



## Characterization of the Methylation Status of *Pax7* and Myogenic Regulator Factors in Cell Myogenic Differentiation

Zhe Chao, Xin-Li Zheng, Rui-Ping Sun, Hai-Long Liu, Li-Li Huang, Zong-Xi Cao, Chang-Yan Deng<sup>1</sup>, and Feng Wang\*

Institute of Animal Sciences and Veterinary, Hainan Academy of Agricultural Sciences, Haikou 571100, China

**ABSTRACT:** Epigenetic processes in the development of skeletal muscle have been appreciated for over a decade. DNA methylation is a major epigenetic modification important for regulating gene expression and suppressing spurious transcription. Up to now, the importance of epigenetic marks in the regulation of *Pax7* and myogenic regulatory factors (MRFs) expression is far less explored. In the present study, semi-quantitative the real-time polymerase chain reaction (RT-PCR) analyses showed *MyoD* and *Myf5* were expressed in activated and quiescent C2C12 cells. *MyoG* was expressed in a later stage of myogenesis. *Pax7* was weakly expressed in differentiated C2C12 cells. To further understand the regulation of expression of these genes, the DNA methylation status of *Pax7*, *MyoD*, and *Myf5* was determined by bisulfite sequencing PCR. During the C2C12 myoblasts fusion process, the changes of promoter and exon 1 methylation of *Pax7*, *MyoD*, and *Myf5* genes were observed. In addition, an inverse relationship of low methylation and high expression was found. These results suggest that DNA methylation may be an important mechanism regulating *Pax7* and MRFs transcription in cell myogenic differentiation. (**Key Words:** *Pax7*, Myogenic Regulatory Factors, C2C12, DNA Methylation, CpG Islands)

### INTRODUCTION

The *Pax* gene family defines an evolutionary conserved group of transcription factors that play critical roles during organogenesis and tissue homeostasis (Olguin et al., 2007). Two members of the *Pax* family of transcriptional factors, *Pax3* and *Pax7*, are supposed to be involved in the myogenic cell specification (Horst et al., 2006). The *Pax7* gene is indeed a key regulator of muscle cell differentiation in specific populations of adult stem cells and is a good candidate to distinguish activated satellite cells (Caliaro et al., 2005).

Differentiation of mammalian skeletal muscle cells is regulated by members of the *MyoD* family of myogenic regulatory factors (MRFs). These include *MyoD*, *Myf5*, *myogenin*, and *MRF4* (Weintraub, 1993). Cultured

myoblasts express one or others of these markers as well. *MyoD* and *Myf5* are required for commitment to myogenic lineage. *Myogenin* and *MRF4* have been reported to have a role in terminal differentiation of the myoblasts (Abe et al., 2009). *MRFs* and *Pax7* appear to play critical roles during satellite cell activation, proliferation and differentiation. *Pax7* is expressed almost ubiquitously by quiescent satellite cells and is co-expressed with *MyoD* in their proliferating myoblast progeny (Collins et al., 2009). In C2C12 immortalised myoblasts, *Pax7* has recently been shown to induce chromatin modifications through association with a histone methyltransferase complex and direct binding to regulatory regions of the *Myf5* locus. Although some results provide evidence for a functional relationship between *Pax7* and the *MyoD* family of transcription factors, the exact nature of this relationship is controversial.

DNA methylation at CpG dinucleotides is a well-studied epigenetic mechanism, defined as a heritable and enzyme-induced chemical modification of DNA, not altering the DNA sequence (Wu et al., 2007). The frequency of CpG dinucleotides is strikingly low in the mammalian genome.

\* Corresponding Author: Feng Wang. Tel: +86-898-65314685, Fax: +86-898-65314685, E-mail: 503911940@qq.com

<sup>1</sup> College of Animal Science, Huazhong Agricultural University, Wuhan 430070, China.

Submitted May 25, 2015; Revised Oct. 1, 2015; Accepted Nov. 13, 2015

However, approximately half of the human gene promoter regions contain dinucleotide clusters of CpGs, known as "CpG islands". A large number of experiments have shown that methylation of promoter CpG islands plays an important role in the regulation of gene expression, genomic imprinting, mammalian development, inactivation of the X chromosome, and carcinogenesis (Takai and Jones, 2002; Palacios and Puri, 2006; Nakamura et al., 2014).

Epigenetic changes occur within muscle cells during development, maturation and regeneration. During development, the determination of mesenchymal cells to either a myogenic, adipogenic, osteogenic or chondrogenic lineage is in part due to stable modifications made to the methylation pattern of DNA that prevents the transcription of genes from other lineages (Barr, 2010). The DNA methylation states of *Pax7* and *MRFs* may serve as an effective indicator of the degree of cell myogenic differentiation. To this purpose, we examined the methylation profile expression of *Pax7* and *MRFs* in the mouse skeletal muscle precursor cell line C2C12. Real-time polymerase chain reaction (RT-PCR) and bisulfite genomic sequencing show a correlation between the state of DNA methylation within the promoter or exon1 CpG-islands and the levels of *Pax7* and *MRFs* expression in cell myogenic differentiation. These findings suggest that DNA methylation is a major regulator of *Pax7* and *MRFs* transcription in cell myogenic differentiation.

## MATERIALS AND METHODS

### Cell culture

The C2C12 (mouse) skeletal muscle cell line was obtained from the American Type Culture Collection (Nos. CRL-1772). The cell line was cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen, USA), 100 U/mL penicillin, and 100

µg/mL streptomycin (Invitrogen, USA) as recommended by the supplier. C2C12 cells were cultured in DMEM containing 2% horse serum (Invitrogen, USA) for differentiation, and harvested at various times (0, 1, 3, 5, 8, or 10 days).

### RNA extraction, cDNA synthesis, and semi-quantitative RT-PCR

Total RNA was isolated from the cells with TRIzol Reagent (Invitrogen, USA) according to standard protocols, and the reverse transcription was performed based on the method described previously by Qiao et al. (2010). The PCR primer sequences used for semi-quantitative RT-PCR for *Pax7* (NM\_011039.2), *MyoD* (NM\_010866.2), *Myf5* (NM\_008656.5), *myogenin* (NM\_031189.2), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (NM\_008084.2) are presented in Supplemental Table 1. The sequences of these genes were obtained from GenBank database.

The *Pax7* and *MRFs* genes mRNA expression in cell myogenic differentiation were detected by semi-quantitative RT-PCR with the *GAPDH* gene as an internal control, and all PCRs were performed in triplicate. PCR amplifications were carried out in a 50 µL reaction mixture containing 2 µL normalized template cDNA, 1.0 µL of each primer (5 µM), 4.0 µL of each dNTP (2 mM), 5.0 µL of 10×PCR buffer, 4.0 µL of (25 mmol/L) Mg<sup>2+</sup> and 2 µL (1 U/µL) of Taq DNA polymerase (Fermentas, Burlington, ON, Canada) and 31.0 µL sterile water. The PCR amplification profiles were as follows: 94°C initial denaturation for 4 min, 35 cycles of 94°C denaturation for 40 s, 59°C to 65°C annealing for 40 s, and 72°C extension for 15 to 20 s (according the length of the target fragments), followed by a 10 min extension at 72°C.

### DNA isolation and bisulfite modification

Genomic DNA was extracted from the cells according

**Table 1.** Primers used for nested polymerase chain reaction

Gene	Primer name	Primer sequence(5'→3')	AT (°C)	Size (bp)
<i>MyoD</i>	Outside forward	ATTTAGGAATTGGGATATGGAG	62	402
	Outside reverse	AAAACCTCATTCACCTTACTCAAA		
	Inside forward	TAGGAATTGGGATATGGAGTTTT	63	309
	Inside reverse	TTACAAACCCACAACAACAAC		
<i>Myf5</i>	Outside forward	GGTATTTTTGAAAGTTTTTGTT	58	417
	Outside reverse	AAATAAACCAAATAACCTTCCC		
	Inside forward	GGTGTTAGTTGGTTTGAAAAGT	60	300
	Inside reverse	AAATAAACCAAATAACCTTCCC		
<i>Pax7</i>	Outside forward	TAGGGTGTGAGGGTTAGTAGAA	60	297
	Outside reverse	ACAAAAAATTCAAACAAACAAA		
	Inside forward	GGTGTGAGGGTTAGTAGAAAGA	61	291
	Inside reverse	AAAAAATTCAAACAAACAAACACT		

AT, annealing temperature.

to the standard phenol-chloroform method and stored at  $-20^{\circ}\text{C}$ . About 0.5 to 1  $\mu\text{g}$  of genomic DNA was used for subsequent modification. Bisulfite treatment converting all cytosine residues except 5-methylcytosine to uracil was performed by established protocols (Frommer et al., 1992).

### PCR amplification of bisulfite-modified DNA and sequencing

All mutagenized DNA was subjected to three independent nested amplifications, to eliminate the sampling bias of PCR. About 2  $\mu\text{L}$  of bisulfite-treated DNA was amplified using specific primers (Table 1), which were designed by Methyl Primer Express Software v1.0 (ABI, Foster, CA, USA). Taq DNA polymerase (Fermentas, Canada) was used for the two rounds of PCR, following the annealing temperatures described in Table 1.

For sequence analysis, the PCR products were cloned into the pMD 18-T cloning vector (TaKaRa, Dalian, China) and 10 individual clones were sequenced.

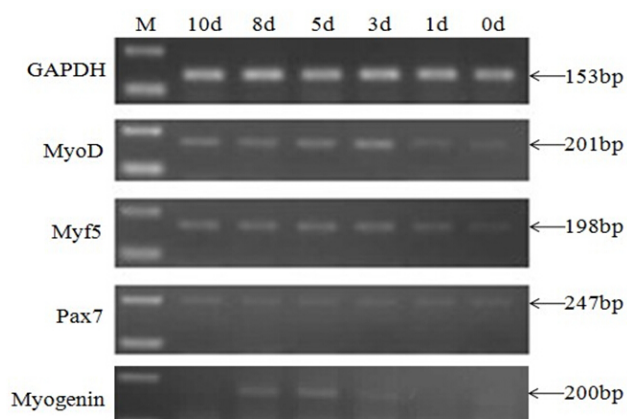
### CpG islands identification

CpG islands were predicted using an online software (<http://www.urogene.org/methprimer/index1.html>).

## RESULTS

### Expression analysis of *Pax7* and *MRFs* genes in myogenic differentiation in C2C12 cells

To investigate the differential expression of *Pax7* and *MRFs* during myoblasts differentiation, cultured C2C12 myoblasts were induced to differentiate. Myotubes started to appear at day 3 and became more evident at days 5, 7, and 10 (Supplementary Figure S1). As shown in Figure 1, *MyoD* and *Myf5* were expressed at 0 d with a relatively low level then increased to a peak at 3 d. *Myogenin* was hardly expressed at 0 d and 1 d, and then increased within the first



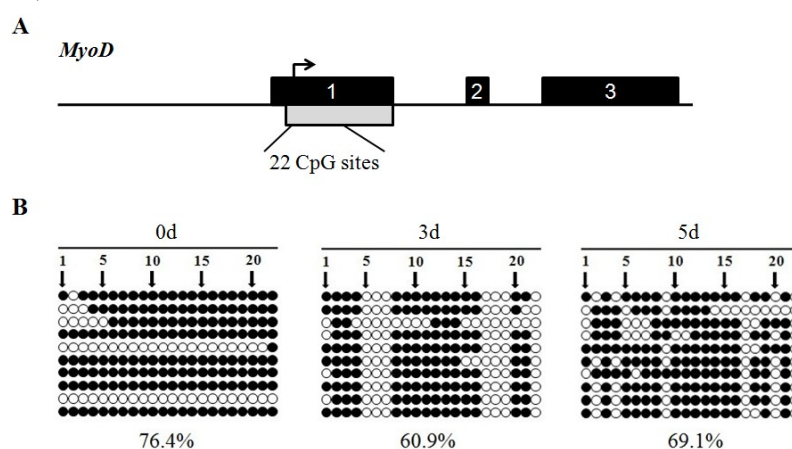
**Figure 1.** Expression analyses of *MRFs* and *Pax7* genes in differentiated C2C12 cells. M: DNA Ladder. *MRFs*, myogenic regulatory factors.

5 d after the induction of differentiation. Increased *MyoD/Myf5/myogenin* mRNA levels from 0 d to 5 d confirmed that the cells had undergone differentiation. During the whole duration of cell differentiation, a low level mRNA of *Pax7* can be detected.

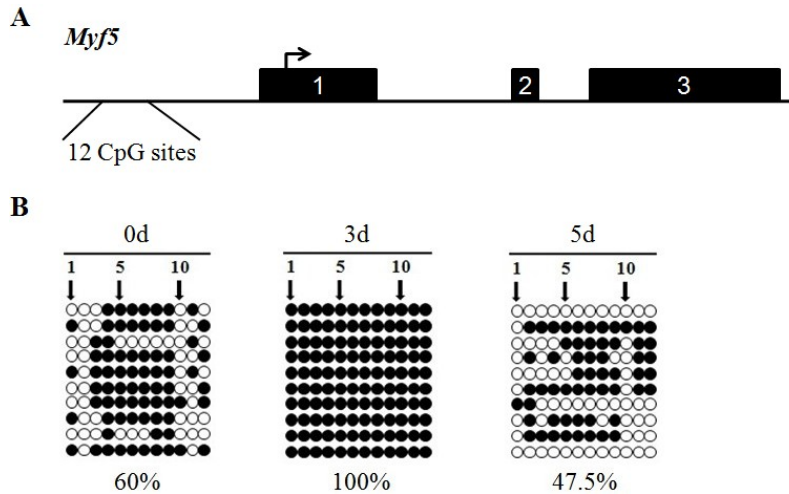
### DNA methylation pattern of the *MyoD* in myogenic differentiation

To determine DNA methylation changes during myogenic differentiation of C2C12 cells, we analyzed the methylation states around the promoter regions of several marker genes (*MyoD*, *Myf5*, and *Pax7*). Total genomic DNA was isolated from C2C12 cells of 0 d, 3 d, and 5 d (differentiation), respectively. DNA was treated with sodium bisulfite, chemically introduced a C to T conversion at unmethylated cytosines.

Firstly, the methylation status of the *MyoD* gene was investigated. The mouse *MyoD* gene sequence was determined including three exons and two introns. Figure



**Figure 2.** Genomic structure of mouse *MyoD* gene and relative CpG islands (A). Exons are depicted as solid boxes. Right-angled arrowhead indicates the translation start site. Open grey box represents CpG islands; the results of bisulfite sequencing analysis (B). Each line represents an individual sequence molecule, with each circle corresponding to a separate CpGs. Black and white circles indicate methylated and unmethylated CpGs respectively.



**Figure 3.** Genomic structure of mouse *Myf5* gene (A). Exons are depicted as solid boxes. Right-angled arrowhead indicates the translation start site; the results of bisulfite sequencing analysis (B). Each line represents an individual sequence molecule, with each circle corresponding to a separate CpGs. Black and white circles indicate methylated and unmethylated CpGs respectively.

2A illustrates the exon 1 region of the *MyoD* gene where we might obtain a big CpG island in DNA sequence of *MyoD* gene exon 1 from -50 to +618 bp (translation start site: +1). Bisulfite sequencing analysis indicated that a differentially methylated region was found from -13 to +296 bp, which contains a total of 22 CpG sites (Figure 2A). Methylation levels reached 76.4%, 60.9%, and 69.1% at 0 d, 3 d, and 5 d, respectively (Figure 2B).

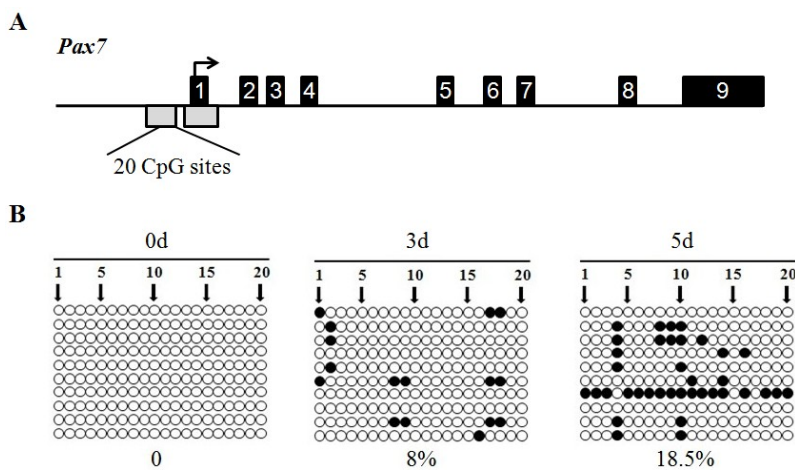
**DNA methylation pattern of the *Myf5* in myogenic differentiation**

Next, the research focused on the methylation status of the *Myf5* gene, which contains three exons and two introns. *Myf5* is devoid of CpG islands near the promoter and exon 1. The region (-1,105 to -806 bp) with relatively high CpG density (12 CpG sites) was selected for methylation

analysis (Figure 3A). A significant variation in methylation status of this region in C2C12 cells of different stages of myogenic differentiation was observed. The percentage methylation of this region at 0 d, 3 d, and 5 d was 60%, 100%, and 47.5%, respectively (Figure 3B).

**DNA methylation pattern of the *Pax7* in myogenic differentiation**

Analysis of the sequencing data revealed that the mouse *Pax7* gene contains nine exons and eight introns. The structure of the analysed CpG islands and their locations within the *Pax7* gene is shown in Figure 4A. We examined both of the first CpG islands, extending from -777 to -210 positions, and the second CpG island, extending from -106 to +529 positions, in the promoter and exon 1 of the *Pax7* gene. A differentially methylated region was found in DNA



**Figure 4.** Genomic structure of mouse *Pax7* gene and relative CpG islands (A). Exons are depicted as solid boxes. Right-angled arrowhead indicates the translation start site. Open grey boxes represent CpG islands; the results of bisulfite sequencing analysis (B). Each line represents an individual sequence molecule, with each circle corresponding to a separate CpGs. Black and white circles indicate methylated and unmethylated CpGs respectively.

sequence of *Pax7* gene promoter from -517 to -227 bp, which contains a total of 20 CpG sites (Figure 4A). Methylation levels reached 0%, 8%, and 18.5% at 0 d, 3 d, and 5 d, respectively (Figure 4B).

## DISCUSSION

Skeletal muscle development occurs in several defined steps. Mesoderm-derived precursor cells become committed to the myogenic lineage and develop into myoblasts, which subsequently fuse to form myotubes. Later, myotubes mature into multinucleated, highly specialized muscle fibers that show cross striation (Steffens et al., 2011). Differentiation involves various cellular changes, including physiology, structural architecture and functions (Xiong et al., 2011). Every event in the course of differentiation should be accompanied by coordinated expression and/or repression of certain subsets of genes (Yeo et al., 2007). DNA methylation can regulate the expression of many vital genes while the base compositions of these genes do not change. It is very important to the growth and development of organism. Consequently, clarifying the relationship between gene expression and DNA methylation during C2C12 myoblasts fusion process should enhance our understanding of the molecular mechanism underlying muscle development.

Great care was taken in all the optimization steps for validating the semi-quantitative PCR method used in this study, and the appropriate controls were included. GAPDH was used as an internal housekeeping gene to normalize total RNA input and reaction efficiency. GAPDH has been used as an internal control because it is ubiquitously expressed, in moderately abundant levels in almost all cell types and in general, its expression is not influenced by any hormonal treatment (Ghai et al., 2010). In present system, the expression pattern of control gene in all samples studied was found to be uniform.

Previous work concerning *MyoD* has indicated a constitutive expression at the myoblast stage (Dedieu et al., 2002). Some observations showed that the MRFs investigated are all expressed during postnatal phases of pig development although a descending trend was detected from day 1 towards day 20 and day 50 after birth for *MyoD*, *Myf5*, and *myogenin* but not *myostatin* and *Pax7* (Caliaro et al., 2005). Our results complete these studies and show that *MyoD* is expressed throughout the process of fusion and even at the later stages of differentiation. The methylation patterns we observed may be relevant to transcriptional silencing of the *MyoD* gene, higher methylation being correlated with lower expression. Studies have shown that CpG islands are mainly distributed in the promoter and first exon of the genes (Butcher and Beck, 2008). In skeletal muscle tissues, the exon 1 hypermethylation status of

*myostatin* gene was found to be negatively correlated with the gene expression (Liu et al., 2010). In addition, Kim et al. (2008) demonstrated prominent hypermethylation of CpG dinucleotides in *TCF4* exon 1, which significantly correlates with gene inactivation in early stage gastric cancers and in intestinal-type gastric cancers.

The expression patterns of *MyoD/Myf5/myogenin* have proven that the cells undergo differentiation. Our data showed that CpG methylation in *Pax7* promoter gradually increased in an day-dependent manner during differentiation of C2C12—the average percent of methylated CpG sites ranging from 0% at 0 d to 18.5% at 5 d. Fan et al. (2002) reported that CpG island methylation was a gradual process, that was, a cytosine base existing in each CpG island was gradually being methylated. The methylation of critical CpG sites decreases gene expression and causes gene silence. Over the past decade, a large number of studies focused on the mechanism of mRNA expression levels and methylation. Methyl groups of CpG sites can directly interfere with binding of transcription factors to their recognition *cis* elements or, on the other side, can facilitate interaction with a family of methyl-binding proteins. Upon binding to methylated DNA, these proteins can mask binding sites for transcription factors or recruit chromatin-modifying enzymes to silence particular DNA loci (Zelko et al., 2010).

There is not one standard CpG island in *Myf5* gene sequence. So we chose to examine one region with relatively high CpG density. In the present study, methylation of the *Myf5* gene is a dynamic process. Nevertheless, expression level of *Myf5* is not inversely correlated with DNA methylation status. It was reported that a third of the genes analyzed show inverse correlation between the state of DNA methylation in the 5'-regulatory regions and gene expression, whereas the methylation state did not correlate with mRNA expression levels for 63% of the genes (Eckhardt et al., 2006; Fan et al., 2011). These data suggest that chromosomal regions near the promoters of *Myf5* gene do not contain the necessary *cis*-acting regulatory factors required for its expression, and that further studies are necessary to determine the mechanism of *Myf5* expression. In addition, only one region with relatively high CpG density in promoter region of *Myf5* gene was investigated. The importance of the other regions needs further analysis and comparison.

The observed expression pattern of *myogenin* is in agreement with the published data. *Myogenin* is the only MRFs gene expressed in all skeletal muscle cell lines. With the onset of *myogenin* expression, cells are withdrawn from the cell cycle and start to fuse into myofibres (Verner et al., 2007). Although there is a weak CpG island in *myogenin* promoter, it has been shown that methylation status of the weak CpG island plays an important role in the activation

of *myogenin* (Steffens et al., 2011).

Collectively, our results indicate that DNA methylation within CpG islands during cell differentiation is associated with a variety of gene regulatory processes, including transcriptional regulation. However, one representative gene, *Myf5*, which does not contain canonical CpG islands within the promoter region, displays some degree of methylation upon differentiation. The gene expression seems to also be regulated by several mechanisms in addition to direct DNA methylation, suggesting that novel approaches are required to gain a comprehensive understanding of transcriptional regulation in myogenic differentiation.

### CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

### ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31560628), National “863” project of China (2011AA100302), Hainan science projects (13-214002-0001, KYYS-2014-33, KYYS-2015-02) and Hainan Agricultural Science and Technology Innovation Project (CXZX201414).

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