Biochemical and Biophysical Research Communications 445 (2014) 255–262

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reversine induces multipotency of lineage-committed cells through epigenetic silencing of miR-133a

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ARTICLE INFO

Article history:
Received 24 January 2014
Available online 7 February 2014

Keywords:
Reversine
miR-133a
Dedifferentiation
Myogenesis
Histone modifications

ABSTRACT

Reversine has been shown to induce dedifferentiation of C2C12 murine myoblasts into multipotent progenitor cells. However, little is known about the key regulators mediating the dedifferentiation induced by reversine. Here, we show that large scale miRNA gene expression profiling of reversine-treated C2C12 myoblasts identifies a down-regulated miRNA, miR-133a, involved in dedifferentiation of myoblasts. Reversine treatment results in up- and down-regulated miRNA profiles. Among miRNAs affected by reversine, the level of muscle-specific miR-133a, which has been shown to be up-regulated during muscle development and to suppress differentiation into other lineages, is markedly reduced by treatment of C2C12 myoblasts with reversine. In parallel, reversine decreases the expression and recruitment of myogenic factor, SRF, to the enhancer regions of miR-133a. Sequentially, down-regulation of miR-133a by reversine is accompanied by a decrease in active histone modifications including trimethylation of histone H3K4 and H3K36, phosphorylation of H3S10, and acetylation of H3K14 on the miR-133a promoter, leading to dissociation of RNA polymerase II from the promoter. Furthermore, inhibition of miR-133a by transfection of C2C12 myoblasts with miR-133a inhibitor increases the expression of osteogenic lineage marker, Ogn, and adipotenic lineage marker, ApoE, similar to that in response to reversine. In contrast, the co-overexpression of miR-133a mimic reversed the effect of reversine on C2C12 myoblast dedifferentiation. Taken together, the results indicate that reversine induces a multipotency of C2C12 myoblasts by suppression of miR-133a expression through depletion of active histone modifications, and suggest that miR-133a is a potential miRNA regulating the reversine-induced dedifferentiation. Collectively, our findings provide a mechanistic rationale for the application of reversine to dedifferentiation of somatic cells.

1. Introduction

MicroRNAs (miRNAs) are 18–22 nucleotide non-coding RNAs that post-transcriptionally regulate gene expression and control diverse cellular processes including proliferation, differentiation, tissue morphogenesis, and apoptosis [1]. There are approximately one thousand miRNAs in the human genome, each one targeting multiple RNAs and exerting an influence on their turnover and translation to different degrees, depending on the specific characteristics of the miRNA–mRNA interaction [2]. miRNAs have been found to play important roles in the regulation of muscle development and heart disease [3–7]. Muscle miRNAs are composed of two distinct families, the miR-1 family and the miR-133 family. The muscle-specific miR-1 and miR-133 are encoded in the same bicistronic transcriptional unit, under the control of the muscle-specific transcription factor, myocyte enhancer factor 2 (MEF2) and serum response factor (SRF). Both miRNAs are expressed in skeletal and cardiac muscles and have been shown to be involved in the control of the expression of muscle specific proteins [8]. Additionally, miR-133 and miR-1 are key regulators of muscle development by modulating myoblast proliferation and differentiation, assuming antagonistic roles in these processes. Whereas miR-1 promotes

Abbreviations: ADM, adipogenic differentiation medium; ALP, alkaline phosphatase; miRNA, microRNA; MEF2, myocyte enhancer factor 2; MRF, muscle regulatory factor; ODM, osteogenic differentiation medium; Pol II, RNA polymerase II; SRF, serum response factor.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.002
0006-291X/

differentiation of embryonic stem cells toward a cardiac fate, miR-133 inhibits differentiation into cardiac muscle. Indeed, a loss-of-function mutation of miR-133 in mouse heart results in lethal ventricular-septal defects in embryos or neonates and cardiomyopathy and heart failure in adulthood [9,10]. It has been reported that the function of miR-133 is mediated by its target mRNAs, like SRF, Krüppel-like factor 15 (KLF15), RhoA and uncoupling protein 2 (UCP2), respectively [3,11–14].

Dedifferentiation of somatic cells to multipotent progenitor cells gives great potency to treat incurable or degenerative diseases by patient-specific stem cell therapy. There are four major mechanisms by which dedifferentiation of somatic cells is induced: nuclear transfer, cell fusion, cell explantation and forced expression of certain genes [15,16]. However, each induction strategy has challenges that need to be overcome such as ethical problems or genetic mutations. As an alternative approach is the use of reversine, one of the 2,6-disubstituted purine analogues, which is a small molecule that can induce dedifferentiation of C2C12 myoblasts to multipotent progenitor cells [17]. C2C12 myoblasts treated with reversine can differentiate into not only mesodermal-lineage cells but also neuroectodermal-lineage cells under appropriate stimuli [18]. Reversine treatment induces reprogramming of primary murine and human dermal fibroblasts into skeletal muscle both *in vitro* and *in vivo* [19].

The identification of the miRNA expression profile induced by reversine and its role in muscle development has never been systematically addressed. We report the first miRNA expression profile of reversine-treated C2C12 myoblasts, focusing on a subset of miRNAs known to be involved in the regulation of muscle development and dedifferentiation processes. In reversine-treated myoblasts, myogenic factor SRF was dissociated from miR-133a enhancer and then active histone modifications decreased at its promoter, leading to down-regulation of miR-133a. Thus, these data indicate that induction of multipotency of C2C12 myoblasts by reversine might be mediated by suppression of miR-133a expression. Collectively, our findings provide new insight into the molecular mechanisms by which reversine promotes dedifferentiation of the muscle cells into multipotent progenitor cells through suppression of a muscle specific miR-133a.

2. Materials and methods

2.1. Western blot analysis

C2C12 myoblast cells were lysed in lysis buffer and kept on ice for 30 min and 30 μg of each protein was subjected to SDS–polyacrylamide gel electrophoresis. Proteins were blotted on PVDF membranes and the membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with primary antibodies, followed by incubation with secondary antibodies coupled to horseradish peroxidase, and visualization with an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

2.2. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using easy-Blue reagent (iNtRON Biotechnology) and 1 μg of RNA with oligo dT primers was subjected to reverse transcription using the ImProm-II™ Reverse Transcription System (Promega). cDNA were amplified using Super Premix Sapphire PCR master mix (mbiotech). Primer sequences used for PCR were: SRF 5′-ATGCCCCATCCCTTAAAATC-3′ and 5′-CGCAGAAGTAGGCTTGTTCC-3′; myogenin 5′-CTTCCTCTTACACACCTTGC-3′ and 5′-GACATCCCCCTATTTCTACC-3′; ApoE 5′-TGCTGTTGGTCACATTGCTG-3′ and 5′-GGAGCTCTGCAGCTCTTCCT-3′; Ogn 5′-AACCTGTGCAAAGCCAAGTG-3′ and 5′-CCCTTTCCTTGGGCTAAGTG-3′; Gapdh 5′-TGATGACATCAAGAAGGTGAAG-3′ and 5′-TCCTTGGAGGCCATGTAGGCCAT-3′. For qRT-PCR for miR-133a, a RT reaction was performed with stem-loop primer to generate strand-specific cDNA. cDNA were amplified using miRNA-specific forward primer and reverse primer in conjunction with KAPA SYBR FAST qPCR Kits (Kapa Biosystems). Primer sequences were: stem-loop primer for miR-133a-specific RT reaction 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG-3′; primers for miR-133a specific PCR 5′-GCCTGTTTGGTCCCCTTCAA-3′ and 5′-GTGCAGGGTCCGAGGT-3′. The expression level of miR-133a was normalized to U6 small nuclear RNA.

2.3. Mesodermal lineage differentiation

C2C12 cells were treated with 5 μM reversine for 4 days and washed in DMEM. For adipogenic differentiation, cells were cultured for 3 days in DMEM medium supplemented with 10% FBS, 0.1 μM dexamethasone, 50 μg/ml indomethacin, 0.45 mM 3-isobutyl-1-methylxanthine, 50 μg/ml ascorbate-2-phosphate and 0.01 mg/ml insulin. Cells were fixed with 10% formalin and placed in 100% propylene glycol and stained for 15 min with Oil-Red-O. For osteogenic differentiation, cells were cultured for 6 days in DMEM medium supplemented with 10% FBS, 0.1 μM dexametasone, 50 mM ascorbate-2-phosphate and 10 M β-glycerophosphate. Cells were fixed with 3.7% formaldehyde and 90% ethanol solution and staining with BCIP/NBT staining solution to detect alkaline phosphatase.

2.4. Chromatin immunoprecipitation (ChIP) assay

Chromatin from 1×10^6 cells/mL sheared by a sonicator was cross-linked and sheared chromatin solution was reserved as the input DNA and the remainder was subject to immunoprecipitation overnight at 4 °C using antibodies. After immunoprecipitation, recovered chromatin fragments were subjected to qRT-PCR using a primer pair specific for miR-133a-1 promoter and enhancer. The primer sequences were as follows: promoter (E1) 5′-GGGAGAATCTGGGAAATGTA-3′ and 5′-AAAGCTGAGGAGGATTCTAT-3′; enhancer (E2) 5′-AGCAAGATAGAATCCTCCTCA-3′ and 5′-AGGCAGCTAAGCATTTGAAACA-3′: enhancer (E3) 5′-GGACCGCTGTCAATGGTGCC-3′ and 5′-CCCTTGGATCAGGAGCGACC-3′.

2.5. miRNA microarray analysis

Total RNA was isolated from control and reversine-treated C2C12 cells using easy-Blue reagent (iNtRON Biotechnology). Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (Thermo). Labeled cRNA samples (750 ng) were hybridized to each Illumina Sentrix BeadChip U1536-16 bead array for 16–18 h at 58 °C, according to the manufacturer's instructions (Illumina, Inc.). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis was performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8). Experiments for miRNA array analysis were done in duplicate.

2.6. Data acquisition and statistical analysis

The hybridized images were scanned using an Illumina Bead Array Reader and quantified with BeadScan software according to the manufacturer's instructions (Illumina, Inc.). Intensity files were analyzed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8). Datasets were then further normalized to the median value for each RNA transcript. Statistically significant genes were determined using two-group *t*-tests. The comparative analysis between test group and control group was carried out using fold-change, LPE test adjusted false discovery rate (FDR) *p*-value. For miRNAs, we used a *p*-value cutoff of less than 0.08. The data set was further filtered, and the ratio of the group median needed to be greater than twofold for an RNA transcript to be stated as significant. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. For miRNA target prediction analysis, the target mRNAs of specific miRNAs were predicted by searching public databases with prediction algorithms, such as MicroCosm (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/).

3. Results

3.1. Reversine inhibits myogenesis of C2C12 myoblasts and induces differentiation into other lineages

To check the reprogramming activity of reversine, we tested whether reversine-treated C2C12 myoblasts decreased expression of known myogenic factors. Two transcription factors, myogenin and SRF, control muscle gene expressions and consequently activate myogenesis. RNA and protein levels of myogenin and SRF were markedly decreased in C2C12 myoblasts after treatment with 5 μM of reversine for 4 days in a dose-dependent manner (Fig. 1A). To confirm reprogramming of C2C12 into myogenic competent cells, C2C12 cells were treated with reversine for 4 days as indicated; after this, the compound-containing media was removed and cells were cultured in compound-free medium suitable for the development of adipocyte (adipogenic differentiation medium; ADM) and osteoblasts (osteogenic differentiation medium; ODM), respectively. These cells differentiated into mesodermal lineage, adipocytes or osteoblasts, which were positively stained for lipid droplets (Oil red O) or alkaline phosphatase (ALP) (Fig. 1B). Consistent with the result, reversine-treated C2C12 caused a significant increase in expression levels of *ApoE* and *Ogn*, which are maintained during adipogenic and osteogenic differentiation, respectively (Fig. 1C). These results indicate that reversine down-regulates specific myogenic factors and blocks normal myogenesis of C2C12 myoblasts, resulting in increased plasticity of C2C12 myoblasts.

3.2. miR-133a, muscle-specific miRNA, is down-regulated in the reversine-treated C2C12 myoblasts

To understand the potential involvement of miRNAs in muscle dedifferentiation induced by reversine, we analyzed the large-scale miRNA expression profiling of reversine-treated C2C12 myoblasts. As shown in Fig. 2, from among 380 mouse miRNAs, reversine induced up- and down-regulation of miRNAs with more than two-fold significant changes relative to control C2C12 myoblasts, respectively ($p < 0.05$). From this, we classified miRNAs that had previously been studied and were known to be involved in myogenesis. Based on these criteria, we did not detect muscle-specific miRNAs. To attempt to solve this issue, we reset the selection criteria for miRNAs changed by reversine ($p < 0.08$ and fold change: $\geqslant 2$). Based on this filtering step, miRNA-133a, which is identified as a muscle-specific miRNA, was markedly down-regulated upon treatment of C2C12 myoblasts with reversine (p value = 0.08, fold change: −10.60). A detailed list of the miRNA signatures affected by reversine treatment is presented in Tables 1 and 2. In order to assess the robustness of the microarray analysis, qRT-PCR analysis was performed to validate miR-133a, using the specific primer sets. U6 small nuclear RNA was used to normalize qPCR data. qPCR

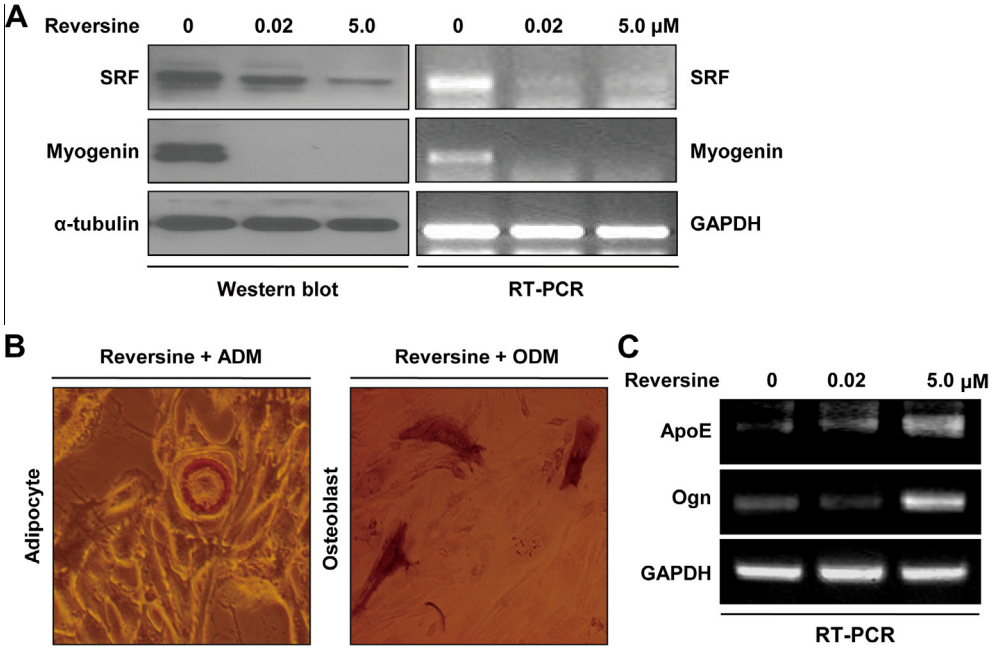

Fig. 1. Reversine down-regulates myogenin and SRF expression and induces C2C12 dedifferentiation. (A) C2C12 cells were treated with 20 nM or 5 μM reversine for 4 days. Levels of myogenin and SRF were determined by RT-PCR and Western blot. Expression levels of myogenin and SRF were normalized to GAPDH and α-tubulin. (B) C2C12 cells were treated with 5 μM reversine for 4 days. After removal of reversine, cells were cultured in either ADM or ODM and stained with Oil-red O or ALP, respectively. (C) C2C12 cells were treated with 20 nM or 5 μM reversine for 4 days, and then the mRNA levels of *ApoE* and *Ogn* were determined by RT-PCR analysis.

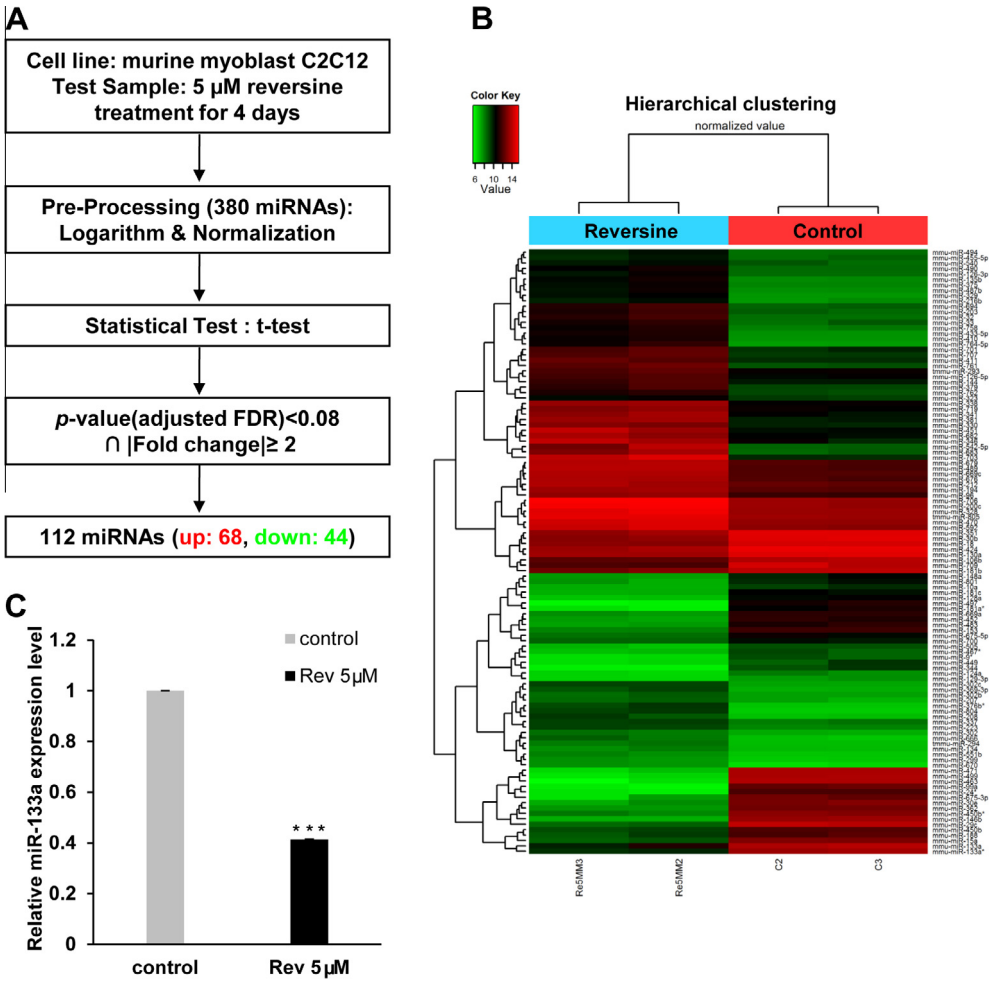

Fig. 2. Microarray analysis of miRNA expression in reversine-treated C2C12 myoblasts. (A) and (B) C2C12 cells were treated with 5 μM reversine for 4 days and the alteration of the miRNA expression pattern by reversine is described in Section 2. All data are >2.0-fold change and p-value < 0.08. miRNAs that were up-regulated relative to controls are shown in red and those that were down-regulated are in green. (B) The heat maps generated from the microarray analysis were subjected to hierarchical clustering analysis. The number of color bar indicates log 2 normalized value. (C) The expression level of miR-133a was determined by qRT-PCR and normalized to U6 small nuclear RNA. Error bars denote ± s.d. of mean: $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

validation of miR-133a (41.32%) was consistent with the microarray analysis (Fig. 2C).

3.3. Reversine abolishes binding of SRF to the miR-133a enhancer region

Muscle miRNAs consist of two families, the miR-1 family (miR-1-1, miR-1-2, and miR-206) and the miR-133 family (miR-133a-1, miR-133a-2, and miR-133b). Although pre-miR-133a-1 and pre-miR-133a-2 are transcribed from different chromosome, mature miR-133a possesses the same sequence. A 4.3 kb muscle-specific enhancer encompassing MEF2, SRF and Yin Yang 1 (YY1) binding sites, was previously identified between miR-1–1 and miR-133a-2 coding regions [20,21] (Fig. 3B). Like the miR1–1/miR-133a-2 locus, the miR1-2/miR-133a-1 locus possesses two enhancers based on genomic sequence conservation and is controlled by multiple muscle-specific regulatory elements. E1 (enhancer 1/upstream enhancer/promoter) is located approximately 2 kb upstream of miR-1-2 while E2 (enhancer 2/intragenic enhancer) is located in an intron separating miR-1-2 and miR-133a-1 coding regions. The enhancer regions of this locus contains a conserved MEF2-like site (CTATTTTAG), a MyoD-binding E-box (CANNTG) [22] and YY1 [20] (Fig. 3A). It was demonstrated that a CArG box (CC(A/T)6GG) is in the enhancer region of miR-133a-2 [23], but not in the enhancer region of miR-133a-1. To delineate the CArG box, the binding site for SRF within the enhancer regions, we investigated the upstream region of miR-133a-1. The CArG box sequence was not found in the promoter region of miR-133a-1 but the CArG box-like sequence, CCATATTAGT, was identified in the intragenic enhancer region of miR-133a-1 (1.1 Kb upstream of miR-133a-1 coding region). As shown in Fig. 3C, SRF was able to bind the enhancer region of miR-133a-1. Next, to examine the effect of reversine on the binding of SRF to the miR-133a-1 enhancer region, we carried out a chromatin immunoprecipitation (ChIP) analysis. Our ChIP results showed that the SRF occupancy was decreased in the enhancer regions of miR-133a-1 and miR-133a-2 following treatment with reversine (Fig. 3C and E). Thus, we suggest that reversine suppresses miR-133a expression by preventing SRF binding to the enhancer region.

3.4. Reversine causes depletion of active histone modifications and RNA polymerase II (pol II) on the miR-133a promoter

Histone modifications such as acetylation, phosphorylation and methylation play an important role in regulation of gene expression through altering chromatin structure. Previous work

Table 1
Analysis of up-regulated miRNAs in reversine-treated C2C12 cells.

miRNA name	*P*-value	Fold change	miRNA name	*P*-value	Fold change
mmu-miR-703	0.03	27.89	mmu-miR-126-3p	0.06	3.92
mmu-miR-542-5p	0.06	27.51	mmu-miR-706	0.02	3.88
mmu-miR-683	0.04	26.90	mmu-miR-369-3p	0.04	3.62
mmu-miR-346	0.06	13.64	mmu-miR-200c	0.02	3.60
mmu-miR-764-5p	0.04	12.39	mmu-miR-302c	0.02	3.57
mmu-miR-451	0.04	12.04	mmu-miR-676	0.03	3.53
mmu-miR-381	0.02	11.64	mmu-miR-669c	0.01	3.51
mmu-miR-761	0.03	11.10	mmu-miR-144	0.07	3.48
mmu-miR-433-5p	0.02	9.79	mmu-miR-96	0.02	3.43
mmu-miR-32	0.04	9.34	mmu-miR-666	0.06	3.19
mmu-miR-410	0.02	9.25	mmu-miR-494	0.05	3.14
mmu-miR-330	0.02	9.00	mmu-miR-328	0.04	2.93
mmu-miR-682	0.04	8.69	mmu-miR-540	0.07	2.80
mmu-miR-341	0.04	8.41	mmu-miR-194	0.03	2.75
mmu-miR-694	0.05	7.35	mmu-miR-805	0.02	2.73
mmu-miR-758	0.04	7.19	Mmu-let-7d*	0.08	2.73
mmu-miR-707	0.03	7.12	mmu-miR-805	0.03	2.59
mmu-miR-338	0.02	7.02	mmu-miR-788-5p	0.07	2.58
mmu-miR-135b	0.04	6.83	mmu-miR-455-5p	0.07	2.58
mmu-miR-329	0.02	6.66	mmu-miR-470	0.04	2.54
mmu-miR-804	0.02	6.53	mmu-miR-212	0.06	2.53
mmu-miR-487b	0.05	6.46	mmu-miR-294	0.03	2.50
mmu-miR-411	0.03	6.36	mmu-miR-207	0.06	2.48
mmu-miR-376b*	0.04	5.86	mmu-miR-223	0.05	2.37
mmu-miR-379	0.08	5.82	mmu-miR-323	0.07	2.28
mmu-miR-719	0.03	5.65	mmu-miR-323b	0.07	2.23
mmu-miR-216b	0.04	5.16	mmu-miR-551b	0.06	2.23
mmu-miR-490	0.04	4.72	mmu-miR-337	0.04	2.14
mmu-miR-762	0.03	4.48	mmu-miR-299	0.04	2.05
mmu-miR-489	0.02	4.29	mmu-miR-134	0.06	2.02
mmu-miR-679	0.02	3.93			

The mature sequences are assigned names of the form miR (the predominant product) and miR* (from the opposite arm of the precursor).

Table 2
Analysis of down-regulated miRNAs in reversine-treated C2C12 cells.

miRNA name	*P*-value	Fold change	miRNA name	*P*-value	Fold change
mmu-miR-130a	0.02	−2.33	mmu-miR-344	0.04	−10.05
mmu-miR-129-3p	0.04	−2.36	mmu-miR-133a	0.08	−10.60
mmu-miR-181b	0.06	−2.44	mmu-miR-153	0.01	−12.74
mmu-miR-424	0.04	−2.62	mmu-miR-128a	0.02	−12.74
mmu-miR-18	0.02	−2.75	mmu-miR-669a	0.02	−12.98
mmu-miR-124a	0.06	−2.84	mmu-miR-483	0.02	−13.58
mmu-miR-700	0.04	−2.88	mmu-miR-452	0.02	−14.51
mmu-miR-30b	0.03	−3.15	mmu-miR-133a*	0.02	−14.92
mmu-miR-106b	0.02	−3.23	mmu-miR-15a	0.02	−17.64
mmu-miR-467*	0.06	−3.99	mmu-miR-181a*	0.03	−26.33
mmu-miR-505	0.03	−4.10	mmu-miR-450b*	0.02	−33.58
mmu-miR-675-5p	0.04	−4.70	mmu-miR-362	0.01	−34.47
mmu-miR-10a	0.04	−4.98	mmu-miR-497	0.02	−37.99
mmu-miR-9*	0.04	−5.76	mmu-miR-30e	0.03	−43.82
mmu-miR-801	0.06	−5.87	mmu-miR-29c	0.02	−49.09
mmu-miR-148a	0.05	−6.16	mmu-miR-675-3p	0.05	−54.88
mmu-miR-449	0.07	−6.61	mmu-miR-24*	0.02	−61.45
mmu-miR-450b	0.02	−7.55	mmu-miR-146b	0.01	−80.90
mmu-miR-188	0.05	−7.97	mmu-miR-99a	0.02	−97.34
mmu-miR-181c	0.02	−9.33	mmu-miR-471	0.02	−149.12

The mature sequences are assigned names of the form miR (the predominant product) and miR* (from the opposite arm of the precursor).

demonstrated that inhibition of histone H3 phosphorylation by reversine results in chromatin remodeling and down-regulation of muscle differentiation genes in C2C12 myoblasts [24,25]. To examine whether down-regulation of miR-133a by reversine is associated with alteration of histone modifications in the upstream regions of miR-133a-1 (E1) and miR-133a-2 (E3), we determined the level of histone acetylation and methylation in the presence or absence of reversine using ChIP analysis. Trimethylation of histone H3 lysine 4 (H3K4me3) and H3K36 (H3K36me3) and phospho-S10/acetyl-K14 histone H3 (H3S10phK14ac), the active markers, on the promoter, were enriched in the absence of reversine. Conversely, reversine treatment caused a significant depletion of active histone modifications. However, the repressive markers, dimethylated H3K9 (H3K9me2) and trimethylated H3K27 (H3K27me3) were not affected while trimethylated H3K9 (H3K9me3) was increased by reversine. In addition, these alterations of histone modification led to the dissociation of Pol II from promoter region (Fig. 3D and E). Taken together, the results indicate that down-regulation of miR-133a by reversine might be attributed to depletion of active histone modifications and Pol II.

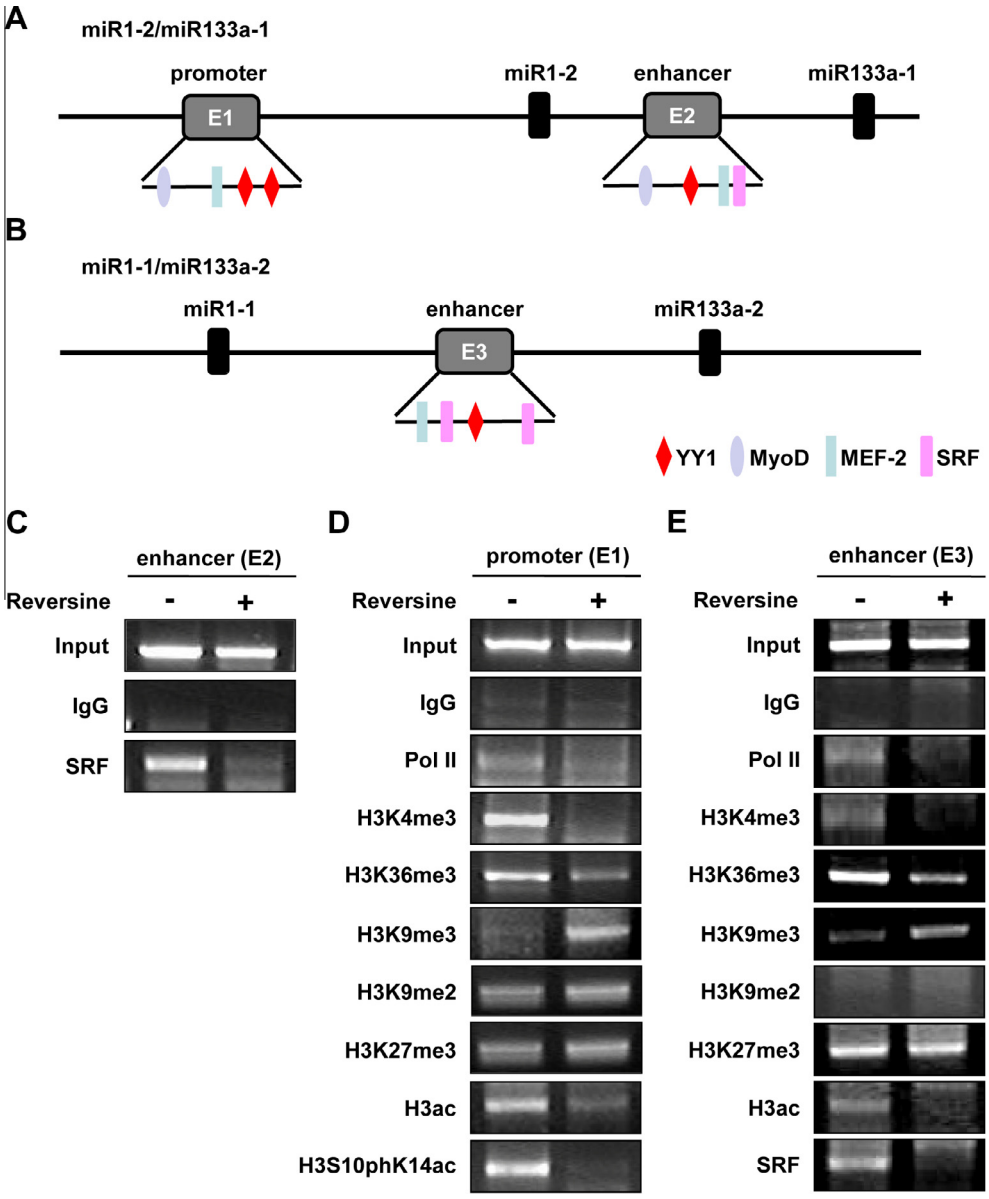

Fig. 3. Down-regulation of miR-133a by reversine is accompanied by dissociation of SRF from enhancer region and alteration of epigenetic modifications and Pol II in the promoter. The schematic presentation of genomic structure of miR-1-2/miR-133a-1 (A) and miR-1-1/miR-133a-2 (B): upstream enhancer (E1, promoter) and intragenic enhancer (E2 in A and E3 in B). The E2 region contains a conserved a MyoD binding E-box, YY1, a MEF2-like site and a CArG box like sequence. C2C12 cells were treated with 5 μM reversine for 4 days. The change in the binding level of SRF by reversine from the upstream region of miR-133a-1 (C) and miR-133a-2 (E) in C2C12 cells was determined by ChIP analysis using SRF antibody. Changes in histone modifications or the binding level of pol II in the promoter (E2) and enhancer (E3) region by reversine were determined by ChIP analysis (D and E).

3.5. Dedifferentiation of C2C12 myoblasts by reversine is mediated by suppression of miR-133a expression

Our results raise the possibility that miR-133a might play an essential role in the dedifferentiation in C2C12 myoblast. To address this possibility, we modulated the expression of miR-133a by transfection of C2C12 myoblasts with miR-133a inhibitor or mimic and assessed its effect on the induction of multipotency. Consistent with reversine-treated C2C12 cells, the expression levels of *ApoE* and *Ogn* were markedly increased following knockdown of miR-133a, while the expression levels of two genes were decreased following overexpression of miR-133a in C2C12 myoblasts (Fig. 4A–C). Furthermore, the overexpression of miR-133a in reversine-treated C2C12 cells reversed the effect of reversine on the expression of those genes (Fig. 4D). These results indicate that the dedifferentiation of C2C12 myoblasts induced by reversine is attributed to the suppression of miR-133a expression.

4. Discussion

Reversine, a purine derivative, was identified by virtue of its ability to increase the plasticity of C2C12 myoblasts. This small molecule prevents normal myoblast differentiation. Reversine causes C2C12 cells to dedifferentiate to a multipotent state, from which they can redifferentiate by reversine-treatment into other cell lineages. Interestingly, reversine is a potent Aurora B kinase inhibitor that causes dephosphorylation of histone H3S10 [24,25]. Phosphorylated H3S10 remodels chromatin structure to active chromatin by dissociating HP1 protein from methylated

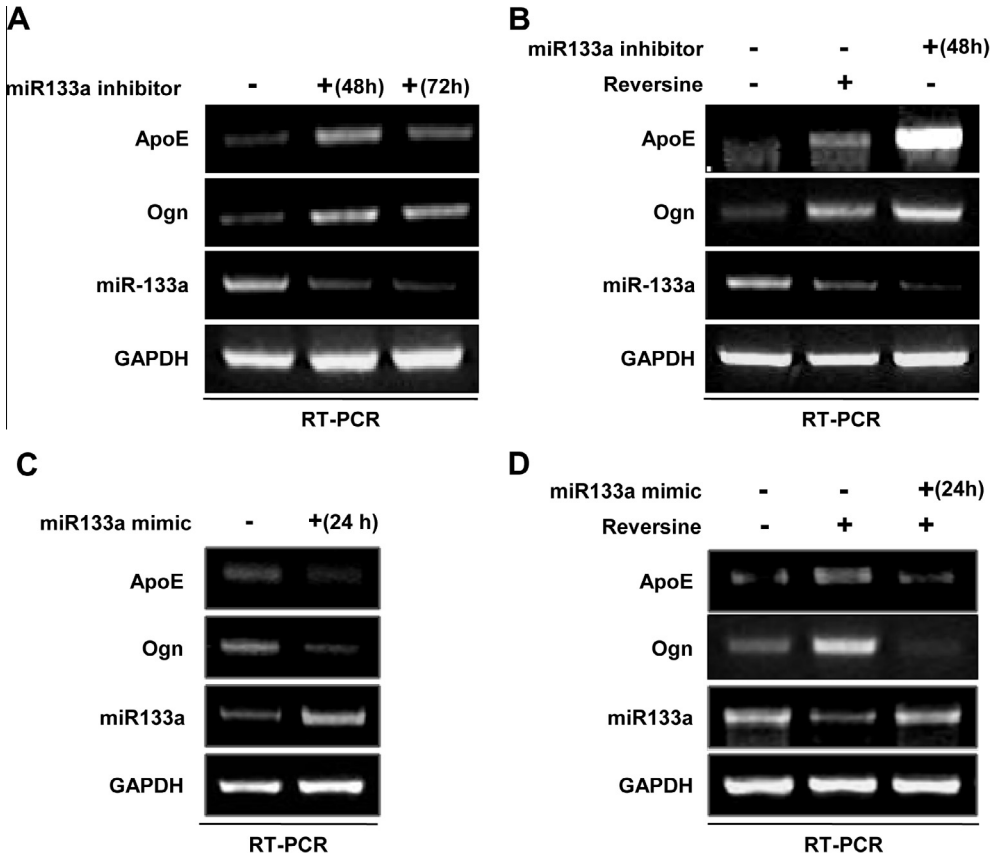

Fig. 4. Effect of miR-133a inhibitor or mimic in C2C12 dedifferentiation. (A) C2C12 cells were transfected with miR133a inhibitor (30 nM) for indicated times. (B) C2C12 cells were treated with miR133a inhibitor (30 nM, 48 h) or reversine (5 μM, 4 days). (C) C2C12 cells were treated with miR-133a mimic (50 nM, 24 h). (D) C2C12 cells were pretreated with 5 μM of reversine for 3 days, followed by 50 nM of miR-133a mimic for 24 h. The mRNA levels of *ApoE, Ogn*, and *miR-133a* were determined by RT-PCR and normalized to GAPDH.

histone H3K9 at the onset of mitosis [26,27]. Indeed, Amabile et al. showed that reversine down-regulates the expression level of muscle regulatory factors (MRFs) such as MyoD, Myf5 and myogenin by altering histone modifications of their promoter region [24]. Here, we show that reversine treatment decreases expression of miR-133a with significant down-regulation of phospho-H3S10 in its promoter region, which is probably mediated by Aurora B inhibition (Fig. 3D). On the basis of our result, reversine also decreased the occupancy of other active markers such as H3K36me3 and H3K4me3 at the miR-133a promoter. These data demonstrates that reversine is involved in chromatin remodeling as well as inhibition of Aurora B.

SRF acts as a transcription factor of genes required for myogenesis by binding to a DNA sequence known as the CArG box (CC(A/T)6GG) as well as the serum response element (SRE) [23,28,29]. SRF binds to these sequences and recruits various transcriptional coactivators like myocardin through conserved MADS box sequence. It has been reported that there is a CArG box in the enhancer region of miR-133a-2 [23], but not in the enhancer region of miR-133a-1, which has an identical sequence to that of miR-133a-2 that is located on another chromosome. In the present study, we investigated the upstream region of miR-133a-1 and verified SRF binding of its upstream region. In fact, the CArG box sequence was not found in the promoter region of miR-133a-1 but the enhancer region of miR-133a-1 has a CArG box-like sequence, CCATATCAGG. These findings suggest that SRF binds to the enhancer region of miR-133a-1 as well as miR-133a-2 similar to its binding to other muscle-related genes and controls its expression.

A previous report showed that miR-133a enhances myoblast proliferation and inhibits myogenesis by repressing SRF [9]. Intriguingly, we found that reversine decreases the expression level of miR-133a by inhibition of SRF binding to its enhancer region. Our results reveal an integral role for reversine in the transcriptional circuits controlled by SRF and miR-133a in the myoblast. The regulation of miR-133a by SRF and the targeting of SRF by miR-133a provide negative feedback regulation to precisely titrate the actions of SRF. Based on our results, reversine might modulate a negative feedback loop of SRF and miR-133a, enhancing the dedifferentiation of C2C12 cells.

In conclusion, our findings suggest that down-regulation of miR-133a following treatment with reversine blocks normal myoblasts differentiation and leads myoblasts to express markers that enhance multi-lineage, and consequently, provide myoblasts with multipotency. The chromatin at the level of miR-133a promoter is drastically remodeled with markers typical of inactive genes by reversine. Therefore, reversine provides an alternative strategy to reprogram somatic cells to multipotent progenitor cells.

Acknowledgments

This research was supported by Medical Research Center programs (2012-0009849) and the Basic Science Research Program (2012013998) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology.

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