Overexpression of Angiopoietin-1 Increases CD133+/c-kit+ Cells and Reduces Myocardial Apoptosis in db/db Mouse Infarcted Hearts

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Abstract
Hematopoietic progenitor CD133+/c-kit+ cells have been shown to be involved in myocardial healing following myocardial infarction (MI). Previously we demonstrated that angiopoietin-1 (Ang-1) is beneficial in the repair of diabetic infarcted hearts. We now investigate whether Ang-1 affects CD133+/c-kit+ cell recruitment to the infarcted myocardium thereby mediating cardiac repair in type II (db/db) diabetic mice. db/db mice were administered either adenovirus Ang-1 (Ad-Ang-1) or Ad-β-gal systemically immediately after ligation of the left anterior descending coronary artery (LAD). Overexpression of Ang-1 resulted in a significant increase in CXCR-4/SDF-1α expression and promoted CD133+/c-kit+, CD133+/CXCR-4+ and CD133+/SDF-1α/c-kit+ cell recruitment into ischemic hearts. Overexpression of Ang-1 led to significant increases in number of CD31+ and smooth muscle-like cells and VEGF expression in bone marrow (BM). This was accompanied by significant decreases in cardiac apoptosis and fibrosis and an increase in myocardial capillary density. Ang-1 also upregulated Jagged-1, Notch3 and apelin expression followed by increases in arteriole formation in the infarcted myocardium. Furthermore, overexpression of Ang-1 resulted in a significant improvement of cardiac functional recovery after 14 days of ischemia. Our data strongly suggest that Ang-1 attenuates cardiac apoptosis and promotes cardiac repair by a mechanism involving in promoting CD133+/c-kit+ cells and angiogenesis in diabetic db/db mouse infarcted hearts.

Introduction
Ang-1 is an oligomeric-secreted glycoprotein, which binds to Tie-2 and induces Tie-2 phosphorylation. Ang-1 is recognized as a survival factor for endothelial cells (EC). Treatment with Ang-1 prevents EC apoptosis via activation of the PI3K/Akt pathway.[1,2] Ang-1 has also been shown to prevent diabetic retinopathy by attenuating retinal permeability in the streptozotocin (STZ)-induced diabetic model.[3] Our previous studies revealed that overexpression of Ang-1 in diabetic db/db mouse heart restores Tie-2 expression and significantly increases myocardial capillary formation; this is accompanied by a dramatic decrease in myocardial hypertrophy and cardiac fibrosis.[4] These data implicate Ang-1 as a potential therapeutic target in the treatment of diabetic cardiovascular complications.

Endothelial progenitor cells (EPCs) home to sites of ischemia and contribute to neoangiogenesis in ischemic tissue.[5] Experimental and clinical studies demonstrate that treatment of acute myocardial infarction with EPCs results in a reduction in infarct size.[6,7] Vascular progenitor cells have been shown to differentiate into cardiomyocytes and vascular smooth muscle cells (VSMC), which may contribute to cardiac and/or vascular regeneration following myocardial infarction.[8,9] Intriguingly, the differentiation of EPCs is impaired in both diabetic patients with coronary artery disease and in diabetic mouse models.[10,11] Previously we demonstrate that the level of EPCs is significantly decreased in STZ-induced diabetic mouse compared to non-diabetic mice.[12] Our previous studies also reveal that disruption of BM-EPC differentiation and impairment of angiogenesis after myocardial ischemia are associated with larger myocardial infarct size in the diabetic STZ mice.[12] These studies suggest that impaired vascular progenitor cell recruitment and failure of BM differentiation to EPCs after MI may contribute to insufficient angiogenesis and exacerbation of MI in diabetes. Thus, an agent that promotes vascular progenitor cell recruitment and angiogenesis will be beneficial for ischemic injury repair and cardiac remodeling after MI in diabetic hearts. This notion is supported by our previous work demonstrating that overexpression of Ang-1 significantly increased myocardial angiogenesis and reduced myocardial infarction size in the STZ diabetic mouse model.[12] However, the underlying molecular mechanism by which Ang-1 attenuates myocardial ischemic injury in the diabetic heart following MI remains poorly understood.

Ang-1 has been shown to have a critical role in the maintenance of hematopoietic stem cell in the bone marrow through its binding to the Tie-2 receptor.[13] The hematopoietic stem cell cytokine SDF-1α and its receptor CXCR-4 have been identified as the central signaling axis that regulates recruitment of hematopoietic stem cells into the injured area of myocardial infarction and in improvement of cardiac function after MI.[14] Using diabetic db/db mice subjected to myocardial ischemia, the present study...
investigates whether overexpression of Ang-1 promotes recruitment of hematopoietic progenitor cells into ischemic sites and whether this leads to attenuation of hematopoietic ischemic injury through SDF-1α/CXCR4 signaling. Our data suggest that Ang-1/Tie-2 plays a crucial role in regulation of hematopoietic progenitor cell recruitment and cardiac repair in the diabetic infarcted heart.

Methods

Ethics Statement

All procedures conformed to the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee (Protocol ID: #1280).

Diabetic mouse myocardial ischemia model

db/db mice (12–14 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, Maine). Experimental mice were anesthetized with ketamine (100 mg/kg) plus xylazine (15 mg/kg), intubated and artificially ventilated with room air. Adequate anesthesia was monitored by toe pinch. A left thoracotomy was performed and myocardial ischemia was achieved by ligation of the left anterior descending coronary artery (LAD) [12,15]. Sham controls underwent the surgery without LAD ligation. Experimental mice were divided into three groups: (1) sham control (n = 12 mice); (2) Ad-β-gal + ischemia 24 hours (n = 22 mice) and 14 days (n = 13 mice) and (3) Ad-Ang-1 + ischemia 24 hours (n = 16 mice) and 14 days (n = 19 mice). Experimental mice were sacrificed under anesthesia with isoflurane at 24 hours and 14 days after LAD ligation for the analyses described below.

Systemic delivery of Ang-1 to experimental mice

After surgery, db/db mice received an intravenous tail vein injection of either Ad-Ang-1 (1×10^9 PFU) or Ad-β-gal (1×10^9 PFU) [4,12].

Western analysis of Ang-1, SDF-1α, VEGF, apelin, APLNR (APJ), Jagged 1 and Notch3 expression

After 24 hours of myocardial ischemia, the hearts and BM were harvested and homogenized in lysis buffer. The membranes were immunoblotted with SDF-1α, VEGF, apelin, APLNR, Jagged1 and Notch3 (1:1000, Santa Cruz, CA) or Ang-1 (1:1000, Sigma, MO) antibodies. The membranes were washed and incubated with a secondary antibody coupled to horseradish peroxidase and with a secondary antibody coupled to horseradish peroxidase and analyzed with an image acquisition and analysis software (TINA 2.0).

Myocardial Ang-1, Tie-2, CD133, CD45, C-kit, CXCR-4, SDF-1α, SMA and Notch3 expression

Heart tissue sections (8 μm thick) were incubated with Ang-1, CD133, Tie-2 and Notch3, CXCR4, CD45, C-kit and SDF-1α (1:200 Santa Cruz, CA) antibodies overnight. Ang-1, CD133, Tie-2 and Notch3 were visualized using FITC labeled goat anti-mouse IgG antibodies; CXCR4, CD45 and SDF-1α were visualized with Fluorolink™ Cy3™ labeled goat anti-mouse IgG antibodies (1:200), Cy3-conjugated anti-β smooth muscle actin (SMA, 1:100; Sigma, MO) was used to identify smooth muscle cells. Myocardial CD133, CD45 and CXCR4 expression was assessed by counting the number of positive cells per square millimeter (mm²) of tissue. Co-localization of CD133 with either CXCR-4 or SDF-1α, or Ang-1 with SMA was visualized with a fluorescence microscope (Nikon TE 2000, Japan). Sections were counterstained with DAPI.

Bone marrow (BM) cell differentiation into CD31+ and SMA+ cells

At 24 hours of myocardial ischemia, BM cells were obtained by flushing the tibias and femurs with 10% FBS EGM. Immediately after isolation, 10^5 bone marrow–derived mononuclear cells were plated on 6-well culture plates coated with fibronectin (50 μg/ml, Sigma). After 4 days of culture, the nonadherent cells were removed and the adherent cells were incubated with FITC-labeled CD31 antibody (1:100, BD Biosciences) and -Cy3-conjugated anti-α smooth muscle actin (SMA, 1:100; Sigma, MO). Positively-stained cells were counted per 10^5 BM cells.

Analysis of myocardial capillary density

After 14 days of myocardial ischemia, the hearts were harvested and immediately flash frozen. Five μm sections were incubated with Isolectin B4 (1:200, B4, Molecular Probe, Invitrogen) to identify capillaries. The number of capillaries was counted and expressed as capillary density per square millimeter (mm²) [12,16].

Analysis of VSMC and arteriole formation

To assess VSMC in the border zone of the ischemic area after 24 hours of MI, VSMC were stained with Cy3-conjugated anti-α smooth muscle actin (SMA, 1:100; Sigma, MO). Arterioles (Ang-1+/SMA+) was examined at 14 days of MI.

Myocardial cell/endothelial apoptosis

Heart tissue sections underwent transferrase deoxyuridine nick end labeling (TUNEL) following the manufacturer’s instructions (Promega, WI). Sections were counterstained with DAPI. Apoptosis was indexed by counting TUNEL positive cells per square millimeter (mm²). To determine endothelial or SMC apoptosis, sections of myocardium were incubated with TUNEL and vWF (1:200, Santa Cruz, CA) or Cy3-conjugated anti-α smooth muscle actin (SMA, 1:100; Sigma, MO) overnight, followed by incubation with Fluorolink™ Cy3™ for vWF. Co-localization of TUNEL with vWF or SMA was visualized with a fluorescence microscope (Nikon TE 2000, Japan). Sections were counterstained with DAPI. Endothelial cell/SMC apoptosis was indexed by counting TUNEL/vWF or TUNEL/SMA positive cells per square millimeter (mm²).

Assessment of cardiac function

Briefly, experimental mice were anesthetized with ketamine (100 mg/kg) plus xylazine (15 mg/kg), intubated and artificially ventilated with room air. A 1.4-Fr pressure–conductance catheter (SPR-839, Millar Instrument, Houston, TX) was inserted into left ventricle (LV) to record baseline hemodynamics of hearts. Data were digitized with a sampling rate of 1000 Hz. All data were analyzed with LabChart software package from AD instruments.

Myocardial fibrosis and heart weight to body weight ratio (HW/BW)

To determine cardiac fibrosis formation, 8 μm sections from hearts after 14 days of myocardial ischemia were stained with Mason’s trichrome (MT, Sigma, MO). Myocardial fibrosis was quantified by measuring the MT staining area (blue) in the infarcted area using NIH Image analysis software [4]. Cardiac hypertrophy was assessed by measuring heart-to-body weight ratio at 14 days post-myocardial ischemia. Each heart weight was
divided by the total body weight of the mouse, resulting in a ratio representative of cardiac hypertrophy.

**Statistical analysis**

All results are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by the multiple-comparison test (Student–Newman–Keuls or Dunn’s test). A p value <0.05 denoted significance.

**Results**

**Overexpression of Ang-1 in diabetic db/db mouse hearts and bone marrow**

Systemic delivery of Ad-Ang-1 led to dramatic induction of Ang-1 expression in db/db mouse hearts and bone marrow after 24 hours of myocardial ischemia (**Figure 1A**). Fluorescent immunohistochemical analysis confirmed the overexpression of Ang-1 in the Ad-Ang-1 treated db/db mouse hearts and bone marrow, but not in the Ad-β-gal treated db/db mouse hearts and bone marrow (**Figure 1B**). We also examined the location of Ang-1 expression in Ad-Ang-1–treated db/db mouse hearts. Our merged images revealed that Ang-1 localized with Isolectin B4 (IB4) on ECs and SMA on vessel SMCs (**Figure 1C**). To examine transfection efficiency of Ad-Ang-1 in vivo, experimental mice were systemic administrated with Ad-Ang-1 at dosage of 10^7–10^9 pfu for 24 hours. Western blot analysis showed that Ang-1 protein expression was gradual increased in a dose dependent fashion both in the heart and bone marrow (**Figure 1D**).

**Overexpression of Ang-1 promotes hematopoietic stem cell recruitment via SDF-1α/CXCR-4 signaling in the ischemic hearts of db/db mouse**

Next, we investigated whether overexpression of Ang-1 affects recruitment of hematopoietic progenitor cells into infarcted db/db mouse hearts. CD133 and c-kit cells were examined in the ischemic hearts at 24 hour and 14 days. No CD133 cells were observed in the sham control db/db mouse hearts without ischemia. As shown in **Figure 2A and 2B**, CD133 cells were recruited into diabetic mouse infarcted hearts after 24 hours ischemia as compared to Ad-β-gal diabetic mouse infarcted hearts. Overexpression of Ang-1 led to a significant increase in CD133 cells in diabetic infarcted hearts in comparison with Ad-β-gal treated db/db mice. Furthermore, no c-kit^+^ cells were detected in the sham control, db/db+ ischemia and db/db + ischemia+Ad-Ang-1 at 24 hours. Intriguingly, c-kit^+^ cells were detected in the Ad-Ang-1 diabetic mouse infarcted hearts at day 14 of ischemia, but not in Ad-β-gal diabetic mouse. The recruited c-kit^+^ cells were co-localized with CD133^+^ cells in db/db mouse infarcted hearts treated with Ad-Ang-1 (**Figure 2C**).

CD45^+^ positive cells were also dramatically increased in diabetic infarcted hearts treated with Ad-Ang-1 at 24 hours after MI as compared to Ad-β-gal diabetic mouse infarcted hearts. Co-
Localization studies further revealed that the increased CD45+ cells co-localized with Tie-2+ in diabetic infarcted hearts treated with Ad-Ang-1 (Figure 2D and E).

To examine the roles of SDF-1α/CXCR-4 in Ang-1-induced CD133+ cell recruitment into db/db mouse hearts, we examined the number of CXCR-4+ and SDF-1α/CXCR-4+ protein expression in the border zone of infarcted myocardium after 24 hours of MI. As shown in Figure 3A and B, overexpression of Ang-1 in db/db mice led to a significant increase in the number of CXCR-4+ cells in the border zone of infarcted hearts in comparison with Ad-β-gal treated/db/db mice. No CXCR-4+ cells were found in the sham control db/db mouse hearts. Western blot analysis revealed that overexpression of Ang-1 resulted in significant increases in CXCR-4 and SDF-1α expression in diabetic infarcted hearts as compared to Ad-β-gal diabetic mouse infarcted hearts (Figure 3 C and D). Co-localization studies further revealed that the increased CD133+ co-localized with CXCR-4+ and SDF-1α+ cells in diabetic infarcted hearts treated with Ad-Ang-1 (Figure 3E).

Overexpression of Ang-1 attenuates myocardial apoptosis and reduces cardiac fibrosis in diabetic db/db mouse hearts

We further sought to determine whether increased recruitment of CD133+/c-kit+ cells into the ischemic area by Ang-1 would minimize myocardial apoptosis. TUNEL staining and quantitative analysis revealed a significant increase in TUNEL-positive cells in the infarcted area of db/db mice after 24 hours and 14 days of MI compared to sham control db/db mice. db/db mice treated with Ad-Ang-1 had significantly fewer TUNEL-positive cells in the infarct area compared to db/db mice that received Ad-β-gal.
Co-staining of TUNEL with vWF further revealed that treatment of db/db mice with Ad-Ang-1 significantly attenuated the number of TUNEL/vWF-positive cells in the infarct area compared to db/db mice (n = 6) compared to that of Ad-β-gal-treated db/db mice (n = 9). CXCR-4+ cells are expressed as the total number per square mm. Sham control (n = 3). All data represent mean ± SD; *p<0.05. ND = not detected. C: Western blot and densitometric analysis of myocardial CXCR-4 expression showing that overexpression of Ang-1 significantly increased CXCR-4 expression in db/db mice compared to that of Ad-β-gal-treated db/db mice. All data represent mean ± SD (n = 4 mice); *p<0.05. D: Western blot and densitometric analysis of myocardial SDF-1α expression. Overexpression of Ang-1 significantly increased SDF-1α expression in db/db mice compared to that of Ad-β-gal-treated db/db mice. All data represent mean ± SD (n = 4 mice); *p<0.05. E: Co-localization of CD133 with CXCR-4 and CD133 with SDF-1α in db/db mouse infarcted hearts. CD133+ cells (green); CXCR-4+ or SDF-1α+ cells (red) and nuclei were stained by DAPI (blue, 40X). Merged images show that CD133+ cells co-localized with both CXCR-4 (upper panel) and SDF-1α (bottom panel) in the Ad-Ang-1 treated db/db mice, but little co-localization was seen in the Ad-β-gal-treated db/db mice. No specific staining was observed in the sham control group.

At 14 days, treatment with Ad-Ang-1 led to a significant decrease in the HW/BW ratio in db/db mice subjected to MI as compared with db/db mice treated with Ad-β-gal (Figure 4G). Overexpression of Ang-1 increases the number of BM-CD31+ cells and myocardial capillary density in db/db mouse hearts.

New evidence indicates that BM-CD31+ cells have angiogenic properties and implantation of BM-CD31+ cells into ischemic hind-limb promotes recovery of tissue from ischemic injury [17]. Therefore, we examined the number of BM-CD31+ cells after...
Figure 4. Ang-1 attenuates myocardial/endothelial apoptosis, and reduces cardiac fibrosis and hypertrophy in db/db mouse. A. TUNEL-stained heart sections from sham control db/db mouse, db/db mouse treated with Ad-β-gal or Ad-Ang-1 at 24 hours and 14 days of MI. Myocardial and endothelial apoptotic cells in the infarcted area of the ischemic hearts were identified by TUNEL staining (green) and TUNEL/vWF (red) positive staining, and total nuclei by DAPI counterstaining (blue). B and C. Quantitative analysis of apoptotic cells in sham control db/db mouse and infarcted area of db/db mouse treated with Ad-β-gal or Ad-Ang-1 at 24 hours and 14 days after ischemia. TUNEL or TUNEL/vWF positive cells are expressed as the total number of per mm². Apoptotic cells were significantly decreased in Ad-Ang-1 compared to Ad-β-gal mice. All data represent mean ± SD (n = 4 mice); *p<0.05. D. Representative images and quantitative analysis of apoptotic cells in the remote area of infarction of db/db mouse at 24 hours after ischemia. Apoptotic cells were significantly decreased in Ad-Ang-1 treated db/db mouse hearts (n = 5) compared to Ad-β-gal treated db/db mouse (n = 7). All data represent mean ± SD; sham control n = 5, *p<0.05. E. Representative images of TUNEL and SMA-stained heart
24 hours of myocardial ischemia. The number of BM-CD31\(^+\) was significantly increased in db/db mice treated with Ad-\(\beta\)-gal or Ad-Ang-1 compared to db/db mice treated with Ad-\(\beta\)-gal or Ad-Ang-1 stained by Masson’s trichrome. Ad-Ang-1 significantly attenuated area of cardiac fibrosis in db/db mice compared to Ad-\(\beta\)-gal mice. All data represent mean ± SD (n = 5 mice); \(^*p<0.05\). G. Heart weight/body weight (HW/BW) ratio in db/db and Ad-Ang-1-treated db/db mouse hearts at 14 days after MI. Treatment with Ad-Ang-1 significantly attenuated cardiac hypertrophy in db/db mouse hearts (n = 5) compared to Ad-\(\beta\)-gal mice (n = 4). All data represent mean ± SD; \(^*p<0.05\).
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Figure 5. Ang-1 increases CD31\(^+\) cells and VEGF expression in the BM and promotes capillary formation in db/db mouse hearts. A. Representative images showing that BM cells differentiate into CD31\(^+\) cells in Ad-\(\beta\)-gal treated db/db mice subjected to myocardial ischemia for 24 hours (left panel) and in the Ad-Ang-1-treated db/db mice (right panel). B. Quantitative analysis of BM differentiation into CD31\(^+\) after myocardial ischemia. The number of BM cells that differentiate into CD31\(^+\) was significantly increased in the Ad-Ang-1-treated db/db mice compared to the Ad-\(\beta\)-gal treated db/db mice. There was not difference between sham control and ischemic group. All data represent mean ± SD (n = 3 mice); \(^*p<0.05\). C. Western blot densitometric analysis of VEGF expression revealed that systemic delivery of Ad-Ang-1 resulted in a significant increase in VEGF expression in the BM of db/db mouse compared to Ad-\(\beta\)-gal db/db treated mice. All data represent mean ± SD (n = 3 mice); \(^*p<0.05\). D and E. Representative images and quantitative analysis showing that treatment with Ad-Ang-1 significantly increased capillary formation in db/db mouse subjected to ischemia compared to Ad-\(\alpha\)-gal-treated db/db mice. All data represent mean ± SD (n = 7 mice); \(^*p<0.05\).
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Overexpression of Ang-1 increases BM-SMA\(^+\) cells and number of VSMC in the db/db mouse hearts

The number of BM-SMA\(^+\) cells was significantly increased in db/db mice treated with Ad-Ang-1 compared to db/db mice treated with Ad-\(\beta\)-gal after MI (Figure 6A & B). Next, the number of VSMC in the ischemic area was examined after 24 hours of ischemia by the co-localization of Ang-1 with SMA. Ang-1 expression co-localized with SMA (middle panel). After 2 weeks of MI, Ang-1-positive arteriole with large diameter was also found in the border zone of infarcted myocardial area (Figure 6C). Sham control had little effect on BM-SMA\(^+\) and VSMC cell number (Data not shown).
Overexpression of Ang-1 upregulates Jagged1/Notch3 and apelin expression in diabetic db/db mouse hearts

To explore the potential intracellular molecular mechanisms by which overexpression of Ang-1 increased angiogenesis in diabetes, myocardial Jagged 1/Notch3 and apelin/APLNR expression in db/db mice was examined. Systemic delivery of Ad-Ang-1 resulted in a significant increase in Jagged 1 and Notch3 expression 24 hours after myocardial ischemia compared to Ad-β-gal (Figure 7A and B). Quantitative analysis showing a significant increase in number of SMA+ cells after myocardial ischemia for 24 hours following Ad-Ang-1 compared to Ad-β-gal in db/db mice subjected to MI. All data represent mean ± SD (n = 3 mice); *p < 0.05. C. Representative images of myocardial VSMC in infarcted areas in db/db+IS+Ad-β-gal and db/db+IS+Ad-Ang-1 mice stained by Ang-1+/SMA+ cells. Ang-1 was stained with monoclonal anti-angiopoietin-1 linked to FITC (green, 40X). VSMCs were stained with smooth muscle actin (SMA, Red, 40X) and nuclei were stained with DAPI counterstaining (blue, 40X). Merged image showed that over-expressed Ang-1 co-localized with VSMC. Top and middle panel: representative images showing that systemic delivery of Ad-Ang-1 led to a dramatic increase in the number of Ang-1+/SMA+ cells in db/db mouse subjected to myocardial ischemia for 24 hours compared to Ad-β-gal in db/db mice. Bottom panel: representative images showing the newly formed arteriole (Ang-1+/SMA+) in the Ad-Ang-1 treated db/db mouse heart after ischemia for 14 days.

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Overexpression of Ang-1 improves diabetic cardiac function after myocardial ischemia

To determine whether increased homing of hematopoietic stem cell and angiogenesis by Ang-1 promoted cardiac functional recovery, cardiac function was measured by P-V loop at 14 days of myocardial ischemia. As shown in Fig 8 A and B, exposure of diabetic hearts to ischemia for 14 days resulted in a dramatic reduction of cardiac output (CO) and ejection fraction (EF%). Overexpression of Ang-1 led to a significant improvement of CO and EF% in diabetic infarcted hearts.

Discussion

Our data establish, for the first time, that Ang-1 plays a critical role in the recruitment of hematopoietic stem cell CD133+ /c-kit+ and CD45+ cells into ischemic areas of the diabetic infarcted heart. This is accompanied with a significant reduction in myocardial/endothelial apoptosis and cardiac hypertrophy as well as fibrosis formation in diabetic heart. Our study also demonstrates that...
upregulation of SDF-1α/CXCR-4 expression in the diabetic infarcted hearts is a novel downstream signaling pathway of Ang-1-mediated recruitment of CD133+ cells. Further, our studies reveal that overexpression of Ang-1 results in increases in capillary density and arteriole formation together with a dramatic improvement of cardiac functional recovery in the diabetic infarcted hearts. These studies strongly suggest that Ang-1 gene therapy protects the diabetic heart from ischemic injury by promoting

Figure 7. Ang-1 upregulates Jagged1, Notch3 and apelin expression in db/db mouse hearts. A. Western blot densitometry analysis of myocardial Jagged 1 expression showing that systemic delivery of Ad-Ang-1 resulted in a significant increase in Jagged 1 expression in the db/db mice subjected to myocardial ischemia for 24 hours compare to the db/db mice treated with Ad-β-gal. All data represent mean ± SD (n = 4 mice); *p<0.05. B. Western blot densitometric analysis of myocardial Notch3 expression revealing that systemic delivery of Ad-Ang-1 (n = 5) resulted in a significant increase in Notch3 expression in db/db mice subjected to myocardial ischemia for 24 hours compare to the db/db mice treated with Ad-β-gal (n = 4). All data represent mean ± SD; *p<0.05. C. Immunofluorescence microscopy showing expression of Notch3 in the vascular wall of db/db mice infarcted hearts treated with Ad-Ang-1. Merged images showed that Notch3 expression was expressed in newly formed arteriole of the Ad-Ang-1 treated db/db mice (right panel), but not the Ad-β-gal-treated db/db mice (left panel). Notch3 was stained with Notch antibody conjugated to FITC (green, 40X) and nuclei were stained by DAPI (blue). No specific staining was observed in the sham control group. D. Western blot densitometry analysis showing that apelin expression was significantly increased in the db/db mice treated with Ad-Ang-1 compare to the Ad-β-gal treated db/db mice at 24 hours of ischemia. All data represent mean ± SD (n = 4 mice); *p<0.05. E. APLNR expression was increased in the Ad-Ang-1 treated db/db mice compare to the Ad-β-gal treated db/db mice at 24 hours of ischemia, but did not achieve a statistical significantly difference. All data represent mean ± SD (n = 4 mice); p>0.05.

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CD133⁺ and c-kit⁺ stem cell recruitment, and enhancing angiogenesis via a mechanism involving SDF-1α/CXCR-4 signaling.

Increasing evidence reveals that Ang-1 has a burgeoning role in heart disease and possesses cardioprotective effects against myocardial ischemia [10][19]. Previously, we showed that overexpression of Ang-1 increases myocardial angiogenesis and decreases myocardial infarction in STZ mouse model, indicating a potential link between angiogenesis and reduction of MI in diabetes [12]. The present studies further reveal that overexpression of Ang-1 in db/db mouse hearts results in a significant reduction in myocardial apoptosis as well as endothelial cell apoptosis. This was accompanied by significant increase in capillary density and cardiac functional recovery. Although overexpression of Ang-1 alleviates myocardial ischemic injury in the STZ and db/db diabetic animal models, [4,12] the underlying mechanisms by which overexpression of Ang-1 attenuates myocardial apoptosis and promotes cardiac repair are not completely understood.

Previous studies showed that Tie-2 expression was detected in hematopoietic stem cell and Tie-2 signaling was crucial for the maintenance of the hematopoietic microenvironment. Ang-1 activates Tie-2 on hematopoietic stem cell in the niche, maintains the in vivo repopulation ability of hematopoietic stem cell.[13] Recent evidence suggests that hematopoietic stem cell CD133⁺ cells are involved in healing the ischemic myocardium and thus may be important in functional recovery of the myocardium following acute infarction (AMI) [20–22]. In non-diabetic patients with AMI, there is often a surge of CD133⁺ cells in the circulation. However, in diabetic patients experiencing AMI, CD133⁺ cell recruitment and function was significantly impaired [20]. CD133⁺ cells have been reported to release large amounts of VEGF, which activates PI3K/Akt and exerts pro-angiogenic and pro-survival effects. Transplantation of a low number of CD133⁺ human fetal aorta-derived vascular progenitor cells promotes reparative neovascularization and skeletal myocyte regeneration in a non-diabetic ischemic hind-limb model [22]. Intriguingly, local therapy with CD133⁺ cells promotes angiogenesis and significantly accelerates healing of ischemic hind-limb skin wounds in a diabetes mellitus model [21]. To our knowledge, the present study is the first demonstration showing that overexpression of Ang-1 significantly increases the recruitment of CD133⁺ and c-kit⁺ into myocardial ischemic areas in diabetic db/db mice. Previously, we have shown that overexpression of Ang-1 significantly increases myocardial VEGF, Akt and eNOS expression in db/db mice [4]. Based upon these findings, we postulate that Ang-1 promotes CD133⁺ cell and c-kit⁺ recruitment into ischemic areas, leading to secretion of VEGF and activation of the Akt/eNOS signaling pathway, which exert beneficial effects on the diabetic infarcted hearts. This notion is supported by our findings that CXCR-4 and SDF-1α, the chemokine for hematopoietic progenitor cells, were significantly increased within the ischemic myocardium of Ang-1 treated db/db mouse. Furthermore, our data reveal that the increased CD133⁺ co-localize with CXCR-4⁺ and SDF-1α⁺ cells in the area of myocardial ischemia. In addition, SDF-1α/CXCR-4 has been shown to protect the hearts after myocardial infarction [14,23,24]. These findings suggest that activation of SDF-1α/ CXCR-4 signaling mediates Ang-1-induced recruitment of CD133⁺ progenitor cells into the infarcted ischemic heart leading to attenuation of myocardial apoptosis. In addition, our present study shows that Ang-1 protects myocardial endothelial cell against ischemia-induced endothelial apoptosis. Our previous study showed that Ang-1 increases myocardial angiogenesis via activation of Tie-2/Akt/eNOS pathway in coronary endothelial cells [25]. These direct protective effects of Ang-1 on endothelial cells may also contribute to the attenuation of myocardial ischemic injury and increase in myocardial angiogenesis and cardiac function in diabetes.

Ang-1/Tie-2 signaling plays a predominant role in controlling both VSMC and pericyte maturation and is essential for the maintenance of vascular stabilization [1,26]. We have demonstrated that overexpression of Ang-1 increases VSMC recruitment and promotes mature neovessel formation; this is accompanied by a significant increase in capillary density in db/db mouse hearts [4]. However, the intracellular molecular mechanisms by which Ang-1 increases VSMC recruitment and maturation remains unexplored. The Notch ligand Jagged1 is essential for vascular remodeling and has been linked to congenital heart failure in humans [27]. Jagged1 plays a critical role in the regulation of VSMC recruitment/maturation via Notch3 during early embryonic development [28]. Deficiency of Notch3 has been shown to disrupt VSMC differentiation and to increase infarct size in ischemic stroke [29–31]. Recent studies reveal that ethanol and cyclic strain stimulate endothelial cell angiogenic activity via a Notch/Ang-1/Tie-2 pathway suggesting potential crosstalk between Notch signaling and the Ang-1/Tie-2 pathway [32,33]. The present data show that overexpression of Ang-1 significantly increased Jagged1 and Notch3 expression in the db/db mouse.
hearts. Immunohistochemical analysis further confirmed that the Notch3 expression was localized to the wall of larger arteries. Apelin is an endogenous ligand of the human orphan G-protein-coupled receptor APJ [34,35]. Apelin exerts a variety of cardiovascular effects and particularly acts as an activator of angiogenesis [36-38]. A recent study also reported that the apelin/APJ system was involved in the regulation of blood vessel diameter during angiogenesis [40]. Our present data also demonstrated that treatment with Ad-Ang-1 significantly increase apelin expression. These data suggest potential role of Jagged1/Notch3 and apelin in Ang-1-mediated angiogenesis. Most intriguingly, overexpression of Ang-1 increases the number of BM-SMA+ cells as well as Ang-1+/SMA+ cells in the diabetic infarcted hearts, suggesting that Ang-1 over-expressing SMA+ cells in the BM may also be recruited to the ischemic myocardium rather than their homing to Ang-1+ cells in the heart itself.

Recruited CD45+ cells represent a rich source of hematopoietic stem cell cytokines in the ischemic heart.[41] Our data showed that CD45+ cells were dramatically increased in diabetic infarcted hearts after Ad-Ang-1 treatment. Furthermore, the abundance of Tie-2-expressing CD45+ cell was increased implying that these cells may contribute to the Ang-1-mediated angiogenic response in diabetic infarcted hearts. Our data also demonstrate that overexpression of Ang-1 significantly increased BM-derived CD31+ cells and upregulated VEGF expression in BM. Recent studies showed that BM-CD31+ cells were enriched with remarkably higher levels of angiogenic and hematopoietic genes as compared to BM-CD31- cells. Furthermore, BM-CD31+ cells induced the activation of angiogenic, anti-apoptotic and chemotactic factors in ischemic hind-limbs via a paracrine effect. Trans-plantation with BM-CD31+ cells significantly increased angiogenesis and improved recovery from ischemia [42,43]. Jagged1 has been shown to drive immature BM progenitor cells to differentiate into functional EPCs [44]. Jagged1 and Notch3 were increased during the differentiation of BM-derived mononuclear cells into VSMC and EC [45]. Further studies are needed to elucidate the molecular mechanisms and crosstalk between the Jagged1/Notch3 and Ang-1/Tie-2 pathways in the regulation of BM/stem cell recruitment/differentiation into VSMC/EC and vascular maturation.

The present study demonstrates that following myocardial ischemia, Ang-1 gene therapy promotes hematopoietic stem cell recruitment. These changes are associated with an increase in CXCXR-4/SDF-1 expression and myocardial angiogenesis and with a reduction in cardiac hypertrophy and improvement of cardiac functional recovery after ischemia. Given the roles of Ang-1/Tie-2 signaling in hematopoietic stem cell development as well as EC/VSMCs differentiation, our data provide novel insights into the intracellular pathways that regulate the formation of mature vasculature in the diabetic infarcted heart and mechanisms that promote cardiac repair. Our studies may aid in the development of novel therapeutic avenues to ameliorate the impaired stem cell function and insufficient angiogenesis that are characteristic of the diabetic state.

Author Contributions
Conceived and designed the experiments: JX. Performed the experiments: HZ LL. Analyzed the data: HZ LL JX. Contributed reagents/materials/ analysis tools: JX. Wrote the paper: JX.

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