SUPPLEMENTAL MATERIAL

Supplemental Methods

In vitro Experiments

Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) was obtained from Lonza, Walkersville, MD, USA and they were serially passaged. Cells were maintained in EGM-2 culture medium supplemented with growth factors and antibiotics according to company specifications.

Cytochemistry for transfection efficiency of *Ad.LacZ*:

To test the transfection efficiency of *Ad.LacZ in vitro*, cultured HUVECs were treated with *Ad.LacZ* (1×10^7 pfu) and then stained for β-galactosidase (β-gal) activity. In brief, the cells were cultured in 8 well chamber slides and the cells were treated with *Ad.LacZ* and were compared with untreated cells. After 48 hours (hr) of treatment the cells were fixed with 4% paraformaldehyde, washed with PBS thrice for 5 min each. The slides were air dried for 20 min and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 30 min and stained for β-galactosidase activity using X-gal substrate. The cells were incubated with X-gal solution (0.1% X-gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$ and 2mM $MgCl_2$ in PBS, pH-7.0) overnight at 30°C. After incubation, the slides were mounted and the images were digitally captured using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.

Immunocytochemistry for transfection efficiency of *Ad.Trx1*:

To test the transfection efficiency of *Ad.Trx1 in vitro*, cultured HUVECs were treated with *Ad.Trx1* (1 x 10^7 pfu) and stained for Trx1. In brief, the cells were cultured in 8 well chamber slides and the cells were treated with *Ad.Trx1* and were compared with untreated cells. After 48hr of treatment the cells were fixed with 4% paraformaldehyde, washed with PBS thrice for 5 min each and blocked with 1% BSA for 1 hr. After blocking the cells were again washed with PBS for three times 5 min each and incubated with primary antibody for rabbit anti-Trx1 (1:100 in PBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After incubation the slides were again washed with PBS thrice for 5 min each and incubated with secondary anti-rabbit Alexa Fluor 488 (1:200 in PBS, Invitrogen, Carlsbad, CA, USA) for 1 hr. After incubation the cells were again washed in PBS and stained with TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, USA) for nuclear staining. After nuclear staining the slides were mounted with using Vectashield mounting medium (Vector laboratories, Burlingame, CA). The sections were observed and images were acquired using Zeiss LSM510 *Meta* confocal microscope and stored as digital TIFF file format.

Matrigel Tube Formation Assay:

HUVECs were cultured in 12-well cell culture plates and were transfected with *Ad.Trx1* (1 x 10^7 pfu) or adeno-sh-Trx-1 (*Ad.sh.Trx1*, 1 x 10^7pfu) 48 hr prior to matrigel (BD Bioscience, Bedford, MA, USA) assay. These cells were then used for matrigel assay. In brief, 250µl of ice cold matrigel was coated on a 24 well cell culture plate as a base for tube formation. After allowing the gel to settle for 30min in a 37°C, 5% CO_2 incubator, the endothelial cells (4 x 10^4) from normal and adeno pre-transfected cells were seeded onto the matrigel and incubated

overnight at 37°C in a 5% CO_2 incubator. After 18h, the extent of tube formation was recorded (200X) using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.

Effect of Adeno-Trx-1 gene transfection on Trx1, HO-1 and VEGF expression in HUVECs:

HUVECs were maintained in EGM-2 culture medium supplemented with growth factors and antibiotics according to company specifications. HUVECs were subcultured in 12 well cell culture plates and were transfected with *Ad.LacZ* (1 x 10^7 pfu), *Ad.Trx1* (1 x 10^7 pfu) and *Ad.Trx1* + 10 μM tin-protoporphyrin IX (SnPP) (HO-1 activity inhibitor) (Porphyrin Products, Logan, Utah, USA). Following 48 hr of adeno transfection the total cell lysate protein was isolated for Western blot analysis of Trx-1, HO-1 and VEGF. The Trx1 antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, HO-1 antibody was purchased from Stressgen Bioreagents, Ann Arbor, MI, USA and VEGF antibody was obtained from R & D systems Inc. Minneapolis, MN, USA. Primary antibody binding was visualized by respective horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

***In vivo* Experiments**

Induction of Diabetes:

Male SD rats (300-325gm) were randomly separated into normal and diabetic rats as they received an (i.p.) injection of vehicle (0.1mol/l citrate buffer, pH 4.5) alone or streptozotocin (STZ) at a dosage of 65mg/kg body weight dissolved in 0.1mol/l citrate buffer. Five days after STZ injection, hyperglycemia was documented by measuring the glucose content of tail vein blood with Freestyle Flash blood glucose monitoring system. Rats with blood glucose concentrations ≥ 300mg/dl were used for the study. MI was induced in these animals 30 days

after the induction of diabetes. Age matched non-diabetic animals were used as comparable controls.

Surgical Procedure:

Myocardial Infarction (MI) was induced by and permanent Left Anterior Descending (LAD) coronary artery ligation, as previously described [1, 2]. The rats were randomized into eight groups 1) Non-diabetic Control Sham (CS), 2) Non-diabetic Control MI (CMI), 3) Non-diabetic Control MI + *Ad.LacZ* (1 x10^9 pfu; CMI-AdLacZ), 4) Non-diabetic Control MI + *Ad.Trx1* (1 x10^9 pfu; CMI-AdTrx1), 5) Diabetic Sham (DS), 6) Diabetic MI (DMI), 7) Diabetic MI + *Ad.LacZ* (1 x10^9 pfu; DMI-AdLacZ), and 8) Diabetic MI + *Ad.Trx1* (1 x10^9 pfu; DMI-AdTrx1). Briefly, the rats were anesthetized with ketamine HCl (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Cefazolin (25 mg/kg i.p.) was administered as a preoperative antibiotic cover. After endotracheal intubation and initiation of ventilation (Harvard Apparatus Rodent Ventilator, model 683), the heart was exposed through a left lateral thoracotomy (fourth intercostal space) [1]. A 6-0 polypropylene suture was passed with tapered needle under the LAD just below the tip of the left atrium and MI was induced by permanent LAD occlusion in the MI groups [1, 3]. Immediately after LAD ligation the adenoviral vector encoding Trx1 (*Ad.Trx1*, 1 x 10^9 pfu) [4] was intramyocardially administered (in 100µl of PBS, using a 30g needle) at 4 sites at the border zone of the infarct in the CMI-AdTrx1 and DMI-AdTrx1 groups. Adeno-LacZ (*Ad.LacZ*, 1 x10^9 pfu) [4] was used as the control adenoviral vector encoding β-galactosidase in the CMI-AdLacZ and DMI-AdLacZ groups, in order to nullify any possible effects exerted by the vector itself. The animals in the Sham groups underwent the same time matched surgical procedure but for fact that the 6-0 suture was passed beneath the LAD and was removed without

ligation. After application of buprenorphine (0.1 mg/kg s.c.) and weaning from the respirator, the rats were placed on a heating pad while recovering from anesthesia [1, 3].

Kloner RA, *et al.* have assessed and measured the efficiency of adenoviral vector mediated gene transfer and expression in the infarcted myocardium [5]. In their study they have placed the animals in 4 groups based on time interval between induction of MI and the Adenovirus mediated transgene delivery (*Ad.LacZ*). *Ad.LacZ* was administered either immediately or after 7, 22 and 30 days of MI and the expression of the reported gene was assessed 7 days after gene delivery in each case. *The rats those were administered Ad.LacZ, 7, 22 and 30 days after MI underwent a second thoracotomy.* They have observed that the expression of transgene was significant in the rats which received the therapy either immediately or 22 and 30 days after MI, while it was reduced in the rats those received *Ad.LacZ*, 7 days after MI [5].

In our study, we have used STZ induced diabetic rats and MI was induced 30 days after the induction of hyperglycemia. These rats even before the first surgical intervention tend to lose weight, become weak, shows lesser physical activity and vital sign indices deteriorate. There was significant reduction in the heart rate in these animals in the sham group as well as after MI [6]. Overall there was a higher rate of mortality in the diabetic animals after MI (45-52%) than compared to the non-diabetic MI controls (8-12%). Though we considered a second thoracotomy for gene delivery at a later time after the induction of MI, owing to the higher rate of mortality and the stress and pain that the diabetic animals have to undergo, we have administered the adenoviral vectors immediately after MI.

For molecular analysis the left ventricular tissue sections from the border zone/risk area surrounding the infarct were harvested and flash frozen in liquid nitrogen and stored at -80℃

while for histochemical analysis the hearts were removed and the tissue was horizontally sectioned between the point of ligation and the apex and fixed overnight in 4% paraformaldehyde for paraffin sections or directly embedded in Optimum Cutting Temperature (OCT) medium for frozen sectioning. The heart tissue was harvested after 4 days for analysis of Dihydroethidium (DHE) staining, Masson's trichrome staining, cardiomyocyte and endothelial cell apoptosis, for immunohistochemistry and Western blot analysis. Echocardiography was performed to assess the ventricular functions 30 days after gene therapy after which the hearts were harvested for immunohistochemical analysis of capillary and arteriolar density and Masson's trichrome staining.

Effect of SnPP on the expression of VEGF

Alternatively, another group of non-diabetic and diabetic animals were treated with tin-protoporphyrin IX (SnPP, an inhibitor of HO-1 activity, 50µmol/kg, i.p.) to determine whether HO-1 plays an important role in Trx1 mediated VEGF expression *in vivo* [1].

Each animal in this group received five i.p. injections of 50µmol/kg SnPP, spaced on alternated days, 2 injections prior to MI and *Ad.Trx1* gene therapy, 1 injection on the day of the intervention and 2 injections after the MI and gene therapy. The hearts were harvested 4 days after the surgery for Western blot analysis.

Histochemsitry for the transfection efficiency of *Ad.LacZ* and *Ad.Trx1*: In order to examine the efficiency of our gene transfer technique, *in vivo*, we have evaluated the adenoviral gene expression 4 days after intramyocardial adenoviral gene administration in the Sham operated non-diabetic control groups. The heart was exposed through a left lateral thoracotomy as described above and we injected the adenoviral vector encoding β-galacatosidase (β-gal, *Ad.LacZ*) at 4 different sites (in 100µl of PBS, using a 30g needle) on the left anterior wall of the

myocardium. The rats were sacrificed 4 days after gene transfer, hearts were removed and the tissue was horizontally sectioned and embedded in OCT medium. In brief, the slides were air dried for 20 min and fixed in 2% formaldehyde and 0.2% glutaraldehyde for 30 min and stained for β-galactosidase activity using X-gal substrate. The sections were incubated with X-gal solution (0.1% X-gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$ and 2mM $MgCl_2$ in PBS, pH-7.0) overnight at 30°C. After incubation, the slides were mounted and the images were digitally captured at (400X) using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope [6].

Similarly, *Ad.Trx1* was injected at 4 different sites (in 100µl of PBS, using a 30g needle) on the left anterior wall of the myocardium. The rats were sacrificed 4 days after gene transfer, hearts were removed and fixed overnight in 4% paraformaldehyde. Horizontal sections of the heart tissue were embedded in paraffin and 5µm sections were prepared. Briefly, the tissue sections were then cleared in 2 washes of histoclear and rehydrated in gradients of alcohol (twice in 100%, once each in 90%, 80% and 70% alcohol for 5 min each). The tissue sections were washed three times in PBS and antigen retrieval was performed in antigen retrieval buffer (Dako). Following endogenous peroxidase blocking with 0.3% H_2O_2 in PBS for 30min, non-specific binding sites were blocked with buffered casein solution (Power Block Universal Blocking Reagent; Bio Genex, San Ramon, USA) for 10 min at room temperature. After PBS wash (3 times) the sections were stained for Trx1 using mouse monoclonal anti-Trx1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation in biotinylated anti-mouse secondary antibody using the Vectastain Elite ABC Kit-Mouse IgG (Vector Laboratories, Inc, Burlingame, CA, USA) and bound antibody was visualized with 3,3'-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc,

Burlingame, CA, USA). The images were digitally acquired at (400X) using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.

Assessment of Myocardial Fibrosis (Masson's Trichrome staining):

To determine the effect of Trx1 gene therapy on myocardial fibrosis (4 days and 30 days after MI/gene therapy, respectively), the paraffin embedded sections of the hearts from each group were stained as per the Masson's Trichrome staining protocol, as previously described [6]. In brief, the paraffin sections were deparaffinized in two washes of Histoclear rehydrated through alcohol gradients (100%, 90%, 80% and 70%) at room temperature. The slides were then washed thrice in distilled water and fixed in Bouin's fixative overnight. The slides were then stained with Weigert's iron hematoxylin (10 min) and placed under running tap (10 min). The sections were again washed in distilled water and then stained with Biebrich scarlet-acid fuschin solution for 15 min. Following another wash in distilled water the sections were further differentiated in phosphomolybdic-phosphotungstic acid solution for 15 min. The sections were then directly transferred to aniline blue solution and stained for 10 min, followed by a brief wash in distilled water and was then treated with 1% acetic acid solution for 5 min. The sections were then dehydrated quickly in 90% and 100% alcohol, cleared in Histoclear and mounted using Permount. The heart tissue sections were digitally imaged in high pixel resolution on an Epson Scanner and the images were stored as JPEG file format [6].

Reactive Oxygen Species (ROS) detection in the myocardium:

4 days after MI and gene therapy the animals were sacrificed and heart tissue sections were harvested and directly embedded in OCT medium as described above. Superoxide production in the hearts 4 days after MI and gene therapy was detected by dihydroethidium (DHE) staining

(*Invitrogen*, Carlsbad, CA, USA). 10μm frozen heart sections were incubated with 30μM DHE for 45min at 37°C in a humidified chamber protected from light [7]. Fluorescent images were obtained using Zeiss LSM510 *Meta* confocal laser scanning microscopy. All images were treated equally and background corrections were performed. The average fluorescent intensity of the nuclei were then analyzed using AutoQuant X2 software (MediaCybernetics, Bethesda, MD, USA).

Determination of cardiomyocyte and endothelial cell apoptosis:

The rats were sacrificed after 4 days of MI and gene therapy, hearts were removed and fixed overnight in 4% paraformaldehyde and then preserved in 70% ethanol. The heart tissue was horizontally sectioned between the point of ligation and the apex and the sections were embedded in paraffin to prepare the block from paraffin embedded tissue sections were made. The heart tissue sections (5μm) were then cleared in 2 washes of histoclear, rehydrated in gradients of alcohol (twice in 100%, once each in 90%, 80% and 70% alcohol for 5 min each) and washed thrice in 1X PBS. Immunohistochemical detection of apoptotic cells was carried out using TUNEL reaction using In Situ Cell Death Detection Kit, Fluorescein as per the kit protocol (Roche Diagnostics, Mannheim, Germany) [8]. In brief, the TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis which can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction catalysed by Terminal deoxynucleotidyl transferase (TdT). Fluorescein labels incorporated in nucleotide polymers are detected by fluorescence microscopy. The sections were washed in PBS three times, blocked with 1X Power Block for 10min and incubated with cardiomyocyte specific mouse monoclonal anti-α sarcomeric actin (1:100 in PBS; Sigma Aldrich, St. Louis, MO, USA) and endothelial cell specific rabbit monoclonal anti-von-Willebrand factor (anti–vWF, 1:100 in PBS; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) followed by staining with the respective secondary Alexa Flour-555 antibodies (1:200 in PBS; Invitrogen, Carlsbad, CA, USA). After incubation, the sections were rinsed thrice in PBS and mounted with Vectashield fluorescent mounting medium (Vector, Burlingame, CA, USA). The sections were observed and images were captured using a confocal laser Zeiss LSM 510Meta microscope. For the quantitative purpose, the number of TUNEL-positive cardiomyocytes and endothelial cells were counted from the endocardium through the epicardium of the mid portion of the left ventricular free wall in from each heart [3, 8].

Immunohistochemistry for capillary and arteriolar density:

The rats were sacrificed 30 days after MI and gene therapy and hearts were removed and paraffin embedded tissue sections were prepared as described previously and were used for capillary and arteriolar density staining and analysis.

Briefly, the sections were stained for capillary density using goat polyclonal anti-CD31/PECAM-1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with secondary anti-goat ImmPRESS reagent as per manufacturer's specifications (ImmPRESS Anti-Goat Ig (peroxidase) Kit, Vector Laboratories, Inc, Burlingame, CA, USA). The antigen antibody interaction was visualized using 3,3'-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc, Burlingame, CA, USA). The images were digitally acquired at 400 x magnification using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope and were used for CD-31 counting. Counts of capillary density per square millimeter of the area at risk from the endocardium through the epicardium of the mid portion of the left ventricular free wall were counted after superimposing a calibrated morphometric grid on each digital image using Adobe Photoshop CS4 Software [9].

For arteriolar density the vascular smooth muscle cells were labeled using mouse monoclonal anti-smooth muscle actin (1:100 in PBS; Abcam Inc, Cambridge, MA) followed by incubation in donkey anti-mouse Alexa Flour 488 (1:200 in PBS; Invitrogen, Carlsbad, CA, USA). To-Pro 3 iodide (Invitrogen, Carlsbad, CA, USA) was used as nuclear stain. After incubation, the sections were again washed in PBS three times and mounted with coverslip using a Vectashield fluorescent mounting medium (Vector, Burlingame, CA, USA). The images were captured using Zeiss LSM510 *Meta* confocal microscope and the arteriolar density was assessed and was expressed in per square millimeter of the area at risk [3, 9].

Immunohistochemistry for expression of Trx-1, HO-1 and VEGF:

The rats were sacrificed 4 days after MI and gene therapy and hearts were removed and paraffin embedded tissue sections were prepared as described previously and were used for immunohistochemical analysis of Trx1, HO-1 and VEGF.

Briefly, the separate sections were stained for Trx1 and HO-1 using mouse monoclonal anti-Trx1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-HO-1 (1:100 in PBS; Abcam Inc, Cambridge, MA), respectively. This was followed by incubation in biotinylated anti-mouse secondary antibody using the Vectastain Elite ABC Kit-Mouse IgG (Vector Laboratories, Inc, Burlingame, CA, USA) and bound antibody was visualized with 3,3'-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc, Burlingame, CA, USA).

For VEGF staining, paraffin embedded tissue sections were stained for VEGF using rabbit polyclonal anti-VEGF (Lab Vision Products, Thermo Fisher Scientific, Fremont, CA, USA) followed by incubation with secondary anti-rabbit ImmPRESS reagent as per manufacturer's

specifications (ImmPRESS Anti-rabbit Ig (peroxidase) Kit, Vector Laboratories, Inc, Burlingame, CA, USA). The antigen antibody interaction was visualized using 3,3'-diaminobenzidine (DAB) substrate using DAB Substrate Kit (Vector Laboratories, Inc, Burlingame, CA, USA). The images for Trx1, HO-1 and VEGF staining were digitally acquired at 400 x magnification using Olympus QColor 3TM digital camera mounted on an Olympus BH2 microscope.

Western blot analysis for Trx1, HO-1, VEGF, p-JNK, JNK, p38MAPK (α and β):

The rats were sacrificed 4 days after MI and gene therapy and the hearts were removed and left ventricular risk area sections were frozen in liquid nitrogen and stored at -80°C. To quantify the Trx1 (12kDa), HO-1 (32kDa), VEGF (46kDa), p38MAPKα (38kDa), p38MAPKβ (38kDa) and p-JNK (54kDa and 46kDa) standard SDS/PAGE Western blot technique was performed [10]. Heart tissue sections from each treatment group were homogenized and suspended (50 mg/ml) in sample buffer (10 mM Tris.HCl, pH 7.3, 11.5% sucrose, 1mM EDTA, 10μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The cytosolic protein was isolated and total protein concentration was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockville, IL). The cytosolic proteins were run on polyacrylamide electrophoresis gels (SDS-PAGE) typically using 12% for Trx1 & 10% for HO-1, VEGF, p38MAPKα, p38MAPKβ, p-JNK and JNK [10]. GAPDH was used as loading control for Trx1, HO-1, VEGF, p38MAPKα, and p38MAPKβ, while JNK was used as loading control for p-JNK. The Trx1 and p38MAPKβ antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, HO-1 antibody was purchased from Stressgen Bioreagents, Ann Arbor, MI, USA, VEGF antibody was obtained from R & D systems Inc. Minneapolis, MN, USA, and the p38MAPKα, p-JNK (Thr183/Tyr185) and JNK antibodies were purchased from Cell Signaling, Danvers, MA, USA. Primary antibody

binding was visualized by respective horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

Echocardiography:

Echocardiogram analysis was carried as previously described [6, 9]. After 30 days of MI each rat was sedated using isoflurane (3%, inhaled). When adequately sedated, the rat was secured with tape in the supine position in a custom-built mold designed to maintain the rat's natural body shape after fixation. The hair on the chest wall was removed with a chemical hair remover. Ultrasound gel was spread over the precordial region, and ultrasound biomicroscopy (Vevo 770, Visual-Sonics Inc., Toronto, ON, Canada) with a 25-MHz transducer was used to visualize the left ventricle. The left ventricle was analyzed in apical, parasternal long axis, and parasternal short axis views for LV systolic function, LV cavity diameter, wall thickness, diastolic function, and LV end-systolic and end-diastolic volume determination. MI segments were determined according to the kinetics: hypokinetic (reduction in wall motion), akinesis (no wall motion), and dyskinesis (unsynchronized movement of segment with normal myocardium). Two-dimensional directed M-mode images of the LV short axis were taken just below the level of the papillary muscles for analyzing ventricular wall thickness and chamber diameter. All the LV parameters were measured according to the modified American Society of Echocardiography–recommended guidelines. Ejection fraction and fractional shortening were assessed for LV systolic function. Diastolic function was assessed by measuring mitral peak flow velocity of the E-wave and A-wave in centimeters per second (cm/s), as was the ratio between the two waves (E/A). All the measurements represent the mean of at least three consecutive cardiac cycles. Throughout the procedure, ECG, respiratory rate, and heart rate were monitored [3, 6, 9].

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