Enhancing Repair of the Mammalian Heart

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Abstract—The injured mammalian heart is particularly susceptible to tissue deterioration, scarring, and loss of contractile function in response to trauma or sustained disease. We tested the ability of a locally acting insulin-like growth factor-1 isoform (mIGF-1) to recover heart functionality, expressing the transgene in the mouse myocardium to exclude endocrine effects on other tissues. Supplemental mIGF-1 expression did not perturb normal cardiac growth and physiology. Restoration of cardiac function in post-infarct mIGF-1 transgenic mice was facilitated by modulation of the inflammatory response and increased antiapoptotic signaling. mIGF-1 ventricular tissue exhibited increased proliferative activity several weeks after infusion. The canonical signaling pathway involving Akt, mTOR, and p70S6 kinase was not induced in mIGF-1 hearts, which instead activated alternate PDK1 and SGK1 signaling intermediates. The robust response achieved with the mIGF-1 isoform provides a mechanistic basis for clinically feasible therapeutic strategies for improving the outcome of heart disease. (Circ Res. 2007;100:1732-1740.)

Key Words: cardiac muscle ■ insulin-like growth factor-1 ■ regeneration ■ wound healing

The insulin/insulin-like growth factor signaling pathway arose early in the evolution and is highly conserved among invertebrates and vertebrates. Mammalian IGF-1 acts predominantly as a growth, survival, and differentiation factor. The pleiotropic functions of IGF-1 are reflected in the complicated structure and regulation of Igf-1 gene. The products include variable amino-terminal signal peptides and different carboxy-terminal E peptides, the precise function of which is still unclear. Injury of mammalian tissues induces transient production of locally acting IGF-1 isoforms that control growth, survival, and differentiation. By contrast, high levels of circulating IGF-1, produced by the liver, has been implicated in the restriction of lifespan and predisposition to neoplasia.

When expressed as transgenes, different IGF-1 isoforms have contrasting effects on the mouse heart. Transgenic mice generated with a minor human IGF-1 cDNA (IGF-1Eh) under the control of the rat α-myosin heavy chain (α-MHC) promoter showed no striking differences in size and cell volume when compared with control mice, but harbored an increased number of cardiomyocytes, coupling IGF-1 overexpression with myocyte proliferation. The hearts of these animals responded to coronary ligation with attenuated diastolic wall stress, cardiac weight, ventricular dilatation, and hypertrophy, attributable mainly to a prevention of cardiac cell death. In another report, cardiac expression of a modified human IGF-1 cDNA produced no hyperplasia but instead induced physiologic, then pathologic, cardiac hypertrophy in transgenic mice.

Here, we have used the mIGF-1 isoform, comprising a Class I signal peptide and a C-terminal Ea extension peptide. This isoform is expressed at high levels in neonatal tissues and in the adult liver, but decreases during aging in extrahepatic tissues, where its expression is activated transiently in response to local damage. The regenerative properties of the mIGF-1 isoform and its dramatic promotion of cell survival and renewal in senescent muscle have been extensively documented, making it an attractive candidate for possible enhancement of cell-based regenerative therapies in injured postmitotic organs. We tested the potential of the mIGF-1 isoform to repair the injured heart and analyzed the signaling pathways induced by transgenic mIGF-1 under physiological and pathological conditions. By challenging the hearts of mice carrying a cardiac-restricted mIGF-1 transgene with 2 different modes of injury, we show that this isoform restores cardiac function by blocking scar formation and enabling myocardial reconstruction.

Materials and Methods

An expanded Materials and Methods section can be found in the online supplement, available at http://circres.ahajournals.org.
Generation of α-MHC/mIGF-1 Transgenic Mice

Transgenic FVB mice carrying a rat mIGF-1 cDNA driven by the mouse α-MyHC promoter were generated by standard methods and selected by positive PCR analysis of tail DNA. αMyHC/mIGF-1 transgenic mice were maintained as heterozygotes. All animals were housed in a temperature-controlled (22°C) room with a 12:12 hour light-dark cycle. All analyses were performed on male mice.

Isolation of Cardiac Cells

Adult mouse cardiomyocytes were isolated and cultured following the instructions of www.signaling-gateway.org.

Statistics

All comparisons between WT and TG mice were performed by means of paired Student t tests. A significant difference was considered when P<0.05, set as a double side value.

Results

The mIGF-1 Isoform Accelerates Cardiac Growth

We generated transgenic mice with a rat mIGF-1 cDNA driven by the mouse α-myosin heavy chain (α-MHC) promoter to restrict expression of mIGF-1 to the mouse myocardium and exclude possible endocrine effects on other tissues (Figure 1A). Cardiac-restricted mIGF-1 expression levels increased with age in all founders (F010, F022, and F018) tested, and reached a steady level at 1 month (Figure 1B). Expression of the α-MHC/mIGF-1 transgene in adult mice was restricted to the heart (Figure 1C) and was undetectable in other tissues using the rat IGF-1 probe. A single transgenic line was selected for further analysis (F018).

Postnatal 2-month mIGF-1 transgenic hearts displayed accelerated cardiomyocyte hypertrophy, precociously attaining wild-type adult heart size (Figure 1D), attributable to a significant increase in cell size compared with wild-type hearts (Figure 1E and supplemental Figure I). Importantly, analysis of cardiomyocytes cross-sectional area (CSA) showed that cells overexpressing mIGF-1 had a comparable size at 2 and 4 months (supplemental Figure I), indicating that heart growth was no longer induced by transgene overexpression. Cardiac hypertrophy was related to higher expression levels of ANP at 1 and 2 months, without any further significant change (Figure 1F). Other markers underlining cardiac hypertrophy, such as BNP, α-skeletal actin, α-myosin heavy chain, and glutamate transporter 1, were not affected (data not shown). Assessment of cardiac function by echocardiography at 4 months showed that mIGF-1 induced a 20% concentric left ventricular hypertrophy (supplemental Table I). In transgenic male mice, echocardiography identified a small but significant decrease in cardiac contractility (13% decrease in ejection fraction [EF] and fractional shortening [FS]). Mildly compromised diastolic function was identified by the 21% decrease of the E/A ratio and the prolongation of the A wave duration (+14%). Nevertheless, cardiac output and chamber diameters remained at normal levels throughout postnatal stages (supplemental Table I).
The mIGF-1 Transgene Promotes Effective Myocardial Repair and Functional Restoration

The restorative capacity of mIGF-1 transgenic hearts was analyzed by ligation of the left coronary artery (LCA) of 4-month-old mice. In wild-type LCA induced infarcts characterized by progressive and extended fibrotic tissue formation (Figure 2A, upper panel), accompanied by functional impairment after 1 month that worsened after 2 months (Figure 2B and supplemental Figure IIA). Percent values of FS and EF were significantly decreased compared with sham operated mice (supplemental Table II and Figure IIB). In contrast, infarcted mIGF-1 transgenic hearts showed a moderate but significant decrease in the percentage of EF and FS after 1 month, with no significant changes after 2 months compared with mIGF-1 transgenic sham-operated mice and wild-type ligated mice (Figure 2B, supplemental Figure IIA and Table II). The mIGF-1–mediated blockade of the normal progressive impairment in infarcted heart function was accompanied by reduced scar formation (Figure 2A, lower panel), and a significantly smaller infarct size compared with control hearts (supplemental Figure IIC, lower panel). Recovery of cardiac function as well as morphological restoration of infarcted mIGF-1 transgenic hearts was confirmed by normal left ventricular motion in systolic and diastolic phases (supplemental Movie II) compared with mIGF-1 transgenic uninjured hearts (supplemental Movie I). In contrast, wild-type hearts (supplemental Movie IV) presented chamber enlargement and a significant decrease in wall motility near the infarct, when compared with uninjured wild-type hearts (supplemental Movie III).

An alternate injury method involving direct cardiotoxin (CTX) injection into the cardiac left ventricle wall produced similar results to those obtained with LCA, although the CTX model produces a well-delineated transmural lesion and reduces the risk of ventricular fibrillation. This technique was used in subsequent experiments. Single CTX injections in 4-month wild-type and mIGF-1 transgenic hearts induced reproducible, small, and localized damage with evident cell death and marked inflammation after 48 hours and 1 week (Figure 2C). To test whether CTX injection reduced the variability of the extent of myocardial injury normally inherent to LCA models, we injected mice with Evans Blue dye (EBD), as vital stain of myocytes permeability and membrane-associated fragility (supplemental Figure IIIA). We found an equivalent increase in myocardial damage in wild-type and mIGF-1 hearts, indicating that the extent of initial injury is similar in both animals tested (supplemental Figure IIIIB). In contrast to the characteristic progression of scar formation in wild-type hearts after 1 month (Figure 2C), mIGF-1 transgene expression induced repair of the injured tissue with minimal scar formation (Figure 2C), and a significant reduction of infarct size (supplemental Figure IIC, upper panel).

Although the overall cardiac function of wild-type and transgenic injured hearts was not dramatically affected by a single shot of CTX injection, analysis of mean values of EF with TG hearts (P<0.05). E, Real time PCR of mIGF-1 transcript in physiological conditions and 24 hours after CTX injection, using IGF-1Ea Taqman probe (Applied Biosysm). PCR values were normalized for GAPDH content in each sample. Asterisk (*) indicates significant increasing values compared with WT uninjured hearts, whereas § represents significant decreasing values compared with TG uninjured hearts.

![Figure 2. Enhanced cardiac repair and functions in mIGF-1 transgenic mice after myocardial infarction. A, Whole mount and histological analysis of sham-operated control (WT) heart (left) and LCA WT and TG hearts (right) 2 months after operation. Arrows indicate fibrotic tissue. LA indicates left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. Histological analysis by trichrome staining is shown for each treatment. B, Functional recovery of mIGF-1 transgenic mice after LCA. Mean percentage values of FS (upper panel) and EF (lower panel) are representative of 3 readings on each animal and averaged among groups. Asterisks (*) show significant values (P<0.05) between uninjured and injured hearts in WT (yellow square) and TG (red square) mice. § shows significant values between WT and TG injured hearts. C, Histological analysis (trichrome) of WT and TG hearts at 48 hours, 1 week, and 1 month after CTX injection in the left ventricular wall. Comparable results were obtained with similar analyses on 6 different groups. D, Functional recovery of mIGF-1 transgenic mice 1 month after CTX injection. Mean percentage values are representative of 3 readings on each animal and averaged among groups. E, Real time PCR of mIGF-1 transcript in physiological conditions and 24 hours after CTX injection, using IGF-1Ea Taqman probe (Applied Biosystem). PCR values were normalized for GAPDH content in each sample. Asterisk (*) indicates significant increasing values compared with WT uninjured hearts, whereas § represents significant decreasing values compared with TG uninjured hearts.](http://circres.ahajournals.org/cover)
and FS by high-resolution echocardiography were moderately but significantly impaired in wild-type hearts when compared with transgenic hearts (EF 61% ± 7% compared with 78% ± 3%; FS 34% ± 4% compared with 47% ± 3%; Figure 2D and supplemental Figure IIB), indicating that mIGF-1 induced both morphological and functional repair.

Variations in the level of IGF-1 transcript induced by CTX injection in both wild-type and transgenic hearts were evaluated by real time PCR with a Taqman probe recognizing both endogenous mouse IGF-1Ea and the rat transgene (Figure 2E). mIGF-1 is 35-fold higher in transgenic hearts compared with wild-type hearts. After CTX injection (24 hours), the level of the transgene significantly decreased (30%), likely attributable to CTX-induced downregulation of the mouse αMHC promoter (Figure 2E). Interestingly, this IGF-1 isoform increased significantly in wild-type hearts after CTX injection (19%), as reported in previous analysis.9

mIGF-1 Mediates Heart Repair by Modulation of the Inflammatory Response

The early events characterizing postmyocardial infarction include complement activation, free radical generation, chemokine upregulation, and activation of cytokine cascades.13 Real time PCR analysis showed that at 24 hours after CTX injection, proinflammatory IL6 and IL1β transcript levels moderately increased or remained unchanged, respectively, in transgenic hearts (Figure 3A). Contrarily, wild-type hearts showed a significant increase of both interleukins (Figure 3A). Other cytokines involved in the inflammatory response, such as IL12A and B, IFNγ, TGFβ, and MCP1 were not affected in wild-type and mIGF-1 transgenic hearts by CTX-induced injury (supplemental Figure IV), highlighting a selective role for certain cytokines in the injured heart. In contrast, antiinflammatory IL4 transcript levels were downregulated in wild-type hearts after CTX injection (42% after 24 hours, and 32% after 1 week), but were significantly increased in transgenic hearts 1 week after injury (50% compared with wild-type; Figure 3B, left panel). Transcripts encoding IL10, another antiinflammatory cytokine that suppresses injury and blocks scar formation,14 were rapidly increased in mIGF-1 transgenic hearts 24 hours after injury (90% compared with wild-type), and to a greater extent at 1 week (113% compared with wild-type; Figure 3B, right panel). Interestingly, the Cdk inhibitor p21WAF1/CIP1 was upregulated up to 1 week after injury in the mIGF-1 transgenic hearts (Figure 3C), opening a novel and so far unexpected role for this Cdk inhibitor in the initial stages of cardiac repair induced by mIGF-1.

mIGF-1 Activates Survival Signaling Pathway in Myocardial Repair

Many of the cellular growth responses attributed to IGF-1 are mediated by activation of the PI3K/Akt/mTOR phosphorylation cascade, leading to upregulation of the translational machinery15 and to cardiac prosurvival signaling in vivo.16 Notably, Akt acts as protective agent in cardiomyocyte survival and function,17,18 although its activation may not be sufficient for long-term cardioprotection and may even have adverse chronic effects.19

To dissect the intracellular signaling induced by supplemental mIGF-1, we performed phosphoprotein profiling on wild-type and mIGF-1 transgenic heart lysates. As seen in supplemental Table III, the canonical Akt phosphorylation cascade was not activated in mIGF-1 transgenic hearts, and phosphorylation levels of downstream mTOR and p70S6K intermediates were downregulated and unaffected, respectively, with wild-type littermates. Nevertheless, mIGF-1 transgenic expression sustained S6 ribosomal protein phosphorylation at 2, 4, and 6 months compared with the wild-type hearts (Figure 4A). The relative activation of S6 in
mIGF-1 transgenic hearts appeared to be independent of canonical Akt signaling, which decreased postnatally in both wild-type and mIGF-1 transgenic hearts (Figure 4B and supplemental Table III).

PI3K also signals through PDK1, a master regulator of the AGC kinase family. PDK1 can directly phosphorylate p70S6K independently of Akt, and promotes cell survival through other intermediates, such as SGK1, a P13K-dependent kinase that is highly expressed in the heart and promotes survival in cardiomyocytes. In the heart, PDK1 regulates glucose uptake and glycogen synthase through Akt and GSK3α/β intermediates. Increased levels of phosphorylated PDK1 were present in mIGF-1 transgenic hearts, although no corresponding increase in GSK3α or GSK3β activation was detected (supplemental Table III). Coimmunoprecipitation analysis showed that PDK1 complexed with SGK1 in mIGF-1 transgenic hearts, whereas in wild-type hearts this interaction was much less apparent (Figure 4C). No interaction between PDK1 and any of the Akt isoforms was detected in mIGF-1 transgenic hearts (Figure 4D), indicating that the cardiac signaling cascade induced by mIGF-1 is independent of Akt and p70S6K, and preferentially uses the PDK1/SGK1 pathway to increase protein synthesis and growth.

To determine whether the activation of the translational machinery and the interaction between PDK1 and SGK1 observed in physiological conditions were modulated or shifted to an Akt-dependent pathway in response to injury, we analyzed S6 ribosomal protein phosphorylation levels, as well as potential interactions between PDK1 and Akt or SGK1, in regenerating mIGF-1 hearts (Figure 4E through 4G). Phosphorylation of S6 was initially more pronounced in wild-type hearts 24 hours after injury, but by 1 week was higher in mIGF-1 transgenic hearts (Figure 4E), indicating the persistence of protein synthesis. As in uninjured mIGF-1 transgenic hearts, PDK1 was found complexed with SGK1 after CTX injury (Figure 4G) but not with any Akt isoform (Figure 4F), indicating that in both physiological and pathological conditions mIGF1 signals specifically through a PDK1/SGK1 intermediate pathway.

To test whether the PDK1-SGK1 signaling cascade in mIGF-1 transgenic hearts increased survival pathways, we analyzed the presence of TUNEL-positive nuclei around the area of injury 1 week after CTX injection. The amount of TUNEL-positive cells (Figure 5A, upper and lower panels and Figure 5B) increased significantly in wild-type hearts compared with mIGF-1 transgenic hearts. Expression levels of the proapoptotic proteins Bax and Bcl-xL were not affected (Figure 5C), although their activation by mitochondrial membrane translocation cannot be excluded.

To better elucidate the survival signaling regulated by mIGF-1 on cardiac damage we performed Affymetrix analysis 24 hours after CTX injection (supplemental Figure VA through VC). A specific gene expression profile, verified also by real time PCR (Figure 5D), revealed increased levels of the mitochondrial protein UCP1 (uncoupling protein 1) in transgenic hearts compared with injured wild-type hearts (Figure 5E). Interestingly, UCP1 transcripts are highly expressed in TG hearts in physiological conditions (Figure 5D), indicating that the transgene regulates UCP1 independently from cardiac damage.

**Figure 4.** mIGF-1 induces interaction of PDK1 with SGK1 but not with Akt to phosphorylate S6. A and B, Western blot analysis of Akt and S6 ribosomal protein phosphorylation. Each analysis is representative of 3 independent experiments with no significant variation (data not shown). C and D, IP-Western analysis of PDK1 interaction with SGK1 and Akt isoforms. No PDK1/Akt interaction was observed. TTE indicates total tissue extract. Additional bands present in Akt1 and Akt2 blots are attributable to antibody cross-reaction and to nonspecific interaction with IgG. E, Western blot analysis of S6 ribosomal protein phosphorylation after CTX cardiac injury. Note persistence of pS6 levels in TG hearts at 1 week after injury. F and G, IP-Western analysis of PDK1 interaction with SGK1 and Akt isoforms after CTX cardiac injury. No PDK1/Akt interaction was observed.
Increased Cell Proliferation in mIGF-1 Transgenic Hearts

To test whether mIGF-1 could induce a proliferative response on CTX injection, we assessed nuclear incorporation of continuously administered bromodeoxyuridine (BrDU), a marker of DNA synthesis, 48 hours, 1 week, and 1 month after CTX injection. No differences in BrDU incorporation were found in injured hearts 48 hours and 1 week after CTX injection (Figure 6B). At 1 month after infarct induction, however, total amount of BrDu-positive cells in the infarct border zone of the mIGF-1 hearts were significantly higher (35% compared with WT values) than in the wild-type hearts (Figure 6A and 6B), although a time-dependent increase of BrdU-positive nuclei has been observed in both wild-type and mIGF-1 transgenic hearts (Figure 6B). Frequent incorporation of BrdU in cardiomyocyte nuclei was seen in both cardiac tissue and individual cells after injury (Figure 6C left and middle panels, D and F), although abundant nonmuscle cells of diverse morphologies were also labeled in the vessels and surrounding myocardial tissue of mIGF-1 transgenic hearts (Figure 6C right panel, E and F). Although the origin and fate of these proliferative cells is still under investigation, they likely contribute to mIGF-1–mediated cardiac recovery in response to tissue damage, contrarily to the BrdU-positive cells in wild-type hearts, which are probably unable to elicit tissue restoration in the unfavorable infarcted environment. Notably, cardiomyocytes isolated from injured wild-type and mIGF-1 transgenic hearts did not show signs of evident hypertrophy or polyploidy (Figure 6D), indicating that neither CTX injection neither mIGF-1 overexpression elicited a hypertrophic response to the viable myocardium (Figure 6D).

Discussion

In this study we have intervened in the normal signaling mechanisms at work in the cardiac repair process to increase the efficiency of mammalian morphological and functional tissue restoration. The beneficial effects of the mIGF-1 isoform have been previously documented in mammalian skeletal muscle, a tissue with considerable endogenous regenerative capacity. The present work extends these observations to the mammalian heart, and elucidates the molecular mechanisms whereby the mIGF-1 isoform improve cardiac function on tissue damage.

Despite shifts in signaling pathways accompanied by modest effects on morphology and hemodynamic parameters, continuous expression of mIGF-1 throughout postnatal life did not produce significant perturbations in normal heart physiology, and in contrast to previous studies with other IGF-1 transgenes did not progress to a pathological phenotype. In response to injury, however, the pathways activated by mIGF-1 were sufficient to restore form and function of damaged cardiac tissue. The program induced by mIGF-1 followed a sequential course (Figure 7), involving early resolution of inflammation at the site of injury to prevent scar formation and to make way for the subsequent tissue replacement. Interestingly, the mIGF-1 transgene activates p21, which has been reported to be important for IGF-1–mediated cell survival on UV irradiation and to suppress production of IL6 in rheumatoid arthritis. Notably, prolonging the initial induction of p21 in damaged cardiac tissue enhances DNA repair and genome stability, without precluding cell replacement. Modulating expression of these downstream effectors of inflammation is important for providing a conducive environment for cell replacement and tissue restoration.

The increased expression of antioxidant and survival transcripts, such as UCP1, adiponectin, and metallothionein 2 (Figure 5C and 5D and supplemental Figure V) during the first hours after CTX injection indicates that mIGF-1 overexpression protects the heart from toxins, generally produced in pathophysiological conditions. Interestingly, several stud-
ies have shown that UCP family members (UCP1, -2, and -3) are involved in decreasing ROS formation when overexpressed in pathological conditions. Our analysis for the first time showed that UCP1 is expressed in the infarcted hearts of mIGF-1 mice, most likely playing an important role in oxidant detoxification.

The novel intracellular signaling cascades set in motion by mIGF-1 isoform (Figure 7) presumably underlie the improved healing capacity conferred on the transgenic hearts. Under both physiological and pathological conditions, transgenic mIGF-1 upregulated S6 ribosomal protein activity through PDK1, an important downstream mediator of IGF1 signaling, rather than through Akt as previously reported for other IGF-1 isoforms. Thus, enhanced protein synthesis is likely to play a critical role during the regenerative process. Because this pathway does not involve the canonical activation of p70S6 kinase in the mIGF-1 transgenic hearts, other as yet unidentified mediators may come into play during cardiac growth and regenerative processes.

One promising candidate is SGK, which is implicated in different cell signaling leading to cell survival, apoptotic response, and osmoregulation. In C elegans, SGK functions in an insulin/IGF-I receptor–mediated signaling pathway to regulate metabolism, development, and longevity. In the heart, SGK1 is dynamically regulated and promotes cardiomyocyte survival. Decreased SGK1 phosphorylation in transgenic hearts expressing chronically active Akt provides further evidence for its independent signaling capacity. The fact that PDK1 interacts with SGK1 but with none of the Akt isoforms in mIGF-1 transgenic hearts suggests that it may be directly implicated in S6 ribosomal protein phosphorylation during physiological growth and in enhancing repair signaling. Alternatively, downstream mediators of PDK1/SGK1 interaction, such as the forkhead family member FKHRL1, the B-Raf kinase, and the sodium channels, may mediate more complex pathways in mIGF-1 response.

The delayed cell proliferative response seen in mIGF-1 transgenic hearts stands in contrast to the effects of direct myocardial injection of fully processed IGF-1 protein, which rapidly induces the appearance of small new myocytes within the infarct at 1 to 2 days after coronary ligation. In that system the maximum benefit required a dual stimulation by IGF-1 injection together with the chemotactic effects of coinjected hepatocyte growth factor. In the present study, full cardiac restoration without additional hepatocyte growth factor points either to a different mode of action used by an expressed IGF-1 transgene product or to a qualitative difference in the action of the mIGF-1 isoform itself. Interestingly, a recent study suggests that induction of IGF-1 may account for the favorable effect of Sonic Hedgehog gene therapy on recovery of myocardial ischemia. Elucidation of the roles played by native signal and E peptides in enhancing the beneficial response in
mIGF-1 transgenic hearts, without the requirement for additional growth factors, will inform the design of clinically feasible therapeutic strategies to counteract the normal fibrotic tissue formation and consequent cardiac functional impairment in heart disease.

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Disclosure

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References

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SUPPLEMENTAL MATERIAL AND METHODS

Cardiac injury

Cardiotoxin injury: 3-4 month-old wild-type and transgenic mice were anesthetized by Avertin injection (0.1ml/10g of a 2.5% solution). The tongue was retracted and a tracheal cannula (1.3 x 1mm, OD x ID, Harvard Apparatus) was inserted into the trachea. The cannula was attached to the mouse ventilator (Model 687, Harvard Apparatus) via the Y-shaped connector. Ventilation was performed with a tidal volume of 200 µl and a respiratory rate of 120/min. The chest cavity was opened in the left fourth intercostal space. The heart was exposed and 25 µl of CTX 10 µmol/L (Latoxan) were injected in the heart wall of the left ventricle. The chest cavity, muscle, and skin were then closed by a 6-0 silk suture (Ethicon). Ligation: LCA was performed on avertin anesthetized mice as described above. Ventilation was performed with a tidal volume of 300 µl and a respiratory rate of 120/min. The chest cavity was opened in the left fourth intercostal space and the left coronary artery (LCA) was ligated with a 8.0 no absorbable suture (ethicon) below the left atrium to produce a 40% infarct size. The chest cavity, muscle, and skin were then closed by a 6-0 silk suture (Ethicon). Mice were kept under ventilation until they were completely awake from anesthetic.

Histological analysis

Mice at different ages and different treatment were anesthetized before cervical dislocation, and hearts were perfused with 4% paraformaldehyde (PFA), then excised and embedded in paraffin. Paraffin sections (10µm) were stained with hematoxylin and eosin and analyzed morphologically. Connective tissue was visualized by using Masson’s Trichrome stain as described by Manufacture (Sigma).
10 sections (10µm) from wild-type and transgenic left ventricles were used for cell size measurement. Cell size was analyzed by measuring the cross-sectional area (CSA) of cardiomyocytes with a centrally located nucleus. CSA was measured in 60 myocytes of 2 month and 4 month wild-type and transgenic hearts. The area was measured with MetaMorph™ (Universal Imaging Corporation), and statistical analysis was performed as described in the manuscript. The CSA was measured on three different animals in each group.

Infarct size was analyzed on Trichrome stained sections, as reported previously. The lengths of the infarcted surface (planimetry), comprising both epicardial and endocardial regions, and of the total left ventricle, were measured in each section with MetaMorph™ (Universal Imaging Corporation). Infarct size was expressed as percent of total left ventricular area in eight wild-type and transgenic mice that received CTX injection or LCA. The analysis was performed 1 month after CTX injection and 2 months after LCA.

**Immunohistochemistry and BrdU analysis**

BrdU (Sigma) was administered *ad libitum* at 0.1% in the drinking water each 24 hour for a month or injected intraperitoneally at 100µg/g each 24 hours for a week. Hearts were perfused with 4% PFA and embedded in paraffin. Sections were stained with anti-BrdU (BD-Pharmingen) as prescribed by the Manufacture. Positive nuclei were quantified by counting all nuclei and BrdU positive nuclei in 10 sections (10µm) of wild-type and transgenic hearts bordering and covering the CTX injured side. Statistical analysis was performed as in the manuscript. Immunofluorescence was performed on frozen sections (10 µm) of wild-type and transgenic hearts 1 month after CTX injection. BrdU was analyzed with a mouse anti-BrdU purchased from Amersham Biosciences, and cardiac muscle cells were stained with an anti-myosin antibody from Sigma (M7648). Nuclei were visualized by Hoechst dye (Sigma). Images were processed with a Leica DM RHC fluorescent microscope and a DC500 Digital Camera.
Echocardiography

Eight 13 and 23 week-old males from wild-type and transgenic lines were weighed and lightly anaesthetized with pentobarbital (30mg/kg i.p.) to allow analysis of cardiac anatomy and function on a Sonos 5500 (Hewlett Packard) with a 15MHz linear transducer (15L6) (Philips Ultrasound, USA). The images were stored in a digital format on a magnetic optical disk for review and analysis. The left hemithorax was shaved and an ultrasound transmission gel was applied to the precordium. The heart was first imaged in the two-dimensional mode (2D) in the parasternal long-axis view to obtain the aortic root dimensions. The aortic flow velocity and the heart rate (HR) were measured with pulsed-wave Doppler on the same section. The sample volume cursor was placed in the aortic root and the transducer angled slightly, which allowed aortic flow parallel to the interrogation beam so that maximum aortic flow velocity was obtained easily. The cardiac output (CO) was calculated from the following equation: $CO=0.785xD^2xVTIxHR$ where $D$ is the internal diameter of the aortic root and $VTI$ is the velocity-time integral of the Doppler aortic spectrum. Then the pulsed Doppler window was placed between the tip of the mitral valve leaflets to record the mitral inflow velocities. The maximal speed of the early (E) and late (A) mitral filling were measured as well as the mean deceleration time of the E wave (DT) and the duration of the A wave (Adur). By placing the Doppler between aortic flow and mitral valve, the isovolumetric relaxation (IVRT) time was measured. Left ventricular cross sectional internal diameters in end-diastole (LVEDD) and in end-systole (LVESD) were obtained by an M-mode analysis of a 2D-short axis view at the papillary muscle level. The ejection and shortening fractions were calculated. From this view, the diastolic septum (S) and posterior wall (PW) thicknesses were measured. The left ventricular mass (LVM) was calculated with the following formula: $LVM=1.055x[(S+PW+LVEDD)3-(LVEDD)3]$. All the measurements were performed on at least three beats, according to the guidelines of the American Society of Echocardiography.
Echocardiographic analysis of injured hearts

Eight 13 week-old males from wild-type and transgenic lines were analyzed by echocardiography 1 month after CTX injection in the left ventricle wall or after 1 month and 2 months after LCA ligation. The mice were weighed and lightly anaesthetized by Avertin injection (0.1ml/10g of a 2.5% solution). Cardiac anatomy and function were measured with a Vevo 660 (VisualSonics) Ultrasound, and by the use of a 630 RMV (real-time-micro-visualization) scanhead (Visualsonics). The analysis was very sensitive due to the high-resolution images that the VisualSonics Ultrasound can acquire. The left hemithorax was shaved and an ultrasound transmission gel (Parkers Laboratories Inc.) was applied to the precordium. The heart was imaged in the two-dimensional mode (2D) in the parasternal short-axis view to obtain left ventricular cross sectional internal diameters in end-diastole (LVEDD) and in end-systole (LVESD) by an M-mode analysis. The ejection and shortening fractions were calculated. Movie recordings of left ventricle motion were analysed in B-mode and in parasternal short-axis (PSA). 300 different frames covering cycles of ventricular contraction and distension (systole and diastole) were recorded.

Western blot analysis and Co-immunoprecipitation

Hearts from wild-type and transgenic mice with or without CTX injection were excised and excess blood was removed by washing in PBS 1X. Hearts were lysed in buffer containing 20mM Tris-HCl (pH 8.0), 150mM NaCl, 5mM MgCl2, 10% glycerol, 1% Triton, 0.5% NP40, supplemented with 1mM proteases and phosphatase inhibitor cocktail. 50 μg of proteins were loaded onto 10% SDS-PAGE gel and blotted on PVDF membrane. Bax and Bcl-xL (Cell Signaling) were used at 1:500 in 5% milk. Phospho-Akt (Pharmingen) and Phospho-S6 (Cell Signaling) were used at a concentration of 1:500 and 1:1000 respectively in 5% BSA. Mouse monoclonal p21 antibody was purchased for Santa Cruz and used at a concentration of 1:250 in 5% milk. The blots were normalized for Akt (Transduction Laboratories), S6 ribosomal protein (Cell Signaling), actin (goat polyclonal, Santa Cruz) and tubulin (Sigma) at 1:1000. Co-immunoprecipitation was
performed using Seize X Protein A Immunoprecipitation Kit as prescribed by Manufacture (Pierce). The kit was used to avoid contamination of immunoglobulins during immunoprecipitation procedure. Briefly, wild-type and transgenic hearts with or without injury were lysed with a buffer containing 20mM Tris-HCl (pH 8.0), 150mM NaCl, 5mM MgCl2, 10% glycerol, 0.1% Triton, 1% NP40, supplemented with 1mM proteases and phosphatases inhibitor cocktail. Rabbit polyclonal PDK1 antibody was cross-linked to Protein A by incubation with disuccinimidyld suberate (DSS) as described by the Manufacture (Pierce). Four milligrams of lysates were gently rotating overnight at 4°C in a solution containing protein A and the immobilized PDK1 antibody (rabbit polyclonal from Cell Signaling). Proteins bound to PDK1 were eluted in different fractions as prescribed by the Manufacture. 40 µl of each fraction was loaded onto 8% SDS-PAGE gel and analysed with the sheep anti-human SGK1 antibody (generous gift of Dr. Philip Cohen) diluted at 5 µg/ml in 5% milk. Akt isoforms were analysed in the same immunoprecipitated lysates by Akt sampler antibody kit (Cell Signaling) at a concentration of 1:500 for each Akt isoform antibody in 5% milk. Co-immunoprecipitated SGK1 was found in the first fraction eluted.

Tunel assay

CTX was injected as described previously in the manuscript. One week after injections wild-type and transgenic hearts were excised and frozen in OCT. 10 sections bordering the injury side were analysed for each animal by Tunel assay as described by Manufacture (La Roche Inc.). Three different animals were analysed in both wild-type and transgenic. Percentage amount of Tunel-positive cells was quantified by counting all nuclei and Tunel-positive nuclei in 10 sections (10µm) of wild-type and transgenic hearts bordering and covering the CTX injured side, using a 40X magnification in a Leica DM RHC fluorescent microscope and a DC500 Digital Camera.
Cardiac injury extension

Analysis of cardiac injury extension was performed on three wild-type and three mIGF-1 transgenic mice after CTX injection into the left ventricle wall. 50µl Evans blue dye (10mg/ml) each 10g body weight was injected intraperitoneally (i.p.) 24 hours after injury. Hearts were harvested and processed for histological analysis of frozen sections 24 hours after dye injection. Total cross-sectional area (mm$^2$) was calculated in each section as well as the area of injury extension. The relatively low number of animal analyzed reflects the high mortality of FVB mice during LCA and CTX-induced cardiac damage. Wild-type and mIGF-1 trangenic injured areas were compared considering the same extension of total myocardial area.

Phosphoprotein analysis

All biochemical analysis was performed on myocardial tissue samples, and not from myocyte preparations. Hearts were excised and washed in PBS 1X to remove the excess of blood. Each gram of chopped tissue was lysated in 4 ml of lysis buffer as described by Manufacture (Kinexus, www.kinexus.ca/pdf/informationpackage.pdf). Analysis was performed at Kinexus Bioinformatics Corporation Suite (402, 6190 Agronomy Road Vancouver, British Columbia, Canada V6T 1Z3, www.kinexus.ca/corporate.html). Briefly, 500µg of total protein amount was used to perform the KPSS-4.1 phosphoprotein screen. The Kinetworks analysis involves resolution of a single lysate sample by SDS-PAGE and subsequent immunoblotting with panels of up to three primary antibodies per channel in a 20-lane Immunetics multiblotter. The trace quantity of the known protein from each screen type was compared against all relevant samples. The corrected data were obtained by trace quantity standardization for all samples.

Real Time PCR and Reverse Transcriptase PCR

1µg of RNA was used in reverse transcription reactions as prescribed by Manufacture (Promega). Real time PCR was performed using 10 µl of the Syber Green DynamoTM Master Mix
(Finnzymes, Espoo, Finland), along with 1µl of cDNA and 0.75µmol/L of each primer in a total reaction volume of 20 µl. Where indicated, Taqman real time PCR was performed as indicated by the Manufacture (Applied Biosystem). Duplicated samples were incubated at 95º for 3 min, followed by 45 cycles of amplification (95º, 10 sec; 56º, 20 sec; 72º, 30 sec). Results for each cytokine were normalized to GAPDH expression.

**Affymetrix Analysis**

CTX-injured wild-type and transgenic hearts were excised and washed in PBS 1X and immediately processed for RNA extraction by Trizol (Gibco-Brl). RNA was further purified by passage in mini columns as prescribed by the Manufacture (Quiagen). RNA was processed for Affymetrix GeneChip analysis by the GeneCore Facility at EMBL (EMBL, http://www.genecore.embl.de/), and a mouse 430A 2.0 chip was used for the analysis. The data were processed by GeneSpring.

**Primers**

Anti-inflammatory cytokines were detected with primers generously gift by Dr Sancho:
- IL10 forward 5’-ccaagccttateggaatg-3’ reverse 5’-tgccctgtacaccc-3’;
- IL4 forward 5’-catggcatatggaa-3’ reverse 5’-cgtttgacatcc-3’.
- ANP forward 5’-atgggtccttcatccacctctg-3’ reverse 5’-tgggtacggagcttgctgcagcc-3’;
- GAPDH forward 5’-tggaatakccacgagcc-3’ reverse 5’-ccaccttcgctcg-3’;
- β-actin forward 5’-taaaacgcagctcagtaacagtccg-3’ reverse 5’-tggaatctgtggcatccatgaa-3’;
- UCP1 forward 5’-caagtgcgctctcagcatcagaaggg-3’ reverse 5’-gtgtgatccacttcgctgtgtgt-3’.

Pro-inflammatory cytokines were detected with primers generously gift by Dr Sancho:
- MCP1 forward 5’-caccagcaagatgatcc-3’ reverse 5’-ataaagttgtagttctct-3’;
- TGFβ forward 5’-ccgaagcgactactat-3’ reverse 5’-gtaacgccaggaatgt-3’;
- INFγ forward 5’-tggtcgtgagtttacg-3’ reverse 5’-tcaagtggcatatgtggga-3’;
- IL12A forward 5’-cacagatgatggtaag-3’ reverse 5’-
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agttccagtggtaacagg-3’. IL12B forward 5’-gtcctcagaagctacca-3’, reverse 5’-cagagctatgacctc-3’;
Class 1 IGF-1Ea forward 5’-attaagatctgctctgtctt-3’, reverse 5’-ctttgtccctgcacttctact-3’ and
probe (FAM-labeled): 5’-tttacttcaacaagcc-3'; IL6, forward 5’-aggataacctcaacagaagct-3’,
reverse 5’-gtagctatgtgcaagacctc-3’; IL1β expression was determined using commercial
Taqman probe from Applied Biosystem.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Analysis of mIGF-1-induced hypertrophy
Hematoxylin and Eosin staining of wild-type (WT) and transgenic (TG) hearts at 2 (upper panels) and 4 months (lower panels). Cross-sectional (CSA) cardiomyocytes area significantly increased in WT hearts (§, p 6.46E-05) during adult heart development. At 4 months TG mice showed no further cell size increase compared to the heart of 2 month old mice (p 0.11). CSA increased significantly in TG myocytes at 2 (*, p 8.3E-12) and 4 months (*, p 0.0062) compared to WT myocytes. Values are the average of 6 independent analyses.

Supplemental Figure 2: Functional recovery of infarcted mIGF-1 TG hearts
(A) Representative echocardiographic recordings of sham operated and LCA heart function in WT (upper panels) and TG hearts (lower panels). The heart function of WT mice 2 months after LCA was dramatically impaired, confounding the reading and the recording in all animals tested (data not shown). (B) Representative echocardiographic recordings of sham-operated and CTX heart function in WT and TG hearts. (C) Infarct size measurement in eight WT and eight TG hearts 1 month after CTX injection (upper panel) and 2 months after LCA (lower panel). Infarct size was expressed as percent of total left ventricular area, as described in Supplemental Material and Methods. TG hearts showed a significant reduction of infarct size in both model of cardiac damage (*, p<0.05).

Supplemental Figure 3: Analysis of CTX-induced injury size in WT and mIGF-1 TG hearts
(A) Microscopic analysis of CTX-induced injury, with Evans Blue dye. Three CTX-injured WT and TG mice were injected with Evans Blue dye 24 hours after infarct induction. Hearts were harvested 48 hours later and frozen in OCT for further processing. (B) Measurement of injury size. Different sections were analysed for injury size, as described in Materials and Methods. Total myocardial area was measured together with extension of the injured side in different sections. Graph shows
that no significant differences in extent of injury were found between WT and TG animals at this early time point.

**Supplemental Figure 4: Analysis of additional inflammatory markers after CTX-induced damage of WT and mIGF-1 TG hearts**

Real time PCR analysis of the inflammatory markers interleukin 12 A and B (ILA, ILB), interferon gamma (IFN\(\gamma\)), monocyte chemoattractant protein 1 (MCP1), and transforming growth factor beta 1 (TGF\(\beta\)1) in WT (white bars) and TG (grey bars) hearts. Each cytokine value is normalized by GAPDH, and the fold increase is the average of three independent experiments.

**Supplemental Figure 5: Upregulation of antioxidant and antiapoptotic genes in mIGF-1 TG hearts after CTX induced-infarct**

A) Logarithmic scale of normalized intensity in flagged genes of WT and TG hearts 24 hours after CTX injection. 22691 genes were normalized and analysed by “Filter on Flags”. The analysis reduced the amount of genes to 11564 genes present in two independent experiments. B, C) Genes Filtered on Flags were further filtered on “Fold Change”. The analysis was performed to select genes that were upregulated (B, red scattered diagram) and downregulated (C, blue scattered diagram) by setting the fold differences to 2. Two independent experiments were compared for similarity in gene expression by Venn diagram. The comparison showed 99 genes upregulated and 128 genes downregulated in TG hearts compared to WT. D) Normalized intensity in logarithmic scale of specific antioxidant (metallothionein), antiapoptotic (Ubce-7 interacting protein 3, ubiquitin conjugating enzyme 7 interacting protein 3), and vessel protecting (adiponectin) genes overexpressed in two TG hearts compared to two WT hearts.
Movie S1: TG left ventricle motion

Movie recording of left ventricle motion was analysed in B-mode and in parasternal short-axis (PSA). 300 different frames covering cycles of ventricular contraction and distension (systole and diastole) were recorded. TG animals were anesthetized and left ventricle echocardiography was performed as described in Materials and Methods with VisualSonic Ultrasound. The movie shows the normal heart beat of a TG animal.

Movie S2: left ventricular motion in systolic and diastolic phases of TG hearts 1 M after LCA

Movie recording was performed as described for Movie S1. TG animals 1 M after LCA were anesthetized and left ventricle echocardiography was performed as described in Materials and Methods with VisualSonic Ultrasound. Ventricular wall motion is lightly impaired when compared to TG uninjured hearts, indicating a good functional performance of mIGF-1 mice after infarct induction. The movie is representative of one out of 8 TG animals analysed.

Movie S3: WT left ventricle motion

WT heart motion was recorded as described for Movie S1. Animals were anesthetized and left ventricle echocardiography was performed with VisualSonic Ultrasound. The movie shows the normal heart beat of a WT animal.

Movie S4: Left ventricular motion in systolic and diastolic phases of WT hearts 1 M after LCA

Recordings were performed as described for Movie S1. Ventricular wall motion is dramatically impaired in WT hearts 1M after infarct induction compared to WT uninjured hearts and TG injured hearts. The movie is representative of one out of 8 WT animals analysed.
## Supplemental Table 1

<table>
<thead>
<tr>
<th>Echocardiography</th>
<th>13 week-old</th>
<th>23 week-old</th>
<th>13 week-old</th>
<th>23 week-old</th>
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<tbody>
<tr>
<td></td>
<td>WT (n=8)</td>
<td>TG (n=8)</td>
<td>WT (n=8)</td>
<td>TG (n=8)</td>
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<tr>
<td>LVM (mg)</td>
<td>98±5</td>
<td>119±6*</td>
<td>101±3</td>
<td>118±4*</td>
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<td>LWM/BW (mg/g)</td>
<td>3.1±0.1</td>
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<td>3.3±0.2</td>
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<td>S (mm)</td>
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<td>0.74±0.02</td>
<td>0.84±0.02*</td>
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<td>PW (mm)</td>
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<td>0.70±0.02*</td>
<td>0.64±0.02</td>
<td>0.70±0.01*</td>
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<td>LVEDD (mm)</td>
<td>4.17±0.11</td>
<td>4.19±0.08</td>
<td>4.10±0.06</td>
<td>4.13±0.13</td>
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<td>LVESD (mm)</td>
<td>2.76±0.10</td>
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<td>2.64±0.06</td>
<td>2.82±0.15</td>
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<td>FS (%)</td>
<td>34±1</td>
<td>27±1*</td>
<td>35±1</td>
<td>32±2</td>
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<td>EF (%)</td>
<td>69±2</td>
<td>60±2*</td>
<td>71±2</td>
<td>66±3</td>
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<td>28±2</td>
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<td>E/A</td>
<td>1.61±0.08</td>
<td>1.27±0.11*</td>
<td>1.49±0.08</td>
<td>1.21±0.07*</td>
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<td>Adur (ms)</td>
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<td>40±1*</td>
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<td>DT (ms)</td>
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<td>Nd</td>
<td>Nd</td>
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<td>SAP (mmHg)</td>
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<td>HR_{conscious} (bpm)</td>
<td>599±15</td>
<td>607±12</td>
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</table>

**Supplemental Table 1**: Echocardiographic measurements of WT and TG hearts in physiological conditions. SAP: systolic arterial pressure, HR: heart rate, PR: PR interval, QT: QT interval, LVM: left ventricular mass, BW: body weight, S: septal thickness, PW: posterior wall.
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thickness, LVEDD: left ventricular end-diastolic diameter, LVESD: left ventricular end-systolic diameter, FS: fractional shortening, EF: ejection fraction, CO: cardiac output, E/A: maximal speed of early to late mitral filling ratio, Adur: duration of the mitral A wave, DT: mean deceleration time of the E wave, IVRT: isovolumetric relaxation time and RV/LV: right to left ventricular diameters ratio. Nd: not determined. All results are expressed as means±sem. *: p<0.05 (paired Student’s t tests for comparisons)
### Supplemental Table 2

<table>
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<tr>
<th>Measurement</th>
<th>WT</th>
<th>WT 1M LCA</th>
<th>TG</th>
<th>TG 1M LCA</th>
<th>TG 2M LCA</th>
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<tbody>
<tr>
<td>% EF</td>
<td>68.1 ± 7.7 *</td>
<td>48.7 ± 6.5 §</td>
<td>70.8 ± 6.0 *</td>
<td>62.7 ± 1.3</td>
<td>57.47 ± 7.1</td>
</tr>
<tr>
<td>% FS</td>
<td>37.7 ± 5.6 *</td>
<td>24.0 ± 4.4 §</td>
<td>39.1 ± 5.0 *</td>
<td>33.6 ± 1.1</td>
<td>30.68 ± 5.45</td>
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</table>

**Supplemental Table 2: Cardiac functional parameters in WT and TG mice.** Ejection fraction (EF) and fractional shortening (FS) were measured in WT and TG mice 1 and 2 months after LCA, as compared to sham-operated controls. Anesthetized mice were analysed with high resolution ultrasound. Each value is the average of three different readings on the same animal from eight male mice in each group. The heart function of WT mice 2 months after LCA was dramatically impaired, confounding the reading and the recording in all animals tested (data not shown). Significant values are calculated with Student t-test setting p<0.05 as a double side value. Asterisk (*) indicates significant decrease of EF and FS of WT or TG hearts compared to respective injured hearts. § marks significant decrease of EF and FS of WT injured hearts compared to TG injured hearts.
### Supplemental Table 3

<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>Abbreviation</th>
<th>WT Heart (CPM)</th>
<th>TG Heart (CPM)</th>
<th>WT Heart (%)</th>
<th>TG Heart (%)</th>
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<tr>
<td>90 kDa Ribosomal S6 Kinases (S380)</td>
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<td>90 kDa Ribosomal S6 Kinases (T573)</td>
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<td>AMP-activated protein kinase alpha (T172)</td>
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<td>Control</td>
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<td>Bone marrow X (Eph-like) kinase (Y40)</td>
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<td>Bruton's tyrosine kinase (Y223)</td>
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<td>Calcium/calmodulin-dependent kinase II (T286)</td>
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<td>Cyclin-dependent kinase 1 (T161)</td>
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<td>eIF4E binding protein (S65) (17)</td>
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<td>Extracellular signal-regulated kinase 1 (T202/Y204)</td>
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<td>Lyn (Y507) (44)</td>
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<td>Lyn (Y507) (46)</td>
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<td>14%</td>
</tr>
</tbody>
</table>
**Supplemental Table 3: Phosphorylation profiling in wild-type and mIGF-1 transgenic heart tissue.**

A screen of 31 phosphoproteins in WT and TG heart tissues from 4 month-old mice was performed by Kinetworks analysis (Kinexus Bioinformatic Corp.). The trace quantity of each band is defined as CPM and is measured under its intensity profile curve. Each value is normalized by the amount of protein in each sample. Each lane corresponds to a specific protein and the phosphorylated Tyrosine or Serine position is indicated. Increase or decrease of band intensity in TG compared to WT is expressed in percentage. Single asterisks (*) mark canonical IGF-1 downstream mediators whose phosphorylation status is unchanged or repressed in mIGF-1 transgenic cardiac muscle, as opposed to PDK1, marked with a double asterisk (**), which is prominently phosphorylated in the presence of mIGF-1.

| Protein kinase C zeta (T410)/lambda (T403) | PKCz/l Lane 3 |
| Protein kinase D (Protein kinase mu) (S916) | PKCm/PKD Lane 3 |
| Protein kinase theta (T538) | PKCt Lane 10 |
| Raf (S259) (60) | Raf1 (60) Lane 8 |
| Raf (S259) (70) | Raf1 (70) Lane 8 |
| Retinoblastoma Protein (S780) | Rb Lane 18 |
| Retinoblastoma Protein (S807/S811) | Rb Lane 7 |
| The mammalian target of Rapamycin (S2448) | mTOR Lane 13 |
| Type1 protein phosphatase alpha (T320) | PP1a Lane 8 |
| Zap70 (Y319)/Syk (Y352) | Zap70/Syk Lane 20 |

Control values are represented as 100%. Decrease in band intensity is noted as -72% for PKCz/l, -2% for Raf1, -14% for Raf1, -23% for Rb, -36% for mTOR, and -8% for PP1a. Single asterisks (*) mark unchanged or repressed phosphorylation status in mIGF-1 transgenic cardiac muscle, as opposed to PDK1, marked with a double asterisk (**), which is prominently phosphorylated in the presence of mIGF-1.
REFERENCES


Supplemental Figure 1

WT

TG

2M

40X

4M

40X

CSA (μm²)

WT

TG

2M

4M

* Significant difference

$ Significant trend