이학박사 학위논문

골격근 분화에서 메틸화에 의한 MEF2 의 조절

Modulation of lysine methylation in myocyte enhancer factor 2 during skeletal muscle cell differentiation

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최 진 미

A thesis of the Degree of Doctor of Philosophy

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February 2014

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Modulation of lysine methylation in myocyte enhancer factor 2 during skeletal muscle cell differentiation

by
Jinmi Choi

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Science at Seoul National University Graduate School

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ABSTRACT

Myocyte enhancer factor 2 (MEF2) is a family of transcription factors that regulates many processes, including muscle differentiation. Due to its many target genes, MEF2D requires tight regulation of transcription activity over time and by location. Epigenetic modifiers have been suggested to regulate MEF2-dependent transcription via modifications of histones and MEF2. However, the modulation of MEF2 activity by lysine methylation, an important posttranslational modification that alters the activities of transcription factors, has not been studied. We report the reversible lysine methylation of MEF2D by G9a and LSD1 as a regulatory mechanism of MEF2D activity and skeletal muscle differentiation. G9a methylates lysine-267 of MEF2D and represses its transcriptional activity, but LSD1 counteracts it. This residue is highly conserved between MEF2 members in mammals. The methylation status of MEF2D modulates chromatin binding affinity. During myogenic differentiation of C2C12 mouse skeletal muscle cells, the G9a-mediated methylation of MEF2D decreased. Concordantly, MEF2D-dependent myogenic genes were upregulated. The methylation defective mutant of MEF2D readily activated *myogenin* gene transcription. Thus, we have identified lysine-267 as a methylation/demethylation site and demonstrate that the lysine methylation state of MEF2D regulates its transcriptional activity and

skeletal muscle cell differentiation.

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Keywords: MEF2, G9a, methylation, C2C12, muscle cell differentiation

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LIST OF ABBREVIATIONS

basic Helix Loop Helix.....bHLH

Calcineurin-binding protein-1 ..... Cabin1

Chromatin Immunoprecipitation .....ChIP

Differentiation Medium .....DM

Growth Medium .....GM

Lysine specific demethylase 1 ..... LSD1

Muscle Creatine Kinase ..... MCK

Myocyte Enhancer Factor 2 .....MEF2

Myosin Heavy Chain ..... MHC

Posttranslational modification ..... PTM

Protein Lysine Methyltrasnferase .....PKMT

Quantitative Real Time Polymerase Chain Reaction.....qRT-PCR

I. INTRODUCTION

1-1. Posttranslationial modification (PTM)

Chromatin-modifying enzymes regulate gene expression by modifying histones and interacting with master transcription factors (1). Modifications of N-terminal tails of histones alter chromatin structure, leading to the activation of inactivation of gene expression. In addition, many non-histone proteins have been identified as substrates of chromatin modifiers. Posttranslational modifications of proteins affects the stabilities, interactions with proteins/DNA, transcription activities and promoter specificities (Table1). Although phosphorylation, acetylation and ubiquitination have been exclusively studied, only few non-histone proteins were reported to be methylated on lysine residue.

EHMT2/G9a is a histone methyltransferase that mediates mono- and dimethylation of histone H3K9 in euchromatic regions (2). G9a also targets many nonhistone proteins to control transcriptional activities during cell fate decisions and cellular responses to environmental stressors (2). In hypoxia, G9a methylates reptin and pontin to regulate hypoxia-targeted gene expression (ref). G9a also represses p53 function in DNA damage, apoptosis and oncogenesis (ref). In addition, G9a has

PTM type	Function
Phosphorylation pTyr, pSer, pThr	regulation of enzyme activity, molecular interaction, signalling, promoter selectivity
Acetylation	protein stability, protein-DNA interaction, promoter selectivity
Methylation meArg, meLys	protein stability, protein-DNA interaction, enzyme activity, transcriptional activity
Glycosylation O-linked, N-linked	excreted protein, cell-cell recognition, signalling, transcriptional activity
Hydroxyproline	protein stability, protein-ligand interaction
Ubiquitination	Destruction signal
Acylation, Fatty acid modification	cellular localization and targeting signal, membrane tethering, protein-protein interaction

Table 1. Posttranslational modifications and their fuctions

been implicated in embryonic development, based on the embryonic lethality of G9a knockout mice (3). The regulation of G9a function affects the generation of induced pluripotent stem cells (iPSCs), and H3K9me2 is dynamically controlled during stem cell differentiation (4, 5).

Lysine specific demethylase 1 (KDM1; LSD1; AOF2), a flavin adenine dinucleotide (FAD)-dependent amine oxidase, is the first histone demethylase identified (6). LSD1 demethylates mono-, di-methyl group on H3K4 and H3K9 (7). LSD1 also takes non-histone proteins as substrates, such as p53, DNMT1 (8, 9). A critical role of LSD1 during development has been implied by the embryonic lethality of mouse lacking LSD1 (10). In fact, LSD1 is involved in various differentiation processes such as neuronogenesis, hematopoiesis, plasma cell differentiation and pituitary development (6, 11, 12). Especially, LSD1 modulates muscle cell differentiation by acting on H3K9 in myogenic gene promoters (Figure 1) (13).

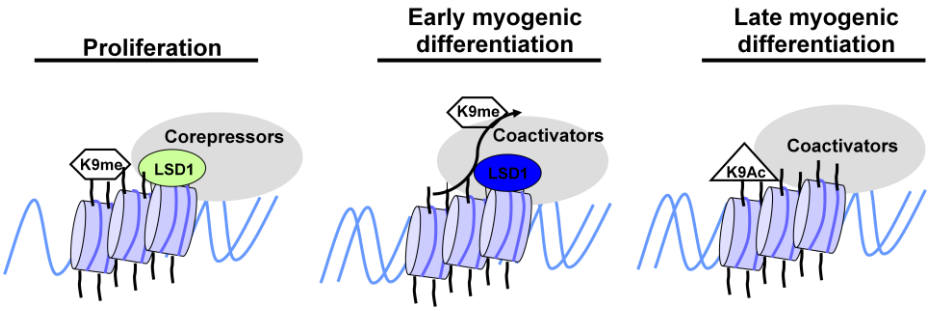


Figure 1. LSD1 regulates myogenic factors by demethylating H3K9 methylation on myogenic promoters (13).

1-2. Skeletal muscle differentiation

Skeletal muscle differentiation is a well-orchestrated process that is not only important in organogenesis, but also in postnatal growth and adult skeletal muscle regeneration. In the developing embryo, undifferentiated cells committed to muscle lineage in the mesoderm is called myoblast cells that are precursors of muscle cells (14). To form the myofibres, myoblast cells proliferate and fuse together into multinucleated myotubes . The differentiation process starting from myoblasts to multinucleated myotubes is called myogenesis (14). In adult skeletal muscle, myoblast cells reside on the surface of myofibre, referred to as satellite cells (15). Upon injury, regeneration begins with myoblast cell proliferation, recapitulating developmental process. The myoblast cells are then differentiated and fused into injured muscle fibres (15).

In vitro, C2C12 murine myoblast cells are used to study skeletal muscle cell differentiation. The C2C12 cells spontaneously differentiate after serum removal.

The series of events in myogenesis is tightly regulated by two major classes of transcription factors, myogenic basic helix-loop-helix (bHLH) proteins and Mef2 (16). Myogenic

bHLH proteins include MyoD, MRF4, Myf5, myogenin and are referred to as myogenic regulatory factors (MRFs) (15). They are expressed exclusively in the skeletal muscle lineage and activate the muscle differentiation program. They bind to E-box consensus sequence (CANNTG) on many muscle specific gene promoters (15). The basic domain of the MRFs interact with DNA while the helix loop helix domain mediates the heterodimerization. bHLH proteins interact with MEF2 transcription factors, synergizing on muscle differentiation.

1-3. Myocyte enhancer factor 2 (MEF2)

The myocyte enhancer factor 2 (MEF2) family of transcription factors, which comprises 4 members (A-D), belongs to MADS (MCM1, agamous, deficiens, SRF) family, and binds to A/T-rich DNA sequence. MEF2 mediates several processes, including the differentiation, proliferation, survival, and apoptosis of various cell types (17-20). Particularly during muscle differentiation, MEF2 targets downstream myogenic genes and is regulated over time and by location (19, 21, 22). Loss of function mutations of the single *Mef2* gene in *Drosophila* result in complete loss of all muscle tissues (19). Mice that lack *MEF2C* die at about E9.5 from cardiovascular defects, while mice lacking *MEF2D* die prior to gastrulation (23). Skeletal muscle specific deletion of *MEF2C* showed abnormal muscle development (23).

To modulate MEF2 activity and effect its precise regulation of target genes, corepressors and coactivators are recruited to MEF2 target promoters. Calcineurin-binding protein-1 (Cabin1) recruits histone methyltransferases and deacetylases, such as Suv39h1 and HDACs, to repress MEF2 activity through chromatin remodeling (24-28).The histone demethylase LSD1

and acetyltransferase p300 activate MEF2 transcriptional activity by modifying the histones in MEF2 target promoters (13, 29). Moreover, a histone chaperone, HIRA, in cooperation with Asf1, stimulates MEF2 transcriptional activity during muscle differentiation (30).

MEF2 activity is also regulated by posttranslational modifications, including sumoylation, phosphorylation, and acetylation (Figure 2). Several kinases, including mitogen-activated protein kinase p38 and extracellular signal-regulated kinase 5 (ERK5), phosphorylate MEF2 to modulate its transcriptional activity (20, 31, 32). Moreover, acetylation at several sites in MEF by p300 and deacetylation by HDAC3 regulate such activity (33-35).

Although many regulatory mechanisms have been suggested to govern its function, how MEF2 regulates an extensive array of target genes during complex cellular processes remains unknown (36-38). Thus, we examined lysine methylation as a novel regulatory mechanism that enables MEF2 to orchestrate the expression profiles of target genes.

We report that MEF2D is methylated and demethylated by G9a and LSD1, respectively, which effects the dynamic

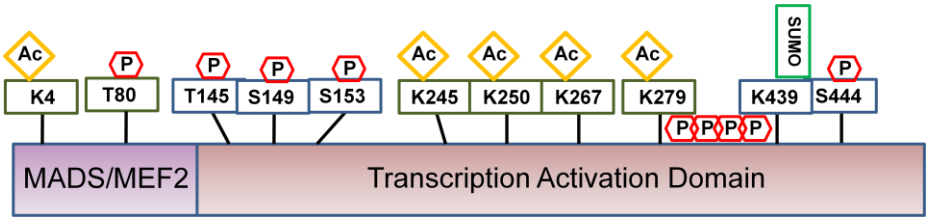


Figure 2. MEF2 is regulated by posttranslational modifications

regulation of MEF2D transcriptional activity and the expression of its target genes during skeletal muscle differentiation. During myogenic differentiation, MEF2D dissociates from G9a, and its methylation is reduced, upregulating myogenic genes that are targeted by MEF2D. Conversely, aberrant MEF2D methylation by overexpression or knockdown of G9a results in the dysregulation of muscle cell differentiation, implicating MEF2D as a master regulator in this process.

II. MATERIALS AND METHODS

2-1. Cell culture and transient expression

The C2C12 mouse myoblast cells and HEK 293 cells have been described (13). Polyethylenimine (PEI, Polysciences, Inc.) was used to transfect HEK293 cells. C2C12 cells were electroporated with the Neon Transfection System (Invitrogen) per the manufacturer's instructions. Plat-E cells, E14 cells (39), and DO11.10 cells have been described (24).

2-2. DNA constructs and site-directed mutagenesis

Flag-MEF2D was generated by subcloning the HindIII-XhoI-digested PCR products from Myc-tagged MEF2D into pcDNA3.0/Flag (Invitrogen). Mutations were introduced by site-directed mutagenesis using ipfu (iNtRON Biotechnology). HA-MEF2D, HA-MEF2D (1-130), and Myc-MEF2C have been described (13). pRSET(B)-MEF2D was generated by subcloning the XhoI-HindIII-cut PCR products from Myc-tagged MEF2D into pRSET(B) (Invitrogen). pCAG-MEF2D was generated by subcloning the XhoI-digested PCR product from HA-MEF2D into pCAG-IP or pMIG (Addgene) (39). Flag-G9a has been described (37). PCR products of truncated mutants of G9a were obtained from full-length G9a and inserted into

pSG5-Flag. pMIG-G9a and pMSCV-G9a were generated by subcloning the EcoRI-digested PCR products from Flag-G9a into pMIG or pMSCV (Clonetech).

2-3. Antibodies and reagents

BIX01294 was purchased from Santa Cruz, and pargyline was purchased from Sigma. Anti-Flag (M2) and anti-G9a were purchased from Sigma; anti-Myc (9E10) and anti-HA (16B12) were obtained from Covance; anti-methyl lysine and anti-G9a were purchased from Abcam; anti-Ezh2 was obtained from Cell Signaling; anti-MEF2, anti-MHC, and anti-myogenin were from Santa Cruz; and anti-MEF2D was from BD Biosciences. ImmunoPure® Goat Anti-Mouse IgG, (H + L) and ImmunoPure® Peroxidase-Conjugated Goat Anti-Rabbit IgG, (H + L) were purchased from Pierce, and anti-mouse Alexa 488, anti-rabbit Alexa 568 (Molecular Probes), and DAPI were obtained from Calbiochem. Anti-K267me was generated from Abmart by immunization with 263-APSR(meK)PDLR-271. Unmodified and mono-methyl-K267 MEF2D peptides were synthesized chemically (Abmart).

2-4. Immunoprecipitation and reporter gene assay

Immunoprecipitation and reporter gene assays were performed as described (13). For the reporter gene assays using pOF-MEF2-luc, containing multimerized MEF2 binding sites and p*Myogenin*-luc, HEK293 cells were transfected with a *luciferase* reporter plasmid with HA-MEF2D and Flag-G9a. Cells were harvested 48 h after transfection, and luciferase activity was measured with an Infinite M200 (Tecan Group Ltd.).

2-5. Retroviral infection

Empty or G9a, MEF2 (WT) or MEF2 (K267R) -expressing viral vectors were transfected into the packaging cell line plat-E cells with Lipofectamine Plus (Invitrogen). Viral supernatants were harvested and used to infect C2C12 cells. Cells that were infected with pMSCV or pMSCV-G9a were selected with puromycin (4 μg/ml) for 2 days before use.

2-6. Lentivirus production

To knock down G9a, lentiviral vectors that contained the mouse G9a-targeting sequences pLKO.1-sh-G9a #1

(TRCN0000054543) and #2 (TRCN0000054545) were purchased from Open Biosystems. pLKO.1 was used as a control. Lentivirus was produced per the manufacturer's protocol using the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen). Twenty-four hours after lentiviral infection, infected cells were selected with puromycin (4 μg/ml) for 2 days and used for experiments. pLKO.1-shG9a #2 was more effective and used all subsequent experiments. Knockdown of LSD1 has been described (13).

2-7. Immunofluorescence

C2C12 cells grown on the gelatin-coated cover glasses were cultured in differentiation medium for indicated time. Cells were firxed with 4% (w/v) paraformaldehyde and permeabilized them with 0.5% (w/v) Triton X-100. Then, we stained the cells with indicated antibodies. We observed immunofluorescence under a Zeiss LSM 510 laser scanning microscope.

2-8. In vitro methylation and demethylation assay

Methylation assay was performed as described (41) using bacterially purified GST-G9a and His-MEF2D. Demethylation

assay was performed as described (9) using bacterially purified GST-LSD1.

2-9. ESI-LC-MS analysis

MEF2D was immunoprecipitated and separated on SDS-polyacrylamide gels. Gels were stained with Coomassie blue. Sliced gel pieces or MEF2D peptides were digested with trypsin or chymotrypsin and analyzed by ESI-LC-MS (Diatech Korea, Co. LTD).

2-10. Quantitative real-time PCR and chromatin immunoprecipitation assay

ChIP and quantitative real-time polymerase chain reaction (qRT-PCR) were performed as described (13). Primers for RT-PCR and the position and sequence of the primers that were used to amplify ChIP-enriched DNA that spanned the MEF2-response elements have been described (13). Specific primers for qRT-PCR are listed in Table 1. Primers for the mouse *Gapdh* promoter were 5'-GCACAGTCAAGGCCGAGAAT-3' and 5'-GCCTTCTCCATGGTGGTGAA-3'.

2-11. Statistical analysis

Data in the bar graphs were expressed as mean and standard deviation of 3 independent experiments. P-values were calculated using a student's t-test calculator (http://www.physics.csbsju.edu/stats/t-test.html). $P < 0.05$ was considered statistically significant. All data are representative of at least 3 independent experiments.

MCK	sense	CACCATGCCGTTCGGCAACA
	antisense	GGTTGTCCACCCCAGTCT
Ezh2	sense	TTTGCTGCTGCTCTTACTGC
	antisense	CCAGTTTCAGTCCCTGCTTC
G9a	sense	ATCCTTAAGCGGGAGACCAT
	antisense	CAGTGGGGACAGAAGACCAT
Mll2	sense	TGTTCGCATGAAAACGCCC
	antisense	TGCAAGTGGCAGCAAAGGA
Setdb1	sense	GATTCTGGGCAAGAAGAGGA
	antisense	GTACTTGGCCACCACTCGAC
Setd6	sense	GGAGATGGTAGGGGAAGAGG
	antisense	TGCCAAACTGTCGTCTTCTG
Setd7	sense	TGAGGATGGAGGTGTTCTCC
	antisense	TCTCCCGTCATCTCTCCATC
Suv39h1	sense	CTGTGCCGACTAGCCAAGC
	antisense	ATACCCACGCCACTTAACCAG
Gapdh	sense	CCCACTAACATCAAATGGGG
	antisense	CCTTCCACAATGCCAAAGTT

Table 2. RT-PCR primer list

III. RESULTS

3-1. MEF2D is methylated at K267

The regulation of MEF2 by various posttranslational modifications has been reported (19). However, lysine methylation of MEF2 and its effects on MEF2-dependent transcription during muscle differentiation have not been examined. Thus, we determine whether MEF2 is methylated at lysine residue(s) using MEF2D.

By immunoprecipitation of overexpressed MEF2D with anti-methyl lysine, MEF2D is methylated at a lysine residue (Figure 3a). Methylation of overexpressed MEF2D in HEK293 cells was confirmed by ESI-LC-MS. A molecular shift (+28 Da) of the modified peptide (left upper panel) compared with the unmodified peptide (lower left panel) indicated dimethylation of lysine 267 (K267) of MEF2D (Figure 3b). Further, monomethylated MEF2D was detected by ESI-LC-MS (right panel) (Figure 3b). Endogenous MEF2D from C2C12 mouse myoblast cells was also methylated at K267 by ESI-LC-MS (Figure 4).

To examine MEF2D methylation, a specific antibody for MEF2D with methylated K267 was generated (anti-K267me) and tested by dot blot assay against unmodified and chemically

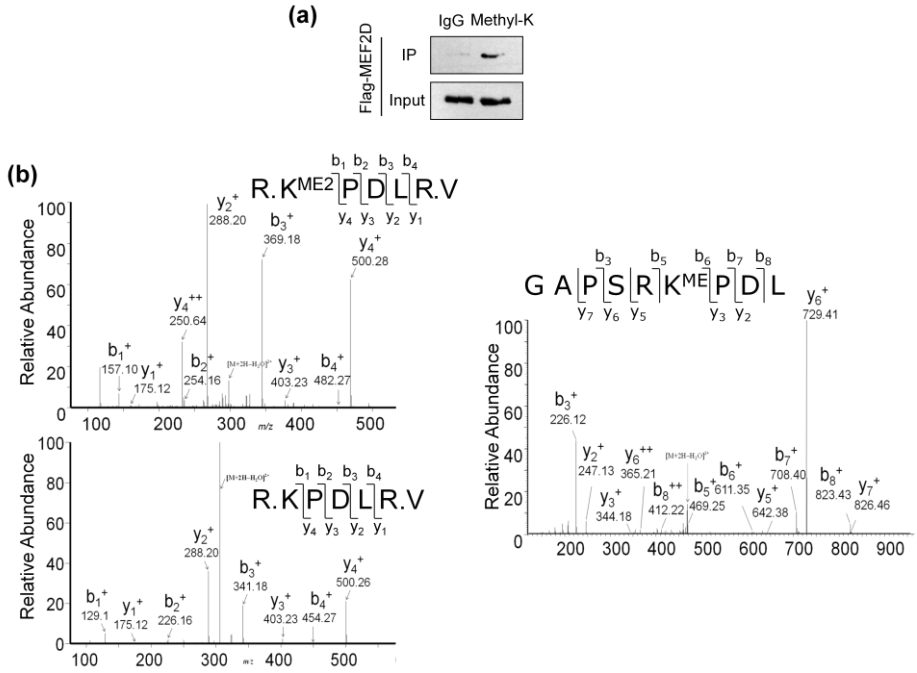


Figure 3. Overexpressed Flag-MEF2D is methylated at K267.

(a) Transiently transfected Flag-MEF2D in HEK293 cells were immunoprecipitated with normal rabbit IgG or anti-methylated lysine antibody (Methyl-K) and immunoblotted with anti-Flag antibody. (b) Overexpressed Flag-MEF2D in HEK293 cells were immunoprecipitated and analyzed with ESI-LC-MS. Di-methylated (left upper panel), unmodified (left lower panel) and mono-methylated (right panel) MEF2D were detected.

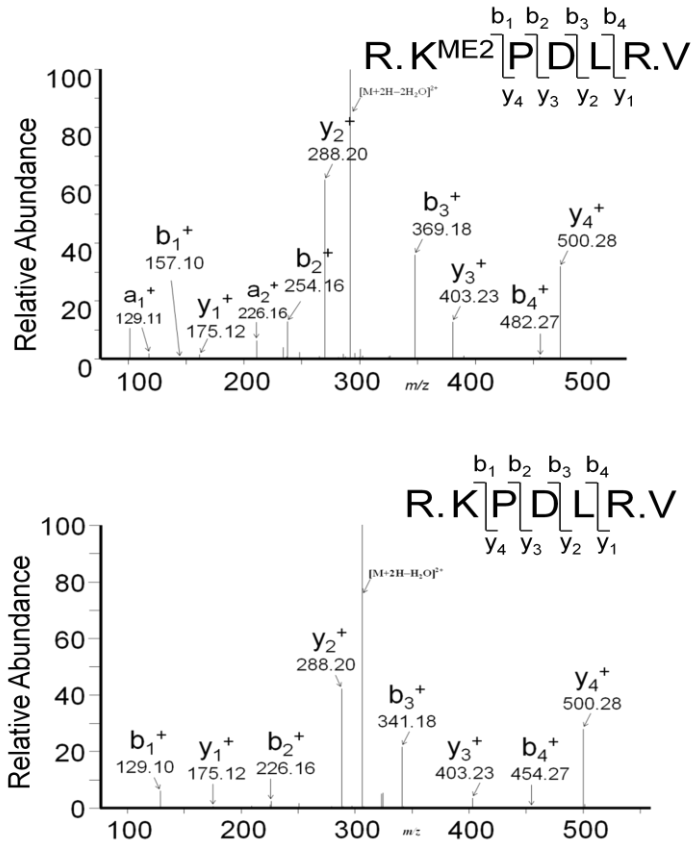


Figure 4. Endogenous MEF2D is methylated at K267.

MEF2D immunoprecipitated from C2C12 cells were analyzed with ESI-LC-MS. Di-methylated (left panel) and unmodified (right panel) MEF2D were detected.

monomethylated K267 containing peptides (263-271) (Figure 5). By ESI-LC-MS analysis, immunoprecipitation of overexpressed HA-MEF2D with anti-K267me pulled down mono- and di-methylated MEF2D, demonstrating that anti-K267me recognizes the mono- and dimethylated forms of MEF2D (Figure 6). Wild-type (WT) HA-MEF2D was detected using anti-K267me, but the K267R mutant was not (Figure 7a). The detection of methylated MEF2D by anti-K267me was blocked with a chemically methylated K267-containing peptide (Figure 7b). Endogenous MEF2D immunoprecipitated with anti-K267me, demonstrating that MEF2D in proliferating C2C12 cells is methylated at K267 (Figure 8a). Further, the proportion of methylated MEF2D in proliferating C2C12 cells was determined by immunodepletion assay (Figure 8b). On immunoprecipitation with anti-K267me, About half of MEF2D was depleted, suggesting the significance of this modification. MEF2D methylation was also observed in DO11.10 T cells and mouse embryonic stem cells (E14) (Figure 9a and b). Notably, K267 was highly conserved between MEF2 isoforms and species, suggesting critical functions for this residue and its modification (Figure 10a and b). Moreover, lysine methylation

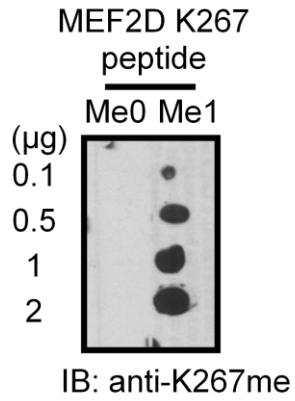


Figure 5. Anti-methyl K267 (anti-K267me) antibody detects mono-methylated MEF2D.

Anti-K267me antibody was tested by dot blot assay using unmodified and chemically mono-methylated K267 containing MEF2D peptides (263-271). Anti-K267me antibody detected methylated peptide only.

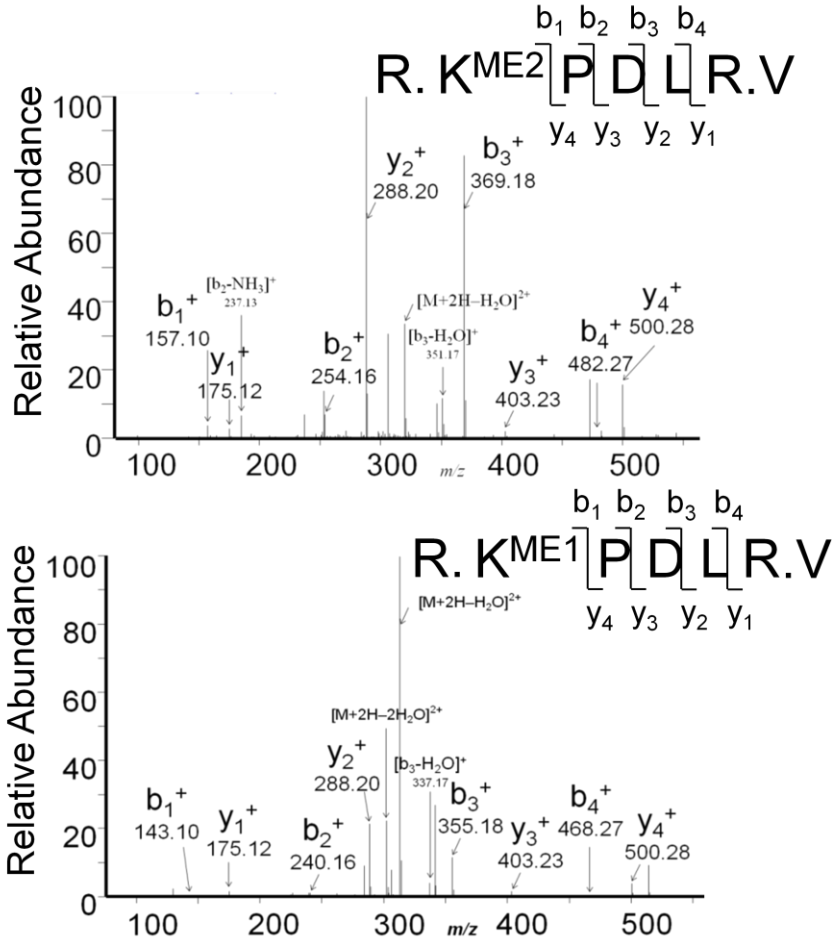


Figure 6. Anti- K267me antibody pulls down mono-, di-methylated MEF2D.

HA-MEF2D, transiently expressed in HEK293 cells, was immunoprecipitated with anti-methylated K267 MEF2D (anti-K267me) and subjected to ESI-LC-MS analysis. Dimethylation (upper panel) and monomethylation (lower panel) of MEF2D were detected.

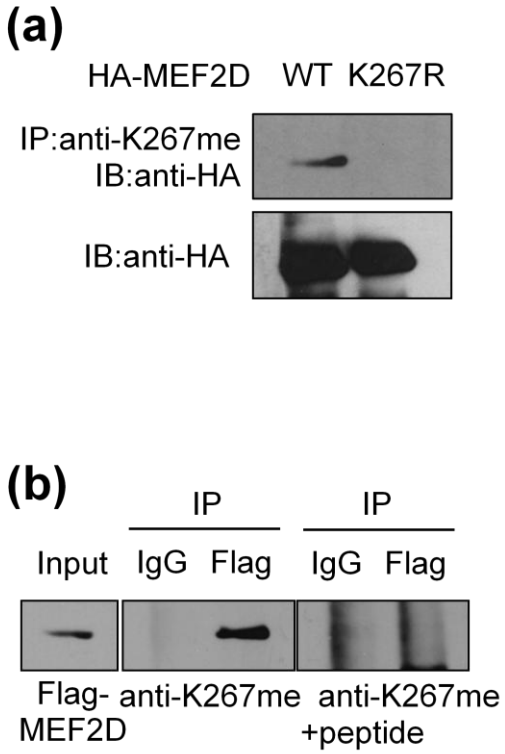


Figure 7. Anti- K267me antibody specifically detects methylated MEF2D.

(a) Transiently expressed HA-MEF2D wild-type (WT) or K267R mutant (K267R) was immunoprecipitated with anti-K267me, followed by immunoblotting with anti-HA. (b) Transiently expressed Flag-MEF2D (WT) was immunoprecipitated with anti-Flag, followed by immunoblotting with anti-K267me with or without chemically methylated K267 containing peptide blocking.

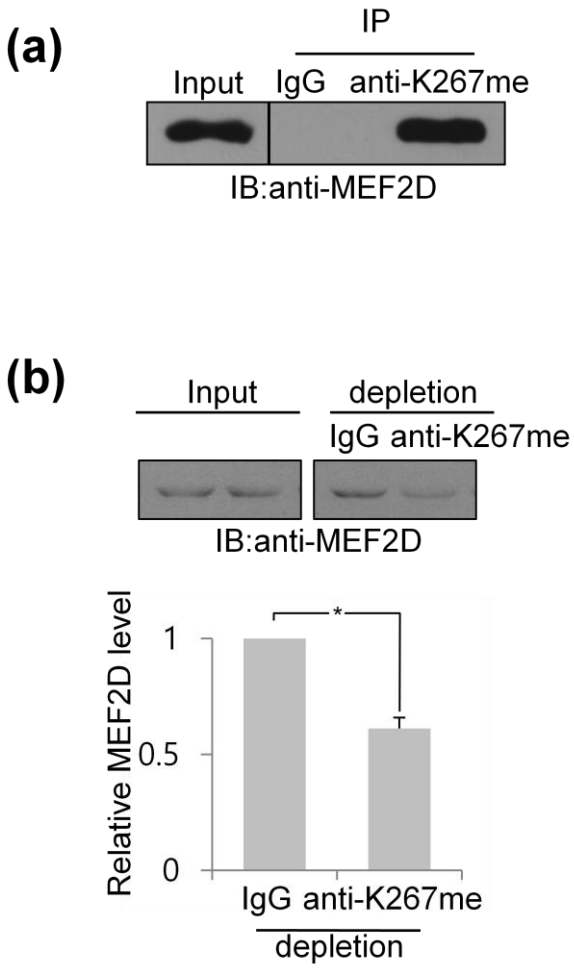


Figure 8. Endogenous MEF2D is methylated in C2C12 cells.

(a) Endogenous MEF2D was immunoprecipitated from C2C12 cells with anti-K267me and immunoblotted with anti-MEF2D. (b) C2C12 whole-cell lysates were immunoprecipitated with anti-K267me or IgG. Supernatants of immunodepleted C2C12 cell lysates were analyzed by immunoblotting with anti-MEF2D (upper panel). Quantification of MEF2D after normalization to input (lower panel).

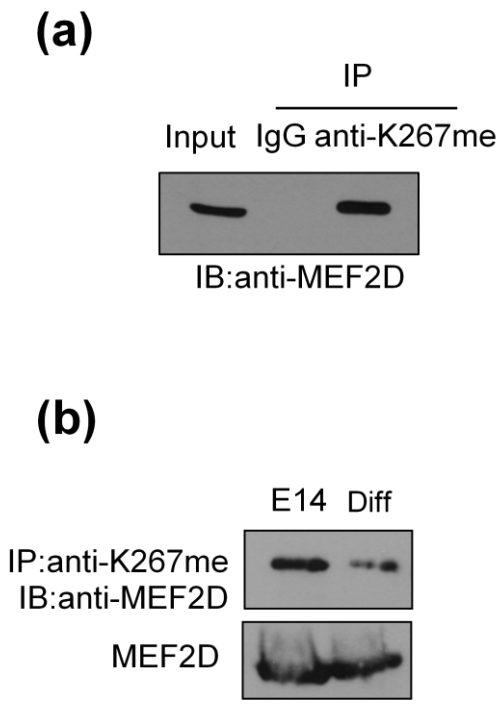


Figure 9. Endogenous MEF2D from various cell lines are methylated.

(a) Endogenous MEF2D was immunoprecipitated from DO11.10 cells with anti-K267me and immunoblotted with anti-MEF2D antibody. (b) mouse embryonic stem cell (E14) were randomly differentiated (Diff) and immunoprecipitated with anti-K267me antibody.

(a)

```
mMEF2A          LGMNSRKPDLRVV
hMEF2A          LGMNSRKPDLRVV
mMEF2C          LGMNNRKPDLRVL
hMEF2C          LGMNNRKPDLRVL
mMEF2D          LGAPSRKPDLRVI
hMEF2D          LGAPSRKPDLRVI
```

(b)

```
H.sapiens       LGAPSRKPDLRVI
M.musculus      LGAPSRKPDLRVI
P.troglodytes   LGAPSRKPDLRVI
R.norvegicus    LGAPSRKPDLRVI
G.gallus        LASNSRKPDLRVI
D.rerio         M-ANSRKPDLRVI
```

Figure 10. The methylated lysine residue is well-conserved,

(a) Sequence alignment of mouse and human MEF2 family proteins. The methylated lysine residues are highlighted in red. (b) Sequence alignment of MEF2 family proteins between species. The methylated lysine residues are highlighted in red.

at K267 in other MEF2 isoforms implicates a significant function of this modification in the regulation of MEF2 activity (Figure 11a). The methylation-defective mutants MEF2A and MEF2C could not be immunoprecipitated with anti-K267me (Figure 11b and c).

To examine the significance of MEF2methylation, methylation levels of MEF2 during C2C12 cell differentiation were measured by western blot. MEF2 methylation levels declined during myogenesis, whereas total MEF2D levels increased (Figure 12). Similarly, methyl-MEF2D levels decreased on ionomycin treatment or random differentiation in DO11.10 T cells and E14 cells (Figure 9b and 12b). Alterations in MEF2D methylation level due to environmental changes implicate methylation as a regulatory mechanism of MEF2D.

3-2. MEF2D is methylated by G9a

To identify the lysine methyltransferase (PKMT) that methylates MEF2D at K267, we screened PKMTs that are differentially expressed during C2C12 cell differentiation, during which MEF2D transcriptional activity is dynamically regulated. Using previous microarray data, we selected PKMTs

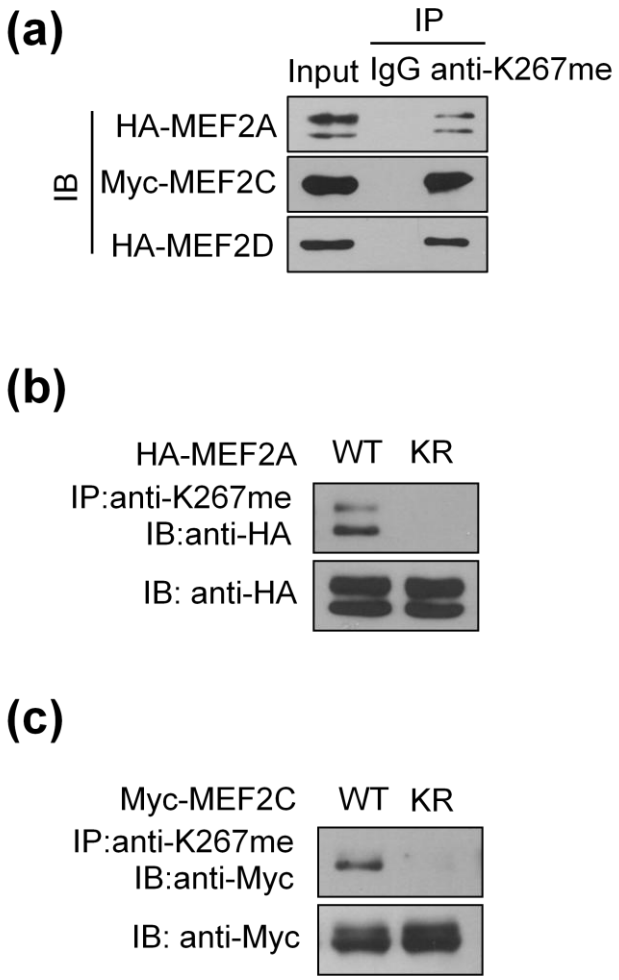


Figure 11. MEF2 family members are methylated.

(a) HA-MEF2A, Myc-MEF2C and HA-MEF2D were overexpressed in HEK293 cells and immunoprecipitated with anti-K267me antibody. (b, c) Transiently expressed HA-MEF2A (b) or Myc-MEF2C (c) wild type (WT) or KR mutant (KR) was immunoprecipitated with anti-K267 methylated MEF2 antibody (anti-K267me) followed by immunoblotting with anti-HA (b) or anti-Myc antibody (c).

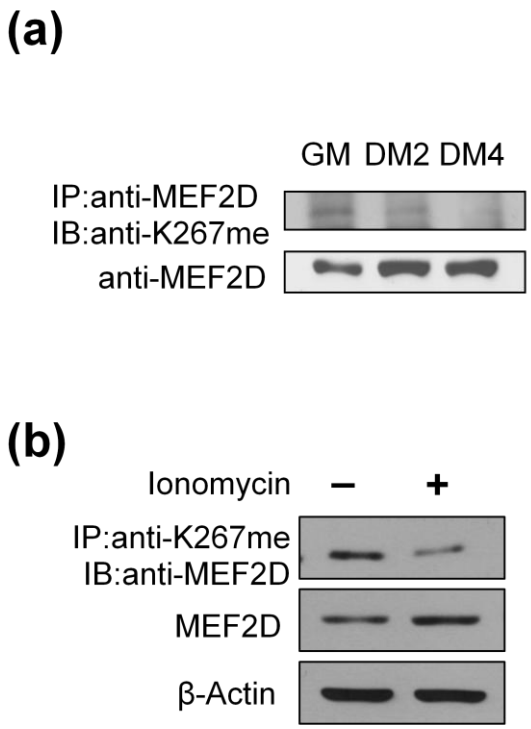


Figure 12. MEF2 methylation level dynamically affected by cell stimulation.

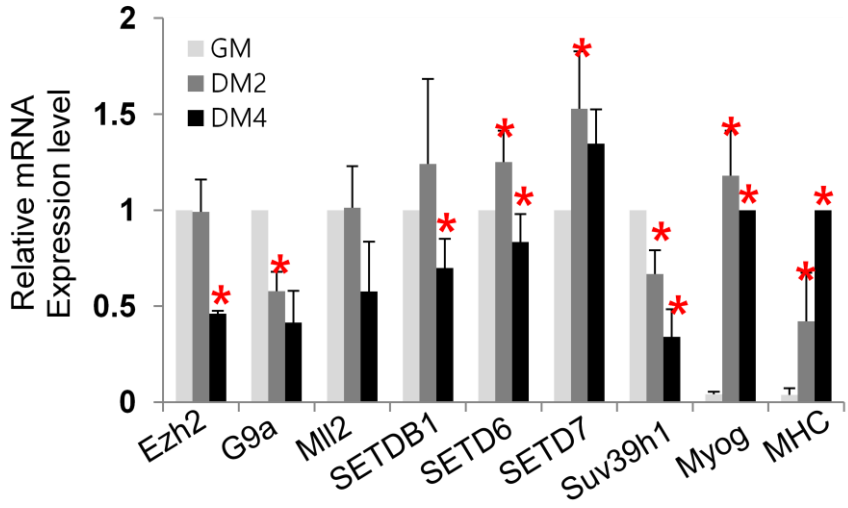
(a) C2C12 cells, differentiated for up to 4 days, were immunoprecipiated with anti-MEF2D and western blotted with anti-K267me (GM; growth medium, DM2, DM4; differentiation medium for 2 or 4 days). MEF2D methlation was decreased during differentiation. (b) The methylation level of MEF2D K267 in DO11.10 cells treated with Ionomycin (500nM for 3 hours) was analyzed by western blot. MEF2D methylation was decreased upon Ionomycin treatment.

that were differentially expressed during myogenesis. mRNA levels of these PKMTs during C2C12 cell differentiation were confirmed (42). C2C12 cells were cultured in differentiation medium (DM) for 2 or 4 days (DM2 or DM4) and harvested for RNA extraction.

Of the PKMTs, *Ezh2*, *G9a,* and *Suv39h1* mRNA levels changed dramatically and were selected as candidates for MEF2D methylation (Figure 13a). Suv39h1 as a methyltransferase that mediates trimethylation (2), but MEF2D is mono- and dimethylated (Figure 6); thus, Suv39h1 is unlikely the enzyme that methylates MEF2D.

We examined the remaining candidates, Ezh2 and G9a. Ezh2 and G9a protein levels decreased during differentiation of C2C12 cells (Figure 13b). To determine whether Ezh2 or G9a methylated MEF2D at K267, we performed an *in vitro* methylation assay, in which MEF2D peptide (263-271) was the substrate. G9a methylated unmodified peptide (K267me0) (Figure 14a) while Ezh2 could not methylate MEF2D peptide (Figure 14b). Methylation of MEF2D peptide at K267 by G9a was also detected by extracted ion chromatography (Figure 14c). To validate the enzymatic activity of G9a on MEF2D,

(a)


Relative mRNA Expression level
2
1.5
1
0.5
0
GM
DM2
DM4
Ezh2
G9a
Mll2
SETDB1
SETD6
SETD7
Suv39h1
Myog
MHC

(b)

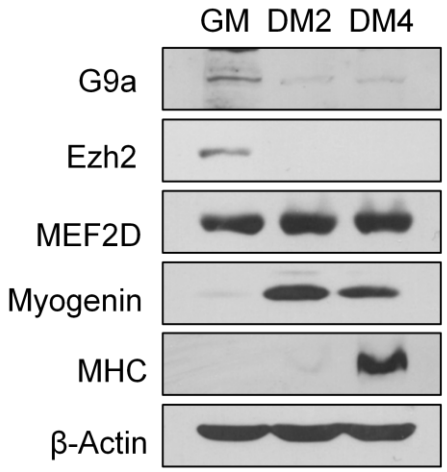

GM
DM2
DM4
G9a
Ezh2
MEF2D
Myogenin
MHC
β-Actin

Figure 13. Protein lysine methyltransferases (PKMT) for MEF2 methylation are screened.

(a) Protein lysine methyltransferases (PKMT) mRNA expression level in differentiating C2C12 cells was analyzed by qRT-PCR. mRNA level was normalized with *Gapdh*, and relative expression level to GM or DM4 has been depicted. (*) $p < 0.05$ (b) Immunoblot of whole cell lysates with indicated antibodies shows differentially expressed PKMT level in differentiating C2C12 cells. Protein expression of G9a and Ezh2 were diminished upon differentiation.

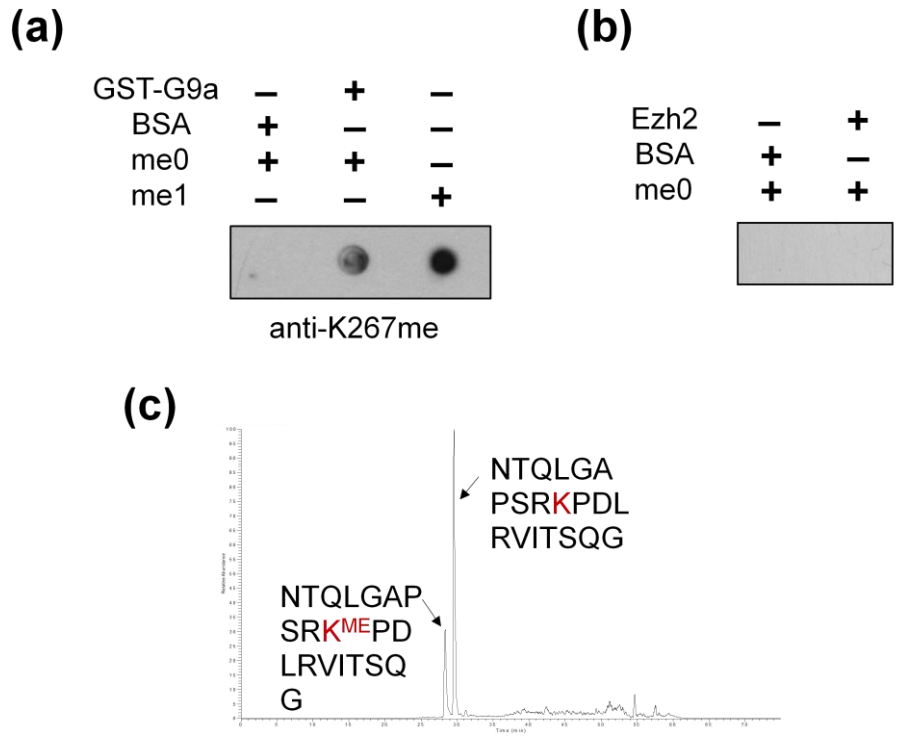


Figure 14. G9a methylates MEF2D peptide.

(a) *In vitro* methylation of MEF2D peptide (263-271) (me0) by G9a was analyzed by dot blot assay. Chemically methylated MEF2D peptide (me1) was used as a positive control for antibody detection. (b) *In vitro* methylation of MEF2D peptide (263-271) (me0) by Ezh2 was analyzed by dot blot assay. (c) Extracted ion chromatography of G9a mediated methylation of MEF2D peptide *in vitro* is depicted.

full-length MEF2D protein was bacterially purified and used as the substrate in an *in vitro* methylation assay. By ESI-LC-MS of MEF2D that was incubated with G9a, MEF2D was dimethylated by G9a *in vitro* (Figure 15a). MEF2D methylation by G9a *in vitro* was confirmed by immunoprecipitation and western blot with anti-K267me (Figure 15b).

To determine whether G9a methylated MEF2D K267 in cells, HA-MEF2D (WT) was transiently expressed in HEK293 cells with or without the G9a-specific inhibitor BIX01294 (43). Methylation of HA-MEF2D decreased on inhibition of G9a activity by BIX01294 (Figure 16). Moreover, by immunoprecipaition with anti-K267me, endogenous methylation of MEF2D in C2C12 cells fell in a BIX01294 concentration-dependent manner (Figure 17a). This is also confirmed by westernblotting with anti-K267me of immunoprecipitated MEF2D (Figure 17b). By immunostaining, we noted decreased methylation of MEF2D on treatment with BIX01294 (Figure 17c).

To confirm the G9a-mediated methylation of MEF2D, G9a was knocked down with lentiviral shRNA. On downregulation of G9a, methylation levels decreased (Figure 18). Thus, MEF2D is

a

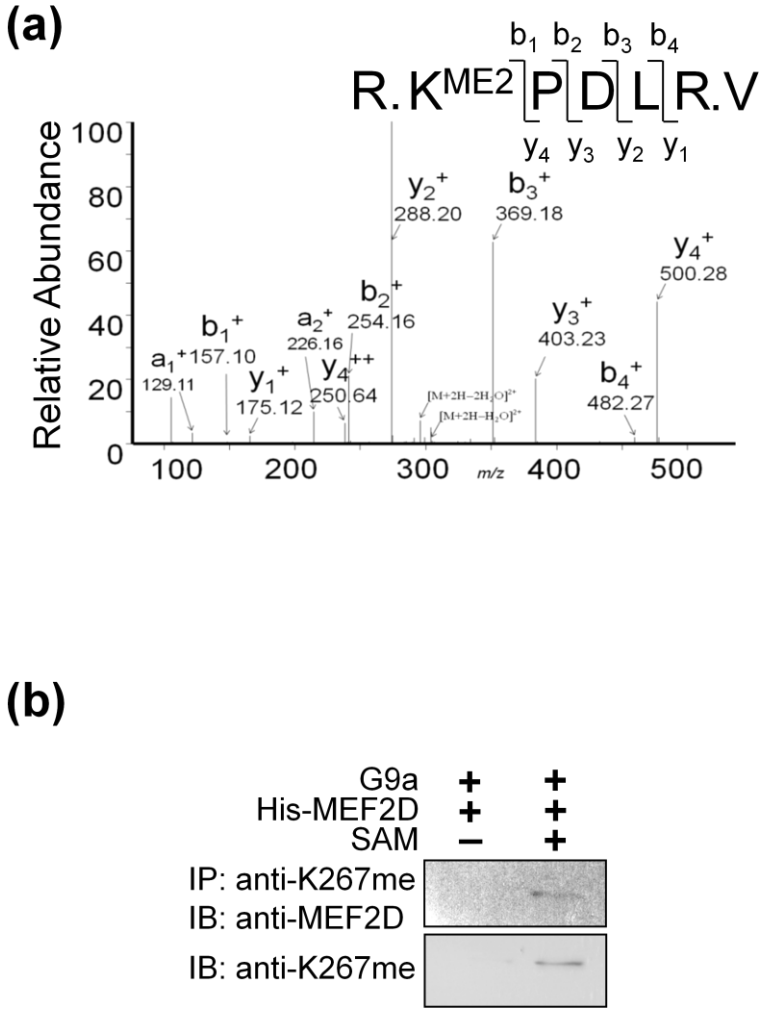

(a)
b1 b2 b3 b4
R.KME2PDLR.V
y4 y3 y2 y1
Relative Abundance
100
80
60
40
20
0
100
200
300
m/z
400
500
y2+ 288.20
b3+ 369.18
y4+ 500.28
b2+ 254.16
b1+ 157.10
a2+ 226.16
y3+ 403.23
a1+ 129.11
y1+ 175.12
y4++ 250.64
b4+ 482.27
(b)
G9a + +
His-MEF2D + +
SAM − +
IP: anti-K267me
IB: anti-MEF2D
IB: anti-K267me

Figure 15. G9a methylates MEF2D at K267.

(a) *In vitro* methylation of bacterially purified full-length His-MEF2D with G9a was analyzed by ESI-LC-MS. (b) Bacterially purified His-MEF2D was incubated with G9a with or without methyldonor, SAM. *In vitro* methylated MEF2D was immunoprecipiated with anti-K267me antibody and immunoblotted with anti-MEF2D antibody (upper panel) or immunoblotted with anti-K267me antibody (lower panel).

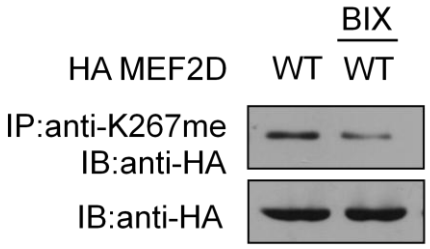
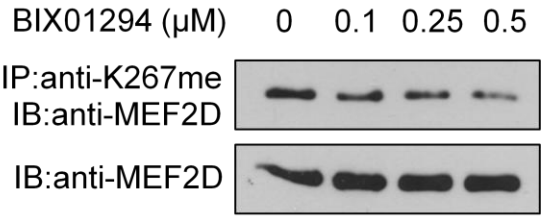


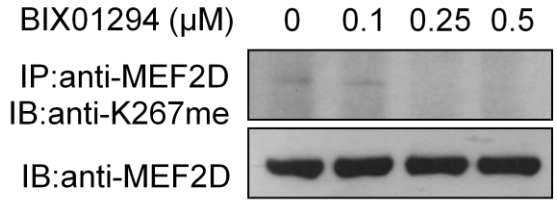
Figure 16. BIX01294 inhibits methylation of overexpressed MEF2D.

HA-MEF2D (WT) overexpressed in HEK293 cells was immunoprecipitated with anti-K267me antibody with or without 4 μM BIX01294. G9a inhibition with BIX01294 decreased overexpressed MEF2D methylation level.

(a)


BIX01294 (μM) 0 0.1 0.25 0.5
IP:anti-K267me
IB:anti-MEF2D
IB:anti-MEF2D

(b)


BIX01294 (μM) 0 0.1 0.25 0.5
IP:anti-MEF2D
IB:anti-K267me
IB:anti-MEF2D

(c)

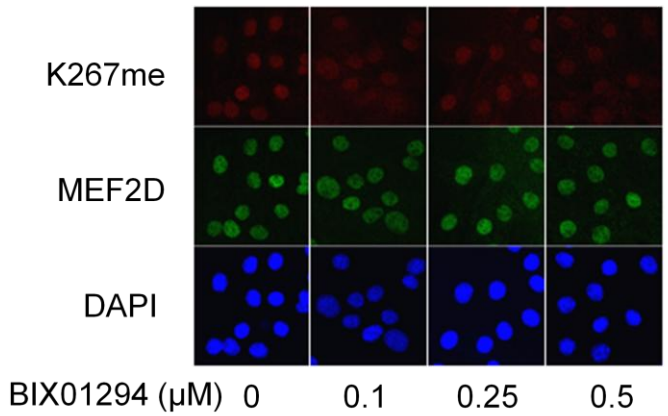

K267me
MEF2D
DAPI
BIX01294 (μM) 0 0.1 0.25 0.5

Figure 17. BIX01294 inhibits methylation of endogenous MEF2D in C2C12 cells.

(a) C2C12 cells were treated with BIX01294 at the indicated concentrations. MEF2D and its methylation levels were analyzed by immunoprecipitation. (b) C2C12 cells were treated with BIX01294 at the indicated concentrations. Methylation level of immunoprecipitated MEF2D was analyzed by western blot with anti-K267me antibody. (c) C2C12 cells were treated with BIX01294 at the indicated concentrations. MEF2D and its methylation level were analyzed by immunostaining. MEF2D methylation level was decreased in a BIX01294 concentration dependent manner.

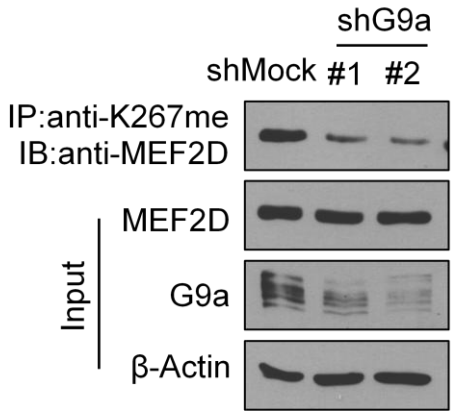


Figure 18. Knockdown of G9a decreases MEF2D methylation. MEF2D methylation in C2C12 cells infected with shMock or shG9a was analyzed by immunoprecipitation. Knockdown of G9a resulted in a reduced MEF2D methylation level.

substrate of G9a, which methylates it at K267.

3-3. MEF2D interacts with G9a

To confirm that MEF2D is a substrate of G9a, the interaction between MEF2D and G9a was observed by His pulldown assay with overexpressed Flag-G9a (Figure 19a). MEF2 family members share a highly conserved N-terminal domain, the MADS/MEF domain, which is important for DNA binding and protein-protein interactions (19). The N-terminus of MEF2D is alternatively spliced during muscle differentiation. A ubiquitously expressed isoform, MEF2Dα1, binds to corepressors, and a muscle-specific isoform, MEF2Dα2, interacts with Ash2L (Figure 20) (44).Thus, we examined whether the binding domain of MEF2Dα1 interacted with G9a.

Truncated mutants of MEF2D—1-270 amino acids (N270) and 1-130 amino acids (N130)—and G9a were overexpressed in HEK293 cells and subjected to coimmunoprecipitation assay. The interaction between the MEF2D MADS/MEF2 domain (N130) and G9a suggested that G9a binds to all MEF2 family members (Figure 19b). Whereas 464-1001 amino acids (464C) and 685-1001 amino acids (685C) truncated mutants of G9a

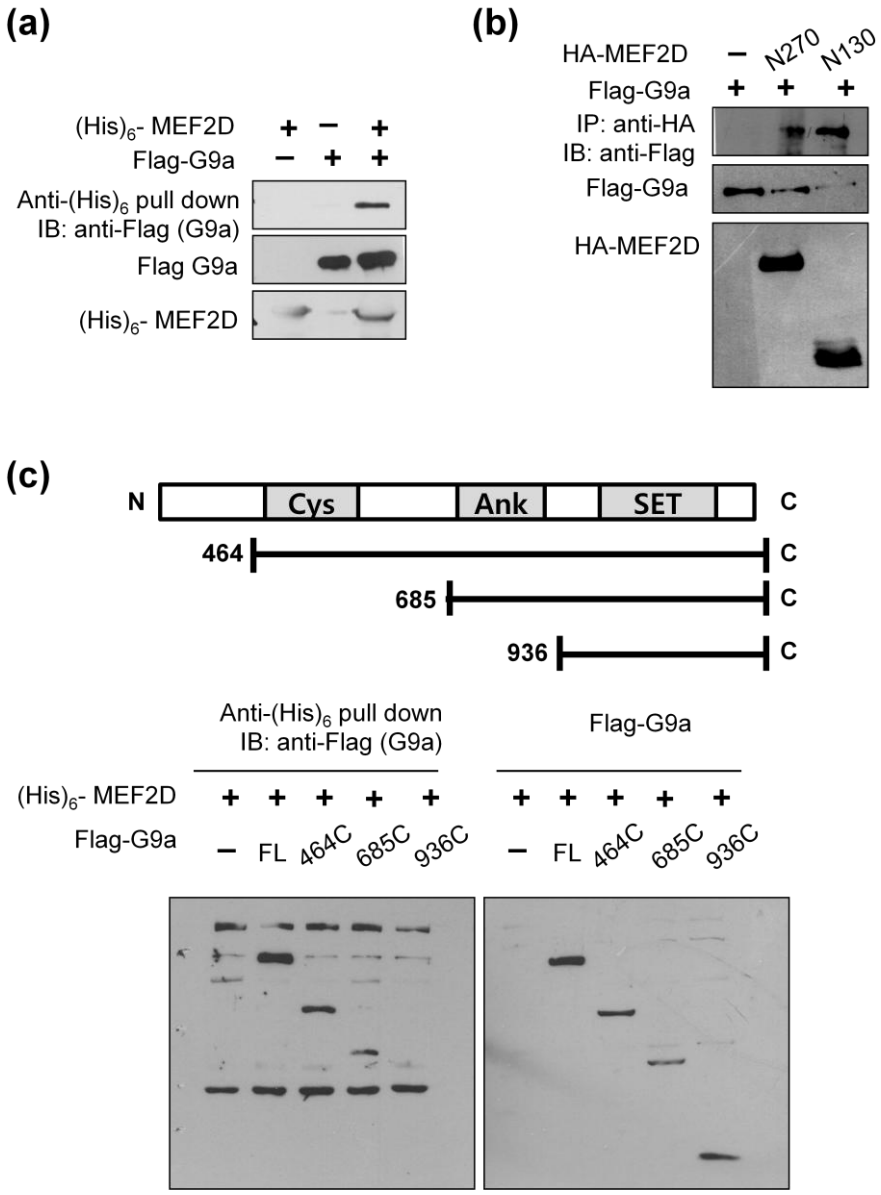

(a)
(His)6- MEF2D + − +
Flag-G9a − + +
Anti-(His)6 pull down
IB: anti-Flag (G9a)
Flag G9a
(His)6- MEF2D
(b)
HA-MEF2D − N270 N130
Flag-G9a + + +
IP: anti-HA
IB: anti-Flag
Flag-G9a
HA-MEF2D
(c)
N
Cys
Ank
SET
C
464
685
936
Anti-(His)6 pull down
IB: anti-Flag (G9a)
Flag-G9a
(His)6- MEF2D + + + + + + + + + +
Flag-G9a − FL 464C 685C 936C − FL 464C 685C 936C

Figure 19. Overexpressed G9a and MEF2D interact.

(a) Flag-G9a overexpressed in HEK293 cells were co-immunoprecipitated with bacterially purified His-MEF2D and immunoblotted with indicated antibodies. (b) Truncated HA-MEF2D (N270, N130) and Flag-G9a were overexpressed and immunoprecipitated with anti-HA antibody. N-terminus of MEF2D interacted with G9a. (c) Truncated mutants of Flag-G9a (464C, 685C, 936C) were co-immunoprecipitated with bacterially purified His-MEF2D. Ankyrin domain of G9a interacted with MEF2D.

interacted with MEF2D, truncated Flag-G9a, 936-1001 amino acids (936C) mutant was unable to bind, indicating that the ankyrin repeat domain (amino acids 685-936) of G9a is required for the interaction (Figure 19c).

The endogenous interaction between G9a and MEF2D was verified in proliferating C2C12 cells (Figure 21a). G9a and MEF2D colocalized in the nucleus of C2C12 cells by immunostaining (Figure 22b).

3-4. MEF2D is demethylated by LSD1

We have reported that LSD1 activates MEF2 during skeletal muscle differentiation (13). Thus, we hypothesized that LSD1 increases MEF2 transcriptional activity by demethylating K267, counteracting the function of G9a. *By in vitro* demethylase assay using a chemically modified K267-containing peptide, LSD1 demethylated K267 (Figure 22). Immunoprecipitated MEF2D from proliferating C2C12 cells were westernblotted with anti-K267me, and showed increased methylation at K267 in LSD1-knockdown C2C12 cells (Figure 23a). This was also confirmed by immunoprecipitating with anti-K267me followed by westernblotting with anti-MEF2D (Figure 23b left panel).

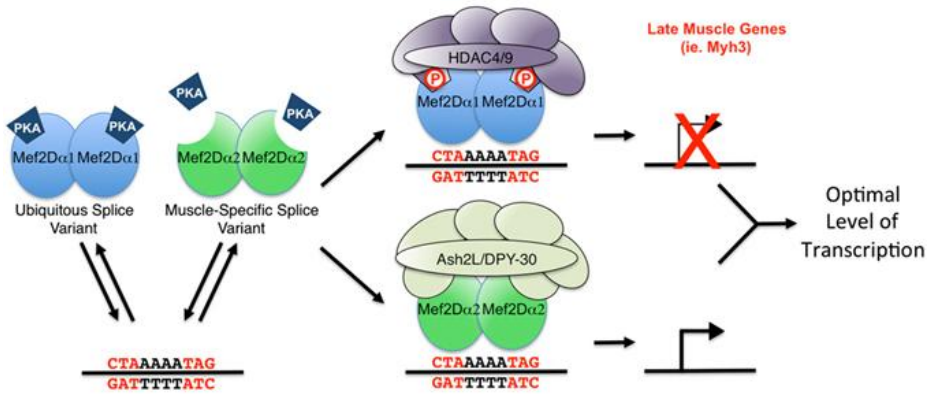


Figure 20. Alternatively spliced MEF2D isoforms associate with different regulatory complexes during muscle differentiation (modified from ref. 44).

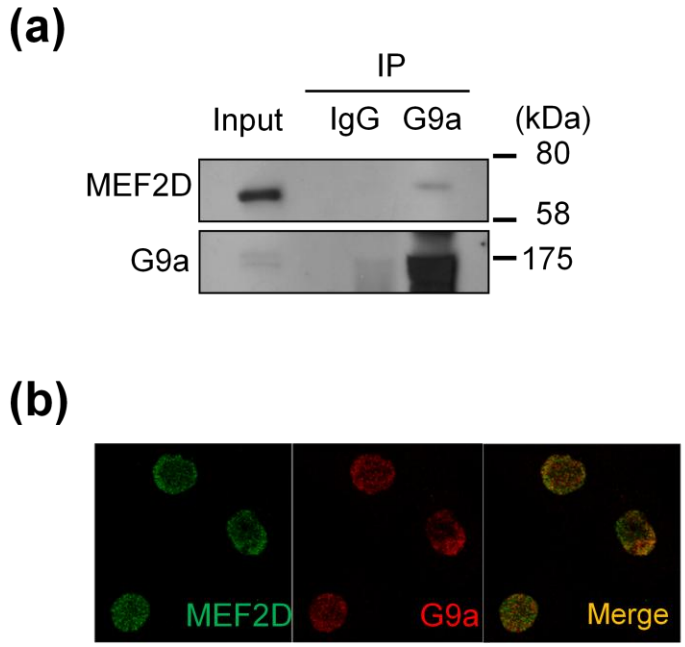


Figure 21. Endogenous G9a and MEF2D interact.

(a) Whole-cell lysates of C2C12 cells in growth medium (GM) were immunoprecipitated with anti-G9a or rabbit normal IgG. G9a and MEF2D interacted endogenously. (b) Colocalization of G9a and MEF2D in proliferating C2C12 cells was analyzed by immunostaining.

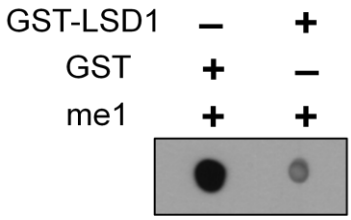


Figure 22. LSD1 demethylates MEF2D peptide.

In vitro demethylation assay of monomethylated MEF2D peptide (263-271) (me1) by LSD1 was analyzed by dot blot assay. MEF2D methylation was demethylated by LSD1.

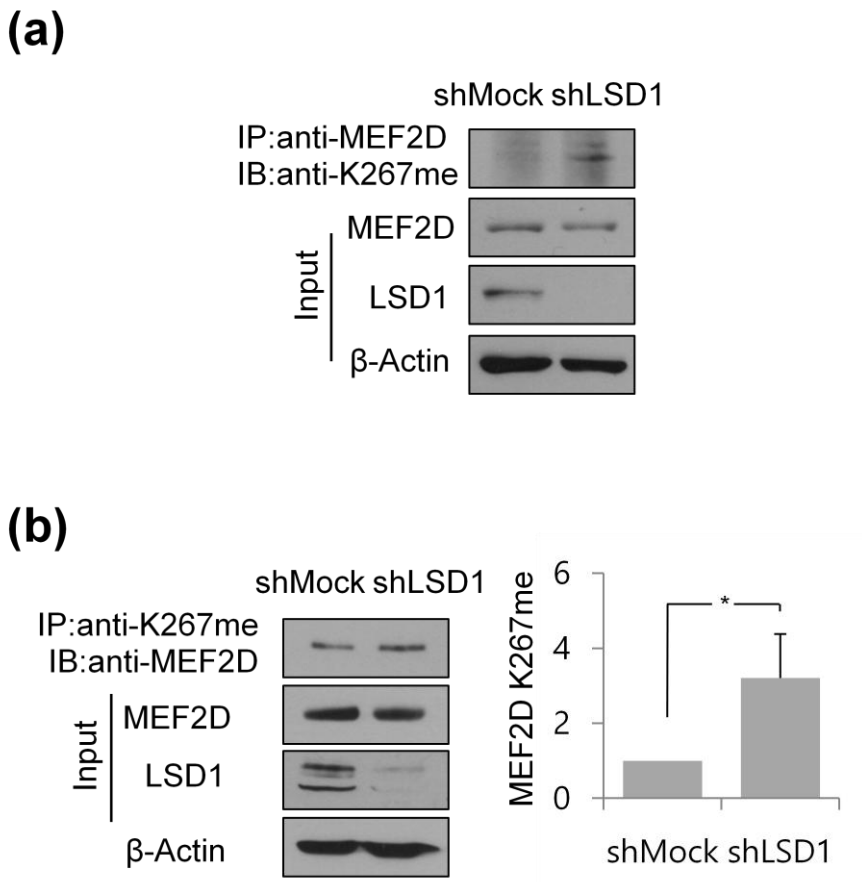


Figure 23. Knockdown of LSD1 increases MEF2D methylation.

(a) Methylation level of immunoprecipitated MEF2D in C2C12 cells infected with shMock or shLSD1 was analyzed by western blot. (b) MEF2D methylation in C2C12 cells infected with shMock or shLSD1 was analyzed by immunoprecipitation (left panel). Quantification of MEF2D methylation was depicted after normalization to total MEF2D levels (n=3). Knockdown of LSD1 increased MEF2D methylation.

Quantification of K267 methylation level demonstrated significantly increased methylation level in LSD1-knockdown C2C12 cells (Figure 23b right panel). Further, MEF2 methylation was elevated on treatment with an LSD1 inhibitor, pargyline, in a concentration-dependent manner by immunoprecipitation (Figure 24a). Immunostaining of pargyline treated C2C12 cells verified increased K267 methylation level while total MEF2D level was not changed (Figure 24b). This demethylase activity against MEF2D indicates that LSD1 regulates MEF2D transcriptional activity by modulating a histone modification, as reported (13), and by directly regulating lysine methylation of MEF2D.

3-5. G9a inhibits MEF2D transcriptional activity by regulating its recruitment to chromatin

Next, we examined whether G9a modulates MEF2D transcriptional activity by methylating it. The luciferase gene, controlled by a promoter with an artificial MEF2 element, was transfected into HEK293 cells with or without MEF2D, alone and with G9a. On increased expression of G9a, MEF2D transcriptional activity on the MEF2 element-containing

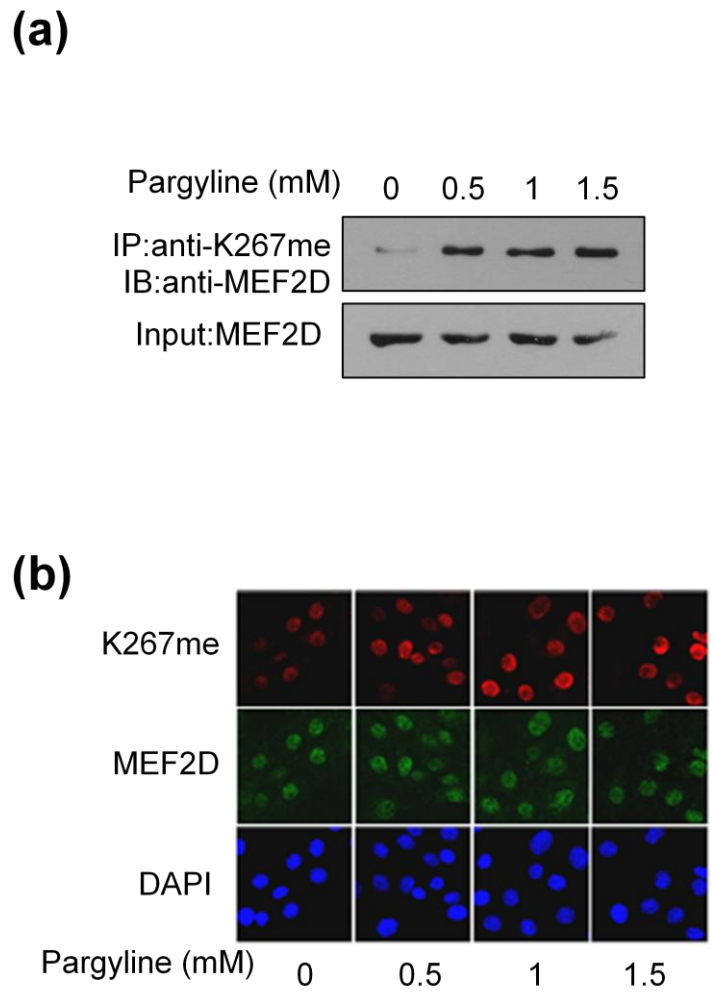


Figure 24. Pargyline increases MEF2D methylation.

(a and b) C2C12 cells were treated with pargyline at the indicated concentrations. MEF2D and its methylation were analyzed by immunoprecipitation (a) and immunostaining (b). Inhibition of LSD1 increased MEF2D methylation.

promoter (Figure 25a). Repression of MEF2D transcription activity by G9a was also verified with *myogenin* promoter declined (Figure 25b).

To determine the significance of the regulation of MEF2D activity by G9a and its methylation, methylation levels and the expression of MEF2D target genes were monitored during C2C12 cell differentiation, during which MEF2D transcriptional activity is elevated. Increased transcriptional activity of MEF2D was reflected by the upregulation of MEF2D target genes and myogenic markers. Consequently, with enhanced MEF2 activity, MEF2D K267 methylation decreased, as did its interaction with G9a (Figure 26a). By immunostaining, it was clearly demonstrated that K267 methylation diminished upon differentiation while total MEF2D increased (Figure 26b).

Moreover, we noted the inverse relationship between MEF2D activity and methylation (Figure 27). On Day 2 of differentiation, cells with methylated MEF2D did not express myogenin, whereas myogenin-expressing cells showed no MEF2D methylation (Figure 27). These results indicate that G9a inhibits MEF2D transcriptional function through K267 methylation.

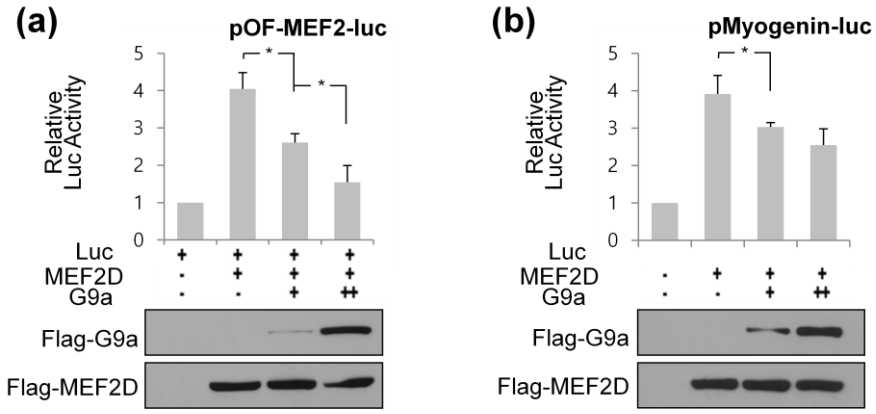


Figure 25. MEF2D transcription activity is repressed by G9a.

(a) pOF-MEF2-luc was transiently transfected with empty vector or Flag-MEF2D and increasing amount of Flag-G9a. (b) pMyogenin-luc was overexpressed with an empty vector or Flag-MEF2D and increasing amount of Flag-G9a. G9a repressed MEF2D transcription activity.

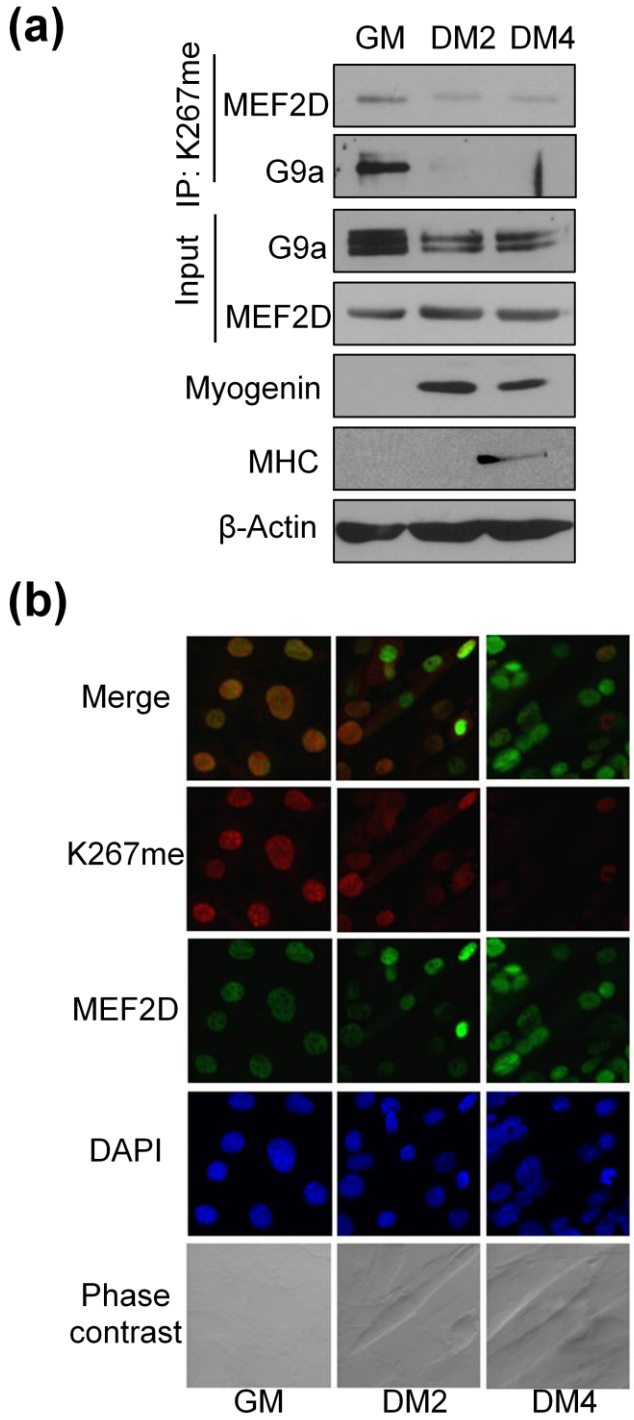

(a)
GM
DM2
DM4
IP: K267me
MEF2D
G9a
Input
G9a
MEF2D
Myogenin
MHC
β-Actin
(b)
Merge
K267me
MEF2D
DAPI
Phase contrast
GM
DM2
DM4

Figure 26. K267 methylation decreases upon MEF2D activation.

(a) C2C12 cells were differentiated for up to 4 days. Whole-cell lysates of C2C12 cells in GM, DM2, and DM4 were immunoprecipitated and immunoblotted with the indicated antibodies. MEF2 methylation decreased during myogenesis. (b) Differentiated C2C12 cells were analyzed by immunostaining with the indicated antibodies. MEF2 methylation was not observed in cells with increased MEF2D expression.

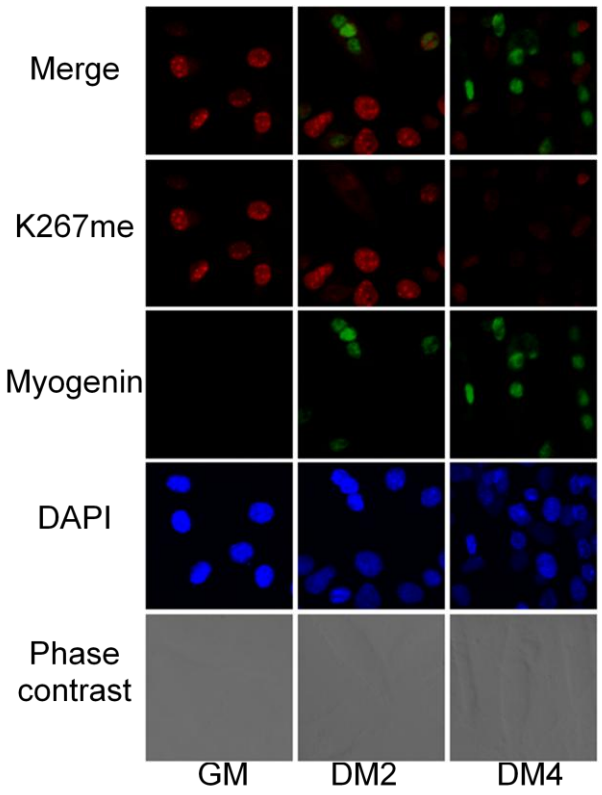


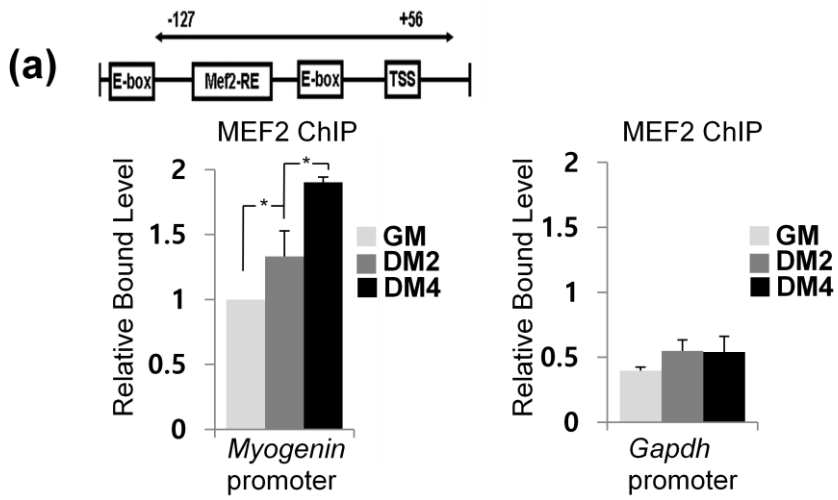
Figure 27. K267 methylation is not detected in myogenin-expressing cells.

C2C12 cells were differentiated for up to 4 days and analyzed by immunostaining with the indicated antibodies. MEF2 methylation was not observed in cells with myogenin expression.

Next, we investigated whether G9a occupied MEF2D binding sites and regulated MEF2D activity on target promoters. The *myogenin* promoter, which contains an MEF2 binding site, was activated by MEF2D during myogenesis (Figure 28a). MEF2 bound to the promoter in C2C12 cells in growth medium (GM); this binding increased in differentiating C2C12 cells (Figure 28a). By ChIP assay, G9a bound to the *myogenin* promoter in proliferating C2C12 cells but dissociated as C2C12 cells differentiated (Figure 28b).

Moreover, K267 methylation of MEF2D was observed in the *myogenin* promoter in undifferentiated C2C12 cells but disappeared as C2C12 cells differentiated, similar to the binding pattern of G9a (Figure 28b). Thus, G9a represses MEF2D activity by methylating it on target promoters, depending on the stage of differentiation. G9a and MEF2 were also detected on the MCK promoter, another MEF2 target gene (Figure 29).

Next, the methylation-defective mutant MEF2D (K267R) was overexpressed in C2C12 cells to assess its activity on target genes. The transcriptional activity of MEF2D (K267R) was derepressed, as evidenced by the increased expression of *myogenin* compared with C2C12 cells that overexpressed


(a)
-127
+56
E-box
Mef2-RE
E-box
TSS
MEF2 ChIP
Relative Bound Level
2
1.5
1
0.5
0
GM
DM2
DM4
*
*
Myogenin
promoter
MEF2 ChIP
Relative Bound Level
2
1.5
1
0.5
0
GM
DM2
DM4
Gapdh
promoter

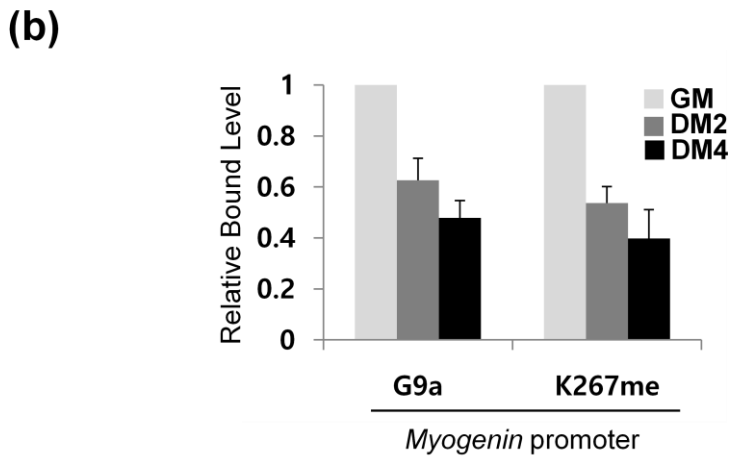

(b)
Relative Bound Level
1
0.8
0.6
0.4
0.2
0
GM
DM2
DM4
G9a
K267me
Myogenin promoter

Figure 28. G9a co-occupies with MEF2D on *myogenin* promoter.

(a and b) ChIP assays were performed with indicated antibodies using differentiated C2C12 cells. (a) Immunoprecipitated DNA fragments were analyzed for *the myogenin* or *gapdh* promoter and expressed, relative to the level of *myogenin* promoter bound in GM. MEF2 was bound on myogenin promoter in GM, and the binding increased upon differentiation. (b) Immunoprecipitated DNA fragments were analyzed for *the myogenin* promoter and expressed, relative to the bound level in GM. G9a was bound on the *myogenin* promoter in GM, and was dissociated as differentiated. MEF2 methylation was also decreased during differentiation.

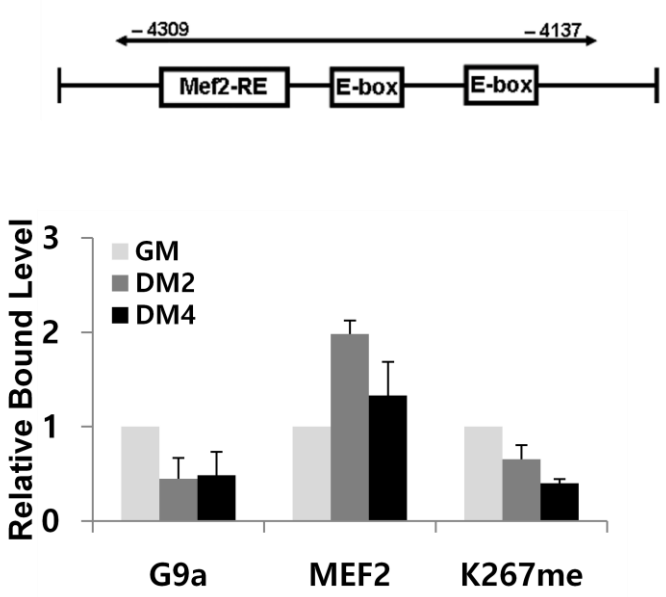


Figure 29. G9a co-occupies with MEF2D on *MCK* promoter.

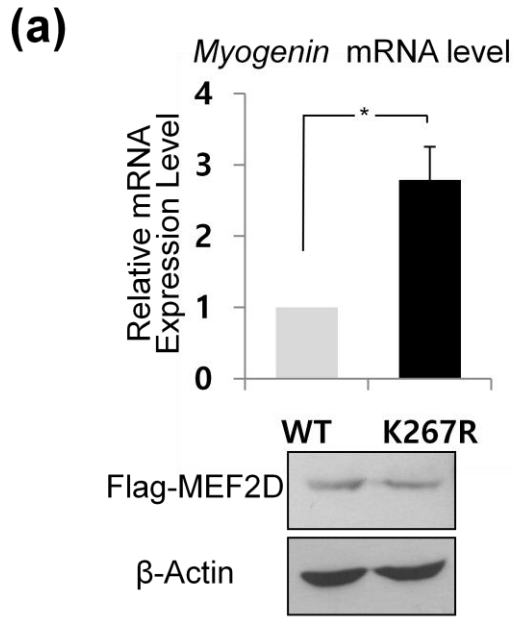
ChIP assays were performed using C2C12 cells differentiated for up to four days with antibodies indicated. Immunoprecipitated DNA fragments were analyzed for *MCK* promoter. All data are expressed relative to the bound level in cells cultured in GM. G9a binding and MEF2 methylation decreased, while MEF2 interaction increased during differentiation.

MEF2D (WT) (Figure 30a).

Because a lysine residue that corresponds to MEF2D K267 is a site of acetylation that affects DNA binding by MEF2C (33), we sought to determine the effects of methylation on the recruitment of MEF2D to chromatin. The DNA binding of Flag-MEF2D (WT) and Flag-MEF2D (K267R) in proliferating C2C12 cells was measured by ChIP assay. MEF2D (K267R) demonstrated enhanced DNA binding compared with MEF2D (WT) (Figure 30b). These results indicate that G9a-mediated MEF2D methylation regulates its recruitment to chromatin and transcriptional activity.

3-6. Knockdown of G9a enhances MEF2D-dependent transcription

To determine the effects of the dysregulation of G9a and MEF2D methylation, G9a was knocked down in C2C12 cells using shRNA-expressing lentiviral vectors. On downregulation of G9a, MEF2D methylation decreased by immunostaining (Figure 31a). Also, knockdown of G9a lowered MEF2D methylation levels on the *myogenin* promoter (Figure 31b). Consistent with previous results, the level of MEF2 that bound


(a)
Myogenin mRNA level
Relative mRNA Expression Level
4
3
2
1
0
*
WT
K267R
Flag-MEF2D
β-Actin

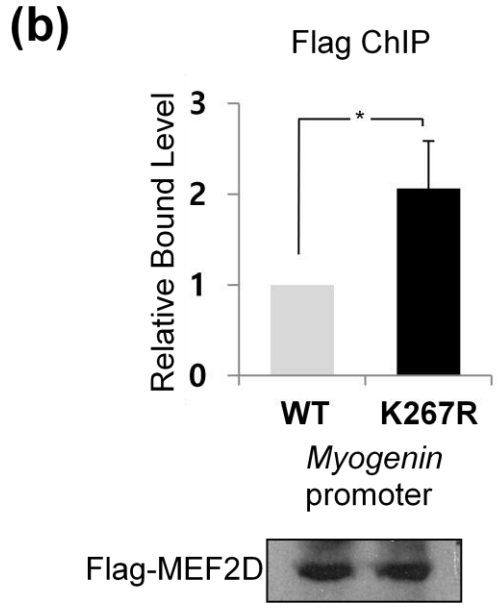

(b)
Flag ChIP
Relative Bound Level
3
2
1
0
*
WT
K267R
Myogenin
promoter
Flag-MEF2D

Figure 30. Methylation-defective mutant activates *myogenin* promoter in GM.

(a) C2C12 cells overexpressing Flag-MEF2D (WT) or Flag-MEF2D (K267R) were harvested for RNA preparation. *myogenin* mRNA level was analyzed by qRT-PCR. Transcribed level of *myogenin* was higher in cells overexpressing Flag-MEF2D (K267R) than Flag-MEF2D (WT). (b) C2C12 cells overexpressing Flag-MEF2D (WT) or (K267R) were harvested for ChIP assay. Immunoprecipitated DNA fragments were analyzed for *the myogenin* promoter and expressed, relative to the level of MEF2D bound (WT). (*) $p < 0.05$. Flag-MEF2D (K267R) was bound to the *myogenin* promoter more than Flag-MEF2D (WT).

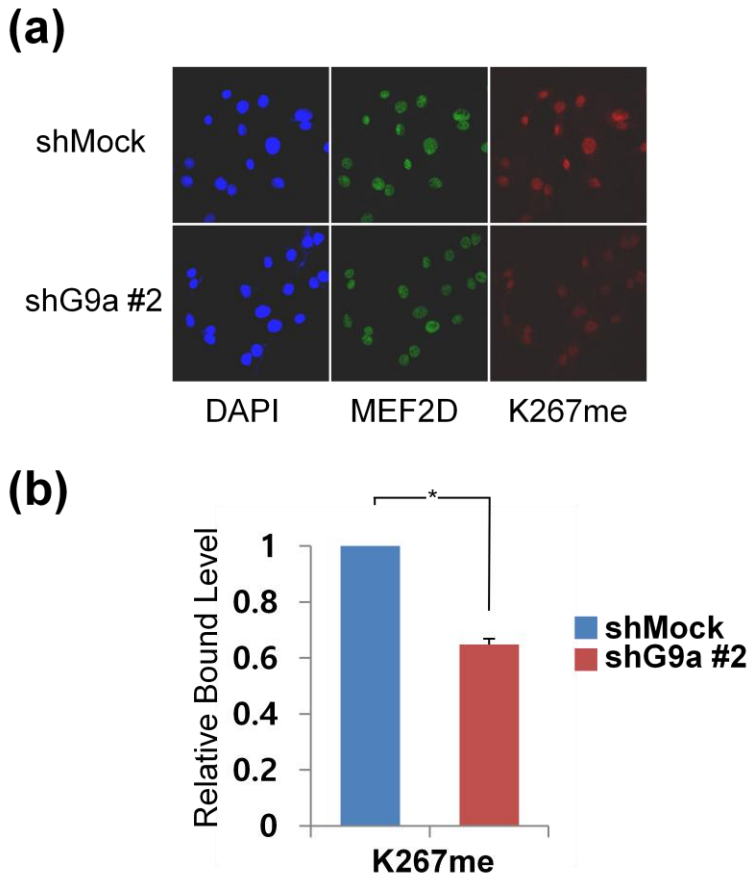

(a)
shMock
shG9a #2
DAPI
MEF2D
K267me
(b)
Relative Bound Level
1
0.8
0.6
0.4
0.2
0
*
K267me
shMock
shG9a #2

Figure 31. Knockdown of G9a decreases MEF2 methylation.

(a) MEF2D methylation in C2C12 cells infected with shMock or shG9a was analyzed by immunostaining with the indicated antibodies. MEF2D methylation was decreased in G9a-knockdown cells. (b) ChIP assays were performed with anti-K267me using proliferating C2C12 cells. Immunoprecipitated DNA fragments were analyzed for *myogenin* promoter and expressed, relative to the bound level in shMock. (*) $p < 0.05$. MEF2 methylation on the *myogenin* promoter was decreased in G9a-knockdown cells.

to the *myogenin* promoter rose in G9a knockdown C2C12 cells (Figure 32). Consequently, the mRNA of MEF2D target genes and the myogenic markers *myogenin*, *MCK,* and *MHC* was upregulated (Figure 33a–c). Moreover, by western blot, Myogenin levels increased (Figure 34a). Expression of myogenin were stimulated in C2C12 cells with lower levels of G9a (Figure 34b). Moreover, with knockdown of G9a, expression of differentiation marker, MHC was increased as well as the myotube formation (Figure 34c).

To confirm the effect of MEF2D methylation with regard to the enhanced transcriptional activity of MEF2D by G9a knockdown, MEF2D (WT) or MEF2D (K267R) was overexpressed in G9a knockdown and control C2C12 cells. Proliferating cells were harvested, and *myogenin* mRNA was analyzed. Overexpressed MEF2D (WT) had higher transcriptional activity in G9a knockdown cells versus control cells (Figure 35). Moreover, *myogenin* mRNA levels in MEF2D (K267R)–overexpressing control cells were comparable with those in G9a knockdown cells that overexpressed MEF2D (WT) and MEF2D (K267R) (Figure 35). Further, the transcriptional activities of MEF2D (K267R) were similar, regardless of the

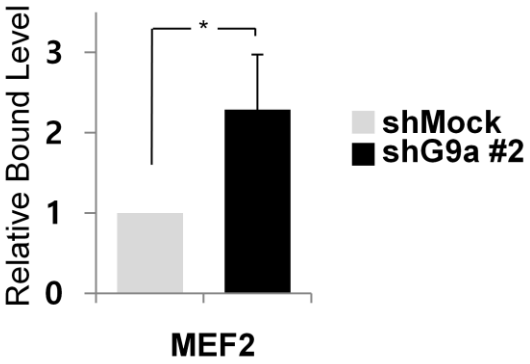


Figure 32. Knockdown of G9a decreases chromatin association of MEF2.

ChIP assays were performed with anti-MEF2 using proliferating C2C12 cells. Immunoprecipitated DNA fragments were analyzed for *myogenin* promoter and expressed, relative to the bound level in shMock. (*) $p < 0.05$. DNA binding affinity of MEF2 increased with the knockdown of G9a.

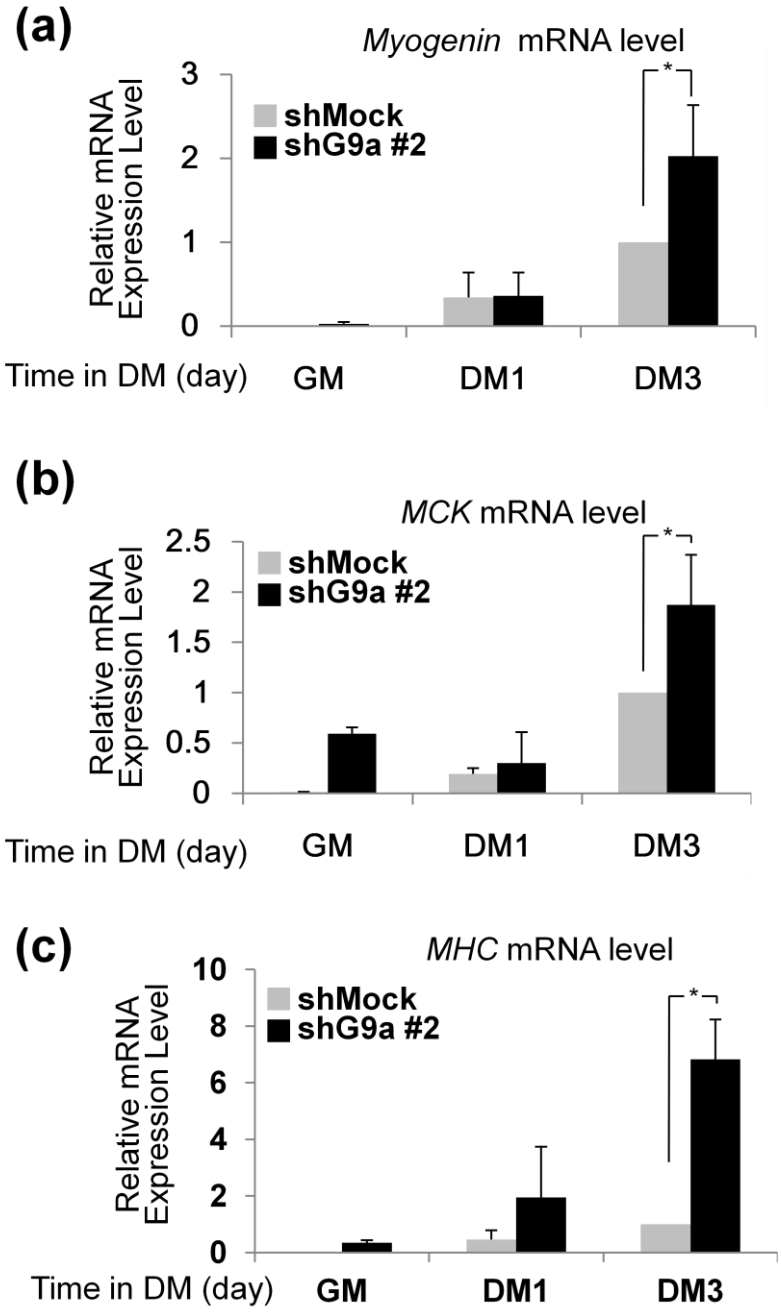

(a)
Myogenin mRNA level
Relative mRNA Expression Level
shMock
shG9a #2
3
2
1
0
*
Time in DM (day)
GM
DM1
DM3
(b)
MCK mRNA level
Relative mRNA Expression Level
shMock
shG9a #2
2.5
2
1.5
1
0.5
0
*
Time in DM (day)
GM
DM1
DM3
(c)
MHC mRNA level
Relative mRNA Expression Level
shMock
shG9a #2
10
8
6
4
2
0
*
Time in DM (day)
GM
DM1
DM3

Figure 33. Knockdown of G9a enhances myogenic gene expression.

(a–c) shMock– or shG9a–infected C2C12 cells were differentiated and harvested at the times indicated. *Myogenin* (a), *MCK* (b) and *MHC* (c) mRNA levels were analyzed by qRT–PCR. (*) $p < 0.05$. Myogenic gene expressions increased with the knockdown of G9a.

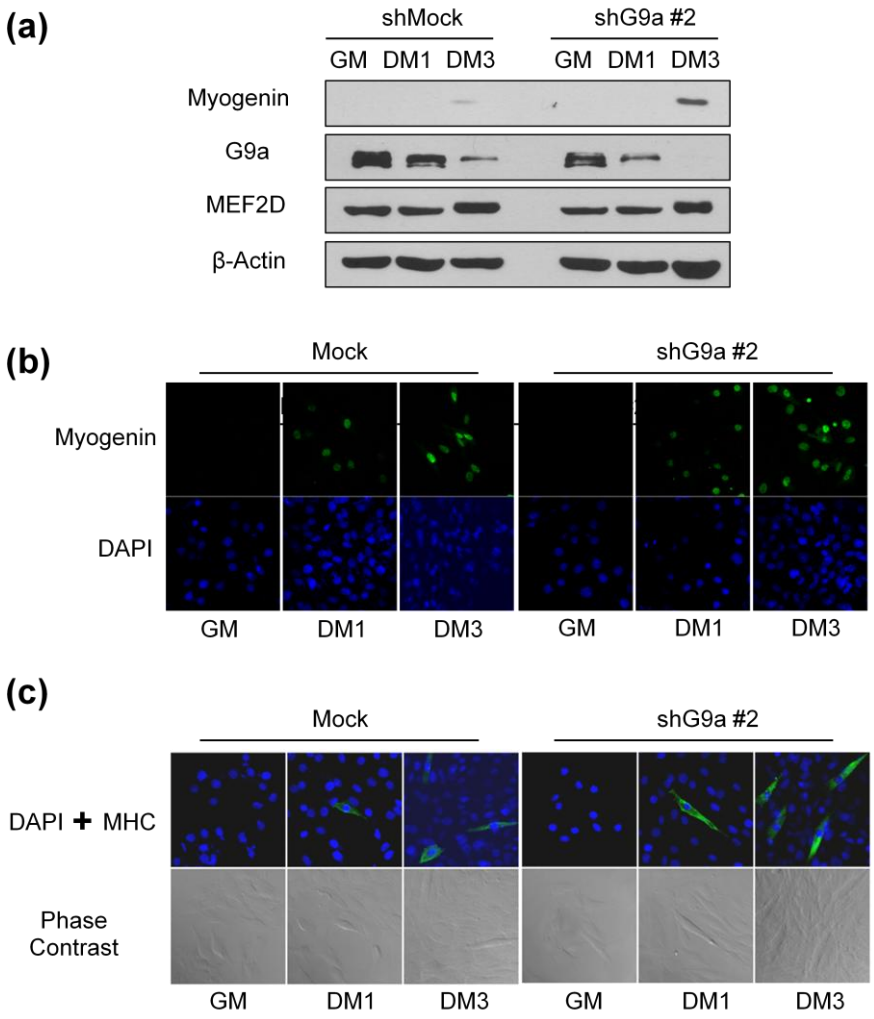


Figure 34. Knockdown of G9a enhances C2C12 cell differentiation. (a–c) C2C12 cells were differentiated and harvested at the times indicated. Differentiated cells were analyzed by immunoblotting (a) or immunostaining (b and c) with the indicated antibodies. Expression of the myogenic genes were enhanced in G9a-knockdown cells.

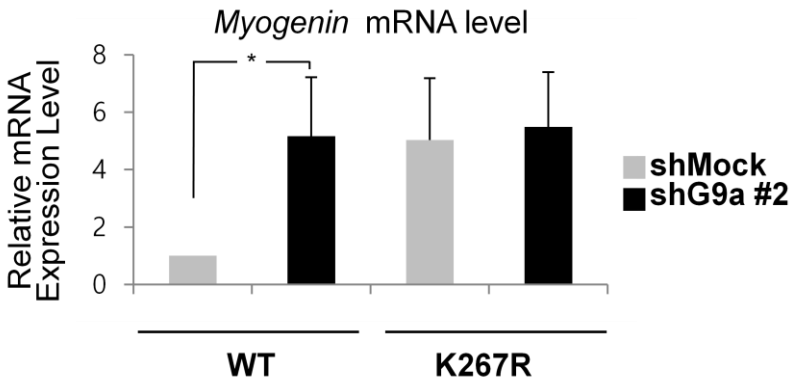


Figure 35. Knockdown of G9a enhances MEF2D (WT), but not MEF2D (K267R).

C2C12 cells infected with shMock or shG9a were infected with pMIG-MEF2D (WT) or MEF2D (K267R). *Myogenin* mRNA level was analyzed by qRT-PCR. (*) $p < 0.05$. The further increase of *myogenin* mRNA level in G9a knockdown cell was not observed when MEF2D (K267R) was overexpressed.

presence of G9a (Figure 35). These data indicate that C2C12 cells in which G9a has been knocked down show enhanced transcriptional activity of MEF2D due to inadequate methylation at K267. Thus, our findings confirm that G9a knockdown significantly decreases MEF2 methylation, inducing muscle differentiation.

3-7. Overexpression of G9a attenuates MEF2D transcriptional activity

To validate the function of G9a with regard to MEF2D transcriptional activity, the effects of G9a overexpression on MEF2D targets were determined during skeletal muscle cell differentiation. G9a was overexpressed in C2C12 cells by infecting them with a retrovirus that coexpressed GFP and G9a. By immunostaining, MEF2D methylation was induced in cells that were infected with G9a-expressing retrovirus (Figure 36a). MEF2D methylation also rose with exogenous expression of G9a by western blot (Figure 36b). Moreover, overexpression of G9a increased MEF2D methylation in the *myogenin* promoter (Figure 37a). The enhanced methylation of MEF2D by G9a overexpression resulted in a decrease in promoter-bound

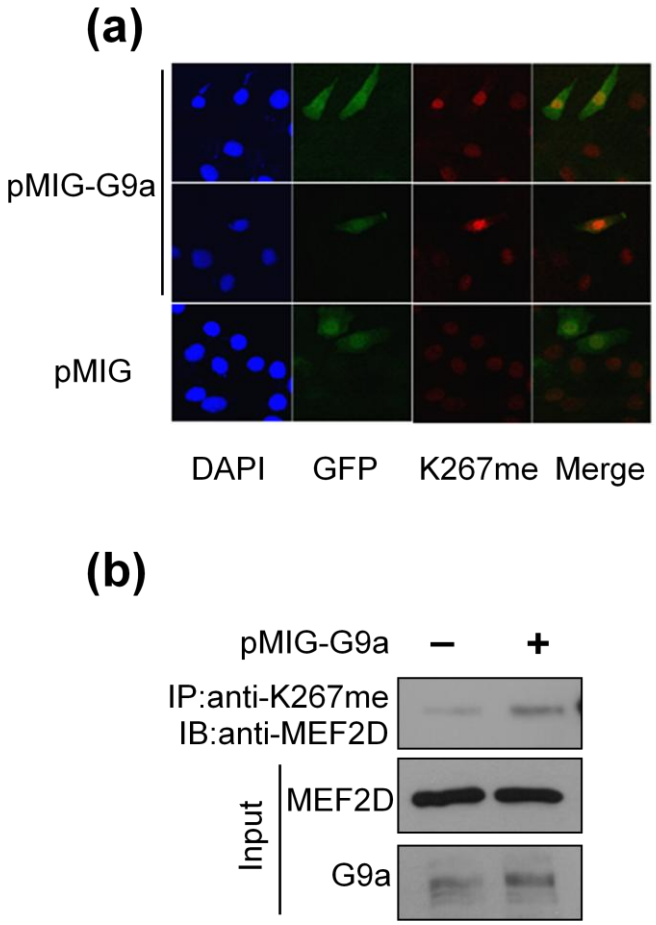


Figure 36. Overexpression of G9a increases MEF2D methylation.

(a and b) Retrovirus expressing G9a (pMIG-G9a) was used to infect C2C12 cells. Methylation of MEF2D K267 was analyzed by immunostaining (a) and immunoprecipitation (b). MEF2D methylation level was elevated with the overexpression of G9a.

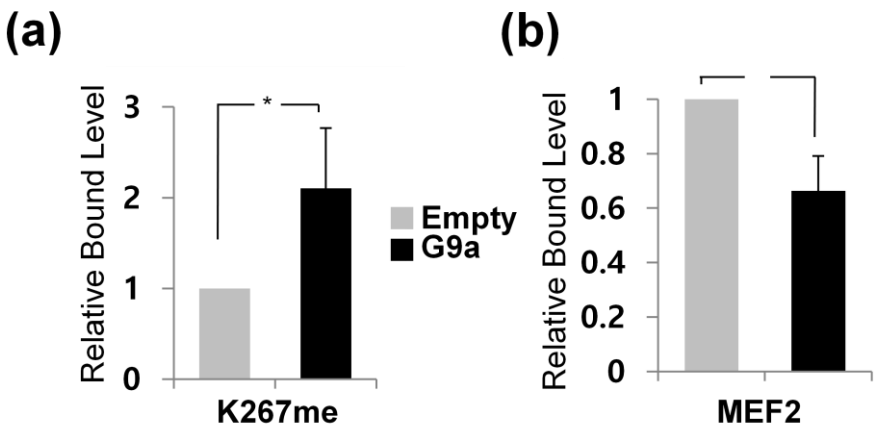


Figure 37. Overexpression of G9a decreases MEF2 chromatin association.

(a) ChIP assays were performed with anti-K267me using proliferating C2C12 cells. Immunoprecipitated DNA fragments were analyzed for *myogenin* promoter and expressed, relative to the bound level in GM. MEF2 methylation increased in G9a overexpressed cells. (b) C2C12 cells stably overexpressing G9a were differentiated and harvested for ChIP assay with anti-MEF2. The DNA binding affinity of MEF2 decreased with the overexpression of G9a.

MEF2 in differentiated C2C12 cells (Figure 37b). Consequently, MEF2-dependent transcription was repressed indicated by MEF2 target gene expression (Figure 38a). The mRNA and protein levels of myogenin were downregulated in G9a-overexpressing C2C12 cells (Figure 38a and b). Moreover, decrease in MHC level demonstrated repression of MEF2 activity in G9a-overexpression C2C12 cells (Figure 38b). The reduction in myogenin was also observed by immunostaining (Figure 39a). Further, C2C12 cells that experienced increased MEF2D methylation due to forced expression of G9a underwent impaired differentiation, as evidenced by immunostaining with anti-MHC (Figure 39b).

To determine the significance of MEF2 methylation in G9a-mediated inhibition of myogenesis, MEF2D (WT) or MEF2D (K267R) was overexpressed in C2C12 cells that stably expressed empty vector (Empty) or G9a (Figure 40a), and *myogenin* mRNA was measured after 24 hr of differentiation. Activity of transiently overexpressed MEF2D (WT) was inhibited by G9a, as reflected by the decrease in *myogenin* in G9a-expressing C2C12 cells compared with empty vector-expressing C2C12 cells (Figure 40b). However, MEF2D

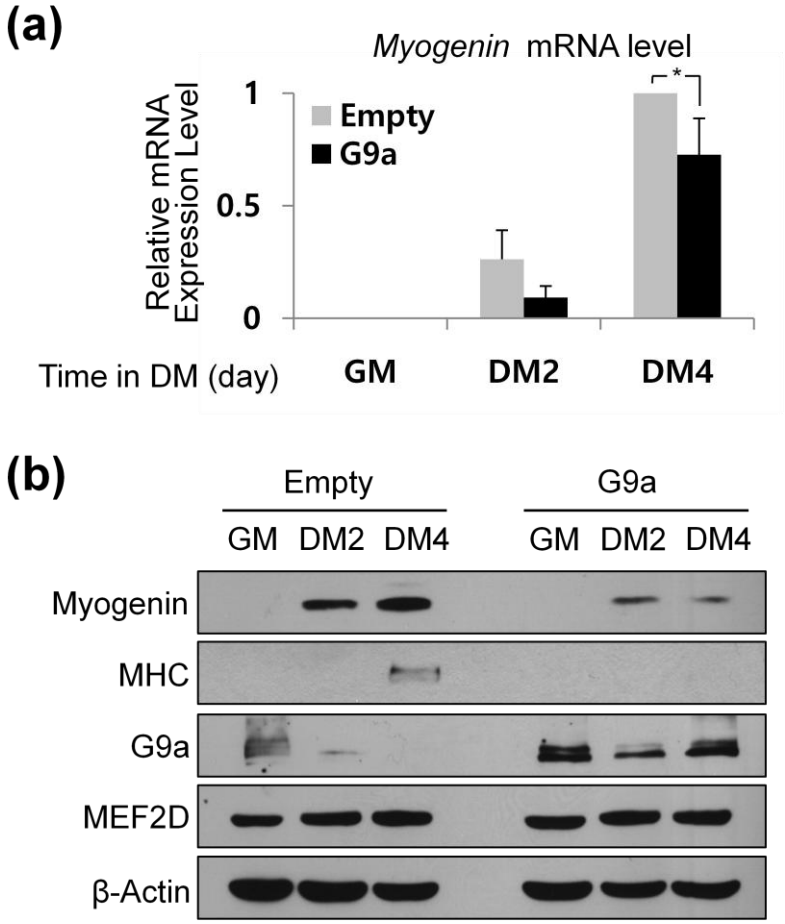


Figure 38. Overexpression of G9a represses myogenic genes.

(a) C2C12 cells stably overexpressing G9a were differentiated and harvested at the times indicated. *Myogenin* mRNA levels were analyzed by qRT-PCR. (b) C2C12 cells were differentiated as in (a) and analyzed by immunoblotting with the indicated antibodies. The expression of myogenic genes repressed by the overexpression of G9a.

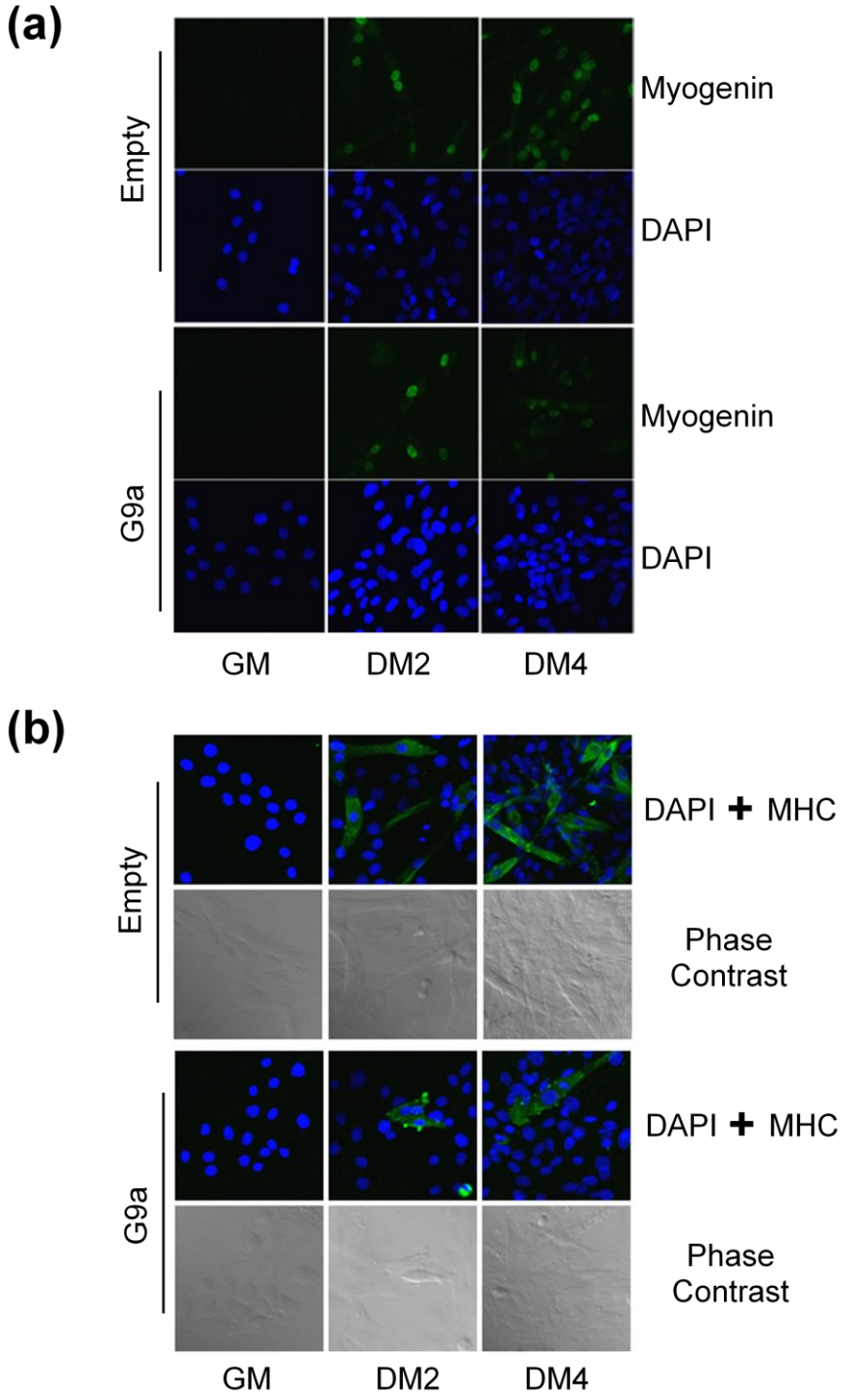

(a)
Empty
G9a
Myogenin
DAPI
Myogenin
DAPI
GM
DM2
DM4
(b)
Empty
G9a
DAPI + MHC
Phase Contrast
DAPI + MHC
Phase Contrast
GM
DM2
DM4

Figure 39. Overexpression of G9a impairs C2C12 cell differentiation.

(a) C2C12 cells stably overexpressing G9a were differentiated and analyzed by immunostaining with anti-Myogenin antibody. The expression of myogenin decreased with the overexpression of G9a. (b) G9a overexpressing C2C12 cells were differentiated and analyzed by immunostaining with anti-MHC antibody. Reduced expression of MHC and morphological changes were observed in G9a overexpressing cells.

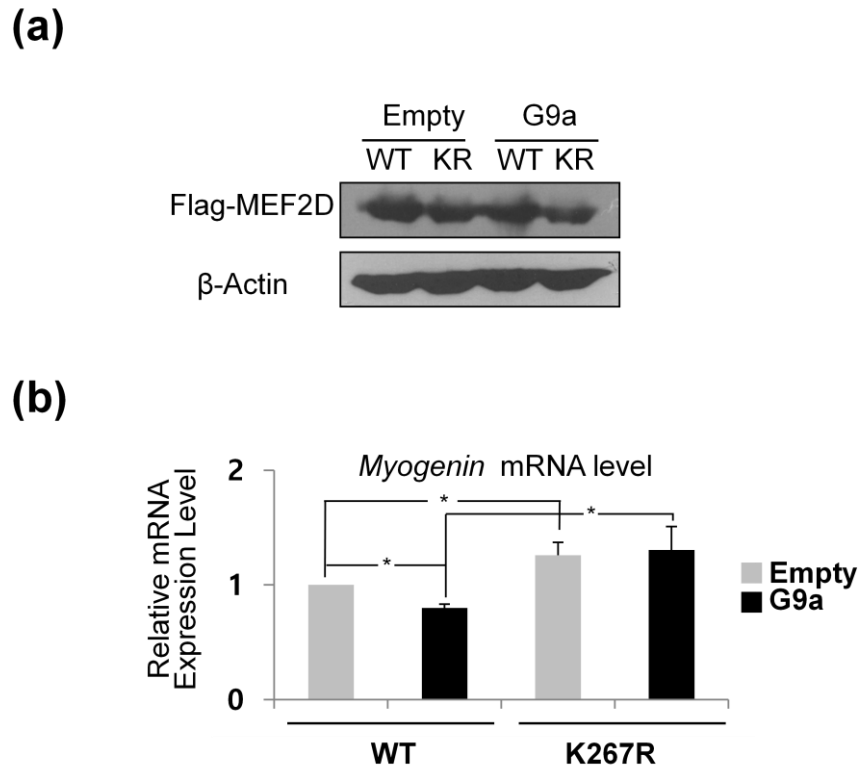


Figure 40. Methylation defective mutant of MEF2D is not repressed by overexpressed G9a.

(a) Wild type MEF2D or K267R mutant was overexpressed in C2C12 cells stably expressing empty vector or G9a. (b) C2C12 cells stably overexpressing G9a were transfected with Flag-MEF2D (WT) or Flag-MEF2D (K267R) and differentiated for 24 hr. *Myogenin* mRNA level was analyzed by qRT-PCR. (*) $p < 0.05$. Flag-MEF2D (K267R) showed the elevated transcription activity even with the overexpression of G9a.

(K267R) activity was unaffected by G9a, as shown by the comparable *myogenin* levels between C2C12 cells that expressed empty vector and G9a (Figure 40b). These data demonstrate that in G9a-overexpressing C2C12 cells, MEF2D-dependent transcription and differentiation are inhibited by MEF2 methylation on K267.

IV. DISCUSSION

In this study, we determined that MEF2 activity is regulated by reversible lysine methylation, a novel posttranslational modification, and newly identified G9a as a corepressor that methylates and regulates MEF2. The methylation of lysine in the transactivation domain, governed by G9a and LSD1, represses MEF2D transcriptional activity, effecting the downregulation of target genes. Our previous and current findings demonstrate that dysregulation of MEF2D methylation due to aberrant expression of G9a and LSD1 impedes and enhances myogenesis, respectively (13).

Transcription factors that lie upstream in transcription cascades are regulated by such mechanisms as posttranslational modifications to obtain strict control over a broad range of target genes. In *Drosophila*, in which a single dMEF2 regulates muscle development, MEF2 levels differentially regulate diverse patterns of target gene expression (21, 45). In vertebrate, 4 isoforms of MEF2 exist and are heavily modified by various kinases and acetyltransferases to regulate its activity over time and by location (20). In particular, the lysine residue of MEF2C that corresponds to residue 267 of MEF2D is acetylated by p300

(33), increasing its DNA binding and transcriptional activity (33), implicating the interactive regulation of MEF2 activity by methylation and acetylation. In myoblasts, MEF2 activity remains repressed by methylation, and MEF2 is demethylated on the initiation of differentiation cues. To fully activate MEF2, p300 is recruited and acetylates MEF2 in myogenesis, constituting a methylation–acetylation switch (Figure 41).

In addition, methylation might suppress the phosphorylation of nearby sites that are targeted by p38α. The region that encompasses lysine 267 is called the D–domain—ie, the p38α docking domain (31). Thus, G9a–dependent methylation of lysine 267might block the p38α–MEF2 interaction in myoblasts to maintain MEF2 activity at basal levels. Thus, it is possible that lysine methylation cooperates with other posttranslational modifications in determining the promoter– and differentiation stage–specific transcriptional activity in a complex gene activation program during myogenesis.

Among the corepressors that are recruited to MEF2 target promoters, Cabin1 has been reported to coordinate histone–modifying enzymes to regulate transcription of MEF2 target genes (24, 27). G9a, a novel corepressor of MEF2 that we have

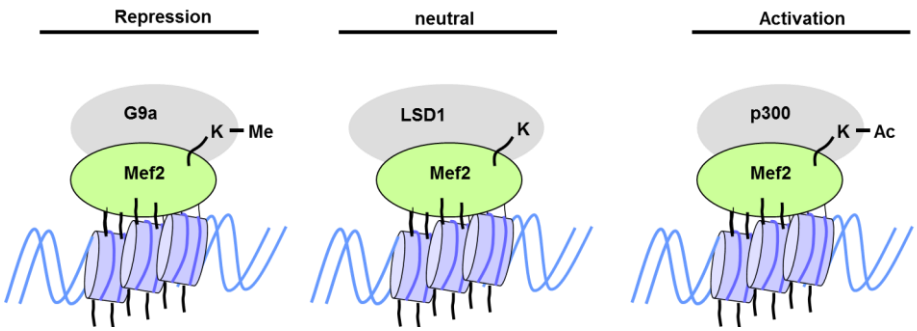


Figure 41. MEF2 is dynamically regulated by methylation and acetylation during myogenesis.

identified, interacts with Cabin1 (46). Thus, we speculate that Cabin1 forms a complex with G9a, in addition to Suv39h1 and HDACs, to modulate MEF2 activity. Sharp-1, a repressor of muscle differentiation (47), is another possible cofactor that mediates the repression of MyoD by G9a. Sharp-1 augments MyoD methylation by G9a and suppresses MyoD activity (48). Thus, Sharp-1 might function as an adaptor protein in G9a-dependent MEF2 methylation and repression.

Among the non-histone proteins that are targeted by G9a, MyoD is a substrate of G9a (2, 49). MyoD is a myogenic regulator that shares target genes with MEF2 to synergistically induce skeletal muscle differentiation (26). Binding elements of MyoD and MEF2 on common target promoters are positioned closely, allowing them to bind as a dimer (29). It is likely that MyoD and MEF2 share cofactors and are regulated similarly, because LSD1 also interacts and both MyoD and MEF2 to enhance myogenic differentiation (13). Thus, having demonstrated the mechanism by which G9a- and LSD1-mediated methylation regulates MEF2, our data support that G9a and LSD1 are the critical epigenetic regulators that govern 2 major classes of myogenic transcription factors and control

skeletal muscle differentiation precisely.

Our findings indicate that G9a and LSD1 regulate MEF2D transcriptional activity through methylation of lysine 267. In myoblast cells with basal MEF2 activity, G9a is expressed and bound to MEF2 target promoters. MEF2 methylation is high, and its target genes are undetected at the mRNA and protein levels. During differentiation, G9a mRNA and protein levels decrease. Consequently, the amount of G9a that binds to MEF2 target promoters declines. Moreover, the interaction between G9a and MEF2D decreases, while MEF2D methylation diminishes. This study implicates a mechanism in which G9a and LSD1 regulate MEF2D activity over time and by location by a novel posttranslational modification, lysine methylation.

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VI. Abstract in Korean

국문 초록

Myocyte enhancer factor 2 (MEF2) 는 MEF2A-D로 이루어져 있으며, 근육 분화를 비롯한 세포의 다양한 활동을 조절하는 전사인자로 잘 알려져 있다. 이들의 하위유전자를 조절하기 위하여, ME2D의 전사활성은 시간적, 공간적으로 조절되어야 한다. 에피제네틱 조절인자들이 히스톤 표지나 MEF2의 전사 후 변형에 관여하여 MEF2의 전사활성을 조절한다는 것이 밝혀진바 있지만, 전사인자의 활성을 조절하는데 중요하다고 알려진 라이신 잔기 메틸레이션에 의한 MEF2의 조절은 아직 알려진 바 없다. 우리는 골격근 분화 과정 중, G9a와 LSD1에 의해 MEF2D의 라이신 잔기가 메틸화 또는 탈메틸화 되는 것을 확인하였다. G9a가 MEF2D의 267번째 라이신 잔기를 메틸화 시킴으로써 전사활성을 억제하는 반면, LSD1은 이 메틸기를 탈메틸화 시켰고, MEF2D의 267번 라이신 잔기는 종간에 유지가 잘 되어있는 것을 확인하였다. 또한 생쥐 골격근 세포 분화 모델인 C2C12 세포의 분화과정 중, G9a에 의한 MEF2D의 메틸화가 감소하고 MEF2D에 의해 조절받는 근육 특이적 유전자의 발현이 증가하였는데 이러한 사실을 메틸화가 되지 못하는 MEF2D (K267R)을 이용하여 G9a에 의한 MEF2D 활성 조절이 메틸레이션에 의한 것임을 확인하였다. 따라서 이러한 MEF2D 라이신 잔기의 메틸/탈메틸화가 MEF2D의

전사활성을 조절하고, 이를 통해 크로마틴에 대한 결합력에 영향을 준다는 사실을 확인하였다. 본 연구를 통해 MEF2D의 267번째 라이신 잔기가 역동적으로 메틸화/탈메틸화 되는 것을 증명하였고, 이것이 MEF2D의 전사활성 조절과 골격근 분화과정에 있어 매우 중요한 조절기전임을 입증하였다.

주요어 :Myocyte enhancer factor 2 (MEF2), G9a, 메틸화, C2C12, 골격근 분화

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