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Endothelial Nitric Oxide Synthase Promotes Bone Marrow Stromal Cell Migration to the Ischemic Myocardium via Upregulation of Stromal Cell-Derived Factor-1α

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ABSTRACT

The aim of this study was to investigate the role of endothelial nitric oxide synthase (eNOS) in the host myocardium on bone marrow mesenchymal stromal cells (MSC) migration to the ischemic myocardium and whether stromal cell-derived factor-1α (SDF-1α) contributes to eNOS-mediated MSC migration. MSCs and coronary microvascular endothelial cells were isolated from adult wild-type (WT) mouse bone marrow and hearts, respectively. Cultured neonatal cardiomyocytes from WT, eNOS$^{-/-}$, and eNOS overexpressing transgenic (Tg) mice were subjected to anoxia and reoxygenation (A/R), and the conditioned medium was used as a chemoattractant for in vitro trans-endothelial migration assay. MSC migration was decreased in the presence of conditioned medium derived from eNOS$^{-/-}$ cardiomyocytes but increased in the presence of eNOS-Tg conditioned medium. SDF-1α expression was decreased in eNOS$^{-/-}$ but increased in eNOS-Tg cardiomyocytes following A/R and in the myocardium following ischemia/reperfusion (I/R). SDF-1α expression was cGMP-dependent as inhibition of soluble guanylyl cyclase decreased SDF-1α expression in WT cardiomyocytes. MSCs expressed very low levels of eNOS proteins compared with the adult myocardium. To examine MSC migration in vivo, MSCs derived from mice expressing enhanced green fluorescence protein (EGFP^{+}) were intravenously administered to WT mice subjected to myocardial I/R. EGFP^{+} cells in the ischemic region were decreased in eNOS$^{-/-}$ but increased in eNOS-Tg compared with WT hearts. MSC treatment improved cardiac function following I/R in WT but not in eNOS$^{-/-}$ mice. In conclusion, eNOS in the host myocardium promotes MSC migration to the ischemic myocardium and improves cardiac function through cGMP-dependent increases in SDF-1α expression. STEM CELLS *2009;27:961–970*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Adult bone marrow mesenchymal stromal cells (MSCs) are multipotential nonhematopoietic stem cells that are able to regenerate mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and adipose tissues [1]. Accumulating evidence suggests that MSCs are attractive candidates for cardiovascular therapy because of their capacity to facilitate myocardial repair and neovascularization in models of cardiac injury [2]. Although the ability of MSCs to differentiate into cardiomyocytes in vivo is still debatable, studies have consistently shown that MSCs are able to migrate into the injured myocardium from the circulation and contribute to cardiac repair post myocardial infarction (MI) [3, 4]. However, the molecular mechanisms governing MSC migration into the ischemic myocardium are not fully understood.

Endothelial nitric oxide synthase (eNOS) is expressed in the heart by several cell types including the vascular endothelium, the endocardium, and cardiomyocytes [5]. Nitric oxide (NO) production from eNOS promotes neovascularization, cardiomyogenesis, and cardiomyocyte proliferation [5–7]. Furthermore, eNOS has been shown to be essential in the mobilization of stem and progenitor cells from the bone marrow [8]. The effect is thought to be through the activation of matrix metalloproteinase 9 and cleavage of stem cell factor from the MSCs, which promotes the release of progenitor cells from the bone marrow [8]. However, whether eNOS in the host myocardium promotes MSC migration toward the ischemic myocardium remains to be determined.

Stromal-derived factor-1 alpha (SDF-1α, also known as CXCL12) is a member of the chemokine family of chemotactic proteins. SDF-1α and its receptor CXCR4 are required for hematopoietic stem cell migration and homing to the bone

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marrow [9]. SDF-1α induces neovascularization by recruiting the endothelial progenitor cells (EPCs) toward the ischemic tissues [10]. Interestingly, cardiomyocytes express SDF-1α, and this expression is upregulated in the heart following MI [11]. Recent studies suggest that SDF-1α/CXCR4 interaction promotes bone marrow stem cell recruitment to the heart following MI [12]. Furthermore, SDF-1α-induced EPC migration in the Boyden chamber is mediated by PI3K/protein kinase B (Akt)/eNOS signaling in the EPCs [13]. However, the role of eNOS in the host myocardium on MSC migration to the ischemic heart is still not known.

In this study, we hypothesized that eNOS-derived NO production from the host myocardium promotes MSC migration toward the ischemic myocardium via upregulation of SDF-1α. To test this hypothesis, in vitro MSC transendothelial migration assays were performed using the conditioned media (CM) of neonatal cardiomyocytes from wild-type (WT), $eNOS^{-/-}$, and cardiac-specific eNOS transgenic (Tg) mice subjected to anoxia/reoxygenation (A/R). The role of eNOS signaling on SDF-1α expression in cardiomyocytes was investigated. The effects of eNOS levels in the heart on MSC migration toward the ischemic myocardium were further studied in vivo using mice subjected to myocardial ischemia and reperfusion (I/R). Our results demonstrated a pivotal role of host eNOS in MSC migration toward the ischemic myocardium, leading to improvement in cardiac function. The effect of eNOS was found to be mediated by an upregulation of SDF-1α expression via cGMP.

Materials and Methods

Animals

WT, $eNOS^{-/-}$, and enhanced green fluorescence protein transgenic (EGFP-Tg) mice of C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). Cardiomyocyte specific eNOS transgenic (eNOS-Tg, line 23) mice were obtained from Dr. Friedrich Brunner (University of Graz, Graz, Austria) [14]. The eNOS-Tg mice were backcrossed to C57BL/6 for more than 15 generations. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996).

Neonatal Mouse Cardiomyocyte Culture

Neonatal mouse cardiomyocyte culture was performed as previously described [7, 15, 16]. Briefly, ventricle myocardial tissues from WT, $eNOS^{-/-}$, and eNOS-Tg mice born within 24 hours were minced in Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution, and cardiomyocytes were dispersed in 22.5 μg/ml liberase blendzyme IV (Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com) at 37°C for 40 minutes. The isolated cells were preplated for 90 minutes to remove noncardiomyocytes. The cardiomyocytes were plated in 24-well plates, cultured in M199 medium containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 incubator and used for the experiments after 72 hours of culture.

Anoxia and Reoxygenation

The in vitro model of A/R used in this study was similar to that described previously [17]. Briefly, confluent beating mouse cardiomyocyte monolayers were exposed to anoxia for 60 minutes in phenol red-free M199 medium containing 1% FBS and then reoxygenated for 6 hours. Anoxic conditions were obtained by perfusion of a small humidified plexiglass chamber with 100% N_2. Oxygen concentration inside the chamber was confirmed to be 0.0% by an oxygen sensor. Control conditions consisted of cardiomyocytes treated identically except that they were cultured under normal normoxic (N/N) conditions, rather than the A/R protocol. The CM from A/R or N/N-treated cardiomyocytes were collected and served as the chemoattractant for the in vitro transendothelial migration assays.

Isolation and Culture of Bone Marrow MSCs

Bone marrow cells were isolated from femurs of adult male WT or EGFP-Tg mice. MSCs were maintained in Dulbecco's modified Eagle's medium consisting 1 g/L glucose, 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Burlington, ON, http://www.invitrogen.com). The nonadherent cell population was removed after 48 hours, and the adherent MSC layer was washed twice with fresh medium. The cells were passed and cultured for three generations. WT MSCs were used for in vitro transendothelial migration assays, and EGFP-Tg MSCs were used for in vivo migration assays.

Flow Cytometry

The immunophenotype of cultured MSCs was analyzed using a FACScan (BD Biosciences, Mississauga, ON, http://www.bdbiosciences.com). The MSCs were incubated with fluorescein isothiocyanate-conjugated monoclonal antibodies against mouse CD31 and CD90, and phycoerythrin (PE)-conjugated antibodies against mouse CD45, CD73, CD105, and CXCR4. Mouse IgG1 and IgG2a were used as isotype controls. All antibodies were purchased from eBIOSCIENCE (San Diego, CA, http://www.ebioscience.com).

In Vitro Differentiation Assay

Osteogenic and adipogenic differentiation assays were performed similar to a previous report with modifications [18]. For osteogenic differentiation, subconfluent MSCs were cultured in 24-well plates in osteogenic medium containing 10 nM dexamethasone, 0.2 mM ascorbic acid, and 10 mM β-glycerolphosphate. The medium was replaced every 3 days for 14 days. Cultures were fixed in ice–cold 70% ethanol, and stained for Alizarin red (40 mM, pH 4.1; Sigma, Oakville, ON, http://www.sigmaaldrich.com). For adipogenic differentiation, subconfluent MSCs were cultured on cover glass in complete medium supplemented with 0.5 μM hydrocortisone, 0.5 mM isobutylmethylxanthine, and 60 μM indomethacin. The medium was replaced every 3 days for 21 days. Cells were fixed in 10% formalin and stained with fresh oil red O solution and hematoxylin (Fisher Scientific, Ottawa, ON, http://www.fisherscientific.com).

In Vitro MSC Transendothelial Migration

MSC transendothelial migration was assessed as previously described [18]. Mouse coronary microvascular endothelial cells (CMEC) were isolated using Dynabeads (Invitrogen) with an anti-CD31 antibody as described previously with modifications [6]. Following collection, CMECs coupled to the beads were separated with a magnetic separator (StemCell Technologies, Vancouver, BC, http://www.stemcell.com) and incubated in endothelial basal medium-2 media supplemented with EGM™-2 (Endothelial cell growth medium-2 bulletKit®: Cedarlane, Burlington, ON, http://www.cedarlanelabs.com). To assess the purity of endothelial cells, cells were incubated with the fluorescence labeled acetylated low-density lipoprotein (DiI-Ac-LDL, 10 μg/ml for 4 hours at 37°C) and counterstained with Hoechst 33342. CMECs were used at passage 2, and subcultured into Transwell cell culture inserts (8-μm diameter pores). Once endothelial cells reached confluence on the inserts, 5×10^4 MSCs were added to the upper compartment of the endothelial cell monolayer, whereas CM from A/R or N/N treated cardiomyocytes were placed in the basal compartment. The cells were coincubated for 6 hours at 37°C and 5% CO_2. The number of cells that migrated to the basal compartment was quantified by CyQuant Cell Proliferation Kit (Invitrogen) as per manufacturer's instructions.

Migration from confluent endothelial monolayer without MSCs in the upper compartment was <0.3% during stimulation by A/R CM.

In Vivo Myocardial I/R

Male WT, eNOS$^{-/-}$, and eNOS-Tg mice (2-3 months old) were anesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg). Mice were intubated and artificially ventilated with a respirator (SAR-830; CWE Inc., Ardmore, PA, http://www.cwe-inc.com). Subsequently, a left intercostal thoracotomy was performed, and the left side of the heart was exposed. The pericardium was opened, and the left coronary artery was occluded by positioning a suture (8-0) around it together with a PE-10 tubing similar to our previous report [19]. After 45 minutes, the PE-10 tubing was removed to allow reperfusion. Third-generation cultured EGFP$^+$ MSCs (5×10^5 in 0.1 ml mouse serum) were intravenously injected through the tail vein 30 minutes after reperfusion. The hearts were harvested 24 hours after reperfusion for immunofluorescent staining, real-time polymerase chain reaction (PCR), and Western blot analysis.

Immunofluorescent Staining

To identify EGFP$^+$ MSCs migration to the heart, frozen sections of WT, eNOS$^{-/-}$, and eNOS-Tg hearts were incubated with an anti-α-actinin antibody (Sigma) overnight at 4°C followed by a rhodamine-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). Hoechst 33342 was used for nuclear staining. Sections were examined under a Zeiss fluorescence microscope. EGFP$^+$ MSCs in the ischemic area were quantified.

Assessment of Cardiac Function

WT and eNOS$^{-/-}$ male mice (2-3 months old) were subjected to 45 minutes of myocardial ischemia followed by 5 days of reperfusion. A total of 5×10^5 MSCs in 0.1 ml mouse serum or mouse serum alone (vehicle) were injected through the tail vein 30 minutes after beginning reperfusion. Sham-operated mice served as controls. The mice were sacrificed 5 days after surgery, and cardiac function was assessed using a Langendorff system as previously described [20].

Real-Time PCR Analysis

Total RNA was extracted from the left ventricle (LV) myocardium or cultured cardiomyocytes with Trizol reagent (Invitrogen). cDNA was synthesized using moloney murine leukemia virus reverse transcriptase with random primers (Invitrogen). Real-time PCR was conducted using SYBR® Green PCR Master Mix as per manufacture's instructions (Eurogentec, San Diego, CA, http://www.eurogentec.be). 28S rRNA (a house keeping gene) was used as a loading control. The primer sequences were as follows: SDF-1α upstream: 5′ TCA GCC TGA GCT ACC GAT GC 3′; downstream: 5′ TCT TCA GCC GTG CAA CAA TC 3′; 28S rRNA upstream: 5′ TTG AAA ATC CGG GGG AGA G 3′; downstream: 5′ ACA TTG TTC CAA CAT GCC AG 3′. Samples were amplified for 34 cycles using a real-time PCR machine (Opticon™, Bio-Rad, Mississauga, ON, http://www.bio-rad.com). Relative SDF-1α mRNA expression is presented as SDF-1α/28S ratios.

Measurement of SDF-1α Protein Expression

SDF-1α protein expression in CM and in heart tissue subjected to I/R was assessed by Quantitine Mouse SDF-1α enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Burlington, ON, http://www.rndsystems.com) according to the manufacturer's recommendations. Heart tissues were homogenized in phosphate buffered saline, and the total protein concentration was measured by Lowry assay. Ten micrograms protein per sample was used for ELISA analysis.

Measurement of NO Production

NO production was measured in live cardiomyocytes using the membrane-permeable fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (Invitrogen) as we have recently described [19]. The fluorescence produced by NO was measured using 485 nm excitation and 515 nm emission (SpectraMax M5; Molecular Devices Corp., Union City, CA, http://www.moleculardevices.com). The results are expressed in arbitrary fluorescence units.

Inhibition of SDF-1α and CXCR4 Interaction

Cultured neonatal cardiomyocytes were treated with murine SDF-1α small interfering RNA (siRNA) to knockdown SDF-1α mRNA expression (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) using the TransMessenger Transfection Reagent Kit (Qiagen, Mississauga, ON, http://www1.qiagen.com). Following treatment, SDF-1α mRNA expression was measured by real-time PCR to determine the efficiency of transfection. Cardiomyocytes treated with scrambled siRNA (Santa Cruz Biotechnology Inc.) were used as controls. The CM was collected after A/R treatment and served as a chemoattractant for the in vitro transendothelial migration assay.

The effect of SDF-1α on MSC migration after I/R in vivo was investigated by using an anti-CXCR4 neutralizing antibody (20 μg/ml, R&D Systems Inc.) to block SDF-1α/CXCR4 binding. EGFP$^+$ MSCs were treated with anti-CXCR4 antibody for 30 minutes before they were injected into mice subjected to myocardial ischemia and reperfusion. The presence of EGFP$^+$ MSC in the ischemic region was assessed using fluorescence microscopy.

Statistical Analysis

All data are expressed as mean ± SEM. For analysis of differences between two groups, Student's t test was performed. For multiple group comparisons, one-way or two-way analysis of variance followed by Bonferroni post hoc test was employed. $p < .05$ was considered statistically significant.

Results

Characterization of Cultured MSCs

MSCs were isolated from the bone marrow and cultured for three generations before they were harvested for all subsequent experiments. The cultured MSCs were CD31 and CD45 negative (supporting information Fig. S1B), but CD73, CD90, and CD105 positive with a purity of >94% (supporting information Fig. S1C, S1D). These staining characteristics represent the classical phenotype of MSCs [21]. More than 99% of these cells were CXCR4 positive (supporting information Fig. S1E). The MSCs were multipotent as they were able to differentiate toward adipocytes and osteoblasts after 21 days culture in the respective differentiation media (supporting information Fig. S2A, S2B).

eNOS Promotes MSC Transendothelial Migration

To determine the effect of eNOS in cardiomyocytes on MSC transendothelial migration, neonatal cardiomyocytes from WT, eNOS$^{-/-}$, and eNOS-Tg mice were subjected to A/R or normoxia/normoxia (N/N), and the CM were collected and served as chemoattractant for the in vitro transendothelial migration assay. Purity of CMECs was 93% as assessed by DiI-Ac-LDL positivity (supporting information Fig. S3). Under N/N condition, MSC migration did not show any significant difference among the three groups (Fig. 1A). Following an A/R challenge, MSC migration was significantly increased by media collected from WT cardiomyocytes compared with N/N condition ($p < .05$; Fig. 1A). Moreover, migration was significantly decreased by eNOS$^{-/-}$ CM and

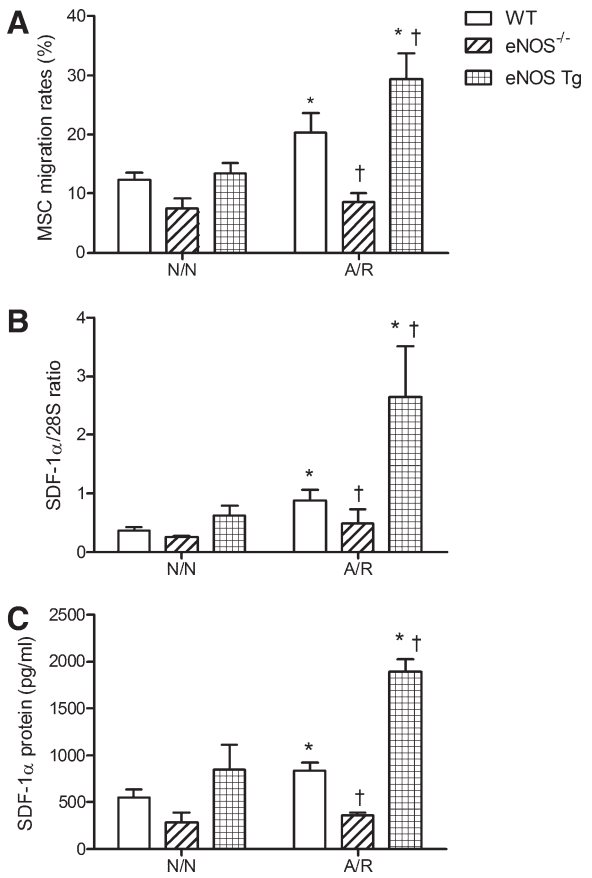

Figure 1. eNOS upregulates SDF-1α expression and promotes in vitro MSC transendothelial migration induced by A/R. **(A):** The conditioned media (CM) from A/R- or N/N-treated WT, eNOS$^{-/-}$, or eNOS-Tg cardiomyocytes were collected and served as the chemoattractant for the in vitro transendothelial migration assay. **(B):** SDF-1α mRNA levels were determined by real-time polymerase chain reaction. **(C):** SDF-1α protein expression in the CM. Data are mean ± SEM from 3 to 5 independent experiments. *, $p < .05$ versus corresponding N/N controls. †, $p < .05$ versus WT A/R. eNOS-Tg, cardiomyocyte specific eNOS overexpression. Abbreviations: A/R, anoxia and reoxygenation; eNOS, endothelial nitric oxide synthase; MSC, mesenchymal stromal cells; N/N, normal normoxic; SDF-1α, stromal cell-derived factor-1α; Tg, transgenic; WT, wild-type.

significantly increased by eNOS-Tg CM compared with WT CM ($p < .05$; Fig. 1A). These results indicate that eNOS promotes MSC transendothelial migration induced by an A/R challenge on cardiomyocytes.

eNOS Enhances SDF-1α Expression Induced by A/R

SDF-1α has previously been shown to be increased in cardiomyocytes and play an important role in bone marrow stem cell recruitment to the heart [12]. To determine whether SDF-1α was involved in the enhanced MSC migration by eNOS, SDF-1α mRNA expression in the neonatal cardiomyocytes and SDF-1α protein in the CM was assessed by real-time PCR and ELISA, respectively. Under N/N conditions, SDF-1α mRNA and protein levels were not significantly altered among WT, eNOS$^{-/-}$, and eNOS-Tg cardiomyocytes (p = NS, Fig. 1B, 1C). Following A/R, SDF-1α mRNA and protein expression was significantly increased in WT and eNOS-Tg cardiomyocytes ($p < .05$; Fig. 1B, 1C) but not in eNOS$^{-/-}$ cardiomyocytes compared with N/N. Furthermore, A/R-induced SDF-1α expression was significantly decreased in eNOS$^{-/-}$ but increased in eNOS-Tg cardiomyocytes when compared with WT following A/R ($p < .05$; Fig. 1B, 1C).

Effects of NO on SDF-1α Expression and MSC Migration

To clarify the role of NO in SDF-1α expression, WT cardiomyocytes were treated with a NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME, 300 μM). L-NAME significantly inhibited NO production, SDF-1α mRNA, and protein levels in WT cardiomyocytes during N/N and A/R treatment ($p < .05$; Fig. 2A–2C). L-NAME treatment also decreased SDF-1α mRNA expression in eNOS Tg cardiomycytes in both N/N and A/R conditions (Fig. 2D). To further demonstrate the effects of NO on SDF-1α expression, the NO donor diethylenetriamine-NO (DETA-NO) was used. DETA-NO treatment (2 μM) significantly increased SDF-1α expression in eNOS$^{-/-}$ cardiomyocytes ($p < .05$; Fig. 2E, 2F). Finally, inhibition of NO production by L-NAME significantly decreased MSC transendothelial migration induced by A/R-CM from WT cardiomyocytes ($p < .05$; Fig. 2G).

SDF-1α siRNA Inhibits MSC Transendothelial Migration

To determine whether SDF-1α mediates MSC migration by eNOS, SDF-1α siRNA was used to inhibit SDF-1α expression in the cultured neonatal WT cardiomyocytes. Following SDF-1α siRNA treatment, SDF-1α mRNA levels were decreased by 80% (Fig. 3A). Furthermore, SDF-1α siRNA significantly decreased MSC migration induced by A/R ($p < .05$; Fig. 3B).

eNOS Increases SDF-1α Expression Through a cGMP-Dependent Mechanism

A major signaling pathway by which NO exerts its biological effects is through activation of soluble guanylyl cyclase and subsequent production of cGMP. To determine whether eNOS regulates SDF-1α expression by a cGMP-dependent mechanism, a soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was employed. Treatment with ODQ (100 μM) significantly decreased A/R-induced SDF-1α mRNA and protein expression in WT cardiomyocytes ($p < .05$; Fig. 4A, 4B). Furthermore, treatment with a cGMP analogue, 8-Br-cGMP (2 mM), significantly increased SDF-1α mRNA and protein expression in eNOS$^{-/-}$ cardiomyocytes following A/R ($p < .05$; Fig. 4C, 4D). These results suggest that eNOS increases cardiomyocyte SDF-1α expression through activation of soluble guanylyl cyclase and production of cGMP.

In Vivo SDF-1α Expression and MSC Migration Following Myocardial I/R

Cardiac SDF-1α expression in vivo was examined in mice subjected to 45 minutes of myocardial ischemia followed by 24 hours of reperfusion (I/R). Myocardial SDF-1α mRNA and protein levels were significantly increased in the ischemic region of WT mice compared with sham-operated mice ($p < .05$; Fig. 5A, 5B). Moreover, SDF-1α expression levels were decreased in eNOS$^{-/-}$, but further increased in eNOS-Tg compared with WT hearts ($p < .05$). No significant difference in SDF-1α expression was observed following sham surgery among WT, eNOS$^{-/-}$, and eNOS-Tg (p = NS, Fig. 5A, 5B). These results are consistent with the in vitro data and support the notion that eNOS enhances myocardial SDF-1α expression following myocardial I/R.

To assess in vivo MSC migration to the ischemic myocardium, WT mice were subjected to 45 minutes of myocardial ischemia and 24 hours of reperfusion (I/R). Thirty minutes

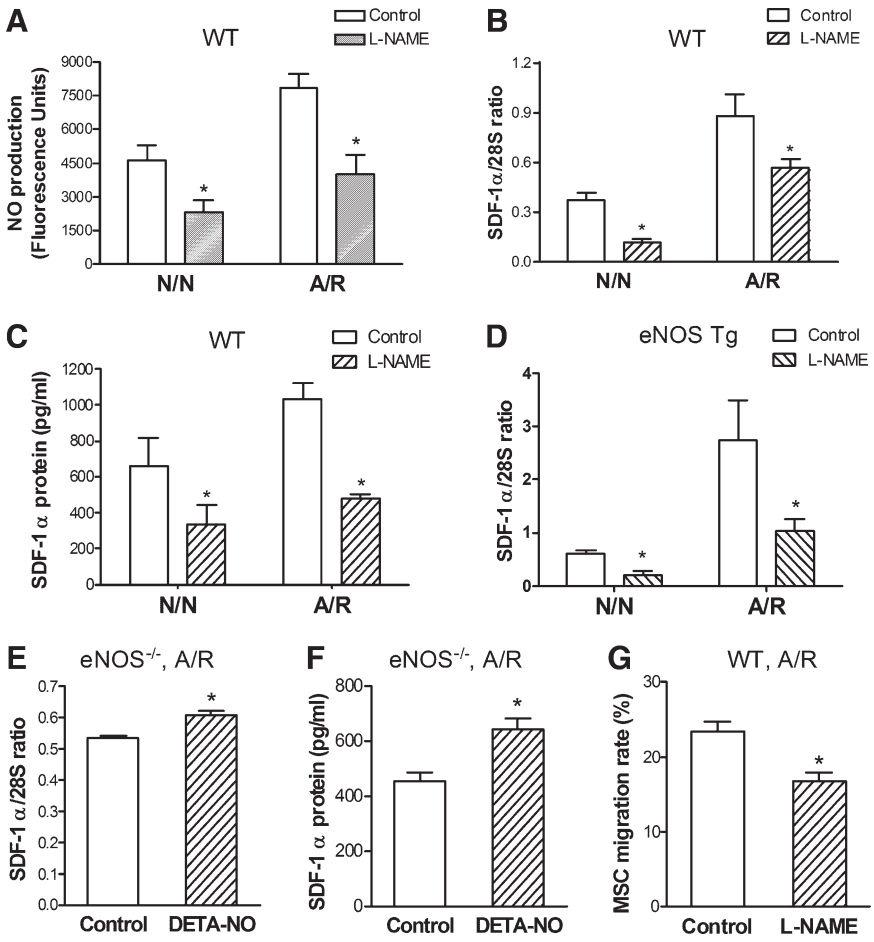

Figure 2. Effects of NO on A/R-induced SDF-1α expression and MSC migration. NOS inhibitor L-NAME (300 μM) decreased NO production **(A)** SDF-1α mRNA **(B)** and SDF-1α protein expression **(C)** both in N/N and A/R conditions. NO donor, DETA-NO (2 μM) increased SDF-1α mRNA **(D)** and protein **(E)** expression during A/R in eNOS$^{-/-}$ cardiomyocytes. **(F):** MSC transendothelial migration induced by conditioned media from A/R-treated WT cardiomyocytes was decreased by L-NAME. Data are mean ± SEM from 3 to 5 independent experiments. *, $p < .05$ versus corresponding controls. Abbreviations: A/R, anoxia and reoxygenation; DETA-NO, diethylenetriamine-NO; eNOS, endothelial nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; MSC, mesenchymal stromal cells; NO, nitric oxide; N/N, normal normoxic; SDF-1α, stromal cell-derived factor-1α; Tg, transgenic; WT, wild-type.

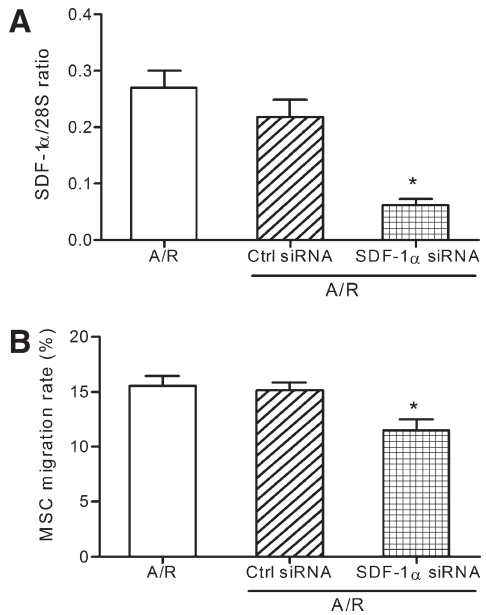

Figure 3. Effects of SDF-1α siRNA on SDF-1α mRNA expression and MSC transendothelial migration. A/R-induced SDF-1 mRNA expression in wild-type (WT) cardiomyocytes **(A)** and MSC migration toward WT conditioned media **(B)** were decreased by SDF-1α siRNA. Data are mean ± SEM of three independent experiments. *, $p < .05$ versus control. Abbreviations: A/R, anoxia and reoxygenation; ctrl, control; MSC, mesenchymal stromal cells; SDF-1α, stromal cell-derived factor-1α; siRNA, small interfering RNA.

after reperfusion, EGFP$^+$ MSCs were administered via a tail vein injection. The number of EGFP$^+$ MSCs migrated from the circulation to the ischemic myocardium was quantified using a fluorescence microscope 24 hours following reperfusion. EGFP$^+$ MSCs were observed in the ischemic region in WT, eNOS$^{-/-}$, and eNOS Tg hearts (Fig. 6A–6C). WT mice subjected to I/R without MSC treatment served as negative controls (Fig. 6D). Cultured EGFP$^+$ MSCs showed strong green fluorescence (Fig. 6E). The number of EGFP$^+$ MSCs was significantly decreased in eNOS$^{-/-}$ but increased in eNOS-Tg compared with WT hearts ($p < .05$; Fig. 6F). These results support the notion that eNOS in the host myocardium promotes MSC migration toward the ischemic myocardium in vivo.

To confirm the role of SDF-1α/CXCR4 binding in MSC migration to the ischemic myocardium, EGFP$^+$ MSCs were treated by an anti-CXCR4 neutralizing antibody before they were administered intravenously to the WT mice. Following 24 hours of reperfusion, the number of EGFP$^+$ MSCs that migrated into the ischemic myocardium was quantified under a fluorescence microscope. Neutralization of CXCR4 significantly decreased the number of EGFP$^+$ MSCs in the ischemic myocardium compared with vehicle treatment ($p < .05$; Fig. 6F). The data support an important role of SDF-1α/CXCR4 interaction in MSC migration to the myocardium following I/R.

MSC Transplantation and Cardiac Function Following Myocardial I/R

To study the role of myocardial eNOS in cardiac function following MSC transplantation, WT and eNOS$^{-/-}$ mice were

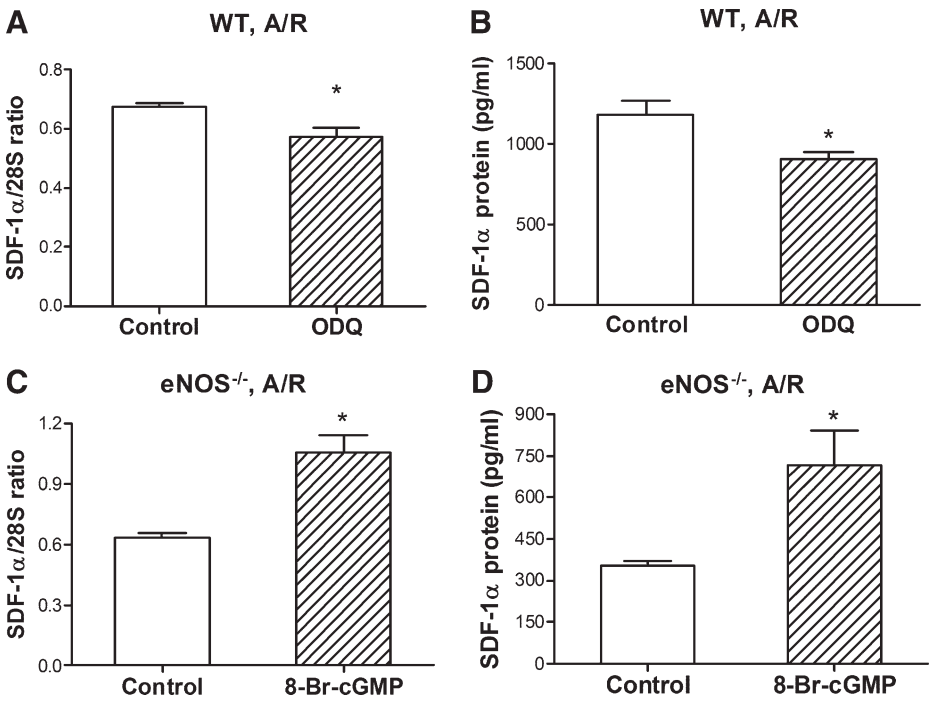

Figure 4. Involvement of soluble guanylyl cyclase and cGMP in A/R-induced SDF-1α expression in WT and eNOS$^{-/-}$ cardiomyocytes. Guanylyl cyclase inhibitor ODQ (100 μM) decreased SDF-1α mRNA **(A)** and protein **(B)** levels induced by A/R in WT cardiomyocytes. cGMP analogue 8-Br-cGMP (2 mM) increased SDF-1 mRNA **(C)** and protein **(D)** expression induced by A/R in eNOS$^{-/-}$ cardiomyocytes. ODQ and 8-br-cGMP were added to the culture media before A/R. Data are mean ± SEM from three independent experiments *, $p < .05$ versus controls. Abbreviations: A/R, anoxia and reoxygenation; eNOS, endothelial nitric oxide synthase; ODQ, oxadiazolo[4,3-a]quinoxalin-1-one; SDF-1α, stromal cell-derived factor-1α; WT, wild-type.

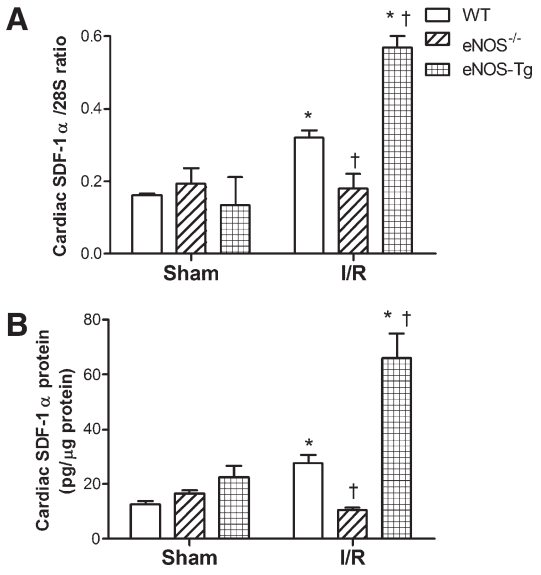

Figure 5. Myocardial SDF-1α expression following ischemia and reperfusion (I/R) in WT, eNOS$^{-/-}$ and eNOS-Tg mice. (**A**): SDF-1α mRNA levels assessed by real-time RT-PCR. (**B**): SDF-1α protein levels determined by ELISA. Sham-operated mice served as controls. Data are mean ± SEM from 5 to 6 mice per group. *, $p < .05$ versus corresponding sham. † $p < .05$ versus WT I/R. Abbreviations: eNOS, endothelial nitric oxide synthase; I/R, ischemia/reperfusion; SDF-1α, stromal cell-derived factor-1α; Tg, transgenic; WT, wild-type.

subjected to myocardial I/R. MSCs were administered via a tail vein injection 30 minutes after reperfusion. Five days following reperfusion, cardiac function was assessed using a Langendorff preparation. This time point after I/R was chosen because functional improvement in mice takes about 3 days to occur following MSC transplantation [22]. In WT hearts, myocardial I/R significantly decreased the rate of contraction and relaxation, and heart work compared with sham operation ($p < .05$; Fig. 7A–7C). MSC transplantation resulted in a significant improvement in the rate of contraction and relaxation, and heart work ($p < .05$). In eNOS$^{-/-}$ hearts, however, the decreased rate of contraction and relaxation, and heart work induced by myocardial I/R were not significantly improved by MSC transplantation (p = NS, Fig. 7A–7C). Heart rate was not different among any study groups (p = NS, Fig. 7D). In agreement with eNOS-induced MSC migration observed in vitro and in vivo, these results suggest that eNOS in the host myocardium is essential in the improvement of cardiac function by MSC transplantation following myocardial I/R.

eNOS protein levels in MSCs, adult LV myocardium, and cultured neonatal cardiomyocytes from WT mice were analyzed by Western blotting. Results showed that eNOS protein in MSCs was very low or barely detectable compared with the adult LV myocardium and cultured neonatal cardiomyocytes ($p < .01$; Fig. 7E, 7F). The low levels of eNOS in MSCs may explain the lack of improvement of cardiac function in eNOS$^{-/-}$ mice following MSC treatment (Fig. 7A–7C) and further support an essential role of host eNOS in MSC migration to the ischemic myocardium.

Discussion

In this study, we showed for the first time that eNOS expression in the host myocardium promotes migration of intravenously delivered MSCs to the ischemic myocardium. The effects of eNOS on MSC migration are mediated in part through upregulation of SDF-1α, leading to increased SDF-1α/CXCR4 coupling and MSC homing to the myocardium. We also demonstrated that the enhanced expression of SDF-1α by eNOS in cardiomyocytes during A/R is dependent on activation of soluble guanylyl cyclase and subsequent production of cGMP. Furthermore, our data showed that increased

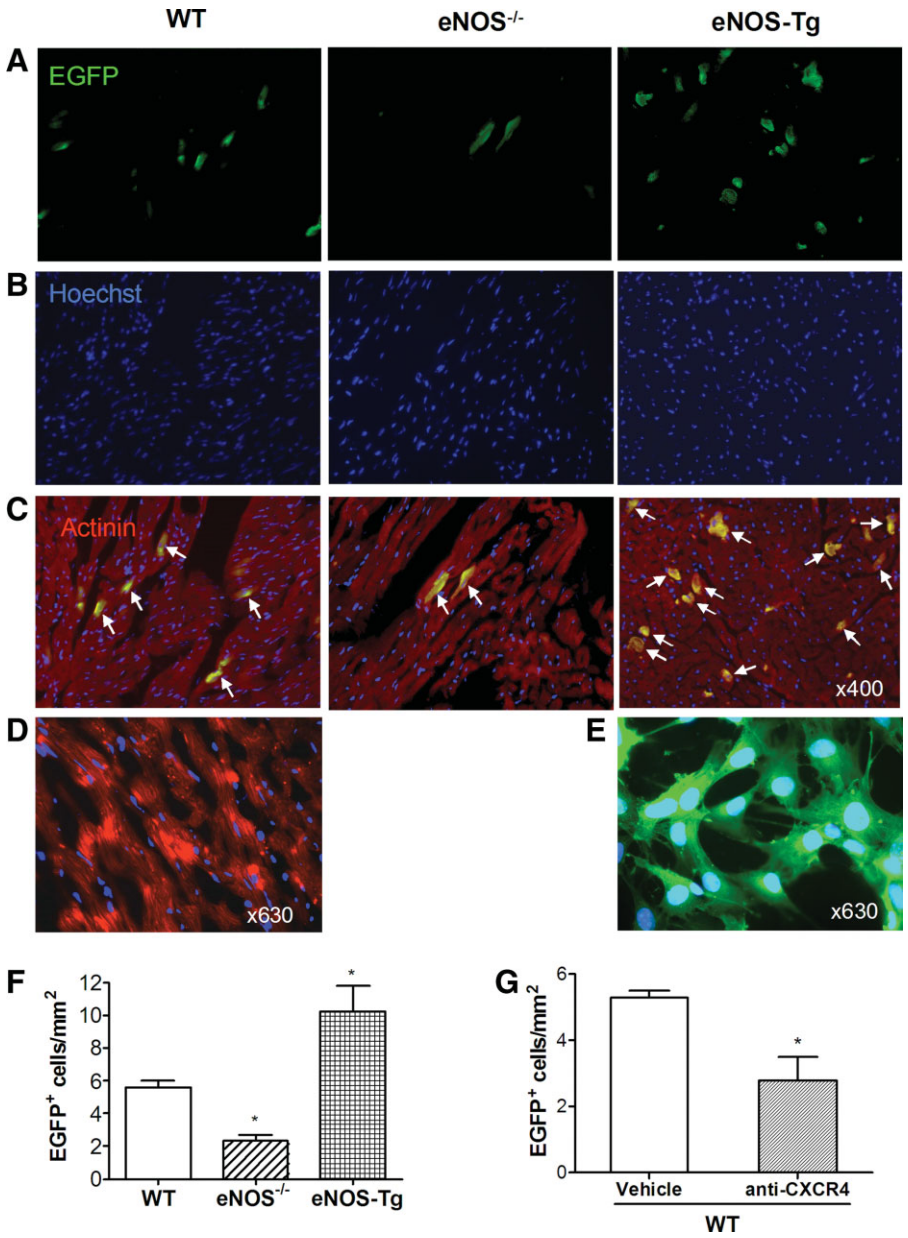

Figure 6. In vivo migration of $EGFP^+$ mesenchymal stromal cells (MSCs) toward the myocardium following ischemia and reperfusion (I/R). **(A–C):** WT, $eNOS^{-/-}$ and eNOS-Tg mice were subjected to 45 minutes of ischemia followed by 24 hours of reperfusion. Frozen sections of the hearts were stained with α-actinin (cardiomyocytes, red) and Hoechst 33342 (nuclei, blue). **(D):** WT mice subjected to I/R without MSC treatment served as negative controls (no green fluorescence). **(E):** Cultured $EGFP^+$ MSCs on cover glasses showed strong green fluorescence. **(F):** Quantification of $EGFP^+$ cells per mm^2 heart tissue. **(G):** Effects of CXCR4 neutralization on in vivo migration of $EGFP^+$ MSCs to the WT hearts following I/R. $EGFP^+$ MSCs were incubated with a neutralizing antibody anti-CXCR4 before intravenously injected to the WT mice following myocardial I/R. MSCs without neutralizing antibody treatment served as controls. Data are mean ± SEM from 5 to 8 mice per group. *, $p < .05$ versus WT or controls. Original magnifications: **(A–C)**, ×400; **(D–E)**, ×630. Abbreviations: EGFP, enhanced green fluorescence protein; eNOS, endothelial nitric oxide synthase; Tg, transgenic; WT, wild-type.

MSC migration and homing to the ischemic myocardium contribute to improvement of cardiac function following I/R. Our study suggests that eNOS expression in the host myocardium is essential in MSC migration and improvement of cardiac function following myocardial I/R.

Cardiomyocytes constitutively express eNOS under normal physiological conditions [19]. NO production from eNOS decreases myocardial oxygen consumption, enhances cardiac relaxation, and protects cardiomyocytes from apoptosis [23]. Cardiomyocyte specific eNOS overexpression in mice decreases LV remodeling and improves cardiac function post MI [24]. Furthermore, it has been demonstrated that eNOS is important for the mobilization of stem and progenitor cells from the bone marrow [8]. However, how myocardial eNOS levels affect MSC homing to the heart was not known. This study provided several lines of evidence that eNOS expression in cardiomyocytes promotes MSC migration toward the ischemic myocardium. First, A/R-induced MSC transendothelial migration was decreased by CM collected from $eNOS^{-/-}$ cardiomyocytes, and increased by CM collected from eNOS overexpressing cardiomyocytes. Second, pharmacological inhibition of NOS activity using L-NAME significantly inhibited A/R-induced MSC transendothelial migration by CM collected from WT cardiomyocytes. Finally, in vivo MSC migration to the heart following myocardial I/R was decreased in $eNOS^{-/-}$ but increased in cardiomyocyte specific eNOS overexpressing mice. These data suggest that eNOS-derived NO production in cardiomyocytes is essential for MSC migration to the ischemic myocardium.

SDF-1α, a member of the chemokine CXC subfamily, plays an important role in stem cell migration, proliferation, and survival [9, 25]. SDF-1α via binding to its cognate receptor CXCR4 promotes stem cell homing to the infarcted myocardium, and activation of SDF-1α/CXCR4 signaling protects

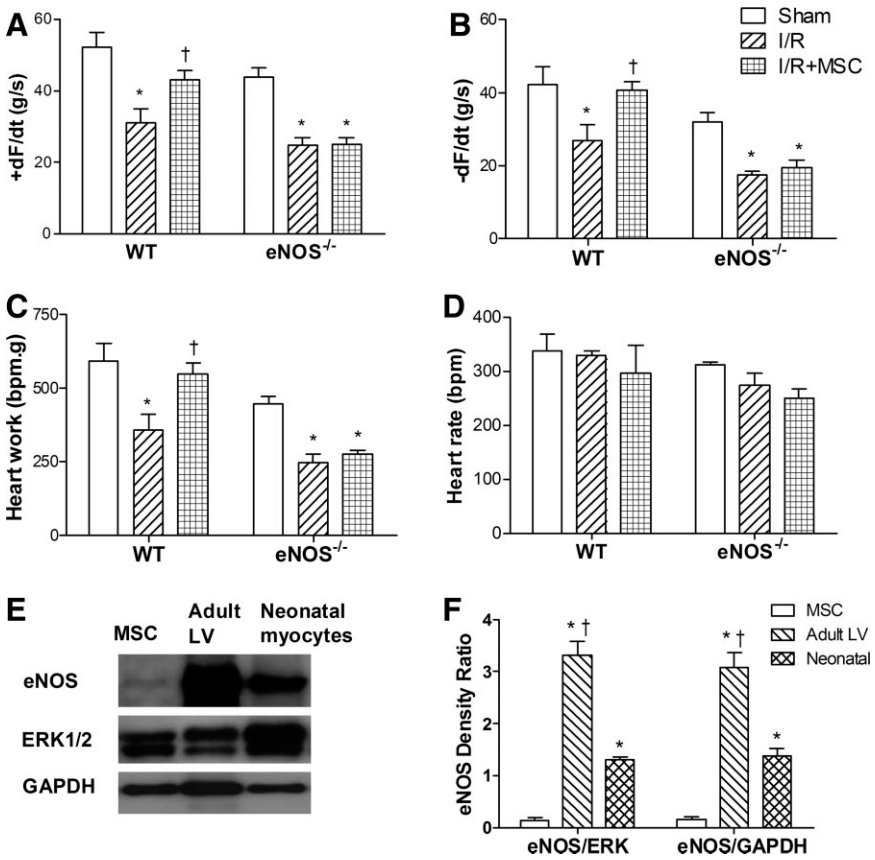

Figure 7. Cardiac function and eNOS expression. **(A–D)**: Cardiac function in WT and eNOS$^{-/-}$ mice after 45 minutes of myocardial ischemia followed by 5 days of reperfusion with or without MSC administration. Sham-operated mice served as controls. Contractile function of the heart was determined using a Langendorff system. Changes in rate of contraction ($+dF/dt_{max}$, **A**), and relaxation ($-dF/dt_{min}$, **B**), heart work **(C)** and heart rate **(D)** were determined. Data are mean ± SEM from 5 to 7 mice per group. *, $p < .05$ versus corresponding sham. † $p < .05$ versus WT I/R. **(E–F)**, eNOS protein expression in MSCs, adult LV myocardium, and cultured neonatal cardiomyocytes from WT mice. **(E):** Representative Western blots. GAPDH and total ERK1/2 were used as loading controls. **(F):** Quantitative analysis of optical density. Data are mean ± SEM from $n = 3$ in each group. *, $p < .001$ versus MSC. † $p < .001$ versus neonatal cardiomyocytes. Abbreviations: eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I/R, ischemia/reperfusion; LV, left ventricle; MSC, mesenchymal stromal cells; WT, wild-type.

against myocardial I/R injury [26, 27]. A recent study showed that eNOS promotes c-kit$^+$ stem cell adhesion to the vascular endothelium in a cremaster muscle preparation via SDF-1/CXCR4 signaling [28]. In this study, we demonstrated that eNOS enhanced MSC transendothelial migration. To further investigate the mechanisms by which eNOS exerts its effects, we studied the SDF-1α/CXCR4 pathway. Using both in vitro and in vivo approaches, we demonstrated that eNOS enhances myocardial SDF-1α expression leading to increased SDF-1α/CXCR4 interaction and MSC migration toward the myocardium during I/R. Apart from SDF-1α, other chemokines and growth factors such as RANTES and insulin-like growth factor-1 also promote MSC migration [29]. Whether eNOS enhances the effects of these factors on MSC migration remains to be investigated.

The mechanisms by which eNOS-derived NO regulates SDF-1α production are not known. NO produced from eNOS exerts its biological effects through both cGMP-dependent and cGMP-independent signaling pathways. In this study, A/R-induced SDF-1α expression was decreased by the soluble guanylate cyclase inhibitor, ODQ in WT cardiomyocytes and increased by the cGMP analogue, 8-Br-cGMP in eNOS$^{-/-}$ cardiomyocytes. Our data demonstrated that NO production from eNOS increases SDF-1α expression at least in part via activation of soluble guanylyl cyclase and production of cGMP. NO has also been shown to upregulate CXCR4 expression in CD34$^+$ progenitor cells [30]. Thus, NO may promote the expression of both SDF-1α and CXCR4, leading to increased progenitor cell migration.

Transplantation of MSCs into the infarcted myocardium has been shown to improve cardiac function following MI or chronic ischemia in rats and dogs [31, 32]. Furthermore, overexpression of Akt1 in MSCs aimed at improving viability of transplanted MSCs in the infarcted myocardium enhances their therapeutic efficacy [33]. Although myocardial MSC transplantation improves cardiac function post-MI, the invasiveness of cell delivery may limit its clinical applications. Interestingly, MSCs are able to sense sites of tissue injury or inflammation, and migrate and engraft in damaged tissues. In this regard, intravenously administered MSCs have been shown to engraft the infarcted myocardium in animal models [34, 35]. More importantly, i.v. administration of MSCs improves cardiac function in patients with acute MI [36, 37]. Although the exact mechanisms are still not completely understood, soluble factors released from MSCs causing paracrine actions on the myocardium and angiogenesis are implicated [22, 38]. Furthermore, i.v. administration of MSCs overexpressing eNOS improves right ventricular remodeling caused by pulmonary hypertension, suggesting that eNOS in the donor MSCs is important for their beneficial effects [39]. In this study, we assessed the effects of host eNOS on MSC migration and cardiac function 5 days following I/R. Our data demonstrated that MSCs have very low or barely detectable eNOS protein expression compared with the adult myocardium, and that myocardial eNOS levels are important for the cardiac migration of intravenously administered MSCs. Furthermore, using a Langendorff preparation that eliminates potential confounding effects of loading conditions of the heart and reflex influences, we demonstrated that intravenously administered MSCs improved cardiac function in WT but not in eNOS$^{-/-}$ mice post myocardial I/R. The results suggest that the ability of MSCs to improve cardiac function following I/R depends on eNOS expression of the host, which promotes MSC migration toward the ischemic myocardium.

However, the role eNOS in long-term cardiac remodeling and function of ischemic hearts following MSC treatment requires further investigation.

Conclusion

In summary, this study demonstrated that eNOS expression in the host myocardium promotes MSC migration in the ischemic myocardium. The effects of eNOS on MSC migration are mediated in part through upregulation of SDF-1α via cGMP, leading to increased SDF-1α/CXCR4 coupling. Our data suggest that eNOS in the host myocardium is essential in MSC migration and improvement in cardiac function following i.v. delivery of MSCs during I/R. Our study may have implications in MSC treatment in conditions such as patients with diabetes, obesity, and hypercholesterolemia where the host eNOS activity is decreased, and myocardial MSC migration and regenerative/repair efficacy may be negatively affected.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Endothelial nitric oxide synthase promotes bone marrow stromal cell migration to the ischemic myocardium via upregulation of SDF-1α

Na Li, Xiangru Lu, Xiaohui Zhao, Fu-Li Xiang, Anargyros Xenocostas, Morris Karmazyn, Qingping Feng

Supplemental Data

Fig. S1. Flow cytometric analysis of cultured bone marrow stromal cells (MSCs). **A**. Isotype controls. MSCs were negative for CD31 and CD45 (**B**), but positive for CD90, CD73 and CD105 (**C, D**). Over 99% of MSCs expressed CXCR4 (**E**).

Fig. S2. MSC Differentiation *in vitro*. A. Osteogenic differentiation. MSCs were cultured in 24-well plate in osteogenic medium for 14 days. Calcium deposits in the extracellular matrix were stained red by Alizarin Red (original magnification x100). B. Adipogenic differentiation. MSCs were cultured on cover glasses in adipogenic medium for 21 days. Lipid vacuoles (arrows) were stained red by Oil Red O. Nuclei were stained by hematoxylin (original magnification x630).

Fig. S3. Uptake of fluorescence-labelled acetylated low-density lipoprotein (DiI-Ac-LDL) by the isolated coronary microvascular endothelial cells (CMECs). Cultured CMECs were incubated with 10 μg/mL DiI-Ac-LDL for 4 hours at 37°C. Cells positive for DiI-Ac-LDL (red) were endothelial cells. Nuclei were stained by Hoechst 33342 (blue, original magnification x200).

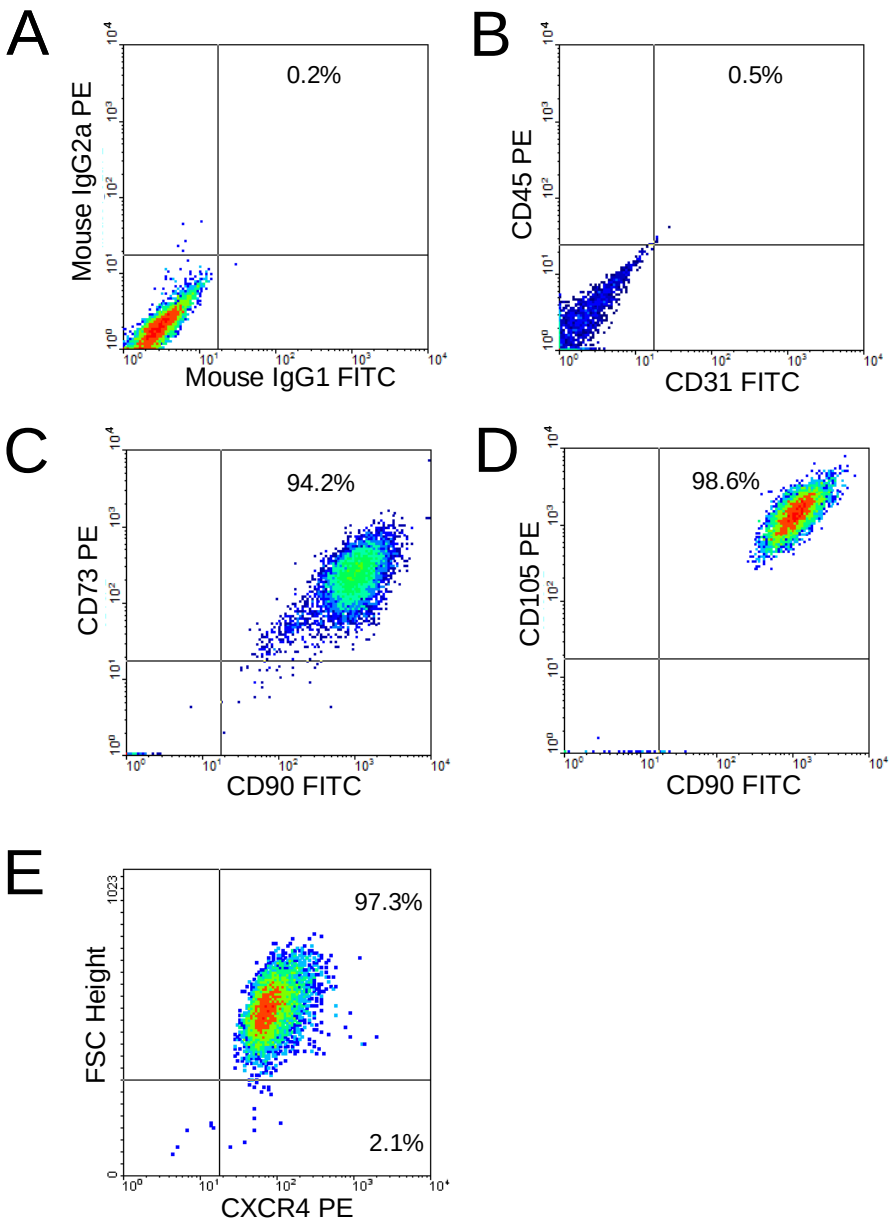
A
0.2%
Mouse IgG2a PE
Mouse IgG1 FITC
B
0.5%
CD45 PE
CD31 FITC
C
94.2%
CD73 PE
CD90 FITC
D
98.6%
CD105 PE
CD90 FITC
E
97.3%
2.1%
FSC Height
CXCR4 PE

Figure S1

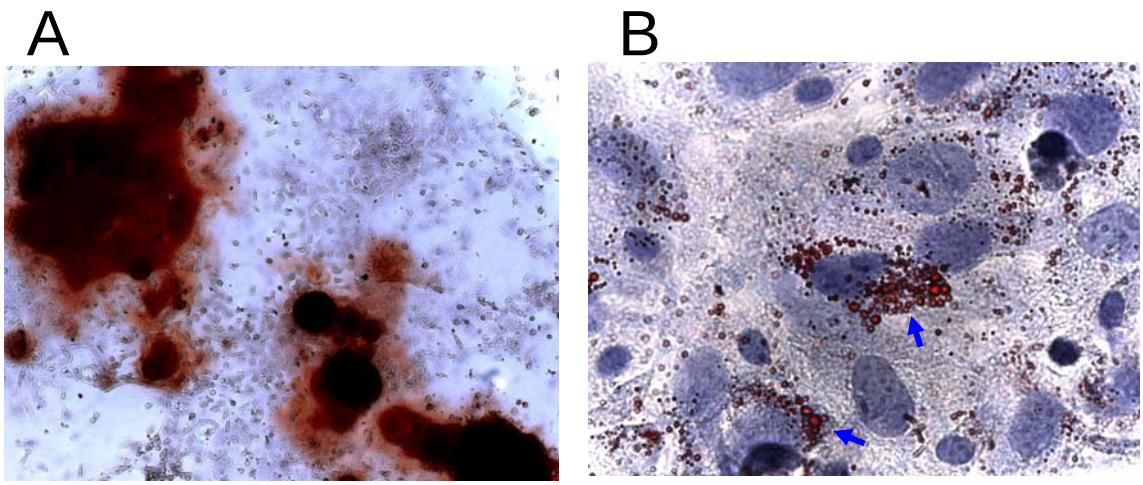

Figure S2

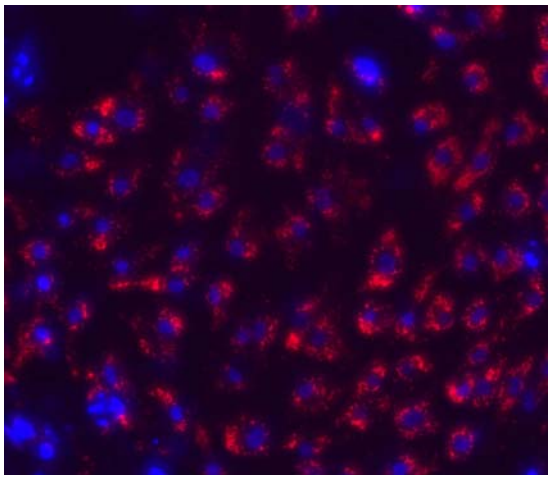

Figure S3