Identification of *lgf2bp1* gene family and effect on chicken myoblast proliferation

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**ABSTRACT**

The function of *lgf2bp1* regulated muscle development in Lueyang black-bone chickens is to be investigated. The insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) belongs to the family of single-stranded RNA-binding proteins (IGF2BP1-3). IGF2BP1 is abundantly expressed in a broad range of foetal tissues, but only in a limited number of normal adult tissues, and is involved in biological regulation processes, including cell proliferation, differentiation, metabolism and migration, especially in tumour cells. Recent study has reported *lgf2bp1* was a key gene that regulated the body size of Pekin ducks. We found that the expression level of *lgf2bp1* gradually decreased with age in the leg muscle of Lueyang black-bone chicken, but its function in chicken muscle development is unknown. Firstly, the evolutionary relationship of *lgf2bp1* gene family was analysed in chicken. Additionally, *lgf2bp1*-overexpressed myoblasts of Lueyang black-bone chicken were constructed. The results showed that *lgf2bp1* plays a negative role in cell proliferation by the CCK8 and 5-ethynyl-2’-deoxyuridine assay. Moreover, *lgf2bp1* inhibited myogenesis by detecting the myoblast proliferation markers of pax7, the expression of myogenic differentiation markers, MyoD, MyoG and Myf5 in myoblasts. Thus, *lgf2bp1* can inhibit chicken myoblast proliferation and differentiation by regulating key gene expression of myogenesis, which is not conducive to the growth and development of skeletal muscle in Lueyang black chickens.

**1. Introduction**

Lueyang black-bone chicken is a low-quality broiler in China, which has been domesticated in Lueyang County, Shaanxi province for 1600 years. In the long history of domestication and breeding, it was always free range around hilly areas in Lueyang County, which contributed unique genetic characteristics and excellent meat quality (Jianqin et al. 2015). However, the factors that affect muscle production in Lueyang black-bone chicken are still unclear. Skeletal muscle is one of the most dynamic and plastic tissues in animal body, which is the main component of poultry carcass that determines the slaughter traits and meat quality of poultry. Skeletal muscle formation is a complex multistage developmental process, which begins with multipotent precursor cells differentiating into myoblast, following proliferation, differentiation, and fusion into multinuclear myotubes and then forming mature muscle fibres (Bentzinger et al. 2012; Yusuf and Brand-Saberi 2012). Skeletal muscle cell proliferation is the basis of muscle growth and development, and is a determinant factor of poultry muscle production.

The insulin-like growth factor-2 mRNA-binding protein family (IGF2BP family) is a conserved family of single-stranded RNA-binding proteins (Huang et al. 2018). IGF2BP family has three members (IGF2BP1-3) (Nielsen et al. 1999), which are also named as IGF-II mRNA binding proteins, coding region instability determinant binding proteins, zinc finger proteins, VICKZ proteins, Vg1RBP/Vera or KOC (Ross et al. 1997; Doyle et al. 1998; Havin et al. 1998; Bell et al. 2013). Several studies demonstrated that these proteins played important roles in cell proliferation, differentiation, polarization, migration, morphology and metabolism. Interestingly, IGF2BP1 in the IGF2BP family showed a significant effect on cell proliferation.

*lgf2bp1* was firstly identified in human rhabdomyosarcoma (RMS) cell line RD (Faye et al. 2015). Recent studies demonstrated that *lgf2bp1* expressed in a broad range of foetal tissues but only in a limited number of normal adult tissues (Huang et al. 2018). It is reported that knockdown of *lgf2bp1* gene caused high levels of perinatal death (60%) and tissues dysplasia in almost every organ in mice (Hansen et al. 2004). Mice with *lgf2bp1* deficiency exhibited dwarfism, severely decreased viability, and impaired gut development. Therefore, *lgf2bp1* is very important for embryonic development in mice. In addition, multiple research studies also unveiled the important roles of *lgf2bp1* in a variety of cancers including lung adenocarcinoma (Kato et al. 2007), hepatocellular carcinoma (Gutschner et al. 2014), breast cancer (Fakhraldeen et al. 2015), glioblastoma (Wang et al. 2015), melanoma (Kim et al. 2018) and others. *lgf2bp1* regulated post-transcriptionally mRNAs of MYC, KRAS, MDR1, PTEN and other genes, those coding key players involved in cell proliferation, survival, adhesion and migration control in different tumours (Stöhr...
and Hüttelmaier 2012; Bell et al. 2013). Therefore, Igf2bp1 may be viewed as a cancer facilitator. A recent study found that Igf2bp1 was a key gene that regulated the body size of Pekin ducks by genome-wide association analysis (Zhou et al. 2018). However, the functions and underlying mechanisms of Igf2bp1 during the proliferation of chicken myoblast remain largely unknown.

In the present study, we conducted transcriptome sequencing analysis of the thigh muscles in Lueyang black-bone chicken at 20, 60 and 120 days of age, and Igf2bp1 was identified as a common down-regulated gene. Additionally, the effect of Igf2bp1 on the proliferation of chicken myoblasts was investigated. These results can provide good knowledge of the molecular mechanisms associated with muscle production in Lueyang black-bone chicken or other poultry species.

2. Materials and methods

2.1 Ethics statements

All animal experiments were performed following the ‘Guide for the Care and Use of Laboratory Animal’ of the Institutional Animal Care and Use Committee of Shaanxi University of Technology (SLGQD/09/2019), and were in accordance with a protocol approved by the animal use committee of the Chinese Ministry of Agriculture.

2.2. Animals and sample collection

All Lueyang black-bone chickens were euthanized by cervical dislocation at 20, 60 and 120 days of age (3 cocks per group), respectively. After euthanasia, roughly 0.3–0.5 g muscle from the left posterior thigh muscle was collected and immediately snap-frozen in liquid nitrogen, which was sent to Shanghai Personal Biotechnology.

2.3. RNA isolation and collection of the transcriptome profile of the Igf2bp1 gene family of chickens

Total RNA was isolated from the thigh muscle using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The purity and amount of RNA were detected by a nanodrop 2000c spectrophotometer (Thermo Scientific). To obtain an RNA-seq library of Lueyang black-bone chicken muscle, the fragments with adaptors were enriched and amplified by PCR. Quantification and quality control of the sample libraries were evaluated with the Bioanalyser 2100 (Agilent Technologies) and the ABI StepOnePlus Real-time PCR System, then sequenced in one lane using an Illumina NextSeq 500 instrument. The assembled RNA-seq data were used to quantify these gene expression levels, based on their fragments per kilobase of exon per million reads mapped values, using Cufflinks with default parameters. For each member’s expression level of the Igf2bp1 gene family, we used the Heml 1.0 software with default parameters. For the expression level of several Igf2bp1 genes with error bars, the ggplot2 R package was used.

2.4. Identification of Igf2bp1 gene family and phylogenetic analysis

The whole chicken (Gallus gallus) genome sequences were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/). Human IGF2BP1 protein sequence also was downloaded from NCBI, and as query by blastp (a local basic local alignment search tool) to search the chicken similar sequence, with -1 E threshold. Finally we obtained Igf2bp1 candidate sequence of chicken. From the pfam database, we had the Hidden Markov Model profile of the K homology (KH) domain; meanwhile those candidate sequences were submitted to the SMART online software (http://smart.embl-heidelberg.de/), identified the final sequence of Igf2bp1 in chicken.

The alignment of chicken IGF2BP1 protein sequences was carried out by ClustalX version 2.1 with default parameters (Edgar 2004). Then, MEGA 7.0 (Molecular evolutionary genetics analysis, Pennsylvania State University: State College, PA, USA) (Kumar et al. 2008) was used to construct the unrooted phylogenetic tree for the evolutionary relationship of Igf2bp1 gene in chicken, by the neighbour-joining (NJ) method with 1000 bootstrap replications to be constructed.

2.5. Cell isolation and culturing

The fertilized eggs (E10) from Lueyang black-bone chicken were chosen to isolate myoblasts. Chicken skeletal muscles were washed in PBS supplemented with antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) (CORNING, Corning city, NY, USA) and minced carefully. Samples were put into a 15-mL centrifuge tube and digested with collagenase Type I (0.2% w/v) (MP Biomedicals, Santa Ana, CA, USA) for 40 min and then digested with 0.25% trypsin (MP Biomedicals, Santa Ana, CA, USA) for 5 min at 37°C. Then, enzymatic reactions were terminated by the addition of growth medium with 10% FBS (Gibco, Grand Island, NY, USA). Cells were suspended and filtered by cell sieves (100 and 400 mesh), and centrifuged at 1000 rpm for 8 min and re-suspended in growth medium. This was repeated two times. Mixed cells, containing both myoblasts and fibroblasts, were transferred to culture dishes. As fibroblasts readily adhere, a differential attachment technique was used to separate myoblasts from the mixture. Mixed cells were cultured at 37°C and 5% CO₂ for 4–6 h to allow fibroblasts to adhere, while non-adherent myoblasts were transferred into new culture dishes with a fresh medium containing 10% FBS. Myoblasts were maintained in an incubator with 5% CO₂ at 37°C. All experimental procedures were approved by the Laboratory Animal Ethics Committee of Shaanxi University of Technology.

2.6. Primer design and cloning Igf2bp1 gene

The sequence of chicken Igf2bp1 mRNA (GenBank accession number: NM_205071.1) was used as the template for primer design with Primer Premier 5.0 software. BamH I and EcoR I restriction enzyme sites were added to the primer set (Table 1). The PCR was performed in the following system:
4 μL of cDNA, 10 μL of Q5 Reaction Buffer, 5 μL of dNTP, 0.6 μL of Q5 DNA polymerase (NEB, USA), 3 μL of each primer and 24.4 μL of ddH2O. The PCR reaction programme was as follows: 95°C pre-denaturation for 5 min; 34 cycles of 95°C denaturation for 30 s, 60°C annealing for 30 s, 72°C extension for 2 min, 72°C extension for 10 min, 12°C preservation. PCR products were identified by electrophoresis on 1% agarose gel and purified with a gel extraction kit (BioFlux, Hangzhou, China). Purified PCR products were cloned into pMD19-T vector (Takara, Tokyo, Japan), and candidate clones were checked by double-directional sequencing (General Biosystems, Anhui, China).

2.7. Construction and transfection of pCDNA3.1-Igf2bp1 expression vector
The Igf2bp1 gene in pMD19-T-Igf2bp1 plasmid was sub-cloned into pCDNA3.1 between BamHI and EcoRI sites, resulting in the expression vector pCDNA3.1-Igf2bp1. Subsequently, pCDNA3.1-Igf2bp1 was transfected into chicken myoblasts when the cell confluence reached 70–80%. Meanwhile, cells treated with plasmid pCDNA3.1 were taken as control. Transfection was carried out using Superfect (HEART, Xi’an, China) according to the manufacturer’s instructions. In each well, cells were transfected with the following transfection mixture: 2 μg of DNA, 400 μL of opti-MEM (Gibco, Grand Island, NY, USA) and 4 μL of Superfact. After 48 or 72 h cells were harvested for further assays.

2.8. Isolation of total RNA and real-time quantitative PCR
TRIzol reagent (TaKaRa, Dalian, China) was used to extract total RNA from cell cultures or tissue samples. First-strand cDNA synthesis was performed using a reverse transcription kit (TaKaRa, Dalian, China) and random primers. Real-time quantitative PCR was performed in triplicate samples using a SYBR Green kit (TaKaRa, Dalian, China) on the Step One Plus™ (Applied Biosystems, Foster City, CA, USA). Chicken β-actin was used as a reference gene. The 2-ΔΔCT algorithm was employed to estimate the relative expression level of each gene. The sequences of the primers used are listed in Table 2.

2.9. Antibodies and western blotting
Antibodies directed against the following proteins were used: IGF2BP1 (Abcam, USA), Pax7 (Abcam, USA), MyoD (DSHB, USA), GAPDH (proteintech, China). The myoblasts were collected after cultivation and adequate treatment, and total protein extracts were isolated using lysis buffer (RIPA, Beyotime, China) containing protease inhibitor (Pierce, USA). Briefly, 25–50 μg aliquots of each protein sample were separated on a SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% BSA in TBS/0.5% Tween-20 (TBST) and then incubated with appropriate primary antibodies. The next day, the PVDF membranes were washed with TBST, incubated with secondary antibody (goat anti-mouse IgG, Invitrogen) at room temperature for 1 h and then washed three times with TBST. Image acquisition and quantitative analysis were performed using ImageJ.

2.10. Cell viability assay
The chicken myoblasts transfected with pcDNA3.1 or pCDNA3.1-Igf2bp1 were seeded in 96-well plates at a density of 5 × 10³ per well and cultured for 24, 48, 72 and 96 h. Cell viability was assessed by the Cell Counting Kit-8 (Sangon Biotech, Shanghai, China). All data were obtained by the average of five independent experiments. For the 5-ethynyl-2′-deoxyuridine (EdU) assay, the proliferation state of myoblasts was performed with Cell-Light™ EdU kit (Beyotime, China) according to the manufacturer’s instructions. The quantities of EdU-positive cells were calculated by using a fluorescence microscope (Leica, Wetzlar, Germany).

2.11. Immunocytochemistry
The chicken myoblasts cultured in 12-well plates were washed with PBS and fixed in 4% paraformaldehyde for 10 min, followed by 0.1% Triton X-100 for 5 min. Nonspecific reactivity was blocked by incubating the cells with 5% goat serum for 30 min. Then, the cells were incubated with monoclonal antibody (Pax7, Abcam, USA) at 37°C for 1–2 h. Next, the secondary antibody was added, and the cells were incubated at 37°C for 40 min. The nuclei were stained with DAPI for 10 min. Images

Table 1. Primers used for cloning.

| Name   | Primer sequences (5’-3’) | Product length (bp) | Annealing temperature (°C) | Genebank accession number |
|--------|-------------------------|---------------------|----------------------------|----------------------------|
| Igf2bp1-F | cggGAATCTCAGGCAAAAGCTGTTACCTCAGCCTCACCA | 1770                | 60                          | NM 205071.1                |
| Igf2bp1-R | cggGAATCTCAGGCAAAAGCTGTTACCTCAGCCTCACCA | 1770                | 60                          | NM 205071.1                |

Table 2. Primers for real-time quantitative PCR.

| Name   | Primer sequences (5’-3’) | Product length (bp) | Annealing temperature (°C) | Genebank accession number |
|--------|-------------------------|---------------------|----------------------------|----------------------------|
| Igf2bp1-q-F | GAAAGGAGTACACGATCCACTCCCT | 155                 | 60                          | NM 205071.1                |
| Igf2bp1-q-R | ATCAAGGCGCGACCACCA | 155                 | 60                          | NM 205071.1                |
| β-actin-F | CTGACACTCCATCTGCTGACA | 139                 | 60                          | NM 205071.1                |
| β-actin-R | TCCAGAAGGCGCTGCTGACA | 139                 | 60                          | NM 205071.1                |
| Pax7-q-F | GAAAGGAGTACACGATCCACTCCCT | 98                  | 60                          | NM 205071.1                |
| Pax7-q-R | ATCAAGGCGCGACCACCA | 98                  | 60                          | NM 205071.1                |
| MyoD-q-F | GAAAGGAGTACACGATCCACTCCCT | 204                 | 60                          | NM 205071.1                |
| MyoD-q-R | ATCAAGGCGCGACCACCA | 204                 | 60                          | NM 205071.1                |
were captured using a fluorescence microscope (Leica, Wetzlar, Germany).

2.12. Data analysis

The statistical analysis was done using SPSS software, version 22 (SPSS Inc.). Differences were considered statistically significant when the $p$-values were less than 0.05. Statistical significance was defined as $^*p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. All experiments were performed three times independently.

3. Results

3.1. Identification and phylogenetic analysis of Igf2bp1 gene in chickens

Based on the transcriptome data of leg muscle from Lueyang black-bone chicken in the three-age stages, under the criteria of $\text{FDR} \leq 0.001$ and absolute log2 ratio > 1, comparison of 20-day-old, 60-day-old and 120-day-old animals showed that 944 genes were significantly differential expressed genes. Of the 944 DEGs, 9 and 1 were co-up-regulated and co-down-regulated, respectively, in the three-age stages of 20, 60 and 120 days (Figure 1(a,b)). In particular, we focused on the co-down-regulation gene, Igf2bp1. Previous studies have found that Igf2bp1 had an essential effect in modulating cancer cell proliferation, differentiation and migration (Stöhr and Hüttelmaier 2012; Bell et al. 2013). However, there have been few studies on IGF2BP of chickens. First, identification and phylogenetic analysis of Igf2bp1 gene in chickens were performed. To identify all genes that may be a gene family with Igf2bp1 gene, using the seed files for the KH domain from G. gallus, we obtained 174 candidate genes. Then, those genes were analysed by online software the SMART and Pfam, and identified 30 genes that are structurally similar to Igf2bp1, all of which contained a KH domain. Therefore, we defined these genes as Igf2bp1 gene family. According to the standardized gene nomenclature, we assigned each member of the Igf2bp1 gene family a unique name. Next, according to the protein ID, we retrieved the comments and functions of

![Figure 1. Phylogenetic analysis of chicken IGF2BP1 proteins. These 30 sequences were used to construct a NJ tree. The tree was divided into three subfamilies; the names of different groups were displayed. Relative genes were listed in the brackets. (a) Intersection of up-regulated genes in different days Lueyang black-bone chicken muscle issue data analyses. (b) Intersection of down-regulated genes in different days Lueyang black-bone chicken muscle issue data analyses. (c) Phylogenetic tree of IGF2BP1 proteins in chicken.](image-url)
these genes in NCBI (Table 3). Of these 30 genes, the function of 16 genes has been elucidated and the other 14 genes were not annotated in chicken. It was notable that the GgIgf2bp1-25 gene represented IGF2BP1 in IGF2BP1 gene family.

To further understand the evolutionary relationship and function of these genes from IGF2BP1 gene family in chicken, we carried out phylogenetic analysis of IGF2BP1 proteins by MEGA based on NJ and ML methods. The 30 predicted genes were classified into three groups based on sequence similarity, signed group A, group B and group C (Figure 1(c)). Group A contained 8 GgIgf2bp1s, including Mex3d, Mex3a, Bicc1, Nova1 and 4 un-annotated genes. Group B contained 11 GgIgf2bp1s; we detected Pcbp2, Pcbp3, Pcbp4, Hnrnpk, Hnrnpkl, Loc426023 and 5 un-annotated genes in this branch. Group C contained 11 GgIgf2bp1s, including 6 annotated genes and 5 un-annotated genes. In group C, IGF2BP1 (GgIgf2bp1-25), IGF2BP2 (GgIgf2bp1-26) and IGF2BP3 (GgIgf2bp1-14) and 3 un-annotated genes were distributed from a relative branch. In addition, Fupb1, Fupb3, Hd1bp and other 2 un-annotated genes formed another clade. In summary, we found some genes, whose structure and functions are related to IGF2BP1, provided insight into the gene family of IGF2BP1 and un-annotated genes in chicken.

3.2. Transcription analysis of IGF2BP1 gene family in chicken muscle tissue

To further investigate the function of the IGF2BP1 gene family in chickens, we retrieved the transcriptional expression data of these genes in the IGF2BP1 gene family. The heat map displayed gene expression level of members in the IGF2BP1 gene family at different development stages (Figure 2(a)). We found that the expression level of IGF2BP1 (GgIgf2bp1-25) gradually decreased with chicken growth, and there was a dramatic difference among the three-age stages of 20, 60 and 120 days. Furthermore, the expression level of IGF2BP3 (GgIgf2bp1-14) was significantly decreased at the age of 60 and 120 days compared with age of 20 days.

To confirm the transcriptome data of genes in the IGF2BP1 gene family, we performed RT-qPCR verification of the IGF2BP1 gene expression in leg muscles from Lueyang black-bone chicken at different ages. The RT-qPCR result showed that the mRNA expression level of IGF2BP1 decreased gradually with the increase of age, and were consistent with the transcriptome data (Figure 2(b)). In eukaryotes, the expression of a gene varies in time and space, so revealing the expression difference of chicken IGF2BP1 gene in different tissues is very necessary to explore gene functions. To validate the effect of IGF2BP1 in muscles, three muscle tissues and four other tissues from Lueyang black-bone chicken at the age of 6 days were harvested to analyse the expression pattern of the IGF2BP1 gene. The results showed that IGF2BP1 expression was widely distributed in all the chosen tissues. Moreover, the expression level of IGF2BP1 gene was significantly lower in breast muscle, leg muscle and cardiac muscle than that in the stomach, liver, skin and gizzard (Figure 2(c)). Therefore, we speculated that IGF2BP1 is involved in skeletal muscle growth and development.

3.3. Isolation and identification of the myoblast cells from chickens

To examine whether IGF2BP1 affects skeletal muscle growth and development, the myoblasts from Lueyang black-bone chicken
were isolated first in vitro. The results showed that the isolated cells began adherent growth 24 h later, and most of the cells were spindle shaped (Figure 3(a)). After 48 h, myoblasts become longer and polar (Figure 3(a)). The number of cells increased significantly, and a few cells began to fuse after 72 h (Figure 3(a)). Immunofluorescence cell staining showed positive expression of Pax7, a biomarker of skeletal muscle cells, indicating that chicken myoblasts were obtained (Figure 3(b)). Next, we investigated the expression pattern of Igf2bp1 during chicken myoblast proliferation. Igf2bp1 expression was...
obviously detectable, but its expression decreased gradually during cell proliferation (Figure 3(c)). These data suggested that Igf2bp1 down-regulation might benefit the myoblast cells. For this reason, the Igf2bp1 overexpression plasmid was transfected to chicken myoblasts, and the cells were harvested at 48 and 72 h post-transfection, RT-qPCR and Western blot were performed, respectively. Notably, the Igf2bp1 expression levels of mRNA and protein were significantly elevated in the overexpressed group compared to the control group treated with empty vector (p < 0.01) (Figure 3(d,e)).

3.4. The presence of Igf2bp1 slowed the rate of chicken myoblast proliferation

Even though the expression levels of Igf2bp1 decreased gradually during chicken myoblast proliferation, how Igf2bp1 affects the proliferation of chicken myoblasts remained unexplored. Therefore, cell proliferation rate was measured by the CCK8 assay after overexpressed Igf2bp1 in chicken myoblasts. Remarkably, Igf2bp1 weakened the rate of cell proliferation compared to the control group at 48 h (p<0.01) and 72 h (p<0.05) (Figure 4(a)). Besides, the number of EdU-staining cells was detected. The results showed that the number of EdU positive cells in the Igf2bp1 overexpressed group was reduced by 11.8% compared with the control group (Figure 4(b,c)), which implied that Igf2bp1 can play a negative regulatory role during chicken myoblast proliferation.

Then, we evaluated the expression of Pax7, MyoD, MyoG and Myf5, markers of early proliferation and differentiation of skeletal muscle cells, respectively. The mRNA levels of four genes all were down-regulated in the Igf2bp1 overexpressed group compared to those of the control group (Figure 4(d)). Meanwhile, Pax7 expression was analysed by immunofluorescence to examine the proliferation of myoblasts at 72 h post-transfection. We observed a marked decrease in the positive expression of compared with the control (Figure 4(e)). Pax7 and MyoD protein levels were also analysed by western blotting, which showed that a significant decrease after Igf2bp1 was overexpressed. Taken together, our data indicated that the presence of Igf2bp1 slowed the rate of chicken myoblast proliferation.

4. Discussion

Previous studies have shown that Igf2bp1 is expressed during the embryonic stage and it is essential for early development (Hansen et al. 2004). A recent report further confirms that Igf2bp1 was not expressed in most postnatal organs of human, mouse, zebrafish, chicken, or mallard, except in some reproductive organs (Zhou et al. 2018). Nevertheless, the mRNA expression of Igf2bp1 decreased with the increase of age in Pekin ducks (Zhou et al. 2018). Intriguingly, we found that Igf2bp1 is a common down-regulation gene on the leg muscle from Lueyang black-bone chicken in the three-age stages by RNA-seq, and the mRNA expression of Igf2bp1 also decreased gradually with the increase of age, suggesting that common mechanisms may exist in Pekin ducks and Lueyang black-bone chicken. Although studies focused on chicken Igf2bp1 are of great importance and interest, they are currently lacking.

To better understand the basic characteristics of Igf2bp1 in chicken, phylogenetic analysis of Igf2bp1 gene in chickens was performed first. Igf2bp1 consists of six canonical RNA-binding domains, including four KH domains and two RNA recognition motifs (Wächter et al. 2013). Importantly, previous
studies indicated that RNA binding was mainly facilitated by the KH domain (Farina 2003). The KH domain was firstly identified in the protein human heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Siomi et al. 1993). KH domain could bind RNA or ssDNA, particularly, the nucleic acid-binding activity of KH domain is central to various cellular processes such as early development and cell differentiation (Valverde and Regan 2008; Nazarov et al. 2019). In the present study, we analysed the evolutionary relationship of chicken lgf2bp1 basing on the KH domains. Results showed that 30 genes of G. gallus contained the KH domain, and we defined these 30 genes as members of lgf2bp1 gene family. In the lgf2bp1 gene family, 14 genes were not annotated, which laid the foundation for the identification and research of new genes and provided research ideas. Additionally, we analysed the gene expression level of lgf2bp1 gene family from the skeletal muscle transcriptome data of Lueyang black-bone chicken. In 16 annotated genes, 9 genes expressed only. Among them, the expression of lgf2bp1 was significantly changed in the leg muscle from Lueyang black-bone chicken with age, suggesting that lgf2bp1 may function in muscle development.

Previous studies have shown that lgf2bp1 is an oncofetal protein that was predominantly expressed in embryonic tissues and cancer cells (Leeds et al. 1997; Ross et al. 2001; Ioannidis et al. 2001, 2004), and lgf2bp1 had an essential effect in modulating cancer cell proliferation. In particularly, lgf2bp1 enhanced tumour cell proliferation, survival, adhesion-independent growth and invasion, and chemoresistance (Huang et al. 2018). However, little has been reported about the role of lgf2bp1 in regulating muscle proliferation. Chicken is believed to be one of the optimal models for researching the development of embryonic skeletal muscle, because it has a similar muscle developmental anatomy to that of mammals (Berti et al. 2015). To elucidate the underlying function of lgf2bp1 in myogenesis, chicken myoblasts were treated with lgf2bp1 overexpressed vector. The reduction in myoblast proliferation was observed by CCK-8 and EdU assays, implying that lgf2bp1 inhibited chicken myoblast proliferation. Pax7 is the marker gene of satellite cells and participates in the processes of myogenesis (Seale et al. 2000). Pax7, highly expressed in quiescent, active, and proliferating satellite cells, plays critical roles in satellite cell regeneration, survival, anti-apoptosis, as well as self-renewal (Relaix et al. 2006; Kuang et al. 2007; Morrison et al. 2010). Satellite cells proliferate in the form of myoblasts after entering the cell cycle, so Pax7 is also a marker gene for the proliferation phase of myoblasts. In the present study, we show that Pax7 expression was markedly reduced at protein levels in chicken myoblasts in lgf2bp1 overexpressed cells. Furthermore, we explored expression levels of other genes relevant to skeletal muscle development. The mRNA expression levels of myogenic differentiation (MyoD), myogenin (MyoG) and the myogenic factor S (Myf5) were drastically reduced, as were as the protein level of MyoD in lgf2bp1 overexpressed cells, implying an essential function of lgf2bp1 in the differentiation process. These results indicated that the high expression of lgf2bp1 in chicken muscles might weaken muscle proliferation. This explains why the expression level of lgf2bp1 in skeletal muscle gradually decreases with the increase of age in Lueyang black-bone chicken.

5. Conclusion

We showed in this study that lgf2bp1 can inhibit chicken myoblast proliferation and differentiation by regulating key gene expressions of myogenesis, which is not conducive to the growth and development of skeletal muscle. This study revealed that regulation mechanism led to the growth and development of skeletal muscle by lgf2bp1 and provided good knowledge of the functional molecular mechanisms associated with meat production in Lueyang black-bone chicken. Our findings provide new insights into the function of lgf2bp1 in poultry meat production.

Disclosure statement

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