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Development-related expression patterns of protein-coding and miRNA genes involved in porcine muscle growth

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ABSTRACT. Muscle growth and development is associated with remarkable changes in protein-coding and microRNA (miRNA) gene expression. To determine the expression patterns of genes and miRNAs related to muscle growth and development, we measured the expression levels of 25 protein-coding and 16 miRNA genes in skeletal and cardiac muscles throughout 5 developmental stages by quantitative reverse transcription-polymerase chain reaction. The Short Time-Series Expression Miner (STEM) software clustering results showed that growth-related genes were downregulated at all developmental stages in both the psoas major and longissimus dorsi muscles, indicating their involvement in early developmental stages. Furthermore, genes related to muscle atrophy, such as *forkhead box 1* and *muscle ring finger*, showed unregulated expression with increasing age, suggesting a decrease in protein synthesis during the later stages of skeletal muscle development. We found that development of the cardiac muscle was a complex process in which growth-related genes were highly expressed

during embryonic development, but they did not show uniform postnatal expression patterns. Moreover, the expression level of miR-499, which enhances the expression of the *β-myosin heavy chain*, was significantly different in the psoas major and longissimus dorsi muscles, suggesting the involvement of miR-499 in the determination of skeletal muscle fiber types. We also performed correlation analyses of messenger RNA and miRNA expression. We found negative relationships between miR-486 and *forkhead box 1*, and miR-133a and *serum response factor* at all developmental stages, suggesting that *forkhead box 1* and *serum response factor* are potential targets of miR-486 and miR-133a, respectively.

Key words: Muscle growth; Porcine; Expression pattern; qRT-PCR

INTRODUCTION

The muscle tissue is one of 4 fundamental types of tissues present in animals and mainly consists of skeletal muscle and cardiac muscle (CM). Both skeletal muscle and CM are striated because they contain sarcomeres that are packed into highly regular arrangements of bundles. Skeletal muscles, the heaviest tissues that account for ~40% of total body mass (Pedersen and Febbraio, 2012), are the predominant sites of energy metabolism and the dominant organ system involved in locomotion. Conversely, CM is controlled by the autonomic nervous system. In animal growth and developmental processes, muscle mass is the most important determinant of the quality of carcass, which is directly related to efficient production. The gain of muscle mass is a complex process dependent on protein and muscle fiber turnover. Notably, the gain of skeletal muscle fibers mainly occurs during embryonic development, whereas hypertrophy of the skeletal muscle occurs after birth. CM shows sustained growth from the embryonic period to adulthood and then almost completely halts and maintains a relatively stable state thereafter. Many studies have revealed the important roles of protein-coding and microRNA (miRNA) genes in the regulation of muscle growth (Sandri, 2008; van Rooij et al., 2008). However, the changes in expression of these genes with increasing age and the interactions of miRNAs with protein-coding genes have not been fully defined in muscle development.

Pigs are commonly used as animal models in biomedical research because their physiology and anatomy show a considerable resemblance to those of humans (Garthoff et al., 2002). We explored the expression patterns of 25 protein-coding (Table 1) and 16 miRNA genes related to the growth and development of 3 porcine muscle tissues [i.e., CM, psoas major muscle (PMM), and longissimus dorsi muscle (LDM)] throughout 5 developmental stages [i.e., embryonic day (E) 90; postnatal days (P) 0, 30, and 180; and 7 years (Y) of age] to investigate variations in the expression levels of these genes with increasing age.

MATERIAL AND METHODS

Sample collection and measurement

The female Jinhua pigs across E90, P0, P30, P180, and 7Y were used in this study, and 3 individuals were randomly selected from each developmental stage. Animals were humanely

sacrificed to ameliorate suffering. CM, PMM, and LDM were collected from each animal; the samples were frozen in liquid nitrogen immediately and stored at -80°C.

Table 1. Primers for protein-coding genes.

Gene symbol	Forward	Reverse	Product length
PAX3	atcggctaatcctgacatgc	acggtgggaaacttttgatg	140
MSTN	aacagcgagcaaaaggaaaa	atcaatcagttcccggagtg	201
MTOR	ctcgtcactcctctcaac	ccgcttccttatggttct	99
NFκB	gcaccctgatcttgcttatt	gaggtccatctccttcgtct	115
SMAD2	cttctggctcagtccgtta	ctgtctgccttcggtattct	115
FOXO1	gctttacaagtgcctctgcc	tctccatccatgaggtcgtt	197
MRF4	cttgagggtgcggatttcctg	ctgaagactgctggaggctg	132
IGF1	ctggtggacgctcttcagtt	acatctccagcctcctcaga	151
MURF	gtgacaaaggcaagaccc	acacggcaagatgaccacc	195
MYF4	caaccaggaggagcgagacctccg	aggcgctgtgggatatgcattcact	86
MYOD	gcactacagcggtgactcag	cacgatgctggacagacagt	196
SMAD3	ggagaagtggtgcgagaagg	cacaggcggcagtagatgac	195
S6K1	gaggacatggcaggagtgtt	cctttaccaagtacccgaag	236
MyHC	ctggatgccagtgagcgtgtc	gcgttgcgagcttcctgaata	134
PDK1	aatcaccaggacagccaata	ctcggtcactcatcttcaca	186
Follistatin	ggcctatgagggaaagtgta	ctcggtgtcttctgaaatgg	274
MYF5	tgccagttctcgccttctga	tttcctcttgcacgctttgc	216
IGF1R	tggatgccgtgtccaat	gtgtcgttgtcgggtgc	256
SRF	gactggcaaggcactgattc	tgctgtctggattgtggagg	220
PI3K	tgaaagtagattggctggac	cactatctcaaagcccgtta	197
GSK3	tacgggacccaaatgtcaaa	acgcagaagcggtgttattg	216
MEF2A	gaccctgatacttcctatgtgc	tgaactccctgggttagtgtag	189
AKT1	atcgtgtggcaggatgtgta	ctggccgagtaggagaactg	200
MAPK14	gattctccgaggtctcaa	gccacatagcctgtcatt	153
IRS1	ctgattggcatctaccgc	gcctccaggattgtctcat	233

RNA extraction

Total RNA was isolated from the muscle tissues using RNAisoPlus (TaKaRa, Dalian, China), purified using RNeasy columns (Qiagen, Duesseldorf, Germany), and RNA concentrations were measured by the NanoDrop 2000 (Thermo Scientific, USA) according to the manufacturer protocol.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was reverse transcripted following manufacturer recommendations for: 1) mRNA transcribed into complementary DNA (cDNA) using the oligo(dT) and random 6-mer primers provided in the PrimeScript RT MasterMix kit (TaKaRa); and 2) miRNA using the oligo(dT) and poly(A) polymerase provided in the PrimeScript miRNA RT-PCR Kit (TaKaRa). qRT-PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa) on a CFX96 real-time PCR detection system (Bio-Rad, Richmond, Canada).

Statistical data and biological analysis

According to **Figure S1**, all mRNA expression levels were normalized by the 3 housekeeping genes with greater stability (i.e., *PPIA*, *RPL4*, and *YWHAZ*), while miRNA

expression levels were normalized by 3 other housekeeping genes (i.e., *18S*, *5S*, and *U6*). All experiments contained a negative control, and each qRT-PCR was performed in triplicate.

RESULTS AND DISCUSSION

Protein-coding gene expression patterns at various developmental stages

Growth of muscle mass is a time-dependent process regulated by protein-coding genes and other factors. To investigate the expression levels of these genes, we used the Short Time-Series Expression Miner (STEM) software for protein coding and miRNA gene clustering.

Formation and function of the heart occur at early stages during embryonic development, which accounts for all subsequent events in the life of an organism (Olson, 2004). Although functional development of the heart is completed during the embryonic period, it grows actively after birth into adulthood (Manabe et al., 2002; McMullen et al., 2004). Growth factors cooperate to regulate the early growth of CM. These factors exhibited increasing expression levels from the prenatal to early postnatal stages but decreased at later stages (Figure 1a; **Table S1**) and showed extremely similar expression patterns ($P < 0.001$). Incidentally, we found that the muscle ring finger (*MURF*), *follistatin*, and mitogen-activated protein kinase (*MAPK*) genes were significantly clustered into one group ($P = 0.040$), indicating a unique expression pattern throughout the 5 developmental stages. This expression patterns in CM showed strong upregulation from E90 to P0, downregulation from P0 to P30, upregulation from P30 to P180, and downregulation at 7Y. During CM development, the *follistatin* gene may participate in positive regulation of muscle mass. Postnatally, the gain of muscle slows and is regulated by numerous factors, which is a complicated regulatory process.

The gain or loss of skeletal muscle mass involves a delicate balance between the production of new myofibrils and degradation of existing proteins (Sandri, 2008), which is directly regulated by protein-coding genes. In both the LDM and PMM, muscle regulatory factor 4 (*MRF4*), forkhead box 1 (*FOXO1*), and *MURF* exhibited slow rises in expression levels throughout the 5 stages (Figure 1b and c; **Table S1**). *MRF4* is the only gene of the *MYOD* family that is expressed in embryonic myotomes to promote muscle mass and continues to increase postnatally (Hinterberger et al., 1991). Moreover, *FOXO1* can reduce muscle mass and fiber atrophy (Xu et al., 2012), and *MURF* ubiquitinates and degrades myosin heavy chains (Clarke et al., 2007); all of these changes negatively regulate muscle mass. Skeletal muscle shows remarkable changes in metabolism with increasing age, which is reflected by the distribution and size of the muscle fibers and a general deceleration in muscle mass gain (Carmeli and Reznick, 1994). We found that the expression patterns of *MURF* and *FOXO1* were upregulated with age, suggesting a decrease in the deposition of protein with increasing age. β-myosin heavy chain (*β-MYHC*), the determinant gene of skeletal muscle isoforms (fast or slow) (Agbulut et al., 2003), showed different expression patterns in LDM (fast muscle) and PMM (slow muscle), which is consistent with previous studies (Figure 2).

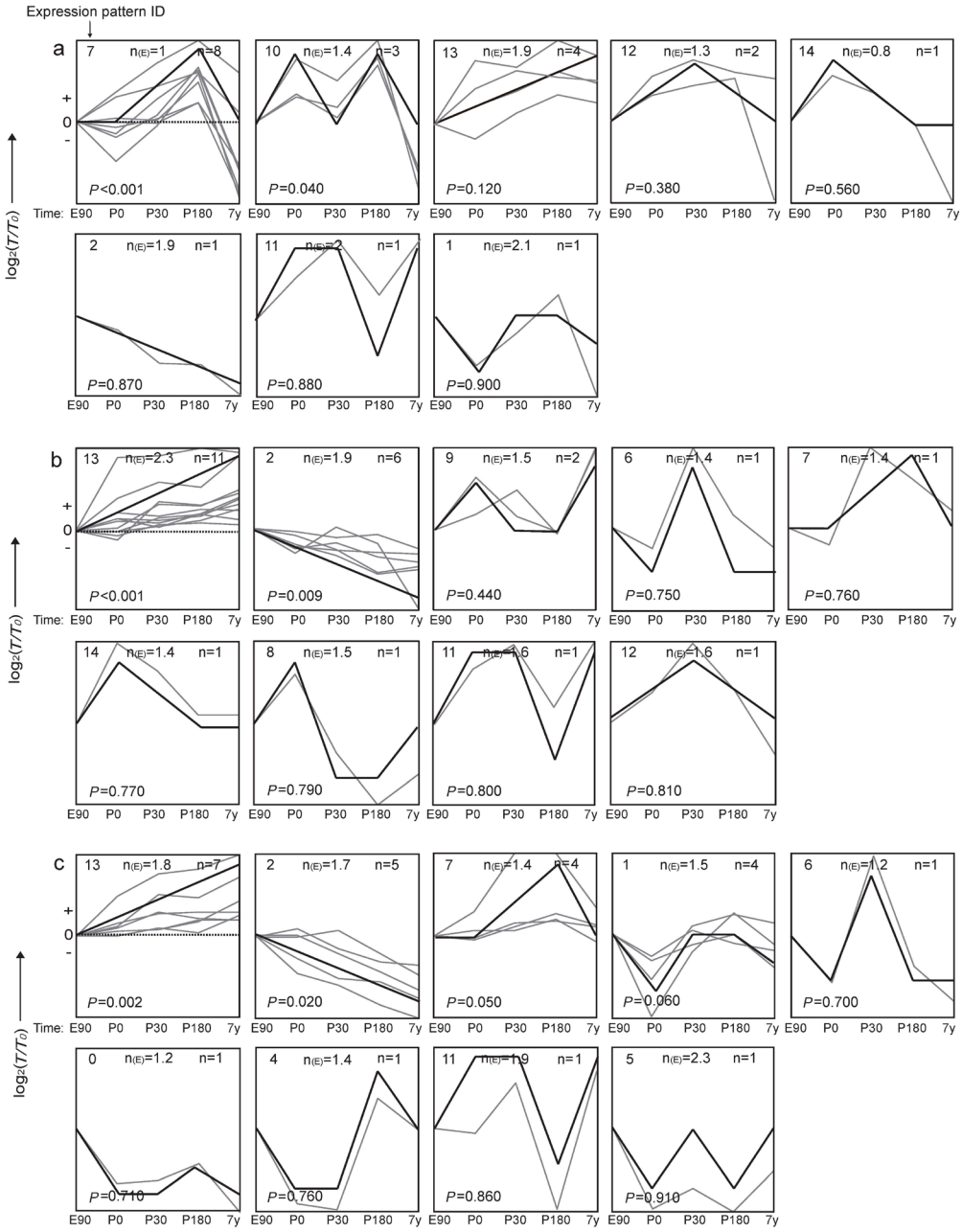

Figure 1. Short Time-Series Expression Miner (STEM) clustering profiles for protein-coding genes in: (**a**) cardiac muscle (CM); (**b**) longissimus dorsi muscle (LDM); and (**c**) psoas major muscle (PMM). The dashed line indicates no change in expression among the different stages. The number at the top left of each square is the expression pattern ID. The black, bold-type lines in the squares represent the trend lines of the expression patterns, and the gray lines represent the variation in expression of the protein-coding genes from embryonic day 90 (E90) to 7 years (7Y).

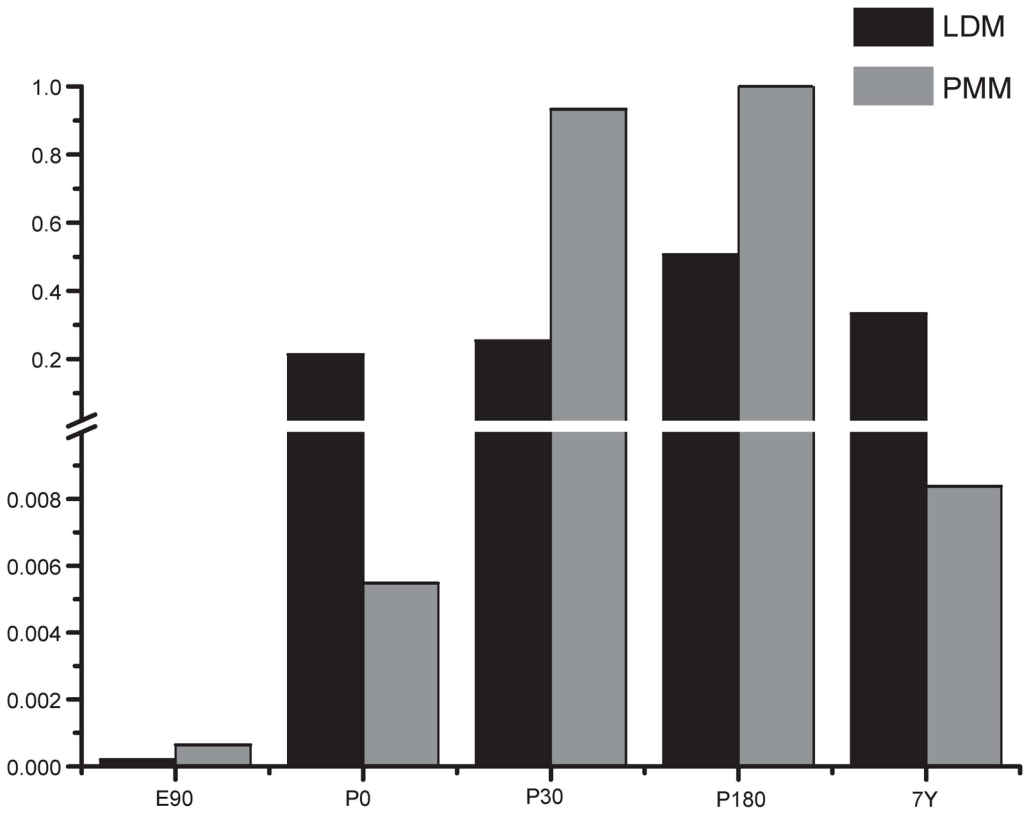

Figure 2. β-myosin heavy chain (*β-MYHC*) expression pattern in longissimus dorsi muscle (LDM) and psoas major muscle (PMM).

miRNA expression patterns at various developmental stages

miRNAs play important roles in the regulatory networks of protein-coding genes related to muscle development. They participate in diverse aspects of muscle function, including myoblast proliferation, differentiation, and muscle atrophy (van Rooij et al., 2008; Townley-Tilson et al., 2010; Xu et al., 2012).

In CM, among the 16 miRNAs, 7 miRNAs showed a dramatic decrease from E90 to 7Y (Figure 3; **Table S2**). miR-1, miR-206, miR-486, miR-221, miR-195, miR-320, and miR-24, which play important roles in muscle myogenesis, differentiation, or proliferation, showed higher expression levels during the early developmental stages when compared to those during later developmental stages (Callis et al., 2007; Carè et al., 2007; Townley-Tilson et al., 2010). For example, miR-486, which directly suppresses *Pax7* expression, modulates myoblast development from the proliferation stage to the subsequent differentiation phase during embryonic development (Dey et al., 2011). Furthermore, it has been shown that this process is completed during the embryonic period; then, the expression level of miR-486 decreases postnatally (Xi et al., 2007).

Notably, in skeletal muscle, we found that miR-1 and miR-499 showed the highest

expression levels at E90 in both the LDM and PMM (Figure 3). According to previous reports, miR-1 plays an important role in skeletal muscle development of *Drosophila* and *Xenopus laevis* embryos (Chen et al., 2005; Nguyen and Frasch, 2006) and is highly expressed during muscle cell differentiation. Our results suggest a role of miRNA-1 in the regulation of muscle cell differentiation during the embryonic period of pigs. Moreover, miR-499 is expressed in skeletal muscle during mouse embryogenesis (van Rooij et al., 2009). In this study, we identified a high expression level of miR-499 during the embryonic period, but its precise function in early skeletal muscle development is currently unknown. miR-499 is expressed in skeletal muscle and controls the skeletal muscle fiber type after birth, which has been validated in transgenic mice (van Rooij et al., 2009). We found that miR-499 had a higher ($P < 0.001$) expression level in PMM than that in LDM, suggesting its role in the formation of type I myofibers in pigs.

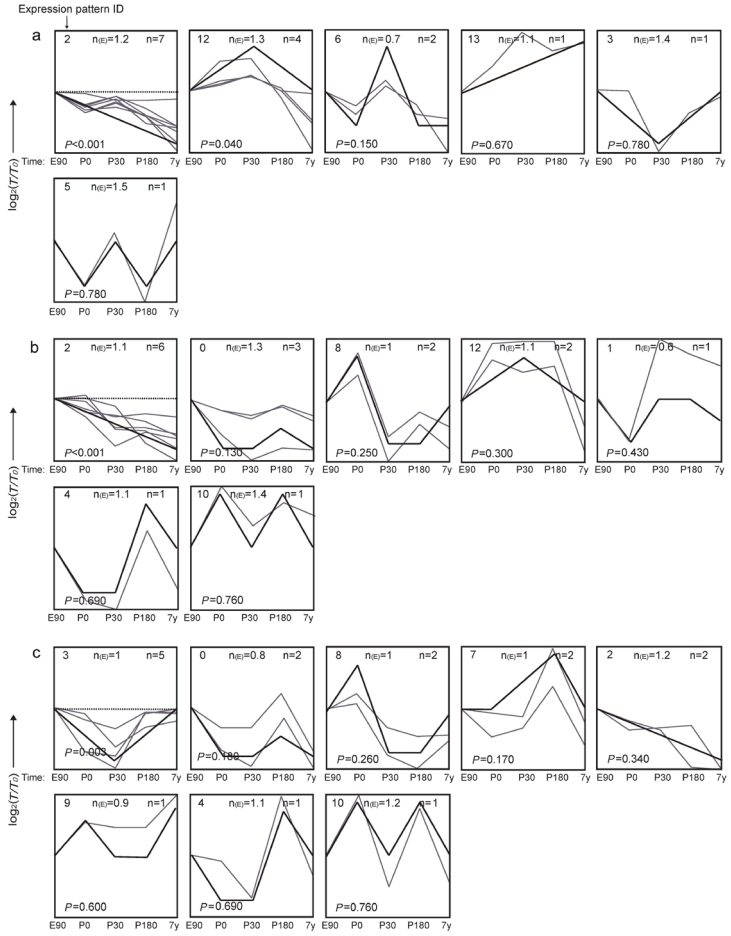

Figure 3. STEM clustering profiles for microRNAs (miRNAs) in CM (**a**), LDM (**b**) and PMM (**c**) The dashed line indicates no change in expression among the different stages. The number in the top left of each square is the expression pattern ID. The black, bold-type lines in the squares indicate the trend lines of the expression patterns, and the gray lines represent the variation in expression of miRNAs from E90 to 7Y.

Relationship between protein-coding genes and miRNAs at various developmental stages

miRNAs negatively regulate gene expression by promoting target mRNA degradation and inhibiting mRNA translation (van Rooij et al., 2008). However, these interactions have generally been elucidated in transgenic mouse models or at the cellular level. To determine whether these interactions were present during various developmental stages, we constructed a correlative network of genes and miRNAs during muscle growth and development (**Figure S2**).

We found that in insulin-like growth factor 1 (*IGF1*)-miR-1, *IGF1*-miR-320, serum response factor (*SRF*)-miR-133a, and *FOXO1*-miR-486 pairs (Figure 4), the expression level of miRNA and its target gene showed significant negative correlations. *IGF1* plays an important role in muscle cell proliferation and myogenic differentiation, and its expression level decreases with increasing age. Previous studies have shown that *IGF1* is a target of miR-1 and miR-320 (Musarò et al., 2001; Wang et al., 2009). *SRF* inhibits muscle cell proliferation and differentiation *in vitro* and *in vivo* (Wang et al., 2002; Li et al., 2005), and its expression level increases after birth and subsequently stabilizes in adulthood. miR-133 is mainly expressed during embryogenesis and promotes muscle cell proliferation by directly repressing the expression of *SRF*. *FOXO1* is usually associated with muscle waste disease or aging under normal conditions, and its expression level increases during the late developmental stages. In addition, *FOXO1* is a target of miR-486 (Small et al., 2010), and they jointly participate in protein degradation. The decreasing level of miR-486 with age suggests an increasing expression level of *FOXO1*, which may promote muscle atrophy during the later stages of pig muscle development.

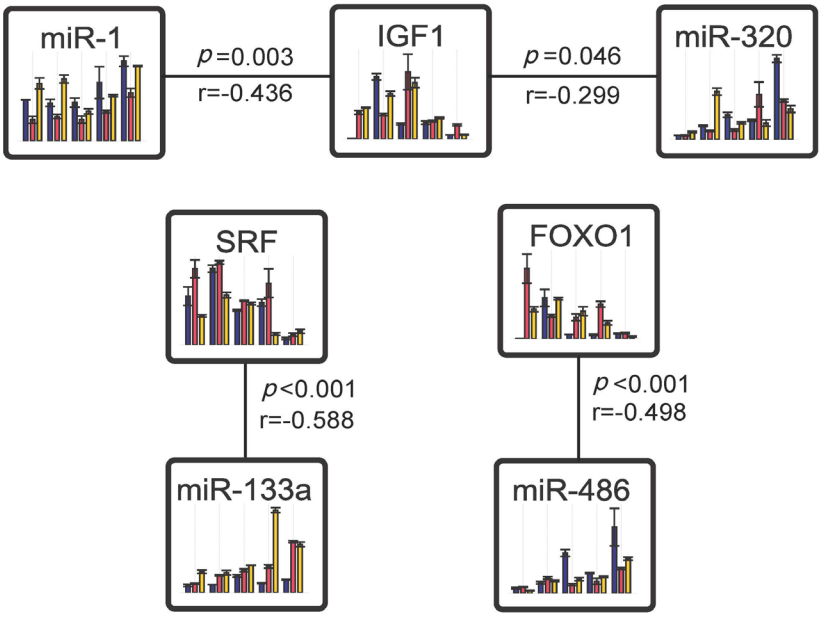

Figure 4. Expression patterns of protein-coding gene-miRNA pairs. The squares represent the expression patterns of miRNA or protein-coding gene in CM (blue bars), LDM (red bars), and PMM (yellow bars). From left to right, E90, P0, P30, P180, and 7Y are represented, respectively.

In summary, we investigated the expression changes of porcine protein-coding and miRNA genes related to muscle development by qRT-PCR. Combined with STEM clustering, we found that growth-related genes are highly expressed during the embryonic period in CM, but do not show uniform expression patterns after birth. Moreover, most genes related to muscle hypertrophy are downregulated with increasing age in skeletal muscle, while the genes related to muscle atrophy are upregulated. In addition, some mRNAs and miRNAs exhibited significant negative correlations throughout all developmental stages. At present, we have constructed only a crude genetic blueprint of muscle development, with a multitude of details that have yet to be clarified. Further study utilizing more accurate methods, such as RNA-seq or small RNA-seq, will be needed to delineate the complicated mechanisms of gene regulation during muscle development.

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Supplementary material

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