MicroRNA-1 and -499 Regulate Differentiation and Proliferation in Human-Derived Cardiomyocyte Progenitor Cells

Joost P.G. Sluijter, Alain van Mil, Patrick van Vliet, Corina H.G. Metz, Jia Liu, Pieter A. Doevendans, Marie-José Goumans

Objective—To improve regeneration of the injured myocardium, it is necessary to enhance the intrinsic capacity of the heart to regenerate itself and/or replace the damaged tissue by cell transplantation. Cardiomyocyte progenitor cells (CMPCs) are a promising cell population, easily expanded and efficiently differentiated into beating cardiomyocytes. Recently, several studies have demonstrated that microRNAs (miRNAs) are important for stem cell maintenance and differentiation via translational repression. We hypothesize that miRNAs are also involved in proliferation/differentiation of the human CMPCs in vitro.

Methods and Results—Human fetal CMPCs were isolated, cultured, and efficiently differentiated into beating cardiomyocytes. miRNA expression profiling demonstrated that muscle-specific miR-1 and miR-499 were highly upregulated in differentiated cells. Transient transfection of miR-1 and -499 in CMPC reduced proliferation rate by 25% and 15%, respectively, and enhanced differentiation into cardiomyocytes in human CMPCs and embryonic stem cells, likely via the repression of histone deacetylase 4 or Sox6. Histone deacetylase 4 and Sox6 protein levels were reduced, and small interference RNA (siRNA)-mediated knockdown of Sox6 strongly induced myogenic differentiation.

Conclusion—miRNAs regulate the proliferation of human CMPC and their differentiation into cardiomyocytes. By modulating miR-1 and -499 expression levels, human CMPC function can be altered and differentiation directed, thereby enhancing cardiomyogenic differentiation. (Arterioscler Thromb Vasc Biol. 2010;30:859-868.)

Key Words: human progenitor cells | differentiation | miRNA | gene regulation | repression

The old paradigm that the heart is a terminally differentiated organ, lacking the capacity for self-renewal and regeneration of the damaged myocardium, is being challenged, because resident cardiac progenitor cells have been identified1–3 that are able to differentiate into cardiomyocytes, as well as other cardiac cell types, such as endothelial cells or fibroblasts.4–7 These progenitor cells can be isolated, expanded in vitro, and transplanted into the damaged rodent myocardium, thereby improving cardiac performance. Recently, we have isolated human cardiomyocyte progenitor cells (hCMPCs) from fetal hearts and adult biopsies that can be expanded in culture and efficiently differentiated into beating cardiomyocytes, without the need for coculture with neonatal cardiomyocytes.8–10 We tested the functional relevance of these hCMPCs by transplanting them into ischemic murine cardiac tissue, which resulted in improved cardiac performance up to 3 months.11 Human cells were still present, including in situ differentiated cardiomyocytes and vascular structures. Little is known about what drives proliferation and differentiation of hCMPCs in vitro, and their exact role and regulation in vivo are still unexplored.

MicroRNAs (miRNAs) were shown to be important for the posttranscriptional regulation of target genes and serve important regulatory functions in a range of biological processes, including maintenance of stem cell–ness and modulation of differentiation.12 miRNAs are short (19 to 23 nucleotides), noncoding small regulatory RNAs that are loaded into the RNA-induced silencing complex, recognize the 3′-untranslated region (UTR) of target mRNAs, and thereby regulate their expression by translational repression or mRNA degradation.13 Recently, several papers focused on the differential expression of miRNAs in cardiac pathology, identifying clusters of differentially expressed miRNAs among different human cardiomyopathies.14,15 Furthermore, the contribution of specific miRNAs in muscle differentiation and cardiac hypertrophy was described.16,17 Here, we analyzed miRNA expression during the growth and after cardiomyogenic differentiation of hCMPCs. We
### miRNA ID | Proliferating CMPC | Differentiated CMPC | log2 fold induction | Proliferating CMPC | Differentiated CMPC | log2 fold repression
--- | --- | --- | --- | --- | --- | ---
1. hsa-miR-1 | 26.1 | 24058.8 | 9.91 | 962.3 | 10,000 | 100,000
2. hsa-miR-499 | 10.9 | 1372.2 | 7.07 | 133.9 | 10,000 | 100,000
3. hsa-miR-133a | 9.3 | 715.5 | 6.28 | 77.6 | 10,000 | 100,000
4. hsa-miR-133b | 15.6 | 727.8 | 5.72 | 1000 | 10,000 | 100,000
5. hsa-miR-335 | 128.9 | 5829.0 | 5.42 | 42.8 | 10,000 | 100,000
6. hsa-miR-450 | 32.2 | 643.7 | 4.40 | 21.2 | 10,000 | 100,000
7. hsa-miR-542-3p | 12.5 | 245.1 | 4.21 | 18.5 | 10,000 | 100,000
8. hsa-miR-143 | 1143.5 | 19731.0 | 4.04 | 16.5 | 10,000 | 100,000
9. hsa-miR-217 | 8.8 | 122.1 | 3.79 | 13.8 | 10,000 | 100,000
10. hsa-miR-204 | 27.9 | 330.7 | 3.68 | 12.8 | 10,000 | 100,000
11. hsa-miR-203 | 20.0 | 181.2 | 3.54 | 11.6 | 10,000 | 100,000
12. hsa-miR-210 | 31.3 | 295.4 | 3.35 | 10.2 | 10,000 | 100,000
13. hsa-miR-145 | 2015.3 | 19156.3 | 3.34 | 10.2 | 10,000 | 100,000
14. hsa-miR-422b | 68.4 | 630.5 | 3.21 | 9.3 | 10,000 | 100,000
15. hsa-miR-334-5p | 1709.7 | 10020.7 | 3.34 | 10.2 | 10,000 | 100,000
16. hsa-miR-30d | 918.3 | 4009.8 | 2.07 | 4.2 | 10,000 | 100,000
17. hsa-miR-374 | 860 | 734 | 2.06 | 4.2 | 10,000 | 100,000

### miRNA ID | Proliferating CMPC | Differentiated CMPC | log2 fold repression | Proliferating CMPC | Differentiated CMPC | log2 fold induction
--- | --- | --- | --- | --- | --- | ---
1. hsa-miR-146a | 7683.9 | 155.1 | -5.59 | 48.1 | 10,000 | 100,000
2. hsa-miR-155 | 6457.5 | 127.6 | -5.53 | 46.2 | 10,000 | 100,000
3. hsa-miR-126 | 8375.6 | 380.6 | -4.48 | 22.3 | 10,000 | 100,000
4. hsa-miR-19a | 192.5 | 12.1 | -3.99 | 15.9 | 10,000 | 100,000
5. hsa-miR-31 | 10019.8 | 781.9 | -3.66 | 12.7 | 10,000 | 100,000
6. hsa-miR-222 | 13140.2 | 1124.8 | -3.48 | 11.2 | 10,000 | 100,000
7. hsa-miR-221 | 11128.8 | 1057.9 | -3.32 | 10.0 | 10,000 | 100,000
8. hsa-miR-20b | 4227.6 | 439.9 | -3.27 | 9.6 | 10,000 | 100,000
9. hsa-miR-542-5p | 181.9 | 20.2 | -3.13 | 8.7 | 10,000 | 100,000
10. hsa-miR-625 | 121.3 | 13.3 | -3.08 | 8.4 | 10,000 | 100,000
11. hsa-miR-663 | 9058.1 | 1057.9 | -3.04 | 8.2 | 10,000 | 100,000
12. hsa-miR-602 | 303.5 | 42.3 | -2.95 | 7.7 | 10,000 | 100,000
13. hsa-miR-621 | 226.6 | 29.3 | -2.93 | 7.6 | 10,000 | 100,000
14. hsa-miR-323 | 309.9 | 44.1 | -2.93 | 7.6 | 10,000 | 100,000
15. hsa-miR-29b | 121.7 | 17.9 | -2.84 | 7.2 | 10,000 | 100,000
16. hsa-miR-299-5p | 331.7 | 47.2 | -2.81 | 7.0 | 10,000 | 100,000
17. hsa-miR-494 | 2099.8 | 353.4 | -2.60 | 6.1 | 10,000 | 100,000
18. hsa-miR-212 | 190.2 | 33.6 | -2.57 | 5.9 | 10,000 | 100,000
19. hsa-miR-18a | 591.4 | 99.8 | -2.57 | 5.9 | 10,000 | 100,000
20. hsa-miR-638 | 37354.8 | 7219.1 | -2.33 | 5.0 | 10,000 | 100,000
21. hsa-miR-329 | 540.3 | 110.4 | -2.32 | 5.0 | 10,000 | 100,000
22. hsa-miR-106a | 5998.6 | 1252.7 | -2.27 | 4.8 | 10,000 | 100,000
23. hsa-miR-17-5p | 8154.2 | 1645.0 | -2.22 | 4.6 | 10,000 | 100,000
24. hsa-miR-20a | 9392.7 | 2119.2 | -2.21 | 4.6 | 10,000 | 100,000
25. hsa-miR-19b | 2901.6 | 660.8 | -2.14 | 4.4 | 10,000 | 100,000
26. hsa-miR-505 | 202.7 | 50.0 | -2.09 | 4.3 | 10,000 | 100,000
27. hsa-miR-15b | 7703.7 | 1734.1 | -2.03 | 4.1 | 10,000 | 100,000
explored 2 identified miRNAs, miR-1 and -499, in more detail for their function in progenitor cells, and we show that miR-1 and -499 repress hCMPC proliferation and enhance in vitro differentiation into cardiomyocytes.

Methods

hCMPCs
Human fetal heart tissue was collected after elective abortion (weeks 15 to 19 of gestation); single cells were obtained; and hCMPCs were isolated, differentiated, and characterized as described.8–10 Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. Lentiviral expression of Sox6 and histone deacetylase 4 (HDAC4) (Hdac4) RNA interference (RNAi) was used to knock down their expression before the start of differentiation. Cells proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich).

Expression Analysis
Total RNA was isolated with the mirVana RNA Isolation Kit (Ambion) or TriPure isolation reagent (Roche Applied Science). miRNA profile was determined by microarray analysis using the ρParaflo microfluidic chip (LC Sciences) and validated with TaqMan MicroRNA Assays (Applied Biosystems). miRNA expression was determined using the SuperScript First-Strand Synthesis System (Invitrogen) or Tripure isolation reagent (Roche Applied Science). Expression Analysis

hCMPC Transfection
Precursor molecules for miR-1 (PM10660), miR-499 (PM10496), and a scrambled miRNA control (AM17121) with or without FAM dye label were obtained from Ambion. Inhibitors for miR-1 (H-300586-06), miR-499 (H-300837-06), and scrambled controls (SCR) (IN-001005-01) were obtained from Dharmacon Scientific (Thermo Fisher Scientific Inc.) and transfected with siPORT NeoFX transfection agent (Ambion).

Embryoid Body Assay
Mouse embryonic stem cells (E14-IB10) were cultured in BRL-conditioned medium as described before18 and transfected with precursor miRNA (premiR)-1, premiR-499, and SCR before body formation.

In Situ Hybridization
In situ hybridization to determine hsa-miR-499 localization was performed as previously described19 by using locked nucleic acid (LNA) digoxigenin (DIG)-labeled probes (Scramble-miR, Exiqon, 99001-01, and hsa-miR-499, Exiqon, 38306-01).

Luciferase Experiments
Primers were generated for the 3′ UTR of Sox6 (human and mouse, 87% homology; all sites conserved), a putative target of miR-499, and inserted into pMIR-REPORT miRNA expression reporter vector (#5795, Ambion) to determine suppression efficiency of miR-499.

Mutational cloning in the seed region of predictive sites was performed with the QuikChange site-directed mutagenesis kit (200518, Stratagene), according to the manufacturer’s instructions (see Supplemental Figure IIC).

Statistical Analysis
Data are presented as mean±SEM of at least 3 independent experiments and were compared using the 2-tailed paired Student t test or 1-way ANOVA. Differences were considered statistically significant at P<0.05.

Results
Expression of miRNAs in Proliferating and Differentiated hCMPCs
miRNA expression was determined in both proliferating and differentiated hCMPCs by comprehensive miRNA microarray analysis. From all human targeted miRNAs (total, 453), 188 miRNAs (42%) were detectable in proliferating hCMPCs and 195 (43%) in differentiated hCMPCs. Of these, 19 showed a 4-fold or higher increase (Figure 1A and 1B) and 27 showed a 4-fold or higher decrease (Figure 1A and 1C) in expression on differentiation. Among the highly upregulated miRNAs (Figure 1B), several cardiac and skeletal muscle–specific miRs were present, such as miR-1, miR-133a, and miR-133b. In addition to these known muscle-specific miRs, other miRs were highly regulated as well, including miR-499, a miRNA that is functional but has not yet been studied.

To confirm miRNA expression changes, we performed quantitative TaqMan-based RT-PCR. After normalization to RNU19, miR-1, miR-133a, and miR-499 expression was significantly increased and miR-126 and miR-155 significantly decreased in differentiated hCMPCs (Figure 1D–1H). Some discrepancy exists between the observed fold increase in array and quantitative RT-PCR, most likely because of calculation of a ratio with the relative low values in proliferating cells. Although they were not detected by the microarray because of technical limitations, miR-206 and -208 are induced in differentiated cardiomyocyte progenitor cells (CMPCs) (Supplemental Figure I). We selected the strongest regulated miRs, miR-1 and miR-499, for functional follow-up studies to test their significance for myogenic differentiation in hCMPCs.

Localization and Tissue Distribution of miR-499
miR-1 is a muscle-specific miRNA that is known to control myogenic differentiation in the embryonic mouse heart.20 However, function of miR-499 is not currently known, and tissue distribution not extensively reported.21 This highly conserved miRNA (Supplemental Figure IIA) is located in intronic region 20 of human MYH7B (myosin heavy chain B) on chromosome 20 (Supplemental Figure IIB). Because we observed miR-499 expression in differentiated CMPCs, we confirmed the endogenous presence of miR-499 in cardiomyocytes in vivo by in situ hybridization for miR-499 in fetal and adult human hearts, as demonstrated by containing

Figure 1. A, miRNA profiling of proliferating hCMPCs and hCMPCs differentiated to cardiomyocytes. Variation in detection of each miRNA on the array is plotted in the figure (axes are arbitrary expression units). B and C, miRNAs are presented that are highly upregulated (>log2 difference) (B) or downregulated (>log2 difference) (C) in differentiated hCMPCs (numbers in proliferating and differentiated hCMPCs are arbitrary expression units). D–H, Quantitative RT-PCR for miR-1 (D), miR-133a (E), miR-499 (F), miR-126 (G), and miR-155 (H) in proliferating hCMPCs (prol. CMPC) and hCMPCs differentiated to cardiomyocytes (diff. CMPC). Data are presented as fold induction±SEM; *P<0.05.

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with cardiac α-actinin (Figure 2A, 2C, and 2F); however, miR-499 was not present in endothelial cells (Figure 2E). This myocyte-specific expression was confirmed when analyzing several cell types for miR-499 expression (Figure 2G). Subsequently analysis of mouse heart, brain, spleen, liver, lung, quadriceps muscle, kidney, and gut tissues showed that the mature miR-499 is abundantly expressed in cardiac tissue and almost absent in other tissues, including skeletal muscles (Figure 2H). Interestingly, MYH7B mRNA is also restricted to heart muscle (Figure 2I), indicating that both MYH7Bβ and miR-499 are cardiac enriched.

**Functional Analysis of PremiR-1 and PremiR-499 In Vitro**

**Transfection Efficiency and Cellular Proliferation** Because expression of miR-1 and miR-499 increased on differentiation, we used a gain-of-function approach to study their roles in proliferating hCMPCs using premiR molecules.

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**Figure 2.** In situ hybridization for miRNA-499 (A and C) (red dots) and a scrambled control probe (B and D) in human fetal heart, double stained for α-actinin (A through D, green) or PECAM (E, green). F, miRNA-499 (red) localization in adult human heart, double stained for α-actinin (green). Nuclei are stained blue. Bar=50 μm in A, B, E, and F and 15 μm in C and D. G, Quantitative RT-PCR for miR-499 in human fetal cardiomyocytes (HFCM), cardiac human fibroblasts (CHF), adventitial fibroblasts (HF), human microvascular endothelial cells (HMECS), endothelial progenitor cells (EPC), mesenchymal stem cells (MSC), and vascular smooth muscle cells (VSMC) (G, all human origin). H and I, miR-499 (H) and MYH7Bβ (I) expression in different mouse samples. Expression is presented as fold induction±SEM and compared with the lowest expression (N=5).
miR-1 resulted in a significant reduction in cellular hCMPCs (Figure 3C and 3D).

compared with endogenous expression levels in control miR-1 and -499 with increasing transfection concentrations RT-PCR. We observed an increase in expression of mature the mature miR-1 and -499 expression by TaqMan-based processing of the premiR into the mature miR, we analyzed a signal was observed on transfection of FAM-premiRs, visible transfection procedures (Figure 3A and 3B). A dose-dependent but not by the SCR miR. *

Figure 3. A and B, Immunofluorescent detection of untransfected (A) and 100 nM (B) FAM-labeled control miR, transfected in hCMPCs. Nuclei are stained blue. Bar = 25 μm. Transfection with increased concentration of miR-1 (C) or miR-499 (D) premiR molecules resulted in increased detectable expression of the mature miR-1 or -499, respectively, by quantitative RT-PCR. Data are expressed as ΔΔCt SEM. E, Proliferation of hCMPCs is determined 4 days after transfection of increased concentrations premiR-1, premiR-499, and SCR miRNA. Proliferation was significantly decreased after transfection of premiR-1 and -499 but not by the SCR miR. *P<0.01.

A FAM-labeled negative control premiR was used to optimize transfection procedures (Figure 3A and 3B). A dose-dependent signal was observed on transfection of FAM-premiRs, visible up to 12 days after transfection in proliferating hCMPCs.

To verify the transfection efficiency and functional cellular processing of the premiR into the mature miR, we analyzed the mature miR-1 and -499 expression by TaqMan-based RT-PCR. We observed an increase in expression of mature miR-1 and -499 with increasing transfection concentrations compared with endogenous expression levels in control hCMPCs (Figure 3C and 3D).

Four days after transfection, increased concentration of premiR-1 resulted in a significant reduction in cellular proliferation up to 25% (100 nM) compared with SCR-transfected control cells (Figure 3E), as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Transfection of miR-499 in hCMPCs resulted in a significant reduction in cell proliferation as well (15% with 100 nM; Figure 3E). When using similar concentrations of the scrambled control miRNA, no significant effect on proliferation was observed (Figure 3E). These effects could still be observed after 6 days, but a dilution of the miRNA resulted in a less pronounced effect (data not shown). This effect was not caused by reduced cell viability (see Supplementary Figure IIIA) or by an increase in cellular apoptosis/necrosis (data not shown). Moreover, by overexpressing a different miRNA expressed in CMPCs, miR-155, we did not observe an inhibitory effect on proliferation (Supplemental Figure IIIB).

hCMPC Differentiation Into Cardiomyocytes
A prerequisite for differentiation is inhibition of proliferation. Because addition of miR-1 and -499 reduced CMPC proliferation, and increased levels of these miRs are present in differentiated hCMPCs, we studied whether we could enhance cardiomyocyte differentiation by the addition of miR-1 and -499 to our differentiation protocol (only 5-azacytidine [5-aza] stimulation).

Figure 4A through 4H. Combining the 2 miRNAs did not result in an enhanced synergistic myogenic differentiation (Figure 4G and 4H). To study whether the presence of these miRNAs is a prerequisite for cardiomyogenic differentiation, we used specific inhibitors for miR-1 and miR-499. Compared with the control (SCR-anti), cardiomyogenic differentiation was blocked, as determined by troponin T mRNA, Mlc-2v mRNA, and cardiac α-actinin protein expression, 2 weeks after the start of differentiation (Figure 4G through 4I).

Interestingly, miR-1 and -499 also enhanced cardiac differentiation of mouse embryonic stem cells. After treatment with premiR-499 and premiR-1, increases in the percentage of beating embryoid bodies of 2.7- and 6.2-fold, respectively, were observed compared with SCR miR (Supplemental Figure IVA). Enhanced differentiation was confirmed by increased immunofluorescent α-actinin staining (Supplemental Figure IVB through IVE) and by increased levels of GATA4 mRNA expression at day 4 (Supplemental Figure IVF) as an early marker, and Mlc-2v at day 8 (Supplemental Figure IVG) as a late marker.

miRNA Targets and Potential Mechanism
miR-1, which has been shown to enhance hCMPC differentiation into cardiomyocytes, has previously been reported to pro-
Figure 4. hCMPCs were differentiated into cardiomyocytes by 5-aza stimulation and also transfected with 100 nM SCR miR, miR-1 (A, C, and E), or miR-499 (B, D, and F). mRNA expression was determined after 2 weeks by RT-PCR for troponin T (A and B), cardiac actinin (C and D), and Mlc-2v (E and F) and compared with SCR miR-transfected cells. Troponin T mRNA (G), Mlc-2v mRNA (H), and α-actinin protein (I) levels were determined 2 weeks after transfection of hCMPCs with premiR-1 or -499, inhibitors for miR-1 or -499 (anti), a combination of premiRs or inhibitors, or their controls (scr and scr-anti). The expression of cardiac markers was significantly increased on transfection of miR-1 and -499 and blocked by inhibitors of these miRs. Data are presented as mean fold increase ± SEM; N=4. β-Tubulin was used as protein loading control.
mote myogenesis by targeting HDAC4, a transcriptional negative regulator of muscle gene expression. On transfection of miR-1 in hCMPCs, we confirmed that HDAC4 protein levels were indeed reduced by miR-1 transfection (Figure 5A).

Because miR-499 function has not been studied before, we used online algorithms (TargetScan and Sanger) to predict potential miR-499 targets that could be involved in myogenic differentiation. Both algorithms predicted that 4 conserved seed sites in the transcription factor SOX6 (sex determining region Y-box 6), involved in muscle differentiation (online Figure II). To verify the potential inhibitory effect of miR-499 on Sox6 translation, we cloned the 3′ UTR of Sox6 behind a cytomegalovirus-driven luciferase construct. Transient transfection of HEK293T cells with the luciferase construct in combination with premiR-499 showed a reduction in luciferase activity of miR-499 that could be restored when a specific miR-499 inhibitor was also included (Figure 5B). Interestingly, the 3′ UTR of Sox6 also contains a putative site targeted by miR-1 (Supplemental Figure II), and cotransfection of miR-1 and cytomegalovirus-luciferase-3′ UTR-Sox6 led to a mild reduction in luciferase activity. When using a scrambled miR, this effect was not observed (Figure 5B). To confirm the specific binding of miR-499, we mutated 2 nucleotides in the seed region of the predicted sites. Although single mutations (Figure 5C; 1, 2, 3, and 4) did not show a reduction in luciferase activity, mutating 2 sites did result in a significant reduction in suppression capacity of miR-499 (Figure 5C). In addition, mutating the miR-1 site reduced the inhibitory effect that miR-1 had on the Sox6 UTR (Supplemental Figure V).

Sox6 is expressed in our proliferating hCMPCs. To demonstrate that Sox6 is a target of miR-1 and -499, we transfected miR-499 and miR-1 in the hCMPCs and observed reduced Sox6 protein levels (Figure 5D). We analyzed miR-1 and -499 expression in proliferating, 5-aza-treated, and fully differentiated hCMPCs. On 5-aza treatment, miR-1 and -499 expression were induced, whereas miR-499 expression remains similar. Their target genes, HDAC4 and Sox6, were reduced upon 5-aza treatment (Supplemental Figure VI), suggesting that this is a prerequisite for differentiation. To confirm that Sox6 is indeed important for myogenic differentiation of hCMPCs, Sox6 expression was knocked down via lentiviral transduction of short hairpin RNA (Figure 6A). hCMPCs, deficient for Sox6, stopped proliferating and displayed enhanced muscle markers (Figure 6B and C) after 14 days of differentiation. The induction of differentiation was even higher than with standard 5-aza treatment.

We were unable to evaluate the role of HDAC4 because cell viability was dramatically affected and myogenic differentiation was not achieved when HDAC4 expression was knocked down (data not shown).

Discussion

The transcripational regulation of cardiomyocyte differentiation is highly conserved and requires sequential activation and/or repression of different genetic programs. Specific disruption of Dicer, essential for processing of mature miRNAs, in mouse cardiac progenitor cells resulted in embryonic lethality due to cardiac failure, pointing to an essential role of miRNAs in cardiac development and cardiomyocyte differentiation. We observed that hCMPCs express many different miRNAs, some of them highly regulated after differentiation. Here, we focused on miR-1 and -499 and on whether they could be used in vitro to induce cardiomyocyte differentiation.

miR-1 is a highly conserved miRNA with a cardiac and skeletal muscle–specific expression pattern, able to bind to the promoters of several essential cardiac transcription factors, such as Mef2, SRF, Nkx2.5, and GATA4. Several miR-1-targeted genes play a role in cardiac development or function and have been experimentally confirmed, such as Hand2, TMSB4X, HDAC4, GJA1, and KCNJ2. In miR-1 transgenic mice, the total number of cycling myocardial cells was decreased, whereas in miR-1 knockout mice, an increased proliferation of cardiomyocytes was observed, resulting in severe heart defects. This suggests that miR-1 fine-tunes the balance between cardiomyocyte proliferation and differentiation, thereby repressing mouse cardiac progenitor cell proliferation. Here, we demonstrated that also introducing miR-1 into human cardiac-derived CMPCs resulted in a reduction in proliferation rate.

In Drosophila, loss of dmIR-1 is lethal, because it is required for determination or differentiation of cardiac or somatic muscle progenitor cells. Similar observations were reported in embryonic stem cell differentiation, demonstrating that miR-1 and miR-133 promote mesoderm formation but have opposing functions during further differentiation into cardiac muscle progenitors. Furthermore, introducing miR-1 in C2C12 myoblasts promoted myogenesis by targeting HDAC4. Accordingly, we were able to induce cardiac differentiation via miR-1 in our hCMPCs and mouse embryonic stem cells, which was also mediated through repression of HDAC4 protein levels. Moreover, we demonstrated that miR-1 functioning is a prerequisite for hCMPC cardiogenic differentiation, because using a specific miR-1 inhibitor blocks differentiation. Unfortunately, full knockdown of HDAC4 leads to cell death, and therefore myogenic differentiation could not be studied. Interestingly, miR-1 was also capable of partially blocking Sox6 protein expression, suggesting that a single miRNA can bind to more targets to direct cell fate, in this case myogenic differentiation.

Taking these data together, miR-1 seems to be a highly conserved miRNA that plays a role in muscle differentiation and maintenance of cardiomyocyte homeostasis, not only in mouse and Drosophila but also in human-derived cardiac progenitor cells.

We explored the role of miR-499, as well as that of miR-1, in hCMPC. We observed that miR-499 is expressed in differentiated hCMPCs and, together with its gene MYH7β, is strongly enriched in cardiac tissue. Van Rooij et al suggested that miR-499 is expressed in cardiac and slow skeletal muscles. This difference might be caused by different percentages of fast and slow fibers being expressed in different skeletal muscles used. miR-499 localization and cellular expression suggest a role in cardiomyocyte homeostasis or maintaining the differentiation state of cardiomyocytes. Indeed, by transfection of miR-499 into our hCMPCs and mouse embryonic stem cells, we could enhance their differentiation into cardiomyocytes. We identified Sox6 as a...
Figure 5. A, Proliferating hCMPCs were transfected with increased concentrations of miR-1, resulting in decreased concentrations of HDAC-4 protein expression. B, A cytomegalovirus-luciferase plasmid was transfected in HEK293 cells containing the 3'UTR of Sox6. Cells were cotransfected with miR-499 (30 nM), a specific inhibitor of miR-499 (anti-499, 50 nM), miR-1, or a scrambled miR (30 nM). By transfection of premiR-499, luciferase expression was reduced and could be restored on specific inhibition of miR-499 (LNA-499). C, Mutations were created in the 4 putative binding sites (sites 1 to 4) of Sox6 and confirmed by sequencing. Reduced luciferase suppression capacity was not observed after creating single mutations; however, 2 mutated sites demonstrated a reduction in suppression of miR-499. * indicates 3'UTR sox6; 1 to 4 indicate mutated seed regions as in online Figure IC. D, Sox6 expression is determined via flow cytometric analysis in CMPCs after 3 days of transfection of 100 nM scr-miR, miR-499, and miR-1. miR-499 transfection resulted in a strong reduction in Sox6 expression, whereas miR-1 transfection demonstrated a lower reduction. *P<0.05.
potential target for miR-499. Sox6, which is expressed in the heart and skeletal muscle, was predicted to contain 4 seed regions (see Supplemental Figure IIC). Although Sox6 is a member of the Sox gene family of transcription factors (Sry-related HMG box) that play a key role in embryonic development and cell fate determination, its exact role is not clear.

Mice homozygous for a Sox6 null mutation show delayed growth and die within 2 weeks after birth, having abnormal muscle architecture and developing cardiomyopathies. In P19Cl6 cells, a embryonic carcinoma cell line able to differentiate into beating cardiomyocytes, Sox6 is regulated by bone morphogenic protein and is expressed only when the cells are committed to differentiate into beating cardiomyocytes, suggesting an association with the initiation of the cardiomyogenic program. Furthermore, because the L-type Ca\(^{2+}\) channel, which is critical for cardiomyocyte contraction, is repressed by Sox6, this protein needs to be tightly regulated in developing and differentiating cardiomyocytes.

Sox6 is involved in muscle development and the tight balance between different muscle isoforms, because in the skeletal muscle of Sox6 null mice, an isoform-specific change in muscle gene expression has been observed. As indicated by our results, induction of miR-499 represses the expression of Sox6 in hCMPCs, leading to a reduction in cell proliferation and enhanced myocyte differentiation. Moreover, knockdown of Sox6 induces cardiogenic differentiation of hCMPCs, confirming the role of Sox6 in muscle differentiation. In addition, we demonstrated by blocking miR-499 that Sox6 expression is required for cardiac differentiation. Thus, our data suggest that when cells are committed to the cardiac lineage and start to express MYH7B\(\beta\), miR-499 is coexpressed, thereby repressing Sox6 to further induce differentiation and modulate or fine-tune fiber expression in developing cardiomyocytes.

In summary, miRNA levels are highly regulated in cultured and differentiated cardiac-derived hCMPCs, with miR-1 and miR-499 expression being significantly higher in differentiated hCMPC. Our results demonstrate that a single miRNA, miR-1, induces muscle differentiation via repression of HDAC4. Furthermore, miR-499, a miR whose function has not yet been explored, is highly enriched in cardiac tissue, targets Sox6, and enhances cardiomyocyte differentiation in hCMPCs in vitro. These results demonstrate that miRNAs are powerful regulators driving hCMPC differentiation and they can be used to influence cell fate. In addition, hCMPCs can be used as a model to study human in vitro developmental processes in order to better understand cardiac development and cardiomyocyte homeostasis.

Acknowledgments

We appreciate the technical assistance for generating mutated 3’UTR constructs by Maarten M.G. van den Hoogenhof, Medical Physiology, UMC Utrecht.

Sources of Funding

This work was supported by a VIDI grant (016.056.319, to M.-J.G.) from the Netherlands Organization for Scientific Research, the Netherlands Heart Foundation (2003B07304, to J.P.G.S.), the van Ruyven foundation (to P.v.V.), and BSIK program “Dutch Program for Tissue Engineering” Grant 6746 (to J.P.G.S.), and a Bekalis price (to P.A.D.).

Disclosures

None.

References

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Arterioscler Thromb Vasc Biol. 2010;30:859-868; originally published online January 15, 2010; doi: 10.1161/ATVBAHA.109.197434
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Material

Methods

*Human cardiomyocyte progenitor cells (hCMPCs)*

Human fetal heart tissue was collected after elective abortion, followed by Langendorff perfusion with Tyrode’s solution, collagenase, and protease treatment. Single cells were obtained and hCMPCs were isolated and characterized as described.\(^1\)\(^-\)\(^3\) Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht were obtained. Cells were cultured in M199 (Gibco)/EGM (3:1) supplemented with 10% FCS (Gibco), 10 ng/ml basic Fibroblast growth factor (bFGF), 5 ng/ml epithelial growth factor (EGF), 5 ng/ml insulin like growth factor (IGF-1) and 5 ng/ml hepatocyte growth factor (HGF). To induce differentiation, cells were treated with 5 μM 5’-azacytidine (Sigma) for 72 hours in differentiation medium (Iscove's Modified Dulbecco's Medium /HamsF12 (1:1) (Gibco)) supplemented with L-Glutamine (Gibco), 2% horse serum, non-essential amino acids, Insulin-Transferrin-Selenium supplement , and 10-4 M Ascorbic Acid (Sigma)), followed by TGF-β stimulation.\(^1\)\(^-\)\(^3\) In addition, lentiviral expression of Sox6 and HDAC4 RNAi was used to knock down their expression prior to the start of differentiation. Knock down efficiency was confirmed by flow cytometry as explained below.

*Analysis of miR expression by microarray*

Low-molecular-weight RNA was isolated from proliferating and differentiated hCMPCs by using the mirVana™ RNA Isolation Kit (Ambion). MiRNA expression profile was determined by microarray analysis using the μParaflo™ microfluidic chip.
Quantitative RT-PCR for miRNA expression

Total RNA was isolated with mirVana™ RNA Isolation Kit or Tripure isolation reagent (Roche Applied Science). In brief, 10 ng purified total RNA was used for reverse transcriptase (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) in combination with Taqman® MicroRNA Assays for quantification of miR-1, 133a, 499, 126, 155, 206, and 208 and RNU19 control transcripts (Applied Biosystems: 4373161, 4373142, 4373224, 4378064, 4373124, 4427975, 4427975, and 4373378, respectively), according to the manufacturer's conditions. Amplification and detection of specific products were performed in a MyiQ single-color real-time PCR system (Bio-Rad) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. The threshold cycle (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to control RNU19 (ΔCt value). The relative difference in expression levels of each miRNA in proliferating and differentiated hCMPCs (ΔΔCt) was calculated and presented as fold induction (2^ΔΔCt).

hCMPC transfection

Pre-miR™ miRNA Precursor molecules for miR-1 (5’-UGGAAUGUAAAGAAGUAUGUA-3’, PM10660), miR-499 (5’-UUAAGACUUGCAGUGAUGUUUAA-3’, PM10496), and a scrambled miR control (AM17121) with or without FAM™ dye-label were obtained from Ambion. Inhibitors for
miR-1, miR-499, and scrambled controls (SCR) are obtained from Dharnacon Scientific (Thermo Fisher Scientific Inc., IH-300586-06, IH-300837-06, and IN-001005-01, respectively). hCMPCs were transfected with siPORT™ NeoFX™ Transfection Agent (Ambion) and pre- and anti-miRs (0,-100nM), according to the manufacturer.

Transfection efficiency of pre-miR was confirmed by RT-PCR (Taqman miRNA assay) and visually by means of FAM-labeled negative control pre-miR. For fluorescence microscope analysis of FAM-labeled pre-miR expression, cells were washed with PBS and fixated with 4% paraformaldehyde in PBS for 15 min at RT. Nuclei were stained with 0.2 μg/ml Hoechst (Invitrogen) and mounted in VectaShield (Molecular Probes, Amsterdam).

To test whether miR-1 and 499 have an effect on cardiomyocyte differentiation, CMPCs were stimulated 3 consecutive days with 5’aza and followed by miR-transfection as described above. After overnight transfection, differentiation medium was changed every 3 days, but without TGF-beta stimulation.

**hCMPC proliferation assay**

Cells were seeded in a 0.1% gelatin coated 96-well plate and transfected as described above. After 4 and 6 days, culture medium was removed and cells were treated with 0.5 mg/ml of Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich) in RPMI serum free medium (Gibco) and incubated at 37°C for 3 hours. Subsequently, purple formazan crystals, formed by mitochondrial reductase enzymes, were solubilized in 0.1 ml DMSO and absorbance was measured at 540 nm with a reference filter of 690 nm in a photometric plate reader (Labsystems Multiskan Ascent).
**Cell viability assay**

Cells were transfected with 100nM SCR-miR, miR-1, and miR-499 for 3 days and cellular viability was measured by staining for 7-AAD and Annexin V (BD Pharmingen 556421, 559925), followed by flow cytometric analysis according to manufacturers protocol.

**Flow cytometry assay**

HEK293T cells were transfected with pLKO.1-puro-shRNA-Sox6 or HDAC4 (Mission library, Sigma) to produce lentiviral transduction particles. hCMPCs were transduced with shRNA-Sox6 or a scrambled (off target) control for 24 hours, washed and grown in the presence of puromycin (2 ug/ml) for 7 days, followed by flow cytometric analysis for the expression of Sox6. Selected cells were trypsinized, fixated in 2% PFA for 15 minutes, followed by permeabilization for 15 minutes in 0.5% NP-40 in PBS, washed with PBS and incubated with Sox6 antibody (1:200, S7193, Sigma) for 1 hour at 4 °C, washed and incubated with anti-FITC secondary antibody (1:600, R&D systems), and measured using a flow cytometer (BD Biosciences). To quantify total cell number expressing Sox6, an isotype control was used to set the negative gate. Total number of cells expressing Sox6 / total number of cells was calculated.

**Embroid body assay**

Mouse Embryonic Stem Cells (mESCs), E14-IB10, were cultured in BRL-conditioned medium as described before4. mESCs were differentiated by generating
embryoid bodies (EBs) via hanging drops. mESCs were transfected with pre-miRNA-1, -499, and scrambled control (SCR) prior to body formation. Cells were monitored at day 7 for percentage of beating EBs, followed by plating them on gelatin coated coverslips allowing visualization of alpha-actinin fibers via immunohistochemistry. Fifty EBs were collected per condition after 4 and 8 days to check differentiation rate by RT-PCR, as described below.

**Western blot**

Total protein was extracted using TriPure, and 33μg, reduced with DTT, was separated by PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked (5% non-fat-dry milk, PBS-0.1% Tween), probed with polyclonal rabbit-anti-human HDAC4 (12171, GeneTex), Sox6 (S7193, Sigma), and alpha-actinin (sarcomeric, EA-53, A7811, Sigma) and goat anti-rabbit IgG secondary antibodies (P0448,Sigma-Aldrich) in blocking-buffer. Signal was visualized with enhanced chemiluminescence (Amersham) and bands were scanned using a ChemiDoc XRS system (Bio-Rad).

**Quantitative RT-PCR**

Total RNA was isolated with TriPure reagent, cDNA was prepared using the SuperScript First-Strand Synthesis System for RT-PCR (170-8890, Bio-Rad), and qRT-PCR amplification was detected in a MyIQ single-color real-time PCR system. Amplifications were performed with iQ™ SYBR® Green Supermix (170-8884, Bio-Rad). Used qRT-PCR conditions are: 5 min at 95 °C, followed by 40 cycles of 15s at 95
°C, 30s at specific annealing temperature (Online Table A), and 45s at 72 °C, followed by melting curve analysis to confirm single product amplification. mRNA expression levels were normalized to β-actin mRNA expression ($\Delta$Ct value), relative differences were calculated ($\Delta\Delta$Ct value) and presented as fold induction ($2^{\Delta\Delta\text{Ct}}$).

**In Situ hybridization**

*In situ* hybridization to determine hsa-miR-499 localization was performed as previously described. In brief, ten µm thick sections of human fetal heart tissue were hybridized O/N at 46 ºC with 5 nM LNA DIG-labeled probes (Scramble-miR, Exiqon, 99001-01 and hsa-miR-499, Exiqon, 38306-01) in denaturizing hybridization buffer, incubated with an anti-DIG-alkaline phosphatase antibody (1:1500, Roche 11093274910), an anti-PECAM (1:200, Santa Cruz, sc-1505), and an anti α-actinin antibody (Sigma) at RT for 2 hours. After washing, sections were incubated with fluorescent-labeled secondary antibodies, exposed to Fast-Red Substrate-Chromogen (DAKO, K0597) O/N at RT and mounted in VectaShield (Molecular Probes, Amsterdam).

**Luciferase experiments**

Primers were generated for the 3’UTR of Sox6 (human and mice 87% homology, all sites conserved), a putative target of miR-499, and extended with SpeI restriction sites (see online table 1). After RT-PCR, the 3’UTR of Sox6 was inserted into pMIR-REPORT™ miRNA Expression Reporter Vector (#5795, Ambion), by SpeI directed ligation, at the 3’UTR of CMV-Luc.
In a 24-wells plate, HEK293T cells were transfected with 200 ng plasmid, containing the CMV-Luc and 3’UTR of Sox6, with and without 30 nM of miR-499, miR-1, or Scr-miR, and 200 ng CMV-Bgal for normalization. To block miR-499 activity, the LNA-probe for miR-499 was used at 30 nM. After 48 hours, luciferase and β-galactosidase activity was measured with the Luciferase Assay System and β-Galactosidase Enzyme Assay System (Promega), respectively. Cells transfected with the LUC plasmids only are normalized to 1.

To confirm specificity of miR-499 and its putative sites, mutational cloning in the seed region of predictive sites was performed with QuikChange® Site-Directed Mutagenesis Kit (200518, Stratagene), according manufacturer (see online figure 1C for sites). The successful creation of mutations was confirmed by sequencing (data not shown).

Statistical analysis

Data is presented as mean ± SEM of at least three independent experiments and were compared using the two-tailed paired Student’s $t$ test or one-way ANOVA, a difference with $p<0.05$ was considered to be statistically significant.
References:


Figure legends:

Online table I: Primer sequences that are used to determine mRNA expression and generate Sox6 3’-UTR (F=forward primer, R=reverse primer).

Online figure I: Quantitative RT-PCR for (A) miRNA-206 and (B) miRNA-208 in proliferating hCMPCs (prol. hCMPC) and hCMPCs differentiated to cardiomyocytes (diff. hCMPC). Data is presented as fold induction +/- sem, * = p<0.05.

Online figure II: The sequence of miR-499 is well conserved among different species (A), moreover, also its localization within intron 20 of MYH7B, beta (myosin heavy chain, cardiac muscle beta isoform 7B) is conserved among human and mice (B). The untranslated region (UTR) of human and mouse Sox6 has four predicted binding sites for miR-499, one 8-mer and three 7mer-m8. Moreover, one additional miR-1 binding site is present, one 7mer-1A (C).

Online figure III: (A) Transfection of 100nM SCR-miR, miR-1, and miR-499 did not reduce cellular viability as measured by 7-AAD and Annexin V staining and flow cytometric analysis. (B) Increasing concentrations of pre-miR-155 in hCMPC did not significantly change their proliferation rate.

Online figure IV: Mouse embryonic stem cells are transfected with 100nM miRNAs and embryoid bodies are generated via hanging drops. (A) After 7 days, number of beating aggregates are counted. Introduction of miR-499 and miR-1 increased number of beating bodies significantly as compared to the SCR-miR control. (B-D) Embryoid
bodies are plated after 7 days, grown for additional 4 days and stained for alpha-actinin (green, arrows), (B) not, (C) SCR-miR, (D) miR-499, and (E) miR-1 transfected. Upon miR-499 and 1, increased alpha-actinin staining was present. (Bar=50μm, nuclei are stained with Hoechst). RNA was isolated 4 and 8 days after embryoid body generation. GATA4 expression (F) was increased after 4 days, and Mlc-2v expression (G) after 8 days of introducing miR-499 and miR-1.

Online figure V: A CMV-Luciferase plasmid was transfected in HEK293 cells, containing the full 3’UTR of Sox6 or containing different mutations (mut 1: substitution of 1 nucleotide in seed region, mut 2: deletion of 3 sites in seed region). Cells were co-transfected with miR-1 (30nM). Changes in the seed region reduced the inhibitory effect significantly. (* p<0.05 full UTR vs LUC control, Δ p<0.05 mut 1 and 2 vs full UTR)

Online figure VI: miR-1 (A), miR-499 (B), HDAC4 (C) and Sox6 (D) mRNA expression in proliferating, 5’aza-treated and fully differentiated CMPCs. 5’aza treatment resulted in an increase in miR-1 (miR-499 is not significantly changed), and a decrease in HDAC4 and Sox6 expression.
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Online Table I
Online figure I

**Graph A**: Fold induction of miR-206 in Prol. hCMPC and Diff. hCMPC. The expression level in Diff. hCMPC is significantly higher than in Prol. hCMPC, indicated by the star (*).

**Graph B**: Fold induction of miR-208 in Prol. hCMPC and Diff. hCMPC. The expression level in Diff. hCMPC is significantly higher than in Prol. hCMPC, indicated by the star (*).
Online figure II

myosin, heavy chain 7B, cardiac muscle, beta

chr 20

intron 20

SOX6 3’ UTR

1 = 123-125, 7mer-1A
2 = 287-289, 7mer-m8
3 = 600-602, 8mer
4 = 623-625, 7mer-m8
Online figure III
Online figure V
Online figure VI