a rich source of angiogenic cytokines. They may contribute to the recipient’s angiocrine response because the abundance VEGF-B-expressing CD45^{+} monocytes was significantly increased in the peri-infarct zone of SVP-transplanted hearts at 5 days post-MI (P<0.01 versus vehicle, Online Figure VII). In addition, SVP transplantation upregulated the expression of CX3C chemokine receptor 1 (data not shown), an antigenic marker shared by proangiogenic murine Ly-6C^{low} and human CD16^{+} monocytes. Consistent with its content of interleukin-8 (IL-8; 397±138 pg/mL/10^5 cells, n=8) and monocyte chemotactic protein-1 (1082±473 pg/mL/10^5 cells, n=6), SVP-CM stimulated by 2.0±0.3-fold the migration of human CD14^{+}CD16^{+} cells in a transwell assay (P<0.05 versus UCM, n=5, data not shown).

VEGF and Ang-1 induce angiogenic and survival responses through Akt and extracellular signal regulated kinase (Erk). Consistently, Western blot analysis showed the activation of Akt/eNOS/Erk1/2/Bcl-2 signaling pathway and increased phosphorylation of Bad and FOXO1 in SVP-treated hearts, which was mirrored by the reduction of cleaved caspase 3 levels (Figure 3C). These data indicate that SVPs stimulate myocardial healing through paracrine and host-intrinsic angiogenic and survival mechanisms.

**SVPs Exert Paracrine Angiogenic Effects on Cultured ECs Through miR-132**

Angiogenic GFs, like VEGF, and cellular kinases, like Akt, stimulate the expression of target genes by inducing the phosphorylation of the transcription factor cAMP response element binding protein (CREB). By this mechanism VEGF induces the sustained upregulation of miR-132 in ECs, resulting in suppression of p120RasGAP and consequent Ras-dependent induction of EC proliferation and angiogenesis in vitro and in vivo. Hence, we next investigated if miR-132 is implicated in promotion of angiogenesis by SVPs.

Importantly, we found that miR-132 is expressed by SVPs under basal conditions and upregulated by 3.0±0.3-fold following H/S (P<0.01 versus normoxia, Figure 4A).
effect being associated with increased CREB phosphorylation (data not shown). Likewise, stimulation with VEGF-B increases miR-132 expression in SVPs by $1.30 \pm 0.02$-fold ($P < 0.05$ versus vehicle), while VEGF-A is ineffective ($P = \text{N.S.}$, data not shown).

To investigate the functional relevance of miR-132, we transfected SVPs with anti-miR-132 and confirmed the inhibition of miR-132 (Figure 4Ai) and consequent upregulation of miR-132 target gene, p120RasGAP (Figure 4Aii). The anti-miR-132 slightly reduced SVP proliferation and survival in vitro (Online Figure VIII, A). We previously reported that SVPs remarkably improve the network-forming capacity of human ECs through physical and paracrine interactions.\(^{20}\) Here, we show that inhibition of miR-132 abrogates the capacity of SVPs to support the network formation by HUVECs in coculture (Online Figure VIII, B). We measured miR-132 levels in SVP-CM and found that SVPs not only produce but also release miR-132 especially under H/S (Figure 4B). Anti-miR causes miRNA sequestration and/or degradation.\(^{29}\) Hence, we found miR-132 to be remarkably reduced in CM of anti-miR–transfected SVPs (Figure 4B). Furthermore, SVP-CM upregulates miR-132 expression in HUVECs (Figure 4Ci), resulting in concurrent inhibition of p120RasGAP (Figure 4Cii). These effects might be attributable to miR-132 transfer from SVP-CM to ECs and/or stimulation of de novo miRNA synthesis by paracrine factors contained in CM, ie, VEGF-A and Ang-1.

Next, we investigated the consequences of miR-132 inhibition on SVP-CM-induced promotion of angiogenesis. Consistent with our previous study,\(^{20}\) we found that SVP-CM stimulates HUVEC proliferation (Online Figure IX, A) and network formation capacity (Figure 4D), both effects being negated here by transfecting SVPs with anti-miR-132. Altogether, these data indicate that the miR-132, either taken up from CM or synthesized on CM stimulation, is crucial for EC growth and networking. VEGF and Akt act as CREB-
dependent activators of miR-132; in turn, miR-132 can activate Akt unlocking Ras, a well-acknowledged inducer of PI3K/Akt pathway. In line, we found that pAkt levels are increased in HUVECs exposed to SVP-CM and reduced by miR-132 inhibition (Online Figure IX, B).

Comparison of CMs from SVPs and MSCs revealed that the former contains ~3-fold higher levels of VEGF-A and Ang-1 (Online Figure X, Ai–iii). Likewise, miR-132 expression was superior in SVP-CM compared to MSC-CM (P<0.01, Online Figure XAiv). However, both SVP-CM and MSC-CM stimulate HUVEC network formation compared to their respective unconditioned media (P<0.01, Online Figure X, B).

Involvement of miR-132 in SVP-Induced Reparative Vascularization of Infarcted Heart

Immunohistochemical analyses of cardiac p120RasGAP revealed that the miR-132 target is mainly expressed in coronary ECs of sham-operated hearts. After MI, p120RasGAP was also expressed on cardiomyocytes and fibroblasts (Online Figure XI, A). SVP transplantation remarkably reduced p120RasGAP as assessed by confocal microscopy (Figure 5A) and Western blot (Online Figure XI, B). This effect was abrogated when SVPs were transfected with anti-miR-132 prior to transplantation (Figure 5A and Online Figure XI, B). We verified the sustained inhibition of miR-132 in SVPs transfected with anti-miR-132 (Online Figure XII).

Importantly, anti-miR-132-transfected SVPs were inferior to naïve SVPs or scrambled-transfected SVPs in improving echocardiographic endpoints (Figure 5Bi and 5Bii and Online Figure XI, Ci), dP/dt (Online Figure XI, Ciii) and angiogenesis (Figure 5Ciii and 5Civ) and EC survival and proliferation (Online Figure XI, Cii and Online Figure XI, Civ). Nonetheless, the improvement afforded by SVPs was not completely abrogated, thus suggesting participation of miR-132-
Figure 4. SVPs exert paracrine effects through miR-132. A, Bar graphs showing induction of miR-132 (Ai) and inhibition of its target p120RasGAP (Aii) in SVPs exposed to H/S. AntimiR-132 transfection (50nmol/L) suppressed miR-132 expression while inducing p120RasGAP expression (Aii). Control SVPs were transfected with scrambled sequence (Scr, 50nmol/L), or exposed to transfection-vehicle (V).

B. Levels of miR-132 in SVP-CM. Data are means±SE from experiments performed in quadruplicates. *P<0.05, **P<0.01, and ***P<0.001 vs corresponding group under normoxia; §§§P<0.001 vs vehicle; ##P<0.01 and ###P<0.001 vs Scr.

C, Bar graphs showing miR-132 levels (Ci) and p120RasGAP protein expression (Cii) in HUVECs exposed to SVP-CM collected under different conditions described above. D, Representative images and bar graphs showing the ability of SVP-CM to enhance HUVEC network formation and abrogation of this effect by CM of antimiR-132-transfected SVPs. HUVECs were cultured on matrigel with SVP-CM for 24 hours. Data are means±SE of experiments performed in quadruplicates. αP<0.05; ααP<0.01 vs unconditioned medium (UCM); αααP<0.001; §§P<0.01 vs corresponding group under normoxic SVP-CM; ##P<0.01 and ###P<0.001 vs Scr.
dependent and independent mechanisms. We transplanted equal numbers of naïve or anti-miR-132-transfected SVPs and verified that anti-miR-132 does not influence SVP engraftment (Online Figure XIII). Hence, reduction in SVP therapeutic activity by miR-132 inhibition has to be ascribed to functional rather than quantitative reduction. Looking at the impact of miR-132 inhibition on molecular endpoints, we found a reversal of Akt/Bcl-2 upregulation in hearts transplanted with anti-miR-132-transfected SVPs (Figure 6A&B). Akt inhibits GSK3β by phosphorylating it at Ser-9. Furthermore, GSK3β represents a predicted inhibitory target of miR-132. Consistently, we found that naïve SVPs remarkably increase Ser-9-phosphorylated GSK3β, while reducing total GSK3β; both the effects were inhibited by anti-miR-132 (Figure 6C). In contrast, Erk1/2 activation and FOXO1 phosphorylation (Figure 6D and 6E), as well as VEGF-B and Ang-1 upregulation (data not shown) were not affected following miR-132 inhibition. Altogether, these data indicate the crucial contribution of miR-132 in the proangiogenic and healing action of SVPs in the infarcted heart.

**Involvement of miR-132 in SVP-Induced Prevention of Cardiac Remodelling**

We next investigated the impact of SVPs on cardiomyocytes and cardiac fibroblasts. In SVP-treated mice, the infarct scar was reduced (25.4±1.7% in SVP versus 32.3±1.3% in vehicle, P<0.05). Furthermore, SVP-transplanted hearts showed reduced cardiomyocyte apoptosis, this effect being blunted by SVP transfection with anti-miR-132 (P<0.05, Figure 7A). To verify if cardiomyocytes are a direct target of SVP paracrine action, we exposed adult rat cardiomyocytes to hypoxia in the presence of SVP-CM or UCM and found that the former reduces the levels of caspase-3/7 activity. AntimiR-132-SVP-CM produced similar antiapoptotic effects (Online Figure XIV), thus suggesting that SVPs promote cardiomyocyte survival independently of miR-132 or through other in vivo actions of miR-132, eg, angiogenesis.

Although SVPs acquire cardiomyocyte markers under inductive conditions in vitro (see above), we could not find any Dil-labeled SVP coexpressing cardiac antigens in vivo, thus discounting the possibility that improvement of cardiac function is due to in vivo transdifferentiation into cardiomyocytes. On the other hand, SVP transplantation increased the number of cardiac stem cells in the infarct border zone (Online Figure XV).

SVP-CM markedly reduced mouse cardiac fibroblasts proliferation (Figure 7D) and differentiation into myofibroblasts as well as MeCP2 expression (Figure 7Dii). These effects were abrogated when exposing fibroblasts to CM of anti-miR-132-transfected SVPs (Figure 7D and Online Figure XVI).

Finally, extensive histological investigation excluded the presence of tumors or calcification in SVP-transplanted hearts, whereas calcification was frequently observed in MSC-transplanted hearts (Online Figure XVII). Altogether, these data indicate the positive action of SVPs in cardiac cell survival and fibrosis and are reassuring on a safety standpoint.
Discussion

The present study newly shows the prolonged therapeutic benefit of SVPs from coronary artery disease patients in a mouse model of MI. Furthermore, we report for the first time that miR-132 is constitutively expressed and released by human SVPs and implicated in SVP proangiogenic activity in vitro and in vivo. Hence, this is, to the best of our knowledge, the first report of human PCs being therapeutically beneficial through an miR-132-mediated mechanism.

Recent findings from our laboratory indicate that human SVPs can build new vessels in ischemic limbs more efficiently than endothelial progenitor cells. The present study extends the application to a mouse model of MI and, in line with recommendations of advisory boards (Somatic Cell Therapy for Cardiac Disease, http://www.fda.gov), establishes the optimal dosage and long-term therapeutic benefit of SVP transplantation on clinical, hemodynamic, and mechanical endpoints. At variance with MSCs, which reportedly differentiate into multiple cell types on transplantation into the infarcted heart, SVPs maintain their original antigenic phenotype on engraftment. This represents an important difference, but certainly not a disadvantage, when considering that differentiation could trigger a switch in MSC antigen composition rendering them susceptible to both humoral and cell-mediated cytotoxicity, eventually resulting in loss of therapeutic efficacy at late stages of recovery. In the present study, MSCs were used as a cellular control and not for direct comparison; hence, further investigation is warranted to establish the relative therapeutic potency and immunologic properties of the 2 cell populations.

By confocal microscopy, we documented the peri-vascular localization of transplanted SVPs. This physical association suggests that transplanted cells improve myocardial recovery mainly by nurturing reparative angiogenesis. This possibility is further supported by the observation of increased myocardial blood flow and improved vascular barrier function. Moreover, long-term follow up of vascular remodeling documented a biphasic response, consisting of the early augmentation of both capillaries and arterioles and late potentiation of arteriole formation. Noteworthy, the founder population originates from PCs surrounding arterioles in the adventitia of the human saphenous vein. The capacity of SVPs to relocate around and support the growth of coronary arterioles opens new perspectives for predictable delivery of PCs to specific regenerative targets in the infarcted myocardium.

Figure 6. Inhibition of miR-132 attenuates SVP-induced activation of survival signaling. A–E. Representative immunoblots and bar graphs showing the expression of pAkt (A), Bcl-2 (B), pGSK3-beta (C), pErk1/2 (D), and pFOXO1 (E) at 14 days post-MI in the myocardium of mice treated with naïve, scrambled-transfected or antimiR-132-transfected SVPs. Data are means±SE (n=5 mice per group). #P<0.05 and ##P<0.01 vs sham; *P<0.05 and **P<0.01 vs vehicle; δP<0.05 and δδP<0.01 vs nontransfected SVPs; φP<0.05 and φφP<0.01 vs Scr-transfected SVPs.
Our previous study indicates that SVPs stimulate EC proliferation by a paracrine mechanism involving Ang-1 and that ECs reciprocally promote SVP recruitment through PDGF-BB.\(^20\) SVPs also release large amount VEGF-A. Using mouse-specific primers, here we found that SVP transplantation upregulates VEGF-B and to a less extent Ang-1 at mRNA level. Both VEGF-B and Ang-1 are potent stimulators of arteriogenesis,\(^8,34,35\) and VEGF-B also exerts antiapoptotic effects in the ischemic heart.\(^35\) Immunohistochemistry studies suggest that the source of endogenous VEGF-B were the infiltrating CD45 positive cells. Although not demonstrated, other angiogenic factors could have been released from same cell source, because the recruited monocytes abundantly express various angiogenic chemokines.\(^36\) In addition, we found that SVP-transplanted hearts express high levels of the CX3C chemokine receptor 1, a typical receptor of proangiogenic Ly-6Clow monocytes that are recruited during phase 2 of post-MI recovery.\(^26\) Furthermore, SVPs secrete interleukin-8, a potent chemoattractant for CXCR1- and CXCR2-positive leukocytes, and monocyte chemotactic protein-1, a cytokine that exerts chemotactic activity for CCR2- and CCR4-positive monocytes. Accordingly, the SVP-CM increases the migration of human CD14+CD16+ cells in a transwell assay.

Our study newly documents the implication of miR-132 in the paracrine activation of reparative vascularization and inhibition of fibrogenesis by SVPs. MicroRNAs are small noncoding RNAs that regulate a wide spectrum of processes including physiological and pathological angiogenesis.\(^37\) The highly conserved miR-132 is expressed in ECs following GF-induced activation of proliferation.\(^28\) On VEGF stimulation, miR-132 is rapidly transcribed by CREB to suppress endothelial p120RasGAP expression, leading to Ras activation and induction of neovascularization.\(^28\) Apart from the endothelium, miR-132 is expressed in neuronal cells, circulating angiogenic cells (Emanuelei et al, unpublished observations, 2011), and as shown here SVPs and bone marrow-
derived MSCs. We also found that SVPs secrete miR-132, especially under H/S, and that SVP-CM is more abundant in angiogenic GFs and miR-132 compared with MSC-CM. Inhibition of miR-132 by SVP transfection with complementary anti-miR results in mild inhibition of proliferation and survival, thus indicating an intracrine homeostatic mechanism involving the miR-132 and its target p120RasGAP in SVPs. Furthermore, miR-132 inhibition abrogated the SVP-CM induced stimulation of HUVEC proliferation and tube formation, thus showing for the first time the pivotal participation of miR-132 in the angiocrine action of SVPs. These data are in keeping with recent evidence indicating that miRNAs are released through a ceramide-dependent secretory machinery and that the secreted miRNAs are transferable and functional in recipient cells. The tight juxtaposition of SVPs with ECs, including the establishment of peg-socket contacts, makes them ideally positioned for such transfer of miRNA signals.

Mutagenesis studies of the seed sequences of 2 predicted miR-132 binding sites showed that miR-132 exerts a synergistic repression of p120RasGAP. We newly document the upregulation of p120RasGAP in infarcted myocardium and identify the wide distribution of this miR-132 target on vascular cells, cardiomyocytes, and fibroblasts, suggesting that all of them could be potentially influenced by locally synthesized or exogenously produced miR-132. Our finding that miR-132 inhibition blunts the benefits of SVP transplantation on contractility indexes, angiogenesis and cell survival is in keeping with universal effects of miR-132 on a wide spectrum of cardiac cells. However, in vitro studies showed that the SVP-CM induces cardiomyocyte survival independently of miR-132, thus suggesting that other paracrine factors contained in CM may be relevant for cardiomyocyte protection. Paracrine factors released by transplanted cells have been acknowledged to play a role in preservation of mature cardiomyocytes and cardiac PCs, although the exact nature of this interaction remains incompletely understood. In addition, both cardiomyocytes and cardiac PCs may benefit from improved perfusion in the area at risk. Hence, miR-132 might support cardiomyocytes indirectly through potentiation of reparative vascularization.

Data from anti-miR-132 inhibition indicate that miR-132 is instrumental to SVP capacity to alleviate interstitial fibrosis in infarcted hearts. Experiments using SVP-CM on isolated murine fibroblasts under basal and stimulated conditions confirm a direct paracrine inhibition of fibroblast growth and differentiation into myofibroblasts through a miR-132-mediated mechanism. Previous studies described the translation repressive influence of miR-132 on profibrotic MeCP2 in neuronal cells and hepatic cells. Unlocking the miR-132 translational block on MeCP2 leads to a series of methylation events culminating in myofibroblast transdifferentiation and liver fibrogenesis. Intriguingly, elevated MeCP2 expression has been reported to result in cardiac and skeletal abnormalities during development. To the best of our knowledge, however, this is the first report of MeCP2 upregulation in infarcted hearts and of cell therapy resulting in concurrent inhibition of interstitial fibrosis, myofibroblast differentiation, and MeCP2 expression.

Importantly, in the perspective of clinical application, no adverse effect or tumorigenesis has been observed to date with SVPs in small animal preclinical models. Ectopic calcification of blood vessels and heart valves represents a concern of vascular regenerative medicine as many angiogenic factors, cytokines, and PCs might exert both direct and indirect effects on bone and cartilage formation. Furthermore, osteoprogenitor cells may be derived from the circulation, neovessels themselves, or adventitial myofibroblasts; however, the relation of these cells with pericytes remains unclear. The present study and our previous one in a limb ischemia model are reassuring with respect to promotion of calcification. Additional studies in larger animals are however warranted.

Figure 8. Cartoon image showing the mechanism of SVP induced protection in the myocardium post-MI.
In conclusion, we found that human SVPs are potent inducers of reparative vascularization and cardiac healing through an integrated mechanism that involves reciprocal interactions between donor cells and the ischemic environment (summarized in Figure 8). Hence, this specialized PC population should be considered for future applications of cardiovascular regenerative medicine.

Acknowledgments

We thank Andrea Caporal (Bristol University) for his expert advice in miRNA data interpretation.

Sources of Funding

This study was supported by the British Heart Foundation (BHF: PG/06/096/21325), the FP7 program of the European Community (Resolve Chronic Inflammation and Achieve Healthy Ageing, 202/047), and the National Institute for Health Research, Bristol Biomedical Research Unit in Cardiovascular Medicine. Rajesh Katare is supported by a project grant from British Heart Foundation (PG/09/086). Costanza Emanueli is a BHF Senior Research Fellow.

Disclosures

None.

References

We demonstrate that this action is principally due to secretion of paracrine factors and release of microRNA-132 (miR-132), which activates angiogenic and antifibrotic mechanisms in the recipient heart by inhibiting Ras-GTPase activating protein and methyl-CpG-binding protein 2, respectively.

Novelty and Significance

What Is Known?
- Arteries and veins contain progenitor cells with proangiogenic properties and clonogenic cells expressing mesenchymal and pericytic markers have been localized in the adventitia around the vasa vasorum.
- Pericyte-like cells from human saphenous veins stimulate endothelial cell growth and reparative angiogenesis in a mouse model of peripheral ischemia through a paracrine mechanism involving angiopoietin-1 and platelet-derived growth factor.
- Pericyte recruitment is fundamental for stabilization of neovascularization and functional recovery of infarcted mouse hearts.

What New Information Does This Article Contribute?
- Pericytes expanded from leftovers of human saphenous veins stimulate neovascularization and functional recovery of infarcted mouse hearts.
- We demonstrate that this action is principally due to secretion of paracrine factors and release of microRNA-132 (miR-132), which activates angiogenic and antifibrotic mechanisms in the recipient heart by inhibiting Ras-GTPase activating protein and methyl-CpG-binding protein 2, respectively.

Although current cell therapy is dominated by bone marrow-derived cells, additional research on other types of stem cells is needed for optimal treatment of cardiovascular disease. This study focuses on human pericytes as potential candidates for vascular stabilization in the infarcted heart. We demonstrate that transplanted pericytes relocate around and support the growth of coronary arterioles, suggesting a peculiar tropism of these cells instrumental to therapeutic benefit. The physical contact between pericytes and resident endothelial cells may strengthen the nascent vascularization, thus reducing microvascular permeability and myocardial edema, which negatively impact cardiac function. Growing evidence indicates that miRNAs can be transferred between cells, thereby modulating functional activities in the target cell. Human pericytes express and secrete miR-132, which is taken up by endothelial cells to enhance their angiogenic activity. Moreover, transplantation of human pericytes promotes proangiogenic and antifibrotic effects in the infarcted heart through the release of miR-132, resulting in the inhibition of miR-132 target genes. Knocking-down miR-132 in pericytes abrogates these beneficial actions. Human pericytes could be a valuable source of angiogenic cells for future use in cardiovascular regenerative medicine.
Transplantation of Human Pericyte Progenitor Cells Improves the Repair of Infarcted Heart Through Activation of an Angiogenic Program Involving Micro-RNA-132

Rajesh Katare, Federica Riu, Kathryn Mitchell, Miriam Gubernator, Paola Campagnolo, Yuxin Cui, Orazio Fortunato, Elisa Avolio, Daniela Cesselli, Antonio Paolo Beltrami, Gianni Angelini, Costanza Emanueli and Paolo Madeddu

Circ Res. 2011;109:894-906; originally published online August 25, 2011; doi: 10.1161/CIRCRESAHA.111.251546

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/109/8/894

Data Supplement (unedited) at:
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Supplement Material

Supplemental Methods

Ethics
Studies complied with the principles stated in the “Declaration of Helsinki” and were covered by approval (06/Q2001/197) from Bath Research Ethics Committee. Patients gave written informed consent to be recruited in the study.

Experiments involving live animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (the Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol.

Isolation and culture of pericyte progenitor cells
Pericyte progenitor cells (SVPs) were isolated from vein leftovers of patients undergoing coronary artery bypass graft surgery as described earlier. In brief, saphenous veins, collected in PBS containing penicillin and streptomycin, were carefully dissected from surrounding tissues using a sterile scalpel and then thoroughly washed in abundant PBS, containing antibiotics. Veins were then manually minced with a scalpel before being incubated for 4 hours with 3.7mg/mL Liberase (Roche). The remaining aggregates were eliminated by passing the cell suspension through 30μm cell strainer. Cells were then incubated with anti-CD31 conjugated beads (Miltenyi) and passed through a magnetic column, following manufacturer’s instruction. This allowed efficient removal of mature endothelial cells. After depletion of CD31 positive cells, remaining cells were further incubated with anti-CD34 beads (Miltenyi) for 30min at 4°C and then processed to obtain purified CD34 positive cells. Sorted cells were plated on fibronectin (10μg/mL) coated plates in presence of differentiation medium (EGM2-2% FBS, Lonza). Adherent cells appeared 5-10 days later which were fed every three days with fresh medium and passed to new culture dishes once they reached 60-70% confluence. Trypsin-EDTA (Invitrogen) was utilized to detach cells from the growth substrate. For in vitro and in vivo experiments, cells were used at passage 7.

Flow cytometry analysis
SVPs were stained for surface antigen expression using combinations of the following antibodies: anti-CD90, anti-CD-105, anti-CD34, anti-CD31, anti-CD45 (all from BD biosciences), anti-CD14 and anti-CD16 (both from Invitrogen) After staining, fluorescence was analyzed using a FACS Canto II flow cytometer and FACS Diva software (both BD Biosciences, UK). To control for specificity, an aliquot of cells was stained with secondary antibody only.

Isolation of circulating mononuclear cells and bone marrow mesenchymal stem cells
Mesenchymal stem cells were isolated from the bone marrow of the patients undergoing hip joint replacement. 1.0 - 1.5 x 10^7 BM-MNCs were resuspended in 10 mL of MSCGM Single Quots medium (Lonza) and plated in a T-25 cm^2 flask for 7-10 days. The resulting mesenchymal stem cells (MSCs) were adherent to plastic, positive for surface antigen markers such as CD90, CD105 and CD73 and negative for CD45, CD34 and CD14. In vitro, MSCs were able to differentiate into osteoblasts, adipocytes and chondroblasts. Cells were used at 4th passage for in vivo studies.

Isolation and culture of rat adult cardiomyocytes
Male Wistar rats were killed by cervical dislocation, the heart dissected and rinsed in cold solution A containing (in mM): 137 NaCl, 5 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 20 N-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES), 16 glucose, 5 Na pyruvate and 1.8 MgCl2 (pH 7.25 with NaOH) + 0.75 mM CaCl2. The heart was cannulated via the
aort and perfused for 4 min with solution A + 0.75 mM CaCl₂ (all perfusing solutions were oxygenated and maintained at 37 °C). This was followed by a 4-min perfusion with solution A + 0.09 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA). Next the heart was digested with 50 ml of enzyme solution containing: solution A + 0.09 mM EGTA, 50 mg collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey, USA. Type I), 5 mg protease (Sigma, Poole, Dorset, UK. Type IV), with (glutamate loaded) or without (control) 6.4 mM potassium L-glutamate until the tissue felt soft. There was a final 4-min perfusion with solution A + 0.15 mM CaCl₂ before the ventricles were cut down and sliced. The sliced ventricles were suspended in approximately 20–25 ml solution A + 0.15 mM CaCl₂ and shaken for 6 min at 37 °C. After filtration, cells were allowed to sediment, the supernatant was discarded, and the remaining cell layer suspended in solution A + 0.5 mM CaCl₂. This sedimentation, removal of supernatant and resuspension step was repeated, but this time the cells were suspended in solution A + 1 mM CaCl₂. This technique typically produced a yield of over 90% rod-shaped cells with the ability to exclude Trypan Blue.² The resulting cells were then washed repeatedly with medium 199 (Invitrogen) supplemented with 0.2% BSA, 10% FBS, 5 mM creatine, 5 mM taurine, 2 mM carnitine, 10 μM cytosine-D-arabinofuranoside (all from Sigma chemicals), ITS and antibiotics (both from Invitrogen). After the final wash cells were resuspended in the same medium and plated on laminin coated culture dish according to the experiments.

**Isolation and culture of adult mouse cardiac fibroblasts**

Adult fibroblasts were isolated by the principle of selective plating as previously described, with some minor modifications. In brief, ventricles from 8 weeks old male CD1 mice (Harlan, UK) were minced and digested twice for 10 min with 1000U Collagenase Type 1 (Worthington), followed by 9500 U Collagenase Type 2 (Worthington) for 70 min. Red blood cells were lysed with a standard ammonium chloride/EDTA buffer and the resulting suspension was plated for 1 h in cell culture dishes in DMEM/10% FBS/1% Penicillin-Streptomycin. The cultures were washed thoroughly with PBS and the remaining adherent cells were cultivated for 3 days without signs of myofibroblast differentiation. Cells were passaged once confluent and used for the experiments. For differentiation experiments, fibroblasts were treated with Angiotensin II (100nM) for 24h in a 8th passage, or phosphate buffered saline (PBS) at 3 different sites along the infarct border zone with final volume of 10µL at each site. Animals were allowed to recover with aseptic precautions and received analgesic medication (Buprenorphine, 0.1mg/kg s.c.) to reduce post-operative pain. Sham operated animals underwent a similar procedure without LAD ligation.

**Mouse model of myocardial infarction and SVP transplantation**

Myocardial infarction (MI) was induced in 8 weeks-old immune deficient CD1-FOXO₅₅/nu mice (Charles River) or immune competent CD1 (Harlan) by permanent ligation of the left anterior descending coronary artery (LAD) as described.², ³ In brief, with mice under anesthesia (2,2,2 tribromo ethanol, 0.3gm/kg, i.p.) and artificial ventilation, the chest cavity was opened and, after careful dissection of the pericardium, LAD was permanently ligated using a 7-0 silk suture. This was followed by injection of Dil-stained SVPs (3X10⁵ or 1X10⁶, 7th passage), MSCs (1X10⁶, 4th passage) or phosphate buffered saline (PBS) at 3 different sites along the infarct border zone with final volume of 10µL at each site. Animals were allowed to recover with aseptic precautions and received analgesic medication (Buprenorphine, 0.1mg/kg s.c.) to reduce post-operative pain. Sham operated animals underwent a similar procedure without LAD ligation.

**Effect of miR-132 antagonism on SVP reparative activity in vivo**

To investigate the importance of miR-132 in SVP-induced promotion of post-MI recovery, SVPs were transfected with anti-miR-132 (50nmol/L) or scramble sequence (50nmol/L, both from Applied Biosystems, UK) for 48h using commercially available transfection agent Lipofectamine 2000 (Invitrogen, UK). After 48h, SVPs were trypsinized, labelled with Dil and transplanted into mouse infarcted heart (3 X 10⁵ cells/animal). Effective antagonism of miR-132 was tested in vitro and in vivo by RT-PCR and western blotting for target gene p120RasGAP.
**Echocardiography and hemodynamic measurements**

Measurements of dimensional and functional parameters were performed before and at different time points after MI using a high-frequency, high resolution echocardiography system (Vevo 770, Visual Sonics, Toronto, Canada) (n=at least 6 mice per group). Briefly, mice were anesthetized using tribromo-ethanol and transferred to an imaging stage equipped with a warming pad for controlled maintenance of mouse body temperature at 37°C and a built-in electocardiography system for continuous heart rate (HR) and respiratory rate monitoring. The thickness of the left ventricle (LV) was measured at the level of the papillary muscles in parasternal short axis at end-systole and end-diastole. LV ejection fraction (LVEF) and fractional shortening (LVFS) were determined as described by De Simone et al. Following the final echocardiography measurement, under anaesthesia, intraventricular pressure measurement was done using a high-fidelity 1.4F transducer tipped catheter (Millar Instruments, Houston, TX, USA) inserted into the left ventricle through right carotid artery (n=at least 6 per group). The position of transducer into the heart was confirmed by the rapid deflection of the diastolic pressure wave without any change in systolic pressure. After 5min stabilization, baseline data were collected, including the HR, Peak LV systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), and maximal rates of LV pressure rise (dP/dt max) and fall (dP/dt min). To calculate pressure volume relationship, the recording from Millar catheter was synchronized with echocardiography measurements as per manufacturer instructions.

**Measurement of blood flow using fluorescent microspheres**

Myocardial perfusion was measured using fluorescent microspheres (n= at least 6 mice per group). A polyethylene (PE10) catheter was inserted through the right carotid artery for the reference blood withdrawal. Microspheres, 0.1µm in diameter (Molecular Probes, Invitrogen) were injected into the LV cavity over 1min and flushed with 0.15ml of 0.9% NaCl. Reference blood was collected via the carotid catheter starting 15sec before to 1min after the microsphere injection. The animals were sacrificed 2min later and the heart was removed and separated into LV and right ventricle (RV). The kidneys were also collected and analyzed as internal control organs to demonstrate homogenous distribution of the microspheres throughout the bloodstream. Each sample was weighed, cut into small pieces and digested in 10ml of 2M ethanolic KOH containing 0.5% Tween 80 at 60ºC for 48h with constant shaking. After complete digestion of tissues, the microspheres were collected by centrifugation at 2,000 x g for 20min and sequential washing with 10ml of deionized water with or without 0.25% Tween 80. Finally, microspheres were dissolved in 3ml of 2-ethoxyethylacetate and the fluorescence intensity was determined using a fluorophotometer (Fluostar Optima, BMG labtech). Regional blood flow was calculated as the absolute blood flow in ml/min/g of tissue as described earlier.

**Measurement of vascular permeability**

In vivo myocardial vascular permeability was measured using modified fluorescent labeled dextran method (n= 6 mice per group). FITC-labeled 70kD dextran was injected intravenously followed 5min later by TRITC-labeled dextran. Mice were sacrificed 30sec from last injection. Cardiac tissue was dissected, homogenized and the fluorescence of the supernatant measured using fluorophotometer (Fluostar Optima, BMG labtech). The amount of TRITC-labeled dextran was subtracted from the amount of FITC-labeled dextran, to correct for the intravascular space and give the total amount of extravasated dextran.

**Biochemical measurements**

To verify the impact of cell therapy of neurohormonal activation after MI, angiotensin II (Ang II) and norepinephrine (NE) levels in plasma and LV myocardium were measured using commercially available ELISA kits (SPI bio and IBL international respectively). The expression of Ang II receptor, type 1 (AT1R) was quantified by western blot (vide infra).
**Immunohistochemistry**

**Whole mount sections preparation**

After completion of hemodynamic measurements, hearts were stopped in diastole by intramyocardial injection of cadmium chloride. Hearts were then washed free of blood by retrograde perfusion with PBS-2% EDTA solution, followed by fixation with freshly prepared ice-cold 4% paraformaldehyde (PFA) solution under physiological pressure from abdominal aorta. Hearts were immediately cut in to 5mm-thick cross sections and embedded in tissue tech O.C.T compound for cryosectioning. Cryosections were made at 60µm thickness, placed on superfrost ultra plus (Thermo, UK) slides, air dried for 30min and stored in -80°C till immunostaining. In some experiments, animals received an intravenous injections of biotinylated isolectin-B4, and after 10min hearts were stopped in diastole, perfused and processed as above.

**Graft Localization and characterization**

We used whole mount sections for localization and characterization of injected SVPs at day 5, 14 and 42 post-MI. Sections, were post-fixed with acetone at -20°C for 10min and air dried at room temperature for 30min. Following permeabilization with 1% triton-X 100 and blocking of non-specific antigens, sections were incubated overnight at 4°C with biotinylated isolectin-B4 to stain endothelial cells (1:50, Invitrogen, UK), followed by incubation with streptavidin Alexa Flour 488 (1:100, Invitrogen, UK) for 3h at room temperature. To characterize the injected SVPs, some sections were subjected to secondary staining by overnight incubation with pericytes markers NG2 (1:100, mouse monoclonal, Millipore) or PDGFβR (1:100, rabbit polyclonal, Santa Cruz, USA), followed by Alexa Flour goat anti mouse or rabbit (1:100, Invitrogen, UK). Serial z-stack images of myocardium were generated using Leica SP5 AOBS confocal laser scanning microscope (Wolfson Bioimaging facility, University of Bristol).

**Vascular density profiling**

For capillary density, 5µm thick LV cryosections were incubated with biotinylated Isolectin B4 (Invitrogen, UK, 1:50, 2h at 37°C in a humidified chamber), followed by streptavidin Alexa Fluor 488 (Invitrogen, UK, 1:100, 1h at room temperature). For arteriole density, same sections were probed with anti-mouse α-smooth muscle cell actin antibody conjugated with Cy3 (Sigma chemicals, UK, 1:400, 1h at room temperature). To recognize cardiomyocytes, sections were also stained with mouse monoclonal primary antibody for the cardiomyocyte marker α-sarcomeric actin (Abcam, UK, 1:100, overnight at 4°C), which was revealed by counterstaining with the secondary antibody conjugated to Alexa 568 (Invitrogen, UK, 1:100, 1h at room temperature). Capillaries and arterioles were calculated in at least 20 fields at X200 magnification and the final data expressed as the number of capillaries or arterioles per square millimetre. Arterioles were also categorized according to their luminal size.4,11

**Analysis of inflammation**

For analysis of inflammatory cells, 5µm serial cryosections were incubated overnight with polyclonal VEGF-B antibody (Abcam, UK, 1:100) followed by Goat anti-rabbit Alexa Flour 488 antibody (Invitrogen, UK, 1:100). Same sections were then probed with either mouse monoclonal CD45 antibody (BD Biosciences, UK, 1:40) or rat anti-mouse Mac-3 antibody (BD Biosciences, UK, 1:40) overnight, followed by goat anti-mouse Alexa Flour 568 antibody (Invitrogen, UK, 1:100).

**Proliferation and apoptosis**

For analysis of proliferation, sections were incubated overnight with mouse monoclonal PCNA antibody (BD Biosciences, UK, 1:100), followed by goat-anti mouse secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, UK, 1:100). Injected SVPs were identified by their Dil staining. The data were expressed as percentage of PCNApos Dilpos SVPs. To identify the proliferating endothelial cells, after staining with PCNA, sections were stained with isolectin-B4 as described above.

Cardiomyocytes and endothelial cells apoptosis was quantified on LV cryosections (5µm) by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (in situ cell death detection kit Fluorescein, Roche applied science,
USA). Following treatment of slides with proteinase K (20µg/ml, 30min at 37°C), TUNEL assay was performed according to the manufacturer’s instruction. Same sections were then stained with DAPI to recognize nuclei. Cardiomyocytes and endothelial cells were stained as described above. Twenty fields were randomly evaluated in each section at X400 magnification. The fraction of TUNEL positive nuclei over total cardiomyocyte or endothelial cell nuclei was then calculated.

Identification of p120RasGAP expression in myocardium

For identification of p120RasGAP, 5µm cryosections were incubated overnight with monoclonal p120RasGAP antibody (SantaCruz, USA, 1:50) followed by Goat anti-mouse Alexa Flour 488 antibody (Invitrogen, UK, 1:100). Same sections were then probed with biotinylated Isolectin B4 (Invitrogen, UK, 1:50) to identify the endothelial cells or α-sarcomeric actin (Abcam, UK, 1:100) to identify cardiomyocytes or s100A4 (Abcam, Uk, 1:50) to identify fibroblasts. To understand the distribution of p120RasGAP in the myocardium, multiple low magnification images (50X) were captured using Leica SP5 AOB confocal laser scanning microscope (Wolfson Bioimaging facility, University of Bristol), and then merged together using Velocity imaging software. For identification of the type of cells expressing p120RasGAP, images were captured.

Identification of cardiac stem cell pool in myocardium

For identification of cardiac stem cell pool in the myocardium, 5µm paraffin sections were incubated with rabbit polyclonal GATA4 antibody (SantaCruz, USA, 1:40, 2h at 37°C, followed by donkey anti-rabbit Alexa Flour 555 antibody (Invitrogen, USA, 1:800)), goat polyclonal c-Kit antibody (R&D, USA, 1:50, 2h at 37°C, followed by donkey anti-goat Alexa Flour 488 antibody (Invitrogen, USA, 1:400)), mouse monoclonal mast cell tryptase (Abcam, USA, 1:50, 2h at 37°C, followed by donkey anti-mouse Alexa Flour 555 antibody (Invitrogen, USA, 1:800)) and mouse monoclonal α-sarcomeric actin (Sigma Chemicals, Italy, 1:100, 1h at 37°C, followed by donkey anti-mouse DYLight 649 antibody (Invitrogen, USA, 1:400)). Proliferating c-Kit cells were identified using rabbit monoclonal Mcm-5 antibody (Abcam, USA, 1:100, over night incubation at 4°C, followed by donkey anti-rabbit Alexa Flour 555 antibody, Invitrogen, USA, 1:800)

Assessment of myocardial fibrosis

Myocardial fibrosis was analyzed by Azan Mallory or Sirius red staining followed by morphometric analysis using the Image Pro analysis software (MediaCybernetics, USA) and the data were expressed as the percentage of scar size or fibrotic area.

Assessment of calcification

Myocardial calcification was assessed using Von Kossa staining as described earlier. In brief 5µm cryosections were incubated with 1% silver nitrate solution and exposed to ultraviolet light for 60 minutes. After serial washes with distilled water, un-reacted silver was removed with 5% sodium thiosulfate for 5 minutes and finally counterstained with nuclear fast red. Images were obtained using Olympus light microscope fitted with camera. Three weeks olde mice fibula was used as positive control.

Immunocytochemistry

For immunocytochemical analysis of SVPs and cardiac fibroblasts, cells were fixed with freshly prepared 4% PFA after different treatment protocols. SVPs were probed with one of the following antibodies – pericytes markers NG2 (1:100, Millipore, UK) and PDGFRβ (1:100, Santa Cruz, USA). Furthermore, in differentiation assays, cells were analysed for expression of cardiomyocytes marker α-sarcomeric actin (1:100, Abcam, UK), and connexin-43 (1:3000, Sigma Chemicals, UK), cardiac transcription factor GATA-4 (1:20, Santacruz, USA), vimentin (1:20, Abcam, UK) and α-smooth muscle actin (1:400, Sigma Chemicals, UK). Cardiac fibroblasts were probed with α-smooth muscle actin (1:400, Sigma Chemicals, UK) or MeCP2 (1:200, Abcam, UK).

RNA isolation and quantitative RT-PCR

Total RNA was extracted from flash-frozen LV samples (Trizol, Invitrogen, UK), SVPs, SVP-CM or HUVECs using Trizol according to the manufacturer’s instructions.
One microgram of total RNA was reverse transcribed using qiagen reverse transcriptase kit, followed by amplification of cDNA using quantitect primers for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, CX3CR1, platelet derived growth factor–β receptor (PDGFβ), angiopoietin, NG2, CD40, CD80, Fas Ligand and internal control 18S (all from Qiagen, UK).

For analysis of miR-132, 10ng of total RNA was reverse transcribed using TaqMan reverse transcriptase kit and specific reverse transcription primers for miR-132 and internal control RN6 (all from Applied Biosystems, UK). Amplification of cDNA was performed using TaqMan universal PCR mix kit (Applied Biosystems, UK) in Light Cycler 480 (Roche, UK).

For quantification, the amount of RNA/miRNA was normalized to the amount of 18S/U6 miRNA using the 2−DDCT method. Each reaction was performed in triplicate.

Protein extraction, western blotting and ELISA

Proteins were extracted from LV, SVPs or HUVECs using ice-cold RIPA buffer. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, UK). Detection of proteins by western blot analysis was done following separation of whole tissue/cell extracts (50μg) on SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Amersham-Pharmacia, Germany) and probed with the following antibodies: Ser473-phospho-Akt (Cell Signaling, UK, 1:1000), Akt (Cell Signaling, UK, 1:1000), pGSK3β (Cell Signaling, UK, 1:1000), GSK3β (Cell Signaling, UK, 1:1000), Ser1177-phospho-eNOS (Cell Signaling, UK, 1:1000), eNOS (Cell Signaling, UK, 1:1000), Thr202/Tyr204-phospho-Erk1/2 (Cell Signaling, UK, 1:1000), Erk1/2 (Cell Signaling, UK, 1:1000), Ser112-phospho-Bad (Cell Signaling, UK, 1:1000), Bad (Cell Signaling, UK, 1:1000), Bcl-2 (Cell Signaling, UK, 1:1000), cleaved-caspase-3 (Cell Signaling, UK, 1:1000), Ser133-phospho-CREB (Cell Signaling, UK, 1:1000), CREB (Cell Signaling, UK, 1:1000), p120RasGAP (SantaCruz, USA, 1:500) and Angiotensin type II receptor 1 (SantaCruz, USA, 1:500). Actin (Cell Signaling, UK, 1:1000) was used as loading control. For detection, secondary antibody goat anti-rabbit or anti-mouse or donkey anti-goat conjugated to horseradish peroxidase (all from SantaCruz, USA 1:5000) were used, followed by chemiluminescence reaction (ECL, Amersham Pharmacia, Germany). Density of the bands was analyzed using Image-J (NIH, USA) software and data expressed as fold changes.

ELISA were performed with 25μg of total protein using the antibodies against VEGF, VEGFβ166, VEGF and angiopoietin-1 using standard ELISA protocol. For ELISA with CM (vide infra), 100μl of total CM was used.

In vitro Experiments

Hypoxia-starvation and sample collection

For hypoxia-starvation experiments, SVPs were serum starved for 8h followed by exposure to 1% O2 for 18h. At the end of hypoxia, conditioned supernatant medium was collected from SVPs for further experiments (vide infra). Cells were then used for measurement of caspase-3/7 activity using caspase-glo assay (Promega, UK) or protein and RNA extraction.

In vitro inhibition of miR-132

For the inhibition of miR-132 in vitro, SVPs were transfected with anti-miR-132 (50nmol/L) or scrambled sequence (50nmol/L, both from Applied Biosystems, UK) using commercially available transfection agent Lipofectamine 2000 (Invitrogen). Forty eight hours later, cells were exposed to hypoxia/starvation and sample collected for measurement of caspase activity, protein and RNA analysis and BRDU incorporation assay as below.
Collection of conditioned medium (CM)

CM were collected from SVPs (SVP-CM) or MSCs (MSC-CM) transfected with either anti-miR-132 or scrambled oligonucleotide after normoxia or H/S for 48h. Forty-eight hours later, medium was replaced with fresh one deprived of growth factors and the cells were cultured under normoxia or hypoxia. At the end of another 48h, CM was collected and concentrated (50X, Amicon ultra, Millipore). By this method we confirmed the higher yield of miR-132 in the CM.15

Experiments with CM

HUVECs, adult rat cardiomyocytes or fibroblasts were cultured in 96-well plate (caspase-3/7 activity, BrdU proliferation assay), 6-well plates (expressional analyses) or 8-well chamber slides (immunocytochemistry). For HUVECs and fibroblasts, after 24h, medium was replaced with SVP- or MSC-CM collected after different treatments to SVP. Thirty-six hours later, cells were used for different assays. For cardiomyocytes, after 4h of plating in the laminin coated culture plates to allow the cells to settle down, medium was replaced with SVP-CM and cells were exposed to hypoxia for 12h, to mimic in vivo ischemia situation to the cardiomyocytes. After 12h, cells were used for caspase-3/7 activity measurement. In addition experiments were also performed to evaluate the ability of network formation by HUVECs after treatment with SVP- or MSC-CM (vide infra).

BrdU incorporation assay

To study the effect of hypoxia on SVP proliferation, we used the BrdU incorporation assay. Following starvation, BrdU (10μmol/L) was added to the medium before cells were exposed to hypoxia. BrdU incorporation by SVPs was measured using a BrdU immunofluorescence assay kit from Roche, according to the manufacturer’s instructions. Briefly, SVPs were fixed and made permeable with FixDenat solution for 20min, then incubated with monoclonal anti-BrdU peroxidase-conjugated antibody (anti-BrdU-POD) for 90min. Bound anti-BrdU-POD was detected by a substrate reaction, then quantified by an ELISA plate reader. Each experiment was performed in triplicate and repeated 5 times. Similar experiments were performed with SVP-CM.4,16

Migration assays

Mononuclear cells (MNC) were prepared from the peripheral blood collected from healthy volunteers. 5X10^6 MNC were placed in the upper chamber of 3 μm pore-size filter-equipped transwell chambers (Corning) and allowed to migrate toward conditional or unconditional medium collected from SVPs subjected to hypoxia/starvation. The assays were stopped after 18h at 37°C, and cells that had invaded the membrane and migrated through the filter were fixed on the lower side of the filter and mounted with Vectashield containing DAPI. Five random fields were counted at 20X magnification for each chamber. Cells were then stained for antibodies against CD14 (FITC conjugated) and CD16 (APC conjugated, both from Invitrogen, UK) and the enrichment of CD14^+CD16^+ cells among the migrated cells were assessed by flowcytometry (Canto II, BD Biosciences, UK).

In vitro matrigel assay

SVPs pre-treated with anti-miR-132 or scrambled sequence and serum starved mixed with human umbilical vein endothelial cells (HUVEC, 1:4 ratio of SVPs to HUVEC respectively) were seeded in 8-well chamber slides at 25000 cells/well on top of 250μl gelified Matrigel (BD Biosciences, UK) and exposed to normal or 1% O2 for 18h. At the end of experiment, floating cells were removed by washing and the endothelial networks were fixed with 2% PFA. Number of branches and total length of the networks were calculated using the Image-Pro Plus software on images taken at 40X magnification. Each condition was run in quadruplicates and assay was repeated three times. Similar experiments were performed with SVP-CM.
**Statistical Analysis**

Results are expressed as mean ± standard error. Difference between multiple groups was analyzed using one-way ANOVA and difference between two groups using t-test (paired or unpaired as appropriate). For myocardial BF and expressional studies, when normality test failed, differences between groups were analyzed using Siegel-Tukey test. A P value of <0.05 was considered statistically significant.
References


Supplemental Figure Legends

Online Table I
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Representative images and bar graph showing EC proliferation (A, arrow points PCNA positive cells) and apoptosis (B, arrow points TUNEL positive cells) in the peri-infarct zone, assessed by PCNA and TUNEL staining, respectively. Dotted lines delineate the infarct zone. Scale bars are 50µm. Data are means±SE (n=6 mice per group). *P<0.05 and **P<0.01 vs. vehicle.

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Effect of MSC transplantation on LV angiogenesis, remodeling, apoptosis. Bar graphs showing the capillary (A) and arteriole density (B), scar dimension (C) and cardiomyocyte apoptosis in the border zone (D) and interstitial fibrosis in the remote zone at 42d post-MI. Data are means±SE (n=4 mice per group). *P<0.05 and **P<0.01 vs. vehicle.

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Bar graphs showing the levels of GFs in SVP-CM under normoxia (normoxic-CM) or hypoxia/serum starvation (H/S-CM); unconditioned medium (UCM); *P<0.05 vs. normoxic-CM.

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Representative images showing the expression of VEGF-B in Mac-3 (1) and CD45 positive cells (2) in hearts harvested at 5d post-MI and bar graph showing the average VEGF-B expression in CD45 positive and negative cells, expressed as fluorescence intensity. ***P<0.001 vs. vehicle. Scale bars are 50µm.

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A. Bar graphs showing the effect of anti-miR-132 or scrambled sequence (Scr) transfection on SVP proliferation (BrdU incorporation) and apoptosis (Caspase 3/7 activity) under normoxia or hypoxia/serum starvation (H/S). Vehicle of transfection (V). Data are means±SE from experiments performed in quadruplicates. *P<0.05, **P<0.01 and ***P<0.001 vs. corresponding group under normoxia; §§P<0.01 vs. vehicle and §§§P<0.01 vs. Scr under corresponding culture conditions. B. Representative images (i to iv) and bar graphs (v) showing the attenuation of the SVP ability to enhance network formation by HUVECs. SVPs were pre-treated with anti-miR-132, scrambled sequence (Scr) or vehicle and co-cultured with human umbilical vein endothelial cells (HUVEC) in 1:4 ratio respectively on matrigel. Values are expressed as cumulative tube length per field and are means±SE. ###P<0.001 vs. HUVECs alone and §§P<0.01 vs. scrambled sequence. Experiments are performed in quadruplicates and repeated three times. Scale bars are 100µm.

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Bar graphs showing BrdU incorporation (A) and representative western blots and bar graphs showing Akt phosphorylation (B) in HUVECs treated with SVP-CM. Data (means±SE) are represented as fold changes to UCM from experiments performed in quadruplicates. ###P<0.001 vs. UCM; §P<0.05 and §§P<0.01 vs. corresponding group under normoxic SVP-CM; ##P<0.01 and ###P<0.001 vs. Scr.

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A. Bar graphs showing the level of angiogenic growth factors and miR-132 in SVP- and MSC-CM. Unconditioned medium (UCM) for SVPs is EBM-2 with 2% FBS and UCM for MSCs is mesenchymal stem cell media with 2% FBS and without growth factors. Data are means±SE. miR-132 levels are expressed as fold change vs. normoxic SVP-CM. *P<0.01 vs. UCM; §P<0.05 and §§P<0.01 vs. normoxic CM; §§§P<0.01 vs. SVP-CM under the same culture conditions. B. Representative images (i-vi) and bar graphs (vii) showing the network formation by HUVEC after treatment with UCM (i&iv) and SVP- (ii&iii) or MSC-CM (v&vi). Values are expressed as cumulative tube length per field and are means±SE. Experiments were performed in quadruplicates and repeated three times. Scale bars are 100µm. *P<0.01 vs. UCM; ###P<0.001 vs. SVP-CM under the same culture conditions.

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A. Representative confocal images showing the localization of p120RasGAP in endothelial cells (ii), cardiomyocytes (iii) and fibroblasts (iv) in the myocardium at 14d post-MI. Negative control (i) without primary antibody to p120RasGAP was used as reference. Scale bars are 50µm. B. Representative western blots and bar graph showing the levels of miR-132 target gene p120RasGAP in myocardium. C. Bar graphs showing hemodynamic data (i&ii), EC apoptosis (iii) and proliferation (iv) at 14d post-MI. Data are means±SE (n=5 mice per group except for hemodynamic measurements which consisted of 6 mice). ###P<0.001 and §§§P<0.001 vs. sham; *P<0.01 and ***P<0.001 vs. vehicle; §P<0.05 and §§P<0.01 vs. non-transfected SVPs; *P<0.05 and ###P<0.01 vs. Scr-transfected SVPs.
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Bar graphs showing long-term inhibition of miR-132 expression in SVPs transfected with anti-miR-132. Experiments were performed in triplicates. Data are means±SE; ***P<0.001 vs. scrambled sequence.

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Bar graph showing the number of Dil-positive SVPs in the myocardium at 14d post-MI. Data expressed as number of Dil-positive cells per million cell nuclei and represented as means±SE (n=6 mice per group).

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Bar graph showing caspase-3/7 activity in adult rat cardiomyocytes exposed to hypoxia after treatment with SVP-CM. Data are means±SE from experiment performed in quadruplicate. ##P<0.01 vs. unconditioned medium (UCM).

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Online Figure XVI. Representative images showing Angiotein-II differentiated myofibroblasts staining positive for α-smooth muscle actin and MeCP2. Scale bars are 50µm.

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Legend for Online Videos

Online video I
Serial z-stack images of myocardium showing the alignment of transplanted Dil-labelled SVPs (stained in red) along the perfused vessels, which were identified by staining with intracardially-injected Isolectin (green). Nuclei are identified with Dapi.
<table>
<thead>
<tr>
<th>Echocardiographic parameters</th>
<th>Pre-MI</th>
<th>SVP</th>
<th>Post-MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVAWs (mm)</td>
<td>1.27±0.02</td>
<td>1.26±0.03</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>LVAWd (mm)</td>
<td>0.99±0.03</td>
<td>0.91±0.05</td>
<td>0.27±0.02</td>
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<tr>
<td>LVPWs (mm)</td>
<td>1.21±0.05</td>
<td>1.24±0.06</td>
<td>1.27±0.08</td>
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<tr>
<td>LVPWd (mm)</td>
<td>0.97±0.05</td>
<td>1.01±0.04</td>
<td>1.08±0.04</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.22±0.15</td>
<td>2.24±0.13</td>
<td>4.68±0.15</td>
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<tr>
<td>LVEDD (mm)</td>
<td>3.61±0.16</td>
<td>3.70±0.08</td>
<td>5.16±0.12</td>
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<tr>
<td>LVESV (µl)</td>
<td>12.9±0.7</td>
<td>14.9±2.4</td>
<td>108.6±8.3</td>
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<tr>
<td>LVEDV (µl)</td>
<td>53.4±1.5</td>
<td>56.7±2.9</td>
<td>132.1±7.7</td>
</tr>
<tr>
<td>LVSV (µl)</td>
<td>40.4±1.7</td>
<td>40.6±3.7</td>
<td>23.5±2.4</td>
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<tr>
<td>LVEF (%)</td>
<td>75.6±1.6</td>
<td>73.7±2.9</td>
<td>18.2±2.2</td>
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<td>LVFS (%)</td>
<td>38.6±1.8</td>
<td>39.4±0.9</td>
<td>11.8±1.9</td>
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<tr>
<td>HR (b/min)</td>
<td>455±14</td>
<td>447±5</td>
<td>534±9</td>
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<tr>
<td>CO (ml/min)</td>
<td>18.4±2.1</td>
<td>17.9±1.7</td>
<td>12.5±1.2</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>-</td>
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<td>10.0±0.7</td>
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<tr>
<td>dP/dt&lt;sub&gt;max&lt;/sub&gt; (mmHg/Sec)</td>
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<td>4370±154</td>
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<tr>
<td>dP/dt&lt;sub&gt;min&lt;/sub&gt; (mmHg/Sec)</td>
<td>-</td>
<td>-</td>
<td>-3434±121</td>
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</tbody>
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A. 

(i) p120RasGAP Lectin Dapi  

(ii) p120RasGAP Lectin Dapi  

(p120RasGAP colocalized with lectin) 

(iii) p120RasGAP α-sarcomeric actin Dapi  

(iv) p120RasGAP S100A4 α-sarcomeric actin Dapi  

B. 

[Graph showing fold changes]
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