The genetic regulation of skeletal muscle development: insights from chicken studies

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Abstract Skeletal muscle development is a complex multi-process trait regulated by various genetic factors. The chicken embryo is an ideal model system for studying skeletal muscle development. However, only a small proportion of the genetic factors affecting skeletal muscle development have been identified in chicken. The aim of this review is to summarize recent knowledge about the genetic factors involved in the regulation of skeletal muscle development in the chicken, such as gene polymorphisms, epigenetic modification, noncoding RNAs and transcription factors, which can influence skeletal muscle development at the genome, epigenome, transcriptome and proteome levels. Research on the regulation of skeletal muscle development in chicken is not yet comprehensive and most of the candidate genes and single nucleotide polymorphisms related to chicken muscle growth remain to be verified in experimental studies. In addition, the data derived from transcriptome sequencing and genome-wide association studies still require further investigation and analysis and comprehensive studies on the regulation of chicken skeletal muscle development will continue as a major research focus.

Keywords chicken, epigenetic modification, miRNAs, skeletal muscle development, SNP, transcription factor

1 Introduction

Skeletal muscle is a form of striated muscle tissue, distributed mainly in the limbs, chest and hips of vertebrates. Most skeletal muscles are attached to bones by tendons, and are important for movement, strength,

metabolism and body posture of the animal. The skeletal muscle is made up of thousands of muscle fibers, each containing multiple nuclei, which come from the fusion of myoblasts. Skeletal muscle development is a complex trait, influenced by genetic regulation and environmental cues[1,2]. The developmental process from muscle precursor cell to muscle fiber formation is regulated by various genetic factors, including gene polymorphism, transcription factors, DNA methylation and noncoding RNAs[3–7]. These genetic factors cooperate with each other to ensure the normal development of skeletal muscle.

Skeletal muscle development is a multi-step process that includes myofiber formation and hypertrophy. The formation of myofiber occurs mainly in embryogenesis. During the embryonic stage, the myofibers are generated by the following four major processes: myogenic precursor differentiation from somites, myoblast development from myogenic precursors, myoblast proliferation and fusion to form the multinucleated myotubes, and finally maturation of myotubes into myofibers[3,8]. After myofibers are formed, they undergo hypertrophy at the postnatal stage. This stage comprises protein turnover, satellite cell proliferation, differentiation and fusion with myofibers to stimulated the hypertrophy of myofibers[9]. In addition to these complex cell developmental processes during myofiber formation and hypertrophy, the fine-tuned regulation of numerous myogenic genes is also important for the development of skeletal muscle.

Members of three families of transcription factors have important roles during skeletal muscle development. The paired box proteins, Pax-3 and Pax-7, are essential for muscle precursor cell proliferation, myoblast determination and the specification of myogenic satellite cell[10–14]. The myogenic regulatory factor family includes MyoD, myogenin, myogenic factor 5 and myogenic regulatory factor 4, which are muscle-specific transcription factors that are indispensable for myoblast determination and

differentiation[15–17]. The myocyte enhancer factor 2 family of transcription factors includes MEF2A, -B, -C and-D, which function mainly in myoblast differentiation and muscle-specific gene transcription[18–20]. These three transcription factor families cooperate with each other to facilitate myogenesis. In addition to the three transcription factor families, many other genetic factors can also regulate skeletal muscle development. DNA polymorphism in these muscle development-related genes can result in abnormal muscle growth[7,21]. MicroRNAs (miRNAs) have been found to have an important regulatory role during skeletal muscle development[3,22]. The long non-coding RNAs and the circular RNAs also regulate myogenesis through their own regulatory mechanisms[23,24]. Epigenetic modification, such as histone modifications and DNA methylation, can also control skeletal muscle development by epigenetic mechanisms[5,6,25]. Studies on all of the above genetic factors have become well established in research on myogenesis regulation. However, the precise regulatory mechanisms and networks involved in skeletal muscle development still need to be elucidated and more and more genetic factors involved in skeletal muscle development remain to be explored.

Myogenic proteins are highly conserved among mammalian and avian embryos[26] and many major discoveries in muscle development depend on avian model systems, especially the chick[27]. The chicken is the first model organism to be used and is excellent for developmental investigations[28,29]. Additionally, the skeletal muscle of chicken is an important food source for humans. However, the genetic regulation of chicken skeletal muscle development is still poorly understood. In this review, the genetic factors regulating the programs of chicken skeletal muscle development are summarized, and the recent progress in the investigation of chicken myogenic-related regulatory factors newly identified in our laboratory are discussed.

2   DNA polymorphisms affecting chicken skeletal muscle development

DNA polymorphisms are a useful tool for finding genetic markers related to chicken phenotypic traits. In this section, single nucleotide polymorphisms (SNPs) that related to chicken skeletal muscle development are summarized and discussed. Most of these SNPs are detected by GWAS, direct genome sequencing and/or SNP genotyping technologies. Since growth traits can to some extent also explain or reflect the status of skeletal muscle development, and skeletal muscles that attached to the trunk, wings and hips have been used by researchers for estimating bodyweight, in this part we also discussed the SNPs that are significantly associated with several production traits related to chicken bodyweight.

2.1   Single nucleotide polymorphisms in the myogenic genes related to chicken skeletal muscle performance

Many genes, called myogenic genes, from three families of transcription factors, myostatin (*MSTN*) and growth axis related genes have been found to be involved in skeletal muscle development. These myogenic genes are ideal candidate genes for genetic marker screening for broiler breeding. Many polymorphisms have been found to be significantly associated with skeletal muscle growth traits through the screening of genetic markers of myogenic genes (Table 1). These SNPs can be located in the 5′UTR, intron, exon and 3′UTR region of the gene. SNPs in the 5′ UTR or intron may influence gene expression, or may be in linkage with some other causative polymorphisms that influences skeletal muscle growth and development[36]. In addition, a synonymous mutation, which does not cause an amino acid change, can also affect gene function and muscle development[44]. SNPs at the *MSTN* gene promoter might result in the downregulation of this gene, which is a negative regulator of skeletal muscle development[42]. However, the mechanism whereby these SNPs affect chicken skeletal muscle development remain unclear. The major task for the future is to find the relationship between genotype and phenotype. Further exploration of the molecular mechanism underling this relationship is still needed.

2.2   Genome-wide association studies reveal single nucleotide polymorphisms significantly related to chicken muscle growth traits

Skeletal muscle development is a complex trait that can be regulated by multiple genetic factors. As a useful method for discovering complex trait-related gene loci or genomic regions, genome-wide association study (GWAS) has yielded more reproducible associations than many other approaches[49]. Five SNPs have been found to be significantly associated with the chicken muscle growth trait in the 1.5 Mb karyopherin subunit alpha 3- forkhead box O1 a (*KPNA3-FOXO1A*) region at chicken chromosome 1 (GGA1), which were detected by using GWAS in a chicken F_2 resource population (White Recessive Rock × Xinghua)[50]. One of the 5 SNPs, rs15497910, is significantly associated with breast and leg muscle weight, indicating its potential role in the regulation of skeletal muscle development. However, no studies have reported indicating that the *KPNA3* gene, which is the nearest gene to rs15497910, is able to influence muscle development in any species. So rs15497910 might influence chicken muscle growth trait by another mechanism that is independent of the function of *KPNA3* gene. The other two SNPs, rs13973515 and GGaluGA055359, were significantly associated with average daily weight gain at 15 to 28 d and wing weight, respectively. In addition, the nearest gene to these two SNPs is *FOXO1A*, which is an

Table 1 Single nucleotide polymorphisms located in myogenic genes associated with chicken skeletal muscle development

Polymorphism	Gene ID	Skeletal muscle development related trait	Reference
A17299834G	*IGF1R*	Eviscerated weight	[30]
G729T	*IGFBP2*	Breast muscle weight	[31]
A663T	*IGFBP2*	Breast muscle weight	
G738A	*IGFBP2*	Breast muscle weight and leg muscle weight	
C/T SNP in intron 2	*IGFBP2*	Bodyweights	[32]
g.570C > A	*IGF1*	Breast muscle weight	[33]
C51978309T	*IGF1*	Transversal area of the leg and breast muscle fiber	[34]
G6631778A	*GHR*	Bodyweights	[35]
G + 1705A	*GH*	Bodyweights	[36]
MR2	*PIT1*	Bodyweight at 28 d, 42 d	[37]
MR4	*PIT1*	Bodyweight at 84 d	
MR5	*PIT1*	Bodyweight at 21 d, 28 d	
g.3051C > T	*GHSR*	Final bodyweight	[38]
c.739 + 726T > C (M2)	*GHSR*	Breast muscle weight and leg muscle weight	[39]
G1215A	*GHRL*	Bodyweight	[40]
8bp indel	*GHRL*	Bodyweight	
C71T	*GHRL*	Bodyweight	[41]
A241T	*MSTN*	Bodyweight at hatching	[42]
c.234G > A	*MSTN*	Bodyweights	[43]
G2283A	*MSTN*	Bodyweights	[44]
T46023C	*MEF2A*	Leg muscle weight	[45]
T89232G	*MEF2A*	Semi-eviscerated percentage	
87T > C	*Myf5*	Eviscerated weight	[46]
154T > C	*MSTN*	Breast muscle weight	
A446G	*MSTN*	Breast muscle percentage	[47]
31-bp indel	*PAX7*	Breast muscle fiber diameter, leg muscle fiber diameter, breast muscle fiber density	[48]

important transcription factor during skeletal muscle development[51–53]. The question whether and how these two SNPs can influence expression or function of *FOXO1A* needs further study.

Two other GWAS of a chicken F$_2$ resource population derived from the reciprocal crosses between slow- and fast-growing chicken breeds, have also identified some loci or genes related to muscle growth[54,55]. Unlike the results described above, these two studies showed that many SNPs on GGA4 are significantly associated with chicken growth traits. The genes near significant SNPs, such as ligand dependent nuclear receptor corepressor-like protein 1, LIM domain binding 2 and microtubule-associated protein tau, have lower expression in breast muscle of slow-growing chickens compared to fast-growing chickens, indicating their potential roles in the regulation of muscle development[54]. By GWAS of local chicken breeds, a 0.65 Mb region on GGA3 was identified as associated with breast muscle weight (BrW) and breast muscle percentage (BrP)[56]. The gap junction protein

alpha 1 (*GJA1*) gene which is located in this region may be a functional gene for skeletal muscle development, because the expression of this gene was upregulated with the increase of breast muscle weight across development[56]. Additionally, four SNPs (GGaluGA225255, Gga_rs16-287013, Gga_rs14366866 and Gga_rs14366948) located near *GJA1* are significantly associated with BrW and BrP. Another GWAS, using Jinghai yellow chickens, found that 15 SNPs are significantly associated with five carcass traits[57]. However, there has been no SNP identified by GWAS that is significantly associated with breast muscle weight or leg muscle weight, which are more representative of skeletal muscle development.

Even though increasing numbers of SNPs, genes and regions have been found to be associated with chicken skeletal muscle development by GWAS, none of the new candidate genes has been verified to be involved in skeletal muscle development. The significant associated loci identified in the genome by these studies are very different, indicating that the loci associated with muscle

development are not consistent between breeds. Thus GWAS cannot fully exploit all loci associated with muscle development and a more comprehensive and deeper analysis of these GWAS results is still needed.

3   Transcriptional level regulation of chicken skeletal muscle development

3.1   Identification of candidate genes involved in chicken skeletal muscle development by microarray and high-throughput sequencing

Gene expression profiling chips and high-throughput sequencing are effective methods for screening differentially expressed genes between groups. By using skeletal muscle from slow- and fast-growing chicken breeds as experimental materials, or using skeletal muscle from different developmental stages of chicken, researchers have found many candidate genes related to skeletal muscle development using microarray or high-throughput sequencing experiments. By sequencing RNA to detect gene expression difference between the skeletal muscle of recessive white rock (WRR) and Xinghua (XH) chicken, higher expression of *FOXO3* was found to potentially inhibit skeletal muscle development of XH chicken[58]. This could be because the mRNA level of *FOXO3* is expressed more highly in the skeletal muscle of XH than that in WRR chickens, and reduced *FOXO3* expression can upregulate growth-related genes expression in DF-1 cells[58]. In addition to WRR and XH chickens, skeletal muscle from sex-linked dwarf (SLD) chicken and normal chicken have been used to discover genes or pathways related to chicken muscle development[59,60]. The SLD chicken has lower bodyweight and smaller muscle fibers than those of normal chicken because of a recessive mutation of the growth hormone receptor (*GHR*) gene. The *GHR* mutation leads 55 genes and 173 genes to be differentially expressed between the two chicken breeds in embryonic day 14 (E14) and week 7 (W7), respectively, and these genes are mainly related to the regulation of cell division[59]. By comparing the differentially expressed genes between E14 and W7 in SLD and normal chickens, it was also found that many genes enriched in the MAPK, PI3K-Akt, Wnt and insulin pathways were related to chicken skeletal muscle development, and these genes can interact with miRNAs to construct a miRNAs-mRNAs regulatory network during skeletal muscle development[59]. The above study was performed using microarray experiments to investigate gene expression. Similarly, microarrays were used to study Qingyuan partridge and Cobb 500, representing slow- and fast-growing chicken breeds, respectively[61]. In that study, many differentially-expressed genes were also found, and it was argued that

energy metabolism-related genes might influence muscle development, such as protein kinase AMP-activated non-catalytic subunit gamma 1, protein kinase AMP-activated non-catalytic subunit gamma 3 and protein kinase cAMP-dependent type II regulatory subunit beta[61].

Broilers and layers are normally different chicken breeds that differ enormously in their growth rates and body sizes. By comparing gene expression in the skeletal muscle of these two types of chicken, researchers have found that many genes related to muscle fiber types, satellite cell proliferation and differentiation are different in the two kinds of chicken[62]. The dickkopf homolog 3 gene could be a potential regulator of skeletal muscle development, because the expression pattern of this gene was significantly correlated with the natural increased rate of body mass of chickens, and it had higher expression level in broilers than in layers[62]. In general, although many potential candidate genes were found to be involved in chicken skeletal muscle development, large-scale analyses are still required to validate their roles during skeletal muscle development, to confirm that a candidate gene that can directly affect chicken skeletal muscle development is important for application in broiler breeding.

3.2   MiRNAs and long non-coding RNAs involved in chicken skeletal muscle development

MiRNAs regulate target gene expression at the post-transcription level. During skeletal muscle development, many miRNAs have been found to have regulatory roles at each step[3,22]. However, most of this research has been in mammals, and the question whether these miRNAs can regulate chicken skeletal muscle development still remains to be elucidated. To investigate the functional miRNAs in chicken skeletal muscle development, miRNAs and mRNAs were sequenced from the skeletal muscle of both broilers and layers[62]. A miRNA-target gene interaction network related to skeletal muscle development was constructed by using integrative miRNA target-prediction and network analysis. In that network, many genes related to muscle development were found to be targeted by miRNAs, for example, gga-miR-1a can directly inhibit activin A receptor type 2B expression, and reversion inducing cysteine rich protein with kazal motifs is a putative target of gga-miR-200b[62]. A similar research strategy has been used in the study of miRNA deep sequencing in the skeletal muscle of WRR and XH chickens, and to construct a miRNAs-mRNAs interaction network for chicken muscle development[63]. However, the detailed functional validation of the genes or miRNAs related to muscle development is still lacking in both of these two studies.

In other work, expression of miRNAs and mRNAs was detected in the skeletal muscle of dwarf and normal

chickens, and it was found that let-7b can regulate skeletal muscle development by directly inhibiting the *GHR* gene[60]. Even though detailed functional verification of let-7b was not performed, many other candidate miRNAs were found from the microarray results, such as miR-203, miR-20a-5p and miR-20b-5p. By using *in vivo* detection and *in vitro* experiments, miR-203 was confirmed to inhibit chicken myoblast proliferation and differentiation by repressing Jun proto-oncogene (*c-JUN*) and *MEF2C*, respectively[64]. Similarly, both miR-20a-5p and miR-20b-5p have been comprehensively validated as regulating chicken myoblast proliferation and differentiation through specifically interacting with E2F transcription factor 1 (*E2F1*)[65]. This work has revealed the regulatory mechanisms of candidate miRNAs during muscle development and also furthered understanding of the regulatory networks of chicken skeletal muscle development.

The candidate gene validation approach is a useful strategy to find a functional miRNA acting during chicken skeletal muscle development. As a well-known myogenic miRNA in mammals, miR-206 has important roles during muscle development. However, its function in chicken still remains unknown. Aiming to investigate miR-206 roles in chicken, miR-206 was overexpressed in chicken myoblasts and found to significantly increase myogenin and muscle creatine kinase expression, which are important genes for muscle differentiation[66]. Additionally, eight variants have been identified in the promoter region of *miR-206* gene that exhibit significant effects on chicken birthweight, indicating an important role of miR-206 in chicken development. Myomaker is a new gene that was found to be involved in muscle development[67]. To find out which miRNA can directly bind to Myomaker 3′UTR and regulate its expression, RNAhybrid software and dual-luciferase reporter assay were used. It was found that miR-140-3p directly inhibited Myomaker expression and by targeting the Myomaker gene inhibits chicken myoblast fusion.

In addition to miRNAs, long non-coding RNAs (lncRNAs) have attracted increasing attention in the study of skeletal muscle development. The first chicken lncRNA catalog in skeletal muscle has been constructed[68]. However, although novel lncRNAs in chicken were identified, no single lncRNA has been validated to function during chicken skeletal muscle development. Recently, differences in lncRNAs expression were detected in the skeletal muscle between slow- and fast-growing chicken breeds, and it was found some lncRNAs can regulate myoblast proliferation and differentiation by interacting with miRNAs and mRNAs[69]. Additionally, some lncRNAs have also been found to regulate gene expression by cis-regulation (unpublished data). Besides lncRNA, the functions of some circular RNAs during chicken skeletal muscle development have been investigated using circle-RNA sequencing. This research has provided further understanding of the regulation of chicken skeletal muscle development.

4　Epigenetic modification regulates chicken skeletal muscle growth

Epigenetics is the study of stably heritable traits or gene expression caused by mechanisms other than underlying DNA sequence changes[70]. During skeletal muscle development, epigenetic regulators are able to promote the transcription of a selective group of gene and drive myogenesis[5]. Gene expression during myogenesis can also be regulated by epigenetic modification, such as DNA methylation. To identify candidate genes and genomic methylated regions for chicken skeletal muscle development, the genome-wide DNA methylation pattern of skeletal muscle was investigated using methylated DNA immunoprecipitation-sequencing in high and low body-weights of 7-week-old WRR and XH chickens[71]. Many well-known growth-related genes, such as insulin like growth factor 1 receptor (*IGF1R*), fibroblast growth factor 12 (*FGF12*), *FGF14* and fibroblast growth factor receptor 2, were found to exhibit altered DNA methylation in all comparisons, indicating that DNA methylation in several growth-related genes may affect chicken skeletal muscle development.

Another study investigating DNA methylation status in chicken skeletal muscle was conducted in a three-yellow chicken population[72]. By using fluorescence-labeled methylation-sensitive amplified polymorphism analysis, it was found that the differences in DNA methylation levels are significantly associated with muscle fiber density and muscle drip loss[72]. The level of DNA methylation in the majority of the genome changes dramatically during early development[73]. Using broilers as experimental animals, the genomic DNA methylation status was examined during chicken embryogenesis. An increasing genomic DNA methylation level was found in muscle, while the methylation level of the *IGF2* promoter gradually decreased[74]. As IGF2 is a positive regulator of skeletal muscle development, and DNA methylation in gene promoters can inhibit gene transcription and expression[75], the decreased promoter methylation levels of *IGF2* could release the expression of *IGF2*, and therefore promote skeletal muscle development.

5　Transcription factor regulates chicken skeletal muscle development through interaction with miRNAs

The critical roles of the three transcription factor families during skeletal muscle development have been introduced above. Undoubtedly these transcription factors also have roles in chickens, and the polymorphisms in these factors are associated with chicken muscle growth traits (Table 1). In chicken myoblasts, it was found that MyoD and

myogenin can promote the transcription of muscle-specific genes, such as Myomaker, by directly binding to the E-box region located in the gene promoter[76]. In addition to regulating the transcription of their downstream genes, these transcription factors can also interact with miRNAs and then influence chicken muscle development. Recently, a miRNA that exhibits gradually upregulated expression during chicken myoblast differentiation was found to be regulated by MyoD because of the binding of MyoD to the promoter of the miRNA gene (unpublished data). The MyoD promotes the expression of this miRNA and facilities myoblast differentiation. In other work, it was found that a small RNA called miR-203 can inhibit the expression of *MEF2C*, a member of the three transcription factors families, by directly binding to its 3′UTR[64]. The inhibition of *MEF2C* by miR-203 repressed chicken myoblast differentiation, and reduced the formation of myotubes[64].

In addition to the members of these three transcription factor families, many other transcription factors also have important roles in the regulation of chicken skeletal muscle development (Fig. 1). As important regulators of cell proliferation, c-JUN and E2F1 promote chicken myoblast proliferation by the regulation of their downstream target genes[64,65]. Similarly, both can be regulated by miRNA. MiR-203 and miR-20a-5p/20b-5p directly inhibit *c-JUN* and *E2F1* expression, respectively, and therefore repress

chicken myoblast proliferation[64,65]. However, E2F1 can in turn directly regulate the transcription of miR-20a-5p/20b-5p and thus form an E2F1-miR-20a-5p/20b-5p auto-regulatory feedback loop[65]. In addition to *c-JUN* and *MEF2C*, miR-203 can also bind to the 3′UTR of tumor protein p63 (*p63*), which is able to promote muscle cell proliferation and differentiation[77,78]. The binding of miR-203 to the *p63* 3′UTR inhibits its mRNA expression and functions[64]. Recently, it was observed that v-myc avian myelocytomatosis viral oncogene homolog (c-Myc) transcription factor can bind to the promoters of a large number of genes, *miRNAs* and *lincRNAs* in chicken muscle cells (unpublished data). *In vitro* experiments showed that c-Myc regulates muscle cell proliferation and differentiation by controlling the transcription of these downstream genes, miRNAs and long intergenic non-coding RNAs (lincR-NAs). Notably, some of c-Myc target miRNAs can in turn bind to the 3′UTR of *c-Myc* mRNA and inhibit *c-Myc* expression. Therefore, the interaction of c-Myc and its downstream miRNAs provide a potentially important feedback loop during chicken skeletal muscle development.

6   Conclusions and future perspectives
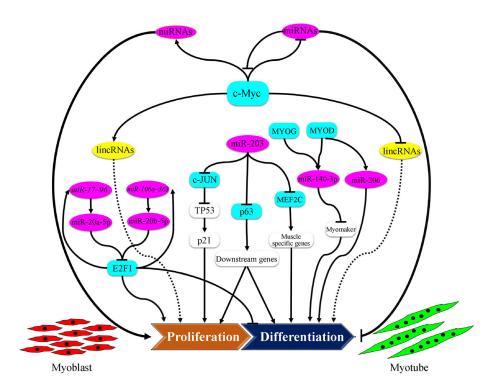
Chicken embryos are a perfect model system for research

Fig. 1 The network of transcription factors and miRNAs interacting during chicken myoblasts differentiation to myotubes. Light blue represents transcription factors; red represents miRNAs; yellow represents lincRNAs; white represents genes.

on skeletal muscle development. Studies on the regulation of chicken skeletal muscle development not only provide further understanding of the process of muscle fiber formation, but also allow the identification of potential candidate genes and molecular markers that could improve chicken muscle mass. In recent years, many genes, SNPs, DNA methylation regions and noncoding RNAs have been found to be involved in chicken skeletal muscle development, especially during the processes of myoblast proliferation and differentiation. These two processes are important for the formation of muscle fiber and determination of muscle fiber number. However, only a few genetic regulators involved in the control of these two processes were found in chicken, including some miRNAs and transcription factors. Dozens of genes, ncRNAs and epigenetic modification involved in the regulation of chicken skeletal muscle development still need to be validated.

The regulatory function of DNA methylation of gene promoters, lncRNAs and circRNAs should be the next area of research focus for chicken muscle development. Also, the interaction between transcription factors and noncoding RNAs should be a priority for further study. Additionally, the reduced costs of sequencing and microarray have enabled the wider use of GWAS for identifying complex trait-related gene loci and genomic regions. Unlike humans and mice, the functions of most of the candidate genes or loci identified by GWAS in chickens are still to be validated empirically. Therefore, experimental validation of function of the candidate genes and loci involved in chicken skeletal muscle development should also be a priority. In addition, gene imprinting, histone acetylation, protein modification and protein structural polymorphism have gradually been attracting more attention in cell development research[79]. The way in which these genetic factors regulate skeletal muscle development should also receive more attention. Finally, the rate of skeletal muscle development is usually associated with meat quality in the production of livestock and poultry. However, the challenge is to improve the growth rate of skeletal muscle while in parallel ensuring the meat quality. To achieve this the characteristics of skeletal muscle during the process of development that affect meat quality must be understood. All of these issues deserve further study.

Compliance with ethics guidelines Wen Luo, Bahareldin A. Abdalla, Qinghua Nie, and Xiquan Zhang declare that they have no conflicts of interest or financial conflicts to disclose.

This article is a review and does not contain any studies with human or animal subjects performed by any of the authors.

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