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Identification of candidate genes for porcine meat quality and investigation of effects of sulforaphane on porcine satellite cells

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Dedicated to my beloved family

Identification of candidate genes for porcine meat quality and investigation of effects of sulforaphane on porcine satellite cells

Meat quality has received more and more customer's attention. Muscle growth rate is closely related with the efficiency of pig production. Meat quality and muscle growth are two important issues in pig production. The present work identified a potential candidate gene, SOX-6, for pig breeding and investigated the effect of sulforaphane on muscle growth.

The genetic work identified a candidate gene, SOX-6, for porcine growth, carcass and meat quality traits. In this work, we investigated single nucleotide polymorphisms (SNPs) and expression of the candidate gene SOX-6, which is selected based on our previous work. The first SNP from porcine SOX-6, rs81358375, was found to be associated with pH 45 min *post mortem* (p.m.) in loin ($pH1_L$), the thickness of backfat and side fat and carcass length in a Pietrain population and was related with backfat thickness and daily gain in a Duroc × Pietrain (DuPi) F_2 population. The other SNP of porcine SOX-6, rs321666676, was related with meat colour in the Pietrain population. In the DuPi population, the SOX-6 mRNA expression is not significant different in the divergent $pH1_L$ pigs. However, the protein expression of SOX-6 in pigs with high $pH1_L$ was significantly less abundant compared with low $pH1_L$ pigs. Based on these results, SOX-6 shows to be a promising candidate for porcine growth, carcass and meat quality traits.

In the epigenetic work, we investigated effects of sulforaphane (SFN) on porcine satellite cells. Our previous study has found SFN treatment inhibited the expression of myostatin in porcine satellite cells. In line with this previous study, we found that SFN enhanced the proliferation of the porcine satellite cells and modulated the expression of myogenic regulatory factors. SFN treatment changed the expression of HDAC members and supressed their activity. The activity of TGF-β signalling was depressed by SFN, which also up-regulated the expression of Smad7, an endogenous suppressor of TGF-β signalling. Furthermore, we found that SFN increased transcription factors of Smad7 and decreased microRNAs targeting Smad7. In summary, our studies found the positive effects of SFN on the proliferation of porcine satellite cells and revealed the underlying

mechanisms. This supports that SFN may serve as a nutritional supplement to increase the muscle growth.

Identifizierung von Kandidatengenen für Merkmale der Fleischqualität und Untersuchung der Auswirkungen von Sulforaphan auf Satellitenzellen beim Schwein

Fleischqualität erhält mehr und mehr Aufmerksamkeit durch den Konsumenten. Die Muskelwachstumsrate steht in enger Beziehung zur Effizienz der Schweineproduktion. Die vorliegende Arbeit zielte darauf ab ein potenzielles Kandiatengen, SOX-6, für Fleischqualität funktionell zu charakterisieren und den Effekt von Sulforaphan auf das Muskelwachstum zu untersuchen.

Diese genetische Studie identifizierte SOX-6 als Kandidatengen für das Muskelwachstum sowie die Schlachtkörper- und Fleischqualitätsmerkmale. Dafür wurden Single-Nukleotid-Polymorphismen (SNPs) im SOX-6 Gen sowie seine Gen- und Proteinexpression im Muskel untersucht. Der SNP, rs81358375, zeigte eine Assoziation mit den Merkmalen pH 45 min post mortem (pm) im Schinken (pH1L), der Rückenspeckdicke, des Seitenfetts und der Schlachtkörperlänge in der verwendeten *Piétrain* Population sowie mit der Rückenspeckdicke und der tägliche Zunahme in einer Duroc × *Piétrain* (DuPi) F2-Population. Der zweite SNP von Schweine-SOX-6, rs321666676, konnte mit der Fleischfarbe in der *Piétrain* Population in Beziehung gesetzt werden. In der DuPi Population, zeigte zwar die Genexpression der mRNA keinen signifikanten Unterschied in Proben mit divergenten pH1L Werten. Jedoch was die Proteinexpression von SOX-6 in Proben mit hoher pH1L signifikant niedriger im Vergleich zu Proben mit niedrigen pH1L Wert. Basierend auf diesen Ergebnissen, ist SOX-6 ein vielversprechender Kandidat für Schlachtkörper- und Fleischqualitätsmerkmale.

In der epigenetischen Studie wurden die Auswirkungen von Sulforaphan (SFN) auf Schweine-Satellitenzellen untersucht. In früheren Studien konnte festgestellt werden, dass eine SFN-Behandlung von Schweine-Satellitenzellen die Expression von Myostatin inhibiert. Im Einklang mit dieser Studie haben wir festgestellt, dass SFN die Proliferation der Schweine-Satellitenzellen verbessert und die Expression von myogenen regulatorischen Faktoren beeinflusst. SFN verändert die Expression von HDAC-Mitgliedern und unterdrückt ihre Aktivität. SFN unterdrückte auch die Aktivität von TGF-β und führte zu einer Erhöhung der Expression von Smad7, einem endogenen

Suppressor des TGF-β-Signalwegs. Des Weiteren konnte festgestellt werden, dass SFN die Expression von Transkriptionsfaktoren von Smad7 einschließlich NF-E2-related Faktor 2 (Nrf2), erhöht und die Expression von mikroRNAs mit Smad7 als Zielgen verringert. Zusammenfassend zeigte die Studie, SFN grundlegende Mechanismen der Wirkung auf die Proliferation von Schweine-Satellitenzellen. Dies unterstützt die Aussage das SFN als Nahrungsergänzungsmittel zur Erhöhung des Muskelwachstum dienen könne.

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List of abbreviations

ACSL	Acyl-CoA synthetase long-chain family
ANGPTL4	Angiopoietin-like 4
ANK1	Ankyrin 1
BVES	Blood vessel epicardial substance
CAST	Calpastatin
Cbg	Cortisol-binding globulin
CDK	Cyclin dependent kinase
$Con1_L$	Conductivity 45 min p.m. in loin
$Con24_L$	Conductivity 24 hours p.m. in loin
COQ9	Coenzyme Q9
CS	Citrate synthase
CSC	China scholarship council
CSRP3	Cysteine and glycine-rich protein 3
CTSB	Cathepsin B
DFD	Dark, firm, dry
DLY	Duroc × (Landrace × Yorkshire)
DNMT	DNA methyltransferase
DRIP	Drip loss
DuPi	Duroc × Pietrain
EGFR	Epidermal growth factor receptor
eQTL	Expression quantitative trait loci
FADS2	Fatty acid desaturase 2
FBS	Fetal bovine serum
gDNA	Genomic DNA
GSK3B	Glycogen synthase kinase 3 beta
GWAS	Genome wide association study
h^2	Heritability
HDAC	Histone deacetylase
HPRT1	Phosphoribosyltransferase 1
HSP90	90 kDa heat shock protein
IFI6	Interferon-α inducible protein 6
IL	Interleukin

IMF	Intramuscular fat
LD	Longissimus dorsi
LMP	Lean meat percentage
lncRNA	Long noncoding RNA
LTL	Longissimus thoracis et lumborum
MAS	Marker-assisted selection
MC4R	Melanocortin 4 receptor
Mef2	Myocyte enhancer factor 2
miRNA	microRNA
ML	Meishan x Large White
MSTN	Myostatin
MYOG	Myogenin
NICD	Notch intracellular domain
Nrf2	NF-E2-related factor 2
NUDT7	Nudix (nucleoside diphosphate linked moiety X)-type motif 7
p.m.	Post mortem
Pax3	Paired box 3
Pax7	Paired box 7
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
$pH1_L$	pH 45 min p.m. in loin
pHu	pH value measured 24 hours p.m.
Pi	Pietrain
Pig QTLdb	Pig QTL database
PKM2	Pyruvate kinase muscle isozyme
pQTL	Phenotypic quantitative trait loci
PRKAG3	Protein kinase, AMP-activated, gamma 3
PSE	Pale, soft, exudative
qRT-PCR	Quantitative real time PCR
QTLs	Quantitative trait loci
SCD	Stearoyl-CoA desaturase
SE	Standard error
SFN	Sulforaphane
SLC3A2	Solute carrier family 3, member 2
SM	Semimembranosus

SNPs	Single nucleotide polymorphisms
SOX-6	SRY (sex determining region Y)-box 6
SREBF1	Sterol regulatory element binding protein gene 1
TBP	TATA-binding protein
TBST	TBS containing 0.1% Tween-20
TGF-β	Transforming growth factor-beta
TSA	Trichostatin A
UBE3B	Ubiquitin protein ligase E3B
UBXN1	UBX domain protein 1
WHC	Water-holding capacity
ZDHHC5	Zinc finger, DHHC-type containing 5
ZRANB1	Zinc finger, ran-binding domain containing 1
ZYX	Zyxin

Chapter 1 General introduction

1.1 Porcine meat quality

Domesticated over 7,000 years ago, pigs are one of the most important farm animals now and pork takes about 40% of the red meat in the market. As a significant food source worldwide, nearly one billion pigs are kept for the pork production every year in the whole world. The main objective of pork industry is to supply enough amount of pork with acceptable meat quality. Thus the improvement of meat quality and muscle growth is one of main objectives in pig production. Beside these, pig also is taken as a very important research model because of the similar physiologies between pig and human.

Meat quality describes the appealingness of meat to consumers and can be traditionally classified into water holding capacity (WHC), pH, marbling, meat colour, tenderness and flavor. Meat quality is composed of nutritional quality and eating quality. Pork, a central part of daily food, has a great nutritional value and is enriched in protein, fat, vitamin, minerals, etc. However, the consumption of processed meat might be associated with the cancer risk (Linseisen *et al.*, 2006). The consumers' awareness of the relationship between food quality and health has significantly increased (Grunert, 2006). Meat quality has become a very important factor influencing the consumer purchasing decision.

On the other side, eating quality of pork is another significant aspect of meat quality. Eating quality is related with a lot of palatability factors, like firmness, tenderness, visual appearance, etc. Eating quality is affected by many factors, like breed, muscle type, fat and ultimate pH as well as environmental influences (Ngapo and Gariepy, 2008). Compared with Landrace and Large White, Duroc is normally used as the sire in pig breeding for its good performance in texture, juiciness and flavour (Candek-Potokar *et al.*, 1998; Jeremiah *et al.*, 1999; Wood *et al.*, 1996). Additionally, the Chinese purebreds maintain the higher intramuscular fat (IMF) than that of European and European × Chinese crossbred pigs (Suzuki *et al.*, 1991). However, the advantage of Chinese purebreds in the IMF fails to commit a increment of eating quality (Ellis *et al.*, 1995). For heritability (h^2) estimates, pork tenderness has moderate heritability (h^2 = 0.45), while the heritability estimates for pork juiciness (h^2 = 0.13) and flavour intensity (h^2 = 0.03) are quite low (Verbeke *et al.*, 1999). This indicates a big potential for genetic improvement in pork eating quality.

Up to now, the genetic basis of meat quality has been investigated with many methods, like quantitative trait loci (QTL) and the candidate gene approach. Additionally, the expression quantitative trait loci (eQTL) mapped by integrating the gene expression data with genotype data was also applied to meat quality studies. One study carried out in one Duroc × Pietrain (DuPi) resource population combined the eQTL data with the traditional phenotypic QTL (pQTL) data to identify candidate genes for traits of interest and tried to uncover the causative relationship from genetic variation to gene expression and further to phenotypic variation (Steibel *et al.*, 2011).

1.1.1 Water holding capacity and drip loss (DRIP)

The WHC describes the capacity of meat to keep the water under external forces. Nearly 75% of muscle weight is made up of water, most of which exists in the space between thick and thin filaments. DRIP is a red proteinaceous fluid consisting of water and a high concentration of proteins like myoglobin and glycolytic enzymes and is released from the cut surface of meat without external forces (except gravity) (Hamm, 1985). WHC and DRIP are moderately heritable and negatively correlated with each other.

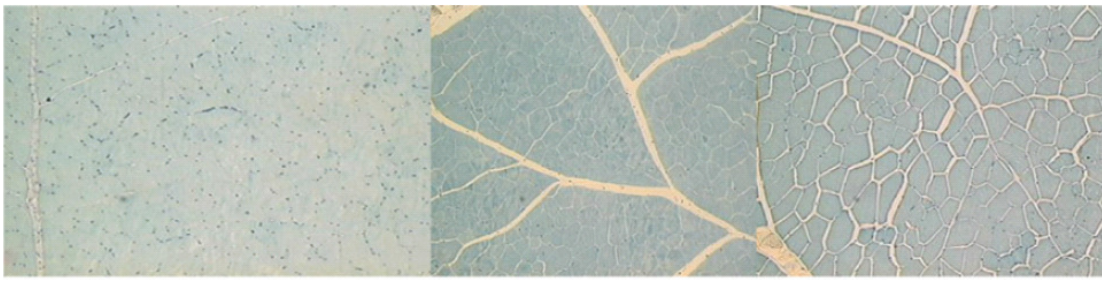
The formation of DRIP is closely related with the energy consumption after slaughter. Oxygen supply stops after slaughter and cells shift to the anaerobic metabolism and produce ATP by converting glycogen to lactate. The muscle pH decreases from 7.0 to around 5.4 as the accumulation of lactate. This pH condition denatures muscle proteins, mainly myosin and actin and leads to the shrinkage of myofibrils and to lose their ability to keep water in interfilament space (Offer and Cousins, 1992). The shrinkage of the interfilament space is one of the main force to drive muscle water into the extra-myofibrillar space (Offer and Trinick, 1983). The formation of DRIP also relies on the permeability of cell membrane, which is supported by the correlation between the DRIP and the integrity of cell membrane, shown in figure 1 (Hughes *et al.*, 2014). After expelled from the myofiber, water needs to reach the cut surface of meat to form DRIP, where DRIP channels play an essential role. As shown in figure 1, larger DRIP channels are found in the higher DRIP muscle.

The amount of DRIP has an important significance in the industrial interest, consumer appeal and eating quality. The WHC can be estimated by assessing the DRIP directly through the hanging bag method (Honikel, 1998) and Danish drip tube method

(Rasmussen, 1996) or indirectly by the filter press method or cooking loss (Honikel, 1986).

A: Low drip loss muscle

Time (hr)	0	6	9
Impedance (Py)	70	70	70



B: High drip loss muscle

Time (hr)	0	6	9
Impedance (Py)	70	20	10

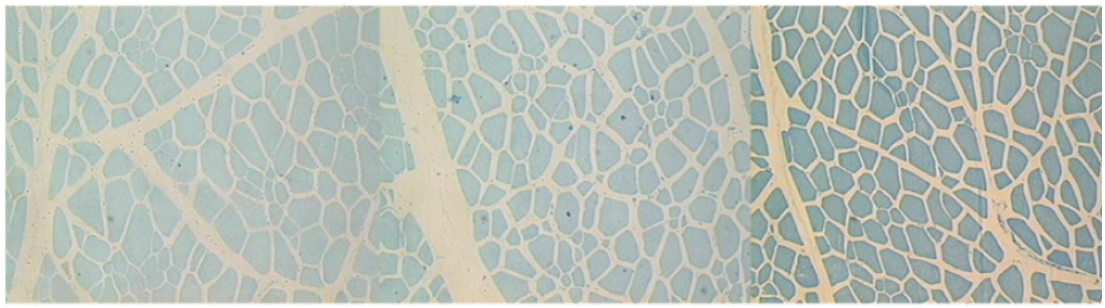


Figure 1: Light microscope images of transverse sections of porcine longissimus in (a) low drip loss muscle and (b) high drip loss muscle. Samples were taken at 0, 6 or 9 hours p.m.. Corresponding impedance values (as a measure of membrane integrity) are shown at each time point. Each panel represents a cross-section of approximately 1.2 mm × 1 mm, and are all at the same magnification. Annette Schäfer & Peter Purslow, unpublished results.

1.1.2 pH

The meat pH value is an important influencing factor and a good indicator for pork quality. After slaughter, the breakdown of glycogen in the muscle under an anaerobic situation produces lactic acid and then leads to the pH decline. A great amount of glycogen stored in muscles potentiates a rapid decline of pH, which is significantly associated with the insufficient WHC (Huff-Lonergan and Lonergan, 2005). The pH value influences many traits of meat quality, which includes tenderness, WHC and meat colour (Fernandez *et al.*, 1994; Fernandez and Tornberg, 1994; van Laack *et al.*, 2001). The pH value measured 24 hours p.m. (pHu) of porcine *longissimus dorsi* (LD) muscle is strongly associated with the juiciness and tenderness of cooked meat (Eikelenboom *et al.*, 1996). In another study, it was found that the meat pH value at 1 and 2 hours p.m. were related with both the WHC of raw meat and the tenderness of cooked meat (Purslow *et al.*, 2008). The pH at 48 h p.m. of the ham muscle is negatively associated with lightness (L*) values (surface lightness of the meat), DRIP and shear force (Purslow *et al.*, 2008).

The low pH caused by high carcass temperature leads to the extensive protein denaturation and produces meat with deficient WHC. The muscle pH at 45 min is associated with meat colour of the LD and the SM muscle and the high muscle pH value is related with low L* value (Duan *et al.*, 2013). A faster pH decrease is observed and the pHu is lower in the LD and SM muscle of the RN^- pig compared with the same muscle from the rn^+ pig (Lindahl *et al.*, 2006a).

1.1.3 Meat colour

Meat colour is an important trait affecting the purchase decision at the point of sale (Mancini and Hunt, 2005). Since consumers are not able to directly feel the texture or odor of the fresh meat in packages, meat colour in bright red is normally used by consumers as an indicator for good meat quality. In the live muscle, myoglobin is the oxygen carrier and is responsible for oxygen transportation for the whole body muscle tissue (Wittenberg and Wittenberg, 2003). Meat colour results from combinations of different proportion of myoglobin (purple), oxymyoglobin (light red) and metmyoglobin (brown-gray) in muscle tissue (Feldhusen *et al.*, 1995). In the meat cooling process, the myoglobin in muscle surface is transformed to oxymyoglobin or metmyoglobin, while a fast cooling method can decrease the oxygenation of the

myoglobin (Feldhusen *et al.*, 1995). In order to create a good first impression, meat colour should be uniform for the entire cut. At the same time, it should not be too light, like pale, soft, exudative (PSE) meat or too dark as dark, firm, dry (DFD) meat. The variation of meat colour can be partially attributed to the genetic basis. The meat of a RN^- pig is initially lighter and then redder and more yellow than that of a rn^+ pig. Finally, the meat of a RN^- pig is redder compared to the meat from a rn^+ pig (Lindahl *et al.*, 2006a). The L* measured at 20 hours p.m. for the m. adductor is a good indicator of the loin muscle quality (Warriss *et al.*, 2006). Compared with industrial genotype pigs, the LD muscles from Iberian pigs are much redder (Estevez *et al.*, 2003). Furthermore, meat colour is also influenced by dietary supplements, like creatine monohydrate, rapeseed oil (canola), $CuSO_4$ and vitamin E (Houben *et al.*, 1998; Jensen *et al.*, 1998; Lindahl *et al.*, 2006b).

1.1.4 Marbling and intramuscular fat

Marbling is another important factor influencing consumers' acceptability of pork. Marbling level is highly correlated with the IMF content (L. Faucitano, 2004), which refers to the total lipid within muscle tissue and can be divided into the extramyocellular lipids and the intramyocellular lipids. Marbling or the IMF content is one of the most important determinants of eating quality (Verbeke *et al.*, 1999) and accounts for the large part of variation in eating quality of pork (Fernandez *et al.*, 1999). According to a study in Spain, a good taste requires a minimum IMF content between 2.2% and 3.4% (Font-i-Furnols *et al.*, 2012). However, the relationship between marbling and consumer acceptance is not consistent and depends on the country, diet custom and even different consumers (Font-i-Furnols *et al.*, 2012).

1.2 QTL and genome wide association study (GWAS) for meat quality

Most of economical traits are genetically controlled by multiple genes or QTLs. The first paper on QTL identification for pig was published in 1994 and identified one QTL on chromosome 4 for the fatness (Andersson *et al.*, 1994). QTL studies identify chromosomal regions associated with interested quantitative traits. QTL studies supply useful molecular markers for the marker-assisted selection (MAS). Compared with the traditional selective pig breeding based on the direct phenotype value, the MAS is much easier, faster and less costly for the improvement of meat quality traits (Meuwissen and Goddard, 1996). The pig QTL database (Pig QTLdb) compiles identified QTL data

from published papers (Hu *et al.*, 2013). Up to now, Pig QTLdb has collected 13030 QTLs for 663 traits from 477 publications. The QTL study has been greatly enhanced by the arrival of GWAS, which uses a porcine SNP chip from Illumina and genotypes 62000 SNPs distributed across the whole pig genome (Ramos *et al.*, 2009).

1.2.1 QTL studies for the water holding capacity and drip loss

WHC and DRIP are important traits for meat quality and have a large economic impact. In pig QTLdb, 1049 QTLs have been discovered for DRIP, which is the trait associated with the most QTLs. Compared with DRIP, only 23 QTLs are identified for the WHC and they are located on SSC1, SSC2, SSC5, SSC6, SSC7, SSC8 and SSC13.

Normally, the identification of QTL uses a F2 population by crossing two breeds. Sanchez et al. genotyped 91 microsatellite markers distributed through the whole genome in a Duroc x Large White F2 population. They identified two suggestive QTLs on SSC11 and SSC14 for the WHC of *biceps femoris* and *gluteus superficialis* respectively (Sanchez *et al.*, 2011). In another similar work, 124 microsatellite markers were genotyped in a DuPi F2 population and one QTL in SSC9 was identified for DRIP (Edwards *et al.*, 2008). In a population crossed between Hampshire and Landrace, one QTL in SSC6 and two QTLs in SSC16 affected the DRIP in LD muscle (Markljung *et al.*, 2008). In a population of 3883 progenies based on the cross between Danish Duroc and Danish Landrace or Danish Large White, Li et al. genotyped 462 SNPs selected from porcine expressed sequence tags and identified five QTLs located in SSC2, SSC4, SSC6, SSC11 and SSC15 for DRIP (Li *et al.*, 2010). Koning et al. genotyped 132 microsatellite markers in a F2 population based on the cross between Chinese Meishan and commercial Dutch. In this study, four suggestive QTLs in SSC4, SSC6, SSC14 and SSC18 were identified for DRIP (de Koning *et al.*, 2001).

1.2.2 GWAS studies for the water holding capacity and drip loss

One GWAS work was conducted in Chinese Erhualian pigs and a Western Duroc × (Landrace × Yorkshire) (DLY) population. This study has identified four SNPs on SSC4 for DRIP of LM muscle and one SNP on SSC1 for DRIP of SM muscle in Erhualian pigs. For DLY population, two SNPs on SSC9 and SSC13 were found for DRIP of LD muscle (Liu *et al.*, 2015). In Chinese Laiwu pigs, one GWAS study was performed to investigate genetic polymorphisms and candidate genes for meat quality.

During this study, one QTL in SSC4 was found to be related with both the moisture content and DRIP of LD muscle (Xiong *et al.*, 2015). Another GWAS study was done in a White Duroc × Erhualian F2 population and Chinese Sutai pigs. This work found one SNP on SSC3 and three SNPs on SSC15 at the genome wide significant level for DRIP (Ma *et al.*, 2013). In our DuPi resource population (Liu *et al.*, 2007), 169 F2 pigs were genotyped with the 60K Illumina porcine SNP chip. Based on these genotyping results, GWAS was performed to identify candidate genes for DRIP. Based on GWAS results and gene function, the porcine SOX-6 was selected as a candidate gene for further work and was validated in a bigger pig population.

1.3 Candidate gene identification for meat quality traits

Most of meat quality traits are complex quantitative traits and are regulated by multiple genes. RYR1 and RN are the two generally accepted candidate genes for meat quality traits (Fujii *et al.*, 1991; Milan *et al.*, 2000a).

1.3.1 Water holding capacity and drip loss

The WHC and DRIP are correlated with the cooking loss, pH value 45 min p.m. and pHu value. A lot of works have been done to reveal the genetic basis for WHC and DRIP. The QTLs for WHC or DRIP are identified on chromosome 1, 2, 3, 4, 5, 6, 11, 13, 14, 15 and 18 (de Koning *et al.*, 2001; Malek *et al.*, 2001; Qu *et al.*, 2002; Su *et al.*, 2004; Thomsen *et al.*, 2004). RYR1 (Fujii *et al.*, 1991) and RN (Milan *et al.*, 2000b) are two famous genes influencing WHC. Additionally, phosphoglycerate mutase 2 was found to be greatly associated with DRIP (Fontanesi *et al.*, 2003). The 90 kDa heat shock protein (HSP90) is a chaperone protein playing critical role in protein stabilization. The expression level of HSP90 was positively associated with WHC and negatively associated with DRIP (Zhang *et al.*, 2014b). UBX domain protein 1 (UBXN1) is required for proteasome-mediated degradation of misfolded proteins. The polymorphisms of UBXN1 were associated with DRIP in the German Landrace and Pietrain × (German Large White × German Landrace) pigs and the expression of UBXN1 was negatively associated with WHC in the early period after slaughter (Loan *et al.*, 2014). Ubiquitin protein ligase E3B (UBE3B) promotes the protein degradation by transferring the ubiquitin to the targeted protein and the ubiquitin thioesterase zinc finger ran-binding domain containing 1 (ZRANB1) inhibits the protein degradation by specifically hydrolyzing 'Lys-29'-linked and 'Lys-33'-linked diubiquitin. The

polymorphism and expression of UBE3B and ZRANB1 were associated with WHC (Huynh *et al.*, 2013). Brunner et al. genotyped the SNPs located in or closed to six candidate genes, including blood vessel epicardial substance (BVES), solute carrier family 3 (amino acid transporter heavy chain) member 2 (SLC3A2), zinc finger DHHC-type containing 5 (ZDHHC5), citrate synthase (CS), coenzyme Q9 (COQ9) and epidermal growth factor receptor (EGFR) in around 1800 pigs (Brunner *et al.*, 2012). They found that BVES, SLC3A2 and CS were associated with DRIP and WHC based on the positional and genetic evidences (Brunner *et al.*, 2012). Xu et al. investigated the polymorphisms in the loci of cysteine and glycine-rich protein 3 (CSRP3) in a Berkshire x Yorkshire F2 population. This work found one substitution of C1924T in CSRP3 significant associated with WHC (Xu *et al.*, 2010). Pyruvate kinase muscle isozyme (PKM) was taken as a candidate gene for the glycolytic potential and pork quality. The TT genotype of PKM was associated with low pH and larger DRIP (Sieczkowska *et al.*, 2010).

1.3.2 pH

The meat pH value greatly influences the pork quality. A SNP in the promoter of protein kinase, AMP-activated, gamma 3 (PRKAG3) was significantly associated with the pHu in the Yorkshire and another SNP I199V in the coding region of PRKAG3 was associated with pH in the Landrace (Uimari and Sironen, 2014). Deoxyhypusine synthase and WD repeat domain 83 partly share 3 prime untranslated region (3'UTR). One SNP located in this shared 3'UTR was significantly associated with pHu in Italian Large White pigs (Zambonelli *et al.*, 2013).

1.3.3 Marbling and intramuscular fat

The IMF level is positively associated with sensory traits of pork (Fernandez *et al.*, 1999). The variation in promoter of insulin-like growth factor 2 was associated with IMF and its expression level was positively associated with a higher IMF content (Aslan *et al.*, 2012a). The SNP in the 5' regulatory region of the gene FABP4 was associated with the marbling and IMF content in the Chinese Songliao black swine (Gao *et al.*, 2011). Angiopoietin-like 4 (ANGPTL4) is expressed in liver and white adipose tissues, interferes with lipoprotein lipase activity and activates white adipose tissue lipolysis. The ANGPTL4 mRNA was more abundant in the backfat of Large White pigs than that in the backfat of Meishan pigs, while one SNP in its third intron had a significant effect

on the IMF content (Ren *et al.*, 2014). In one GWAS in a White Duroc × Erhualian F2 intercross with Chinese Sutai pigs, two QTLs on SSC9 and SSCX and 13 candidate genes were identified for IMF (Ma *et al.*, 2013). A QTL for the IMF content of *longissimus dorsi* was identified at 16 cM of the porcine SSCX and sulfatase isozyme S gene embedded within this QTL was suggested to be a functional and positional candidate gene for the IMF content (Fernandez *et al.*, 2014). In a Large White x Meishan F2 population, the polymorphisms of troponin I were associated with the IMF content (Yang *et al.*, 2010). Liu et al. identified 40 different expressed genes between pigs with divergent IMF (Liu *et al.*, 2009a).

1.3.4 Candidate genes for other porcine meat quality traits

Compared with the genome-wide scanning approach, it is much more effective and economical to identify candidate genes for interested traits (Zhu and Zhao, 2007). However, the traditional method for candidate genes identification largely depends on the existing information about gene function, which restricts its application. The porcine SNP chip and GWAS has greatly accelerated the identification of SNP markers and candidate genes for traits of interest (Fontanesi *et al.*, 2012; Ma *et al.*, 2013).

Calpastatin (CAST) is an endogenous inhibitor of the proteolytic enzyme, calpains, which is responsible for the degradation of structural proteins p.m. and plays an important role in the meat tenderization (Wendt *et al.*, 2004). The polymorphisms of CAST were found to be associated with meat colour, pH, WHC and texture parameters for the LD and SM muscle of pig breeds in Poland (Ropka-Molik *et al.*, 2014). Meat colour, external fatness and tenderness are the most important characters for consumers (Fortomaris *et al.*, 2006). CAST is suggested to be a genetic marker in breeding programs to improve the porcine meat quality (Ropka-Molik *et al.*, 2014). The polymorphisms of CAST had effects on its expression and the tenderness of LD muscle in Duroc-Landrace-Yorkshire pig population (Lindholm-Perry *et al.*, 2009).

Stearoyl-CoA desaturase (SCD) is an enzyme responsible for catalyzing a double bond in the saturated fatty acid to produce the monounsaturated fatty acid (Ntambi, 1999). Another enzyme, fatty acid desaturase 2 (FADS2), introduces a double bond into the linoleic acid (Ge *et al.*, 2003). Sterol regulatory element binding protein 1 (SREBF1) is a transcription factor and regulates the gene expression required for fatty acid and lipid metabolism, including SCD and FADS2 (Renaville *et al.*, 2013). The polymorphisms of

SCD, FADS2 and SREBF1 were associated with the fatty acid composition and dietary quality of the heavy pig breeds meat in Italy (Renaville *et al.*, 2013).

Long-chain acyl-CoA synthetase (ACSL) synthesizes acyl-CoA by activating fatty acids with chain lengths of 12 to 20 carbon atoms. ACSL is an important enzyme in lipid synthesis and fatty acid catabolism. In an Iberian x Landrace cross population, one SNP in the 3'UTR of ACSL4 was related with the percentage of oleic and monounsaturated fatty acids (Mercade *et al.*, 2006). Furthermore, this SNP had effect on the expression of ACSL4 in liver (Corominas *et al.*, 2012).

Ankyrin 1 (ANK1) is an adaptor protein and plays an important role in sustaining the integrity of plasma membrane (Ackermann *et al.*, 2011). The expression level of ANK1 was associated with DRIP in the Large White breed. Two SNPs in the promoter of ANK1 were connected with IMF of both *longissimus thoracis et lumborum* (LTL) muscle and SM muscle (Aslan *et al.*, 2012b). A haplotype of polymorphisms in the ANK1 promoter was associated with DRIP in the Pietrain (Pi) breed and with IMF in the Large White breed (Aslan *et al.*, 2012b).

Interferon-α inducible protein 6 (IFI6) is a mitochondria-targeted protein and inhibits the apoptotic process (Cheriyath *et al.*, 2007). The polymorphism of IFI6 was associated with meat colour, pH, conductivity, DRIP and carcass length in the DuPi population and with meat colour and muscle area in the Pietrain population (Kayan *et al.*, 2011).

Cysteine and glycine-rich protein 3 (CSRP3) is a positive regulator of muscle differentiation (Kong *et al.*, 1997). One synonymous SNP of porcine CSRP3 was associated with the firmness, loin pH and WHC in a Berkshire x Yorkshire F2 population (Xu *et al.*, 2010).

The nudix (nucleoside diphosphate linked moiety X)-type motif 7 (NUDT7) is a positional candidate gene for meat colour. More than one Hundred SNPs are discovered in the coding region and 5' regulatory region of NUDT7. Compared with Japanese wild-boar, the expression of NUDT7 was higher in Large White (Taniguchi *et al.*, 2010a). Over-expression of NUDT7 led to the suppressed heme biosynthesis in rat L6 myoblasts (Taniguchi *et al.*, 2010b). Thus, NUDT7 is a positional and functional candidate gene for meat colour.

Zyxin (ZYX) is also a positional and functional candidate gene for meat quality. ZYX plays an important role in the signal transduction pathway and cytoskeletal organization of actin bundles. The polymorphism of ZYX was associated with meat colour and pH in Pietrain pigs and was related with DRIP in the Pietrain x (German Large White x German Landrace) F1 population (Srikanchai *et al.*, 2010).

Cortisol-binding globulin (Cbg) regulates the bioavailability and metabolic clearance of glucocorticoids, which are protected from absorption and degradation by the binding of Cbg. A substitution SNP of Cbg was associated with the DRIP percentage in gilts of Meishan x Large White (ML) intercross population (Guyonnet-Duperat *et al.*, 2006). The maximum binding capacity of Cbg was associated with loin yields in the Large White and Landrace gilts, with ham yields in Duroc gilts, with the fat depth and leaf fat in ML gilts, with the meat colour in Land-race gilts and Large White gilts, with pHu of the lumbar multifidus muscle and SM muscle in Large White gilts and with DRIP and IMF in the SM muscle of ML pigs (Geverink *et al.*, 2006).

Melanocortin 4 receptor (MC4R) is a G-protein coupled receptor and is expressed in the appetite-regulating brain areas. In murine models, MC4R has been found to be involved in the regulation of feeding behavior, metabolism and sexual behavior (Fan *et al.*, 1997; Huszar *et al.*, 1997; Van der Ploeg *et al.*, 2002). MC4R was associated with the fatness, high daily gain and feed intake in pigs (Davoli *et al.*, 2012; Fan *et al.*, 2009; Houston *et al.*, 2004; Kim *et al.*, 2000).

Cathepsin B (CTSB), a lysosomal cysteine proteinase, is an important enzyme in the post mortem tenderization. Russo et al. discovered four alleles in porcine CTSB locus and found CTSB was significantly associated with the backfat thickness (Russo *et al.*, 2002).

However, the relationship between the polymorphisms of candidate gene is normally restricted in the studied population, which means the effects of SNP on the phenotype variation largely depend on the genetic background (Srikanchai *et al.*, 2010).

1.3.5 SOX-6, a candidate gene for meat quality

SRY (sex determining region Y)-box 6 (SOX-6) is a multiple-faced transcription factor in the vertebrate development. SOX-6 is a member of SoxD family, which also contains

Sox5 and Sox13. Distinguishing from other Sox proteins, SoxD members have a leucine zipper and glutamine-rich domains close to the N-terminus (Kamachi *et al.*, 2000). Besides these two domains, SOX-6 contains one DNA-binding domain (Lefebvre *et al.*, 1998). SOX-6 does not contain any activator or repressor transcriptional regulatory domain. Thus, SOX-6 has to corporate with different transcription factors to regulate gene activity (Kamachi *et al.*, 2000). The leucine zipper and glutamine-rich domains located in the N-terminal part of SOX-6 is responsible for protein-protein interaction (Cohen-Barak *et al.*, 2003; Iguchi *et al.*, 2007; Lefebvre *et al.*, 1998). SOX-6 is expressed in brain, heart, lung, liver, spleen, pancreas, skeletal muscle, kidney and testis (Cohen-Barak *et al.*, 2001; Hagiwara *et al.*, 2000).

In the skeletal muscle development, SOX-6 regulates the muscle fiber differentiation (Hagiwara *et al.*, 2005; Hagiwara *et al.*, 2007; von Hofsten *et al.*, 2008). SOX-6 suppresses slow fiber gene activity and the loss of SOX-6 during mouse embryo development led to the up-regulation of slow fiber genes (Hagiwara *et al.*, 2007; von Hofsten *et al.*, 2008). The expression of SOX-6 is normally inhibited by the Hedgehog signalling, which permits the formation of slow muscle fiber (Liew *et al.*, 2008; von Hofsten *et al.*, 2008). On the post-transcriptional level, the translation of SOX-6 mRNA is suppressed by two muscle specific microRNAs (miRNAs), miR-499 and miR-208b (Bell *et al.*, 2010; Rossi *et al.*, 2010; van Rooij *et al.*, 2009). On the other side, the overexpression of SOX-6 decreases the expression of miR-499 (van Rooij *et al.*, 2009). Thus, SOX-6 can inhibit the expression of its own suppressor and its expression is modulated by a feedback loop.

Based on our previous GWAS study, four SNPs located in porcine SOX-6 loci are significant associated with DRIP in the DuPi F2 population. Combined with its important role in muscle fiber differentiation, SOX-6 is selected as a potential candidate gene for porcine meat quality and is validated in the chapter 2.

1.4 Muscle stem cell and muscle growth

1.4.1 Muscle stem cells

The muscle stem cells, also called satellite cells, are a group of mononucleated cells located between the plasma membrane and the basal lamina of muscle fibers (Mauro, 1961). The name, satellite cells, describes their cellular location, surrounding mature

myofibers. Satellite cells express a group of specific genes, including paired domain transcription factors Paired box 7 (Pax7) (Seale *et al.*, 2000), Paired box 3 (Pax3) (Buckingham *et al.*, 2003) and myogenic factor 5 (Myf5) (Cornelison and Wold, 1997). A study in chick and quail demonstrates satellite cells might originate from embryonic somites (Armand *et al.*, 1983). However, non-satellite cells or mature myofibers can also transform or dedifferentiate into satellite cells and contribute to the muscle regeneration (Brockes and Kumar, 2002).

Satellite cells are a group of heterogeneous cells and are composed of major fast-cycling cells and minor slow-cycling reserve cells, where the latter cells are proven to be true stem cells (Conboy *et al.*, 2007; Schultz, 1996). The heterogeneity is not only found in the satellite cells from the same muscle tissue. Satellite cells derived from different type of muscle tissue also show the different potential in proliferation and differentiation (Collins *et al.*, 2005).

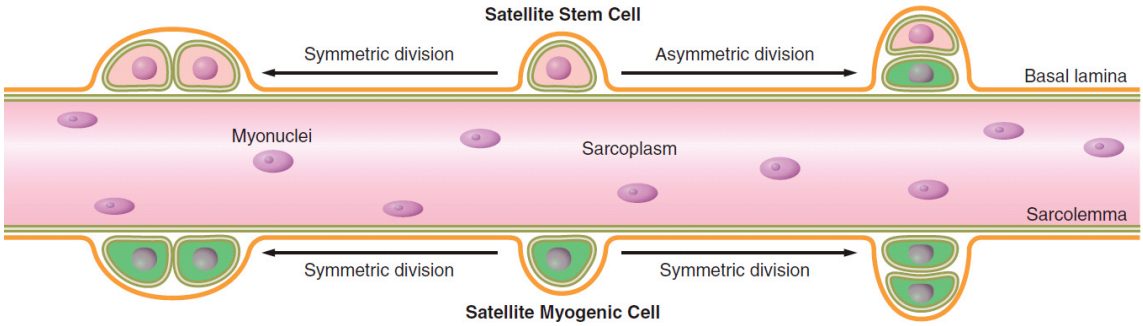


Figure 2: Characteristics of the satellite cell

As adult stem cells, satellite cells have the self-renewal ability to replenish themselves through the asymmetric and symmetric division. One parental stem cell produces one daughter stem cell and one daughter cell destined for myogenic differentiation. During the symmetric cell division, one parental stem cell gives birth to two same daughter stem cells (figure 2). Satellite cells can renew itself in both asymmetric and symmetric divisions (Kuang *et al.*, 2007). For example, $Pax7^{+}/Myf5^{-}$ satellite cell can asymmetrically produce one $Pax7^{+}/Myf5^{-}$ satellite stem cell and one $Pax7^{+}/Myf5^{+}$ satellite myogenic cell. However, both $Pax7^{+}/Myf5^{-}$ and $Pax7^{+}/Myf5^{+}$ satellite cells can undergo the symmetric division. The factor Wnt7a in the satellite cell niche can boost the symmetric division (Le Grand *et al.*, 2009). Transplant experiments has proven that only the $Pax7^{+}/Myf5^{-}$ cells are real muscle stem cells and are able to reconstruct the stem cells pool (Kuang *et al.*, 2007).

Along the myofiber, the density of satellite cells is not homogenous. More satellite cells are found at the ends of the myofiber or perisynaptic regions (Gibson and Schultz, 1982; Kelly, 1978). Nearly 90% of human satellite cells are closely located to capillaries (Christov *et al.*, 2007). The division way of satellite cell is determined by their relative location in a myofiber. Not all satellite cells can undergo asymmetric divisions. One possible mechanism of asymmetric division could be due to the asymmetrical distribution of certain factors during mitosis, like the cell fate determinant Numb (Conboy and Rando, 2002). Furthermore, it is also found that the real muscle stem cell tend to reserve the template chromosome during the asymmetric division.

1.4.2 The role of satellite cells in muscle growth and regeneration

1.4.2.1 The role of satellite cells in muscle growth

Satellite cells can be activated by injury or other external signals and give rise to myoblasts, which are incorporated into growing (Cardasis and Cooper, 1975; Moss and Leblond, 1971) and injured muscle fibers (Montarras *et al.*, 2005), or fuse with each other to form new myofibers.

In a new born mouse, more than 30% of the nuclei in a myofiber comes from the satellite cell population and this proportion is going to gradually decrease while the muscle mass increases. During the postnatal muscle growth, satellite cells contribute new myonuclei to the enlarged muscle fibers. The average duration of cell cycle for a satellite cell is around 32 hours (Schultz, 1996). The satellite cell population consists of two subpopulations: the fast-cycling satellite cells and the slow-cycling satellite cells. The fast-cycling satellite cells account for 80% of the satellite cell population and these satellite cells undergo limited cell divisions before fusing with the growing myofibers. The slow-cycling satellite cells are able to generate new fast-cycling satellite cells in the asymmetric division way (Schultz, 1996).

Satellite cells in juveniles are not the exactly same as those in adults. This difference can be revealed by the distinct function of Pax7 in these two stages. The mutation of Pax7 leads to a impairment in the growth and regeneration ability of juvenile muscle (Oustanina *et al.*, 2004). However, the similar phenotype is not observed in the adult mouse, when Pax7 is conditionally knocked out (Lepper *et al.*, 2009). Thus, based on

aforementioned, Pax7 might be only required for the establishment of satellite cell pool at the beginning stage of postnatal growth.

1.4.2.2 The role of satellite cells in muscle regeneration

Muscle regeneration is a very important process for normal exercise or pathological muscle necrosis. Satellite cells paly a vital role in muscle regeneration. A muscle without satellite cells completely loses the ability of regeneration (Lepper *et al.*, 2011). Muscle regeneration consists of three steps: 1) the inflammatory response; 2) the activation, differentiation and fusion of satellite cells; and 3) the maturation and remodeling of newly formed myofibers.

For the first stage of muscle regeneration, macrophages with the surface marker $CD68^{+}/CD163^{-}$ reach the inflammation area and release proinflammatory cytokines, like tumor necrosis factor-α and interleukin-1 (IL1). The $CD68^{+}/CD163^{-}$ macrophages reaches the highest level at 24 hours after the myofiber necrosis. Another type macrophages with the surface marker $CD68^{-}/CD163^{+}$ secrete IL10 and regulate the proliferation and differentiation of satellite cells (Cantini *et al.*, 2002; Merly *et al.*, 1999). The second stage is characterized by satellite cells proliferation, which produces enough myonuclei for muscle regeneration (Snow, 1977). The new produced myoblasts fuse with damaged myofibers or with other myoblasts to form new myofibers. The cell proliferation and cell fusion during muscle regeneration normally happen in a focal manner. Finally, the repaired myofibers return to a normal condition.

In the normal condition, the quiescent satellite cells are $Pax7^{+}/MyoD^{-}$ (Cornelison and Wold, 1997). The activated satellite cells are $Pax7^{+}/MyoD^{+}$ and some of these $Pax7^{+}/MyoD^{+}$ myoblasts can return to $Pax^{+}/MyoD^{-}$ cells in a quiescent state (Day *et al.*, 2007; Halevy *et al.*, 2004). Thus the balance between Pax7 and MyoD determines the satellite cell fate (Olguin *et al.*, 2007). The regional damage signal activates satellite cells in the whole myofiber (Schultz *et al.*, 1985). Cell mobility is another important part of muscle regeneration. The activated satellite cells can move around between myofibers across basal lamina and even in muscles (Hughes and Blau, 1990; Watt *et al.*, 1987).

After activation, the satellite cells start proliferation and produce new myoblasts. The activation of satellite cells is regulated by many factors. MyoD or Myf5 is expressed in

satellite cells 24 hours after activation and both of them are co-expressed in the same satellite cell by 48 hours (Cooper *et al.*, 1999; Cornelison and Wold, 1997). MyoD and Myf5 have different roles in the regulation of satellite cells proliferation and differentiation. MyoD mainly drives the differentiation process (Montarras *et al.*, 2000) and Myf5 primarily promotes the proliferation of satellite cells (Sabourin *et al.*, 1999). The co-expression of MyoD and Myf5 keeps the balance between differentiation and proliferation during muscle growth and regeneration. Thus, MyoD and Myf5 cooperatively regulate the muscle development.

After the proliferation period, the fresh myoblasts enter the differentiation stage by fusing with other myoblasts to form new myofibers or fusing with existing myofibers. The expression of myogenin (MYOG) and Myf6 indicates the initiation of differentiation stage (Smith *et al.*, 1994; Yablonka-Reuveni and Rivera, 1994).

MYOG is a myogenic regulatory factor and holds a critical importance in muscle development (Hasty *et al.*, 1993). MYOG is associated with both muscle growth traits like total fiber number, muscle fiber cross-sectional area and loin eye area and is also related with the meat quality traits such as meat colour and firmness (Kim *et al.*, 2009).

The expression of MYOG is activated by MyoD and strengths the myogenic effect of MyoD to drive the muscle regeneration (Cao *et al.*, 2006). Myocyte enhancer factor 2 (MEF2) is another downstream target gene of MyoD and drives the myoblast differentiation program. E proteins heterodimerize with MyoD and aid its binding on the E-response element, which is located in the upstream of most MyoD downstream genes (Lassar *et al.*, 1991; Murre *et al.*, 1989). Another target gene of MyoD is the cyclin dependent kinase (CDK) inhibitor $p21^{(Waf1/Cip1)}$. p21 induces the cell cycle arrest before S-phase (Weinberg, 1995). By the end of differentiation, myoblasts fuse with each other to form new myofiber. The new myofiber will incorporate more myonuclei and become a mature myofiber.

1.4.3 The regulatory signalling pathways in muscle stem cell biology

Myogenesis is regulated by multiple signalling pathways. The Wnt signalling regulates the satellite cell proliferation and differentiation. The β-catenin is the effector of Wnt signalling pathway. The binding of Wnt to its receptor pair activates β-catenin. The activated β-catenin transfers into nucleus and cooperates with Tcf/Lef to manipulate the

activity of downstream genes. The basal degradation speed of *β*-catenin is determined by glycogen synthase kinase 3 beta (GSK3B). Firstly, *β*-catenin is phosphorylated by GSK3B. Then phosphorylated *β*-catenin is recognized by ubiquitin ligase complex and is submitted to proteasome for degradation. The Wnt signalling is able to activate Dishevelled, an inhibitor of GSK3B. The inactivation of GSK3B frees *β*-catenin to mediate the Wnt signalling (Nelson and Nusse, 2004). The *β*-catenin can increase the affinity of MyoD to its binding sites and drives the myogenic cell differentiation (Kim *et al.*, 2008). Wnt signalling increases the satellite cell proliferation and is in favour of muscle regeneration. It is found that Wnt1, Wnt3a and Wnt5a enhance the satellite cell proliferation, while Wnt4 and Wnt6 show the opposite effect. The Wnt7a administration leads to the elevated muscle mass and enlarged myofiber size and significantly enhances the muscle regeneration.

Notch signalling regulates the skeletal muscle regeneration (Luo *et al.*, 2005). Delta and Jagged family members bind to Notch transmembrane receptors and release the Notch intracellular domain (NICD), the active form of Notch, into the cytoplasm. NICD translocates into the nucleus and regulates the expression of target genes. The activation and proliferation of satellite cells are enhanced by Notch signalling (Conboy and Rando, 2002). Numb, an inhibitor of Notch signalling, leads to the ubiquitination of the NICD and discourages the activation of satellite cells. On the other side, Numb promotes satellite cell fusion by downregulating the Notch signalling (Conboy and Rando, 2002). At the end of myogenesis, Notch signalling is also inhibited by the Wnt signalling, which empowers the beginning of myoblast differentiation (Brack *et al.*, 2008).

The transforming growth factor-beta (TGF-β) signalling plays an important role in myogenesis. The mature TGF-β1 is released from the latency-associated peptide and binds to type I and II receptors, which leads to the phosphorylation and activation of type I receptor by type II receptor. The activated type I receptor sequentially phosphorylates the Smad2/3 (Rahimi and Leof, 2007). The phosphorylated Smad2/3 binds to Smad4 and switches into the nucleus and regulates downstream genes (Guo and Wang, 2009). Smad7, another member of Smad gene family, is induced by the TGF-β signalling and also regulates the activity of TGF-β signalling by impeding the phosphorylation of Smad2/3 in a negatively feedback way (Rahimi and Leof, 2007).

TGF-β1 hinders the activation of satellite cell and inhibits myoblast proliferation and fusion (Allen and Boxhorn, 1987). TGF-β1 regulates the muscle fiber type differentiation. TGF-β1 promotes the development of fast switch muscle fiber and its absence empowers the formation of slow muscle fiber (McLennan, 1993). TGF-β1 impedes the differentiation of fetal myoblast but has no effects on the embryonic myoblast development (Cusella-De Angelis *et al.*, 1994). TGF-β1 harms the skeletal muscle regeneration (Allen and Boxhorn, 1987). Myostatin (MSTN), one member of TGF-β super family, maintains a high level in skeletal muscle (Moustakas and Heldin, 2005). MSTN has been shown to harm the activation, differentiation, and self-renewal of satellite cells (Carnac *et al.*, 2007; McCroskery *et al.*, 2003; McCroskery *et al.*, 2005).

1.4.4 Epigenetic regulation of muscle growth and regeneration

Epigenetics studies the heritable variation of gene expression that is not the result of the mutation of the DNA sequence. Epigenetic mechanisms include DNA methylation, noncoding RNA, histone modification and chromatin remodelling.

1.4.4.1 The role of histone modification in myogenesis

Histone acetylation is related with the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Many transcription activators, like p300, have an intrinsic HAT activity, while HDAC is generally associated with transcriptional suppression. The activity of MyoD and MEF2 is suppressed by their interaction with class I HDACs and class IIa HDACs respectively, which stops the myoblast from the terminal differentiation (Lu *et al.*, 2000b; Puri *et al.*, 2001).

1.4.4.2 The role of DNA methylation in myogenesis

DNA methylation is one mechanism in regulating gene activity. The methylation modification of the cytosine in a CpG site is catalyzed by DNA methyltransferase (DNMT), including DNMT1, DNMT3A and DMNT3B. DNMT1 is responsible for the maintenance of genome methylation pattern, while DNMT3a and DNMT3b are the enzymes for *de novo* methylation. The DNA methylation inhibitor, 5-azacytidine, induces the myogenic differentiation of fibroblast (Lassar *et al.*, 1989; Taylor and Jones, 1979). The treatment of 5-azacytidine increased the expression of myogenic transcription factors (Montesano *et al.*, 2013). The inhibition of DNA methylation on

the transcription of MYOG exists until the onset of myogenic differentiation (Palacios *et al.*, 2010).

1.4.4.3 The role of noncoding RNAs in myogenesis

Based on the transcriptome studies, only 2% of the genome sequence is transcribed to the protein coding RNA (Djebali *et al.*, 2012; Mortazavi *et al.*, 2008; Shiraki *et al.*, 2003). The rest RNAs produced from genome are called noncoding RNAs. The miRNAs are a group of around 22-nucleartides small noncoding RNAs and post-transcriptionally regulate the stability of target mRNAs by binding to their 3'UTR (Ha and Kim, 2014). Another group of noncoding RNAs is called long noncoding RNA (lncRNA), which is normally > 200 nucleotides and is much longer than miRNA. The expression of lncRNA is tightly controlled both spatially and temporally. The lncRNA can be found in both the nucleus and the cytoplasm (Batista and Chang, 2013; van Heesch *et al.*, 2014). In the nucleus, lncRNA interacts with many chromatin remodelling complexes and guides these complexes to target chromatin region via RNA : DNA complementarity (Chu *et al.*, 2011). The lncRNA in the cytoplasm regulates the stability of transcripts (Beltran *et al.*, 2008; Carrieri *et al.*, 2012; Gong and Maquat, 2011; Kretz *et al.*, 2013; Poliseno *et al.*, 2010; Yoon *et al.*, 2012).

Dicer is a key enzyme in miRNA biogenesis and cleaves the precursor miRNA into the mature miRNA. The knockout of Dicer in satellite cell leads to its exit from quiescence and the beginning of proliferation (Cheung *et al.*, 2012). It is shown that miR-195 and miR-497 target Cdc25a, Cdc25b and Ccnd2 to keep satellite cell in a quiescent state (Sato *et al.*, 2014). In addition, lncRNA Uc.283+A can inhibit the expression of miR-195 through interfering its biogenesis to activate satellite cells (Liz *et al.*, 2014). The mRNA of Myf5 is detected in the quiescent satellite cell and is subject to the degradation induced by miR-31 (Crist *et al.*, 2012). The activation of satellite cell releases the post-transcriptional inhibition from miR-31 on Myf5. The expression of Myf5 and MyoD in turn induces the transcription of miR-133a/b (Chen *et al.*, 2006). miR-133a/b keeps the satellite cell from the terminal differentiation (Chen *et al.*, 2006; Huang *et al.*, 2011).

1.4.5 The effects of HDAC inhibitors on myogenesis

HDAC is a very important regulator in myogenesis. The HDAC inhibitors have significant effects on muscle growth and regeneration. Trichostatin A (TSA) and sulforaphane (SFN) are two inhibitors of HDAC activity and have been investigated in many works. In mice, TSA could relief the unloading-induced soleus muscle atrophy (Dupre-Aucouturier *et al.*, 2015). In C2C12, TSA treatment upregulated the expression of Myf5 and MEF2, but inhibited the expression of MYOG (Hagiwara *et al.*, 2011). In addition, TSA treatment upregulated the expression of follistatin, enhanced the myoblast fusion and led to the bigger muscle cell size in C2C12 (Iezzi *et al.*, 2004). However, TSA was also found to inhibit the proliferation of smooth muscle cell by inducing p21 (Okamoto *et al.*, 2006). SFN, another potent HDAC inhibitor, significantly suppressed the expression of MSTN in porcine satellite cells (Fan *et al.*, 2012). In contrast, MSTN was activated by TSA in differentiated C2C12 (Han *et al.*, 2010). One specific class II HDAC inhibitor, MC1568, retarded myogenesis by interfering the activity of MEF2 (Nebbioso *et al.*, 2009).

SFN is a compound found in cruciferous vegetables. SFN is a potent activator of Nrf2 signalling (Fahey and Talalay, 1999) and inhibits the HDAC activity (Myzak *et al.*, 2004). Our previous work has found positive effects of SFN on the activity of porcine satellite cells (Fan *et al.*, 2012). The detailed mechanisms are investigated in the work described in the chapter 3.

1.5 The relationship between meat quality and muscle growth

Muscle growth and meat quality are two important issues in the pig production. On one side, maximal meat yield or high growth rates is one decisive economical factor. On the other side, consumers always prefer the pork products with higher meat quality. Thus, the balance between meat quality and muscle growth probably will generate a new concept in pig breeding to supply the market with enough meat with high meat quality.

Muscle growth consists of two parts: the prenatal muscle growth and the postnatal muscle growth. The prenatal muscle growth is to increase in cell number, termed by hyperplasia and the postnatal muscle growth is to increase in cell size, called hypertrophy. After the prenatal muscle development, piglets are born with a certain number of myofibers, which is constant for the whole life (Koohmaraie *et al.*, 2002).

The postnatal muscle growth is characterized by the increment of myonuclei, which is achieved by the intensive amplification of satellite cells (Moss and Leblond, 1971).

The rate of muscle growth significantly depends on the protein turnover and the myofiber type. Furthermore, muscle growth is also closely related to many meat traits, like IMF, tenderness, muscle fiber size, muscle colour, etc. It was reported that pigs with higher growth rate tended to have a heavier final body weight, carcass weight, dressing percentage, backfat thickness and a higher concentration of fat in muscle (Yang *et al.*, 2012). Another study stated that the higher growth rate in pigs was associated with a higher calpain activity and a higher tenderization rate (Kristensen *et al.*, 2002). In poultry, the increased breast muscle was associated with the bigger myofiber size and showed paler meat colour, higher pHu value and ameliorated WHC (Remignon *et al.*, 1995).

In summary, muscle growth and muscle quality are closely related with each other and are important topics in pig breeding. The present work is trying to supply some molecular markers and basic strategies to improve the muscle growth and meat quality, as stated in the chapter 2 and 3.

1.6 Scope of the thesis

Meat quality and muscle growth are two valuable and determining objectives in pig production. The pig breeding strategies always try to balance the demands of the higher pork production and the better meat quality. The present work tries to supply some suggestive evidences for the pig breeding development in the future from the genetic and epigenetic aspects:

Experiment I:

Polymorphisms and expression analysis of SOX-6 in relation to porcine growth, carcass and meat quality traits

Experiment II:

Sulforaphane enhances proliferation of porcine satellite cells through suppression of TGF-β signalling pathway

1.7 Experiment I:

Polymorphisms and expression analysis of SOX-6 in relation to porcine growth, carcass and meat quality traits

This study is to supply a useful candidate gene and applicable molecular maker for marker-assisted selection in pig breeding. The candidate gene, SOX-6, was selected based on the data in house. SOX-6 was proven to be a valuable candidate gene on multiple levels. The methodologies and results of this study are briefly described here.

1.7.1 Animals

This study was conducted in a commercial Pietrain and an experimental DuPi population. The information about the Pietrain and DuPi population was stated in details by Srikanchai *et al.* (2010) and Liu *et al.* (2007), respectively. The DuPi pigs were kept at the experimental research farm "Frankenforst", Institute of Animal Science, University of Bonn (Germany).

1.7.2 Genomic DNA extraction and polymorphisms genotyping by PCR-RFLP

Genomic DNA (gDNA) was extracted from muscle of the Pietrain and DuPi pigs using the phenol–chloroform method. Finally, a working solution of 50 ng/µl gDNA was prepared for all samples and stored at 4 °C until used. PCR was performed to amplify the interested fragment. The PCR product was digested with the respective restriction enzyme. The digestion products were separated by electrophoresis in the agarose gel or the polyacrylamide gel, visualized with ethidium bromide under ultraviolet light and analyzed with Bio-Rad Quantity One software.

1.7.3 Association study

The association studies between studied SNPs with interested traits in the Pietrain and DuPi population were performed by fitting generalized linear models using R software:

$$Grotrait_{ijkl} = \mu + Genotype_i + BS_j + fam_k + \beta_{Age}(Age_{ijkl} - \overline{Age}) + e_{ijkl}$$

$$Cartrait_{ijkl} \text{ or } Meatrait_{ijkl} = \mu + Genotype_i + YS_j + fam_k + \beta_{SW}(SW_{ijkl} - \overline{SW}) + e_{ijkl}$$

where Grotrait, Cartrait and Meatrait are the observation of traits; μ is the population mean; Genotypei is the effect of i-th genotype (i = 1, 2 and 3); BSj and YSj are the effect of j-th season of birth and slaughter (j = 1 to 8 or 9); famk is the random effect of k-th family in DuPi population (k = 1 to 22); βAge(Ageijkl - $\overline{Age}$) and βSW(SWijkl - $\overline{SW}$) are the linear effect of age and slaughter weight as covariate; and eijkl is the random residual error. Because of skewness, DRIP values were log-transformed. The least-square mean for each genotype was estimated using R software and pairwise comparisons were performed among different genotypes, where *P* value was adjusted by using the Tukey's method.

1.7.4 Results

1. The first SNP, rs81358375, in the porcine SOX-6 was associated with $pH1_L$, the thickness of backfat and side fat and carcass length in the Pietrain population, and was related with the backfat thickness and daily gain in the DuPi population.

2. The second SNP, rs321666676, was associated with meat colour in the Pietrain population.

3. In the DuPi population, the protein, not mRNA, level of SOX-6 in high $pH1_L$ pigs was significantly less abundant than that of low $pH1_L$ pigs.

4. MiRNAs targeting SOX-6 were also differently regulated in pigs with divergent $pH1_L$ value.

1.8 Experiment II:

Sulforaphane enhances the proliferation of porcine satellite cells through suppression of TGF-β signalling pathway

1.8.1 Porcine satellite cells isolation and cell culture

Porcine satellite cells were isolated form SM muscles of 21 days purebred Pietrain piglets. All piglets were kept and slaughtered according to German performance test directions. The muscle samples were quickly obtained, treated with 70% ethanol, rinsed in the cold phosphate-buffered saline, minced and digested with 0.25% trypsin for 1 - 2 hours at 37°C with a continuous shaking. The trypsin digestion was stopped by the addition of 20% fetal bovine serum (FBS). The cell suspension was then filtered through 2 × 70 μm and 1 × 40 μm cell strainer. Satellite cells were enriched by using a Percoll gradient (25%, 40% and 90%) centrifugation. Then the enriched satellite cells were collected and diluted with minimum essential medium α supplemented with 4 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone and 10% FBS.

1.8.2 Cell proliferation assay

Cell proliferation was measured with the WST-1 cell proliferation assay kit. After treated with SFN for 48 hours, 10 μl of the reconstituted WST-1 mixture was added to each well and cells were cultured for additional 2 hours at 37 °C in a CO_2 incubator. After gently shaking, the absorbance of the supernatant from each well was measured using a microplate reader at a wavelength of 450 nm.

1.8.3 In vitro HDAC activity assay

HDAC activity of porcine satellite cells was determined with the Color-de-Lys® HDAC colorimetric activity assay kit (Enzo Life Science, Farmingdale, NY). The same amount of protein from porcine satellite cells was diluted into 25 μl assay buffer, warmed to 37 °C before mixed with 25 μl pre-warmed Color-de-Lys ™ substrate and incubated for 1 hour at 37 °C. Then the reaction was stopped by adding 50 μl of Color-de-Lys™ developer containing 2 μM TSA and incubated for 15 min at 37 °C. Signal was read with a microtiter-plate reader at 405 nm and the data was normalized to No Enzyme Control.

1.8.4 Bisulfite sequencing

The 5´-regulatory region of Smad7 (Gene ID: 100521305) was submitted to the online program Methprimer (Li and Dahiya, 2002) for CpG islands identification and bisulfite sequencing PCR primers design. Genomic DNA (1 μg) was subjected to bisulfite modification using EZ DNA Methylation-Direct Kit. The Smad7 5´-regulatory region containing CpG island was amplified by PCR. PCR products were cloned into the pGEM T-easy vector. A minimum of six different positive clones were randomly selected for sequencing with M13 primers with the CEQ8000 sequencer system. The final sequence results were processed by the BiQ Analyzer software.

1.8.5 Results

1. SFN enhanced the proliferation of the porcine satellite cells and modified the expression of myogenic regulatory factors.

2. SFN altered the mRNA expression of HDAC members and inhibited the HDAC activity.

3. The activity of TGF-β signalling was suppressed by the SFN treatment, which was accompanied with up-regulated Smad7, an endogenous suppressor of TGF-β signalling.

4. SFN increased the transcription factors, including Nrf2, of Smad7 and decreased miRNAs targeting Smad7.

Chapter 2 Polymorphisms and expression analysis of SOX-6 in relation to porcine growth, carcass and meat quality traits

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2.1 Abstract

This study was to investigate single nucleotide polymorphisms (SNPs) and expression of SOX-6 to support its candidacy for growth, carcass and meat quality traits in pigs. The first SNP, rs81358375, was associated with pH 45 min *p.m.* in loin ($pH1_L$), the thickness of backfat and side fat, and carcass length in Pietrain population, and related with backfat thickness and daily gain in DuPi population. The other SNP, rs321666676, was associated with meat colour in Pietrain population. In DuPi population, the protein, not mRNA, level of SOX-6 in high $pH1_L$ pigs was significantly less abundant compared with low $pH1_L$ pigs, where miRNAs targeting SOX-6 were also differently regulated. This paper shows that SOX-6 could be a potential candidate gene for porcine growth, carcass and meat quality traits based on genetic association and gene expression.

Keywords: SOX-6, growth, carcass, meat quality, single nucleotide polymorphism, microRNA

2.2 Introduction

Pig is one of the most important farm animals and serves as a valued source of food worldwide. Modern pork production system tries to balance the requirements of animal welfare, cost of production and meat quality. Among diverse parameters of meat quality, pH during p.m. period has been shown to be one of the best predictors according to the accuracy and precision (Monin, 1998) and influences many meat quality traits like tenderness, WHC and meat colour (Fernandez *et al.*, 1994; Fernandez and Tornberg, 1994; van Laack *et al.*, 2001). Marker-assisted selection in pig breeding programmes has contributed to the improvement of many aspects of porcine production, including growth, carcass, meat quality, reproduction and disease resistance (Ernst and Steibel, 2013). Application of porcine SNP chip and GWAS has accelerated the identification of SNP markers and candidate genes for traits of interest (Fontanesi *et al.*, 2012; Ma *et al.*, 2013). Many candidate genes, like a-1-microglobulin/bikunin precursor, triosephosphate isomerase 1 and Long-chain acyl-CoA synthetase 4, have been suggested for pork traits (Corominas *et al.*, 2012; Heidt *et al.*, 2013).

SOX-6 is a versatile transcription factor and plays an important role in muscle fiber differentiation. SOX-6 represses the specification of slow fiber type during skeletal muscle differentiation by inhibiting the transcription of multiple myosin and sarcomeric genes (Hagiwara, 2011; Quiat *et al.*, 2011). Muscle fiber is an important determinant for meat quality (Karlsson *et al.*, 1999; Klont *et al.*, 1998). The distinct biochemical and physiological properties of slow- and fast-twitch muscle fiber lead to their divergent responses to pre-slaughter stress, varied p.m. pH decline and different meat quality (Karlsson *et al.*, 1999). As a key regulator of muscle fiber type specification, SOX-6 could be greatly associated with muscle growth and meat quality characteristics. Human population genetic studies revealed that the genetic polymorphisms in SOX-6 gene are associated with blood-pressure traits (Franceschini *et al.*, 2013), bone mineral density (Rivadeneira *et al.*, 2009), wrist bone mass (Tan *et al.*, 2010), obesity phenotypes in males (Liu *et al.*, 2009b) and carotid plaque traits (Dong *et al.*, 2010). Up to now, thousands of SNPs were identified in pig SOX-6 loci, including SOX-6 and SOX-6-like; however, there is no genetic association study about SOX-6 in pigs.

MiRNA is a post-transcriptional regulator and induces mRNA degradation or translation inhibition of target genes. The polymorphisms of miRNA and its binding sites have been

proven to be associated with pig litter size and fatness (Lei *et al.*, 2011; Shao *et al.*, 2011). In human, mouse and zebrafish, SOX-6 has been experimentally validated as a target gene of miR-208b and miR-499-5p (Sluijter *et al.*, 2010; van Rooij *et al.*, 2009; Wang *et al.*, 2011).

The present work is to investigate the candidacy of SOX-6 for growth, carcass and meat quality traits in Pietrain and DuPi population through genetic association and gene expression study.

2.3 Materials and methods

2.3.1 Animals and phenotypes

This study was conducted in the commercial Pietrain and experimental DuPi population. The information about the Pietrain and DuPi population was stated in details by Srikanchai *et al.* (2010) and Liu *et al.* (2007), respectively. The DuPi pigs were kept at the experimental research farm "Frankenforst", Institute of Animal Science, University of Bonn (Germany). The phenotype of interest, including muscle pH and conductivity, carcass composition and growth traits, were collected for Pietrain and DuPi population, and mean values and standard deviations are shown in Table 1.

2.3.2 Genomic DNA extraction and polymorphisms genotyping by PCR-RFLP

The gDNA was extracted from muscle of the Pietrain and DuPi pigs using the phenol–chloroform method. Finally, a working solution of 50 ng/µl gDNA was prepared for all samples and stored at 4 °C until used.

Two SNPs were genotyped in this study. The first SNP (dbSNP ID: rs81358375), located in the intron 3 of SOX-6-like (Entrez Gene ID: 100738152), was selected based on our GWAS study (unpublished data). The other SNP (dbSNP ID: rs321666676) is located in the exon 7 of SOX-6 (Entrez Gene ID: 397173), which is the 3' neighbor gene of SOX-6-like. These two SNPs were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The details of used primers and enzymes for the PCR-RFLP are shown in Table 2.

PCR was performed in a 20 µl volume containing 100 ng gDNA, 1 × PCR buffer (with 1.5 mM MgCl2), 0.25 mM of dNTP, 5 pmol of forward and reverse primers, and 1 U of Taq

DNA polymerase (Genecraft, Münster, Germany). The PCR program consisted of an initial denaturing at 95 °C for 5 min, 40 cycles of 95 °C for 30 s, annealing temperature for 30 s and 72 °C for 1 min, and a final elongation of 72 °C for 10 min. The PCR product was restriction enzyme digested in 20 µl volume containing 10 µl of PCR product, 2 µl of 10 × reaction buffer and 2 units of BsmBI (R0580S, New England Biolabs, Herts, UK). The digestion products were separated by electrophoresis in 2% agarose gel or 8% polyacrylamide gel, visualized with ethidium bromide under ultraviolet light, and analyzed with Bio-Rad Quantity One software.

2.3.3 Association study

The association studies between these two SNPs with growth (Grotrait), carcass (Cartrait) and meat quality (Meatrait) in the Pietrain and DuPi population were performed by fitting generalized linear models (Model 1 and 2) using R software:

$$Grotrait_{ijkl} = \mu + Genotype_i + BS_j + fam_k + \beta_{Age}(Age_{ijkl} - \overline{Age}) + e_{ijkl} \quad \text{(Model 1)}$$

$$Cartrait_{ijkl} \text{ or } Meatrait_{ijkl} = \mu + Genotype_i + YS_j + fam_k + \beta_{SW}(SW_{ijkl} - \overline{SW}) + e_{ijkl} \quad \text{(Model 2)}$$

where Grotrait, Cartrait and Meatrait are the observation of traits; µ is the population mean; Genotypei is the effect of i-th genotype (i = 1, 2 and 3); BSj and YSj are the effect of j-th season of birth and slaughter (j = 1 to 8 or 9); famk is the random effect of k-th family in DuPi population (k = 1 to 22); βAge(Ageijkl - $\overline{Age}$) and βSW(SWijkl - $\overline{SW}$) are the linear effect of age and slaughter weight as covariate; and eijkl is the random residual error. Because of skewness, DRIP values were log-transformed. Least-square mean for each genotype was estimated using R software and pairwise comparisons were performed among different genotypes, where P value was adjusted by using Tukey's method.

2.3.4 mRNA and miRNA expression study by quantitative real time PCR (qRT-PCR)

For expression assay, total RNA was isolated from the muscle of low (5.99 ± 0.07) and high (6.97 ± 0.02) pH1L DuPi pigs (n = 4 per group) with TriFast (PeqLab, Erlangen, Germany). For miRNA cDNA synthesis, a RNA mixture consisted of the same amount of total RNA from each sample was prepared for either group. The cDNA for mRNA and miRNA was synthesised with First Strand cDNA Synthesis Kit (Thermo Scientific, NH, USA) and miScript Reverse Transcription Kit (Qiagen, CA, USA), respectively. For qRT-

PCR, primers for SOX-6 and GAPDH were designed using the Primer3 software (Untergasser *et al.*, 2012). The qRT-PCR was performed in 20 µl volume containing 1 µl of 10 times diluted cDNA, 5 pmol forward and reverse primers, and 10 µl iTaq SYBR Green Supermix with Rox (Bio-Rad, CA, USA). The qRT-PCR was conducted with the following program: 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s, and a melt curve step on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, CA, USA). The mean of Ct value for each sample was used for analysis using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Expression of SOX-6 was normalized with the housekeeping gene GAPDH, while miRNAs data were normalized with U6 and ssc-let-7a. The information and primers sequence for mRNA and miRNA qRT-PCR are shown in Table 2.

2.3.5 Protein expression study by western blot

Total protein was extracted from the muscle of low (5.99 ± 0.06) and high (6.88 ± 0.19) pH1L DuPi pigs (n = 5 per group). The protein samples were loaded to a (5.4 + 12%) SDS-PAGE gel, transferred to nitrocellulose membrane (Bio-Rad, CA, USA). Then the blot was incubated with blocking buffer (A151.1, Carl Roth, Karlsruhe, Germany) at room temperature for 1 hours before the incubation with 1:500 diluted SOX-6 antibody (sc-17332, Santa Cruz, CA, USA) in the blocking buffer overnight at 4 °C. The blot was washed for five times with TBS containing 0.1% Tween-20 (TBST), incubated with 1:5000 diluted HRP-conjugated anti-goat IgG (sc-2020, Santa Cruz, CA, USA) at room temperature for 1 h and rinsed with TBST for five times. The blot was visualized with Clarity Western ECL Substrate (170-5060, Bio-Rad, CA, USA) and ChemiDoc XRS system (Bio-Rad, CA, USA). The band intensities were analysed using ImageJ (http://rsb.info.nih.gov/ij) with GAPDH (sc-20357, Santa Cruz, CA, USA) as a loading control. The data of qRT-PCR and western blot were analysed with Student's t-test, and $P < 0.05$ and $P < 0.01$ were set as statistically significant.

2.4 Results

2.4.1 Alleles and genotypes frequencies and association analysis of SNPs in SOX-6

Two SNPs, rs81358375 and rs321666676, of SOX-6 were investigated in the present study. We have genotyped rs81358375 in 262 Pietrain pigs and 249 DuPi pigs. Allele frequencies for rs81358375, A and G, were 0.41 and 0.59 in Pietrain population, and 0.57 and 0.43 in

DuPi population, respectively. Genotype frequencies for rs81358375, AA, AG and GG, were 0.17, 0.47 and 0.35 in Pietrain population, and 0.33, 0.48 and 0.19 in DuPi population (Table 3), respectively. The association analysis was performed for rs81358375 with growth, carcass and meat quality traits in Pietrain and DuPi population. It was shown that rs81358375 was significantly associated with pH1L, carcass length, the thickness of backfat (middle and average) and side fat, and also had high tendency to be related with lean meat percentage (LMP), conductivity 45 min p.m. in loin ($Con1_L$), conductivity 24 hours p.m. in loin ($Con24_L$) and the thickness of front backfat in Pietrain population (Table 4). In DuPi population, the front backfat thickness was associated with rs81358375. In addition, daily gain from born to 30 kg body weight was also likely to be influenced by rs81358375 (Table 4). It was found in Pietrain population that rs81358375 had dominance effects for meat quality traits like pH1L, Con24L and meat colour, while, for carcass traits, it appeared to be an additive effect for backfat (middle and average) but a dominance effect for side fat and LMP (Table 4).

The second SNP, rs321666676, was genotyped in 293 Pietrain pigs and 128 DuPi pigs. Allele frequencies for rs321666676, G and C, were 0.92 and 0.08 in Pietrain population, respectively. Genotype frequencies for rs321666676, GG, GC and CC, were 0.84, 0.16 and 0.00 in Pietrain population (Table 5), respectively. The genotyped 128 DuPi pigs turned out to be homogeneous as GG. The association study for rs321666676 was performed in Pietrain population. The results showed that only meat colour was significantly associated with rs321666676, while pH1L and front backfat thickness also tended to be related with rs321666676 (Table 6).

2.4.2 The mRNA and protein level of SOX-6 in DuPi pigs with divergent pH1L

SOX-6 mRNA was quantified by qRT-PCR. The results showed that there was no significant difference for SOX-6 mRNA expression between low pH1L and high pH1L group (figure 3). Furthermore, western blot was used to measure protein abundance of SOX-6. Inconsistent with mRNA results, SOX-6 protein was significantly decreased in high pH1L group compared to low pH1L group ($P < 0.05$) (figure 4).

2.4.3 Expression of miRNAs targeting SOX-6 in DuPi pigs with divergent pH1L

Since miRNAs post-transcriptionally modulate gene expression, it is interesting to check whether the discordance of mRNA and protein level of SOX-6 is caused by miRNA-

induced translational repression. Based on the prediction of algorithms, TargetScan (Lewis *et al.*, 2005) and PITA (Kertesz *et al.*, 2007), and conservation of miRNA binding site, five porcine miRNAs, including ssc-miR-18a, ssc-miR-19a, ssc-miR-19b, ssc-miR-208b and ssc-miR-499-5p, were quantified by qRT-PCR. The results showed that ssc-miR-208b ($P < 0.01$) and ssc-miR-499-5p ($P < 0.01$) were significantly up-regulated in high pH1L group and ssc-miR-18a ($P < 0.05$) was down-regulated in high pH1L group (figure 5).

2.5 Discussion

The present study is the first report about the relationship between SNPs of SOX-6 and porcine growth, carcass and meat quality traits. Pig SOX-6 is located at 46 megabase pair of *Sus scrofa* chromosome 2 and is embedded in or close to a number of reported QTLs (Table 7). Given nine pig breeds involved in the listed QTLs studies (Table 7), the association between QTLs and traits is quite consistent in these diverse pig breeds. In this work, the polymorphisms of pig SOX-6 were found to be associated with $pH1_L$, carcass length, the thickness of backfat and side fat in Pietrain population, and with front the backfat thickness and daily gain from birth to 30kg body weight ($P = 0.06$) in DuPi population. As shown in Table 7, these QTLs are associated with backfat, abdominal fat, body weight and daily gain. Thus, both SOX-6 and its surrounding QTLs are associated with backfat and daily gain, which strongly supports the candidacy of SOX-6. For rs81358375, it was associated with backfat thickness in both Pietrain and DuPi population, which agrees with reported backfat and abdominal fat weight QTLs (Table 7). At the same time, the relationship between rs81358375 and daily gain from born to 30 kg body weight ($P = 0.06$) or from birth to weaning ($P = 0.14$) in DuPi population is in line with average daily gain QTLs (Table 7). As for rs321666676, the CC genotype is missed from Pietrain and DuPi population. This could be owing to its lethal mutation or molecular selection by farmers. Thus, the genetic association of SOX-6 reported in this study and its chromosome position together support its candidacy for traits of interest.

For past several decades, the pig breeding with aim of leanness and growth has led to the reduction of carcass fatness and meat quality. It is worthwhile to notice that rs81358375 shows pleiotropic effect and is associated with pig meat quality ($pH1_L$, $Con1_L$ and $Con24_L$), carcass (backfat, side fat, lean meat percentage and carcass length) and growth (daily gain) traits in Pietrain or DuPi population. The Pietrain pigs carrying genotype AA of rs81358375 have high $pH1_L$, thick backfat, but short carcass length. It has been shown high

pH1 value normally indicates for good meat quality, like meat colour and water-holding capacity (Popp *et al.*, 2014). On the other hand, fat deposition negatively affects production efficiency, consumer acceptance and meat quality. Thus, rs81358375 maintains a complex relationship with meat quality and fat deposit. Thus, it could be a valuable marker in breeding for pig with balanced meat quality and fat deposit.

SOX-6 is a key transcription factor in specification of muscle fiber and maintains the highest mRNA level in mouse skeletal muscle (Hagiwara *et al.*, 2000). We found that SOX-6 protein was much less abundant in high $pH1_L$ pigs, although DuPi pigs with low $pH1_L$ shared the similar mRNA expression of SOX-6 with high $pH1_L$ pigs. In mice, SOX-6 mutant resulted in dramatic disturbance of fiber type-specific genes expression pattern (Hagiwara *et al.*, 2005), which might lead to varied characteristics of muscle fibers. Because SOX-6 is one transcriptional suppressor of slow fiber-specific isoforms (Hagiwara *et al.*, 2007), decreased SOX-6 protein in high $pH1_L$ pigs could enhance expression of slow fiber-type isoforms and, conversely, inhibit fast fiber-type isoforms expression, which is in favour of the improvement of muscle oxidative capacity. The oxidative capacity of muscle is positively correlated with pork quality, which has been reviewed in details (Karlsson *et al.*, 1999; Klont *et al.*, 1998). In pigs, fast-twitch muscle fibers normally contain higher glycogen level and have lower ultimate pH (Fernandez and Tornberg, 1991), which is negatively related with pork quality. Thus, the disturbance of SOX-6 expression might influence pork quality by changing the characteristics of muscle fibers and, furthermore, energy metabolism of muscle fiber peri- and post-mortem through modifying muscle fiber-specific isoforms expression pattern.

MiRNA is a small post-transcriptional regulator binding to 3' UTR of the target gene. Generally, a single miRNA could target several genes and one gene can be regulated by multiple miRNAs. MiRNAs are differently expressed among pig breeds and tissues (Li *et al.*, 2012; Timoneda *et al.*, 2013). Pig SOX-6 mRNA has a long, ~ 5 kb, 3' UTR, which contains hundreds of potential miRNA binding sites. SOX-6 mRNA is detected in many tissues and its protein abundance is closely related to tissue-specific miRNAs (Hagiwara, 2011). As called MyomiRs, miR-208b and miR-499-5p are respectively transcribed form the intron of MYH7 and MYH7B in striated muscle cells (van Rooij *et al.*, 2008). Together with miR-17/20a/92a-1, miR-18a/19a/19b belongs to the miR-17-92 cluster, which plays a key role in cardiovascular development and function (Chen *et al.*, 2013; Danielson *et al.*, 2013). As reported in pigs, although LTL muscle (mainly glycolytic muscle) and *psoas*

major muscle (mainly oxidative muscle) shared the majority of detectable miRNAs, they shown different miRNAs expression profile, which contributed to their differentiated physiological characters (Liu *et al.*, 2013). It was found that the sequence of miR-208b and miR-499-5p and binding sites in SOX-6 3' UTR are strongly conservative between human and pig. In the present study, we found that ssc-miR-208b and ssc-miR-499-5p were up-regulated in high $pH1_L$ group, while ssc-miR-18a was down-regulated. Thus, the different expressed SOX-6 and distinct phenotypes between low and high $pH1_L$ pigs may be partially attributed to the altered expression of ssc-miR-208b, ssc-miR-499-5p and ssc-miR-18a.

In conclusion, this is the first work to associate pig SOX-6 with growth, carcass and meat quality traits. This paper supports the candidacy of SOX-6 on the levels of genetic association, chromosome position and gene expression. However, the available studies about pig SOX-6 are still limited. Thus, it demands more work to elucidate the role of SOX-6 in pig production.

Table 1: Descriptive data for the Pietrain and DuPi populations. Mean ± standard deviation is shown.

Traits	Pietrain (n = 296)	DuPi (n = 330)
$pH1_L$[1]	6.35 ± 0.31	6.57 ± 0.22
$pH24_L$[2]	5.44 ± 0.06	5.5 ± 0.09
$Con1_L$ (mS)[3]	5.51 ± 3.09	4.36 ± 0.63
$Con24_L$ (mS)[4]	4.65 ± 2.72	2.79 ± 0.82
Drip loss (%)	3.75 ± 3.34	2.08 ± 0.97
Shear force (kg)	35.23 ± 6.61	-
Meat colour	66.95 ± 9.14	69.01 ± 5.76
Backfat (front) (cm)	2.81 ± 0.3	3.38 ± 0.45
Backfat (middle) (cm)	1.52 ± 0.21	1.65 ± 0.31
Backfat (average) (cm)	1.73 ± 0.19	2.13 ± 0.31
Side fat (cm)	1.77 ± 0.4	2.67 ± 0.66
Carcass length (cm)	98.08 ± 2.79	96.6 ± 2.56
Lean meat percentage (%)	65.45 ± 1.48	-
Daily gain (from birth to weaning)	-	232.26 ± 60.12
Daily gain (born to 30kg) (g)	-	319.69 ± 63.42
Feed conversion (30 to 105kg) (kg feed fed per kg weight gain)	2.42 ± 0.15	2.65 ± 0.27

[1,2] $pH1_L$ and $pH24_L$: pH 45 min and 24 hours *post mortem* (p.m.) in loin; [3,4] $Con1_L$ and $Con24_L$: conductivity 45 min and 24 hours p.m. in loin.

Table 2: Restriction enzymes and primer sequences used in the chapter 2.

SNP/ Gene Name	Accession no.	Application	Primer (5′ - 3′)	Size (bp)	Annealing temperature (°C)	Enzyme	Alle	RFLP pattern (bp)
rs81358375	rs81358375	Genotyping	F: CCAGTCCATCCTTTCCTTGA R: GTTTCCAAAAGGGAATGCAG	402	50.7	BsmBI	A/G	402/305+97
rs321666676	rs321666676	Genotyping	F: CAATGCCATCGTTGAGTCTG R: GTTGTACTGCACATCTTCTCCCTG TTGGATCGTCT	258	56	BsmBI	C/G	258/217+41
SOX-6	XM_003122960	qRT-PCR	F: CGGATTGGGGAGTATAAGCA R: CATCTGAGGTGATGGTGTGG	159	60	-	-	-
GAPDH	NM_001206359	qRT-PCR	F: ACCCAGAAGACTGTGGATGG R: ACGCCTGCTTCACCACCTTC	247	60	-	-	-
ssc-miR-18a	MIMAT0002161	qRT-PCR	F: TAAGGTGCATCTAGTGCAGATA	-	60	-	-	-
ssc-miR-19a	MIMAT0002128	qRT-PCR	F: TGTGCAAATCTATGCAAAACTGA	-	60	-	-	-
ssc-miR-19b	MIMAT0013950	qRT-PCR	F: TGTGCAAATCCATGCAAAACTGA	-	60	-	-	-
ssc-miR-208b	MIMAT0013912	qRT-PCR	F: ATAAGACGAACAAAAGGTTTGT	-	60	-	-	-
ssc-miR-499-5p	MIMAT0013877	qRT-PCR	F: TTAAGACTTGCAGTGATGTTT	-	60	-	-	-
ssc-let-7a	MIMAT0013865	qRT-PCR	F: TGAGGTAGTAGGTTGTATAGTT	-	60	-	-	-

Table 3: Frequencies of alleles and genotypes for rs81358375 of the porcine SOX-6 in the Pietrain and DuPi populations.

Population	Number	Genotype frequency			Allele frequency	
		AA(n)	AG(n)	GG(n)	A	G
Pietrain	262	0.17(45)	0.47(124)	0.35(93)	0.41	0.59
DuPi	249	0.33(82)	0.48(119)	0.19(48)	0.57	0.43

Table 4: Least square means of growth, carcass and meat quality traits across genotypes of rs81358375 in the Pietrain and DuPi populations. [a, b, *] $P < 0.05$; [c, d, **] $P < 0.01$.

Trait	Pop.	P-value	Explained variance	Genotype (μ±S.E.)			Effect (μ±S.E.)	
				AA	AG	GG	Additive	Dominance
pH1L	Pi	*	0.020	6.43±0.04a	6.30±0.03b	6.35±0.03	0.08±0.06	-0.10±0.04*
Con1L	Pi	0.09	0.012	4.79±0.30	5.56±0.18	5.37±0.21	-0.63±0.39	0.50±0.27
Con24L	Pi	0.07	0.014	3.88±0.27	4.59±0.16	4.25±0.19	-0.4±0.36	0.55±0.24*
Drip loss	Pi	0.20	0.005	3.45±0.16	3.79±0.10	3.69±0.11	-0.26±0.21	0.23±0.15
Meat colour	Pi	0.11	0.010	69.17±1.31	65.99±0.79	67.26±0.91	2.07±1.70	-2.31±1.16*
Backfat (front)	Pi	0.07	0.014	2.76±0.06	2.82±0.03	2.71±0.04	0.06±0.07	0.10±0.05
Backfat (middle)	Pi	*	0.028	1.56±0.03a	1.52±0.02	1.47±0.02b	0.11±0.04**	0.01±0.03
Backfat (average)	Pi	*	0.020	1.76±0.03	1.74±0.02	1.68±0.02	0.09±0.04*	0.01±0.02
Fat (side)	Pi	*	0.025	1.76±0.06	1.84±0.03a	1.69±0.04b	0.08±0.07	0.12±0.05*
Muscle-fat-relation	Pi	0.20	0.005	0.18±0.01	0.19±0.00	0.18±0.00	0.00±0.01	0.01±0.01
Lean meat percentage	Pi	0.06	0.014	65.60±0.22	65.26±0.13	65.72±0.15	-0.14±0.28	-0.41±0.19*
Carcass length	Pi	*	0.018	96.70±0.30	96.62±0.18a	97.28±0.21b	-0.65±0.38	-0.38±0.26
Feed conversion rate	Pi	0.21	0.005	2.41±0.02	2.44±0.01	2.41±0.01	0.01±0.02	0.03±0.02
Drip loss	DuPi	0.19	0.001	0.64±0.06	0.56±0.05	0.66±0.08	-0.02±0.08	-0.09±0.06
Cooking loss	DuPi	0.23	0.003	24.65±0.23	24.74±0.21	25.08±0.3	-0.43±0.32	-0.13±0.22
Shear force	DuPi	0.12	0.015	34.60±0.83	35.95±0.77	37.02±1.06	-2.43±1.09	0.14±0.76
Backfat (front)	DuPi	*	0.016	3.30±0.05	3.42±0.05	3.43±0.07	-0.13±0.08	0.06±0.06
Dressing percentage	DuPi	0.18	0.001	77.06±0.27	77.11±0.25	76.65±0.35	0.40±0.37	0.25±0.25
Daily gain (from birth to weaning)	DuPi	0.14	0.006	225.70±8.78	232.84±8.36	219.02±10.41	6.68±9.54	10.48±6.44
Daily gain (from birth to 30kg)	DuPi	0.06	0.009	319.85±7.36	326.90±6.85	310.86±9.22	8.99±9.57	11.54±6.49

Table 5: Frequencies of alleles and genotypes for rs321666676 of the porcine SOX-6 in the Pietrain and DuPi populations.

Population	Number	Genotype frequency			Allele frequency	
		GG(n)	GC(n)	CC(n)	G	C
Pietrain	293	0.84(245)	0.16(47)	0.00(1)	0.92	0.08
DuPi	128	1.00(128)	0.00(0)	0.00(0)	1.00	0.00

Table 6: Least square means of growth, carcass and meat quality traits across genotypes of rs321666676 in the Pietrain population. [c, d, **] $P < 0.01$

Trait	Population	*P*-value	Explained variance	Genotype (μ±S.E.)			Effect (μ±S.E.)	
				CC	CG	GG	Additive	Dominance
$pH1_L$	Pi	0.09	0.011	6.74±0.30	6.42±0.05	6.33±0.02	0.43±0.31	-0.12±0.16
$pH24_L$	Pi	0.23	0.004	5.42±0.06	5.46±0.01	5.45±0.00	-0.03±0.06	0.03±0.03
$Con1_L$	Pi	0.11	0.010	4.04±1.99	4.76±0.32	5.47±0.13	-1.44±2.07	-0.02±1.09
Meat colour	Pi	**	0.050	71.80±5.89	70.83±0.96[c]	66.92±0.40[d]	4.81±6.10	1.67±3.22
Backfat (front)	Pi	0.07	0.013	3.03±0.29	2.91±0.05	2.80±0.02	0.23±0.30	0.00±0.16
Backfat (average)	Pi	0.13	0.008	1.92±0.19	1.77±0.03	1.71±0.01	0.21±0.19	-0.04±0.10
Fat (side)	Pi	0.11	0.010	1.91±0.38	1.89±0.06	1.75±0.03	0.15±0.39	0.07±0.21
Fat area	Pi	0.19	0.005	13.35±1.70	11.45±0.28	11.07±0.11	2.35±1.76	-0.78±0.93
Muscle-fat-relation	Pi	0.17	0.006	0.21±0.04	0.20±0.01	0.18±0.00	0.03±0.04	0.00±0.02
Lean meat percentage	Pi	0.13	0.008	64.36±1.38	64.95±0.22	65.41±0.09	-1.05±1.43	0.05±0.76

Table 7: Quantitative trait loci (QTLs) summary around the SOX-6 loci on Sus scrofa chromosome 2.

QTL Trait	*P*-values	QTL Peak (cM)	QTL Span (cM)	Additive	Dominance	Associated breeds or studied animals	Reference
Backfat at tenth rib	-	37.6	31.2-42	-	-	Three F2 families based on crosses of Meishan, European Wild Boar, and Pietrain	(Lee et al. 2003)
Backfat at tenth rib	<0.01	44.8	-	-	-	Duroc, Berkshire	(Stearns et al. 2005a)
Backfat at tenth rib	<0.01	44.8	-	0.12	-0.047	Duroc, Berkshire	(Stearns et al. 2005b)
Backfat at first rib	0.45	4.3	0-60.6	-	-	Animals were crosses of the following breeds: Landrace, Large White, and Pietrain	(Harmegnies et al. 2006)
Abdominal fat weight	<0.01	84.1	2.5-88.9	-0.05	0.02	Duroc, Erhualian	(Ai et al. 2012)
Abdominal fat weight	<0.01	18.6	0.6-98.3	-0.05	0.02	Duroc, Erhualian	(Ai et al. 2012)
Body weight (16 days)	<0.05	60	46-74.8	-0.00	0.44	A three-generation family created by crossing two Berkshire sires with nine Yorkshire dams	(Thomsen et al. 2004)
Average daily gain (on weaning)	<0.05	61	46-88.5	-0.00	0.02	A three-generation family created by crossing two Berkshire sires with nine Yorkshire dams	(Thomsen et al. 2004)
Average daily gain	-	31.2	9.8-42	-15.5	19.1	Three F2 families based on crosses of Meishan, European Wild Boar, and Pietrain	(Lee et al. 2003)
Average daily gain	<0.05	53.7	42-77.9	-	-	Pietrain, Wild boar	(Ruckert and Bennewitz 2010)

Figure 3: Relative expression of SOX-6 mRNA between low and high $pH1_L$ DuPi pigs.

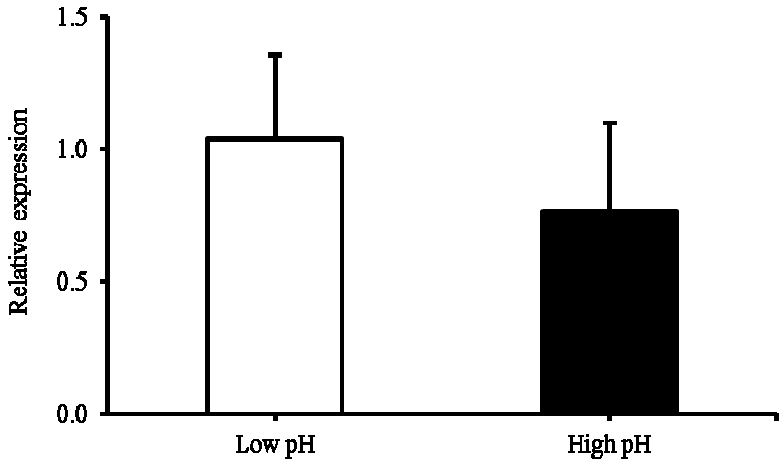
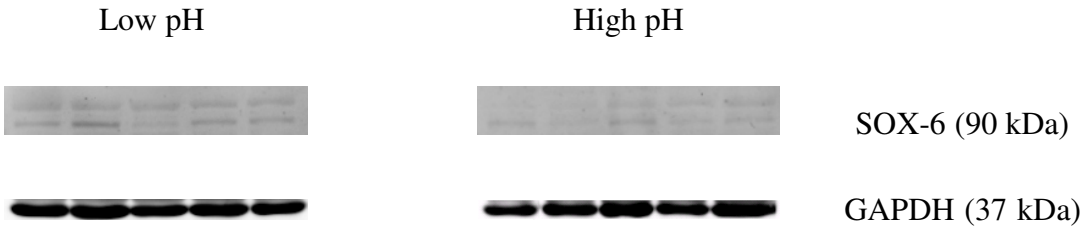


Figure 4: SOX-6 protein analysis in low and high $pH1_L$ DuPi pigs.

(A) Western blot analysis of SOX-6 and GAPDH protein expression. (B) The relative intensity of SOX-6 normalized by GAPDH. * $P < 0.05$.

A



B

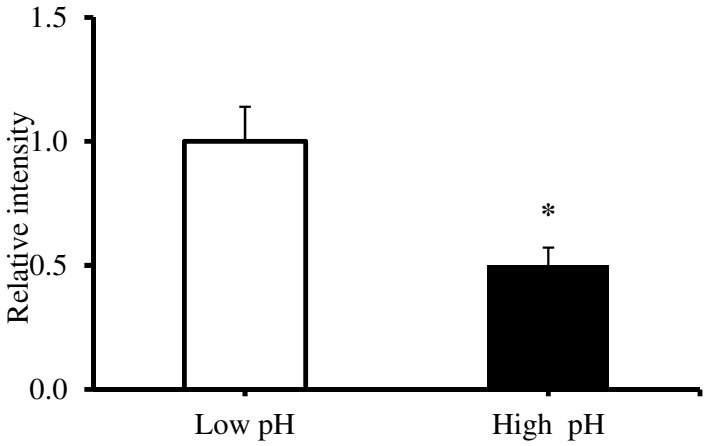
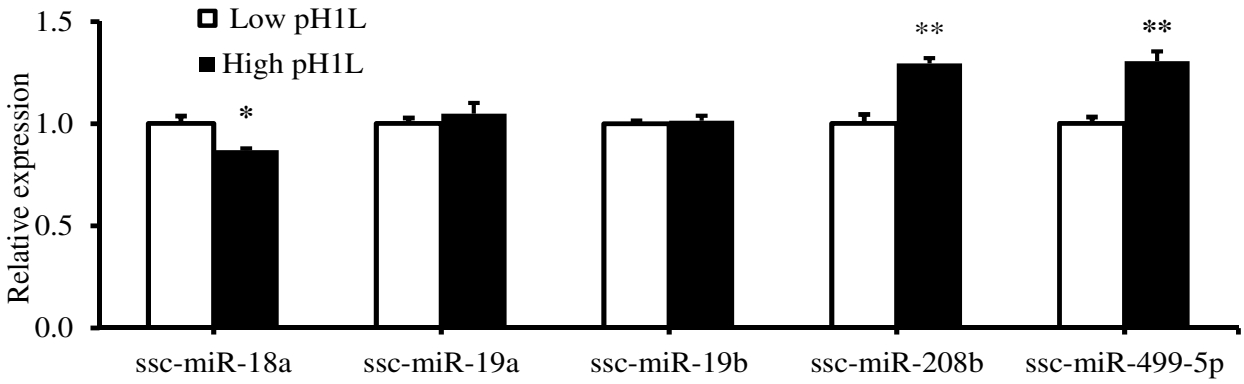


Figure 5: Expression analysis of miRNAs targeting SOX-6 in low and high $pH1_L$ DuPi pigs. * $P < 0.05$, ** $P < 0.01$.



Chapter 3 Sulforaphane enhances proliferation of porcine satellite cells through suppression of TGF-β signalling pathway

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3.1 Abstract

Satellite cells, which are also considered as the muscle stem cells, play a critical role in muscle growth, maintenance and regeneration. Our previous study has found sulforaphane (SFN), a histone deacetylases inhibitor, epigenetically supressed the transcription of MSTN in porcine satellite cells. However, the effect of SFN on the activation and proliferation of porcine satellite cells and the related mechanisms are far from understood. In the present study, we report that SFN enhanced the proliferation of the porcine satellite cells and modified the expression myogenic regulatory factors. SFN altered the expression of HDAC members and inhibited their activity. The activity of TGF-β signalling was supressed by SFN treatment, which was accompanied by up-regulating Smad7, an endogenous suppressor of TGF-β signalling. Furthermore, we found that SFN increased the transcription factors, including Nrf2, and decreased miRNAs targeting Smad7. In summary, our studies evidenced that SFN enhanced the proliferation of porcine satellite cells by suppressing TGF-β signalling through activation of Smad7 in an Nrf2-dependent way.

Keywords: sulforaphane, satellite cell, porcine satellite cells, TGF-beta

3.2 Introduction

Satellite cells, which are considered as the muscle stem cells, are discovered by Alexander Mauro at 1961 and are known to be a group of mononucleated cells lied between the plasma membrane and the basal lamina of muscle fibers (Mauro, 1961). Satellite cells can be activated by injury or other external signals and give rise to myoblasts, which are incorporated into growing (Cardasis and Cooper, 1975; Moss and Leblond, 1971) and injured muscle fibers (Montarras *et al.*, 2005), or fuse with each other to form new myofibers. Satellite cells have an indispensable role in the postnatal growth, maintenance and regeneration of skeletal muscle, where MyoD and MEF2 gene families are master regulators (Berkes and Tapscott, 2005; Molkentin *et al.*, 1995; Naya and Olson, 1999). A lot of muscle diseases result from defective function of satellite cells, which is caused by the abnormal microenvironment of stem cell niche (Yin *et al.*, 2013). TGF-β signalling is a critical component of this microenvironment (Oshimori and Fuchs, 2012; Ten Broek *et al.*, 2010). In the canonical TGF-β signalling pathway, the signal transductors, Smad2/3, are phosphorylated by activated TGF-β receptor (Miyazono *et al.*, 2000), which is suppressed by the Smad7 in a negative feedback way (Yan and Chen, 2011). Inhibition of TGF-β signalling by a TGF-β receptor kinase inhibitor is in favor of muscle regeneration (Carlson *et al.*, 2009).

Porcine satellite cells are a good model for studying the mechanisms of muscle development. Pork, the main economic product of pig, is the consumed meat in the largest amount in the world. As mentioned above, satellite cells contribute myonuclei for the hypertrophy of skeletal muscle (Cardasis and Cooper, 1975; Moss and Leblond, 1971). Thus enhancement of porcine satellite cell proliferation should be in favour of the increment of pork production. On the other hand, defective satellite cells impair the muscle regeneration and maintenance and cause a lot human muscle diseases (McCullagh and Perlingeiro, 2014). SFN, a natural molecule rich in cruciferous vegetables, is a potent Nrf2 dependent inducer of phase II detoxification enzymes (Fahey and Talalay, 1999) and also inhibits the HDAC activity of (Myzak *et al.*, 2004), which impedes the myogenic program by interfering the function of MEF2 (Dressel *et al.*, 2001; McKinsey *et al.*, 2000). SFN is considered to be a promising cancer chemopreventive agent (Guerrero-Beltran *et al.*, 2012; Houghton *et al.*, 2013). Recently, we have found that SFN epigenetically suppressed the transcription activity of MSTN in porcine satellite cells (Fan *et al.*, 2012). Sulforaphane is also reported to

activate Nrf2 and alleviate muscular dystrophy in *mdx* mice (Sun *et al.*, 2015). One of main therapeutic strategies of the human muscle diseases is to modulate the activity of satellite cells. Collectively, the present study provides a model that SFN enhances the proliferation of porcine satellite cells by suppressing of TGF-β signalling through up-regulation of Smad7.

3.3 Materials and methods

3.3.1 Porcine satellite cells isolation and cell culture

Porcine satellite cells were isolated from SM muscles of 21 days purebred Pietrain piglets. All piglets were kept and slaughtered according to German performance test directions. The isolation of porcine satellite cells has been described in our previous report (Fan *et al.*, 2012). In order to investigate the effects of SFN on the proliferation of porcine satellite cell (McCroskery *et al.*, 2003), cell culture procedure was modified as follow: cell medium was changed everyday after cells were plated 2 days and porcine satellite cells were treated with the vehicle (DMSO) or SFN (S8044, LKT) at day 4 for 48 hours, and then harvested for the further analysis.

3.3.2 Cell proliferation assay

Cell proliferation was measured with WST-1 cell proliferation assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Porcine satellite cells were cultured in 96-well plate following the procedure shown in figure 6A. After treated with SFN for 48 hours, 10 µl of the reconstituted WST-1 mixture was added to each well and cells were cultured for additional 2 hours at 37 °C in incubator with 5% CO_2. After mixed, the absorbance of the supernatant from each well was measured using a microplate reader at a wavelength of 450 nm.

3.3.3 In vitro HDAC activity assay

HDAC activity of porcine satellite cells was determined with Color-de-Lys® HDAC colorimetric activity assay kit (Enzo Life Science, Farmingdale, NY). 10 µg protein of porcine satellite cells was diluted into 25 µl assay buffer, warmed to 37 °C before mixed with 25 µl warmed Color-de-Lys ™ substrate, and incubated for 1 hour at 37 °C. Then the reaction was stoped by adding 50 µl of Color-de-Lys™ developer containing 2 µM

TSA and incubated for 15 min at 37 °C. Signal was read with a microplate reader at a wavelength of 405 nm and the data was normalized to No Enzyme Control.

3.3.4 Prediction of miRNAs targeting Smad7

To determine the differentially expressed miRNAs targeting Smad7, we used two miRNA target prediction algorithms: PITA (Kertesz *et al.*, 2007) and TargetScan (Lewis *et al.*, 2005). The putative binding sites were further verified by RNAHybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) and RNA22 (https://cm.jefferson.edu/rna22/Interactive/).

3.3.5 qRT-PCR of mRNA and miRNA

Genomic DNA and total RNA, including miRNA, from porcine satellite cells were simultaneously isolated with AllPrep DNA/RNA/miRNA Universal Kit (80224, Qiagen, Germany). Total RNA was reverse transcripted with First Strand cDNA Synthesis Kit (K1612, Thermo Scientific) and miScript PCR Starter Kit (218193, Qiagen) respectively for mRNA and miRNA cDNA synthesis. Quantitative real time PCR (qRT-PCR) was performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with iTaq SYBR Green Supermix (172-5850, Bio-Rad) and miScript PCR Starter Kit for the detection of mRNA and miRNA, respectively. All primers for mRNA were designed using the Primer3web version 4.0.0 (Koressaar and Remm, 2007), while the primers for mRNA and miRNA qRT-PCR are listed in Table 8. The data of qRT-PCR were analyzed using a comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with hypoxanthine phosphoribosyltransferase 1 (HPRT1) and TATA-binding protein (TBP) for mRNAs and U6 small nuclear RNA and 18s for miRNAs as endogenous references.

3.3.6 Western blotting

A protocol for Western blotting was described previously (Fan *et al.*, 2011). The following primary antibodies for the following proteins were used in this study: acetyl-histone H3 (0.05 μg/ml, 06-599, Millipore), acetyl-histone H4 (1:1000, 06-866, Millipore), H4 acetylated lysine residue (K) 5, K8, K12 and K16 (1:1000, 06-759, 06-760, 06-760 and 06-762, respectively, Millipore), TGF-β1(1:1000, sc-146, Santa Cruz Biotechnology), Smad7 (1:1000, sc-11392, Santa Cruz Biotechnology), C/EBPβ

(1:1000, sc-150, Santa Cruz Biotechnology), NF-κB p50 (1:1000, sc-114, Santa Cruz Biotechnology), Dicer (1:1000, sc-30226, Santa Cruz Biotechnology). The secondary antibody used in this study included donkey anti-goat and goat anti-rabbit (1:5000, sc-2020 and sc-2004, Santa Cruz Biotechnology). Finally, the specific signals were detected by Clarity Western ECL Substrate (170-5060, Bio-Rad). Images were acquired by ChemiDoc XRS system (Bio-Rad, CA, USA).

3.3.7 Bisulfite sequencing

The 5´-regulatory region of Smad7 (Gene ID: 100521305) was submitted to the online program Methprimer (Li and Dahiya, 2002) for CpG islands identification and bisulfite sequencing PCR primers design. Genomic DNA (1 μg) was subjected to bisulfite modification using EZ DNA Methylation-Direct Kit (D5020, Zymo Research). The Smad7 5´-regulatory region containing CpG island was amplified by PCR. PCR products were cloned 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.

3.3.8 Statistical analysis

Pairwise comparisons were made between SFN treatment groups and the vehicle control, using Student's t test. The data were expressed as mean ± standard error (SE) and (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ were set statistically significant.

3.4 Results

3.4.1 SFN affected porcine satellite cell proliferation and MyoD and MEF2 expression

In the present study, the cell culture procedure, as shown in figure 6A, was adapted from previously described methods (Fan *et al.*, 2012). Herein, we found that SFN modulated the proliferation of porcine satellite cells in a dose-dependent manner (figure 6B). The proliferation of porcine satellite cells was enhanced by low levels (5 and 10 μM) of SFN, but inhibited by high dose (20 μM) of SFN. Based on these results, SFN treatment at 10 μM was selected for further work. In the previous report, we have found that expression of MSTN was supressed by the SFN treatment. Additionally, we also found that SFN treatment down-regulated the myogenic regulatory factors, including

MyoD, MYF5 and myogenin. In contrast, another important myogenesis regulators, MEF2 family including MEF2A1, MEF2A2 and MEF2C, were up-regulated in the presence of SFN.

3.4.2 SFN altered the expression and activity of HDAC and global histone acetylation

SFN metabolites were proven to impair the HDAC activity as a competitive inhibitor (Myzak *et al.*, 2004). As expected, SFN caused a down-regulation of the activity of HDAC in the porcine satellite cells (figure 7A). Beside these, we investigated the effects of SFN on the mRNA expression of HDAC members. After SFN treatment, the expression of HDAC1 and HDAC8 of class I and HDAC7 and HDAC10 of class II were increased, while HDAC9 of class II and HDAC11 of class IV was decreased (figure 7B). Furthermore, global acetylation level of histone H3 and H4 was greatly increased (figure 7C). Based on these results, we measured acetylation status of each lysine residue of histone H4 tail. It is interesting that only acetylation of histone H4K12 was up-regulated, while acetylation of histone H4 K5, K8 and K16 was significantly reduced (figure 7D).

3.4.3 Effects of SNF on TGF-β signalling pathway

TGF-β harms skeletal muscle regeneration by suppressing satellite cell proliferation and myofiber fusion (Allen and Boxhorn, 1987). To check whether TGF-β signalling pathway is involved in the influence of SFN on porcine satellite cells, we assessed the activity of TGF-β signalling pathway in porcine satellite cells. Surprisingly, SFN greatly increased the expression of TGF-β1 and had no effect on the expression of Smad2/3 (figure 8A). However, both monomer and homodimer forms of TGF-β1 were down-regulated on the protein level after SFN treatment (figure 8B). Additionally, we found that SFN treatment increase the expression of Smad7 on both mRNA and protein level (figure 8C and D).

3.4.4 SFN activated transcription factors of Smad7

As mentioned above, SFN at 10 μM doubled mRNA level of Smad7. We tried to investigate the mechanisms how SFN activated the transcription of Smad7. Firstly, we checked the expression of five transcription factors of Smad7. Except Sp1, the other four transcription factors, especially C/EBPβ and Nrf2, were up-regulated by SFN

treatment (figure 9A). However, the protein level of C/EBPβ and NF-κB1 was unchanged or even slightly decreased by SFN treatment (figure 9B).

3.4.5 SFN supressed miRNAs targeting Smad7

In human, Smad7 has been experimentally validated as target gene for multiple miRNAs, including miR-15b, miR-20a, miR-21, miR-92a and miR-106b (Bhagat *et al.*, 2013; Chang *et al.*, 2013; Ezzie *et al.*, 2012; Smith *et al.*, 2012) and also is predicted to be targeted by miR-17-5p (Kertesz *et al.*, 2007; Lewis *et al.*, 2005). In the present work, we found most of miRNAs to be inhibited by SFN treatment (figure 10A). Moreover, we found that SFN could not change the mRNA expression of Dicer (figure 10B), but down-regulated the protein abundance of Dicer by nearly 50% (figure 10C), which might explain the decreased level of miRNAs.

3.4.6 Effects of SFN on the expression of DNMTs and the methylation status of Smad7 promoter

DNA methylation is normally negatively associated with transcriptional activity. We measured the expression of DNMT and the CpG island methylation of Smad7 promoter with bisulfite sequencing. We found that DNMT1, not DNMT3a, was supressed by SFN (figure 11A), where DNMT3b was undetectable. However, the studied CpG sites were found to be unmethylated and their methylation level was unaffected by SFN (figure 11B).

3.5 Discussion

Diminished satellite cell regenerative capacity and decreased satellite cell number underline many muscle related diseases, like Duchenne muscular dystrophy. Transplantation of the satellite cells is a way to cure these diseases. Another potential therapy is to increase the activity of satellite cells. In present study, we found that SFN at low level could greatly increase the activity and proliferation of porcine satellite cells by supressing TGF-β signalling pathway. Herein, we may supply a new clue for therapy for muscle diseases related with defected satellite cells. On the other hand, satellite cells play a critical role of in the postnatal muscle growth. Thus, it is possible to improve the meat production with SFN as a forage supplement.

SFN has received substantial attention because of its potential application in cancer therapy. The present study, for the first time, investigated the effect of SFN on the activation and proliferation of muscle stem cells and the underlying mechanisms. The mechanisms in which SFN influences cells are complex. The responses of cells to SFN treatment depend on the time, dose and also the cell genetic background. In this study, low doses of SFN (5 and 10 μM) enhanced the proliferation of porcine satellite cell, but, in contrast, high dose of SFN (20 μM) inhibited the proliferation of porcine satellite cells. It is probable that SFN at 15 μM has already shown its cytotoxicity by inducing the apoptosis of porcine satellite cells, which is consistent with the inhibited proliferation of porcine satellite cells at a dose 20 μM. The similar results are also observed in study about human mesenchymal stem cells (Zanichelli *et al.*, 2012). This dose-dependent effect reflects multiple signalling pathways that SFN could influence. The different responses of cells might result form the integration of multiple signalling pathways.

SFN is reported to inhibit the differentiation of C2C12 cells (Whitman *et al.*, 2013) and protect rat from the exercise-induced muscle damage (Malaguti *et al.*, 2009). In C2C12 cells, TSA, another well-studied HDAC inhibitor, increased genes related to myoblast proliferation but inhibited genes associated with myoblast differentiation (Hagiwara *et al.*, 2011). Myogenic regulatory factors, including MyoD, Myf5, MRF4 and myogenin, are the master regulators in the myogenic lineage commitment and activation and differentiation of satellite cells (Tapscott, 2005). Myf5 mRNA, not protein, is present in the quiescent satellite cells (Crist *et al.*, 2012). After satellite cells are activated, Myf5 protein is accumulated (Crist *et al.*, 2012) and the transcription of MyoD is turned on as well. The transcription of MYOG is induced when myoblast enters in the differentiation program. Additionally, a small part of activated satellite cells stop expressing MyoD and return to a quiescent state. Based on our results, SFN treatment impaired myogenic differentiation by decreasing the expression of Myf5, MyoD and MYOG. It is reported that early up-regulation of MyoD leads to pre-maturation of satellite cells and the formation of tiny muscle groups (Brohl *et al.*, 2012). Thus, SFN decelerated the progress of myogenesis and, at the same time, enhances the proliferation of satellite cells, resulting in abundant amount of myoblasts for differentiation.

MEF2 family is MADS (MCM1, agamous, deficiens, serum response factor)-box transcription factor. In vertebrates, MEF2 family includes four members, MEF2A,

MEF2B, MEF2C and MEF2D. MEF2D protein can be found in the undifferentiated myoblasts (Breitbart *et al.*, 1993), and MEF2A and MEF2C protein are only present in differentiated myotube (McDermott *et al.*, 1993; Yu *et al.*, 1992). In Drosophila, MEF2 is essential for myogenesis (Bour *et al.*, 1995). In mammals, however, MEF2 alone can not drive myogenesis and has to cooperate with myogenic transcription factors, like MyoD or myogenin, to promote myogenic differentiation (Molkentin *et al.*, 1995). In this study, although SFN increased MEF2 family members, the myogenic potential of MEF2 was compromised by the reduced expression of MyoD and MYOG in the presence of SFN. The recumbent of MEF2 to the promoter of MYOG and MyoD activity were inhibited by HDAC inhibitors (Nebbioso *et al.*, 2009). These were evidenced by depressed MYOG, whose transcription is induced by MEF2 and MyoD. Besides cooperation with myogenic transcription factors, it is found that MEF2 and Notch synergistically cause general hyperproliferation in Drosophila (Pallavi *et al.*, 2012). Thus, another potential pathway in which SFN enhances proliferation of porcine satellite cells is to strengthen the synergy between MEF2 and Notch, which needs to be further investigated.

SFN is a potent inhibitor of HDACs (Myzak *et al.*, 2004) and inhibits the HDAC activity in a competitive way. However, the effects of SFN on expression of HDAC members are not consistent among the previous reports. In HeLa cells, SFN decreased the protein abundance of HDAC3 and HDAC6 (Rajendran *et al.*, 2013). In human embryonic kidney 293 cells, SFN had no effect on the protein level of nuclear located HDAC1 (Myzak *et al.*, 2004). In line with previous study, we also found that SFN significantly inhibited the HDAC activity in porcine satellite cells (figure 7B). Furthermore, we measured the mRNA level of different HDAC members. Results indicated that SFN might indirectly regulate the transcription of individual HDAC members. The activity of individual HDAC member is selectively regulated by different mechanisms (Di Giorgio *et al.*, 2015; Gupta *et al.*, 2012; McKinsey, 2011), as the transcription regulation of HDAC members. This could explain the divergent responses of HDAC members to SFN treatment. The members of class IIa HDACs can physically interact with MEF2 family members and inhibit the MEF2-dependent transcription to interfere the myogenic program (Lemercier *et al.*, 2000; Lu *et al.*, 2000a; Miska *et al.*, 1999; Zhou *et al.*, 2001). In the present work, the activity of HDACs was significantly

inhibited by SFN, which might sequentially relief the inhibition of HDAC on MEF2-dependent transcription.

TGF-β signalling has been shown to inhibit many steps of myogenesis (Burks and Cohn, 2011). Both TGF-β1 and MSTN belong to TGF-β superfamily and activate the same intracellular downstream mediator, Smad2/3. MSTN inhibits the activation and self-renewal of satellite cells and halts myoblast in G_1 phase of cell cycle by up-regulating p21 (McCroskery *et al.*, 2003). Excessive level of TGF-β, instead of MSTN, in aged muscle impairs the stemness of satellite cell by decreasing the proliferation of satellite cells by upregulating the CDK inhibitors, including p15, p16, p21 and p27, in a pSmad3 dependent way (Carlson *et al.*, 2008). Inhibition of TGF-β signalling by a TGF-β receptor kinase inhibitor was in favor of muscle regeneration (Carlson *et al.*, 2009). We found that the protein, not mRNA, level of TGF-β1 was inhibited by SFN, which was concomitant with enhanced proliferation of porcine satellite cells. Thus, SFN probably regulates TGF-β1 expression in a post-transcriptional way and TGF-β signalling pathway might play an important role in the influence of SFN on the proliferation of porcine satellite cells.

Smad7 negatively regulates the activity of TGF-β signalling pathway by inhibiting the phosphorylation of Smad2/3. Smad7 was upregulated by HDAC inhibitors in nasal polyp-derived fibroblasts (Cho *et al.*, 2012) and breast cancer cell lines (Salot and Gude, 2012). In the present study, both mRNA and protein level of Smad7 were greatly increased by SFN. Thus, besides down-regulating TGF-β1 protein abundance, SFN also inhibited the activity of TGF-β signalling by increasing the expression of Smad7. It has been shown that over-expression of Smad7 could lead to enhanced skeletal muscle differentiation and cellular hypertrophy (Kollias *et al.*, 2006). The cellular location of Smad7 is very important for the Smad7 function. Smad7 is a nuclear protein and could translocate to cytoplasm to repress the activity of TGF-β signalling. Independent of TGF-β signalling, nucleus located Smad7 was able to enhance MyoD transcriptional activity to enhance myogenesis (Miyake *et al.*, 2010). However, the expression of MyoD was supressed by SFN in the present study. Based on these results, the effects of SFN is mainly mediated by the function of cytoplasm located Smad7, instead of nuclear located Smad7. Thus, it will be very interesting to investigate the effect of SFN on the shuttle of Smad7 between nucleus and cytoplasm.

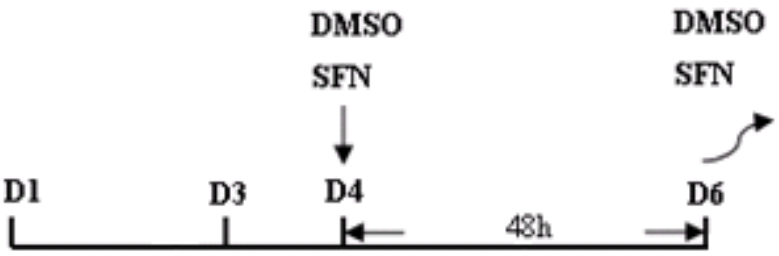
As discussed above, Smad7 may represent a key nodal point in the effects of SFN on the porcine satellite cells. On one side, the up-regulated Smad7 enhanced proliferation of porcine satellite cells by supressing the TGF-β signalling and, on the other side, myogenic function of Smad7 was disturbed by SFN to retard the progress of myogenesis. Thus it is very interesting to dissect the mechanisms in which SFN positively regulates expression of Smad7. The transcription of Smad7 can be induced by a number of transcription factors (Band *et al.*, 2009; Dole *et al.*, 2009; Freudlsperger *et al.*, 2013; Nagarajan *et al.*, 1999; von Gersdorff *et al.*, 2000) and the binding sites for multiple transcription factors, like STAT5, NF-κB1, CEBPbeta, and Nrf2, were found in the promoter of Smad7 (Messeguer *et al.*, 2002). As shown in the results, the mRNA level of STAT5, NF-κB1, CEBPbeta and Nrf2 was increased by SFN, although the protein abundance CEBPbeta and NF-κB1 were unaffected or slighted reduced. The Nrf2 signalling is a major pathway SFN activates (Kensler *et al.*, 2012). Thus, to a certain degree, the increased Nrf2 may contribute to the transcription activation of Smad7. In addition to transcription level, Smad7 is also regulated on the post-transcriptional level. Up to now, Smad7 has been proven to be the target gene for multiple miRNAs (Bhagat *et al.*, 2013; Chang *et al.*, 2013; Dattaroy *et al.*, 2015; Ezzie *et al.*, 2012; Jia *et al.*, 2014; Kan *et al.*, 2015; Li *et al.*, 2013; Li *et al.*, 2014; Lin *et al.*, 2014; Liu *et al.*, 2014; Smith *et al.*, 2012; Xia *et al.*, 2013; Zhang *et al.*, 2014a). In the present work, most of measured miRNAs were down-regulated by SFN, which could be partly due to the decreased Dicer by SFN. It is first time to state that SFN might inhibit the miRNA biogenesis by suppressing Dicer. Furthermore, we also found SFN could inhibit the expression of DNMT1, which was also shown in our previous study (Fan *et al.*, 2012) and in the work with human breast cancer cells (Meeran *et al.*, 2010). Thus, we used the bisulfite sequencing to analyze the methylation of CpG island in the Smad7 promoter region. However, CpG sites in Smad7 promoter turned out to be unmethylated and their methylation level was not sensitive to SFN treatment. This result is normal since most of CpG sites are unmethylated (Eckhardt *et al.*, 2006) and DNA methylation, compared with histone modifications, is much more stable [12-14]. However, it is still a good idea to analysis the effect of SFN on the whole genome methylation without bias.

In summary, our studies provide a model how SFN retards the progress of myogenesis and enhances the proliferation of porcine satellite cells, where SFN alters expression of

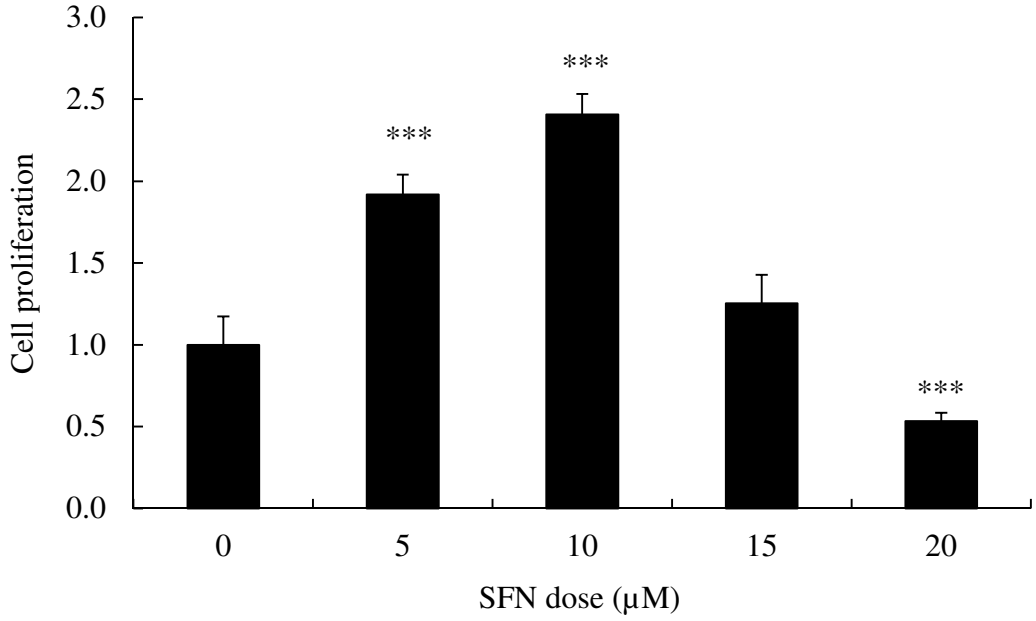
myogenic regulator factors, the expression and activity of epigenetic enzymes, and suppresses TGF-β signalling by increasing Samd7.

Figure 6

A:


DMSO
SFN
DMSO
SFN
D1
D3
D4
48h
D6

B:


Cell proliferation
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
0
5
10
15
20
SFN dose (μM)

C:

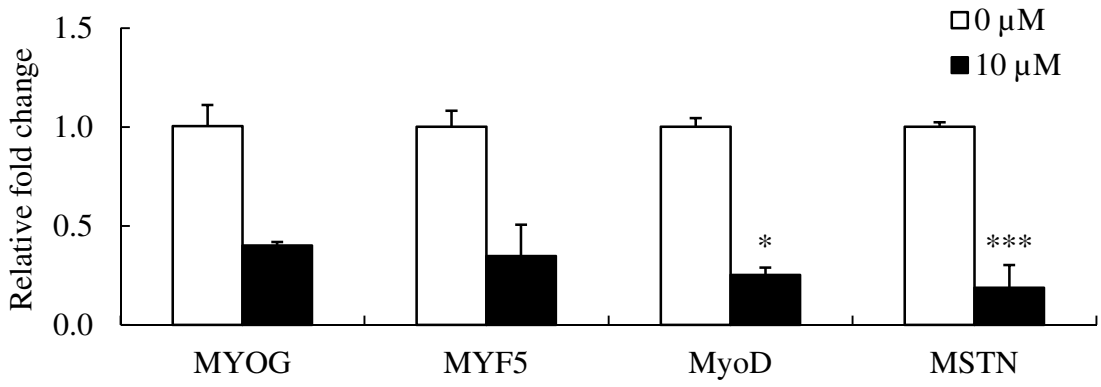

Relative fold change
1.5
1.0
0.5
0.0
0 μM
10 μM
*
***
MYOG
MYF5
MyoD
MSTN

Figure 6 continued

D:

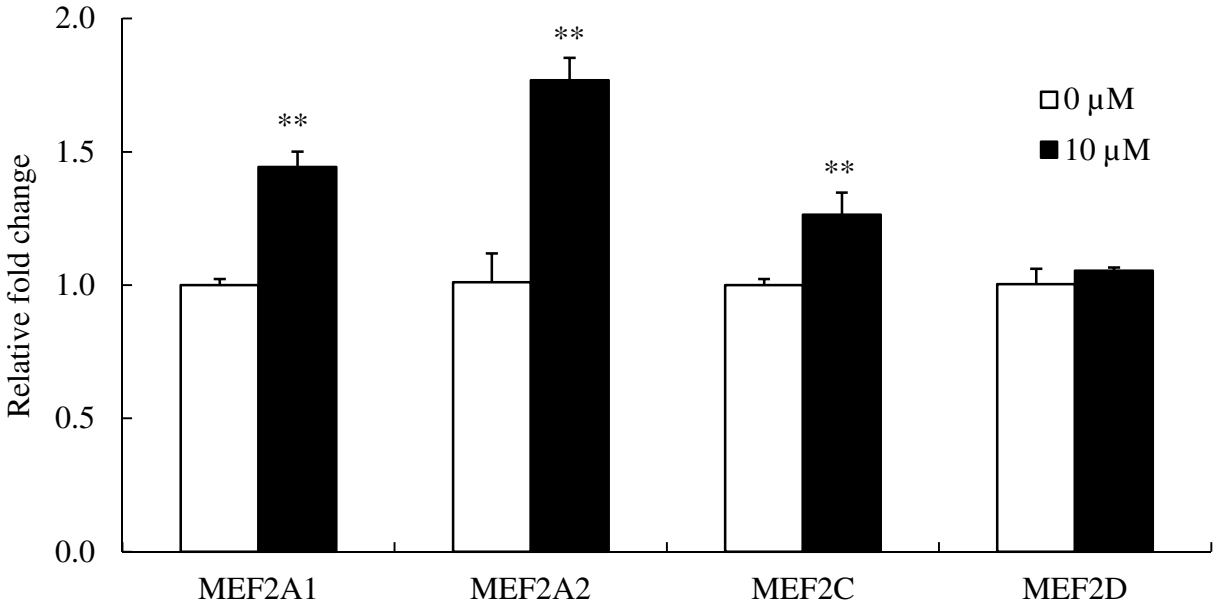
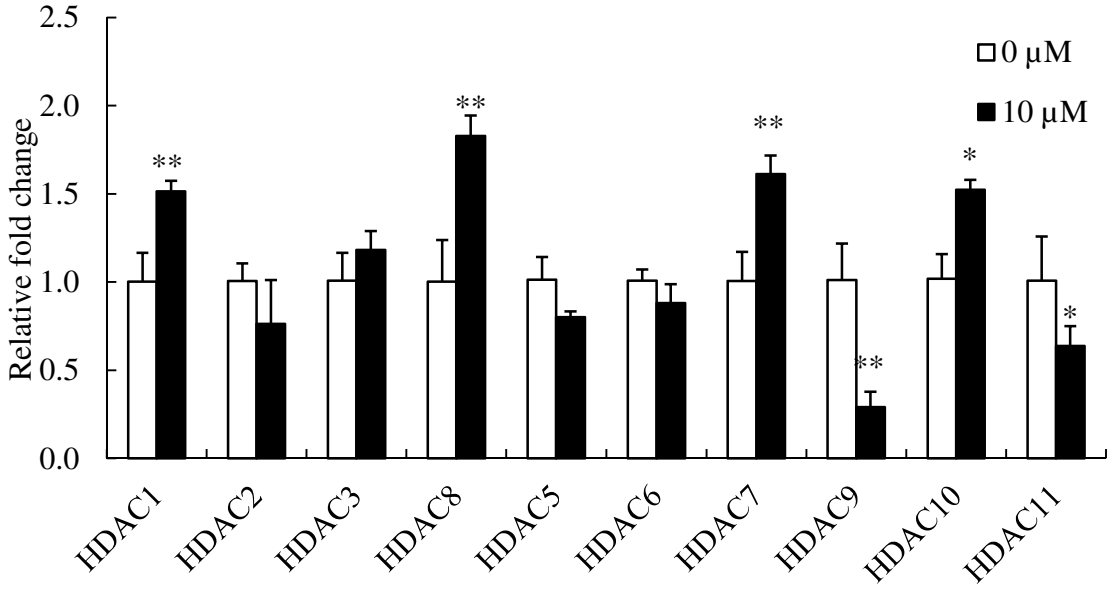


Figure 6: SFN affected porcine satellite proliferation and myogenesis related factors. (A) Cell culture program is shown in figure 6A. Cell proliferation was determined by WST-1 kit (B). qRT-PCR was carried out to quantify Myogenic regulatory factors (C) and MEF2 family members (D). The results represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate.

Figure 7

A:


2.5
2.0
1.5
1.0
0.5
0.0
Relative fold change
0 μM
10 μM
**
**
**
**
*
*
HDAC1
HDAC2
HDAC3
HDAC8
HDAC5
HDAC6
HDAC7
HDAC9
HDAC10
HDAC11

B:

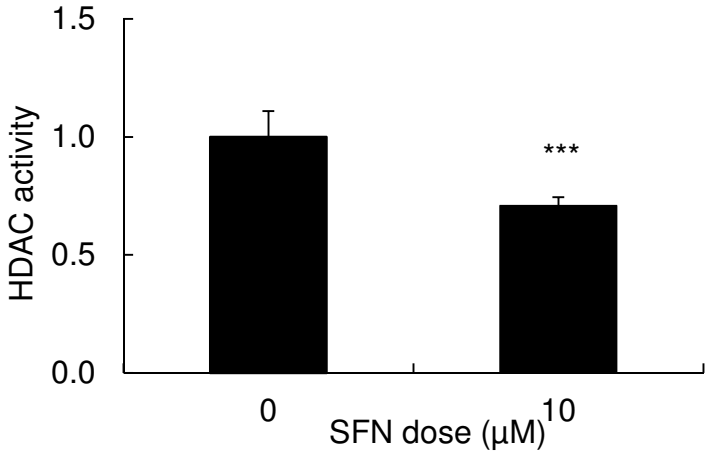
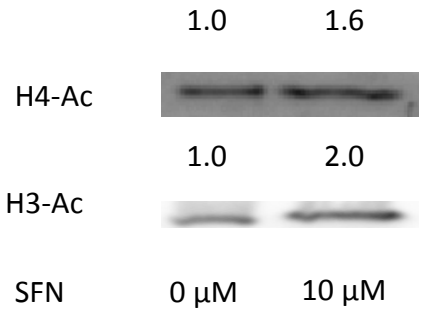

1.5
1.0
0.5
0.0
HDAC activity
***
0
10
SFN dose (μM)

Figure 7 continued

C:



D:

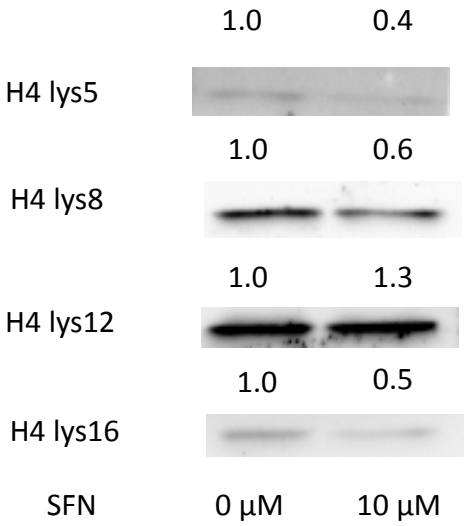
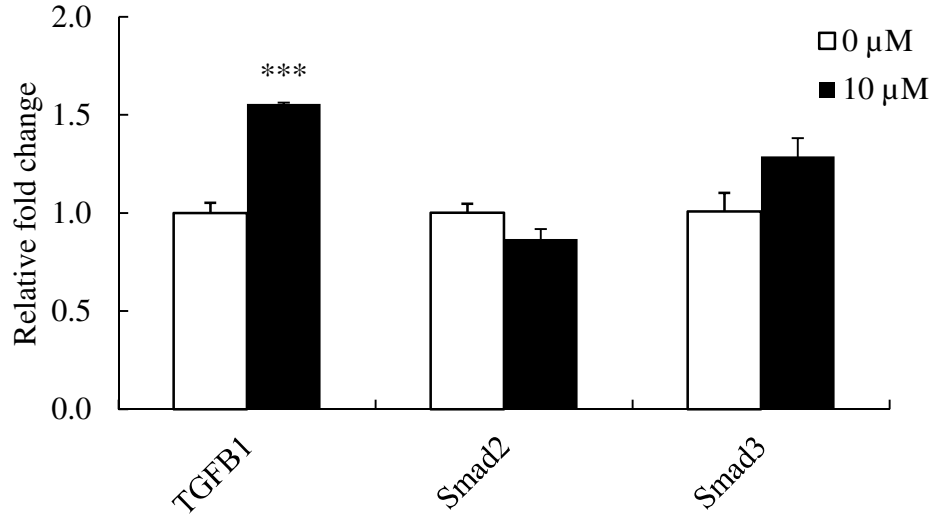


Figure 7: Effects of SFN on the expression of HDAC members and the acetylation of Histone H3 and H4. Relative HDAC mRNA expression (A) and activity (B) was examined using qRT-PCR and the Color-de-Lys HDAC colorimetric activity assay kit, respectively. Global and local acetylation of H3 and H4 (C) and individual lysine site of H4 (D) were measured by Western blot. The results represent the mean ± standard deviations (SD) of three independent experiments each performed in duplicate.

Figure 8

A:


2.0
1.5
1.0
0.5
0.0
Relative fold change
***
0 μM
10 μM
TGFB1
Smad2
Smad3

B:

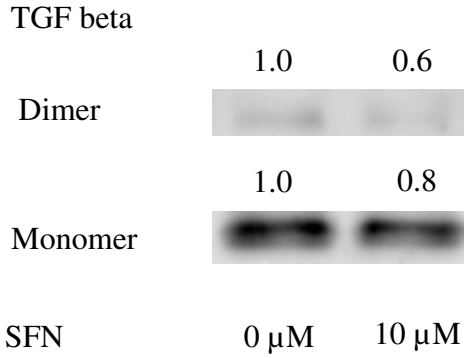
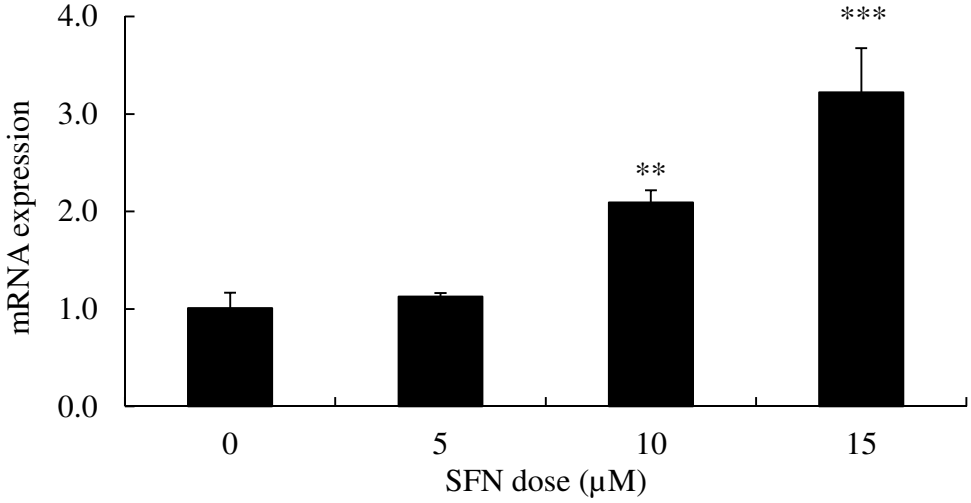

TGF beta
1.0
0.6
Dimer
1.0
0.8
Monomer
SFN
0 μM
10 μM

Figure 8 continued

C:



D:

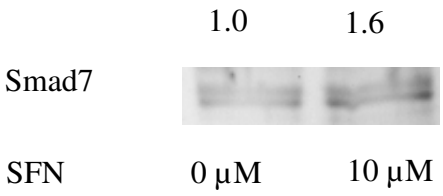
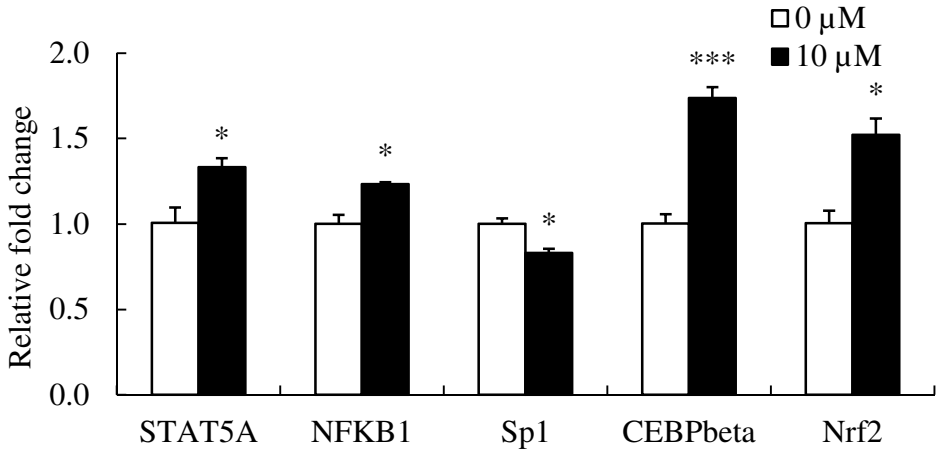


Figure 8: Effects of SFN on TGF-β signalling pathway. Relative TGF- β1 and Smad2/3 mRNA expression (A) and Smad7 (C) were examined using qRT-PCR. The protein level of TGF- β1 (B) and Smad7 (D) measured by Western blot.

Figure 9

A:



B:

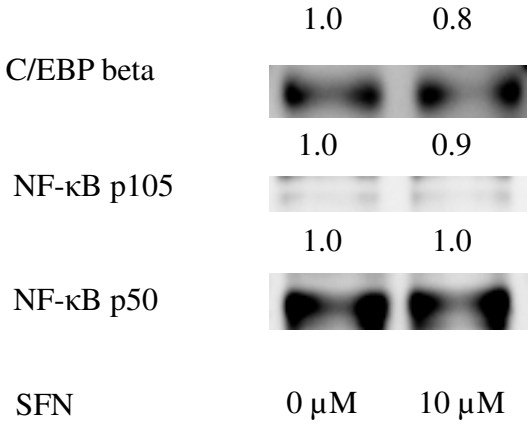
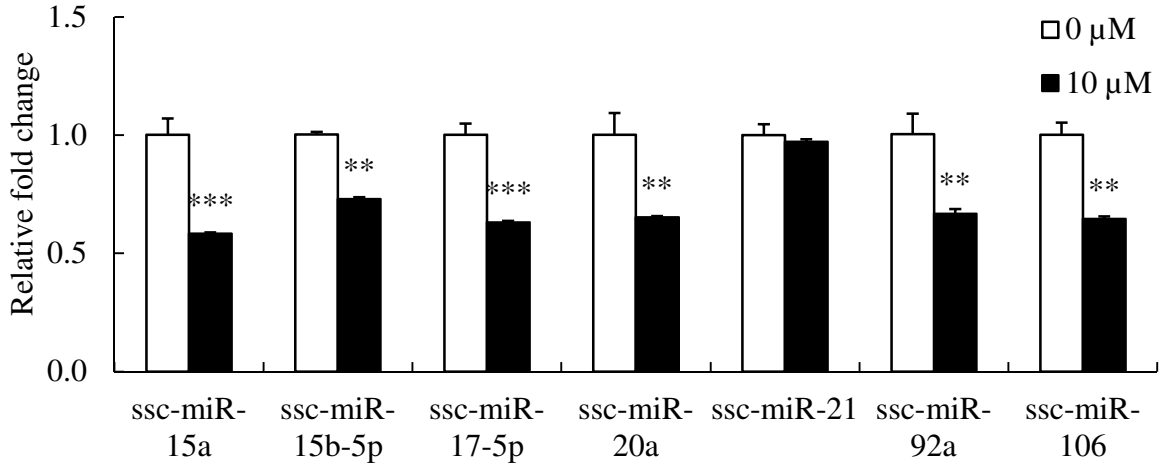


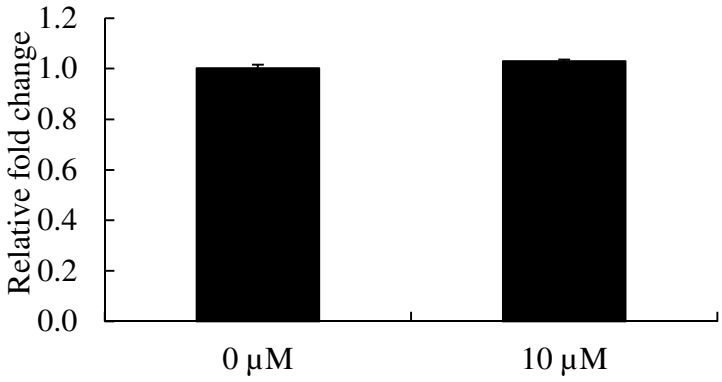
Figure 9: Effects of SFN on transcription factors of Smad7. Relative STAT5A, NF-κB1, Sp1, C/EBPbeta and Nrf2 mRNA expression (A) were examined using qRT-PCR. The protein level of C/EBPβ and NF-κB1 (B) were measured by Western blot.

Figure 10

A:



B:



C:

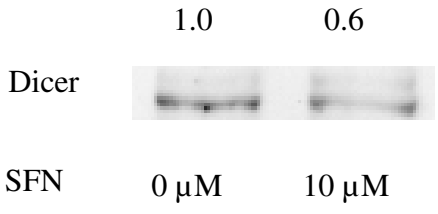
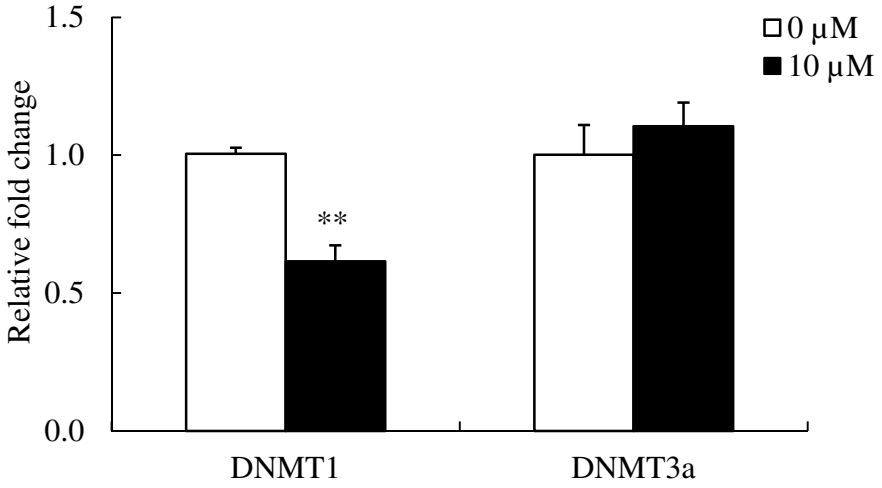


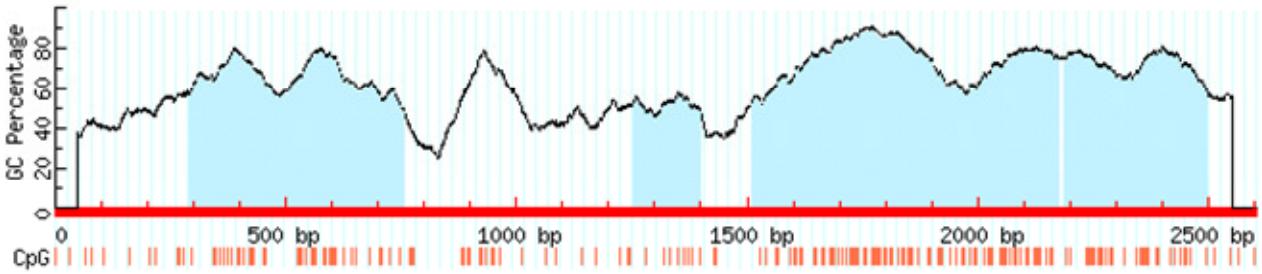
Figure 10: MiRNAs targeting Smad7 were down regulated by SFN treatment. qRT-PCR was carried out to quantify ssc-miR-15a, ssc-miR-15b-5p, ssc-miR-17b-5p, ssc-miR-20a, ssc-miR-21, ssc-miR-92a and ssc-miR-106 expression level (A). The mRNA (B) and protein (C) of Dicer was measured with qRT-PCR and Western blot respectively.

Figure 11

A:



B:



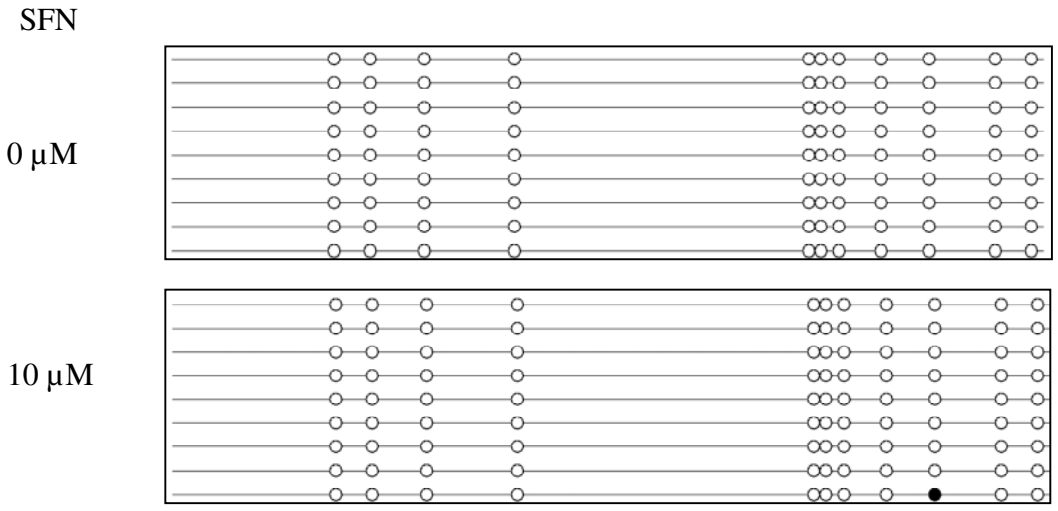


Figure 11: Effects of SFN on DNMTs expression and the methylation status of Smad7 promoter. Relative DNMT1 and DNMT3a mRNA expression (A) were examined with qRT-PCR. (B) The methylation status of CpG island in Smad7 promoter was measured with bisulfite sequencing.

Table 8: List of primer sequences used in the Chapter 3

Gene	Sequence (5' - 3')	Accession
Smad7	F: CCAACTGCAGACTGTCCAGA	HM803236
	R: CAGGCTCCAGAAGAAGTTGG	
HDAC1	F: GGAAATCTATCGCCCTCACA	XM_003356305.2
	R: AAACACCGGACAGTCCTCAC	
HDAC2	F: AACCTGCTGCTTGGAGAAAA	XM_001925318.3
	R: ACCATCAGGATGCAAAGCTC	
HDAC3	F: CAACCAGGTGGTGGACTTCT	NM_001243827.1
	R: GCAGAGGGATGTTGAAGCTC	
HDAC5	F: AGATGCACTCCTCCAGTGCT	XR_135351.1
	R: GGATGATGGCAAATCCATTC	
HDAC6	F: ATGGACGGGTATTGCATGTT	XM_003360315.2
	R: GCGGTGGATGGAGAAATAGA	
HDAC7	F: CGTCCCCTACAGAACTCTCG	XM_003355640.2
	R: TCAGGTTGGGCTCAGAGACT	
HDAC8	F: GGTGACGTGTCTGATGTTGG	XM_003360365.2
	R: AGCTCCCAGCTGTAAGACCA	
HDAC9	F: AACTGAAGCAACCAGGCAGT	XM_003122063.2
	R: CCCAACTTGTCCCAGTGAGT	
HDAC10	F: TCCATCCGAGTACCTTCCAC	XM_003362070.1
	R: GGCTGCTATGGCCACACTAT	
HDAC11	F: GACAAGCGCGTGTACATCAT	XM_003483230.1
	R: AGGTTCCTCTCCACCTTCGT	
DNMT1	F: GCGGGACCTACCAAACAT	DQ060156
	R: TTCCACGCAGGAGCAGAC	
Dnmt3a	F: CTGAGAAGCCCAAGGTCAAG	NM_001097437
	R: CAGCAGATGGTGCAGTAGGA	
Dnmt3b	F: AATCGCAACAGGGTACTTGG	NM_001162404
	R: TGATATTCCCCTCGTGCTTC	
MEF2A1	F: TCCCACACTAGCTTGCAGAA	NM_001097421.1
	R: TGCTTTCTTGGTTCCTGCTT	
MEF2A2	F: TGATGCGGAATCATAAAATCG	NM_001099698.1
	R: GCACCAGTAGTTCCAACCAAA	
MEF2C	F: CGAGATACCCACAACACACG	NM_001044540.1
	R: CGCTTGACTGAGGGACTTTC	
MEF2D	F: TCACTGCAGTTCAGCAATCC	XM_003125698.3
	R: AGGCCAGGAGACACACTGTT	
STAT5A	F: GAGGTGCTGAAGAAGCATCA	NM_214290.1
	R: GGCTTCAGATTCCACAGGTT	
NF-kB1	F: TGGGAAAGTCACAGAAACCA	NM_001048232.1
	R: CCAGCAGCATCTTCACATCT	

Sp1	F: TGCAGCAGAATTGAGTCACC	XM_003355406
	R: ACTGCTGCCACTTTGTTCCT	
CEBPbeta	F: GCTTGAACAAGTTCCGFAGG	XM_003483983.1
	R: CAAGAAGACCGTGGATAAGC	
TBP	F: GCAGCACAGTACGAGCAACT	DQ845178.1
	R: ACGTTCGGTTTAGGTTGCAG	
Nrf2	F: GTGCCTATAAGTCCCGGTCA	XM_003483682.1
	R: ATGCAGAGCTTTTGCCCTTA	
MYOG	F: CAGTGAATGCAGTTCCCACA	NM_001012406.1
	R: GGTGAGGGAGTGCAGATTGT	
MYF5	F: AGACGCCTGAAGAAGGTCAA	NM_001278775.1
	R: TCCTGCAGGCTCTCAATGTA	
MyoD	F: TGCAAACGCAAGACCACTAA	GU249575
	R: GCTGATTCGGGTTGCTAGAC	
MSTN	F: GATTATCACGCTACGACGGA	AY448008
	R: CCTGGGTTCATGTCAAGTTTC	
MEF2A1	F: TCCCACACTAGCTTGCAGAA	NM_001097421.1
	R: TGCTTTCTTGGTTCCTGCTT	
MEF2A2	F: TGATGCGGAATCATAAAATCG	NM_001099698.1
	R: GCACCAGTAGTTCCAACCAAA	
MEF2C	F: CGAGATACCCACAACACACG	NM_001044540.1
	R: CGCTTGACTGAGGGACTTTC	
MEF2D	F: TCACTGCAGTTCAGCAATCC	XM_003125698.3
	R: AGGCCAGGAGACACACTGTT	
TGFB1	F: CGTGCTAATGGTGGAAAGCG	NM_214015.1
	R: AGAGCAATACAGGTTCCGGC	
Smad2	F: GCAATCTTTGTGCAGAGCCC	NM_001256148.1
	R: ACACGGCTTCAAAACCCTGA	
Smad3	F: GCTGGACGACTACAGCCATT	NM_214137.1
	R: TGTGGTTCATCTGGTGGTCG	
Dicer	F: AACCCGAGAGTTGCCTGATG	NM_001197194
	R: ACAGCAAATGAGAGCCACGA	
HPRT1	F: AACCTTGCTTTCCTTGGTCA	NM_001032376.2
	R: TCAAGGGCATAGCCTACCAC	
ssc-miR-15a	F: TAGCAGCACATAATGGTTTGT	MIMAT0007753
ssc-miR-15b	F: TAGCAGCACATCATGGTTTACA	MIMAT0002125
ssc-miR-17-5p	F: CAAAGTGCTTACAGTGCAGGTAG	MIMAT0007755
ssc-miR-20a	F: TAAAGTGCTTATAGTGCAGGTA	MIMAT0002129
ssc-miR-21	F: TAGCTTATCAGACTGATGTTGA	MIMAT0002165
ssc-miR-92a	F: TATTGCACTTGTCCCGGCCTGT	MIMAT0013908
ssc-miR-106	F: AAAAGTGCTTACAGTGCAGGTAGC	MIMAT0002118
Smad7Bis	F: TGATTTTTAAGTATTTTGAAAGTTG	
Smad7Bis	R: TCTCTAAAATACATTTAACTAACTAAC	

Chapter 4 General discussion and conclusion

Pork is the world's most widely eaten meat and takes around 40% of meat in the market. As the animal welfare is receiving more and more attention, the pig industry has tried to decrease the required pigs to meet the demand of market. The objective of pig production is to supply the market with enough pork of high quality. Muscle growth and meat quality are two important economic traits in the pig breeding program. The muscle growth and meat quality are closely associated with each other, as discussed in the section 1.5 of chapter I. The objective of this study is to supply some suggestive clues to increase muscle growth and meat quality from the genetic and epigenetic aspect.

Nowadays, meat quality has received more and more attention. Meat quality greatly influences consumer purchasing decisions (Papanagiotou *et al.*, 2013). One objective of the pig breeding project is to increase the production of pork with high meat quality. The farmers select high productive breeds with good meat character for pig industry by using pig breeding systems. Compared with traditional phenotype-based selection systems, MAS is an indirect and more efficient selection (Dekkers, 2004). One critical step in MAS is to identify molecular markers related with interested traits. It will be great valuable to identify molecular markers that are related with multiple interested phenotypes. Those pleiotropic markers can be used to optimize the breeding program for the balanced improvement of discrete traits.

SOX-6 is a transcriptional factor and also plays a vital role in muscle fiber differentiation. Multiple myosin and sarcomeric genes are transcriptionally impeded by SOX-6 to inhibit the slow muscle fiber differentiation (Hagiwara, 2011; Quiat *et al.*, 2011). Thousands of SNPs have been found in the porcine SOX-6 locus (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?chooseRs=all&locusId=397173&mrna=XM_003122960.4&ctg=NW_003609572.1&prot=XP_003123008.4&orien=forward&refresh=refresh). The chapter II is the first work to focus on the relationship between polymorphisms of porcine SOX-6 with porcine growth, carcass and meat quality traits. The two studied SNPs, rs81358375 and rs321666676, of porcine SOX-6 are associated with multiple indices of growth, carcass and meat quality traits, as shown in Chapter II.

In the chapter II, SOX-6 is associated with meat quality, carcass and growth traits at the same time. In Pietrain population, pigs with AA for rs81358375 had high $pH1_L$, high back fat deposition and short carcass length, which indicate high meat quality but low

production efficiency. These suggest that rs81358375 could be a valuable molecular maker for pig breeding for high meat quality. The genotype CC of rs321666676 could not be detected in the Pietrain and DuPi population, which can be due to the genetic drift or its lethal effect. The genomic location of porcine SOX-6 is closed to many QTLs, which are related with backfat, abdominal fat, body weight and daily gain. Especially for backfat and daily gain, the genomic location of SOX-6 overlaps with several related QTLs (Table 7). The association between QTLs and interested traits is found in nine pig breeds, which strongly supports the candidacy of SOX-6.

Furthermore, SOX-6 had lower protein abundance in meat with high $pH1_L$ value, in spite of the not significant different mRNA expression. SOX-6 transcriptionally inhibits the differentiation of slow myofibers. The mutation of SOX-6 in mice led to the loss of fetal fast muscle characteristics, in term of slow myosin heavy chain MyHC-beta expression (Hagiwara *et al.*, 2007). The decrement of SOX-6 abundance might increase the amount of muscle fiber with slow-fiber characteristics. Compared with the fast muscle fiber, the slow muscle fiber has higher oxidative capacity and lower glycogen storage (Fernandez and Tornberg, 1991), which is positively associated with meat quality (Karlsson *et al.*, 1999; Klont *et al.*, 1998). SOX-6 might contribute to the formation of meat quality by modulating the differentiation of muscle fiber.

On the other side, SOX-6 regulates the cell proliferation. Down-regulation of SOX-6 enhanced the proliferation of insulinoma INS-1E and NIH-3T3 cells (Iguchi *et al.*, 2007). SOX-6 reduced the transcription activity of cyclin D1 by direct interaction with HDAC1 (Iguchi *et al.*, 2007). SFN treatment might also have an impact on the activity of SOX-6 by inhibiting the HDACs activity. We quantified the expression of SOX-6 in the porcine satellite cell after SFN treatment for 48 hours. However, we did not found a significant effect of SFN treatment on the mRNA level of SOX-6 (unpublished data), although the protein level of SOX-6 remains to be investigated.

SOX-6 has a long 3'UTR and is a target gene for multiple miRNAs, including miR208a, miR-208b and miR-499 (van Rooij *et al.*, 2009). miR-208a, miR-208b and miR-499 are transcribed from the intron of α-MHC, β-MHC and Myh7b. The polymorphisms of miRNA genes and their binding sites have been proven to be associated with the pig litter size and fatness (Lei *et al.*, 2011; Shao *et al.*, 2011). Furthermore, the divergence of trait performance among different pig breeds is partially due to the breed-specific

miRNA expression profile (Chen *et al.*, 2012; Li *et al.*, 2012; Timoneda *et al.*, 2013). In the SOX-6 mutant mice p^{100H}, compared with wild type, the expression level of slow fiber isoform genes were greatly higher and fast fiber isoform genes were significantly lower (Hagiwara *et al.*, 2005). Furthermore, SOX-6 can repress the expression of β-MHC by binding to its promoter (Hagiwara *et al.*, 2007). As aforementioned above, SFN treatment has no effect on the transcriptional activity of SOX-6 in the porcine satellite cells. However, the expression of ssc-miR-499-5p was enhanced by the SFN treatment (unpublished data), which is also observed in the high $pH1_L$ pig group as described in the Chapter II. Thus, the expression of SOX-6 might be manipulated by SFN in a post-transcriptional way through miRNA pathway. This could be one possible mechanism in which SFN as a dietary supplement influences muscle characteristics and meat quality. Furthermore, the effect of SFN on the slow/fast fiber genes, like β-MHC and Myh7b, is also interesting to be investigated.

Muscle growth consists of prenatal growth and postnatal growth. The main part of postnatal growth is muscle hypotrophy, which is characterized by the great increment of myofiber size and myonuclei amount (Koohmaraie *et al.*, 2002). Satellite cells, the muscle stem cell, are responsible to supply new myonuclei for enlarging myofibers during postnatal growth (Moss and Leblond, 1971). TGF-β signalling regulates the activation, proliferation and differentiation of satellite cells (Allen and Boxhorn, 1987). MSTN, a member of TGF-β superfamily, inhibited the proliferation of myoblast through the induction of p21, a CDK inhibitor (Thomas *et al.*, 2000). The mutation of MSTN led to the generalized hyperplasia and hypertrophy of skeletal muscle (McPherron *et al.*, 1997). In the previous study, we found that SFN could inhibit the expression of MSTN (Fan *et al.*, 2012).

It was found that low dose (0.25 – 5 μM) SFN enhanced the proliferation of human mesenchymal stem cells, while the high dose (25 μM) SFN shown the opposite effect (Zanichelli *et al.*, 2012). In line with this, we also found that SFN (5 and 10 μM) increased the proliferation of satellite cells and SFN (20 μM) shown the cytotoxicity by inhibiting the activity of satellite cells. As we know, TGF-β negatively regulated the proliferation of satellite cells (Allen and Boxhorn, 1987) and the inhibition of Smad2/3 enhanced the muscle growth (Sartori *et al.*, 2009). Thus, we suspect that SFN enhanced the proliferation of satellite cells by suppressing the activity of TGF-β signalling pathway.

SFN was found to inhibit the activity or expression of TGF-β in human hepatic stellate cell line (Oh *et al.*, 2012) and the type 2 diabetic mice (Zhang *et al.*, 2014c). However, SFN also was shown to induce the TGF-β signalling in porcine monocyte-derived dendritic cells (Qu *et al.*, 2015) and colorectal cancer cells (Kaminski *et al.*, 2010). In the present work, we found that SFN inhibited the TGF-β signalling by decreasing the protein level of TGF-β and up-regulating the expression of Smad7. The inconsistent effects of SFN on TGF-β signalling pathway might be due to the different cell genetic background, applied dosage and treatment duration. Besides modulating the activation and proliferation of satellite cells, TGF-β can also influence the muscle fiber type formation. In the present of TGF-β, myotubes differentiate into slow muscle fibers, while the fast muscle fiber differentiation takes the priority when the TGF-β is absent (McLennan, 1993).

The muscle fiber type is also an important parameter for meat quality. Muscle fiber type can be divided into four categories: slow-oxidative or type I, fast oxido-glycolytic or type IIA and fast glycolytic IIX and IIB (Schiaffino and Reggiani, 1996). Higher proportion of fast-twitch glycolytic fibers is positively associated with postmortem pH decline (Choi *et al.*, 2007; Ryu and Kim, 2006). The fast-twitch glycolytic (IIB) is accompanied by the increased lightness and decreased WHC (Kim *et al.*, 2013b). The amount and size of type IIB fibers are related with the accumulation of IMF (Kim *et al.*, 2013a; Kim *et al.*, 2013b). The slow-twitch muscle has more collagen and is related with decreased meat tenderness (Kovanen *et al.*, 1984). The relative composition of fast-twitch and slow-twitch myofiber greatly influences the meat quality. As observed in our work, SFN treatment decreased the activity of TGF-β signalling, which regulates the differentiation of myofiber type and in turn influences the formation of meat quality.

Based on the above discussion, SFN not only modulates the activation of myogenic differentiation, but also might have effects on the formation of meat quality. Some of nutritional supplements have already been found to affect the meat quality. The dietary with additional vitamin D3 for young steers before slaughter ameliorated the DRIP of aged steaks (Hope-Jones *et al.*, 2012). It was also found that the dietary with magnesium significantly reduced DRIP (Alonso *et al.*, 2012). The oxidative capacity of meat is positively associated with meat quality. Compared with Large White pigs, Chinese Laiwu pigs have higher antioxidative enzymes activities, lower grade of lipid oxidation and higher antioxidative ability in muscle. Phaffia rhodozyma, an antioxidant,

is potential diet supplement for animal industry. The corn-soybean meal with phaffia rhodozyma could improve feed efficiency and meat quality of the finishing pig (Lei and Kim, 2014). SFN is a potent activator of Nrf2 signalling, which regulates the cellular antioxidant system. Thus, SFN also maintains the potential to be used as the diet supplement to improve the meat quality.

Smad7 is an endogenous inhibitor of TGF-β signalling. Smad7 suppresses the activity of TGF-β by interacting with cell membrane located TGF-β type I receptor in a negative feedback way (Hanyu *et al.*, 2001; Yan *et al.*, 2009). We found that SFN treatment increased the expression of Smad7 at both mRNA and protein level. Besides the cytoplasmic role of Smad7 in the regulation of TGF-β signalling, the nuclear located Smad7 has the ability to drive the myogenic genes expression, like MyoD, and promote myogenesis (Miyake *et al.*, 2010). The overexpression of Smad7 is in favor of the myotube differentiation and leads to cellular hypertrophy (Kollias *et al.*, 2006), which means the bigger size of myofiber. The size of myofiber or fiber bundle is associated with meat tenderness (Karlsson *et al.*, 1993). Thus, SFN might not only modulate the proliferation of satellite cells, but also has potential effects on the differentiation of satellite cells. However, combined with decreased MyoD by SFN treatment, the nuclear function of Smad7 was seemingly not enhanced in the present of SFN, while the translocation of Smad7 from nucleus into cytoplasm might be enhanced by SFN. In a recent study, SFN treatment by gavage significantly reduced the muscular dystrophy in mdx mice by increasing skeletal muscle mass, muscle force and running distance (Sun *et al.*, 2015). The observed enhanced proliferation of satellite cells by SFN in the present work could be one of the mechanisms underling effects of SFN on muscular dystrophy. In summary, SFN as diary supplement probably produces effects on muscle growth and meat quality through modulating the proliferation of satellite cells and muscle fiber differentiation.

Additionally, the protein level of Smad7 was increased by KEAP1 knockdown, which activated the Nrf2 signalling (Ryoo *et al.*, 2014). Nrf2 binding sites are found in the promoter region of porcine Smad7. SFN is a potent activator of Nrf2, which regulates the cellular oxidative stress response (Nguyen *et al.*, 2009). The expression of Nrf2 was increased up to nearly two times by SFN treatment in the present work. Thus, the activation of Nrf2 could be one of the possible molecular mechanisms, in which SFN increased the level of Smad7 mRNA and protein. However, the effects of SFN on the

protein level of Nrf2 and binding activity of Nrf2 on the promoter of Smad7 in porcine satellite cells still need further investigation.

Besides working as an activator of Nrf2, SFN is also an inhibitor of HDACs activity. Normally, HDACs negatively regulate the myogenesis. HDAC4, HDAC5 and HDAC7 interact with MEF2 and repress MEF2-dependent genes expression. The onset of myoblast differentiation was accompanied by the disassociation of HDAC4/5/7 from MEF2 (Dressel *et al.*, 2001; McKinsey *et al.*, 2000). TSA, one of the most potent HDAC inhibitors, promoted the proliferation of myoblast by increasing the expression of Myf5 and MEF2, but suppressed myoblast differentiation by impeding myogenin expression (Hagiwara *et al.*, 2011). In the present work, the activity of HDACs was inhibited by the SFN treatment, which was coincided with the enhanced proliferation of satellite cells. The significant inhibition of SFN by oral gavage on HDAC activity was found in mouse colonic mucosa (Myzak *et al.*, 2006). However, it was also found that SFN had little direct effects on the HDACs activity, but its main metabolites, SFN-cysteine and SFN-N-acetylcysteine, could strongly suppress HDACs activity in vitro (Myzak *et al.*, 2004). Thus, the transformation of SFN to its metabolites is a critical for the inhibition of SFN on HDACs activity.

In our study, the transcription levels of HDAC members were disturbed by SFN treatment. However, the effects of SFN on the expression of HDAC family members are inconsistent. In human embryonic kidney 293 cells, SFN had no effect on the protein level of nuclear located HDAC1 (Myzak *et al.*, 2004). However, the SFN treatment could decrease the protein level of HDAC1/4/6 in the human breast cancer cells (Meeran *et al.*, 2012). In human colon cancer cells, SFN decreased the protein level of HDAC3 and HDAC6, but not the other class I and II HDAC members (Rajendran *et al.*, 2013).

This thesis is composed of two parts, genetic part and epigenetic part. My study tries to supply the applicable molecular markers and suggestive clues for pig industry in both genetic and epigenetic way. Genetics and Epigenetics describe two ways of phenotype transgenerational inheritance, which assist living organisms in adapting to their environment. Genetic variation is relatively stable and does not directly relay on the external environment, while epigenetic variation is easily modified and closely related with environment cues (Mazzio and Soliman, 2014). Compared with the genetic

variation, epigenetic modification is flexible and reversible. Epigenetic variation improves the adaptation of living organism in a relative short time, which variates from one to several generations (Skinner and Guerrero-Bosagna, 2009; Titus-Ernstoff *et al.*, 2008). However, the mechanism how epigenetic modification interacts with genetic variation is not yet fully understood. It is a tendency to apply the genetic and epigenetic technologies in pig industry.

In conclusion, my work identified one useful candidate gene, SOX-6, for pig breeding and investigate the effects of SFN on the proliferation of muscle stem cells, satellite cells. This thesis has tried to provide some preliminary clues for the increment of meat quality and muscle growth in the genetic and epigenetic way.

Summary

Both animal welfare and the efficiency of pork production are important in the pig industry. The objective of pig industry is to supply high quality and sustainably pork. Muscle growth and meat quality are two important factors directing the pig industry. The variation of meat quality can be partially contributed to genetic variations (Sellier and Monin, 1994). For the early stage of artificial selection, marker-assisted selection shows big efficiency (Lande and Thompson, 1990). One of the important steps in marker assisted breeding is to identify more polymorphisms, candidate genes and QTLs for interested traits. Besides the genetic factors, the environmental factors, like dietary supplement, also greatly influence the muscle protein accretion and muscle growth rate (Bell *et al.*, 1998). Furthermore, the nutrients, like methylating micronutrients, in F0 pigs has significant effects on the meat quality and carcass traits of F2 pigs, which is termed as transgenerational epigenetic inheritance (Braunschweig *et al.*, 2012). Genetics and epigenetics are two important strategies in pig breeding.

In the first study, SOX-6 was selected based on the in-house GWAS data. SOX-6 is a multi-faced transcription factor and regulates the myofiber differentiation. We studied two valuable SNPs located in the SOX-6 loci. The first SNP, rs81358375, was associated with backfat thickness and daily gain in DuPi population, and related with $pH1_L$, the thickness of backfat and side fat, and carcass length in Pietrain population. The pleiotropic effects of rs81358375 increase its contribution to pig breeding. For the other SNP, rs321666676 was merely associated with meat colour in Pietrain population. The missed CC genotype of rs321666676 in Pietrain and DuPi population could be due to its lethal effect or genetic drift. In addition, the genomic location of porcine SOX-6 overlaps or is closed to several QTLs that are identified in previous studies. The protein level of SOX-6 in low was significantly more abundant than that of high $pH1_L$ pigs, while the mRNA expression of SOX-6 was not significant different in divergent $pH1_L$ pigs. Furthermore, different expressed miRNAs targeting SOX-6 were found between these two groups, which maybe explain the inconsistent mRNA and protein level of SOX-6 in different $pH1_L$ pigs. In summary, this work identifies two valuable SNPs in the candidate gene, SOX-6, for pig breeding.

In the second study, we investigated the effects of SFN on the proliferation of satellite cells. Satellite cells, the muscle stem cells, play a crucial role in muscle growth, maintenance and regeneration. Our previous study has found SFN epigenetically supressed the transcription of MSTN in porcine satellite cells. In the present study, we

found that SFN enhanced the proliferation of the porcine satellite cells and modified the expression of myogenic regulatory factors and MEF2s. SFN altered the mRNA level of HDACs and supressed the HDACs activity. The activity of TGF-β signalling pathway was supressed by SFN treatment, which was accompanied with up-regulated Smad7, the endogenous suppressor of TGF-β signalling. Furthermore, to find out how SFN modified the transcription of Smad7, we check the transcription factors of Smad7, miRNAs targeting Smad7, and the methylation status of Smad7 promoter. The mRNA expression of STAT5A, NFKB1, CEBPbeta and Nrf2 was up-regulated by SFN treatment. The expression of miRNAs, including ssu-miR-15a/15b-5p/17-5p/20a/92a/106, was inhibited by SFN, while the protein level of Dicer was also decreased in the present of SFN. In summary, this study investigated the mechanisms in which SFN regulated the proliferation of satellite cells and provided the fundamental and scientific evidences for SFN applied as dietary supplement in pig breeding.

In conclusion, in Pietrain and DuPi pig population, the candidacy of porcine SOX-6 for growth, carcass and meat quality was supported by its genetic association study, its expression pattern and its genomic location. Furthermore, SFN, an epigenetic chemical, regulated the proliferation of the muscle stem cells, satellite cells, through suppression of TGF-β signalling by up-regulating Smad7.

Zusammenfassung

Der Tierschutz als auch die Effizienz der Schweinefleischproduktion sind wichtige Bestandteile der Schweinefleischindustrie. Die Aufgabe der Schweinefleischindustrie ist es hochwertiges und nachhaltiges Schweinefleisch anzubieten. Das Muskelwachstum und die Fleischqualität sind zwei wichtige Faktoren die die Schweinefleischerzeugung lenken. Genetische Variationen können teilweise die Variation der Fleischqualität erklären (Sellier und Monin, 1994). Im Anfangsstadium der künstlichen Selektion zeigt die markergestützte Selektion eine große Effizienz (Lande und Thompson, 1990). Einer der wichtigsten Schritte der markergestützen Zucht ist es mehr Polymorphismen, Kandidatengene und QTLs für das zu untersuchende Merkmale zu identifizieren. Neben den genetischen Faktoren beeinflussen auch Umweltfaktoren wie Nahrungsergänzungsmittel den Muskelproteinzuwachs und die Muskelwachstumsrate (Beil et al., 1998). Darüber hinaus haben Nährstoffe, wie methylierende Spurenelemente, in der F0 Population einen erheblichen Effekt auf die Fleischqualität und die Schlachtkörpermerkmale der F2 Schweine. Dieser Effekt wird als transgenerationale epigenetische Vererbung bezeichnet (Braunschweig et al., 2012).

Das SOX-6 Gen wurde für die erste Studie aufgrund der im Institut erhobenen GWAS Daten ausgewählt. SOX-6 ist ein vielseitiger Transkriptionsfaktor, der die Myofibrillen Differenzierung reguliert. Wir untersuchten zwei interessante SNPs im SOX-6 Gen. Der erste SNP, rs81358375, war mit den Merkmalen Rückenspeckdicke und tägliche Zunahme in der DuPi Population assoziiert. Zusätzlich zeigte der SNP eine Assoziation zu den Merkmalen $pH1_L$, Rückenspeckdicke, Seitenfett und Schlachtkörperlänge in der Pietrain Population. Die pleiotropen Wirkungen des rs81358375 erhöhen seinen Beitrag in der Schweinezucht. Der andere SNP, rs321666676, zeigte lediglich eine Verbindung zu dem Merkmal Fleischfarbe in der Pietrain Population. Aufgrund eines letalen Effekts oder der genetischer Drift konnte es zu dem fehlenden CC Genotyp des rs321666676 in den Pietrain und DuPi Populationen kommen. Die genomische Region des porcinen SOX-6 zeigte zusätzlich Überschneidungen mit mehreren QTLs aus früheren Studien oder liegt in der Nähe dieser QTLs. Der Proteingexpression von SOX-6 zeigte bei Schweinen mit einem geringen $pH1_L$ eine signifikante Veränderung als bei Tieren mit einem hohen $pH1_{L.}$ Darüber hinaus konnten unterschiedlich exprimierte miRNAs für das Zielgen SOX-6 zwischen diesen beiden Gruppen ermittelt werden. Dieses Ergebnis kann vielleicht den Unterschied zwischen der mRNA und der Protein Expression von

SOX-6 in den verschiedenen $pH1_L$ Gruppen erklären. Zusammenfassend erbrachte diese Studie zwei nützliche SNPs in der Kandidatengenregion SOX-6 für die Schweinezucht.

In der zweiten Studie untersuchten wir die Wirkung von SFN auf die Proliferation der Satellitenzellen. Satellitenzellen sind Muskel-Stammzellen, diese spielen eine entscheidende Rolle im Muskel-Wachstum, in der Muskel-Erhaltung und in der Muskel-Regeneration. In einer früheren Studie konnten wir festgestellt, dass SFN die MSTN Transkription in porcinen Satellitenzellen epigenetisch unterdrückt. In der vorliegenden Studie fanden wir heraus, dass SFN die Proliferation von porcinen Satellitenzellen verbesserte und die Expression des myogenen Regulationsfaktors, einschließlich der Expression des MEF2s modifizierte. SFN veränderte den HDACs mRNA Expressionslevel und unterdrückt die HDACs Aktivität. Die Aktivität des TGF-β Signalwegs wurde durch die SFN Behandlung ebenfalls unterdrückt. Diese Veränderung ging mit der Hochregulierung des Smad7 einher, welcher sich als ein endogener Suppressor des TGF-β Signalwegs darstellt. Zusätzlich wollten wir herausfinden wie SFN die Transkription von Smad7 modifizierte. Deswegen untersuchten wir den Transkriptionsfaktor von Smad7, die Smad7 Ziel miRNAs und den Methylierungsstatus des Smad7 Promotors. Die Expressionsprofile von STAT5A, NFKB1, CEBPbeta und Nrf2 waren nach der Behandlung mit SFN hoch reguliert. Die miRNA Expressionen von ssu-miR-15a / 15b-5P / 17-5p / 20a / 92a / 106 wurden durch SFN gehemmt, auch die Proteinexpression von Dicer war während der SFN Behandlung verringert. Zusammenfassend konnten in dieser Studie die Mechanismen untersucht werden durch die SFN die Proliferation der Satellitenzellen regulierte und lieferte grundlegende sowie wissenschaftliche Beweise dafür, dass SFN als Nahrungsergänzungsmittel in der Schweinezucht eingesetzt werden könne.

Abschließend zeigte sich, dass das porcine SOX-6 in der Pietrain und DuPi Population ein Kandidatengen für Wachstum, Schlachtkörper- und Fleischqualität sein kann. Dies wurde durch die genetische Assoziationsstudie, sein Expressionsmuster und die genomische Lage bekräftigt. Zusätzlich kann festgehalten werden, dass SFN, als epigentische Chemikalie, die Proliferation der Muskel-Stammzellen, Satellitenzellen, regulierte. Diese Regulierung entstand durch das hoch regulierte Smad7 welches den TGF-β-Signalweg unterdrückte.

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