Nerve Growth Factor Promotes Cardiac Repair following Myocardial Infarction

Marco Meloni, Andrea Caporali, Gallia Graiani, Costanza Lagrasta, Rajesh Katare, Sophie Van Linthout, Frank Spillmann, Ilaria Campesi, Paolo Madeddu, Federico Quaini, Costanza Emanueli

Rationale: Nerve growth factor (NGF) promotes angiogenesis and cardiomyocyte survival, which are both desirable for postinfarction myocardial healing. Nonetheless, the NGF potential for cardiac repair has never been investigated.

Objective: To define expression and localization of NGF and its high-affinity receptor TrkA (tropomyosin-related receptor A) in the human infarcted heart and to investigate the cardiac roles of both endogenous and engineered NGF using a mouse model of myocardial infarction (MI).

Methods and Results: Immunostaining for NGF and TrkA was performed on heart samples from humans deceased of MI or unrelated pathologies. To study the post-MI functions of endogenous NGF, a NGF-neutralizing antibody (Ab-NGF) or nonimmune IgG (control) was given to MI mice. To investigate the NGF therapeutic potential, human NGF gene or control (empty vector) was delivered to the murine perifarct myocardium. Results indicate that NGF is present in the infarcted human heart. Both cardiomyocytes and endothelial cells (ECs) possess TrkA, which suggests NGF cardiovascular actions in humans. In MI mice, Ab-NGF abrogated native reparative angiogenesis, increased EC and cardiomyocyte apoptosis and worsened cardiac function. Conversely, NGF gene transfer ameliorated EC and cardiomyocyte survival, promoted neovascularization and improved myocardial blood flow and cardiac function. The prosurvival/proangiogenic Akt/Foxo pathway mediated the therapeutic benefits of NGF transfer. Moreover, NGF overexpression increased stem cell factor (the c-kit receptor ligand) expression, which translated in higher myocardial abundance of c-kitpos progenitor cells in NGF-engineered hearts.

Conclusions: NGF elicits pleiotropic beneficial actions in the post-MI heart. NGF should be considered as a candidate for therapeutic cardiac regeneration. (Circ Res. 2010;106:1275-1284.)

Key Words: myocardial infarction ■ angiogenesis ■ gene therapy ■ apoptosis

Myocardial infarction (MI) remains a major cause of morbidity and mortality and it is responsible for about one third of heart failure cases worldwide. Sudden occlusion of a major coronary artery and acute myocardial ischemia causes rapid death of cardiomyocytes and vascular cells in the area of interest. Loss of cardiomyocytes and vasculature leads to progressive fibrous replacement of myocardium, hypertrophic growth of the spared myocardium, and left ventricular (LV) dilatation. Maladaptive ventricular remodeling contributes to post-MI heart failure, and prognosis of heart failure patients is still poor. Myocardial ischemia triggers a spontaneous angiogenic response aimed at reestablishing myocardial blood flow. Nonetheless, this protective response is usually not sufficient.

Nerve growth factor (NGF) is a secreted glycoprotein of the neurotrophin family. NGF elicits its biological effects mainly by binding the high-affinity TrkA receptor (tropomyosin-related receptor A, which is a tyrosine kinase). We and others previously demonstrated that NGF, via TrkA, promotes angiogenesis and endothelial cells (ECs) survival through a mechanism involving the serine/threonine kinase Akt (also known as protein kinase B). Akt regulates a variety of cellular functions and it is strongly implicated in angiogenesis and cell survival. Akt also demonstrated that cultured cardiomyocytes express TrkA and release NGF. Moreover, NGF is an autocrine prosurvival factor for the cardiomyocyte through the Akt/Forkhead box-O transcription factors (Foxo) pathway. Foxo factors stimulate cell death and are downstream targets of Akt. Akt-mediated phosphorylation regulates Foxo-3a subcellular localization and activity: unphosphorylated Foxo-3a resides in the nucleus, whereas Foxo-3a phosphorylation leads to its nuclear exclusion and inactivation. Moreover, Foxo-1 and -3a represses angiogenesis by downregulating endothelial nitric oxide synthase.

Experimental evidences suggest that myocardial repair is, at least in part, dependent on c-kit receptor–expressing (c-kitpos) progenitor cell populations. Lineage negative...
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activation.17 It was recently reported that transgenic mice is predominant in vivo, is able to induce more persistent c-kit mediated cardiac repair after MI.

mice with an adenoviral vector carrying human NGF overexpression, mice were injected in the periinfarct myocar-

heart-derived Linnegc-kitpos cells can generate new vascular

associated form.16 The membrane-bound SCF isoform, which is predominant in vivo, is able to induce more persistent c-kit activation.17 It was recently reported that transgenic mice with cardiomyocyte-specific membrane-associated SCF over-expression have improved post-MI cardiac function and angiogenesis in comparison to wild types.18

In the present study, we firstly investigated NGF and TrkA expression in the human infarcted heart. We then evaluated the functions of endogenous NGF and the therapeutic potential of its overexpression in the post-MI heart using mice. Finally, we studied the potential involvement of the Akt/ Foxo-3a pathway and of cardiac progenitor cell populations, with particular emphasis on Lin<sup>neg</sup>c-kit<sup>pos</sup> cells, in the NGF-mediated cardiac repair after MI.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Immunohistochemical Analysis of Human Cardiac Samples**

Cardiac samples were collected at autopsy from human subjects (described in Online Table I) died after MI or causes other than cardiovascular diseases. NGF expression and TrkA expression and localization in cardiomyocytes and endothelial cells (ECs) were determined.

**MI Protocol in Mice**

In vivo experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and with the prior approval of the UK Home Office and the University of Bristol. MI was induced in anesthetized CD1 male mice, as described.19 To study the actions of endogenous NGF in the postinfarct myocardium, mice received IP a goat-raised NGF-neutralizing antibody (Ab-NGF) or control non immune goat-IgG.6,9 To investigate the therapeutic potential of local NGF overexpression, mice were injected in the periinfarct myocardium with an adenoviral vector carrying human NGF (VS-tagged) or Ad.Null (control).9 Sham-operated mice received Ad.Null. Survival curves of mice enrolled in the NGF neutralization and the hNGF gene therapy protocols were studied. To verify whether NGF-induced therapeutic effect depends on the Akt/Foxo-3a pathway activation, additional MI mice were injected in the perifibract with a Foxo-3a mutant form which is resistant to Akt phosphorylation (Ad.AAFoxo3a) or Ad.Null in combination with Ad.hNGF or Ad.Null. LV function and dimensions were measured by echocardiography at 14 and 30 days post-MI. LV function at 14 days was additionally measured with a miniaturized 1.4F transducer Millar tip-catheter. Myocardial blood flow (mL/min per gram of tissue) at 14 days postsurgery was measured using fluorescent microspheres.20 Myocardial capillary and arteriolar densities were measured at 14 and 30 days postsurgery. EC and cardiomyocyte apoptosis was evaluated at 14 days. Putative cardiomyocyte progenitor cells were identified in myocardial sections as those cells expressing both c-kit and the cardiomyocyte transcription factor GATA-4.21 The rate of c-kit<sup>pos</sup> cell proliferation was determined by double-staining of sections for c-kit and the cell cycle marker minichromosome maintenance protein (MCM)-2.22

The effect of SCF neutralization on NGF-induced improvement of cardiac function and angiogenesis was studied at 14 days postoperation.

**Analyses of Cardiac Progenitor Cells**

Cardiac flow cytometry analyses of LV-resident Lin<sup>neg</sup>c-kit<sup>pos</sup> and Lin<sup>neg</sup>sca-1<sup>pos</sup> progenitor cells were performed at 3 and 14 days (Lin<sup>neg</sup>c-kit<sup>pos</sup> only) postoperation. Expression of TrkA and p75<sup>TR</sup> (low-affinity NGF receptor) by isolated c-kit<sup>pos</sup> cells was evaluated by RT-PCR. The effect of neutralizing the c-kit receptor ligand SCF on Ad.hNGF-induced Lin<sup>neg</sup>c-kit<sup>pos</sup> expansion was assessed at 3 and 14 days postsurgery by flow cytometry.

**Expression Analyses**

At 3 days postsurgery, mRNA levels of transgenic (human) NGF and murine NGF, TrkA and SCF were evaluated in the perifibract zone. Western blot analyses for VS-tag, TrkA, total and phospho(Ser473)-Akt, total and phospho(Thr32)-Foxo-3a, total and phospho(Tyr730)-c-kit, SCF, and GAPDH (loading control) were performed. Human and murine NGF and SCF levels were measured by ELISA.

**Results**

**NGF and TrkA Expression in the Human Heart**

Immunohistochemical analyses detected NGF presence in the human myocardium. As shown in Figure 1A, according to a semiquantitative analysis, the majority of cardiomyocytes of both not infarcted hearts and the remote area of infarcted hearts expressed NGF weakly to moderately. By contrast, most cardiomyocytes in the perifibract area express high NGF levels, thus suggesting that NGF is upregulated by ischemia. Figure 1B shows NGF expression in a human infarcted heart. Figure 1C and 1D shows TrkA expression by cardiomyocytes and ECs in an infarcted heart, respectively.

**Myocardial NGF and TrkA Levels Are Increased by MI in Mice**

To confirm that MI increases NGF myocardial expression, we used a mouse MI model. At 3 days post-MI, relative NGF and TrkA mRNA expression in the perifibract LV increased by 2.0-folds and 4.2-folds in comparison versus sham-operation (Online Figure I, A and B). Furthermore, protein expression of NGF and TrkA increased in the perifibract myocardium, as assessed by ELISA and western blot, respectively (Online Figure I, C and D).
NGF Neutralization Increases Apoptosis, Abrogates Spontaneous Neovascularization, and Worsens LV Function After MI in Mice

To investigate the role of endogenous NGF in the spontaneous responses to MI, we neutralized NGF. Postoperation survival of mice was monitored for 14 days. Whereas survival of sham-operated mice was 100%, mortality in MI mice was significantly increased, although no differences were observed between MI/Ab-NGF- and MI/goat-IgG-injected mice (Figure 2A). Importantly, NGF neutralization aggravated the MI-induced deterioration of cardiac function (Online Table II). In comparisons with non immune goat-IgG, Ab-NGF further decreased LV pressure (Figure 2B), dP/dt max (Figure 2C), dP/dt min (Figure 2D) and LV ejection fraction (LVEF) (Online Table II). Moreover, Ab-NGF increased LV chamber volume and LV internal diameter (Online Table II), which are features of maladaptive LV remodeling. Histological analyses of the periinfarct myocardium (14 days post-MI) indicated that, in comparison with nonimmune goat-IgG, Ab-NGF increased apoptosis of ECs (Figure 2E) and cardiomyocyte (Figure 2F), abrogated the spontaneous capillary growth (Figure 2G), and reduced the density of small (diameter <50 μm) arterioles (Figure 2H).

NGF Overexpression in the Infarcted Myocardium Improves LV Function After MI

To test the therapeutic potential of NGF, we delivered Ad.hNGF or Ad.Null in the mouse perinfarct myocardium.

As expected, at 3 days from Ad.hNGF, human NGF mRNA was expressed in the mouse LV, with higher expression in the perinfarct zone (Online Figure II, A). Moreover, the V5-tag was detected in the perinfarct zone of Ad.hNGF-injected mice (Online Figure II, B). Furthermore, ELISA documented the presence of transgenic NGF in the plasma of Ad.hNGF-injected MI mice, only (Online Figure II, C).

Importantly, Ad.hNGF reduced mortality at 30 days post-MI (P<0.01 versus MI/Ad.Null; Figure 3A) and improved LV function at both 14 and 30 days post-MI. In fact, in comparisons to MI/Ad.Null, at both time points, NGF overexpression increased LV pressure (Figure 3B), decreased LV end-diastolic pressure (Figure 3C), and improved dP/dt max and dP/dt min (Figure 3D and 3E), as well as LVEF and LV fractional shortening (LVFS) (Figure 3F and 3G). Furthermore, Ad.hNGF reduced systolic LV internal diameter and LV chamber volume (Figure 3H and 3I), indicating the preventive effect of NGF on post-MI LV chamber dilatation. All measured functional and dimensional data are reported in Online Table III.

NGF Overexpression Reduces Apoptosis and Improves Angiogenesis and Cardiac Perfusion

At 14 days post-MI, in comparison with Ad.Null, Ad.hNGF reduced the percentage of TUNEL-positive apoptotic ECs and cardiomyocytes in the perinfarct area (Figure 4A and 4B). Moreover, Ad.hNGF increased the densities of both...
capillaries and small (diameter, <50 μm) arterioles at both 14 and 30 days post-MI and gene transfer (Figure 4C and 4D). Figure 4E shows mouse myocardial sections were capillaries are stained by isolectin-B4 (red fluorescence) and arterioles (indicated by arrows) are identified by both isolectin-B4 and α-smooth muscle actin staining (green fluorescence). The proangiogenic effect of Ad.hNGF was associated with improved LV perfusion, as myocardial blood flow was increased to 152% versus MI/Ad.Null (Figure 4F).

NGF Therapeutic Effects Are Regulated by the Akt/Foxo-3a Pathway

Ad.hNGF increased phospho-Akt and phospho–Foxo-3a levels in the perifarct myocardium (Figure 5A). Moreover, Ad.hNGF-induced neovascularization (Figure 5B and 5C) and LV function improvement (Figure 5D and 5E) at 14 days post-MI were abolished by coinjection with Ad.AAAFoxo-3a. All functional and LV dimensional data of this experiment are reported in the Online Table IV.

NGF Increases the Abundance of c-kitpos Progenitor Cells in the Infarcted Heart

To investigate whether NGF overexpression may increase the myocardial abundance of c-kitpos/GATA-4pos, putative cardiomyocyte progenitor cells were counted at 3 days postsurgery.13,23 Results show that hNGF increases the abundance of c-kitpos cells in the periinfarct area (Figure 6A), but not in the infarct area (Online Figure III, A). In addition, Ad.hNGF increased the number of c-kitpos cells in both the perifarct and the infarct area at 3 days post-MI (Online Figure III, B). To address whether NGF overexpression induced proliferation of c-kitpos cells, a double-staining for c-kit and the proliferation marker MCM-2 was performed. As shown by Figure 6B, proliferating c-kitpos cells were higher in NGF-
engineered hearts. Heart-resident Lin^{-}\text{neg}c-kit^{+}\text{pos} cells have been proposed to represent cardiac stem cells.\textsuperscript{24} To investigate the hypothesis that NGF overexpression increases the abundance of Lin^{-}\text{neg}c-kit^{+}\text{pos} cells, flow cytometric analysis of cardiomyocyte-depleted cardiac cell populations was performed. As shown in Figure 6C, MI increased the number of Lin^{-}\text{neg}c-kit^{+}\text{pos} cells. The abundance of Lin^{-}\text{neg}c-kit^{+}\text{pos} cells was further enhanced after NGF overexpression. We also evaluated whether Ad.hNGF selectively enriches for Lin^{-}\text{neg}c-kit^{+}\text{pos} cells without affecting other putative progenitor cell populations. To this aim, flow cytometric analysis for Lin^{-}\text{neg}sca-1^{+}\text{pos} cells was performed at 3 days postsurgery and gene transfer. The percentage of heart-resident Lin^{-}\text{neg}sca-1^{+}\text{pos} cells was increased by MI, without being affected by Ad.hNGF (Online Figure III, C), thus suggesting that NGF activates an enhancement mechanism, which selectively impacts on c-kit^{+}\text{pos} cells. To determine whether the increase of Lin^{-}\text{neg}c-kit^{+}\text{pos} cells was attributable to a direct effect of NGF on these cells, we evaluated whether heart-resident c-kit^{+}\text{pos} cells possess NGF receptors, but RT-PCR showed that neither TrkA nor the NGF low-affinity p75NTR receptor were expressed (Online Figure IV). As the activation of c-kit receptor occurs by SCF binding,\textsuperscript{13,21} we next analyzed whether Ad.hNGF increases SCF levels in the myocardium and in cultured cardiomyocytes. Real-time RT-PCR showed that Ad.hNGF increased SCF mRNA in both the mouse infarcted heart (Figure 7A) and rat neonatal cardiomyocytes (RNCMs) (Online Figure V). Moreover, Western blot analyses showed that Ad.hNGF increased the myocardial level of the 30-kDa membrane-associated SCF isoform (Figure 7B).\textsuperscript{18} ELISA confirmed increased SCF and increased c-kit receptor phosphorylation in NGF-overexpressing myocardium (Figure 7C and 7D). These findings suggest that NGF-induced increase in c-kit^{+}\text{pos} progenitor cells may be dependent on the enhancement of the SCF ligand/c-kit receptor signaling. To further investigate this hypothesis, we neutralized SCF in mice with MI or sham-operation and gene transfer. We verified the efficacy of our SCF neutralization approach by
measuring (by ELISA) the LV amount of immunoreactive SCF, which resulted decreased by Ab-SCF at 3 days post-MI (Figure 7C). Next, we observed that SCF neutralization prevented the Ad.hNGF-induced increase in Lin<sup>neg</sup>c-kit<sup>pos</sup> cells (Figure 7E) and diminished Ad.hNGF-mediated increase in c-kit phosphorylation (Figure 7D) at 3 days post-MI. These data provide further evidence that SCF mediates NGF overexpression-induced increase in heart-resident Lin<sup>neg</sup>c-kit<sup>pos</sup> cells at 3 days post-MI. The MI- and Ad.hNGF-induced increase in heart-resident Lin<sup>neg</sup>c-kit<sup>pos</sup> cells was not maintained up to 14 days from MI and gene transfer, and it was also not affected by long-term SCF neutralization (Online Figure VI).

Finally, NGF neutralization did not change the abundance of Lin<sup>neg</sup>c-kit<sup>pos</sup> cells at 3 or 14 days post-MI (Online Figure VII, A and B), thus suggesting that endogenous factors other than NGF may support Lin<sup>neg</sup>c-kit<sup>pos</sup> homeostasis in the infarcted heart.

Blocking SCF-Mediated Increase in Lin<sup>neg</sup>c-kit<sup>pos</sup> Cells Early After MI Does Not Impair NGF Therapeutic Effects in MI Mice

To investigate whether the rise in Lin<sup>neg</sup>c-kit<sup>pos</sup> cells at 3 days post-MI plays a role in NGF gene therapy-induced therapeutic benefits, we treated mice with the short-term SCF neutralization protocol, earlier (Figure 7E) shown to prevent NGF-induced increase in Lin<sup>neg</sup>c-kit<sup>pos</sup> cells. We did not prolong SCF neutralization because we knew that MI- and Ad.hNGF-induced initial increase in Lin<sup>neg</sup>c-kit<sup>pos</sup> cells are not maintained at later time points (Online Figure VI). Short-term SCF neutralization did not affect the Ad.hNGF-induced improvement of cardiac function (for LVEF, Online Figure VIII, A; for LVFS, Online Figure VIII, B; other parameters are not shown) and myocardial angiogenesis (Online Figure VIII, C) at 14 days post-MI. These results suggest that although NGF overexpression, via SCF, is able
to increase c-kitpos progenitor cells in the infarcted heart, this response is dispensable for the full therapeutic potential of NGF-based therapy.

**Discussion**

NGF was initially considered with respect of its neural functions, but we and others have provided seminal evidence of cardiovascular actions of NGF (for review, see elsewhere). The present study has demonstrated a series of novel and important cardiovascular features of NGF and unveiled the therapeutic potential of this neurotrophin for ischemic heart disease.

NGF neutralization reduced the native angiogenic response to MI. Spontaneous neovascularization is one of the compensatory responses through which the infarcted heart attempts to salvage the spared myocardium. In fact, reduced blood flow (ischemia) is cause of progressive cardiomyocyte and EC depletion by apoptotic death, which contributes to cardiac dysfunction. We found apoptosis of both cardiomyocytes and ECs increased by NGF blockade and this may account for impaired LV function and LV chamber dilatation observed at 14 days post-MI in Ab-NGF mice.

In consideration of the worsened outcome caused by NGF neutralization, we hypothesized that intramyocardial NGF gene therapy could preserve cardiomyocyte/ECs viability and promote reparative processes in the infarcted heart, thereby sustaining cardiac function. Angiogenesis gene therapy has received severe criticisms, but it still remains a promising approach to resolve or at least improve ischemic cardiovascular disease.

In our study, adenovirus-mediated NGF overexpression stimulated angiogenesis at both capillary and arteriolar levels and reduced apoptosis of ECs and cardiomyocytes in the peri-infarct myocardium. The significant angiogenic effect of NGF was also emphasized by increased myocardial perfusion in NGF-engineered hearts. The proangiogenic effect of NGF might be explained by its capacity to directly induce survival, proliferation and migration/invasion of ECs, all essentials for the angiogenic process. In fact, the capacity of NGF to induce EC survival and proliferation has been already reported.

We previously demonstrated that NGF-induced angiogenesis in ischemic limb muscles is mediated by Akt and that NGF promotes in vitro cardiomyocyte survival via the Akt/Foxo-3a pathway. In line with these previous findings, we show here that overexpressing NGF increases Akt and Foxo-3a phosphorylation in the myocardium. Importantly, cotransduction of the MI heart with a mutant form of Foxo-3a which cannot be phosphorylated by Akt prevented NGF gene therapy-induced improvements in myocardial angiogenesis and cardiac function. The impact of NGF on the Akt/Foxo-3a pathway may explain both the proangiogenic and antiapoptotic effects of NGF gene therapy.

We newly identified that NGF increases the expression level of the c-kit receptor ligand SCF and promotes c-kit phosphorylation in the infarcted heart. SCF reportedly induces survival, migration, and tube-like structure formation of human fetal umbilical vein ECs, which express c-kit. SCF was also
shown to induce neovascularization in the adult myocardium.18 However, we did not observe c-kit receptor expression by adult myocardial capillary ECs.15 A possible explanation for this discrepancy is that SCF-induced neovascularization in adult hearts is exclusively dependent on vasculogenesis.18 In our study, SCF resulted fundamental for NGF-induced expansion of heart-resident c-kitpos progenitor cells. Solid experimental evidence originating from the Anversa laboratory, and confirmed by others, suggests that c-kitpos stem and progenitor cells participate in the native myocardial repair and regeneration after MI.13,14,36 Few agents able to modulate in vivo expansion of cardiac c-kitpos progenitor cells have been identified to date14,37; thus, the understanding that NGF is one of such factors represents a novel and relevant finding in the perspective of therapeutic cardiac regeneration. Heart-derived c-kitpos cells do not express the mRNA for NGF receptors, thus pinpointing SCF as the mediator of NGF indirect actions on these cells. In agreement with this theory, SCF promotes proliferation of c-kitpos cells38 and proliferating c-kitpos cells were increased in the NGF-engineered infarcted hearts. Of note, SCF neutralization blunted NGF-induced increase in resident Lin<sup>-</sup>c-kitpos cells at 3 days post-MI. Furthermore, NGF appears to act selectively or preferentially on the Lin<sup>-</sup>c-kitpos cells, because it did not affect the cardiac abundance of Lin<sup>-</sup>sca-1pos cells, a different population of putative progenitor cells. We then tried to rule out the contribution of Lin<sup>-</sup>c-kitpos cells in the overall therapeutic benefit induced by NGF gene therapy. To this aim, we used a short-term SCF neutralization protocol, which proved able to prevent NGF-induced increase in Lin<sup>-</sup>c-kitpos. Under these experimental conditions, NGF overexpression maintained its capacity to improve cardiac function and to promote angiogenesis at 14 days post-MI. This suggests that the increase in Lin<sup>-</sup>c-kitpos early after MI is not important for the therapeutic benefit induced by NGF overexpression. However, we cannot exclude that NGF-induced increase in SCF expression level and thus in c-kitpos progenitor cells could be utilitarian under different experimental and clinical circumstances.

In conclusion, this study provides strong evidence for the therapeutic potential of NGF in the post-MI heart and furthermore reinforces the concept that neurotrophins have important cardiovascular actions.

Acknowledgments

We thank Drs Nicolle Kraenkel, Atsuhiko Oikawa, Mauro Siragusa (all from the University of Bristol) and Beatrice Testa (University of Parma) for technical assistance.
Sources of Funding

The study was supported by British Heart Foundation grants BS/05/001 and PG/06/146/21946 (to C.E.).

Disclosures

None.

References


NGF has historically been implicated in several functions of the nervous system. More recently, we and others have introduced the new concept that NGF can also elicit cardiovascular actions, including angiogenesis and cardiomyocyte survival. However, whether NGF plays a role in the healing of the infarcted heart is unknown. Here we show that NGF expression is upregulated by myocardial infarction (MI) in both humans and mice and that endogenous NGF protects the murine infarcted heart by inhibiting cardiovascular apoptosis and increasing coronary microvasculature. Enhancing this endogenous mechanism by intramyocardial NGF gene therapy reduces post-MI mortality and improves left ventricular perfusion, function, and remodeling. This study also uncovers the previously unknown capacity of NGF to promote expansion of Lin<sup>−</sup>ckit<sup>−</sup> cardiac progenitor cells through an indirect mechanism involving the c-kit receptor ligand, stem cell factor (SCF). These results reveal novel pleiotropic properties of NGF and suggest that NGF-based therapy may be a useful approach to alleviate post-MI remodeling.
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*Circ Res.* 2010;106:1275-1284; originally published online April 1, 2010; doi: 10.1161/CIRCRESAHA.109.210088

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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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: +++.

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. CD34 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 5th 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 (Ab-NGF, kindly provided by Dr. Luigi Aloe, Consiglio Nazionale delle Ricerche, Roma, Italy) at the dose of 100 µg in 100 µl 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 10⁸ 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, 100 µg in 100 µl PBS, i.p. every 5 days starting from the day of surgery) (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 Linneg-cutpos 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, 880 mmol/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 (µl³), 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<sub>max</sub>, mmHg/s) and minimal rate of LVP fall (dP/dt<sub>min</sub>, 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 mm² 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 mm² 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. 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<sup>pos</sup> 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<sup>pos</sup> cells, c-kit<sup>pos</sup>GATA-4<sup>pos</sup> cells and c-kit<sup>pos</sup>MCM-2<sup>pos</sup> cells per mm² 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). 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, Klinikum 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 sca1 (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 ckitpos cells from the murine heart.**

Cardiac ckitpos 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-kitpos 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. 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.

**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-β forward 5'-GGCTGCTGGCGGTTTAT-3', reverse 5'-GCCAGTCAAGGTCTCT TCTCA-3' (which amplify human NGF cDNA but do not amplify murine NGF cDNA); mouse NGF-β forward 5'-AGACTTCCAGGCCCATGTA-3', reverse 5'-GAACCTCCCCATGTGAAAGA-3'; mouse TrkA forward 5'-CTTTGTGCACCCGGATCTG-3', reverse 5'-TCATGCAAAAGCTCTCAATCTTC-3'; mouse SCF forward 5'-CCCTGAAGACTGGCCCTA-3', reverse 5'- CAATTACAAGCGAATGAGGC-3'. Gene expression was additionally evaluated in c-kitpos cells, isolated from mice hearts as described previously, using the following set of primers: mouse TrkA forward 5'-CTTTGTGCACCCGGATCTG-3', reverse 5'-TCATGCAAAAGCTCTCAATCTTC-3'; mouse p75NTR forward 5'-CTAGGGTGTGCTCTTGGAGGT-3', reverse 5'-CAGGGTTACACACCGTGCT-3'. Finally, gene expression for rat SCF (forward 5'-TTGCTTTGGAATTTGCGTTTG -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(Ser473)-
Akt, total and phospho(Thr<sup>32</sup>)-Foxo-3a (all from Cell Signaling), total and phospho(Trp<sup>730</sup>)-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:**


### Supplemental Table I

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

<table>
<thead>
<tr>
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<th>MI</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Age (year)</td>
<td>69±10</td>
<td>62±12</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>6/2</td>
<td>5/3</td>
</tr>
<tr>
<td>Cause of death</td>
<td>MI</td>
<td>Acute trauma (n=6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastrointestinal hemorrhage (n=1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pulmonary thromboembolism (n=1)</td>
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</table>
### 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).

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>14 days</th>
<th>30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham/Ad.Null</td>
<td>Ad.Null</td>
</tr>
<tr>
<td>LV pressure (mmHg)</td>
<td>88.50±3.9</td>
<td>63.52±2.7**</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.20±0.33</td>
<td>10.79±1.08**</td>
</tr>
<tr>
<td>LV dP/dt max (mmHg/s)</td>
<td>476±6.128</td>
<td>2969.5±146**</td>
</tr>
<tr>
<td>LV dP/dt min (mmHg/s)</td>
<td>3513.9±227</td>
<td>-2377.5±97.3**</td>
</tr>
<tr>
<td>Heart rate (beats per minute)</td>
<td>441.8±22.1</td>
<td>387.68±12.2*</td>
</tr>
</tbody>
</table>

### Hemodynamics

- **Ejection fraction (%)**
  - 14 days: Sham/Ad.Null 70.17±2.43, Ad.Null 31.69±2.75**, Ad.hNGF 46.87±4.25** †
  - 30 days: Sham/Ad.Null 70.87±1.62, Ad.Null 27.38±2.18**, Ad.hNGF 39.38±2.21** ††

- **Fractional shortening (%)**
  - 14 days: Sham/Ad.Null 39.76±2.35, Ad.Null 15.11±1.47**, Ad.hNGF 24.15±2.63** †
  - 30 days: Sham/Ad.Null 41.75±1.49, Ad.Null 12.92±1.13**, Ad.hNGF 19.27±1.19** ††

### LV remodelling

- **LV posterior wall (ED; mm)**
  - 14 days: Sham/Ad.Null 0.88±0.07, Ad.Null 1.00±0.19, Ad.hNGF 1.08±0.18
  - 30 days: Sham/Ad.Null 0.85±0.06, Ad.Null 0.49±0.05**, Ad.hNGF 0.81±0.04 ††

- **LV posterior wall (ES; mm)**
  - 14 days: Sham/Ad.Null 1.23±0.08, Ad.Null 1.20±0.10, Ad.hNGF 1.43±0.06
  - 30 days: Sham/Ad.Null 1.24±0.07, Ad.Null 0.89±0.09, Ad.hNGF 1.36±0.06 †

- **LV anterior wall (ED; mm)**
  - 14 days: Sham/Ad.Null 0.82±0.09, Ad.Null 0.59±0.17*, Ad.hNGF 0.58±0.22*
  - 30 days: Sham/Ad.Null 0.78±0.02**, Ad.Null 0.28±0.02**, Ad.hNGF 0.37±0.03** ††

- **LV anterior wall (ES; mm)**
  - 14 days: Sham/Ad.Null 1.29±0.09, Ad.Null 0.75±0.15**, Ad.hNGF 0.78±0.07**
  - 30 days: Sham/Ad.Null 1.34±0.03, Ad.Null 0.28±0.02**, Ad.hNGF 0.52±0.06** ††

- **ED LV internal diameter (mm)**
  - 14 days: Sham/Ad.Null 3.60±0.09, Ad.Null 4.80±0.08**, Ad.hNGF 4.39±0.08**
  - 30 days: Sham/Ad.Null 3.60±0.10, Ad.Null 5.62±0.11**, Ad.hNGF 4.76±0.15** ††

- **ES LV internal diameter (mm)**
  - 14 days: Sham/Ad.Null 2.17±0.14, Ad.Null 4.05±0.16**, Ad.hNGF 3.36±0.11** †
  - 30 days: Sham/Ad.Null 2.11±0.08, Ad.Null 4.84±0.11**, Ad.hNGF 3.71±0.21* ††

- **ED LV chamber volume (µl³)**
  - 14 days: Sham/Ad.Null 49.26±4.87, Ad.Null 102.27±8.95**, Ad.hNGF 85.76±10.65*
  - 30 days: Sham/Ad.Null 54.95±3.70, Ad.Null 156.02±7.30**, Ad.hNGF 112.37±5.66** ††

- **ES LV chamber volume (µl³)**
  - 14 days: Sham/Ad.Null 16.05±2.02, Ad.Null 70.07±6.91**, Ad.hNGF 49.28±8.65**
  - 30 days: Sham/Ad.Null 17.62±0.74, Ad.Null 110.4±6.33**, Ad.hNGF 68.99±6.31** ††
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 (AAA-Foxo-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 + AAA-Foxo-3a; 4) hNGF + AAA-Foxo-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).

<table>
<thead>
<tr>
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<th>MI/Ad.Null/Ad.Null</th>
<th>MI/Ad.Null/Ad.hNGF</th>
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<tr>
<td>Ejection fraction (%)</td>
<td>28.99±1.94</td>
<td>42.56±0.84*</td>
<td>25.90±1.96</td>
<td>26.35±2.23††</td>
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<td>Fractional shortening (%)</td>
<td>13.84±0.99</td>
<td>21.69±0.87*</td>
<td>12.17±1.02</td>
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<td><strong>LV remodelling</strong></td>
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<tr>
<td>LV posterior wall (ED; mm)</td>
<td>0.93±0.03</td>
<td>0.99±0.05</td>
<td>0.72±0.07</td>
<td>0.85±0.05</td>
</tr>
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<td>LV posterior wall (ES; mm)</td>
<td>1.28±0.06</td>
<td>1.39±0.06</td>
<td>0.96±0.07</td>
<td>1.07±0.07†</td>
</tr>
<tr>
<td>LV anterior wall (ED; mm)</td>
<td>0.53±0.01</td>
<td>0.53±0.03</td>
<td>0.31±0.03**</td>
<td>0.38±0.03</td>
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<tr>
<td>LV anterior wall (ES; mm)</td>
<td>0.62±0.02</td>
<td>0.71±0.05</td>
<td>0.96±0.07</td>
<td>0.41±0.07†</td>
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<td>ED LV internal diameter (mm)</td>
<td>4.78±0.14</td>
<td>4.41±0.09</td>
<td>5.29±0.18</td>
<td>4.98±0.13†</td>
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<tr>
<td>ES LV internal diameter (mm)</td>
<td>4.15±0.08</td>
<td>3.53±0.12**</td>
<td>4.60±0.19</td>
<td>4.36±0.21†</td>
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<tr>
<td>ED LV chamber volume (µl)</td>
<td>111.48±4.21</td>
<td>92.47±2.35**</td>
<td>130.98±11.16</td>
<td>132.09±6.68†</td>
</tr>
<tr>
<td>ES LV chamber volume (µl)</td>
<td>81.79±2.93</td>
<td>53.50±2.06**</td>
<td>98.93±9.73</td>
<td>97.58±6.33†</td>
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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$^{\text{pos}}$ cells, while it does not affect the abundance of Lin$^{\text{neg}}$sca-1$^{\text{pos}}$ cells putative progenitor cells. (A) Bar graph shows the number of c-kit$^{\text{pos}}$GATA-4$^{\text{pos}}$ 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$^{\text{pos}}$ cells are increased by Ad.hNGF-in both the infarct and peri-infarct areas. (C) Identification and quantification of cardiac Lin$^{\text{neg}}$sca-1$^{\text{pos}}$ 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$^{\text{neg}}$ cells (in P2) were gated from the total population of extracted cells. (iii) Representative graphs show the negative control (NC) for sca-1$^{\text{pos}}$ cells. (iv) Bar graph and representative microphotographs show the number of Lin$^{\text{neg}}$sca-1$^{\text{pos}}$ cells per mg of LV tissue. The number of c-kit$^{\text{pos}}$ cells (in P3) was analyzed within the Lin$^{\text{neg}}$ 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<sup>NTR</sup> receptors for NGF are not expressed in c-kit<sup>pos</sup> cell isolated from mouse heart. RT-PCR shows the lack of expression of TrkA (high affinity receptor for NGF) (A) and p75<sup>NTR</sup> (p75, low affinity receptor for NGF) (B) in c-kit<sup>pos</sup> 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. 1Ad.βGal.
Supplemental Figure VI. The early MI- and Ad.hNGF-induced increase in Lin<sup>neg</sup>c-kit<sup>pos</sup> putative progenitor cell numbers is not maintained at 14 days post-MI. Bar graph shows the number of Lin<sup>neg</sup>c-kit<sup>pos</sup> 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\textsuperscript{pos} progenitor cells after MI. Identification and quantification of cardiac Lin\textsuperscript{neg}c-kit\textsuperscript{pos} cells by flow cytometry shows that the number of Lin\textsuperscript{neg}c-kit\textsuperscript{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).