Evolutionary analysis and functional characterization reveal the role of the insulin-like growth factor system in a diversified selection of chickens (*Gallus gallus*)

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ABSTRACT The insulin-like growth factor (**IGF**) system plays an indispensable role in embryonic and postnatal development in mammals. However, the effects of the system on growth, carcass, and egg-laying traits, and diversified selection have not been systematically studied in chickens. In the present study, firstly the composition and gene structures of the chicken IGF system were investigated using phylogenetic tree and conserved synteny analysis. Then the effects of the genetic variations in the IGF system genes on breeding of specialized varieties were explored by principal component analysis. In addition, the spatiotemporal expression properties of the genes in this system were analyzed by RT-qPCR and the functions of the genes in egg production performance and growth were explored by association study. Moreover, the effects of IGF-binding proteins 3 (***IGFBP3***) on skeletal muscle development in chicken were investigated by cell cycle analysis, 5-ethynyl-2′-deoxyuridine (**EdU**) and Cell Counting Kit-8 (**CCK-8**) assays. The results showed that the chicken IGF system included 13 members which could be classified into 3 groups based on their amino acid sequences: IGF binding proteins 1 to 5 and 7 (**IGFBP1−5** and **7**) belonged to the first group; IGF 1 and 2 (**IGF1** and **IGF2**), and IGF 1 and 2 receptor (**IGF1R** and **IGF2R**) belonged to the second group; and IGF2 binding proteins 1−3 (**IGF2BP1−3**) belonged to the third group. The *IGF2BP1* and *3*, and *IGFBP2, 3*, and *7* genes likely contributed more to the formation of both the specialized meat-type and egg-type lines, whereas *IGFBP1* and *5* likely contributed more to the formation of the egg-type lines. The SNPs in the *IGF2BP3* and *IGFBP2* and *5* genes were significantly associated with egg number, and SNPs in the *IGFBP3* promoter region were significantly associated with body weight, breast muscle weight and leg muscle weight. The *IGFBP3* inhibited proliferation but promoted differentiation of chicken primary myoblasts (**CPMs**). These results provide insights into the roles of the IGF system in the diversified selection of chickens. The SNPs associated with egg-laying performance, growth, and carcass traits could be used as genetic markers for breeding selection of chickens in the future.

Key words: chicken, IGF system, evolution, expression regulation, *IGFBP3*

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INTRODUCTION

The insulin-like growth factor (**IGF**) system consists of IGF 1 and 2 (**IGF1** and **IGF2**), IGF 1 and 2 receptor (**IGF1R** and **IGF2R**), IGF binding proteins 1 to 6 (**IGFBP1−6**), IGF2 binding proteins 1 to 3 (**IGF2BP1−3**), and IGFBP-related proteins 1 to 10 (**IGFBP-rP1−10**). There are many tissues that produce IGF1 and IGF2; these proteins have functions in autocrine and paracrine secretion (Menuelle et al., 1999) and mediate the biological function of growth hormone (Yakar et al., 1999; Zhu et al., 2021).

The biological functions of IGFs are achieved by binding to their receptors (Payet et al., 2003; Juengel et al., 2010). There are two types of glycoprotein receptors for IGFs on the cell membrane: IGF1R and IGF2R. IGF1 and IGF1R have the highest affinity, and the long-term effect of IGF1 on promoting cell growth is mainly mediated by IGF1R (Jones and Clemmons, 1995; Valentinis

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et al., 1999; Kalista et al., 2012). Only a few IGFs (approximately 1%) bind to IGF1R and IGF2R on the cell surface and trigger downstream biological processes. The great majority of IGFs (more than 97%) form complexes with IGFBPs in serum and tissue gaps, which can prolong the half-life of IGFs and regulate the activity of IGFs (Clemmons et al., 1998; Firth and Baxter, 2002).

There are 6 IGFBPs that have been identified in mammals (Shimasaki and Ling, 1991), and their primary structure contains 3 distinct domains of roughly equivalent sizes. The amino (N)- and carboxy (C)-termini are highly conserved and required to maintain their high affinity to IGFs (Baxter, 2000). The central linker domain is the least conserved region; this region brings the N- and C-terminal domains into close proximity to create a high affinity IGF-binding pocket and includes sites affected by post-translational modifications, such as proteolysis, glycosylation, and phosphorylation (Haywood et al., 2019; Cai et al., 2020). IGFBP-rPs can also bind to IGFs, but with lower affinity than IGFBPs. IGFBP-rPs act as key signaling and regulatory molecules and they are involved in many important biological functions, including cell proliferation, angiogenesis, tumorigenesis, and wound healing (Artico et al., 2021; Wang et al., 2022c).

Accumulated evidence has demonstrated that the IGF system is closely related to muscle development through ligand-dependent or -independent mechanisms in embryos and after birth in mammals (Tilley et al., 2007; Ghanipoor et al., 2018). For example, IGFBPs have different expression patterns during myogenesis, which can positively or negatively regulate myoblast proliferation and differentiation (Ewton et al., 1998; Foulstone et al., 2003; Wang et al., 2019). *IGFBP1* inhibits protein synthesis in skeletal muscle (Vaccaro et al., 2022), whereas *IGFBP2* and *6* inhibit skeletal muscle development (Mierzejewski, et al., 2022; Safian, et al., 2012). Additionally, *IGFBP3* has a bidirectional regulation effect on myoblast proliferation and differentiation (Foulstone et al., 2003; Wang et al., 2022d). These differences may reflect the variety of different models and experimental conditions used in those studies (Murphy, 1998).

Moreover, IGFBP5 can exercise inhibition of myoblast proliferation by activating the downstream p38 MAPK pathway and the Erk1/2 pathway through activation of Gαi3 (Flynn et al., 2009), or it can regulate the autoregulatory function of IGF2 by binding to IGF2, which in turn promotes muscle differentiation (Ren et al., 2008). *IGFBP7*, the first discovered member of the IGFBP superfamily, is widely expressed in the body, and can bind with IGFs to regulate the IGF signaling pathway, or function independently to participate in the regulation of cell growth, differentiation, proliferation, apoptosis, tissue remodeling, and other important physiological processes (Cai et al., 2018b; Wu et al., 2019). It was also widely reported that the IGF system is an essential intraovarian regulator and plays important roles in the regulation of steroidogenesis, growth, follicular development and selection, and atresia in mammals (Jones and Clemmons, 1995; Spicer and Echternkamp, 1995; Spicer, 2004; Sudo et al., 2007; Neirijnck et al., 2019) and fish (Jia et al., 2019; Li et al., 2021; Zheng et al., 2021). It is thus clear that the IGF system is involved in various biological processes, including muscle growth and reproduction performance.

Research on chickens indicated that the genes in the IGF system are expressed in various organs in early embryonic developmental stages, and play important fundamental roles in chicken embryo development (Schmid, et al., 1983; Schoen et al., 1995; Tomas et al., 1998; Allan et al., 2003; Honda et al., 2021 Zhu, et al., 2021). Those findings indicate that the IGF system is equally important in mammals and chickens. However, its effects on chicken reproduction traits and diversified selection have not yet been reported.

In the present study, the composition of the chicken IGF system was investigated. The SNPs that were mapped within 2-kb promoters, 5′ untranslated regions (**UTRs**), exons, introns, and 3′ UTRs of all genes in the chicken IGF system were screened, and the genetic variations associated with the domestication and divergent selection of chickens were analyzed. The effects of genes in the chicken IGF system on growth and egg-laying performance were confirmed. These results both provide insight into the functions of the IGF system in growth and egg-laying performance, and revealed genetic markers that could be used for breeding selection in the chicken industry in the future.

MATERIALS AND METHODS

Ethics Statement

The Animal Care Committee of Henan Agricultural University (Zhengzhou, People's Republic of China) approved this study (approval number 11-0085). All chickens were killed by cervical dislocation that is considered acceptable for euthanasia of birds according to the Laboratory Animal Guidelines for Euthanasia (T/CALAS 31-2017; Chinese Association for Laboratory Animal Sciences). All experimental animals were female Arbor Acres broiler (**AA**, a larger fast-growing chicken breed) and Lushi Blue-Shelled-Egg (**LS**, a slow-growing chicken breed) chickens from the Animal Center of Henan Agricultural University. All chickens were fed in the same environmental conditions and provided with food and water ad libitum.

Experimental Animals and Sample Collection

To explore the spatiotemporal expression profiles of genes in the chicken IGF system, 10 embryos at each embryonic stage were arbitrarily selected at the following times: embryonic day (d) 10 (**E10**), embryonic d 14 (**E14**), and embryonic d 18 (**E18**). Additionally, 10 healthy birds were arbitrarily selected at each post-hatch stage at the following ages: 1 d old (**D1**), 1 week

(wk) old (**W1**), 3 wk old (**W3**), and 5 wk old (**W5**). The pectoral muscle was dissected from all the embryos and post-hatch birds, and 10 other tissue samples, including heart, liver, spleen, lung, kidney, pancreas, muscular stomach, glandular stomach, leg muscle, and duodenum, were collected from 1-wk-old birds, snap-frozen in liquid nitrogen, and then stored at −80°C until use.

Sequence Retrieval and Bioinformatics Analysis of the IGF System Genes

The protein sequences of the genes in the chicken IGF system (GRCg6a. protein. fa) were downloaded from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/genome/?term=CHICKEN). The isoelectric point and molecular weight of proteins in the IGF system were analyzed using ExPASy (http://expasy.org). The consensus conserved genomic synteny of the genes in the chicken IGF system was analyzed using PhyloViewin Genomicus v71.01 (http://www.genomicus.biologie.ens.fr/genomicus-71.01/cgi-bin/search.pl). The gene gain/loss tree was constructed with the Ensembl Genome Browser online software (https://asia.ensembl.org/index.html). The phylogenetic tree was constructed with amino acid sequences using the Neighbor-Joining method in Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6.0) with 1,000 bootstrap replications (Huang et al., 2009). Exon−intron structures of genes were analyzed based on coding and genomic DNA sequences using the online software GSDS (http://gsds.cbi.pku.edu.cn/). The conserved domains of genes in the chicken IGF system were analyzed using the online tool MEME (https://meme-suite.org/meme/index.html).

Principal Component Analysis of Genetic Variations in the IGF System Genes in Multiple Chicken Breeds

The whole-genome sequences (**WGS**) of various chicken breeds were used to screen SNPs within the 2-kb promoters, 5′ UTRs, exons, introns, and 3′ UTRs of the genes in the IGF system. The chicken breeds analyzed included: commercial broiler Cobb (N = 30); commercial layer White Leghorn (**WL**, N = 20); the native local breeds Gushi chicken (**GS**, N = 10), Lushi chicken (LS, N = 10), Henan Game chicken (**HNG**, N = 10), Xichuan Black Bone chicken (**XCBB**, N = 10), and Zhengyang San Huang chicken (**ZYSH**, N = 10); and a wild breed, Red Jungle Fowl (**RJF**, N = 5). The WGS of the five native breeds were obtained from our previous sequencing data, the WGS of white leghorn and red jungle fowl were retrieved from from published dataset (Wang et al., 2022a), and the WGS of Cobb was obtained from China Agricultural University. Principal component analysis (**PCA**) was used to investigate the response of genes in the IGF system to domestication, from wild to indigenous breeds, and to specialization, from local breeds to commercialized varieties.

Association Studies Between SNPs Within the Genes of the IGF System and Egg Production, Growth, Carcass, and Muscle Fiber Traits

The GS chicken, a Chinese indigenous breed, was obtained from the core breeding population of the GS Chicken Breeding Farm at Henan Sangao Agriculture and Animal Husbandry Co., Ltd., Henan Province, China. The raising conditions and egg production performance of the GS chicken were previously described in details (Wang et al., 2022b). The selected SNPs (Table S1) in *IGF2BP3*, and *IGFBP2* and *5* were genotyped in 640 GS hens by Kompetitive Allele Specific PCR (He et al., 2014), and association analysis was conducted between SNP genotypes and egg production traits in different laying periods (21−25, 26−30, 31−35, 36−43, and 21−43 wk) was carried out using a mixed linear model.

The selected SNPs (Table S2) in *IGFBP3* and *7* were genotyped by Kompetitive Allele Specific PCR for 730 individuals from the Gushi × Anka F_2 resource population; this was conducted as previously described in details (Zhang et al., 2021). The association studies between the SNPs and 7 growth, 17 carcass, and 12 muscle fiber traits were conducted by mixed linear model.

Chicken Primary Myoblast Isolation, Culture and Treatment

A modified procedure based on the previous method reported by Cai et al. (2018a) was used to isolate chicken primary myoblasts (**CPMs**) from leg muscle at embryonic day 11. Briefly, the leg muscle tissues were cut into pieces and swirled for 40 s after removing the bone. The mixture was filtered through 200-mesh sieves to remove all solids. Then, single cells were collected by centrifugation at 1,500 r/min for 5 min. The CPMs were obtained by the differential adhesion method (Luo et al., 2014) and maintained in DMEM high glucose media (BioInd, Kibbutz Beit Haemek, Israel) with 15% fetal bovine serum (BioInd, Kibbutz Beit Haemek, Israel) and 1% penicillin−streptomycin (Solarbio, Beijing, China). After CPM density reached approximately 90%, cell differentiation was induced by replacing 15% fetal bovine serum with 2% horse serum. Cells were cultured at 37°C with 5% CO_2.

RNA Extraction, cDNA Synthesis and Real-Time Quantitative PCR Analysis (RT-qPCR)

Total RNA was extracted from tissues or cells using the RNA Isolation Kit (Vazyme Biotech, Nanjing, China). After measuring the optical density using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE), the qualified total RNA was stored at −80°C. The first strand of cDNA was synthesized according to the manufacturer's protocol using the HiScript reverse transcription kit (Vazyme Biotech, Nanjing, China) and then stored at −20°C until use.

To detect the expression levels of genes in the IGF system, RT-qPCR was conducted using a Roche LightCycler 96 Instrument (Roche, Basel, Switzerland) in a 10-μL reaction volume including 5 μL 2 × ChamQ Universal SYBR Qpcr Master Mix (Vazyme Biotech, Nanjing, China), 3.0 μL RNase-free water, 0.5 μL each of forward and reverse primers (10 μM), and 1 μL cDNA. All reactions were performed in triplicate with the following procedure: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The housekeeping genes *β-actin* and *GAPDH* served as internal control to normalize the mRNA expression levels. The specific primers for RT-qPCR were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Sangon Biotech (Shanghai, China) (Table S3). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative mRNA expression (Bubner and Baldwin, 2004).

Overexpression Plasmid Vector Construction and Short Interfering RNA Synthesis of IGFBP3 and Cell Transfection

The coding region of *IGFBP3* was amplified by PCR and cloned into HindIII and EcoRI sites of pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) vector. The correctness of the *IGFBP3* overexpression vector was confirmed by sequencing, and designated as pcDNA3.1-IGFBP3. Three short interfering RNAs (**siRNAs**) targeting different *IGFBP3* regions, designated as si-IGFBP3-1, -2, and -3, were designed and synthesized by Tsingke Biotechnology Co., (Beijing, China). The sequences of si-IGFBP3-1, -2, and -3 are listed in Table S4.

Transfection was carried out using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To improve the knockdown efficiency of *IGFBP3*, the three different siRNAs were mixed in our study. The transfection concentrations of siRNAs were 100 nM. Then, the transfection efficiencies for *IGFBP3* siRNA interference fragments and overexpression vectors were checked by RT-qPCR.

Cell Proliferation Assays

After 24 h of transfection, cells were exposed to 50 μM 5-Ethynyl-2′-Deoxyuridine (**EdU**) (RiboBio, Guangzhou, China) for 2 h at 37°C. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min, neutralized using 2 mg/mL glycine solution, and then permeabilized by adding 0.5% Triton X-100. A solution containing EdU (Apollo Reaction Cocktail, RiboBio, Guangzhou, China) was added and the cells were incubated at room temperature for 30 min. The nuclear stain Hoechst 33342 was then added, and incubation was continued for another 30 min. The inverted fluorescence microscope (Olympus, Tokyo, Japan) was used to capture three arbitrarily selected fields to count the number of EdU-stained cells. Cell proliferation was evaluated by Cell Counting Kit-8 (**CCK-8**) assay (Dojindo, Kumomoto, Japan). First, 10 μL of CCK solution was added to the cells when the myoblasts were transfected with overexpression plasmid or siRNA for 12 h, 24 h, 36 h, and 48 h. The absorbance at 450 nm was measured using Multimode Reader (BioTek, Winooski, VT) after incubation for 1 h.

Flow Cytometric Analysis

The CPM cells were collected and fixed in 80% ethanol overnight at −20°C after the cells were transfected with overexpression plasmid or siRNA for 24 h. The cells were then rinsed by pre-cooled PBS and centrifuged. The flow cytometry analysis was performed on a BD AccuriC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) with the Cell Cycle Detection Kit (KeyGEN Biotech, Nanjing, China).

Immunofluorescence Analysis

The myoblasts cultured in 24-well plates were treated with 4% formaldehyde for 30 min after the cells were transfected with overexpression plasmid or siRNA for 48 h. Immunofluorescence experiments were conducted according to previously published procedures (Li et al., 2019). Additionally, immunofluorescence images were captured with an inverted fluorescence microscope (Olympus, Tokyo, Japan). The percentage of the total image area covered by myotubes as the total myotube area was calculated by Image Proplus 6.0 (Media Cybernetics Inc., Bethesda, MD).

Statistical Analysis

All experimental results are presented as the mean ± SEM. The statistical significance of differences between means was assessed by an unpaired Student's t-test. $P < 0.05$ was considered as statistically significant.

RESULTS

Members of the Chicken IGF System

A total of 13 members of the chicken IGF system were identified by HMM analysis and BLASTP against the protein sequences of known members of the IGF system in mammals. The features of the IGF system genes and their corresponding proteins are listed in Table 1.

IGFBP6, a member of the IGF system in mammals, was not found in the chicken and Japanese quail genomes from NCBI and other public genome databases; this indicates that *IGFBP6* was evolutionarily lost in chicken. To confirm this, a syntenic analysis of the *IGFBP6* neighboring genes was performed in eight representative genomes from mammals (human, mouse, rat, pig), birds (chicken, Japanese quail), a reptile (Chinese turtle), and a fish (zebrafish). The results indicated that *IGFBP6* neighboring genes in mammals were

Table 1. The features of the IGF system genes and their corresponding proteins in chicken.

Name	Gene ID	Chr.	Genomic location	ORFs	Exon	AA	MW (kDa)	pI
IGF1	NM_001004384.2	1	55326300−55374745	8	4	153	17.27	9.25
IGF2	XM_015286525.3	5	13374440−13394080	6	4	226	24.84	9.3
IGF1R	NM_205032.2	10	16697228−16844485	58	21	1363	154.1	5.76
IGF2R	NM_204970.1	3	45169351−45223492	105	48	2470	275.65	5.67
IGF2BP1	NM_205071.1	27	3259117−3283321	19	15	576	63.27	9.21
IGF2BP2	XM_040679797.1	9	4079118−4096159	18	17	611	66.75	9.2
IGF2BP3	XM_015281444.3	2	31057514−31164997	26	15	584	64.37	9.05
IGFBP1	NM_001001294.1	2	54589745−54595971	5	4	269	29.64	7.78
IGFBP2	NM_205359.1	7	22879236−22951723	9	4	311	33.54	6.59
IGFBP3	NM_001101034.1	2	54605655−54621232	8	4	282	31.17	8.9
IGFBP4	NM_204353.1	27	4441013−4446307	5	4	260	27.73	7.79
IGFBP5	XM_422069.7	7	22854820−22876649	6	4	269	30.07	8.97
IGFBP7	XM_015276448.2	4	48670018−48686876	11	4	320	33.07	9.23

distributed at a conserved genomic region in chicken and Japanese quail genomes (Figure 1), which provided strong evidence that *IGFBP6* was indeed evolutionarily lost in chicken and Japanese quail, and perhaps other avian species as well. The gene gain/loss tree depicts the phylogenetic history of *IGFBP6* by showing new gene additions and deletions (Figure S1).

Structure and Conserved Motifs in Members of the Chicken IGF System

A phylogenetic tree based on the amino acid sequences, exon−intron gene structure, and conversed motifs of the amino acid sequences in members of the chicken IGF system was constructed to explore the genetic origins of this multigene family. The results indicated that there were three large, distinct branches in the chicken IGF system. IGFBP1−5 and 7 formed the first clade; IGF1 and 2, and IGF1 and 2R formed the second clade; and IGF2BP1−3 formed the third clade (Figure 2A). The IGFBP family members had 44.26% overall amino acid sequence similarity with conserved residues that are primarily present in the N- and C-terminal domains. Additionally, they contained two conserved fingerprint domains (Figure S2) that had the same exon−intron distribution and 5 conversed motifs. IGF1 and 2 had the same exon−intron distribution and 2 conversed motifs. In addition, the exon−intron distribution and conversed motifs of IGF1 and 2R were different (Figure 2B, C). IGF2BP1 to 3 had 76.76% overall amino acid sequence similarity and a similar protein structure characterized by 6 RNA-binding domains, which included N-terminal RNA recognition motif and 4 hnRNPK homology domains (Figure S3). The lengths of these conserved motifs varied from 9 to 50 amino acids (Table S5).

To clarify the evolutionary characteristics of genes in the IGF system among species, a phylogenetic tree was reconstructed according to the amino acid sequence alignment of different species, including humans (*Homo sapiens*), mice (*Mus musculus*), chickens (*Gallus gallus*), Japanese quail (*Coturnix japonica*), green sea turtles (*Chelonia mydas*), and zebrafish (*Danio rerio*). The NCBI accession numbers of amino acid sequences from different species are shown in Table S6. These data indicated that *IGFBP6* was lost in chicken and Japanese

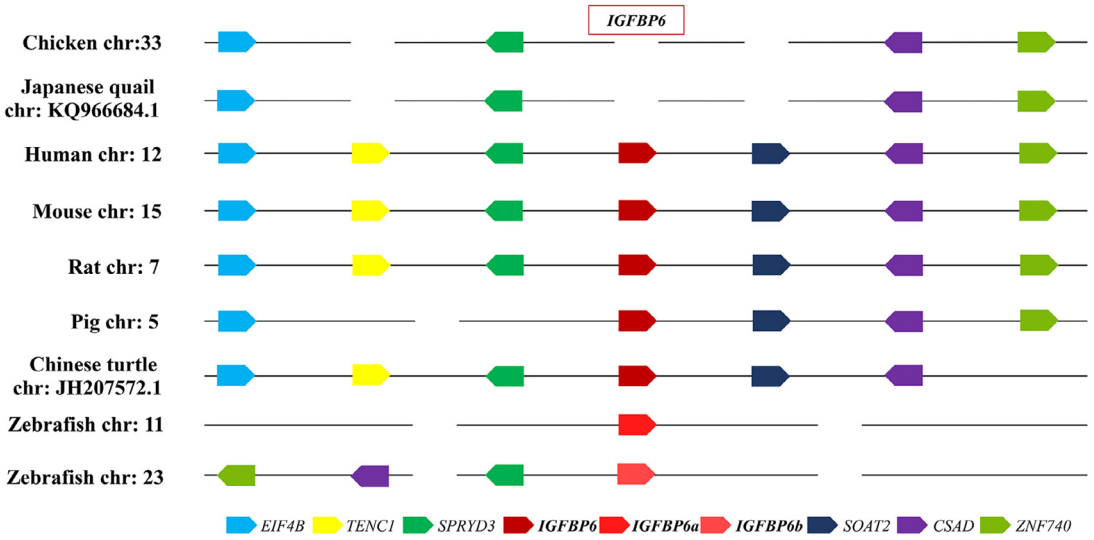

Figure 1. Conserved synteny analysis for the genomic region of *IGFBP6* gene. The species and chromosome distribution are listed on the left. Every pentagon refers to a gene, and the same color in the column means the same gene existing in the corresponding species. The direction represents DNA strand for gene transcription, right refers to sense strand, left refers to antisense strand.

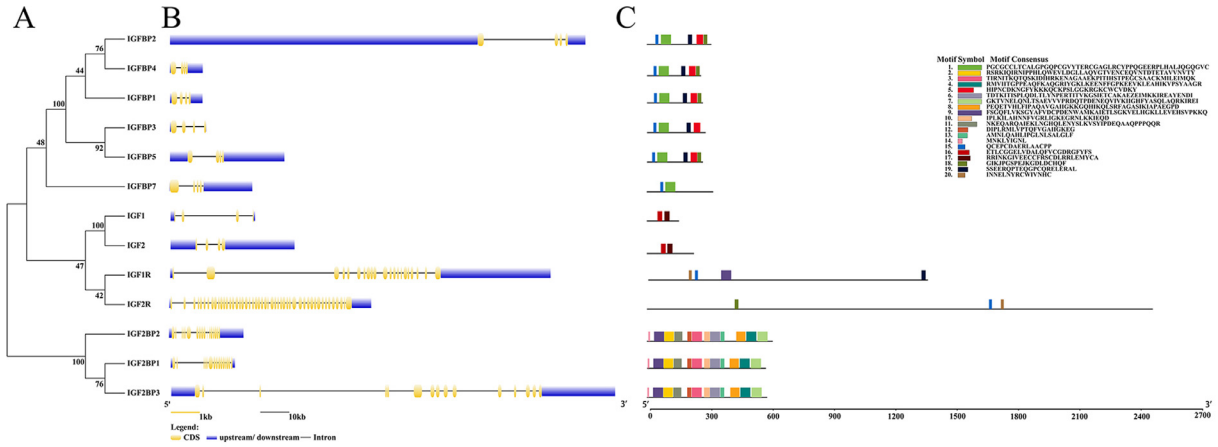

Figure 2. Phylogenetic relationship, gene structure and conserved motif analysis of the genes in the IGF system. (A) Phylogenetic tree of 13 proteins in the IGF system. The unrooted neighbor-joining phylogenetic tree was constructed with MEGA7 using full-length amino acid sequences of 13 IGF system proteins, and the bootstrap test replicate was set as 1000 times. (B) Exon/intron organization of IGF system genes. Yellow boxes represent exons and black lines with same length represent introns. The upstream/downstream region of IGF system genes are indicated in blue boxes. The length of exons can be inferred by the scale at the bottom. (C) Distributions of conserved motifs in IGF system genes. Twenty putative motifs are indicated in different colored boxes.

quail genomes, and that *IGFBP4* was lost from the zebrafish genome during evolution. Every system member in birds was clustered into its respective group with other species (Figure 3). This phylogenetic analysis provided an indication of the overall evolution of the IGF system in multiple species.

Genetic Variations in the IGF System Genes Resulting from Divergent Selection in Chickens

To investigate the response of the genes in the IGF system to domestication, from wild to indigenous breeds, and to specialization, from local breeds to commercialized varieties, the SNPs that were mapped within 2-kb promoters, 5′ UTRs, exons, introns, and 3′ UTRs of all 13 genes in the IGF system were screened, and PCA was performed. A total of 132,121 SNPs were identified among multiple chicken breeds. The PCA results showed that the commercial broilers and layers could be explicitly separated from wild and native chicken by PC1 (Figure 4A). Genetic variation in the genes of the IGF system was produced in response to the intensive artificial selection during the breeding process of specialized commercial chicken lines. This indicates that genes in the IGF system contributed to growth and egg-laying performances in chickens.

To further investigate the effects of individual genes in the IGF system on specialization of commercialized varieties, The PCA was performed using the SNPs identified in each member of the IGF system. The results showed that *IGF2BP1* and *3*, and *IGFBP2, 3*, and *7* genes likely contributed more than other genes to the formation of both the specialized meat-type and egg-type lines under specific directional selection during modern chicken breeding, whereas *IGFBP1* and *5* likely contributed more than other genes to the formation of the egg-type line (Figure 4B–N).

Associations Between SNP Genotypes of the IGF System Genes and Egg-Laying Performance, Growth, and Carcass Traits

According to the results of the above PCA, the genetic variations in *IGF2BP1* and *3*, and *IGFBP1, 2, 3, 5*, and *7* were produced in response to selection of egg-laying traits. To confirm the function of the genes on egg-laying performance, the selected SNPs of these genes were genotyped and association study was performed using 640 GS hens. The results demonstrated that *IGF2BP3* and *IGFBP2* and *5* were associated with egg-laying traits (Table S1). SNP 2_31231347 in the *IGF2BP3* intron region was associated with egg-laying traits except for egg number from 21–25 weeks, and SNP 7_23325125 in the *IGFBP2* promoter and SNP 7_23238154 in the *IGFBP5* intron region were associated with the total egg number.

According to the results of the above PCA, the genetic variations in *IGF2BP1* and *3*, and *IGFBP2, 3*, and *7* were produced in response to growth trait selection. Therefore, only the selected SNPs in the *IGFBP3* and *7* were genotyped, and association study was conducted using 730 individuals in the Gushi × Anak F_2 population. The results confirmed that SNP 2_55119951 in the *IGFBP3* promoter region was associated with 12-wk-old body weight, breast muscle weight, and leg muscle weight, and the SNPs 4_49499548 and 4_49503186 in the *IGFBP7* intron were associated with the 4-wk-old body weight, breast muscle weight, and leg weight (Table S2).

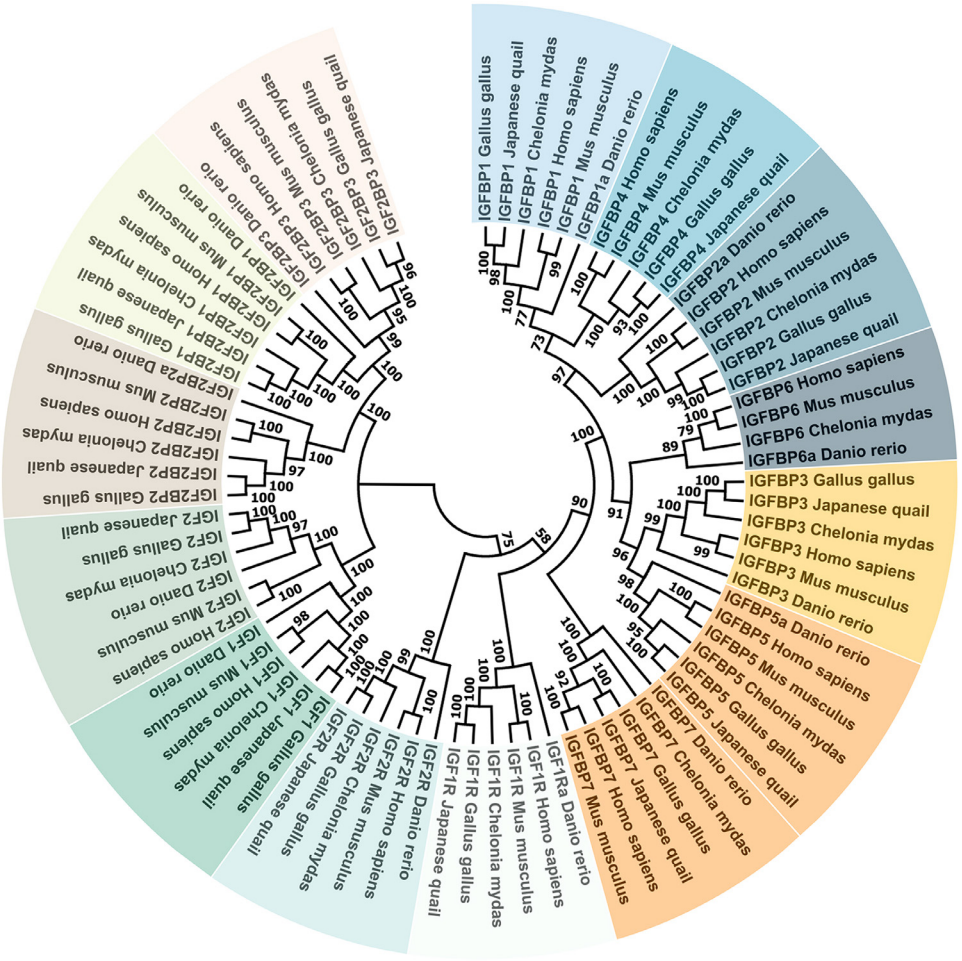

Figure 3. Phylogenetic analysis of amino acid sequences of IGF system genes. The species used for the analysis include human (Homo sapiens), mouse (Mus musculus), chicken (Gallus gallus), Japanese quail (Coturnix japonica), green sea turtles (Chelonia mydas) and zebrafish (Danio rerio). The 14 gene clusters were distinguished in different colors. The unrooted neighbor-joining phylogenetic tree was constructed with MEGA6.0 using full-length amino acid sequences of 81 IGF system proteins from 6 species, and the bootstrap test replicate was set as 1,000 times.

Tissue Distribution and Dynamic Expression Profiles of the IGF System Genes in the Pectoral Muscle of Chickens

To determine the expression pattern of the genes in the chicken IGF system, cDNAs synthesized using total RNAs isolated from 11 different tissues of 1-wk-old LS hens were used for PCR. The results showed that most of the genes in the IGF system were universally expressed in various tissues (Figure S4).

To further accurately analyze the function of the IGF system genes, the expression levels of these genes in the pectoral muscle at different developmental stages were compared between 2 distinct chicken breeds: fast-growing meat-type commercial AA chickens and typical dual-purpose Chinese indigenous LS chickens. The results showed that the expression levels of all IGF system members sharply dropped after hatching in both breeds; this indicated that the IGF system plays a much greater role in the embryonic stage and early period after hatching. The expression levels of some genes in the IGF system showed significant difference ($P < 0.05$) between the two breeds in various developmental stages; in most cases, expression levels were higher in AA than LS chickens, except for *IGF1* and *IGF2BP1* at E14, and *IGF1R* and *2R*, and *IGFBP2* and *4* at E10. It is worth noting that the *IGFBP3* expression levels were almost always significantly higher in AA than LS chickens at nearly all developmental stages (Figure 5).

IGFBP3 Represses Proliferation but Promotes Differentiation of Myoblasts

The mRNA level of *IGFBP3* increased by 22-fold (Figure 6A) and decreased by 55.86% (Figure 6B) when the CPM cells were transfected by the *IGFBP3* overexpression vector pcDNA3.1-IGFBP3 and its siRNA, si-IGFBP3, respectively. The EdU assay results indicated that *IGFBP3* overexpression significantly inhibited CPM cell proliferation (Figure 6C, D) and lowered the number of S phase cells, but increased the number of G0/G1 cells (Figure 6F, S5). In contrast, *IGFBP3* knockdown significantly promoted CPM cell proliferation (Figure 6C, E) and increased the number of S phase cells, but reduced the number of G0/G1 cells (Figure 6G). Moreover, CCK-8 assay showed that *IGFBP3* overexpression reduced cell

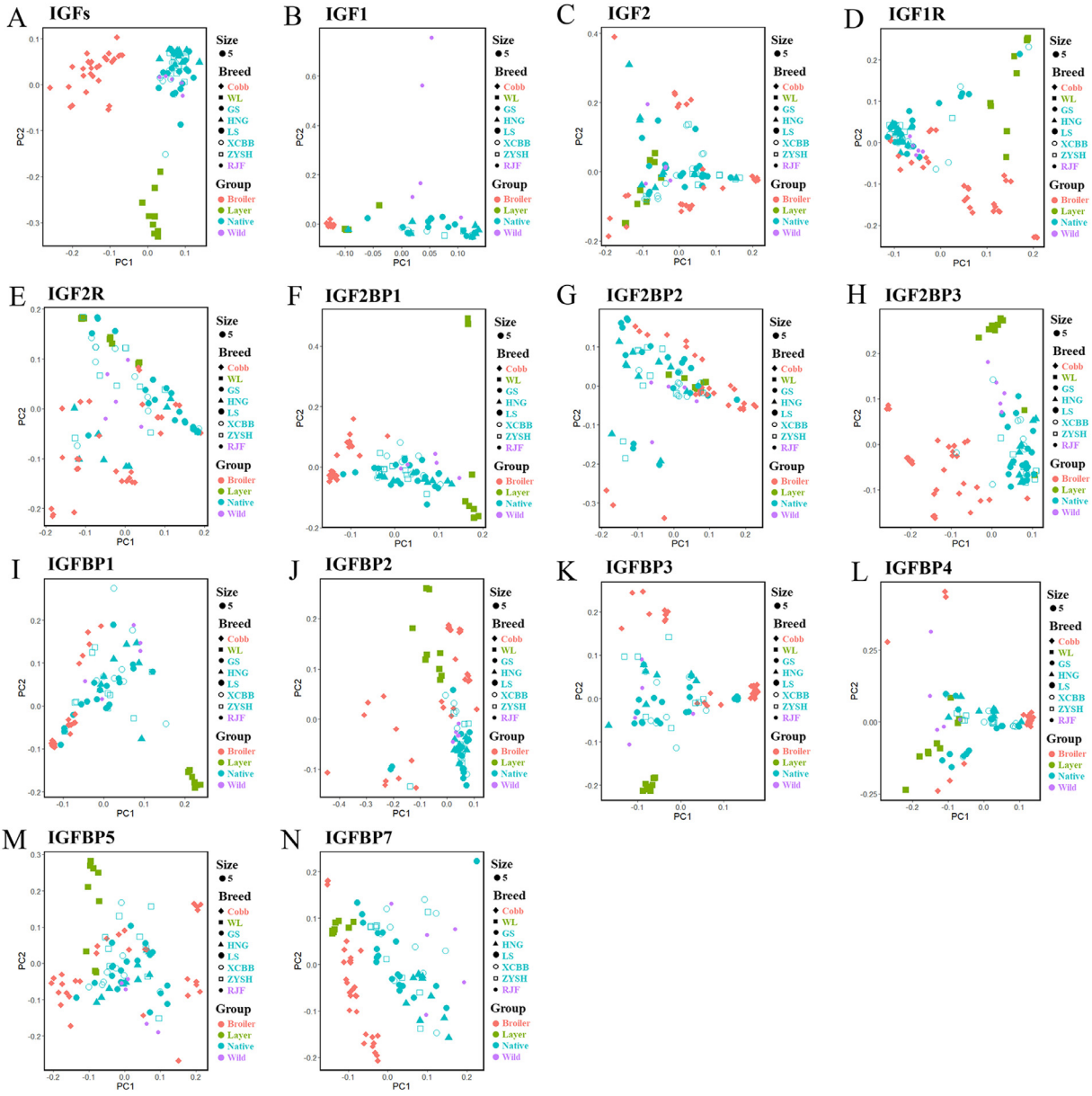

Figure 4. Principal component analysis. The plots were established according to the SNPs mapped to (A) the whole-genome in IGF system genes, (B) *IGF1* gene, (C) *IGF2* gene, (D) *IGF1R* gene, (E) *IGF2R* gene, (F) *IGF2BP1* gene, (G) *IGF2BP2* gene, (H) *IGF2BP3* gene, (I) *IGFBP1* gene, (J) *IGFBP2* gene, (K) *IGFBP3* gene, (L) *IGFBP4* gene, (M) *IGFBP5* gene, (N) *IGFBP7* gene.

viability (Figure 6H), but *IGFBP3* knockdown showed an opposite trend (Figure 6I). Collectively, these data revealed that *IGFBP3* represses myoblast proliferation.

To investigate the effect of *IGFBP3* on myoblast differentiation, MyHC immunofluorescence staining was performed. The results showed that *IGFBP3* overexpression significantly increased the myotube area (Figure 7A, C), whereas *IGFBP3* knockdown prevented myotube formation (Figure 7B, C). Additionally, the expressions of myoblast differentiation marker genes (*MyoD, MyHC, MyoG*, and *Myomaker*) were significantly upregulated when *IGFBP3* was overexpressed (Figure 7E), but significantly downregulated when of *IGFBP3* was knocked down in myoblasts (Figure 7F). During the myoblast differentiation process, *IGFBP3* expression was gradually upregulated until DM5 and then decreased after the induction of myoblast differentiation (Figure 7D). Therefore, *IGFBP3* could promote myoblast differentiation.

DISCUSSION

In the present study, it was found that the IGF system was generally highly conserved in the chicken genome with the exception of *IGFBP6*, which was evolutionarily lost, based on the consensus conserved genomic synteny and a gene (*IGFBP6*) gain/loss tree. Phylogenetic evolutionary analysis revealed that the chicken IGF system clustered into three distinct groups, which is consistent with previous reports on the IGF system in mammals (Daza et al., 2011) and fish (Zou et al., 2009; Macqueen et al., 2013). Further amino acid sequence alignment and structural domain analysis of the IGF2BPs

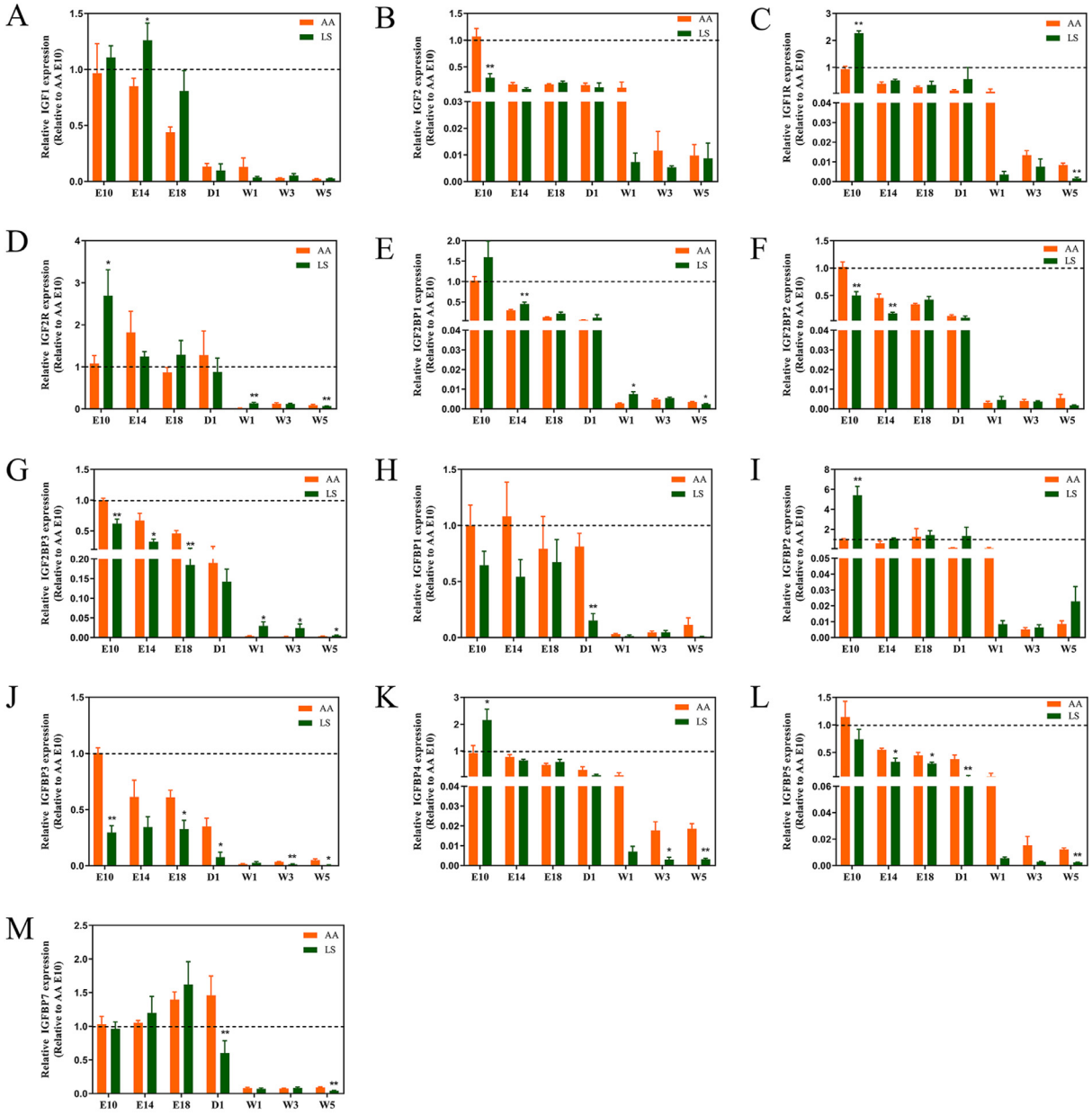

Figure 5. The expression profiles of chicken IGF system genes in chicken skeletal muscle from 10-day embryonic to 5-weeks after hatching. The mRNA expression levels of chicken (A) *IGF1*, (B) *IGF2*, (C) *IGF1R*, (D) *IGF2R*, (E) *IGF2BP1*, (F) *IGF2BP2*, (G) *IGF2BP3*, (H) *IGFBP1*, (I) *IGFBP2*, (J) *IGFBP3*, (K) *IGFBP4*, (L) *IGFBP5* and (M) *IGFBP7* based on RT-qPCR analysis. Each value is represented the mean ± SEM (n = 6). Student's t-test was used to determine the statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$.

indicated that an IB structural domain of 78 amino acids at the N-terminal and a TY structural domain of 53 amino acids at the C-terminal occurred in the chicken IGF2BPs, and all of them contained two RNA recognition motifs and four hnRNPK homology structural domains; this was similar to the IGF2BPs in mammals.

The indigenous chicken breeds and specialized commercial broilers and layers have been subjected to strong human-driven selection, leading to remarkable phenotypic changes in morphology, physiology, and behavior. The commercial broiler, commercial layer, indigenous chicken breeds, and wild red jungle fowl populations could be clearly separated when PCA was conducted with SNP datasets of the IGF system genes. These findings indicated that the genetic variations contributed to the formation of the specialized traits in the modern commercial broilers and layers. Moreover, our association study further supported that these genes have functions in egg production performance and growth. Our results were consistent with several previous studies, which showed that variations in the IGF system genes could have functions in chicken growth performance (Geng and Zhang, 2008; Zhou et al., 2018) and egg-laying traits (Nagarajs et al., 2000; Tang et al., 2010).

A previous study showed that the IGF system genes were expressed in many tissues of the embryo and played an important role in cell proliferation and differentiation (Stewart and Rotwein, 1996). Our spatiotemporal expression spectrum analysis revealed that the genes in the IGF system were extensively expressed and their expression levels continuously decreased from embryonic stages to early birth stages, which was generally

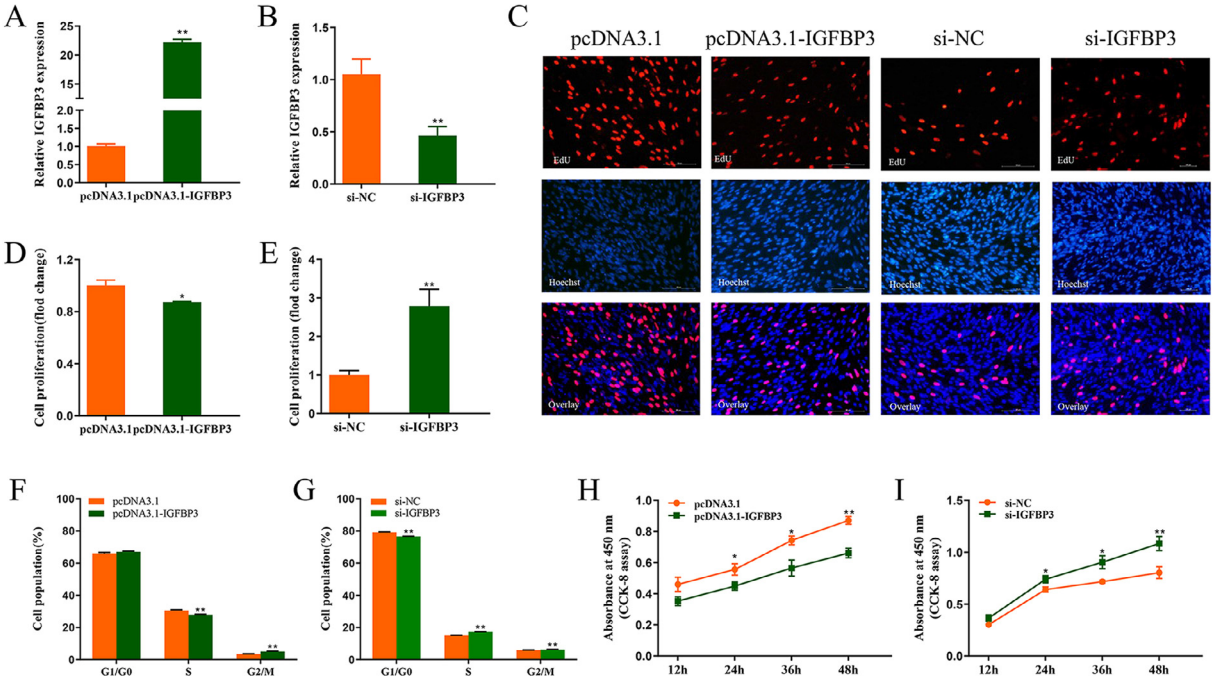

Figure 6. *IGFBP3* represses cell proliferation. (A, B) *IGFBP3* relative expression in *IGFBP3*-overexpressed and *IGFBP3*-knockdown CPM cells. (C) EdU proliferation assays for the *IGFBP3*-overexpressed and *IGFBP3*-knockdown cells. (D, E) The proliferation rate of CPM cells transfected with pcDNA3.1-IGFBP3 or si-IGFBP3 according to the statistical results of C, and the proliferation rate was calculated by the ratio of the number of EdU-stained cells to the number of Hoechst 33342-stained cells. (F, G) Cell cycle analysis after *IGFBP3*-overexpressed and *IGFBP3*-knockdown. (H, I) CCK-8 assays for the *IGFBP3*-overexpressed and *IGFBP3*-knockdown. Results are shown as mean ± SEM and the data are representative of at least three independent assays. Student's t-test was used to determine the statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$.

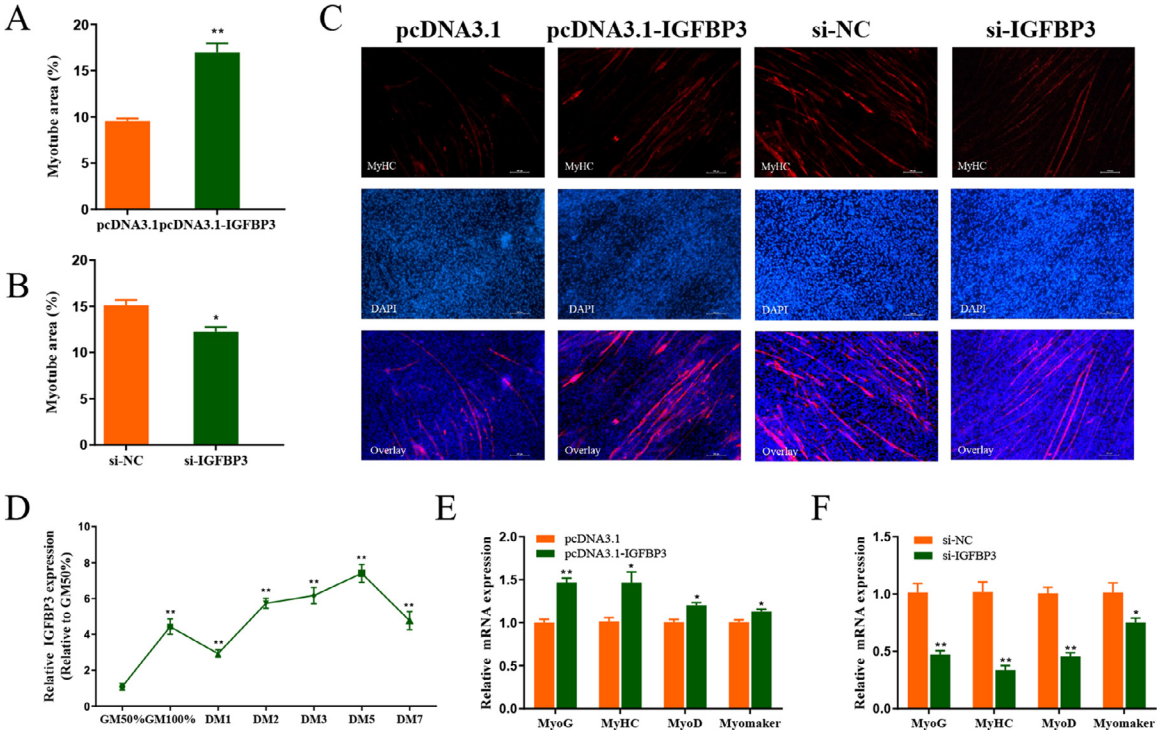

Figure 7. *IGFBP3* promotes cell differentiation. (A, B) Myotube area (%) of CPM cells transfected with pcDNA3.1-IGFBP3, pcDNA3.1, si-IGFBP3 or si-NC. (C) MyHC Immunofluorescence staining of CPM cells. (D) The relative expression of *IGFBP3* during CPM differentiation. (E, F) The relative expression of *MyoD, MyoG, MyHC*, and *Myomaker*. GM stands for myoblasts in the proliferative stage, and DM1, 2, 3, 5, 7 stands for myoblasts that successfully induced differentiation from d 1 to d 7 (at different time points). Results are shown as mean ± SEM. and the data are representative of at least three independent assays. Student's t-test was used to determine the statistical significance. $^{*}P < 0.05$, $^{**}P < 0.01$.

consistent with the findings in other studies on chickens (Armstrong and Hogg, 1994; Karcher et al., 2009; Saneyasu et al., 2016; Saneyasu et al., 2017); this indicated that the IGF system also plays a positive role in embryonic and early post-hatch stage growth and development in chickens. Interestingly, the expression patterns of the genes in the IGF system were very similar throughout the developmental stages; however, the expression levels were somewhat different at some stages between two distinct breeds.

It was well documented that IGFBP3 is the main IGF-binding protein, and can inhibit cell proliferation by an IGF-independent mechanism (Luo et al., 2015) or stimulate cell differentiation through IGF-dependent mechanisms in vitro (Gómez, 2006; Al-Khafaji et al., 2018). Although the expression levels of *IGFBP3* mRNA in the epiphyseal cartilage of chickens at different developmental stages were preliminarily investigated (Lu et al., 2010), the potential role in skeletal muscle remains unclear.

In this study, it was also found that SNPs in the *IGFBP3* promoter region were significantly associated with body weight, breast muscle weight, and leg muscle weight, and expression levels of *IGFBP3* in pectoral muscle at multiple developmental stages were always significantly higher in AA broiler than LS chicken, which indicated that *IGFBP3* participates in myogenic cell proliferation and myoblast differentiation. Further in vitro study revealed that *IGFBP3* overexpression suppressed the cell cycle progression of myoblasts by inhibiting the transition of myoblasts from G0/G1 phase to S phase; however, *IGFBP3* overexpression facilitated myotube formation by promoting *MyHC, MyoD, MyoG*, and *Myomaker*, which were functionally identical to those found in humans (Foulstone et al., 2003) and pigs (Pampusch et al., 2003).

In conclusion, the IGF system is highly conserved, and is involved in the regulation of egg production, growth, and carcass traits in chickens. *IGF2BP1* and *3*, and *IGFBP2, 3*, and *7* genes might have largely contributed to the formation of both specialized meat-type and egg-type chicken lines, whereas *IGFBP1* and *5* likely contributed more to the formation of the egg-type line. The SNPs in the *IGFBP3* promoter regions were significantly associated with breast muscle weight. The *IGFBP3* mRNA expression level was significantly higher in the pectorales of AA than LS chickens across embryonic and post-hatch periods. Moreover, *IGFBP3* could repress the proliferation, but promote the differentiation of chicken primary myoblasts. Our results provide a systematic understanding of the roles of the IGF system genes, and the SNPs related to growth and egg-laying performance could be used as genetic markers for breeding selection of chickens in the future.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102411.

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