Human Cardiac Stem Cell Differentiation Is Regulated by a Mircrine Mechanism

Toru Hosoda, MD, PhD, Hanqiao Zheng, MD, Mauricio Cabral-de-Silva, PhD, Fumihiro Sanada, MD, Noriko Ide-Iwata, MD, Barbara Ogórek, PhD, João Ferreira-Martins, PhD, Christian Arranto, MD, Domenico D’Amario, MD, Federica del Monte, MD, Konrad Urbanek, MD, David A. D’Alessandro, MD, Robert E. Michler, MD, Piero Anversa, MD, Marcello Rota, PhD, Jan Kajstura, PhD, and Annarosa Leri, MD

Departments of Anesthesia and Medicine, and Cardiovascular Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA; Cardiovascular Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; Montefiore Medical Center, Albert Einstein College of Medicine, New York, NY

Abstract

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

Methods and Results—Based on 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 post-mitotic 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.

Correspondence: Toru Hosoda, MD, PhD, or Annarosa Leri, MD, Departments of Anesthesia and Medicine, Brigham and Women’s Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115; phone: 617-525-8174; fax: 617-264-6320; thosoda@partners.org or aleri@partners.org.

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Disclosures
None.
Keywords

stem cells; cardiomyogenesis; microRNA; gap junctions; mircrine

Myocyte turnover in the adult heart is regulated by cardiac stem cells (CSCs) that generate new myocytes integrated structurally and functionally with the adjacent myocardium. 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. This recovery of the integrity of the myocardium does not occur with acute and chronic infarcts following the injection of CSCs, or their local activation by growth factors. A large number of myocytes is formed but the new cells consist predominantly of clusters of small, proliferating, mononucleated myocytes. Myocyte differentiation is prevented partly, 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.

The occasional migration of CSCs from the border zone to the remote myocardium results in the formation of fully-mature myocytes, mimicking cell turnover. Although the mechanism regulating the growth of CSCs into small versus large myocytes is currently unknown, coupling of CSCs with post-mitotic myocytes may be critical in determining their fate. microRNAs (miRs) are small RNAs that have the ability to traverse gap junctions 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 translocation of miRs, which promote CSC differentiation. Additionally, the effects of CSCs overexpressing miR were evaluated in vivo following myocardial infarction.

Methods

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

Human Cardiac Stem Cells (hCSCs) and Rat CSCs (rCSCs)

Human and rat myocardial samples were enzymatically dissociated and c-kit-positive hCSCs and rCSCs were obtained.

miR Microarray, qRT-PCR and Western Blotting

RNA was extracted and subjected to microRNA Microarray or qRT-PCR. Protein lysates of hCSCs, human myocytes, rCSCs and rat myocytes were employed for the measurement of Sox6 and Rod1.

miR-499 Transfer

Cardiomyocytes were co-cultured with hCSCs and luciferase-reporter-assays were employed to document the functional competence of translocated miR-499.

Myocardial Infarction

Myocardial infarction was produced in rats and hCSCs infected with a miR-499-EGFP-lentivirus were injected in the border zone. At sacrifice, ventricular function was determined and the heart was fixed for histology.
**Statistical Analysis**

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 based on one-way analysis of variance was statistically significant. In all cases, variances were assumed to be equal and variables were assumed to be normally distributed. P values less than 0.05 were considered significant.

**Results**

**Expression of miR-499 in the Heart**

miRs are a class of small non-coding RNAs, which 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 (Supplemental Table 1). The rat model was then employed to isolate a larger number of myocytes and rCSCs for the evaluation of the differential expression of miRs in these two cell classes. By array analysis (Supplemental Table 2), miR-1, miR-22, miR-30a, miR-30b, miR-30c, miR-133a, miR-133b and miR-499 were abundant in myocytes (Figure 1A). While 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; Supplemental Figure 1).

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 (MHC) 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 a 70% homology with α-MHC and β-MHC (Supplemental Figure 2A), suggesting that Myh7b represents a distinct myosin gene with unknown function.

miR-499 and Myh7b are encoded in the same orientation in all species (Supplemental Figure 2B), 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; Supplemental Figure 1). 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 whether a similar differential expression of miR-499 was present in human myocytes and hCSCs. miR-499 was essentially undetectable in hCSCs, while extremely high levels were found in cardiomyocytes (Figure 2A; Supplemental Figure 2). A web-based target-prediction program was then employed to identify putative target genes of miR-499 (Supplemental Table 3). Among the genes that scored as strong predicted-targets, based on high complementarity and evolutionary conservation, we selected two 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 which negatively modulates cell differentiation. The 3’ untranslated region (3’-UTR) of Sox6 contains 5 evolutionary conserved miR-499 binding sites. Human Sox6 possesses one additional non-conserved miR-499 binding site. Two evolutionary conserved sites are located in the 3’-UTR of Rod1. These putative binding
sequences show high complementarity with the seed region of miR-499 (Supplemental Figure 3), 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 cardiomycocytes (Figure 2B; Supplemental Figure 1). Importantly, Sox 6 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. 3T3-fibroblasts were employed to minimize the influence of endogenous miR-499. The 3'-UTR of Sox6 and Rod1 was obtained by PCR and ligated downstream the luciferase coding sequence in two reporter plasmids. The interaction of miR-499 and the 3'-UTR of Sox6 and Rod1 was expected to downregulate luciferase expression. 3T3-cells were transfected with a plasmid carrying miR-499; the expression of miR-499 was assessed by qRT-PCR and found to be 2,300-fold higher than in cells transduced with blank plasmid (Figure 2D; Supplemental Figure 1). Cells overexpressing miR-499 were then co-transfected 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 employed 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: a) Translocation of miR-499 from myocytes to neighboring hCSCs via gap junctions; b) Transcript and protein levels of the miR-499 target genes, Sox6 and Rod1, in hCSCs; and c) Expression of transcription factors specific of myocytes in hCSCs. These in vitro assays were complemented with in vivo studies to document the effects of hCSCs overexpressing miR-499 on: a) Myocardial regeneration after infarction; and b) Phenotypic properties and mechanical behavior of new myocytes, and their impact on ventricular function.

To establish whether miR-499 had the ability to migrate from donor cells to recipient hSCs, C2C12 myoblasts were first employed as donor cells. To obtain high levels of expression, C2C12 myoblasts were transfected with a plasmid carrying miR-499 (Figure 3A; Supplemental Figure 1). C2C12 myoblasts were then co-cultured with EGFP-tagged hCSCs and the presence of miR-499 was documented by Q-FISH. Initially, miR-499 was restricted to C2C12 myoblasts (Supplemental Figure 4), but 36 hours later, miR-499 was identified in EGFP-positive hCSCs. Connexin 43 was expressed at the interface between the two 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 hCSCs. 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 non-physiological level of miR-499 in transfected C2C12 cells, a similar protocol was employed with neonatal rat cardiomyocytes. EGFP-labeled hCSCs were sorted 5 days after being co-cultured with cardiomyocytes and compared with hCSCs seeded alone. Expression of miR-499, measured by qRT-PCR, was ~200-fold higher in the co-cultured cells (Figure 3D). This process was markedly attenuated by the gap junction inhibitor α-GA. Exposure of hCSCs to the medium obtained from myocyte cultures failed to increase miR-499 expression in hCSCs. The
culture medium showed only in ~60% of the cases minimal quantities of miR-499 by qRT-PCR, although snoRNA was found in all samples (Supplemental Figure 4).

The localization of miR-499 in hCSCs was documented further by in situ hybridization and confocal microscopy. Thirty-six hours after co-culture of these cells with cardiomyocytes, 35% of hCSCs were positive for miR-499 and this value was markedly decreased in the presence of α-GA (Figure 3E and F). Additionally, the use of a scrambled probe failed to label hCSCs and cardiomyocytes. At the concentration used, α-GA had no impact on the viability of hCSCs 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 hCSCs.

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 hCSCs were loaded with Cy3-miR-499 together with the blue fluorescent dye, cascade blue. Transfer of cascade blue between adjacent cells occurs through gap junctions; it was utilized as an indicator of functional cell-to-cell coupling. hCSCs were employed because they express connexin 43 mRNA but lack the protein (Supplemental Figure 5). However, following interaction with other cells, hCSCs 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 cells was followed by acquisition of serial images with an inverted microscope. The appearance of blue and red fluorescence, i.e., cascade blue and Cy3-miR-499, in unlabeled hCSCs adjacent to the injected hCSCs was considered indicative of the formation of functional gap junctions, critical for miR-499 translocation (Supplemental Figure 6). To test whether the translocated miR retained its function, hCSCs, transfected with a plasmid carrying miR-499, were used as donor cells and hCSCs, transfected with a luciferase plasmid containing the 3'-UTR of Sox6, were employed as recipient cells. Donor and recipient cells were co-cultured for 3 days. Luciferase activity decreased more than 40% in hCSCs co-cultured with donor cells transduced with miR-499. This effect was abolished by α-GA (Figure 3H). Thus, miR-499 enters hCSCs via gap junction channels, preserving its function. This biological phenomenon has been termed “mircrine.”

### Function of miR-499 in hCSCs

To establish the role of miR-499 and its target genes in hCSC 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 transduced hCSCs increased ~4,000-fold, but this change did not affect Sox6 and Rod1 transcripts (Supplemental Figure 7; see also Supplemental Figure 1). However, protein levels of Sox6 and Rod1 were markedly decreased (Figure 4A). Importantly, upregulation of miR-499 decreased BrdU incorporation in hCSCs, and this attenuation in hCSC growth was coupled with enhanced expression of Nkx2.5 and GATA4 (Figure 4B; Supplemental Figure 8).

To establish further whether the function of miR-499 in hCSC differentiation was mediated by Sox6 and Rod1, siRNA was employed to block the synthesis of these two 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; Supplemental Figure 1). Additionally, the fraction of Nkx2.5- and GATA4-positive cells increased 2-fold. A 3–4-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 mediated 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 acutely after infarction in the border zone of immunosuppressed rats. The high level of expression of miR-499 was confirmed by qRT-PCR (Supplemental Figure 9). Infarcted rats injected with EGFP-positive hCSCs (EGFP-hCSCs) were used as controls. Animals were exposed continuously to BrdU and killed 10–14 days later.

In all hearts, infarct size involved 50% of left ventricular (LV) myocytes (Supplemental Figure 10). Myocardial regeneration was detected in the two 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 10–14 day period following coronary occlusion and cell implantation (Figure 5B). Similarly, the level of myocyte formation, measured by the cell cycle protein Ki67, was comparable in these two groups of hearts (Supplemental Figure 10) documenting that myocyte regeneration was still ongoing at sacrifice (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). The specificity of immunolabeling for EGFP, α-sarcomeric actin, BrdU, Ki67 and Alu was confirmed by spectral analysis (Supplemental Figure 11). 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 1.44-fold (P<0.005) greater in animals injected with miR-499-EGFP-hCSCs, respectively (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, 600 µm³ in volume or larger, since myofibrils were not seen in smaller cells.

Treatment with EGFP-hCSCs or miR-499-EGFP-hCSCs resulted in an improvement in LV function, consisting of a better preservation of LV developed pressure, 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. But, whether this biological role of miR-499 in vivo was completed at this early stage of ventricular remodeling remained unclear.

Because of this question, infarcted hearts injected with EGFP-hCSCs and miR-499-EGFP-hCSCs were sacrificed 21 days later at 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 current study indicate that the commitment of hCSCs to the myocyte lineage and the generation of functionally-competent adult cardiomyocytes is influenced by miR-499, which is barely detectable in primitive cells, but is highly expressed in post-mitotic 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 role of these two types of junction remains 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 microrna mechanism may be impaired partly, 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 following 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 observations raise challenging questions on 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. Although significant differences have been reported on the extent of myocyte turnover in humans, 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. hCSCs maintain the balance between cell death and regeneration in the young adult heart, but this compensatory mechanism is no longer effective later in life or following severe myocardial injury. 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 post-mitotic 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. 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 organized myofibrillar compartment, typically present in fetal-neonatal cardiomyocytes. The demonstration that myogenic miRs, including miR-1 and miR-499, favor their evolution towards a more mature phenotype is consistent with the results of the current study in which the critical role of miR-499 was documented at the level of the controlling cell, i.e., the hCSC. Similar findings have been reported in embryonic stem cells.23

A group of miRs has been found to be highly represented in the intact mammalian heart including humans.9 These miRs are dynamically regulated in cardiac diseases, suggesting that inhibition of their target genes may be involved in the control of cardiac hypertrophy and fibrosis. However, the actual role of the muscle specific miR-1 in human heart failure is open to question and the function of miR-21 in pathological ventricular remodeling has been challenged.24 Importantly, miR-29 inhibits a cluster of genes regulating tissue fibrosis, while miR-195, miR-199a and miR-320 are critical determinant of cell viability in myocardial ischemia.9

Myosin-encoded miRs form a hierarchically organized network of small RNAs that control the expression of contractile genes.8 Although miR-208a appears to occupy an upstream position in the pathway regulating the shift in myosin expression, miR-499 replaces its function in miR-208a-null-animals.25 Unexpectedly, deletion of miR-208b and miR-499 lacks an overt cardiac phenotype, suggesting that a significant degree of redundancy exists among myomiRs.

Our findings document that miR-499 inhibits the expression of Sox6 and Rod1 only at the protein level. The incomplete base-pairing between the nucleotides flanking the seed region of miR-499 and its binding sequences on Sox6 and Rod1 mRNA indicates that repression of translation rather than mRNA degradation was the major mechanism of miR-499 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, 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

Clinical Perspective
Effective repair of the damaged heart requires the recovery of the normal architecture and function of the organ. Myocardial regeneration mediated by activation and differentiation

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of human cardiac stem cells (hCSCs) commonly results in the formation of small myocytes with fetal-neonatal characteristics. The number of new cells exceeds the number of myocytes lost, but the replacement of cardiac mass is incomplete since the regenerated myocytes fail to acquire the adult phenotype. This limitation in myocyte maturation has been overcome partly by enhancing the expression of miR-499 in hCSCs; miR-499 represses genes responsible for the primitive state of these cells, promoting the program of myocyte differentiation, structurally and functionally. In the presence of miR499, the regenerated parenchymal cells increase in size and acquire electrical and mechanical properties of adult cells. This process occurs by the physiological translocation of miR-499 from myocytes to hCSCs via gap junction channels. Importantly, the potentiated reconstitution of the infarcted myocardium has significant effects on the anatomical remodeling and hemodynamic performance of the injured heart. Understanding the molecular control that regulates the differentiation of hCSCs into functionally-competent cardiomyocytes may be critical for the implementation of stem cell therapy in the diseased heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Figure 1. Expression of miRs in cardiomyocytes and CSCs. A, Expression of selected miRs in rat cardiomyocytes (Myo) and CSCs. B and C, miR-499 in rCSCs and rMyo parallels Myh7b mRNA expression. Small nucleolar snoRNA, normalization. Gapdh: housekeeping-gene.
Figure 2.
Target genes of miR-499. A, miR-499 in hCSCs and human-cardiomyocytes (hMyo). Small nucleolar RNA RNU44, normalization. B, Sox6 and Rod1 mRNA. Fold-changes vs. hCSCs or rCSCs. C, Protein levels of Sox6 and Rod1. Loading, Gapdh and Ponceau red. D, qRT-PCR of miR-499 in transfected 3T3 cells. E, Repressive activity of miR-499 on Sox6 and Rod1 3’-UTRs by luciferase assay. *,**,†P<0.05 vs. 0, 0.1 and 0.4 μg, respectively.
Figure 3.
Translocation of miR-499 through gap junctions. A, qRT-PCR of miR-499 in transfected C2C12 cells (+miR). C2C12: non-transfected cells. B, Co-culture of C2C12 cells and hCSCs. Upper panels: hCSCs show red and green fluorescence (miR-499-EGFP, arrows) and C2C12 cells show red fluorescence only (miR-499). Lower panels: connexin 43 (white, arrowheads) at the interface of hCSCs and C2C12 cells. C, Fraction of hCSCs expressing miR-499. +α-GA, gap junction inhibitor. *P<0.05 vs. -α-GA. D, miR-499 expression in hCSCs seeded alone (Control), hCSCs exposed to medium collected from myocyte cultures (+Medium), EGFP-positive hCSCs sorted by FACS 5 days after co-culture with myocytes in the absence (+Myo) and presence (+Myo +α-GA) of α-GA. *,**,†P<0.05 vs. Control, +Medium, and +Myo, respectively. E, Co-culture of cardiomyocytes (α-SA, yellow) and EGFP-positive hCSCs (green). At 2 hours (left panel), miR-499 (red) is confined to myocytes. At 36 hours (central panel), hCSCs show miR-499 (red) and EGFP (miR-499-EGFP, arrowheads). Connexin 43 is present at the interface of hCSCs and myocytes (white). Scrambled probe (right panel). F, Results are mean±SD. +α-GA, gap junction inhibitor, 10 µM. *P<0.05 vs. -α-GA. G, hCSC and myocyte apoptosis and α-GA. *,**,†P<0.05 vs. Control and 10 µM α-GA, respectively. H, Repressive activity of translocated miR-499 on Sox6 UTR in hCSCs is abolished by α-GA, 10 µM. Blank: donor cells transfected with empty plasmid. *,**,P<0.05 vs. blank and miR-499, respectively.
Figure 4.
Function of miR-499 in hCSCs. A, Sox6 and Rod1 expression in hCSCs infected with a lentivirus carrying DsRed only (control) or DsRed-miR-499. Loading: Ponceau red. *P<0.05 vs. Control.
B, hCSCs positive for BrdU, Nkx2.5 and GATA4. *P < 0.05 vs. Control.
C, Sox6 and Rod1 mRNA in hCSCs transfected with negative siRNA (Neg) or specific siRNA (Spec). *P<0.05 vs. Neg.
D, hCSCs positive for Nkx2.5 and GATA4. *P<0.05 vs. siRNA-Neg.
**Figure 5.**
Cardiac repair with miR-499-EGFP-hCSCs. **A**, Regenerated myocardium (arrowheads) within the infarct 10 days after surgery and cell implantation. The area included in the rectangle is shown at higher magnification in the inset. Myocytes: α-sarcomeric-actin, α-SA, red. EGFP: green. *Dead myocytes. **B** and **C**, Localization of BrdU (B, red) and Ki67 (C, magenta) in EGFP-positive human-myocytes. Cell boundaries are defined by laminin (white). **D**, Human-myocytes, stained by α-SA (red) and EGFP (green), exhibit sarcomere striation and are positive for Alu (white).
Figure 6.
Regenerated myocytes. A, Connexin 43 and N-cadherin (white) are expressed in EGFP-positive α-SA-positive human-myocytes (arrowheads). B, Aggregate myocyte volume and average cell volume of human-myocytes. Infarcted hearts treated with EGFP-hCSCs (Ctrl) or miR-499-EGFP-hCSCs (miR). *P<0.05 vs. Ctrl. C, Size distribution of human-myocytes derived from differentiation of EGFP-hCSCs (green-bars) and miR-499-EGFP-hCSCs (red-bars).
Figure 7.
Ventricular function and mechanics of human myocytes. A, LV end-diastolic pressure (LVEDP), LV systolic pressure (LVSP), LV developed pressure (LVDevP), and LV+ and -dP/dt in sham-operated (Sham), infarcted-untreated (MI), and infarcted hearts treated with EGFP-hCSCs (Ctrl) or miR-499-EGFP-hCSCs (miR). *,**,†P<0.05 vs. Sham, MI and Ctrl, respectively.
B, Human-myocytes derived from the differentiation of EGFP-hCSCs (left panels) and miR-499-EGFP-hCSCs (right panels).
C, Volume of myocytes in Ctrl and miR.
D, Fractional-shortening of regenerated human-myocytes (New) and surviving rat-myocytes (Surv): tracings of surviving and regenerated myocytes derived from miR-499-EGFP-hCSCs. Spared myocytes had depressed fractional shortening. *P<0.05 vs. Surv.