Supplemental material

Detailed methods

Patients

The hearts of 8 people deceased of myocardial infarction (MI) were obtained at autopsy performed at the Department of Pathology, University-Hospital of Parma. Coronary atherosclerosis was severe and affected the left and right coronary arteries in all cases. Myocardial infarction consistently involved the anterior and inferior aspects of the left ventricle (LV). None of the patients had a history of systemic hypertension or diabetes. Control hearts (n=8) were from subjects of similar age who did not have primary heart disease or major risk factors for coronary artery disease, including hypertension, diabetes, obesity, and severe atherosclerosis. Autopsy and histologic examination of all organs ruled out the presence of diffuse metastatic malignant neoplasms and chronic inflammation. The control (not MI) group consisted of 6 people died from acute trauma, one from gastrointestinal hemorrhage and one from pulmonary thromboembolism. Patient data are shown in Supplemental Table I.

Immunohistochemical staining in human hearts.

Immunohistochemical staining to detect NGF was performed using a polyclonal rabbit antibody (Abcam, Cambridge, UK) and the peroxidase Advance Herp method (Dako, Milan, Italy). The reaction was revealed by diaminobenzidine (DAB). The analyses were performed in randomly chosen fields using the following semi-quantitative scoring system: no staining: 0; weak staining: +; weak to moderate staining: ++; strong staining: +++.[1] The expression of TrkA in cardiomyocytes and endothelial cells (ECs) was assessed in α-sarcomeric actin (α-sarc) and CD34 positive cells, respectively. Heart sections were incubated with a monoclonal antibody anti-TrkA (Cell Signaling, Danvers, MA, USA) revealed with anti-rabbit FITC and a monoclonal mouse anti-α-sarc (Sigma, Milan, Italy) antibody followed by anti-mouse TRITC TrkA expression in ECs was recognized by double staining for TrkA and CD34 (monoclonal mouse antibody, Neomarkers) revealed by anti-mouse TRITC. CD34 is not exclusively expressed by ECs. Therefore, CD34-positive cells were classified as ECs also in base at their morphology and vascular localization. Nuclei were visualized by DAPI (4`,6-diamidino-2-phenylindole, Sigma) staining.

Preparation of NGF adenoviral vector.

The pCMV-Sport 6 containing the complete cDNA of human *NGF*-β (*p.NGF*), cloned into NotI and SalI restriction sites, was purchased (LGC Promochem, Teddington, UK). To prepare an adenoviral vector carrying human NGF-β (*Ad.hNGF*), the coding sequence for the NGF-β from *p.NGF* was amplified (KOD proofreading DNA polymerase, Novagen, Darmstadt, Germany) using the following primers: 5'-GCTAGCGTAATGTCCATGTTGTTCTAC-3' (NheI site) and 5'-GGATCCTCTCACAGCCTTCCT-3' (BamHI site and stop signal removed) to allow the inclusion of a V5-tag at the C-terminus. The fragment was excised and subcloned in the shuttle vector pDC515 (Microbix Biosystems, Toronto, ON, Canada) modified to contain the V5 coding sequence. A replication-deficient adenovirus was generated by site-specific FLP-mediated recombination of the cotransfected shuttle and genomic plasmids in 293 cells. Viral stocks were amplified, CsCl banded, and titrated. Preparation of *Ad.hNGF* was already published by us.[2]

Myocardial infarction (MI).

Mice were anesthetized by intraperitoneal injection of ketamine (50mg/kg) and xylazine (2.5mg/kg), orally intubated and artificially ventilated using a Minivent mouse ventilator (Harvard Apparatus, Kent, UK). The tidal volume was set at 8-9 µl/g and the respiratory rate was set at 140 breaths per minute. Under a surgical microscope, an incision was made at the level of the left $5^{th.}$ intercostal space and MI was induced by permanent ligation of the proximal left anterior descending coronary artery (LAD) by using a 7.0 Mersilene suture (Ethicon, Somerville, NJ, USA). Coronary occlusion was confirmed by pallor and regional wall motion abnormality of the left ventricle. To study the effect of endogenous NGF after MI, one group of mice (n=38 mice) was i.p. injected with a goat-raised antibody neutralizing NGF[3-5] (Ab-NGF, kindly provided by Dr. Luigi Aloe, Consiglio Nazionale delle Ricerche, Roma, Italy) at the dose of 100µg in 100µl PBS, starting 10 minutes after induction of MI and every 5 days thereafter. Control MI mice (n=30 mice) and sham-operated (n=20 mice) received the same volume of non immune goat-IgG. For analyses at 3 days post-MI, mice received only one i.p. injection of Ab-NGF or its control (10 minutes after MI or sham operation; n=10 mice/group). To investigate the therapeutic potential of NGF overexpression, 2 minutes after the induction of MI, gene transfer in the peri-infarct area was performed by intra-myocardial multiple injections with an adenoviral vector carrying human NGF (*Ad.hNGF*) at the dose of 10^8 plaque-forming unit (p.f.u.)/10 µl (n=90 mice; 55 mice for analyses at 14 and 30 days, 35 mice for analyses at 3 days). Under the surgical microscope, we entered the infarct zone using a 30G needle bent at the right angle and injected the genetic material in 3 equidistant points of the MI border zone. Control MI mice (n=75 mice; 42 mice at 14 and 30 days, 33 mice at 3 days) were injected with an empty vector (*Ad.Null*). Sham-operated mice underwent the same procedure except LAD was circled with a 7-0 Mersilene but not occluded (n=60 mice; 32 mice at 14 and 30 days, 28 mice at 3 days). They also received intra-myocardial *Ad.Null* in LV. Surgical wound was sutured and animals were allowed to recover. Post-operative survival after MI and NGF neutralization or overexpression was monitored for 14 or 30 days, depending on the duration of the experimental protocol. Furthermore, additional groups of MI mice given *Ad.hNGF* or *Ad.Null* were treated with/out a goat-raised antibody neutralizing the c-kit receptor ligand stem cell factor (SCF) (Ab-SCF, R&D System, 100µg in 100µl PBS, i.p. every 5 days starting from the day of surgery)[6] (n=30 mice/group). This approach was used in order to investigate whether NGF-induced therapeutic benefit after MI depends at least in part on the SCF-induced cardiac expansion of $Lin^{neg}c\text{-}kit^{pos}$ progenitor cells at 3 days post-MI. Sham-operated mice received *Ad.Null* (n=15 mice). Finally, in order to verify whether the Akt/Foxo-3a pro-angiogenic and anti-apoptotic pathway plays a role in the NGF-induced therapeutic effects after MI, additional MI mice were injected in the peri-infarct with a constitutive active mutant form of Foxo-3a, which has Alanine residues in the three phosphorylation sites of Akt and it is consequently resistant to Akt phosphorylation (*Ad.AAAFoxo3a*, Vector Biolabs, Philadelphia, PA, USA) or with a combination of *Ad.hNGF* and *Ad.AAAFoxo3a*. Control mice received *Ad.Null* alone or combined with *Ad.hNGF* (*Ad.Null*/*Ad.hNGF*) (n=15 mice/group).

Measurement of LV remodelling and function by echocardiography.

Cardiac dimensional and functional parameters were analyzed in anesthetized (Tribromoethanol, 880mmol/kg i.p., Sigma) mice at 14 and 30 days after MI by using a High Resolution Echocardiography System (Vevo 770, Visual Sonics) with a 30-MHz phased-array transducer. After a short-axis two-dimensional (2D) image of the LV was obtained at a level close to the papillary muscles, a 2D guided M-mode image crossing the anterior and posterior walls was recorded. The following parameters were investigated: LV ejection fraction (EF, %), LV fractional shortening (FS, %), LV chamber volume ($µl^3$), LV internal diameter (LVID, mm) and LV anterior and posterior

wall thickness (mm) during both systole and diastole. Echocardiographic analyses were performed in at least n=10 mice/group.

Measurement of LV function by Millar catheter.

At 14 and 30 days after induction of MI or sham operation, mice were anaesthetized with Tribromoethanol, intubated and ventilated. A miniaturized 1.4F transducer tip-catheter (Millar Instrument, Huston, TX) was inserted in the right carotid artery and advanced to the LV cavity to simultaneously measure heart rate (HR, beats/min), peak systolic LV pressure (LVP, mmHg), LV end-diastolic pressure (LVEDP, mmHg), maximal rate of LVP rise (dP/dt_{max}, mmHg/s) and minimal rate of LVP fall (dP/dt_{min}, mmHg/s). Data were digitally recorded and analyzed with a dedicated software (PowerLab/4SP data acquisition system and LabChart Reader Software, ADInstrument). Hemodynamic analyses were performed in at least 10 mice/group.

Measurement of myocardial perfusion.

Myocardial blood flow (BF) was measured using fluorescent microspheres of 0.02µm in diameter (Invitrogen, Paisley, UK). Anesthetized mice were intubated and a polyethylene catheter (PE10) was inserted into the right carotid artery and connected to a syringe pump for collection of reference BF. Then the chest was opened and microspheres were injected into the LV cavity at the rate of 0.15ml/min (200µl of total volume). Animals were sacrificed 2 minutes later and the heart removed and the LV separated. To verify homogenous distribution of microspheres into the bloodstream, both kidneys were also collected and analyzed as internal controls. Each sample was weighted, cut in small pieces and digested in 10ml of 2M ethanolic KOH at 60°C for 48 hours. Finally, microspheres were collected and fluorescence intensity determined by a fluorometer. Regional absolute BF was calculated and expressed in ml/min/g of tissue.[7] Myocardial perfusion was assessed in at least 10 mice/group.

Mouse heart preparation for histological analyses.

Histological analysis were assessed in perfusion/fixed hearts collected from mice at 3, 14 and 30 days after MI or sham-operation (n= 5-6 mice/group). Briefly, mice were anesthetized (Tribromoethanol, 880 mmol/Kg i.p.) and intubated. The abdominal cavity was opened and the aorta was cannulated with a PE-50-catheter connected to a perfusion apparatus. The chest was opened and the heart arrested in diastole by intraventricular injection of cadmium chloride (100 nmol). The right atrium was then cut and the myocardial vasculature was perfused with a heparinized PBS-solution at a pressure similar to the mean arterial pressure, followed by 10 min perfusion with 10% formalin. After removal of the atria and the major thoracic vessels, the hearts were excised and fixed in 4% formalin. After 24-hours fixation in 4% buffered formalin, the LV, inclusive of the septum, was separated from the right ventricle (RV). Next, the heart was sliced into 4 transversal sections, perpendicularly at the major axis: the first section contained the atrio-ventricular level (part 1), the second section showed the following healthy tissue (part 2), the third section showed the peri-infarct zone up to the middle part of the scar, thereby including LV posterior wall which belongs to the remote zone (part 3), and the fourth section showed the apical scar-part of the heart (part 4). Transverse LV slices were embedded in paraffin. LV sections (3 µm in thickness) from part 3 were prepared onto poly-lysin coated slides for subsequent histological or immunohistochemical analyses.

Analysis of capillary and arteriolar density.

Analyses of capillary and arteriolar density were performed in transverse sections of the LV peri-infarct zone and remote zone from hearts collected 14 or 30 days after MI or sham operation (n= 5-6 diastole-arrested hearts per group). Arteriolar and capillary densities were evaluated after fluorescent immunohistochemical staining for α-smooth muscle actin (α-SMA, Sigma) and with isolectin B4 (Sigma), which

recognizes ECs. Slides were observed under a fluorescence microscope. High power fields were captured (at 400X). Arterioles were recognized as vessels with one or more continuous layer of α-SMA-positive vascular smooth muscle cells and isolectin B4 positive lumen. The number of arterioles per mm^2 was counted in blind. According to their luminal size, arterioles were also divided in different categories: small (luminal diameter <50μm), medium (50μm-100μm) and large (>100μm; including arteries) arterioles. The number of capillaries per mm^2 was evaluated in the same sections by counting the number of isolectin B4-positive and α-SMA-negative microvessels.

Apoptosis of ECs and cardiomyocytes.

Apoptosis was quantified at 14 days after MI on paraffin embedded LV sections (3μm) by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (in situ cell death detection kit Fluorescein, Roche applied science, USA). Following treatment of slides with proteinase K (20 μg/ml, 30min at 37°C), TUNEL assay was performed according to the manufacturer's instruction. The same sections were then stained with DAPI to recognize nuclei. To recognize cardiomyocytes, sections were also stained with mouse monoclonal primary antibody for the cardiomyocyte marker α-sarc (Dako), which was revealed by counterstaining with the secondary antibody conjugated to fluorophore (Alexa 568, Invitrogen, Molecular probes). Apoptosis of ECs was investigated by combining TUNEL with von Willebrand Factor (Abcam) staining. Ten fields from the peri-infarct zone were analysed at 1000X magnification. The number of TUNEL-positive nuclei of ECs and cardiomyocyte was expressed as percent of total EC or cardiomyocyte nuclei. The fraction of TUNEL positive nuclei over total nuclei was then calculated for both cardiomyocytes and ECs.

Identification of cardiac cells populations by immunohistochemistry.

Putative cardiomyocyte progenitor cells were identified as those cells expressing both the stem cell receptor marker c-Kit on the surface and the nuclear cardiac transcription factor GATA-4.[8] Heart sections were incubated with an antibody for c-kit (R&D Systems, Abingdom, UK) followed by incubation with an antibody for GATA-4 (Santa Cruz). In order to evaluate the impact of NGF overexpression on the proliferative potential of c-kit^{pos} progenitor cells, heart sections were double-stained for c-kit and the cell cycle marker MCM-2 (minichromosome maintenance protein-2; Santa Cruz). The density of c-kit^{pos} cells, c-kit^{pos}GATA-4^{pos} cells and c-kit^{pos}MCM-2^{pos} cells per mm^2 of myocardium was evaluated at 1000X magnification in the three different portions of myocardium: peri-infarct zone, remote zone and infarct zone (recognized as the area entirely occupied by the scar).[8] Analyses were performed at 3 days post-MI.

Identification of cardiac progenitor cells (CPCs) by flow cytometry.

These analyses were performed adapting a protocol kindly provided by Prof. Wolfgang-Michael Franz (Ludwig-Maximilians-University, Klinukum Grosshadern, Munich, Germany). Cardiac flow cytometry analysis was performed at 3 and 14 days after MI or sham operation. Hearts were explanted and blood was washed out with PBS. The LV was separated from RV and septum, weighted and minced. A "myocyte-depleted" cardiac cell population was prepared by enzymatic digestion of LV in 0.1% collagenase IV (30 minutes at 37°C) and filtration through a 70-μm mesh. The expression of c-kit (APC-Cy7 conjugated, BD Biosciences, Oxford, UK) and sca-1 (FITC-conjugated, BD) was evaluated in isolated cells stained for anti-lineage markers antibodies (FITC-conjugated, Caltag, Buckingham, UK). Unstained and single stained controls were performed to define positivity. Fluorescence was analysed in a Canto II flow cytometer using the Diva software (BD). In order to determine the absolute number of progenitor cell populations per gram of heart, flow cytometry analyses were performed using fluorescent counting beads (Invitrogen).

Following manufacturer instruction, 20,000 counting beads in 100µl (same volume as sample) were added to the myocardial sample immediately before use. The final absolute count was determined by the formula: final absolute count = [(number of cells counted/ total number of beads counted) x number of beads per ml)]/ mg of tissue.

Isolation of ckit^{pos} cells from the murine heart.

Cardiac c-kit^{pos} cells were isolated from hearts harvested at 3 days after MI or sham operation and gene transfer. The LV was separated and minced. Cardiomyocytes were depleted by enzymatic digestion (0.1% collagenase IV for 30 minutes at 37ºC) followed by filtration through a 70µm mesh. Remaining cells were labeled with c-kit monoclonal antibody conjugated with magnetic beads (Miltenyi Biotec, Germany) and c-kit^{pos} cells were selected by using MACS separation columns (Miltenyi Biotec) according to the manufacture instructions.

Isolation of rat neonatal cardiomyocytes (RNCMs) and NGF transfer.

RNCMs were isolated from 2- to 3-day-old Wistar rats (bred at the University of Bristol), as previously described.[9] Briefly, heart was excised and minced and RNCMs were prepared by four cycles (15 minutes) of digestion in 0.1% trypsin containing 0.02% EDTA in PBS. Digestion was stopped by the addition of 20% fetal calf serum (FCS). The dispersed cells were resuspended in DMEM supplemented with 10% FCS, 100 µg/ml streptomycin, and 100 U/ml penicillin and pre-plated for 2h to exclude fibroblasts which adhere earlier than CMs. The suspended RNCMs were seeded on gelatin-coated plates (1% gelatine in PBS) in serum-free 4:1 DMEM-M199 medium. The level of purity of RNCM cultures was assessed by staining for the cardiac marker α-sarc. RNCMs were growth in 4:1 DMEM-M199 and were infected with *Ad.hNGF or Ad.βGal* (each at 50 M.O.I. in PBS) for 48 hours, as previously described.[9]

RT-PCR and Q-PCR.

Total RNA was extracted using the RNeasy plus mini kit (Qiagen, Crawley, UK) and RNA concentration was measured with the NanoDrop spectrophotometer. Gene expression was evaluated in the per-infarct zone of hearts collected at 3 days after MI or sham operation. The following set of primers has been used: human NGF-ß forward 5'-GGCTGCCTGGCGGTTTAT-3', reverse 5'-GGCAGGTCAGGCTCT TCTCA-3' (which amplify human NGF cDNA but do not amplify murine NGF cDNA); mouse NGF-ß forward 5'-AGACTTCCAGGCCCATGGTA-3', reverse 5'-GAACTCCCCCATGTGGAAGA-3'; mouse TrkA forward 5'-CTTTGTGCACCGGGATCTG-3', reverse 5'-TCATGCCAAAGTCTCCAATCTTC-3'; mouse SCF forward 5'-CCCTGAAGACTCGGGCCTA-3', reverse 5'-CAATTACAAGCGAAATGAGAGCC-3'. Gene expression was additionally evaluated in c-kit^{pos} cells, isolated from mice hearts as described previously, using the following set of primers: mouse TrkA forward 5'-CTTTGTGCACCGGGATCTG-3', reverse 5'-TCATGCCAAAGTCTCCAATCTTC-3'; mouse p75^{NTR} forward 5'-CTAGGGGTGTCCTTTGGAGGT-3', reverse 5'-CAGGGTTCACACACGGTCT-3'. Finally, gene expression for rat SCF (forward 5'-TTCGCTTGTAATTGGCTTTGC -3', reverse 5'- TTCAACTGCCCTTGTAAGACTT -3') was also assessed in RNCMs. 18S ribosomal RNA was used for normalization.

Western blot analyses.

Western blot analyses were performed on proteins extracted from LV peri-infarct tissues isolated at 3 days after surgery. The equivalent of 50 µg of total proteins was loaded, resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA). Primary antibodies were: TrkA (Santa Cruz), V5 (Novus Biological, Littleton, CO, USA), total and phospho(Ser^{473})-

Akt, total and phospho(Thr^{32})-Foxo-3a (all from Cell Signaling), total and phospho(tyr^{730})-c-kit, SCF (all from R&D Systems) and GAPDH (loading control, Research Diagnostics Inc., Flanders, NJ,USA).). Secondary antibodies were: anti-rabbit, anti-mouse, or anti-goat IgG- horseradish peroxidase conjugate (Amersham Bioscience).

Statistical analyses.

Values are presented as mean±standard error of the mean (SEM). Statistical significance was evaluated through the use of an unpaired *t* test for comparisons between 2 groups. For comparison among more than 2 groups, ANOVA was used, followed by an unpaired *t* test. Survival curves were analyzed by Log-rank test. Analyses were performed using the SigmaStat 3.1 software. A P value <0.05 was interpreted to denote statistical significance.

Detailed methods references:

1. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM, Hayes DF. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol.* 2007;25:118-145.

2. Caporali A, Sala-Newby GB, Meloni M, Graiani G, Pani E, Cristofaro B, Newby AC, Madeddu P, Emanueli C. Identification of the prosurvival activity of nerve growth factor on cardiac myocytes. *Cell Death Differ.* 2008;15:299-311.

3. Vigneti E, Bracci-Laudiero L, Aloe L. Production and characterization of a monoclonal antibody against nerve growth factor (NGF) which recognizes rodent and human NGF. *The Year in immunology.* 1993;7:146-149.

4. Torcia M, Bracci-Laudiero L, Lucibello M, Nencioni L, Labardi D, Rubartelli A, Cozzolino F, Aloe L, Garaci E. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell.* 1996;85:345-356.

5. Emanueli C, Salis MB, Pinna A, Graiani G, Manni L, Madeddu P. Nerve growth factor promotes angiogenesis and arteriogenesis in ischemic hindlimbs. *Circulation.* 2002;106:2257-2262.

6. Colmone A, Amorim M, Pontier AL, Wang S, Jablonski E, Sipkins DA. Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science.* 2008;322:1861-1865.

7. Cardinal TR, Hoying JB. A modified fluorescent microsphere-based approach for determining resting and hyperemic blood flows in individual murine skeletal muscles. *Vascul Pharmacol.* 2007;47:48-56.

8. Graiani G, Lagrasta C, Migliaccio E, Spillmann F, Meloni M, Madeddu P, Quaini F, Padura IM, Lanfrancone L, Pelicci P, Emanueli C. Genetic deletion of the p66Shc adaptor protein protects from angiotensin II-induced myocardial damage. *Hypertension.* 2005;46:433-440.

9. Sala-Newby GB, Freeman NV, Curto MA, Newby AC. Metabolic and functional consequences of cytosolic 5'-nucleotidase-IA overexpression in neonatal rat cardiomyocytes. *Am J Physiol Heart Circ Physiol.* 2003;285:H991-998.

Online Supplemental Tables and Figures

	MI	Controls
Number	8	8
Age (year)	69±10	62±12
Sex (male/female)	6/2	5/3
Cause of death	MI	Acute trauma (n=6) Gastrointestinal hemorrhage (n=1) Pulmonary thromboembolism (n=1)

Supplemental Table I. Age, sex and cause of death of human MI and control subjects. Age data are expressed as mean±SEM.

		MI	
	Sham/Goat IgG	Goat IgG	Ab-NGF
Hemodynamics			
LV pressure (mmHg)	81.93±1.6	67.12±1.2*	56.02±1.2** †
LVEDP (mmHg)	2.24±0.25	10.31±0.97**	10.26±0.41**
LV dP/dt max (mmHg/s)	4881.6±111	3304.7±115**	2887.8±109** †
LV dP/dt min (mmHg/s)	-3574.2±185	-2536.1±98.4*	-2137.6±94.7** †
Heart rate (beats per minute)	463.3±16.8	380.87±13.5**	409.69±9.5*
Echocardiography			
Ejection fraction (%)	75.27±4.55	31.31±3.75**	27.26±2.07** †
Fractional shortening (%)	45.0±4.78	15.15±2.00**	12.93±1.02**
LV remodelling			
LV posterior wall (ED; mm)	1.08±0.06	0.96±0.07	0.91±0.07
LV posterior wall (ES; mm)	1.50±0.13	1.15±0.09	1.25±0.09
LV anterior wall (ED; mm)	0.82±0.04	0.34±0.05**	0.33±0.02**
LV anterior wall (ES; mm)	1.33±0.08	0.38±0.08**	0.42±0.04**
ED LV internal diameter (mm)	3.31±0.20	4.85±0.18**	5.75±0.26** †
ES LV internal diameter (mm)	1.79±0.29	4.10±0.26**	4.91±0.30**
ED LV chamber volume (μl^3)	42.06±7.40	118.77±11.9**	165.26±19.4** †
ES LV chamber volume (μl^3)	13.90±3.50	83.51±10.7**	121.78±16.4** †

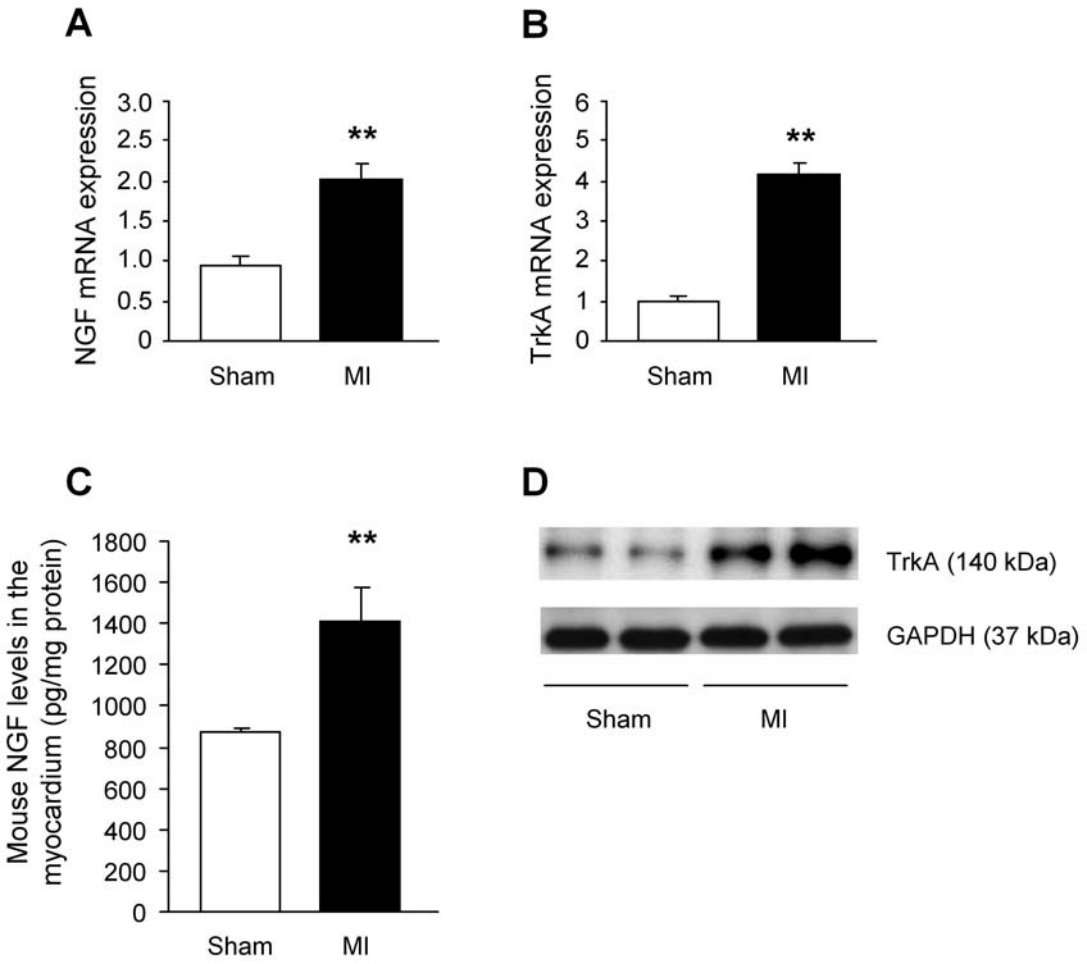
Supplemental Table II. Effect of endogenous NGF neutralization on the left ventricle functional and dimensional parameters at 14 days post-MI. NGF neutralization was obtained by using a goat-raised Ab-NGF. Non immune goat IgG was used for control. Left ventricle functional and dimensional parameters were measured by Millar tip-catheter and echocardiography at 14 days post-MI. ED: end diastolic; ES: end systolic. Data are expressed as mean±SEM. *p<0.05 and **p<0.01 vs. sham/Goat IgG; †p<0.05 vs. MI/Goat IgG. (n= at least 10 mice/group).

	14 days			30 days		
		MI			MI	
	Sham/*Ad.Null*	*Ad.Null*	*Ad.hNGF*	Sham/*Ad.Null*	*Ad.Null*	*Ad.hNGF*
Hemodynamics						
LV pressure (mmHg)	88.50±3.9	63.52±2.7**	73.77±2.5** †	84.46±1.2	59.76±2.15**	70.45±1.7** ††
LVEDP (mmHg)	2.20±0.33	10.79±1.08**	5.06±0.41** ††	2.13±0.18	11.01±0.67**	6.79±0.79** ††
LV dP/dt max (mmHg/s)	4706.7±128	2969.5±146**	3935.1±193** †	4807.6±63	2855.4±194**	3813.22.1±103** ††
LV dP/dt min (mmHg/s)	-3513.9±227	-2377.54±97.3**	-3060.81±140** †	-3697.97±183	-2363.02±83.9**	-2984.75±127* ††
Heart rate (beats per minute)	441.8±22.1	387.68±12.2*	413.15±17.1	467.6±19.2	383.08±5.18**	407.19±12.9*
Echocardiography						
Ejection fraction (%)	70.17±2.43	31.69± 2.75**	46.87±4.25** †	70.87±1.62	27.38± 2.18**	39.38±2.21** ††
Fractional shortening (%)	39.76±2.35	15.11±1.47**	24.15±2.63** †	41.75±1.49	12.92±1.13**	19.27±1.19** ††
LV remodelling						
LV posterior wall (ED; mm)	0.88±0.07	1.00±0.19	1.08± 0.18	0.85±0.06	0.49±0.05**	0.81± 0.04 ††
LV posterior wall (ES; mm)	1.23±0.08	1.20±0.10	1.43±0.06	1.24±0.07	0.89±0.09	1.36±0.06 †
LV anterior wall (ED; mm)	0.82±0.09	0.59±0.17*	0.58±0.22*	0.78±0.02	0.28±0.02**	0.37±0.03* ††
LV anterior wall (ES; mm)	1.29±0.09	0.75±0.15**	0.78±0.07**	1.34±0.03	0.28±0.02**	0.52±0.06** †
ED LV internal diameter (mm)	3.60±0.09	4.80±0.08**	4.39±0.08**	3.60±0.10	5.62±0.11**	4.76±0.15** ††
ES LV internal diameter (mm)	2.17±0.14	4.05±0.16**	3.36±0.11** †	2.11±0.08	4.84±0.11**	3.71±0.21* †
ED LV chamber volume (μl^3)	49.26±4.87	102.27±8.99**	85.76±10.65*	54.95±3.70	156.02±7.30**	112.37±5.66** ††
ES LV chamber volume (μl^3)	16.05±2.02	70.07±6.91**	49.28±8.65**	17.62±0.74	110.4±6.33**	68.99±6.31** †

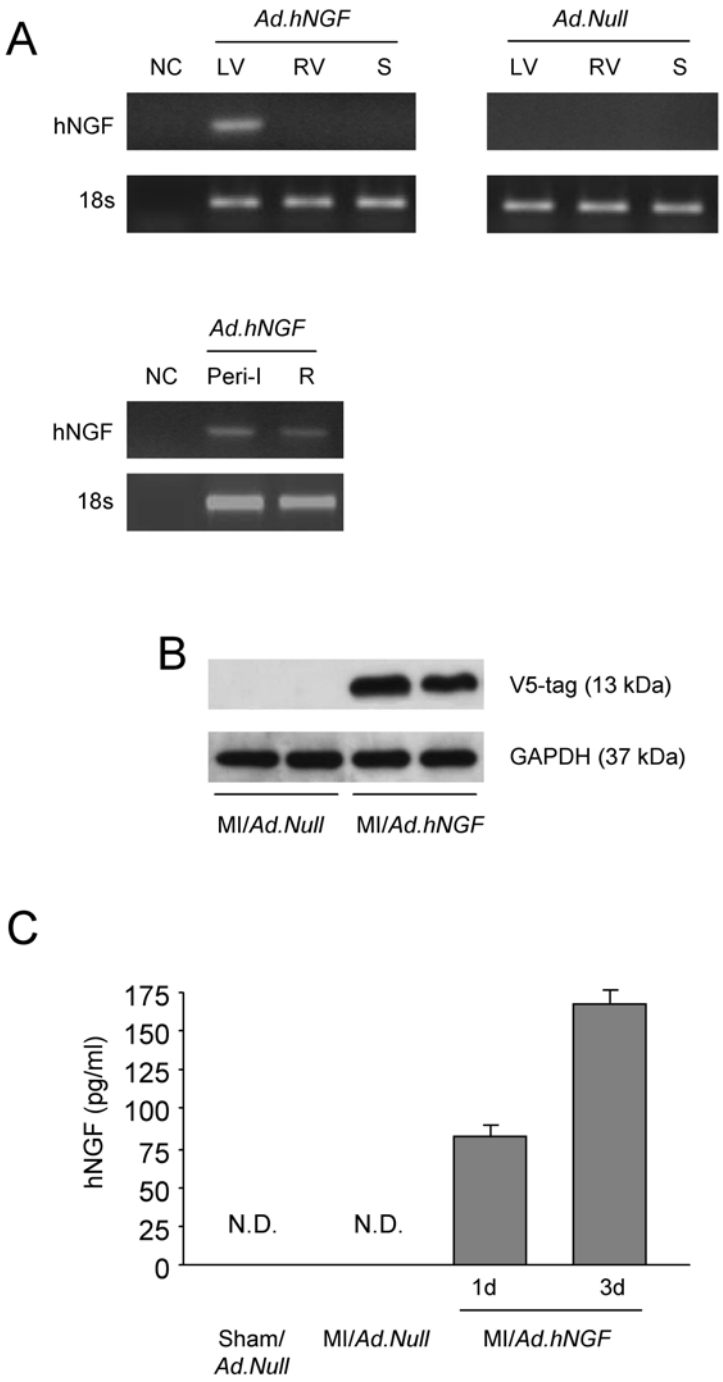
Supplemental Table III. Cardiac functional and dimensional parameters measured by Millar tip-catheter or echocardiography at 14 and 30 days post-surgery and *hNGF* or *Null* gene transfer. ED: end diastolic; ES: end systolic. Data are expressed as mean±SEM. *p<0.05 and **p<0.01 vs. sham/*Ad.Null*; †p<0.05 and ††p<0.01 vs. MI/*Ad.Null*. (n= at least 10 mice/group/time point).

	MI/*Ad.Null*/ *Ad.Null*	MI/*Ad.Null*/ *Ad.hNGF*	MI/*Ad.Null*/ *Ad.AAAFoxo-3a*	MI/*Ad.AAAFoxo-3a*/ *Ad.hNGF*
Echocardiography				
Ejection fraction (%)	28.99±1.94	42.56± 0.84*	25.90±1.96	26.35±2.23††
Fractional shortening (%)	13.84±0.99	21.69±0.87*	12.17±1.02	12.40±1.14††
LV remodelling				
LV posterior wall (ED; mm)	0.93±0.03	0.99±0.05	0.72± 0.07	0.85±0.05
LV posterior wall (ES; mm)	1.28±0.06	1.39±0.06	0.96±0.07	1.07±0.07†
LV anterior wall (ED; mm)	0.53±0.01	0.53±0.03	0.31±0.03**	0.38±0.03
LV anterior wall (ES; mm)	0.62±0.02	0.71±0.05	0.96±0.07	0.41±0.07†
ED LV internal diameter (mm)	4.78±0.14	4.41±0.09	5.29±0.18	4.98±0.13†
ES LV internal diameter (mm)	4.15±0.08	3.53±0.12**	4.60±0.19	4.36±0.21†
ED LV chamber volume (μl^3)	111.48±4.21	92.47±2.35**	130.98±11.16	132.09±6.68†
ES LV chamber volume (μl^3)	81.79±2.93	53.50±2.06**	98.93±9.73	97.58±6.33†

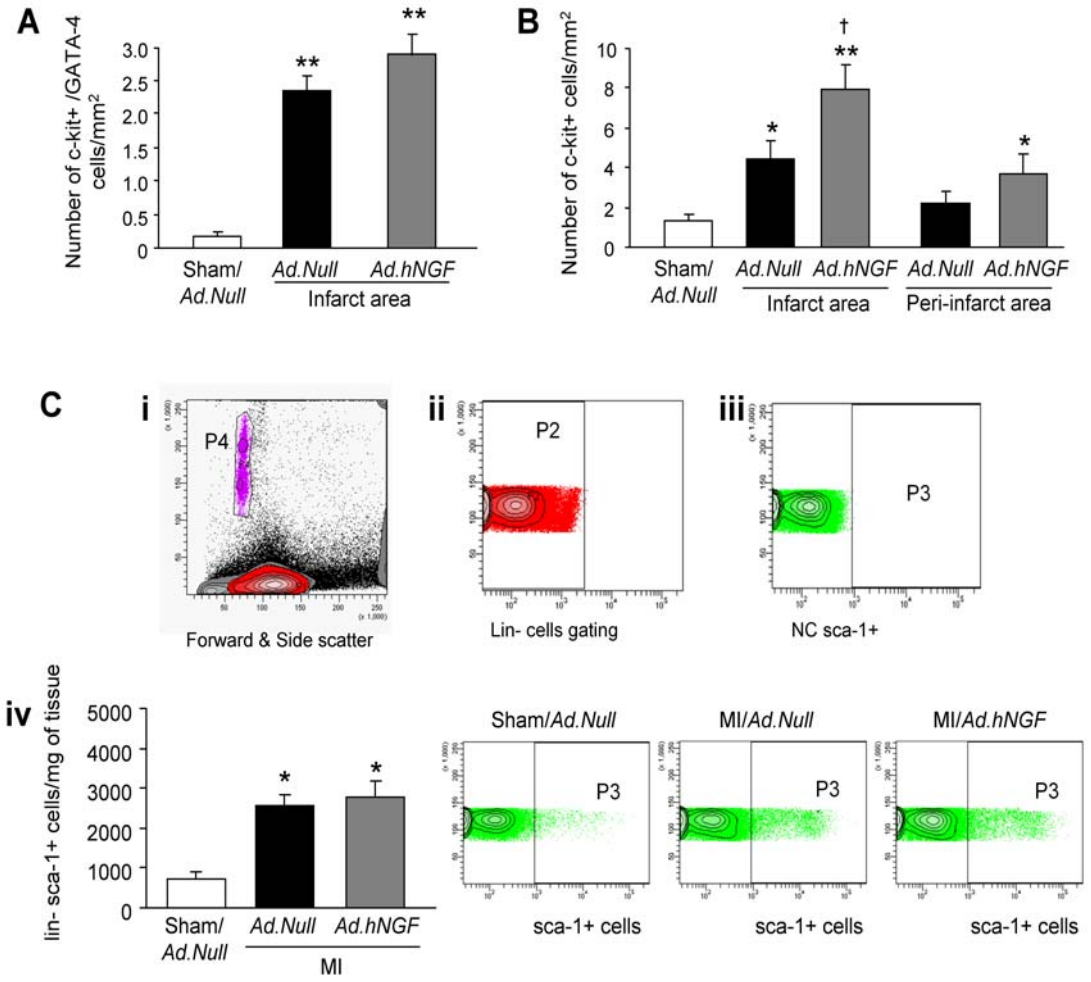
Supplemental Table IV. Inhibition of the Akt-Foxo-3a pathway prevents the beneficial effect of *NGF* gene therapy on left ventricle cardiac function and remodelling at 14 days post-MI. The Akt-Foxo-3a pathway was inhibited in the MI heart by expressing a Foxo-3a mutant form (AAAFoxo-3a), which is resistant to phosphorylation by Akt. MI was induced in mice and the peri-infarct hearts were infected (*via* adenoviral vectors) with one of the following treatments: 1) *Null* (control of *hNGF*) + *Null* (control of *AAA-Foxo-3a*), 2) *Null* + *hNGF*; 3) *Null* + *AAAFoxo-3a*; 4) *hNGF* + *AAAFoxo-3a*. Cardiac functional and dimensional parameters were measured by echocardiography at 14 days post-MI and gene transfer. ED: end diastolic; ES: end systolic. Data are expressed as mean±SEM. *p<0.05 and **p<0.01 vs. MI/*Ad.Null*/*Ad.Null*; †p<0.05 and ††p<0.01 vs. MI/*Ad.Null*/*Ad.hNGF*. (n= at least 10 mice/group).



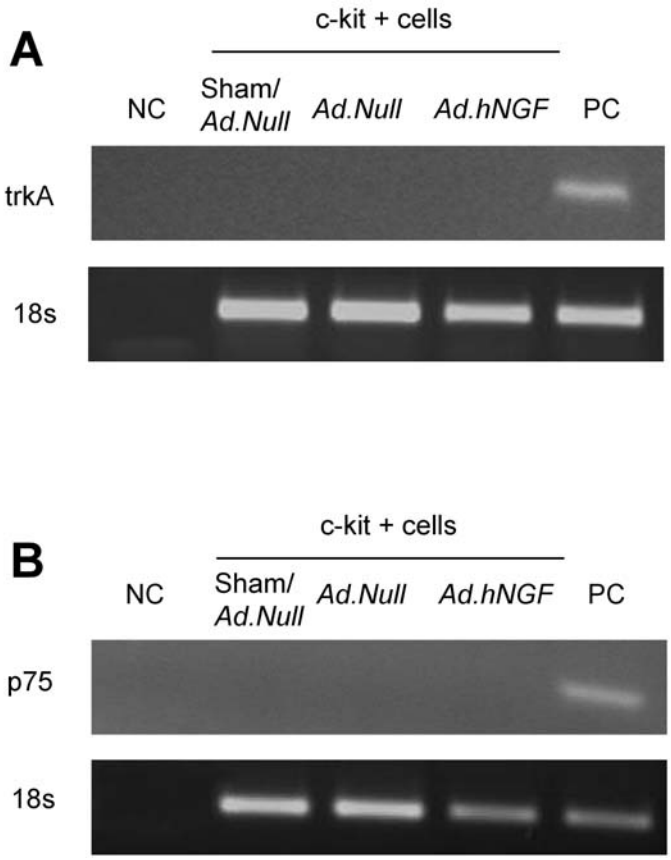
Supplemental Figure I. NGF and TrkA increase after MI in mice. Bar graph represents mouse NGF (A) and TrkA (B) mRNA expression (normalized to 18S ribosomal RNA) in the LV peri-infarct zone at 3d after MI. (C) ELISA for NGF confirmed increased protein expression after MI. (D) Western blot for TrkA showing the increased protein expression in the peri-infarct area at 3 days post-MI. Data are expressed as mean±SEM. **p<0.01 vs. Sham.



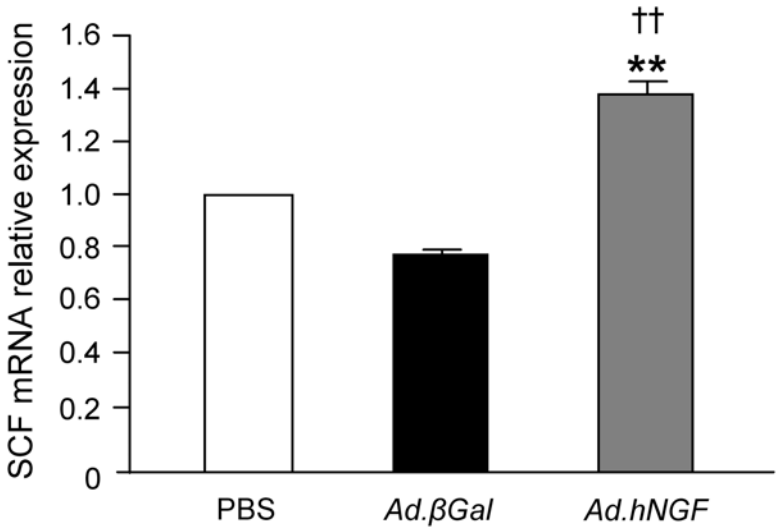
Supplemental Figure II. Efficiency of adenovirus-mediated *hNGF* (V5-tagged) transfer. (A) RT-PCR shows the expression of transgenic human NGF (*hNGF*) in the LV at 3 days post-MI and gene transfer. The upper microphotograph shows the presence of *hNGF* restricted to the LV of mice injected with *Ad.hNGF* (NC: negative control; RV: right ventricle; S: septum). The lower microphotograph shows the expression of *hNGF* in the LV peri-infarct (Peri-I) and remote (R) zones. 18S ribosomal RNA was used for normalization. (B) Representative western blot for V5-tag showing the presence of *hNGF* restricted to the LV of *Ad.hNGF*-injected mice. (C) Bar graph shows the presence of human NGF (measured by ELISA) in the plasma of *Ad.hNGF*-injected mice at 1 and 3 days post-MI and gene transfer Plasma of sham-operated mice and of mice at 3 days after MI and *Ad.Null* delivery was assayed for reference. Data are expressed as mean±SEM. N.D.= not detectable.



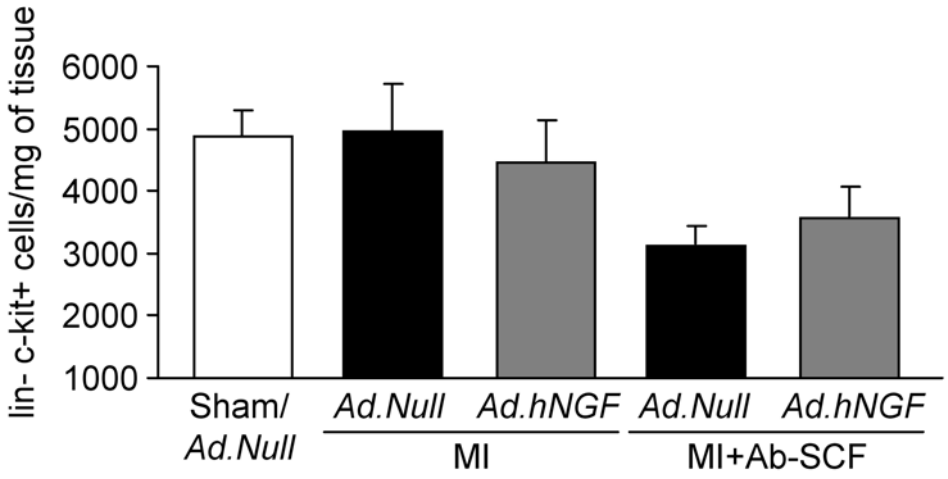
Supplemental Figure III. At 3 days-post MI, *NGF* gene transfer selectively increases the number of heart-resident c-kit^{pos} cells, while it does not affect the abundance of Lin^{neg}sca-1^{pos} cells putative progenitor cells. (A) Bar graph shows the number of c-kit^{pos}GATA-4^{pos} cells in the infarct area at 3 days after surgery. (B) Bar graph quantifies analyses after immunostaining for c-kit at 3 days after surgery and it shows that c-kit^{pos} cells are increased by *Ad.hNGF*-in both the infarct and peri-infarct areas. (C) Identification and quantification of cardiac Lin^{neg}sca-1^{pos} cells by flow cytometry. Forward and side scatter (i) shows the total population analyzed (in red) after LV digestion and cardiomyocyte depletion. For data analysis, counting beads (purple) were identified by their size (in P4 in the forward and side scatter). (ii) Lin^{neg} cells (in P2) were gated from the total population of extracted cells. (iii) Representative graphs show the negative control (NC) for sca-1^{pos} cells. (iv) Bar graph and representative microphotographs show the number of Lin^{neg}sca-1^{pos} cells per mg of LV tissue. The number of c-kit^{pos} cells (in P3) was analyzed within the Lin^{neg} cell population and the absolute number of cells in the heart samples was established using fluorescent counting beads. Data are expressed as mean±SEM. *p<0.05 and **p<0.01 vs. sham/*Ad.Null*; †p<0.05 vs. MI/*Ad.Null*. (n=5 mice per group for histological analyses; n= 6 mice per group for flow cytometry analyses).



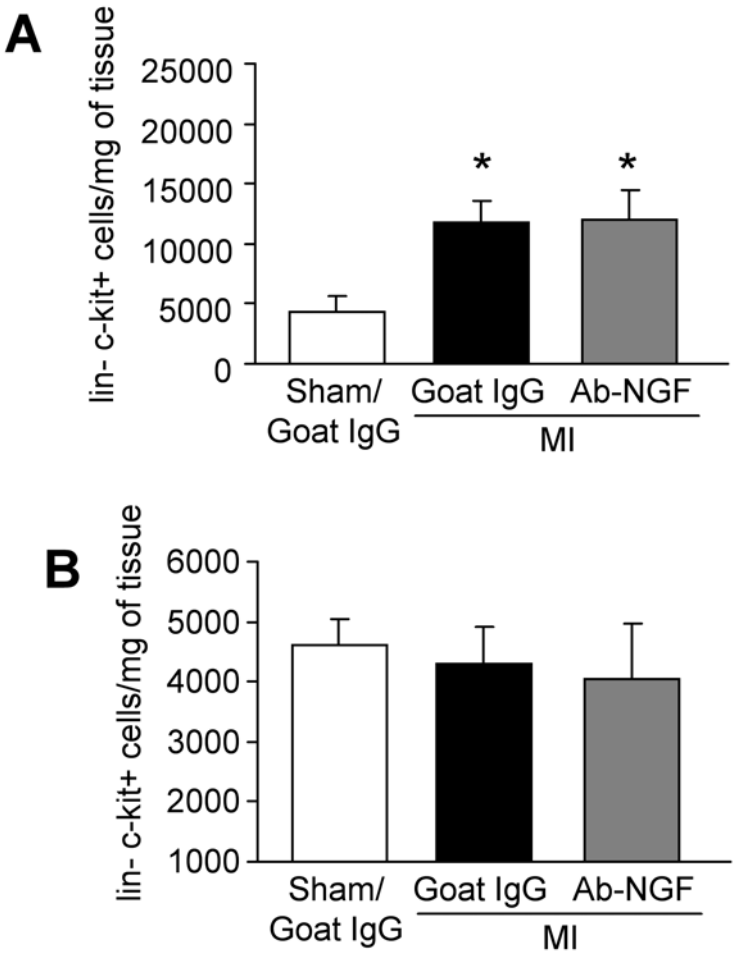
Supplemental Figure IV. TrkA and $p75^{NTR}$ receptors for NGF are not expressed in c-kit^{pos} cell isolated from mouse heart. RT-PCR shows the lack of expression of TrkA (high affinity receptor for NGF) (A) and $p75^{NTR}$ (p75, low affinity receptor for NGF) (B) in c-kit^{pos} cells isolated from mouse heart at 3 days post-MI. PC: The positive control (PC) consists of mouse brain extract.



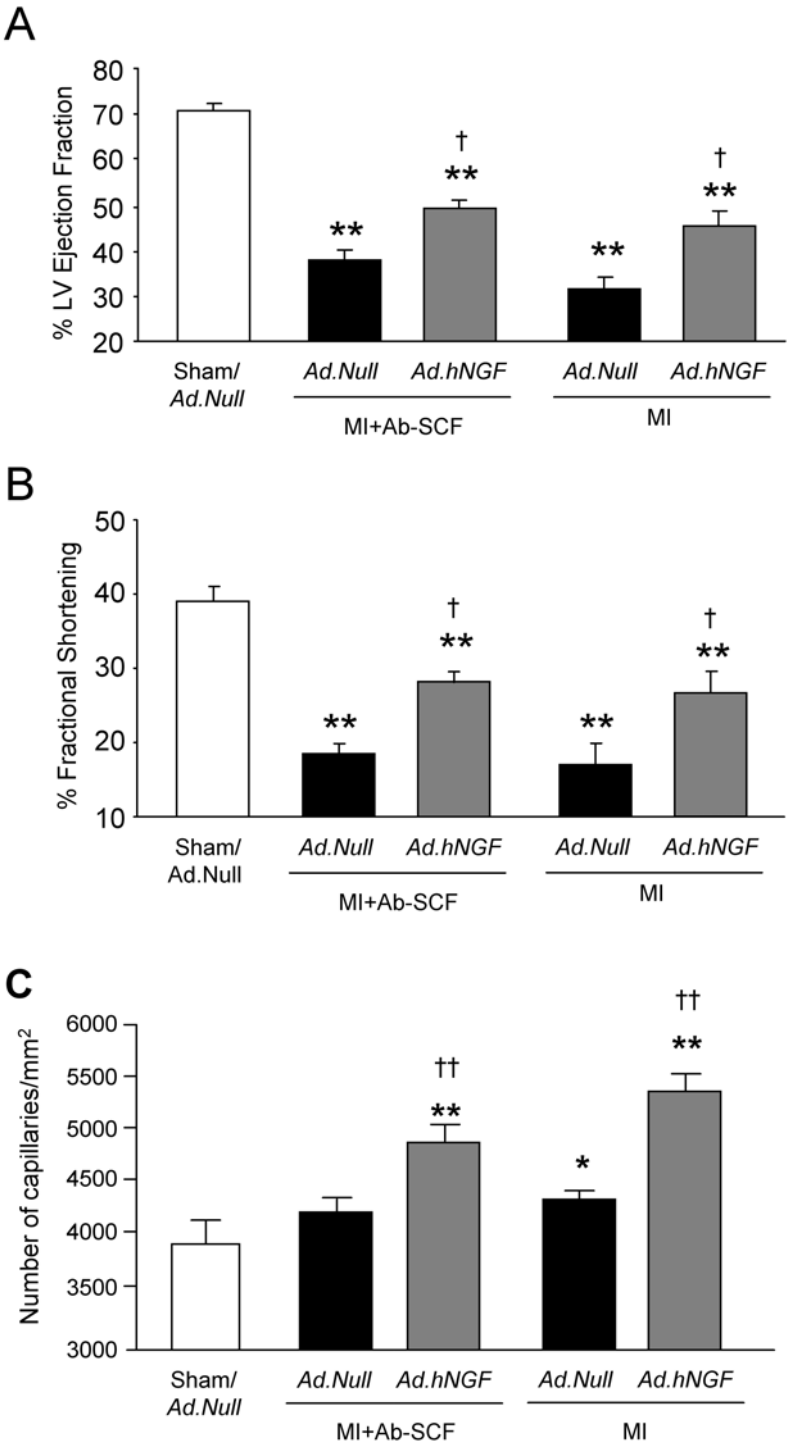
Supplemental Figure V. SCF mRNA level increases in cultured rat neonatal cardiomyocytes (RNCMs) after NGF overexpression. Bar graphs show the mRNA relative expression of SCF in RNCMs at 48h after *NGF* gene transfer. Data are expressed as mean±SEM. **p<0.01 vs. Sham, ††p<0.01 vs. 1*Ad.βGal*.



Supplemental Figure VI. The early MI- and *Ad.hNGF*-induced increase in $Lin^{neg}c\text{-}kit^{pos}$ putative progenitor cell numbers is not maintained at 14 days post-MI. Bar graph shows the number of $Lin^{neg}c\text{-}kit^{pos}$ cells analysed by cardiac flow cytometry at 14 days post-intervention, including SCF neutralization or not. Data are expressed as mean±SEM. (n= 6 mice/group).



Supplemental Figure VII. NGF neutralization does not affect the number of putative c-kit^{pos} progenitor cells after MI. Identification and quantification of cardiac Lin^{neg}c-kit^{pos} cells by flow cytometry shows that the number of Lin^{neg}c-kit^{pos} cells per mg of LV tissue after MI was not influenced by Ab-NGF at either 3 (A) or 14 (B) days post-MI. Data are expressed as mean±SEM. *p<0.05 vs. sham-operated mice given non immune goat IgG. (n= 6 mice/group).



Supplemental Figure VIII. Blocking SCF does not impair the NGF therapeutic actions in mice after MI. (A, B) Bar graphs show the LV ejection fraction (A) and LV fractional shortening (B) measured by echocardiography after *NGF* or *Null* gene transfer with/out associated neutralization of SCF. (C) Bar graphs show the capillary density in the peri-infarct area of the same mice used for panels A and B. All analyses were performed at 14 days after surgery. Data are expressed as mean±SEM. **p<0.01 and *p<0.05 vs. Sham/*Ad.Null*, †p<0.05 and ††p<0.01 vs. MI/*Ad.Null* (within the same group of treatment with/out Ab-SCF). (at least n=6 mice per group for echocardiographic analyses; n=5 mice per group for histological analyses).