Human Cardiac Stem Cell Differentiation Is Regulated by a Mir346b Mechanism

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Background—Cardiac stem cells (CSCs) delivered to the infarcted heart generate a large number of small fetal-neonatal cardiomyocytes that fail to acquire the differentiated phenotype. However, the interaction of CSCs with postmitotic myocytes results in the formation of cells with adult characteristics.

Methods and Results—On the basis of results of in vitro and in vivo assays, we report that the commitment of human CSCs (hCSCs) to the myocyte lineage and the generation of mature working cardiomyocytes are influenced by microRNA-499 (miR-499), which is barely detectable in hCSCs but is highly expressed in postmitotic human cardiomyocytes. miR-499 traverses gap junction channels and translocates to structurally coupled hCSCs favoring their differentiation into functionally competent cells. Expression of miR-499 in hCSCs represses the miR-499 target genes Sox6 and Rod1, enhancing cardiomyogenesis in vitro and after infarction in vivo. Although cardiac repair was detected in all cell-treated infarcted hearts, the aggregate volume of the regenerated myocyte mass and myocyte cell volume were greater in animals injected with hCSCs overexpressing miR-499. Treatment with hCSCs resulted in an improvement in ventricular function, consisting of a better preservation of developed pressure and positive and negative dP/dt after infarction. An additional positive effect on cardiac performance occurred with miR-499, pointing to enhanced myocyte differentiation/hypertrophy as the mechanism by which miR-499 potentiated the restoration of myocardial mass and function in the infarcted heart.

Conclusions—The recognition that miR-499 promotes the differentiation of hCSCs into mechanically integrated cardiomyocytes has important clinical implications for the treatment of human heart failure. (Circulation. 2011;123:1287-1296.)

Key Words: cardiomyogenesis ■ gap junctions ■ microRNA ■ mir346b ■ stem cells

M yocyte turnover in the adult heart is regulated by cardiac stem cells (CSCs) that generate new myocytes integrated structurally and functionally with the adjacent myocardium.1 A similar phenomenon occurs with aging when cell renewal is enhanced by translocation, growth, and differentiation of CSCs, which lead to the formation of cardiomyocytes with characteristics indistinguishable from the neighboring cells.2 This recovery of the integrity of the myocardium does not occur with acute and chronic infarcts after the injection of CSCs or their local activation by growth factors.3,4 A large number of myocytes are formed, but the new cells consist predominantly of clusters of small, proliferating, mononucleated myocytes.5 Myocyte differentiation is partly prevented, suggesting that incomplete silencing of the gene program that governs the primitive state of CSCs interferes with the acquisition of the adult cell phenotype. Alternatively, CSCs may be arrested in the early committed phase by prolonged upregulation of the Notch1 receptor telomerase and Nkx2.5, which are the molecular determinants of amplifying myocytes.6

Clinical Perspective on p 1296

The occasional migration of CSCs from the border zone to the remote myocardium results in the formation of fully mature myocytes, mimicking cell turnover.6 Although the mechanism regulating the growth of CSCs into small versus large myocytes is currently unknown, coupling of CSCs with postmitotic myocytes may be critical in determining their fate. MicroRNAs (miRs) are small RNAs that have the ability to traverse gap junctions7 and by this means may migrate from cardiomyocytes to CSCs dictating their destiny. In the present study, we tested whether the commitment of CSCs to the myocyte lineage depends on their interaction with the surrounding myocytes with

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translocation of miRs, which promote CSC differentiation. Additionally, the effects of CSCs overexpressing miR were evaluated in vivo after myocardial infarction.

Methods

Detailed methods are available in the online-only Data Supplement.

Human CSCs and Rat CSCs

Human and rat myocardial samples were enzymatically dissociated, and c-kit–positive human CSCs (hCSCs) and rat CSCs (rCSCs) were obtained.

miR Microarray, Quantitative Reverse-Transcription Polymerase Chain Reaction, and Western Blotting

RNA was extracted and subjected to miR microarray or quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Protein lysates of hCSCs, human myocytes, rCSCs, and rat myocytes were used to measure Sox6 and Rod1.

miR-499 Transfer

Cardiomyocytes were cocultured with hCSCs, and luciferase reporter assays were used to document the functional competence of translocated miR-499.

Myocardial Infarction

Myocardial infarction was produced in rats, and hCSCs infected with an miR-499–enhanced green fluorescence protein (EGFP) lentivirus were injected into the border zone. At the time of death, ventricular function was determined and the heart was fixed for histology.

Statistical Analysis

Continuous measurements are presented as mean±SD. Comparisons between 2 groups were determined by the unpaired Student t test and >2 groups by the Newman-Keuls method. The latter test was performed only if the overall comparison based on 1-way ANOVA was statistically significant. In all cases, variances were assumed to be equal, and variables were assumed to be normally distributed. Values of P<0.05 were considered significant.

Results

Expression of miR-499 in the Heart

miRs are a class of small noncoding RNAs that negatively regulate gene expression by repressing protein translation or promoting mRNA degradation. These short RNAs are not uniformly distributed in the organism but display a preferential localization that is organ and cell specific. Initially, the miR profile of the mouse myocardium was determined (Table I in the online-only Data Supplement). The rat model was then used to isolate a larger number of myocytes and rCSCs for the evaluation of the differential expression of miRs in these 2 cell classes. By array analysis (Table II in the online-only Data Supplement), miR-1, miR-22, miR-30a, miR-30b, miR-30c, miR-133a, miR-133b, and miR-499 were abundant in myocytes (Figure 1A). Although miR-1, miR-133a, and miR-133b were well expressed in rCSCs, the level of miR-499 was much higher in myocytes than in rCSCs. A 400-fold difference was found by qRT-PCR (Figure 1B and Figure 1 in the online-only Data Supplement).

Myogenic miRs are positioned within introns of myosin genes. In a manner similar to miR-208, which is located within the intron of the α-myosin heavy chain gene, miR-499 is placed in the intron of the recently identified Myh7b gene. Myh7b is present in the brain, heart, skeletal muscle, and testis. It has only 70% homology with α- and β-myosin heavy chain (Figure IIA in the online-only Data Supplement), suggesting that Myh7b represents a distinct myosin gene with unknown function.

miR-499 and Myh7b are encoded in the same orientation in all species (Figure IIB in the online-only Data Supplement), indicating that they are transcribed concurrently. The level of Myh7b measured by qRT-PCR was 33-fold higher in rat cardiomyocytes than in rCSCs (Figure 1C and Figure 1 in the online-only Data Supplement). A similar behavior was observed for miR-499, raising the possibility that translocation of miR-499 from cardiomyocytes to structurally coupled CSCs conditions their commitment to the myocyte lineage and cell differentiation.

miR-499 Target Genes

The findings in the rodent heart raised the question of whether a similar differential expression of miR-499 was present in human myocytes and hCSCs. miR-499 was essentially undetectable in hCSCs, whereas extremely high levels were found in cardiomyocytes (Figure 2A and Figure II in the online-only Data Supplement). A Web-based target-prediction program was then used to identify putative target genes of miR-499 (Table III in the online-only Data Supplement). Among the genes that scored as strong predicted targets, on the basis of high complementarity and evolutionary conservation, we selected 2 genes: SRY (sex determining region Y)-box 6 (Sox6) and regulator of differentiation 1 (Rod1).

Sox6 is a transcription factor implicated in early myocyte commitment in embryos, and Rod1 is an RNA binding protein that negatively modulates cell differentiation. The 3′
untranslated region (UTR) of Sox6 contains 5 evolutionarily conserved miR-499 binding sites. Human Sox6 possesses 1 additional nonconserved miR-499 binding site. Two evolutionarily conserved sites are located in the 3’ UTR of Rod1. These putative binding sequences show high complementarity with the seed region of miR-499 (Figure III in the online-only Data Supplement), suggesting that miR-499 negatively regulates Sox6 and Rod1 in hCSCs.

Sox6 and Rod1 transcripts were measured in human and rat myocytes and CSCs. Sox6 mRNA was 49-fold higher in human myocardium than in hCSCs, but Rod1 mRNA did not differ in these cell classes. Sox6 was 5-fold more abundant in rat myocytes than in rCSCs, and Rod1 was 8-fold higher in rCSCs than in cardiomyocytes (Figure 2B and Figure I in the online-only Data Supplement). Importantly, Sox6 and Rod1 proteins were barely detectable in human and rat myocytes but were apparent in both hCSCs and rCSCs (Figure 2C).

To test whether miR-499 binds to Sox6 and Rod1 mRNA interfering with translation, a reporter assay was performed. We used 3T3 fibroblasts to minimize the influence of
endogenous miR-499. The 3' UTRs of Sox6 and Rod1 were obtained by PCR and ligated downstream the luciferase coding sequence in 2 reporter plasmids. The interaction of miR-499 and the 3' UTRs of Sox6 and Rod1 was expected to downregulate luciferase expression. The 3T3 cells were transfected with a plasmid carrying miR-499; the expression of miR-499 was assessed by qRT-PCR and found to be 2300-fold higher than in cells transfused with blank plasmid (Figure 2D and Figure I in the online-only Data Supplement). Cells overexpressing miR-499 were then cotransfected with reporter plasmids containing luciferase and the 3' UTR of Sox6 or Rod1. Cells transfected with plasmids carrying luciferase only were used as controls. Luciferase activity decreased in a dose-dependent manner with the quantity of plasmid used in the assay (Figure 2E), suggesting that Sox6 and Rod1 were target genes of miR-499.

miR-499 Transfer and Gap Junctions

The in vitro experiments included the evaluation of several parameters: translocation of miR-499 from myocytes to neighboring hSCCs via gap junctions; transcript and protein levels of the miR-499 target genes, Sox6 and Rod1, in hSCCs; and expression of transcription factors specific to myocytes in hSCCs. These in vitro assays were complemented with in vivo studies to document the effects of hSCCs overexpressing miR-499 on myocardial regeneration after infarction and on phenotypic properties and mechanical behavior of new myocytes, as well as their impact on ventricular function.

To establish whether miR-499 had the ability to migrate from donor cells to recipient hSCCs, C2C12 myoblasts were first used as donor cells. To obtain high levels of expression, C2C12 myoblasts were transfected with a plasmid carrying miR-499 (Figure 3A and Figure I in the online-only Data Supplement). C2C12 myoblasts were then cocultured with EGFP-tagged hSCCs, and the presence of miR-499 was documented by quantitative fluorescence in situ hybridization. Initially, miR-499 was restricted to C2C12 myoblasts (Figure IV in the online-only Data Supplement), but 36 hours later, miR-499 was identified in EGFP-positive hSCCs. Connexin 43 was expressed at the interface between the 2 cell types, suggesting that translocation of miR-499 required the formation of functional gap junctions (Figure 3B and 3C). miR-499 was detected in 62% of hSCCs. Importantly, this value decreased to 18% with the gap junction inhibitor 18α-glycyrrhetinic acid (α-GA), documenting the critical role of gap junction channels in miR-499 transfer.

To exclude that the translocation of miR-499 was influenced by the nonphysiological level of miR-499 in transfected C2C12 cells, a similar protocol was used with neonatal rat cardiomyocytes. EGFP-labeled hSCCs were sorted 5 days after being cocultured with cardiomyocytes and compared with hSCCs seeded alone. Expression of miR-499, measured by qRT-PCR, was 2000-fold higher in the cocultured cells (Figure 3D). This process was markedly attenuated by the gap junction inhibitor α-GA. Exposure of hSCCs to the medium obtained from myocyte cultures failed to increase miR-499 expression in hSCCs. The culture medium showed only in ~60% of the cases minimal quantities of miR-499 by qRT-PCR, although small nucleolar RNA was found in all samples (Figure IV in the online-only Data Supplement).

The localization of miR-499 in hSCCs was documented further by in situ hybridization and confocal microscopy. Thirty-six hours after coculture of these cells with cardiomyocytes, 35% of hSCCs were positive for miR-499, and this value was markedly decreased in the presence of α-GA (Figure 3E and 3F). Additionally, the use of a scrambled probe failed to label hSCCs and cardiomyocytes. At the concentration used, α-GA had no impact on the viability of hSCCs and cardiomyocytes (Figure 3G). These findings minimize the effects of the negligible amount of miR-499 present in the medium and the occasional observation of myocyte death on the translocation of miR-499 from cardiomyocytes to hSCCs.

For real-time transfer, translocation of miR-499 through gap junctions was studied in living cells by fluorescent microscopy. The 5' side of mature miR-499 was labeled with the fluorescent dye Cy3 (Cy3–miR-499). Individual hSCCs were loaded with Cy3–miR-499 together with cascade blue, a blue fluorescent dye. Transfer of cascade blue between adjacent cells occurs through gap junctions; it was used as an indicator of functional cell-to-cell coupling. hSCCs were used because they express connexin 43 mRNA but lack the protein (Figure V in the online-only Data Supplement). However, after interaction with other cells, hSCCs form gap junctions. Conversely, connexin 43 protein is widely expressed in mature cardiomyocytes, possibly confounding the protocol. Additionally, the unlabeled endogenous miR-499 in myocytes would compete with the labeled exogenous miR-499.

The translocation of cascade blue and Cy3–miR-499 to neighboring was followed by acquisition of serial images with an inverted microscope. The appearance of blue and red fluorescence, ie, cascade blue and Cy3–miR-499, in unlabeled hSCCs adjacent to the injected hSCCs was considered indicative of the formation of functional gap junctions, critical for miR-499 translocation (Figure VI in the online-only Data Supplement). To test whether the translocated miR retained its function, hSCCs transfected with a plasmid carrying miR-499 were used as donor cells, and hSCCs transfected with a luciferase plasmid containing the 3' UTR of Sox6 were used as recipient cells. Donor and recipient cells were cocultured for 3 days. Luciferase activity decreased >40% in hSCCs cocultured with donor cells transfected with miR-499. This effect was abolished by α-GA (Figure 3H). Thus, miR-499 enters hSCCs via gap junction channels, preserving its function. This biological phenomenon has been called micrRNA.

Function of miR-499 in hSCCs

To establish the role of miR-499 and its target genes in hSCC growth and differentiation, these cells were transfected with an expression vector or infected with a lentivirus carrying miR-499. The quantity of miR-499 in transfected hSCCs increased ~4000-fold, but this change did not affect Sox6 and Rod1 transcripts (Figures I and VII in the online-only Data Supplement). However, protein levels of Sox6 and Rod1 were markedly decreased (Figure 4A). Importantly, upregulation of miR-499 decreased BrdU incorporation in hSCCs, and this attenuation in hSCC growth was coupled with enhanced expression of Nkx2.5 and GATA4 (Figure 4B; Figure VIII in the online-only Data Supplement).
To establish further whether the function of miR-499 in hCSC differentiation was mediated by Sox6 and Rod1, siRNA was used to block the synthesis of these 2 target genes. hCSCs transfected with siRNA-Sox6 and siRNA-Rod1 showed a 62% and 69% reduction in mRNA for Sox6 and Rod1, respectively (Figure 4C and Figure I in the online-only Data Supplement). Additionally, the fraction of Nkx2.5- and GATA4-positive cells increased 2-fold. A 3- to 4-fold increase in the percentage of Nkx2.5- and GATA4-positive cells was found when hCSCs were transfected with both siRNA-Sox6 and siRNA-Rod1 (Figure 4D). Thus, miR-499 promotes hCSC differentiation, which is medi-
ated by the inhibitory function of this miR on Sox6 and/or Rod1 expression.

**miR-499 and Myocardial Regeneration**

Our in vitro results suggested that miR-499 may have significant implications in myocardial regeneration, favoring the formation of functionally competent myocytes. To test this possibility, hCSCs infected with a lentiviral vector carrying miR-499 and EGFP (miR-499–EGFP-hCSCs) were injected soon after infarction in the border zone of immunosuppressed rats. The high level of expression of miR-499 was confirmed by qRT-PCR (Figure IX in the online-only Data Supplement). Infarcted rats injected with EGFP-positive hCSCs (EGFP-hCSCs) were used as controls. Animals were exposed continuously to BrdU and euthanized 10 to 14 days later. In all hearts, infarct size involved 50% of left ventricular myocytes (Figure X in the online-only Data Supplement). Myocardial regeneration was detected in the 2 cell-treated groups and consisted of clusters of closely packed human cardiomyocytes (Figure 5A). The fraction of human myocytes that incorporated BrdU was \(95\%\) in both cell-treated infarcted hearts, indicating that human cells were continuously added over the 10- to 14-day period after coronary occlusion and cell implantation (Figure 5B). Similarly, the level of myocyte formation, measured by the cell cycle protein Ki67, was comparable in these 2 groups of hearts (Figure X in the online-only Data Supplement), documenting that myocyte regeneration was ongoing at the time of death (Figure 5C).

The human origin of these regenerated structures was confirmed by the detection of EGFP and human DNA sequences with an Alu probe (Figure 5D). Connexin 43 and N-cadherin were present between regenerated human myocytes (Figure 6A). Myocytes derived from differentiation of miR-499–EGFP-hCSCs appeared to be larger, and sarcomere striation was more evident (see Figure 5D). The aggregate volume of the regenerated myocyte mass and myocyte cell volume were 2.1-fold \( (P<0.02)\) and 1.4-fold \( (P<0.005)\) greater, respectively, in animals injected with miR-499–EGFP-hCSCs (Figure 6B). Moreover, the frequency distribution of the volume of human myocytes formed by miR-499–EGFP-hCSCs was shifted to the right to higher values (Figure 6C). This analysis included EGFP-labeled myocytes \( \geq 600 \mu m^3 \) in volume because myofibrils were not seen in smaller cells.

Treatment with EGFP-hCSCs or miR-499–EGFP-hCSCs resulted in an improvement in left ventricular function, consisting of better preservation of left ventricular developed pressure and positive and negative dP/dt. An additional positive effect on cardiac performance was found in the presence of miR-499–EGFP-hCSCs (Figure 7A). Collectively, these findings point to enhanced myocyte differentiation/hypertrophy as the mechanism by which miR-499 potentiated the restoration of myocardial mass and function after infarction. However, whether this biological role of miR-499 in vivo was completed at this early stage of ventricular remodeling remained unclear.

Because of this question, rats with infarcted hearts injected with EGFP-hCSCs and miR-499–EGFP-hCSCs were euthanized 21 days later at the completion of the healing process, and myocytes were enzymatically dissociated from the infarcted region of the wall for the assessment of their volume and mechanical properties. Human myocytes derived from miR-499–EGFP-hCSCs were 50% larger than those formed by EGFP-hCSCs (Figure 7B and 7C). Additionally, peak shortening of regenerated human myocytes was significantly higher than that of the hypertrophied rat myocytes collected from the...
surviving myocardium (Figure 7D). This observation is consistent with the enhanced contractile function of young myocytes and the depressed mechanical behavior of hypertrophied cells. Thus, overexpression of miR-499 promotes the progression of amplifying myocytes to the functionally competent adult phenotype.

**Discussion**

The results of the present study indicate that the commitment of hCSCs to the myocyte lineage and the generation of functionally competent adult cardiomyocytes are influenced by miR-499, which is barely detectable in primitive cells but is highly expressed in postmitotic human cardiomyocytes. Consistent with our in vitro observations, miR-499 traverses gap junction channels and may translocate in vivo from cardiomyocytes to structurally coupled hCSCs favoring the activation of the differentiation program.

The control of stem cell growth is not intrinsic to stem cells but depends on communications with the microenvironment. Interstitial structures with the architectural organization of stem cell niches are present in the mammalian heart. hCSCs form clusters and interact with the adjacent supporting cells through adherens and gap junctions, which are located at the interface between undifferentiated cells and myocytes or fibroblasts. Although the roles of these 2 types of junction remain to be defined, it is reasonable to assume that they allow stem cells to sense environmental stimuli. Attenuation in cell-to-cell communication may occur with age, as supported by data in animal models. The microrine mechanism may be partly impaired, interfering with the activation of CSCs and progeny formation in the old heart.

In several organs, gap junctions favor replication, migration, and maturation of progenitor cells. The lack of an appropriate connection between hCSCs and the surrounding myocytes may oppose hCSC differentiation. Experimentally,
the injection of hCSCs results in cardiac repair with significant recovery of muscle mass, but the human myocytes resemble fetal-neonatal cells. Additionally, foci of small differentiating myocytes have been found after acute myocardial infarction or chronic aortic stenosis in humans. Conversely, newly formed cardiomyocytes in the context of a preserved myocardium typically show an adult phenotype, being indistinguishable from the adjacent cells. These obser-
vations raise challenging questions about the control of cardiac homeostasis and tissue regeneration.

Scattered myocyte dropout by wear and tear, aging, or cardiotoxicity is readily recovered by activation of CSCs.\(^2,19,20\) Although significant differences have been reported on the extent of myocyte turnover in humans,\(^19–21\) there is general agreement that this process preserves the integrity of the myocardium. Conversely, segmental losses of tissue after infarction exceed the growth reserve of the organ, precluding cardiac repair.\(^18\) Human cardiac stem cells maintain the balance between cell death and regeneration in the young adult heart, but this compensatory mechanism is no longer effective later in life\(^21\) or after severe myocardial injury.\(^18\) The recognition that miR-499 enhances the hypertrophic response of hCSC-derived cardiomyocytes, promoting a more effective functional and structural recovery of the damaged heart, offers a novel strategy for the management of the human disease.

The factors that govern hCSC growth appear to be regulated by junctional coupling and the translocation of miR-499 from postmitotic myocytes. The inhibitory function of miR-499 on Sox6 and Rod1 in hCSC differentiation may be mediated by repression of stemness-related genes and/or upregulation of lineage-specific genes. miR-499 expression and Sox6 inhibition enhance the differentiation of a heterogeneous population of fetal cardiac human cells.\(^22\) However, these cells were erroneously considered a class of cardiac progenitors; they express the myocyte-specific transcription factors Nkx2.5, GATA4, and MEF2C, together with a poorly myosin expression, miR-499 replaces its function in miR-1 and miR-499, favor their function. This is consistent with the relatively large quantity of Sox6 and Rod1 transcripts in cardiomyocytes. Moreover, overexpression of miR-499 in hCSCs did not affect the mRNA level of Sox6 and Rod1 but abrogated protein accumulation. The apparent discrepancy between Sox6 and Rod1 mRNA in cardiomyocytes may be due to the presence of 5 and 2 miR-499 binding sequences in the 3' UTR of human Sox6 and Rod1 mRNA, respectively. The stronger inhibitory effect of miR-499 on Sox6-luciferase reporter assay supports this contention.

The existence of circulating miRs has raised the possibility that the function of these small RNAs may not be limited to the intracellular control of gene expression.\(^26–29\) Initially believed to be a passive leakage from dead cells, miR release is an organized and controlled process of secretion involving cell-derived microvesicles.\(^28,30\) Microvesicles may allow communication with cells located in organs distant from the cell of origin of miRs.\(^31,32\) In contrast, the exchange of small RNAs through gap junctions may be part of the complex cross-talk between neighboring functionally connected cells. To exclude that the presence of miR-499 in hCSCs was mediated by a paracrine mechanism, the level of miR-499 in the growth medium was measured and found to be negligible. Moreover, the gap junction inhibitor α-GA dramatically decreased the number of hCSCs positive for miR-499, pointing to gap junction channels as the major mediator of miR-499 transfer from cardiomyocytes to hCSCs. The recent documentation that miRs can traverse gap junction channels, a phenomenon confirmed here, has raised the intriguing possibility of a novel modality of intercellular control of gene expression.\(^7\)

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Disclosures
None.

References


15. Montecino-Rodriguez E, Dorshkind K. Regulation of hematopoiesis by microRNA-499 represses genes responsible for the primitive state of these cells, promoting the program of myocyte differentiation, structurally and functionally. In the presence of microRNA-499, the regenerated parenchymal cells increase in size and acquire electric and mechanical properties of adult cells. This process occurs by the physiological translocation of microRNA-499 from myocytes to human cardiac stem cells via gap junction channels. Importantly, the potentiated reconstitution of the infarcted myocardium has significant effects on the anatomic remodeling and hemodynamic performance of the injured heart. Understanding the molecular control that regulates the differentiation of human cardiac stem cells into functionally competent cardiac myocytes may be critical for the implementation of stem cell therapy in the diseased heart.
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Supplemental Methods

Human Cardiac Stem Cells (hCSCs)

Discarded myocardial specimens were obtained from patients who underwent cardiac surgery. We had no knowledge concerning the age, type of the cardiac disease and duration of the disease. Additionally, we had no information concerning the atrial or ventricular origin of the specimens. Myocardial samples were enzymatically dissociated and small cardiac cells were sorted for c-kit, expanded and characterized by FACS analysis to obtain lineage negative hCSCs.\textsuperscript{1,2} hCSCs at P4-P5 were employed in the in vitro and in vivo studies described in the subsequent sections.

Human Cardiomyocytes

Explanted human hearts and donor hearts declined for transplantation were utilized to obtain cardiomyocytes. In large myocardial samples, a branch of the left main coronary artery was cannulated and perfused at 37°C with a Ca\textsuperscript{2+}-free solution gassed with 85% O\textsubscript{2} and 15% N\textsubscript{2}. After 5 minutes, 0.015 mM CaCl\textsubscript{2}, 274 units/mL collagenase (type 2, Worthington Biochemical Corp.) and 0.57 units/mL protease (type XIV, Sigma) were added to the solution which contained (mM): NaCl 126, KCl 4.4, MgCl\textsubscript{2} 5, HEPES 5, Glucose 22, Taurine 20, Creatine 5, Na Pyruvate 5 and NaH\textsubscript{2}PO\textsubscript{4} 5 (pH 7.4, adjusted with NaOH). At completion of digestion, the myocardium was cut in small pieces and re-suspended in Ca\textsuperscript{2+} 0.015 mM solution. Myocytes were enriched by centrifugation and washed. Smears were made to control the purity of the cell suspension.\textsuperscript{3}

Rat CSCs (rCSCs) and Cardiomyocytes

The heart of Fischer 344 rats at 3 months of age was excised and placed on a stainless steel cannula for retrograde perfusion through the aorta.\textsuperscript{4,6} A protocol similar to that described above was utilized with some additional steps. At the time of centrifugation, the supernatant was collected and small cardiac cells were obtained and sorted for c-kit to obtain rCSCs.\textsuperscript{7} Pellets of myocytes and rCSCs were frozen and stored. Additionally,
clonogenic rCSCs previously obtained in our laboratory were used in some of the studies.⁴

**miR Microarray**

RNA was extracted from mouse myocardium, rCSCs and rat myocytes with mirVana miRNA isolation kit (Applied Biosystems). Five microgram each of total RNA was subjected to microRNA Microarray (LC Sciences).

**qRT-PCR for miR-499**

RNA was extracted from hCSCs, human myocytes, rCSCs, rat myocytes, 3T3 cells and C2C12 myoblasts with mirVana miRNA isolation kit. One ng of total RNA including the small RNA fraction was reverse transcribed utilizing specific primers for miR-499 and the TaqMan MicroRNA reverse transcription kit (Applied Biosystems). The cDNA was subsequently amplified with forward and reverse primers together with FAM-labeled probe (Applied Biosystems). Equal amounts of RNA were used for the quantification of small nucleolar RNA RNU44 (human), snoRNA (rat), and snoRNA412 (mouse). They were employed for normalization of miR-499 expression. PCR products were run on 12.5% acrylamide/0.5X TBE gel and stained with 0.5 μg/ml ethidium bromide for 30 minutes.

**qRT-PCR for mRNA**

Total RNA was extracted from hCSCs, rCSCs and rat myocytes with mirVana miRNA isolation kit or TRIzol Reagent (Invitrogen) and employed for the measurement of the quantity of transcripts for α-MHC, Myh7b, and the target genes of miR-499, Sox6 and Rod1. RNA extracted from human myocardium was also employed. cDNA for mRNAs was obtained from 2 μg total RNA in a 20 μl reaction using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 100 pmole of oligo(dT)₁₅ primer.¹⁶⁻⁹ This mixture was incubated at 37°C for 2 hours. Quantitative RT-PCR was performed with primers (see below) designed using the Primer Express v2.0 or Vector NTI (Invitrogen) software employing the 7300 Real-Time PCR system. cDNA synthesized
from 100 ng total RNA was combined with Power SYBR Green PCR Master Mix (Applied Biosystems) and 0.5 μM each of forward and reverse primers. Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of amplification (95°C denaturation for 15 sec, and 60°C annealing-extension for 1 min). The melting curve was then obtained. To avoid the influence of genomic contamination, forward and reverse primers for each gene were located in different exons. Reactions with primers alone were also included as negative controls. Quantified values were normalized against the input determined by the housekeeping gene β-actin or Gapdh. Real-time PCR products were run on 2% agarose/1X TBE gel.

- rat α-MHC-F: 5’- ACC AAC CTG TCC AAG TTC CG -3’
- rat α-MHC-R: 5’- AAC AGC GAG GCT CTT TCT GC -3’
- rat Myh7b-F: 5’- AGC ACG AGT TAG ACG ACG CTG -3’
- rat Myh7b-R: 5’- ATC CCT CGT CAG AGC TGG AG -3’
- rat Sox6-F: 5’- GAA GGC AGG AGA TGC GAC A -3’
- rat Sox6-R: 5’- AAT AGC ACC AGG ATA CAC AAC ACC -3’
- rat Rod1-F: 5’- AAA CAT CTT CCC TCC GTC AGC -3’
- rat Rod1-R: 5’- GAA CAT CCA GCT TCC GTG AAC -3’
- rat Gapdh-F: 5’- CCA TTC TTC CAC CTT TGA TGC -3’
- rat Gapdh-R: 5’- CAT ACC AGG AAA TGA GCT TCA CAA -3’
- human Sox6-F: 5’- CCG ACA TGC ATA ACT CCA ACA T -3’
- human Sox6-R: 5’- CGG TCG GGG TTT GTA TTT ATA GTT -3’
- human Rod1-F: 5’- GTC CAA ACA TCA AGC AGT ACA GC -3’
- human Rod1-R: 5’- AGA TCA TCC ACT GTA ACA GAA GGG -3’
- human Cx43-F: 5’- CAA GGA GTT CAA TCA CTT GGC G -3’
- human Cx43-R: 5’- TGT CCC CAG CAG CAG GAT TC -3’
- human β actin-F: 5’- CCG CGA GAA GAT GAC CCA GAT -3’
- human β actin-R: 5’- GTC CATCAC GAT GCC AGT GG -3’
Western Blotting for Sox6 and Rod1

Protein lysates of hCSCs, human myocytes, rCSCs and rat myocytes were obtained using RIPA buffer (Sigma) and protease inhibitors. Equivalents of 20-50 μg of proteins were separated on 8-12% SDS-PAGE, transferred onto PVDF membranes (Bio-Rad) and subjected to Western blotting with rabbit polyclonal anti-Sox6 (Millipore) and mouse monoclonal anti-Rod1 (Abcam) diluted 1:200 in TBST overnight at 4ºC. HRP-conjugated anti-IgG were used as secondary antibodies. Proteins were detected by chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and optical density was measured. Loading conditions were determined by the expression of Gapdh (Millipore) and/or by Ponceau S (Sigma) staining of the membrane after transfer. HeLa cells were used as positive control. An identical protocol was employed for the measurement of the quantity of Sox6 and Rod1 proteins in hCSCs infected with a lentivirus carrying miR-499 (see below).

Gene Reporter Activity Assay

3T3 mouse fibroblasts, ~80% confluency, were co-transfected with reporter and expression plasmids.

a) Luciferase reporter plasmids: The pMIR-REPORT Luciferase and pMIR-REPORT β-gal Control plasmids (Applied Biosystems) were utilized for transfection with or without modifications. Mouse genomic DNA was amplified to obtain the entire 3’ untranslated region (3’-UTR) of Sox6 (5.6 kb) and Rod1 (5.0 kb) genes using the primers indicated below. The sequence was partly modified (see underlined nucleotides) to introduce restriction sites in the amplicons.

- mouse Sox6-Mlu1-F: 5’- TGT TTG ACG CGT TAA AAC ACT CTG ACA TTT CGC TCC -3’
- mouse Sox6-Pme1-R: 5’- AGT CCT GTT TAA ACT TCT CTT TAT CAC TAT CCA GAG -3’
• mouse Rod1-Mlu1-F: 5’- TGA CCT ACG CGT GAA ATT GTC TCC TTA TAC TGG AC-3’
• mouse Rod1-Pme1-R: 5’- AAA AGG GTT TAA ACA ATG CTA TAT GTG TTA GGA AAA GAG GC -3’
The reaction mixture consisted of 22.5 μl of AccuPrime Pfx SuperMix (Invitrogen), 5 pmole each of Forward and Reverse primers and 50 ng of genomic DNA extracted from adult mouse heart using QIAamp DNA Mini Kit (Qiagen). PCR cycling conditions were as follows: 95°C for 5 min followed by 35 cycles of amplification (95°C for 15 sec, 60°C for 30 sec and 68°C for 5 min 30 sec) and the last step at 68°C for 5 min. PCR products were run on 0.6% agarose/1X TBE gel. The DNA bands were cut out and DNA was extracted using QIAEX II Gel Extraction Kit (Qiagen). Following restriction digestion with Mlu1 and Pme1 for Sox6 3’-UTR and Rod1 3’-UTR, DNA fragments were directionally ligated to pMIR-REPORT Luciferase plasmid (Applied Biosystems). This plasmid has a multiple cloning site downstream of the luciferase gene which is placed under the control of the CMV promoter. To exclude the possibility of erroneous amplification events, constructs were fully sequenced utilizing the pMIR-REPORT-1 primer (5'- AGG CGA TTA AGT TGG GTA -3’) which is located downstream of the multiple cloning site in the antisense orientation. Additional primers were designed at a distance of 500 bp each within the amplicons and employed to verify construct sequences.

b) miR-499 expression plasmid: The pMIR-REPORT β-gal Control plasmid was modified by digesting with BamH1 and Hind3, blunted with T4 DNA polymerase, and then self-ligated with T4 DNA ligase to serve as blank plasmid (CMV-blank). Mouse genomic DNA coding the stem loop of miR-499 together with the two flanking regions of ~100 bp each was amplified with the primers indicated below. The construct was ligated to replace the β-gal gene in the Control plasmid and obtain the miR-499 expression plasmid (CMV-miR-499).
• miR-499-BamH1-F: 5'- CCT AAG GAT CCC ACG CCC CCT ACA GGC TGC CAC -3'
• miR-499-Hind3-R: 5'- ACC TAA AGC TT C ACC GCC CCC CCA CCC CCA G -3'

To assess the degree of miR-499 overexpression, 3T3 fibroblasts, 10^5, were transfected with 1 μg of CMV-blank or CMV-miR-499; 3 days later, RNA was extracted and miR-499 and snoRNA-412 were measured by quantitative RT-PCR (see above).

For the gene reporter assay, 8 3T3 cells were co-transfected with increasing quantities (up to 0.8 μg) of CMV-miR-499 and 0.1 μg of reporter plasmids containing the luciferase coding sequence alone or conjugated with the 3’-UTR of Sox6 and Rod1. CMV-blank was used as negative control and to adjust the total amount of DNA to be transfected. The siPORT XP-1 Transfection Agent (Applied Biosystems) was employed. A β-gal-expressing plasmid, 0.1 μg, was employed to normalize luciferase activity. Two days later, co-transfected 3T3 cells were incubated with 150 μl of 1X Passive Lysis Buffer. The β-Galactosidase Enzyme Assay System and Luciferase Assay System (Promega) were employed to assess β-galactosidase and luciferase activities, respectively. The ratio of these two measurements was calculated to normalize luciferase activity.

**miR-499 Transfer**

Five approaches were employed to demonstrate that functionally competent miR-499 translocated from donor cells to hCSCs via gap junctions.

a) C2C12 myoblasts were transfected with CMV-miR-499 plasmids, employed as donor cells and co-cultured with recipient EGFP-labeled hCSCs at a ratio of 2:1 in the absence or presence of the gap junction inhibitor 18α-Glycyrrhetinic acid (α-GA), 10μM. Thirty-six hours later, the presence of miR-499 was documented by quantitative fluorescence in situ hybridization^{1,11} utilizing a 3’-digoxigenin-labeled Locked-Nucleic Acid (LNA) probe (Exiqon), 10 nM, with a sequence complementary to that of mature miR-499. The LNA probe forms a stable complex with the target molecule. Cells were fixed in 4%
paraformaldehyde, treated with 0.1% Triton X, 0.2 N HCl and proteinase K (5 μg/ml), incubated in acetylation solution containing 1.3% Triehanolamine, 0.018N HCl and 0.25% Acetic Anhydride for 10 minutes, and washed in PBS. Samples were pre-hybridized for 2 hours in hybridization buffer: 50% formamide (Sigma), 5X SSC (Sigma), 5X Denhardt’s solution (Sigma), 62.5 μg/ml yeast tRNA (Sigma), 125 μg/ml salmon sperm DNA (Invitrogen), and 20 mg/ml Blocking reagent (Roche). After denaturation of the probe at 80°C for 5 minutes, cells were hybridized at 50°C for 16 hours in the presence of 50% formamide, 5X SSC, 5X Denhardt’s solution, 62.5 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA, 20 mg/ml Blocking reagent, 0.25% CHAPS (Sigma), 0.5% Tween 20 (Sigma), and 10 nM digoxigenin-labeled miR-499 probe. Immunological detection was carried out with anti-digoxigenin Fab conjugated to rhodamine (Roche). The presence of connexin 43 at the interface between C2C12 myoblasts and hCSCs was documented by immunolabeling with a mouse monoclonal Cx43 antibody (Sigma). Negative controls consisted of omission of the LNA.

b) Neonatal rat cardiomyocytes were isolated by exposing minced heart at 37°C in a solution containing 116 mM NaCl, 20 mM Heps, 0.8 mM Na₂HPO₄, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM MgSO₄, 0.6 mg/ml pancreatin, and 0.4 mg/ml Collagenase Type II. Cell suspension was pre-plated for 10 min to remove fibroblasts and then plated onto 1%-gelatin-coated dishes with medium consisting of DMEM supplemented with 10% horse serum, 5% fetal bovine serum, and 100 μg/l penicillin and streptomycin. Myocytes were co-cultured with EGFP-labeled hCSCs at a ratio of ~10:1 in the absence and presence of 10 μM α-GA. EGFP-positive hCSCs were FACS-sorted 5 days after co-culture and employed for the measurement of miR-499 by qRT-PCR (see above). EGFP-labeled hCSCs exposed for 5 days to conditioned medium obtained from myocyte cultures were employed to evaluate the influence of microvesicles. EGFP-labeled hCSCs seeded alone were used as control.

c) Neonatal rat myocytes and EGFP-labeled hCSCs were co-cultured at a ratio of ~10:1 in
the absence and presence of 10 μM α-GA, and fixed 2 or 36 hours later for fluorescence in situ hybridization with the miRCURY LNA microRNA ISH Optimization Kit (Exiqon). Cells were fixed in 4% paraformaldehyde, treated with proteinase K, 0.5 μg/ml, and subsequently dehydrated with ethanol. The hybridization mix consisted of 1X microRNA ISH buffer containing either 40 nM of double digoxigenin-labeled LNA probe against miR-499 or 40 nM double digoxigenin-labeled scrambled LNA control. Cells were exposed to the hybridization solution and incubated at 48.5°C for 90 min. After sequential washing in 5X SSC, 1X SSC and 0.2X SSC, anti-digoxigenin Fab conjugated to rhodamine (Roche) was used for the detection of digoxigenin.

d) For real-time transfer of miR-499, translocation of miR-499 through gap junctions was studied in living cells by two-photon microscopy.14 The 5’-side of mature miR-499 (21 nucleotides) was labeled with the fluorescent dye Cy3 (Sigma). One day after plating, individual hCSCs were loaded with Cy3-miR-499 and cascade blue (Molecular Probes) by microinjection (FemtoJet, Eppendorf AG) of a medium containing: K2HPO4, 27 mM, NaHPO4, 8 mM, KH2PO4, 26 mM, Cy3-miR-499, 75 μM, and cascade blue, 2 mM (pH 7.3). Transfer of the fluorescent dye to neighboring cells was followed by acquisition of serial images with an inverted microscope (IX-71, Olympus).

e) To establish the functional competence of transferred miR-499, hCSCs were transfected with a plasmid carrying miR-499 (see above) and utilized as donor cells. Recipient cells corresponded to hCSCs transfected with luciferase reporter plasmid containing the 3’-UTR sequences of the Sox6 gene ligated at a position downstream the luciferase coding sequence (CMV-Luc-Sox6-UTR). Donor miR-499-hCSCs were plated at a density of 6x10⁴ cells in each well of a 6-well dish and transfected with 1 μg of CMV-miR-499 or CMV-blank. Recipient hCSCs were plated at a density of 1.2x10⁴ cells in each well of a 6-well dish and transfected with 0.9 μg each of CMV-Luci-Sox6-UTR (see above). A pRL-CMV plasmid (Promega), 0.1 μg, was co-transfected for the normalization of luciferase activity.8 Six hours later, cells were washed in PBS three
times. Donor transfected cells were trypsinized and plated together with recipient cells at a ratio of 5:1 for 3 days. Cells were incubated in 1X Passive Lysis Buffer (Promega) and luciferase activity measured with the Dual-Luciferase Reporter Assay Systems (Promega). hCSCs transfected with the same reporter plasmids but cultured with donor hCSCs carrying the CMV-blank plasmid were used as baseline. Downregulation of luciferase activity in co-cultured cells was considered indicative of translocation of miR-499 from donor to recipient hCSCs. The gap junction inhibitor α-GA, 10 μM, was added in a separate set of experiments.

**Microvesicles in co-culture**

Medium was recovered after co-culturing neonatal rat myocytes and hCSCs for 2-3 days in the absence and presence of 10 μM α-GA. Medium was centrifuged at 160,000 g for 1 hour at 4°C to collect microvesicles. RNA extraction and qRT-PCR analysis were performed as above.

**Toxicity of α-GA**

Neonatal rat myocytes and EGFP-labeled hCSCs were co-cultured for 3 days in the absence and presence of α-GA, 10 μM or 100 μM. The fraction of apoptotic hCSCs and myocytes was evaluated using In Situ Cell Death Detection Kit, TMR red (Roche) following manufacturer’s instruction.

**miR-499 Lentivirus for In Vitro Assays**

We generated a lentiviral vector carrying miR-499 and DsRed. The amplification of human genomic DNA coding miR-499 was performed as described above for the expression plasmid CMV-miR-499. Following restriction digestion with NotI and XbaI at the sites of modified nucleotides (underlined), the amplified fragment was ligated to a position downstream the DsRed coding sequence in pLVX-DsRed-Monomer-N1 Vector (Clontech) to obtain the DsRed-miR-499 plasmid:

- hsa-miR-499-Not1-F: 5’- GGT GTC GCG GCC GC ACAA GGT AAG GCC CCA TCT GG -3’
- hsa-miR-499-Xba1-R: 5’- GGT GAT TCT AGA CCC TTC GCT GTC TCC CAT CAC CAC -3’

After validation by DNA sequencing, HEK293T/17 cells cultured in DMEM, 10% FBS, were used as packaging cells for lentiviral production. Three plasmids, DsRed-miR-499, delta 8.9, and pMD.G, were mixed at a ratio of 4:3:1 and diluted in OPTI-MEM (Invitrogen) for co-transfection using Lipofectamine 2000 (Invitrogen). Six hours later, the medium was removed and fresh medium added. Medium was collected at 24 and 48 hours and frozen after filtration. pLVX-DsRed plasmid not carrying miR-499 was utilized to generate the control virus. For infection, hCSCs were incubated overnight in culture medium containing the lentiviral solution and 8 μg/ml Sequabrene (Sigma). The following day, the medium with lentiviral particles was discarded and replaced with fresh medium.

siRNA Transfection of hCSCs
siRNAs for human Sox6 and Rod1 were obtained from Dharmacon and corresponded to a mixture of 4 different duplexes for each gene. siRNAs had highly complementary sequence to induce the degradation of the target mRNAs. For transfection, a final concentration of 25 or 50 nM siRNAs against Sox6 or Rod1 was used together with NTER Nanoparticle siRNA Transfection System (Sigma) to induce interference in hCSCs. hCSCs transfected with Non-Targeting siRNA (Dharmacon) were used as baseline.

Growth and Differentiation of hCSCs
Replication of hCSCs infected with a lentiviral vector carrying DsRed and miR-499 or DsRed only was determined by BrdU labeling. BrdU, 10 μM, was added once to the medium and cells were fixed in 70% ethanol/50 mM glycine one hour later. BrdU incorporation was determined by immunostaining with monoclonal antibody (Roche). In similar samples, hCSC commitment was assessed by immunolabeling for the myocyte-specific transcription factors Nkx2.5 (goat polyclonal antibody, Santa Cruz) and GATA4 (rabbit polyclonal antibody, Santa Cruz).
miR-499 Lentivirus for In Vivo Assays

We have generated a lentivirus carrying miR-499 together with EGFP. The human genomic DNA fragment coding miR-499 was amplified by PCR utilizing the primers:

- hsa-miR-499-EcoR1-F: 5’- CAA GGG AAT TCC CCA TCT GGG AGA CAG ACC CTC -3’
- hsa-miR-499-EcoR1-R: 5’- GAT GGG AAT TCC TTC GCT GTC TCC CAT CAC CAC -3’

The PCR product was ligated into a CAD plasmid vector carrying EGFP. The direction of insertion was confirmed by sequencing. Lentiviral production and hCSC infection were performed as described above.

Myocardial Infarction

All protocols were approved by the Institutional Animal Care and Use Committee of the Brigham and Women’s Hospital. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” as described by the Institute of Laboratory Animal Research Resources, Commission on Life Sciences, National Research Council. Under ketamine (100 mg/kg b.w., i.p.) and acepromazine (1 mg/kg b.w., i.p.) anesthesia, myocardial infarction was produced in female Fischer 344 rats at 3 months of age by ligation of the left coronary artery near its origin. Animals were treated with a standard immunosuppressive regimen. Shortly after coronary occlusion, ~50,000 hCSCs infected with the miR-499-EGFP-lentivirus were injected at multiple sites in proximity of the border zone. A group of infarcted animals received hCSCs infected with a lentivirus carrying EGFP only. Untreated infarcted rats received saline and were used as controls. Animals were sacrificed ~10 and 21 days after infarction and cell implantation.

Ventricular Hemodynamics

At sacrifice, animals were anesthetized with chloral hydrate (300 mg/kg b.w., i.p.) and the right carotid artery was cannulated with a microtip pressure transducer (SPR-612, Millar
Instruments) connected to an A/D converter (iWork 214) to record data on a PC. Subsequently, the catheter was advanced into the LV cavity for the evaluation of LV pressures and $+\text{ and } -\frac{dP}{dt}$ in the closed -chest preparation. Subsequently, the abdominal aorta was cannulated with a polyethylene catheter, PE-50, filled with a phosphate buffer, 0.2 M, pH 7.4, and heparin, 100 U/ml. In rapid succession, the heart was arrested in diastole by the injection of 0.15 ml of CdCl$_2$, 100 mM, through the aortic catheter, the thorax was opened, perfusion with phosphate buffer was started, and the vena cava was cut to allow drainage of blood and perfusate. After perfusion with buffer for 2 min, the coronary vasculature was perfused for 15 min with fixative. The heart was then excised, and weights were recorded.

**Infarct Size**

The number of myocytes in the LV of control and infarcted hearts was measured by employing a methodology well-established in our laboratory. The percentage of myocytes lost provides a quantitative measurement of infarct size while the percentage of myocytes left correlates with ventricular function. The volume of the regenerated myocardium was determined by measuring in three sections, obtained from the base to the apex of the heart, the area occupied by the restored tissue and section thickness. The product of these two variables yielded the volume of tissue repair in each section. Values in the three sections were added, and the total volume of formed myocardium was obtained. Additionally, the volume of newly generated myocytes was measured in each heart. Sections were stained with $\alpha$-SA, EGFP, laminin and DAPI. Only longitudinally oriented cells with centrally located nuclei were included. The length and diameter across the nucleus were collected in each myocyte to compute cell volume, assuming a cylindrical shape.

**Immunohistochemistry and In Situ Hybridization**

Antibodies were used to detect human myocytes and coronary vessels within the recipient rat heart. Human cells were detected by EGFP localization and by in situ
hybridization with a probe (Biogenex) against the human-specific Alu repeat sequences.\textsuperscript{1,2} Gap and adherens junctions were characterized by connexin 43 (Sigma) and N-cadherin (Santa Cruz). Cycling cells were detected by Ki67 (Vector) and BrdU labeling (Roche). Images were assembled with Adobe Photoshop 7.0 software according to the standard protocol detailed in the Nature guidelines for digital images. Processing included changes in brightness and was applied uniformly across the entire image; it was used exclusively to equalize the appearance of multiple panels in a single figure.

**Spectral Analysis**

This methodology was performed with a Zeiss LSM510 Meta confocal microscope (Zeiss) utilizing the meta detector and the lambda acquisition mode.\textsuperscript{6,8,22} Myocardial sections, labeled by EGFP and Alu conjugated with FITC, α-SA, BrdU and Ki67 conjugated with TRITC, were examined. Nuclei were stained by DAPI. The emission signal for DAPI was excited at 405 nm with a blue diode laser and its fluorescence intensity was recorded generating a lambda stack ranging from 417 to 748 nm at 10.7 nm intervals. The emission signal for FITC was excited at 488 nm with an argon laser and its fluorescence intensity was recorded generating a lambda stack ranging from 502 to 748 nm at 10.7 nm intervals. The emission signal for TRITC was excited at 568 nm with an argon laser and its fluorescence intensity was recorded generating a lambda stack ranging from 577 to 748 nm at 10.7 nm intervals. The lens and corresponding numerical aperture were 60X and 1.4, respectively. For each region of interest, a graph plotting mean pixel intensity and the emission wavelength of the lambda stack was generated. The spectrum obtained from EGFP-positive cells exhibited a major peak at ~525 nm, with a smaller peak at ~560 nm. In contrast, the spectrum of autofluorescence was more uniformly spread across the range of wavelengths and did not show a clearly defined peak of emission. To compare the shape of each curve obtained from EGFP-positive and EGFP-negative structures, the values of emission spectra were normalized by dividing the intensity of each wavelength by the peak signal. In an identical manner, we analyzed
myocardial sections labeled by Alu probe conjugated with FITC and α-SA, BrdU, and Ki67 antibody conjugated with TRITC. Myocyte nuclei negative for each of the antigens and present in the same samples were used as control to discriminate background autofluorescence from specific labeling.

**Myocyte Isolation and Contractility**

In two groups of animals sacrificed 21 days after infarction and cell implantation, myocytes were enzymatically dissociated (see above). At completion of digestion, the LV was dissected in surviving and infarcted area. The two parts were then cut in small pieces and resuspended in Ca\(^{2+}\) 0.1 mM solution. Isolated myocytes obtained from the infarcted and surviving region were placed in a bath on the stage of an inverted microscope (IX71, Olympus) for physiological determinations\(^1\,2\,23\,24\) Human myocytes were recognized by the green fluorescence of EGFP. Experiments were conducted at room temperature. Cells were bathed continuously with a Tyrode solution containing (mM): NaCl 140, KCl 5.4, MgCl\(_2\) 1, HEPES 5, Glucose 5.5 and CaCl\(_2\) 1 (pH 7.4, adjusted with NaOH).

Contractility measurements were performed in field-stimulated cells by using a video edge-track detection system (Crescent Electronics). Signals were digitized using a 500 kHz 16-bit resolution A/D converter (Digidata 1322, Axon Instruments) and recorded using pCLAMP 9.0 software (Axon Instruments). Contractions were elicited by rectangular depolarizing pulses, 2 ms in duration, and twice-diastolic threshold in intensity, by platinum electrodes\(^1\,2\,24\). Changes in cell length were computed by edge-track detection. Isolated myocytes were fixed in 4% paraformaldehyde for the measurement of cell volume. The volume of freshly isolated paraformaldehyde–fixed myocytes in each cell class was obtained by three-dimensional optical section reconstruction\(^4\,14\,20\,21\) by confocal microscopy (Bio-Rad Radiance 2100). Three-dimensional reconstruction of isolated myocytes was computed utilizing Imaris software (Bitplane AG).

**Data Analysis and Statistics**
The magnitude of sampling utilized in each in vitro and in vivo determination is listed in Table IV. Continuous measurements are presented as means±SD. Comparisons between two groups were determined by unpaired Student’s t-test and in more than two groups by Newman-Keuls method. The latter test was performed only if the overall comparison was statistically significant. In all cases, variances were equal and the variables were normally distributed. P values less than 0.05 were considered significant.25

Supplemental References
Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res. 2004; 94:514-524.


Supplemental Table 1. miR Expression in Mouse Myocardium

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**In vivo studies**

**Hemodynamics**

<table>
<thead>
<tr>
<th>Group</th>
<th>Hemodynamics</th>
<th>Infarct size</th>
<th>Magnitude of regeneration</th>
<th>Ki67 in newly formed myocytes</th>
<th>New myocyte volume</th>
<th>Function of isolated myocytes</th>
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**Infarct size**

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<th>Ki67 in newly formed myocytes</th>
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**Magnitude of regeneration**

<table>
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<td>control</td>
<td>8</td>
<td>184(2)</td>
<td>23 ± 4</td>
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<td>283(2)</td>
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**Ki67 in newly formed myocytes**

<table>
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**New myocyte volume**

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<td>Tissue sections</td>
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**Function of isolated myocytes**

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<tr>
<td>control - surviving</td>
<td>23(1)</td>
</tr>
<tr>
<td>control - newly formed</td>
<td>7(1)</td>
</tr>
<tr>
<td>miR-499 - surviving</td>
<td>16(1)</td>
</tr>
<tr>
<td>miR-499 - newly formed</td>
<td>9(1)</td>
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</table>

(1) Number of cells analyzed; (2) Area of tissue analyzed, mm²; (3) Number of nuclei analyzed; N/A, not applicable.
Supplemental Figure 1, Part A
Supplemental Figure 1, Part B
Supplemental Figure 1, Part C
Supplemental Figure 1, Part D
A

Similarity of myosin heavy chain proteins in different species

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<th>Mouse</th>
<th>Rat</th>
<th>Human</th>
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<tr>
<td>Myh7b vs α-MHC</td>
<td>69%</td>
<td>69%</td>
<td>68%</td>
</tr>
<tr>
<td>Myh7b vs β-MHC</td>
<td>70%</td>
<td>69%</td>
<td>69%</td>
</tr>
<tr>
<td>α-MHC vs β-MHC</td>
<td>93%</td>
<td>93%</td>
<td>92%</td>
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B

Conserved miR-499 sequences in different species

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<td>hsa-miR-499</td>
<td>GUGCUUGGGCGGCACGUG</td>
<td>UUAAGACGUUGCAGUGAUGUU</td>
<td>(Chr 20, forward strand)</td>
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<tr>
<td>ptr-miR-499</td>
<td>GUGCCUUGGGGCGGCACGUG</td>
<td>UUAAGACGUUGCAGUGAUGUU</td>
<td>(Chr 20, forward strand)</td>
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<tr>
<td>mml-miR-499</td>
<td>GUGUCUUGGGCGGCACGUG</td>
<td>UUAAGACGUUGCAGUGAUGUU</td>
<td>(Chr 10, forward strand)</td>
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<tr>
<td>bta-miR-499</td>
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<td>UUAAGACGUUGCAGUGAUGUU</td>
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<tr>
<td>cfa-miR-499</td>
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<td>UUAAGACGUUGCAGUGAUGUU</td>
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<tr>
<td>rno-miR-499</td>
<td>CUGUCUUGGGCGGCACGUG</td>
<td>UUAAGACGUUGCAGUGAUGUU</td>
<td>(Chr 3, forward strand)</td>
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<td>mmu-miR-499</td>
<td>GUGUCUUGGGCGGCACGUG</td>
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<td>gga-miR-499</td>
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<td>xtr-miR-499</td>
<td>UCUUGUGAGAGGAGGGCAG</td>
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********** **********

Supplemental Figure 2
### 5 conserved binding sites in the 3'-UTR of Sox6

| xtr  | 5'-... UCCAUCA GCAAAAGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| gga  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| mmu  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| rno  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| cfa  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| bta  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| mml  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| ptr  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |
| hsa  | 5'-... UACAUCAGCAAAUGUCUUAAGACUGCUAAGCUUAGCCGACGUCUUAAGCUUAA -3' |

**miR** 3'- UUGUGAGUGACGCUUCAGAAAUU

### 1 poorly conserved binding site in the 3'-UTR of Sox6

| xtr  | 5'-... | |
| gga  | 5'-... | |
| mmu  | 5'-... | |
| rno  | 5'-... | |
| cfa  | 5'-... | |
| bta  | 5'-... | |
| mml  | 5'-... | |
| ptr  | 5'-... | |
| hsa  | 5'-... | |

**miR** 3'- UUGUGAGUGACGCUUCAGAAAUU

### 2 conserved binding sites in the 3'-UTR of Rod1

| xtr  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| gga  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| mmu  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| rno  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| cfa  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| bta  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| mml  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| ptr  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |
| hsa  | 5'-... AUAAGAA- GCUUACAGCUUAGAGAGAGAA- GCUUACAGCUUAGAGAGAA -3' |

**miR** 3'- UUGUGAGUGACGCUUCAGAAAUU

---

**Supplemental Figure 3**
Supplemental Figure 4
Supplemental Figure 5
Supplemental Figure 11, Part B
Supplemental Figure 11, Part C
Supplemental Table Legends

Table 1. miR expression in mouse myocardium. The expression of miRs was determined by miR microarray. miRs with expression level >200 are listed.

Table 2. miR expression in rat myocytes and CSCs. The expression of miRs was determined by miR microarray. miRs included in the table fulfill two criteria: a) expression levels >1,000 in myocytes; and b) higher myocyte-to-CSCs ratio of expression (P<0.05 vs. CSCs). All data are available upon request.

Table 3. Predicted target genes of miR-499.

Table 4. Magnitude of sampling.

Supplemental Figure Legends

Figure 1. Representative curves of miRs and transcript expression measured by qRT-PCR.

Figure 2. Myh7b and miR-499. A, Degree of homology among myosin heavy chain proteins. B, Myh7b and miR-499 are encoded in the same orientation in all species. Red corresponds to mature miR-499. Asterisks indicate conserved nucleotides among species. Chr: chromosome; Scaf: scaffold.

Figure 3. Seed region of miR-499 and 3’-UTR of Sox6 and Rod1. miR-499 predicted binding sites among species. Red corresponds to the seed region of miR-499; blue indicates binding sites to the seed region, and green additional binding sequences. For symbols, see Figure IIB.

Figure 4. Q-FISH for miR-499. A, At the beginning of the co-culture assay, miR-499 (red) is restricted to transfected donor C2C12 myoblasts and is not present in recipient EGFP-positive (green) hCSCs. B, Expression of miR-499 and snoRNA in conditioned medium. Data are presented as number of cycles (Ct). Undet, undetectable. NTC, Non-template control.

Figure 5. Connexin 43 and hCSCs. A, hCSCs express the transcript for connexin 43. Human myocardium was used as positive control. B, hCSCs (c-kit, green) do not express
connexin 43 protein (Cx43). Human myocytes (α-SA, red) were employed as positive control. Cx43: white.

**Figure 6.** Real-time transfer of miR-499. Individual hCSCs (left panel) were loaded with miR-499 conjugated with Cy3 (red) and cascade blue (blue). Adjacent hCSCs are shown by phase contrast microscopy (left panel). Yellow dotted lines define the profile of the cells. The appearance of cascade blue (mid panel) together with the red fluorescence of miR-499 (right panel) in the hCSCs adjacent to the injected cell indicates miR-499 translocation through gap junctions.

**Figure 7.** miR-499 and transcripts for Sox6 and Rod1. qRT-PCR for Sox6 and Rod1 mRNA in hCSCs transfected with an empty plasmid (blank) or a plasmid carrying miR-499.

**Figure 8.** miR-499 and hCSC growth and commitment. hCSCs overexpressing miR-499 display nuclear localization of BrdU (A, white) Nkx2.5 (B, green) and GATA4 (C, yellow). Phalloidin: magenta. DsRed (red) identifies hCSCs infected with a lentivirus carrying miR-499.

**Figure 9.** Overexpression of miR-499 in hCSCs to be delivered in vivo. qRT-PCR for miR-499 showing the efficiency of infection of hCSCs with miR-499 lentivirus; RNU44 was employed for normalization.

**Figure 10.** Infarct size and cardiac repair. A, Percentage of LV myocytes lost as a result of coronary ligation. MI: non-treated; Ctrl: treated with EGFP-hCSCs; miR: treated with miR-499-EGFP-hCSCs. Results are mean±SD. B, Fraction of regenerated human myocytes positive for Ki67 at 10 days after infarction. Ctrl: treated with EGFP-hCSCs; miR: treated with miR-499-EGFP-hCSCs.

**Figure 11.** Validation of EGFP, α-SA, BrdU, Ki67 and Alu labeling. Spectral analysis of EGFP- (A), α-SA- (B), BrdU- (C), Ki67- (D), and Alu- (E) positive human cardiomyocytes. To validate the specificity of the recorded signals, spectral analysis was performed to discriminate tissue autofluorescence from actual immunostaining of myocytes and myocyte nuclei. Examples of myocytes and myocyte nuclei are illustrated
in the insets. Emission spectra are shown by the green lines, while emission spectra for tissue autofluorescence of myocytes and myocyte nuclei are shown by the blue lines. Note the difference in the intensity of the signals at wavelength corresponding to maximum fluorescence in each case (upper panels). Following normalization for the intensity of the signals, all emission spectra for specific labeling were essentially superimposable (lower panels). In contrast, emission spectra for tissue autofluorescence of negative structures had different shapes and were easily distinguishable from specific labeling.