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Biochemical and Biophysical Research Communications 350 (2006) 1006–1012
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MicroRNA-1 facilitates skeletal myogenic differentiation without affecting osteoblastic and adipogenic differentiation

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Received 26 September 2006
Available online 6 October 2006

Abstract

MicroRNAs (miRNAs) are small non-coding RNAs emerging as important post-transcriptional gene regulators. In this study, we examined the role of miR-1, an miRNA specifically expressed in cardiac and skeletal muscle tissue, on the myogenic, osteoblastic, and adipogenic differentiation of C2C12 cells. Upon induction of myogenic differentiation, miR-1 was robustly expressed. Retrovirus-mediated overexpression of miR-1 markedly enhanced expression of muscle creatine kinase, sarcomeric myosin, and α-actinin, while the effects on myogenin and MyoD expression were modest. Formation of myotubes was significantly augmented in miR-1-overexpressing cells, indicating miR-1 expression enhanced not only myogenic differentiation but also maturation into myotubes. In contrast, osteoblastic and adipogenic differentiation was not affected by forced expression of miR-1. Thus, the muscle-specific miRNA, miR-1, plays important roles in controlling myogenic differentiation and maturation in lineage-committed cells, rather than functioning in fate determination.

Keywords: MicroRNA; Differentiation; Skeletal muscle cells; Adipocytes; Osteoblasts; C2C12 cells

MicroRNAs (miRNAs) are a class of small non-coding RNAs that play an important role in the post-transcriptional regulation of protein-coding gene expression. They anneal to the complementary sequences in the 3'UTRs of target mRNAs and cause degradation or, more notably, translational inhibition of target transcripts [1]. Although the functions of only a handful of miRNAs have been identified, it is emerging that miRNAs are involved in a wide variety of biological functions such as developmental patterning, lineage differentiation, cell death, proliferation, insulin secretion, and antiviral defense [2]. MiRNA-1 (miR-1) is an miRNA that is specifically expressed in cardiac and skeletal muscle [3]. Transfection of miR-1 in HeLa cells, a human epithelial cell line, has been shown to shift the gene expression profile toward that of muscle cells [4]. It has also been shown that transgenic expression of miR-1 in mouse hearts results in a proliferation defect and a failure of cardiac myocyte expansion, suggesting premature differentiation of cardiac myocytes by miR-1 overexpression [3]. A recent study revealed that miR-1 promotes myogenesis of myoblasts while repressing proliferation [5], although only relatively early steps of myoblast differentiation were examined in this study. These studies suggest that miR-1 regulates the balance between differentiation and proliferation, but the roles of miR-1 in lineage specification and terminal differentiation remain to be clarified.

The C2C12 cell line is a subclone isolated from parental C2 cells established from the regenerating thigh muscle of an adult mouse. Although C2C12 cells are widely used as a myoblast cell line, these cells are also well characterized as mesenchymal progenitor cells, and can differentiate into several mesenchymal cell types including myocytes,

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doi:10.1016/j.bbrc.2006.09.153

osteoblasts, and adipocytes [6,7]. Incubation of C2C12 cells under low serum conditions induces muscle differentiation and fusion of cells into multinucleated myotubes. Treatment of C2C12 cells with bone morphogenetic protein (BMP)-2 blocks myotube formation and induces osteogenic differentiation instead [6,8,9]. Culturing the cells with adipogenic medium, treatment with long-chain fatty acids, or treatment with thiazolidinediones also blocks myotube formation and leads to typical adipocyte differentiation [7,10]. During differentiation into these cell types, the cells capture important aspects of their respective differentiation programs such as expression of tissue-specific transcription factors and functional gene products, providing unique opportunities to study the mechanisms of differentiation into these mesenchymal cell types.

Understanding the molecular mechanisms that control differentiation into various specialized types of cells is crucial not only for the advancement of stem or progenitor cell biology, but also for developing its clinical potential as a tissue regeneration therapy. Formation of specialized cells is a multistep process of specific cellular events that includes commitment into specific lineages, differentiation, and maturation. In this study, we used C2C12 cell differentiation as a model system to determine whether miR-1 plays a role in myogenic, osteoblastic, and adipogenic differentiation.

Materials and methods

Cell culture and differentiation induction. C2C12 cells (a kind gift from A. Takahashi) and 3T3-L1 cells (Japanese Collection of Research Bioresources) were maintained as described previously [11,12]. Myogenesis was induced by changing the growth medium to DMEM supplemented with 2% horse serum after the cells reached confluency [11]. Osteoblastic differentiation was induced by treating cells with 300 ng/ml recombinant human BMP-2 (Astellas Pharma) [13]. For adipogenic differentiation, the growth medium was switched to adipogenic induction medium for 3 days and subsequently to adipogenic maintenance medium for 7 days as described previously [10].

Northern blot analysis. Total RNA samples extracted using TRIZOL (Invitrogen) were electrophoresed on denaturing 15% polyacrylamide gels and electroblotted onto GeneScreen Plus membranes (Perkin-Elmer). The membranes were UV-crosslinked, baked, and hybridized with ^{32}P end-labeled oligonucleotide DNA probes in ULTRAhyb-Oligo (Ambion). After washing, hybridization signals were detected using the Bio-imaging analyzer system BAS5000 (Fuji Film). Mouse U6 was used as an internal control.

Immunofluorescent microscopy and quantitative analyses of myotubes. Cells were stained with an antibody against sarcomeric myosin (MF20; Developmental Studies Hybridoma Bank) followed by Alexa Fluor 555-conjugated anti-mouse IgG antibody (Invitrogen) with nuclear staining with DAPI. The average number of nuclei per myotube was determined by counting randomly chosen myosin-positive cells containing two or more nuclei, and 1000 nuclei per culture were counted. The fusion index was calculated as the ratio of the number of nuclei in myotubes with two or more nuclei to the total number of nuclei, and 5000 myotube nuclei were counted.

Oil red O staining and alkaline phosphatase (ALP) assays. Cells were fixed and stained with Oil Red O solution as described previously [12]. Oil Red O was eluted with 100% 2-propanol and measured at 490 nm absorbance for quantification. For ALP staining, cells were stained with a mixture of 0.01% (w/v) naphthol AS-MX phosphate and 0.25 mg/ml fast violet B salt (Sigma–Aldrich) and counterstained with Mayer's Hematoxylin Solution. ALP activity was determined with *p*-nitrophenyl phosphate as a substrate.

DNA constructs. To express miR-1 under the control of the U6 promoter, miR-1 precursor sequences were synthesized, annealed, and ligated into the pENTR/U6 vector (Invitrogen). An miR-1 expression plasmid under the control of the long terminal repeat of PCMV virus was constructed using genomic sequences of miR1-2 containing pre-miR-1 gene sequences with 50 bp flanking each side, and the pMSCV-puro vector (Clontech). For use as a control, a pMSCV-puro vector expressing EGFP was also made.

Retrovirus production and infection. GP2-293 cells were cotransfected with the envelop vector pVSV-G and pMSCV-puro vectors using FuGENE6 (Roche). The medium supernatant was collected and centrifuged to concentrate virus stocks according to the manufacturer's instruction. Cells were infected with the retrovirus in the presence of 4 μg/ml polybrene for 24 h, and the infected cells were selected with 2.5 μg/ml puromycin.

Reverse transcriptase (RT)-polymerase chain reaction (PCR). cDNA was synthesized and analyzed by kinetic real-time PCR using the ABI Prism 7700 Sequence Detector system (Applied Biosystems) with Platinum SYBR Green qPCR SuperMix (Invitrogen). Mouse β tubulin was used for normalization, and comparative threshold (C_T) method was used to assess relative abundance of the targets. Primers used were myogenin-f: TACGTCCATCGTGGACAGCAT, myogenin-r: TCAGCTAAATTCCCTCGCTGG; myoD-f: ACATAGACTTGACAGGCCCCGA, myoD-r: AGACCTTCGATGTAGCGGATGG; muscle creatine kinase (MCK)-f: CACCTCCACAGCACAGACAG, MCK-r: ACCTTGGCCATGTGATTGTT; β-tubulin-f: GGAACATAGCCGTAAACTGC, β-tubulin-r: TCACTGTGCCTGAACTTACC; osterix-f: GGGTTAAGGGGAGCAAAGTCAGAT, osterix-r: CTGGGGAAAGGAGGCACAAAGAAG; osteocalcin-f: CTGAGTCTGACAAAGCCTTC, osteocalcin-r: GCTGTGACATCCATACTTGC; ALP-f: AACCCAGACACAAGCATTCC, ALP-r: GCCTTTGAGGTTTTTGGTCA; PPARγ-f: CCCTGGCAAAGCATTTGTAT, PPARγ-r: GAAACTGGCACCCTTGAAAA; C/EBPα-f: GAACAGCAACGAGTACCGGGTA, C/EBPα-r: GCCATGGCCTTGACCAAGGAG; aP2-f: CCGCAGACGACAGGA, aP2-r: CTCATGCCCTTTCATAAACT.

Immunoblot analysis. Cell lysates containing equal amounts of protein were electrophoresed on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Blots were immunoblotted with the primary antibody against sarcomeric myosin, α-actinin (EA-53; Sigma–Aldrich) or α-tubulin (Sigma–Aldrich), and horseradish peroxidase-labeled donkey anti-mouse IgG as a secondary antibody, followed by enhanced chemiluminescence (GE Healthcare) [14].

Statistical analysis. All experiments were performed at least three times. Data were expressed as means ± standard error and analyzed by one-way ANOVA with post hoc analysis. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

MiR-1 is a muscle-specific miRNA that is expressed during myogenic differentiation

We first examined the expression of miR-1 in C2C12 cells during differentiation into myocytes, osteoblasts, and adipocytes. Although myotube formation was completely abolished when cells were induced to differentiate into osteoblasts, myotube formation was evident upon adipogenic differentiation (Fig. 1A). In undifferentiated cells, miR-1 was not expressed, while its expression was robustly increased when cells were induced to differentiate into myotubes, but not into osteoblasts (Fig. 1B). MiR-1 expression was also observed upon adipogenic differentiation, which

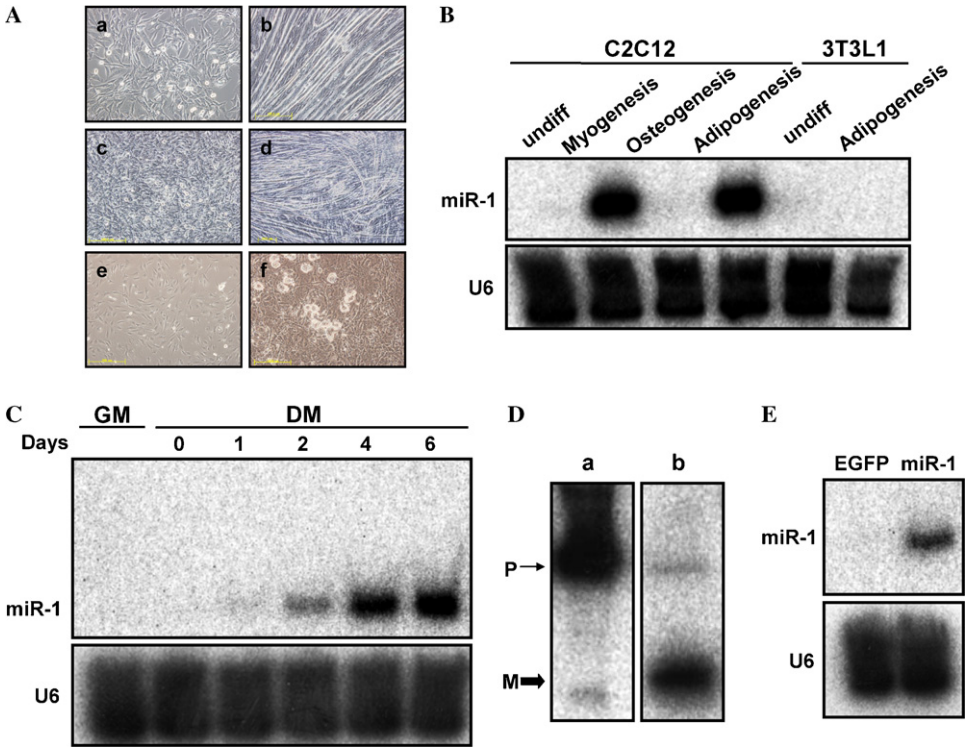

Fig. 1. MiR-1 is a muscle-specific miRNA that is expressed during myogenic differentiation. (A) Myogenic, osteoblastic, and adipogenic differentiation was induced in C2C12 cells (a–d) and 3T3-L1 cells (e,f). (a,e) Undifferentiated, (b) myogenic differentiation, (c) osteogenic differentiation, (d,f) adipogenic differentiation. (B) MiR-1 expression was analyzed in C2C12 cells or 3T3-L1 cells treated as indicated. (C) Myogenic differentiation was induced in C2C12 cells for the indicated periods of time (DM) or cells were cultured in growth medium (GM). Northern blot analysis was performed for miR-1 expression. (D) 293 cells were transfected with the pre-miR-1 (a) or the pri-miR-1-like molecule (b) expression vector, and miR-1 expression was analyzed. P, pre-miR-1; M, mature miR-1. (E) C2C12 cells were infected with EGFP or miR-1-expressing retrovirus vector, and miR-1 expression was analyzed. U6 was used as a loading control.

might reflect concomitant differentiation into myotubes in the adipogenic condition used in this study (Fig. 1A and B). MiR-1 expression was not observed in adipogenic differentiation of 3T3-L1 pre-adipocytes, where myotube formation was not observed (Fig. 1A and B). The observation that miR-1 expression was restricted to conditions that induced myotube formation was consistent with the previously observed restriction of miR-1 expression to cardiac and skeletal muscle in adult mice [3]. Kinetic analysis of miR-1 expression in myogenic differentiation revealed that miR-1 expression was readily detectable 2 days after induction of differentiation and reached its maximum at around days 4–6 (Fig. 1C). This time course correlated well with the expression of myogenic markers such as myogenin, a myogenic regulatory factor (MRF), and muscle type creatine kinase (MCK), a well-characterized marker for mature myocytes (Fig. 2), suggesting that miR-1 plays a role in controlling myogenic differentiation programs.

Overexpression of miR-1 facilitates myogenic differentiation

To analyze the role of miR-1 in C2C12 cell differentiation, we developed a vector-based expression system which efficiently expressed exogenous mature miR-1 in cells, since transient expression by synthetic RNA molecule transfection is not suitable for stable expression during the time course of C2C12 cell differentiation. When a precursor of miR-1 (pre-miR-1) was expressed under the control of the RNA polymerase III promoter, processing from the precursor to mature miR-1 was largely impaired, as revealed by much less abundance of mature miR-1 than pre-miR-1 (Fig. 1D). We then expressed a primary miR-1 (pri-miR-1) like molecule, consisting of the pre-miR-1 plus an additional 50 nucleotides taken from its genomic sequence on each end, under the control of the RNA polymerase II promoter. With this system, efficient expression of mature miR-1 was achieved (Fig. 1D), indicating that exogenous expression of pre-miR-1 is not sufficient for entering the proper processing mechanism, whereas expression of a pri-miR1-like molecule facilitates mature miR-1 expression. Therefore, we made a retroviral vector with this construct for efficient production of mature miR-1 in C2C12 cells (Fig. 1E).

Myogenic differentiation is a multistep dynamic process, during which the cells are defined to be myogenic (terminal commitment), differentiate into myocytes expressing muscle-specific structural and enzymatic proteins (biochemical differentiation), and subsequently fuse to form mature multinucleated myotubes (terminal differentiation). Progression through myogenic differentiation is controlled by

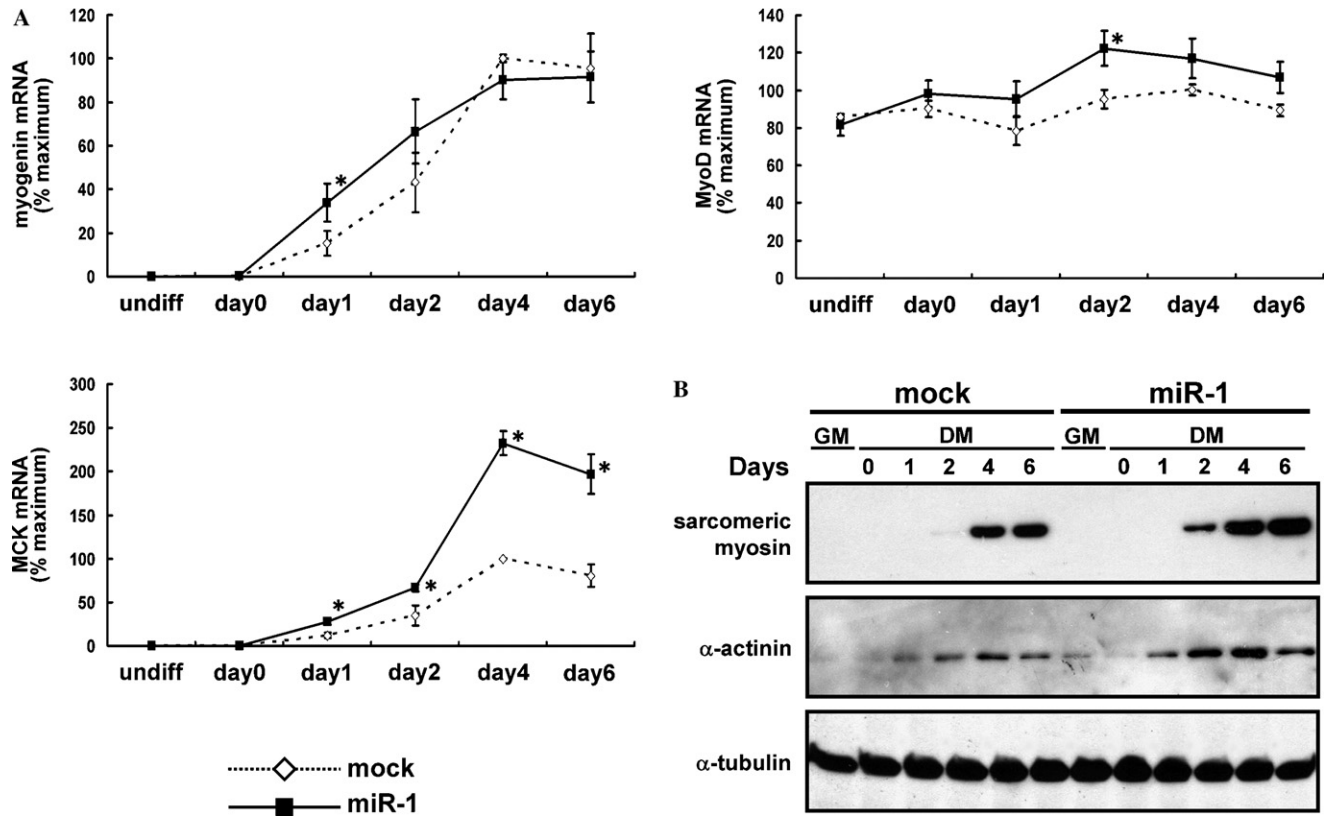

Fig. 2. Overexpression of miR-1 facilitates myogenic differentiation. Myogenic differentiation was induced for the indicated periods of time in C2C12 cells infected with mock or miR-1-expressing retrovirus. (A) Myogenin, MyoD, and MCK expression was analyzed with kinetic real-time PCR. The results were expressed as relative expression to β-tubulin and plotted as percentages of the maximum levels seen in mock-infected cells. $^{*}P < 0.05$ versus control. (B) Immunoblot analysis was performed using anti-sarcomeric myosin, anti-sarcomeric α-actinin, and anti-α-tubulin antibodies.

sequential activation of members of muscle-specific basic helix-loop-helix proteins called MRFs [15]. Among them, MyoD is expressed in undifferentiated myoblasts, while myogenin is activated during differentiation into myocytes. Myogenin expression in miR-1-overexpressing cells was accelerated at day 1, exhibiting a 2.1-fold increase in miR-1-overexpressing cells over mock-infected cells (Fig. 2A). A recent study [5] reported a similar observation, where the effect of transfection of a synthetic miR-1 duplex on myogenin expression was evaluated up to 24 h after induction of differentiation. Later time points were then examined with the aid of retrovirus-mediated stable expression of miR-1. Myogenin expression was comparable between miR-1-overexpressing cells and control cells after day 4, and a modest increase in MyoD expression was observed by miR-1 overexpression only in the differentiating state (Fig. 2A). However, a striking increase was observed in MCK expression in miR-1-overexpressing cells compared to mock-infected cells in the late phase (Fig. 2A). About a 2.7- and 3.8-fold enhancement in MCK expression in miR-1 cells was observed at day 4 and 6, respectively. Western blot analysis with anti-sarcomeric myosin and anti-sarcomeric α-actinin antibodies revealed that expression of these structural proteins was not only accelerated but also augmented by miR-1 overexpression (Fig. 2B). These results indicated that miR-1 overexpression enhanced the biochemical differentiation of myocytes.

MiR-1 overexpression leads to enhanced formation of multinucleated mature myotubes

Fusion of individual myocytes to form multinucleated mature myotubes is a unique feature of skeletal myogenic differentiation, and myoblast fusion has been shown to be regulated by mechanisms genetically dissociated from other myogenic processes such as biochemical differentiation [16–19]. Therefore, we analyzed the effect of miR-1 expression on the formation of mature myotubes and observed that in miR-1-overexpressing cells, myotubes were both higher in number and larger in size compared to mock-infected cells (Fig. 3A). This observation was quantified by the average number of nuclei per myotube (Fig. 3B), and by the percentage of all nuclei present in myotubes (fusion index) [20] (Fig. 3C). The results showed about a 1.9-fold increase in nuclei per myotube and a 1.6-fold increase in the fusion index.

Taken together, these results indicate that in addition to its role in the early steps of myogenic differentiation [5], miR-1 also plays an important role in late biochemical differentiation and in terminal differentiation. This was

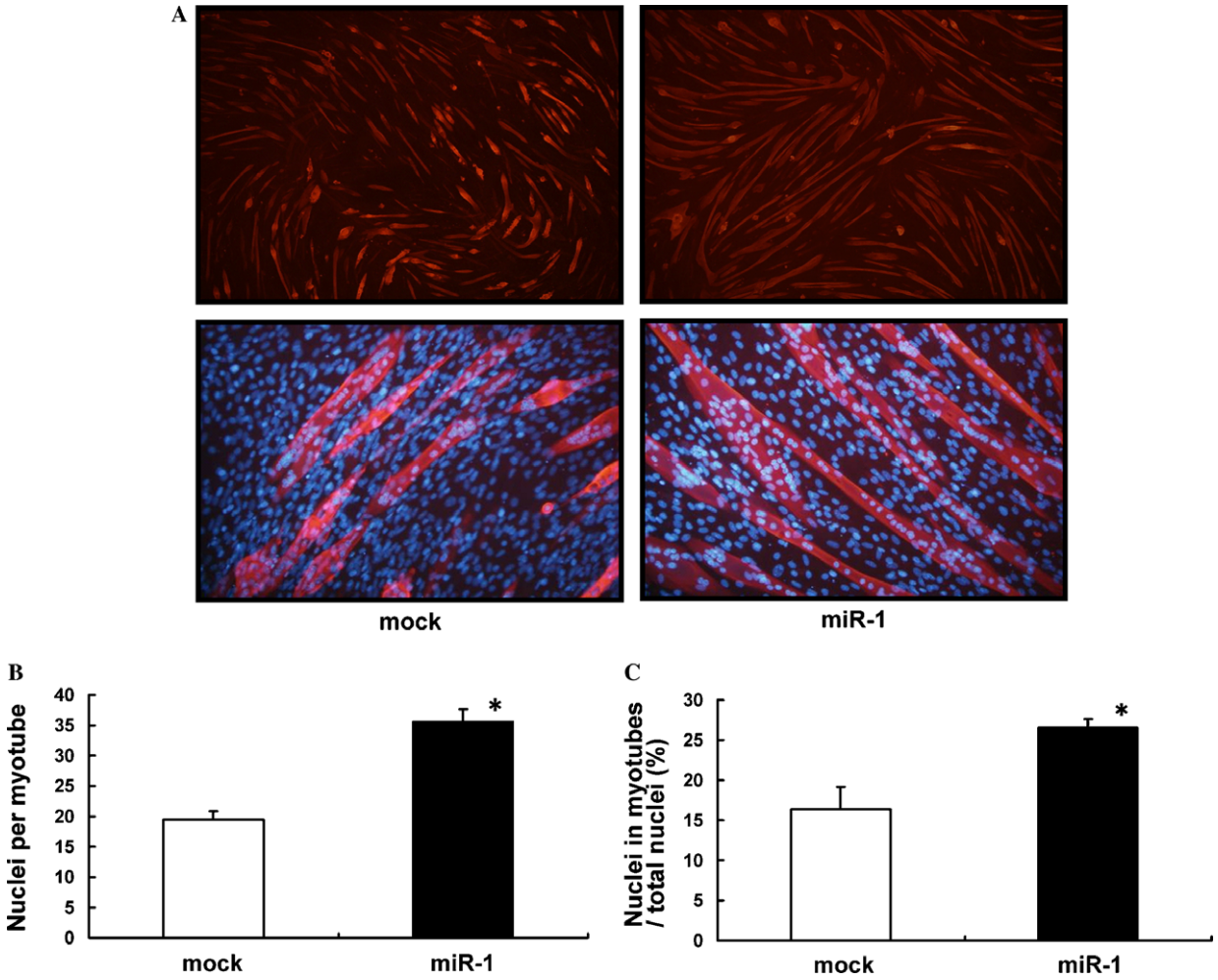

Fig. 3. MiR-1 overexpression leads to enhanced formation of multinucleated mature myotubes. Cells infected with mock or miR-1-expressing retrovirus were induced for myogenic differentiation for 6 days. (A) Myotubes were stained with anti-sarcomeric myosin antibody, and nuclei were stained with DAPI. (B) The mean number of nuclei per myotube was determined by counting 1000 nuclei per culture in three independent cultures. (C) The fusion index was defined as the ratio of nuclei within myotubes (cells containing two or more nuclei) to total number of nuclei, and percentages were plotted. Five thousand nuclei per culture were counted in three independent cultures. $^{*}P < 0.05$ versus control.

supported by: (i) the similar kinetics of endogenous miR-1 expression (Fig. 1C) with the expression of MCK, sarcomeric myosin, and α-actinin (Fig. 2A and B), and the formation of myotubes, which all peaked at days 4–6 after induction; and (ii) the enhancement of expression of mature myocyte markers and myotube fusion with overexpression of miR-1.

In the heart, which also endogenously expresses miR-1, it has been reported that overexpression of miR-1 in mouse hearts leads to a decrease in proliferating ventricular cardiac myocytes [3]. Although the role of miR-1 in the determination of myocyte fate could not be evaluated in this study, as a cardiac-specific β-myosin heavy chain promoter was used to drive miR-1 expression in cardiac myocytes, these results suggest that miR-1 expression in cardiac myocytes results in enhanced or premature differentiation of cardiac myocytes that impairs the balance between differentiation and proliferation. CHIP assays have demonstrated that MyoD and myogenin bind to regions upstream to miR-1 genes, suggesting these MRFs regulate expression of miR-1 [21]. These results with our observations suggested that miR-1 plays an important role in the relatively late stages of myogenic differentiation, although further studies are needed to fully clarify the functions of miR-1 in myogenesis.

MiR-1 does not influence osteoblastic or adipogenic differentiation

Since it is not known whether miR-1 plays a role in specification of cell fate to myogenic lineages, we analyzed the effects of miR-1 overexpression on the osteoblastic and adipogenic differentiation of C2C12 cells. The osteoblastic and adipogenic differentiation programs are also multistep processes [10,12,22,23], so we evaluated the expression of transcription factors involved in determination and initial differentiation of these lineages such as osterix, PPARγ, and C/EBPα, relatively late differentiation markers such

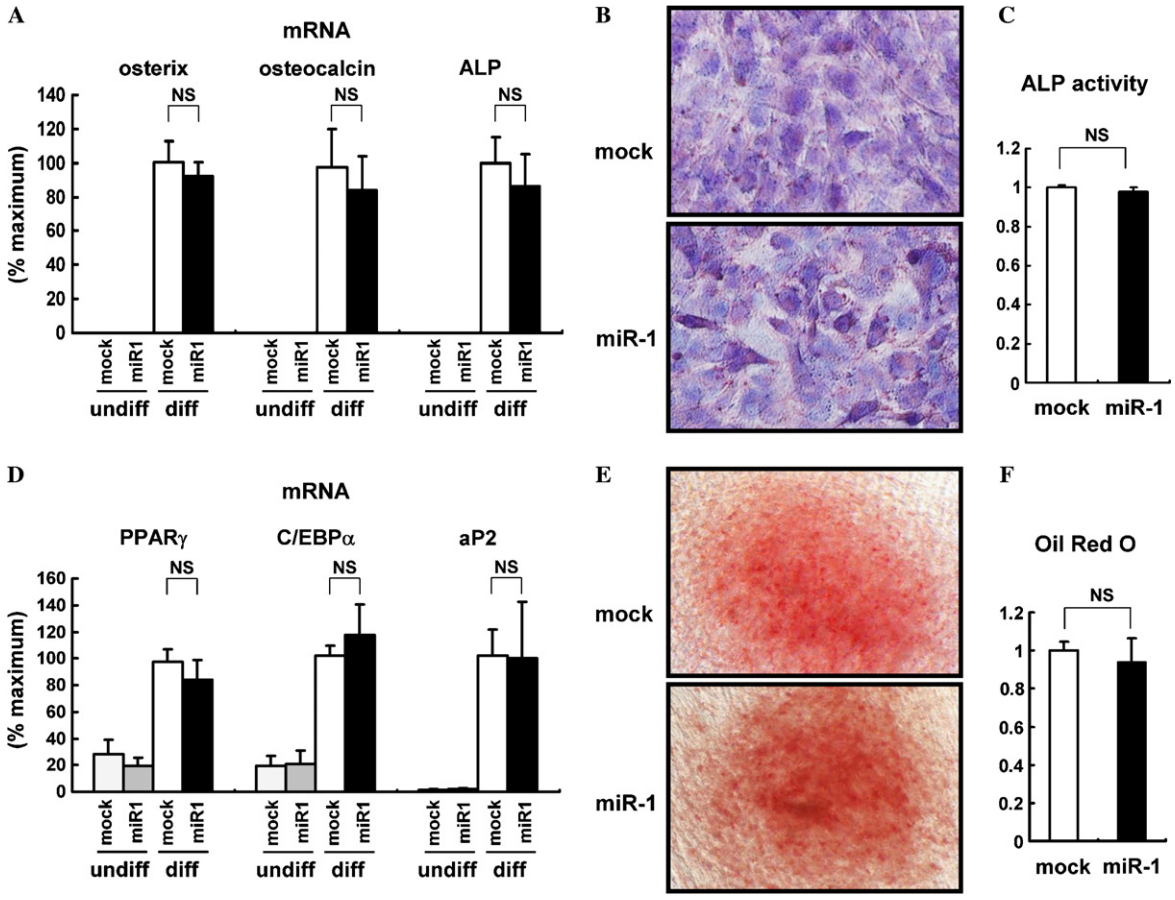

Fig. 4. MiR-1 does not influence osteoblastic or adipogenic differentiation. Osteoblastic (A–C) or adipogenic (D–F) differentiation was induced in mock-infected and miR-1-overexpressing C2C12 cells. (A) Osterix, osteocalcin, and ALP expression was measured by kinetic real-time PCR in undifferentiated and differentiated cells. (B) ALP staining was performed in differentiated cells. (C) ALP activity was determined with *p*-nitrophenyl phosphate as a substrate. (D) Kinetic PCR analysis was performed to analyze expression of PPARγ, C/EBPα, and aP2 in undifferentiated and differentiated cells. (E) Differentiated cells were stained with Oil Red O. (F) To quantify lipid accumulation, Oil Red O was extracted, and optical density was measured at 490 nm. NS; not significant.

as osteocalcin, ALP, and aP2, and characteristic biochemical features of these cells such as ALP activity and lipid accumulation. The osteoblastic markers osterix, osteocalcin, and ALP were absent in undifferentiated C2C12 cells, but markedly induced upon induction of osteoblastic differentiation (Fig. 4A). Neither the expression of these osteoblastic markers nor ALP staining and activity was altered by the exogenous expression of miR-1 during osteoblastic differentiation (Fig. 4A–C). When cells were cultured in adipogenic condition, adipogenic markers such as PPARγ, C/EBPα, and aP2 and lipid accumulation were significantly induced, and the forced expression of miR-1 did not alter the expression of these adipogenic marker genes or lipid accumulation in the cells (Fig. 4D–F). It has been reported that exogenous miR-1 expression in non-muscle cells shifts the mRNA expression profile towards muscle by downregulating the expression of genes not expressed in muscle [4]. Although these results suggested that miR-1 might act to prevent cells from differentiating into lineages other than muscle, our results showed that osteoblastic and adipogenic differentiation was not modulated by the expression of miR-1, implying that miR-1 does not function in determination of cell fate.

Conclusion

In this study, we analyzed the role of miR-1 in myogenic, osteoblastic, and adipogenic differentiation of C2C12 cells, and found that miR-1 enhanced myogenic differentiation and maturation into myotubes, but did not affect osteoblastic and adipogenic differentiation. These results suggest that miR-1 plays important roles in controlling the myogenic differentiation and maturation in lineage-committed cells, rather than functioning in fate determination. Identification of downstream targets of miR-1 will be an important issue to fully clarify the roles of miR-1 in myogenesis, which could be coordinately regulated by multiple miR-1 targets, since bioinformatic predictions indicate that each miRNA regulates on average ~200 target transcripts [24].

Acknowledgments

We thank Akihiro Takahashi for providing C2C12 cells, Astellas Pharma for BMP-2, Parth Patwari for critical reading of the manuscript, and Atsumi Kosugi and Mami Nishikawa for expert technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from the Mitsubishi Pharma Research Foundation and Grants-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

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