Netrin-1 Ameliorates Myocardial Infarction-Induced Myocardial Injury: Mechanisms of Action in Rats and Diabetic Mice

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Abstract

Netrin-1 is typically known as a neuronal guidance factor. Studies have reported the proangiogenic, anti-apoptotic, and antiinflammatory properties of Netrin-1. A critical role for Netrin-1 in ischemic organ damage, myocardial infarction (MI) in particular, has also been demonstrated, making Netrin-1 a potential therapeutic target for the treatment of cardiovascular diseases (CVDs). Mesenchymal stem cells (MSCs) have shown promising therapeutic efficacy in preclinical studies. However, limited clinical success was observed, mainly due to poor MSC survival. Given the reported beneficial impact of Netrin-1 in tissue repair and cell survival, we examined the effects of Netrin-1 in MSC therapy against MI-induced ischemic cardiac injury in rats and type 2 diabetic (T2D) mice. MSCs were isolated and Netrin-1-expressing MSCs were obtained by transduction with a Netrin-1-encoding retroviral vector. The Netrin-1-MSCs were then delivered intramyocardially to the infarct sites of rats and T2D mice with MI. Thirty days after MSC implantation, changes at the infarct area, level of collagen deposition, and cardiac hypertrophy were assessed. Molecular mechanisms underlying the effects of Netrin-1 were also investigated. Attenuated MI-induced myocardial dysfunction was observed after Netrin-1-MSC treatment. Protective effects of the Netrin-1-MSCs were attributable primarily to better MSC survival and migration, which is mediated by Netrin-1-induced phosphorylation of p44/42 mitogen-activated protein kinase. Netrin-1-stimulated nitric oxide production was also responsible, which could promote neovessel formation and progenitor cell mobilization *in vivo*. We report a protective role for Netrin-1 against MI-induced ischemic injuries, reinstating its promising potential as a therapeutic target for CVDs and, more importantly, for patients with CVD with coexisting diabetes.

Introduction

ACUTE MYOCARDIAL INFARCTION (MI) is a common form of ischemic cardiac disease. It is characterized by limited cardiac blood flow, which often leads to irreversible necrosis and scar formation within the ischemic myocardium. Heart failure as a result of MI is the primary cause of death throughout the world. Despite considerable progress that has been made to reduce age-adjusted cardiovascular mortality, restoration of myocardial function after ischemic insult remains a challenge. Cell-based therapies have shown promising therapeutic potential in post-MI myocardial recovery. In particular, results from preclinical studies have shown that intramyocardial delivery of mesenchymal stem cells (MSCs)

was able to reduce myocardial loss, improve left ventricular function, and prevent disease progression toward heart failure (Shake *et al.*, 2002; Zimmet and Hare, 2005; Zeng *et al.*, 2007).

MSCs are multipotent cells that could differentiate into tissues of mesenchymal origin (Fraser *et al.*, 2007; Schaffler and Buchler, 2007). Ample evidence has indicated a beneficial role for MSC therapy against MI and other organ injuries (Lee *et al.*, 2006; Billon *et al.*, 2007; Valina *et al.*, 2007; Heydarkhan-Hagvall *et al.*, 2008). Although the exact mechanisms via which MSCs restore the function of ischemic myocardium are still unidentified, it has been proposed that MSCs prevent myocardial loss via paracrine secretion of bioactive factors such as vascular endothelial growth factor

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(VEGF), monocyte chemotactic protein (MCP)-3, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) (Gnecchi *et al.*, 2005; Uemura *et al.*, 2006; Huang *et al.*, 2011; Figeac *et al.*, 2014). Disappointingly, however, MSC administration failed to exert any significant effects in post-MI patients (Scheubel *et al.*, 2003; Heeschen *et al.*, 2004; Walter *et al.*, 2007). This lack of therapeutic efficacy of MSC treatment in preliminary clinical trials is attributable mainly to inflammation, ischemia, and hypoxia, which are characteristics of the infarcted myocardium, resulting in poor MSC survival. Thus, approaches to enhance MSC viability during MSC implantation have been employed (Gomillion and Burg, 2006).

Netrin-1 belongs to the extracellular matrix protein family. It is a diffusible protein that is structurally similar to laminin, and has initially been identified as a chemotropic molecule responsible for axon guidance during development. An anti-apoptotic property of Netrin-1 was also unveiled on the discovery of its association with the deleted in colorectal cancer (DCC) receptor. Indeed, Netrin-1 has been identified as a survival factor, the absence of which could lead to DCC-mediated cell apoptosis under normal physiological circumstances (Arakawa, 2004). Additional roles for Netrin-1 in angiogenesis, vascular regeneration, and inflammation have since then been observed (Hebrok and Reichardt, 2004; Park *et al.*, 2004; Ly *et al.*, 2005; Chen *et al.*, 2012; Ranganathan *et al.*, 2013). More importantly, studies also reported a stimulatory impact of Netrin-1 on nitric oxide (NO) synthesis and secretion from both myocardial and endothelial cells (Ly *et al.*, 2005), implicating Netrin-1 as a potential therapeutic target for cardiovascular disorders.

Considering the dual benefits of Netrin-1 on cell survival and cardiac tissue regeneration, we combined MSC therapy together with Netrin-1 by MSC transduction with a Netrin-1 vector. The obtained Netrin-1-MSCs were then delivered to the site of ischemic injury after MI insult, and changes in cardiac function were subsequently evaluated. Furthermore, diabetes is a major risk factor for cardiovascular disorders and NO deficiency is now known to be correlated with aging, diabetes, and the onset of cardiovascular diseases (Yan *et al.*, 2009). Indeed, NO has been reported to promote peripheral glucose uptake in addition to its well-known vasodilatory effect in blood vessels (Vicent *et al.*, 2003). The positive impact of Netrin-1 on NO production may therefore be beneficial to post-MI recovery in patients with diabetes. Given the prevalence of type 2 diabetes (T2D) and the frequency of MI occurrence among diabetic individuals, we also evaluated the effects of Netrin-1-MSC therapy against MI-induced injury in diabetic mice.

Materials and Methods

Materials

All cell culture reagents were obtained from Gibco (Beijing, China). Collagenase V, tryptone, yeast extract, agar, ampicillin, ethidium bromide, 4',6-diamidino-2-phenylindole (DAPI), streptozocin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Polybrene, and Masson trichrome stain kit were purchased from Sigma-Aldrich (Dorset, UK); *EcoRI*, *XhoI*, T4 DNA ligase, *Taq* polymerase (2 U/ μ l), and dNTP mixture were from Takara Biotechnology (Dalian, China); and Moloney murine leukemia

virus (M-MLV) reverse transcriptase was purchased from Promega (Beijing, China). TRIzol and RNase inhibitor were obtained from TransGen Biotechnology (Beijing, China). Mayer's hematoxylin was purchased from AbCam (Hangzhou, China) and eosin Y was from Solarbio (Beijing, China). The plasmid Maxi kit and QIAquick gel extraction kit were from Qiagen (Shanghai, China). The Griess reagent kit was from Roche (Shanghai, China). Rabbit anti-human von Willebrand factor (vWF) antibody was obtained from Dako (Shanghai, China). Anti-phospho-p44/42 mitogen-activated protein kinase (MAPK), total p44/42 MAPK, and cNetrin-1 affinity-purified goat IgG antibodies were from R&D Systems (Shanghai, China). Agarose, Alexa 486-conjugated isolectin-B4, Alexa 488-conjugated wheat germ agglutinin (WGA) antibodies, fluorescein isothiocyanate (FITC)-conjugated secondary antibody, and horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (H+L) were from Invitrogen (Beijing, China). SuperSignal West Pico chemiluminescence substrate was purchased from Pierce Biotechnology/Thermo Fisher Scientific (Rockford, IL). PCR primers were from AuGCT DNA-SYN Biotechnology (Beijing, China) and standard PCR was carried out with a thermal RT-PCR cycler (Bio-Rad, Shanghai, China). All animals were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). Procedures performed in the present study were in compliance with the regulations of the Tianjin Committee on the Use and Care of Laboratory Animals and the overall project protocol was approved by the Animal Ethics Committee of Nankai University (Tianjin, China).

Mouse models of T2D and MI

Male C57BL/6J mice (3–4 weeks old; body weight, 12–14 g) were put on a high-fat diet (55% calories from fat) for 6 weeks. At week 8, T2D was induced by a single injection of 1% streptozocin (STZ, 85 mg/kg body weight). An intraperitoneal glucose tolerance test (IPGTT) was carried out to assess the onset of T2D. Once the T2D mouse model was set up, left anterior descending (LAD) coronary artery chronic ligation was performed to induce MI (Liu *et al.*, 2013). Mice with left ventricular (LV) fractional shortening less than 44% were immediately treated with saline (MI+saline group), MSCs (MI+MSC group), or Netrin-1-expressing MSCs (MI+Netrin-1-MSC group). A sham group was also included in which all experimental procedures were executed other than LAD artery ligation. Animals were killed on day 30 after MSC implantation.

Rat models of MI

Sprague-Dawley rats (male; body weight, 200–230 g) were anesthetized, after which ligation of the LAD coronary artery was carried out as detailed previously. The rats were also randomly allocated to four treatment groups (sham, MI+saline, MI+BMSCs [bone marrow-derived MSCs], and MI+Netrin-1-BMSCs) and killed on day 30.

MSC isolation from mice

To obtain adipose tissue-derived MSCs, epididymal fat of each T2D C57BL/6J mouse (male, 8 weeks old) was cut and washed with serum-free low-glucose Dulbecco's modified Eagle's medium (DMEM). The adipose tissue was then

finely macerated and digested with 0.075% collagenase I at 37°C for 1 hr. The cell suspension was passed through a 40- μ m pore size filter before being washed with DMEM twice. Each cell pellet was resuspended in α -MEM (minimal essential medium) supplemented with 20% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μ g/ml), nonessential amino acids (0.1 mM), and L-glutamine (2 mM) and plated in a 100-mm plate to be maintained under standard culturing condition (37°C, 95% air–5% CO₂).

MSC isolation from rats

Bone marrow MSCs were isolated from compact bones of chow-fed Sprague-Dawley rats (male; body weight, 100–150 g) to minimize hematopoietic cell contamination for better multipotency and differentiation capacity (Zhu *et al.*, 2010). Briefly, the hind limbs of each rat were dissected. Excessive muscle and tendons were removed and the bones were sterilized and kept in α -MEM supplemented with 0.1% penicillin–streptomycin and 2% FBS for no more than 2 hr. The bone cavity was flushed three times with α -MEM to obtain bone marrow, which was then maintained in culture for BMSC expansion. The humeri, tibias, and femurs were then fragmented into 1- to 3-mm³ pieces and digested with collagenase I (1 mg/ml) at 37°C for 45 min. The bone fragments were washed with phosphate-buffered saline (PBS) and cultured in α -MEM supplemented with 20% FBS and penicillin (100 units/ml)–streptomycin (100 μ g/ml) under standard tissue culture conditions. After 3 days, 8 ml of the culturing medium was added and the cells were passaged as previously reported (Valina *et al.*, 2007; Constantin *et al.*, 2009).

Retroviral vector construction

The pMSCV-IRES-GFP, pMD.MLV.gag.pol, and pVSV.G retroviral vectors were provided courtesy of the Institute of Biomedical Engineering, Chinese Academy of Medical Science and Peking Union Medical College (Beijing, China), and the Netrin-1-encoding pGNET1myc retroviral vector was obtained from M. Tessier-Lavigne (Rockefeller University, New York, NY). The pMSCV-Netrin-1-GFP vector was constructed by double enzyme digestion with *Eco*RI and *Xho*I. Ligation of Netrin-1 was identified by PCR, using pGNET1myc and pMSCV-Netrin-1 as templates against primers that are specifically designed for Netrin-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). After 30 cycles of amplification, the PCR products were separated on a 1.5% agarose gel and visualized under UV light. The pMSCV-Netrin-1-GFP retroviral vector was then enriched and purified with QIAGEN-tip 500 (Qiagen) according to the manufacturer's instruction. The concentrated

retroviral particles were prepared in 293T packaging cell lines according to a previous report (Cheng *et al.*, 2008).

MSC transduction

MSCs were suspended and plated in 10-cm culturing dishes to a confluency of 60–80%. The cell transduction mixture was composed of fresh culturing medium and an appropriate amount of retroviral particles, and Polybrene was added to a final concentration of 4 μ g/ml. For each culturing dish, 3 ml of the transduction mixture was added and the cells were then kept under standard culturing conditions for 5 hr. At the end of the transduction process, the transduction mixture was replaced with fresh culturing medium. Successful cell transduction could be observed by confocal microscopy, due to the green fluorescent protein (GFP) reporter tag. The efficacy of viral transduction was determined by Western blotting, using an anti-Netrin-1 antibody, and flow cytometry.

Protein extraction and Western blotting

Protein samples were extracted from MSCs of the various treatment groups and fractionated by SDS-PAGE, using 12% polyacrylamide gels. The separated proteins were then transferred to polyvinylidene difluoride membranes and the expression of phosphorylated MAPK was assessed by immunoprobings, using antibodies raised against phosphorylated p44/42-MAPKs (diluted 1:1000) and tubulin (diluted 1:2500). After incubation with a horseradish peroxidase-linked goat anti-rabbit secondary antibody (diluted 1:5000), the blots were developed by enhanced chemiluminescence (ECL).

Histomorphological and immunofluorescence analysis

Rat and mouse hearts were obtained on day 30, fixed in Carnoy's fixative solution, and paraffin embedded, and consecutive sections (thickness, 5 μ m) were cut onto microscope slides. Hematoxylin and eosin staining was employed to examine the infarct size and Masson's trichrome staining was used to assess the level of collagen deposit after MI. For immunofluorescence staining, the sections were dewaxed, rehydrated, and permeabilized with 0.1% (v/v) Triton X-100 in Tris-buffered saline (TBS) followed by antigen retrieval using Tris-EDTA. The sections were then incubated at 4°C overnight with FITC-conjugated antibodies against isolectin (1:70 dilution, to examine capillary density), von Willebrand factor (vWF; 1:200 dilution, to determine capillary formation), and wheat germ agglutinin (WGA; 1:400 dilution, for cardiac hypertrophy assessment) and DAPI for counterstaining. The immunofluorescence was examined with a confocal microscope (Leica, Wetzlar,

TABLE 1. PRIMERS USED IN RT-PCR

	Primer sequence	Annealing temperature (°C)	Product size (bp)
Netrin-1	F: 5'-ATTGCCCCCTGCATAAAGAT-3' R: 5'-TGGATCTGCACAGCGTAGTC-3'	58	153
GAPDH	F: 5'-TCCCTCAAGATTGTCAGCAA-3' R: 5'-AGATCCACAACGGATACATT-3'	58	308

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Germany) and quantified with ImageJ software (W. Rasband, National Institutes of Health, Bethesda, MD).

Effects of Netrin-1 on cell proliferation and migration

MSCs and Netrin-1-MSCs (passage 10) were seeded in a 48-well plate at a density of ~ 5000 cells per well and maintained under standard culturing conditions for 5 days. On days 1, 3, and 5, the proliferative activity of the cells was determined by MTT assay and the relative levels of cell proliferation were determined as absorbance at a wavelength of 492 nm. The effect of Netrin-1 on MSC migration was also assessed by scratch wound assay (Park *et al.*, 2004). MSCs were seeded into 6-well plates at a density of $\sim 3 \times 10^5$ cells per well. After cell adhesion, the middle section (~ 1 cm in width) of each well was scraped off with a 1-ml pipette tip. Wound closing was examined under a light microscope at 0, 12, 18, and 24 hr and the images were analyzed with ImageJ.

Quantification of NO production

Total NO produced by MSCs was determined with a Griess reagent kit. Briefly, 48 hr after transduction, MSCs transduced with noncoding GFP vectors and Netrin-1-MSCs ($\sim 5 \times 10^5$ each) were plated in 10-cm culturing plates and maintained under standard culturing conditions. Supernatant from each group was collected and aliquoted into 96-well plates. The determination of nitrite within each samples were then carried out with the aliquots according to the procedures provided by the manufacturer (Roche).

Statistical analysis

Numerical data are expressed as means $\pm$ SD of multiple independent sets of experiments. Differences between two groups were analyzed by Student *t* test and analysis of variance (ANOVA) was used for multiple comparisons followed by Bonferroni's test, as appropriate. Values of $p < 0.05$ were considered statistically significant.

Results

MSC transduction

To examine the potential benefit of Netrin-1 on MSCs, MSCs were isolated and a Netrin-1-encoding vector or a noncoding control vector was used for MSC transduction. Protein lysates and cDNA samples were obtained from transduced MSCs. As shown in Fig. 1a, only MSCs transduced with the Netrin-1 vector express Netrin-1 mRNA, which was absent in nontransduced MSCs and MSCs transduced with control vector. Western blotting with an antibody raised against Netrin-1 protein also showed positive expression of Netrin-1 from the Netrin-1-MSCs, whereas no immunoreactivity was detected from MSCs transduced with control vector (Fig. 1b). Because a GFP tag is incorporated within each vector, successful cell transduction with the Netrin-1 vector or control vector could be visualized by confocal microscopy (Fig. 1c). The fluorescence intensity for each group of MSCs was subsequently quantified by flow cytometry (Fig. 1d). An average fluorescence intensity of 60.02% was obtained for Netrin-1-MSCs, which corresponds to 60% transduction efficiency in comparison with 76.7% for control vector.

In vitro assessment of Netrin-1-MSCs

In vitro assays evaluating MSC proliferation and migration were then carried out with Netrin-1-MSCs and MSCs transduced with the control vector. The MTT assay showed elevated cell proliferation from the Netrin-1-MSCs (128.6 ± 0.28 and $115.32 \pm 0.81\%$ on days 1 and 3, respectively; data shown as a percentage relative to control MSCs; $p < 0.01$ vs. control). However, the increase in Netrin-1-MSC proliferation was not sustained as no detectable changes could be observed between Netrin-1-MSCs and control MSCs on day 5 (Fig. 2a). Increased cell migration was also detected from the Netrin-1-expressing MSCs because widths of the scratch damage decreased by 29, 41, and 75% after 12, 18, and 24 hr, respectively (percentage relative to scratch width at time 0; $p < 0.01$ vs. control group). No difference was detected from the control MSCs (Fig. 2d and e).

Furthermore, because upregulation of NO release was reported of Netrin-1 by binding to its receptor DCC (deleted in colorectal cancer), which then activates downstream p44/42 mitogen-activated protein kinases (MAPKs) and endothelial nitric oxide synthase (eNOS) (Zhang and Cai, 2010), we also examined NO release and p44/42 MAPK phosphorylation associated with Netrin-1-MSCs. Supernatant was collected from the culture medium of Netrin-1-MSCs or control MSCs for NO measurement. It can be seen from Fig. 2c that the level of NO within the Netrin-1-MSC medium was approximately three times higher than the NO content of the MSC culture medium. Increased p44/42 MAPK phosphorylation was also observed from the Netrin-1-MSCs (Fig. 2b), confirming the Netrin-1/DCC/MAPK/eNOS signaling pathway. Given the positive impact of Netrin-1 overexpression on MSC proliferation, migration, and endogenous NO production, *in vivo* effects of the Netrin-1-MSCs on cardiac repair were assessed.

Intramyocardial injection of Netrin-1-MSCs reduces collagen deposition and prevents cardiac hypertrophic remodelling in rats and T2D mice

Collagen deposit and chamber enlargement were evident from MI-affected rats (Fig. 3a and b) and diabetic mice (Fig. 3c and d). In addition, anterior wall thinning and left ventricular (LV) chamber dilation could also be observed, characteristic of MI-induced ischemic injury. The therapeutic efficacies were comparable between the MSC and Netrin-1-MSC treatment groups for both rats and T2D mice (Fig. 3e–h). It could be observed that rats treated with MSCs or Netrin-1-expressing MSCs exhibited significantly reduced infarct size (41.7 ± 1.6 and $35.4 \pm 2.6\%$ of total cardiac area, $p < 0.05$ vs. saline group) and collagen content (MSCs, $93.5 \pm 2.3\%$ of saline group, $p > 0.1$; Netrin-1-MSCs, $76.6 \pm 2.1\%$ of saline group, $p < 0.05$). Protective effects of MSC and Netrin-1-MSC treatments were also observed in T2D mice, resulting in a $10 \pm 1.7\%$ (MSC treated) and $15 \pm 2.5\%$ (Netrin-1-MSC treated) reduction of infarct size ($p < 0.05$ vs. saline group), in addition to decreased collagen deposition (MSCs, $92.4 \pm 3\%$ of saline group, $p > 0.1$; Netrin-1-MSCs, $76.2 \pm 2.8\%$ of saline group, $p < 0.05$).

To determine the potential effect of MSC or Netrin-1-MSC therapy on cardiac hypertrophy, immunofluorescence staining with an antibody raised against wheat germ agglutinin (WGA; Fig. 4) was employed. Quantitative analyses revealed

FIG. 1. Mesenchymal stem cell (MSC) transduction with pMSCV-GFP and pMSCV-Netrin-1-GFP vectors. **(a)** PCR amplification of Netrin-1 (*top*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; *bottom*) from nontransduced MSCs (*left lane*), Netrin-1-transduced MSCs (*middle lane*), and MSCs transduced with control vector (*right lane*). Amplified product was detectable only from the Netrin-1-transduced MSCs whereas the positive control, GAPDH, could be observed from all treatment groups, demonstrating selective and successful transduction of the Netrin-1 gene. **(b)** Protein expression of Netrin-1 from Netrin-1-transduced MSCs and MSCs transduced with control vector. Positive immunoreactivity against an anti-Netrin-1 antibody was observed from the Netrin-1-transduced MSCs, but not from MSCs that had been transduced with the control vector. **(c)** Confocal images of Netrin-1-transduced MSCs (*bottom left*) and MSCs transfected with control vector (*top left*). The corresponding bright-field images of the two groups are shown on the *right*. Green fluorescence could be observed from both groups of MSCs, due to the GFP tag within each vector, demonstrating successful transduction for both groups. **(d)** Measurement of fluorescence intensity from the Netrin-1-transduced MSCs (*left*) and MSCs transduced with control plasmid (*right*). For Netrin-1-transduced MSCs, the fluorescence intensity was 60% (outlined in red), indicating excellent transduction efficiency. Color images available online at www.liebertpub.com/hum

a $17.3 \pm 5.9\%$ reduction in relative cross-sectional area of cardiomyocytes within the periinfarct area of the Netrin-1-MSC-treated rats (Fig. 4a and c). In contrast, the reduction was not observed in the MSC-treated group, suggesting an independent effect of Netrin-1 against MI-induced cardiac hypertrophy. A similar pattern was also observed in T2D mice (Fig. 4b and d), where Netrin-1-MSCs significantly attenuated cardiac hypertrophy at the periinfarction site ($16.1 \pm 4.95\%$ of saline-treated control group, $p < 0.01$).

Intramyocardial injection of Netrin-1-MSCs promotes neovessel formation

Immunostaining was employed to assess capillary formation within the ischemic myocardium, using an antibody raised against vWF, which is a well-known endothelial cell marker of neovessel formation. As shown in Fig. 5a and b, hardly any neovessel was detectable in the sham-operated animals. For MI-affected rats, limited revascularization could be observed in the saline control group and increased total vessel formation was shown 30 days after MSC or Netrin-1-MSC injection (34.72 ± 13.9 and $76.39 \pm 6.9\%$

increases relative to the saline control group, respectively, $p < 0.01$). Although the increase in revascularization may be attributable in part to the paracrine regulation of MSCs, the independent proangiogenic impact of Netrin-1 was also implicated because the Netrin-1-MSC-treated rats exhibited significantly elevated capillary formation ($130.9 \pm 2.4\%$ relative to the MSCs-only group, $p < 0.01$). Similar observations were made in MI-affected diabetic mice, in which 30 days after MSC or Netrin-1-MSC implantation, 23 ± 4.4 and $75.8 \pm 7\%$ increases in revascularization were detected, respectively (data presented as a percentage relative to the saline control group, $p < 0.05$).

Average capillary densities of the infarct, periinfarct, and remote areas were also estimated by isolectin staining. Elevated capillary density in the infarct and periinfarct areas was observed in the MSC and Netrin-1-MSC treatment groups for both rats and diabetic mice (Fig. 6), confirming a positive role for MSCs in cardiac revascularization. Elevated capillary density was observed in the Netrin-1-MSC-treated animals, which despite the lack of statistical significance was at least supportive of a beneficial role for Netrin-1 in revascularization.

FIG. 2. Netrin-1-MSCs exhibit enhanced proliferation, migration and nitric oxide (NO) production. **(a)** MTT assay of Netrin-1-MSCs and MSCs transduced with control vector on days 1, 3, and 5. Significant increase in cell growth is shown for Netrin-1-MSCs in comparison with control MSCs on days 1 and 3. OD, optical density. **(b)** Expression of phosphorylated p44/42 mitogen-activated protein kinases (MAPKs) and actin by Western blotting. Increased phosphorylation of the p44/42 MAPKs from Netrin-1-MSCs is shown compared with control MSCs, indicating Netrin-1-dependent upregulation of p44/42 MAPKs. Phos-ERKs, phosphorylated extracellular signal-regulated kinases. **(c)** Significant increase in NO release was observed from Netrin-1-MSCs compared with control MSCs. **(d)** Representative images of MSCs and Netrin-1-MSCs during a time-course scratch-wound assay. Increased MSC migration is shown from the Netrin-1-MSCs (*bottom*) as indicated by gradual closing of the scratched gap (border highlighted by red dotted lines). No change can be detected from the control MSCs (*top*). **(e)** The width of each scratch gap was measured and the average gap width was plotted against each time point for both Netrin-1-MSC and control MSC groups. Results are the means of three independent experiments and data are shown as means and standard errors. Color images available online at www.liebertpub.com/hum

Discussion

Globally, ischemic cardiac disease is the leading cause of death. Despite the clinical application of drug therapy, reperfusion strategies, and the development of implantable cardioverter defibrillators and cardiac assistant devices, ischemic heart disorders continue to be the primary health concern, often resulting in irreversible loss of cardiac function, which ultimately leads to heart failure. As a terminally differentiated organ, the adult human heart exhibits limited regenerative capacity. Although previous observations have demonstrated that certain cardiomyocytes are able to reenter the cell cycle from a resting state or to initiate endogenous cardiac progenitor cell differentiation after MI insults (Beltrami *et al.*, 2001; Ptaszek *et al.*, 2012; Faucherre and Jopling, 2013), this *per se* is insufficient to overcome myocyte necrosis. Moreover, post-MI cardiac hypertrophy and myocardial fibrosis are also commonly detected as a result of cardiac compensatory mechanisms, leading to the development of heart failure.

Given the limitations of the currently available MI therapies, several regenerative approaches, including tissue engineering,

gene therapy, and stem cell therapy, have been extensively investigated to facilitate myocardial repair and regeneration. To date, many cell types, including embryonic stem cells (ESCs), endothelial progenitor cells (EPCs), peripheral blood cells (PBCs), and MSCs have been considered as treatment for MI (Zimmet and Hare, 2005; Heydarkhan-Hagvall *et al.*, 2008; Ptaszek *et al.*, 2012; Liu *et al.*, 2013), among which the MSCs possess unique advantages including easy accessibility, *in vitro* expandability, excellent multipotency, and lower immunogenicity, making it a popular choice for cell therapy. As previously reported, besides its reported potential to differentiate into cardiomyocytes and endothelial cells, MSCs are highly secretory cells capable of producing a wide range of intracellular mediators, which play a more essential role in cardiac remodeling. However, preliminary data from clinical trials were discouraging regarding the efficacy of MSC therapy, mainly due to loss of implanted cells. Indeed, poor cell survival and retention is a major challenge for cell therapy because as much as 10% of implanted stem cells could be lost by mechanical ejection from the heart during implantation or through induced apoptosis as a result of the hostile microenvironment within the injured myocardium.

FIG. 3. Intramyocardial delivery of MSCs or Netrin-1-MSCs ameliorates myocardial infarction (MI)-induced cardiac damage. **(a and b)** Histological analyses of heart sections obtained from MI-affected rats: **(a)** Representative images of hematoxylin–eosin (H&E) staining of rat heart sections from sham (*left*), saline-treated control (*center left*), MSC-treated (*center right*), and Netrin-1-MSC-treated (*right*) groups; **(b)** Masson's trichrome staining of rat heart sections from corresponding treatment groups. **(c and d)** Histological analyses of heart sections from MI-affected diabetic mice: **(c)** Representative images of H&E staining of mice hearts from sham (*left*), saline-treated control (*center left*), MSC-treated (*center right*), and Netrin-1-MSC-treated (*right*) groups; **(d)** Masson's trichrome staining of mouse hearts from corresponding treatment groups. Scale bar: 2 mm. **(e and g)** Quantitative representation of infarction sizes of MI-affected rats **(e)** and diabetic mice **(g)** treated with saline, MSCs, or Netrin-1-MSCs. **(f and h)** Measurement of collagen deposition levels of MI-affected rats **(f)** and diabetic mice **(h)** treated with saline, MSCs, or Netrin-1-MSCs, or untreated (sham group). Data demonstrated beneficial effects of MSC and Netrin-1-MSC injection in cardiac protection in rats and diabetes mice post-MI. Superior cardiac protection was further shown from the Netrin-1-MSC-treated groups in comparison with the MSCs-only groups. Images are representative of three independent experiments using five to nine animals for each experiment. Data are shown as means and standard errors. Color images available online at www.liebertpub.com/hum

Netrin-1, the laminin-related neuronal guidance protein, has been implicated in apoptosis regulation (Arakawa, 2004). Several signaling cascades were proposed to be involved, including activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, direct inhibition of caspase-3 activity, and Netrin-1-activated DCC-induced activation of the p44/42 MAPK network. In addition, earlier evidence indicated a protective role for Netrin-1 against acute kidney injury, acute lung injury, inflammation-mediated tissue injury, hypoxia challenge, and MI (Ly *et al.*, 2005; Rosenberger *et al.*, 2009; Mirakaj *et al.*, 2010; Zhao *et al.*, 2013; Mao *et al.*, 2014). More importantly, the Netrin-1/DCC/MAPK/eNOS pathway has been shown to be responsible for the protective effects of Netrin-1 against ischemic MI injuries (Zhang and Cai, 2010), making Netrin-1 a promising target for therapy development against cardiovascular diseases. Given the regulatory role of Netrin-1 in apoptosis and myocyte recovery, we have investigated the potential of Netrin-1-overexpressing MSCs for MI treatment and the molecular action of Netrin-1 during post-MI regeneration.

Subsequent *in vivo* results showed reduced cardiac collagen deposition and infarct area from Netrin-1-MSC-treated rats, in addition to a milder attenuation of cardiac hypertrophy (Fig. 2). The improvement is, at least in part, attributable to the paracrine mechanisms of MSCs (Shake *et al.*, 2002; Zimmet and Hare, 2005; Uemura *et al.*, 2006; Valina *et al.*, 2007; Figeac *et al.*, 2014), although better MSC migratory ability and significantly enhanced cell growth were also evident with the Netrin-1-MSCs (Fig. 2), demonstrating a positive role for Netrin-1 in regulating MSC mobilization and proliferation. Moreover, myocardial dysfunction was markedly ameliorated in the Netrin-1-MSC-treated animals compared with the MSCs-only treatment group (Figs. 3–5), which may be explained by the proangiogenic impact of Netrin-1. In addition, increased NO release and upregulation of p44/42 MAPK phosphorylation were observed with the Netrin-1-overexpressing MSCs (Fig. 2b), further confirming the importance of the Netrin-1-activated DCC/MAPKs/eNOS pathway in post-MI myocardial restoration.

FIG. 4. Intramyocardial delivery of MSCs or Netrin-1-MSCs reduces cardiac hypertrophy after MI insult. (a and b) Wheat germ agglutinin staining shows the myocyte boundaries in the remote and periinfarct areas of heart sections obtained from MI-affected rats (a) and diabetic mice (b). (c and d) Relative transverse area of myocyte in the remote and periinfarct areas of hearts obtained from MI-affected rats (c) and diabetic mice (d). Images are representative of three independent experiments using five to nine animals for each experiment. Scale bar: 50 μ m. Data are shown as means and standard errors. Color images available online at www.liebertpub.com/hum

FIG. 5. Intramyocardial delivery of MSCs or Netrin-1-MSCs increases blood vessel density in the infarct and periinfarct sites. (a and b) Immunohistochemical staining of vWF in heart sections obtained from MI-affected rats (a) and diabetic mice (b) reveals increased capillary density in Netrin-1-MSC-treated groups in comparison with the MSC-treated and saline-treated control groups. (c and d) Average fluorescence density of fluorescein isothiocyanate (FITC) within the infarct area was quantified and plotted against each treatment group for MI-affected rats (c) and diabetic mice (d). Images are representative of three independent experiments, using five to nine animals for each experiment. Scale bar: 50 μ m. Data are shown as means and standard errors. Color images available online at www.liebertpub.com/hum

FIG. 6. Intramyocardial delivery of MSCs or Netrin-1-MSCs enhances capillary formation at infarct and periinfarct sites. (a and b) Isolectin staining of heart sections obtained from MI-affected rats (a) and diabetic mice (b) shows upregulated neocapillary formation at the infarct and periinfarct sites in MSC- and Netrin-1-MSC-treated animals in comparison with the saline-treated control group. (c and d) Average fluorescence density of FITC within the remote, infarct, and periinfarct areas was quantified and plotted against each treatment group for MI-affected rats (c) and diabetic mice (d). Images are representative of three independent experiments, using five to nine animals for each experiment. Scale bar: 50 μ m. Data are shown as means and standard errors. Color images available online at www.liebertpub.com/hum

The endothelial nitric oxide synthase (eNOS)-derived NO is a key modulator in vascularization, especially capillary formation (Aicher *et al.*, 2003; Park *et al.*, 2010; Zhao *et al.*, 2013). It also functions as a molecular communicator between the cardiovascular system and metabolism (Cabou *et al.*, 2007). NO deficiency is implicated as a main defect responsible for dysregulation of stem cell mobilization during sustained hyperglycemia, which often leads to impaired vascular regeneration after ischemic injury in diabetes (Yan *et al.*, 2009). A decrease in NO production is also responsible for vascular dysfunction during insulin resistance (Cabou *et al.*, 2007). Furthermore, systemic glucose disposal may also be partly influenced by endothelial NO in response to insulin signaling (Vicent *et al.*, 2003). Studies further reported a causal relationship between NO deficiency and diabetic nephropathy (Claybaugh *et al.*, 2014). Indeed, type 2 diabetes (T2D), which accounts for 90–95% of all cases of diabetes, is associated with compromised myocyte function, vascular endothelial dysfunction, abnormal blood rheology, and changes in reactive oxygen species (ROS) susceptibility, making it a major risk factor for cardiovascular and microvascular diseases (Haffner *et al.*, 1998). One other concern is that although a considerable number of preclinical studies have shown markedly reduced infarct size, and improved systolic function and left ventricular remodeling after cell therapy, the MI models used in

these studies did not have the comorbidities that are often present in MI-affected individuals. Given the positive impact of Netrin-1-MSCs on eNOS-dependent NO production and post-MI revascularization in rats, we further examined the impact of Netrin-1-MSC therapy in T2D mice after MI injury.

Netrin-1 has been implicated in pancreatitis (Chen *et al.*, 2012), vascular inflammation (Ly *et al.*, 2005), neutrophil infiltration, leukocyte migration, and macrophage polarization (Ranganathan *et al.*, 2013; Mao *et al.*, 2014). These antiinflammatory effects of Netrin-1 are also beneficial because T2D is now considered a chronic inflammatory disorder resulting mainly from overactivated inflammatory responses. Thus, we used adipose tissue-derived MSCs (ADMSCs), which have been reported to be less immunogenic (Fiorina *et al.*, 2009; Polchert *et al.*, 2008; Bassi *et al.*, 2012) for Netrin-1 transduction. Effects of Netrin-1-ADMSCs were evaluated *in vivo* and our results demonstrated a facilitative role for Netrin-1 during post-MI recovery in T2D mice. Briefly, normalization of parameters including infarct size, level of collagen deposition, and cardiac hypertrophy was observed, as well as enhanced capillary density, indicating attenuated myocardial dysfunction. Moreover, in addition to the superior therapeutic outcome with the Netrin-1-ADMSCs, better glycemic control was also observed (data not shown), partly due to the

antiinflammatory property of Netrin-1. Furthermore, another report proposed a Netrin-1/PPAR γ (peroxisome proliferator-activated receptor γ) mechanism (Mao *et al.*, 2014). Because PPAR γ is a well-known therapeutic target for thiazolidinediones (TZDs), a class of antidiabetic medications currently in use, this is particularly relevant to MI therapy in patients with T2D. Further studies are required to confirm the association between Netrin-1 and PPAR γ and the molecular impact of this signaling cascade.

The present study demonstrated a protective role for Netrin-1 in MSC therapy against acute MI-induced ischemic injury. Several molecular actions of Netrin-1 may be responsible for the positive impact of Netrin-1-MSCs. Thus, Netrin-1 overexpression resulted in increased phosphorylation of p44/42 MAPK *in vitro*, accounting for the enhanced proliferation and migration of the Netrin-1-MSCs. Increased NO release was also detected from the Netrin-1-MSCs, beneficial for enhanced myocardial protection, endothelial function, and neovascularization. Immunohistochemical analyses indicated that Netrin-1 stimulated capillary formation, which may increase the blood supply and also lead to ameliorated MI-induced cardiac dysfunction. For MI that is coexistent with T2D, attenuation of MI-initiated cardiac injury and T2D was observed, attributable to the antiinflammatory property of Netrin-1 and its potential association with the PPAR γ signaling pathway.

In summary, we report here a protective role for Netrin-1-MSCs in post-MI myocardial recovery. Given the previously reported proangiogenic, antiapoptotic, and antiinflammatory properties of Netrin-1, this paper reports and highlights the potential of Netrin-1 as a therapeutic target for cardiovascular diseases in the presence of other metabolic disorders.

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Author Disclosure Statement

No competing financial interests exist for any of the authors.

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