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Local Gene Transfer with Nerve Growth Factor Promotes Reparative Neovascularisation in a Mouse Model of Myocardial Infarction

Coordinatore:

Chiar.mo Prof. EZIO MUSSO

Tutori:

Prof. DONATELLA STILLI

Dott.ssa COSTANZA EMANUELI

Dottorando:

MARCO MELONI

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Abbreviation and acronyms

Ang II	**Angiotensin II**
BDNF	**Brain-Derived Neurotrophic Factor**
BW	**Body Weight**
CM	**Cardiomyocyte**
EC	**Endothelial cells**
EPC	**Endothelial progenitor cells**
FGFs	**Fibroblast growth factors**
GT	**Gene Therapy**
HR	**Heart Rate**
H/R	**Hypoxia/ Reoxygenation**
HW	**Heart Weight**
LAD	**Left Anterior Descending Coronary Artery**
LV	**Left Ventricle**
LVP	**Left Ventricular Pressure**
MI	**Myocardial Infarction**
NGF	**Nerve Growth Factor**
NTs	**Neurotrophins**
NT-3	**Neurotrophin-3**
NT-4/5	**Neurotrophin-4/5**
$p75^{NTR}$	**p75 neurotrophin receptor**
RNCM	**Rat Neonatal Cardiomyocytes**
RV	**Right Ventricle**
Trk	**Tropomyosin-related kinase receptor**
SDF-1	**Stromal Derived Factor-1**
VEGF-A	**Vascular Endothelial Growth Factor-A**

Introduction

Myocardial infarction

Myocardial infarction (MI) is the leading cause of death in the United States as well as in most developed countries throughout the world.[1, 2] According to the American Heart Association, around 13 million people suffer some form of coronary artery diseases in the United States.[3] More than 50% of death in Europe in 2001 has been related to cardiovascular diseases, and the 80% of these events were associated with ischemic conditions.[4]

The pathological substrate of MI is atherosclerosis, a chronic inflammatory disorder of the artery wall characterized by the accumulation of cholesterol in the artery wall. Several risk factors predispose to MI. They include aging, hyperglycemia (diabetes), dyslipidemia, cigarette smoking, hypertension, obesity, male gender and genetic predisposition.[5, 6]

These risk factors can promote the occurrence of MI by triggering atherosclerosis or aggravating its progression, therefore increasing the possibility of accumulating cholesterol and fibrous tissue which are essential for the formation of atherosclerotic plaques in the inner lining of a coronary artery.

The coronary vascular system functions as main supplier of oxygen and nutrition for the heart tissue, and as transportation system for waste products from the underlying heart tissue, i.e. the myocardium.

MI occurs as the consequence of occlusion of one of the branches of the coronary artery system, which is caused by the sudden disruption of an atherosclerotic plaque that prevents blood flow supply to a part of the heart. Once a coronary artery is occluded,

a region of the myocardium is deprived of oxygen and nutrition, which results in myocardial ischemia (Figure 1). As result, ischemia leads to the death of heart muscle.

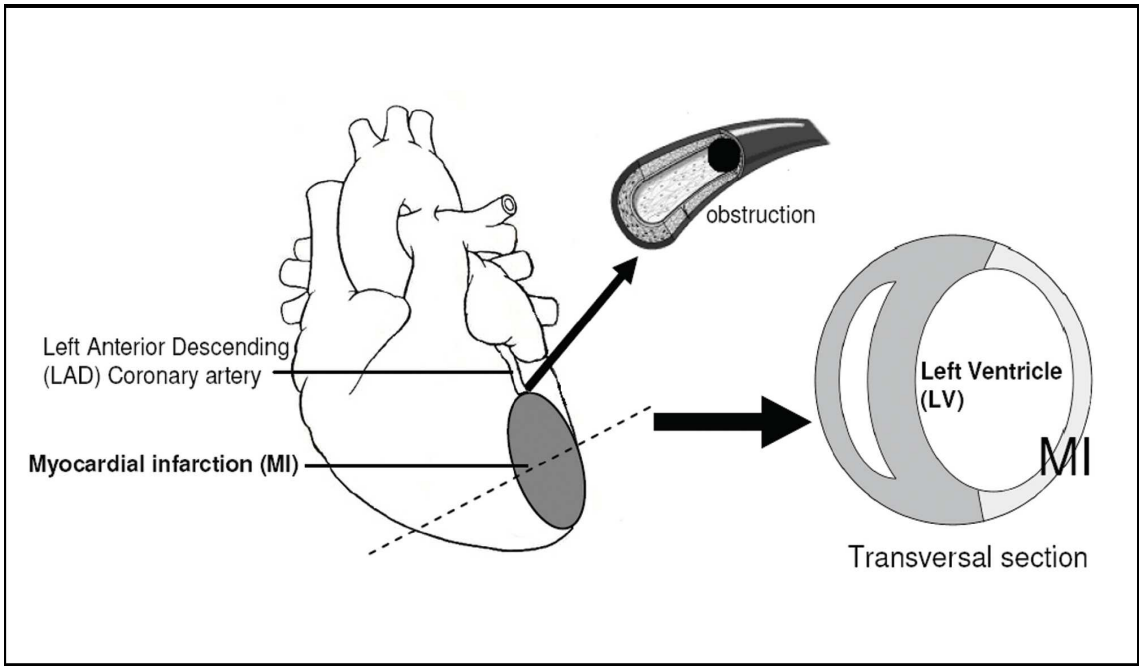


Figure 1. Schematic example of myocardial infarction

The angiogenesis process

Angiogenesis is a general term for describing the process of growth of new vessels. In the past, the term angiogenesis was used to describe the growth of endothelial sprouts from pre-existing post-capillary venules.[7] More recently, this term has been used to indicate the growth and remodeling process of the primitive vascular network into a complex network.

There are three modes that contribute to the process: vasculogenesis, angiogenesis (true angiogenesis) and arteriogenesis.

Vasculogenesis is the *de novo* formation of blood vessels starting from endothelial progenitor cells (EPC). It is a crucial process for embryonic development, but it has also been considered to contribute to vessel growth in adult.[8-10] In the adult, EPC originate from the bone marrow.

Angiogenesis is defined as a process by which new blood capillaries grow from pre-existing vessels. It plays an important role during embryonal development and it is also involved in adult life in both physiological and pathological condition, like tumor and chronic inflammation.[11]

Arteriogenesis is the maturation of capillaries or arterioles and the formation of arteriole collaterals.[7, 12-15]

During embryogenesis, mesoderm-derived cells differentiate into endothelial precursors, like EPC and angioblasts, and aggregate to form the so called blood islands. Fusion of blood islands leads to the formation of primary capillary plexi, the primitive and uniform vascular structures, that are remodeled to develop into a network of arteries, capillaries and veins. Other cell types participate to the process, like vascular smooth-muscle cells that associate with arteries and veins, and pericytes that cover capillaries making a direct contact with endothelial cells and stabilizing the vascular network.

Similar remodeling processes are thought to be important for the formation of blood vessels in both development and angiogenesis in adult life.

It is now accepted that there are at least two different types of angiogenesis: sprouting angiogenesis, that consists in the true sprouting of capillaries from pre-existing vessels, and non-sprouting angiogenesis like intussusception.[16]

Sprouting angiogenesis is a complex process that involves many types of cells, growth factors and signaling pathways. It is initiated by activation of endothelial cells and vasodilatation of the vessel, a process that involves nitric oxide, followed by increased vascular permeability. The increase in vascular permeability allows extravasation of plasma proteins that provide a stimulus for endothelial cells migration. Several proteases, like matrix metalloproteinases (MMPs), chymase or plasminogen activator, participate to the process by degrading the extracellular matrix and by activating growth factors like the vascular endothelial growth factor-A (VEGF-A) and fibroblast growth factors (FGFs). The degradation of the extracellular matrix allows the endothelial cells to migrate to the sites where new capillaries are needed and to proliferate, leading to the formation of a lumen and functional maturation of the endothelium. Recruitment of pericytes and deposition of new extracellular matrix into the subendothelial basement membrane promote vessel maturation and quiescence, stabilizing the new vascular connection (Figure 2a).

The best characterized type of non-sprouting angiogenesis is intussusception, which was first described in the pulmonary capillary network.[17, 18]

In this process, the opposing microvascular walls protrude into the capillary lumen creating a contact zone between endothelial cells. The endothelial bilayer is perforated, intracellular contacts are

recognized and a transluminal pillar with an interstitial core is formed. The core is invaded by interstitial cells leading to its rapid enlargement through deposition of collagen fibrils.[19] An important advantage of intussusception capillary growth is that it permits a rapid expansion of the capillary network (Figure 2b).

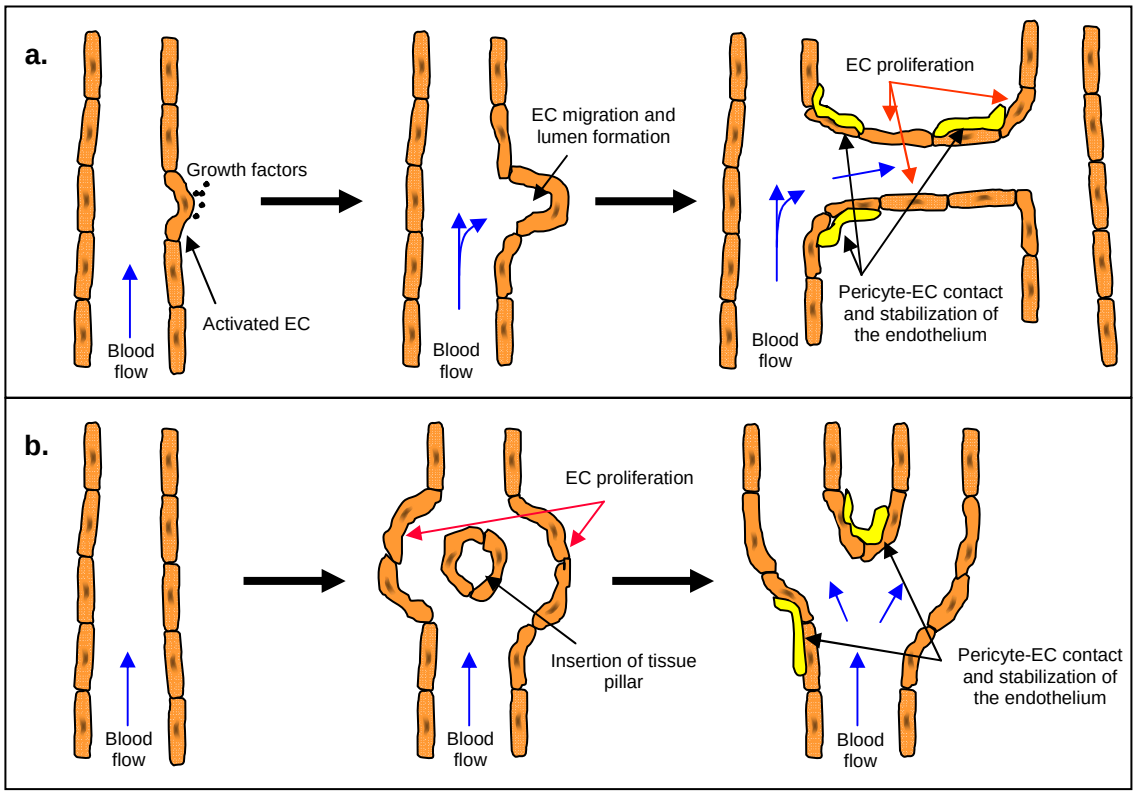


Figure 2. Sprouting angiogenesis and intussusception
a.) During sprouting angiogenesis, once activated, EC migrate and proliferate leading to the formation of a lumen. Fusion processes at the interfaces between EC cells establish a continuous lumen. The final step requires also other cells type like pericytes that stabilize the new vascular connection. **b.)** Intussusception is the splitting of a vessel through the insertion of tissue pillars. This process requires also EC proliferation and their final contact with pericytes for the stabilization of the endothelium.

The driving force of angiogenesis is hypoxia in the surrounding tissue. Sprouting of capillaries leads to an increase of their density improving blood perfusion of hypoxic tissue which is necessary to maintain or restore local oxygen and nutrition supply.[14]

Myocardial infarction *per se* provides a potent stimulus to angiogenesis and the subsequent development of collateral vasculature that in part maintains and/or revitalizes cardiac tissue.[20-22]

EPC and their implication in angiogenesis

EPC are bone marrow-derived cells that circulate in the blood and have the ability to differentiate into endothelial cells. Numerous studies have demonstrated that EPC can be mobilized from the bone marrow into the peripheral circulation after various stimuli, like cytokines, growth factors, hormones and tissue injury, including MI.

The mobilization and differentiation of bone marrow–derived EPC has been shown to be important in the process of adult neovascularization.[8, 9, 23, 24]

In animal model of ischemia, EPC were shown to incorporate into sites of active neovascularisation in ischemic tissue. After transplantation of human peripheral blood-[25] or bone marrow-derived EPC,[26] blood flow recovery and capillary density were markedly improved and the rate of limb loss was significantly reduced. Improved neovascularisation was also noticed after infusion of peripheral blood–derived EPC or implantation of bone marrow mononuclear cells after myocardial infarction.[27,28] These findings provide evidence that exogenously administered EPC can augment

naturally occurring neovascularisation in animal models of experimentally induced ischemia.[29]

Moreover, transplantation of EPC into patients has been demonstrated to induce blood flow recovery in ischemic limbs[30] and increase myocardial left ventricular ejection fraction after myocardial infarction.[29, 31-34]

The number and migratory activity of circulating EPC has also been shown to inversely correlate with risk factors for coronary artery disease[35] and to serve as a surrogate biological marker for vascular function and cumulative cardiovascular risk.[36]

The mechanism of EPC-induced neovascularisation is still a matter of debate. Several studies showed that bone marrow–derived cells incorporate into the newly formed capillaries and express endothelial markers, suggesting that EPC enhance neovascularisation by physically contributing to the newly formed capillaries.

However, some studies speculate that paracrine effects are responsible for the pro-angiogenic effects of EPC. It has been shown that bone marrow-derived cells have the ability to secrete potent angiogenic ligands (like FGFs, VEGF-A and Angiopoietin-1) as well as to be incorporated in the site of neovascularisation,[28] while others reported that bone marrow–derived cells were able to support tumor neovascularisation and growth without physically building endothelial structures.[37]

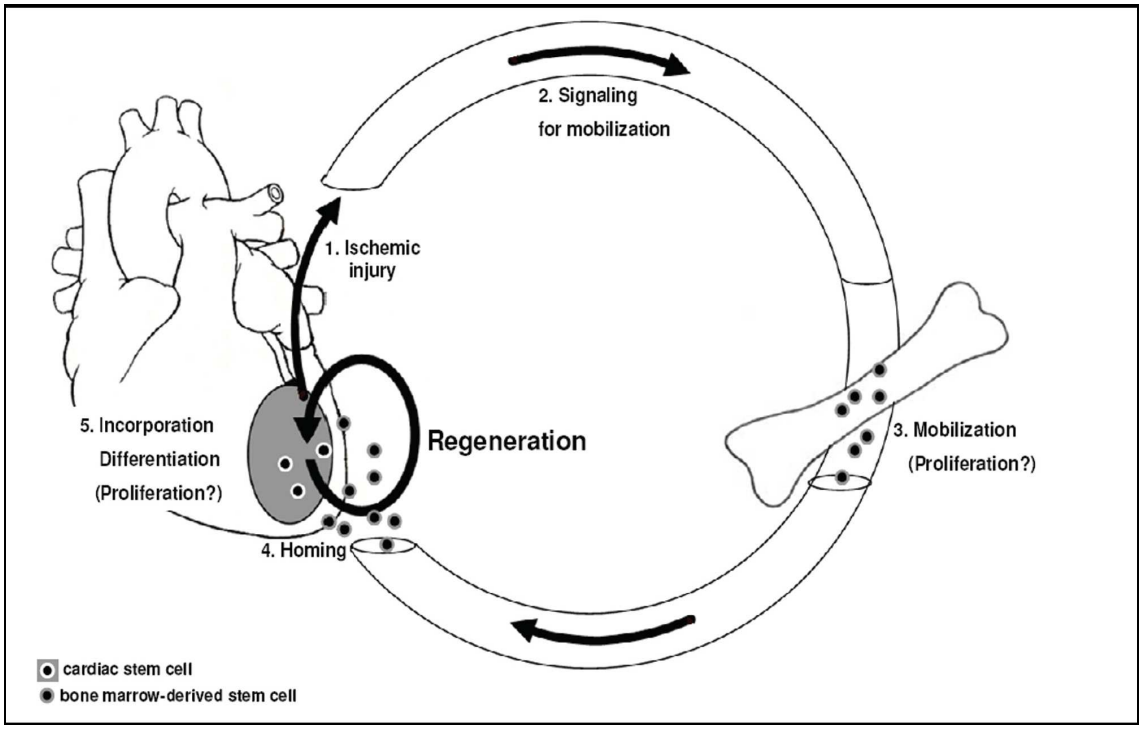


Figure 3. Schematic example of bone marrow-derived EPC mobilization and homing
After ischemic injury **(1.)**, a variety of factors, including cytokines and growth factors, are produced **(2.)**. These factors can stimulate bone marrow-derived EPC mobilization **(3.)**, leading to EPC homing **(4.)** and incorporation **(5.)** into the ischemic tissue.

Gene therapy and therapeutic angiogenesis

Different treatments are available for patients who show symptoms suggesting possible myocardial infarction. First line agents are aspirin and nitroglycerin that usually are administered immediately after first symptoms. Once the diagnosis of MI is confirmed, other pharmacologic agents, like β-blockers and anti-coagulant, are often given.

Patients with suspected occlusive thrombosis in a coronary artery are candidates for immediate reperfusion therapies with thrombolytics, like streptokinase or tissue plasminogen activator (tPA). An alternative to thrombolytics is percutaneous coronary intervention (PCI), also known as coronary angioplasty. This is an

invasive procedure in which a guidewire and balloon catheter is used to mechanically compress the thrombus and at the same time deal with the underlying stenotic atheromatous plaque.

When these reperfusion therapies are not successful, another alternative could be coronary artery bypass graft surgery (CABG). In this invasive surgical procedure, arteries or veins from the patient`s body are grafted to the coronary arteries to bypass atherosclerotic narrowing and improve blood supply to the coronary circulation.

These treatments can be used to reduce or eliminate the symptoms of coronary artery disease, including angina (chest pain), dyspnea (shortness of breath) and congestive heart failure, as well PCI and CABG can also be used to abort an acute MI.

Even so, there is a large number of patients who have incomplete revascularisation using these available techniques and, as a result, many of these patients have residual symptoms of myocardial ischemia despite medical therapy.[38]

However, alternative therapies are emerging for the treatment of myocardial infarction and other ischemic diseases, like stem cell therapy and gene therapy. In fact, after over a decade of preclinical studies and recent clinical trials, gene therapy has been established as a potential method to induce therapeutic angiogenesis in patients with ischemic cardiovascular disease.

Gene therapy (GT) potentially represents one of the most important evolutions in medicine, since it can be used to correct genetic defects or to express gene products that can be therapeutically useful.

GT can be defined as the transfer of nucleic acid to the somatic cells of an individual to obtain a specific therapeutic effect.[39, 40]

One of the most important advantages in the use of gene therapy is that the beneficial therapeutic effects can be obtained with relatively low doses of the delivered genetic material. Another advantage of gene transfer compared to classical pharmacological approaches is the possibility to continuously produce endogen proteins, and that a single local administration of gene may induce a long-term therapeutic effect in the target tissue without systemic side-effects.

Two different approaches have been used to deliver DNA to cells, namely viral vectors and non-viral vectors. Both have different advantages, but also different grade of efficiency and side-effects.

Viral vectors are very efficient in transfecting their DNA into the host cell and the size of DNA they can normally deliver is bigger compared to non-viral vectors. The most common used viral vectors are retroviruses, lentiviruses, adenoviruses and adeno-associated viruses. Even if they have a high transduction rate, their capacity is limited. For example, first generation-adenoviral vectors are immunogenic, although in adenoviral vectors of the third generation all viral encoded genes are removed and thereby they mediate lower immune response. Moreover, both adeno-associated viruses and lentivirus can integrate into the genome at a random position therefore increasing safety risks, such as insertional mutagenesis.

On the other side, non-viral vectors have no infectious or mutagenic capability, but they still have a low efficiency. At the moment there are three methods of non-viral DNA transfer, namely

naked DNA (in the form of a plasmid), liposomes and molecular conjugates.

Plasmids can be directly injected into the target tissue, for example muscle cells[41] and, although their low efficiency, numerous experimental *in vivo* studies have demonstrated that the direct introduction of plasmid-DNA in the myocardium have no side-effects and have an organ specific gene expression.[42, 43]

One of the most interesting purpose and possible utilization of GT in the cardiovascular field is the application of exogenous genes capable for the stimulation of growth of capillary and arteriole density, leading to a better perfusion of ischemic limb muscle and heart.[40, 44-48]

Therapeutic angiogenesis holds promises for the cure of myocardial ischemia. Experimental evidence indicates that supply-side delivery of angiogenic growth factors improves the perfusion of ischemic heart, seemingly resulting in preservation of cardiac function. Accordingly, some controlled clinical trials involving intra-myocardial gene transfer for short-term expression of VEGF-A and FGFs showed sufficient levels of safety and a trend towards improved myocardial perfusion exercise and performance.[49, 50] The primary physiological target of the strategy consists of promoting the local growth of capillaries and arteriole collaterals, with preference for arteriogenesis that more efficiently compensates for occlusion of a medium-size coronary artery. In addition, the optimal agent should be capable of preventing cardiomyocyte death, while fostering regeneration mechanisms for resuscitation of hibernating myocardium in the area at risk.

Several factors contribute to the angiogenic process, like VEGF, FGF, platelet-derived growth factor (PDGF) and transforming growth factors (TGFs). On the contrary, anti-angiogenic molecules like angiostatin or endostatin lead to the inhibition of angiogenesis. If the vessel growth is dysregulated, excessive angiogenesis or insufficient vessel growth can lead to a variety of diseases, such as cancer and retinopathy.

During the last years, it has been demonstrated that, in addition to these “classical“ angiogenic factors, many other endogenous peptides play an important regulatory role in angiogenesis. These endogenous regulators include erythropoietin (EPO), endothelins (ETs), adrenomedullin (AM), adiponectin, neuropeptide-Y (NPY), vasoactive intestinal peptide (VIP), substance-P (SP), tissue kallikrein (TK) and neurotrophins such as nerve growth factor (NGF).[51, 52]

Table 1 shows a list of factors implicated in angiogenesis.

GROWTH FACTORS	***CHEMOKINES***	***TRANSCRIPTION FACTORS***	***OTHERS***
VEGF-A, B,C,D,E **FGF-1,2,4,5** **Angiopoietin-1,2** **HGF, PDGF** **IGF-1,2** **Neurotrophins**	**MCP-1**	**HIF-1α** **EGR-1** **Prox-1**	**Del-1** **PR39** **Tissue kallikrein** **eNOS** **iNOS**

Table 1. Factors with known angiogenic properties
Modified from Ylä-Herttuala S. & Alitalo K., Nat Med. 2003 Jun;9(6):694-701

Neurotrophins and their receptors

Neurotrophins (NTs) are secreted molecules responsible for numerous functions in the development, differentiation and survival especially of neurons, although many of them are now known to affect other cell type and tissues.

The history of neurotrophins started 50 years ago when Levi-Montalcini, Cohen and Hamburger discovered nerve growth factor (NGF),[53] the prototypical neurotrophin, as a factor required for axonal growth. The other neurotrophins, brain-derived neurotrophic factor (BDNF),[54] neurotrophin-3 (NT-3),[55] and neurotrophin-4/5 (NT-4/5),[56] were identified 30 years later.

NGF is a small secreted protein (13.5 KDa) mainly synthesized in specific areas of the central nervous system in mammals, principally in the cortex, hippocampus and hypothalamus, but also from extra-neuronal cells like fibroblast, epithelial cells and cells from the endocrine and immuno system.

Initially, NGF was considered as a critical molecule implicated in the survival and maintenance of sympathetic and sensory neurons as well as involved in neuronal regeneration. Recent studies have revealed the potential of NGF and other NTs for vascular biology and it is now known that NGF contributes to pro-angiogenic activities and is able to promote endothelial cells survival and proliferation.[57-61]

Previous study from our group showed that NGF promotes neovascularisation in a murine model of hind-limb ischemia and in diabetic skin wounds.[57, 58, 60]

We demonstrated that the over-expression of NGF is able to accelerate the tissue recovery and reperfusion through the improvement of angiogenesis and arteriogenesis. We also showed that NGF protects endothelial cells and skeletal myocytes from apoptosis.[57, 58, 60]

Neurotrophins mediate their effects through binding to two different receptors, the tropomyosin-related kinase (Trk) receptors and the p75 neurotrophin receptor ($p75^{NTR}$).

The $p75^{NTR}$ belongs to the tumor necrosis factor (TNF) superfamily and was the first identified receptor for NGF.[62, 63] The $p75^{NTR}$ integral membrane protein is the founding member of the TNF family of receptors and contains a cysteine-rich domain and a cytoplasmic death domain.

The first Trk receptor was originally discovered as a rearrangement of non-muscle tropomyosin and a then unknown tyrosine kinase. This tyrosine kinase was referred to as TrkA and subsequently identified as a receptor for NGF.[64-67] The identification of TrkB and TrkC, based on their similarity to TrkA, followed thereafter.[68]

For a long time, neurotrophins were believed to bind preferentially to specific Trk receptors: NGF to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC. However, some promiscuity exists, as NT-3 can also bind with lower affinity to TrkA and TrkB receptors that contain an additional short insert in the extracellular domain.[69-71] All NTs bind to $p75^{NTR}$ with equal affinity.

Trk receptors are typical tyrosine kinase receptors, which contain an extracellular domain composed of three leucine-rich motifs flanked by two cysteine clusters, two immunoglobulin-like C2

type domains (Ig-C2), a single transmembrane domain, and a cytoplasmic region with a kinase domain. Binding of NTs to Trk receptors occurs mainly through the Ig-C2 domains, with the domain closer to the transmembrane region playing a more prominent role, as described using chimeras between different Trk receptors, deletions, and point mutation analyses.[72-76]

In addition to ligand binding, the Ig-C2 domains can also stabilize the monomeric form of the Trk receptor to prevent spontaneous dimerization and activation in the absence of NTs (Figure 4).

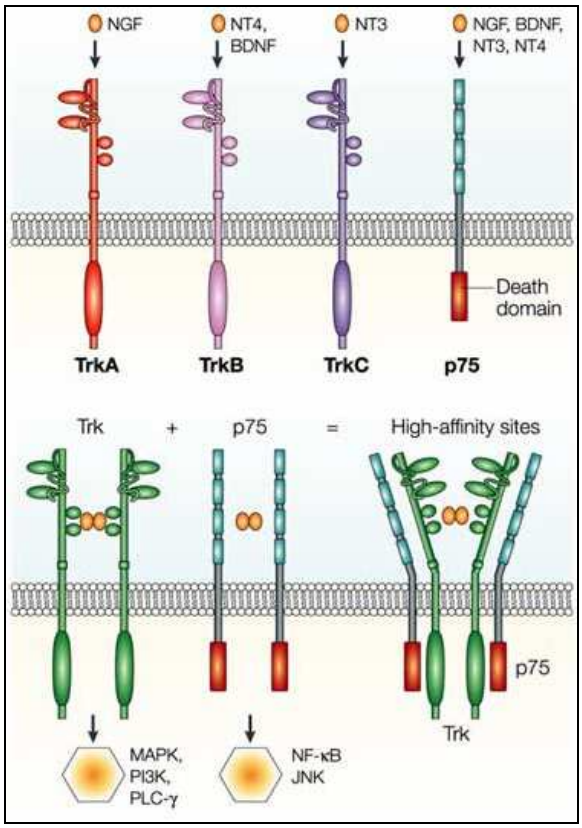


Figure 4. Model of TrkA and p75^{NTR} activation
Neurotrophins selectively bind to specific TrkA receptors leading to the activation of several trasductional pathways. All neurotrophins bind with lower affinity to p75^{NTR} receptor. MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase C-γ; NF-kB, nuclear factor-kB; JNK, Jun N-terminal kinase. From Chao, M.V., Nat Rev Neurosci., 2003 Apr;4(4):299-309.

Neurotrophins signaling mediated by Trk receptors

The signaling pathways activated by NTs through Trk receptors result in many neuronal functions, such as cell survival, differentiation, dendritic arborization, synapse formation, plasticity, axonal growth, and axonal guidance. Binding of NTs to Trk receptors leads to dimerization, phosphorylation in *trans* of the receptors, recruitment of different adaptors and enzymes, and activation of several signaling pathways (Figure 5).

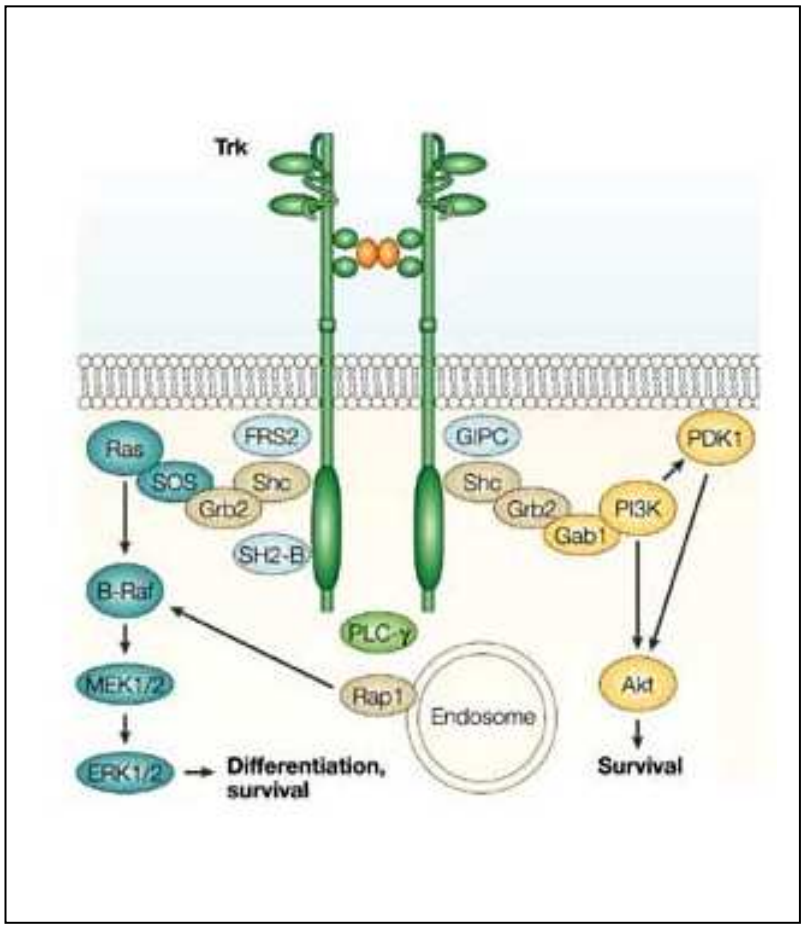


Figure 5. TrkA receptor signaling
Several signaling pathway can be activated by the binding of NTs to Trk receptors. Among these, the PI3K-Akt, Shc-Ras-MAPK, Rap-MAPK, and PLCγ-protein kinase C (PKC) pathways are the most known. Adapted from Chao, M.V., Nat Rev Neurosci.,2003,Apr;4(4):299-309.

These events are shared by other receptor tyrosine kinases, but the unique combination of Trk receptor docking sites, recruitment of different adaptors and enzymes, and regulated receptor trafficking elicits specific responses for NTs. Two tyrosines in Trk receptors (Y490 and Y785 in the human TrkA receptor and their corresponding tyrosines in TrkB and TrkC receptors) are phosphorylated in response to NTs and serve as the major docking sites for binding of adaptor proteins and enzymes. Y490 and Y785 primarily recruit Shc and phospholipase C (PLC), respectively. The phosphorylated tyrosines located at the kinase domain, Y670, Y674, and Y675, can also engage adaptor proteins, including SH2B, APS, and Grb2.[77, 78]

Among the signaling pathways activated by Trk receptors in response to NTs, the PI3K-Akt, Shc-Ras-MAPK, Rap-MAPK, and PLCγ-protein kinase C (PKC) pathways are the most studied.

- PI3K-Akt

NTs play an important role in the survival of several neuronal populations during development, and signaling through the PI3K-Akt pathway is critical for this function. However, PI3K/Akt is strongly implicated in angiogenesis,[79] vasculogenesis[80] and survival of endothelial cells[81] and cardiomyocytes.[82, 83] We showed that Akt mediates NGF-induced neovascularisation in ischemic limb muscles.[57]

Active Trk receptors engage Shc, which associates with Grb2 and Gab1 to activate PI3K and, subsequently, Akt. PI3K binding to Gab1 is required for PI3K activation, as disruption of this association decreases the NGF-dependent survival of PC12 cells.[84]

Several different pro-apoptotic and pro-survival effectors downstream of Akt can mediate the survival actions of NTs. For example, Bad phosphorylation by Akt leads to additional phosphorylation of Bad by other kinases, which prevents the pro-apoptotic effects of the protein.[85] Similarly, the forkhead transcription factor Foxo–3a, which regulates the expression of several pro-apoptotic proteins, is phosphorylated by Akt in response to NTs, thus preventing its transcriptional activity.[86] Moreover, the NF-kB pro-survival pathway is activated via Akt phosphorylation of its inhibitor IkB, which decides for IkB degradation. PI3K-Akt can also exert a positive effect on the retrograde signaling that modulates neuronal survival.[87]

Signaling through PI3K-Akt mediates functions other than survival. Active Akt at the growth cone causes axonal growth and increases axon caliber and branching in sensory neurons by phosphorylating and inactivating glycogen synthase kinase-3β (GSK-3β) locally, leading to microtubule assembly that promotes axonal growth.[88]

Similarly, Akt has been implicated in cardiomyocyte hypertrophy. Stimulation and chronic activation of the PI3K-Akt pathway by insulin-like growth factor-1 (IGF-1) in the heart, as well as over-expression of constitutively active Akt, leads to cardiac hypertrophy.[89, 90]

Akt phosphorylates substrates including regulators of apoptosis and growth in both cytoplasmic and nuclear compartments. Inactive Akt is located principally in the cytoplasm. During receptor-mediated activation, Akt is recruited to the plasma membrane where it undergoes a double phosphorylation involving auto-phosphorylation (or phosphorylation by an unidentified kinase)

on Ser-473 and by phosphoinositide-dependent kinase-1 (PDK-1) on Thr-308.[91] Phosphorylated Akt leaves the membrane and targets substrates in the cytoplasm and nucleus. Potential cytoplasmic Akt targets are GLUT-4,[92] the main cardiac glucose transporter, many regulators of apoptosis, including Bad and caspase-9, and GSK-3β[93, 94] which, besides its role in both cell cycle and protein synthesis regulation, it is also implicated in the Akt-dependent regulation of cardiac hypertrophy. In fact, when Akt is chronically situated in the cytoplasm it can lead to the phosphorylation/inactivation of GSK-3β resulting in cardiac hypertrophy, whereas when Akt is targeted to the nuclear compartment, GSK-3β is inaccessible and retains activity preventing hypertrophy.

Potential nuclear targets include the Fas ligand (FasL), Foxo, the transcriptional regulator Bcl-6, and the cell cycle regulator p27Kip1.

- Ras-MAPK

Activation of the small GTPase Ras in response to NTs has been linked to signaling and transcriptional regulation implicated in neuronal survival and differentiation. Ras is induced rapidly, but transiently, by NGF in PC12 cells.[95] TrkA receptor stimulation by NGF engages Shc and Grb2 to activate the GEF SOS, which then activates Ras.[96] Raf-1 and B-Raf activation downstream of Ras subsequently triggers the activation of extracellular signal-regulated kinases/mitogen-activated protein kinases (MAPK).[97, 98] Stimulation of Ras through this pathway promotes only transient activation of MAPK.[99] Modulation of Ras signaling can be achieved by a negative feedback loop, as activation of MAPK can stop the signaling of this

pathway by phosphorylating SOS to disrupt the Grb2-SOS complex.[100]

In recent years, NGF-induced activation of MAPK has been also described in endothelial cells. Activation of the Ras/MAPK pathway in response to NGF leads to endothelial cells migration.[101] Moreover, the MAPK pathway is responsible of NGF-induced endothelial cells proliferation through phosphorylation of TrkA followed by phosphorylation of the MAPK $ERK_{1/2}$.[58]

- PLCγ

The first reports indicating that NT actions result in activation of PLCγ showed serine, threonine, and tyrosine phosphorylation of PLCγ directly mediated by the Trk receptor kinase. Trk receptor phosphorylation at Y785 leads to PLCγ recruitment and activation, which results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) to generate inositol tris phosphate (IP3) and diacylglycerol (DAG).[102] IP3 and DAG cause the activation of different PKC isoforms. The effect of IP3 is mediated by release of $Ca2^{+}$ from internal stores that additionally stimulate $Ca2^{+}$-calmodulin-regulated protein kinases (CaM kinases). PKC subsequently activates the ERK_1 signaling pathway via Raf.[103] PLCγ activation in response to NTs has been implicated in growth cone chemotropism and in the potentiation of thermal sensitivity by VR1, a heat-activated ion channel on sensory neurons.[104, 105] Moreover, the prolonged activation of PLCγ by a brief pulse of NGF induces transcription of a sodium channel gene.[106, 107]

Aim of the study and experimental design

Recent studies have demonstrated that therapies designed to stimulate neovascularisation can be indicated to counteract ischemic diseases.[24, 30, 39, 46]

The ability of NGF to promote new vessel growth and formation and to inhibit apoptosis has suggested us that NGF could have an appropriate therapeutic action for the treatment of myocardial infarction.

The aim of the study was to evaluate the action of NGF on cardiomyocytes and EPC and to assess the regional effects of NGF gene transfer into the peri-infarct zone of mouse myocardium. We studied the characteristics of microvascular remodeling induced by NGF in the border and remote zone of the infarcted heart, the impact on endothelial cells and cardiomyocytes apoptosis, and the long-term influence on cardiac contractility. To characterize NGF cellular actions and signaling, a part of the study has been done on isolated rat neonatal cardiomyocytes and human EPC.

The major points of this study are the following:

1. To evaluate the role of NGF on reparative neovascularisation in the ischemic heart *in vivo*.
2. To investigate the effect of NGF gene transfer treatment on cardiomyocyte and endothelial cells survival *in vivo*.
3. To evaluate whether NGF delivery to the peri-infarcted heart could prevent post-MI heart failure.
4. To define NGF signaling in the cardiomyocyte *in vitro*
5. To assess the role of NGF on EPC proliferation and migration *in vitro*.

Materials and Methods

Animals

Experiments were performed in male CD1 mice with initial body weight between 35 to 40 g (Charles River, Comerio, Italy).

Procedures complied with the standards stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Science, Bethesda, MD, 1996). Mice were housed under standard conditions (23℃, 12-h light/dark cycle) and given free access to water and standard food.

Plasmid and adenoviral vector preparation

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.NGF*), 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 co-transfected shuttle and genomic plasmids in 293 cells. Viral stocks were amplified, CsCl banded, and titrated.[108]

Myocardial infarction model

Myocardial infarction was performed in CD1 mice anesthetized with a combination of ketamine (50mg/kg) and xylazine (2.5mg/kg), intubated and artificially ventilated (tidal volume: 8-9µl/g; stroke rate: 165/min) during the procedure.

Under a surgical microscope, an incision was made at the left fourth intercostal space to expose the heart and MI was induced by permanent ligature of the proximal left anterior descending coronary artery (LAD) by using a 7.0 Mersilene suture (Ethicon, Somerville, NJ, USA).

Occlusion was confirmed by pallor and regional wall motion abnormality of the left ventricle.

Gene transfer *in vivo*

Two minutes after MI, gene transfer was performed intramyocardially with multiple injections.

Under the surgical microscope, we enter 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. Sham-operated mice underwent the same procedure except LAD was circled with a 7.0 Mersilene suture but not occluded. Surgical wound was sutured and animals were allowed to recover. Surgical mortality rate was less than 8% in all MI groups.

In the first part of the study, NGF gene transfer was performed by using a plasmid carrying the human NGF gene (*p.NGF*) at the dose of 5µg in 10µl of NaCl. Control animals were treated with the same dose of an empty vector (*p.Null*). The capacity of 5µg *p.NGF*

to induce the transgene expression in the mouse infarcted heart was assessed by RT-PCR three days after MI by using *p.NGF* at the doses of 5μg, 10μg and 25μg (n=6 animals/group).

The effect of NGF gene transfer after MI was also analyzed by using an adenoviral vector carrying the human NGF in the expression cassette. *Ad.hNGF* was injected in the MI border zone at the dose of 10^7, 10^8 and 10^9 p.f.u. (n=6 animals/group). Three days after MI and gene transfer, the peri-infarct zone was isolated. The dose of 10^8 p.f.u. for *Ad.hNGF* was chosen after human NGF expression was assessed by RT-PCR. Control animals received the same volume of *Ad.Null* (10^8 p.f.u.).

Histological analyses

Histological analyses were performed after hearts were perfusion-fixed and embedded in paraffin three days or two weeks after MI.

Briefly, mice were anaesthetized (2,2,2-tribromoethanol, 880mmol/kg body weight IP, Sigma Aldrich, Milano, Italy), the abdominal cavity was opened and the aorta was cannulated with a PE50-catheter connected to a perfusion apparatus. Next, the chest was opened and the heart stopped in diastole by left ventricle (LV) intracavitary injection of cadmium-chloride (100nmol); the right atrium was then opened and the heart was perfused with heparinized PBS for 1 minute, followed by 10 minutes with 10% buffered formalin at 100mmHg through the abdominal aorta.

The heart was excised, placed in 4% formalin for 24 hours[109] then the free walls of the right ventricle (RV) and LV inclusive of the septum were separated and their weight recorded.

Finally, the heart was sliced into four transversal sections of which the first section contained the atrio-ventricular level, the second section showed the following healthy tissue, the third section showed the peri-infarct zone until the middle part of the scar, thereby including the LV posterior wall, which belongs to the remote zone, and the fourth section showed the apical scar-part of the heart. Heart sections of 4 or 5µm thickness were than prepared for histological and immuno-histological staining.

Moreover, LV transversal diameters and wall thickness were analyzed with a stereomicroscope connected to a digital camera (Kodak, DC290 ZOOM). Captured photographs were analyzed by the use of Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA). The cavity volume was calculated with the Dodge equation.[109, 110]

- Immuno-staining for NGF

The capacity of NGF gene delivery to produce the transgenic protein in the infarcted heart was determined at 14 days post-MI by NGF immuno-staining (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immuno-staining for NGF was also performed to determine the effect of myocardial infarction on NGF expression. For this purpose, a rabbit polyclonal antibody (1:50; Santa Cruz Biotechnology), which recognizes both human and murine NGF, has been used. The antigen was visualized by incubation with diaminobenzidine tetrahydrochloride (DAB) (Dako, Glostrup, Denmark). In negative controls the primary antibody was substituted by non immune rabbit IgG. Counterstaining was performed with hematoxylin.

- Analyses of capillary density

At 14 days after MI, capillary density following *p.NGF* gene transfer was determined in both peri-infarct and remote myocardium following Silver-hexamethylenetetramine staining, which defines vascular basal membrane and the cell nucleus.[109]

Analysis was performed with an ocular reticle (9604 μm^2 area) at 1000X magnification. A total of 20 fields were analysed in order to compute the capillary numerical density/mm^2 of tissue.

Moreover, capillary density was also assessed after MI and gene transfer with the adenoviral vector carrying the human NGF (*Ad.NGF* 10^8 p.f.u.). Briefly, in deparaffinized and rehydratated 5µm thick sections, endothelial cells (EC) were recognized by fluorescent immuno-histochemical staining for Factor VIII by using a primary rabbit polyclonal antibody (1:100; Dako) and revealed by a FITC-conjugated goat anti-rabbit secondary antibody (Sigma Aldrich). In the same sections, cardiomyocytes were stained for α-sarcomeric actin by using a primary mouse antibody (1:20; Dako) and revealed by a TRITC-conjugated secondary goat anti-mouse secondary. Nuclei were counterstained by DAPI. Slides were observed under a fluorescence microscope. High power fields were captured (at 400X) from *Ad.NGF* and *Ad.Null* hearts and the number of capillaries per field was counted in blind.

- Evaluation of apoptosis

Two weeks after MI and gene transfer, apoptosis was analyzed by TUNEL assay on 4µm LV sections. Cardiomyocytes were recognized by α-sarcomeric actin (1:30; Dako), and revealed by VIP kit (Vector Laboratories, Burlingame, CA, USA). In separate heart sections, endothelial cells were identified by staining for Factor

VIII (1:20; Dako), and revealed by VIP kit. The numbers of TUNEL-positive apoptotic cardiomyocytes and TUNEL-positive apoptotic EC were separately counted in the peri-infarct myocardium with the aid of ocular reticle (at 1000X). The density of apoptotic cardiomyocytes and apoptotic endothelial cells was calculated per mm^2 of tissue section[111].

Hemodynamic parameters and heart remodeling

Cardiac function and dimensions were performed in artificially ventilated mice under Thiopental anesthesia (125mg/kg body weight) at 5 weeks after MI. Left ventricular catheterization has been performed using a high-fidelity micro-tip pressure transducer (1.4F Millar-Micro-Tip catheter, Millar Instruments, Houston, TX, USA).

The catheter was inserted into the right carotid artery and moved on until the left ventricular cavity. Heart rate (HR, bpm), peak systolic LV pressure (LVP, mmHg), maximal rate of LV pressure rise (dP/dt_{max}, mmHg/s) and fall (dP/dt_{min}, mmHg/s) were digitally recorded and analyzed using a Power-Lab/4SP polygraph (AD Instruments, Colorado Springs, CO, USA).

After intraventricular pressure measurements, hearts were stopped in diastole by intracardiac injection of cadmium chloride (100nM). The heart was excised and separated in LV (including septum) and right ventricle (RV) and the following parameters were analyzed: total heart weight (HW) to body weight (BW) ratio, LVW to BW, RVW to BW, LV free wall thickness and LV transversal diameter and volume.

Rat neonatal cardiomyocytes isolation

To better understand the effects of NGF and its pro-survival and anti-apoptotic features, we decided to analyse the role of NGF in rat neonatal cardiomyocytes (RNCMs) *in vitro*.

Hearts from neonatal Wistar rats (Charles River) were rapidly excised and washed to remove blood and debris. The whole heart was carefully minced and submitted to four cycles of digestion in 0.1% trypsin containing 0.02% EDTA in PBS at 37℃ with gently stirring. Digestion was stopped by adding 20% fetal calf serum (FCS). The dispersed cells were resuspended in MEM supplemented with 10% FCS, 100µg/ml streptomycin, and 100U/ml penicillin and preplated for 1 hour to allow the adhesion of fibroblasts leaving the myocytes in suspension. The suspended cardiomyocytes were resuspended in EMEM with 10% FCS, counted and cultured in 6 well plates previously coated with 1% gelatin in PBS at the density of 10^6 myocytes/well. After 24 hours in culture, the culture medium was replaced to 4:1 DMEM-M199, containing antibiotics and 1% FCS, and the cells were kept in culture until they were used for experiments.

Immunocytochemical analyses of rat neonatal cardiomyocytes

After isolation, rat neonatal cardiomyocytes were cultured in gelatine-coated Labtekt slides (Nalge Nunc Int., Rochester, NY, USA), fixed with 4% paraformaldehyde (PFA, 10min, RT) and permeabilized (0.2% Triton X-100 in PBS, 20min, RT). After blocking with 5% normal goat serum (1h, RT), cells were incubated with the

following primary antibodies (16h, 4℃): α-sarcomeric actin (1:50; DAKO) and NGF (1:50; Santa Cruz Biotechnology).

This was followed by incubation (1h, RT) with rabbit anti-mouse PE conjugated secondary antibody (1:200; Sigma-Aldrich) or goat anti-rabbit Alexas-Green 488-conjugated secondary antibody (1:200; Molecular Probes, Paisley, UK). In negative controls, the primary antibody was substituted with non-immune IgG. Coverslips were mounted using Vectashields (Vector Laboratories) mounting medium containing 4'.6 diamidino-2 phenyindole (DAPI) or propidium iodide (PI). Cells were observed under a fluorescence microscope (Olympus BX40, Southall, UK).

RNA extraction and RT-PCR

Gene expression was assessed by reverse transcription-polymerase chain reaction (RT-PCR) in both hearts isolated from infarcted animals and rat neonatal cardiomyocytes.

- RT-PCR on infarcted heart

Three days after MI, the heart of anaesthetized mice was harvested and processed for RNA extraction. Total RNA was isolated from each heart of mice and one microgram of synthesized complementary DNA (cDNA) was prepared for the measurements.

Total RNA was isolated with an RNeasy Kit from Qiagen (Qiagen, Valencia, CA, USA). Briefly, tissues were homogenized in Trizol using a rotor-stator homogenizer. The homogenates were further centrifuged and the supernatants were precipitated with ethanol and the mixture was applied to a RNeasy mini spin column for adsorption of RNA to a membrane. The RNA bound to the membrane after centrifuging for 15 seconds at 10.000 rpm. After

digestion with DNase-I to eliminate DNA contamination, the total ready-use RNA was eluted with RNase-free water and was stored at –80℃. The concentration of the sample was determined using a DNA/RNA reader and the quality of RNA was evaluated by A260/A280 value.

One microgram of total RNA was used to synthesize cDNA in a reaction mixture in a volume of 20µl which contained 200 units of M-MLV reverse transcriptase in the presence of 1X first–strand buffer, 5ng/µl random primers, 10mM dithiothreitol, 500µM dNTPs, and 2 units/µl of Rnasin Recombinant Ribonuclease Inhibitor. The reaction was carried out in a 0.5 ml eppendorf tube at 25 ℃ for 10 minutes, followed by 37 ℃ for 50 minutes. After reaction, the tube was heated at 70 ℃ for 15 minutes to inactivate the enzyme and to stop the reaction.

The following set of primers has been used: human NGF-ß, forward: 5'-GGCTGCCTGGCGGTTTAT-3', and reverse: 5'-GGCAGGTCAGGCTCTTCTCA-3', which amplify human NGF cDNA but do not amplify murine NGF cDNA; mouse NGF-ß: forward:5'-AGACTTCCAGGCCCATGGTA-3', reverse: 5'-GAACTCCCC CATGTGGAAGA-3'; mouse TrkA: forward: 5'-CTTTGTGCAC CGGGATCTG-3', reverse: 5'-TCATGCCAAAGTCTCCA-ATCTTC-3') and normalized towards murine GAPDH[57] (Table 2).

- RT-PCR on rat neonatal cardiomyocytes

Rat neonatal cardiomyocyte total RNA was extracted using RNAeasy mini kit (Qiagen). First-strand cDNA was synthesized from 1mg of total RNA using 200U of Moloney–murine leukemia virus reverse transcriptase (Invitrogen Ltd, Paisley, UK) and oligo-dT

oligomer (Invitrogen) as primer. One microliter of each cDNA preparation was amplified by PCR.

The following forward and reverse oligonucleotides were used: rat NGF-ß, forward, 5'-CTTCAGCATTCCCTTGACAC-3'; and reverse, 5'-TGAGCACACACACGCAGGC-3', human NGF-ß 5'-GGCTGCCTGGCGGTTTAT-3', and reverse: 5'-GGCAGGTCAG GCTCTTCTCA-3' (primers amplify human NGF cDNA but do not amplify rat NGF cDNA), rat TrkA, forward, 5'-GCATCCTCT TACCGCAAGTT-3', and reverse 5'- CAGACTCCTAGCCC CAGAACG-3'; 18S rRNA forward, 5'-TAGAGGGACAAGTG GCGTTC-3', and reverse 5'-TGTACAAAGGGCAGGGACTT-3' (Table 2).

cDNA		*Sequence*
human NGF-β	**forward** **reverse** **probe size**	**5'-GGCTGCCTGGCGGTTTAT-3'** **5'-GGCAGGTCAGGCTCTTCTCA-3'** **192bp** primers amplify human NGF cDNA but not rat NGF cDNA
mouse NGF-ß	**forward** **reverse** **probe size**	**5'-AGACTTCCAGGCCCATGGTA-3'** **5'-GAACTCCCCCATGTGGAAGA-3'** **412bp**
rat NGF-β	**forward** **reverse** **probe size**	**5'-CTTCAGCATTCCCTTGACAC-3'** **5'-TGAGCACACACACGCAGGC-3'** **592bp**
mouse TrkA	**forward** **reverse** **probe size**	**5'-CTTTGTGCACCGGGATCTG-3'** **5'-TCATGCCAAAGTCTCCAATCTTC-3'** **434bp**
rat TrkA	**forward** **reverse** **probe size**	**5'-GCATCCTCTTACCGCAAGTT-3'** **5'-CAGACTCCTAGCCCCAGAACG-3'** **306bp**
18S rRNA	**forward** **reverse**	**5'-TAGAGGGACAAGTGGCGTTC-3'** **5'-TGTACAAAGGGCAGGGACTT-3'**

Table 2. Sequences of primers used for RT-PCR

Protein level quantification and western blot analyses

- Western blot analyse of infarcted heart

Tissue samples of the peri-infarct zone were homogenized in 600µl of ice-cold protein extraction buffer using a rotor-stator homogenizer. The buffer for homogenization contained 200mmol/l sucrose, 20mmol/l Hepes buffer [pH 7.4] and protease inhibitors. After incubation at 4℃ for 30 minutes, the homogen ate was centrifuged at 10.000 rpm for 10 minutes at 4℃. Th e pellet was discarded and the supernatant was collected and frozen at –80℃. The concentration of protein in the supernatant was determined by the method of Bradford. An equal amount of protein was loaded into a 10% SDS-polyacrylamide gel and subsequently blotted onto a PVDF membrane (Bio-rad, Hercules, CA, USA). Antibodies against phospho-Akt-B (Ser-473) and Akt-B (Cell Signaling) were used at a dilution of 1:1000, phospho-eNOS (Ser-1177) (Cell Signaling, Danvers, MA, USA) and eNOS (Cell Signaling) were used at a dilution of 1:500 and 1:1000 respectively. Specific protein was detected by chemiluminescent reaction (ECL kit, Amersham Biosciences, Little Chalfont, UK) followed by exposure of the membrane to Hyperfilm™ MP (Amersham Biosciences). Quantifications were performed by densitometric analysis after scanning using the software Scion Image 4.0 (Scion Corporation, Frederick, MA, USA). The ratio of phosphorilated to total Akt-B and phosphorylated to total eNOS was calculated.

- Western blot analyses of rat neonatal cardiomyocytes

Serum-starved RNCM were treated with NGF (50ng/ml) before undergoing protein extraction. Some cells were also pre-treated with the TrkA inhibitor K252a (100nM) or the PI3K inhibitor LY294002 (50nM).

Protein extraction from RNCM was carried out using ice-cold RIPA buffer (Pierce, Rockford, IL, USA) with protease inhibitors at time 0 and after 10, 20, and 60 min of NGF or combined treatments. Cells were kept in the lysis buffer for 30 minutes at 4℃ before being centrifuged at 12.000 rpm for 10 minutes at 4℃. The supernatant was collected and frozen at –80℃. Protein concentration was defined by the method of Bradford. Proteins were separated in 10% SDS-polyacrylamide gels and blotted into a PVDF membrane (Bio-rad). The following antibodies were used: phospho-TrkA (Tyr-490), total TrkA, phospho-Akt (Ser-473), total Akt, phospho-Foxo-3a (Thr-32), total Foxo-3a, phospho-Foxo-1 (Ser-256) and total Foxo-1(all from Cell Signaling; dilution at 1:1000).

Proteins were detected by ECL chemiluminescent reaction (Amersham Biosciences) and quantified by densitometric analysis after scanning using the software Scion Image 4.0 (Scion Corporation). The ratio of phosphorylated to total form was calculated.

ELISA

Serum-free conditioned culture medium of rat neonatal cardiomyocytes was used to investigate RNCM-released NGF content by the NGF $E_{max}^{®}$ ImmunoAssay System (Promega, Madison, WI, USA). RNCM were maintained in serum-free medium

for 24 hours before collecting the conditioned medium. ELISA assay was performed according to the manufacture instructions.

Hypoxia/reoxygenation and Angiotensin II apoptosis assays on rat neonatal cardiomyocytes

RNCM were submitted to hypoxia/reoxygenation (H/R) or incubated with Angiotensin II (Ang II), two conditions which reportedly cause cardiomyocyte apoptosis.

- Hypoxia/reoxygenation assay

Rat neonatal cardiomyocytes were infected overnight with *Ad.hNGF* or *Ad.βGal* (50M.O.I. each). Cells were then transferred to an anaerobic chamber which was purged with 95% N_2/5% CO_2. RNCM were kept under hypoxia for 12 hours before being reoxygenated under normoxic conditions for 24 hours. Incubations were performed at 37℃. Normoxic condition was used as control. Apoptosis was detected by cleaved caspase-3 immuno-staining and confirmed by TUNEL assay.

- Angiotensin II apoptosis assay

After overnight infection with *Ad.hNGF* or *Ad.βGal* (50 M.O.I. each), RNCM were incubated with AngII (10^{-7} M) for 24 hours at 37℃. PBS was used as control.

Apoptosis was then detected by cleaved caspase-3 immuno-staining.

Human EPC culture

To prepare EPC, mononuclear cells (MNCs) were isolated by Biocoll density-gradient centrifugation with Histopaque 1077 (Sigma Aldrich) from 20ml of human peripheral blood of healthy volunteers. After separation, cells were washed two times in phosphate buffered saline (PBS) and counted. Peripheral blood-MNCs were suspended in EPC culture medium EGM-2-MV (Cambrex, Milano, Italy) supplemented with 10% FBS, hEGF, hydrocortisone, GA-1000, VEGF, hFGF-B, IGF-1 and ascorbic acid (all from Cambrex) and plated on 10μg/ml human fibronectin-coated tissue culture plates. After 4 days non-adherent cells were removed and fresh medium was added to the adherent EPC. When necessary, EPC were detached with trypsin, counted and subjected to experiments.

EPC migration assay

After 4 days in culture, isolated EPC were washed in PBS (pH 7.4), detached using trypsin for 2 minutes at 37℃. after washing and centrifugation, cells were resuspended in EBM-2 with 0.1% bovine serum albumin (BSA), counted, and $5x10^4$ cells were placed in 150μL of EBM-2 with 0.1% BSA in the upper chamber of a modified Boyden chamber (Costar Transwell, Corning Incorporated, Acton, MA, USA) composed by a 5μm-pores polycarbonate membrane of which the lower surface was pre-coated with fibronectin (10μg/ml, Sigma Aldrich). The chamber was placed in a 24-well culture dish containing EBM-2 (in 0.1%BSA) and human recombinant NGF at different concentration (10ng/ml, 50ng/ml and 100ng/ml). Human recombinant SDF-1 (100ng/ml, R&D System)

was used as positive control for migration while EBM-2 in 0.1% BSA as a negative control.

After overnight incubation in a humidified incubator at 37℃, non-migrated cells were removed from the upper chamber by gently wiping the upper surface of the membrane with a cotton swab, the membrane was isolated and washed with PBS, fixed with methanol and placed on a slide mounted with DAPI-Vectashield (Vector Laboratories). Migrating cells into the lower chamber were counted using a fluorescent microscope in 5 random microscopic fields at 20X magnification. Each experiment was performed in triplicate. Evaluations were performed in blind.

MTS cell proliferation assay

After 7 days in culture, isolated EPC were washed in PBS and detached with trypsin (2 minutes at 37℃). Cells were counted and cultured at the density of $1x10^4$ cells/well in a flat bottomed 96 well plate and stimulated with different doses of NGF (NGF 5ng/ml; NGF 50ng/ml; NGF 100ng/ml) for 24 hours. The viability of cell cultures was monitored over time by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS) colorimetric assay following the manufacturer's instructions (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, Milano, Italy). Cell viability was determined by measuring the absorbance at 490 nm using a 550 BioRad plate-reader (Bio-Rad).

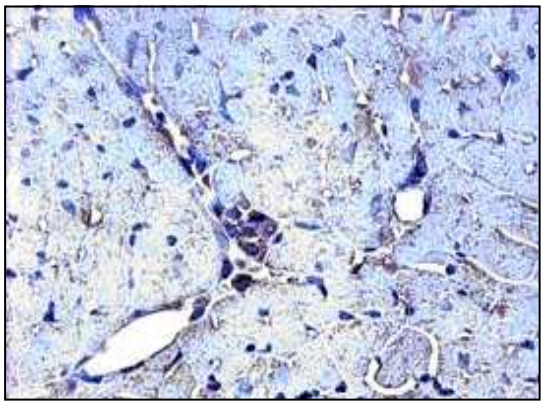
Statistical analyses

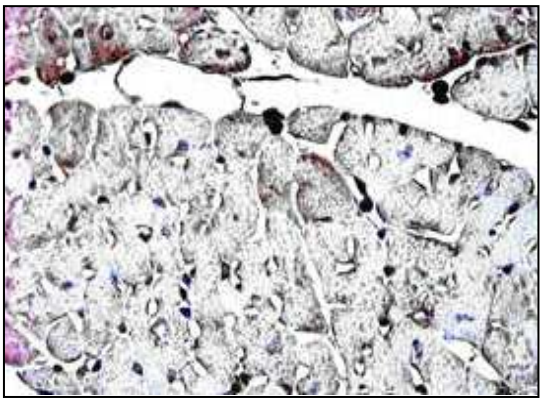
Values are presented as mean±SEM. Statistical significance was evaluated through the use of an unpaired *t* test for comparisons between 2 groups. For comparison among more than 2 groups, ANOVA was used, followed by an unpaired *t* test to compare 2 groups within them. Analyses were performed using the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

Results

NGF and TrkA expression after myocardial infarction

Myocardial infarction increased the cardiac levels of NGF. Immuno-staining for NGF revealed a higher endogenous NGF protein content in the heart of infarcted animals in comparison to sham-operated. Figure 6 shows that sham-operated hearts express low levels of NGF, and the expression of murine NGF increased following myocardial infarction.





Sham **MI**

Figure 6. NGF protein content after myocardial infarction
Myocardial infarction or sham operation was induced in mice. NGF protein content was revealed by immuno-histochemistry using an antibody which recognizes both human and murine NGF. Counterstaining was performed with hematoxylin (Magnification 200X).

Moreover, the analyses of mRNA levels by RT-PCR confirmed an increase of both NGF (NGF/GAPDH cDNA level ratio multiplied per 100: 0.410±0.052 vs. 0.068±0.016 in sham-operation, P<0.05) and TrkA (TrkA/GAPDH cDNA level ratio multiplied per 100: 0.190±0.023 vs. 0.029±0.005 in sham-operation, P<0.05) content in heart submitted to myocardial infarction (Figure 7).

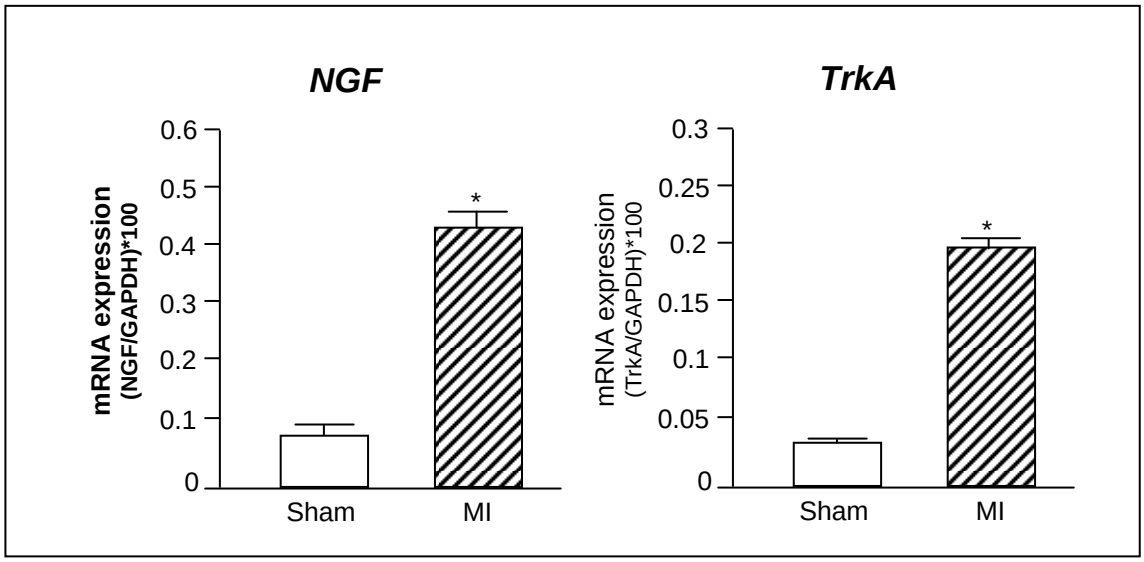


Figure 7. NGF and TrkA mRNA expression after myocardial infarction
Bar graphs showing the ratio between NGF to GAPDH (left panel) and TrkA to GAPDH (right panel) mRNA expression levels determined in the peri-infact zone at 3 days post-MI. Data are expressed as mean±SEM (n=5/group). *P<0.05 vs. sham.

NGF transgene expression after myocardial infarction and plasmid-mediated gene transfer

Intra-myocardial *p.NGF* delivery induced expression of human NGF mRNA (Figure 8) and increase the content of total (murine plus human) NGF protein in comparison to *p.Null* in the peri-infarct myocardium (Figure 9).

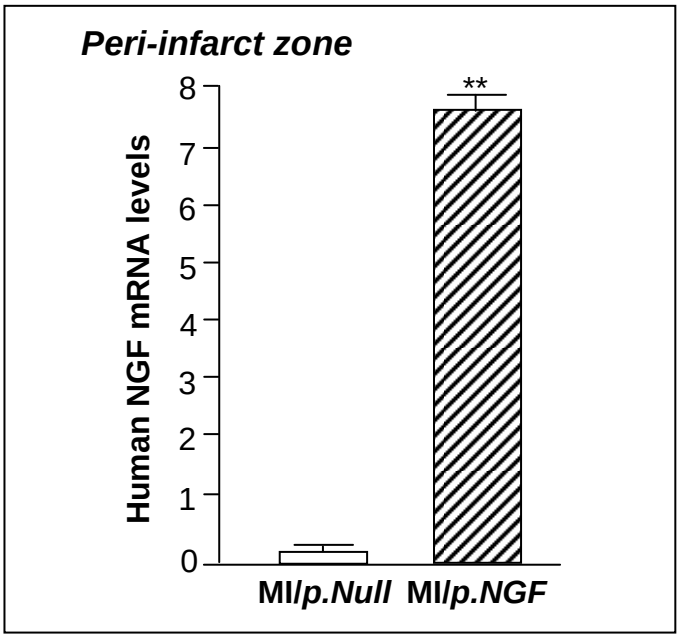
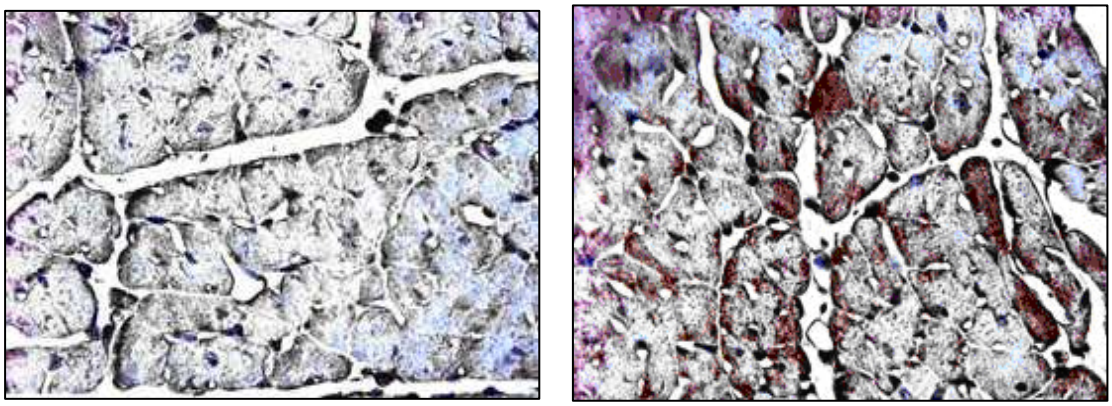


Figure 8. NGF mRNA levels after myocardial infarction and plasmid-mediated gene transfer
Bar graphs showing the mRNA levels of human NGF determined in the peri-infact zone at 3 days post-MI. Data are expressed as mean±SEM (n=5/group). **P<0.01 vs. MI/*p.Null*.



MI/*p.Null* **MI/*p.NGF***

Figure 9. NGF protein content after gene transfer
Immediately after MI, the peri-infarct myocardium was injected with a plasmid carrying the human *NGF* gene (*p.NGF*) or an empty vector (*p.Null*). After 14 days, the NGF protein content was studied by immuno-histochemistry using an antibody which recognized both the murine and human NGF protein (Magnification 200x).

NGF over-expression increases capillary density in the peri-infarct area

Two weeks after myocardial infarction and *p.Null* or *p.NGF* gene delivery, capillary density was evaluated by Silver-hexamethylenetetramine staining in 4µm-thick heart sections. The number of capillary per mm^2 of tissue was similar in the remote zone of hearts harvested from *p.Null*- (3843±358 cap/mm^2) or *p.NGF*-injected mice (4313±407; P=N.S.).

In contrast, capillary density was potentiated by *p.NGF* at the level of the injected peri-infarct zone (5131±242 cap/mm^2) corresponding to a significant 1.57-fold increase vs. *p.Null* (3252±341; P<0.05) (Figure 10).

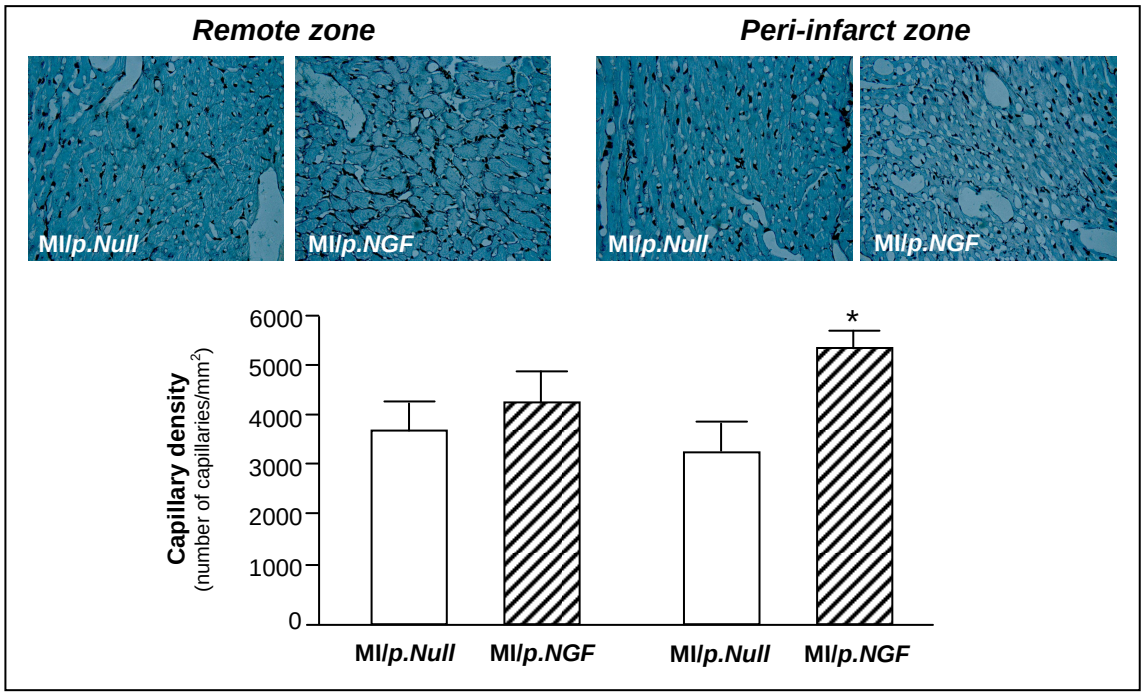


Figure 10. Capillary density in the peri-infarct and remote myocardium following plasmid-mediated gene delivery
Capillary density was investigated two weeks after myocardial infarction and *p.NGF* or *p.Null* gene delivery. The micro-photographs show heart sections after Silver staining (Magnification 200X). Bar graphs show capillary numerical density per mm^2 of tissue in both remote and peri-infarct zones. The number of capillaries per mm^2 was assessed following the analyses of twenty five fields at 1000X magnification. Data are presented as mean±SEM (n=5/group). *P<0.05 vs. MI/*p.Null*.

Similar results were obtained when gene transfer to the mouse peri-infarct myocardium was mediated by adenoviral vectors and capillary density evaluated after fluorescent immuno-histochemical staining of endothelial cells for Factor VIII (Figure 11, green fluorescence) in heart sections. In the same sections, cardiomyocytes were stained for α-sarcomeric actin (red fluorescence) and nuclei counterstained with DAPI (blue fluorescence). As shown in Figure 11, *Ad.NGF* improved capillary density of the peri-infarct myocardium (5348±144 cap/mm^2 vs 4116±264 cap/mm^2 for *Ad.Null*; P<0.05).

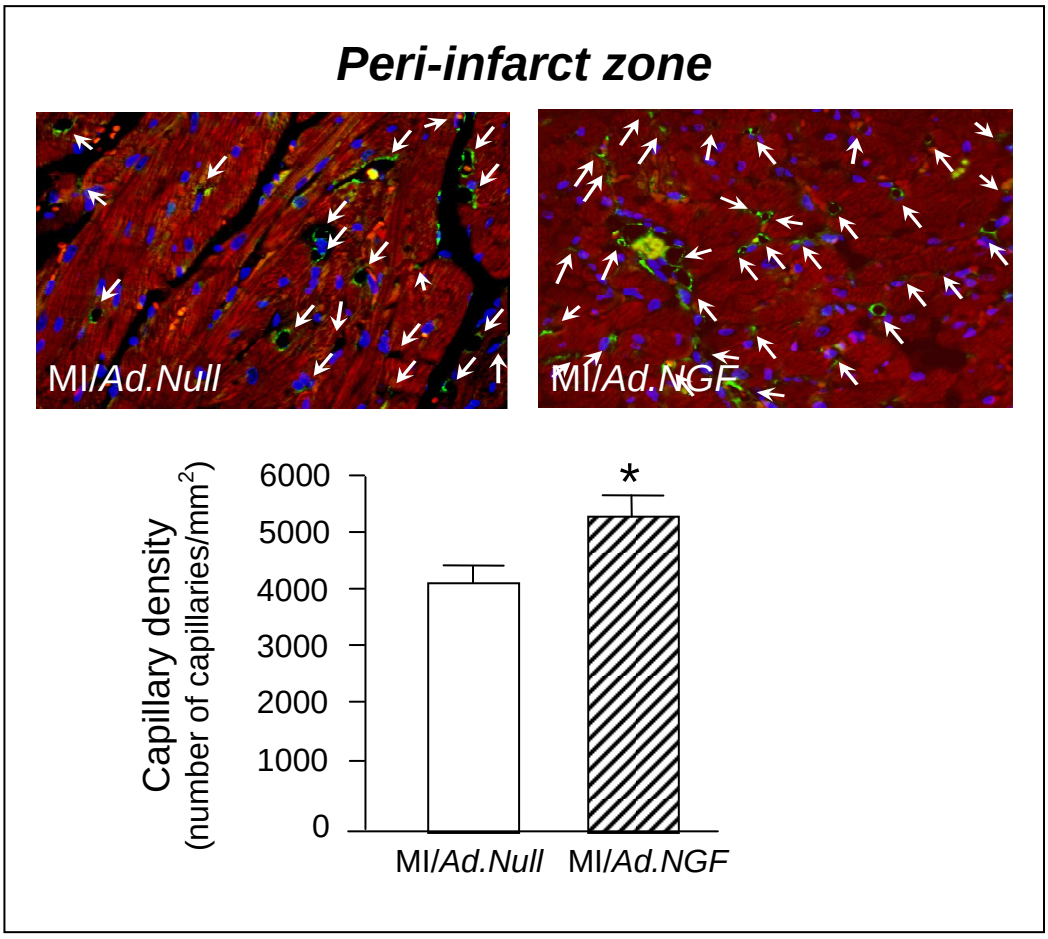


Figure 11. Capillary density in the peri-infarct and remote myocardium following adenovirus-mediated gene transfer
Capillary density two weeks after myocardial infarction and *Ad.NGF* or *Ad.Null* gene transfer was assessed following immuno-fluorescence staining for Factor VIII. The micro-photographs show capillary density in the peri-infarct zone of *Ad.Null* and *Ad.NGF* injected hearts. Capillaries are stained in green fluorescence, cardiomyocytes in red and nuclei in blue (Magnification 200X). Bar graphs show capillary numerical density per mm^2 of tissue in the peri-infarct zone. Data are presented as mean±SEM (n=5/group). *P<0.05 vs. MI/*Ad.Null*.

Collectively, these data indicate that NGF potentiates reparative angiogenesis in the area at risk. The improved capillary density in the peri-infarct area of NGF-treated animals suggests that the neurotrophin is able to stimulate therapeutic neovascularisation and reinforce the idea that neurotrophins are important for the cardiovascular system.

NGF over-expression reduces apoptosis of both endothelial cells and cardiomyocytes after myocardial infarction

Intra-myocardial over-expression of NGF by either a plasmid or an adenoviral vector reduced apoptosis of both endothelial cells and cardiomyocytes in the peri-infarct area two weeks after MI.

Apoptosis of CM was revealed by double staining for TUNEL and the cardiac marker α-sarcomeric actin.

The TUNEL assay revealed a significant reduction of the number of apoptotic cardiomyocytes in the peri-infarct zone of *p.NGF*-injected MI hearts (66±8.2 apoptotic CM/mm^2) compared with *p.Null*-injected hearts (93±5.7 apoptotic CM/mm^2; $P<0.05$) (Figure 12).

No significant group difference was detected in the remote zone (*p.Null*: 55±3.2 apoptotic CM/mm^2; *p.NGF*: 47±4.5 apoptotic CM/mm^2, P=N.S.).

Similar data were obtained in experiments performed with adenoviral vectors.

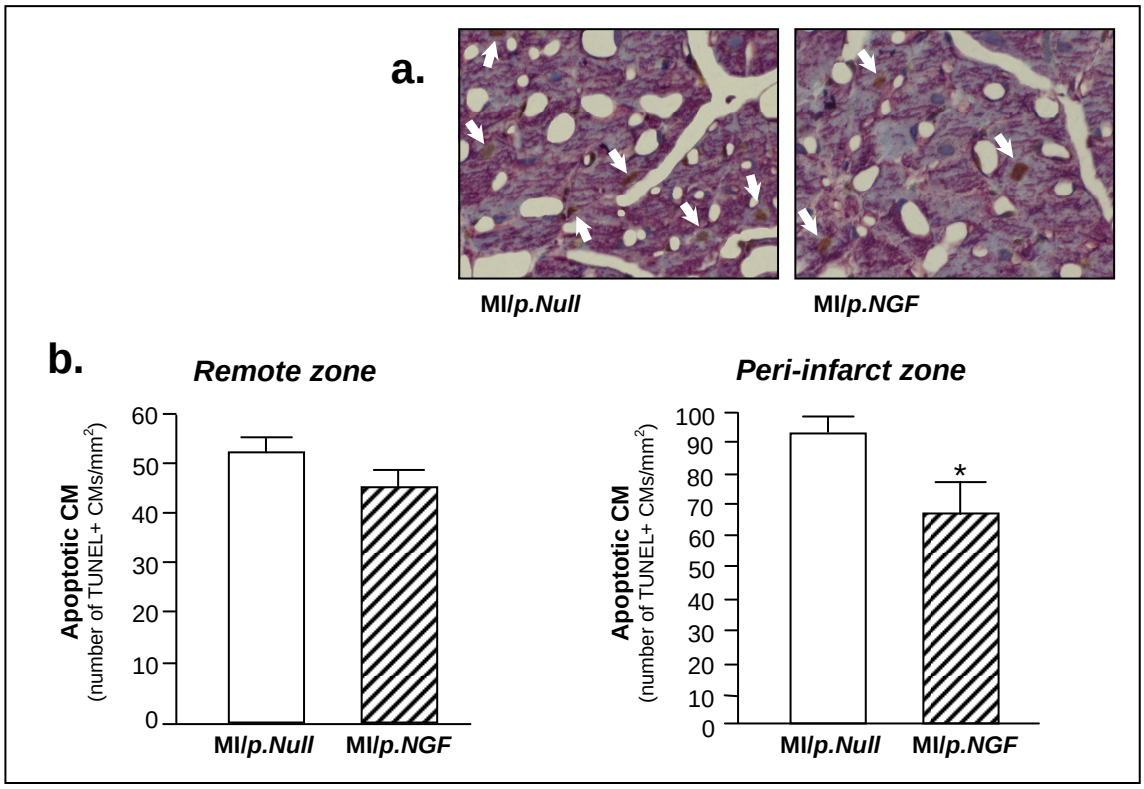


Figure 12. NGF reduces apoptosis of cardiomyocytes *in vivo*
a.) Sections of the peri-infarct area of hearts harvested at 2 weeks post-MI were double stained for TUNEL and α-sarcomeric actin. Micro-photographs show the reduced number of apoptotic cardiomyocytes in the peri-infarct zone of *p.NGF*-injected hearts. Arrows point to apoptotic myocytes (Magnification 200X). **b.)** Bar graphs show the number of apoptotic CM in both remote and peri-infarct area. Data are expressed in number of TUNEL positive cells per mm^2. Data are presented as mean±SEM (n=5/group). *P<0.05 vs. MI/*p.Null*.

Apoptotic endothelial cells were detected after TUNEL staining combined with the endothelial marker Factor VIII. The number of apoptotic cells was calculated for mm^2.

The number of apoptotic endothelial cells was significantly less in the peri-infarct zone of *p.NGF* treated mice (29±7.4 versus 48±5.7 apoptotic endothelial cells/mm^2 in *p.Null*; P<0.01; Figure 13). There were no differences in endothelial cells apoptosis in the remote zone between the groups (*p.Null*, 34±6.4 apoptotic endothelial cells/mm^2; *p.NGF*, 27±3.5 apoptotic endothelial cells/mm^2, P=N.S.).

Similar data were obtained in experiments performed with adenoviral vectors.

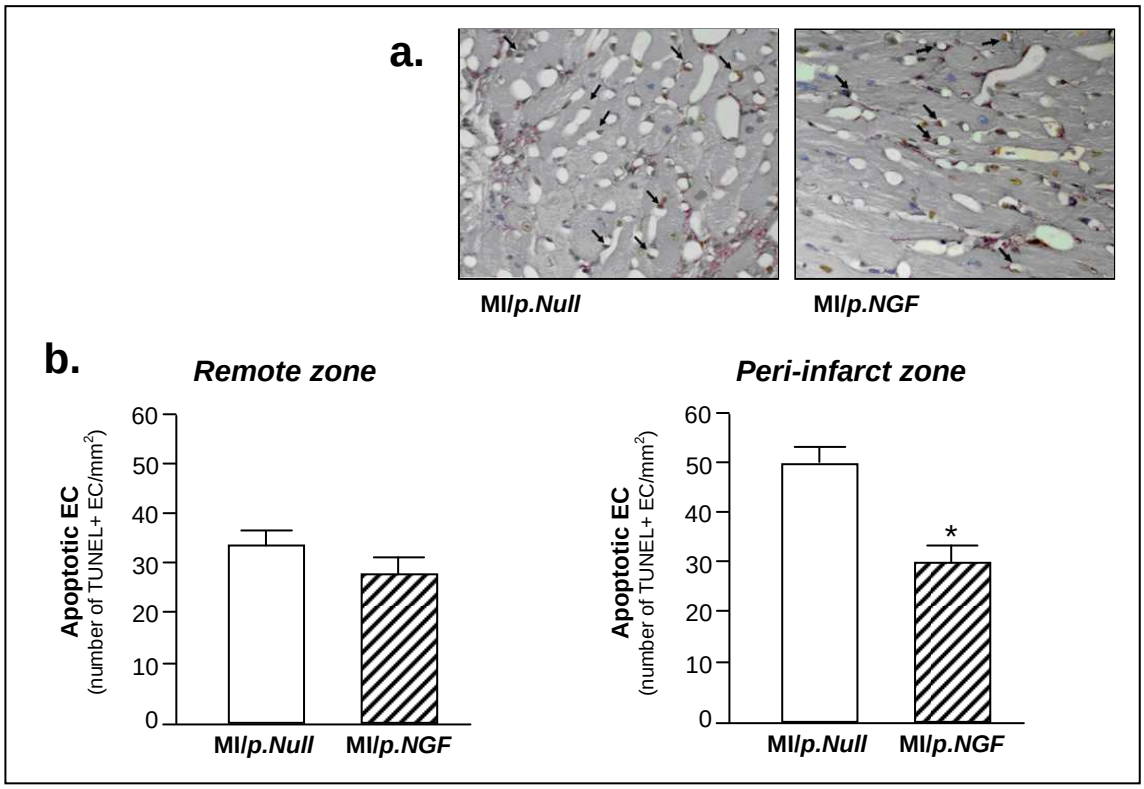


Figure 13. NGF reduces apoptosis of endothelial cells *in vivo*
a.) Representative sections from the peri-infarct myocardium of *p.Null* and *p.NGF* transduced hearts harvested at 2 weeks post-MI. Sections were double stained for TUNEL and Factor VIII. Arrows point to apoptotic endothelial cells (Magnification 200X). **b.)** Bar graphs show the number of apoptotic endothelial cells in both remote and peri-infarct area. Data are expressed in number of TUNEL positive cells per mm^2. Data are presented as mean±SEM (n=5/group). *P<0.05 vs. MI/*p.Null*.

NGF over-expression increases the phosphorylation of eNOS and Akt in the infarcted heart

NGF gene transfer resulted in an increased phosphorylation/ activation of the pro-angiogenic and anti-apoptotic factors Akt and eNOS.

As shown in Figure 14, western blot analyses of heart protein extract three days after myocardial infarction showed that the ratio of phosphorylated to total Akt was higher in *Ad.hNGF*-injected MI hearts than in *Ad.Null*-injected MI hearts or *Ad.Null*-injected sham operated hearts (P<0.05 for both comparison). Moreover, *Ad.hNGF* also up-regulated the phosphorylation of eNOS in the peri-infarct zone compared to controls (P<0.05 for both comparison).

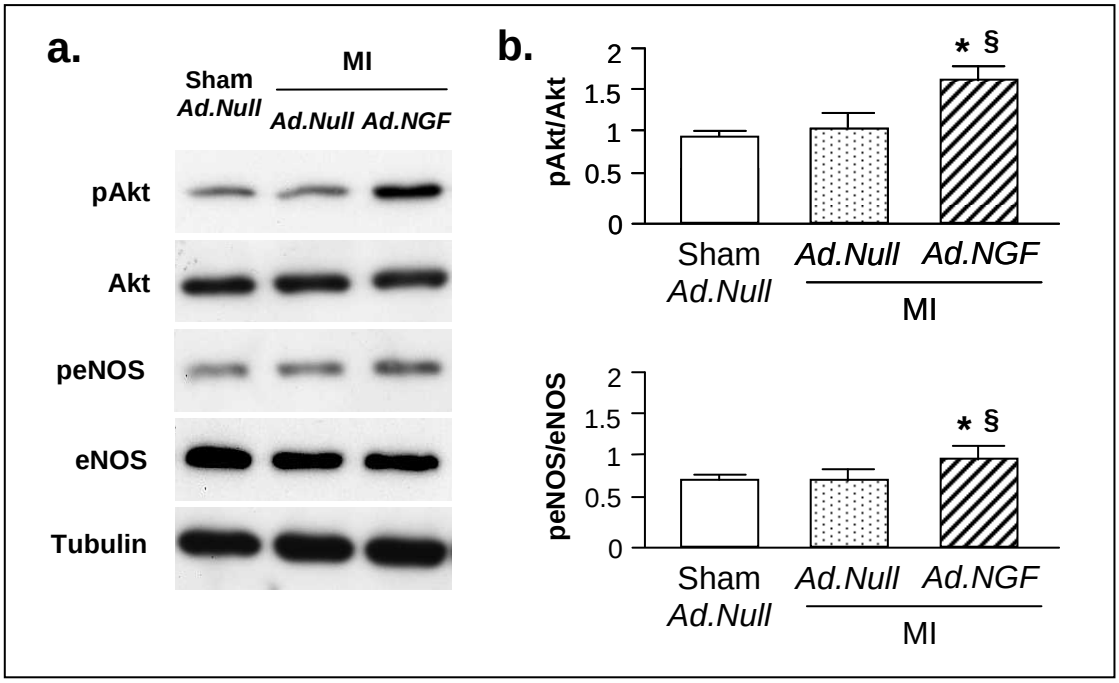


Figure 14. NGF gene transfer increases p-Akt and p-eNOS protein levels
a.) Representative western blot of phospho-Akt (pAkt), total Akt, phospho-eNOS (peNOS) and total eNOS in the peri-infarct zone. Tubulin was used as loading control. **b.)** Bar graphs show the ratio between pAkt to total Akt (upper panel) and peNOS to total eNOS (lower panel) Data are presented as mean±SEM (n=5/group). §<0.05 vs sham-operated; *P<0.05 vs. MI/*p.Null*.

NGF over-expression does not change indexes of cardiac function

The hemodynamic parameters, systolic LV pressure (LVP, mmHg), maximal rate of LV pressure rise (dP/dt_{max}, mmHg/s) and fall (dP/dt_{min}, mmHg/s) and heart rate (HR, bpm), were measured by the use of a Millar-Micro-Tip catheter at five weeks after MI, a time point in which cardiac dysfunction is generally evident.

As shown in Table 3, either MI/*p.Null*- or MI/*p.NGF*-injected mice showed a deterioration of cardiac function in comparison to sham-operated, thus indicating that plasmid-mediated NGF gene transfer did not result in long-lasting positive effects on cardiac function.

	Sham/*p.Null*	**MI/*p.Null***	**MI/*p.NGF***
- LVP (mmHg)			
- dP/dt_{max}			
(mmHg/s)	87.6±2.3	73.7±3.4 *	70.2±3.9 *
- dP/dt_{min}	6078±278	4001±189 *	3986±212 *
(mmHg/s)	-5342±243	-3175±128 *	-3255±219 *
- HR (bpm)	429±19	410±13	432±16

Table 3. Cardiac function at five weeks after myocardial infarction
Mice underwent MI or sham-operation. *p.Null* and *p.NGF* gene delivery was performed immediately after MI. Five weeks after MI, hemodynamic parameters were measured by using a Millar-Tip catheter inserted into the LV cavity. Each group consisted at least of n=12 mice. Values are in mean±SEM. *P<0.05 vs. sham.

At the same time-point the heart remodeling was analysed. To this aim, the heart weight to body weight ratio (HW/BW), left ventricle weight to BW ratio (LVW/BW) and right ventricle to BW ratio (RVW/BW) were determined. Additionally, left ventricle free

wall thickness, left ventricle transversal diameter and chamber volume were also measured.

Data are shown in Table 4.

	Sham/*p.Null*	**MI/*p.Null***	**MI/*p.NGF***
- HW/BW (x1000)	5.39±0.5	5.63±1.2	6.02±0.7 *
- LVW/BW (x1000)	3.78±0.4	3.94±0.5	4.16±0.2 *
- RVW/BW (x1000)	1.21±0.3	1.30±0.3	1.26±0.5
- LV free wall thickness (mm)	1.13±0.2	1.36±0.2 *	1.40±0.2 *
- LV transversal diameter (mm)	3.94±0.7	3.91±1	3.44±0.5 *
- LV chamber volume (mm^3)	71.2±33.3	78.62±44.6	58.6±18.6 *§

Table 4. Heart remodeling five weeks after myocardial infarction
Five weeks after MI or sham-operation, the hearts of mice were perfused-fixed with formalin and stopped in diastole. The RV was separated from the LV and heart remodeling was measured. Each group consisted at least of n=12 mice. Values are in mean±SEM. *P<0.05 vs. sham; §P<0.05 vs. MI/*p.Null*.

Rat neonatal cardiomyocytes express TrkA and produce NGF

The presence of TrkA on rat neonatal cardiomyocytes (RNCM) was determined by both RT-PCR and western blot. As shown in Figure 15, RNCM express TrkA at both mRNA and protein levels. NGF expression was also found by RT-PCR in RNCM-derived mRNA. PC12 cells, which express high level of TrkA, were used as reference.

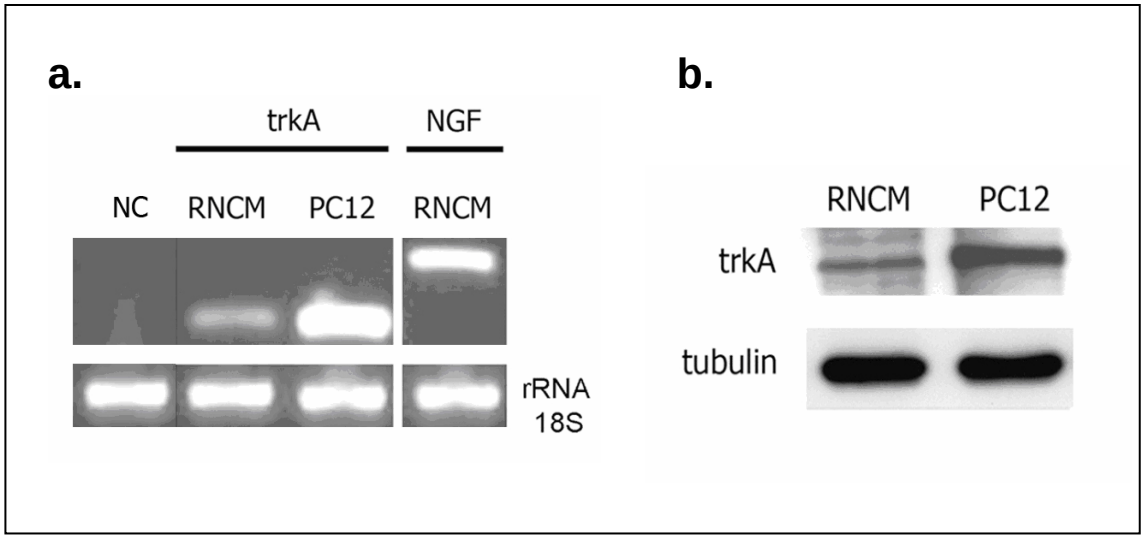


Figure 15. RNCM express TrkA and NGF
a.) RT-PCR demonstrates the presence of mRNA for TrkA and NGF in cultured rat neonatal cardiomyocytes (RNCM). Bands are representative of 3 experiments. Ribosomial RNA (18S) was used as a loading control. **b.)** Western blot analysis of TrkA in lysates of RNCM. PC12 protein extracts were loaded as positive controls for TrkA. Bands are representative of 3 experiments.

Furthermore, the ability of RNCM to produce NGF was also investigated by fluorescence double-staining for NGF and the cardiomyocyte marker α-sarcomeric actin. As shown in Figure 16, more than the 95% of cultured RNCM are stained for α-sarcomeric actin, and many of those cells are also positive for NGF. Importantly, NGF and α-sarcomeric actin co-localize, thus suggesting that cardiomyocytes can produce the neurotrophin.

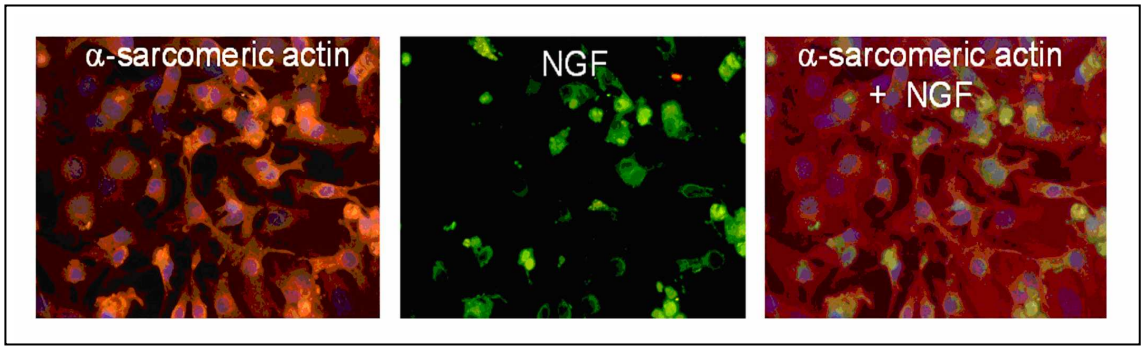


Figure 16. NGF expression in RNCM by immuno-fluorescence
Cultured RNCM were double-stained for the cardiac marker α-sarcomeric actin (in red fluorescence) and NGF (in green fluorescence). Nuclei were counterstained by DAPI (in blue). The co-localization of NGF and α-sarcomeric actin is evident in many cells and this indicates that cardiomyocytes produce NGF.

Moreover, in order to analyze whether NGF is secreted by cardiomyocytes, the presence of NGF in serum-free conditioned culture medium has also been investigated. By ELISA, we could demonstrate that RNCM produce NGF (Figure 17), which is functionally competent, since the serum-free culture medium of RNCM was able to promote neurite outgrowth from PC12 cells. Consistently, a neutralizing antibody for NGF prevented PC12 differentiation (Figure 17).

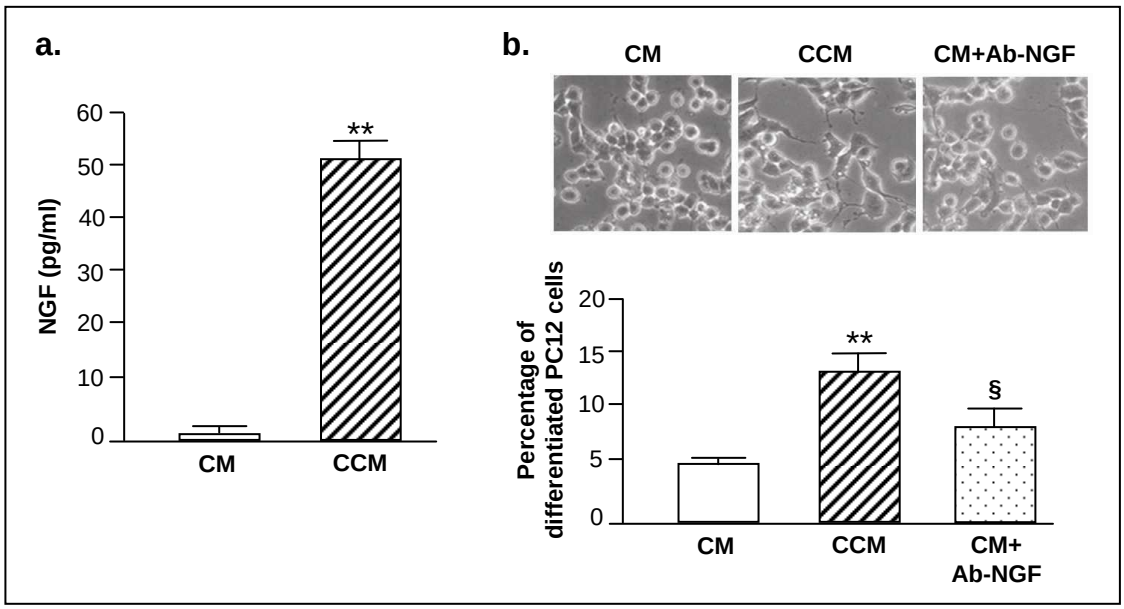


Figure 17. RNCM-produced NGF is functionally competent

a.) NGF content in the RNCM conditioned culture medium (CCM) and in basal culture medium (CM) was measured by ELISA. Data are presented as mean±SEM (n=3). **P<0.01 vs. CM.

b.) RNCM-produced NGF stimulates the differentiation of PC12. PC12 morphology was observed after incubation for 3 days in the RNCM CCM or in CM. CCM induced neurite extension in PC12. A NGF neutralizing antibody (Ab-NGF) prevented PC12 differentiation. Data are presented as mean±SEM (n=3). **P<0.01 vs. CM and §P<0.05 vs. CCM.

NGF signaling in rat neonatal cardiomyocyes

In order to study the impact of NGF on the PI3K/Akt survival pathway, serum-free cultured RNCM were stimulated with NGF (50ng/ml) for 10, 20, or 60 min. As shown in Figure 18, addition of NGF resulted in the phosphorylation of TrkA (Tyr-490, active form), Akt (Ser-473, active form), Foxo-3a (in Thr-32, inactive form), and Foxo-1 (in Ser-256, inactive form). The NGF-induced phosphorylation of TrkA and Akt peaked at 20 min and returned to the basal level by 60 min, whereas the phosphorylation of both Foxo-3a and Foxo-1 was maximal at 60 min.

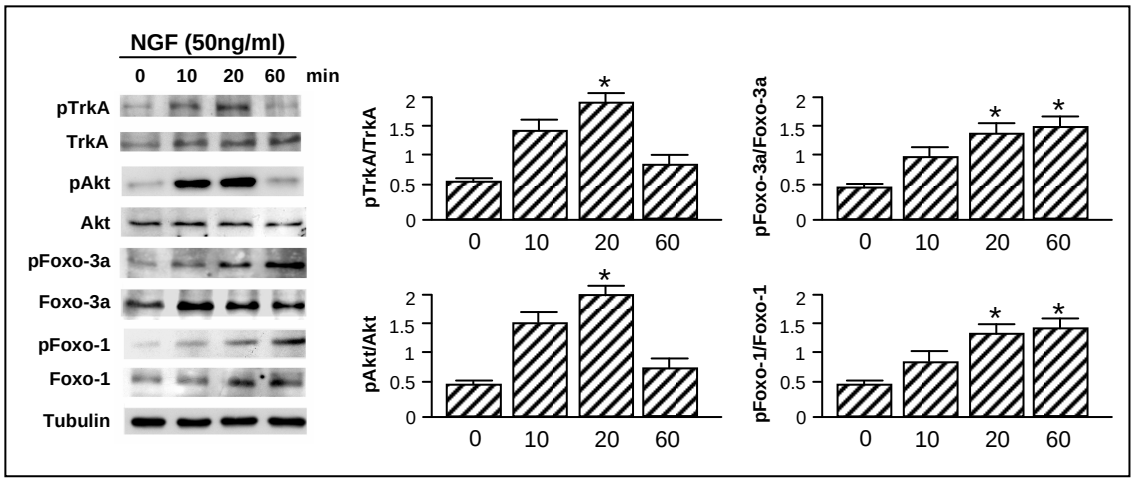


Figure 18. NGF signaling in RNCM
Serum-starved RNCM received NGF (50ng/ml). After 0, 10, 20, and 60 min, the phosphorylation of TrkA (Tyr-490), Akt (Ser-473), Foxo-3a (Thr-32) and Foxo-1 (Ser-256) was determined by western blot. Tubulin was used as loading control. Graphs show the ratio between the densitometric reading of phosphorylated and total TrkA, Akt, Foxo-3a, and Foxo-1. Data are presented as mean±SEM (n=3). *P<0.05 vs. time zero.

In separate experiments, 30 min before NGF, RNCM were pre-treated with the TrkA inhibitor K252a (100nM), or with the PI3K inhibitor LY294002 (50µM), or vehicle (0.1% DMSO). As shown in Figure 19a, K252a totally prevented Akt phosphorylation, thus suggesting that TrkA mediates NGF-induced Akt activation.

Moreover, also LY294002 prevented the phosphorylation of Foxo-3a and Foxo-1 in response to NGF, thus suggesting that Foxo is downstream to Akt in the pathway initiated by NGF in RNCM (Figure 19b).

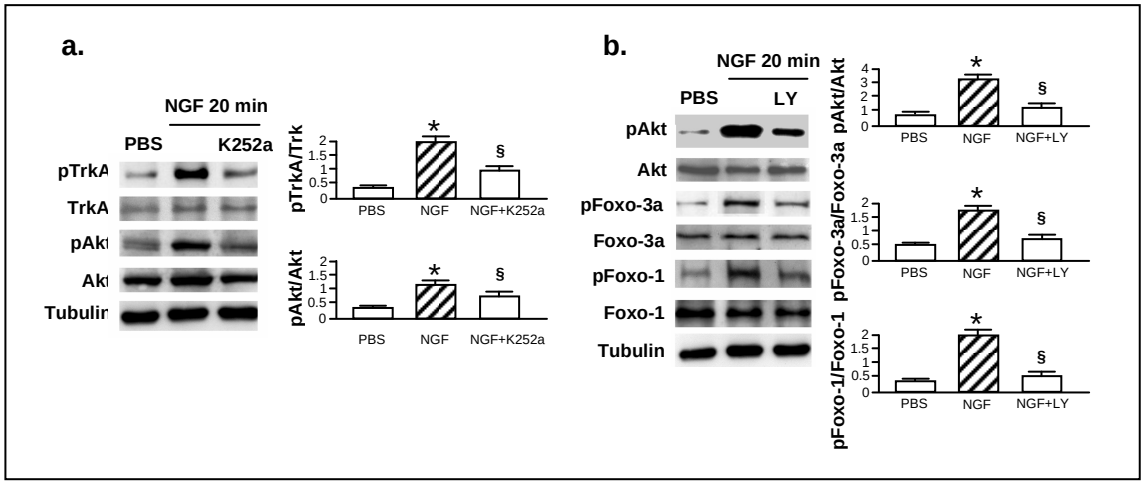
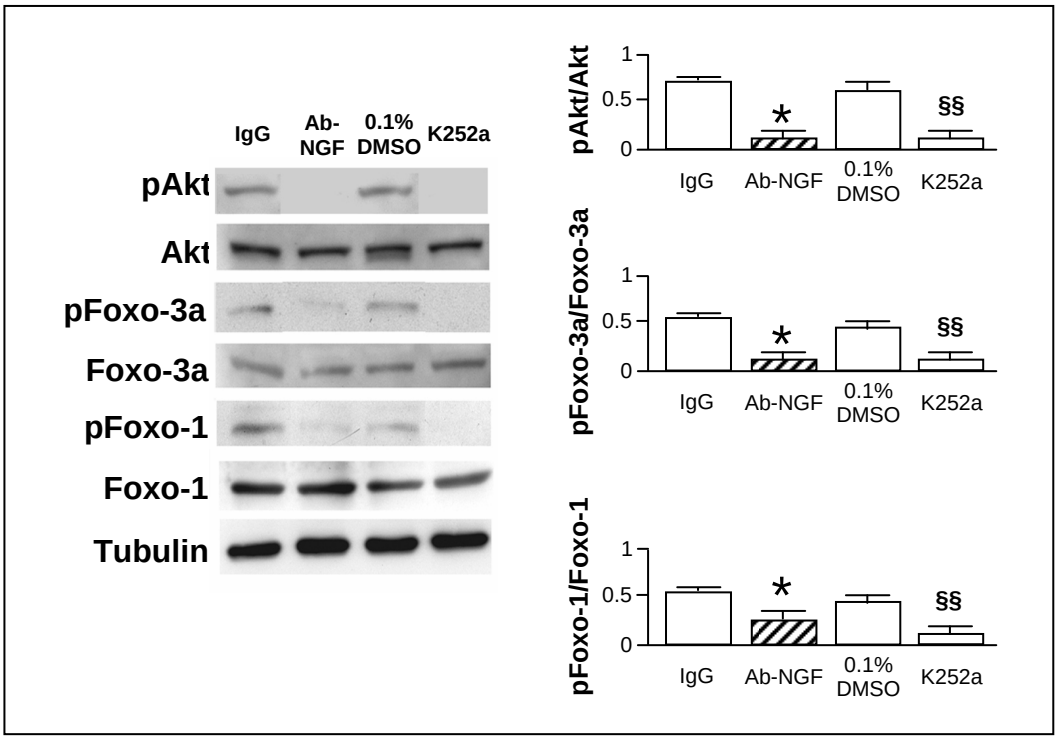


Figure 19. NGF signaling in RNCM
a.) Serum-starved RNCM were pre-treated with the TrkA inhibitor K252a (100nM) or its vehicle (0.1% DMSO) before adding NGF. After 20 min, the phosphorylation of TrkA and Akt was examined. Controls (PBS) received 0.1% DMSO (instead of K252a) and PBS (instead of NGF). Tubulin was used as loading control. K252a blocked the phosphorylation of both TrkA and Akt. **b.)** Serum-starved RNMC were pre-treated with the PI3K inhibitor LY294002 (50nM) or its vehicle (0.1% DMSO) before adding NGF. After 20 min, the phosphorylation of Akt, Foxo-3a, and Foxo-1 was evaluated. Controls received 0.1% DMSO and PBS. Tubulin was used as loading control. LY294002 blocked the phosphorylation of Akt, Foxo-3a, and Foxo-1. Graphs show the ratio between the densitometric reading of phosphorylated and total Akt, phosphorylated and total Foxo-3a, and phosphorylated and total Foxo-1. Data are presented as mean±SEM (n=3). *P<0.05 vs. PBS; §P<0.05 vs. NGF+0.1% DMSO (NGF).

We finally investigated whether the same pathway is activated by cardiomyocyte-released NGF. To this aim, RNCM were treated with Ab-NGF or K252a (or the respective controls) for 48h in serum-free medium. Cells were lysated to perform western blot analyses for phospho-Akt (Ser-473), phospo-Foxo-3a (Thr-32), phospho-Foxo-1 (Ser-256), and for the respective total forms. As shown in Figure 20, both Ab-NGF and K252a impaired the phosporylation of Akt, Foxo-3A and Foxo-1. This strongly suggests that the

NGF/TrkA/Akt/Foxo pathway may physiologically regulate cardiomyocyte survival.



<u>Figure 20</u>. Endogenous NGF regulates phosphorilation of Akr and Foxo via TrkA

Serum-free cultured RNCM were treated for 48h with Ab-NGF or K252a. Controls received 0.1% non-immune goat serum or 0.1% DMSO, respectively. Either Ab-NGF or K252a reduced phosphorylation of Akt, Foxo-3a, and Foxo-1. Tubulin was used as loading control. Graphs show the ratio between the densitometric reading of phosphorylated and total Akt, phosphorylated and total Foxo-3a, and phosphorylated and total Foxo-1. Data are presented as mean±SEM (n=3). *P<0.05 vs. 0.1% goat IgG (IgG); §§P<0.01 vs. 0.1% DMSO.

NGF over-expression prevents apoptosis of rat neonatal cardiomyocytes

As shown in Figure 21, the number of apoptotic RNCM increased following hypoxia/reoxygenation (H/R). Importantly, *Ad.NGF* prevented H/R-induced RNCM apoptosis. The upper panels show typical images of RNCM stained for cleaved caspase-3 (green fluorescence). Nuclei are counterstained by DAPI (blue fluorescence).

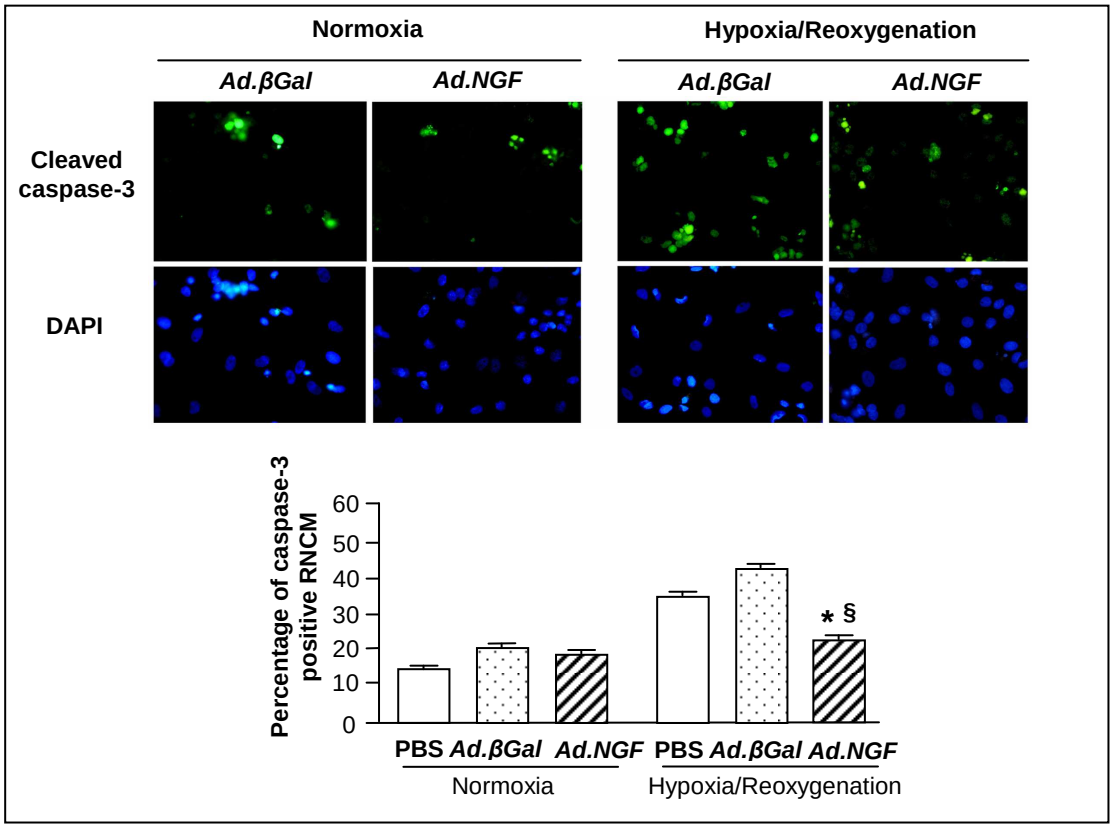


Figure 21. NGF over-expression prevents apoptosis of RNCM
RNCM were transduced with *Ad.NGF* or *Ad.βGal*, or not infected (PBS). After 24h, RNCM underwent 12h of hypoxia followed by 24h reoxygenation. Some RNCM were maintained under normoxia as control. Apoptotic RNCM were detected by cleaved caspase-3 staining (green fluorescence). Bar graph shows the quantitative analysis of apoptosis, expressed as percentage of cleaved caspase 3-positive RNCM. Data are presented as mean±SEM (n=3). *P<0.05 vs. PBS; §P<0.05 vs. *Ad.βGal*.

The pro-survival action of *Ad.NGF* after hypoxia/reoxygenation-induced apoptosis was confirmed by TUNEL assay (Figure 22).

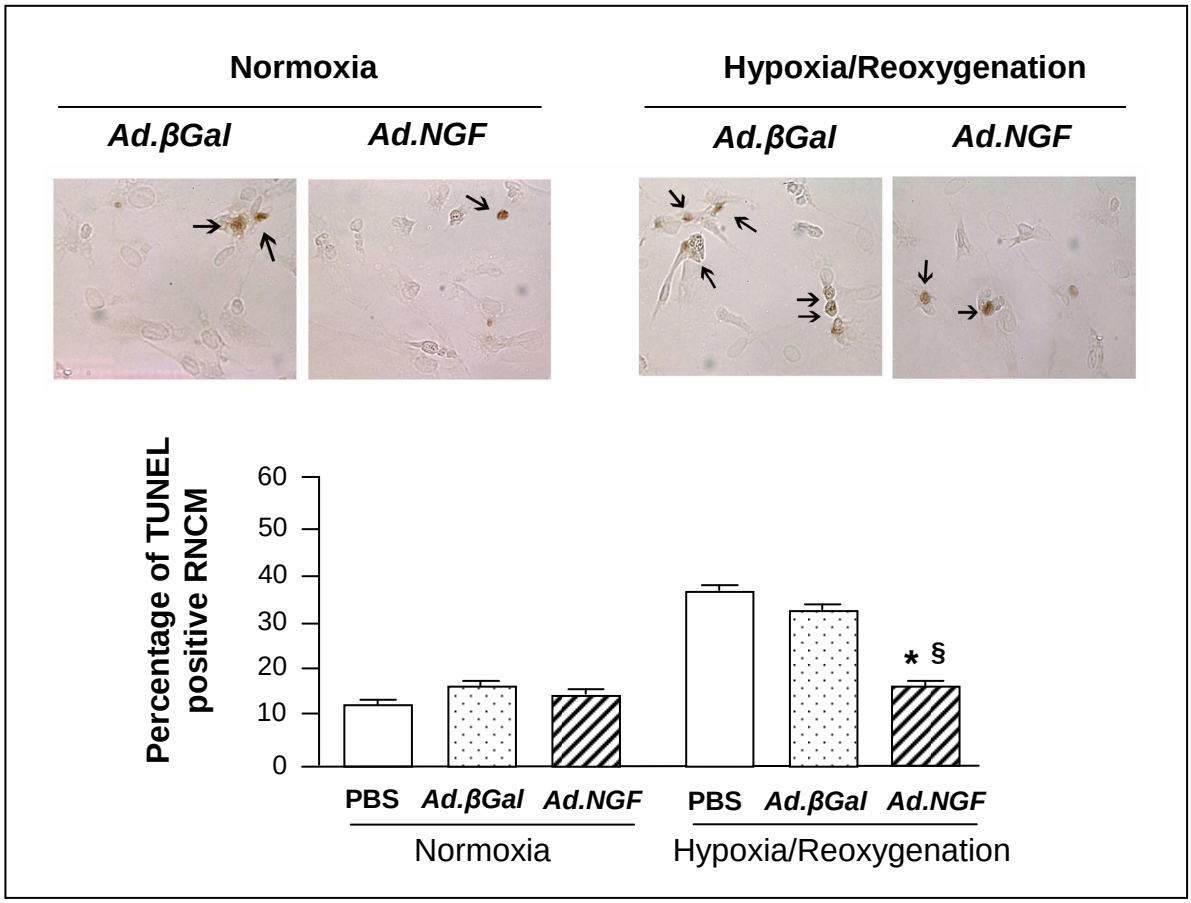


Figure 22. NGF over-expression prevents apoptosis of RNCM after hypoxia/reoxygenation

Apoptosis on RNCM transduced with *Ad.NGF* or *Ad.βGal* (PBS was used as control) and submitted to 12h hypoxia followed by 24h reoxygenation were also analyzed after TUNEL assay. In the representative images, TUNEL-positive RNCM are pointed by the arrows. Bar graph quantifies apoptosis, which is expressed as percentage of TUNEL-positive RNCM. Data are presented as mean±SEM (n=3). *P<0.05 vs. PBS; §P<0.05 vs. *Ad.βGal*.

The anti-apoptotic action of *Ad.NGF* was also observed when apoptosis was induced by Angiotensin II (Figure 23).

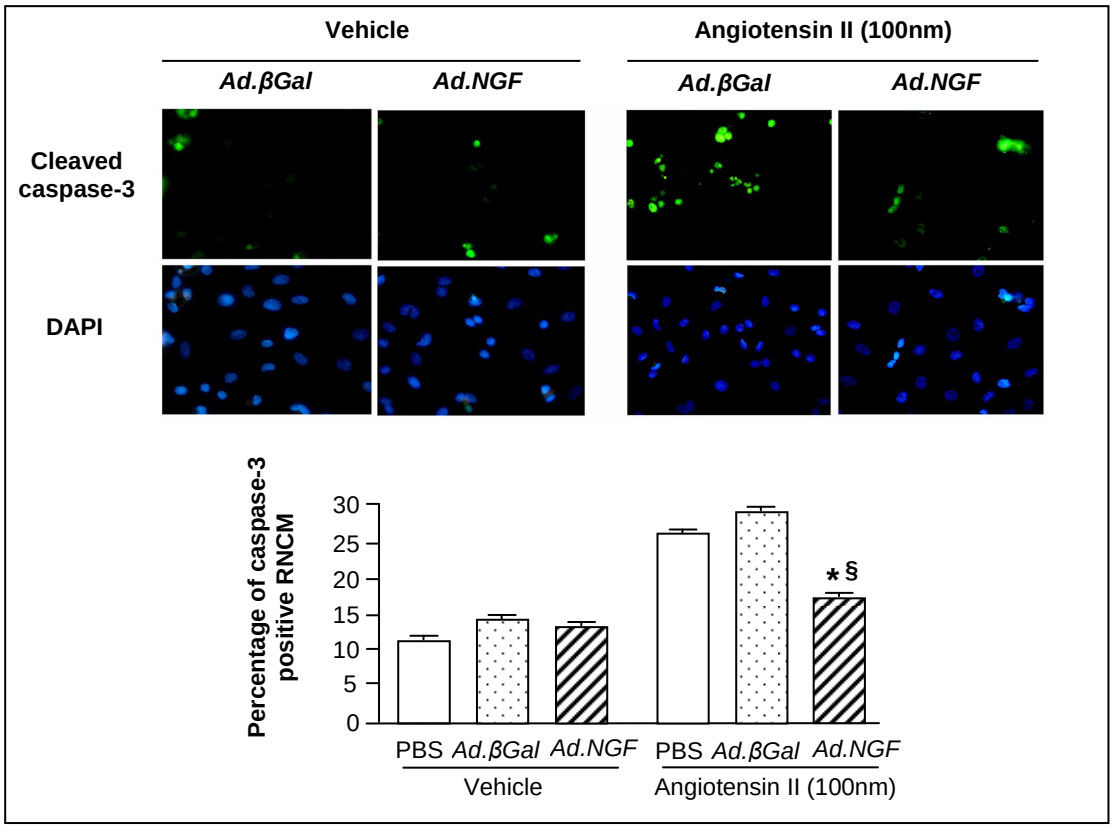


Figure 23. NGF over-expression prevents Angiotensin II-induced apoptosis
After transduction with *Ad.NGF* or *Ad.βGal* RNCM were submitted to Angiotensin II apoptotic assay. Apoptotic cells were detected by cleaved caspase-3 fluorescence staining (green fluorescence). Nuclei were revealed by DAPI (blue fluorescence). Bar graphs show the percentage of RNCM positive for caspase-3. Data are presented as mean±SEM (n=3). *P<0.05 vs. PBS; §P<0.05 vs. *Ad.βGal*.

NGF induces human EPC migration and proliferation

Human EPC migration towards NGF was determined by using a modified Boyden chamber. Figure 24 shows that NGF significantly increases the migration of EPC compared with control. No significantly differences were found considering the three doses of recombinant NGF.

Those results show that human endothelial progenitor cell of peripheral blood origin migrate following exposure to an NGF gradient.

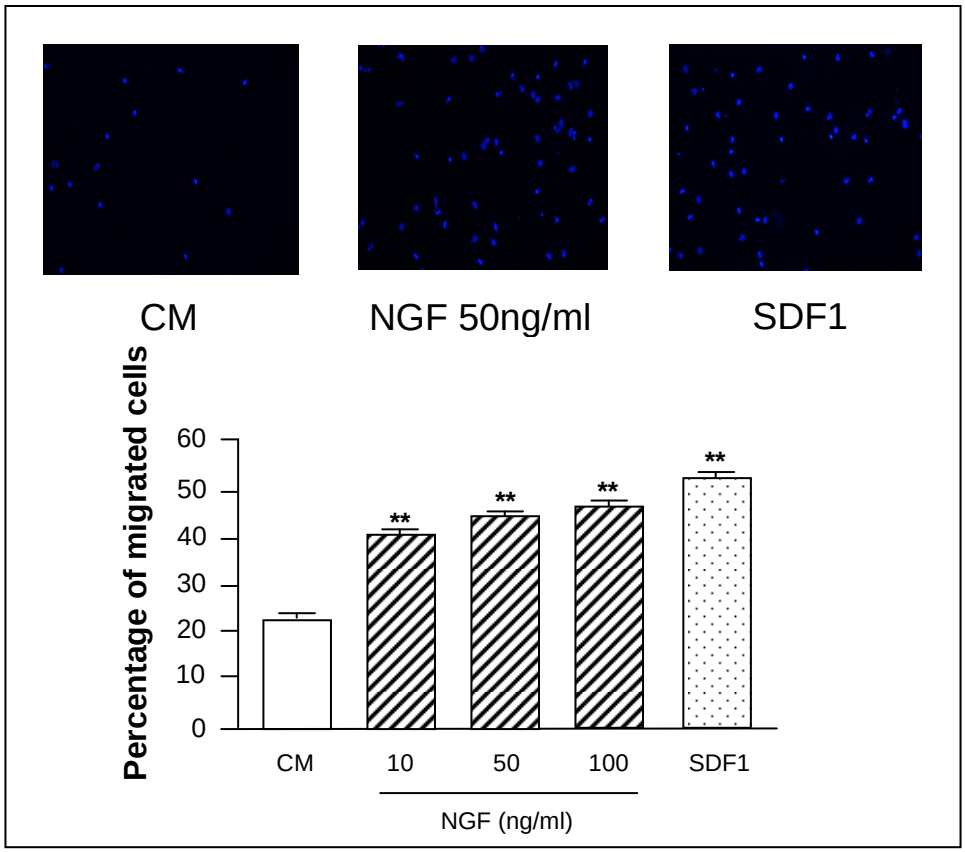


Figure 24. NGF induces EPC migration
EPC migration following stimulation by NGF. Migration studies were conducted in a modified Boyden chamber incubating cells with NGF (10ng/ml, 50ng/ml and 100ng/ml), SDF-1 (100ng/ml) or control medium alone (CM). The fluorescence imaging panels show the DAPI positive cells after migration. EPC migration towards NGF is represented at the dose of 50ng/ml of NGF. The diagram shows the mean (± SEM) number of migrated cells counted in three independent experiments (n=3). **P<0.01 vs. CM.

To further elucidate the biological significance of NGF for endothelial progenitor cells, EPC proliferation in response to NGF has been analysed by an MTS colorimetric assay.

NGF stimulation resulted in a promotion of EPC proliferation compared to control. As shown in Figure 25, the lowest dose of NGF (5ng/ml) induced a significant elevation of EPC viability compared to control medium, while NGF at higher doses did not result in an improvement of cells proliferation.

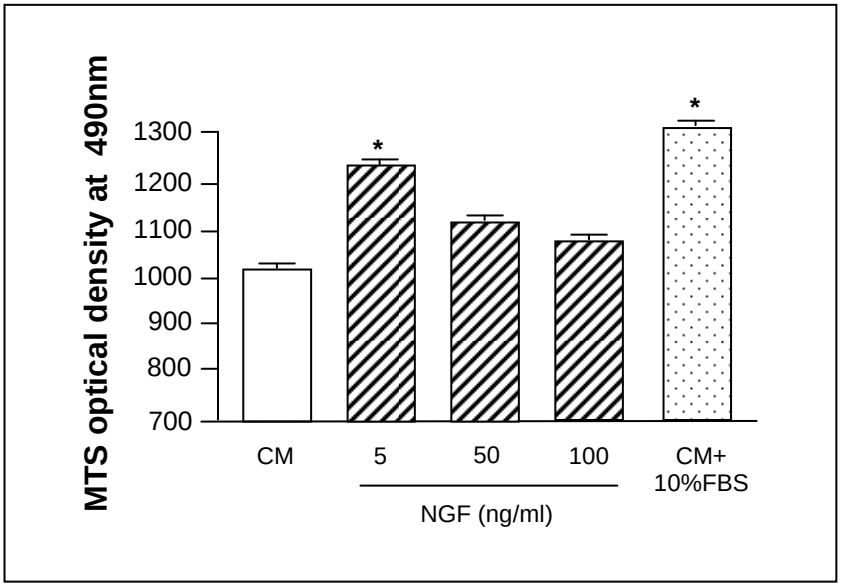


Figure 25. EPC proliferation is induced by NGF
EPC proliferation was studied by MTS Assay. EPC were cultured for 24h with different doses of NGF (5ng/mL, 50ng/mL and 100ng/mL).
Control medium (CM) with 10%FBS was used as positive control. Results are expressed in optical density, which was measured at 490nm. *P<0.05 vs. CM.

Taken together, these data show that NGF is not only involved in angiogenic process but, since it is able to improve EPC migration and proliferation, can also have an impact on vasculogenesis.

Discussion

Therapeutic angiogenesis is a promising strategy for the cure of ischemic heart diseases. The goal of this therapy is to build up sufficient vessels to compensate for decreased blood flow in ischemic tissues.

Angiogenic therapy has been carried out in laboratory animals for more than a decade and a wealth of evidence has shown that several growth factors can induce neovascularisation in ischemic models.[46, 47]

Furthermore, some controlled clinical studies have been carried out using angiogenic factors, especially VEGF-A.[49, 112]

The most commonly used modalities for growth factors delivery are plasmids or adenoviral vectors. Although the optimal application of growth factors and delivery method require further exploration, research in this field showed sufficient levels of safety and efficacy in the use of those vectors.[43, 113]

Recent studies have demonstrated the angiogenic properties of neurotrophins, and in particular NGF[57, 114] and BDNF.[115, 116] Although the functions of neurotrophins were initially considered to be limited at the central and peripheral nervous system, recent studies have revealed their potential for vascular biology.[57, 58, 60] For instance, our group discovered that NGF is able to promote neovascularisation in a murine model of peripheral ischemia and diabetic skin wounds,[57, 59, 60] describing the involvement of protein kinase B-Akt, VEGF-A and nitric oxide in NGF-induced angiogenesis.[57] We also showed that NGF is able to protect endothelial cells and skeletal myocytes from apoptosis.[59]

Based on these data, the aim of my PhD studies was to evaluate the possible therapeutic effect of NGF in a mouse model of myocardial infarction. We investigated whether NGF induces reparative neovascularisation in MI, and promotes the survival of cardiomyocytes and endothelial cells. Finally, we also analyzed the impact of NGF on EPC migration and proliferation, two mechanisms which are relevant for vasculogenesis.

Our study documents that local intra-myocardial NGF gene delivery in the peri-infarct zone immediately after myocardial infarction stimulates capillary growth and reduces apoptosis of endothelial cells and cardiomyocytes.

Moreover, the pro-angiogenic and anti-apoptotic potential of NGF was associated with an increase in eNOS phosphorylation through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. This observation is in line with previous findings of Cantarella *et al.*[58] and our group,[57] which indicated the activation of eNOS by Akt in response to NGF. Collectively, these data suggest that NGF is able to restore the balance between pro-survival and pro-apoptotic factors in the infarcted heart.

These results reinforce the idea of a therapeutic potential of NGF, as improved neovascularisation may ameliorate the oxygenation of the infarcted myocardium, thus preventing further cardiomyocyte loss. Furthermore, we showed the up-regulation of the endogenous NGF and its TrkA receptor in the infarcted heart, and this is in agreement with what previously reported by Hiltunen *et al.*[117] in a rat model of coronary ischemia/reperfusion and also with our results in ischemic hind-limb muscle.[57] The finding that NGF expression level increases after myocardial infarction suggests that

this system may be involved in the spontaneous reparative responses developed by the infarcted heart.

We also analyzed heart remodeling at five weeks post-myocardial infarction after NGF gene delivery. *p.NGF* reduced the left ventricle chamber volume, thus suggesting that NGF may prevent dilatation of the infarcted LV and, through this action, slow the progression versus heart failure. By contrast, indexes of cardiac function (measured by the Millar catheter system) were not different between groups.

To better understand the pro-survival and anti-apoptotic features of NGF on the cardiomyocyte, we analysed its role in rat neonatal cardiomyocytes *in vitro*.

Interestingly, neonatal cardiomyocytes express TrkA and release active NGF, which stimulates the phosphorylation of Akt leading to the phosphorylation and inactivation of Foxo-3a and Foxo-1 in the same cells, a result which is in accordance with the possibility that NGF may exert an autocrine control on cardiomyocyte survival. Moreover, the use of the TrkA inhibitor K252a or the PI3K inhibitor LY294002 could demonstrate the importance of TrkA and Akt in the regulation of the pro-survival and anti-apoptotic properties of NGF, thus strongly suggesting that the NGF/TrkA/Akt/Foxo pathway may physiologically control cardiomyocyte survival.

Afterwards, the pro-survival action of NGF was also demonstrated in hypoxia/reoxygenation and Angiotensin II apoptosis assays. In RNCM, NGF over-expression after gene transfer showed

the capacity of the neurotrophin to combat apoptosis induced by those pro-apoptotic conditions.

Taken together, these data indicate that NGF improved resistance of cardiomyocytes to pro-apoptotic stimuli.

Additionally, the fact that NGF was active in cardiomyocytes from both rats and mice discards the possibility that the observed actions are species-specific, thus raising hope that NGF may be also useful for combating human cardiac diseases.

Finally, since bone marrow-derived EPC have been shown to regenerate the myocardium by enhancing neovascularisation, we also investigated the influence of recombinant NGF on cultured EPC migration and proliferation. Adult bone marrow is a rich reservoir for endothelial and hematopoietic stem and progenitor cells that contribute to revascularization of injured tissue.

Many experimental studies proved that vascular regeneration and angiogenesis require migration of various cells, and that both migration and proliferation of endothelial cells and EPC, among many other factors, contribute to angiogenesis.

We found that both migration and proliferation of EPC were improved after NGF stimulation, thus suggesting that NGF may be able to promote the expansion of EPC and their mobilization and homing to ischemic tissues, thus ultimately favouring the hypothesis that NGF may positively impact on vasculogenesis.

In conclusion, our results suggest that intra-myocardial NGF induces reparative neovascularisation by promoting both angiogenesis and vasculogenesis and by inhibiting the apoptotic death of resident endothelial cells.

In perspective, NGF may represent a potential tool to induce therapeutic angiogenesis in the setting of myocardial infarction.

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