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Epigenetic and molecular mechanisms underlying gene expression in porcine skeletal muscle and satellite cells

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Dedicated to my beloved parents, sister, my beloved wife

Xiaoli, my daughter Xinqi and my son Yunqi

Epigenetic and molecular mechanisms underlying gene expression in porcine skeletal muscle and satellite cells

Epigenetic studies have been conducted in association with skeletal muscle only during the last years. We performed *in vivo* study in skeletal muscle and *in vitro* study in satellite cells with the aims to understand the epigenetic mechanisms regulating muscle gene expression. Duroc and Pietrain pigs, representing two extremes in skeletal muscle phenotypes, were selected for the *in vivo* study. Myogenic factor 6 (MYF6) was selected as one of the contributing factors to the postnatal breed-specific muscle properties in two breed pigs. Quantitative real time PCR (qRT-PCR) and Western blotting results revealed that mRNA and protein expression of MYF6 were dramatically higher in Pietrain pigs compared to Duroc pigs and suggested breed-specific expression. Variations in DNA methylation of the *MYF6* gene 5´-regulatory region, particularly within important transcription factor binding elements, are shown to occur between breeds. However, in Pietrains pigs, higher expression of MYF6 is not consistent with hypermethylation status, but concurrent with enriched E2F1 expression. In addition to its practical implications, this work extends our understanding the role of epigenetic mechanism in postnatal pigs. Satellite cells, acting as the muscle stem cells to support postnatal muscle growth, were selected for the *in vitro* study. Sulforaphane (SFN), a novel and natural histone deacetylase (HDAC) inhibitor, was selected as the treatment epigenetic reagent. Apart from SFN, we also employed the typical epigenetic reagents 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin (TSA). Distinguished from TSA, SFN and 5-aza-dC remarkably suppress *myostatin* (*MSTN)* expression and inhibit HDAC activity as well as *DNA methyltransferase (DNMT)* expression. SFN, 5-aza-dC and TSA exhibited differential mechanisms to repress *MSTN* expression and negative regulators of MSTN pathway. Deregulated miRNA may be excluded from epigenetic repression of *MSTN*. However, epigenetic suppression of *MSTN* by 5-aza-dC and SFN is associated with the decreased myoblast determination protein (MyoD) expression, reduced binding of MyoD on *MSTN* promoter and hypoacetylation in MyoD binding site. These observations manifest a novel mechanism for manipulation of muscle cell phenotypes.

Epigenetische und molekulare Mechanismen die der Genexpression in porcinen Skelettmuskel und Satellitenzellen zugrunde liegen

In den letzten Jahren wurden epigenetische Studien nur in Zusammenhang mit der Skelettmuskulatur umgesetzt. Wir hingegen führten in vivo Studien an der Skelettmuskulatur und in vitro Studien in Satellitenzellen durch, mit dem Ziel ein besseres Verständnis der epigenetischen Mechanismen zu erlangen, die einen Effekt auf die Regulierung von relevanten Muskelgenen haben könnten. Für die in vitro Studie wurden Duroc und Pietrain Schweine, die sich in ihrem Phänotyp der Skelettmuskulatur stark unterscheiden, ausgesucht. Myogener Faktor 6 (Myf6) gilt als ein entscheidender Faktor für postnatale rassenspezifische Muskeleigenschaften in zwei Rassen. Quantitative real time PCR (qRT-PCR) und Western Blot Ergebnisse zeigten, dass die Myf6 mRNA- und Protein-Expression dramatisch höher bei Pietrain im Vergleich zu Duroc Schweinen waren. Dieses kann auf eine rassenspezifische Expression hindeuten. Variationen in der DNA-Methylierung des Myf6 Gens in der 5´regulierenden Region, speziell innerhalb wichtiger Transkriptionsfaktorbindeelementes, konnten zwischen den Rassen ermittelt werden. Doch zeigte sich bei der Rasse Pietrain, dass eine höhere Expression von Myf6 nicht übereinstimmend mit dem Hypermethylierungsstatus ist, sondern mit einer hohen E2F1 Expression konkurriert. Zusätzlich zu den praktischen Implikationen erweitert diese Studie unser Verständnis über die Rolle des epigenetischen Mechanismus in postnatalen Schweinen. Für die in vitro Studie wurden Satellitenzellen als Muskelstammzellen, die das postnatale Muskelwachstum unterstützen, verwendet. Sulforaphane (SFN), ein neuartiger und natürlicher Histon Deacetylase (HDAC) Inhibitor, wurde als ein epigenetisches Behandlungsreagenz ausgewählt. Neben SFN setzten wir weitere typische epigenetische Reagenzien wie 5-Aza-2′-deoxycytidine (5-aza-dC) und Trichostatin (TSA) ein. Im Gegensatz zu TSA zeigen SFN und 5-aza-dC die Eigenschaft, nicht nur die HDAC Aktivität zu unterdrücken sondern auch die DNA-Methyltransferase Expression (DNMT) und die Expression von Myostatin (MSTN). SFN, 5-aza-dC und TSA weisen unterschiedliche Mechanismen auf, um die Expression von MSTN und negativen Regulatoren des MSTN Signalwegs zu unterdrücken. Deregulierte miRNA wird vielleicht von der epigenetischen Unterdrückung von MSTN ausgenommen. Jedoch kann die epigenetische Unterdrückung von MSTN durch 5-aza-dC und SFN mit einer verringerten Myoblast Determination Protein (MyoD) Expression assoziiert werden durch reduzierte Bindung von MyoD an den MSTN Promotor und Hypoacetylierung der MyoD Bindungsstelle. Diese Beobachtungen manifestieren einen neuartigen Mechanismus zur Manipulation des Muskelzellphänotypen.

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List of abbreviations

3'UTR	:	3' untranslated region
5-aza-dC	:	5-aza-2′-deoxycytidine
5'UTR	:	5' untranslated region
18S	:	18S ribosomal RNA
ActRIIB	:	Activine type II receptor
ATF	:	Activating transcription factor
BS	:	Binding site
BSP	:	Bisulfite sequencing PCR
CGIs	:	CpG islands
ChIP	:	Chromatin immunoprecipitation
CREB	:	cAMP response element binding protein
Ct	:	Threshold cycle
DNMT1	:	DNA methyltransferase 1
DMSO	:	Dimethyl sulfoxide
EAAs	:	Essential amino acids
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal bovine serum
FIG	:	Figure
FoxO1	:	Forkhead box O1
FST	:	Follistatin
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GDF8	:	Growth differentiation factor 8
GM	:	Growth medium
GREs	:	Glucocorticoid response elements
H4K5	:	Histone 4 acetylation at lysines 5
H4K8	:	Histone 4 acetylation at lysines 8
HCl	:	Hydrogen chloride
HDAC	:	Histone deacetylase
HPRT1	:	Hypoxanthine phosphoribosyltransferase 1
HRP	:	Horseradish peroxidase

hSGT	:	Human small glutamin-rich tetratricopeptide repeat-containing protein
MEF2	:	Myocyte enhancer factor 2
MEMα	:	Minimum essential medium α
MHC	:	Myosin heavy chain
min	:	Minutes
miRNAs	:	MicroRNAs
MRF	:	Myogenic regulatory factor
MRF4	:	Myogenic regulatory factor 4
mRNA	:	Messenger RNA
MSTN	:	Myostatin
MYF5	:	Myogenic factor 5
MYF6	:	Myogenic factor 6
MyoD	:	Myoblast determination protein
MYOG	:	Myogenin
NaCl	:	Sodium chloride
ng	:	Nanogram
nM	:	Nanomol
NP-40	:	Nonidet-P40
NPDFs	:	Nasal polyp-derived fibroblasts
NTC	:	No template control
^{o}C	:	Degree celsius
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate-buffered saline
qPCR	:	Quantitative polymerase chain reaction
qRT-PCR	:	Quantitative real time polymerase chain reaction
RYR1	:	Rryanodine-receptor gene
S.D.	:	Standard deviation
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.	:	Standard error
SFN	:	Sulforaphane
Smad2	:	Smad family member 2

Smad3	:	Smad family member 3
Smad4	:	Smad family member 4
Smad7	:	Smad family member 7
Smurf1	:	Smad specific E3 ubiquitin protein ligase 1
SRF	:	Serum response factor
TFBS	:	Transcription factor binding site
TGF-β	:	Transforming growth factor-β
TSA	:	Trichostatin A
TTBS	:	Tween-Tris-buffer saline
μg	:	Microgram
μM	:	Micromol

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1.1 Introduction

Skeletal muscle performance is one of the main economic traits in meat production. Pig breeding has focused on selection for improving muscle growth during the past 50 years (Merks, 2000). Pig breeds differ for their muscle phenotypes (i.e., myofiber numbers and myofiber types). Duroc and Pietrain pig breeds represent two extremes of western pig breeds (Cagnazzo et al., 2006). Duroc pigs have slower growing and redder muscle fiber types (Sellier, 1998), and Pietrain pigs have faster growing and whiter muscle fiber types (Jones, 1998; Sellier, 1998). Pietrain pigs are more muscular than Duroc pigs, whereas Duroc pigs are fatter than Pietrain pigs (Cagnazzo et al., 2006). However, the mechanism underlying these breed-specific differences of muscle phenotypes is poorly known.

Skeletal muscle develops from mesodermal stem cells by complex consecutive steps including mesenchymatous cells determination, progenitor cells proliferation, terminal differentiation and myoblasts fusion into myotubes (Joulia-Ekaza and Cabello, 2007; Schnorrer and Dickson, 2004). The amount of muscle fibres originating from myoblast proliferation and fusion is determined at or close to birth in many vertebrates. Postnatal muscle growth relies on the myogenesis of quiescent muscle stem cells — satellite cells located along the fibres which give rise to committed myogenic cells (Joulia-Ekaza and Cabello, 2007). These latter cells (myoblasts) can proliferate, migrate, differentiate and subsequently fuse with the muscle fibres to contribute more myonuclei that sustain postnatal muscle repair and growth (Sambasivan and Tajbakhsh, 2007).

Many genes regulating the well-orchestrated multistep process of myogenesis are known. The muscle regulatory factors (MRF) (e.g. myogenic factor 5 (MYF5), myoblast determination protein (MyoD), myogenin (MYOG) and myogenic factor 6 (MYF6)) take central positions in the regulation of myogenesis (Rudnicki and Jaenisch, 1995). MRFs are expressed in a coordinated and sequential manner when satellite cells are activated. The transcription factors MyoD and Myf5 are expressed in proliferating undifferentiated myoblasts, whereas MYOG and MYF6 are subsequently expressed at the differentiation stage, respectively (Sartorelli and Caretti, 2005; Tapscott, 2005). In contrast to other MRFs, MYF6 is the most abundantly expressed myogenic factor in adult muscle (Bober et al., 1991; Hinterberger et al., 1991) which is also involved in myogenesis, regulation of muscle fiber phenotype and maintainance of the

differentiated skeletal phenotype (Simon et al., 1995). MYF6 tends to be expressed more highly in muscle tissue of the lean selection line. This could be the result of the higher lean mass of Pietrain (te Pas et al., 2000). Accordingly, MYF6 is considered as one promising candidate gene for growth- and meat quality-related traits in adult pigs (Maak et al., 2006).

In comparison with MRFs, myostatin (MSTN) has been considered as a potent negative regulator of muscle development and growth. Knockout of the *MSTN* gene resulted in a dramatic and widespread increase in mstn−/− mice skeletal muscle mass (McPherron et al., 1997). Xu et al. (Xu et al., 2003) report that in zebrafishes, similar as in mice, inhibition of MSTN activity induces an increase in muscle growth. In addition, inhibition of MSTN by its antibody treatment in mice specifically increases skeletal muscle mass without effects on organ size and histology, or various serum parameters (Whittemore et al., 2003). Cattle breeds such as Belgian Blue carry natural *MSTN* gene mutations, exhibiting a notably increased muscle mass (Charlier et al., 1995).

Nowadays, the MSTN pathway is better understood which comprises myoblast progression inhibition in the cell cycle, myoblast terminal differentiation inhibition and association with protection from apoptosis (Joulia-Ekaza and Cabello, 2006). The active dimer of MSTN can bind to the activine type II receptor (ActRIIB) which is capable of recruiting and activating the type I receptor (ALK4 or ALK5). Smad family member 2 and 3 (Smad2 and Smad3) are subsequently activated and form complex with Smad family member 4 (Smad4) which are then translocated to the nucleus, regulating target gene transcription. Many signalling partners of MSTN have been identified, particularly MSTN-binding proteins such as follistatin, human small glutamin-rich tetratricopeptide repeat-containing protein (hSGT), Smad family member 7 (Smad7) and Smad specific E3 ubiquitin protein ligase 1 (Smurf1). In muscle, these partners interact with MSTN to prevent latent complex formation and consequently inhibit MSTN secretion and activation (Joulia-Ekaza and Cabello, 2007).

Evidences have accumulated to support roles for transcription factors in the *trans*-regulation of MSTN expression. Many transcription factor binding sites have been identified and experimentally confirmed in the *MSTN* promoter region. The effects of MyoD on *MSTN* promoter have been characterized and it was observed that MyoD can interact with the *MSTN* promoter and enhance its activity to directly up-regulate MSTN expression (Salerno et al., 2004; Spiller et al., 2002). Moreover, the glucocorticoid

response elements (GREs) were found to be present within the *MSTN* promoter. Using C2C12 myoblasts exposed to dexamethasone, glucocorticoids stimulated both the *MSTN* promoter's transcriptional activity and the endogenous MSTN expression in a dose-dependent way (Ma et al., 2001). Another *in vivo* study also showed that intramuscular MSTN mRNA and protein expression were elevated in rats administrated with dexamethasone (Ma et al., 2003). Furthermore, FoxO1, a member of the Forkhead Box O (FoxO) transcription factor family, could activate *MSTN* promoter activity to inhibit myogenesis through interaction with its binding motifs in the *MSTN* promoter region (Allen and Unterman, 2007).

More importantly, these regulatory myogenesis-related genes do not act in isolation and there are increasing evidences that interactions between epigenetic modulators of chromatin, and microRNAs (miRNAs) are all implicated in myogenesis process (Sousa-Victor et al., 2011). These muscle-specific genes seems to be controlled epigenetically during myogenesis. Indeed, the recruitment of satellite cells is achieved by an integration of genetic and epigenetic events, ranging from transcription factors, DNA methylation, covalent histone modifications and miRNAs which in conjunction implement the regulation of muscle-specific genes expression (Palacios and Puri, 2006; Sartorelli and Caretti, 2005). In mammals, the cytosine of a CpG dinucleotide can be methylated and the methylation of CpG within promoters suppresses genes expression (Lande-Diner et al., 2007). Various histone acetylation at a variety of lysine residues constitutes the complex histone code (Jenuwein and Allis, 2001). Generally, hyperacetylation correlates with gene activation, whereas deacetylation correlates with gene silencing (Fry and Peterson, 2001). miRNAs are a population of non-coding small RNAs that negatively regulate gene expression at posttranscriptional level. miRNAs can silence mRNAs by endonuclease cleavage, translational repression and mRNA degradation (Valencia-Sanchez et al., 2006). However, epigenetic studies have emerged only during the last years in the skeletal muscle field (Perdiguero et al., 2009; Sousa-Victor et al., 2011).

Previous reports show that demethylation within the distal enhancer of *MyoD* and *MYOG* promoter appears essential for the differentiation program to proceed (Lucarelli et al., 2001; Palacios and Puri, 2006). Recent study demonstrates that *MYOG* activation can be restricted by DNA methylation until other two transcription factors are co-expressed during embryonic myogenesis and in myoblasts (Palacios et al., 2010). DNA

methylation pattern of the 5'-flanking of the *MYOG* gene with no CpG island and low CpG density were examined in both C2C12 muscle satellite cells and embryonic muscle. A kinetically controlled equilibrium between CpG and non-CpG demethylation regulates *MYOG* transcriptional activation during muscle differentiation (Fuso et al., 2010).

In addition to DNA methylation, histone acetylation is a major source of epigenetic information. Epigenetic mechanisms that act on chromatin-related histones are more thoroughly delineated. Histone acetylation of various lysine residues of histones H3 and H4 represents the permissive mark of gene expression (Sousa-Victor et al., 2011). In quiescent and proliferating satellite cells, the promoters of genes involved in myoblasts differentiation exhibit hypoacetylated histones which are associated with genes repression (Palacios and Puri, 2006; Sartorelli and Caretti, 2005). A recent genome wide study revealed that MyoD constitutively binds to thousands of additional sites during myogenesis and this genome-wide binding of MyoD was correlated with local histone acetylation (Cao et al., 2010).

Moreover, accumulating evidences describe the post-transcriptional regulatory roles for miRNAs during myogenesis. The miRNA molecules typically target the 3' untranslated region (UTR) of mRNAs by base pairing, which triggers mRNA decay (if the base pairing is perfect) or translational repression (if it is imperfectly matched) (Hutvagner, 2005; Pattanayak et al., 2005). The “myomiRs” or muscle-specific miRNAs such as miR-1 and miR-133 are transcribed together, but manifesting contrasting roles in muscle proliferation and differentiation. MiR-1 stimulates myogenesis by targeting histone deacetylase 4 (HDAC4) whereas miR-133 promotes myoblasts proliferation by suppressing serum response factor (SRF) (Chen et al., 2006). Interestingly, miR-27 is expressed in both activated satellite cells of the adult muscle and differentiating skeletal muscle in the embryonic myotome. This miR-27 defines normal entry into the myogenic differentiation process through specifically targeting Pax3 mRNA (Crist et al., 2009). MiR-29 acts as a positive regulator of myogenesis through suppression of the YY1 transcription factor which repress muscle-specific gene expression (Wang et al., 2008).

More interestingly, several studies have demonstrated miRNAs are implicated in *MSTN* expression. A new target site for miR-1 and miR-206 created by “G” to “A” transition in the 3′UTR of *MSTN* enables MSTN translational inhibition and lead to the muscle

hypertrophy in Texel sheep (Clop et al., 2006). Recently, it was confirmed that miR-27b efficiently target the 3′UTR of *MSTN* which may contribute to glucocorticoid-mediated MSTN expression in skeletal muscle (Allen and Loh, 2011). Drummond et al. (Drummond et al., 2009) show the association of miR-499, -23a, -1, and -206 rapid up-regulation with human skeletal muscle MSTN down-regulation. The increase of miR-499 may account for the reduced MSTN expression based on miR-499 efficiently targeting the 3′UTR of *MSTN* experimentally validated (Bell et al., 2010). In defining the epigenetic regulation of *MSTN* expression, other epigenetic elements such as DNA methylation and histone acetylation may also participate, but still far from being understood.

Sulforaphane (SFN), an isothiocyanate derived from cruciferous vegetables, is identified as antioxidant, anti-carcinogenic and chemotherapeutic agent (Guerrero-Beltran et al., 2010), but evidence is mounting that SFN also has the ability to inhibit type I and II HDAC and functions via epigenetic mechanisms (Dashwood and Ho, 2007). In human colorectal cancer cells and prostate epithelial cells, concomitant global and *P21* promoter-specific histone acetylation increase induced by HDAC inhibition was associated with elevated expression of P21 protein (Myzak et al., 2006b; Myzak et al., 2004). *In vivo* studies in SFN-fed mice, global and local hyperacetylation was accompanied by inhibition of HDAC activity in various tissues (Myzak et al., 2006a). In healthy human volunteers, oral consumption of SFN-rich broccoli sprouts led to a potent HDAC inhibition associated with elevated histone acetylation at 3 and 6 h in peripheral blood mononuclear cells (PBMC) (Dashwood and Ho, 2007; Myzak et al., 2007). These findings revealed that the chemoprevention mechanism of SFN is via epigenetic modifications associated with HDAC activity inhibition.

In the muscle cell context, there is growing interest in dietary HDAC inhibitors and their impact on epigenetic mechanisms affecting muscle cell phenotypes. In comparison to SFN, *in vitro* intervention with another HDAC inhibitor—trichostatin A (TSA) can stimulate myoblast recruitment and fusion with an increased cell size (Iezzi et al., 2002; Iezzi et al., 2004). An *in vivo* study demonstrated that intraperitoneal injections of TSA increase muscle fiber size in dystrophin-deficient (MDX) mice (36). The dissected mechanism underlying HDAC inhibitor-mediated increase of muscle size and satellite cell recruitment were via inducing the expression of the natural *MSTN* antagonist *FST*

(Iezzi et al., 2004; Lee, 2004). However, as the new natural HDAC inhibitor, SFN epigenetically regulating FST or MSTN signalling pathway genes is unknown.

To sum up, we took the following considerations and carried out the *in vivo* and *in vitro* experiments in this thesis:

1. Epigenetic studies are emerging only during the last years in the skeletal muscle research field. Only recently, epigenetic research has been conducted in association with skeletal muscle, thus increasing our interests in the epigenetic mechanisms regulating muscle gene expression.
2. *In vivo* study: Duroc and Pietrain pigs differ remarkably in their skeletal muscle phenotypes. However, the mechanism underlying these breed-specific differences of muscle properties is poorly known. The MYF6 gene is predominantly expressed in the adult muscle. Up to now, no data are available concerning DNA methylation and histone acetylation pattern of *MYF6* 5′-regulatory region in pigs. Therefore, the importance of these epigenetic modifications in the modulation of porcine MYF6 gene expression was aimed to be explored. The potential roles of these epigenetic modifications variations in breed-specific expression of MYF6 in loin eye muscle of 6-month female pure breed Pietrain and Duroc pigs was aimed to be evaluated. (Chapter 1)
3. *In vitro* study: Satellite cells act as the muscle stem cells to support postnatal muscle growth. HDAC inhibitor TSA *in vivo* and *in vitro* studies revealed its roles in promoting myoblast fusion, recruitment of satellite cells and increasing muscle fiber size via inducing MSTN antagonist FST. However, epigenetic regulatory roles of SFN as a novel natural HDAC inhibitor in muscle cells remain undiscovered. Therefore, SFN epigenetically regulating FST or MSTN signalling pathway genes was aimed to be examined. (Chapter 2)

1.2 Materials and methods

To achieve the objectives of this study, multiple materials and methods were used. The details of materials and methods could be found in the respective chapters of this thesis. The principle of the main methods and their applications are briefly described here.

1.2.1 Quantitative Real-time PCR (qRT-PCR)

qRT-PCR is a frequently used new molecular laboratory technique to amplify and simultaneously quantify a targeted DNA molecule. The distinct feature is that the amplified template can be detected during the reaction progress in real time, whereas the conventional PCR detects the product of reaction at its end. The quantity of interested molecule can be either an absolute copies number or a relative amount when normalized to additional reference genes. In the *in vivo* study of this thesis, nine-fold serial dilution of plasmid DNA were prepared and used as template for the generation of the standard curve. qRT-PCR was performed in an ABI prism® 7000 (Applied Biosystems) qPCR system. The amount of transcript of target genes present in each sample were determined using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). Each RT-PCR quantification experiment was performed in duplicate for individual sample. No template control (NTC) was set to monitor the possible contamination of genomic DNA. Melting curves were performed to investigate the specificity of the PCR reaction. Final results were reported as the relative expression normalized with the transcript level of the endogenous reference TOP2B (Erkens et al., 2006; Van Poucke et al., 2001) (Chapter 1). In the *in vitro* study, qRT-PCR was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems) using miScript SYBR Green PCR Kit (218073, Qiagen) and iTaq SYBR Green Supermix with ROX (172-5850, Bio-Rad) for the detection of miRNAs and mRNAs, respectively. qRT-PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Uddin et al., 2011) for mRNAs and 18S ribosomal RNA for miRNAs as endogenous references (Chapter 2).

1.2.2 Western blotting analysis

Western blot is a widely used molecular approach to determine specific interested proteins in the cell or tissue extract. After gel electrophoresis, the proteins are then

transferred to a membrane (nitrocellulose or PVDF), where they are probed with antibody against the target protein. Finally, the membrane was washed and the specific signals were detected by chemiluminescence. In the *in vivo* study of this thesis, enhanced chemiluminescence signals recorded on X-ray film were scanned and visualized by Kodak BioMax XAR film (Kodak). Results were shown as the relative band intensities normalized to the area densities of GAPDH bands for each lane using Image-J software (National Institute of Mental Health, Bethesda, Maryland, USA) (Chapter 1). In the *in vitro* study, the specific signals were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (34077, Thermo Scientific). Images were acquired by Quantity One 1-D analysis software (Bio-Rad) (Chapter 2). The detailed procedure and specific antibodies can be found in the respective chapters.

1.2.3 Bisulfite sequencing PCR

Bisulfite sequencing PCR is a precise and widely used epigenetic method to determine DNA methylation pattern of interested gene. Bisulfite treatment of genomic DNA converts cytosine residues to uracil, but keeps 5-methylcytosine residues unconverted. Hence, bisulfite treatment induces specific changes in the DNA sequence which are based on the methylation status of individual cytosine residues. In this thesis, genomic DNA was subjected to bisulfite modification using EZ DNA Methylation Kit (Zymo Research). The bisulfite PCR products were purified with QIAquick PCR purification kit (Qiagen) and then subcloned into the pGEM T-easy vector (Promega). Different positive clones for each subject were randomly selected for sequencing with M13 primers performed by the CEQ8000 sequencer system (Beckman Coulter). The final sequence results were processed by QUMA (Kumaki et al., 2008) software. Sequences with a conversion rate below 90% were excluded from analysis.

1.2.4 Chromatin immunoprecipitation (ChIP)

ChIP is a type of immunoprecipitation experimental technique used to evaluate DNA and protein interaction. It aims to determine the association of specific proteins with specific genomic regulatory regions, such as transcription factor binding sites on promoters. ChIP is also able to determine the specific histone modifications in the genome. In short, the method is as follows: chromatin and proteins are temporarily

cross-linked, the chromatin-protein complexes are then sheared and DNA fragments interacting with the interested proteins are selectively immunoprecipitated, and the related DNA are isolated and purified which are subject to ChIP quantitative PCR (ChIP-qPCR). These DNA sequences are supposed to interact with the protein of interest *in vivo*. In this thesis, the chromatin immunoprecipitation (ChIP) assay kit (17-295, Millipore) was used. Antibodies against RNA polymerase II (sc-899 X, Santa Cruz) and normal rabbit IgG (2729S, Cell signaling Technology) were used as a positive and a negative control in the assay, respectively. PCR products were separated on 2% agarose gel stained with ethidium bromide for visualization. QPCR data were normalized to and expressed as % of input (Chapter 2).

1.2.5 Statistical analysis

Results from qRT-PCR and Western blotting analysis were expressed as mean ± SEM. The difference between values was analyzed by t-Test in SAS software v.9.2 and $P < .05$ was set statistically significant (Chapter 1). Pairwise comparisons were made between treatment groups and the vehicle-treated control, using Student's t test. The data were expressed as means ± standard deviations (SD) and (*) $P < .05$, (**) $P < .01$, (***) $P < .001$ were set statistically significant (Chapter 2).

1.3 Results

The main results in this thesis are briefly described here. The detailed description of the results can be found in the respective chapters.

1.3.1 MYF6 expression was *trans*-activated and up-regulated by enriched transcription factor E2F1 in Pietrain breed

In the chapter 1, in order to evaluate the potential roles of epigenetic modifications variations in breed-specific expression of MYF6, six months old female Duroc and Pietrain pure breed pigs were used. qRT-PCR and Western blotting results revealed that mRNA and protein expression of MYF6 were dramatically higher in Pietrain pigs compared to Duroc pigs and suggested breed-specific expression. Variations in DNA methylation of the *MYF6* gene 5´-regulatory region, particularly within important transcription factor binding elements, are shown to occur between breeds. Furthermore, DNMT1 exhibited higher levels in both mRNA and protein expression in Pietrain pigs, indicating global hypermethylation status in Pietrain pigs. The histone acetylation levels at both H4K5 and H4K8 sites were not different between two breed pigs. However, in Pietrains pigs, higher expression of MYF6 is not consistent with hypermethylation status, but concurrent with enriched E2F1 expression.

1.3.2 Sulforaphane epigenetically represses MSTN expression in porcine satellite cell

In the chapter 2, in order to assess epigenetic effects of SFN supplementation on satellite cell, satellite cells were isolated from the right and left semimembranosus muscles of 20 days old purebred Pietrain piglets. Apart from SFN, we also employed the typical epigenetic reagents 5-aza-2′-deoxycytidine (5-aza-dC) and TSA. Distinguished from TSA, SFN and 5-aza-dC remarkably suppress *MSTN* expression and inhibit HDAC activity as well as *DNA methyltransferase (DNMT)* expression. Significantly reduced MyoD enrichment associated with hypoacetylation of the MyoD-binding site in the *MSTN* promoter were observed simultaneously in SFN and 5-aza-dC groups.

1.4 Conclusions

In this thesis, we conducted *in vivo* study in skeletal muscle and *in vitro* study in satellite cells with the aims to understand the epigenetic and molecular mechanisms regulating muscle gene expression.

In the *in vivo* study, we selected MYF6 as one of the contributing factors to the postnatal breed-specific muscle properties in two breed pigs. The increased MYF6 transcription in postnatal porcine skeletal muscle is not coordinated with *cis*-activation by epigenetic modifications of *MYF6* 5´-regulatory region, but may be attributed to *trans*-activation through enriched E2F1 expression. In addition to its practical implications, this work extends our understanding the role of epigenetic mechanism in postnatal pigs. In comparison with epigenetic modifications during the embryonic stage, DNA methylation variations of MYF6 in adult pigs may be attributed to the environmental factors (Aguilera et al., 2010), as well as the selection pressure. In conclusion, our findings provide the first evidence that postnatal MYF6 expression and DNA methylation variations within 5´-regulatory region occur differentially between breeds and may lead to novel insights and clues into the selection of lean-type pigs. However, further experiments are required to fully clarify the exact signalling pathway for *MYF6* transcriptional activation by enriched E2F1.

In the *in vitro* study, we found that both 5-aza-dC and SFN dramatically inhibit HDAC activity and *DNMT1* expression. SFN, 5-aza-dC and TSA exhibited differential mechanisms to repress *MSTN* expression and negative regulators of MSTN pathway. Previous studies has shown protective effects of SFN treatment on rat skeletal muscle damage and oxidative stress (Malaguti et al., 2009), but not in the context of epigenetics, and to our knowledge this is the first study with SFN in satellite cells. Deregulated miRNA may be excluded from epigenetic repression of *MSTN*. However, epigenetic suppression of *MSTN* by 5-aza-dC and SFN is associated with the decreased MyoD expression, reduced binding of MyoD on *MSTN* promoter and hypoacetylation in MyoD binding site. These observations manifest a novel mechanism for manipulation of muscle cell phenotypes.

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2.1 Chapter 1 (Published in: *Gene.* 486: 8–14, 2011)

Molecular mechanism underlying the differential MYF6 expression in postnatal skeletal muscle of Duroc and Pietrain breeds

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Abstract

Among modern western pigs, Duroc (high meat fat ratio) and Pietrain (low meat fat ratio) breeds extensively utilized in commercial pork production differ extremely for their muscle phenotypes. The molecular mechanism, especially the epigenetic mechanism, underlying these breed-specific differences is poorly known. Myogenic factor 6 (MYF6) is the most abundantly expressed myogenic factor in adult muscle. Moreover, MYF6 tends to be expressed more highly in muscle tissue of the lean selection line and is supposed to be one promising candidate gene for growth- and meat quality-related traits in adult pigs. Six months old female Duroc and Pietrain pure breed pigs were used in this study. Protein and mRNA levels of MYF6 in loin eye muscle were determined by Western blotting and quantitative Real-time reverse transcription PCR (qRT-PCR), respectively. The DNA methylation status of the *MYF6* 5´-regulatory region was determined by bisulfite sequencing PCR (BSP). The global Histone 4 acetylation at lysines 5 (H4K5) and 8 (H4K8) were examined by Western blotting. Pietrain pigs exhibited significant higher expression of MYF6 and hypermethylated E2F1 binding element within *MYF6* 5´-regulatory region as compared with Duroc pigs. Significant elevation in DNA methyltransferase 1 (DNMT1) expression was observed in Pietrain pigs which is in agreement with hypermethylation of MYF6. Histone acetylation level at neither H4K5 nor H4K8 is significant between two breed pigs. Nevertheless, mRNA and protein expression of E2F1 were significantly elevated in the Pietrain breed. It is thus conceivable that the upregulation of MYF6 transcription in postnatal Pietrain pigs is not associated with *cis*-activation by epigenetic modification of *MYF6* 5´-regulatory region, but may be attributed to *trans*-activation through enriched expression of E2F1.

1. Introduction

Commercial western pig breeds have been selected for meat production over the past 50 years. Pietrain (low meat fat ratio) and Duroc (high meat fat ratio) pig breeds are two extremes in skeletal muscle phenotypes (Cagnazzo, te Pas *et al.* 2006). The myogenic factor 6 (*MYF6*/myogenic regulatory factor 4, *MRF4*) gene codes for the bHLH transcription factor belonging to MyoD family. The expressed MYF6 is involved in the processes of differentiation and maturation of myotubes during embryogenesis and dominates quantitatively over the other MRFs in adult muscle (Bober, Lyons *et al.* 1991; Hinterberger, Sassoon *et al.* 1991; Wyszynska-Koko and Kuryl 2004). Increases in MYF6 mRNA and protein may play a role in the differentiation of adult fibers (Lowe, Lund *et al.* 1998). MYF6 is also involved in regulation of muscle fiber phenotype and maintainance of the differentiated skeletal phenotype (Miner and Wold 1990; Walters, Stickland *et al.* 2000). Interestingly, MYF6 tends to be expressed more highly in muscle tissue of the lean selection line which has higher lean mass and expression of MYF6 in the thicker muscle fibers for maintenance (te Pas, Verburg *et al.* 2000). Therefore, *MYF6* is supposed to be one promising candidate gene for growth- and meat quality-related traits in adult pigs (Maak, Neumann *et al.* 2006).

Numerous studies in the past decade have unveiled that epigenetic control of chromatin structure is essential for eukaryotic gene activation and inactivation. This epigenetic regulation is mainly performed by DNA methylation and histone modification (Matsubara, Takahashi *et al.* 2010). The DNA methylation and histone modification are functionally linked with each other to regulate gene expression (Cedar and Bergman 2009; Matsubara, Takahashi *et al.* 2010). Generally, in mammals the cytosine of a CpG dinucleotide can be methylated. Gene which is hypermethylated is silenced and one gene which is hypomethylated is actively transcribed. A variety of histone acetylation at

various lysine residues constitutes the complex histone code (Jenuwein and Allis 2001). In general, acetylation of histone 4 correlates with gene activation, while deacetylation correlates with gene silencing (Fry and Peterson 2001). In the skeletal muscle field, epigenetic studies have really emerged only during these last years, thereby increasing our understanding of the mechanisms regulating muscle gene expression (Perdiguero, Sousa-Victor *et al.* 2009). It was reported that demethylation including the distal enhancer of *Myod* and *Myog* promoter appears necessary for the differentiation program to proceed (Lucarelli, Fuso *et al.* 2001; Palacios and Puri 2006). However, the precise mechanisms regulating methylation/demethylation during adult myogenesis are still far from being understood (Perdiguero, Sousa-Victor *et al.* 2009).

Duroc and Pietrain pigs differ considerably in their skeletal muscle properties. In addition, the *MYF6* gene is quantitatively dominant over the other MRFs in the adult muscle. Up to now, no data are available concerning methylation and histone acetylation status of *MYF6* 5´-regulatory region in pigs. The present study was aimed to answer the following questions. First, would predominantly expressed MYF6 in the adult muscle be differentially expressed in Duroc and Pietrain breeds? Second, was such differential expression, if any, epigenetically regulated? Third, did epigenetic control involve transcription factors interaction with their target DNA? To address these issues, we first characterized the sequence of *MYF6* and observed its mRNA and protein expression using quantitative Real-time reverse transcription PCR (qRT-PCR) and Western blotting. Bisulfite sequencing PCR (BSP) was performed to quantify DNA methylation status within two putative transcription factor elements. Furthermore, we investigated the global Histone 4 acetylation at lysines 5 (H4K5) and 8 (H4K8) level using Western blotting. Eventually, we examined E2F1 mRNA and protein expression using qRT-PCR and Western blotting, suggesting a *trans*-regulating mechanism in

postnatal porcine skeletal muscle MYF6 activation.

2. Materials and methods

2.1. Experimental animals and samples

Six pure breed Pietrain and four pure breed Duroc female pigs at the age of 6-month were used in this study. All the pigs were kept and slaughtered at a commercial abattoir according to German performance test directions (ZDS, 2004). The loin eye muscle of each pig was dissected and immediately frozen in liquid nitrogen within 5 min after slaughter. Then the samples were stored at –80°C until DNA, RNA and protein isolation.

2.2. *In silico* analysis of *MYF6*

The 5´-regulatory region of the published sequence (GenBank ID: AY327443) which contains the promoter and 5´-untranslated region (5´-UTR) of *MYF6* gene was submitted to the online program: Methprimer (Li and Dahiya 2002) to identify the CpG islands (CGIs). The 5´-regulatory region was screened and analyzed for *cis*-acting elements involved in *trans*-activation within the CpG island using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). The *cis*-acting elements prediction results were confirmed by TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

2.3. Isolation of mRNA, protein and DNA from loin eye muscle

Total RNA was extracted using TRI reagent (Sigma). RNA was purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was then treated using on-column RNase-Free DNase set (Promega) and the concentration was quantified by spectrophotometer (NanoDrop, ND8000). RNA quality was checked by 2% agarose gel electrophoresis. First-strand cDNA was synthesized using Superscript II enzyme (Invitrogen). Genomic DNA was isolated by conventional proteinase K digestion and phenol-chloroform extraction. The DNA concentration was measured by

spectrophotometer (NanoDrop, ND8000). The whole cell protein was extracted using Nonidet-P40 (NP-40) buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% IGEPAL CA-630, 2 mM EDTA, 1 mM dithiothreitol). The protein concentration was quantified using coomassie (Bradford) protein assay kit (Thermo Fisher Scientific) in spectrophotometer (NanoDrop, ND8000) according to the manufacturer's instructions. All the mRNA, protein and DNA were stored at –80°C until assay.

2.4. Quantitative Real-time RT-PCR

Primers were designed using the online Primer3 program (Rozen and Skaletsky 2000) and are listed in Table 1. Nine-fold serial dilution of plasmid DNA were prepared and used as template for the generation of the standard curve. Real-time qRT-PCR was performed in an ABI prism® 7000 (Applied Biosystems) qPCR system. The amount of transcript of target genes present in each sample were determined using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). Each RT-PCR quantification experiment was performed in duplicate for individual sample. Amplification conditions were as follows: 10 min at 95°C, 45 cycles of 15 s at 95°C and 1 min at indicated Tm listed in Table 1. No template control (NTC) was set to monitor the possible contamination of genomic DNA. Melting curves were performed to investigate the specificity of the PCR reaction. Final results were reported as the relative expression normalized with the transcript level of the endogenous reference TOP2B (Erkens, Van Poucke *et al.* 2006; Van Poucke, Yerle *et al.* 2001).

2.5. Western blotting analysis

Forty micrograms of protein extract were diluted 4:1 with 5 × loading buffer (10% glycerol, 2% sodium dodecyl sulphate [SDS], 5% 2-mercaptoethanol, 1% bromophenol blue) and denatured by boiling for 5 min before loading on a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran®,

Schleicher & Schuell Bioscience) and the latter were then blocked with Roti-block solution (Carl Roth GmbH) for 1 h at room temperature. After repeated washing with Tween-Tris-buffer saline (TTBS), the membranes were incubated with primary antibodies specific for MYF6 (rabbit, 1:100; Catalog No. sc-301, Santa Cruz), DNMT1 (mouse, 1 μg/ml; Catalog No. IMG-261A, Imgenex), E2F1 (mouse, 1:200; Catalog No. sc-56662, Santa Cruz), c-myb (rabbit, 1:100; Catalog No. sc-517, Santa Cruz), Acetyl-Histone H4 Antibody Set (acetyl K5 + K8) (rabbit, 1:500; Catalog No. 17-211, Millipore), and GAPDH (goat, 1:3000; Catalog No. sc-20357, Abcam) respectively. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit for MYF6, c-myb and Acetyl-Histone 4, 1:2000, Catalog No. sc-2004, Santa Cruz; goat anti-mouse for DNMT1 and E2F1, 1:3000, Catalog No. sc-2024, Santa Cruz; donkey anti-goat for GAPDH, 1:5000, Catalog No. sc-2020, Santa Cruz). Finally, the membrane was washed and the specific signals were detected by chemiluminescence using the ECL plus Western blotting detection system (Amersham Biosciences). Enhanced chemiluminescence signals recorded on X-ray film were scanned and visualized by Kodak BioMax XAR film (Kodak). Results were shown as the relative band intensities normalized to the area densities of GAPDH bands for each lane using Image-J software (National Institute of Mental Health, Bethesda, Maryland, USA). Relative means were compared between Duroc and Pietrain pigs.

2.6. Bisulfite sequencing PCR

Genomic DNA (1 μg) isolated from loin eye muscle was subjected to bisulfite modification using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. The *MYF6* 5´-regulatory region was PCR amplified with MYF6-met primer pairs (Table 1) designed using the MethPrimer program (Li and Dahiya 2002). PCR products were purified with QIAquick PCR purification kit

(Qiagen) and then subcloned into the pGEM T-easy vector (Promega). A minimum of four different positive clones for each subject were randomly selected for sequencing with M13 primers performed by the CEQ8000 sequencer system (Beckman Coulter). The final sequence results were processed by QUMA (Kumaki, Oda *et al.* 2008) software. Sequences with a conversion rate below 90% were excluded from analysis.

2.7. Data analysis

Results from Real-time qRT-PCR and Western blotting analysis were expressed as mean ± SEM. The difference between values was analyzed by T-Test in SAS software v.9.2 and $P < 0.05$ was set statistically significant.

3. Results

3.1. *In silico* analysis of *MYF6*

The 5´-regulatory region of *MYF6* which contains the promoter and 5´-UTR was submitted to online program: Methprimer and two potential CGIs were predicted. Thirteen CpG sites were mapped and two putative transcription factor binding sites (TFBS for c-myb and E2F1) were found in the MYF6 5´-regulatory region within the CpG island. We found 2 CpG sites located in the putative TFBS for c-myb (CpG5 and CpG6) and 2 within putative TFBS for E2F1 (CpG8 and CpG9) (Figure 1).

3.2. Expression of MYF6, c-myb, E2F1 and DNMT1 in the skeletal muscle

The Real-time qRT-PCR results showed that expression of *MYF6*, *c-myb* and *E2F1* gene were markedly higher in Pietrain pigs compared to Duroc pigs (Figure 2a, 3a and 4a). At protein level, results are in line with the mRNA level except for c-myb that was not significantly different at protein level (Figure 2b, 2c; Figure 3b, 3c; Figure 4b, 4c;). We also evaluated DNMT1 expression which was higher in mRNA and protein levels in Pietrain pigs (Figure 5).

3.3. DNA methylation profile of MYF6

The methylation status of the computationally predicted CpG island in two breeds were shown in Supplementary Figure 1. The bisulfite sequencing analysis revealed that the average methylation percentage was not different between Pietrain and Duroc pigs (data not shown). However, the methylation percentages of 3 CpG sites (CpG7, CpG12 and CpG13) were higher in Pietrain pigs than in Duroc pigs (Figure 6a). Moreover, the methylation percentages of 2 specific CpG sites within TFBS: CpG5 (Figure 6b) and CpG8 (Figure 6c) were significantly higher in Pietrain pigs than in Duroc pigs.

3.4. Global histone acetylation in the skeletal muscle

Our results demonstrated that histone acetylation levels at both H4K5 and H4K8 were not significantly different between two breed pigs (Supplementary Figure 2 and 3).

4. Discussion

The commercial Duroc and Pietrain breeds differ extremely for their muscle phenotypes (i.e., myofiber numbers and myofiber types) (Cagnazzo, te Pas *et al.* 2006). MYF6 is the most abundantly expressed myogenic factor in postnatal muscle where it quantitatively predominates over the other MyoD family transcripts (Miner and Wold 1990). Up to now, the importance of epigenetic marks in the regulation of porcine *MYF6* gene expression is far less explored. In the present study, our Real-time qRT-PCR and Western blotting results demonstrated that mRNA and protein expression of MYF6 were markedly higher in Pietrain pigs compared to Duroc pigs and indicated breed-specific expression. Variations in methylation status of the *MYF6* gene 5´-regulatory region, especially within specific transcription factor binding sites, are shown to occur between breeds. Additionally, DNMT1 exhibited higher levels in both mRNA and protein levels in Pietrain pigs, suggesting hypermethylation status in the loin eye muscle of Pietrain pigs. The histone acetylation levels at both H4K5 and H4K8 sites were not significantly different between two breed pigs. However, in the loin eye

muscle of Pietrains pigs, higher expression of MYF6 is not consistent with hypermethylation status, but concurrent with elevated E2F1 expression. We propose that the upregulation of MYF6 transcription in Pietrain pigs is not associated with *cis*-activation by DNA methylation and histone acetylation in *MYF6* 5´-regulatory region, but may be attributed to *trans*-activation through enriched expression of transcription factor E2F1.

MYF6 is considered as one promising candidate gene for growth- and meat quality-related traits in adult pigs (Maak, Neumann *et al.* 2006). In our studies, the dramatic differences both in MYF6 mRNA and protein expression were observed in the 6-month-old Duroc and Pietrain pigs, suggesting differential expression in breed-specific skeletal muscle. It has been shown that increased MYF6 mRNA and protein were correlated with increased myofiber size (Bodine and Pierotti 1996) and increased mean fiber area (Hespel, Op't Eijnde *et al.* 2001). In addition, the elevations in MYF6 expression could have also facilitated an enhanced transcription of Type I, IIa, and IIx Myosin heavy chain (MHC) mRNA molecules. These alterations dependent on the myogenic regulation of MYF6 result in hypertrophy in muscle (Willoughby and Rosene 2001; Willoughby and Rosene 2003). Therefore, elevated MYF6 may elucidate the fast growing fiber types and more muscular property in Pietrain pigs. In line with our results, MYF6 tends to be expressed more highly in muscle tissue of the lean-type breed (te Pas, Verburg *et al.* 2000). Considering MYF6 expression is dominant in postnatal mature muscle fibers (Olson 1990; Weintraub, Davis *et al.* 1991), faster growing muscles may be more mature at slaughter than slower-growing muscles (te Pas, Verburg *et al.* 2000). Thus, faster-growing muscles in Pietrain pigs show higher MYF6 expression. The increases in MYF6 mRNA and protein expression in Pietrain may provide a possible mechanism for the increase in myofiber size and the change in

myofiber types compared to Duroc pigs.

Epigenetic control of chromatin structure is mainly performed by DNA methylation and histone modification (Caiafa and Zampieri 2005). CGIs occur primarily—although not exclusively—at the 5´ end of genes, particularly promoters and first exon (Butcher and Beck 2008). In our computational analysis, thirteen CpG sites were mapped in one plotted CGI in the *MYF6* 5´-regulatory region. The *MYF6* 5´-regulatory region contains a c-myb and an E2F1 binding site that may serve as the molecular targets. Our bisulfite sequencing analysis revealed that the average methylation percentage was not different between Pietrain and Duroc pigs. However, variations in methylation status of the *MYF6* gene 5´-regulatory region, especially within the two putative transcriptional factor c-myb and E2F1 binding sites in Pietrain pigs, are shown to occur between breeds. These hypermethylated transcripton factor binding elements in the *MYF6* gene 5´-regulatory region of Pietrain pigs, which is in parallel with increased DNMT1 mRNA and protein expression levels, might be associated with breed-specific transcriptional activity and gene expression. Nevertheless, expression level of MYF6 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, Lewin *et al.* 2006). Corresponding to the latter, our observations suggest that DNA methylation in the *MYF6* gene 5´-regulatory region might not correlate with *MYF6* gene expression.

Additional factors that could regulate transcription, such as histone modifications or transcription factors, should not be neglected. Histone 4 acetylation at lysines 5 and 8 is associated with open chromatin leading to gene activation (Hublitz, Albert *et al.* 2009). Our results exibited that global histone acetylation levels at both H4K5 and H4K8 were

not significantly different between two breed pigs. Histone acetylation may also not play a vital role in *MYF6* gene expression. As for *trans*-regulation, transcription factors could exert their transcriptional regulatory effects directly on the gene by specific protein–DNA interaction and elicit trans-activation of promoter activity (Weaver, D'Alessio *et al.* 2007). Indeed, we observed a significant difference in c-myb mRNA expression and a tendency in protein expression between two breeds. However, because of the similar c-myb protein levels for protein-DNA interaction, c-myb may not be involved in the enhanced MYF6 expression in Pietrain pigs. Furthermore, mRNA and protein levels of another transcription factor E2F1 were both significantly higher expressed in Pietrain pigs as opposed to Duroc pigs, indicating a *trans*-activation of skeletal muscle MYF6 transcription in Pietrain pigs. A previous study showed that E2F1, which is included in the E1A promoter bind nuclear protein complexes, mediates E1A transcriptional (auto)activation (Kirch, Putzer *et al.* 1993). Accordingly, resembling other protein-DNA interaction (Bernard, Quatannens *et al.* 2001; Moore, Narayanan *et al.* 2007), upon facilitation of more abundant transcription factor occurring in its binding element, E2F1 can stimulate MYF6 transcriptional activity in Pietrain pigs. What is more, E2F1 is required for adult skeletal muscle regeneration in vivo (Yan, Choi *et al.* 2003) which, to some extent, can account for elevated E2F1 expression in more muscular adult Pietrain pigs. It is thus conceivable that hypermethylation of the *MYF6* promoter does not underlie its overexpression in Pietrain pigs and E2F1 could regulate MYF6 expression in *trans*-activating the *MYF6* promoter containing E2F1 binding site.

In this study, we suggest that MYF6 might be one of the contributing factors to the adult breed-specific muscle properties in two breed pigs. The upregulation of MYF6 transcription in postnatal porcine skeletal muscle is not associated with *cis*-activation by

epigenetic modification of *MYF6* 5´-regulatory region, but may be attributed to *trans*-activation via enriched expression of E2F1. In addition to its practical implications, this work extends our understanding the role of epigenetic mechanism in postnatal pigs. In contrast to epigenetic modifications during the embryo stage, DNA methylation variations of MYF6 in adult pigs may be attributed to the environmental factors (Aguilera, Fernandez *et al.* 2010), as well as the selection pressure. Taken together, our findings provide the first evidence that postnatal MYF6 expression and DNA methylation variations within 5´-regulatory region occur differentially between breeds and may lead to novel insights and clues into the selection of lean-type pigs. However, further experiments are required to fully clarify the exact signalling pathway for MYF6 transcriptional activation by enriched E2F1.

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Table 1. List of primer sequences

Gene name	Primers sequence	Tm	Product Size	GenBank ID
MYF6	F: TGGATCAGCAGGACAAAATG	55°C	171 bp	AY188502
	R: TGTTTGTCCCTCCTTCCTTG			
DNMT1	F: GCGGGACCTACCAAACAT	55°C	133 bp	DQ060156
	R: TTCCACGCAGGAGCAGAC			
E2F1	F: AGTGGCTAGGCAGCCATGCAG	60°C	208 bp	XM_001926880
	R: GCAGGGTCCGCGATGCTACG			
c-myb	F: GTCCGAAACGTTGGTCTGTT	57°C	190 bp	XM_001928926
	R: GGCAGTAGCTTTGCGATTTC			
TOP2B	F: AACTGGATGATGCTAATGATGCT	55°C	137 bp	AF222921
	R: TGGAAAAACTCCGTATCTGTCTC			
MYF6-met	F: TTTTTTTGTTAGGATTAAATGTTTT	57°C	257 bp	AY327443
	R: CTTTAATTAAAATTAACCACAATCC			

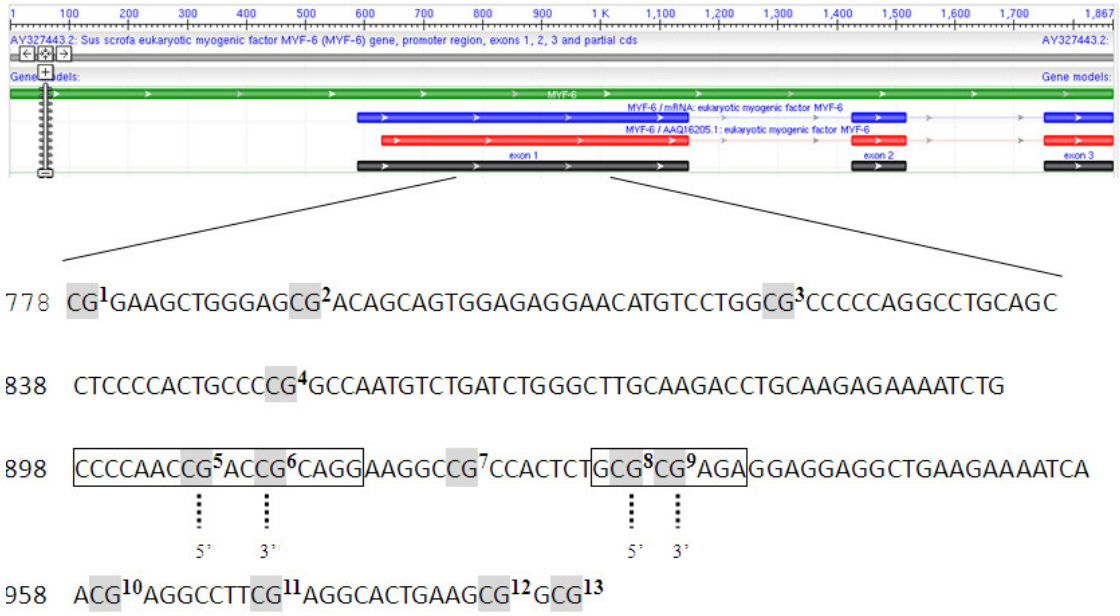


Figure 1. Sequence map of *MYF6* including the 13 CpG dinucleotides (shaded) and the predicted binding sites of c-myb (CpG5 and CpG6) and E2F1 (CpG8 and CpG9) (encircled)

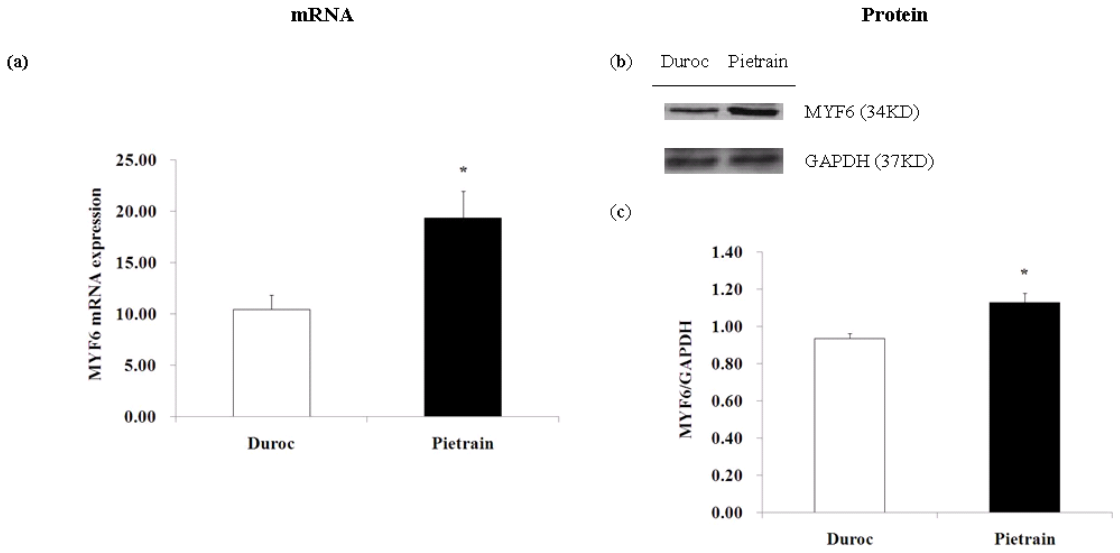


Figure 2. Determination of MYF6 mRNA and protein levels. (a) mRNA expression in Duroc and Pietrain. (b) Representative blots of MYF6 and GAPDH protein. (c) Ratio of relative protein levels of MYF6 expressed to GAPDH. (* $P < 0.05$)

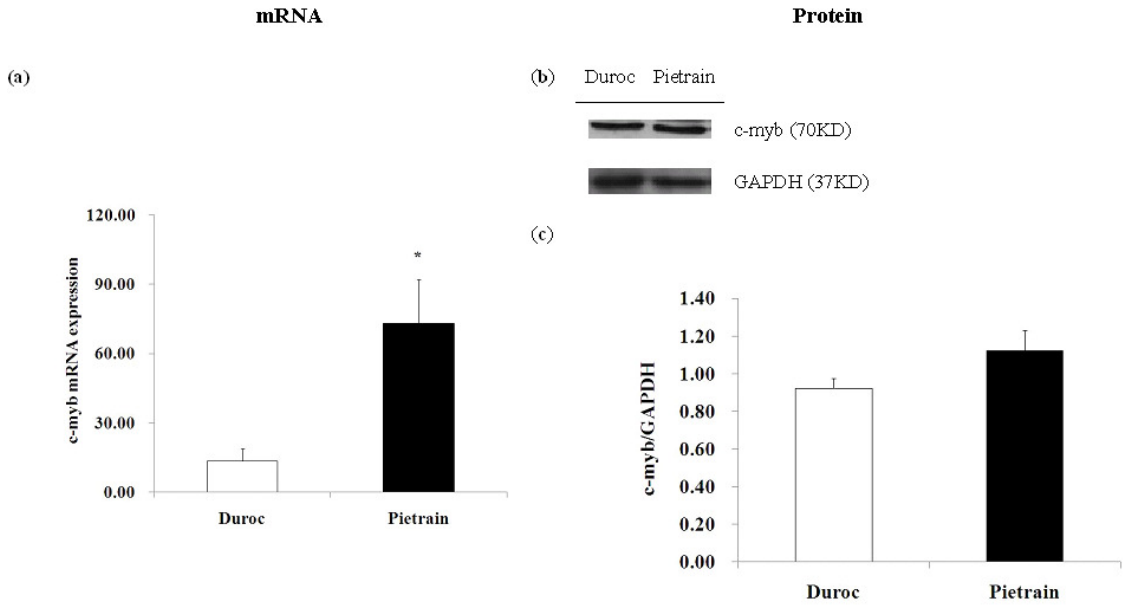


Figure 3. Determination of c-myb mRNA and protein levels. (a) mRNA expression in Duroc and Pietrain. (b) Representative blots of c-myb and GAPDH protein. (c) Ratio of relative protein levels of c-myb expressed to GAPDH. (*$P < 0.05$)

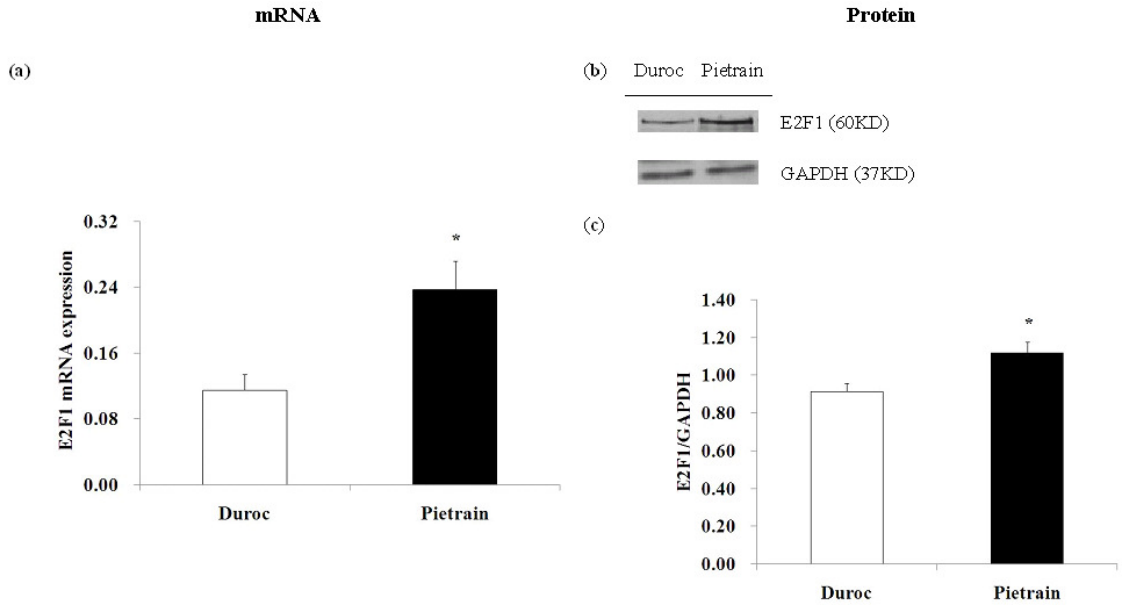


Figure 4. Determination of E2F1 mRNA and protein levels. (a) mRNA expression in Duroc and Pietrain. (b) Representative blots of E2F1 and GAPDH protein. (c) Ratio of relative protein levels of E2F1 expressed to GAPDH. (*$P < 0.05$)

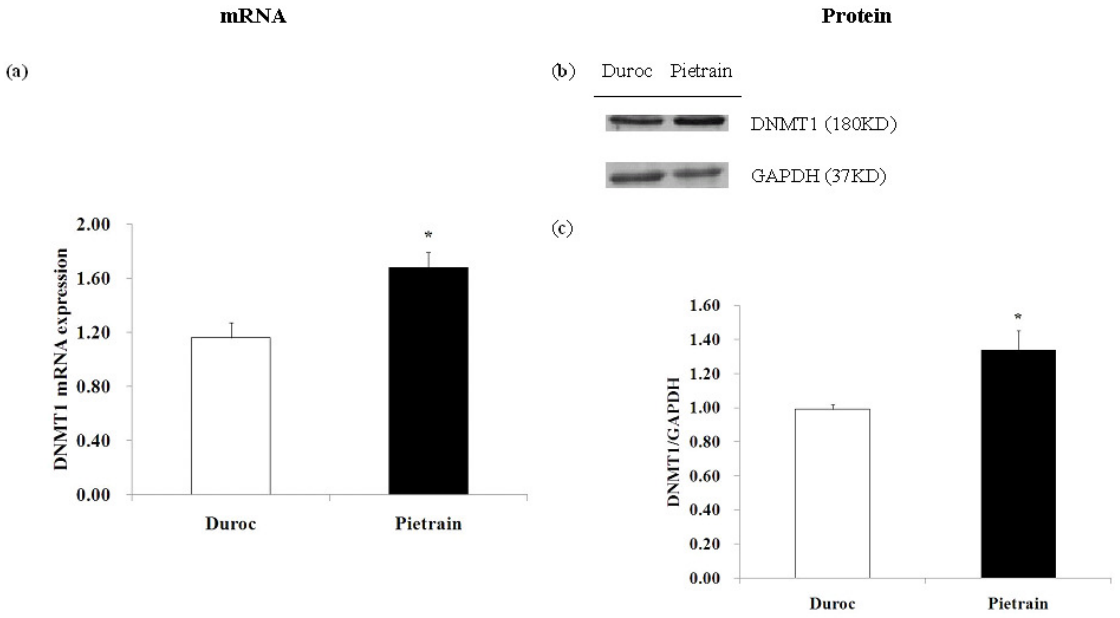


Figure 5. Determination of DNMT1 mRNA and protein levels. (a) mRNA expression in Duroc and Pietrain. (b) Representative blots of DNMT1 and GAPDH protein. (c) Ratio of relative protein levels of DNMT1 expressed to GAPDH. (*$P < 0.05$)

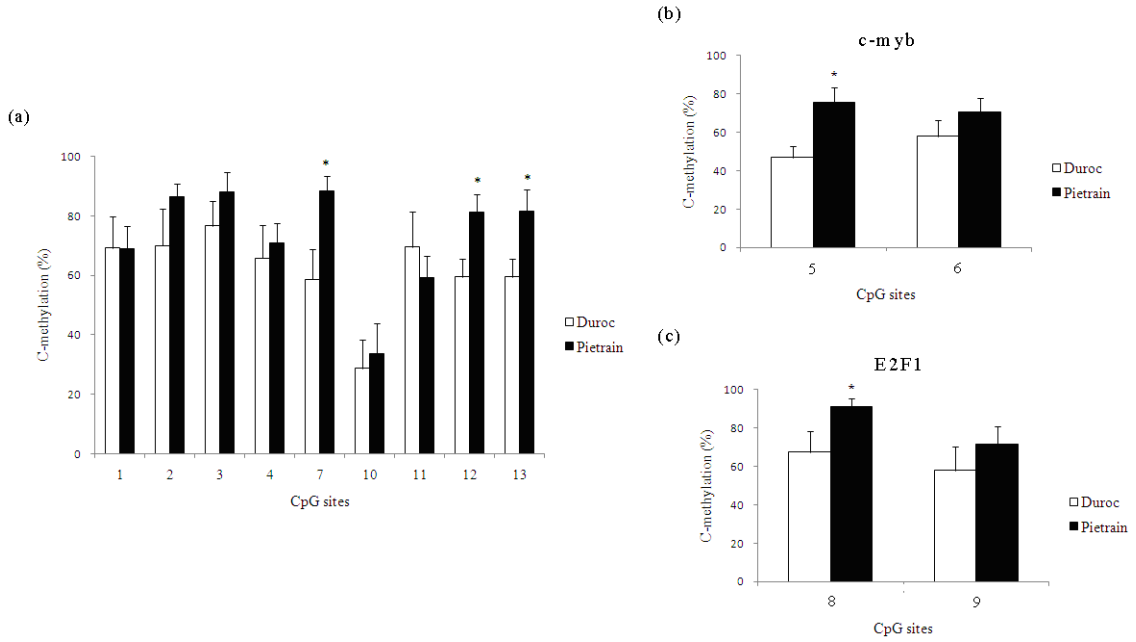
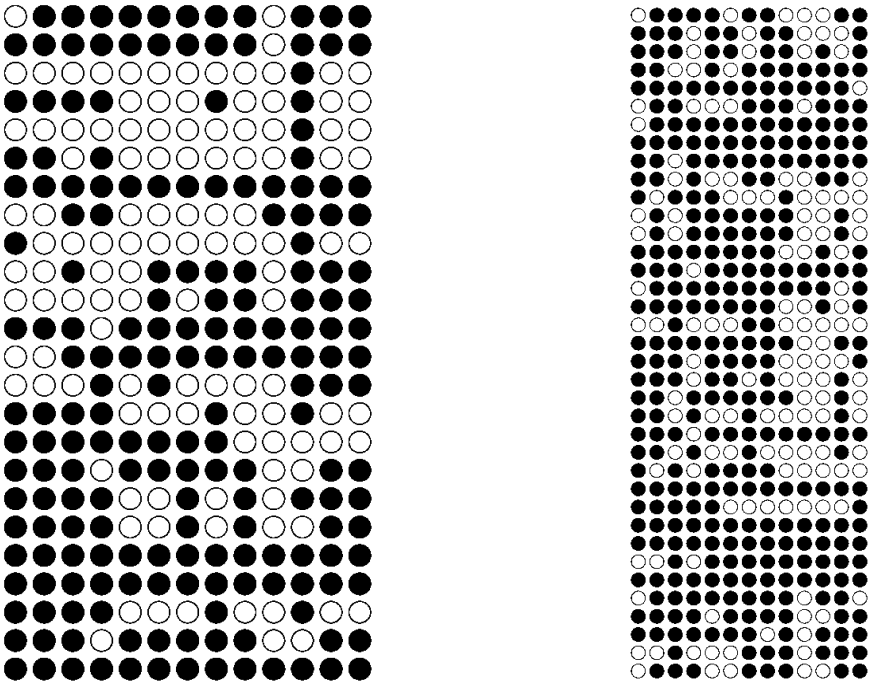
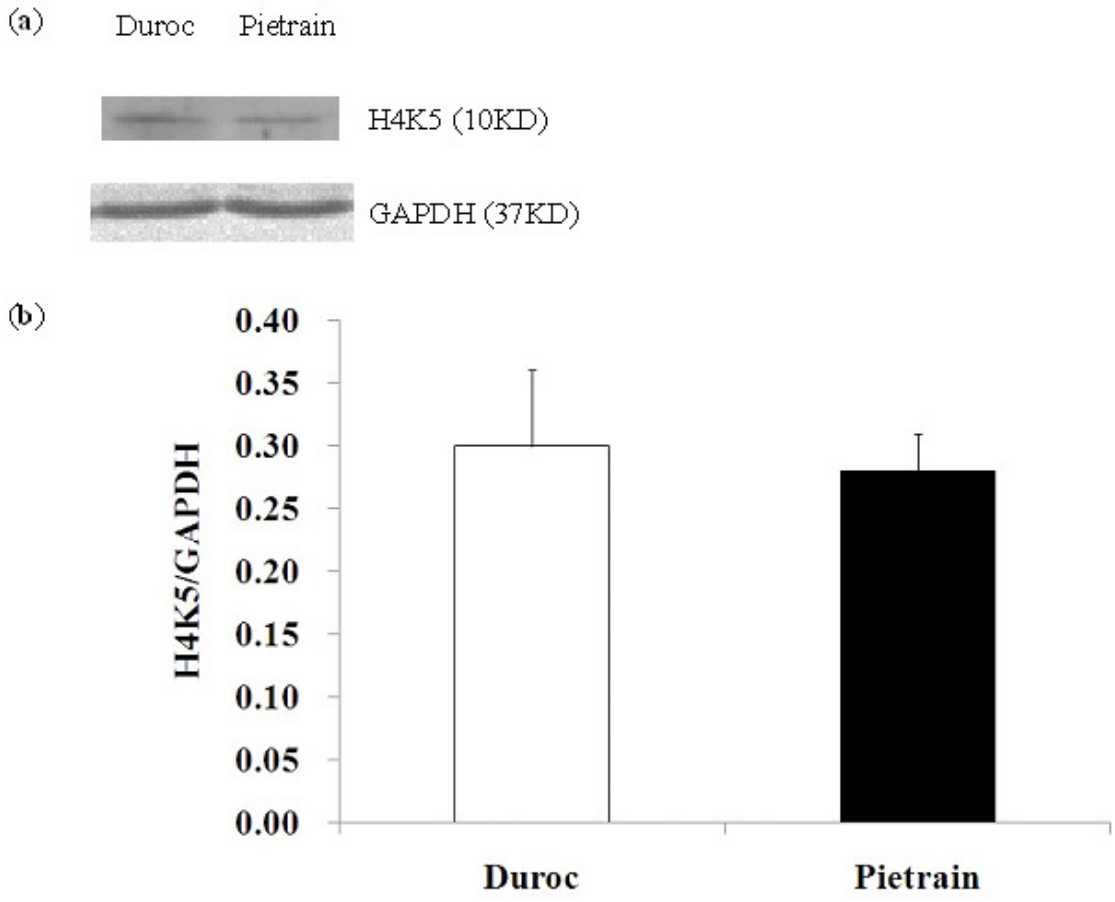


Figure 6. Cytosine methylation of *MYF6* 5´-regulatory region. (a) Percentage of methylated cytosines (mean ± S.E.) for the different CpG dinucleotides. (b) Percentage of methylated cytosines (mean ± S.E.) for the site 5 and site 6 CpG dinucleotides within the c-myb binding sequence. (c) Percentage of methylated cytosines (mean ± S.E.) for the site 8 and site 9 CpG dinucleotides within the E2F1 binding sequence. (*$P < 0.05$)

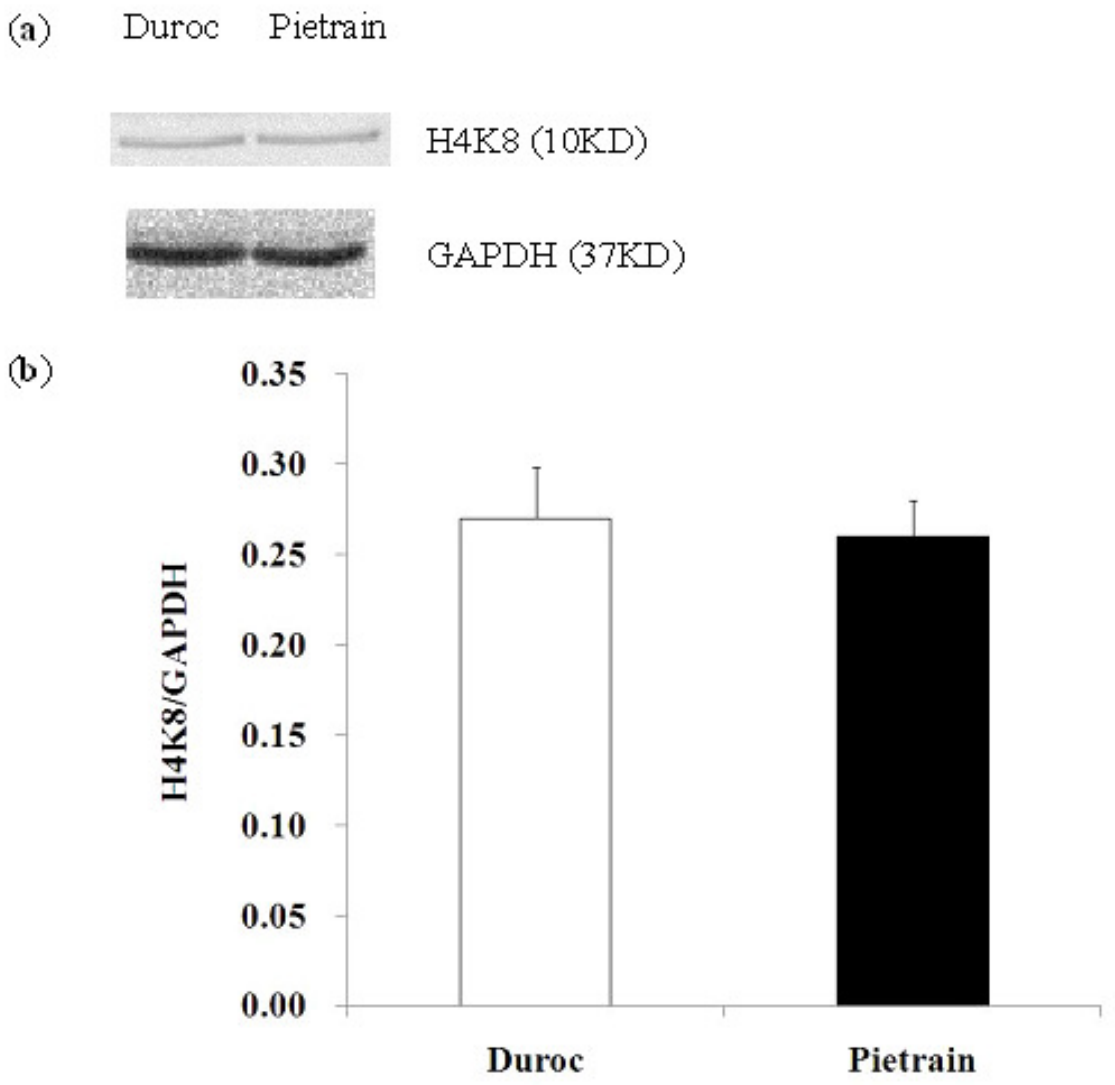
Duroc (24 colonies) Pietrain (37 colonies)



Supplementary Figure 1. Methylation profile of MYF6 (Web-based tool, “QUMA”, http://quma.cdb.riken.jp/). Filled (black) circles correspond to methylated Cs, unfilled (white) circles correspond to unmethylated Cs. Every row indicates a single colony sequence. Every column indicates a single CpG site. Four to five positive colonies were picked for each pig.



Supplementary Figure 2. Determination of global acetyl-Histone H4 (Lys 5, H4K5) level. (a) Representative blots of H4K5 and GAPDH protein. (c) Ratio of relative protein levels of H4K5 expressed to GAPDH. (*$P < 0.05$)



Supplementary Figure 3. Determination of global acetyl-Histone H4 (Lys 8, H4K8) level. (a) Representative blots of H4K8 and GAPDH protein. (c) Ratio of relative protein levels of H4K8 expressed to GAPDH. (*$P < 0.05$)

2.2 Chapter 2 (*Epigenetics*: under revision)

Sulforaphane causes a major epigenetic repression of *myostatin* in porcine satellite cells

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Keywords: Sulforaphane, satellite cell, myostatin, epigenetic repression, pig

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; ATF, activating transcription factor; CREB, cAMP response element binding protein; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; EAAs, essential amino acids; FBS, fetal bovine serum; FST, follistatin; FoxO1, forkhead box O1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase GDF8, growth differentiation factor 8; GM, growth medium; HDAC, histone deacetylase; HRP, horseradish peroxidase; HPRT1, hypoxanthine phosphoribosyltransferase 1; hSGT, human small glutamin-rich tetratricopeptide repeat-containing protein; MEF2, myocyte enhancer factor 2; MEMα, minimum essential medium α; miRNAs, microRNAs; MSTN, myostatin; MyoD, myoblast determination protein; NPDFs, nasal polyp-derived fibroblasts; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; qRT-PCR, quantitative real time PCR; RYR1, ryanodine-receptor gene; SD, standard deviations; SFN, sulforaphane; Smad7, Smad family member 7; Smurf1, Smad specific E3 ubiquitin protein ligase 1; Ct, threshold cycle; TGF-β, transforming growth factor-β; TSA, trichostatin A; UTR, untranslated region

Abstract

Satellite cells function as skeletal muscle stem cells to support postnatal muscle growth and regeneration following injury or disease. There is great promise for improvement of muscle performance in livestock and therapy of muscle pathologies in human targeting myostatin (MSTN) with this cell population. Human diet contains many histone deacetylase (HDAC) inhibitors such as the bioactive component sulforaphane (SFN) and epigenetic effects of SFN on *MSTN* gene in satellite cells are unknown. Therefore, we aimed to investigate the epigenetic influences of SFN on the *MSTN* gene in satellite

cells. The present work provides the first evidence, distinct from effects of trichostatin A (TSA), that SFN supplementation *in vitro* indeed not only acts as HDAC inhibitor but also *DNA methyltransferase (DNMT)* inhibitor in porcine satellite cells. Compared to TSA and 5-aza-2′-deoxycytidine (5-aza-dC), SFN treatment significantly represses *MSTN* expression, accompanied by strongly attenuated expression of negative feedback inhibitors of MSTN signalling pathway. miRNAs targeting *MSTN* are not implicated in post-transcriptional regulation of *MSTN*. Nevertheless, weakly enriched myoblast determination protein (MyoD) associated with diminished histone acetylation in MyoD binding site located in *MSTN* promoter region by SFN may contribute to transcriptional repression of *MSTN*. These findings reveal a new mode of epigenetic repression of *MSTN* by the bioactive compound SFN. Given this new pharmacological, biological activity of SFN in satellite cells, it may thus allow for developing novel approaches to weaken the MSTN signalling pathway both for therapies of human skeletal muscle disorders and livestock production.

Introduction

Pig is an economically important animal in livestock production, as well as in biomedical studies for humans because of the similarity in physiology, organ development and disease progression.[1] Skeletal muscle growth is one of the major economic traits in meat production. Postnatal muscle maintenance and growth rely on activation of a unique population of quiescent 'satellite cells' (referred as muscle stem cells), which are capable of self-renewal and myogenic differentiation to form hypernucleated myotubes.[2, 3] Satellite cells can be also activated under injury and pathological conditions and contribute to muscle repair and regeneration.[3] Nevertheless,

little is known about the effects of bioactive compounds on this multipotent muscle cell population and the mechanisms that mediate their effects.

Sulforaphane (SFN), an isothiocyanate derived from cruciferous vegetables, is a common bioactive compound that has the ability to inhibit type I and II histone deacetylase (HDAC), as well as an antioxidant, anti-carcinogenic and chemotherapeutic agent.[4, 5] In this context, we became interested in the HDAC inhibitor property of SFN. In human colorectal cancer cells and prostate epithelial cells, this HDAC inhibition was accompanied by increased histone acetylation in global histone H3, H4 and *P21* gene promoter region, associated with elevated expression of P21 protein.[5, 6] *In vivo* studies in SFN-fed mice, HDAC activity was inhibited significantly in various tissues with a concomitant increase in global and local histone acetylation.[7] In healthy human volunteers, oral consumption of SFN-rich broccoli sprouts resulted in a strong HDAC inhibition associated with histone hyperacetylation at 3 and 6 h in peripheral blood mononuclear cells (PBMC).[8] However, as the novel natural HDAC inhibitor, the potential epigenetic effects of SFN supplementation on skeletal muscle cells remain undiscovered.

Myostatin (MSTN; previously called growth differentiation factor 8, GDF8) is a member of the transforming growth factor-β (TGF-β) superfamily and a potent inhibitor of skeletal muscle growth.[9] MSTN can also block satellite cell activation and negatively regulate self-renewal of satellite cells.[10] It has been identified that follistatin (FST), Smad family member 7 (Smad7), Smad specific E3 ubiquitin protein ligase 1 (Smurf1) and human small glutamin-rich tetratricopeptide repeat-containing protein (hSGT) are involved in the MSTN pathway and inhibit MSTN activity to attenuate MSTN signalling.[11] In the skeletal muscle field, epigenetic research is emerging only during the last years.[12] Numerous studies have unveiled that epigenetic alterations, including DNA

methylation and histone modifications, are important players in the finely tuned gene expression. Small noncoding microRNAs (miRNAs) capable of inducing stable changes in gene expression without altering the sequence of genes also contribute to the epigenetic landscape.[13] Recently, the evidence is accumulating supporting a post-transcriptional regulatory role for miRNAs in the regulation of *MSTN* expression. Data from Drummond et al. show that a rapid increase of miR-499 expression by essential amino acids (EAAs) results in the suppression of *MSTN* expression in human skeletal muscle.[14] Allen and Loh have shown that miR-27b targets 3′ untranslated region (3′UTR) of *MSTN* efficiently and may contribute to fast-specific and glucocorticoid-dependent *MSTN* expression in skeletal muscle.[15] In a *trans*-regulatory manner, transcription factor forkhead box O1 (FoxO1) could bind to the mouse *MSTN* promoter and activate its activity to up-regulate *MSTN* expression.[16] Transcriptional activity of human *MSTN* promoter was strongly enhanced by myocyte enhancer factor 2 (MEF2) binding to the element present in the promoter region.[17] However, the ability and mechanism of SFN epigenetically regulating the *MSTN* gene in satellite cells are unknown.

Taking these above observations into account, the objective of this study was to test the hypothesis that SFN supplementation influences satellite cells growth and the epigenetic mechanisms account for the *MSTN* gene modulation in response to SFN exposure. In the present study, we also employed the typical epigenetic reagents 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA) compared to SFN treatment. We show that, distinguished from TSA, SFN and 5-aza-dC significantly suppress MSTN expression and inhibit HDAC activity as well as *DNA methyltransferase (DNMT)* expression in porcine satellite cells. Significantly diminished myoblast determination protein (MyoD) enrichment associated with hypoacetylation of the MyoD-binding site

in the *MSTN* promoter results in an epigenetic repression of *MSTN*. Our findings implicate that SFN is able to epigenetically modulate *MSTN* expression in an *in vitro* muscle stem cell model. If verified and applied to the *in vivo* models, it may have therapeutic benefits in human skeletal muscle disorders and practical value in meat production.[18]

Results

Epigenetic reagents affect porcine satellite cell growth and inhibit HDAC activity. In this study, we followed cell culture procedure as shown in Fig. 1A. By design, we selected three serial doses (5 μM, 10 μM and 15 μM) of SFN to avoid oxidative stress and apoptosis, which occurs at higher concentrations *in vitro*. [19, 20] Cells were harvested 48 h after exposure as SFN at 15 μM inhibits HDAC activity and increases histone acetylation level in prostate cell lines after 48 h treatment.[6] For 5-aza-dC, 10 μM concentration was selected because it is the optimal dose to up-regulate both *FST* isoforms (*FST288 and FST315*).[21] For TSA, we used the general concentration 50 nM in accordance with previous reports to serve as a positive control.[22, 23] Almost all of satellite cells were not viable after treatment at day 2 (data not shown), therefore we selected day 3 (the myoblast stage) to start the treatment. To examine the cytotoxic effects of SFN, TSA and 5-aza-dC treatments on porcine satellite cell growth, we determined cell viability (Fig. 1B) and cell proliferation rate (Fig. 1C). No difference in cell viability was observed except an increase in the SFN5 group. Only the 5-aza-dC and SFN15 groups show reduction of cell proliferation two days after treatment. Given that 5-aza-dC is a DNMT inhibitor, we firstly quantified *DNMT1* mRNA expression. In addition to 5-aza-dC group, *DNMT1* transcripts were also remarkably decreased in SFN10 and SFN15 groups (Fig. 1D). These results indicate that SFN10 is the optimal

group for our experiments. To confirm this and pro-apoptotic effect of SFN, we examined caspase 3 and caspase 9 activity which are related to apoptosis induced by SFN.[24, 25] As shown in Fig. 1E and 1F, caspase activities were inhibited except caspase 3 in TSA group. Then we selected SFN10 and analysed the HDAC activity in different treatments. In line with the previous reports, relative HDAC activities were significantly inhibited in all three treatments (Fig. 1G, lower panel). However, global acetyl-histone 3 and 4 levels were notably decreased in 5-aza-dC and SFN10 groups which differ from highly elevated histone acetylation in TSA group (Fig. 1G, upper panel).

FST variants were not involved in epigenetic effects of SFN on satellite cells. Acting as a potent HDAC inhibitor, TSA can induce the natural MSTN antagonist FST.[23, 26] Our initial interest was focused on whether SFN demonstrated the similar mechanism as TSA. Therefore, we quantified the total *FST* (Fig. 2A) and *FST315* (Fig. 2B) expression as it is not possible to distinguish another porcine *FST* isoform *FST288* from *FST315* due to the identical sequence. To gain a full profile of influences of SFN on FST, other two doses (5 μM and 15 μM) were also used. Nevertheless, up-regulated total *FST* and *FST315* were not observed in SFN groups. Considering the *DNMT1* alterations, we plotted 31 CpG sites in the second predicted CpG island (Fig. 2C) of the porcine *FST* 5´-regulatory region and determined the DNA methylation status (Fig. 2D). The bisulfite sequencing analysis revealed that all the 31 CpG sites were sparsely methylated except some sporadic methylation sites. Taken together, these results indicate that *FST* is not induced by SFN and not involved in SFN effects on porcine satellite cells.

MSTN and its signalling pathway were inhibited by SFN treatment. MSTN is a potent negative regulator of myogenesis, and inactivation of MSTN results in heavy muscle growth.[27] Given no induction of *FST* gene expression by SFN, we then

reselected *MSTN* as the candidate gene and determined its expression levels which were significantly up-regulated by TSA and down-regulated by 5-aza-dC and SFN (Fig. 3A). In this experiment, other two doses of SFN (5 μM and 15 μM) were also used to gain a full profile of influences of SFN on MSTN signalling pathway genes. In the skeletal muscle, besides FST, Smad7, Smurf1, and hSGT were also identified as inhibitors of the MSTN signalling pathway.[11] Our results show that *hSGT* expression was up-regulated in TSA, 5-aza-dC and SFN15 groups (Fig. 3B). In comparison, *Smad* (Fig. 3C) and *Smurf1* (Fig. 3D) were only up-regulated in SFN treatment groups. Collectively, our observations thus suggested that MSTN itself and its pathway were more strongly attenuated by SFN.

Predicted miRNAs targeting porcine MSTN 3' UTR region were not involved in epigenetic repression of MSTN. miRNAs can silence mRNAs by endonuclease cleavage, translational repression and mRNA degradation.[28] In order to discover that specific miRNAs play regulatory roles in modulating *MSTN* transcription, we identified several miRNA targeting *MSTN* including miR-21, miR-26a, miR-29abc, miR-181a and also obtained experimentally confirmed miRNAs such as miR-27ab[15], miR-208b[29], miR-499[30] targeting *MSTN* from previous publications. MiR-208b and miR-499 expression was not able to be determined indicated by threshold cycle (Ct) value above 35 cycles (data not shown). All the miRNAs except miR-27b were remarkably down-regulated in 5-aza-dC group (Fig. 4A-H). MiR-29a and miR-29b expression dramatically decreased in the SFN group (Fig. 4E and Fig. 4F). Only miR-29b exhibited notably increased expression in the TSA group (Fig. 4F).

MyoD can bind to MSTN promoter to regulate its transcription involving histone deacetylation by SFN. Accumulating evidences report that *MSTN* expression could be regulated at transcriptional level. Further analysis of porcine *MSTN* promoter

sequence revealed three putative MyoD binding sites (Fig. 5A). To study the transcriptional regulation of the *MSTN* gene, we next examined whether MyoD would be recruited to the promoter regions of *MSTN* with the use of ChIP. ChIP with antibody to MyoD confirmed that endogenous MyoD was present in binding site 1 of *MSTN* promoter region, indicating MyoD interacts with *MSTN* to regulate *MSTN* transcription (Fig. 5B). In order to examine MyoD availability as the transcription factor, we quantified MyoD mRNA and protein level. In accordance with mRNA expression, MyoD protein level was significantly elevated in TSA group but decreased in 5-aza-dC and SFN10 groups (Fig. 5C). To examine recruitment of MyoD in the *MSTN* promoter region, ChIP was conducted to quantify the relative enrichment. ChIP results demonstrated weak recruitment of MyoD in 5-aza-dC and SFN10 group compared to a robust recruitment of MyoD in TSA group (Fig. 5D). We were unable to investigate the epigenetic regulatory role of DNA methylation in *MSTN* gene transcription because no CpG island in the promoter region of porcine *MSTN* is available. Moreover, ChIP assays were carried out to determine the local histone acetylation status of MyoD reponse element. In contrast to dramatic histone hyperacetylation in TSA group, hypo-acetylation status of histone 3 and 4 was observed in 5-aza-dC and SFN10 groups (Fig. 6A and 6B). Taken together, these results suggest diminished MyoD and promoter-specific hypoacetylation could down-regulate *MSTN* expression at transcription level..

Discussion

For understanding human diseases, pig represents a promising model for biomedical research which closely resemble and reflect human biology.[31] In this study, we provided the first evidence for both SFN and 5-aza-dC as inhibitor of HDAC and *DNMT* in porcine satellite cells, demonstrating different epigenetic mechanisms from TSA.

Previous report has described protective effects of SFN treatment on rat skeletal muscle damage and oxidative stress,[32] but not in the context of HDAC inhibition, and to our knowledge this is the first study with SFN in satellite cells. As for 5-aza-dC, there is only one report demonstrating human skeletal muscle *ryanodine-receptor gene (RYR1)* transcription was reactivated after treatment with 5-aza-dC,[33] but without evidence for epigenetic effects of 5-aza-dC on myoblasts.

In this study, we investigated the potential epigenetic effects of SFN, TSA and 5-aza-dC treatments on porcine satellite cell growth. Our results demonstrate that none of SFN, 5-aza-dC and TSA influenced the cell viability except SFN at 5 μM. The increased cell viability in SFN5 group may indicate the protective effects of SFN on satellite cells at lower concentration.[32] We also found that cells in 5-aza-dC and SFN15 groups had a reduction of cell proliferation as compared to negative control. SFN at 10 μM did not have anti-proliferative effect in the satellite cells. The reduction detected after 2 days of treatment may be due to apoptotic response of 5-aza-dC and high-dose of SFN.[6, 24, 25] We then aimed to investigate the effects of 5-aza-dC on DNA methylation status, and how alteration in promoter region methylation affects gene expression. *DNMT1* expression was determined and found to be significantly suppressed in 5-aza-dC group, and also in HDAC inhibitor group (SFN10 and SFN15). These results are in line with previous studies which report that SFN significantly decreased the expression of *DNMT1* in response to SFN in breast cancer cells [34] and prostate cancer cells.[35] Gomyo et al.[24] and Singh et al.[25] reported that 5-aza-dC and SFN-induced apoptosis is associated with activation of caspase-3 and caspase-9. SFN dramatically reduced the activity of caspase-3 in the cortex and hippocampus after hypoxia-ischemia insult[36] and was able to counteract rat skeletal muscle damage induced by acute exercise.[32] In our study, we determined caspase-3 and caspase-9 activities which decreased in 5-aza-dC

and SFN10 groups, suggesting that SFN at 10 μM had no pro-apoptotic effect in the cells. Multiple studies from cell to human have established that SFN is an effective inhibitor of HDAC activity, with evidence for increased global and local histone acetylation status.[37] However our work shows that SFN and 5-aza-dC are potent inhibitor of HDAC activity but decreased global acetylated histones H3 and H4 which differ from TSA. Although HDAC inhibitor is expected to induce hyperacetylation, no changes in the H3 or H4 acetylation[20] and even histone deacetylation[38] were also observed. Following the very similar treatment conditions as ours (48 h exposure to 15 μM SFN), Pledgie-Tracy A et al. demonstrate significantly inhibited HDAC activity in four human breast cancer cell lines without significant changes in the acetylation of H3 or H4.[20] Another two HDAC inhibitors MS-275 or SK-7068 also effectively inhibited cellular HDAC activity in human gastric adenocarcinoma cells (SNU-16) and cause decreased H3 or H4 acetylation after 48 h, 72 h and 96 h exposure.[38] These findings indicate that HDAC inhibitory effects by SFN withdraw and can not last longer to maintain histone acetylation in porcine satellite cells.

FST is one of the regulatory proteins which is capable of binding directly to MSTN, inhibiting its activity and acting as a potent MSTN antagonist.[39] Both *FST315* and total *FST* expression increased remarkably in TSA and 5-aza-dC group, whereas SFN was not able to induce *FST*. The bisulfite sequencing PCR results just demonstrate some sporadic methylated sites. In contrast, a significant increase in *FST* mRNA expression and peptide secretion was detected after 5-aza-dC treatment in human NCI-H295R adrenocortical cells, as well as hypomethylation in *FST* promoter region.[21] The present study suggest that *FST* isoforms were not induced by SFN and DNA methylation may be not involved in regulation of *FST* expression.

MSTN inhibits myoblast proliferation and differentiation via a typical TGF-β pathway.[11] Here we show, for the first time, SFN and 5-aza-dC treatments clearly results in attenuated *MSTN* expression. In keeping with previous report that TSA increased *MSTN* mRNA expression up to 40-fold after treatment for 24 h in C2C12 myoblast[40], a substantial increase in *MSTN* expression was observed in TSA group. These results indicate that these epigenetic reagents affect *MSTN* expression through distinct regulatory mechanisms. In skeletal muscle, several proteins of MSTN signalling pathway have been identified as inhibitor of its secretion, activation, or receptor binding, including FST, Smad7, Smurf1, and hSGT.[11] We determined mRNA expression of these negative regulators in different treatments which illustrates differential mechanisms involved in the inhibition of MSTN signalling pathway. Up-regulated *FST* and *hSGT* may mainly cause MSTN pathway inhibition in TSA and 5-aza-dC group. Moreover, enhanced *Smad* and *Smurf1* could participate in such inhibition in SFN group. A recent study has shown that TSA induced expression of *Smad7* in nasal polyp-derived fibroblasts (NPDFs) exposed to TSA (50 nM–400 nM) with TGF-β1 for 24 hours.[41] Our results show that TSA did not affect expression of *Smad7* which may be due to the different treatment and cells. No data are available for SFN or 5-aza-dC regulating *Smad7* expression. As for *Smurf1*, in contrast to no alterations in TSA and 5-aza-dC group, we report firstly that SFN could up-regulate its expression. With regard to *hSGT*, elevated expressions were also firstly observed in TSA, 5-aza-dC and SFN15 groups.

Recently, decreased expression of miR-136 and miR-500 have been detected in low protein fed pigs at finishing stage, which is related to higher *MSTN* mRNA expression.[42] Here, we demonstrate in porcine satellite cells that miRNAs and *MSTN* were not expressed in a reciprocal manner, suggesting that miRNAs may be not involved in the

post-transcriptional regulation of *MSTN* expression. We speculate that increased miRNAs expression in TSA group and decreased miRNAs expression in 5-aza-dC group and SFN10 group are highly linked with deregulated permissive mark histone acetylation as indicated by global acetylated histone 3 and 4 level (Fig. 1G). Up-regulation of miR-127 has been observed in T24 human bladder cancer cells treated by 5-aza-dC and another HDAC inhibitor 4-phenylbutyric acid. Importantly, acetylated histone 3 restored miR-127 expression, confirming that histone acetylation epigenetically regulates miRNA gene expression.[43] Reduced expression of miR-200 family and miR-205 in bladder cancer cells is also caused by repressive histone marks in their promoter region.[44] miRNA microarray analysis revealed 22 down-regulated miRNA species and 5 up-regulated miRNAs in the breast cancer cell line SKBr3 in response to HDAC inhibitor LAQ824.[45]

Our ChIP assay results confirmed that MyoD was recruited to the first putative binding site *in vivo*. We also demonstrated by quantitative PCR that MyoD was relatively lower enriched in 5-aza-dC and SFN10 groups, which coincides with the lower levels of MyoD mRNA and protein abundance. It can be suggested that MyoD could be regulating *MSTN* gene expression by binding to its response element in the promoter region. Spiller MP et al. [46] reported that one of bovine *MSTN* gene upstream regulatory elements appears to be critical for the *MSTN* promoter activity and that MyoD interacts with this binding motif *in vitro* as well as *in vivo* to regulate *MSTN* gene expression. More recently, Liu XJ et al. [42] has described that increased histone 3 acetylation, an activation mark, may account for transcriptional activation of *MSTN* in response to maternal dietary protein at finishing stage in pigs. In our study, compared to hyperacetylation of histone 3 and 4 in TSA group, hypoacetylation at the MyoD binding

site in 5-aza-dC and SFN10 diminishes binding of MyoD to its element, inactivating *MSTN* transcription.

In summary, this is the first demonstration that SFN can regulate MSTN and inhibitors of MSTN signalling pathway in porcine satellite cells. Our data revealed the following: (i) Both 5-aza-dC and SFN significantly inhibit HDAC activity and *DNMT1* expression. (ii) 5-aza-dC, SFN and TSA demonstrate differential mechanisms to inhibit *MSTN* expression and negative regulators of MSTN pathway. (iii) Deregulated miRNA may be not involved in epigenetic repression of *MSTN*. (iiii) Epigenetic repression of *MSTN* by 5-aza-dC and SFN is associated with the reduced MyoD expression, diminished binding of MyoD on *MSTN* promoter and hypoacetylation in MyoD binding site. These results provide new insight into the manipulation of muscle cell phenotypes. In the future, it will be interesting to verify the functional roles of SFN *in vivo* and evaluate the potentials for therapy of human muscle diseases and livestock muscle growth.

Materials and Methods

Porcine satellite cells isolation. The right and left semimembranosus muscles from 6 purebred Pietrain piglets at 20 days of age were collected for porcine satellite cells isolation. Piglets were slaughtered by intracardiac injection of T61 (Intervet). All the pigs were kept and slaughtered according to German performance test directions.[47] The porcine satellite cells isolation procedure was outlined by Mau et al.[48] Briefly, the muscle samples were quickly removed, sterilized with 70% ethanol, rinsed in cold phosphate-buffered saline (PBS), minced, and digested with 0.25% trypsin (27250-018, Invitrogen) for 1 h at 37°C with continuous shaking. Digestion was stopped by 20% fetal bovine serum (FBS, 10270106, Invitrogen). The resulting cell suspension was then

filtered through 2×70 μm (352350, BD Falcon) and 1×40 μm cell strainer (352340, BD Falcon). Satellite cells were enriched by using a Percoll (P1644, Sigma-Aldrich) gradient (90%, 40%, 25%) centrifugation. Then the enriched satellite cells were collected and diluted with minimum essential medium α (MEMα, M0894, Sigma-Aldrich) supplemented with 4 mM glutamine (25030, Invitrogen), 100 IU/ml penicillin, 100 μg/ml streptomycin (15140, Invitrogen), 2.5 μg/ml fungizone (15290, Invitrogen) and 10% FBS. After counting by a hemocytometer, aliquots of the cells were frozen in liquid nitrogen until making a pool. Before starting experiments, a uniform pool was made from all aliquots obtained from several isolation procedures.

Cell culture, cell viability and cell proliferation rate. Aliquots of uniform porcine satellite cells pool were thawed and reseeded in gelatin-coated (0.1%, G1890, Sigma-Aldrich) CytoOne cell culture dishes or flasks (USA Scientific, Inc.). All incubations were performed at 37°C under a humidified atmosphere of 5% CO_2 in air. The medium was changed every other day and all experiments were repeated for three times with duplicates. Details of cell culture procedure are shown in Fig. 1A. At day 3, the cells were exposed to DMSO (7029.1, Carl Roth GmbH), TSA (T8552, Sigma-Aldrich), 5-aza-dC (A3656, Sigma-Aldrich), and SFN (S8044, LKT) for 48h in MEMα with 10% FBS (growth medium, GM). Treatments were categorized into 0.04% DMSO (vehicle control, equal 0.04% DMSO present in other reconstituted chemicals), TSA (50 nM TSA), AZA (10 μM 5-aza-dC), SFN5 (5 μM SFN), SFN10 (10 μM SFN) and SFN15 (15 μM SFN) groups.

WST-1 kit (10008883, Cayman Chemical) was used to quantify cell viability and cell proliferation rate according to the manufacturer's instruction. Cell viability was measured after treatment. Cell proliferation rate was determined after two additional days in GM without treatment.

Measurement of cell apoptosis. Cells were reseeded in gelatin coated plates, followed by a 48 h treatment. Caspase-3 and 9 activity in cultures were measured using the caspase-3/CPP32 Colorimetric Assay Kit (K106-25, Biovision) and caspase-9 Colorimetric Assay Kit (K119-25, Biovision), according to the manufacturer's instructions. Samples were read at 405 nm in a microtiter plate reader (Molecular Devices).

In vitro HDAC activity assay and histone isolation. After treatment at day 5, *in vitro* HDAC activity was determined using the Color-de-Lys HDAC colorimetric activity assay kit (BML-AK501-0001, Enzo Life Sciences), following the protocol described by the manufacturer. Briefly, approximately 10 μg nuclear extract for each sample was incubated with the HDAC assay buffer and the HDAC colorimetric substrate for 30 min at 37°C. Lysine developer was then added, and the samples were incubated at 37°C for another 30 min. Samples were read at 405 nm using a microtiter plate reader.

Satellite cells were cultured without or with different treatment for 48 h. Histone proteins were then isolated by EpiQuik Total Histone Extraction Kit (OP-0006, Epigentek) according to the manufacturer' manual, followed by Western blotting analysis of acetylated histone 3 and histone 4.

Prediction of miRNAs targeting MSTN. To determine the differentially expressed miRNAs targeting *MSTN*, we used two miRNA target prediction algorithms: MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), and TargetScan[49]. The putative binding sites were further verified by RNAHybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) and RNA22 (http://cbcsrv.watson.ibm.com/rna22.html).

qRT-PCR of miRNA and mRNA. Total RNAs including miRNAs were isolated using miRNeasy Mini Kit (217004, Qiagen) and reverse transcribed using miScript II RT Kit (218161, Qiagen). Total RNAs for mRNA expression were isolated and reverse transcribed as described previously.[4] Primers were designed using the online Primer3 program.[50] Quantitative real time PCR (qRT-PCR) was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems) using miScript SYBR Green PCR Kit (218073, Qiagen) and iTaq SYBR Green Supermix with ROX (172-5850, Bio-Rad) for the detection of miRNAs and mRNAs, respectively. Primers used for the detection of miRNA and mRNA are listed in Table 1. qRT-PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method[51] with *hypoxanthine phosphoribosyltransferase 1 (HPRT1)*[52] for mRNAs and 18S ribosomal RNA for miRNAs as endogenous references.

Western blotting analysis. A protocol for this procedure was described previously.[53] For acetylated histone 3 and histone 4, equal protein was loaded and confirmed by ponceau S staining. For MyoD, blots were probed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody to correct for differences in protein loading. Western blotting was carried out with the following primary antibodies: MyoD (1:1000, sc-31940 X, Santa Cruz), acetyl-histone H3 (0.05 μg/ml, 06-599, Millipore), acetyl-histone H4 (1:1000, 06-866, Millipore), GAPDH (1:3000, sc-20357, Abcam), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (donkey anti-goat, 1:3000, sc-2020, Santa Cruz; goat anti-rabbit, 1:2000, sc-2004, Santa Cruz). Finally, the specific signals were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (34077, Thermo Scientific). Images were acquired by Quantity One 1-D analysis software (Bio-Rad).

DNA methylation study. The 5´-regulatory region of *FST* (GenBank: M19529) was submitted to the online program Methprimer[54] to identify the CpG islands. Genomic

DNA (1 μg) was subjected to bisulfite modification using EZ DNA Methylation-Direct Kit (D5020, Zymo Research) according to the manufacturer's protocol. The *FST* 5´-regulatory region containing CpG island was amplified by nested PCR with FST-met-nest primer pairs (Table 1) designed using PerlPrimer[55] and Methyl Primer Express Software v1.0 (Applied Biosystems Inc.). PCR products were purified with QIAquick PCR purification kit (28104, Qiagen) and then subcloned into the pGEM T-easy vector (A1360, Promega). A minimum of six different positive clones were randomly selected for sequencing with M13 primers performed by the CEQ8000 sequencer system (Beckman Coulter). The final sequence results were processed by QUMA software.[56] Sequences with a conversion rate below 90% were excluded from analysis.

ChIP assays. The chromatin immunoprecipitation (ChIP) assay kit (17-295, Millipore) was used in accordance with the manufacturer's instructions. Soluble chromatin was immunoprecipitated with anti-MyoD (sc-31940 X, Santa Cruz), anti-acetyl-histone H3 (06-599, Millipore) or anti-acetyl-histone H4 (06-866, Millipore). Immunoprecipitates were subjected to quantitative PCR with *MSTN* promoter specific primers spanning the putative binding sites of interest (Table 1). As a negative control, a primer pair of MyoD binding site free region was used to amplify another genomic region that was not expected to interact with MyoD. Antibodies against RNA polymerase II (sc-899 X, Santa Cruz) and normal rabbit IgG (2729S, Cell signaling Technology) were used as a positive and a negative control in the assay, respectively. PCR products were separated on a 2% agarose gel stained with ethidium bromide for visualization. With the percent input method, signals obtained from the ChIP are divided by signals obtained from an input sample. Quantitative PCR data were normalized to and expressed as % of input.

Statistical analysis. Pairwise comparisons were made between treatment groups and the vehicle-treated control, using Student's t test. The data were expressed as means ± standard deviations (SD) and (*) $P < .05$, (**) $P < .01$, (***) $P < .001$ were set statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

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Table 1 List of primer sequences used in this study.

Applications and targets		**Primers sequence (5´-3´)**	**GenBank ID**
qRT-PCR for mRNA expression	DNMT1	F: GCGGGACCTACCAAACAT R: TTCCACGCAGGAGCAGAC	DQ060156
	Myostatin	F: GATTATCACGCTACGACGGA R: CCTGGGTTCATGTCAAGTTTC	AY448008
	FST315	F: AGTGACAATGCCACCTACGC R: CCTCGGTGTCTTCTGAAATGG	M19529.1
	FSTtotal	F: AAAACCTACCGCAACGAATG R: CAGAAAACATCCCGACAGGT	NM_001003662
	MyoD	F: TGCAAACGCAAGACCACTAA R: GCTGATTCGGGTTGCTAGAC	GU249575
	Smurf1	F: CAGCGTCTGGATCTATGCAA R: CTAACAGGCCTCTGCAGTCC	XM_003354460
	Smad7	F: CCAACTGCAGACTGTCCAGA R: CAGGCTCCAGAAGAAGTTGG	HM803236
	hSGT	F: GACCCCGACAATGAGACCTA R: TGATGCCATGCTCATAAAGC	NM_001244392
	HPRT1	F: AACCTTGCTTTCCTTGGTCA R: TCAAGGGCATAGCCTACCAC	NM_001032376.2
qRT-PCR for miRNA expression	ssc-miR-21	F: GCACCTAGCTTATCAGAC	
	ssc-miR-26a	F: TTCAAGTAATCCAGGATAGGCT	
	ssc-miR-27a	F: TTCACAGTGGCTAAGTTCTGC	
	ssc-miR-27b	F: TTCACAGTGGCTAAGTTCTGC	

	ssc-miR-29a	F: CTAGCACCATCTGAAATCGGTTA	
	ssc-miR-29b	F: TAGCACCATTTGAAATCAGT	
	ssc-miR-29c	F: TAGCACCATTTGAAATCGGTTA	
	ssc-miR-181a	F: AACATTCAACGCTGTCGGTGAGTT	
	18S	F: ACGGACAGGATTGACAGATT	
Bisulfite sequencing PCR	FST-met-nest1	F: TATTGGGAGATYGTTTATYGTAAAT R: CTTAAAACRAACCATTCT	M19529
	FST-met-nest2	F: AGATTTTYGTTTAGATTTAAAG R: CARCAAATAATTCCARCAAA	M19529
ChIP assay	MyoD-BSF	F: TGAATCAGCTCACCCTTGACT R: ATGATTGGCTCTTGCTCCAC	AY527152
	MyoD-BS1	F: CCAGACCTTACCCCAAATCC R: GCAGTTTGCCTCAGATTTCC	AY527152
	MyoD-BS2	F: CAGTTGAAAACTGAGCACGA R: TTTAGACAAACATTTGAGGAAAAA	AY527152
	MyoD-BS3	F: GTGGAGCAAGAGCCAATCAT R: ACAACTTGCCACACCAGTGA	AY527152

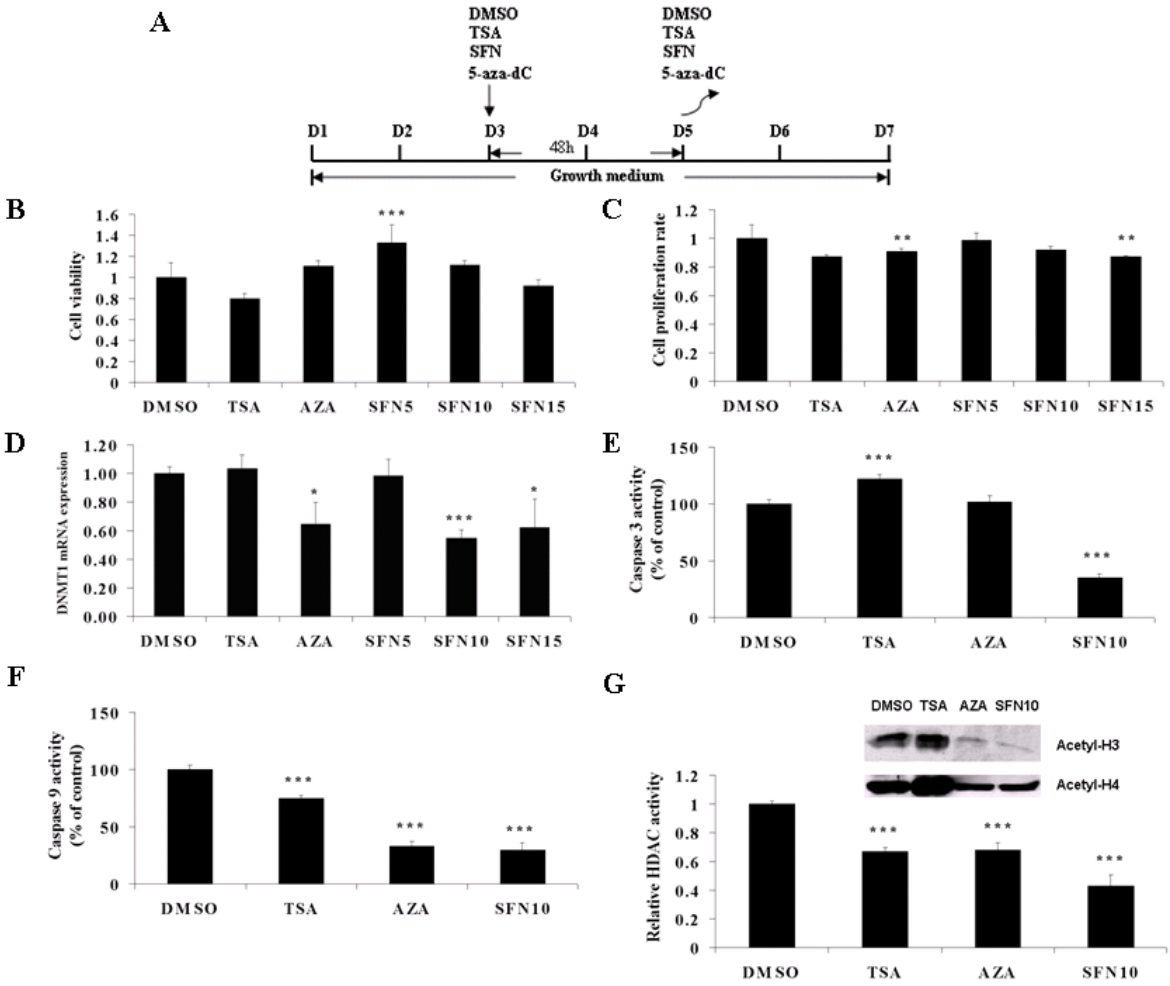

Figure 1. Epigenetic reagents affect porcine satellite cell culture and inhibit HDAC activity. (A) Scheme of porcine satellite cell culture and treatment procedure. (B) Cells were treated following the procedure as shown in (A). Cell viability was determined by WST-1 kit. (C) After removal of epigenetic chemicals, cells were allowed to proliferate for 2 days. Then cell proliferation rate was assessed by WST-1 kit. (D) *DNMT1* relative mRNA expression was quantified by qRT-PCR after treatments. (E) and (F) Caspase-3 and 9 activity in cultures were evaluated using the caspase-3/CPP32 Colorimetric Assay Kit and caspase-9 Colorimetric Assay Kit, respectively. (G) Relative HDAC activity (lower panel) was examined using the Color-de-Lys HDAC colorimetric activity assay kit. Equal amounts of isolated histone protein were subjected to Western blotting analysis to investigate acetylated histone 3 and 4 levels (upper panel). The results

represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

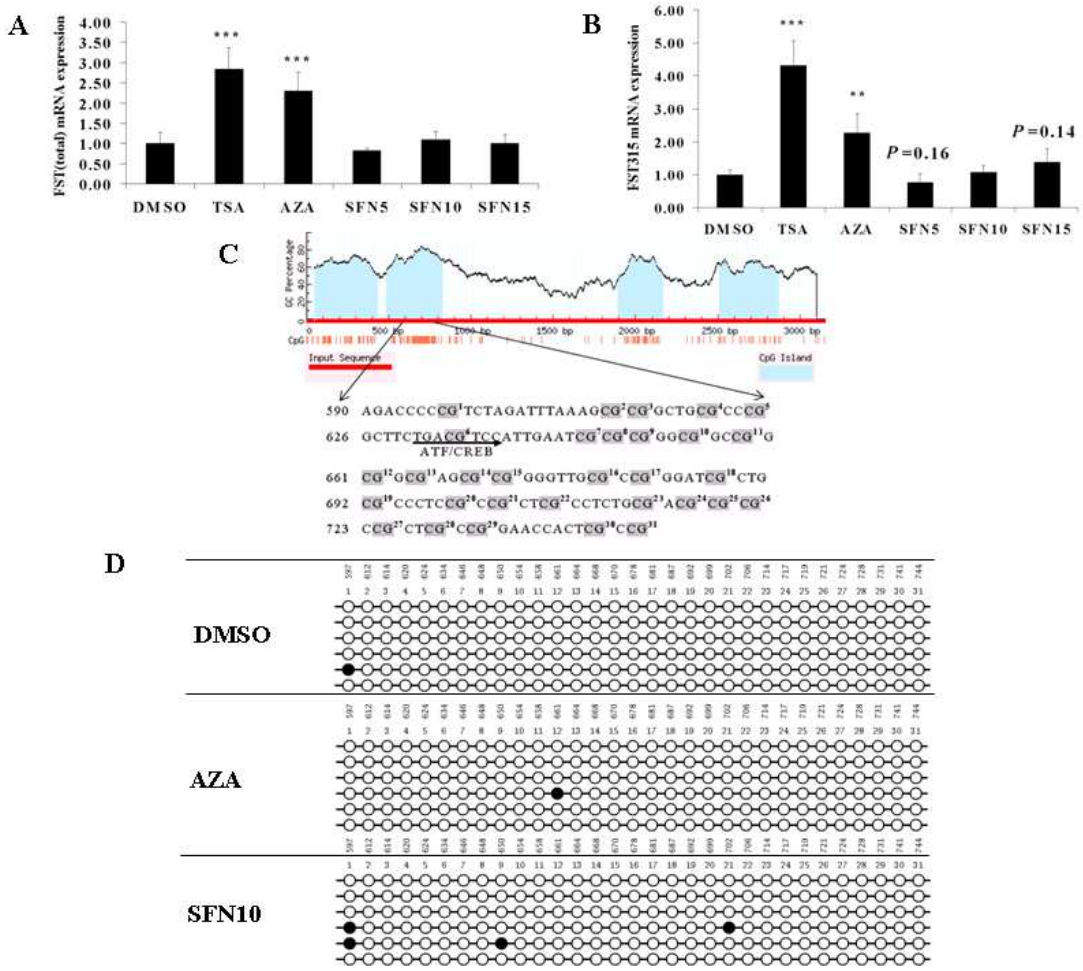

Figure 2. *FST* variants were not involved in epigenetic effects of SFN on satellite cell. (A) and (B) Total *FST* and *FST315* mRNA levels were quantified by qRT-PCR after treatments. The results represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate (** $P < 0.01$; *** $P < 0.001$). (C) CpG islands in the *FST* promoter region were predicted by MethPrimer online (upper panel). Thirty-one numbered CpG dinucleotides were mapped in genomic sequence (lower panel). (D) DNA methylation status within CpG island 2 spanning putative activating transcription factor (ATF)/cAMP response element binding protein (CREB) binding site was quantified by bisulfite sequencing PCR. A minimum of six positive

clones were randomly picked for sequencing with M13 primers. Sequencing results were visualized by QUMA software. Unfilled (white) circles correspond to unmethylated Cs and filled (black) circles correspond to methylated Cs.

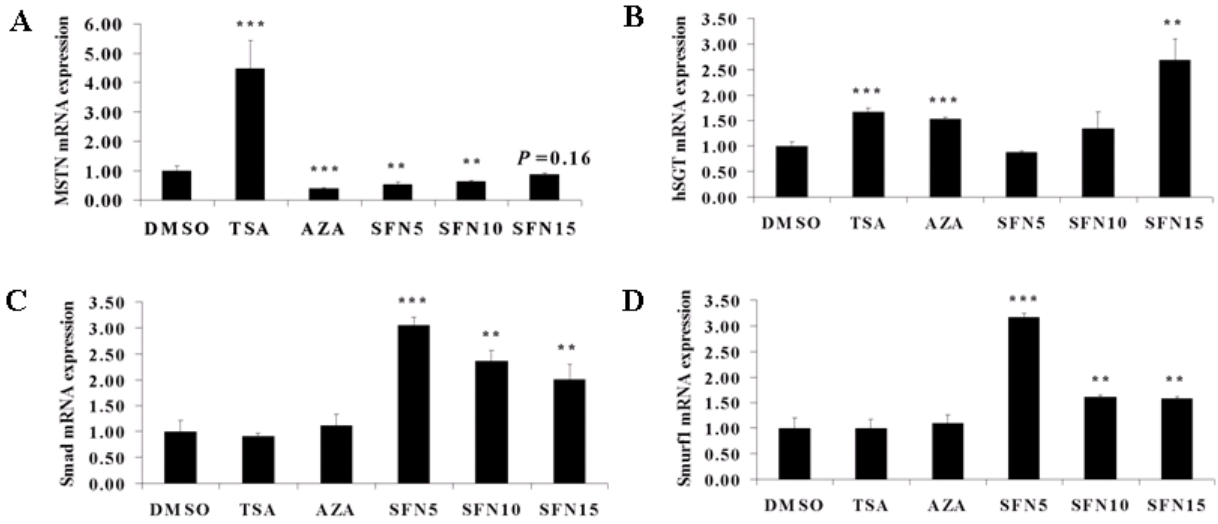

Figure 3. MSTN and its signalling pathway were inhibited by SFN treatment. qRT-PCR was carried out to quantify *MSTN* (A), *hSGT* (B), *Smad7* (C) and *Smurf1* (D) mRNA level. The results represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate (** $P < 0.01$; *** $P < 0.001$).

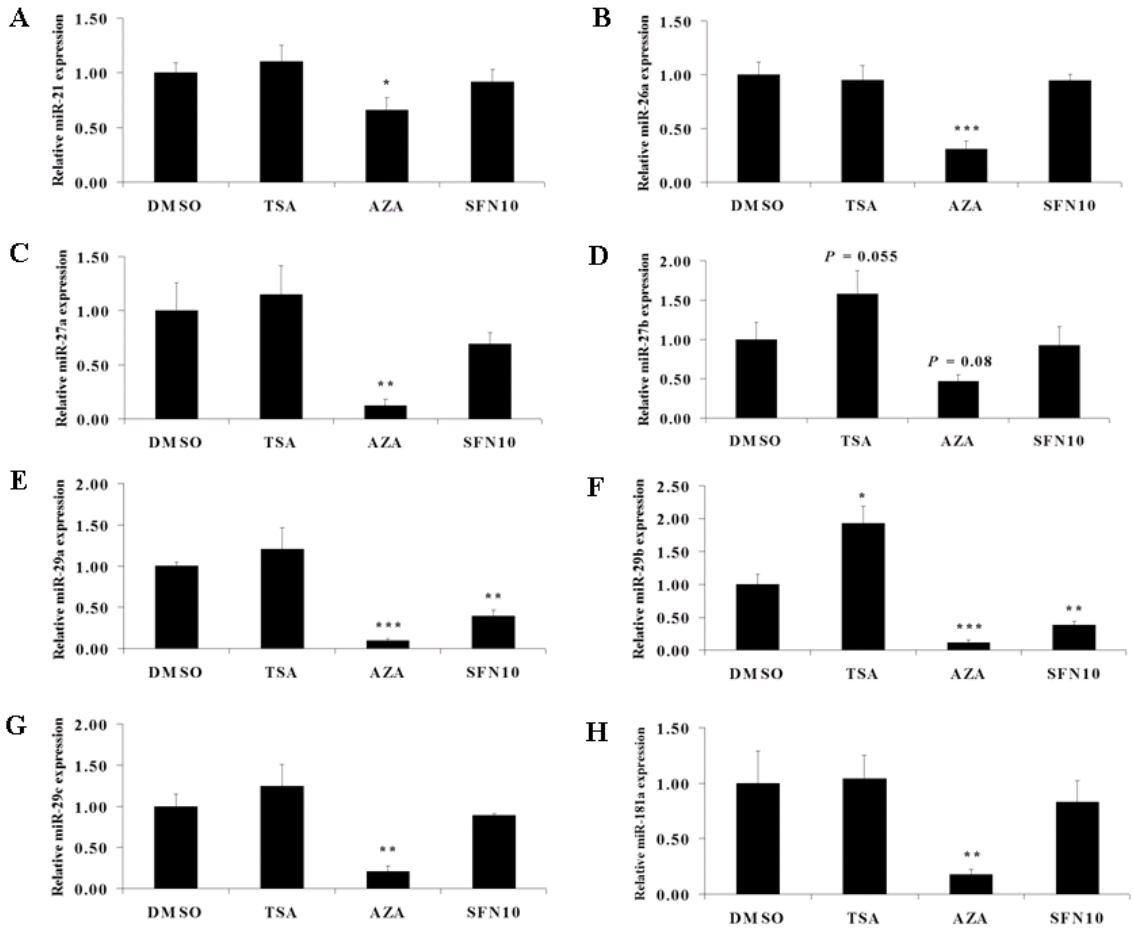

Figure 4. Predicted miRNAs targeting porcine *MSTN* 3'UTR region were not implicated in *MSTN* epigenetic repression. qRT-PCR was undertaken to quantify miR-21 (A), miR-26a (B), miR-27a (C), miR-27b (D), miR-29a (E), miR-29b (F), miR-29c (G) and miR-181a (H) expression level. The results represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

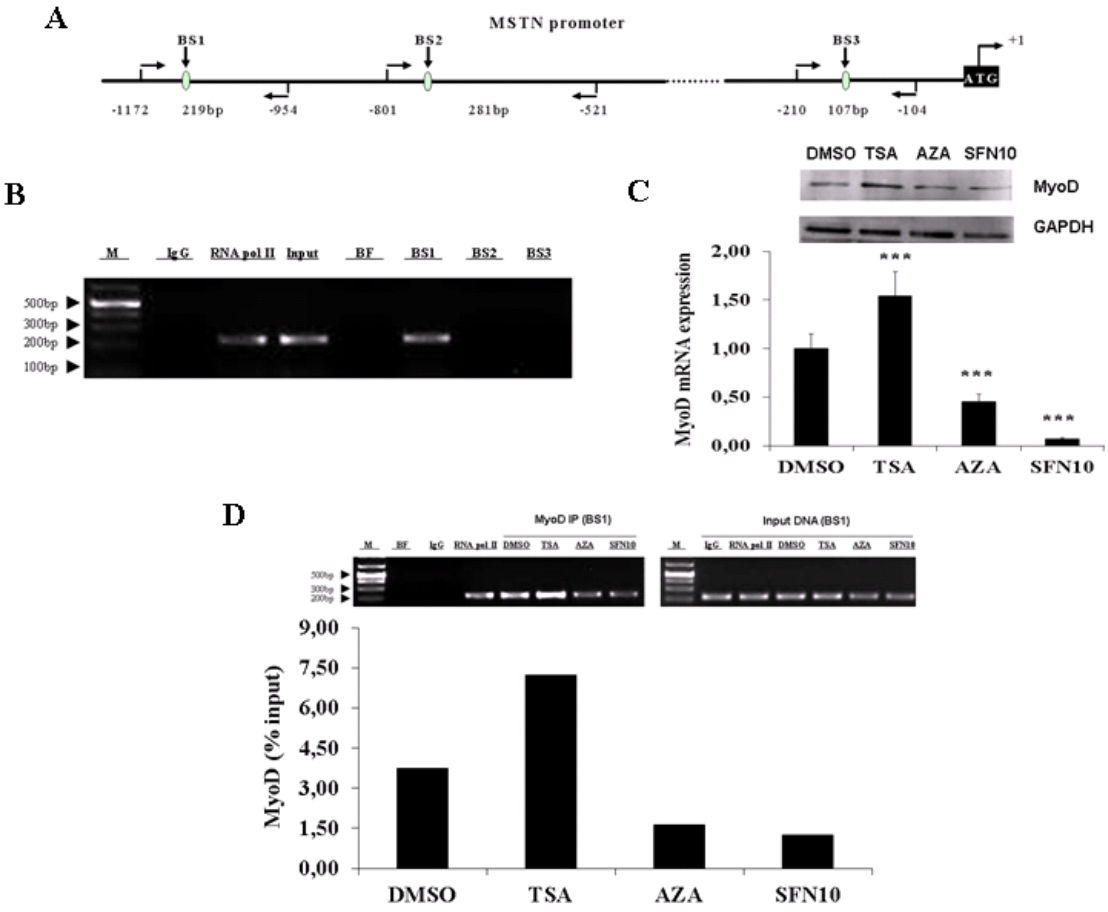

Figure 5. MyoD expression and occupancy in the *MSTN* promoter were significantly diminished by SFN and 5-aza-dC. (A) Schematic representation of three potential MyoD binding sites and PCR-amplified fragments located in *MSTN* promoter region. (B) ChIP was performed for MyoD recruitment to *MSTN* promoter following 48 h treatment. Soluble chromatin was immunoprecipitated with antibodies against rabbit IgG, RNA pol II and MyoD. Immunoprecipitates were subjected to PCR with primer-pairs for each amplicon indicated in (A). The sequences of primer pairs are described in Table 1. As a negative control, a set of primers were used to amplify binding free region that was not expected to interact with the MyoD. Amplification products were resolved in 2% agarose gel stained with ethidium bromide. M, DNA marker. BF, MyoD binding free region. BS1-3, MyoD binding site 1-3. (C) MyoD mRNA (lower part) and protein (upper part) expression were quantified by qRT-PCR and Western blotting,

respectively. The results represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate (*** $P < 0.001$). (D) Abundance of MyoD binding in BS1 of *MSTN* promoter region was determined by quantitative PCR following ChIP assay with MyoD antibody (lower part). Data are shown as a ratio to the input DNA. The PCR products were generated and visualized in 2% agarose gel (upper part). M, DNA marker. BF, MyoD binding free region. BS1, MyoD binding site 1.

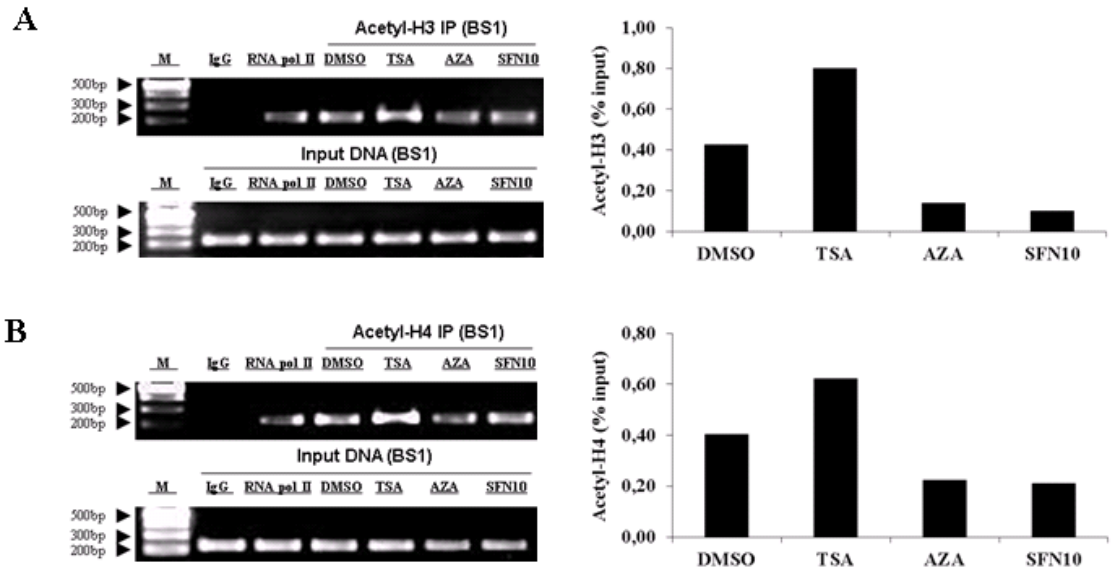

Figure 6. SFN and 5-aza-dC caused weak enrichment of acetylated histones around the *MSTN* BS1. (A) Acetyl-histone 3 was examined by quantitative PCR following ChIP assay with MyoD antibody (right part). Amplification products were visualized in a 2% agarose gel (left part). (B) Acetyl-histone 4 was determined by quantitative PCR following ChIP assay with MyoD antibody (right part). PCR products were visualized in a 2% agarose gel (left part). Data are normalized to the amount of input chromatin. M, DNA marker. BS1, MyoD binding site 1.

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