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MyoD is regulated by the miR-29a-*Tet1* pathway in C2C12 myoblast cells

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Abstract: Skeletal myogenesis is regulated by a considerable number of microRNAs (miRNAs). miRNA regulatory networks are complicated, and details of how they operate remain unclear. In this study, MTT assays confirmed that miR-29a is the most effective miR-29 paralog. Microarray analysis demonstrated upregulation of ten-eleven translocation enzyme-1 (*Tet1*) mRNA in response to miR-29a inhibition in C2C12 murine myoblast cells. We investigated the factors acting downstream in the miR-29a-*Tet1* signal pathway using real-time RT-PCR. *MyoD* expression was upregulated by *Tet1* inhibition and downregulated by miR-29a inhibition, whereas expression of cyclin-dependent kinase 6 (*Cdk6*) was regulated in an opposite manner. These results suggest that the miR-29a-*Tet1* pathway upregulates *MyoD* expression and conversely downregulates *Cdk6* expression. However, changes in the expression of other myogenic factors such as serum response factor (*Srf*), the myocyte enhancer factor 2 family (*Mef2a*, *b* and *c*), *myogenin*, myogenic regulatory factor 4 (*Mrf4*), muscle creatine kinase (*Mck*), and other cell cycle regulators such as *Cdk4* and thymine DNA glycosylase (*Tdg*) cannot be explained in terms of the miR-29a-*Tet1* pathway alone. The miR29a-*Tet1*

pathway may be part of a complex myogenic regulatory network in C2C12 cells.

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Introduction

Skeletal myogenesis is coordinated by various regulatory transcription factors including *MyoD*, myogenic factor 5 (*Myf5*), *myogenin*, and the muscle regulatory factor 4 (*Mrf4*) and myocyte enhancer factor-2 (*Mef2*) families (1). Muscle creatine kinase (*Mck*) and serum response factor (*Srf*) are also known to be skeletal muscle-regulatory factors (1,2). There have been many reports concerning the development, regeneration, differentiation and proliferation of skeletal muscle in various organisms, and various *in vivo* and *in vitro* methods have been used to investigate these processes. However, details of the molecular mechanism of myogenesis have remained unclear. In the last decade, various microRNAs (miRNAs) have been identified as regulators of myogenic factors (3,4).

miRNA is a small, endogenous, non-protein-encoding RNA comprising only 20-24 nucleotides. miRNAs control developmental timing, the maintenance, renewal and differentiation of stem cells, signal transduction, and the pathogenesis of diseases such as cancer (5-7). The mechanism by which animal miRNAs regulate these processes may involve complementary binding to the 3'-untranslated region (UTR) of a target gene mRNA, resulting in repression of translation. miRNAs regulate

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the expression of mRNAs only through a 6-nt seed sequence and one or two neighbouring nucleotides upon complementary binding to the target mRNA (8,9). The major role of mRNAs is the fine-tuning of gene expression (10); however, miRNAs clearly have very important biological functions because the sequences of the mature forms of miRNA are very highly conserved not only in vertebrates but also in lower animals (3,4,11).

In skeletal muscle development and regeneration, miR-1 is a very important muscle-specific miRNA for differentiation, and miR-133 is important for skeletal muscle proliferation (2,12-14). In cultured C2C12 murine myoblast cells, which mimic the development of skeletal muscle cells *in vivo*, miR-1 inhibits the expression of *Hdac4*, which negatively regulates the differentiation of skeletal muscle cells by repressing myocyte enhancer factor-2 (*Mef2*), a positive regulator of differentiation (15-17). Additional muscle-specific myomiRs, including miR-206, -208, -486, and -499, also have important roles in myogenesis (18-21). miR-29 is not muscle-specific, but has been implicated in some important myogenic functions. miR-29 enhances muscle cell differentiation and some downstream genes including v-akt murine thymoma viral oncogene homolog 3 (*Akt3*), and members of the ten-eleven translocation enzyme (*Tet*) family (22-24). However, any direct pathway for regulation of myogenic factors has remained unclear. There are three known murine miR-29 paralogs (miR-29a, miR-29b, and miR-29c) and all appear to have similar functions (23,24). In the present study using C2C12 myoblast cells we focused on miR-29a, an enhancer of myogenesis. In particular, we investigated the downstream regulators of miR-29a, which regulate myogenic factors including serum response factor (*Srf*), the *MyoD* family (*Myf5*, *MyoD*, *myogenin*, and *Mrf4*), the *Mef2* family, muscle creatine kinase (*Mck*), thymine DNA glycosylase (*Tdg*), and cyclin-dependent kinases *Cdk4* and *Cdk6* (25,26).

Materials and Methods

Cell culture for miR-29 and *Tet* inhibition

The C2C12 murine myogenic cell line (Funakoshi Co., Ltd., Tokyo, Japan) was cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) with 1% streptomycin and 10% (v/v) fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) [growth media (for proliferation-inducing culture)] or 5% (v/v) horse serum (Sigma-Aldrich) [differentiation media (for differentiation-inducing culture)]. Cultures were maintained at 37°C in the presence of 5% CO₂ and 95% air.

Cells were treated with miRVana miRNA hairpin

inhibitors for miR-29a or corresponding controls (Ambion, Life Technologies) at 0.187 pM and grown in complete media before being harvested for RNA. An siRNA against murine *Tet1* (*siTet1*; catalog number: #4397701) was purchased from Ambion (Thermo Fisher Scientific Inc., Waltham, MA, USA) and transfected into the cells at 9.0 pM using RNAiMax (Invitrogen, Thermo Fisher Scientific Inc.). Both inhibitors were added on day 0 (1 day after starting the initial culture).

MTT assay

C2C12 cells were cultured in 96-well plates at a density of 5×10^3 cells/well in growth media and differentiation media. The mitogenic activity of the samples was assayed using thiazolyl blue tetrazolium bromide (MTT; 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H tetrazolium bromide) (Promega KK, Tokyo, Japan) in accordance with the manufacturer's instructions. A 20-μL aliquot of MTT (5 μg/mL) was added to each well 0, 1, 2, and 3 days after the miR-29a, 29b, or 29c inhibitors (0.187 pM) had been added, and then the cells were incubated for 1 h. The optical density of each well was measured at 490 nm with background subtraction at 690 nm using an Ultramark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Microarray analysis

C2C12 cells were cultured in six-well plates at a density of 1×10^5 cells/well. The mRNA expression profiles of the C2C12 cells that had been treated with 3.0 pM (manufacturer's recommended concentration) miR-29a inhibitor for 24 h and control C2C12 cells were examined using microarray analysis. Total RNA was extracted from the cells using TRIzol containing phenol/guanidine isothiocyanate (Invitrogen, Thermo Fisher Scientific Inc.) and DNase I treatment. We examined the expression profiles of mRNAs using ChIP-on-chip microarray analysis (Genopal chips for miRNA, Mitsubishi Rayon Co., Ltd., Yokohama, Japan), which employs 180-oligonucleotide DNA probes in hollow plastic fibers for detection of mouse miRNAs. Hybridization signals were analyzed using a DNA-chip analyzer in accordance with the manufacturer's instructions. The quantity of each mRNA was expressed as a ratio relative to the external controls.

Analysis of the target sequences for miR-29s

The miR-29 target sequences were searched using TargetScanMouse 6.2 (www.targetscan.org/mmu_61/) and microRNA.org-Targets and Expression (www.microrna.org/microrna/getGeneForm.do), and also by

a direct search of the 3'-UTR of mRNAs. The target sequences for miR-29a, -29b, and -29c are conserved as 8-mer fully matched (5'-UGGUGCUA), 7-mer matched (5'-UGGUGCU or 5'-GGUGCUA), and 6-mer matched (5'-GGUGCU), which is referred to as the seed sequence. This core 6-mer sequence is indispensable for the target sequences of miR-29s.

miR-29s expression analysis by real-time RT-PCR

C2C12 cell cultures were maintained in six-well plates at a density of 1×10^5 cells/well in growth media. Total miRNA was extracted from the C2C12 cells using a High Pure miRNA Isolation Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Reverse transcription reactions were performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific Inc.). To quantify miRNA expression, real-time polymerase chain reaction (PCR) analysis was performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc., Kusatsu, Japan) with a TaqMan microRNA Assay Kit for mmu-miR-29a, -29b, and -29c (Applied Biosystems, Thermo Fisher Scientific Inc.). Amplification of snoRNA-202 served as an endogenous control for normalizing the expression data for target miRNAs. miR-29a inhibitor was applied at 0.187pM for miR-29a inhibition studies.

mRNA expression analysis by real-time RT-PCR

C2C12 cell cultures were maintained in six-well plates at a density of 1×10^5 cells/well in growth media. Total RNA extraction, treatment with DNase I and reverse transcription were performed as described previously (27). SYBR Green real-time PCR was performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc.) with the following cycling parameters: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation 95°C for 15 s, and annealing and extension at 55°C for 15 s. The quantities of target mRNAs were normalized by the quantity of murine glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA. The resulting ratio was expressed as a percentage relative to the mean value of each target gene in C2C12 cells treated with control oligonucleotides. The dose of si*Tet1* was 9.0 pM and the dose of miR29a inhibitor was 0.187pM. The PCR primer sets used were: *Tet1*: 5'-CGA AAG AAC AGC CAC CAG AT-3' (forward), 5'-TTG CTC TTC TTC CCC ATG AC-3' (reverse); *Tet2*: 5'-GTT GCA AGA AGA AAG CGG AG-3' (forward), 5'-CTC TGC CCT TGC TGA AGG T-3' (reverse); *Tet3*: 5'-TCC GGA TTG AGA AGG TCA TC-3' (forward), 5'-CCA GGC CAG GAT CAA GAT AA-3' (reverse); *Srf*: 5'-GAG CTA CAG

GTG GTG AAC CTG-3' (forward), 5'-CCT GTG AGT GGG AAG GTG GCA-3' (reverse); *Myf5*: 5'-GGA ACA GGT GGA GAA CTA TTA-3' (forward), 5'-CGT GAT AGA TAA GTC TGG AGC-3' (reverse); *MyoD*: 5'-GAC GGC TCT CTC TGC TCC-3' (forward), 5'-AAG TGT GCG TGC TCC TCC-3' (reverse); *Mef2a*: 5'-GGC TGG AGG GCA GTT ATC TCA-3' (forward), 5'-GTA GGG CAG TTC ACT GCA GTA-3' (reverse); *Mef2b*: 5'-CCA GTT GCC CGC AGT CTG TGC-3' (forward), 5'-CTA CCG TGG CCA GCT GTC AGG-3' (reverse); *Mef2c*: 5'-CCT CAG TCA GTT GGG AGC TTG-3' (forward), 5'-CCC ATC CTT CAG AGA GTC GCA-3' (reverse); *myogenin*: 5'-GAG CGC GAT CTC CGC TAC AGA GG-3' (forward), 5'-CTG GCT TGT GGC AGC CCA GG-3' (reverse); *Mrf4*: 5'-GCT GGA TCA GCA AGA GAA GAT-3' (forward), 5'-CTG GCT GAG TTA CTT CTC CAC-3' (reverse); *Mck*: 5'-CAA TAA GCT TCG CGA TAA GGA G-3' (forward), 5'-GAT GGG ATC AAA CAG GTC CTT G-3' (reverse); *Tdg*: 5'-CCA GGC TAT GAG GCA GCT TAC-3' (forward), 5'-GCT CTC TTC TTC CTG CTC TCG-3' (reverse); *Cdk4*: 5'-ACG CCT GTG GTG GTT ACG CT-3' (forward), 5'-CCA TCT CTG GCA CCA CTG AC-3' (reverse); *Cdk6*: 5'-CAC CTC GGA GCT GAA CAC AGC -3' (forward), 5'-GCT GTG TTC AGC TCC GAG GTG-3' (reverse); *Gapdh*: 5'-GAT GCT GGT GCT GAG TAT GTC G-3' (forward), 5'-GTG GTG CAG GAT GCA TTG CTG A-3' (reverse).

Statistical analyses

Tukey's method and Student's *t*-test were used to compare the mean values between the samples and the controls. Differences were considered significant at $P < 0.05$ and $P < 0.01$, respectively.

Results

MTT assay

Changes in the C2C12 myoblast cell growth rate were examined using the MTT assay. The optical density in growth media gradually increased to approximately three times that at day 0 in the control by day 3 (Fig. 1A). The optical density of the control increased gradually at day 2 and day 3 when 0.187 pM miR-29a inhibitor was present (Fig. 1A). However, the optical density decreased at day 2 when 0.187 pM miR-29b or miR-29c inhibitor was present. The optical density increased at day 3 when miR-29a or 29b was present, but no marked changes were observed at day 3 when miR-29c inhibitor was present (Fig. 1A). In differentiation media, few changes in optical density were observed from day 0 to day 3 in the control (Fig. 1B). However, the optical density increased noticeably at day 3 (approximately three times greater

Fig. 1 Changes in the relative optical density of C2C12 myoblast cells cultured with inhibitors of miR-29a, miR-29b, and miR-29c. A, cultured in growth media supplemented with 0.187 pM each miR-29 inhibitor. B, cultured in differentiation media with 0.187 pM each miR-29 inhibitor. Each optical density is represented as a percentage of the mean value at day 0. Columns represent the mean, and error bars represent the SD of six samples. Significant differences, * $P < 0.05$, ** $P < 0.01$.

Table 1 List of growth- and differentiation-related genes detected in the microarray analysis

Gene	Change rate	miR-29 target sequence
<i>Tet1</i>	246%	++
<i>Tet2</i>	196%	++
<i>Tet3</i>	246%	++
<i>Tdg</i>	142%	+
<i>Cdk4</i>	—	—
<i>Cdk6</i>	—	+
<i>Cyclin D1</i>	—	—
<i>Igf1</i>	151%	+
<i>Igf2</i>	126%	—
<i>Fgf1</i>	189%	—
<i>Fgf2</i>	77%	—
<i>Pax3</i>	39%	—
<i>Pax7</i>	—	—
<i>Srf</i>	—	—
<i>myf5</i>	—	—
<i>MyoD</i>	—	—
<i>myogenin</i>	123%	—
<i>Mef2c</i>	—	—
<i>Mef2a</i>	—	—
<i>Mef2b</i>	—	—
<i>Mrf4</i>	156%	—

Changes indicate the percentage of relative expression in the presence of 3.0 pM miR-29a inhibitor for 24 h. ++, genes containing three or more miR-29a target sequences; +, genes containing one or two miR-29a target sequences; —, genes not detected or genes with no miR-29a target sequence.

than the control) when 0.187 pM miR-29a inhibitor was present (Fig. 1B). In contrast, the optical density did not increase when 0.187 pM miR-29b or -29c inhibitor was present (Fig. 1B). The proliferation-inducing effect of the miR-29a inhibitor was confirmed at day 3 in differentiation media; however, the effects of both miR-29b and

-29c inhibitors were not clarified in growth media and differentiation media (Fig. 1B).

Microarray analysis and target sequence analysis of miR-29

Changes in mRNA expression in C2C12 cells cultured in the growth media containing miR-29a inhibitor for 24 h were examined using ChIP-on-chip microarray analysis. A total of 7,013 mRNAs were upregulated by miR-29a inhibition, and 1,152 mRNAs were either downregulated or did not respond. The genes related to myogenic differentiation and proliferation, the changes in their expression rates, and the miR-29a target sequences, are summarized in Table 1. *Tet1*, *Tet2*, *Tet3*, *Tdg* and some growth factors, such as *Igf1*, *Igf2*, and *Fgf1*, and differentiation factors, such as *myogenin* and *Mrf4*, were upregulated. Among them, the expression rates of *Tet1* and *Tet3* showed the largest change (246% in each), followed by *Tet2* (196%). *Pax3* and *Fgf2* were downregulated and other related genes were not detected (Table 1).

Next, the miR-29a target sequences in major upregulated mRNAs were analyzed. Each member of the *Tet* family contains multiple miR-29a target sequences, which are presumably effective when present in their 3'-UTRs. An 8-mer matched target sequence, seven 7-mer matched target sequences, and two 6-mer matched target sequences were found in murine *Tet1* (NM_001253857). Two 8-mer and three 7-mer matched target sequences were found in the 3'-UTR of *Tet2* (NM_001040400). One 8-mer, nine 7-mer, and five 6-mer matched target sequences were found in the 3'-UTR in *Tet3* (NM_007872.4). Representative miR-29a targets (8-mer and 7-mer matched) in the 3'-UTRs of *Tet* family mRNAs are summarized

Table 2 Representative miR-29a target sites in the 3'-UTR of the *Tet* family

Gene (Accession number)	miR-29a target site
<i>Tet1</i> (NM_001253857)	3'-AUUGGCUAAAAGUCUACCACGAU-5' miR-29a 407:5'-UCUACACAGU - UCCUGGUGCUU-3' <i>Tet1</i> (7-mer matched) 3'-AUUGGCUAAAAGUCUACCACGAU-5' miR-29a 6471:5'-UUUCUUGAAGGAGAUGGUGCUA-3' <i>Tet1</i> (8-mer matched)
<i>Tet2</i> (NM_001040400)	3'-AUUGGCUAAAAGUCUACCACGAU-5' miR-29a 384:5'-GUUGCAGGAUCAUCUGGUGCUG-3' <i>Tet1</i> (7-mer matched) 3'-AUUGGCUAAAAGUCUACCACGAU-5' miR-29a 543:5'-GUGUUAACAUAUAAUUGGUGCUA-3' <i>Tet1</i> (8-mer matched)
<i>Tet3</i> (NM_007872.4)	3'-AUUGGCUAAAAGUCUACCACGAU-5' miR-29a 1353:5'-GAAAAUGAGAACGUUGGUGCUU-3' <i>Tet1</i> (7-mer matched) 3'-AUUGGCUAAAAGUCUACCACGAU-5' miR-29a 4244:5'-AGGCCGCUUUUAGUUGGUGCUA-3' <i>Tet1</i> (8-mer matched)

|, complementary bond between miR-29a and *Tet* paralog; :, a C-U bond. Underlined sequences in *Tet* mRNAs indicate the seed sequence for miR-29.

Fig. 2 Expression of endogenous miR-29a, 29b, and 29c in C2C12 cells and changes in miR-29a expression upon addition of miR-29a inhibitor. A, expression of endogenous miR-29a; B, expression of endogenous miR-29b; C, expression of endogenous miR-29c; D, miR-29a expression in the presence of 0.187 pM miR-29a inhibitor. The expression of each miR-29 paralog is represented as a percentage of the mean of the control at day 0. Columns represent the mean and error bars represent the SD of six samples.

in Table 2. The 8-mer matched target in *Tet1* is a novel target sequence for miR-29a; it could not be found in TargetsCanMouse 6.2 or microRNA.org. Few of the genes listed in Table 1 contain miR-29 targets, *Tdg*, *Cdk6* and *Igf1* contain a single miR-29 target sequence, but *Cdk6* was not detected in the microarray analysis. We were unable to find any miR-29 target sequence in major upregulated genes other than those listed in Table 1.

Endogenous expression of miR-29s and the *Tet* family, and the effect of miR-29a inhibitor

Endogenous expression of miR-29 was examined using real-time RT-PCR. The endogenous expression of miR-29a in C2C12 cells cultured in growth media increased approximately 350% (relative to that at day 0) at day 1, which was two days after initial culture, and was decreased at day 2. Expression was diminished even more at day 3, to approximately 20% of that on day 0 (Fig. 2A). The expression patterns of miR-29b and miR-29c were quite similar to those of miR-29a; each of them peaked at day 1, although the rates at which they changed were not greater than that for miR-29a (Fig. 2A-C). The effect of 0.187 pM miR-29a inhibitor was confirmed. The expression of miR-29a was repressed almost completely until day 3 (Fig. 2D).

Tet1 mRNA expression in C2C12 cells cultured with growth media did not change markedly until day 2, but suddenly increased at day 3. *Tet1* expression then decreased at day 4 (Fig. 3A). The expression patterns of *Tet2* and *Tet3* were similar, but the change in *Tet1* expres-

Fig. 3 Expression of endogenous *Tet1*, *Tet2*, and *Tet3* mRNA, and effect of miR-29a inhibition in C2C12 cells. A, endogenous *Tet1* expression; B, endogenous *Tet2* expression; C, endogenous *Tet3* expression; D, *Tet1* expression in the presence of 0.187 pM miR-29a inhibitor; E, *Tet2* expression in the presence of 0.187 pM miR-29a inhibitor; F, *Tet3* expression in the presence of 0.187 pM miR-29a inhibitor; G, *Tet3* expression in the presence of 9.0 pM siTet1. The expression of each *Tet* is represented as a percentage of the mean of the control at day 0 (A-F). The relative expression rate of *Tet1* is shown (G). Columns represent the mean and error bars represent the SD of five or six samples. Significant difference, * $P < 0.05$.

sion was the largest of the three *Tet* genes (Fig. 3A-C). The *Tet1* expression pattern changed substantially when C2C12 cells were incubated in the presence of 0.187 pM miR-29a inhibitor. *Tet1* expression was particularly increased at day 1 to approximately four times that on day 0, and continued to increase at day 2 to approximately 6 times that on day 0 (Fig. 3D). The *Tet2* expression pattern in C2C12 cells cultured in the presence of 0.187 pM miR-29a inhibitor was similar to the expression pattern observed for *Tet1*, but the rate of change was much less. The peak at day 2 was only approximately 150% of the expression on day 0 (Fig. 3E). In the presence of 0.187 pM miR-29a inhibitor, the *Tet3* expression pattern peaked at day 1, being approximately 200% of that on day 0, and then gradually decreased in the following days (Fig. 3F). *Tet1* expression underwent the most pronounced change

among the three *Tet* paralogs. The effect of siTet1 was examined using real-time PCR, and a change in *Tet1* expression in the presence of 9.0 pM siTet1 was demonstrated. *Tet1* expression was reduced to approximately 70% (day 1) and to approximately 55% (day 3) that of the control, respectively (Fig. 3G).

Changes in myogenic factor expression patterns upon inhibition of *Tet1* or miR-29a

Srf mRNA expression in C2C12 cells cultured with 9.0 pM siTet1 was increased markedly at days 1 and 2, and was also increased at days 1 and 2 when cultured with 0.187 pM miR-29a inhibitor (Fig. 4A). In contrast, *Myf5* was markedly downregulated at days 2 and 3 in the presence of siTet1. It was also downregulated in the presence of miR-29a inhibitor, mainly at day 2 (Fig. 4B). *MyoD*

Fig. 4 Changes in the expression of myogenic factors in C2C12 cells upon addition of 9.0 pM siTet1 or 0.187 pM miR-29a inhibitor. A, *Srf* mRNA expression; B, *Myf5* expression; C, *MyoD* expression; D, *myogenin* expression; E, *Mef2c* expression; F, *Mef2a* expression; G, *Mef2b* expression; H, *Mrf4* expression. The expression of each gene is represented as a percentage of the mean of the control on each day and as a percentage of the mean of at day 0 in each sample (A-C, and E-H). The relative expression rate of *myogenin* is shown (D). Columns represent the mean, and error bars represent the SD of five or six samples. Significant differences, * $P < 0.05$, ** $P < 0.01$.

expression was markedly increased at days 2 (approximately 180% that of the control) and 3 (approximately 150% that of the control) in the presence of siTet1 (Fig. 4C). When miR-29 inhibitor was added, *MyoD* expression gradually decreased to approximately 65% that of the control at days 2 and 3 (Fig. 4C). *Myogenin* expression was very faint in the control until day 3 and was unchanged until day 1 when each inhibitor was added. However, it increased notably at day 3 when either siTet1 or also miR-29a inhibitor was added (Fig. 4D).

In the *Mef2* family, *Mef2c* expression was markedly

upregulated in the presence of siTet1 until day 3. In the presence of miR-29a inhibitor, *Mef2c* expression was increased at day 1, but returned to control levels at days 2 and 3. *Mef2a* expression was consistently increased until day 3 in the presence of siTet1 or miR-29a inhibitor (Fig. 4F). *Mef2b* expression was decreased until day 2, but increased suddenly at day 3 in the presence of either siTet1 or miR-29a inhibitor (Fig. 4G). *Mrf4* expression was consistently increased until day 3 in the presence of either siTet1 or miR-29a inhibitor; however, in the presence of miR-29a inhibitor, the expression was consistently

Fig. 5 Changes in the expression of myogenic or proliferation factors in C2C12 cells upon addition of 9.0 pM *siTet1* or 0.187 pM miR-29a inhibitor. A, *Mck* expression; B, *Tdg* expression; C, *Cdk4* expression; D, *Cdk6* expression. The expression of each gene is represented as a percentage of the mean of the control on each day and as a percentage of the mean of at day 0 in each sample (B-D). The relative expression rate of *Mck* is shown (A). Columns represent the mean and error bars represent the SD of five or six samples. Significant differences, * $P < 0.05$, ** $P < 0.01$.

enhanced until day 3 (Fig. 4H). *Mck* expression was very faint until day 3, like that of *myogenin*, but it increased consistently toward day 3 in the presence of *siTet1*. In the presence of miR-29a inhibitor, it was suddenly expressed at day 3 (Fig. 5A).

Tdg expression was increased at days 1 and 2 to more than twice that of the control in the presence of either *siTet1* or miR-29a inhibitor. However, it was decreased at day 3 in the presence of miR-29a inhibitor (Fig. 5B). Expression of the cell cycle regulator *Cdk4* was not changed by *siTet1* (Fig. 5C), but was increased noticeably at days 2 and 3 to approximately 180% that of the control in the presence of miR-29a inhibitor (Fig. 5C). Expression of *Cdk6*, another cell cycle regulator, was markedly decreased to approximately 50% that of the control at days 2 and 3 in the presence of *siTet1* (Fig. 5D). In contrast, *Cdk6* expression was increased from day 1 to day 3 to more than 170% that of control in the presence of miR-29a inhibitor (Fig. 5D).

Discussion

miR-29, like miR-1/206, is known to be an HDAC4 inhibitor (28,29). miR-29b and miR-29c reduce the proliferation and facilitate the differentiation of C2C12 myoblast cells *via Akt3* (22). TET proteins and thymine DNA glycosylase (TDG) are known to be components of the DNA demethylation pathway in smooth muscle cells (24). Moreover, *Tet1*, *Tet2*, and *Tet3* mRNAs are known

to be direct targets of miR-29 (23). However, regulation of myogenesis by miR-29 *via* the *Tet* family has not been reported. In our MTT assays of C2C12 cells grown in differentiation media, proliferation was strongly induced only by inhibition of miR-29a (Fig. 1B). We performed MTT and gene expression assays using 0.187 pM miR-29a inhibitor, and the effect of the inhibitor was confirmed (Figs. 2D, 3D). We were unable to obtain useful MTT and gene expression data around day 3 using 3.0 pM miR-29a inhibitor, which is the manufacturer's recommended concentration. Considerable induction of proliferation was not demonstrated when miR-29b or miR-29c inhibitors were added to either the growth media (Fig. 1A) or differentiation media (Fig. 1B). We then focused on miR-29a, which is the most effective myogenic differentiation inducer among the three miR-29 paralogs. Different effects of members of the miR-29 family in C2C12 cells were suggested for the first time (23,24). The expression patterns of the endogenous miR-29 paralogs were quite similar, but the rates of change in miR-29b and miR-29c did not exceed those of miR-29a (Fig. 2A-C), thus supporting our focus directed on miR-29a.

In the microarray analysis, miR-29a inhibition increased the expression of >7,000 mRNAs in C2C12 cells, including mRNAs related to myogenesis or proliferation (Table 1). However, most of them had no target sequence for miR-29 paralogs in their 3'-UTRs.

Fig. 6 Schematic diagram of the miR-29a-*Tet1* pathway in C2C12 cells. A, *Tet1* suppression by miR-29a (Δ indicates moderate *MyoD* suppression); B, *Tet1* suppression by the *Tet1* inhibitor (× indicates reduced *MyoD* suppression); C, miR-29a suppression by the miR-29a inhibitor and strong *MyoD* suppression by *Tet1* (× indicates reduced *Tet1* suppression).

The exceptions were members of the *Tet* family, *Tdg*, and *Igf1*. The DNA demethylation mediator and cell cycle regulator, *Tdg*, has two miR-29a target sequences (an 8-mer and a 7-mer matched) and is known to be a direct target of miR-29a (23,24). Thus miR-29a inhibition was expected to result in considerable upregulation of *Tdg* expression. However, the expression was not increased as much as the expression of *Tet* family members in the microarray analysis. Therefore, we excluded *Tdg* from the list of most effective candidate mediators of miR-29a. The growth factor *Igf1* also contains an 8-mer matched miR-29 target sequence, and its expression was shown to be moderately increased by microarray analysis. However, *Igf1* is known to have a close relationship with miR-1/206 regulation (30,31). Thus, the importance of *Igf1* could not be explained without understanding the circumstances of miR-1 and miR-206 expression. *Fgf1* was also upregulated in the microarray analysis. However, we did not consider *Fgf1* to be a mediator of miR-29a because it has no miR-29 target sequence in its 3'-UTR.

Tet paralogs contain an extreme number of miR-29 target sequences, and the expression of each paralog was noticeably upregulated in the microarray analysis. Therefore, these paralogs are assumed to be the most likely candidate mediators of miR-29a. Among the *Tet* family, *Tet1* showed the largest change in endogenous expression (Fig. 3A-C) and the greatest upregulation

resulting from miR-29a inhibition (Fig. 3D-F). Thus, we confirmed *Tet1* to be the best mediator of miR-29a. It had not been clarified which *Tet* paralog is the best mediator of miR-29a (23).

Next, we investigated the effects of the miR-29a-*Tet1* signal pathway, focusing on the myogenic factors directly downregulated by *Tet1* upregulation. *MyoD*, which is a myogenesis progressing transcription factor (32), was upregulated by *Tet1* inhibition and downregulated by miR-29a inhibition in C2C12 cells mainly at days 1 and 2. *MyoD* is known to be the target gene of *Pax-3*, *Myf5* and a long non-coding RNA, *MUNC* (32-35). However, neither *Tet1* nor miR-29 inhibition enhanced the expression of *Myf5* in our real-time RT-PCR analysis, and miR-29a inhibition did not upregulate *Pax3* in the microarray analysis. From these data, we concluded the miR-29a-*Tet1* pathway directly regulates *MyoD*. Endogenous miR-29a at concentrations above the level at days 0 and 2 (Fig. 2A) may moderately repress the expression of *Tet1*. Under these conditions, *MyoD* is expressed to a certain degree (Fig. 6A). Addition of si*Tet1* strongly suppressed the expression of *Tet1*, and *MyoD* expression may have been enhanced as a result (Fig. 6B). Inhibition of miR-29a increased *Tet1* expression and suppressed *MyoD* expression to a level less than that of the control (Fig. 6C). The miR-29a-*Tet1* pathway is suggested to regulate *MyoD* and *Cdk6* oppositely. *Cdk6*, which contains a miR-29a target sequence, is thought to be directly repressed by miR-29a. Our PCR analysis showed that *Cdk6* was upregulated by miR-29a inhibition and downregulated by *Tet1* inhibition. Thus, the myogenic factor *MyoD* and the cell cycle regulator *Cdk6* are suggested to be oppositely regulated by the miR-29a-*Tet1* pathway at the same time. The miR-29a-*Tet1* pathway does not regulate the other cell cycle regulators *Cdk4* and *Tdg* in the same way that it regulates *Cdk6*.

MEF2 is known to have various functions in myogenesis. MEF2 directly enhances muscle differentiation (15-17), upregulates HDAC9 (36) and downregulates HDAC4 via miR-1 (2). MEF2C, a member of the MEF2 family, is known to be a direct transcriptional target of *MyoD in vivo* (1,37). Our PCR data revealed that *Mef2c* expression was increased by *Tet1* inhibition, as was *MyoD* expression, but was not suppressed by miR-29a inhibition. Modes of regulation different from that of *Mef2c* were demonstrated for *Srf*, *Mef2a*, *Mef2b*, *Mrf4* and *Mck*. All of them may not be regulated by the miR-29a-*Tet1* pathway, as their expression dynamics cannot be explained by the miR-29a-*Tet1* pathway alone. The regulation of myogenesis, including the effects of various miRNAs, is extremely complicated, and the miR-

29a-*Tet1* signal pathway is just one part of the complex myogenic regulation network in C2C12 cells.

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