MBD2b/DEMETHYLASE IS INVOLVED IN THE MYOGENESIS OF C2C12 MYOBLASTS

Huiping Ren

Department of Pharmacology and Therapeutics
McGill University
Montreal, Quebec, Canada

Submitted: Dec. 2003

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Your file Votre référence
ISBN: 0-612-98726-4
Our file Notre référence
ISBN: 0-612-98726-4

DEDICATION

I dedicate this manuscript to my dear husband Chen Ding. Without his encouragement and support, I will never overcome the difficulties met in my study.

THESIS FORMAT DESCRIPTION

The format of this thesis conforms to the requirements of the National Library of Canada outline in the ***Guidelines for Thesis Preparation*** *of the Faculty of Graduate Studies and Research of McGill University.*

This thesis includes an overall abstract written in English and in French, a general introduction and literature review as well as reference list. Chapter 3 consists of an abstract, an introduction, materials and methods, results and discussion. In addition, the thesis includes a section on the objective of the thesis, general conclusions and suggestions for future research. All references cited are listed at the end.

GENERAL ABSTRACT

CpG methylation is the major epigenetic modification of mammalian genomes and is essential for normal development, most likely due to its important roles in genomic imprinting, X chromosome inactivation, silencing of parasitic elements and tissue-specific gene expression. CpG methylation plays its role in transcriptional repression by recruiting methylated DNA binding proteins that in turn recruit other transcriptional repressors such as histone deacetylase (HDAC). Up to now, five members of methyl-DNA-binding proteins (MBD) have been identified, including MeCP2, MBD1-MBD4.

Initiating the muscle differentiation pathway typically results in activation of muscle-specific genes previously maintained in a silenced state. One of the processes controlling changes in gene expression during differentiation is a global demethylation event. The mechanisms involved in this global hypomethylation are not fully understood.

Among the MBD family, MBD2b is reported to possess a dual function: transcriptional repression as well as promoter specific demethylation. During the differentiation process of C2C12 myoblasts, MBD2b/demethylase is induced. Its induction peak corresponds to the peak of global demethylation and myogenin expression, a unique marker of muscle differentiation. Rapamycin, which inhibits p38 kinase, a kinase involved in the signaling pathway leading to C2C12 differentiation, abolished also the expression of MBD2b completely. This demonstrates that MBD2b/demethylase is a component of the differentiation process of C2C12 myoblasts.

Promoter methylation is one of the normal mechanisms inactivating gene expression. A single CpG site in the 5' flanking region of myogenin is reported to undergo demethylation during C2C12 differentiation. Considering the demethylase feature of MBD2b and its expression profile during C2C12 differentiation, I propose the hypothesis that MBD2b/demethylase is involved in C2C12 differentiation by demethylating the promoter of the myogenin gene as well as other genes involved in myogenic differentiation.

To test this hypothesis, I determined the consequences of up-regulation and down-regulation of MBD2b/demethylase in C2C12 cells. Forced expression of MBD2b/demethylase in C2C12 cells by an adenovirus carrying MBD2b/demethylase cDNA did enhance the expression of myogenin. However, antisense knockdown of MBD2b/demethylase reduced myogenin expression significantly compared with the GFP control. Antisense knoockdown of MBD2b/demethylase also resulted in global hypomethylation as indicated by HpaII digestion and Southern blot analysis with a probe corresponding to repetitive satellite DNA MR150.

The state of the myogenin promoter was also altered as determined using a probe recognizing a single HpaII site, which was previously reported to become demethylated during C2C12 differentiation. The extent of demethylation of the myogenin promoter was reduced by antisense knockdown of MBD2b/demethylase.

In summary, my data are consistent with a model that MBD2b/demethylase is involved in muscle differentiation by bring about the demethylation of the myogenin promoter.

RESUME GENERAL

La méthylation de l'DNA sur les sites CpG est la modification épigénétique majeure dans le génome des mammifères. Cette modification est essentielle pour le développement, probablement grâce à son rôle important dans la mise en place de l'empreinte génomique parentale, l'inactivation du chromosome X, des séquences parasites et l'expression génique tissus spécifiques. La méthylation des sites CpG joue un rôle dans la répression de la transcription par le recrutement des protéines MBD (Methyl Binding Domain), qui a leur tour, recrutent d'autres répresseurs transcriptionnels, tels que les histones déacétylases (HDAC). A ce jour, cinq membres de la famille MBD ont été identifies incluant MeCP2, MBD1 et MBD4.

L'initiation de la différenciation musculaire résulte de l'activation des gènes musculaires, qui étaient préalablement maintenus dans un état inactif. L'hypométhylation a été observée dans des myoblastes pendant leur différenciation afin de contrôler l'expression dans des gènes. Cependant, aucune étude n'est parvenue à identifier les facteurs responsables de ce changement au niveau de la méthylation.

Parmi les membres de la famille des MBD, MBD2b a été identifiée comme une protéine possédant une double fonctionnalité. En effet, elle est impliquée dans la répression de la transcription mais elle a également été identifiée comme un promoteur spécifique de la déméthylation. A la suite de la différenciation des myoblastes C2C12, l'induction de l'expression de la MBD2b déméthylase a été observée. Le pic d'expression de MBD2b correspond a celui de la déméthylation globale. Il concorde également avec l'expression de la Myogénine, l'unique marqueur de la différenciation des cellules musculaires. La rampamycine inhibiteur de la protéine kinase p38, une enzyme impliquée *dans la voie de signalisation conduisant à la différenciation des cellules C2C12, abolit complètement l'expression de MBD2b. Cela démontre que la MBD2b déméthylase est un des composants du mécanisme de différenciation des myoblastes C2C12.*

La méthylation des promoteurs est un des processus classiques permettant l'inactivation de l'expression des gènes. Un seul site CpG situe dans la région 5' du gène de la myogénine été identifie comme subissant une déméthylation au cours de la différenciation des C2C12. Etant donne la fonction déméthylase de MBD2b, et son profil d'expression au cours de la différenciation, je propose l'hypothèse selon laquelle la MBD2b déméthylase joue un rôle dans la différenciation des C2C12, en déméthylant le promoteur du gène de la myogénine.

La surexpression de la déméthylase MBD2b par l'adénovirus portant le gene n'augmente pas l'expression de la myogénine. Cependant, par l'expression d'un ARNm anti-sens de MBD2b, l'expression de la myogénine est significativement réduite. Une réduction de l'hypométhylation globale a pu être observée par l'hybridation de la sonde MR150 après digestion par HpaII.

Les resultats sont en accord avec un article antérieur, dans lequel il est démontré que le promoteur du gène de la myogénine est moins méthylé, en raison d'un mécanisme de déméthylation au cours de la différenciation des cellules C2C12.

En résume, mon travail soutient le modèle selon lequel la déméthylase MBD2b est *impliquée dans la différenciation des cellules musculaires par la déméthylation du promoteur du gène de la myogénine. Ceci est le premier exemple d'un rôle biologique de la déméthylase MBD2b.*

ACKNOWLEDGEMENTS

I am deeply indebted to my research supervisor, Dr. Moshe Szyf, for his support, guidance and advice, as well as for enriching my life both at the personal and scientific level. I highly appreciated his careful review of all of the work.

I am also grateful to Dr. Andrew Slack, for generation the recombinant adenovirus used for infection.

I extend my gratitude to Dr. Qiangli Zhuang, for the help on flow cytometry.

I am grateful to Ms. Qiaoling Cui, for her help on statistic analysis.

I would also like to express my sincere thanks to all my laboratory colleagues. I am especially grateful to Ms. Jingni Ou, for friendship, and her work on the general abstract in French. I also wish to thank Ms. Johanne Theberge, for support and assistance in the laboratory. I would also like to acknowledge Ms. Shelly Brown, Ms. Veronica Bovenzi, Ms. Ann D'alessio, Ms. Nancy Detich, Ms. Snezana Milutinovic, Mr. Stefan Hamm, Mr. Jerome Torrisani and Mr. Iann Weaver for their friendship and help.

I offer my sincere thanks to the Department of Pharmacology and Therapeutics of McGill University for fostering a positive growth environment. I would like to thank all staff members, especially Pam Moore and Helene Duplessis, and professors in the department for their help and support.

I would like to thank Dr. Almazan, for her advice on my study and Dr. Trasler, for her review of my thesis.

I also wish to acknowledge the finical support of the Faculty of Medicine, McGill University.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLE

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Cytosine methylation and gene silencing

Proper methylation is critical for the normal function of the organism

Methylation at CpG dinucleotides is the most abundant epigenetic modification in vertebrate genomes (Razin and Szyf 1984; Yisraeli and Szyf 1984). Two decades of research resulted in the understanding that maintaining correct DNA methylation patterns is essential for the normal function of an organism. A group of genes has been identified to be responsible for establishing and maintaining the DNA methylation patterns, which includes the DNA methyltransferase family (DNMT) and the methylated DNA binding domain (MBD) family of genes. Homozygous deletion of genes belonging to these two families of genes causes embryonic lethality and some defective phenotypes even when the mice are viable (Li 2002). Several methylation related diseases have been reported, including Rett syndrome (Amir, Van den Veyver et al. 1999; Shahbazian, Young et al. 2002), ICF (immunodeficiency, centromere instability, and facial deficiency) (Hansen, Wijmenga et al. 1999; Xu, Bestor et al. 1999) and fragile X syndrome (Kremer, Pritchard et al. 1991; Oberle, Rousseau et al. 1991). Cancer is
also believed to be a methylation related disease. Genome wide hypomethylation and chromosome instability, as well as hypermethylation and silencing of normally expressed genes such as tumor suppressor genes are common in cancer. In addition, the aberrant expression of DNMT and MBD genes has been well documented in a variety of cancers (Esteller and Herman 2002). Elucidating the molecular function of DNA methylation is important for understanding critical biological processes as well as for developing new therapeutics for human diseases.

Cytosine methylation is a common form of DNA modification in mammalian

The information content of mammalian DNA is not limited strictly to a linear sequence of bases. Mammals modify their DNA through methylation of cytosine residues at the 5 position of the pyrimidine ring. This modification occurs at cytosine found in the dinucleotides CpG. Approximately 60-90% of all CpG dinucleotides in the mammalian genome are methylated, the majority of these sites are found in repetitive elements, inactive X-chromosome and at imprinted loci, suggesting its role in silencing of mobile elements, X chromosome inactivation and genomic imprinting (Razin and Riggs 1980; Razin and Szyf 1984). While sparsely distributed CGs are highly methylated, CpG islands which are clusters of unmethylated CpG sites frequently found near the promoter and first exon of protein coding genes are protected from methylation (Wade 2001). The precise mechanisms by which these regions are protected from methylation are not clear. Some reports suggest that an Sp1 site located in the promoter region may prevent de novo methylation (Szyf, Bozovic et al. 1991; Brandeis, Frank et al. 1994; Macleod, Charlton et al. 1994).

The DNA methylation pattern is the result of a dynamic interaction between methylation and demethylation reactions

Genomic methylation patterns in somatic cells are generally stable and heritable. However, in mammals there are at least two stages at which methylation patterns are reprogrammed.

The genomes of the mature sperm and egg in mammals are highly methylated. Genome wide demethylation occurs during development of primordial germ cells and is completed by E13 to E14 in both male and female germ cells. Remethylation takes place several days later. It occurs earlier, however, in the male germ line than in the female. (Reik, Dean et al. 2001).

In embryos, the paternal genome is demethylated immediately after fertilization, prior to the first cell division by an active process. The demethylation of the maternal genome depends on DNA replication and occurs later. It is unclear whether this is a passive or active process. After implantation, most of the parental genome is modified by remethylation. Only genes containing CpG islands, such as housekeeping genes, escape this process (Reik, Dean et al. 2001). The final pattern of methylation of tissue-specific genes is achieved later through site-specific demethylation in expressing tissues.

DNMT members are responsible for the establishment of methylation pattern in vivo

DNMT members can be divided into two groups, depending on their enzymatic activities. De novo methyltransferases catalyze the transfer of a methyl group to previously unmethylated DNA while maintenance methyltransferases show preference for hemimethylated DNA. De novo activities are mainly found during early embryogenesis and are low in somatic tissues. Maintenance methyltransferase activity is coupled to the cell cycle (Bestor 2000). Cellular DNA methylation patterns seems to be established by a complex interplay of at least three DNA methyltransferases: DNMT1, DNMT3A and DNMT3B. The activity and function of DNMT2 are still undefined (Okano, Xie et al. 1998).

DNMT1 is the first methyltransferase to be discovered (Bestor, Laudano et al. 1988), which is believed to be responsible for maintaining DNA methylation patterns after replication. DNMT1 efficiently methylates DNA containing hemi-methylated CpG (Bestor 1992), which is generated during replication since the nascent DNA is unmethylated whereas the parental strands are methylated. DNMT1 poorly methylates DNA that has unmethylated sites on both strands. Thus, it copies methylation patterns from the parental to the nascent strands but does not introduce new methylation (Razin and Riggs 1980; Guenbaum, Stein et al. 1981; Bestor 1992). DNA methylation occurs concurrently with DNA replication (Gruenbaum, Szyf et al. 1983; Araujo, Knox et al. 1998; Araujo, Knox et al. 1999) DNMT1 is found to associate with replication foci in proliferating cells (Leonhardt, Page et al. 1992; Iida, Suetake et al. 2002), ensuring proper methylation of daughter strands during DNA replication. Dnmt1 knockout mice show global demethylation and die before birth (Li, Bestor et al. 1992), which is consistent with the critical role of DNMT1 in maintenance of DNA methylation pattens.

The fact that embryonic stem cells from Dnmt1 knock out mice were viable and maintained residual detectable DNA methylation (Lei, Oh et al. 1996) led to the identification of two new de novo methyltransferases DNMT3a and DNMT3b (Okano, Xie et al. 1998). Both Dnmt3a-/- and Dnmt3b-/- knock out mice showed defects in genome methylation and died before completing proper development (Okano, Bell et al. 1999).

The functional co-operation between DNMT family members is important for maintaining the methylation of repetitive elements in mouse (Liang, Chan et al. 2002) and in human colon cancer cells (Rhee, Bachman et al. 2002). Recently, human DNMT3a

and DNMT3b were reported to form a complex with DNMT1 (Kim, Ni et al. 2002), suggesting that both de novo and maintenance enzymes function together to establish and keep the proper methylation patterns.

Beside the function of generation and maintenance of DNA methylation patterns, some studies coming out recently demonstrated that DNMTs are proteins with multiple functions *in vivo*. Inhibition of DNA methyltransferase by antisense knockdown induces genes in a mechanism that does not involve DNA methylation, suggesting that DNMT1 can regulate gene expression by mechanisms that do not involve DNA methylation (Milutinovic, Knox et al. 2000; Miutinovic, Zhuang et al. 2003). All DNMTs except DNMT2 have been reported to mediate transcriptional repression through HDAC, which is not relying on its catalytic function (Fuks, Burgers et al. 2000; Robertson, Ait-Si-Ali et al. 2000; Rountree, Bachman et al. 2000; Bachman, Rountree et al. 2001; Fuks, Burgers et al. 2001). Newly identified DNMT3L (Aapola, Kawasaki et al. 2000; Aapola, Lyle et al. 2001), lacking methyltransferase activity but functioning as the regulator for maternal imprinting (Bourc'his, Xu et al. 2001; Hata, Okano et al. 2002), is also proved to be associated with HDAC and mediate repression (Aapola, Liiv et al. 2002; Deplus, Brenner et al. 2002).

Cytosine methylation is involved in gene regulation by repressing transcription

It is widely believed that cytosine methylation is an epigenetic mechanism that controls gene expression by repressing transcription. Numerous genes are inactivated by promoter hypermethylation and the list is still growing. There are several potential mechanisms that might lead to transcriptional repression at methylation loci.

First, methylation at cytosine residues in the recognizing element of transcription factor blocks the binding of transcriptional activators to these elements. Several transcription factors were found to be sensitive to the methylation status of their binding sites, including E2F, creB, AP2, cMyc/Myn, NF-kB, cMyb and so on (Bergman and Mostoslavsky 1998). However, this is not the case for all transcription factors.

The second mechanism involves the change in chromatin structure that is precipitated by DNA methylation around the promoter. This mechanism is mediated by proteins which specifically recognize methylated DNA and target them for silencing by recruiting chromatin modification enzymes such as histone deacetylases and histone methyltransferase. Five methylated DNA binding proteins have been identified in mouse and human (Lewis, Meehan et al. 1992; Hendrich and Bird 1998; Hendrich, Abbott et al. 1999). They all contain a conserved methyl-CpG binding domain (MBD), and are able to form complexes with methylated DNA, except for MBD3 (Lewis, Meehan et al. 1992; Hendrich and Bird 1998; Hendrich, Abbott et al. 1999).

How do methyl-CpG-binding proteins repress transcription? MeCP2 was first identified to have selective affinity for methylated DNA (Lewis, Meehan et al. 1992). Besides its MBD domain, a methylation-dependent transcriptional repression domain (TRD) was mapped to its C-terminal. (Nan, Campoy et al. 1997). Upon further analysis, it was found that TRD is associated with a co-repressor complex containing the transcriptional repressor mSin3 and histone deacetylase 1 (HDAC1). The silencing mediated by MeCP2 and methylated DNA can be partially relieved by deacetylase inhibitor trichostatin A (TSA) (Nan, Ng et al. 1998). This finding unraveled a linkage between DNA methylation, histone acetylation and chromatin remodeling, finally

establishing a causal relationship between methylation-dependent transcriptional silencing and the modification of chromatin structure. The associations between MBD1-3 and histone deacetylase were subsequently established using a similar approach (Ng, Zhang et al. 1999; Zhang, Ng et al. 1999; Ng, Jeppesen et al. 2000; Saito and Ishikawa 2002), suggesting transcriptional repression through HDAC at methylated CpG sites is a normally utilized mechanism *in vivo* to inactivate gene expression.

Demethylation and gene activation

Global hypomethylation and local demethylation

Under physiological conditions, global hypomethylation takes place during gametogenesis and in pre-implantation embryos (Reik, Dean et al. 2001). Loss of methylation has also been documented in cells undergoing differentiation (Scarpa, Lucarelli et al. 1996; Jost, Oakeley et al. 2001). In cancer cells, DNA methylation is lost to a large degree, leading to genome instability (Fruhwald and Plass 2002). Site-specific demethylation is proposed as the mechanism to activate the expression of tissue-specific genes. Such demethylation events have been observed in the activation of muscle specific α-actin genes (Paroush, Keshet et al. 1990) and *myogenin* gene (Lucarelli, Fuso et al. 2001), d-crystallin gene in lense fiber cells (Dirks, Klok et al. 1996), GLU4 gene in adipocytes (Yokomori, Tawata et al. 1999), immunoglobulin k locus in cultured B cells (Kirillov, Kistler et al. 1996), and myeloperoxidase gene in myeloid cells (Lubbert, Miller et al. 1991). However, it is still not clear how demethylation happens in a time and spatial manner at a specific locus.

Passive demethylation and active demethylation

Both passive and active mechanisms can lead to demethylation. Passive demethylation arises from the absence of methylation activity after DNA replication. DNMT1 prefers hemimethylated DNA and is targeted to replication foci through its association with PCNA (Leonhardt, Page et al. 1992; Iida, Suetake et al. 2002). Thus loss of function of DNMT1 can cause demethylation and this effect becomes obvious after several rounds of DNA replication. The demethylation of the maternal genome during early mouse embryogenesis seems consistent with this mechanism (Rougier, Bourc'his et al. 1998). However, the fast demethylation of the paternal genome after fertilization (Mayer, Niveleau et al. 2000) indicates the existence of active demethylation.

Up to now, three such mechanisms have been proposed to explain how active demethylation happens *in vivo*. They differ in the first step performed by the putative demethylase: direct replacement of the methyl moiety by a hydrogen atom, excision of the methylated base, or excision of the methylated nucleotide. The latter two mechanisms need further DNA repair activities after the initial step. MBD2b is an example of a direct demethylase which demethylates methylated DNA by removing a methyl group directly from cytosine (Bhattacharya, Ramchandani et al. 1999; Ramchandani, Bhattacharya et al. 1999). Jost et al. observed active demethylation in chicken embryos (Jost, Siegmann et al. 1995) and subsequently isolated a DNA glycosylase preferring hemimethylated CpG and breaking a single DNA strand for the initiation of the demethylation reaction (Zhu, Zheng et al. 2000). Demethylation through excision of the dinucleotide appears to occur *in vitro* (Weiss, Keshet et al. 1996), but the enzyme catalyzing this reaction has not been identified.

DNA demethylation and the activation of tissue specific genes

DNA methylation is generally accepted as a mechanism to silence transcription and turn off gene expression. Consequently, demethylation is believed to reactivate genes silenced by hypermethylation. Site-specific demethylation and gene activation have been documented in a variety of systems, but the molecular mechanism for this correlation is unclear. Szyf proposed a model that the specificity of demethylation is determined by interaction of *cis-* and *trans*-acting factors and demethylation is controlled by the availability of the demethylase (Szyf 1991; Szyf 1994; Szyf 1996). Supporting this model, "hypomethylation" signals are identified in the CpG islands of a variety of tissue-specific genes (Paroush, Keshet et al. 1990; Szyf, Tanigawa et al. 1990; Brandeis, Frank et al. 1994; Santoso, Ortiz et al. 2000). But what is the gating mechanism for demethylase? Chromatin remodeling mediated by histone modification seems to be involved in that control (Selker 1998; Cervoni, Detich et al. 2002; Zhang, McKinsey et al. 2002) . By using the *Neurospora crassa* system, Selker observed that TSA, an inhibitor of histone deacetylase, caused selective loss of methylation and suggested that acetylation of chromatin protein can directly or indirectly control DNA demethylation (Selker 1998). The muscle-specific gene *myogenin* undergoes demethylation and activation upon differentiation (Scarpa, Lucarelli et al. 1996; Lucarelli, Fuso et al. 2001; Fuso, Cavallaro et al. 2001). Recently, the report from Olson's group demonstrated that nucleosomal histones surrounding the promoter region of *myogenin* were highly methylated in undifferentiated myoblasts when the gene was silent, and became acetylated during muscle differentiation when *myogenin* was expressed at a high level (Zhang, McKinsey et al. 2002). The direct evidence that acetylation of histone controls demethylation comes from Cervoni's work (Cervoni and Szyf, 2001). By using a transient transfection system, she has shown that histone acetylation induces DNA demethylation and this replication-independent demethylation is due to MBD2b/demethylase (Cervoni and Szyf, 2001). Forced expression of Set/TAF-1β, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA and over-expression of a mutant Set/TAF-1beta, that does not inhibit histone acetylation, is defective in inhibiting DNA demethylation (Cervoni, Detich et al. 2002). Taking together, the data presented by Selker (Selker 1998), Zhang (Zhang, McKinsey et al. 2002) and Cervoni (Cervoni and Szyf , 2001; Cervoni, Detich et al. 2002) support the model proposed by Szyf (Szyf, 1991; Szyf, 1994; Szyf 1996) that histone acetylation is involved in the control of DNA demethylation by determining the availability of demethylase to the targeted sequences.

MBD2b, a dual function protein, provides a flexible switch between methylation and demethylation

MBD2 was cloned based on its sequence similarity with the methyl-CpG binding domain of MeCP2. High homology exists between human and murine MBD2 genes, and they are both mapped to chromosome 18 (Hendrich, Abbott et al. 1999). MBD2 is widely detected in somatic tissues, but its expression in embryonic cells is absent. An alternative splicing variant is detected in testes. MBD2a and MBD2b are two forms of MBD2, which corresponds to the translation initiation site at either the first (MBD2a, 43.5Kda) or second (MBD2b, 29.1Kda) methionine codon respectively. MBD2b shares up to 70%

sequence similarity with MBD3 (another member of MBD family, lacking methylated DNA binding activity) and they form a heterodimer *in vivo* (Hendrich and Bird 1998). The heterodimer is targeted to replication foci in late S phase through its association with DNMT1 (Tatematsu, Yamazaki et al. 2000). *In vitro*, MBD2 shows specific binding to methylated DNA and GFP tagged MBD2 co-localizes with heavily methylated satellite DNA *in vivo*. This localization is disrupted in cells that have greatly reduced levels of CpG methylation (Hendrich and Bird 1998). The minimal repression domain of MBD2b overlaps with its methyl-CpG binding domain (Boeke, Ammerpohl et al. 2000), and Nan (Nan, Ng et al. 1998) provides the evidence that MBD2 can repress transcription through a histone deacetylase mediated mechanism.

Besides the fact that MBD2 mediates transcriptional repression, studies from our lab also support the demethylase activity of MBD2b. Purified MBD2bdemethylase from human cancer cells can transform methylated cytosine to cytosine by cleaving methyl residue from the pyrimidine ring and release it as methanol (Ramchandani, Bhattacharya et al. 1999). This enzyme shows specificity to both fully methylated and hemi-methylated DNA (Ramchandani, Bhattacharya et al. 1999) and demethylates *in vitro* methylated plasmid when its cDNA is transfected into human host cells (Bhattacharya, Ramchandani et al. 1999). Bisulfite mapping reveals that MBD2b/demethylase is a processive enzyme, and the rate- limiting step is the initiation of demethylation (Cervoni, Bhattacharya et al. 1999). Forced expression of MBD2b/demethylase in HEK293 cells leads to the demethylation and activation of two methylated reporters pSV40-CAT and pGL2T+14xTBRE in a time and dose dependent manner, while no influence is observed in p19-ARF reporter (Detich, Theberge et al. 2002).

Human cancer cells exhibit both a global hypomethylation and some localized hypermethylation, suggesting that the deregulation of the methylation machinery is a central event in tumorigenesis. As the first example of a protein that has a dual function on both gene repression and activation, MBD2b seems to be involved in this process, but its function is gene and tumor type dependent. MBD2b is reported to be associated with hypermethylated GSTP1, MDR1 and p16/p14 (Magdinier and Wolffe 2001; Bakker, Lin et al. 2002; El-Osta, Kantharidis et al. 2002; Lin and Nelson 2003) and to be responsible for their inactivation. Other papers investigated the expression of MBD2 in cancers and found that MBD2b is down-regulated (Kanai, Ushijima et al. 1999; Muller-Tidow, Kugler et al. 2001; Billard, Magdinier et al. 2002; Patra, Patra et al. 2002). However, in some cases, the methylation status of genes and their expression are not related to the expression of MBD2b (Paroush, Keshet et al. 1990; Sato, Horio et al. 2002). Recently, the inhibition of anchorage-independent growth of cancer cells by antisense MBD2b treatment was reported (Slack, Bovenzi et al. 2002). The same treatment also reduced tumor size when injected into xenograft tumor (Slack, Bovenzi et al. 2002).

Myogenesis and methylation

Introduction on myogenesis

The formation of skeletal muscle involves a series of steps in which multi-potential mesodermal precursor cells become committed to a muscle cell fate and then proliferate as myoblasts until they encounter an environment lacking mitogen, at which point they exit the cell cycle and differentiate. This process is accompanied by fusion of mononucleate myoblasts to form multinucleate muscle fibers, transcriptional activation of

muscle-specific genes and repression of genes associated with cell proliferation (Sabourin and Rudnicki 2000).

The process of myogenesis is regulated by four myogenic regulatory factors (MRFs): MyoD, Myf-5, *myogenin* and MRF4. All of them are able to induce myogenic conversion when over expressed in a vast number of non-muscle cell lines (Arnold and Winter 1998; Perry and Rudnicki 2000). The MRF proteins are muscle-specific transcriptional factors, they contain a conserved basic DNA-binding domain, which shows specificity to a sequence known as E-boxes (CANNTG). This DNA motif is present in the promoter of many skeletal muscle-specific genes and mediates gene activation in an MRF-dependent manner. Each MRF is able to form a heterodimer *in vitro* and *in vivo* with E proteins. The heterodimer then selectively recognizes and binds to an E-box, triggering the activation of transcription (Perry and Rudnicki 2000).

MRFs are classified into two groups of factors primary MRFs and secondary MRFs, as determined by targeted inactivation experiments that measure their requirement for muscle development. The primary MRFs, MyoD and Myf-5, are required at the determination step for commitment of the proliferating somatic cells to the myogenic lineage. The secondary MRFs, *myogenin* and MRF4, drive the proliferation of committed myoblasts and their further differentiation into myocytes. In addition, studies have demonstrated that some MRFs can substitute for one another without affecting overall muscle development, suggesting potential redundancy among MRFs (Weintraub 1993).

The activity of MRFs is regulated in a cell cycle dependent manner

The activity of MRFs is strictly controlled by several mechanisms. The activation of MRFs involves the formation of a complex with E-protein. Id factor and mTwist protein have been found to heterodimerize with E proteins, preventing its association with MRFs and subsequent muscle-specific gene activation (Benezra, Davis et al. 1990; Benezra, Davis et al. 1990; Spicer, Rhee et al. 1996). The cell-cycle MRF connection is substantiated by the following observations. First, up-regulation of cell cycle inhibitors p21 and p16 is observed in myoblasts upon the initiation of differentiation. p21 has an E-box in its regulatory region and is activated by MyoD over-expression (Guo, Wang et al. 1995; Halevy, Novitch et al. 1995). Second, the activity of MyoD is compromised by its interaction with cyclin D1 and Cdk4 (Skapek, Rhee et al. 1995; Zhang, Wei et al. 1999). Third, various oncogenes such as c-Jun, c-Fos, c-myc, N-ras and H-ras inhibit muscle differentiation *in vitro*, suggesting that growth-promoting factors negatively regulate MRFs (Olson, Spizz et al. 1987; Li, Chambard et al. 1992; La Rocca, Crouch et al. 1994).

Signal transduction pathways during myogenesis

Myoblasts undergo differentiation upon switching into culture medium with low serum. This process is accelerated by including insulin or insulin like growth factor (IGF) I (Florini, Ewton et al. 1991) or II (Rosenthal, Hsiao et al. 1994) in the medium. Studies on signaling through IGF/IGF-I receptor by using synthetic inhibitors revealed two main pathways by which these signals were transmitted: the phosphatidylinositol 3-kinase (PI3K)/PKB-AKT/p70S6-kinase (P70S6K) pathway and the Ras/Raf/mitogen activated protein kinase (MAPK) pathway (Coolican, Samuel et al. 1997; Jiang, Aoki et al. 1999; Xu and Wu 2000). LY294002, an inhibitor of PI3 kinase, and rapamycin, an inhibitor of p70S6 kinase, completely abolished IGF-I stimulation of myogenesis in rat

L6A1 myoblasts. PD098059, an inhibitor of MAP kinase activation, enhanced myogenesis by inhibiting Ras induced proliferation in the same differentiation systems (Coolican, Samuel et al. 1997). Moreover, data from Conejo and his colleagues identified NF-kB and AP-1 as nuclear factors of the above two pathways. The induction of NF-kB DNA binding activity and down-regulation of AP-1 activity were observed in the differentiation of C2C12 myoblasts (Kaliman, Canicio et al. 1999; Conejo and Lorenzo 2001; Conejo, de Alvaro et al. 2002). In addition, p38-MAPK is also reported to promote C2C12 and L8 cell differentiation (Cuenda and Cohen 1999; Zetser, Gredinger et al. 1999; Cabane, Englaro et al. 2003).

Myogenin during muscle differentiation

Myogenin was first isolated and sequenced by Wright (Wright, Sassoon et al. 1989). It is absent in undifferentiated cells, peaks and then declines following a stimulus of differentiation (Wright, Sassoon et al. 1989). *Myogenin* belongs to the secondary MRFs, and is the only member of the MRF family that is expressed in all skeletal muscle cells. During embryogenesis, the activation of *myogenin* is observed at 8.5 dpc followed by MyoD at about 10.5 dpc along with markers of terminal differentiation. In tissue culture cells, *myogenin* expression is rapidly up-regulated when myoblasts enter into the differentiation pathway in response to withdrawal of growth factors (Cheng, Tseng et al. 1995). The importance of *myogenin* for development has been elucidated by targeted inactivation. Mice lacking *myogenin* display a normal number of myoblasts but die at birth because of an absence of myofibers (Hasty, Bradley et al. 1993; Nabeshima, Hanaoka et al. 1993).

In accordance with the changes in cell-cycle regulation that take place during differentiation, the activation of the *myogenin* gene has been correlated with high expression of p21 during embryogenesis (Parker, Eichele et al. 1995). In addition, c-Fos is down-regulated by direct *myogenin* binding to its E-box located in the promoter region (Trouche, Masutani et al. 1995). The activity of *myogenin* is also negatively regulated by protein kinase C (PKC) directly and protein kinase A through an indirect mechanism (Li, Heller-Harrison et al. 1992; Li, Zhou et al. 1992).

Myogenesis and chromatin remodeling

DNA methylation, histone modification and ATP-dependent remodeling complexes are all associated with chromatin remodeling. Although they function together *in vivo* to control myogenesis, only the latter two components will be discussed in this section. The effect of DNA methylation on myogenesis will be addressed in the next section.

The structure of chromatin is influenced by the acetylation state of histone. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze these two opposite reactions and thereby act as transcriptional activators and repressors respectively. p300 and PCAF are transcriptional coactivators with intrinsic HAT activity (Strahl and Allis 2000). p300 stimulates myogenesis through its association with MyoD (Eckner, Yao et al. 1996), while either antibody against p300 (Eckner, Yao et al. 1996) or the dominant negative form of p300 (Sartorelli, Huang et al. 1997) represses MyoD-mediated transcription and myogenesis. PCAF is also critical for myogenesis, but it functions through the interaction with p300, not MyoD (Puri, Sartorelli et al. 1997). HATs can promote myogenesis by acetylating histone tails since acetylation weakens the interaction

between histone and DNA phosphate backbone. On the other hand, HDACs inhibit this process resulting in a condensed chromatin. HDACs interact with MEF2 proteins, a group of transcriptional factor required for myogenesis (Black and Olson 1998), and repress the expression of MEF2-dependent genes (Lu, McKinsey et al. 2000). Moreover, a subclass of HDACs, HDAC5, is shown to shuttle from the nucleus to the cytoplasm when myoblasts are triggered to differentiate, suggesting its role in the control of myogenesis (McKinsey, Zhang et al. 2000).

The evidence that ATP-dependent chromatin remodeling complexes positively regulates myogenesis is provided by the work from a US group. BRG1 and BRM are the ATPase subunits for two SWI/SNF chromatin remodeling complexes. In the presence of mutant BRG1 and BRM, NIH3T3 fibroblasts lose their ability to differentiate even when MyoD is expressed by a retrovirus vector (de la Serna, Carlson et al. 2001). The upstream region of the *myogenin* promoter is inaccessible in NIH3T3 cells expressing dominant negative BRG1 or BRM, demonstrating that the promotion effect of SWI/SNF on differentiation is due to chromatin opening around the *myogenin* promoter (de la Serna, Carlson et al. 2001; de la Serna, Roy et al. 2001).

Myogenesis and methylation

The initiation of muscle differentiation is accompanied by transcriptional activation of muscle specific genes and transcriptional inactivation of genes involved in DNA replication and cell cycle progression. Demethylation observed during the process of muscle differentiation is possibly involved in the activation of gene expression.

The potential link between methylation and myogenesis was first observed by treating 10T1/2 fibroblasts with 5-azacytidine, an agent which inhibits CpG methylation. The drug treatment converts 10T1/2 fibroblast to myoblasts at high frequency, implying that genome hypomethylation plays a role in myogenic differentiation (Lassar, Paterson et al. 1986). Other investigators subsequently extended these observations to different differentiation systems such as rat L5 myoblasts (Scarpa, Lucarelli et al. 1996) and mouse G8 myoblasts (Jost and Jost 1994; Jost, Oakeley et al. 2001). Supporting the hypothesis that demethylation is involved in differentiation, a rapid drop of DNA methyltransferase activity following by genome wide demethylation was observed during differentiation (Jost, Oakeley et al. 2001). Using antibody against DNA methyltransferase, the same group also showed the loss of nuclear DNA methyltransferase upon differentiation (Liu, Sun et al. 1996). In addition, Szyf et al. reported that expression of the DNA methyltransferase cDNA in an antisense orientation was sufficient to induce muscle differentiation in transfected 10T1/2 cells (Szyf, Rouleau et al. 1992).

The correlation between methylation and myogenesis is also reflected in the activation of two MRFs protein: MyoD and *myogenin*. The CpG island of the MyoD promoter is constitutively unmethylated *in vivo* (Jones, Wolkowicz et al. 1990). However, a conserved distal enhancer element of MyoD undergoes a regulated demethylation before MyoD gene is activated (Brunk, Goldhamer et al. 1996), and the methylation status of this enhancer is tightly correlated with MyoD transcription. In myogenic cells, the identified enhancer is completely unmethylated, but significantly methylated in non-muscle cells and tissues (Brunk, Goldhamer et al. 1996).

As a transcriptional factor responsible for myogenic terminal differentiation, the activation and expression of *myogenin* is extensively studied. The expression of *myogenin* occurs simultaneously with the genome hypomethylation during the differentiation process of L5 myoblasts (Scarpa, Lucarelli et al. 1996). Recently, Scarpa et al. reported demethylation of the *myogenin* promoter during the differentiation process of C2C12 mouse myoblasts. By combining HpaII/MspI digestion and methylation sensitive PCR, they showed a correlation between the demethylation of a single CpG site in the promoter region and the expression of *myogenin*. This CCGG site is hypomethylated *in vivo* in embryonic mouse muscle, while non-muscle tissues have a fully methylated site. The fast demethylation profile suggests that an active demethylation mechanism is involved (Lucarelli, Fuso et al. 2001). S-adenosylmethionine (SAM) inhibits *myogenin* expression and myoblast differentiation by delaying the demethylation of that specific CpG site in differentiating myoblasts (Fuso, Cavallaro et al. 2001).

Besides the demethylation and activation of muscle-specific transcriptional factors, the muscle structural gene α-actin is activated by the same mechanism. Its demethylation requires cis-acting elements (Paroush, Keshet et al. 1990).

Although overall DNA hypomethylation seems to promote myoblast differentiation, methylation at specific loci (for example, genes involved in proliferation) may help to speed up this process. The above hypothesis is supported by the observation that overexpression of DNA methyltransferase in myoblasts accelerates myotube formation (Takagi, Tajima et al. 1995). However, the specific loci responsible for this change have not been identified. Another group also reported that an alternative Dnmt1 isoform is specifically expressed in differentiated myotubes, whereas the ubiquitously expressed isoform is down-regulated during myogenesis (Aguirre-Arteta, Grunewald et al. 2000). Considering all these together, it stands to reason that DNA methylation and demethylation work synergistically to promote myogenesis.

CHAPTER 2: THESIS OBJECTIVES

Cytosine methylation is a common modification in the mammalian genome (Wade 2001), which is generally accepted as an important mechanism for controlling gene expression programs (Nan, Campoy et al. 1997; Nan, Ng et al. 1998). In general, DNA methylation of a gene suppresses its expression while an unmethylated gene is active. Consequently, DNA demethylation is believed to play a role in gene activation (Bergman and Mostoslavsky 1998). Since cellular differentiation is a complex process accompanied with the activation of multiple genes simultaneously, it stands to reason that demethylation of these genes must be involved.

Myogenic cell lines that could be induced to differentiate *in vitro* are well-defined systems to study tissue-specific gene activation during differentiation since many muscle-specific transcriptional factors, which are activated upon differentiation, are characterized (Perry and Rudnicki 2000). In some myogenic cell lines such as C2C12, the time course for the activation of these genes during differentiation is well delineated. Demethylation is correlated with muscle differentiation as demonstrated by the observation that differentiation of myogenic cell lines *in vitro* is associated with global hypomethylation (Jost and Jost 1994; Scarpa, Lucarelli et al. 1996; Jost, Oakeley et al. 2001). Moreover, the activation of some muscle-specific genes such as *MyoD* (Brunk, Goldhamer et al. 1996), *myogenin* (Fuso, Cavallaro et al. 2001) and *α-actin* (Parough, Keshet et al. 1990) is associated with their demethylation. The idea that demethylation plays a causal role in myogenic differentiation is supported by experiments showing that either inhibition of DNA methylation with the DNA methylation inhibitor 5-aza deoxycytidine (5-aza-CdR) (Jones, Wolkowicz et al. 1990) or by antisense knock down of DNA methyltransferase 1 (DNMT1) causes the myogenic differentiation of 10T1/2 cells (Szyf , Rouleu et al. 1992) However, no study had been done to elucidate the protein that catalyzes the demethylation reaction during myogenic differentiation.

Whereas enzymes that methylate DNA such as DNMT1, DNMT3a/3b were known for decades, it was unclear whether enzymes that demethylate DNA exist. The common wisdom in this field is that DNA methylation is an irreversible process and the active removal of methyl groups from DNA is thermodynamically impossible (Smith 2000). Therefore, other mechanisms were sought to explain DNA demethylation such as DNA replication in the absence of DNMT1 (Bester 2000) or mismatch repair glycosylases that could remove the complete methylated base and consequently replace it with an unmethylated base by a repair process (Jost, Siegmann et al. 1995). However three years ago it was shown in our laboratory that the enzyme activity that removes methyl moieties from methylated cytosine was expressed in human cells (Ramchandani, Bhattacharya et al. 1999) and MBD2b/demethylase, the first demethylase candidate was subsequently cloned.

MBD2b/demethylase belongs to methyl DNA binding protein family, the abnormal expression of MBD2b/demethylase is documented in a variety of tumors (Kanai, Ushijima et al. 1999; Muller-Tidow, Kugler et al. 2001; Billard, Magdinier et al. 2002; Patra, Patra et al. 2002). The fact that antisense MBD2b/demethylase abolishes anchorage-independent growth of cancer cells and reduces tumor growth rate demonstrates its role in tumorigenesis (Slack, Bovenzi et al. 2002). The above conclusion is further supported by the following observations. First, Mbd2-deficient mice are viable

and fertile, but resistant to intestinal cancer (Sansom, Berge et al. 2003). Second, electrotransfer of an MBD2-antisense encoding plasmid inhibits tumor growth in *vivo*. This anti-tumor effect is augmented while combined with bleomycin, a cytotoxic drug for anti-tumor chemotherapy (Ivanov, Lamrihi et al. 2003). However, up to now there is no report regarding the role of MBD2b/demethylase in normal physiological processes such as cellular differentiation. In this thesis, I will test the hypothesis that MBD2b/demethylase plays a causal role in myogenic differentiation through catalyzing demethylation and activation of myogenic specific genes.

First, the time course for the expression of MBD2b/demethylase during myogenesis will be defined. This would set up the potential relation between MBD2b/demethylase expression, genome wide hypomethylation and *myogenin* expression. *Myogenin* is a myogenic specific transcription factor, which is induced upon myogenic C2C12 differentiation and is responsible for activating multiple muscle specific genes (Rudnicki and Jaenisch, 1995). Second, I will test whether the expression of MBD2b/demethylase is dependent on the induction of myogenic differentiation by using rapamycin, an inhibitor of myogenesis which blocks PI3K/PKB-APT/p70S6 signal transduction pathway for C2C12 differentiation (Conejo, de Alvaro et al. 2002). Third, the hypothesis that MBD2b/demethylase plays a causal role in myogenesis will be addressed by knocking down MBD2b/demethylase using an adenoviral vector expressing antisense MBD2b/demethylase.

CHAPTER 3

MBD2b/DEMETHYLASE IS INVOLVED IN THE MYOGENIC DIFFERENTIATION OF MOUSE C2C12 MYOBLAST CELLS

ABSTRACT

Muscle differentiation is accompanied with global hypomethylation, demethylation and activation of muscle-specific genes. MBD2b/demethylase is the only enzyme having active demethylation activity characterized to date. In this Chapter, the potential role of MBD2b/demethylase in myogenesis is investigated. MBD2b/demethylase is highly induced upon the initiation of differentiation. It peaks on day 5 of differentiation, fitting the time window of the expression of myogenin (a unique marker for myogenesis) and global demethylation. Rapamycin, an inhibitor of the PI3K/PKB-AKT/p70S6 signal transduction pathway for C2C12 differentiation, abolishes the expression of MBD2b/demethylase completely, implying that MBD2b/demethylase is a downstream effector of this pathway and definitely required for differentiation. Knockdown of MBD2b/demethylase by antisense MBD2b/demethylase RNA, which was introduced into C2C12 myoblasts by an adenoviral tool and subsequently expressed, reduced the expression of myogenin as well as global methylation. Demethylation of a specific site in the promoter region of myogenin is inhibited by the application of antisense MBD2b/demethylase. Taken together, the data support the model that MBD2b/demethylase is involved in myogenesis through demethylation of the promoter of myogenin. This is the ***first study*** *determining the function of MBD2b/demethylase in a physiological process.*

Introduction

DNA methylation contributes to the control of gene expression and plays an essential role in cellular physiology; one such event is muscle myogenesis. Myogenesis is accompanied by genome wide hypomethylation, as methylation inhibition by 5-azacytidine (Lassar, Paterson et al. 1986) and antisense knock down of DNMT1 (the main player for maintaining methylation) (Szyf, Rouleau et al. 1992) converted 10T1/2 fibroblasts to myoblasts at high frequency. The expression and activation of *Myogenin*, the master regulator for terminal differentiation of myoblasts (Cheng, Tseng et al. 1995), were also correlated with genome hypomethylation during differentiation (Scarpa, Lucarelli et al. 1996). However, it was not clear if the activation of *myogenin* was the consequence of genome wide hypomethylation or if it was specifically targeted to be demethylated. Later work revealed that the demethylation of a single CpG site in the promoter region of *myogenin* is responsible for its activation (Lucareli, Fuso et al. 2001). The expression of *myogenin* and muscle differentiation are delayed by exogenous

application of S-adenosylmethionine (SAM), the methyl donor, to the cells, through delaying the demethylation of *myogenin* promoter (Fuso, Cavallaro et al. 2001). The rapid progress of promoter demethylation observed in the process of differentiation suggests that an active mechanism is involved (Fuso, Cavallaro et al. 2001).

Both passive and active mechanisms are involved in demethylation. MBD2b belongs to a methyl-CpG binding protein family, which was first cloned and identified in our lab to be the enzyme catalyzing active demethylation by removing methyl groups from methylated cytosine directly (Bhattacharya, Ramchandani et al. 1999; Ramchandani, Bhattacharya et al. 1999). This discovery is further supported by the work from Cervoni (Cervoni, Bhattacharya et al. 1999), Detich (Detich, Theberge et al. 2002) and Slack (Slack, Bovenzi et al. 2002) respectively.

Since MBD2b/demethylase posseses active demethylation activity, I investigated its role in myogenesis. Following induction of C2C12 myoblast differentiation by serum starvation, induced expression of MBD2b/demethylase is observed. The peak of its expression corresponds to the peak of global demethylation and *myogenin* expression. Rapamycin is an inhibitor for p38 kinase and thus blocks the signal transduction pathway involved in C2C12 differentiation and abolishes the expression of MBD2b/demethylase completely. Antisense knockdown of MBD2b/demethylase reduced *myogenin* levels significantly and a reduction in global methylation was also observed by applying methylation sensitive HpaII/MspI digestion analysis on the highly repetitive and methylated MR150 satellite sequence. Consistent with previous reports, the promoter region of *myogenin* is demethylated during differentiation, as determined by HpaII/MspI methylation sensitive restriction enzyme digestion and Southern blotting hybridization with a probe recognizing a single CpG site in the promoter region of *myogenin*, which is reported to undergo demethylation during C2C12 differentiation. The demethylation of the *myogenin* promoter was reduced by antisense knockdown of MBD2b/demethylase. Taken together, all these data support the model that MBD2b/demethylase is involved in muscle differentiation by demethylating the promoter of *myogenin* as well as other sequences. This is the **first example** of a biological role of MBD2b/demethylase in the process of cellular differentiation.

Materials and Methods

Cell culture and Differentiation: mouse C2C12 myoblasts (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum (FCS, Colorado Serum Co.) (~10^6 cells/100mm plate) in 10% FCS-DMEM at 37°C and 5% CO_2. The cells were initiated to differentiate by serum starvation. Mouse P19 embryonic carcinoma cells (American Type Culture Collection) were maintained in 10% FCS-DMEM at 37°C and 5% CO_2. Aggregates were formed in bacterial culture dishes with the supplementation of 1mM retinoic acid (Sigma) for 48 hours. Aggregates were subjected to differentiation when seeded into cell culture dishes containing 10% FCS-DMEM.

Preparation and Analysis of RNA: At the end of the culture period, cells were washed once with phosphate-buffered saline (PBS). Total RNA was prepared by the guanidinium isothiocyanate method (Chromcyanski and Sacchi, 1987) and mRNA levels were

determined by Northern blot analysis. Approximately 20 μg of total RNA was electrophoresed on a 1.0% denaturing agarose gel and then transferred onto a charged nylon filter (Hybond N+, Amersham Pharmacia Biotech). Blots were probed with the indicated ^{32}P-labelled cDNA probe synthesized using a random priming labeling kit (Roche Molecular Biochemicals). The membranes were hybridized at 65°C for 4 hours in a buffer containing 0.5M sodium phosphate, pH 6.8, 1mM EDTA, 7.0% SDS and 0.2mg/ml herring sperm DNA. Following hybridization, the membranes were washed twice for 10 min each in a 5% SDS, 0.04M sodium phosphate, pH6.8, 1mM EDTA solution and then four times for 10min each in the same solution with only 1% SDS. The level of expression of the different mRNAs was quantified by denstiometric scanning of the relevant autoradiograms. Each experiment was normalized for the amount of total RNA by hybridization with a ^{32}P-labelled 18s ribosomal RNA oligonucleotide probe (Wu, Issa et al. 1993).

The probes for performing Northern blot analysis were generated as follows:

A 0.5-kilobase pair fragment bearing mouse MBD2 cDNA was excised from plasmid pETMbd2b (a gift from Dr. Brain Hendrich) (Hendrich and Bird, 1998) using NcoI.

Full-length mouse MBD3 cDNA (0.8-kilobase pair) was excised by NcoI and SmaI from the pETMbd3 plasmid (a gift from Dr. Brain Hendrich) (Hendrich and Bird, 1998) and sub-cloned into pBluescript SK plasmid in the SmaI site. The MBD3 insert was then excised with NotI and EcoRI to generate the riboprobe.

A 1.3-kilobase pair fragment encoding human MBD2 was excised from plasmid pcDNA3.1 (Bhattachrya, Ramchandani et al. 1999) using NotI.

Myogenin cDNA fragment was a gift from Dr. E.N. Olson and was sub-cloned into EcoRI site of plasmid pCVDV (Szyf, Rouleau et al. 1992).

Genomic DNA isolation and Southern blot analysis: Genomic DNA was prepared following the standard procedure (Ausubel, Brent et al. 1988). In brief, C2C12 cell pellets were treated with protease K at 50°C overnight at a final concentration of 1μg/μl in a buffer containing 50mM Tris-HCl, pH8.0, 100mM EDTA and 0.5% SDS. Crude DNA was extracted twice with phenol/chloroform (1:1, v/v) and the concentration of DNA was quantified spectrophotometrically. 20μg of genomic DNA was digested at 37°C overnight with 40U of XbaI (MBI), followed by methylation-sensitive restriction enzyme HpaII/Msp (MBI) digestion for another eight hours. For Southern blot analysis, 12μg of double digested DNA was fractionated on a 1.0% agarose gel and then transferred onto a charged nylon membrane (Hybond N+; Amersham Pharmacia Biotech). The membrane was pre-hybridized at 42°C for 2h in a solution containing 6× SSC, 5× Denhardt's, 1%SDS, and 0.05% tetrasodium pyrophosphate (100× Denhardt's = 2% Ficoll, 2% polyvinyl pyrrolidine, 2% BSA (bovine serum albumin). The filter was then hybridized in the same solution with the indicated ^{32}P labeled probe synthesized using a random priming labeling kit (Roche Molecular Biochemicals) at 42°C overnight. Following hybridization, the membrane was washed twice for 10mins each in a 2× SSC, 0.5% SDS solution and then four times for 10mins each in a 0.2× SSC, 0.5% SDS solution. The methylation status of each sample was established by measuring the intensity ratio between the indicated bands.

A DNA probe scanning the promoter region (nucleotide 1022-1520) of the mouse *myogenin* gene (GeneBank™ accession number M95800) was generated by *Taq* PCR

amplification using the primer pair as following: the forward primer 5'-TGGAGTGGTCCTGATGTGGTAGTGG-3' (nucleotides 1022-1046) and the backward primer 5'-ACCCAGAGATAAATATAGCCAACGC-3' (nucleotides 1496-1520) (Lucarelli, Fuso et al. 2001). PCR was performed under the conditions of an initial 3 min of denaturation at 94°C, 30 cycles (1 min at 94°C, 1min at 62°C, 1min at 72°C) and a final extension of 10 min at 72°C. The identity of this 499bp PCR product was confirmed by sequencing and it was sub-cloned into the EcoRI site of pCR®2.1 vector using TA cloning kit (Invitrogen).

The minor satellite MR150 probe, a 66-mer oligonucleotide, was oligosynthesized: 5'-GACTGAAAAACACATTCGTTGGAAACGGGATTTGTAGAACAGTGTATATCAA TGAGTTACAATGAG-3' (Dennis, Fan et al. 2001) and ^{32}P labeled.

Nuclear extraction and Western blotting: At the end of the culture period, cells were harvested by centrifugation, the cell pellet was re-suspended in 100µl of ice-cold Buffer A (10mM of Tris-HCl, pH 8.0, 1.5mM $MgCl_2$, 5mM KCl, 0.5mM dithiothreitol (DTT), 0.5mM phenylmethyl sulfonylfluoride (PMSF) and 0.5% NP40) with protease inhibitors (leupeptin 1.0µg/ml, aprotinin 1.0µg/ml, Amersham Biosciences) and incubated on ice for 10mins. The whole cell extracts were recovered from the supernatant after centrifugation for 15mins at 4°C, then re-suspended into 20µl of ice-cold Buffer B (20mM of Tris-HCl, pH8.0, 1.5mM $MgCl_2$, 0.4mM NaCl, 0.5mM DTT, 0.5mM PMSF, 0.2mM EDTA and 25% (v/v) glycerol) and incubated on ice for 15mins. The nuclear extract was recovered by centrifugation at 10,000 × *g* for 30mins at 4°C.

50µg of nuclear extract was resolved by a 10% SDS-PAGE gel electrophoresis. After transferring onto a nitrocellulose membrane (Amersham Pharmacia Biotech) and blocking the non-specific binding with 5% milk in PBS, MBD2b protein was detected with a monoclonal antibody generated against the methylated DNA binding domain of MBD2 (Imgenex) at 1:200 dilution at 4°C overnight, followed by peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch) at 1:20,000 dilution and an enhanced chemiluminsecene detection kit (Amersham Pharmacia Biotech).

Immuno-fluorescence and flow cytometry: C2C12 cells were washed twice with PBST-BSA (1% BSA, 0.1% Tween 20 in PBS) and fixed with 0.25% paraformaldehyde in PBS at 37°C for 10mins. Immunostaining was performed by first blocking the sample with 10% FCS in PBST-BSA at 37°C for 20mins, followed by one hour incubation with anti-methylated cytosine (anti-MeC) antibody (kindly provided by Dr. A. Niveleau in a hybridoma supernatant containing 5µg anti-MeC/ml) diluted 1:5 in PBST-BSA and another hour incubation with fluorescent conjugated anti-mouse IgG (Sigma) diluted 1:100 in PBST-BSA. After washing three times with PBS, flow cytometry was performed following the detailed procedure described before (Habib, Fares et al. 1999).

C2C12 myoblast differentiation assay: C2C12 cells were seeded into 24 well dishes and the differentiation was initiated by serum starvation. The cells were fixed in methanol/acetone (v/v) and immunostained for myosin heavy chain with the anti-myosin monoclonal MF20 (Developmental Studies Hybridoma Bank, Iowa City) (Bader, Masaki et al. 1982) and the secondary bispecific monoclonal anti-mouse IgG anti-horseradish

peroxidase MCC10 (Kenigsberg, Semenenko et al. 1990). The staining was developed by diaminobenzidine (DAB) and H_2O_2 (Kenigsberg, Semenenko et al. 1990).

Amplification of adenovirus and adenoviral infection: The human MBD2 cDNA (a 1.3-kilobase pair fragment) was cloned from a HeLa cell cDNA library and ligated into the NotI site of the pcDNA3.1 expression vector (Invitrogen) (Bhattacharya, Ramchandani et al. 1999). The MBD2 insert was excised with NotI and sub-cloned into a pAdTrack CMV adenoviral shuttle vector both in sense and antisense orientations (He, Zhou et al. 1998). Recombinant adenovirus carrying sense MBD2 insertion (Ad-GFP-MBD2) or antisense MBD2 insertion (Ad-GFP-AS) was subsequently generated by recombination with pAd Easy adenoviral vector (Ad-GFP) (Knox, Araujo et al. 2000). A large-scale preparation of adenovirus stock was achieved by infecting confluent HEK293 cells (American Type Culture Collection) repeatedly. The infected cells were collected, adenovirus was released by freezing and thawing three times, and further purified by ultra-centrifugation through a CsCl2 gradient followed by extensive dialysis (Tollefson, Hermiston et al. 1998). C2C12 cells were infected with Ad-GFP-MBD2, Ad-GFP-AS or Ad-GFP for 24 hours in DMEM with 10% serum at multiplicities of infection (MOI) of 5, 10, 20 respectively. More than 95% of the cells were infected as determined by visualization of the green fluorescent protein (GFP), which was expressed by the adenoviral vector Ad-GFP. Cells were then maintained in freshly replaced DMEM with either 10% or 2% serum and subjected to nuclear extract, genomic DNA or RNA extraction 72 hours post infection.

Results

Induced expression of MBD2/demethylase is observed in two differentiation systems: embryonic carcinoma P19 cells and C2C12 myoblasts.

There is increasing evidence supporting the hypothesis that an active process of demethylation occurs in embryonic cells (Frank, Keshet et al. 1991) and differentiating cells (Paroush, Keshet et al. 1990; Razin, Feldmesser et al. 1985; Szyf, Eliasoon et al. 1985). MBD2b/demethylase is the only identified active demethylase (Bhattacharya, Ramchandani et al. 1999; Ramchandani, Bhattacharya et al. 1999) to date, but its possible role during differentiation is unknown. To answer this question, I focused on two differentiation systems, mouse embryonic carcinoma P19 cells and mouse C2C12 myoblasts. P19 cells were shown before to have a general demethylase activity (Szyf, Theberge et al. 1995), while hypomethylation has been previously shown to accompany muscle differentiation (Jost and Jost 1994; Scarpa, Lucarelli et al. 1996; Jost, Oakeley et al. 2001). Due to its high similarity to MBD2b/demethylase (Hendrich and Bird 1998), the expression of MBD3 was also monitored.

MBD2b/demethylase is rapidly induced in P19 cells upon the initiation of differentiation by retinoic acid. It peaks on the second day of differentiation, and then its expression is reduced sharply (Figure 1A). Compared with the rapid induction of MBD2b/demethylase expression, MBD3 is induced slowly and progressively. No peak is observed on the second day of differentiation as observed with MBD2b, the expression of MBD3 reaches its peak on day 16 (Figure 1B).

MBD2b/demethylase and MBD3 follow a similar expression profile during C2C12 myoblast differentiation. Expression is low in undifferentiated cells, and reaches the first peak 6 days after switching into medium with low serum. Another two comparable peaks are observed on day 8 and day 11 respectively (Figure 2).

The fact that MBD2b/demethylase and MBD3 exhibit different expression profiles during differentiation of P19 cells is consistent with previously published data. Hendrich et al. reported that mbd3 (-/-) mice died before birth whereas mbd2b (-/-) mice were viable and fertile, implying that MBD2b/demethylase and MBD3 play different roles in development (Hendrich, Guy et al. 2001).

Genome-wide demethylation is one of the events associated with the induction of embryonic carcinoma cell differentiation (Frank, Keshet et al. 1991). The rapid and sharp induction of MBD2b/demethylase during P19 differentiation is consistent with the hypothesis that MBD2b/demethylase is involved in this process. However, future experiments are required to determine whether MBD2b/demethylase plays a causal role in differentiation.

MBD2b/demethylase is involved in the differentiation of C2C12 myoblasts, its peak of expression corresponds to global hypomethylation and* myogenin *expression.

Global hypomethylation is associated with myogenic differentiation as reported in L5 and G8 myoblasts (Scarpa, Lucarelli et al. 1996; Jost and Jost 1994; Jost, Oakeley et al. 2001), however the methylation status of C2C12 cells during myogenesis was not determined. I therefore determined the state of methylation of the C2C12 genome during differentiation by staining the cells with a monoclonal antibody directly against methylated cytosine (anti-MeC), followed by a secondary fluorescent anti-mouse IgG and flow cytometry sorting by the intensity of the fluorescent signal. The intensity of fluorescence signal correlates with the level of genomic DNA methylation. As shown in Figure 3A, before differentiation, the intensity of the fluorescence is centered at 11.78. After 24 hours of differentiation, the fluorescent peak appears at 8.34, suggesting loss of methylated cytosines (Figure 3B). The genome wide demethylation continues in C2C12 cells 72 hours and 96 hours post differentiation (Figure 3C and Figure 3D). Table 3.1 summarizes the position of the fluorescence intensity observed in FACS assay every 24 hours after initiation of differentiation. The left-shifting of the fluorescence peaks during differentiation provides strong evidence that genome-wide demethylation is associated with C2C12 myoblast differentiation. The induction in MBD2b/demethylase expression (Figure 2) corresponds to the time course of global hypomethylation (Figure 3), which is consistent with its involvement in genome wide demethylation.

Morphological changes in C2C12 myoblasts are visible by microscopy 72 hours after initiation of differentiation and become clear at day 6 (Figure 4). In accordance with this profile of differentiation, the expression of MBD2b/demethylase is induced up to day 6 and drops on day 7 (Figure 2).

I then correlated the expression of MBD2b/demethylase with the expression of *myogenin*, a unique marker for myogenic differentiation. MBD2b/demethylase is induced 72 hours after initiation, and peaks on day 5 and day 6. Consistent with the previous experiment, MBD2b/demethylase declines on day 7 (Figure 5A). The expression of myogenin is not detected until day 5, at which stage cells are well differentiated (Figure 5B).

The early induction of MBD2b/demethylase supports that it serves an important role in differentiation. I first determined whether MBD2b/demethylase induction is a downstream effector of the signaling pathway that triggers C2C12 differentiation. Two main pathways are essential in the signal transduction for C2C12 myoblast differentiation: the phosphatidylinositol 3-kinase (PI3K)/PKB-AKT/p70S6-kinase (p70S6K) pathway and the Ras/Raf/mitogen activated protein kinase (MAPK) pathway (Conejo, de Alvaro et al. 2002). Rapamycin, an inhibitor of p70S6 kinase, interferes with the first pathway described above and thus abolishes the potential for C2C12 differentiation. C2C12 myoblasts were kept under standard differentiation conditions, but were treated with 25ng/ml of rapamycin (Calbiochem). As seen in Figure 6, 72 hours of treatment with rapamycin completely abolishes the expression of MBD2b/demethylase mRNA as well as MBD2b/demethylase protein determined by a Western blotting analysis. The control, cells without rapamycin treatment underwent differentiation and expressed normal levels of MBD2b/demethylase.

Forced expression or knock down of MBD2b/demethylase is achieved by adenovirus carrying sense or antisense MBD2b/demethylase constructs

The data presented above clearly demonstrated that muscle differentiation was accompanied with the induction of MBD2b/demethylase. The inhibition of MBD2b/demethylase induction by rapamycin suggests that MBD2b/demethylase is a down-stream effector of PI3K/PKB-AKT/p70S6 pathway. The induction of MBD2b/demethylase prior to *myogenin* activation is consistent with the hypothesis that MBD2b/demethylase plays an important role in muscle differentiation.

In order to investigate the function of MBD2b/demethylase during myogenesis, I over expressed MBD2b/demethylase by infecting C2C12 myoblasts with an adenovirus carrying MBD2b/demethylase cDNA under the control of the CMV promoter (Ad-GFP-MBD2) (Knox, Araujo et al. 2000; Slack, Bovenzi et al. 2002). For the evaluation of infection efficiency, GFP (green fluorescence protein) that is engineered into the adenovirus vector (Ad-GFP), was utilized as a reporter. More than 95% of infected cells expressed GFP after 24 hours of infection at MOI of 5, 10, 20 respectively. I then tested whether MBD2b/demethylase was over-expressed in cells infected with Ad-GFP-MBD2. The representative Northern and Western blots are presented in Figure 7A and Figure 7B, which show successful over-expression of MBD2b/demethylase both at the mRNA level and the protein level. No MBD2b/demethylase was detected in cells infected with Ad-GFP. MBD2b/demethylase protein was knocked down by Ad-GFP-AS infection (Figure 7C).

Over-expression of MBD2/demethylase is not sufficient to trigger myogenesis

Can forced-expression of MBD2b demethylase drive C2C12 myoblasts towards differentiation under conditions that normally do not induce differentiation such as in the presence of 10% serum? To address this question, C2C12 cells were infected with Ad-GFP-MBD2 at different multiplicities of infection (MOI) and maintained in growing medium for 72 hours. Cells infected with Ad-GFP were used as a control. As shown in Figure 8 , demethylase was successfully over-expressed in a dose dependent manner, but no *myogenin* expression was detected even under the highest level of expression of

MBD2b/demethylase, suggesting MBD2b/demethylase itself was not sufficient to initiate cell differentiation.

My result shows that MBD2b/demethylase is not the only member determining myoblast differentiation. One explanation is that myogenesis is under the control of several layers of regulatory mechanisms. Histone modification, chromatin remodeling and DNA methylation are all reported to be actively involved in muscle differentiation (Strahl and Allis, 2000; de la Serna, Roy et al. 2001; de la Serna, Carlson et al. 2001, Lassar, Paterson et al. 1986) . Demethylation and MBD2b/demethylase might be only one component in this complex interconnection, its activation and function depends on upstream factors and cofactor(s). Another possibility is the toxicity of adenovirus infection. Adenovirus is a highly efficient tool for gene delivery, but its toxicity to the host is also well recognized (Braithwaite and Russel, 2001). For C2C12 cells infected by the highest MOI, about 10% of the cells detached from the plate after 72 hours of infection.

Antisense knockdown of MBD2/demethylase inhibits the expression of* myogenin *gene and reduces global demethylation

Forced expression of MBD2b/demethylase itself can't trigger the process of myogenesis, suggesting that MBD2b/demethylase is not sufficient for differentiation. The next interesting question is whether MBD2b/demethylase is necessary for muscle differentiation and whether differentiation could be interrupted by antisense knock down of MBD2b/demethylase. C2C12 cells were plated in medium with 10% serum and infected with Ad-GFP-AS. After 24 hours of infection, fresh medium with 2% serum was supplied and cells were differentiated for 72 hours before lysis. My results, presented in Figure 9A, demonstrate that *myogenin* was greatly reduced by increasing the MOI of Ad-GFP-AS. An unpaired t test revealed that at MOI 20, the difference between GFP and Ad-GFP-AS was considered significant ($t = 2.4660$, $p < 0.05$) (Figure 9B).

As an active demethylase involved in myogenesis, the knock down of MBD2b/demethylase inhibits the activation of *myogenin* as shown in Figure 9. I then tested whether the global methylation status was ffected by knock down of MBD2b/demethylase. MR150, a repetitive satellite element widely distributed and usually heavily methylated, is a well-accepted indicator for global methylation status of the genome. Genomic DNA extracted from C2C12 cells was first digested with XbaI (to reduce the size of genomic DNA fragments) and HpaII, and then subjected to Southern blotting and hybridization with a ^{32}P-labelled MR150 probe. After introducing Ad-GFP-AS into C2C12 cells, the genomic DNA MR150 sequences were more resistant to HpaII digestion in a dose-dependent manner, which was reflected by the disappearance of small molecular weight bands relative to the high molecular weight fragments, as indicated by arrows in the agarose gel. In contrast, genomic DNA, extracted from cells infected with Ad-GFP, was easily cleaved with HpaII (Figure 10).

Antisense MBD2b/demethylase reduces the expression of* myogenin *by inhibiting the demethylation of its promoter

Data from Figure 9 and Figure 10 demonstrated that MBD2b/demethylase is involved in the activation and expression of *myogenin* and global demethylation, however the molecular mechanism by which MBD2b//demethylase turns on *myogenin* expression is

unclear. The activation of myogenin occurs simultaneously with its hypomethylation and leads to muscle differentiation (Scarpa, Lucarelli et al. 1996). Recently, Scarpa et al. reported that the demethylation of a single CpG site located at the promoter region of myogenin was responsible for its activation (Lucarelli, Fuso et al. 2001) and this activation was demonstrated later to be inhibited by the application of a demethylation inhibitor SAM (Fuso, Cavallaro et al. 2001). The fast demethylation suggests that an active demethylation mechanism is involved (Fuso, Cavallaro et al. 2001).

MBD2b/demethylase is an active demethylase that was first cloned by our lab (Ramchandani, Bhattachary et al. 1999; Bhattachary, Ramchandani et al. 1999). It is highly induced upon the initiation of differentiation and the peak of its induction corresponds with global hypomethylation and *myogenin* activation (Figure 2, Figure 3 and Figure 4). The introduction of antisense MBD2b/demethylase greatly reduces the expression of *myogenin* (Figure 10). Considering all of these facts, I hypothesized that MBD2/demethylase was involved in C2C12 differentiation by demethylating the promoter of *myogenin.*

A probe spanning the reported demethylated CpG site was designed to monitor the methylation change in the *myogenin* promoter during myogenesis. The assay for detecting the methylation status of the *myogenin* promoter with Southern blotting is illustrated in Figure 11A. Genomic DNA was digested first by XbaI and HpaII, and then subjected to Southern blotting and hybridization with the ^{32}P-labelled *myogenin* promoter probe. H1239* is the CpG site undergoing demethylation during differentiation. A 1.7kb digestion product is detected if H1239* is methylated and resistant to HpaII digestion. On another hand, 1.2kb and 0.5kb fragments will be achieved if H1239* is demethylated. Figure 11B illustrates the change in methylation of the *myogenin* promoter during differentiation. The three possible digestion products are indicated by arrows on the right. Based on an average from three independent experiments, it is concluded that the promoter of *myogenin* is demethylated during myogenesis using two parameters: the intensity ratio 1.2kb/1.7kb and 0.5kb/1.7kb (Figure 11C). Partial demethylation of the *myogenin* promoter exists even before differentiation. However, the promoter demethylation peaks at day 5 following initiation of differentiation, which fits the highest expression time window of *myogenin* as illustrated in Figure 4B. My results are consistent with what was reported by Lucarelli (Lucarelli, Fuso et al. 2001). Antisense MBD2b/demethylase knock down was utilized again to investigate the effect of MBD2b/demethylase on the state of methylation of the *myogenin* promoter. Figure 11D is a representative Southern blot analysis performed using DNA prepared from C2C12 cells infected with either Ad-GFP-AS or Ad-GFP. Out of two expected demethylation products generated by the digestion of XbaI and HpaII, the 0.5kb fragment shows a declining pattern in differentiating C2C12 myoblasts infected with Ad-GFP-AS, and this declining pattern is MOI dose dependent. My result implies that antisense MBD2b/demethylase inhibits the promoter demethylation induced by differentiation since HpaII fails to cut H1239*. A slight decline is also observed in 1.2kb digestion product. As expected, an increase in the methylated and HpaII resistant 1.7kb fragment is observed, demonstrating the H1239* is protected from demethylation and resistant to HpaII digestion (Figure 11E).

Discussion

CpG methylation in mammalian DNA is involved in gene silencing. Tissue-specific genes are methylated in most tissues where they are not expressed, but unmodified in expressing tissues. Global hypomethylation, demethylation and activation of muscle specific genes are well documented in differentiating myoblasts (Scarpa, Lucarelli et al. 1996; Jost and Jost 1994; Jost, Oakeley et al. 2001). However, the mechanisms underlying the process of demethylation during muscle differentiation have not been fully elucidated.

The work from this thesis first establishes a molecular link between demethylation and activation of *myogenin*, a muscle transcription regulatory factor playing a central role in myogenesis, and MBD2b/demethylase. MBD2b/demethylase is up-regulated upon the initiation of differentiation, which peaks at Day 5 and corresponds to the demethylation and activation of *myogenin*. Rapamycin, an inhibitor of PI3K/PKB-AKT/p70S signal transduction pathway for C2C12 differentiation, abolishes the expression of MBD2b/demethylase completely, implying that MBD2b/demethylase is a nuclear target of this pathway. Antisense knock down of MBD2b/demethylase inhibits global demethylation and activation of *myogenin* by protecting the promoter region from demethylation. This is the first example for a biological function of MBD2b/demethylase under physiological environments.

Complex regulation network for myogenesis: MBD2b/demethylase itself is not sufficient to drive myoblasts to terminal differentiation

Muscle differentiation is accompanied with the activation of muscle-specific genes and inactivation of genes required for cell proliferation. CpG methylation, histone modification and ATP-dependent chromatin remodeling form a three-way partnership to regulate gene expression (Razin 1998; Wade 2001). Accumulating evidence suggests that all three mechanisms mentioned above are involved in myogenesis (Strahl and Allis, 2000; de la Serna, Roy et al. 2001; de la Serna, Carlson et al. 2001, Lassar, Paterson et al. 1986). For instance, histone acetyltransferase activity in cell extracts from chicken myoblast cultures increases within 24 hours of differentiation, and steadily decreases to 30-40% of the original level after six to seven days (Liew, thi Man et al. 1980). In addition, exposure of undifferentiated skeletal myoblasts to a HDAC inhibitor, following by incubation in differentiation medium, enhanced the expression of a muscle-specific reporter and led to a dramatic increase in the formation of multinucleated myotubes (Lezzi, Cossu et al. 2001). The activities of some important muscle transcriptional factors such as MyoD and MEF2 are dynamically controlled by both HAT and HDAC (McKinsey, Zhang et al. 2001). It is therefore not surprising to see that forced expression of MBD2b/demethylase in C2C12 cells which are maintained in normal growth medium doesn't initiate the whole program of muscle differentiation, as indicated by two observations that no morphological change is detected under the microscope and no *myogenin* is induced by over expression of MBD2b/demethylase (Figure 8). The up-regulation of MBD2b/demethylase (Figure 4) during the process of differentiation is the result of the activation of PI3K/PKB-AKT/p70S6 pathway, since rapamycin, an inhibitor of this pathway, abolished the expression of MBD2b/demethylase completely (Figure 6). Other factor(s) activated in the same pathway might also be required to cooperate with MBD2b/demethylase to fulfill its role in myogenesis. One such molecule might be

MEF2. The transcriptional activation of myogenin is MEF2 dependent (Fickett 1996; Johanson, Meents et al. 1999; Rogerson, Jamali et al. 2002). However, MEF2 is largely regulated by HAT and HDAC (McKinsey, Zhang et al. 2001). MEF2 is also modified at the post-translational level (Cox, Du et al. 2003); the phosphorylation of MEF2 is the result of the activation of PI3K/PKB-AKT/p70S6 (Xu and Wu, 2000; Naya and Olson, 1999), the same pathway for the activation of MBD2b. This may provide an explanation as to why ectopic over-expression of MBD2b/demethylase itself can't drive myoblasts toward terminal differentiation. In addition, the large number of copies of MBD2b/demethylase in the forced expression system might also interfere with the normal mechanisms responsible for myogenesis.

The function of MBD2/demethylase: global demethylation versus site-specific demethylation

Both global demethylation and site-specific demethylation are observed in the C2C12 muscle differentiation system. The fast genome wide demethylation happens within 24 hours upon the initiation of differentiation (Figure 3 and Table I). The promoter specific demethylation of *myogenin* is observed at Day 5 (Figure 11C). Compared with the expression profile of MBD2b/demethylase, it seems that MBD2b/demethylase has a minor effect on the early global hypomethylation and is mainly responsible for the demethylation of the *myogenin* promoter, because MBD2b/demethylase is not inducible until Day 3 and peaks at Day 5. The expression of *myogenin* is detected in the same time window. The above conclusion is supported by the fact that the introduction of antisense MBD2/demethylase protects *myogenin* promoter from demethylation (Figure 11E) consequently the expression of *myogenin* is reduced significantly (Figure 9).

However, MBD2b/demethylase is reported to be a processive enzyme and it is suggested to be a molecular explanation for global hypomethylation (Cervoni, Bhattacharya et al. 1999). The observation that antisense MBD2b/demethylase reduces global methylation as indicated by the increased methylation of the repetitive element MR150 (Figure 10) also supports an involvement of MBD2b/demethylase in some aspects of global demethylation.

Myogenesis and demethylation: a two-step demethylation model

Two demethylation events are observed in the process of C2C12 myogenesis differentiation. The global demethylation wave happens to a large degree within 24 hours of initiation of differentiation and the genome keeps losing methylation until 72 hours (Figure 3 and Table I). It seems that a passive mechanism is responsible for that event since active demethylase MBD2b is not inducible until Day 3 (Figure 4). Passive demethylation is the consequence of the exit from the cell cycle and a drop in DNMT1 activity (Jost and Jost, 1994). The function of hypomethylation here is to initiate muscle differentiation by releasing transcriptional repression states mediated by histone deacetylation and to open the packaged structure of chromatin. HDAC is reported to interfere with the activation of MyoD and MEF2 (Lu, McKinsey et al. 2000; Dressel, Bailey et al. 2001) and repress muscle differentiation. Myoblasts with a mutated negative form of SWI/SNF chromatin remodeling complex also lose their potential for differentiation (de la Serna, Roy et al. 2001).

In the second step, the central player for myogenesis, such as myogenin, is demethylated by the action of MBD2b/demethylase. The active form of *myogenin* further directs terminal differentiation. MBD2b/demethylase needs to be directed to the *myogenin* promoter by other molecule(s), one potential candidate is MEF2. There is a MEF2 binding site in the promoter region of *myogenin* (Buchberger, Ragge et al. 1994). It seems that MEF2 helps to alter the condensed structure of chromatin because histones around the MEF2 binding site are more acetylated following MEF2 binding (Zhang, *McKinsey et al. 2002).* Moreover, the promoter of *myogenin* becomes more accessible and is easily digested by several endonucleases (de la Serna, Carlson et al. 2001). I therefore propose that MEF2 binding modifies the structure of the chromatin associated with the *myogenin* promoter, allowing the access of MBD2b/demethylase to the methylated CpG sites, resulting in demethylation and activation of the expression of *myogenin.* Cervoni et al. have previously demonstrated that acetylation of chromatin defines the accessibility of MBD2b/demethylase to DNA (Cervoni and Szyf, 2001).

. This two-step model for the involvement of MBD2b/demethylase in muscle differentiation through demethylating the promoter of *myogenin* is illustrated in Figure 12.

Table 3.1: The differentiation of C2C12 myoblasts is accompanied by the genome wide hypomethylation. After the initiation of differentiation, C2C12 cells were harvested according to the indicated time point and fixed with methanol:PBS (88:12, *v/v*). Cells were stained with antibody against methylated cytosine first, followed by fluorescence conjugated anti-mouse IgG antibody. The methylation status of the whole genome is presented by the mean fluorescence intensity of 10,000 gated cells.

	Background	Time Post Differentiation (hour)			
		0	24	72	96
Fluorescence Intensity (mean)	4.38	11.78	8.34	7.30	6.92

Figure legend

Figure 3.1: MBD2b/demethylase is rapidly induced upon P19 differentiation. (a) Mouse P19 embryonic carcinoma cells were induced to differentiation with 1mM retinoic acid. RNA was isolated following the differentiation time course. 20μg isolated RNA was loaded in each lane, fractioned on 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybrization with ^{32}P-labelled MBD2b/demethylase probe. (b) The same membrane was stripped and re-blotted with ^{32}P-labelled MBD3 probe. (c) To normalize the amount of total RNA in each lane, the blot was then hybridized with 18S rRNA oligonucleotide probe. The intensity of signal obtained with the MBD2b/demethylase probe or MBD3 probe was divided by the signal obtained with the 18S probe. (d) The expression of MBD2b/demethylase and MBD3 were determined by quantifying the relative abundance of MBD2b/demethylase and MBD3 fragments.

Figure 3.1

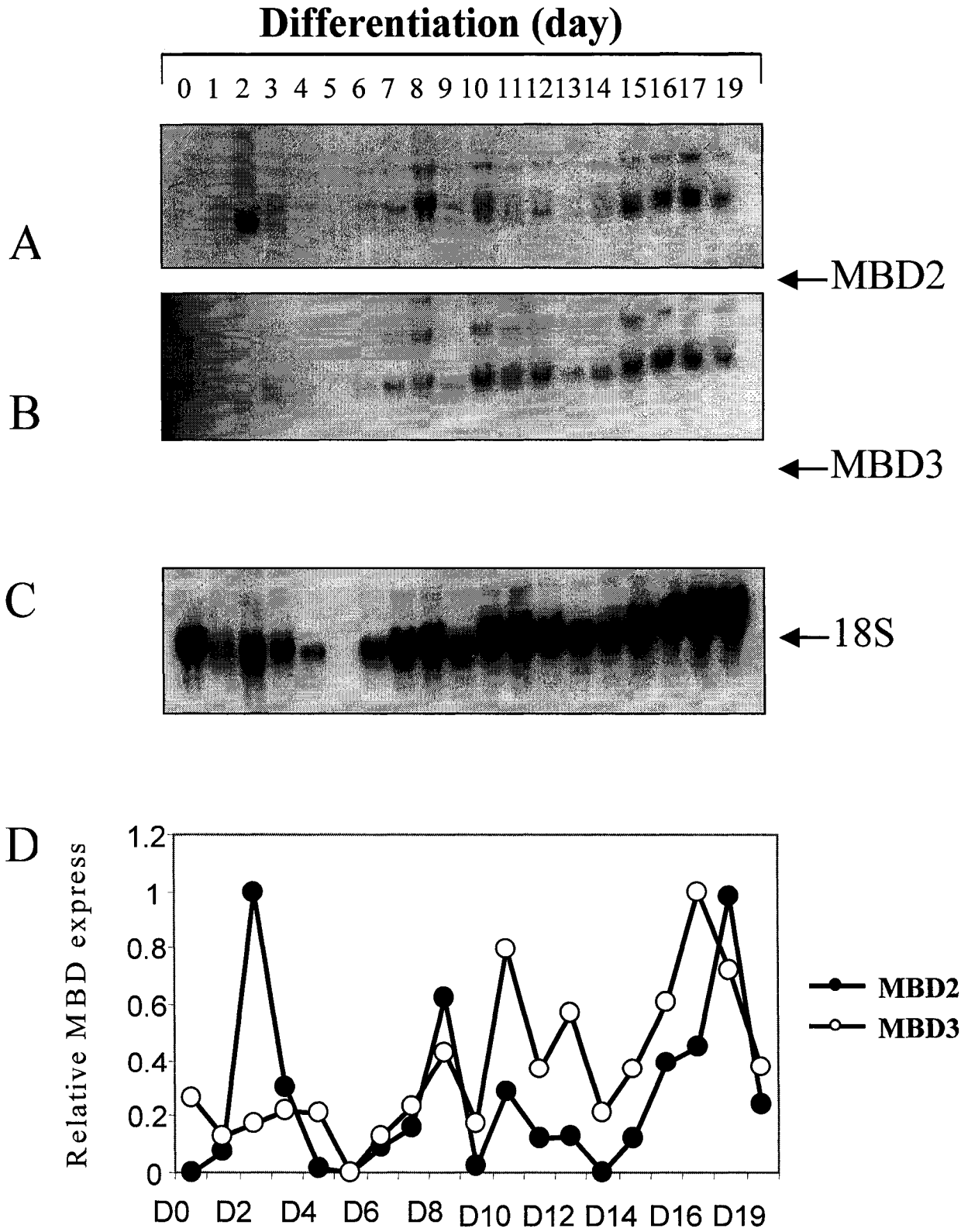

Differentiation (day)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 19
A
MBD2
B
MBD3
C
18S
D
Relative MBD express
1.2
1
0.8
0.6
0.4
0.2
0
D0 D2 D4 D6 D8 D10 D12 D14 D16 D19
MBD2
MBD3

Figure Legend

Figure 3.2: MBD2b/demethylase is progressively induced upon C2C12 differentiation. (a) Mouse C2C12 myoblasts was induced to differentiation with low serum starvation. RNA was isolated following the differentiation time course. 20μg isolated RNA was loaded in each lane, fractioned on a 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybridization with a ^{32}P-labelled MBD2b/demethylase probe. (b) The same membrane was stripped and re-blotted with a ^{32}P-labelled MBD3 probe to detect the expression profile of MBD3. (c) To normalize the amount of total RNA in each lane, the blot was then hybridized with 18S rRNA oligonucleotide probe. The intensity of signal obtained with the MBD2b/demethylase probe or the MBD3 probe was divided by the signal obtained with the 18S probe. (d) The expression of MBD2b/demethylase and MBD3 were determined by quantifying the relative abundance of MBD2b/demethylase and MBD3 fragments.

Figure 3.2

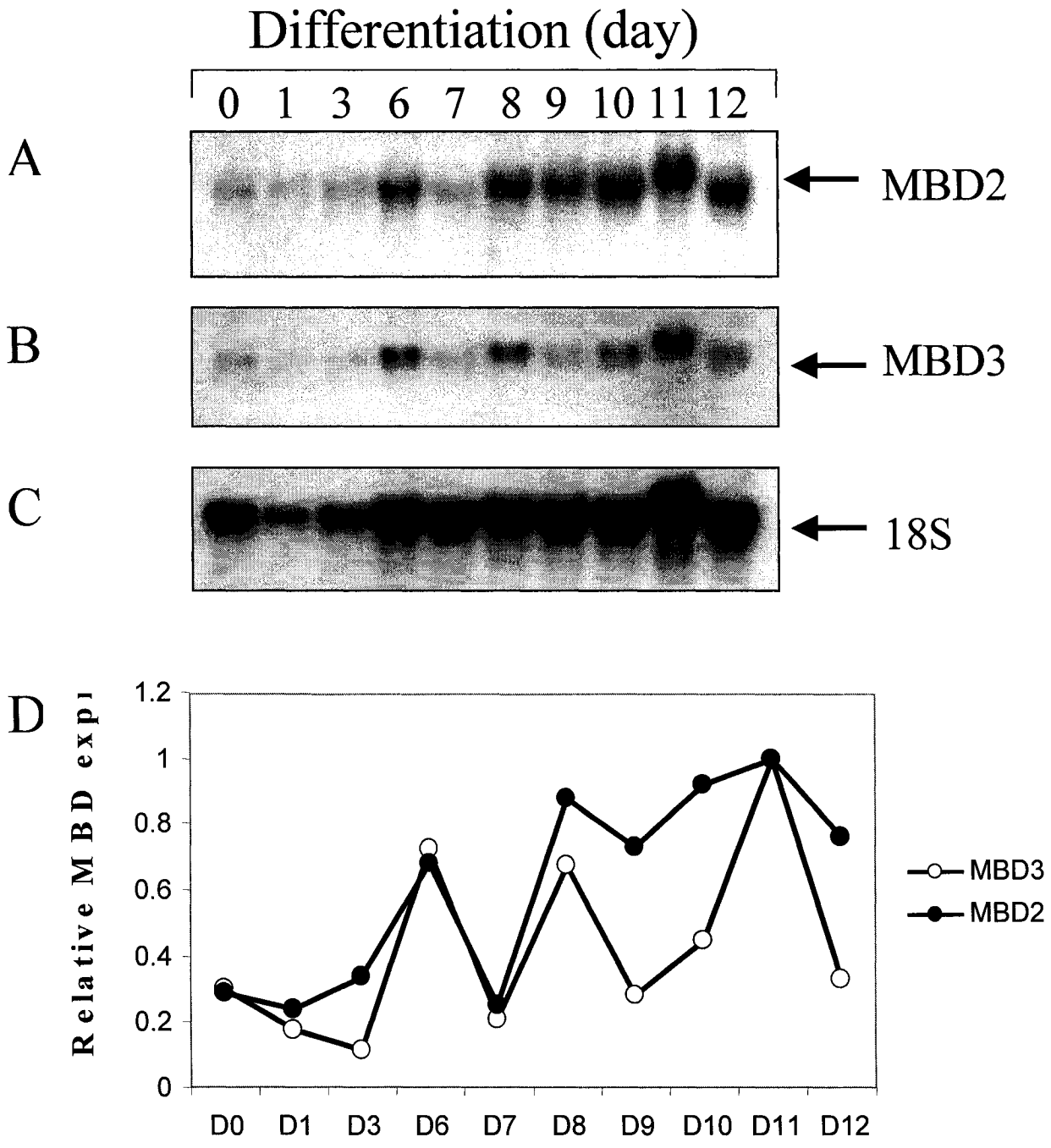

Differentiation (day)
0 1 3 6 7 8 9 10 11 12
A
MBD2
B
MBD3
C
18S
D
Relative MBD expr
1.2
1
0.8
0.6
0.4
0.2
0
D0 D1 D3 D6 D7 D8 D9 D10 D11 D12
MBD3
MBD2

Figure Legend

Figure 3.3: The differentiation of C2C12 myoblasts is accompanied with genome wide hypomethylation. After the initiation of differentiation, C2C12 cells were harvested every day and fixed with methanol: PBS (88:12 *v/v*). Cells were stained with antibody against methylated cytosine first, followed by fluorescence conjugated anti-mouse IgG. antibody. The methylation status of the whole genome was determined by a flow cytometry assay.

Figure 3.3

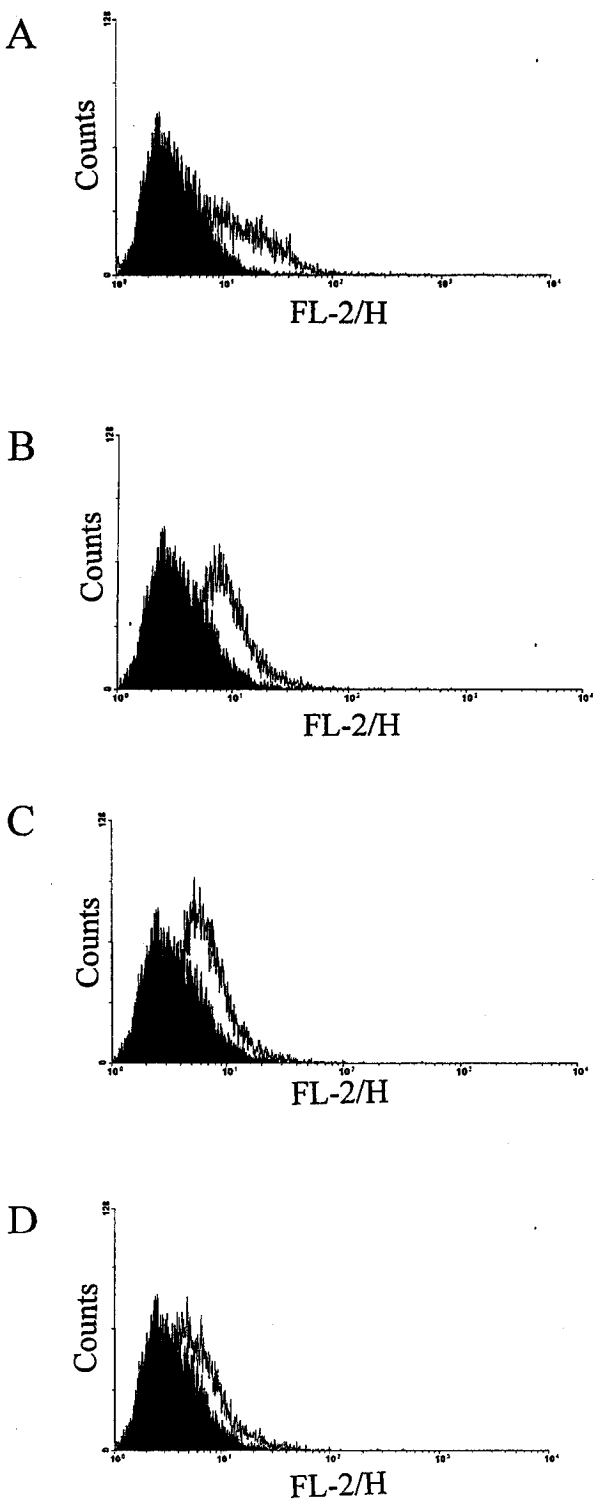
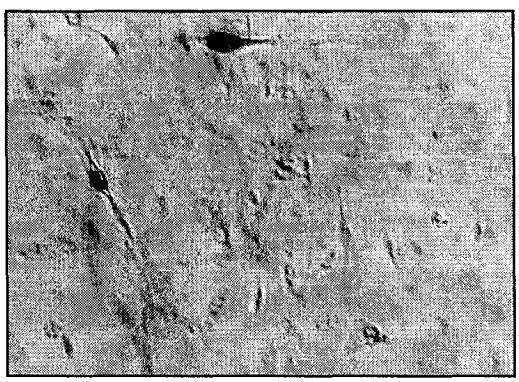


Figure Legend

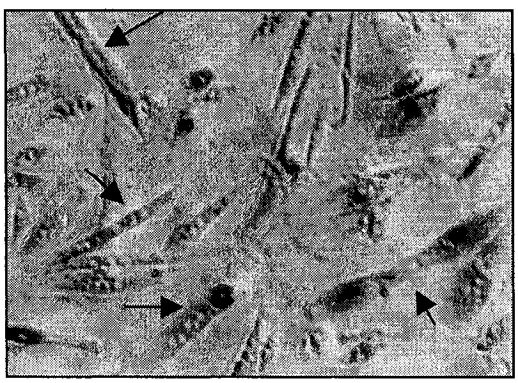
Figure 3.4: Morphological transformation of differentiated C2C12 myoblasts. Myosin immunostaining of C2C12 cells with MF20 antibody in cultures (a) before the initiation of serum starvation. (b) 6 days post serum starvation. (c) enlarged image showing mononucleate myoblasts.

Figure 3.4

A



B



C

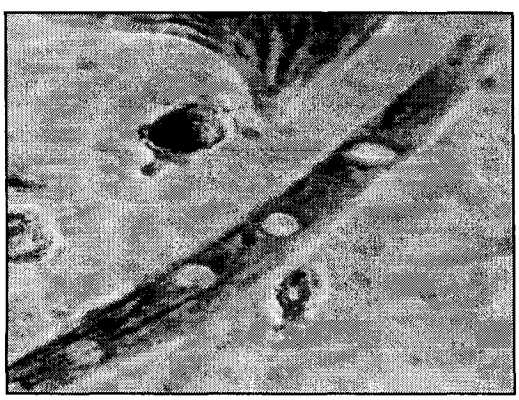


Figure Legend

Figure 3. 5: MBD2b/demethylase is induced earlier than *myogenin* expression during muscle differentiation. (a) C2C12 cells were maintained sparsely in growing medium and plated up to 80% confluence for 24 hour before switching to differentiation medium. Fresh differentiation medium was supplied every other day and RNA is isolated following the differentiation time course. 20μg isolated RNA was loaded in each lane, fractioned on 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybridization with ^{32}P-labelled MBD2/demethylase probe. (b) The same membrane was stripped and re-blotted with ^{32}P-labelled *myogenin* probe to detect the expression profile of *myogenin*. (c) Equal RNA loading as evidenced by ethidium bromide staining. (d) The relative expression of MBD2b/demethylase and *myogenin* were determined by quantifying the relative abundance of *myogenin* fragment and MBD2b/demethylase fragment.

Figure 3.5

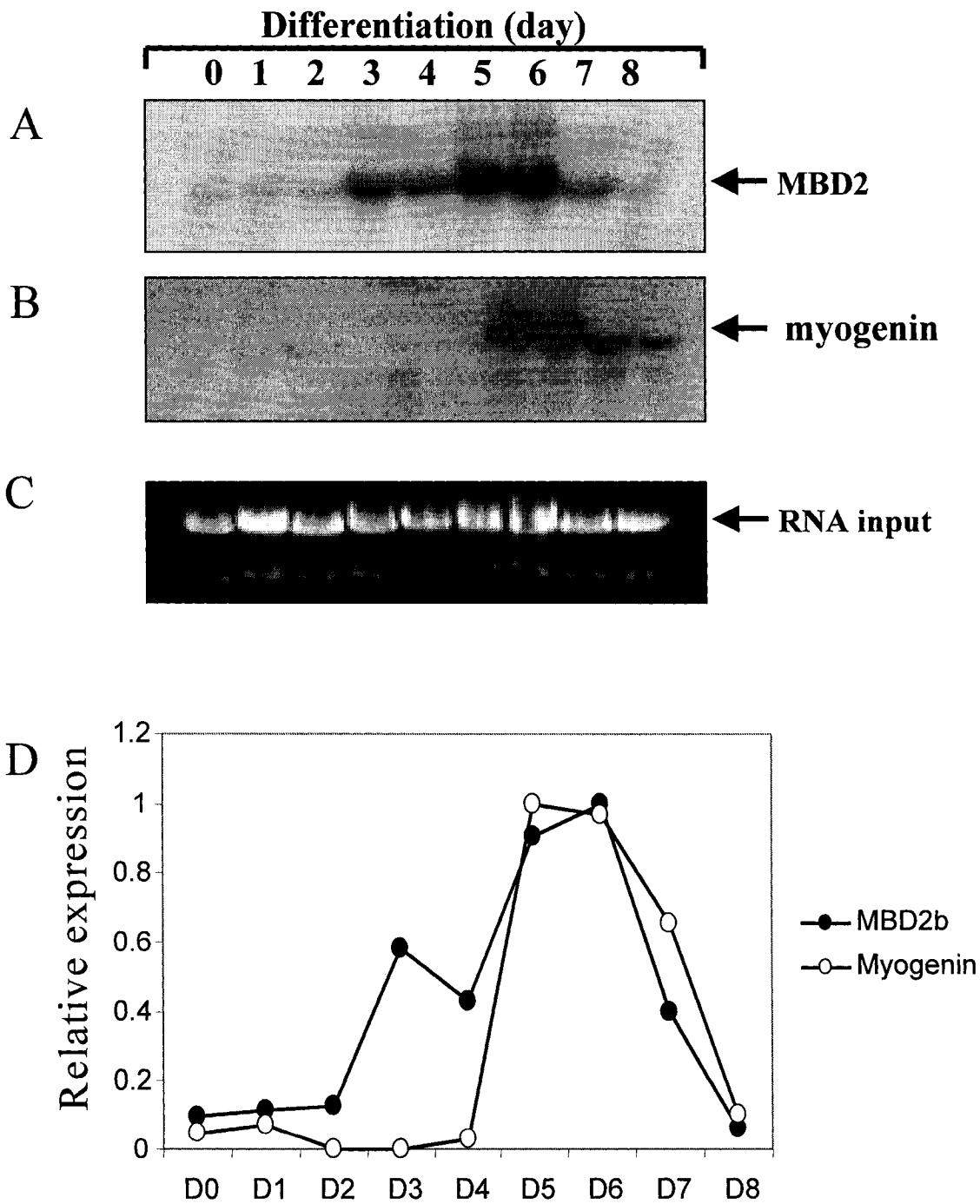
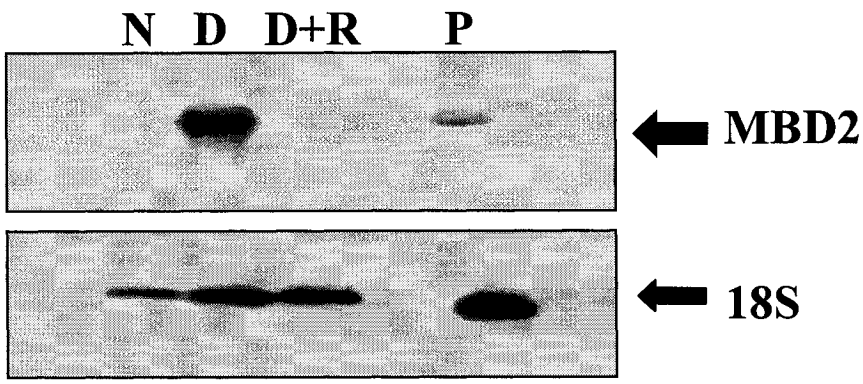

Differentiation (day)
0 1 2 3 4 5 6 7 8
A
MBD2
B
myogenin
C
RNA input
D
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
D0 D1 D2 D3 D4 D5 D6 D7 D8
MBD2b
Myogenin

Figure Legend

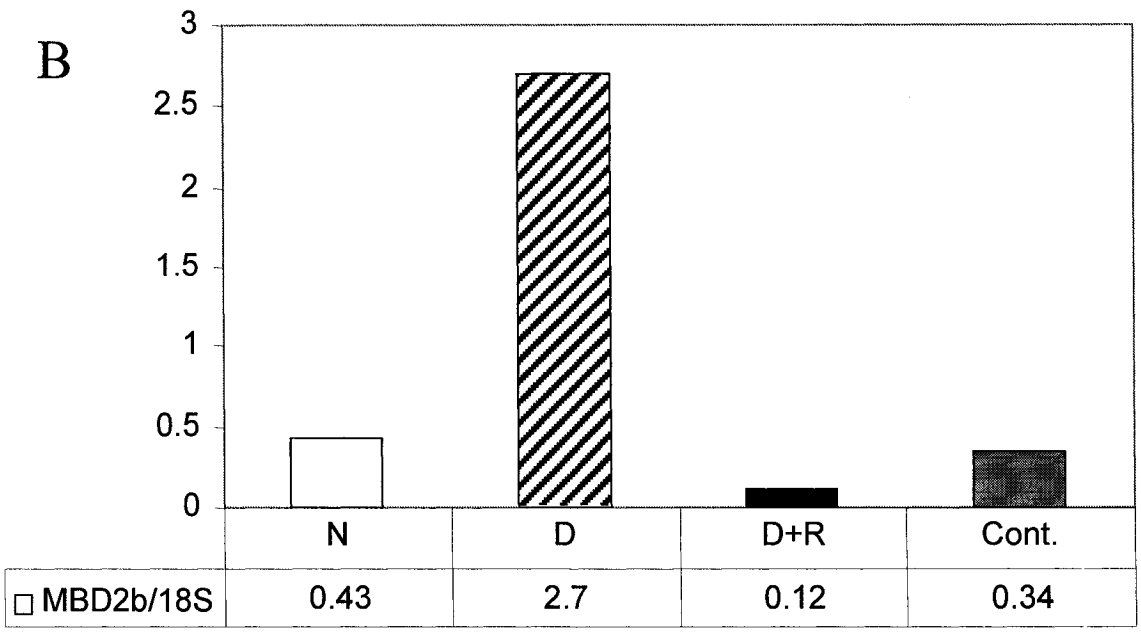
Figure 3. 6: MBD2b/demethylase was completely abolished by rapamycin, an inhibitor of the PI3K/PKT-AKB/p70S6 pathway of C2C12 differentiation. (a) C2C12 cells were maintained in differentiation medium with 25ng/ml of rapamycin supplementation for 96 hours. 20μg of isolated RNA was loaded in each lane, fractioned on 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybrization with a ^{32}P-labelled MBD2b/demethylase probe, as shown in the top panel. The same membrane was stripped and re-blotted with ^{32}P-labelled 18S rRNA probe, as shown in the bottom panel. G: growing medium; D: differentiation medium; D+R: differentiation medium with 25ng/ml of rapamycin supplement. P: RNA isolated from fully differentiated C2C12 myoblasts was used as the positive blotting control for *myogenin.* (b) The relative expression of MBD2b/demethylase was determined by quantifying the relative abundance of MBD2b/demethylase fragment. (c) Nuclear extracts were made from C2C12 cells subjected to different treatments and 50μg of protein was loaded each lane of a 10% SDS-PAGE gel. 1:250 dilution of antibody against MBD2b/demethylase (Imgenex) was used for Western blotting.

Figure 3.6

A



B



C

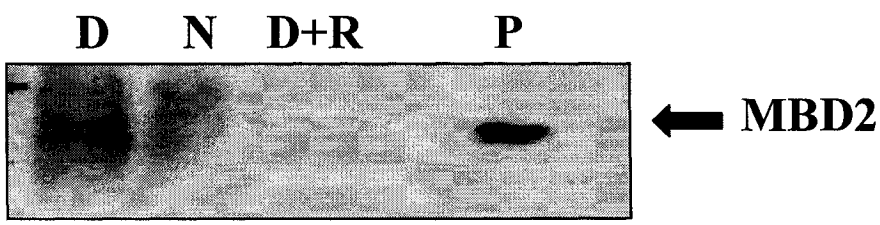


Figure Legend

Figure 3.7: MBD2b/demethylase is over-expressed in C2C12 cells infected with adenovirus carrying a MBD2b/demethylase construct (Ad-GFP-MBD2) and knocked down by antisense MBD2b/demethylase (Ad-GFP-AS). (a) C2C12 cells were infected with Ad-GFP or Ad-GFP-MBD2 respectively and maintained in growing medium for 48 hours. 20μg of isolated RNA was loaded in each lane, fractioned on a 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybrization with ^{32}P-labelled MBD2b/demethylase probe, as shown at the top panel. Equal RNA loading is shown at the bottom panel. (b) 50μg of nuclear extract was loaded in each lane for 10%SDS-PAGE. 1:250 dilution of antibody against MBD2/demethylase (Imgenex) was used for Western blotting. (c) Western blotting for C2C12 cells infected with Ad-GFP or Ad-GFP-MBD2b respectively. 1:250 dilution of antibody against MBD2b/demethylase (Imgenex) was used for Western blotting.

Figure 3.7

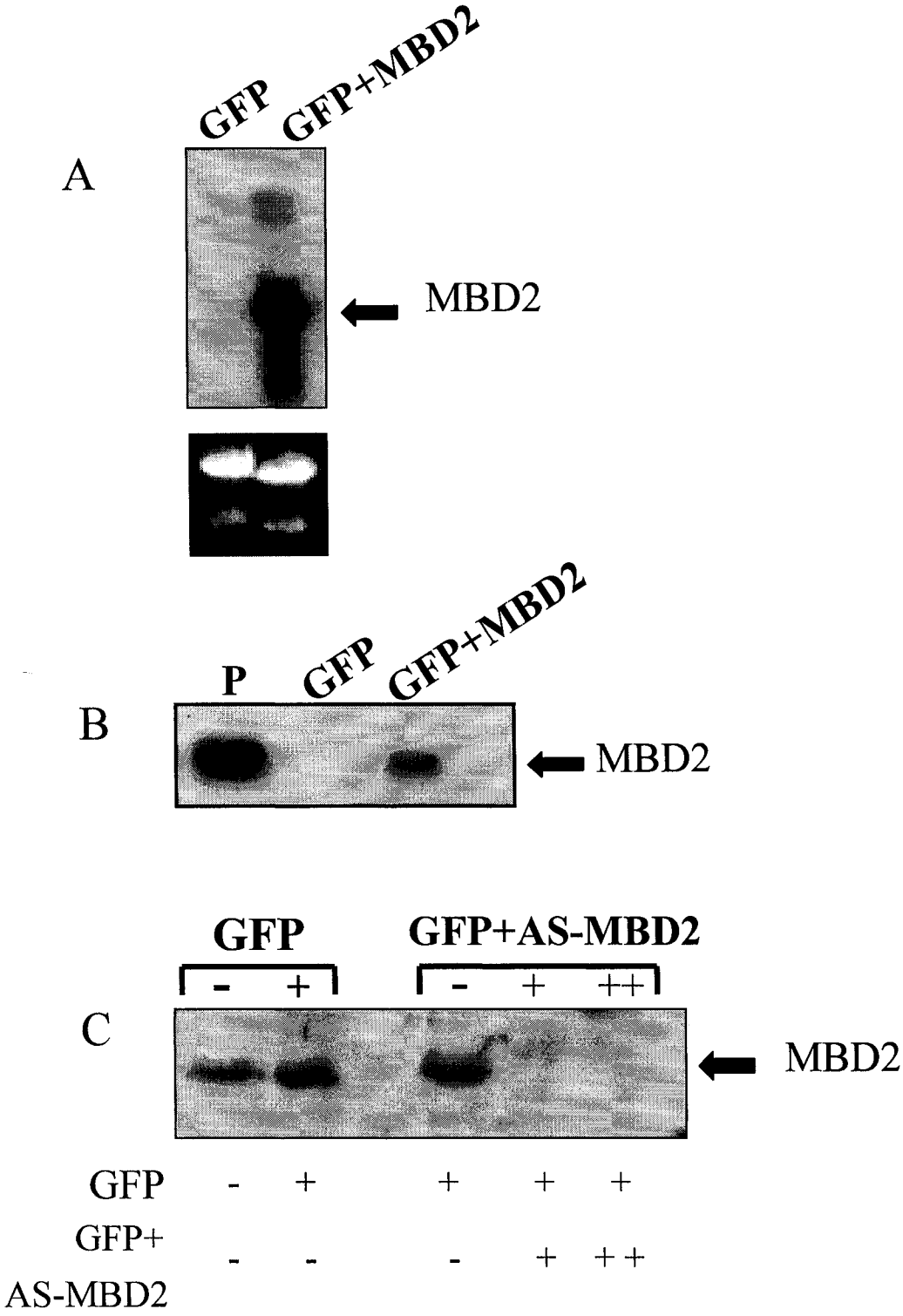

GFP
GFP+MBD2
A
MBD2
P
GFP
GFP+MBD2
B
MBD2
GFP
GFP+AS-MBD2
- + - + ++
C
MBD2
GFP - + + + +
GFP+ AS-MBD2 - - - + ++

Figure legend

Figure 3. 8: *Myogenin* is not induced by forced expression of MBD2b/demethylase. (a) C2C12 cells were infected with Ad-GFP or Ad-GFP-MBD2 respectively and maintained in growing medium for 72 hours. 20μg isolated RNA was loaded into each lane, fractioned on a 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybridization with ^{32}P-labelled *myogenin* probe. RNA isolated from cells maintained in growing medium was run as the negative control, while RNA isolated from normally differentiated C2C12 cells was the positive control. (b) The same membrane was stripped and re-blotted with ^{32}P-labelled MBD2b/demethylase probe, demonstrating a dose-dependent expression of MBD2b/demethylase. (c) Equal RNA loading as stained by ethidium bromide.

Figure 3.8

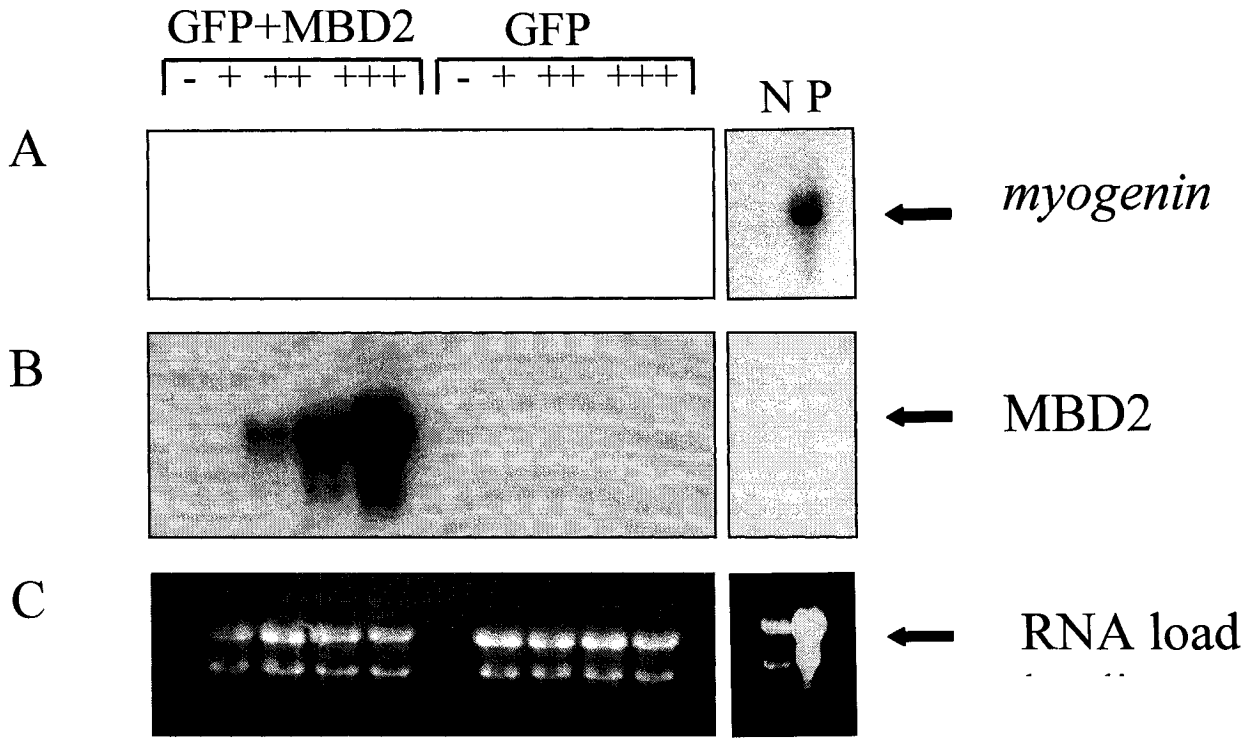


Figure Legend

Figure 3. 9: Antisense knock down of MBD2b/demethylase reduced the expression of *myogenin* significantly. (a) C2C12 cells maintained in growing medium were infected with Ad-GFP or Ad-GFP-AS respectively. 24 hours post infection, cells were supplied with differentiation medium and maintained for 72 hours. 20μg of isolated RNA was loaded in each lane, fractioned on a 1% agarose gel with formaldehyde, and then subjected to Northern blot transfer and hybrization with a ^{32}P-labelled *myogenin* probe, as shown on the top panel. The same membrane was stripped and blotted with a ^{32}P-labelled 18S rRNA probe, as shown at the bottom panel. (b) The expression of *myogenin* was normalized by the intensity of 18S probe blotting. Compared with GFP at same MOI, GFP-AS-MBD2b inhibited the expression of *myogenin* in a dose-dependent manner. The result is the average of three independent experiments with error bars displaying standard deviation.

Figure 3.9

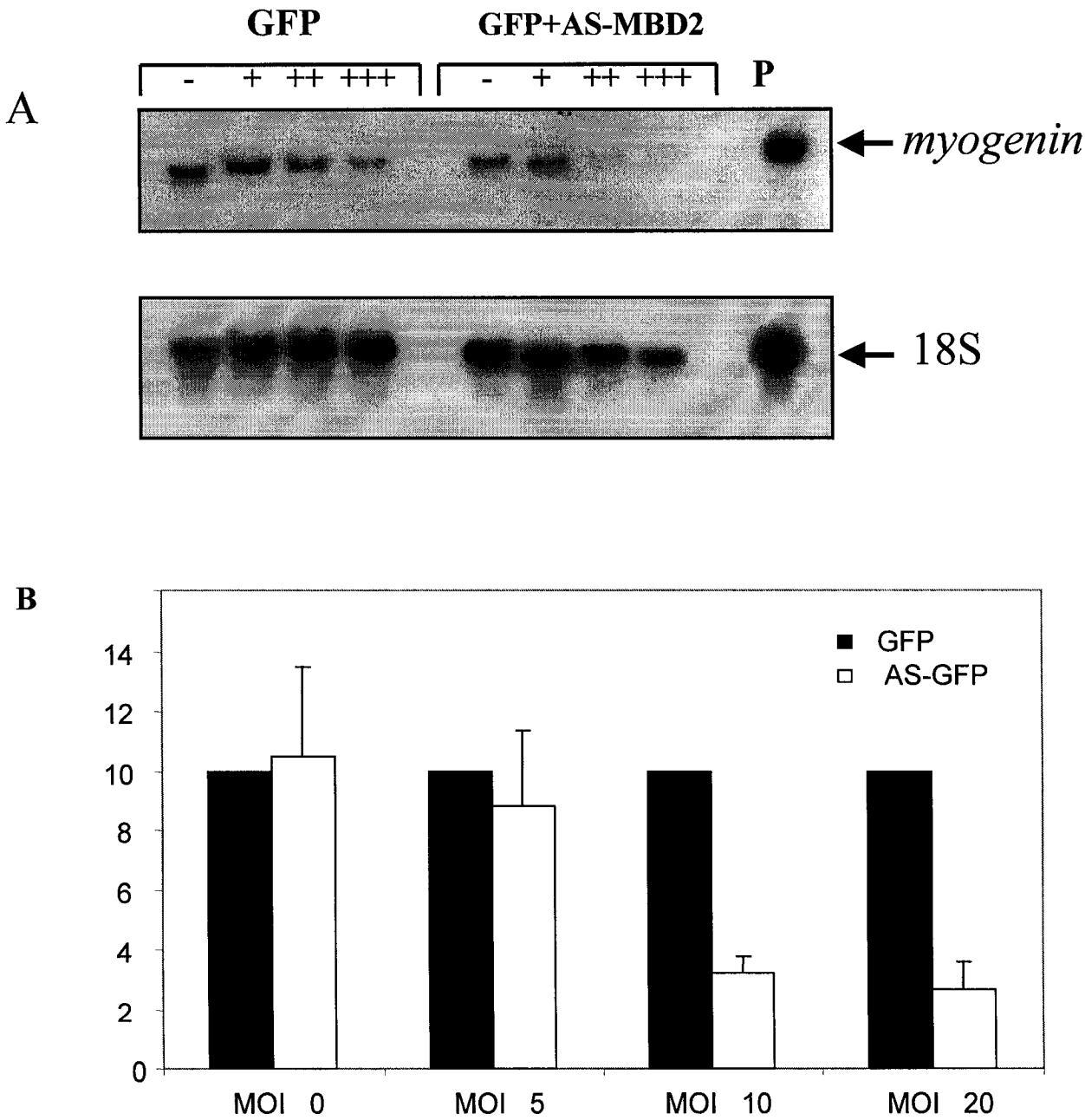

A
GFP
GFP+AS-MBD2
- + ++ +++
- + ++ +++
P
myogenin
18S
B
GFP
AS-GFP
14
12
10
8
6
4
2
0
MOI 0
MOI 5
MOI 10
MOI 20

Figure Legend

Figure 3. 10: Antisense knock down of MBD2b/demethylase leads to increased global methylation. C2C12 cells were infected with Ad-GFP or Ad-GFP-AS respectively. 24 hours post-infection, cells were supplied with fresh differentiation medium and maintained for 72 hours. 50μg isolated genomic DNA was digested with 40 units of XbaI followed by either 40 units of MspI or HpaII, fractionated on a 1.8% agarose gel, and subjected to Southern blot transfer and hybridization with ^{32}P-labelled MR150 probe. The hypomethylation of the genome is represented by the small fragments indicated by the arrows on the right. X=XbaI, M=MspI.

Figure 3.10

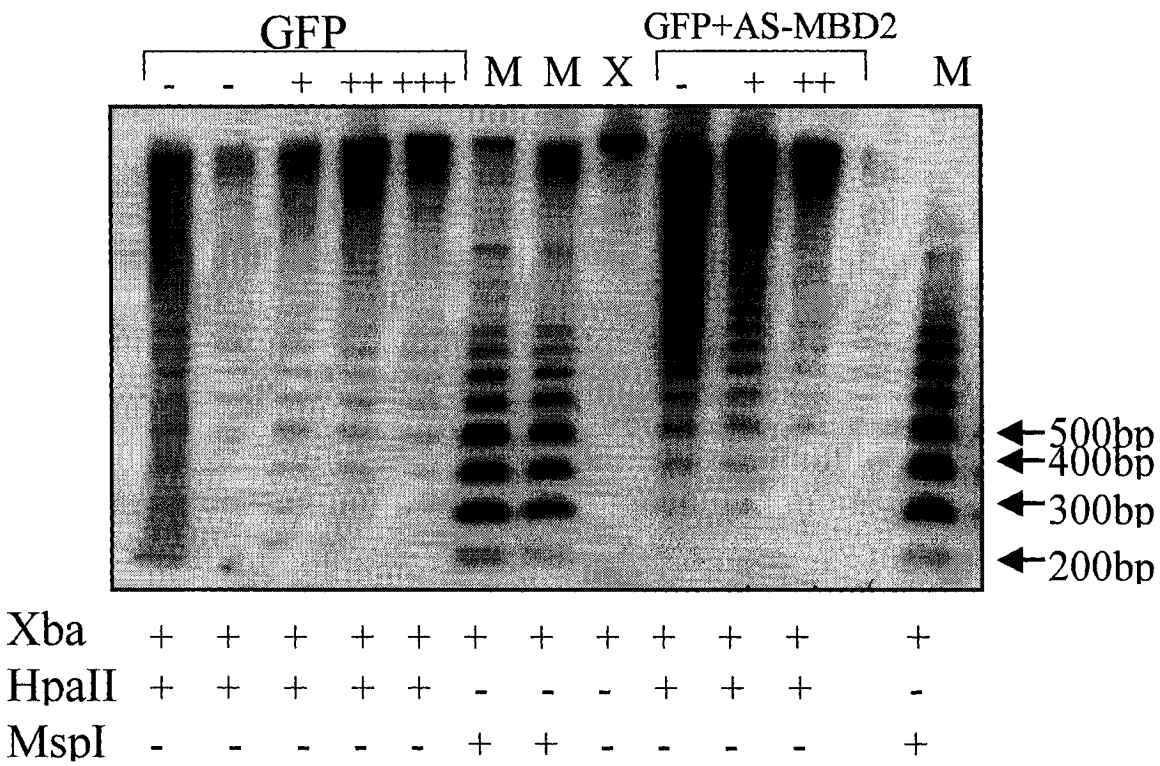
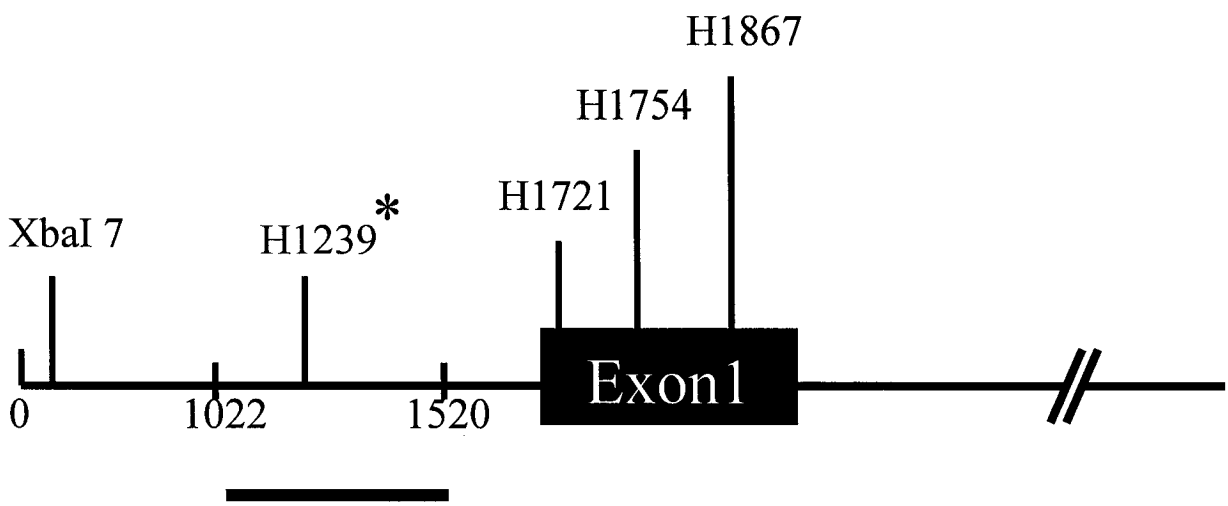


Figure Legend

Figure 3. 11: Antisense knock down of MBD2b/demethylase protects the promoter of *myogenin* from demethylation. (a) The illustration of *myogenin* gene structure. H represents a HpaII site, X represents a XbaI site. The CpG site undergoing demethylation during differentiation is marked by *. The probe recognizing this site is represented by the dark solid line below the structure of *myogenin*. (b) An example of Southern blotting showing the methylation change of the *myogenin* promoter during differentiation. The position and size of the each expected HpaII digested band is indicated by arrow on the right. (c) The demethylation of the *myogenin* promoter during differentiation was determined by quantifying the relative abundance of 1.2kb and 0.5kb fragments after endonuclease digestion and Southern blotting. The result is the average of three independent experiments with error bars displaying standard deviation. (d) An example of Southern blotting showing the methylation change of the *myogenin* promoter after introduction of antisense MBD2b/demethylase. The position and size of each expected HpaII digested band is indicated by arrow on the right. (e) The demethylation of the *myogenin* promoter after the introduction of antisense MBD2b/demethylase was determined by quantifying the relative abundance of 1.7kb, 1.2kb and 1.5kb fragments after endonuclease digestion and Southern blotting. The result is the average of three independent experiments with error bars displaying standard deviation.

Figure 3.11

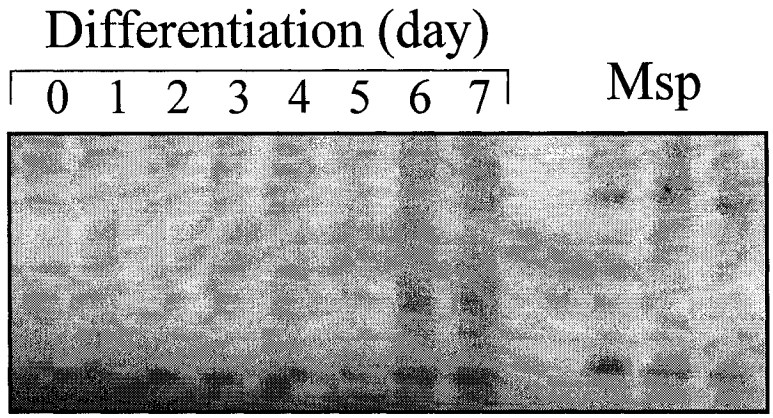
A



H: HpaI

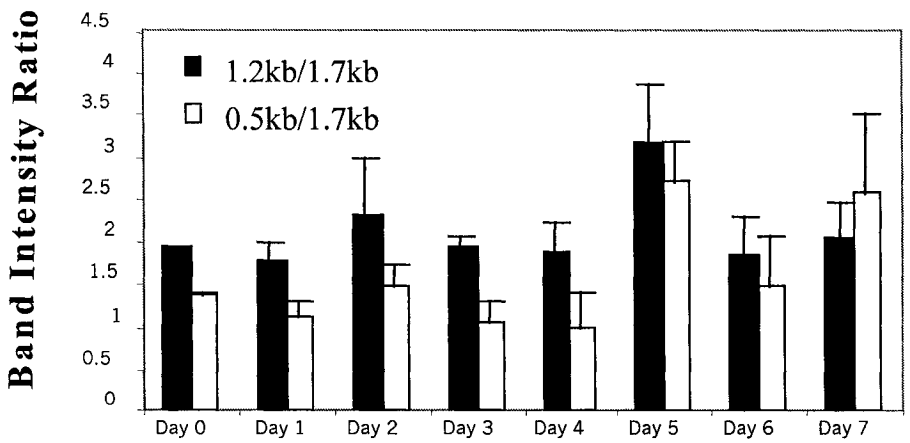
X: XbaI

B

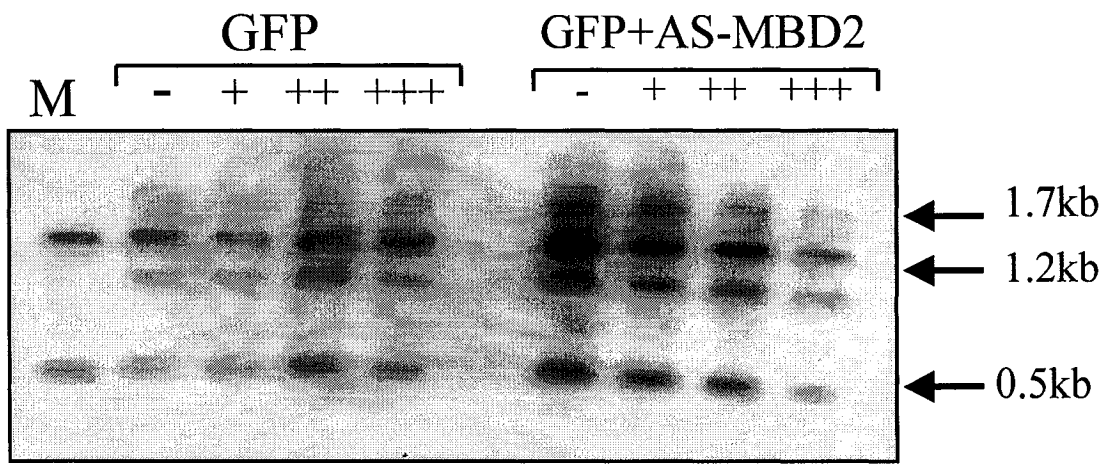


HpaII	+	+	+	+	+	+	+	+	-	-	-
MspI	-	-	-	-	-	-	-	-	+	+	+
XbaI	+	+	+	+	+	+	+	+	+	+	+

C



D


GFP
GFP+AS-MBD2
M
-
+
++
+++
-
+
++
+++
1.7kb
1.2kb
0.5kb

E

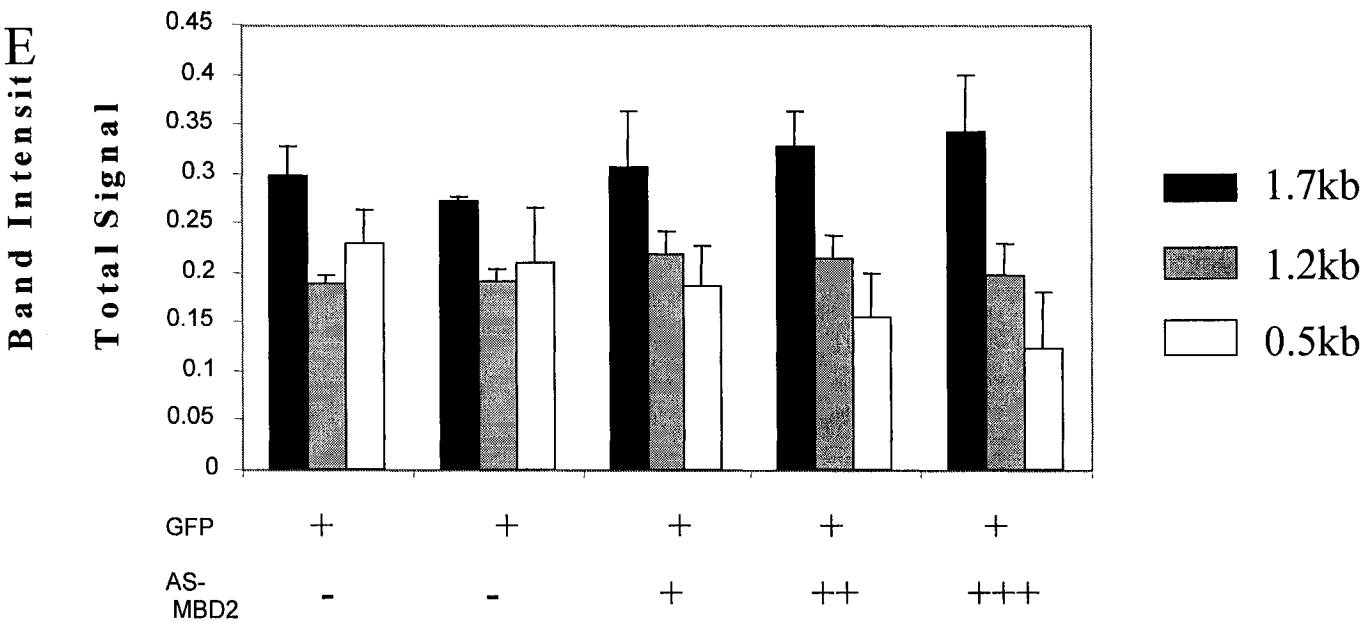
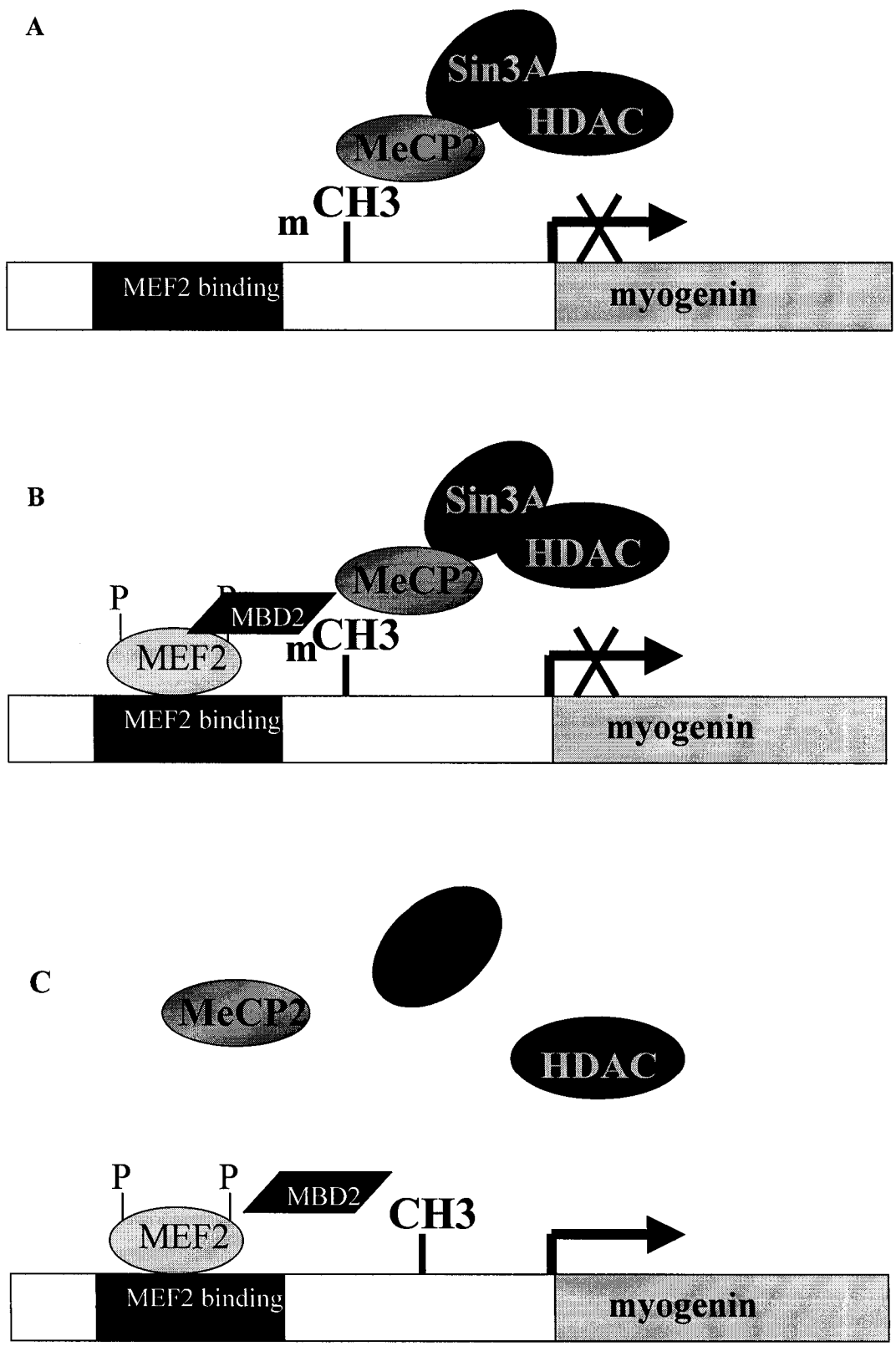

Band Intensity
Total Signal
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
GFP
AS-MBD2
1.7kb
1.2kb
0.5kb

Figure Legend

Figure 3.12: A model for the activation of *myogenin* during differentiation. (a) Before the initiation of C2C12 myoblast differentiation, the chromatin associated with the muscle-specific genes is in a closed configuration, and some critical regulatory factors such as *myogenin* are repressed by methyl-CpG binding protein mediated histone deacetylation. (b) Upon differentiation, C2C12 cells exit the cell-cycle and global hypomethylation serves to open the packaged chromatin. Phosphorylated and activated MEF2 binds to the promoter of *myogenin*, the histones around the promoter region of *myogenin* are acetylated. (c) MBD2b/demethylase gains accessibility to the methylated CpG site, demethylates and turns on the expression of *myogenin*.

Figure 3.12


A
Sin3A
HDAC
MeCP2
mCH3
MEF2 binding
myogenin
B
Sin3A
HDAC
MeCP2
P
MBD2
MEF2
mCH3
MEF2 binding
myogenin
C
MeCP2
HDAC
P
P
MBD2
MEF2
CH3
MEF2 binding
myogenin

CHAPTER 4: CONCLUSION

In this thesis, I established the temporal and causal relationship among MBD2b/demethylase, demethylation/ activation of *myogenin*, and differentiation of myoblasts. MBD2b/demethylase is involved in myogenesis through demethylating and activating the expression of *myogenin*, the master regulator of muscle differentiation. Regulation of MBD2b/demethylase is here observed under a physiological condition: muscle differentiation. The inhibition of MBD2b/demethylase by rapamycin revealed that MBD2b/demethylase is one of the nuclear effectors of pI3K/PKB-AKT/p70S6 pathway. During myogeneis, MBD2b/demethylase plays a role in demethylation of the promoter of *myogenin*, turning on its expression and consequently promoting the down stream events of myogenesis. Besides gene-specific demethylation, MBD2b/demethylase was also demonstrated to play a role in genome-wide demethylation, since antisense knock down of MBD2b/demethylase increased the degree of methylation of the whole genome.

Reversible methylation and demethylation was believed to shape the methylation pattern of the organism. The establishment and maintenance of DNA methylation patterns are studied very well. On the other hand, it is not clear yet how demethylation comes about. In this thesis, the demethylation function of MBD2b/demethylase is ***first*** illustrated under a biological process, which provides an example as of how a tissue-specific gene (*myogenin*) is regulated by the dynamic process of methylation and demethylation.

CHAPTER 5: REFERENCE

Aapola, U., K. Kawasaki, et al. (2000). "Isolation and initial characterization of a novel zinc finger gene, DNMT3L, on 21q22.3, related to the cytosine-5-methyltransferase 3 gene family." Genomics 65(3): 293-8.

Aapola, U., I. Liiv, et al. (2002). "Imprinting regulator DNMT3L is a transcriptional repressor associated with histone deacetylase activity." Nucleic Acids Res 30(16): 3602-8.

Aapola, U., R. Lyle, et al. (2001). "Isolation and initial characterization of the mouse Dnmt3l gene." Cytogenet Cell Genet 92(1-2): 122-6.

Aguirre-Arteta, A. M., I. Grunewald, et al. (2000). "Expression of an alternative Dnmt1 isoform during muscle differentiation." Cell Growth Differ 11(10): 551-9.

Amir, R. E., I. B. Van den Veyver, et al. (1999). "Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2." Nat Genet 23(2): 185-8.

Ammerpohl, O., M. L. Short, et al. (1997). "Complex protein binding to the mouse M-lysozyme gene downstream enhancer involves single-stranded DNA binding." Gene 200(1-2): 75-84.

Araujo, F.D., J. D. Knox, et al. (1998). "Concurrent replication and methylation at mammalian origins of replication." Mol Cell Biol. 18(6): 3475-82.

Araujo, F.D., J. D. Knox, et al. (1999). "Identification of initiation sites for DNA replication in the human Dnmt1 (DNA-methyltransferase) locus." J Biol Chem. 274(14): 9335-41.

Arnold, H. H. and B. Winter (1998). "Muscle differentiation: more complexity to the network of myogenic regulators." Curr Opin Genet Dev 8(5): 539-44.

Bachman, K. E., M. R. Rountree, et al. (2001). "Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin." J Biol Chem 276(34): 32282-7.

Bader, D., T. Masaki, et al. (1982). "Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro." J cell Biol 95(3): 763-70.

Bakker, J., X. Lin, et al. (2002). "Methyl-CpG binding domain protein 2 represses transcription from hypermethylated pi-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells." J Biol Chem 277(25): 22573-80.

Benezra, R., R. L. Davis, et al. (1990). "Id: a negative regulator of helix-loop-helix DNA binding proteins. Control of terminal myogenic differentiation." Ann N Y Acad Sci 599: 1-11.

Benezra, R., R. L. Davis, et al. (1990). "The protein Id: a negative regulator of helix-loop-helix DNA binding proteins." Cell 61(1): 49-59.

Bergman, Y. and R. Mostoslavsky (1998). "DNA demethylation: turning genes on." Biol Chem 379(4-5): 401-7.

Bestor, T., A. Laudano, et al. (1988). "Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases." J Mol Biol 203(4): 971-83.

Bestor, T. H. (1992). "Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain." Embo J 11(7): 2611-7.

Bestor, T. H. (2000). "The DNA methyltransferases of mammals." Hum Mol Genet 9(16): 2395-402.

Bhattacharya, S. K., S. Ramchandani, et al. (1999). "A mammalian protein with specific demethylase activity for mCpG DNA." Nature 397(6720): 579-83.

Billard, L. M., F. Magdinier, et al. (2002). "MeCP2 and MBD2 expression during normal and pathological growth of the human mammary gland." Oncogene 21(17): 2704-12.

Black, B. L. and E. N. Olson (1998). "Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins." Annu Rev Cell Dev Biol 14: 167-96.

Boeke, J., O. Ammerpohl, et al. (2000). "The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A." J Biol Chem 275(45): 34963-7.

Bourc'his, D., G. L. Xu, et al. (2001). "Dnmt3L and the establishment of maternal genomic imprints." Science 294(5551): 2536-9.

Braithwaite A.W. and I. A. Russell (2001). "Induction of cell death by Adenoviruses." Apotosis 6(5): 359-70.

Brandeis, M., D. Frank, et al. (1994). "Sp1 elements protect a CpG island from de novo methylation." Nature 371(6496): 435-8.

Brunk, B. P., D. J. Goldhamer, et al. (1996). "Regulated demethylation of the myoD distal enhancer during skeletal myogenesis." Dev Biol 177(2): 490-503.

Buchberger, A., K. Ragge, et al. (1994). "The myogenin gene is activated during myocyte differentiation by pre-existing, not newly synthesized transcription factor MEF-2. ". J Biol Chem. 269(25): 17289-96.

Cabane, C., W. Englaro, et al. (2003). "Regulation of C2C12 myogenic terminal differentiation by MKK3/p38alpha pathway." Am J Physiol Cell Physiol 284(3): C658-66.

Cervoni, N., S. Bhattacharya, et al. (1999). "DNA demethylase is a processive enzyme." J Biol Chem 274(13): 8363-6.

Cervoni, N., N. Detich, et al. (2002). "The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing." J Biol Chem **277**(28): 25026-31.

Cheng, T. C., B. S. Tseng, et al. (1995). "Activation of the myogenin promoter during mouse embryogenesis in the absence of positive autoregulation." Proc Natl Acad Sci U S A 92(2): 561-5.

Chomczynski, P. and N. Sacchi (1987). "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." Annal Biochem 162(1): 156-9.

Conejo, R., C. de Alvaro, et al. (2002). "Insulin restores differentiation of Ras-transformed C2C12 myoblasts by inducing NF-kappaB through an AKT/P70S6K/p38-MAPK pathway." Oncogene 21(23): 3739-53.

Conejo, R. and M. Lorenzo (2001). "Insulin signaling leading to proliferation, survival, and membrane ruffling in C2C12 myoblasts." J Cell Physiol 187(1): 96-108.

Coolican, S. A., D. S. Samuel, et al. (1997). "The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways." J Biol Chem 272(10): 6653-62.

Cox D. M., M. Du, et al. (2003). "Phosphorylation motifs regulating the stability and function of myocyte enhancer factor 2A." J Biol Chem 278(17): 15297-303.

Cuenda, A. and P. Cohen (1999). "Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis." J Biol Chem 274(7): 4341-6.

Dennis, K., T. Fan, et al. (2001). "Lsh, a member of the SNF2 family, is required for genome-wide methylation." Genes Dev 19(22): 2940-4.

de la Serna I. L., K. A. Carlson, et al. (2001) 'Mammalian SWI/SNF complexes promote MyoD-mediated muscle differentiation." Nat Genet 27(2): 187-90.

de la Serna I. L., K. Roy, et al. (2001). "MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes." Biol Chem 276(44): 41486-91.

Deplus, R., C. Brenner, et al. (2002). "Dnmt3L is a transcriptional repressor that recruits histone deacetylase." Nucleic Acids Res 30(17): 3831-8.

Detich, N., J. Theberge, et al. (2002). "Promoter-specific activation and demethylation by MBD2/demethylase." J Biol Chem 277(39): 35791-4.

Dirks, R. P., E. J. Klok, et al. (1996). "The sequence of regulatory events controlling the expression of the gamma D-crystallin gene during fibroblast growth factor-mediated rat lens fiber cell differentiation." Dev Biol 173(1): 14-25.

Dressel, U., P. J. Bailey, et al. (2001). "A dynamic role for HDAC7 in MEF2-mediated muscle differentiation." J Biol Chem 276(20): 17007-13.

Eckner, R., T. P. Yao, et al. (1996). "Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation." Genes Dev 10(19): 2478-90.

El-Osta, A., P. Kantharidis, et al. (2002). "Precipitous release of methyl-CpG binding protein 2 and histone deacetylase 1 from the methylated human multidrug resistance gene (MDR1) on activation." Mol Cell Biol 22(6): 1844-57.

Esteller, M. and J. G. Herman (2002). "Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours." J Pathol 196(1): 1-7.

Fickett J.W. (1996). "Coordinate positioning of MEF2 and myogenin binding sites." Gene 172(1): GC19-32.

Florini, J. R., D. Z. Ewton, et al. (1991). "Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression." Mol Endocrinol 5(5): 718-24.

Frank, D., I. Keshet, et al. (1991). "Demethylation of CpG islands in embryonic cells." Nature (6323): 239-41.

Fruhwald, M. C. and C. Plass (2002). "Global and gene-specific methylation patterns in cancer: aspects of tumor biology and clinical potential." Mol Genet Metab 75(1): 1-16.

Fuks, F., W. A. Burgers, et al. (2000). "DNA methyltransferase Dnmt1 associates with histone deacetylase activity." Nat Genet 24(1): 88-91.

Fuks, F., W. A. Burgers, et al. (2001). "Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription." Embo J 20(10): 2536-44.

Fuso, A., R. A. Cavallaro, et al. (2001). "Gene silencing by S-adenosylmethionine in muscle differentiation." FEBS Lett 508(3): 337-40.

Guo, K., J. Wang, et al. (1995). "MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation." Mol Cell Biol 15(7): 3823-9.

Guenbaum, Y., M. Szyf, et al. (1983). "Methylation of replicating and post-replicated mouse L-cell DNA." Proc Natl Acad Sci U S A. 80(16): 4919-21.

Guenbaum, Y., R. Stein et al. (1981). "Methylation of CpG sequences in eukaryotic DNA." FEBS Lett. 124(1): 67-71.

Habib, M., F. Fares, et al. (1999). "DNA global hypomethylation in EBV-transformed interphase nuclei." Exp Cell Res 249(1): 446-53.

Halevy, O., B. G. Novitch, et al. (1995). "Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD." Science 267(5200): 1018-21.

Hansen, R. S., C. Wijmenga, et al. (1999). "The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome." Proc Natl Acad Sci U S A 96(25): 14412-7.

Hasty, P., A. Bradley, et al. (1993). "Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene." Nature 364(6437): 501-6.

Hata, K., M. Okano, et al. (2002). "Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice." Development 129(8): 1983-93.

He, T.C., S. Zhou, et al. (1998). "A simplified system for generating recombinant adenoviruses." Proc Natl Acad Sci USA 95(5): 2509-14.

Hendrich, B. and A. Bird (1998). "Identification and characterization of a family of mammalian methyl-CpG binding proteins." Mol Cell Biol 18(11): 6538-47.

Hendrich, B., C. Abbott, et al. (1999). "Genomic structure and chromosomal mapping of the murine and human Mbd1, Mbd2, Mbd3, and Mbd4 genes." Mamm Genome 10(9): 906-12.

Hendrich B, J. Guy, et al. (2001). "Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development." Genes Dev 15(6): 710-723.

Iida, T., I. Suetake, et al. (2002). "PCNA clamp facilitates action of DNA cytosine methyltransferase 1 on hemimethylated DNA." Genes Cells 7(10): 997-1007.

Ivanov, MA., B. Lamrihi, et al. (2003). "Enhanced antitumor activity of a combination of MBD2-antisense electrotransfer gene therapy and bleomycin electrochemotherapy." J Gene Med. 5(10): 893-9.

Jiang, B. H., M. Aoki, et al. (1999). "Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B." Proc Natl Acad Sci U S A 96(5): 2077-81.

Johanson, M., H. Meents, et al. (1999) "Transcriptional activation of the myogenin gene by MEF2-mediated recruitment of myf5 is inhibited by adenovirus E1A protein." Biochem Biophys Res Commun 265(1): 222-32.

Jones, P. A., M. J. Wolkowicz, et al. (1990). "Methylation and expression of the Myo D1 determination gene." Philos Trans R Soc Lond B Biol Sci 326(1235): 277-84.

Jost, J. P. and Y. C. Jost (1994). "Transient DNA demethylation in differentiating mouse myoblasts correlates with higher activity of 5-methyldeoxycytidine excision repair." J Biol Chem 269(13): 10040-3.

Jost, J. P., E. J. Oakeley, et al. (2001). "5-Methylcytosine DNA glycosylase participates in the genome-wide loss of DNA methylation occurring during mouse myoblast differentiation." Nucleic Acids Res 29(21): 4452-61.

Jost, J. P., M. Siegmann, et al. (1995). "Mechanisms of DNA demethylation in chicken embryos. Purification and properties of a 5-methylcytosine-DNA glycosylase." J Biol Chem 270(17): 9734-9.

Kaliman, P., J. Canicio, et al. (1999). "Insulin-like growth factor-II, phosphatidylinositol 3-kinase, nuclear factor-kappaB and inducible nitric-oxide synthase define a common myogenic signaling pathway." J Biol Chem 274(25): 17437-44.

Kanai, Y., S. Ushijima, et al. (1999). "Reduced mRNA expression of the DNA demethylase, MBD2, in human colorectal and stomach cancers." Biochem Biophys Res Commun 264(3): 962-6.

Kenigsberg, R. L. , F. M. Semenenko, et al. (1990). "Development of a bi-specific monoclonal antibody for simultaneous detection of rabbit IgG and horseradish peroxidase: use as a general reagent in immunocytochemistry and enzyme-linked immunosorbent assay." J Histochem Cytochem 38(2): 191-8.

Kim, G. D., J. Ni, et al. (2002). "Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases." Embo J 21(15): 4183-95.

Kirillov, A., B. Kistler, et al. (1996). "A role for nuclear NF-kappaB in B-cell-specific demethylation of the Igkappa locus." Nat Genet 13(4): 435-41.

Knox J. D., F. D. Araujo, et al. (2000) "Inhibition of DNA methyltransferase inhibits DNA replication." J Biol Chem 275(24): 17986-90.

Kremer, E. J., M. Pritchard, et al. (1991). "Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n." Science 252(5013): 1711-4.

La Rocca, S. A., D. H. Crouch, et al. (1994). "c-Myc inhibits myogenic differentiation and myoD expression by a mechanism which can be dissociated from cell transformation." Oncogene 9(12): 3499-508.

Lassar, A. B., B. M. Paterson, et al. (1986). "Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts." Cell 47(5): 649-56.

Lei, H., S. P. Oh, et al. (1996). "De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells." Development 122(10): 3195-205.

Leonhardt, H., A. W. Page, et al. (1992). "A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei." Cell 71(5): 865-73.

Lewis, J. D., R. R. Meehan, et al. (1992). "Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA." Cell 69(6): 905-14.

Li, E. (2002). "Chromatin modification and epigenetic reprogramming in mammalian development." Nat Rev Genet 3(9): 662-73.

Li, E., T. H. Bestor, et al. (1992). "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." Cell 69(6): 915-26.

Liew C. C., N. thi Man, et al. (1980) "Histone acetylation in differentiating chick myoblast cultures." Cell Biol Int Rep. 4(7): 711-6.

Li, L., J. C. Chambard, et al. (1992). "Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression." Genes Dev 6(4): 676-89.

Li, L., J. Zhou, et al. (1992). "FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains." Cell 71(7): 1181-94.

Li, L., R. Heller-Harrison, et al. (1992). "Cyclic AMP-dependent protein kinase inhibits the activity of myogenic helix-loop-helix proteins." Mol Cell Biol 12(10): 4478-85.

Liang, G., M. F. Chan, et al. (2002). "Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements." Mol Cell Biol 22(2): 480-91.

Lin, X. and W. G. Nelson (2003). "Methyl-CpG-binding Domain Protein-2 Mediates Transcriptional Repression Associated with Hypermethylated GSTP1 CpG Islands in MCF-7 Breast Cancer Cells." Cancer Res 63(2): 498-504.

Liu, Y., L. Sun, et al. (1996). "In differentiating mouse myoblasts DNA methyltransferase is posttranscriptionally and posttranslationally regulated." Nucleic Acids Res 24(14): 2718-22.

Lubbert, M., C. W. Miller, et al. (1991). "Changes of DNA methylation and chromatin structure in the human myeloperoxidase gene during myeloid differentiation." Blood 78(2): 345-56.

Lucarelli, M., A. Fuso, et al. (2001). "The dynamics of myogenin site-specific demethylation is strongly correlated with its expression and with muscle differentiation." J Biol Chem 276(10): 7500-6.

Lu. J., T. A. McKinsey, et al. (2000). "Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases." Mol Cell 6(2): 233-44.

Lu. J., T. A. McKinsey, et al. (2000). "Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases." Proc Natl Acad Sci USA 97(8): 4070-5.

Macleod, D., J. Charlton, et al. (1994). "Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island." Genes Dev 8(19): 2282-92.

Magdinier, F. and A. P. Wolffe (2001). "Selective association of the methyl-CpG binding protein MBD2 with the silent p14/p16 locus in human neoplasia." Proc Natl Acad Sci U S A 98(9): 4990-5.

Mayer, W., A. Niveleau, et al. (2000). "Demethylation of the zygotic paternal genome." Nature 403(6769): 501-2.

McKinsey T. A., C. L. Zhang, et al. (2000). "Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5." Proc Natl Acad Sci USA 97(26): 14400-5.

McKinsey, T. A., C. L. Zhang, et al. (2000). "Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation." Nature 408(6808): 106-11.

McKinsey T. A., C. L. Zhang, et al. (2001). "Control of muscle development by dueling HATs and HDACs." Curr Opin Genet Dev 11(5): 497-504.

Milutinovic S, J. D. Knox, et al. (2000). "DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21(WAF1/CIP1/sdi1)." J Biol Chem. 275(9): 6353-9.

Milutinovic, S., Q. Zhuang, et al. (2003). "Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes." J Biol Chem. 278(17): 14985-95.

Muller-Tidow, C., K. Kugler, et al. (2001). "Loss of expression of HDAC-recruiting methyl-CpG-binding domain proteins in human cancer." Br J Cancer 85(8): 1168-74.

Nabeshima, Y., K. Hanaoka, et al. (1993). "Myogenin gene disruption results in perinatal lethality because of severe muscle defect." Nature 364(6437): 532-5.

Nan, X., F. J. Campoy, et al. (1997). "MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin." Cell 88(4): 471-81.

Nan, X., H. H. Ng, et al. (1998). "Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex." Nature 393(6683): 386-9.

Naya F. S. and E. Olson (1999). "MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation." Curr Opin Cell Biol 11(6): 683-8.

Ng, H. H., P. Jeppesen, et al. (2000). "Active repression of methylated genes by the chromosomal protein MBD1." Mol Cell Biol 20(4): 1394-406.

Ng, H. H., Y. Zhang, et al. (1999). "MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex." Nat Genet 23(1): 58-61.

Nickel, J., M. L. Short, et al. (1995). "Methylation of the mouse M-lysozyme downstream enhancer inhibits heterotetrameric GABP binding." Nucleic Acids Res 23(23): 4785-92.

Oberle, I., F. Rousseau, et al. (1991). "Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome." Science 252(5010): 1097-102.

Okano, M., D. W. Bell, et al. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell 99(3): 247-57.

Okano, M., S. Xie, et al. (1998). "Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases." Nat Genet 19(3): 219-20.

Okano, M., S. Xie, et al. (1998). "Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells." Nucleic Acids Res 26(11): 2536-40.

Olson, E. N., G. Spizz, et al. (1987). "The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation." Mol Cell Biol 7(6): 2104-11.

Parker, S. B., G. Eichele, et al. (1995). "p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells." Science 267(5200): 1024-7.

Paroush, Z., I. Keshet, et al. (1990). "Dynamics of demethylation and activation of the alpha-actin gene in myoblasts." Cell 63(6): 1229-37.

Patra, S. K., A. Patra, et al. (2002). "DNA methyltransferase and demethylase in human prostate cancer." Mol Carcinog 33(3): 163-71.

Perry, R. L. and M. A. Rudnick (2000). "Molecular mechanisms regulating myogenic determination and differentiation." Front Biosci 5: D750-67.

Puri, P. L., V. Sartorelli, et al. (1997). "Differential roles of p300 and PCAF acetyltransferases in muscle differentiation." Mol Cell 1(1): 35-45.

Ramchandani, S., S. K. Bhattacharya, et al. (1999). "DNA methylation is a reversible biological signal." Proc Natl Acad Sci U S A 96(11): 6107-12.

Razin A (1998). "CpG methylation, chromatin structure and gene silencing-a

three-way connection." EMBO J 17(17): 4905-8.

Razin A and A. D. Riggs. (1980). "DNA methylation and gene function." Science 210 (4470): 604-10.

Razin A., E. Feldmesser, et al. (1985). "Cell specific DNA methylation patterns; formation and a nucleosome locking model for their function." Prog Clin Biol Res. 198: 239-253.

Razin A and M. Szyf. (1984). "DNA methylation patterns. Formation and function." Biochim Biophys Acta. 782(4): 331-42.

Reik, W., W. Dean, et al. (2001). "Epigenetic reprogramming in mammalian development." Science 293(5532): 1089-93.

Rhee, I., K. E. Bachman, et al. (2002). "DNMT1 and DNMT3b cooperate to silence genes in human cancer cells." Nature 416(6880): 552-6.

Robertson, K. D., S. Ait-Si-Ali, et al. (2000). "DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters." Nat Genet 25(3): 338-42.

Rogerson, P. J., M. Jamali, et al. (2002). "The C-terminus of myogenin, but not MyoD, targets upregulation of MEF2C expression." FEBS Lett 524(1-3): 134-8.

Rosenthal, S. M., D. Hsiao, et al. (1994). "An insulin-like growth factor-II (IGF-II) analog with highly selective affinity for IGF-II receptors stimulates differentiation, but not IGF-I receptor down-regulation in muscle cells." Endocrinology 135(1): 38-44.

Rougier, N., D. Bourc'his, et al. (1998). "Chromosome methylation patterns during mammalian preimplantation development." Genes Dev 12(14): 2108-13.

Rountree, M. R., K. E. Bachman, et al. (2000). "DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci." Nat Genet 25(3): 269-77.

Rudnicki, M.A. and R. Jaenisch. (1995). "The MyoD family of transcription factors and skeletal myogenesis. " Bioessays. 17(3): 203-9.

Sabourin, L. A. and M. A. Rudnicki (2000). "The molecular regulation of myogenesis." Clin Genet 57(1): 16-25.

Saito, M. and F. Ishikawa (2002). "The mCpG-binding domain of human MBD3 does not bind to mCpG but interacts with NuRD/Mi2 components HDAC1 and MTA2." J Biol Chem 277(38): 35434-9.

Sansom, OJ., J. Berger, et al. (2003). "Deficiency of Mbd2 suppresses intestinal tumorigenesis." Nat Genet. 34(2): 145-7.

Santoso, B., B. D. Ortiz, et al. (2000). "Control of organ-specific demethylation by an element of the T-cell receptor-alpha locus control region." J Biol Chem 275(3): 1952-8.

Sartorelli, V., J. Huamg, et al. (1997). "Molecular mechanisms of myogenic coactivation by p300: direct interaction with the activation domain of MyoD and with the MADS box of MEF2C." Mol Cell Biol. 17(2): 1010-26.

Sato, M., Y. Horio, et al. (2002). "The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a) and RASSF1A in human lung cancer cell lines." Oncogene 21(31): 4822-9.

Scarpa, S., M. Lucarelli, et al. (1996). "Simultaneous myogenin expression and overall DNA hypomethylation promote in vitro myoblast differentiation." Cell Growth Differ 7(8): 1051-8.

Selker, E. U. (1998). "Trichostatin A causes selective loss of DNA methylation in Neurospora." Proc Natl Acad Sci U S A 95(16): 9430-5.

Shahbazian, M., J. Young, et al. (2002). "Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3." Neuron 35(2): 243-54.

Skapek, S. X., J. Rhee, et al. (1995). "Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase." Science 267(5200): 1022-4.

Slack, A., V. Bovenzi, et al. (2002). "Antisense MBD2 gene therapy inhibits tumorigenesis." J Gene Med 4(4): 381-9.

Spicer, D. B., J. Rhee, et al. (1996). "Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist." Science 272(5267): 1476-80.

Strahl B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature 403 (6765): 41-5.

Szyf, M. (1991). "DNA methylation patterns: an additional level of information?" Biochem Cell Biol 69 (12): 764-7.

Szyf, M. (1994). "DNA methylation properties: consequences for pharmacology." Trends Pharmacol Sci 15(7): 233-8.

Szyf, M. (1996). "The DNA methylation machinery as a target for anticancer therapy." Pharmacol Ther 70 (1): 1-37.

Szyf, M., G. Tanigawa, et al. (1990). "A DNA signal from the Thy-1 gene defines de novo methylation patterns in embryonic stem cells." Mol Cell Biol 10(8): 4396-400.

Szyf, M., J. Rouleau, et al. (1992). "Induction of myogenic differentiation by an expression vector encoding the DNA methyltransferase cDNA sequence in the antisense orientation." J Biol Chem 267(18): 12831-6.

Szyf M., L. Eliasson, et al. (1985). "Cellular and viral DNA hypomethylation associated with induction of Epstein-Barr virus lytic cycle." Proc Natl Acad Sci USA 82(23): 8090-4.

Szyf M., J. Theberge, et al. (1995). "Ras induces a general DNA demethylation activity in mouse embryonal P19 cells." J Biol Chem 70(21): 12690-6.

Szyf M., V. Bozovic, et al. (1991). "Growth regulation of mouse DNA methyltransferase gene expression." J Biol Chem. 266(16): 10027-30.

Takaji, H., S. Tajima, et al. (1995). "Overexpression of DNA methyltransferase in myoblast cells accelerates myotube formation." Eur J Biochem. 231(2): 282-91.

Tatematsu, K. I., T. Yamazaki, et al. (2000). "MBD2-MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase." Genes Cells 5(8): 677-88.

Trouche, D., H. Masutani, et al. (1995). "Myogenin binds to and represses c-fos promoter." FEBS Lett 361(2-3): 140-4.

Wade, P. A. (2001). "Methyl CpG-binding proteins and transcriptional repression." Bioessays 23(12): 1131-7.

Weintraub, H. (1993). "The MyoD family and myogenesis: redundancy, networks, and thresholds." Cell 75(7): 1241-4.

Weiss, A., I. Keshet, et al. (1996). "DNA demethylation in vitro: involvement of RNA." Cell 86(5): 709-18.

Wright, W. E., D. A. Sassoon, et al. (1989). "Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD." Cell 56(4): 607-17.

Wu, J., J. P. Issa, et al. (1993). "Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells." Proc Natl Acad Sci USA 90(19): 8891-5.

Xu, G. L., T. H. Bestor, et al. (1999). "Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene." Nature 402(6758): 187-91.

Xu, Q and Z. Wu (2000). "The insulin-like growth factor-phosphatidylinositol 3-kinase-Akt signaling pathway regulates myogenin expression in normal myogenic cells but not in rhabdomyosarcoma-derived RD cells." J Biol Chem 275(47): 36750-7.

Yokomori, N., M. Tawata, et al. (1999). "DNA demethylation during the differentiation of 3T3-L1 cells affects the expression of the mouse GLUT4 gene." Diabetes 48(4): 685-90.

Zetser, A., E. Gredinger, et al. (1999). "p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor." J Biol Chem 274(8): 5193-200.

Zhang, C. L., T. A. McKinsey, et al. (2002). "Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation." Mol Cell Biol 22(20): 7302-12.

Zhang, J. M., Q. Wei, et al. (1999). "Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4." Embo J 18(4): 926-33.

Zhang, Y., H. H. Ng, et al. (1999). "Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation." Genes Dev 13(15): 1924-35.

Zhang, C. L., T. A. McKinsey, et al. (2002). "Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation." Mol Cell Biol **22**(20): 7302-12.

Zhu, B., Y. Zheng, et al. (2000). "5-Methylcytosine DNA glycosylase activity is also present in the human MBD4 (G/T mismatch glycosylase) and in a related avian sequence." Nucleic Acids Res 28(21): 4157-65.

APPENDIX