Cardiac Stem Cells Possess Growth Factor-Receptor Systems That After Activation Regenerate the Infarcted Myocardium, Improving Ventricular Function and Long-Term Survival

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Abstract—Cardiac stem cells and early committed cells (CSCs-ECCs) express c-Met and insulin-like growth factor-1 (IGF-1) receptors and synthesize and secrete the corresponding ligands, hepatocyte growth factor (HGF) and IGF-1. HGF mobilizes CSCs-ECCs and IGF-1 promotes their survival and proliferation. Therefore, HGF and IGF-1 were injected in the hearts of infarcted mice to favor, respectively, the translocation of CSCs-ECCs from the surrounding myocardium to the dead tissue and the viability and growth of these cells within the damaged area. To facilitate migration and homing of CSCs-ECCs to the infarct, a growth factor gradient was introduced between the site of storage of primitive cells in the atria and the region bordering the infarct. The newly-formed myocardium contained arterioles, capillaries, and functionally competent myocytes that with time increased in size, improving ventricular performance at healing and long thereafter. The volume of regenerated myocytes was 2200 µm³ at 16 days after treatment and reached 5100 µm³ at 4 months. In this interval, nearly 20% of myocytes reached the adult phenotype, varying in size from 10 000 to 20 000 µm². Moreover, there were 43±13 arterioles and 155±48 capillaries/mm² myocardium at 16 days, and 31±6 arterioles and 390±56 capillaries at 4 months. Myocardial regeneration induced increased survival and rescued animals with infarcts that were up to 86% of the ventricle, which are commonly fatal. In conclusion, the heart has an endogenous reserve of CSCs-ECCs that can be activated to reconstitute dead myocardium and recover cardiac function. (Circ Res. 2005;97:663-673.)

Key Words: cardiac progenitor cells ■ myocardial regeneration ■ mortality

Adult cardiac stem cells (CSCs) and early committed cells (ECCs) express the stem cell antigens c-kit, MDR1, and Sca-1.2-4 c-kit+8 cells are self-renewing, clonogenic, and multipotent and give rise to myocytes, smooth muscle cells (SMCs), and endothelial cells (ECs) in vitro and in vivo.5 A similar category of CSCs-ECCs has been found in the human heart,6,7 suggesting that these undifferentiated cells participate in the normal turnover of cardiac cells and, under favorable conditions, have the ability to form myocytes, coronary arterioles, and capillary structures.1,5,7 The presence of CSCs-ECCs raises the question of why they fail to respond to ischemic injury with regeneration of myocytes and coronary vessels and restoration of function. CSCs-ECCs distributed within the damaged area may die together with parenchymal cells, however, and SMCs and ECs in coronary vessels prevent myocardial repair. For this reason, we have explored the possibility that CSCs-ECCs, if properly activated, can translocate to sites of damage, survive the unfavorable environment, multiply, and differentiate, forming functionally competent myocardium.

Hepatocyte growth factor (HGF) stimulates cell migration8 by expression of metalloproteinases (MMPs)9 that by breaking down the extracellular matrix favor cell locomotion, homing, and tissue reconstitution. The receptor of HGF, c-Met, is expressed in bone marrow progenitor cells, satellite cells, and embryonic myocytes.10,11 Insulin-like growth factor-1 (IGF-1) is mitogenic, antiapoptotic, and necessary for neural stem cell growth.12 IGF-1 promotes myocyte formation and attenuates myocyte death after infarction.13 Together, these findings prompted us to determine whether CSCs-ECCs express c-Met and whether HGF stimulates their migration to the infarcted myocardium. If CSCs-ECCs ex-
press IGF-1 receptor, IGF-1 may protect their viability and enhance their growth within the infarct. Thus, myocardial infarction was produced in mice and HGF and/or IGF-1 were locally injected to trigger migration and homing of CSCs-ECCs to the ischemic area. The impact of this intervention on myocardial regeneration, heart function, and animal survival was determined acutely, at healing and long thereafter to evaluate its therapeutic efficacy.

**Materials and Methods**

Mice were infarcted, treated with IGF-1 and HGF (GFs) and euthanized at different intervals up to 4 months. Myocardial regeneration was determined by confocal microscopy. An expanded Materials and Methods can be found in an online-only data supplement available at http://circres.ahajournals.org.

**Results**

**CSCs**

CSCs did not express markers of cardiac and hemopoietic cell lineages. They were more numerous in the atria and apex than in the ventricle (supplemental Figure S1). ECCs expressed transcription factors of cardiac (GATA-4) and myocyte (MEF2C) lineages, identifying progenitor cells (Figure 1A through 1D). Also, c-kit^POS, MDR1^POS, and Sca-1^POS cells expressed α-sarcomeric actin or cardiac myosin, representing myocyte precursors. SMC progenitors and precursors expressed GATA-6 and -smooth muscle actin and EC progenitors and precursors Ets-1 and von Willebrand factor, respectively (supplemental Figure S2).

**c-Met and IGF-1R**

Most CSCs-ECCs expressed c-Met and IGF-1 receptor together with HGF and IGF-1 (supplemental Figure S3). Migration studies documented that HGF had a chemoattractive effect on CSCs-ECCs that peaked at 100 ng/mL. Invasion assays showed that 100 ng/mL HGF increased by 7-fold the number of CSCs-ECCs that accumulated in the lower well of a modified Boyden chamber. IGF-1 had few motogenic and invasive properties (supplemental Figure S4). After HGF

![Image](http://circres.ahajournals.org/)
administration, CSCs-ECCs had gelatinolytic activity and secreted MMP-2 and MMP-9; IGF-1 had little effects on MMPs (Figure 1E).

IGF-1 and HGF decreased apoptosis of CSCs-ECCs in serum-free medium by 94% and 58%, respectively. Moreover, BrdU<sup>POS</sup> CSCs-ECCs increased 11-fold with IGF-1 and 3-fold with HGF. The effects of both GFs on cell death and growth were not different from those of IGF-1 alone (supplemental Figure S5). Murine mRNA and protein for IGF-1 and HGF were present in non-stimulated CSCs-ECCs and increased in GF-treated infarcted-hearts (Figure 1H). Animals were injected with heterologous proteins, human IGF-1, and HGF.

HGF and IGF-1 in the supernatant of GF-stimulated CSCs-ECCs in serum-free medium increased from 30 minutes to 120 minutes, reaching a 13-fold and 26-fold elevation at 120 minutes, respectively (Figure 1F). The increase in murine HGF and IGF-1 mRNA, together with the significant accumulation of murine HGF and IGF-1 protein in stimulated CSCs-ECCs in vivo, supports the notion that CSCs-ECCs can synthesize these GFs. The time-dependent increase in the secretion of HGF and IGF-1 in vitro strengthens this conclusion. Thus, CSCs-ECCs possess the c-Met-HGF and IGF-1 receptor-IGF-1 systems that promote cell growth and migration, attenuate apoptosis, and increase the secretion of their ligands.

**Migration of CSCs-ECCs**

Because cycling CSCs-ECCs were identified in the atrioventricular (AV) groove (supplemental Figures S6 and S7), a retrovirus expressing enhanced green fluorescence protein (EGFP)<sup>1,5</sup> was injected in this region to label replicating cells before the administration of the GFs. Integration and accumulation of EGFP in the tagged cells required ~48 hours (supplemental Figure S8). Therefore, coronary occlusion was performed 2 days after viral inoculation, and HGF and/or IGF-1 were injected 5 hours later (supplemental Figure S9).

Increasing doses of HGF were used between the AV groove and the infarct to favor migration of CSCs-ECCs to the infarct. Fluorescein-conjugated HGF was injected to establish its distribution; fluorescence intensity was higher adjacent to the infarct than in the distant myocardium (Figure 2A through 2C). As shown by ELISA, HGF quantity was also higher in the border than in the remote tissue (Figure 2D). Autocatalytic phosphorylation of c-Met and IGF-1R, phospho-Akt, phospho-IRS-1, and phospho-FAK in c-kit<sup>POS</sup> CSCs-ECCs showed that these indices of c-Met and IGF-1 receptor activation were barely detectable in untreated hearts. Conversely, their expression in CSCs-ECCs from GF-treated infarcts increased with time (Figure 2E). Phospho-FAK and phospho-c-Met were also detected by immunocytochemistry (data not shown) and fluorescent-activated cell sorter (Figure 2F). Antibody specificity was determined by fluorescence-activated cell sorter, and immunoprecipitation Western blotting of Lewis lung carcinoma cells (Figure 2G and 2H).

The effects of GFs on cell migration were evaluated ex vivo. Microscopic fields were analyzed for 1 to 5 hours to measure movement of EGFP<sup>POS</sup> cells (Figure 3A through 3E). The same EGFP<sup>POS</sup> cells were then characterized after fixation: EGFP<sup>POS</sup> cells expressed antigens typical of CSCs-ECCs (Figure 3F and 3G; supplemental Figure S10). EGFP<sup>POS</sup> cells, which were not CSCs-ECCs, were occasionally seen in the AV groove; these cells did not move after GF administration. EGFP<sup>POS</sup> CSCs-ECCs migrated at ~70 μm/h (supplemental Figure S11) from the AV groove to the border and central infarct in 2 to 3 and 6 to 7 hours, respectively. IGF-1 had minimal effect on locomotion. In GF-treated infarcts, the number of EGFP<sup>POS</sup> CSCs-ECCs and EGFP<sup>POS</sup>-EGFP<sup>NEG</sup> CSCs-ECCs in this region increased 3-fold and 8-fold, respectively (supplemental Figure S12). Most CSCs-ECCs expressed both c-Met and IGF-1 receptor and were committed to cardiac lineages (supplemental Figure S13 and S14).

**Destiny of CSCs-ECCs**

CSCs-ECCs can migrate via the coronary bed, the interstitium, or both. Therefore, the coronary circulation was perfused with rhodamine-labeled dextran, and GFs were administered at the time of observation. The coronary vasculature was recognized by red fluorescence and EGFP<sup>POS</sup> cells by green fluorescence (Figure 4A through 4D; supplemental Figure S15). In all cases, EGFP<sup>POS</sup> cells were located outside the vessels, suggesting that the coronary circulation was not implicated in cell migration; EGFP<sup>POS</sup> cells were found in interstitial tunnels defined by fibronectin (Figure 4E).

The death, survival, and growth of CSCs-ECCs within the infarct was characterized (supplemental Figures S16 through S19) together with the differentiation of EGFP<sup>POS</sup> CSCs-ECCs in the early stages of myocardial restoration. In the absence of GFs, CSCs-ECCs within the infarct died rapidly, whereas the presence of GFs promoted their survival and replication. Moreover, a time-dependent increase in the number of c-kit<sup>POS</sup>, MDR1<sup>POS</sup>, and Sca-1<sup>POS</sup> cells undergoing the transition from undifferentiated cells to small myocytes was detected. At 1 day, EGFP<sup>POS</sup> cells expressed stem cell antigens and MEF2C, but sarcomeric proteins were absent. At 2 days, cells were positive for sarcomeric myosin and at 3 to 4 days had developed into small EGFP<sup>POS</sup> myocytes. Connexin 43 and N-cadherin were present (Figure 5A and 5B). These cells, clustered in patches throughout the infarct, increased with time (Figure 5C). At 1 to 2 days, new arterioles had thick walls and ill-defined lumen. At 3 to 4 days, arterioles and capillaries increased; they were formed by EGFP<sup>POS</sup> and EGFP<sup>NEG</sup> cells (supplemental Figure S20).

**GFs and Infarct Size**

Myocardial infarction rates in GF-treated hearts at 16 days and 4 months were 67% and 63%, respectively. Corresponding values in untreated mice were 42% and 43%. Thus, infarct size was 60% and 47% larger in GF-treated than in untreated mice at the early and late time point, respectively (supplemental Figure S21). This unexpected result was the consequence of a rapid regeneration of dead myocardium induced by GFs, which rescued animals with extremely large infarcts acutely after coronary occlusion. Figure 6A illustrates a 44% infarct, a nearly maximum infarct size compatible with survival in untreated mice at 16 days. The infarct corresponds to a loss of 1.6 million myocytes from a total of 3.6 million. GF-treated mice survived infarcts of 82% (Figure 6B) and
86%; this is a striking result, as 2.9 and 3.1 million myocytes were lost, respectively.

The group of untreated infarcted mice consisted of 123 animals (supplemental Table S1). The 87 animals that died 10 hours to 15 days after surgery had infarctions of 57% to 82%. Twenty-two of the remaining 36 were euthanized at 16 days, and 15 were studied functionally and structurally. The group of GF-treated infarcted mice consisted of 107 animals. The 46 animals that died 10 hours to 15 days after surgery had infarctions of 63% to 79%. Forty-nine of the remaining 61 were euthanized at 16 days, and 26 were studied. None of untreated mice survived 16 days with infarcts larger than 60%. Conversely, 14 of 22 mice in the GF-treated group survived 16 days with infarcts greater than 60% (Figure 6C; 68%). Thus, GF-treatment saved mice with infarcts that are typically fatal.

Myocardial Regeneration

Four of 26 GF-treated mice euthanized at 16 days did not respond to GF administration; they were undistinguishable from untreated infarcted mice. Most likely, they were not properly injected with GFs. Similarly, 2 of 10 GF-treated mice at 4 months had no regenerating band. Thus, successful treatment was obtained in 85% and 80% of treated mice. Unsuccessfully treated mice were characterized structurally (supplemental Table S2).

In untreated mice, the infarcts consisted of collagen type I and III (Figure 6A). In treated mice, the regenerated myocardium occupied 65±8% and 73±7% of the infarct at 16 days (Figure 6B) and 4 months (Figure 6C), respectively. The new band of myocardium was located in the mid-portion of the wall and, in some cases, replaced the entire wall thickness.
At 16 days, the new myocardium recovered 15\% of the infarct, decreasing the infarct size from 67\% to 57\%. At 4 months, the formed myocardium accounted for 54\% of the infarct, reducing infarct size from 63\% to 29\% (supplemental Figure S22).

The regenerated myocardium was composed of myocytes and coronary vessels. At 16 days, myocytes varied in volume from 600 to 7200 \( \mu \text{m}^3 \) and at 4 months, from 700 to 20 000 \( \mu \text{m}^3 \) (Figure 6D). At both intervals, treated hearts generated an average 3.2 million myocytes to compensate for a loss of 2.2 million. There were 43±13 arterioles and 155±48 capillaries/mm² at 16 days, and 31±6 arterioles and 390±56 capillaries at 4 months. For comparison, there are \( \approx 10 \) arterioles and \( \approx 5500 \) capillaries/mm² in myocardium in the adult heart. New vessels contained erythrocytes (supplemental Figure S23).

To document that cells in the band were formed after infarction, mice were exposed to BrdU; 84±9\% and 94±3\% myocytes and vessels in the band were BrdUPOS at 16 days and 4 months, respectively (Figure 6E and 6F; supplemental Figure S23). At 4 months, the spared myocardium adjacent to and distant from the infarct had 17±6\% and 8±3\% BrdUPOS myocyte nuclei, respectively (supplemental Figure S24). New myocytes were smaller than adult myocytes (21 800±2200 \( \mu \text{m}^3 \)) but expressed nestin, desmin, myosin, \( \alpha \)-sarcomeric actin, N-cadherin, and connexin 43 (supplemental Figure S25). These results indicate that, in response to HGF and IGF-1, CSCs-ECCs translocate, grow, and differentiate, resulting in significant myocardial regeneration. A single GF had a smaller impact, however, and the 2 GFS acted synergistically in cardiac repair (supplemental Figure S26).

Myocyte Mechanics and Cell Fusion

Myocytes were isolated from regenerated and spared myocardium (Figure 7A through 7F) of GF-treated hearts at 16 days, and their contractile behavior was determined. New cells were smaller than old cells, had BrdUPOS nuclei, and resembled neonatal myocytes. Connexin 43 and N-cadherin were detected (Figure 7C through 7F). In comparison with surviving myocytes, new cells exhibited higher peak shortening and velocity of shortening and relengthening (Figure 7G; supplemental Figure S27).
The discrepancy in size between old and new myocytes makes it unlikely that fusion of the activated CSCs with spared myocytes was implicated in the differentiation of these cells into myocytes. Moreover, to rule out fusion of CSCs with other cells, the number of X chromosomes was evaluated in the regenerated myocytes of the female infarcted treated mice. At most, 2 X chromosomes were detected in the nuclei, strongly arguing against cell fusion (Figure 7H). Cycling and noncycling new and old myocytes were identified by Ki67, and DNA content was measured in individual nuclei. Mouse lymphocytes were used as control. Nuclei of noncycling myocytes and lymphocytes had 2C DNA content, whereas Ki67POS nuclei had DNA content intermediately between diploid and tetraploid excluding cell fusion (supplemental Figure S28).

Cardiac Performance and Survival
Contraction in the infarct of GF-treated mice reappeared at 15 days and improved at 1, 3, and 4 months (Figure 8A through 8E). Hemodynamics improved in GF-treated mice and deteriorated in untreated mice (Figure 8F). In GF-treated hearts, from 16 days to 4 months, left ventricular end-diastolic pressure decreased and LV-developed pressure and dP/dt increased. Also, chamber volume decreased, attenuating diastolic stress (Figure 8F). Together, these variables were responsible for a 34% decrease in animal mortality (Figure 8G), despite ~50% larger infarcts in GF-treated animals. Thus, activation of CSCs-ECCs acutely after infarction elicits a sustained regenerative response that ameliorates the anatomy and function of the heart, improving animal survival.

Discussion
The results of the current study demonstrate that the mammalian heart contains a population of resident stem cells and progenitor cells that, after activation, can migrate and invade the infarcted myocardium, leading to a significant restoration of the damaged portion of the wall and a remarkable recovery of ventricular function. Myocardial regeneration comprises parenchymal cells and coronary vessels, providing additional evidence in support of the notion that the adult heart possesses an extraordinary growth reserve capable of restoring losses of tissue commonly considered fatal in animals and humans. Commitment of cardiac primitive cells to the myocyte lineage, together with the creation of small actively dividing amplifying myocytes, occurs in the acute phases of cardiac repair, whereas maturation and hypertrophy of the newly formed cells constitute the predominant mechanisms of myocardial regeneration long-term after infarction. Coronary arterioles and capillary structures are produced concurrently with myocytes to ensure adequate oxygenation of the developing myocardium. Differentiation and enlargement of myocytes is paralleled by expansion of the coronary vasculature and microvasculature that tends to preserve blood supply and oxygen diffusion to the surrounding cells. Ultimately, the infarcted heart largely regains its anatomical configuration, dramatically reversing ventricular dilation and thinning of the wall. Although the normal architecture and orientation of myocyte bundles across the wall was not acquired, our findings suggest that local administration of GFs may become a novel powerful therapeutic strategy for the acutely decompensated infarcted heart.

CSCs-ECCs in the Adult Heart
The fact that the heart possesses a stem cell compartment has been shown in several species, including large animals and humans. With one exception, these cells have been characterized by the expression of thetypical stem cell antigens c-kit, MDRI or Sca-1. However, the fact that cardiac primitive cells are self-renewing, clonogenic, and multipotent, which are the critical variables for the definition of a stem cell, has been documented only in rats and dogs. Because of this inevitable premise, it is difficult to reconcile the claim made and terminology used when cells already committed to the myocyte lineage and without established epitopes have been interpreted as a novel class of cardiac...
Importantly, the majority of CSCs in dogs, humans, and here in the mouse heart expresses together c-kit, MDR1, and Sca-1–like proteins. Additionally, these stem cell antigens are not associated with a significant difference in the ability of these primitive cells to acquire the myocyte, endothelial, and smooth muscle cell lineage in vitro and in vivo. The latter has been confirmed in the mouse heart. The ability of clonogenic c-kitPOS cells to create myocardial cells in vitro, however, is several-fold greater than cells expressing MDR1, Sca-1–like protein, or c-kit, MDR1, and Sca-1 together. Whether this in vitro property of c-kitPOS cells has a comparable in vivo counterpart remains an unanswered question.

The Infarcted Heart

Post-infarction cardiomyopathy is the major cause of heart failure, and the current observations offer new insights concerning the onset and evolution of this disease. On the basis of the present results, the progressive nature of ischemic cardiomyopathy appears to be dictated by the inability of CSCs-ECCs to translocate and home to the infarcted myocardium rather than by limitations in the growth reserve of the damaged heart. The resident stem cells distributed in the infarcted region do not survive the ischemic event and die by apoptosis and necrosis in a manner identical to that of myocytes and coronary vessels. CSCs-ECCs are not capable of opposing the death signals activated by permanent coronary occlusion and initiate regenerative growth within the infarcted myocardium. Moreover, these primitive cells cannot escape replicative senescence with severe telomeric shortening and activation of the death program in end-stage failure or premature myocardial aging in humans. Spontaneous myocardial regeneration within acute infarcts, however, appears to be mediated by CSCs-ECCs, which undergo lineage commitment and differentiation. With some exceptions, tissue growth does not invade and replace the infarcted myocardium but is restricted to the noninfarcted portion of the ventricular wall. This is not a peculiar limitation of the heart. Stem cells are efficient in maintaining organ homeostasis but do not prevent the formation of a scar after artery occlusion in the intestine, skin, brain, liver, kidney, and the bone marrow.

Consistent with previous observations of cardiac repair, early myocardial regeneration is characterized by the formation of immature parenchymal cells and coronary vessels that resemble the fetal heart. Cardiomyocytes, resistance arterioles, and capillary structures mature with time, but only ~20% of myocytes reach the adult phenotype and have a volume of 10,000 to 20,000 μm³ 4 months after successful treatment (Figure 6D). This is at variance with the process of postnatal development, as myocytes acquire adult characteristics at 3 to 4 weeks after birth. Interestingly, scattered regeneration of myocytes in the surviving myocardium after infarction results in the formation of parenchymal cells that are indistinguishable from the preexisting partners. This observation has led to the conclusion that contact with differentiated cells and paracrine factors may be implicated in the destiny of lineage-committed CSCs-ECCs.

On the basis of the belief that the heart lacks endogenous regenerative capacity, early strategies of cardiac repair have used the colonization of the myocardium with exogenous cells. The presence of CSCs raises the unique prospect of reconstituting dead myocardium after infarction, repopulating the hypertrophic decompensated heart with new, better-functioning cells and vascular structures, and, perhaps, restoring the physiological and anatomical characteristics of the normal heart. These possibilities have formed the basis of a new paradigm in which the heart is viewed as a dynamic organ that constantly renews its cell populations and has the inherent ability to reconstitute tissue. Understanding cardiac homeostasis would offer the extraordinary opportunity to potentiate this naturally occurring process and promote myocardial repair after injury.

Rescue of the Infarcted Heart

So far, experimental studies and clinical trials have used rather heterogeneous bone marrow cell populations to regen-
erate infarcted myocardium and have consistently shown an improvement in function of the infarcted heart. However, the most logical and potentially powerful cell to be used is the CSC. It is intuitively apparent that if the adult heart possesses a pool of primitive multipotent cells, these cells have to be tested first before more complex and unknown cells are explored. The attraction of this approach is its simplicity. Cells from other organs are not programmed to give rise to

Figure 6. Myocardial regeneration. A through C, Untreated MI (A) and MI-Ts at 16 days (B) and 4 months (C). Arrows show infarcted nonregenerated (A) and infarcted regenerated (B and C) myocardium. BrdU<sup>P0</sup> nuclei (A, fibroblasts). B and C, Four levels of regeneration from base to apex. Groups of 3 panels show areas within rectangles. Each triplet illustrates myocytes (cardiac myosin, red), collagen-type-I/type-III (blue), nuclei (PI, green), and BrdU<sup>P0</sup> nuclei (white). BrdU labeling in regenerated myocardium (B and C) indicates that cells were formed after infarction. D, Myocyte size distribution. E and F, New myocytes (cardiac-myosin, red) in GF-treated infarcted mice at 16 days (E) and 4 months (F). BrdU<sup>P0</sup>-nuclei appear as white dots; laminin appears green.
Cardiomyocytes and coronary vessels, and transdifferentiation requires nuclear reprogramming with chromatin reorganization. This is a time-consuming process\textsuperscript{25} that delays the formation of alternate progeny, affecting the onset and efficiency of myocardial regeneration. Conversely, the intramyocardial injection of GFs promotes the translocation of CSCs-ECCs to the damaged area and activates their growth and differentiation, resulting in the formation of functionally competent myocardium. The blunted response to HGF or IGF-1 emphasizes the need for a dual stimulation of CSCs-ECCs: The survival and growth provided by IGF-1 together with the chemotactic effects of HGF. The presence of small new myocytes within the infarct at 1 to 2 days after coronary ligation demonstrates that tissue reconstitution is an immedi-

**Figure 7.** Regenerated myocytes and cell fusion. A and B, Regenerated (A) and spared (B) myocytes from MI-T. BrdU\textsuperscript{POS} nuclei are bright blue. C-F, Connexin 43 (C and D, yellow) and N-cadherin (E and F, white). BrdU\textsuperscript{POS} nuclei (C and E, green; D and F, bright blue). G, Sarcomere mechanics from new and spared myocytes from GF-treated infarcted hearts at 16 days. Tracings show the changes in length of sarcomeres. H, Only 1 or 2 X chromosomes were detected (white dots).
ate reaction that restores an amount of myocardium larger than any other reported so far in the long term.

At variance with previous strategies, local GF administration benefited animals with infarcts that are typically fatal. However, these results leave unanswered the question of why ischemia does not activate stem cells locally, favoring their homing to the damaged area, or whether the effects of GFs on ischemic myocytes influenced cell viability. Myocardial ischemia is a potent inducer of IGF-1 and HGF. The injection of GFs did not increase the number of bone marrow progenitor cells in the circulation, as shown by the lack of changes in the pool of CD45 and c-kit cells in the peripheral blood (supplemental Figure S29); 240 μg of HGF over a period of 12 days was required to produce a modest increase in circulating BMPCs. This quantity is 170,000-fold higher than the 1.4 ng given here at a single time point (limitations; online).

Cell Migration
The locomotion of CSCs-ECCs occurs through the myocardial interstitium independently from the coronary circulation. CSCs-ECCs traverse in vitro a 3-dimensional substrate of Matrigel and in vivo dense barriers of collagen type I and III. MMP-2 and MMP-9 appear to be the proteolytic enzymes responsible for the invasive phenotype of CSCs-ECCs and their trafficking across the myocardial interstitium. However, MMP-9 may trigger the release of stem cell factor from CSCs-ECCs, although stem cell factor has no effects on the invasive properties of these cells in vitro.

The migratory elements of CSCs-ECCs may include morogenic factors, and ischemia is a potent inducer of the chemotactants HGF, stromal cell-derived factor-1, and vascular endothelial growth factor. Although these cytokines may favor cell movement, it is only with HGF that waves of CSCs-ECCs are engaged in a directional migration toward the injured myocardium. A stop signal may also be present to promote the accumulation of cells within the infarct. This involves the extracellular substrates used by the cells to traverse the interstitium. The ability of CSCs-ECCs to degrade extracellular matrix provides a basis for the tunnel tracks found in the myocardium. CSCs-ECCs adhere to the wall of fibronectin that delimit the interstitial channels. Importantly, fibronectin is implicated in the migration of c-kit cells. Finally, the directional locomotion

Figure 8. Cardiac function and mortality. A through E, Echocardiography of a noninfarcted-SO (A), untreated infarcted heart at 4 months (B), and GF-treated infarcted hearts at 15 days (C), 1 month (D), and 4 months (E). Contraction in the infarcted region is apparent in GF-treated mice at 15 days and thereafter (arrowheads). F, Functional properties of SO, untreated MI and MI-T. * ** IP 0.05 to 0.001 versus SO (*), MI (**) and MI-T at 16 days (†). G, Mortality after infarction was reduced in GF-treated mice (P < 0.0001; log-rank test) although these mice had ~50% larger infarcts.
of cells is regulated by guidance factors. Ligand-receptor systems are potential candidates for defining the trajectory of migrating CSCs-ECCs and, in our conditions, the activation of c-Met by HGF may be the determinant element. MMPs, together with the HGF-mediated changes in integrin phenotype of CSCs-ECCs, are likely to be involved. The long journey of a stem cell toward its final destination, however, remains an intriguing and mysterious event.

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References


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Correction

In an article by Urbanek et al (Circ Res. 2005;97:663–673.) the authors incorrectly included the top panel of Figure 1F (page 664) in the paper. The corrected figure is shown below. The authors state that the corrected figure does not change the legend to Figure 1, nor does it affect the conclusions of the study.

![Corrected Figure 1F](image-url)
SUPPLEMENTARY INFORMATION

Materials and Methods

**Invasion Assays**

c-kit\textsuperscript{POS}, MDR1\textsuperscript{POS} and Sca-1\textsuperscript{POS} cells were collected with immunobeads (Miltenyi). GF-depleted extracellular matrix was spread on the surface of the inserts of a Boyden chamber (Chemicon); 100 ng/ml HGF (n=4) or 100-200 ng/ml IGF-1 (n=4) were placed in the lower chamber. Invading cells clung to the bottom.

**Zymography, MMPs, Cell Death and Growth**

c-kit\textsuperscript{POS}, MDR1\textsuperscript{POS} and Sca-1\textsuperscript{POS} cells were cultured in SFM containing HGF, 100-200 ng/ml, and/or IGF-1, 100-200 ng/ml, for 36 hours. Conditioned media were run onto 10% polyacrylamide gel copolymerized with 0.1% gelatin. Gelatinolytic activity was detected as clear bands against a blue background (Coomassie); MMPs (MMP-2 and MMP-9 antibodies, Chemicon) in conditioned media were detected by Western blot. n=5. Under identical culture conditions, BrdU was added, 1µg/ml. Cells were fixed and BrdU incorporation (n =6) and TdT assay (n=6) were performed.\textsuperscript{1,2}

**GF-Formation and Circulating BMPCs**

c-kit\textsuperscript{POS}, MDR1\textsuperscript{POS} and Sca-1\textsuperscript{POS} cells were collected and exposed to HGF and/or IGF-1 as above. Media containing GFs were removed and, following a 24-hour washout period, fresh SFM was added and the cells were exposed to c-Met and/or IGF-1R blocking antibodies. The antibodies were employed to avoid ligand binding and measure the amount of GFs secreted in the medium. Media were collected immediately after washout and 30, 60 and 120 minutes later. The quantity of HGF and IGF-1 was determined by ELISA (R&D Systems). Similarly, the concentration of HGF in samples of infarcted hearts was determined by ELISA.
HGF and IGF-1 mouse mRNA was detected by RT-PCR using murine specific primers in untreated and GF-treated cultured cells and in CSCs-ECCs isolated from control, untreated and GF-treated mice at 12, 24 and 72 hours after infarction. Moreover, the level of expression of HGF, IGF-1 and phosphorylated proteins was evaluated by Western blot. n=4 separate determination at each time point. Murine specific HGF and IGF-1 antibodies were used for the expression of these GFs in CSCs-ECCs; these cells were collected from hearts injected with human HGF and IGF-1. n=4-6 in all cases. See online Table S3. Mice were injected locally in the myocardium (Figure S9) or intravenously with HGF, 5 µg, and 1 hour later the number of CD45 and c-kitPOS in the peripheral blood was determined by FACS. Positive control mice were injected with G-CSF and SCF for 5 days.3 Expression of phospho-c-Met in c-kitPOS cells was also measured by FACS; Lewis lung carcinoma cells were used as positive control. For antibodies see online Table S3.

Cell Locomotion

EGFP retrovirus was injected in the AV-groove. Two days later, coronary ligation or sham-operation was performed and GFs were injected (see below). The heart was excised, perfused retrogradely through the aorta with Tyrode (30 mM KCl) and rhodamine-labeled dextran, and placed in a bath on a two-photon microscope-stage to view the LV wall.4 EGFP was excited at 940nm with mode-locked Ti:Sapphire femtosecond laser; 4D images were acquired at 515nm. Rhodamine: excitation=840; emission=610nm. n=7 at each time point. HGF was labeled with FITC, mixed with rhodamine-labeled microspheres, and injected in the LV wall.5 The localization and intensity of HGF-FITC were recorded: excitation=840nm; emission=505nm. n=4. In a previous study,4 we have shown by two-photon microscopy that EGFPPOS CSCs injected in the left ventricle entered the coronary circulation and with time traversed the vessel wall reaching areas of infarcted myocardium. Because of the protocol, these results demonstrated the validity of this approach for the recognition of cell movement in the heart. Additional experiments were also performed in vitro in which locomotion of
EGFP<sup>POS</sup> CSCs was promoted by 10% FCS following disruption of a cell monolayer. This in vitro wound-healing assay allowed us to document the ability of two-photon microscopy to detect translocation of cells in a simplified model system (Figure S30).

**Myocardial Infarction**

Under ketamine-acepromazine anesthesia, myocardial infarction was induced in female 129 SV-EV. After 5 hours, 4 injections of HGF-IGF-1 were made from the AV-groove to the border zone (Figure S9). Since the portion of the LV supplied by the occluded coronary artery stops contracting one minute after ligation of the vessel, the border zone and the distant myocardium are easily identified. Sham-operated and infarcted-untreated mice were injected with saline. BrdU (50 mg/kg b.w.) was injected once a day or added to drinking water, 1 mg/ml, to identify newly formed cells. The heart was arrested in diastole and perfused with formalin. The antibodies used for identification of CSCs, ECCs and other nuclear, cytoplasmic and membrane proteins are listed in online Table S4. GF-treated animals were sacrificed at 7-8 hours (n=12) and 1 (n=10), 2 (n=11), 3 (n=5), 4 (n=9), 16 (n=26) and 120 (n=10) days. Corresponding n values for infarcted untreated mice were 5, 3, 3, 2, 15 and 10. In sham-operated controls, n=6-10 from 7-8 hours to 4 days; n=11 at 16 and 8 at 120 days.

**Echocardiography and Hemodynamics**

Echocardiograms were obtained in conscious mice with an Acuson Sequoia 256c equipped with a 13-MHz linear transducer. For hemodynamics, mice were anesthetized and a Millar microtip pressure transducer connected to a chart recorder was employed for the evaluation of LV pressures and + and - dP/dt. Echocardiography of the infarcted mouse heart does not provide consistent reliable measurements but only an indication of the absence or presence of contraction in the wall.

**Infarct Size**

Two processes that vary with time occur simultaneously in the infarcted heart: 1. Shrinkage of the infarcted region with healing and scar formation; and 2. Myocyte growth in the unaffected portion of the ventricle, which expands chronically the surviving
myocardium. These phenomena cannot be separated and independently measured complicating the assessment of infarct size at distinct time points after coronary occlusion. Moreover, scattered apoptotic myocyte death occurs within the viable myocardium after infarction diminishing further the number of contracting cells. Therefore, the number of myocytes lost and remaining provide an appropriate characterization of the dimension of the infarct and extent of recovery with time, respectively. Importantly, these variables are the determinants of cardiac function. To obtain this information, the number of myocytes in the LV of control and infarcted hearts was obtained employing a methodology well-established in our laboratory.\textsuperscript{6,7} The quotient between the number of LV myocytes in sham-operated animals and the number of myocytes present in the infarcted LVs gives the percentages of myocytes lost and remaining after infarction. The percentage of myocytes lost provides a quantitative measurement of infarct size while the percentage of myocytes left correlates with ventricular function. The myocardium formed in treated-hearts was not included in this analysis in order to evaluate the consequences of coronary ligation on infarct size, independently from tissue reconstitution at 16 days and 4 months.

**Myocyte Function**

Myocytes isolated from infarcted treated mice at 16 days (n=8) were placed in a cell bath (25 ± 0.2°C) containing 1.0 mM Ca\textsuperscript{2+} and stimulated at 0.5-2 Hz by square pulses, 3-5 ms in duration and twice diastolic threshold in intensity.\textsuperscript{1} Cell mechanics were measured utilizing a CCD camera (Myocam) and IonWizard 4.4 software (IonOptix) in SarcLength mode, for sarcomere length measurement by fast Fourier transform analysis.

**DNA Content**

DNA content was measured by PI labeling of nuclei and confocal microscopy. Sampling: infarcted-treated mice=600 new myocytes, 1,000 old myocytes; lymphocytes=1000. X-chromosomes were detected by FISH.\textsuperscript{5}

**Data Analysis**
Morphometric data were collected blindly. Results are mean±SD. Significance between two comparisons was determined by Student’s t-test and among multiple comparisons by Bonferroni test; P <0.05 was considered significant. Mortality was measured by log-rank test.

The n values and statistical differences indicated in Figure 1F for the ELISA assay are as follows. n=4 different preparations from 4 separate isolations at each time point. For HGF, P<0.001 between 30 and 120 minutes (*). P<0.01 between 60 and 120 minutes (**). For IGF-1, P<0.05 between 30 and 60 minutes (*). P<0.001 between 30 and 120 minutes (*). P<0.01 between 60 and 120 minutes (**). The n values and statistical differences for the Western blots included in Figure 1F are as follows. n=4 different preparations from 4 separate isolations at each time point. For HGF, P<0.05 at 12 hours, P<0.01 at 24 hours and P<0.001 at 72 hours. For IGF-1, P<0.05 at 12 hours, P<0.05 at 24 hours and P<0.001 at 72 hours. Comparisons were performed between cells grown in SFM and cells stimulated with growth factors.

The n values and statistical differences indicated in Figure 2D are as follows. Atria, n=5; mid-LV, n=5; border zone (BZ), n=4; n values represent separate samples in each region. P<0.05 between atria and BZ (*). P<0.01 between mid-LV and BZ (**). The n values and statistical differences for the Western blots included in Figure 2E are as follows. n=4-10 different preparations from separate isolations at each time point. For phospho-c-Met, P=NS at 12 hours, P<0.01 at 24 hours and P<0.001 at 72 hours. For phospho-IGF-1R, P=NS at 12 hours, P<0.01 at 24 hours and P<0.001 at 72 hours. For phospho-FAK, P<0.05 at 12 hours, P<0.001 at 24 hours and P<0.001 at 72 hours. For phospho-Akt, P<0.01 at 12 hours, P<0.05 at 24 hours and P<0.001 at 72 hours. For phospho-IRS-1, P<0.001 at 12 hours, P<0.001 at 24 hours and P<0.001 at 72 hours. Comparisons were made between CSCs-ECCs from untreated and treated MI.

The n values and statistical differences indicated in Figure 8F are as follows. For ventricular hemodynamics: SO, n=21; untreated MI, 16 days, n=15, 4 months, n=10;
GFs-treated MI-T, 16 days, n=17, 4 months, n=8. For anatomical properties: SO, n=16; untreated MI, 16 days, n=9, 4 months, n=10; and GFs-treated MI-T, 16 days, n=15, 4 months, n=8. **Indicate a difference (P<0.05-0.001) versus SO (*), MI (**) and MI-T at 16 days (†). P values were obtained by the analysis of variance and the Bonferroni method. In Figure 8G, n=123 in untreated and n=107 in treated infarcts.

Limitations

Several limitations in the current study have to be acknowledged. The in vivo studies tested only one concentration of IGF-1 and we cannot exclude that higher or lower quantities of IGF-1 may have different effects on myocardial regeneration after ischemic injury. However, IGF-1 overexpression in myocytes of transgenic mice interferes with myocyte death in the surviving myocardium after infarction in the absence of myocardial reconstitution and reduction of infarct size. The lack of differences in infarct size results in no appreciable differences in cardiac function between wild-type and transgenics. Similar results have been obtained with chronic coronary artery constriction in the same model in which foci of replacement fibrosis are noticeably present in spite that the circulating level of IGF-1 increases from <3 ng/ml in wild-type mice to 5.7 ng/ml in transgenics. Again, ventricular function was impaired in a comparable manner in both groups of animals. Conversely, IGF-1 overexpression has a powerful positive impact when scattered cell death only affects myocardial structure and ventricular performance, a condition present in myocardial aging. Of relevance, migration studies in vitro with concentration of IGF-1 varying from 25 to 400 ng/ml had no appreciable impact on the motility of CSCs-ECCs. Therefore, these findings are consistent with the notion that IGF-1 does not promote a significant migration of CSCs-ECCs after infarction repairing segmental losses of myocardium.

Another issue that has to be recognized involves the difficulty to obtain preparations of CSCs-ECCs, which do not contain a fraction of other cell types including fibroblasts, ECs and SMCs. These cell populations possess the c-Met-HGF and IGF-1R-IGF-1 systems and have to be considered in the interpretation of the properties of
CSCs-ECCs in this regard. Finally, animals used for the isolation and mechanical measurements of regenerated and preexisting myocytes did not receive BrdU. This protocol was implemented because exposure of BrdU labeled cells to light results in DNA damage and death of the cells.\textsuperscript{15}

**References**


Online Figures and Tables

**Figure S1.** Distribution of CSCs in the heart. Number of CSCs per unit volume of myocardium expressing three, two or one surface antigen. The percentage of each population is indicated numerically in each bar. Results are mean±SD; n=8 separate hearts for each region of the myocardium. *••**Indicate a difference (P < 0.001) versus the atria and the LV base-mid region, respectively.

**Figure S2.** Atrial clusters and properties of CSCs-ECCs. Groups of Sca-1POS cells (A-F, yellow; arrowheads) in the atrial myocardium express GATA-6 (B, C, green), Ets1 (B, C, E, F, magenta) and MEF2C (E, F, white) in nuclei; they are surrounded by fibronectin (C, F, magenta). Myocytes are identified by α-sarcomeric actin (C, F, red). *Indicates cells positive for Sca-1 only (B, C, E, F). G, H, Number of Myocyte (M), SMC, EC progenitors and precursors in the heart. Progenitors had one or more surface antigens and expressed in their nuclei MEF2C (myocyte progenitors), GATA-6 (SMC progenitors) or Ets-1 (EC progenitors). Precursors had the same transcription factors and were positive for α-sarcomeric actin or cardiac myosin (myocyte precursors), α-smooth muscle actin (SMC precursors) or von Willebrand factor (EC precursors). Values are per unit volume of myocardium. Results are presented as mean ± SD. *••**Indicate a difference (P < 0.001) from the atria and LV base-mid region, respectively. The antibodies employed may not have the ideal specificity since a high degree of homology exists among members of the GATA and MEF2 family of proteins. However, the localization of GATA-4 and MEF2C in nuclei of adjacent small developing cardiomyocytes (see Figure 1C) and in a fraction of fully differentiated myocytes is consistent with their specificity.

**Figure S3.** Expression of c-Met and IGF-1R on CSCs-ECCs. A-C, Atrial myocardium with four small cells expressing c-kit (A, green; arrowheads), c-Met (B, yellow), and c-kit and c-Met (C, green-yellow). D-F, Six small cells express MDR1 (D, magenta) and two of these IGF-1R (E, white; arrowheads). The coexpression of MDR1 and IGF-1R is shown in panel F (magenta-white). G-I, Six small cells express Sca-1 (G, yellow) and
five of these c-Met (H, green; arrowheads). The coexpression of Sca-1 and c-Met in shown in panel I (yellow-green). J-L, Three small cells express MDR1 (J, magenta) and two of these express c-Met (K, yellow; arrows) and/or IGF-1R (L, white; arrowheads). *Indicates a MDR1\textsuperscript{POS} cell that expresses both c-Met and IGF-1R.

**Figure S4.** Effects of growth factors on matrix invasion of CSCs-ECCs. (A) The preparations included cells collected with c-kit\textsuperscript{POS}, MDR1\textsuperscript{POS} or Sca-1\textsuperscript{POS} immunobeads only. Results are presented as mean±SD; n=4 separate hearts. *Indicates a difference (P<0.001) versus identical cell populations not exposed to the growth factors. (B) The preparation employed had an average of 84% of cells positive for c-kit (B, green), MDR1 or Sca-1. The cell preparation included 84% CSCs-ECCs, 5% ECs, 10% fibroblasts, 1% SMCs.

**Figure S5.** Effects of HGF and/or IGF-1 on the death and growth of CSCs-ECCs. Results are mean±SD; n=6 separate preparations. *Indicates a difference (P<0.001) versus untreated (serum free medium: SFM); **Indicates a difference (P<0.001) versus HGF-treated cells.

**Figure S6.** Proliferation and EGFP tagging of CSCs-ECCs. A-F, Sections of atrial myocardium: BrdU incorporation (A, D; white), and Ki67 (B, E; yellow) and MCM5 (C, F; green) labeling of c-kit\textsuperscript{POS} (green) CSCs (*) and cells committed to the myocyte (D; MEF2C, red), SMC (E; GATA-6, red) and EC (F; Ets-1, red) lineages. They correspond, respectively, to myocyte, SMC and EC progenitors. c-kit\textsuperscript{POS} cells in panels A-C express GATA-4 (red) representing cardiac progenitors.

**Figure S7.** Proliferation of CSCs-ECCs in the atrial myocardium. BrdU was injected twice a day for 3 days to determine the accumulation of newly formed cells during this interval. Ki67 and MCM5 were employed to evaluate the fraction of cycling cells at the time of observation. These measurements were obtained in situ. Results are mean±SD; n=8 animals.
Figure S8. Proliferation and EGFP tagging of CSCs-ECCs. **A-D.** Sections of atrial myocardium: CSCs-ECCs were identified by the presence of c-kit (**A, C, D**, white; arrowheads) and Sca-1 (**B**, yellow; arrowheads). Some of these cells express EGFP (green; asterisks). GATA-4 (**A, B**; red, cardiac progenitors), MEF2C (**C**; yellow, myocyte progenitors), MEF2C together with α-sarcomeric actin (**C**; red, myocyte precursor; arrow) and Ets-1 (**D**; white, EC progenitors) are also found.

Figure S9. Protocol of GF-administration. Schematic representation of the intervention performed in the infarcted mouse heart. Doses of growth factors and sites of injection are indicated numerically. The titer of EGFP retrovirus is also listed. Shaded area corresponds to the cardiac region examined first by two-photon microscopy and then by confocal microscopy.

Figure S10. GF-mediated migration of EGFP$^{POS}$ cells. **A-H,** Four examples of translocation of EGFP$^{POS}$ cells to the mid-region of acute infarcts. Pairs of panels (**A and B, C and D, E and F, and G and H**) illustrate the detection by confocal microscopy of EGFP$^{POS}$ cells (green) within the infarcted myocardium; these fields of myocardium were previously examined by two-photon microscopy to identify the migrated EGFP$^{POS}$ cells (not shown). Panels **A, C, E and G** show viable, hairpin $^{NEG}$ PI-labeled (blue) EGFP$^{POS}$ cells within the infarct. Panel **B** depicts EGFP$^{POS}$ cells expressing Sca-1 (yellow) and GATA-4 (red; cardiac progenitors, arrows); panel **D** shows EGFP$^{POS}$ cells expressing c-kit (white) and MEF2C (yellow; myocyte progenitors, arrows); panel **F** illustrates EGFP$^{POS}$ cells expressing MDR1 (magenta) and GATA-6 (white; SMC progenitors, arrows); panel **H** demonstrates EGFP$^{POS}$ cells expressing Sca-1 (yellow) and Ets-1 (magenta; EC progenitors, arrows).

Figure S11. Effects of GFs on the migration of EGFP-CSCs-ECCs. Results are mean±SD.

Figure S12. Effects of GFs on the number of mobilized CSCs-ECCs. The number of EGFP$^{POS}$-CSCs-ECCs between the AV-groove and mid-LV-wall (length=2.15±0.31 mm; width=1.59±0.28 mm) increased in GF-treated infarcted hearts. Infarcted and sham-
operated (SO) hearts non-treated with GFs had no moving EGFP\textsuperscript{POS}-CSCs-ECCs; they were restricted to the AV-groove. Similarly, EGFP\textsuperscript{NEG}-CSCs-ECCs increased in GF-treated infarcted hearts. The number EGFP\textsuperscript{POS} and EGFP\textsuperscript{NEG} CSCs-ECCs per mm\textsuperscript{3} of myocardium increased from a value of 380±78 in SO to a value of 3,007±789 after infarction and GF-treatment. Results are mean±SD.

**Figure S13.** Expression of GF receptors in CSCs-ECCs. Distribution of c-Met and IGF-1R on CSCs-ECCs in the heart of SO, GF-treated infarcted and untreated infarcted mice acutely after coronary artery occlusion. Results are presented as mean ± SD. A, atria; LV, left ventricle; R, remote region; B, border zone; I, infarct.

**Figure S14.** GF-administration and lineage commitment of CSCs-ECCs. Number of CSCs and number of progenitors-precursors of myocytes, SMCs and ECs per unit volume of myocardium. Results are presented as mean ± SD. *Indicates a difference (P < 0.001) versus sham-operated (SO) animals. MI, myocardial infarction.

**Figure S15.** Pathway of migration of EGFP\textsuperscript{POS} cells. **A-C,** *Ex vivo* heart preparation perfused with an oxygenated Tyrode solution examined by two-photon microscopy. These images correspond to viable myocardium of the border zone (BZ) and dead myocardium of the infarcted region (MI) 8 hours after coronary occlusion and 3 hours after the administration of GFs. The coronary circulation was perfused with rhodamine-labeled dextran (red) for its detection. These 3 images of the same field were taken 20 minutes apart (**A**=baseline; **B**=20 minutes after baseline; **C**=40 minutes after baseline). The edge of the MI and viable BZ are visible in panels **A-C.** There is little labeling of the coronary vessels in the infarcted region. Colored arrowheads indicate EGFP\textsuperscript{POS} cells (green) moving in the direction of the large open arrows in 40 minutes. In all cases, EGFP\textsuperscript{POS} cells were outside of the coronary vessels. In panel **D,** the EGFP\textsuperscript{POS} cells detected in the living myocardium by two-photon microscopy (**C,** square) are shown at higher magnification by confocal microscopy after fixation and staining of the same myocardial region. Green fluorescence identifies the same EGFP\textsuperscript{POS} cells (**C, D,** arrows).
In panel D, EGFP\textsuperscript{POS} cells express Sca-1 (yellow; arrows). Myocytes are labeled by cardiac myosin heavy chain (red). In panel D, bright fluorescence corresponds to hairpin \textsuperscript{1POS} apoptotic myocyte nuclei; viable EGFP\textsuperscript{POS} cells are hairpin \textsuperscript{1NEG} and are labeled by PI only (blue).

**Figure S16.** Myocardial infarction and cell death. Section of LV myocardium of an untreated infarcted heart obtained 8 hours after surgery and 3 hours after saline injection. Apoptosis in myocyte (red, myosin) and c-kit\textsuperscript{POS} cell (green) nuclei is shown by hairpin 1 labeling (white dots, arrows). Viable nuclei are stained by PI alone (blue). One viable c-kit\textsuperscript{POS} cell is present within the infarcted myocardium (arrowhead).

**Figure S17.** Myocardial infarction and cell death. Section of LV myocardium of a GF-treated infarcted heart obtained 8 hours after surgery and 3 hours after GF-administration. The yellow line separates the infarcted myocardium (MI) with apoptotic myocyte nuclei indicated by hairpin 1 labeling (white dots, arrows) from the border zone (BZ) with viable myocyte nuclei stained by PI alone (blue). Viable c-kit\textsuperscript{POS} cells (blue nuclei, PI; c-kit, green) are present in MI and BZ (arrowheads).

**Figure S18.** Distribution and viability of CSCs-ECCs. Number of viable and dead CSCs-ECCs in SO, GF-treated infarcted and untreated infarcted hearts of mice sacrificed 7-8 hours after surgery and 2-3 hours after the administration of GFs. A, atria; LV, left ventricle; R, remote region; B, border zone; I, infarct. Results are presented as mean ± SD. *,**Indicate a difference (\(P < 0.05-0.001\)) from SO and GF-treated infarcted hearts, respectively.

**Figure S19.** Growth of CSCs-ECCs. The upper panel shows cycling c-kit\textsuperscript{POS} cells (green; arrows) in the acutely infarcted myocardium (yellow dots, apoptotic nuclei) of mice treated with GFs. Magenta fluorescence in c-kit\textsuperscript{POS} cells corresponds to MCM5 labeling of their nuclei (arrowheads). The lower panel shows the percentage of CSCs-ECCs labeled by MCM5 in SO, GF-treated infarcted and untreated infarcted hearts of mice.
sacrificed acutely after infarction. Results are presented as mean ± SD. A, atria; LV left ventricle; R, remote region; B, border zone; I, infarct.

**Figure S20.** Differentiation of EGFP<sup>POS</sup>-cells. A, Small-arterioles (red; α-smooth-muscle-actin) with EGFP<sup>POS</sup>-SMCs (yellow-green; arrowheads). TER-119-labeled-erythrocytes (yellow) are present in the lumen. B, capillaries (red, von Willebrand factor; arrows); two of them are EGFP<sup>POS</sup> (yellow-green; arrowheads).

**Figure S21.** Infarct size. To make valid comparisons of infarct size in mice sacrificed at 16 days and 4 months, the number of myocytes lost and remaining in the LV and interventricular septum was measured in untreated (MI) and GF-treated (MI-T) mice. The percent of myocytes lost (infarct size) is indicated in the upper portion of each bar. Untreated mice had smaller infarcts than GF-treated mice ($P<0.001$). Number of mice: SO, n=19; MI 16d, n=15; MI-T 16d, n=22; MI 4m, n=10; MI-T 4m, n=8.

**Table S1.** Number of surviving and dead untreated and treated mice.

**Table S2.** Volume composition of the infarcted myocardium in unsuccessfully GF-treated mice.

**Figure S22.** Myocardial regeneration after infarction. On the basis of the volume of LV in SO mice and infarct size, 42% in untreated mice (MI) and 67% in GF-treated mice (MI-T) at 16 days, the volume of myocardium destined to remain (R) and destined to be lost (L) was computed in the two groups of infarcted mice. The volume of newly formed myocardium (F) was measured quantitatively in treated mice. Myocardial regeneration increased the volume of remaining myocardium (R+F) and decreased the volume of lost myocardium (L minus F) by the same amount. An identical analysis was done at 4 months after infarction, as shown in the lower panel. Results are mean±SD. *Indicates a difference in myocardial mass produced by myocardial regeneration. Number of mice: SO, n=19; MI 16d, n=15; MI-T 16d, n=22; MI 4m, n=10; MI-T 4m, n=8. At 16 days, $P<0.01$; at 4 months, $P<0.001$. 
**Figure S23.** Myocardial regeneration. New-arterioles (A, B; α-smooth-muscle-actin, red) in GF-treated-infarcted-mice at 16-days (A) and 4-months (B). BrdUPos-nuclei (white dots). TER-119-labeled-erythrocytes (A, B, yellow).

**Figure S24.** Myocyte regeneration in the non-infarcted myocardium. BrdU labeling of myocytes distant from (upper panel) and adjacent to (lower panel) the infarct at 4 months. Nuclei (PI, dark blue only); BrdU-PI-labeled nuclei (white; arrowheads). Cardiac myosin (red) and laminin around myocytes (yellow).

**Figure S25.** Cytoplasmic and plasmamembrane markers of developing myocytes. A-F, BrdU labeling of nuclei (A, C, E; green) and localization of nestin (B, red), desmin (D, red), cardiac myosin heavy chain (F, red) in myocytes of tissue sections of regenerating myocardium at 16 days. Nuclei are labeled by BrdU and PI (B, D, F; bright blue). G-J, Identification of connexin 43 (G, H; yellow) and N-cadherin (I, J; yellow) in sections of developing myocardium. Myocytes are stained by cardiac myosin heavy chain (H, J; red) and nuclei by BrdU only (G, I; green) or by BrdU and PI together (H, J; bright blue). White fluorescence corresponds to laminin staining.

**Figure S26.** HGF, IGF-1 and myocardial regeneration. The separate effects of HGF and IGF-1 and their potential synergism on cardiac regeneration were measured 16 days after infarction. In 2 of 8 surviving IGF-1-only treated-mice, a small area of forming myocardium was detected near the border zone (A, arrowheads). Regeneration was absent in the other mice. Conversely, HGF alone led to a regenerating band within the length of the infarct in 6 out of 6 mice (B, arrowheads). However, the new tissue occupied only 35±7% of the infarct and myocytes were smaller, 1,100±280 µm³, than those obtained with both GFs. Myocytes are stained by cardiac myosin (red) and nuclei by PI (green).

**Figure S27.** Contractile performance of myocytes. Sarcomere shortening was evaluated in each cell at three different frequencies of stimulation: 0.5, 1 and 2 Hz. Max –dL/dt,
maximal velocity of shortening; Max +dL/dt, maximal velocity of re-lengthening. Measurements are presented as mean±SD. *Indicates (P<0.05) vs surviving myocytes.

**Figure S28.** Frequency distribution of DNA content in non-cycling (red line) and cycling (green line; Ki67 positive) myocyte and lymphocyte nuclei. New and old myocytes and lymphocytes showed a 2n population. DNA content greater than 2n was restricted to cycling cells.

**Figure S29.** Circulating bone marrow progenitor cells. Scatter plots of CD45 and c-kit positive cells in the peripheral blood of splenectomized mice, at baseline (A); following intramyocardial injection of 1.4 ng HGF or intravenous injection of 5 µg HGF (B); Positive control mice (C) were injected subcutaneously for 5 days with a daily dose of G-CSF, 200 µg/kg, and SCF, 50 µg/kg. The minimal amount of CD45 and c-kit positive cells in the blood of untreated animals (A) was not affected by the local or intravenous (B) injection of HGF. Conversely, G-CSF and SCF together increased significantly the pool of bone marrow progenitor cells in the blood.

**Figure S30.** Wound healing assay. Movement of clonogenic EGFP-positive CSCs following disruption of the monolayer from time 0 to 120 minutes (m).

**Table S3.** Primers and antibodies used for biochemical and FACS analysis.

**Table S4.** Immunostaining, autofluorescence, fluorochromes and digital processing.
Table S1

**Number of surviving and dead untreated and treated infarcted mice**

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<thead>
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<th>Untreated Mice</th>
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hs, hours; ds, days.
### Table S2

**Characteristics of unsuccessfully treated infarcted mice**

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<td><strong>16 days</strong></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Unsuccessfully treated infarcted mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>79.9</td>
<td>4.73</td>
<td>96.4</td>
</tr>
<tr>
<td>2</td>
<td>91.0</td>
<td>4.67</td>
<td>111.6</td>
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<tr>
<td>3</td>
<td>87.2</td>
<td>5.23</td>
<td>149.2</td>
</tr>
<tr>
<td>4</td>
<td>89.7</td>
<td>4.57</td>
<td>99.3</td>
</tr>
<tr>
<td></td>
<td>Untreated infarcted mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average ± SD</td>
<td>85.8 ± 7.8</td>
<td>4.81 ± 0.45</td>
</tr>
<tr>
<td><strong>4 months</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Unsuccessfully treated infarcted mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>84.2</td>
<td>6.25</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>90.8</td>
<td>7.22</td>
<td>265</td>
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<tr>
<td></td>
<td>Untreated infarcted mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average ± SD</td>
<td>84.9 ± 10.3</td>
<td>6.95 ± 0.56</td>
</tr>
</tbody>
</table>

Values obtained in unsuccessfully treated infarcted mice are within the range defined by the standard deviation of the mean in untreated infarcted mice.
Table S3

**Primers and antibodies for biochemical and FACS analyses**

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR conditions</th>
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<tbody>
<tr>
<td>HGF mRNA</td>
<td>94°C 5 min</td>
</tr>
<tr>
<td>GeneBank # XM_131908 (Superarray)</td>
<td>94°C 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50°C 30 sec</td>
</tr>
<tr>
<td></td>
<td>30 cycles</td>
</tr>
<tr>
<td>IGF-1 mRNA</td>
<td>72°C 45 sec</td>
</tr>
<tr>
<td>GeneBank # NM_010512 (Superarray)</td>
<td>4°C ∞</td>
</tr>
</tbody>
</table>

**Antibodies for Western blot**

- Phospho(Ser473)-Akt: rabbit polyclonal
- Phospho(Tyr397)-Focal Adhesion Kinase: rabbit polyclonal
- Phospho(Tyr612)-IRS-1: rabbit polyclonal
- Phospho(Tyr1230/1234/1235)-c-Met: rabbit polyclonal
- Phospho(Tyr1158/1162/1163)-IGF-1R: rabbit polyclonal
- Mouse IGF-1: mouse monoclonal
- Mouse HGF-α: goat polyclonal

**Antibodies for FACS**

- CD45: TRITC-conjugated mouse monoclonal
- CD117: PE-conjugated mouse monoclonal
- Phospho(Tyr1234/1235)-c-Met: rabbit polyclonal/indirect fluorescence
### Immunolabeling protocol

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Labeling</th>
<th>Fluorochrome(s)</th>
<th>Color in illustrations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stem cell markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-kit</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, TRITC</td>
<td>green, white</td>
</tr>
<tr>
<td>MDR1</td>
<td>goat polyclonal</td>
<td>indirect</td>
<td>FITC, TRITC</td>
<td>magenta</td>
</tr>
<tr>
<td>Sca-1</td>
<td>rat monoclonal</td>
<td>direct and indirect</td>
<td>FITC, TRITC</td>
<td>yellow</td>
</tr>
<tr>
<td><strong>Transcription factors of cardiac cell lineages</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GATA-4</td>
<td>rabbit polyclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>yellow, red</td>
</tr>
<tr>
<td>MEF2C</td>
<td>goat polyclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>white, red</td>
</tr>
<tr>
<td>GATA-6</td>
<td>goat polyclonal</td>
<td>indirect</td>
<td>FITC, Cy5</td>
<td>green, white</td>
</tr>
<tr>
<td>Ets-1</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>FITC, Cy5</td>
<td>white, magenta</td>
</tr>
<tr>
<td><strong>Structural proteins of myocardial cells</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-sarcomeric actin</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, TRITC, Cy5</td>
<td>red, green</td>
</tr>
<tr>
<td>cardiac myosin</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, TRITC, Cy5</td>
<td>red</td>
</tr>
<tr>
<td>connexin 43</td>
<td>rabbit polyclonal</td>
<td>direct and indirect</td>
<td>TRITC, Cy5</td>
<td>yellow</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>TRITC, Cy5</td>
<td>white, yellow</td>
</tr>
<tr>
<td>nestin</td>
<td>rat monoclonal</td>
<td>indirect</td>
<td>FITC, TRITC</td>
<td>red</td>
</tr>
<tr>
<td>desmin</td>
<td>mouse monoclonal</td>
<td>indirect</td>
<td>FITC, TRITC</td>
<td>red</td>
</tr>
<tr>
<td>α-smooth actin</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, TRITC, Cy5</td>
<td>red, magenta</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>TRITC, Cy5</td>
<td>red, yellow</td>
</tr>
<tr>
<td>phospho-FAK</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>TRITC, Cy5</td>
<td>white</td>
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<tr>
<td><strong>Extracellular matrix proteins</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Collagen I and III</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>Cy5</td>
<td>blue</td>
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<tr>
<td>laminin</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>Cy5</td>
<td>white, blue, yellow</td>
</tr>
<tr>
<td>fibronectin</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>Cy5</td>
<td>yellow, magenta</td>
</tr>
<tr>
<td><strong>Other stainings</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP</td>
<td>rabbit polyclonal</td>
<td>direct and indirect</td>
<td>FITC, TRITC, Cy5</td>
<td>green</td>
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<tr>
<td>Ki67</td>
<td>rabbit polyclonal</td>
<td>indirect</td>
<td>FITC, TRITC</td>
<td>not shown</td>
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<tr>
<td>BrdU</td>
<td>mouse monoclonal</td>
<td>indirect</td>
<td>TRITC, Cy5</td>
<td>white, blue</td>
</tr>
<tr>
<td>Nuclear DNA</td>
<td>DAPI, PI</td>
<td>direct</td>
<td>blue, red</td>
<td>blue, green</td>
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<tr>
<td>X chromosome</td>
<td>DNA probe</td>
<td>direct</td>
<td>Cy3</td>
<td>white</td>
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<tr>
<td>hairpin 1</td>
<td>oligonucleotide</td>
<td>indirect</td>
<td>FITC</td>
<td>white</td>
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</table>
**Hematopoietic markers**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antibody Type</th>
<th>Labeling Method</th>
<th>Fluorochromes</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-2</td>
<td>rabbit polyclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>not shown</td>
</tr>
<tr>
<td>CD45</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>not shown</td>
</tr>
<tr>
<td>CD45RO</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>not shown</td>
</tr>
<tr>
<td>CD8</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>not shown</td>
</tr>
<tr>
<td>CD20</td>
<td>mouse monoclonal</td>
<td>direct and indirect</td>
<td>FITC, Cy5</td>
<td>not shown</td>
</tr>
<tr>
<td>TER-119</td>
<td>mouse monoclonal</td>
<td>indirect</td>
<td>FITC, Cy5</td>
<td>yellow</td>
</tr>
</tbody>
</table>

*Autofluorescence (expressed in 8-bit intensity scale)*

<table>
<thead>
<tr>
<th>Section</th>
<th>Specific signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3 Section</td>
<td>100-255</td>
</tr>
<tr>
<td>Section + secondary</td>
<td></td>
</tr>
</tbody>
</table>

**Direct labeling**: primary antibody conjugated to the fluorochrome. **Indirect labeling**: species-specific secondary antibody conjugated with the fluorochrome.

*Autofluorescence*. During confocal analysis of the sections, the values of signals were set to be between 100 and 255 on an 8-bit intensity scale. This was achieved by adjusting the power of the lasers and the gain of the photomultipliers. The autofluorescence of the section alone and together with the unspecific fluorescence of the secondary antibody was measured. As shown in the Table, the actual signals were at least 20-fold higher than non-specific fluorescence. For more information, see reference 48 in the manuscript.

**Digital processing**. The need for showing two different structures with the same color (for example Ets1 and c-kit in Fig. 2d are both illustrated by white fluorescence) was due to the fact that only 4 fluorochromes were available and, at times, more than 4 antigens were labeled in the same section. For example, during the examination of the tissue by confocal microscopy, the presence of c-kit which was labeled by FITC (green fluorochrome) was obtained together with EGFP which was labeled by TRITC (red fluorochrome) and α-sarcomeric actin which was labeled by Cy5 (infrared fluorochrome). Subsequently, the section was washed and stained for Ets1 and nuclear DNA. The presence of Ets1 which was labeled by FITC (green fluorochrome) was recorded together with the signal from nuclei which were labeled by propidium iodide (red fluorochrome).

Since 5 distinct recordings were obtained and only 4 fluorochromes were employed, the 5 separate signals were analyzed independently for specificity. The 5 recordings were then converted into grayscale images and assembled as separate layers in 1 micrograph using Adobe Photoshop. This software was utilized to assign the 4 colors to 5 distinct layers. In the example, white was used twice. Finally, Adobe Photoshop was utilized to adjust images in order to create an equivalent signal distribution among various panels. This is a commonly employed procedure in confocal microscopy [Eriksson, P.S., et al. Neurogenesis in the adult human hippocampus. *Nat. Med.* **4**, 1313-1317 (1998)].
Cardiac Stem Cells

Cells/10 mm³ of myocardium

<table>
<thead>
<tr>
<th>c-kit</th>
<th>MDR1</th>
<th>Sca-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ + +</td>
<td>+ + +</td>
<td>+ - +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base-Mid</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apex</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Figure S1
Figure S2G, H

Cardiac Cell Precursors

- Atria
- Base-Mid
- Apex

Cardiac Cell Progenitors

- Atria
- Base-Mid
- Apex
Figure S3 A-F
Invasion Assay

**Figure S4**

(A) Bar charts showing cell number for c-kit\(^{POS}\) Cells, MDR1\(^{POS}\) Cells, and Sca-1\(^{POS}\) Cells under different conditions: C, HGF, IGF-1. The asterisk (*) indicates a significant difference.

(B) Microscope image showing expression of c-kit and PI with a scale bar of 10 \(\mu\)m.
Cycling c-kit$^\text{POS}$, MDR1$^\text{POS}$ and Sca-1$^\text{POS}$ CSCs-ECCs

![Bar graph showing the percentage of BrdU, Ki67, and MCM5](Figure S7)
Figure S8
Figure S9

1: HGF: 50 ng/ml  
IGF-1: 200 ng/ml  

2: HGF: 100 ng/ml  
IGF-1: 200 ng/ml  

3-4: HGF: 200 ng/ml  
IGF-1: 200 ng/ml  

AV-Groove EGFP-Retrovirus (10^6 c.f.u.)

2 DAYS

Two-Photon Microscopy

Myocardial Infarction
Figure S11

Speed of Migration

μm/h

HGF | HGF+IGF-1 | IGF-1 | HGF | IGF-1
---|---------|-------|-----|-----

*
Mobilized CSCs - ECCs

3,000 CSCs - ECCs; 50,000 myocytes; 150,000 cardiac cells

Figure S12
Figure S13
Figure S14
Viable CSCs-ECCs

- Cells/mm³ of myocardium
- SO
  - A LV
- GF-Treated
  - A R B I
  - *
- Untreated
  - A R B I
  - **

Dead CSCs-ECCs

- Cells/mm³ of myocardium
- SO
  - A LV
- GF-Treated
  - A R B I
  - *
- Untreated
  - A R B I
  - **

Figure S18
Cycling CSCs-ECCs

Figure S19
Number of Myocytes Lost (Infarct Size) and Spared

Myocytes, $\times 10^6$

- **SO**: 3.5
  - **MI**: 2.0 (42%)
  - **MI-T**: 1.3 (67%)
- **MI**: 2.0
  - **MI-T**: 1.3 (43%)

**Figure S21**
Viable and Lost Myocardium

16 Days

4 Months

Figure S22
Figure S25 A-F
Myocyte Mechanics

**Sarcomere Shortening**

<table>
<thead>
<tr>
<th></th>
<th>Spared</th>
<th>New</th>
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<tbody>
<tr>
<td>0.5Hz</td>
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<tr>
<td>1.0Hz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0Hz</td>
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<td></td>
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</tbody>
</table>

**Max -dL/dt**

<table>
<thead>
<tr>
<th></th>
<th>Spared</th>
<th>New</th>
</tr>
</thead>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.0Hz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0Hz</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Max +dL/dt**

<table>
<thead>
<tr>
<th></th>
<th>Spared</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5Hz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0Hz</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0Hz</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates significant difference.
DNA Content

- New Myocytes
- Old Myocytes
- Lymphocytes

Figure S28
Figure S29
Figure S30