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RESEARCH ARTICLE

The mRNA Expression Pattern of Skeletal Muscle Regulatory Factors in Divergent Phenotype Swine Breeds [1]

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Summary

Muscle regulatory factors (MRFs) play a major role in muscle growth and development. Thus, they are considered as candidate genes for meat production traits in pigs. These basic helix-loop-helix (bHLH) and paired box transcription factors together with Bone morphogenetic proteins, regulate myogenesis: they initiate the formation of muscle fibers and regulate the transcription of muscle specific genes. The objective of this study was to investigate the mRNA abundance of transcription factor (TF) genes (*MYOD, MYF5, MYOG, PAX3, PAX7, BMP2* and *BMP4*) in the adult skeletal muscle (*longissimus dorsi*) of divergent Duroc (high meat fat ratio) and Pietrain (low meat fat ratio) pig breeds. For this purpose, purebred Duroc and Pietrain animals (for each breed n = 5) were used for mRNA expression study. Highest gene expression was observed for *MYOG*, followed by *BMP2*. The lowest gene expression was observed for *PAX3*. Moreover, we statistically investigated differences in the expression profiles of these genes between the Duroc and Pietrain breeds. Among seven genes *MYF5* and *PAX3* showed different expression (P<0.01) between Duroc and Pietrain breeds. Duroc showed higher *MYF5* and *PAX3* mRNA abundance compared to the Pietrain breed. Although *PAX3* gene was the lowest expressed, results suggest that Duroc breed may have more skeletal muscle regeneration potential compared to Pietrain breed.

Keywords: *Duroc pigs, Pietrain pigs, Myogenic regulatory factors, mRNA expression*

İskelet Kası Düzenleyici Faktörlerin mRNA İfadesinin Farklı Özellikteki Domuzlarda Gösterimi

Özet

Kas düzenleyici faktörler (KDF), kas büyümesi ve gelişmesinde önemli bir rol oynar. Bu nedenle domuzlarda et üretim özellikleri için aday genler olarak kabul edilirler. Bu temel helix-loop-heliks (tHLH) ve eşleştirilmiş kutu transkripsiyon faktörleri ile kemik morfogenetik proteinleri miyogenesisi düzenlerler: kas liflerinin oluşumu başlatmak ve kas spesifik genlerin transkripsiyonunu düzenlerler. Bu çalışmanın amacı, Duroc (yüksek et yağ oranı) ve Pietrain (düşük et yağ oranı) domuz ırklarında yetişkin iskelet kası (*longissimus dorsi*) transkripsiyon faktörü (TF) genlerinin (*MYOD, MYF5, MYOG, PAX3, PAX7, BMP2* ve *BMP4*) mRNA ifadesini araştırmaktır. Bu amaçla, safkan Duroc ve Pietrain hayvanlar (her ırkan cins n = 5) mRNA ifade çalışması için kullanılmıştır. En yüksek gen ifadesi *MYOG* için, ve onu takiben *BMP2* için gözlendi. En düşük gen ifadesi *PAX3* için gözlendi. Ayrıca bu genlerin ifadeleri Duroc ve Pietrain ırklarında istatistiksel olarakta araştırılmıştır. Yedi gen içinde *MYF5* ve *PAX3* genleri Duroc ve Pietrain ırkları arasında (P<0.01) farklı ifade gösterdi. Duroc ırkı hayvanlar Pietrain ırkına kıyasla daha yüksek *MYF5* ve *PAX3* mRNA ifadesi gösterdi. *PAX3* gen ifadesi düşük olmasına rağmen sonuçlar Duroc ırkının Pietrain ırkına kıyasla daha yüksek kas yenileme potensiyeline sahip olabileceğini göstermiştir.

Anahtar sözcükler: *Duroc domuzlar, Pietrain domuzlar, Miyogenik düzenleyici faktörler, mRNA ifadesi*

INTRODUCTION

Skeletal muscle development in vertebrates (also termed myogenesis) is a highly integrated process [1]. Adult muscle tissue is characterized by its capacity to grow and to regenerate after injury, managed by the processes which require stem cells - so called satellite cells [2]. Myogenesis is regulated by a group of muscle-specific trans-

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cription factors, know also as the myogenic regulatory factors (MRFs): Myogenic factor 5 (*MYF5*), muscle-specific regulatory factor 4 (*MRF4*; also known as *MYF6*), myoblast determination protein (*MYOD*) and myogenin (*MYOG*) [3]. MRFs are transcription factors that activate many downstream genes to initiate muscle cell differentiation. MRFs are basic helix-loop-helix (bHLH) proteins that heterodimerize with a ubiquitous class of bHLH transcription factors known as E proteins [4]. These complexes bind to a canonical DNA sequence, CANNTG, called the E-box within the regulatory elements of genes to affect their expression [4]. Beside during prenatal stages of muscle development, MRFs regulating muscle specific expression patterns during postnatal muscle tissue functioning [5]. Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor ß (TGFß) superfamily [6]. Stem cells in the skeletal muscle have the capacity to differentiate into osteogenic cells upon stimulation with BMP. Thus, these cells can act as carriers of BMP as well as participate in new-bone formation by differentiating into osteogenic cells [7]. Although to date, around twenty BMP family members have been identified and characterized [6], in our study, we focused on *BMP2* and *BMP4*. Bone morphogenetic proteins 2 (*BMP2*) directs the development of neural crest cells into neuronal phenotypes [8], while *BMP4* and *BMP7* specifically induce a sympathetic adrenergic phenotype [6]. The implantation of bone morphogenetic protein into muscular tissues induces ectopic bone formation at the site of implantation [9] and recent evidence suggests that perturbation of this cleavage can have profound effects on skeletal muscle growth [10].

The understanding of the growth and development of skeletal muscle is one of the most important goals in animal science [11]. The development of skeletal muscle depends on the number and size of muscle fibers. In pigs, fiber formation ceases around 85-90 days of gestation when the total number of fibers is established [12]. Postnatal muscle growth for pigs is achieved by an increase in number (hyperplasia) and size (hypertrophy) of myofibres. Postnatal muscle growth is accompanied by the proliferative activity of satellite cells which are the source of new nuclei incorporated into the muscle fibres [11]. Muscle fiber hypertrophy is associated with an increased DNA content, yet myonuclei do not retain the ability to synthesize DNA [13]. Satellite cells are the postnatal source of DNA which contributes to muscle fibers growth. DNA accretion occurs through proliferation of satellite cells followed by differentiation and fusion with existing muscle fibers [14]. Similar to prenatal stage [1], postnatal muscle growth also orchestrated by MRFs [15]. Investigating gene expression and gene regulation in phenotypically diverse populations is a useful approach to reveal the effects of genetic variability and to understand the development and regulation of quantitative traits in livestock. Genetic background of skeletal muscle growth in extreme divergent breeds has been shown in several studies [16-19]. Among modern western pigs, Duroc (high meat fat ratio) and Pietrain (low meat fat ratio) breeds extensively utilized in commercial pork production differ extremely for their muscle phenotypes [16]. Although given the critical roles that MRFs play in myogenesis and the expression of MRFs is associated with skeletal muscle growth, a few study was devoted to understand breed dependent molecular mechanism of MRFs and BMPs. Therefore, the aim of the present study was to identify mRNA expression of skeletal muscle related transcription factor (TF) genes (*MYOD*, *MYF5*, *MYOG*, *PAX3*, *PAX7*, *BMP2* and *BMP4*) in the *longissimus dorsi* muscle of divergent swine breeds Duroc and Pietrain.

MATERIALS and METHODS

Animals

Pietrain (n=5) and Duroc (n=5) female pigs at the age of 6-months were used in this study. Details regarding the animals used in this study were given in previous paper [16]. All the pigs were slaughtered at a commercial abattoir according to German performance test directions [20]. The loin eye muscle of each pig was dissected and immediately frozen in liquid nitrogen within 5 min after slaughter. Then the samples were stored at -80°C until RNA isolation.

RNA Isolation and cDNA Synthesis

Total RNA was extracted using TRI-Reagent® (Sigma-Aldrich). RNA was purified using RNeasyMini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was then treated using on-column RNase-Free DNase set (Promega) and the concentration was quantified by spectrophotometer (NanoDrop, ND-8000, Thermo-Scientific). RNA quality was checked by 2% agarose gel electrophoresis. First-strand cDNA was synthesized using Superscript II enzyme (Invitrogen). The cDNA concentration was measured by spectrophotometer (NanoDrop, ND-8000, Thermo-Scientific).

Quantitative Real-time PCR (qRT-PCR)

Primers were designed using the online Primer3 program [21] and are listed in *Table 1*. Experiments were performed in StepOne Plus Real-Time PCR system (Applied Biosystems). The qRT-PCR was set up using 2 µl first-strand cDNA template (at the concentration of 100 ng/µl), 7.4 µl deionized H_2O, 0.3 µM of forward and reverse gene specific primers and 10 µl 1×Power SYBR Green I (Bio-Rad) master mix with ROX as reference dye. The thermal cycling conditions were 3 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. All samples were analyzed twice (technical replication) and the geometric mean of the Ct values were further used for statistical analysis using SAS v9.2 (SAS Institute Inc., Cary, NC). Melting curve analysis was performed to investigate the specificity of the PCR reaction. The no-template control

Table 1. *Primer sequences used for qRT-PCR analysis*
Tablo 1. *qRT-PCR çalışmasında kullanılan primer dizileri*

Gene Name	Primers Sequence (5'-3')	Tm (°C)	Product Size (bp)	GenBank ID
MYOD	F: TGCAAACGCAAGACCACTAA R: GCTGATTCGGGTTGCTAGAC	55	127	NM_001002824.1
MYOG	F: GGTGAGGGAGTGCAGATTGT R: CAGTGAATGCAGTTCCCACA	56	130	NM_001012406.1
MYF5	F: CCGACACAGCTTGTGGAATA R: GCCAATCAACTGATGGCTTT	55	128	XM_001924362.2
PAX3	F: ATCGGCTAATCCTGACATGC R: ACGGTGGGAAACTTTTGATG	54	130	AY579430.1
PAX7	F: GGCAGAGGATCTTGGAGACA R: TGGGTGGGGTTTTCATCAAT	55	144	AY653213.1
BMP2	F: CTTGACGCTTTTCCCTTTTG R: AAGAGGCATGTGCGGATTAG	55	130	NM_001195399.1
BMP4	F: ACGGTGGGAAACTTTTGATG R: ATCGGCTAATCCTGACATGC	57	140	NM_001101031.1
GAPDH	F: ACCCAGAAGACTGTGGATGG R: ACGCCTGCTTCACCACCTTC	60	247	AF017079.1
HPRT1	F: AACCTTGCTTTCCTTGGTCA R: TCAAGGGCATAGCCTACCAC	55	150	NM_001032376.2

F: *Forward,* ***R:*** *Reverse*

(NTC) was included in each run for each gene to check for contamination. The geometric mean of twohouse-keeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were used for normalization of the target genes. The delta Ct (ΔCt) values were calculated as the difference between target gene and geometric mean of the reference genes: $\Delta Ct = Ct_{target\ gene} - Ct_{housekeeping\ gene}$. Ct values were Logarithm (Log) transformed and used for further analysis. The higher Ct values indicated lower expression and vice versa.

Statistical Analysis

Results from qRT-PCR analysis were expressed as mean ± SEM. The difference between values was analyzed by t-test in SAS software v. 9.2 and $P<0.05$ was set statistically significant. The principal component analysis was performed using PAST (Palaeontological Statistics) [22].

RESULTS

Myogenin found to be most abundant mRNA in *longissimus dorsi* muscle *(Fig. 1)*, followed by *BMP2* and *BMP4 (Fig. 1)*. Paired box transcription factor genes *PAX3* and *PAX7* were the lowest expressed genes in pig skeletal muscle *(Fig. 1)*. On the other hand, other myogenic regulatory factor genes *MYOD* and *MYF5* showed moderate mRNA expression in pig skeletal muscle *(Fig. 1)*.

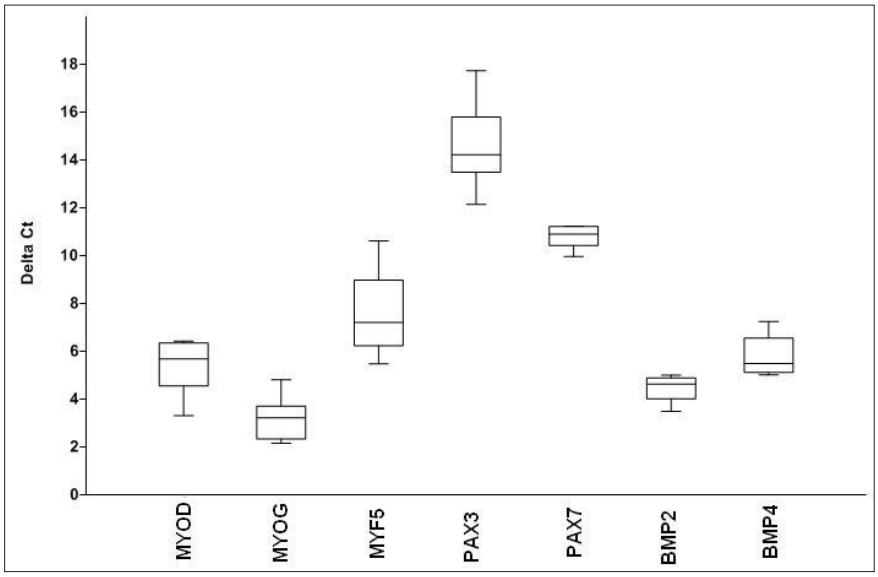

Fig 1. Box-and-whisker plots, representing the expression of genes in the studied groups (Duroc and Pietrain)

The results are calculated as delta Ct values. Whiskers represent median and minimum and maximum values for particular groups. Boxes represent lower quartile and upper quartile. Lower Ct values indicate higher mRNA expression.

Şekil 1. Kutu ve bıyık (box and whisker) diyagramları, incelenen gruplarda (Duroc ve Pietrain) genlerin ifadesini temsil ediyor

Sonuçlar delta Ct değerleri olarak hesaplanmıştır. Bıyıklar belirli gruplar için ortanca, minimum ve maksimum değerleri temsil eder. Kutular, alt dörttebirlik ve üst dörttebirlik değerleri temsil eder. Düşük Ct değerleri yüksek mRNA ifadesini göstermektedir.

Principal component analysis (PCA) of mRNA expression data showed that two breeds (Duroc and Pietrain) well discriminated in case of seven genes mRNA expressions *(Fig. 2)*. Moreover, it was shown that *PAX3* and *PAX7* genes clustered distinctly among seven skeletal muscle development genes *(Fig. 2)*. Results showed that expression of *PAX7* gene has weight on Duroc animals where as *PAX3* has more weight on Pietrain animals *(Fig. 2)*.

The variance analysis results showed that mRNA expression of *MYF5* and *PAX3* showed differential regulation (P<0.01) between Duroc and Pietrain breeds *(Fig. 3)*. Duroc had higher *MYF5* and *PAX3* expression compared to Pietrain breed *(Fig. 3)*. On the other hand, although, Duroc had a slightly higher mRNA expression, no significant difference was detected between Duroc and Pietrain breeds for the mRNA expression of *MYOD*, *MYOG*, *PAX7*, *BMP2* and *BMP4* genes *(Fig. 3)*.

DISCUSSION

Pig breeds differ in muscle traits such as muscularity, muscle fiber type, color, etc.[23]. Here, we reported the mRNA expression of three MRFs, two paired box and two bone morphogenetic protein gene. Among MRFs, mRNA and protein expression of *MYF6* was shown previously by Fan et al.[16] in Duroc and Pietrain pigs. Their results suggested that Pietrain pigs have higher *MYF6* mRNA and protein expression compared to Duroc pigs. In this study, myogenin was the most abundantly expressed gene in young pig skeletal muscle, followed by *BMP2* and *BMP4* *(Fig. 1)*. Paired box transcription factor genes *PAX3* and *PAX7* were the lowest expressed genes in pig skeletal muscle *(Fig. 1)*. On the other hand, other myogenic regulatory factor genes *MYOD* and *MYF5* showed moderate mRNA expression in pig skeletal muscle tissue *(Fig. 1)*. Application of principal component analysis (PCA) to gene expression dataset was shown in a clear manner [24]. PCA is a mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set [24]. When we applied PCA to mRNA expressions of seven genes among Duroc and Pietrain breeds, analysis revealed that two breeds can be separated in case of seven genes mRNA expressions *(Fig. 2)*. Moreover, it was shown that *PAX3* and *PAX7* genes clustered distinctly among seven skeletal muscle development genes *(Fig. 2)*. Results

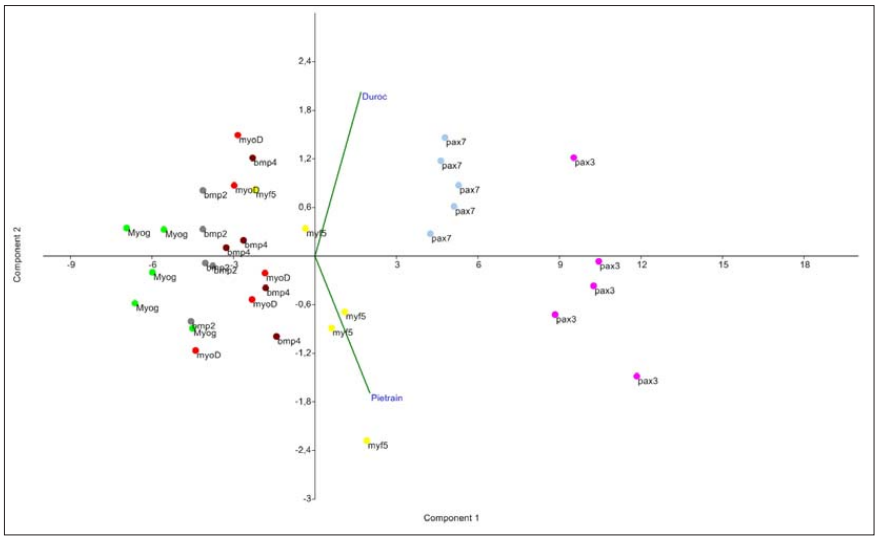

Fig 2. Visual representation of Principal Component Analysis results for the gene expressions

The dots (scores) represent gene expression categories or subgroups, and the arrows (loadings) are the breeds

Şekil 2. Gen ifadeleri icin Temel Öğeler Analizi sonuçlarının grafiksel gösterimi

Noktalar gen ifadesi kategorilerini veya alt grupları, oklar ırkları göstermektedir

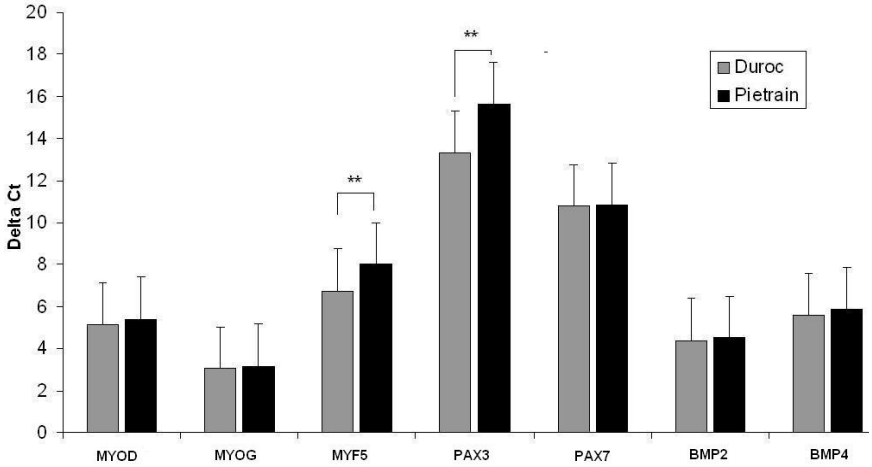

Fig 3. Differential gene expressions among two breeds

The results are calculated as delta Ct values. Lower Ct values indicate higher mRNA expression

Şekil 3. Gen ifadesinin iki ırk arasındaki farklılığı

Sonuçlar delta Ct değerleri olarak hesaplanmıştır. Düşük Ct değerleri yüksek mRNA ifadesini göstermektedir

showed that expression of *PAX7* gene has weight on Duroc animals where as *PAX3* has more weight on Pietrain animals *(Fig. 2)*. mRNA expression of *MYF5* had weight on Pietrain breed compared to Duroc breed. Notably, other myogenic regulatory factors *MYOD* and *MYOG* and Bone morphogenetic proteins *BMP2* and *BMP4* clustered together and their weights were equally distributed on Duroc and Pietrain breeds *(Fig. 2)*.

Different researches have shown that satellite cells play a major role in muscle hypertrophy. These cells represent the oldest known adult stem cells which are involved in normal growth of muscle and regeneration process [25,26]. The variance analysis results showed that mRNA expression of *MYF5* and *PAX3* showed differential regulation (P<0.01) between Duroc and Pietrain breeds *(Fig. 3)*. Duroc had higher *MYF5* and *PAX3* expression compared to Pietrain breed *(Fig. 3)*. On the other hand, although, Duroc had a slightly higher mRNA expression, no significant difference was detected between Duroc and Pietrain breeds for the mRNA expression of *MYOD*, *MYOG*, *PAX7*, *BMP2* and *BMP4* genes *(Fig. 3)*. In mature muscle tissue, satellite cells occur as a small, dispersed population of mitotically and physiologically quiescent cells, marked by their expression of the transcription factor *PAX7* [27]. The *PAX3* gene plays an essential role in regulating the developmental program of embryonic myoblasts [27,28]. Edwards et al.[29,30] evaluated the Duroc and Pietrain sired pigs and recorded their carcass and meat quality traits. They concluded that Duroc sired progeny were heavier and had a greater average daily gain compared to Pietrain sired ones. Their results were in good agreement with our results, showing that Duroc animals had higher *PAX3* mRNA expression than Pietrain pigs which may cause more skeletal muscle regeneration potential in Duroc pigs. Recently, importance of *PAX7* compared to *PAX3* was shown in one review paper [31]. In our experiment although mRNA expression of *PAX7* was found to be higher compared to *PAX3* mRNA, no significant difference was identified between two pig breeds in terms *PAX7* expression.

Analysis of mice depleted of *PAX3* and *MYF5* showed almost complete loss of trunk muscles and a loss of *MYOD* expression; this indicates that *MYOD* expression depends on either *PAX3* or *MYF5* [3]. The role of myogenic factor MYF5 in mammalian myogenesis during development is well defined [32]. Ustanina et al.[33] observed a significant delay in the regeneration of *MYF5* deficient skeletal muscles after injury. They concluded *MYF5* supports efficient skeletal muscle regeneration by enabling transient myoblast amplification. A few studies were devoted to understand breed specific postnatal transcription profile of muscle regulatory family, but most of them dealt with age specific alteration of transcript abundance. For instance Pierzchała et al.[34] used Polish Large White, Polish Landrace, Pietrain, Duroc and Pulawska gilts to investigate the expression *MYF5* at different ages, however, they did not observe any significant differences postnatal expression of *MYF5* in porcine skeletal muscle. In Large Whites *MYOD* and *MYF5* were reported to be expressed regularly up to the third week of age followed by a decreasing trend (detected up to 50 days of age) while the differentiation factors myogenin and *MRF4* seemed to diminish already after the first week from birth [35]. Gayraud-Morel et al.[32] showed that *MYF5* is a regulator of regenerative myogenesis and homeostasis, with functions distinct from those of *MYOD* and *MRF4*. In our research, we observed transcript abundance difference between Duroc and Pietrain pigs for *MYF5* gene but not for *MYOD*. Inconsistency at mode of regulation (up-down) for the transcript abundance of *MYF5* and *MYOD* could be explained by distinction of functionality. Due to the close vicinity of *MYF6* and *MYF5* genes on SSC5, these two genes have a common regulatory region and the expression of both genes is in part activated together [36] and differentially mRNA regulation of *MYF6* between Duroc and Pietrain pigs was shown previously [16]. To the best of our knowledge, no literature is available about the transcriptional regulation of *MYF5*, however, *trans*-regulation for *MYF6* gene was shown in literature [37]. Since *MYF5* and *MYF6* had a common regulatory region, it could be speculated that transcription of *MYF5* is controlled by the same eQTL which *MYF6* is controlled. However, mode of transcription regulation of *MYF5* gene could be investigated with further eQTL studies in larger pig populations. Te Pas et al.[38] genotyped 1.276 animals in five loci of *MYF5* gene for association analysis. However, statistical analysis revealed no association with birth weight, growth rate, weight at slaughter age, carcass meat weight, and backfat thickness. Their experiment showed that *MYF5* did not explain genetic variation in meat (muscle) development in pigs. On the other hand, Zhang et al.[39] found significant association between *MYF5* SNPs and Chinese cattle breeds and suggested *MYF5* as strong candidate genes that influence growth traits in cattle.

In summary, investigation of gene expression in different pig breeds or developmental stages could reveal candidate genes associated with growth and meat quality traits. Up to now, a few studies about transcript abundance of the myogenic regulatory factors in different pig breed were performed. Our research on expression patterns of main myogenic regulatory factors could be helpful to understand the genetic basis of postnatal myogenesis process and its effects on high and low meat fat ratio pig breed. Especially, up regulation of *MYF5* and *PAX3* genes suggest that faster growing Duroc pigs connected with the presence of a larger number of active satellite stem cells compared to Pietrain. However, research on polymorphisms and their association of this polymorphism with growth and meat quality traits is further needed for a better understanding of myogenic regulatory factors in different pig breeds.

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