Online Appendix - Data Supplement

Materials and Methods

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. The diabetic experimental rats used showed elevated blood glucose levels in between 400 and 420 mg/dl. 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:

The experimental animals (non-diabetic control and diabetic) 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 tracheotomy 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 the tapered needle under the left anterior descending (LAD) coronary artery just below the tip of the left atrium and Myocardial Infarction (MI) was produced by permanent LAD occlusion in the MI groups (1; 2). Immediately after LAD ligation a mixture of adenoviral vectors encoding for VEGF (*Ad.VEGF*, 6 x 10^7 pfu) and Ang-1 (*Ad.Ang1*, 1.5 x 10^5 pfu) were intramyocardially administered in combination (in 100µl of PBS, using a 30g needle) at 4 sites at the border zone of the ischemic area in the CVAMI and DVAMI groups. Adeno-LacZ (*Ad.LacZ*, 6x10^7 pfu) was used as the control adenoviral vector encoding β-galactosidase in the CLZMI and DLZMI 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. In our current investigation the adenoviral dosage was used based on Zhou *et al's* observations (3). *Ad.VEGF* and *Ad.Ang1* were generous gifts from Dr. Li C, East Tennessee State University, Tennessee, USA (3). The authors have verified the transfection efficiency of these adenoviral vectors *in vitro*. Moreover Zhou *et al* have demonstrated the synergistic effect of co-administration of VEGF and Ang1 in the ischemic rat myocardium and its effect on the reduction myocardial infarct size through the induction of PI3-K activity and expression of Bcl-2 (3).

After the surgery, the lungs were re-inflated and the chest incision was closed. 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; 2). For molecular analysis the left ventricular tissue sections from the border zone surrounding the infarct were harvested and flash frozen in liquid nitrogen for molecular analysis 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 and then fixed in 70% ethanol. The hearts were harvested after 2 days for gel shift analysis for NFκB and Western blot analysis for p-MK2 (Thr 334) and after 4 days for Western Blot analysis of VEGF, Flk-1, Ang-1, Tie-2, p-MK2 (Thr 334) and anti-apoptotic protein survivin and 7 days for Masson's trichrome staining, immunohistochemical

analysis and capillary and arteriolar density. The myocardial function was measured *in vivo* by echocardiography 30 days after the intervention and the tissue was then harvested for Masson's trichrome staining.

Histochemical analysis for the transfection efficiency of adeno-LacZ (in vivo):

In the present study the adenoviral vectors encoding the transgene of interest were intramyocardially administered (in 100µl of PBS, using a 30g needle) at 4 different sites into the border zone surrounding the infarct, immediately after LAD ligation. In order to examine the efficiency of localized gene transfer we have injected the adenoviral vector encoding β-galactosidase (β-gal, Ad-LacZ) in the non-diabetic SHAM operated animals into the left anterior wall. The reporter gene expression was assessed 4 days after the gene transfer by X-gal staining. The animals were sacrificed and the hearts were fixed overnight in 4% paraformaldehyde and then processed in sucrose gradients (10%, 20% and 30%). The sections were then embedded in Shandon Cryomatrix (Thermo Scientific, Pittsburgh, PA, USA) for frozen sectioning. 10µm frozen sections were made on glass slides and were used for β-gal staining. 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 with a digital camera attached phase contrast microscope and stored in TIFF file format.

Immunohistochemistry for VEGF and Ang-1

The rats were sacrificed 7 days after the surgical procedure, hearts were removed and the tissue was horizontally sectioned between the point of ligation and the apex and fixed overnight in 4% paraformaldehyde. The paraffin embedded tissue sections (4 µm thick) were deparaffinized and hydrated in graded series of xylene (two washes in 100% xylene-5 min each) and ethanol solutions (twice in 100% alcohol followed by one wash each in 90%, 80%, and 70% alcohol) for 5 min each. The tissue sections were washed thrice in PBS after hydration in the graded series of alcohol and then incubated in pre-boiled target retrieval solution. The sections were stained for VEGF using rabbit polyclonal anti-VEGF (1:100 in PBS; Lab Vision Products, Fremont CA, USA) or Ang-1 (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by a respective biotinylated secondary antibody (1:200 in PBS) and the bound antibody was visualized with 3,3′-diaminobenzidine (DAB) substrate using the Vector ABC Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA). Tissue sections incubated in PBS without the primary antibody were used as the negative control. The analysis was performed in the border region of the heart tissue sections. The sections were examined and images were captured at 400 x magnification and stored in TIFF file format.

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

To determine the effect the combination gene therapy on myocardial fibrosis, the paraffin embedded sections of the hearts from each group were stained as per the Masson's Trichrome staining protocol. The rats were sacrificed 7 and 30 days after the surgical procedure; hearts were removed and fixed overnight in 4% paraformaldehyde. After fixation the paraffin embedded sections (of 4 µm thick) were made and the extent of fibrosis was analysed using Masson's trichrome staining. In brief, the paraffin sections were deparaffinized using Histoclear for 6 min

at room temperature, followed by transferring the slides in fresh Histoclear for 6 additional min. Sections were rehydrated using sequential passage through 100, 95, 80 and 70% ethanol for 6 min each followed by washing in distilled water 3 times for 6 min each. The slides were then stained with Weigert's iron hematoxylin for 10 min and placed under tap water for 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 transferred to aniline blue solution and stained for 10 min. The sections were rinsed briefly in distilled water and were treated with 1% acetic acid solution for 5 min. After a final wash in distilled water the sections were dehydrated through sequential gradient of 70-100% alcohol followed by histoclear wash and then mounted using Permount (4). The heart tissue sections were digitally imaged in high pixel resolution on an Epson Scanner and the percentage of myocardial fibrosis was assessed by Image J software (4).

Immunohistochemistry for capillary and arteriolar density:

The rats were sacrificed after 7 days after the surgical procedure, hearts were removed and the tissue was horizontally sectioned between the point of ligation and the apex and fixed overnight in 4% paraformaldehyde and then fixed in 70% ethanol. The paraffin embedded tissue sections (4 μm thick) were deparaffinized and hydrated in graded series of xylene (two washes in 100% xylene-5 min each) and ethanol solutions (twice in 100% alcohol followed by one wash each in 90%, 80%, and 70% alcohol) for 5 min each. The sections were stained for capillary density using goat polyclonal anti-CD31/PECAM-1 (1:200 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by biotinylated horse anti-goat secondary antibody (1:200 in PBS) and bound antibody was visualized with 3,3′-diaminobenzidine (DAB) substrate using the Vector ABC Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA). The images were captured at 400 x magnification and were used for CD-31 counting. For the quantitative measurement, the number of CD-31 was counted on area at risk from the endocardium through the epicardium of the mid portion of the left ventricular free wall (2).

For arteriolar density the tissue sections of the heart were washed thrice in PBS after hydration in the graded series of alcohol and then incubated in pre-boiled target retrieval solution. Vascular smooth muscle cells were labeled using mouse monoclonal anti-smooth muscle actin (1:100 in PBS; Sigma, St. Louis, MO, USA) followed by incubation in donkey anti-mouse Alexa Flour 488 (1:200 in PBS; Invitrogen, Carlsbad, CA, USA). After incubation the sections were again washed thrice in PBS and mounted using fluorescence mounting medium (Vector laboratories, Burlingame, CA, USA) (2). The analysis was performed in the border region of the heart tissue sections. Images were captured using a confocal laser Zeiss LSM 510 Meta microscope and stored in digital TIFF file format.

Counts (in counts/mm^2) of capillary density and arteriolar density were obtained after superimposing a calibrated morphometric grid on each digital image using Adobe Photoshop Software (2).

Western blot analysis for VEGF, Flk-1, Ang-1, Tie-2, survivin and p-MK2:

The rats were sacrificed 2 and 4 days after the surgical procedure and the hearts were removed and left ventricular risk area sections were frozen in liquid nitrogen and stored at -80°C. To quantify the VEGF, Flk-1, Ang-1, Tie-2, survivin and p-MK2, standard SDS-PAGE Western Blot technique was performed. Heart tissue sections (100mg) from each group were homogenized and

suspended in 1ml of 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 protein was isolated and total protein concentration was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockville, IL). The proteins were run on polyacrylamide electrophoresis gels (SDS-PAGE) typically using 10% for VEGF, Ang-1 and p-MK2, 14% for survivin and 7% for Flk-1 and Tie-2. The VEGF antibody was purchased from R & D systems Inc. MN, USA, Flk-1, Ang-1 and Tie-2 antibodies were purchased from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA, threonine 334 p-MK2 antibody was purchased from Cell Signaling, Boston, MA, USA and the survivin antibody was purchased from Abcam Inc. Cambridge, MA, USA**.** GAPDH (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and non-phosphorylated MK2 (Cell Signaling, Boston, MA, USA) were used as loading controls wherever appropriate. Primary antibody binding was visualized by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

Electrophoretic Mobility Shift Assay (EMSA) for DNA binding activity of NFκB:

The nucleoprotein was isolated according to the kit protocol of the CelLytic NuCLEAR Extraction Kit (Sigma, St. Louis, MO, USA). In brief, 100 mg of tissue was homogenized in 1 ml of buffer containing 10.0 mM HEPES (pH 7.9) with 1.5 mM $MgCl_2$, 10.0 mM KCl, and 1.0 mM DTT solution and centrifuged at 10000g for 20 min. The supernatant containing the cytosolic fraction was transferred to a fresh tube. The pellet was resuspended in 150 μL of extraction buffer containing 1.5 μL of 0.1 M DTT and 1.5 μL of protease inhibitor cocktail (104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin A). The solution was allowed to stand on ice for 30 min with shaking at brief intervals followed by centrifugation at 20000*g* for 5 min. The supernatant containing the nucleoprotein fraction was transferred to a clean chilled tube (4). EMSA was performed by using 5 μg of the nuclear extracts for 20 min at room temperature with ^{32}P end-labeled oligonucleotides containing the putative NF-κB (5′AGTTGAGGGGACTTTCCCAGGC-3′) binding site. Reaction products were resolved on 5% non-denaturing polyacrylamide gel. The specificity of the DNA-protein interaction was established using anti-p65 NF-κB antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) to verify the shift in the band. After electrophoresis, gels were dried and visualized by autoradiography (5).

Echocardiographic Analysis:

Echocardiographic analysis was performed 30 days after MI as described previously (2; 6). Briefly rats received isoflurane (3%) mixed with oxygen for anesthesia and when adequately sedated, were placed on the rat scanning platform and secured with tape. Ultrasound gel was spread over the precordial region, and ultrasound biomicroscopy (UBM) (Vevo 770, Visual-Sonics Inc., Toronto) with a 30-MHz transducer was used to visualize the left ventricle and echocardiographic images were obtained. Left ventricle was analyzed in apical, parasternal long axis, and parasternal short axis views for LV systolic function, LV cavity diameter, wall thickness, and diastolic function determination. Myocardial infracted segment was determined according to its kinetics: hypokinetic (reduction in wall thickness), akinesis (no wall motion) and dyskinesis (unsynchronized movement of segment with normal myocardium). All left ventricular parameters were measured according to the modified American Society of Echocardiography–recommended guidelines. Two dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle allowed us to measure the left ventricular dimensions, as well

as septum and posterior wall thickness. Ejection Fraction (EF) and Fractional Shortening (FS) were assessed for left ventricular systolic function and was calculated from the M-mode LV dimensions using the following equations. Fractional Shortening (%) = 100 x (LVID;d – LVID;s) / LVID;d); Ejection Fraction (%) = 100 x ((LV Vol;d-LV Vol;s) / LV Vol;d) (where LVID;d is the Left ventricular internal diameter in diastole, LVID;s is the left ventricular internal diameter in systole, LV Vol;d is the left ventricular end diastolic volume which is equal to ((7.0 / (2.4 + LVID;d)) x LVID;d^3 and LV Vol;s is the left ventricular end systolic volume which is equal to ((7.0 / (2.4 + LVID;s)) x LVID;s^3). All measurements represent the mean of at least 3 consecutive cardiac cycles (5).

Results

Myocardial Gene Delivery and Expression of the Transgene

In order to evaluate the transfection efficiency of the adenoviral vectors, *Ad.LacZ* ($6x10^7$ pfu) encoding the β-galactosidase enzyme, was employed for intramyocardial administration in non-diabetic Sham operated animals.

We have observed a robust expression of the reporter transgene, LacZ, in the myocardium as assessed histochemically by X-gal staining 4 days after the gene transfer in 10μm thick frozen sections from the harvested hearts (Fig. 1A). The expression was predominantly localized in the cardiac tissue surrounding the sites of *Ad.LacZ* transfection (Fig. 1A), in contrast to the only PBS injected Sham operated animals (negative control), which demonstrated lack of any positive X-gal staining. However, the transgene expression was not evident in areas distant from the sites of gene transfer such as the septum or the right ventricle.

References

1. Kaga S, Zhan L, Matsumoto M, Maulik N: Resveratrol enhances neovascularization in the infarcted rat myocardium through the induction of thioredoxin-1, heme oxygenase-1 and vascular endothelial growth factor. *J Mol Cell Cardiol* 39:813-822, 2005
2. Koneru S, Varma Penumathsa S, Thirunavukkarasu M, Vidavalur R, Zhan L, Singal PK, Engelman RM, Das DK, Maulik N: Sildenafil-mediated neovascularization and protection against myocardial ischaemia reperfusion injury in rats: role of VEGF/angiopoietin-1. *J Cell Mol Med* 12:2651-2664, 2008
3. Zhou L, Ma W, Yang Z, Zhang F, Lu L, Ding Z, Ding B, Ha T, Gao X, Li C: VEGF165 and angiopoietin-1 decreased myocardium infarct size through phosphatidylinositol-3 kinase and Bcl-2 pathways. *Gene Ther* 12:196-202, 2005
4. Samuel SM, Thirunavukkarasu M, Penumathsa SV, Paul D, Maulik N: Akt/FOXO3a/SIRT1-mediated cardioprotection by n-tyrosol against ischemic stress in rat in vivo model of myocardial infarction: switching gears toward survival and longevity. *J Agric Food Chem* 56:9692-9698, 2008
5. Thirunavukkarasu M, Penumathsa SV, Samuel SM, Akita Y, Zhan L, Bertelli AA, Maulik G, Maulik N: White wine induced cardioprotection against ischemia-reperfusion injury is mediated by life extending Akt/FOXO3a/NFkappaB survival pathway. *J Agric Food Chem* 56:6733-6739, 2008
6. Penumathsa SV, Koneru S, Samuel SM, Maulik G, Bagchi D, Yet SF, Menon VP, Maulik N: Strategic targets to induce neovascularization by resveratrol in hypercholesterolemic rat myocardium: role of caveolin-1, endothelial nitric oxide synthase, hemeoxygenase-1, and vascular endothelial growth factor. *Free Radic Biol Med* 45:1027-1034, 2008