JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0180114

Effect of IGFBP2 Overexpression on the Expression of Fatty Acid Synthesis Genes in Primary Cultured Chicken Hepatocytes

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The effects of insulin-like growth factor binding protein 2 (IGFBP2) on the expression of fatty acid synthesis regulators and triglyceride production were investigated in primary cultured chicken hepatocytes. The full-length chicken *IGFBP2* coding region was synthesized by overlap extension PCR and cloned into the pcDNA3.1 vector. An *in situ* digestion method was used to prepare the chicken hepatocytes. Primary chicken hepatocytes were maintained in monolayer culture. Real-time PCR was used to detect changes in the expression of *IGFBP2*, *PPARG*, *IGF1*, *IGF1R*, *APOAI*, and *LFABP,* after the overexpression of *IGFBP2* in chicken hepatocytes. Triglyceride production and glucose content were also evaluated using triglyceride and glucose analysis methods. The expression level of *IGFBP2* increased after transfection of the *IGFBP2*-containing vector. The expression levels of *PPARG*, *IGF1*, and *IGF1R* also increased in cultured chicken hepatocytes after the overexpression of *IGFBP2*, whereas the expression of *LFABP* and *APOAI* decreased. Triglyceride production in primary cultured chicken hepatocytes increased after the overexpression of *IGFBP2*. These results suggest that IGFBP2 is involved in lipogenesis, increasing both the expression of fatty acid synthesis regulators, and triglyceride production in primary cultured chicken hepatocytes.

Key words: chicken, hepatocyte, insulin-like growth factor binding protein 2, triglyceride

J. Poult. Sci., 56: 177−185, 2019

Introduction

In birds, the liver is the main site of *de novo* fatty acid synthesis, and accounts for more than 70% of all lipid production (Griffin *et al*., 1992). In liver, synthesized fatty acid are incorporated into triglycerides, which are the major components of lipoproteins (Hillgartner *et al*., 1995). Adipose tissues only serves as a lipid storage site in avians (Saadoun and Leclercq, 1987). Therefore, 60−80% of the triglyceride storage in avian adipose tissues is dependent upon the availability of plasma lipid substrate originating from hepatic lipogenesis (Griffin *et al*., 1992). Understanding lipid metabolic pathways in the liver is crucial for identifying the genes responsible for fat deposition in chickens.

The proper regulation of gene networks in the liver is integral to the maintenance of fatty acid synthesis. Several factors have been identified as key regulators of lipid and lipoprotein metabolism in the liver, including peroxisome proliferator-activated receptors (PPARs), insulin-like growth factor 1 (IGF1), IGF1 receptor (IGF1R), liver fatty acid binding protein (LFABP), and apolipoprotein AI (APOAI). Peroxisome proliferator-activated receptor gamma (PPARG) is a member of the nuclear hormone receptor family, and functions in the liver as a transcription factor that regulates fatty acid catabolism and lipid export (Wahli and Michalik, 2012). IGF1, IGF1R, and their signaling pathways play critical roles in stimulating lipogenesis (Accili *et al*., 1996). APOAI is the main structural component of high-density lipoproteins and a lecithin-cholesterol acetyltransferase activator, and thus plays roles in lipoprotein assembly, lipid transport, and lipid metabolism, by mediating the interactions of these proteins with receptors, enzymes, and lipid transport proteins (Rosenson *et al*., 2011). LFABP delivers the fatty acid substrates required to synthesize triglycerides, and prevents the detergent effects of fatty acids in cells (Storch and McDermott, 2009). These genes profoundly affect various overlapping aspects of fatty acid synthesis, and participate in significant cross-talk to coordinate lipid homeostasis.

IGF binding protein 2 (IGFBP2) is a secreted protein that binds IGFI and IGFII with high affinity (Rajaram *et al*., 1997; Fuller *et al*., 1999). IGFBP2 secreted by chicken preadipocytes can affect IGF activity in adipose tissues (Clemmons *et al*., 1992; Butterwith, 1994). In pigs, IGFBP2 regulates the

Received: October 18, 2018, Accepted: November 22, 2018
Released Online Advance Publication: January 25, 2019
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proliferation and differentiation of preadipocytes, which is indirectly mediated by IGF or transforming growth factor β (TGFβ) signals (Richardson *et al*., 1998; Gonzalez-Fernandez *et al*., 2016). IGFBP2 also interacts with many different ligands, independent of its binding to IGF (Wang *et al*., 2006; Chua *et al*., 2016). In previous studies, IGFBP2 was shown to interact directly with the gene encoding integrin α5 (*ITGB1*), and also to independently activate the EGFR/STAT3 signaling pathway (Wang *et al*., 2006; Chua *et al*., 2016). Our previous studies and other studies have suggested that the *IGFBP2* gene is a candidate locus, or linked to a major gene that affects chicken fatness, growth, and carcass traits (Lei *et al*., 2005; Li *et al*., 2006; Leng *et al*., 2009; Gholami *et al*., 2014). However, the molecular mechanisms underlying the regulation of triglyceride synthesis by IGFBP2 remain unclear.

Based on the available information, this study was undertaken to evaluate the effects of IGFBP2 on the expression of fatty acid synthesis regulators and triglycerides in cultured chicken hepatocytes.

Materials and Methods

Animals

The broilers (18−22 days old) used in this study were derived from Northeast Agricultural University broiler lines divergently selected for high and low abdominal fat content (NEAUHLF) (Li *et al*., 2006; Leng *et al*., 2009). Broilers from low abdominal fat content lines were selected for this study. In each experiment, three broilers were used for hepatocyte culture. All the chickens were kept under the same environmental conditions and had free access to feed and water. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agriculture University (IACUC-02-005).

Test Reagents

All chemicals and reagents were supplied by Sigma–Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Cloning the Chicken IGFBP2 Coding Sequence (CDS)

The chicken *IGFBP2* CDS is 936 bp long, with a high-GC-content region. To isolate the CDS of chicken *IGFBP2*, we used overlap extension PCR. First, a 421-bp region of the *IGFBP2* cDNA with a high GC content was synthesized as 12 fragments (Table 1), and the synthesized oligonucleotides were diluted separately to 50 μmol/*l*. In the first-round PCR, 50 μl of the reaction mixture contained 2 μl each of 10 pmol/μl primers BP2-OF6 and BP2-OR6, 0.4 μl of 5 U/μl Pyrobest^{TM} DNA Polymerase (Takara, Shiga, Japan), 5 μl of 10×Pyrobest Buffer, 4 μl of 2 mmol/*l* dNTPs, and 36.6 μl of H_2O. The PCR cycling conditions were 94 C for 5 min, followed by 20 cycles of 94℃ for 40 s, 55℃ for 40 s, and 72℃ for 40 s, followed by 72℃ for 10 min. In the second-round PCR, the template was the product of the first-round PCR, and the primers were BP2-OF5 and BP2-OR5. The second-round PCR cycling conditions were the same as those for the first-round PCR. In subsequent PCRs, the last PCR product was used as the next PCR template, and the primer pairs BP2-OF4 and BP2-OR4, BP2-OF3 and BP2-OR3, BP2-OF2 and BP2-OR2, and BP2-OF1 and BP2-OR1 were used consecutively. The PCR cycling conditions in these rounds were the same as those in the first-round PCR. Thus, the 421-bp sequence of the *IGFBP2* CDS was obtained after six rounds of PCR.

The remaining 536-bp CDS fragment of *IGFBP2* with a normal GC content was generated with routine reverse transcription PCR. The PCR mixture (50 μl) contained 2 μl of 2 ng/μl cDNA from chicken adipose tissue, 1 μl each of 10 pmol/μl primers IGFBP-2E-F and IGFBP-2E-R (Table 2), 0.4 μl of 5 U/μl Pyrobest^{TM} DNA Polymerase, 5 μl of 10× Pyrobest Buffer, 4 μl of 2 mmol/*l* dNTPs, and 36.6 μl of H_2O. The cycling conditions for the PCR was 94℃ for 5 min, followed by 33 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s, followed by 72℃ for 10 min.

Finally, the entire 936 bp CDS of *IGFBP2* was obtained by fusing the 421 bp and 536 bp cDNA fragments by overlap extension PCR. The PCR mixture (50 μl) contained 2 μl of the 421 bp fragment and 2 μl of the 536-bp fragment, 0.4 μl of Pyrobest^{TM} DNA Polymerase, 5 μl of 10×Pyrobest Buffer, 4 μl of 2 mmol/*l* dNTPs, and 36.6 μl of H_2O. The cycling conditions for the PCR were 94 C for 5 min, followed by 33 cycles of 94℃ for 45 s, 60℃ for 45 s, and 72℃ for 45 s, followed by 72℃ for 10 min. The product was stored at −20℃.

Table 1. **Oligonucleotide primer sequences used to amplify *IGFBP2***

No	Name	Sequence (5′ to 3′)
1	BP2-OF1	ATGGCGCTCGGCGGGGTCGGTCGCGGCGGCGCGGCGCGGG CCGCTTGGCC
2	BP2-OF2	GCGGCGCGGGCCGCTTGGCCGCGGTTGCTGCTGGCGGCGTTGGCGCCGGC GCTGGCGC
3	BP2-OF3	GT TGGCGCCGGC GCTGGCGCTG GCGGGGCCGG CGCTGCCCGA GGTGCTGTTC CGC
4	BP2-OF4	TGCCCGA GGTGCTGTTC CGCTGCCCGC CCTGCACGGC GGAACGCCTG GCC
5	BP2-OF5	GCACGGC GGAACGCCTG GCCGCTTGCT CCCCGGCCGC CCGGCCGCCC TGC
6	BP2-OF6	CGGCCGC CCGGCCGCCC TGCCCCGAGC TGGTGCGGGA ACCGGGCTGC GGCTGCTGTC C
7	BP2-OR1	GACCGGTCAT CACCGTTGTC TGCGGGGGGC TCGGTGCTGG CTCCGTACTC
8	BP2-OR2	TCGGTGCTGG CTCCGTACTC GTCCGTGTCG GGGGGGCGGG CGCAGGTGCC CTGTCCCT
9	BP2-OR3	GG CGCAGGTGCC CTGTCCCTGG ACCAGGGCTT GGGGGGGCAG CTCGGCTCCG GGG
10	BP2-OR4	GGGGCAG CTCGGCTCCG GGGTCGGGGT AGCAGCGCAA ACCGGCGGCG CAG
11	BP2-OR5	AGCGCAA ACCGGCGGCG CAGCGCGGGG TGTACACGCC GCACGCCTCG TCC
12	BP2-OR6	ACACGCC GCACGCCTCG TCCTCCAGGC GGGCGCACAC CGGACAGCAG CCGCAGCCCG G

Construction of Chicken IGFBP2-expressing Plasmid

*Eco*RI and *Xho*I (Takara) restriction endonuclease sites were introduced into the 936 bp chicken *IGFBP2* CDS by PCR. The PCR mixture (50 μl) contained 2 μl of DNA encoding the 936 bp CDS of the chicken *IGFBP2* gene, 1 μl each of 10 pmol/μl primers IGFBP-2EcoRI-F and IGFBP-2XhoI-R (Table 2), 0.4 μl of 5 U/μl Pyrobest^{TM} DNA Polymerase, 5 μl of 10×Pyrobest Buffer, 4 μl of 2 mmol/*l* dNTPs, and 36.6 μl of H_2O. The cycling conditions for the PCR were 94℃ for 5 min, followed by 33 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 30 s, followed by 72℃ for 10 min.

The *IGFBP2* CDS and pcDNA3.1+ vector (Invitrogen, Carlsbad, CA, USA) were both digested with *Eco*RI and *Xho*I. The pcDNA3.1–*IGFBP2* recombinant plasmid was obtained by ligating the prepared chicken *IGFBP2* CDS and the pcDNA3.1 vector.

Chicken Hepatocyte Culture and Transfection

Chicken hepatocytes were prepared and maintained in a monolayer culture, as described previously (Fujii *et al*., 1996). Broiler chickens were starved for 3 h and anesthetized with ether. Their livers were perfused *in situ* with calcium/magnesium-free Hanks' balanced salt solution (pH 7.5) for 20 min, followed by perfusion with Hanks' balanced salt solution (pH 7.5) containing 0.05% collagenase IV, for approximately 10−15 min at 37℃. The hepatocytes were released from the digested livers and suspended in 80 m*l* of precooled (4℃) serum-free Williams' E medium (Invitrogen) and filtered through nylon mesh (150 μm pores). The hepatocytes were then incubated for 30 min at 37℃ and filtered through nylon mesh (75 μm pores) to remove any aggregated hepatocytes. The hepatocytes were washed three times with serum-free Williams' E medium, with centrifugation at 300×*g* for 3 min. Hepatocytes with > 90% viability, verified by Trypan Blue exclusion test, were plated (5.0×10^5 cells/60 mm collagen-coated dish) in incubation medium (Williams' E medium) containing 0.5 μg/m*l* insulin, 5 mg/*l* transferrin, 3 g/*l* glutamate amine, 10^{-7} mol/l dexamethasone, 100 U/m*l* streptomycin, 100 U/m*l* penicillin, and 10% fetal bovine serum (FBS) at 37℃ in a 5% CO_2 atmosphere. After incubation for 4 h, the medium was replaced with fresh incubation medium. The monolayer cultures of hepatocytes were then used in experiments. The incubation medium was changed every 24 h.

To determine the efficacy of *IGFBP2* overexpression, chicken hepatocytes transfected with pcDNA3.1 were used as controls, and untransfected hepatocytes were used as blank controls. To transfect hepatocytes with the pcDNA3.1–*IGFBP2* recombinant plasmid, 2 μg of the plasmid was diluted in 250 μl of serum-free Williams' E medium without antibiotics, mixed gently, and incubated at room temperature for 5 min. Lipofectamine^{TM} 2000 liposomes (10 μl; Invitrogen) were added to 240 μl of serum-free Williams' E medium without antibiotics, mixed gently, and incubated at room temperature for 5 min. The diluted Lipofectamine^{TM} 2000 and pcDNA3.1–*IGFBP2* plasmid were then gently mixed together and incubated at room temperature for 15 min to form liposome complexes. The liposome complexes were added to hepatocyte culture dishes, supplemented with incubation medium (Williams' E medium) containing 0.5 μg/m*l* insulin, 5 mg/*l* transferrin, 3 g/*l* glutamate amine, 10^{-7} mol/*l* dexamethasone, and 10% FBS without antibiotics, and incu-

Table 2. **Sequences of primers used for real-time PCR**

No	Gene	Type	Primers Name	Primer Sequence (5′ to 3′)	Amplicon (bp)
1	IGFBP2	Clone	IGFBP2E-F	GACAACGGTGATGACCGGTC	536
			IGFBP2E-R	AGCAAGCAGGACCACATCC	
2	IGFBP2	Clone	IGFBP2*Eco*RI-F	GAGAATTCATGGCGCTCGGCGGGGTCGG	936
			IGFBP2*Xho*I-R	CGCTCGCGGGACCACATCCATCTACTGGC	
3	PPARγ	Expression	PPARγ-F	GTGCAATCAAAATGGAGCC	170
			PPARγ-R	CTTACAACCTTCACATGCAT	
4	IGF1	Expression	IGF1-F	TTCTTCTACCTGGCCTGTG	147
			IGF1-R	CATACCCTGTAGGCTTACTG	
5	IGF1R	Expression	IGF1R-F	TTGGCTAATGGCTACTTTG	177
			IGF1R-R	GTGCGTCATATTATCTTTCG	
6	LFABP	Expression	LFABP-F	TCACTGGAAAGTACGAGC	390
			LFABP-R	GCATGCAGGGTCTCTAGATT	
7	ApoAI	Expression	ApoAI-F	GCA TTC GGG ATA TGG	133
			ApoAI-R	CTC AGC GTG TCC AGG TTG T	
8	IGFBP2	Expression	IGFBP2-F	TGC CGG ATG AGC GAG GTC	117
			IGFBP2-R	CCA TTC ACC GAC ATC TTG C	
9	18S	Expression	18S-F	TAGATAACCTCGAGCCGATCGCA	321
			18S-R	GACTTGCCCTCCAATGGATCCTC	

bated at 37℃ in a 5% CO_2 atmosphere. After incubation for 6 h, the culture medium was changed to the incubation medium containing antibiotics.

Total RNA Extraction, cDNA Synthesis, and Quantitative Real-time PCR Analysis

To analyze gene expression, the chicken hepatocytes were harvested with 0.25% trypsin–0.04% EDTA digestion solution, and the RNA was extracted with a PureLink™ Micro-to-Midi Total RNA Purification Kit with DNase I treatment (Invitrogen). cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Gene expression was detected with SYBR® *Premix Ex Taq*™ (Perfect Real Time) (Takara) in 50 μl real-time reactions using the oligonucleotide primers shown in Table 2. All transcript levels were normalized to the corresponding level of 18S rRNA. Quantitative real-time PCR was performed with the ABI 7500 Real-Time PCR System. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification. To determine the efficacy of *IGFBP2* overexpression, chicken hepatocytes transfected with pcDNA3.1 were used as controls, and untransfected hepatocytes were used as blank controls. Three culture cells were made from three individual broilers, and real-time PCR was done three times per sample.

Total Protein Determination

The cultured hepatocytes were digested with 0.25% trypsin and suspended in phosphate-buffered saline (PBS). They were washed three times with PBS, and lysed with repeated freeze-thaw cycles. Total protein was determined with the BCA Protein Assay Kit (Beyotime, Beijing, China). Three culture cells were made from three individual broilers, and total protein determination was performed three times per sample.

Glucose Determination

The cultured hepatocytes were digested with 0.25% trypsin and suspended in PBS. They were washed three times with warm PBS to remove any extracellular glucose, and adjusted to 10^6 cells/m*l* with PBS. The hepatocytes were homogenized in a glass homogenizer for 1 min. The glucose concentration was determined with a Total Carbohydrate Assay Kit (Sigma), and results were normalized to cellular protein concentration. Three culture cells were made from three individual broilers, and glucose determination was done three times per sample.

Triglyceride Determination

The cultured hepatocytes were digested with 0.25% trypsin and suspended in PBS. They were washed three times with PBS, adjusted to 10^6 cells/m*l* with PBS, and homogenized in a glass homogenizer for 1 min. The triglyceride concentration was determined with an Adipogenesis Kit (Sigma), and the results normalized to cellular protein concentration. Three culture cells were made from three individual broilers, and triglyceride determination was done three times per sample.

Statistical Analysis

Experimental data are expressed as means±SEM. All statistical analyses were performed with SPSS 19 software (Somers, NY, USA). One-way ANOVA with Student's t test was used to evaluate statistical significance between the different treatment groups. $P<0.05$ indicates significant difference.

Results

Primary Culture of Chicken Hepatocytes

At 1 h after inoculation, the chicken hepatocytes began to exhibit adherent growth. After 4 h in culture, 90% of the hepatocytes displayed adherent growth. After 24 h, the hepatocytes appeared as typical epithelial-like polygons, and the cell bodies had become flat and thin. After 3 days in culture, the hepatocyte cell junctions had become more compact, and the cells were flattened, stretched, and tightly adherent to the bottom surface of the culture dish (Fig. 1).

Cloning Chicken IGFBP2 CDS and Construction of Chicken IGFBP2 Expression Vector

The complete chicken *IGFBP2* CDS was cloned by overlap extension PCR (Fig. 2A). The prepared chicken *IGFBP2* CDS was ligated into the pcDNA3.1 eukaryotic expression vector, generating the pcDNA3. 1–*IGFBP2* recombinant

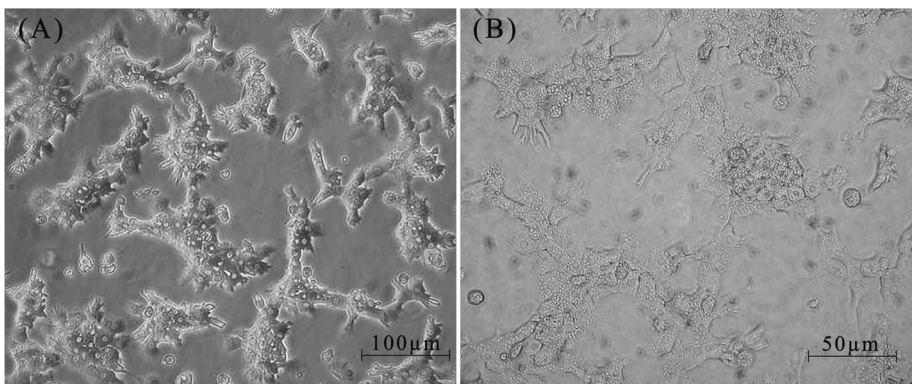

Fig. 1. **Primary culture of chicken hepatocytes.** (A) Chicken hepatocyte morphology was observed by microscopy at 400-fold magnification. (B) Chicken hepatocyte morphology was observed at 1000-fold magnification.

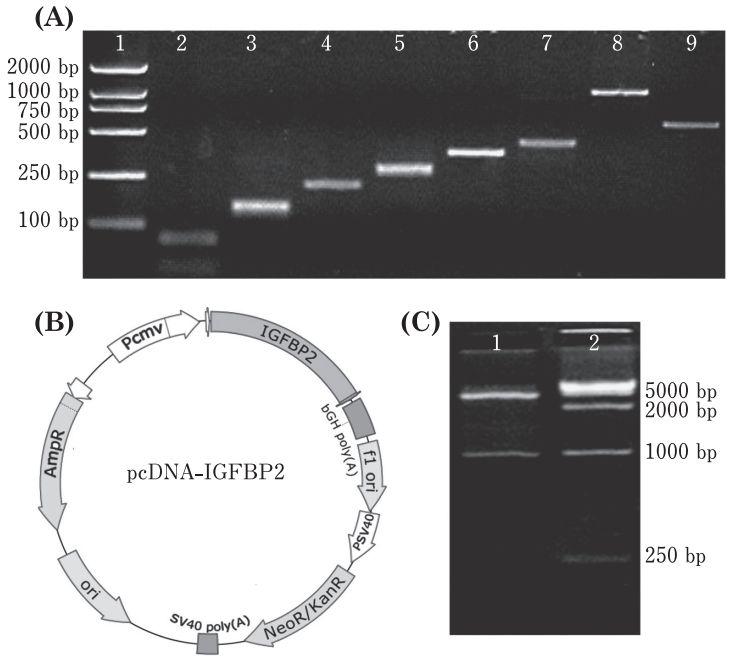

Fig. 2. **Cloned chicken *IGFBP2* CDS and construction of chicken IGFBP2 expression vector.** (A) Cloning the chicken *IGFBP2* CDS. Lane1: DNA Marker DL2000, lane2: first-round PCR product, lane3: second-round PCR product, lane4: third-round PCR product, lane5: fourth-round PCR product, lane6: fifth-round PCR product, lane7: sixth-round PCR product, lane8: 936 bp full length of *IGFBP2* coding sequence, lane9: 536 bp coding sequence of *IGFBP2*. (B) Map of chicken *IGFBP2* expression vector. (C) Recombinant plasmid pcDNA3.1–*IGFBP2* was digested with *EcoRI* and *XhoI*.

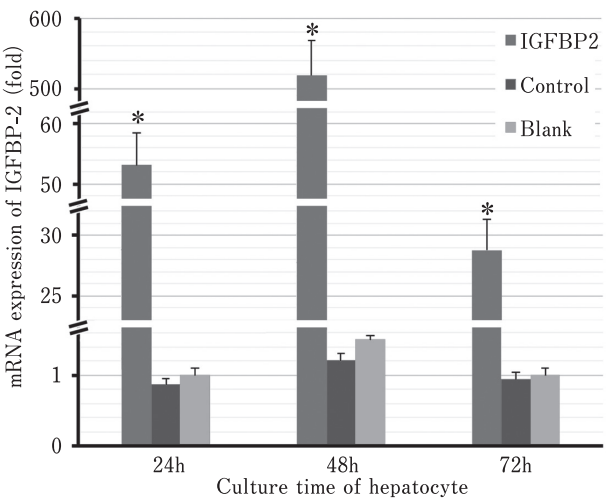

Fig. 3. **Overexpression of IGFBP2 in chicken hepatocytes.** 'IGFBP2' indicates chicken hepatocytes transfected with the pcDNA3.1–*IGFBP2* vector. 'Control' indicates chicken hepatocytes transfected with the pcDNA3.1 vector. 'Blank' indicates untransfected chicken hepatocytes. Statistical analysis was performed on IGFBP2 versus controls. * $P<0.05$, significantly different.

plasmid (Fig. 2B). The recombinant plasmid was digested with *Eco*RI and *Xho*I, yielding two fragments: the 5.5 kb pcDNA3.1 vector fragment, and the 936 bp chicken *IGFBP2* CDS fragment (Fig. 2C).

Overexpression of Chicken IGFBP2 in Hepatocytes

After the hepatocytes were transfected with the pcDNA 3.1–*IGFBP2* eukaryotic expression vector, the expression of *IGFBP2* was significantly higher than in the control group after 24, 48, and 72 h ($P<0.05$; Fig. 3).

Effect of IGFBP2 Overexpression on the Expression of PPARG, IGF1, and IGF1R

After the overexpression of *IGFBP2* in hepatocytes, the expression of *PPARG*, *IGFI*, *IGFIR* genes were significantly higher in the transfected hepatocytes than in the control groups at 24 and 48 h ($P<0.05$; Fig. 4). At 72 h, the expression of *PPARG*, *IGFI*, and *IGFIR* genes in the hepatocytes in the transfected group did not differ significantly from that in control groups (Fig. 4).

Effect of IGFBP2 on the Expression of LFABP and APOAI

After the overexpression of *IGFBP2* in hepatocytes, the expression of the *LFABP* gene was significantly lower in the transfected hepatocytes than in either control group at 24 h ($P<0.05$; Fig. 5A). At 48 and 72 h, the expression of the *LFABP* gene in the transfected hepatocytes did not differ significantly from that in the control groups (Fig. 5A).

After the hepatocytes were transfected with the *IGFBP2*

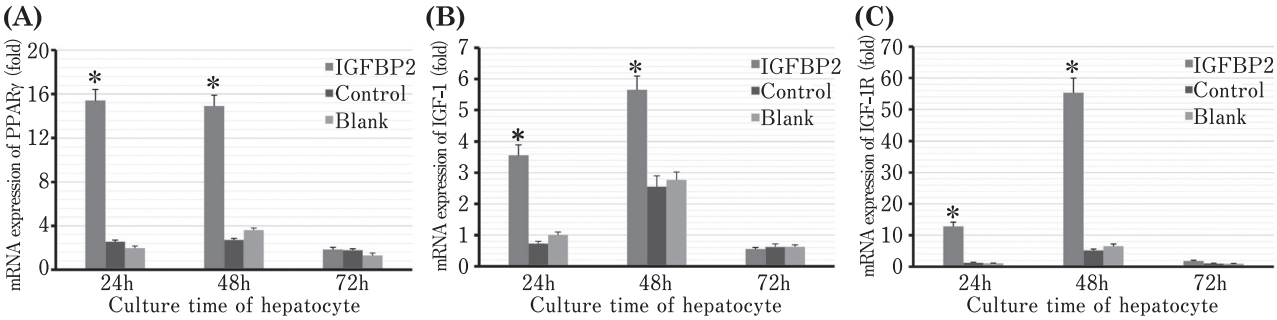

Fig. 4. **Expression of the *PPARG, IGFI*, and *IGFIR* genes in chicken hepatocytes.** (A) Expression of the *PPARG* gene in chicken hepatocytes. (B) Expression of the *IGFI* gene in chicken hepatocytes. (C) Expression of the *IGFIR* gene in chicken hepatocytes. 'IGFBP2' indicates chicken hepatocytes transfected with the pcDNA3.1–*IGFBP2* vector. 'Control' indicates chicken hepatocytes transfected with the pcDNA3.1 vector. 'Blank' indicates untransfected chicken hepatocytes. Statistical analysis was performed on IGFBP2 versus controls. * $P<0.05$, significantly different.

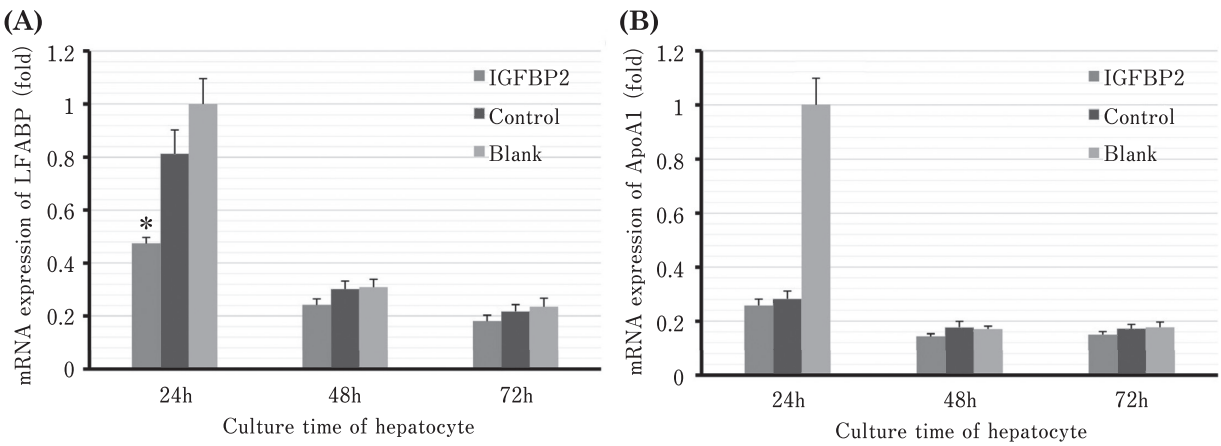

Fig. 5. **Expression of *LFABP* and *APOAI* genes in chicken hepatocytes.** (A) Expression of the *LFABP* gene in chicken hepatocytes. (B) Expression of the *APOAI* gene in chicken hepatocytes. 'IGFBP2' indicates chicken hepatocytes transfected with the pcDNA3.1–*IGFBP*-2 vector. 'Control' indicates chicken hepatocytes transfected with the pcDNA3.1 vector. 'Blank' indicates untransfected chicken hepatocytes. Statistical analysis was performed on IGFBP2 versus controls. * $P<0.05$, significantly different.

expression vector, expression of the *APOAI* gene was significantly lower in the transfected hepatocytes, and the control group, than in the blank group at 24 h. However, the expression of the *APOAI* gene in the transfected hepatocytes did not differ significantly from that in the control group at 24 h (Fig. 5B). At 48 and 72 h, the expression of *APOAI* in the transfected hepatocytes did not differ significantly from that in the control groups (Fig. 5B).

Effect of IGFBP2 Overexpression on Triglyceride and Glucose Contents of Chicken Hepatocytes

After the hepatocytes were transfected with the *IGFBP2* expression vector, the triglyceride content was significantly higher in the hepatocytes than in either control group at 48 and 72 h ($P<0.05$; Fig. 6A). However, there was no significant difference between the treatment group and the control group at 24 h (Fig. 6A).

After the hepatocytes were transfected with the *IGFBP2* expression vector, the glucose content was significantly higher in the hepatocytes than in the control groups at 24 and 48 h ($P<0.05$; Fig. 6B). However, there was no significant difference between the treatment group and the control group at 72 h (Fig. 6B).

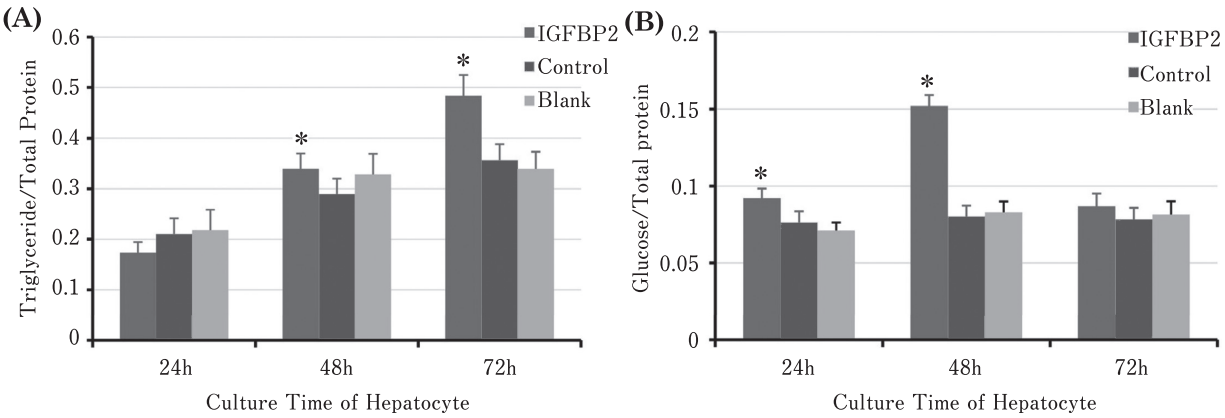

Fig. 6. **Triglyceride and glucose contents in chicken hepatocytes.** (A) Triglyceride content in chicken hepatocytes. (B) Glucose content in chicken hepatocytes. 'IGFBP2' indicates chicken hepatocytes transfected with the pcDNA3.1–*IGFBP2* vector. 'Control' indicates chicken hepatocytes transfected with the pcDNA3.1 vector. 'Blank' indicates untransfected chicken hepatocytes. Statistical analysis was performed on IGFBP2 versus controls. * $P<0.05$, significantly different.

Discussion

IGFBP2 is a secreted protein that functions by interacting with circulating IGFs to modulate IGF-mediated signaling (Baxter, 2000). We previously mapped a QTL significantly influencing abdominal fat weight, and the percentage of abdominal fat of chicken, on chicken chromosome 7, and *IGFBP2* is the only known gene located within this QTL region (Wang *et al*., 2012). We further found that a single nucleotide polymorphism (SNP) in the 3′-UTR of chicken *IGFBP2*, is significantly associated with chicken abdominal fat weight and percentage of abdominal fat in the NEAUHLF and the Northeast Agricultural University F(2) (NEAU F(2)) resource population (Li *et al*., 2006; Leng *et al*., 2009). The purpose of the current study was to assess whether IGFBP2 is directly involved in lipid metabolism of chicken hepatocytes.

Additional functions of IGFBP2 have recently been identified, many of which are IGF-independent, and involve intracellular and nuclear IGFBP2 activities. IGFBP2 has a classic nuclear localization signal sequence that is responsible for nuclear entry (Azar *et al*., 2014), and enters the cell nucleus by a mechanism that involves its binding to importin-β (Azar *et al*., 2014). IGFBP2 can bind integrins (Pereira *et al*., 2004; Wang *et al*., 2006; Holmes *et al*., 2012) and activates PI3K/AKT (Mehrian-Shai *et al*., 2007), NF-κB (Holmes *et al*., 2012), and ERK signaling (Han *et al*., 2014). IGFBP2 also potentiates nuclear EGFR/STAT3 signaling, thereby activating the expression of the corresponding downstream genes (Azar *et al*., 2011; Chua *et al*., 2016). In this study, the overexpression of *IGFBP2* promoted the upregulated expression of the *PPARG*, *IGF1*, and *IGF1R* genes in chicken hepatocytes. While PPARG cross-talks with NF-κB signaling (Ide *et al*., 2003; Yoshikawa *et al*., 2003; Konstantinopoulos *et al*., 2007), both IGF1 and IGF1R belong to the IGF1/AKT/STAT3 signaling axis (Yao *et al*., 2016; Zhang *et al*., 2018). Our results indicate that IGFBP2 influences the expression of *PPARG*, *IGF1*, and *IGF1R*, although it may not interact with them directly, and may indirectly interact through NF-κB, STAT3, and AKT signaling, which affects the expression of PPARG, IGF1, and IGF1R.

The expression of LFABP is regulated by growth hormones and PPARα (Vida *et al*., 2013; Graham *et al*., 2016). In this study, the expression of *LFABP* was reduced by 24 h in the *IGFBP2*-overexpressing group. Therefore, IGFBP2 participates in the regulation of *LFABP* gene expression. The expression of APOAI is repressed by PPARG in HepG2 cells (Shavva *et al*., 2016). In the present study, the expression of *PPARG* was upregulated by the overexpression of *IGFBP2*. However, the expression of *APOAI* decreased at 24 h in the *IGFBP2*-overexpressing cells and in the control cells. This does not reflect the effect of IGFBP2 on the expression of the *APOAI* gene, but that the liposome transfection method affected the expression of the *APOAI* gene relative to that in the blank control group. At 48 and 72 h after transfection, the expression of both *LFABP* and *APOAI* was reduced in all three groups. A possible reason is that the secretory function of primary cultured hepatocytes is compromised *in vitro,* and the expression of secretion-related gene products is reduced (Sasaki *et al*., 2000; Tachibana *et al*., 2005; Bamji-Mirza *et al*., 2014).

The triglyceride content and glucose content were higher in the *IGFBP2*-overexpressing cells than in the control group. In a previous report, the methylation of hepatic *IGFBP2* during infancy predicts the development of fatty liver later in life, and is linked to deterioration in glucose metabolism (Kammel *et al*., 2016). In HEK293 cells, reduced IGFBP2 expression aggravates cell death during glucose deficiency, whereas the overexpression of IGFBP2

prolongs cell survival (Ord *et al*., 2015). In the present study, IGFBP2 activated the expression of genes related to synthetic metabolic processes, such as *PPARG*, *IGF1*, and *IGF1R*. PPARG is the central coordinator of triglyceride synthesis, and the increased expression of PPARG promotes triglyceride synthesis rate (Kershaw *et al*., 2007). The IGF1 signal pathway has multiple effects on glucose metabolism. In rats, IGF1 reduces hepatic glucose production rate and increased peripheral glucose uptake (Simpson et al., 2004). In humans, IGF1 improves whole-body glucose uptake and glucose tolerance, while increasing hepatic glucose production (Rao et al., 2010). These findings indicate that IGFBP2 may participate in triglyceride synthesis and glucose absorption by regulating the expression of *PPARG*, *IGF1*, and *IGF1R*.

In summary, overexpression of the *IGFBP2* gene in chicken primary cultured hepatocytes increased the expression of the *PPARG*, *IGF1*, and *IGF1R* genes, and triglyceride synthesis rates. These results provide evidence that the expression of IGFBP2 in chicken hepatocytes contributes to the progression of triglyceride synthesis, thus implicating *IGFBP2* as a gene potentially affecting fat deposition in chickens.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31201796) and China Agriculture Research System (No. CARS-41).

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