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.