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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

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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 identified[1–3] 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

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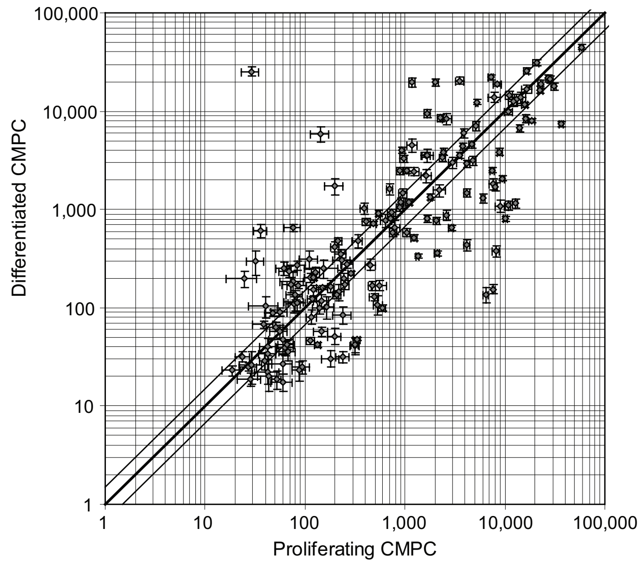

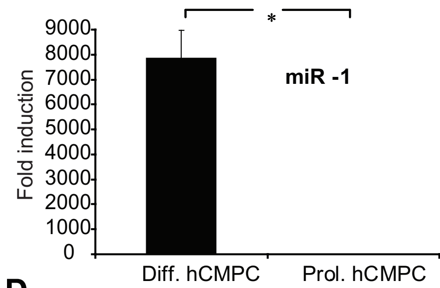

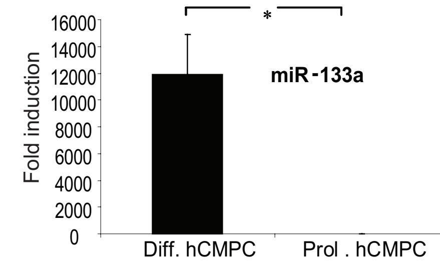

	miRNA ID	Proliferating CMPC	Differentiated CMPC	log2	fold induction
1	hsa-miR-1	26.1	24058.8	9.91	962.3
2	hsa-miR-499	10.9	1372.2	7.07	133.9
3	hsa-miR-133a	9.7	715.5	6.28	77.6
4	hsa-miR-133b	15.6	727.8	5.72	52.9
5	hsa-miR-335	128.9	5829.0	5.42	42.8
6	hsa-miR-450	32.2	643.7	4.40	21.2
7	hsa-miR-542-3p	12.5	245.1	4.21	18.5
8	hsa-miR-143	1143.5	19731.0	4.04	16.5
9	hsa-miR-217	8.8	122.1	3.79	13.8
10	hsa-miR-204	27.9	330.7	3.68	12.8
11	hsa-miR-203	20.0	181.2	3.54	11.6
12	hsa-miR-210	31.3	295.4	3.35	10.2
13	hsa-miR-145	2015.3	19156.3	3.34	10.2
14	hsa-miR-422b	68.4	630.5	3.21	9.3
15	hsa-miR-218	221.0	1798.7	3.10	8.6
16	hsa-miR-214	3645.1	20597.9	2.55	5.9
17	hsa-miR-152	1709.7	10020.7	2.51	5.7
18	hsa-miR-30d	918.3	4009.8	2.07	4.2
19	hsa-miR-374	59.6	249.2	2.06	4.2

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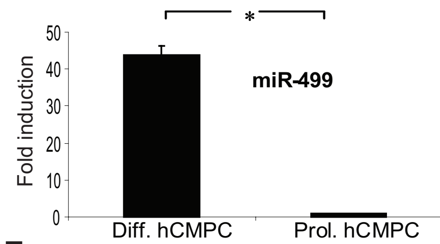
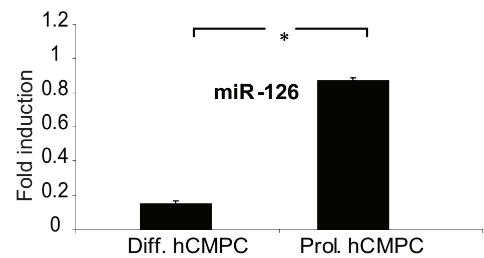
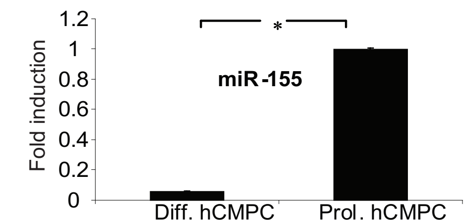


	miRNA ID	Proliferating CMPC	Differentiated CMPC	log2	fold repression
1	hsa-miR-146a	7683.9	155.1	-5.59	48.1
2	hsa-miR-155	6457.5	127.6	-5.53	46.2
3	hsa-miR-126	8375.6	380.6	-4.48	22.3
4	hsa-miR-19a	192.5	12.1	-3.99	15.9
5	hsa-miR-31	10019.8	781.9	-3.66	12.7
6	hsa-miR-222	13140.2	1124.8	-3.48	11.2
7	hsa-miR-221	11128.8	1057.9	-3.32	10.0
8	hsa-miR-20b	4227.6	439.9	-3.27	9.6
9	hsa-miR-542-5p	181.9	20.2	-3.13	8.7
10	hsa-miR-625	121.3	13.3	-3.08	8.4
11	hsa-miR-663	9058.1	1057.9	-3.04	8.2
12	hsa-miR-602	303.5	42.3	-2.95	7.7
13	hsa-miR-612	226.6	29.3	-2.93	7.6
14	hsa-miR-323	309.9	44.1	-2.93	7.6
15	hsa-miR-29b	121.7	17.9	-2.84	7.2
16	hsa-miR-299-5p	331.7	47.2	-2.81	7.0
17	hsa-miR-494	2099.8	353.4	-2.60	6.1
18	hsa-miR-212	190.2	33.6	-2.57	5.9
19	hsa-miR-18a	591.4	99.8	-2.57	5.9
20	hsa-miR-638	37354.6	7219.1	-2.33	5.0
21	hsa-miR-329	540.3	110.4	-2.32	5.0
22	hsa-miR-106a	5998.6	1252.7	-2.27	4.8
23	hsa-miR-17-5p	8154.2	1645.0	-2.22	4.6
24	hsa-miR-20a	9392.7	2119.2	-2.21	4.6
25	hsa-miR-19b	2901.6	660.8	-2.14	4.4
26	hsa-miR-505	202.7	50.0	-2.09	4.3
27	hsa-miR-15b	7703.7	1734.1	-2.03	4.1

C





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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

See online Methods for extended descriptions, available at http://atvb.ahajournals.org.

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) 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 μParaflo microfluidic chip (LC Sciences) and validated with TaqMan MicroRNA Assays (Applied Biosystems). mRNA expression was determined using the SuperScript First-Strand Synthesis System (170 to 8890, Bio-Rad) and quantitative RT-PCR amplification was detected in a MyIQ single-color real-time polymerase chain reaction system using iQ SYBR Green Supermix (170 to 8884, Bio-Rad) and specific primers (Supplemental Table I). For Western blotting, HDAC4- (12171, GeneTex), Sox6- (S7193, Sigma), and α-actinin-(sarcomeric, EA-53, A7811, Sigma) specific antibodies were used.

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 (IH-300586-06), miR-499 (IH-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 before[18] 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 described[19] 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 7B cardiac muscle β) 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 costaining

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 CMPCs 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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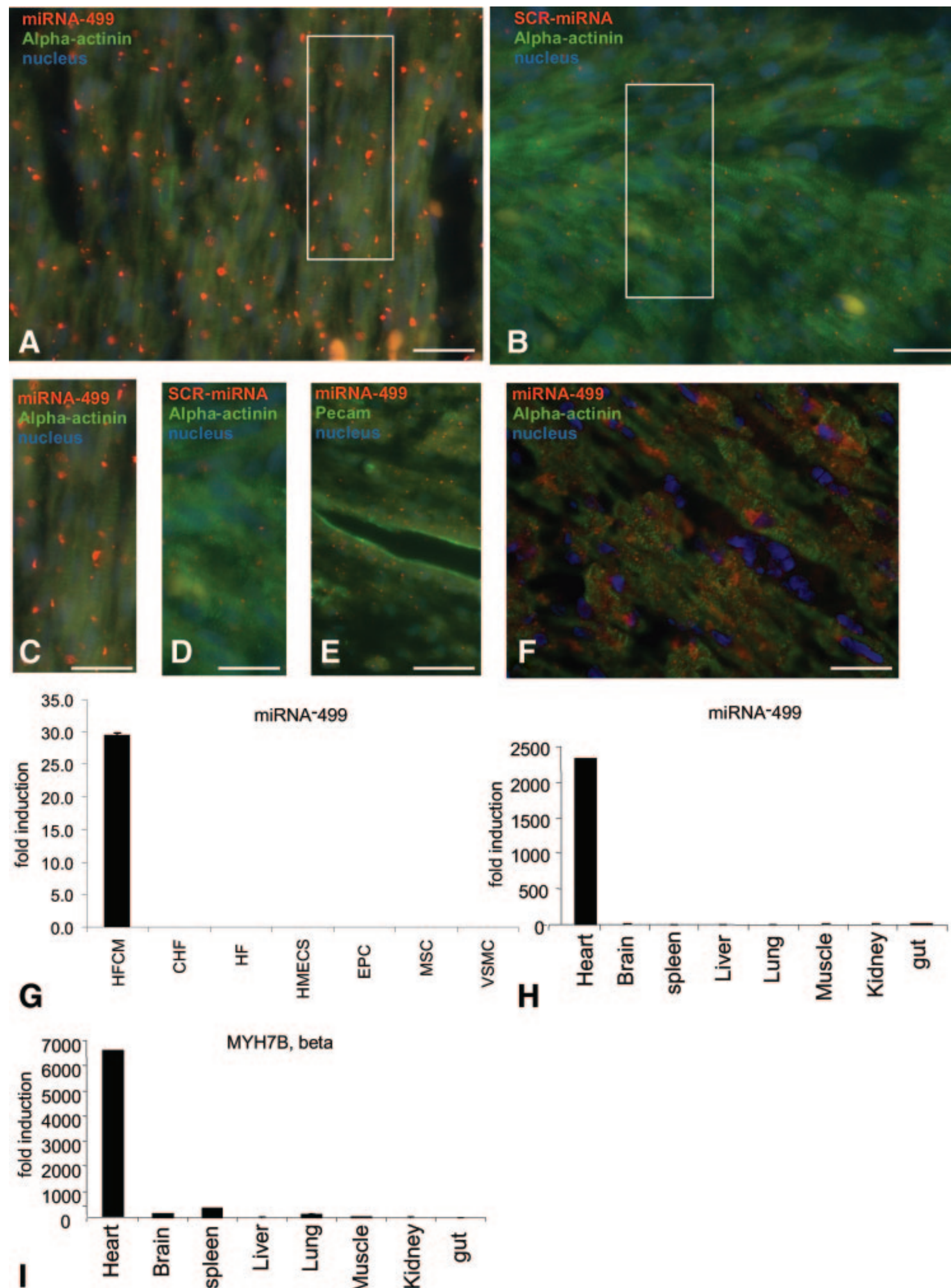

Figure 2. In situ hybridization for miRNA-499 (A and C) (red dots) and a scrambled control probe (B and D) in human fetal heart, double stained for α-actinin (A through D, green) or PECAM (E, green). F, miRNA-499 (red) localization in adult human heart, double stained for α-actinin (green). Nuclei are 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).

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, MYH7Bβ 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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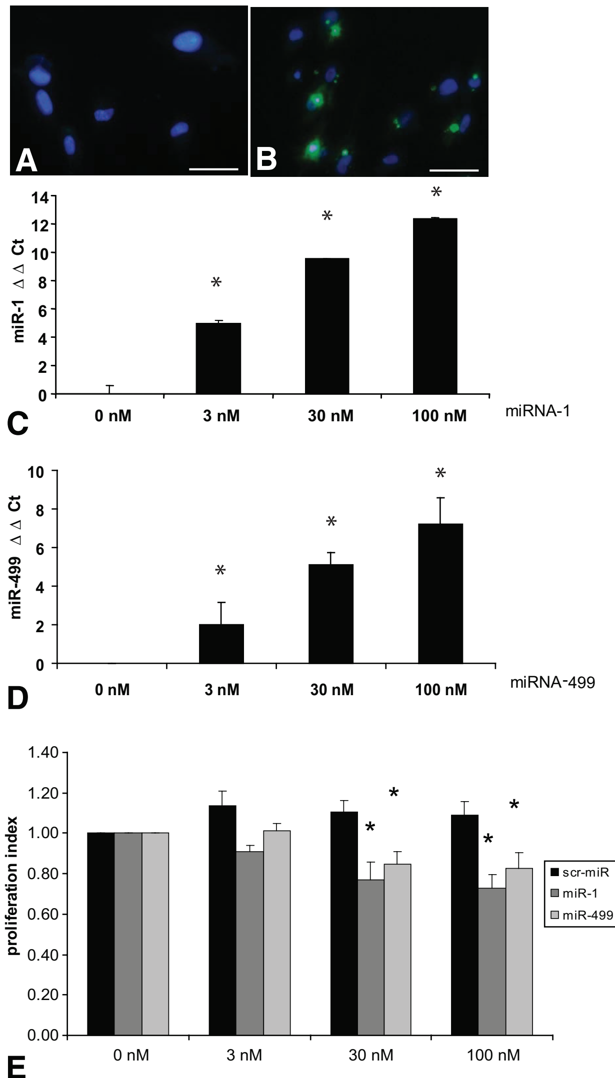

Figure 3. A and B, Immunofluorescent detection of untransfected (A) and 100 nM (B) FAM-labeled control miR, transfected in hCMPCs. Nuclei are stained blue. Bar=25 μm. Transfection with increased concentration of miR-1 (C) or miR-499 (D) premolecules resulted in increased detectable expression of the mature miR-1 or -499, respectively, by quantitative RT-PCR. Data are expressed as $\Delta\Delta$Ct$\pm$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 Supplemental 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).[8,9,10] Normally, spontaneous beating clusters of differentiated cells are observed after 21 days. However, when adding miRNAs individually, we observed spontaneous beating areas as soon as 6 days after initiation of differentiation (Supplemental Video). This suggests that the differentiation of our progenitor cells is greatly enhanced by transfection of miR-1 or miR-499. To quantify this, we studied mRNA levels for different cardiomyocyte-specific genes by quantitative RT-PCR after 2 weeks of differentiation. We observed that the expression of cardiac troponin T, α-cardiac actinin, and Mlc-2v was increased on addition of miR-1 and -499 (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, Mlc2v 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-

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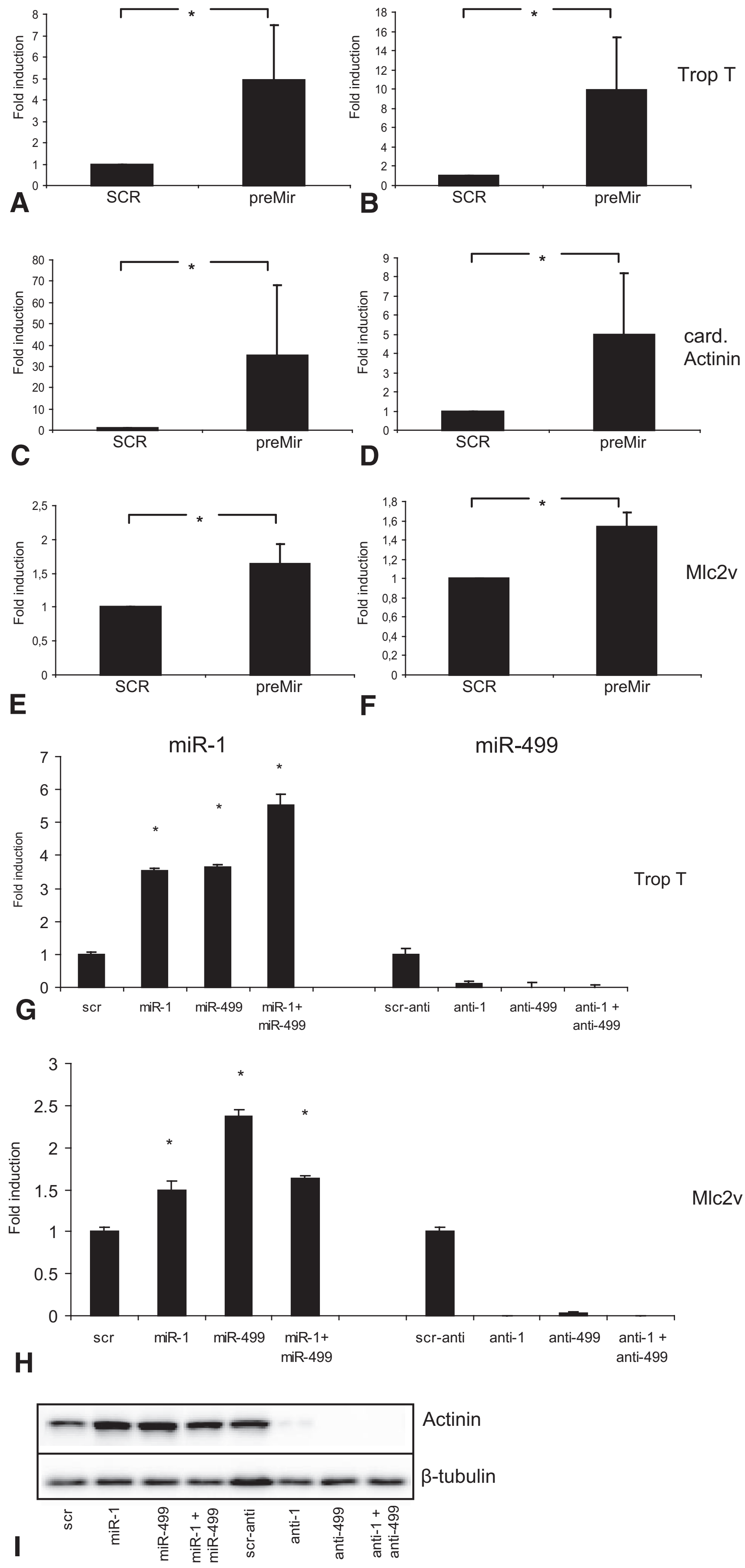

Figure 4. hCMPCs were differentiated into cardiomyocytes by 5-aza stimulation and also transfected with 100 nM SCR miR, miR-1 (A, C, and E), or miR-499 (B, D, and F). mRNA expression was determined after 2 weeks by RT-PCR for troponin T (A and B), cardiac actinin (C and D), and Mlc-2v (E and F) and compared 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.

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mote myogenesis by targeting HDAC4, a transcriptional negative regulator of muscle gene expression.[22] 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).[23] 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 CMPCs. On 5-aza treatment, miR-1 levels are 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 transcriptional regulation of cardiomyocyte differentiation is highly conserved and requires sequential activation and/or repression of different genetic programs.[24,25] 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.[20] We observed that hCMPCs express many different miRNAs, some of them highly regulated after differentiation. Here, we focused on miR-1 and -499[26] 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.[17] Several miR-1-targeted genes play a role in cardiac development or function and have been experimentally confirmed, such as Hand2,[17] TMSB4X,[17] HDAC4,[22] GJA1,[27] and KCNJ2.[27] In miR-1 transgenic mice, the total number of cycling myocardial cells was decreased,[17] whereas in miR-1 knockout mice, an increased proliferation of cardiomyocytes was observed, resulting in severe heart defects.[20] 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.[28] 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.[29] Furthermore, introducing miR-1 in C2C12 myoblasts promoted myogenesis by targeting HDAC4.[22] 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[21] 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

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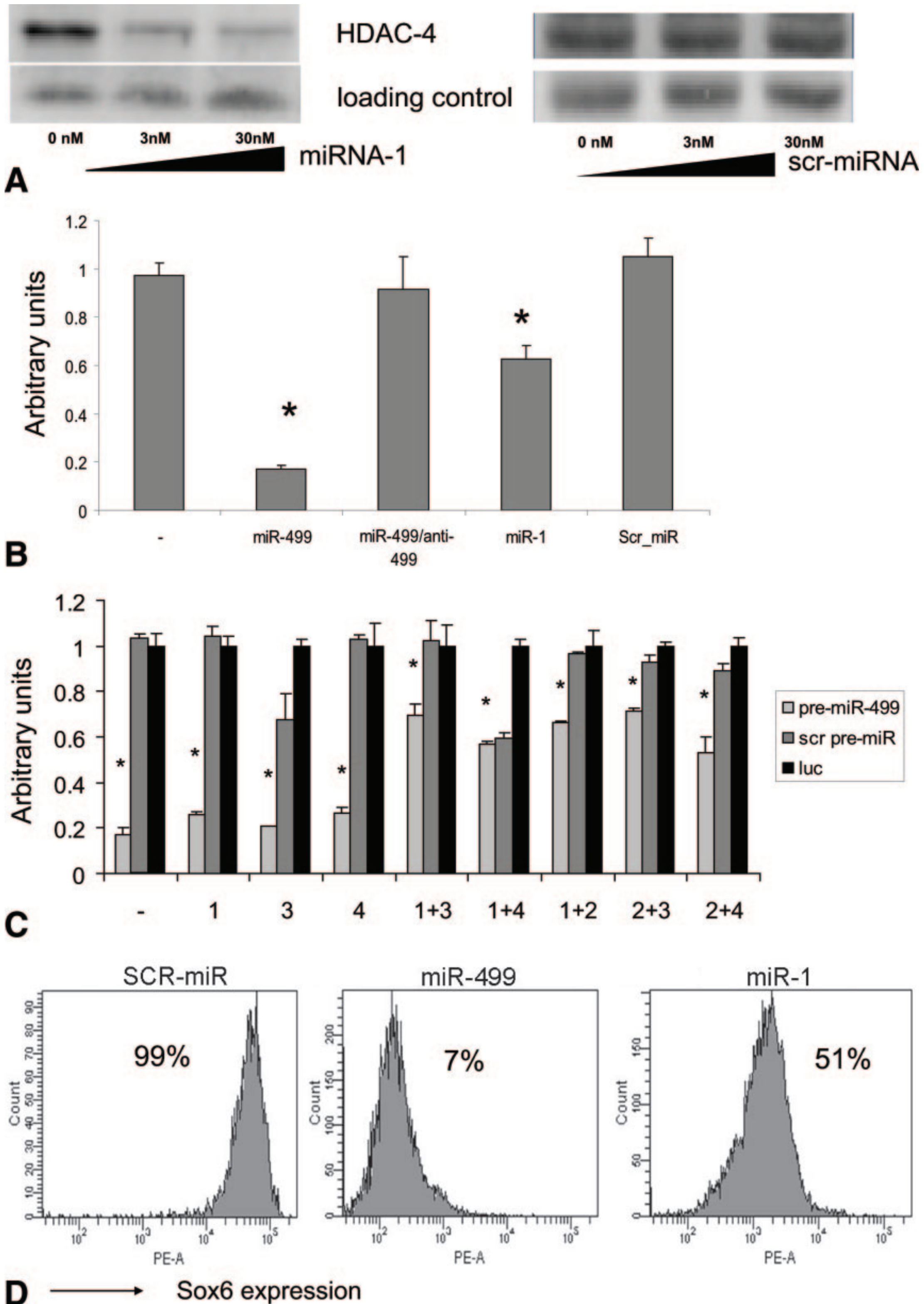

Figure 5. A, Proliferating hCMPCs were transfected with increased concentrations of miR-1, resulting in decreased concentrations of HDAC-4 protein expression. B, A cytomegalovirus-luciferase plasmid was transfected in HEK293 cells containing the 3′UTR of Sox6. Cells were cotransfected with miR-499 (30 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 sox 6; 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. *$P<0.05$.

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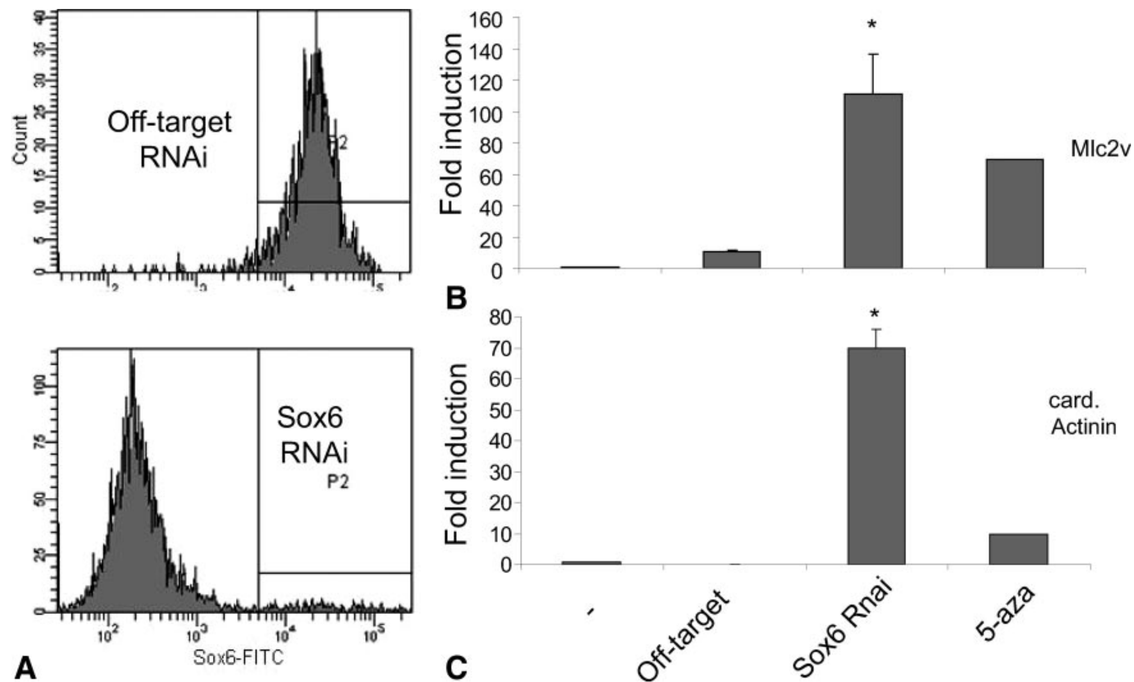

Figure 6. RNA interference (RNAi) mediated Sox6 knockdown in hCMPCs (A), as shown by flow cytometric analysis for Sox6 expression. Muscle differentiation was induced as indicated by increased expression of mlc-2v (B) and cardiac actinin (C) after 14 days of differentiation. The induction after Sox6 RNAi was even higher than with standard 5-aza treatment for 3 days.

potential target for miR-499. Sox6, which is expressed in the heart and skeletal muscle,[30] 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.[23]

Mice homozygous for a Sox6 null mutation show delayed growth and die within 2 weeks after birth, having abnormal muscle architecture and developing cardiomyopathies.[31] 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,[23] 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.[31] 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 MYH7Bβ, 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.

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Disclosures

None.

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