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

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Running title: IGFBP2 overexpression in chicken hepatocytes

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Abstract

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, PPARγ, 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 PPARγ, 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.

Keywords: chicken, hepatocyte, insulin-like growth factor binding protein 2, triglyceride
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 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 (Shenyang, China), 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™ DNA Polymerase (Takara, Dalian, China), 5 μl of 10 × Pyrobest Buffer, 4 μl of 2 mmol/l dNTPs, and 36.6 μl of H₂O. The PCR cycling conditions were 94 C for 5 min, followed by 20 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 40 s, followed by 72°C 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 \textit{IGFBP2} CDS was obtained after six rounds of PCR.

The remaining 536-bp CDS fragment of \textit{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™ DNA Polymerase, 5 µl of 10 × Pyrobest Buffer, 4 µl of 2 mmol/l dNTPs, and 36.6 µl of H₂O. The cycling conditions for the PCR was 94°C for 5 min, followed by 33 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min.

Finally, the entire 936 bp CDS of \textit{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™ DNA Polymerase, 5 µl of 10 × Pyrobest Buffer, 4 µl of 2 mmol/l dNTPs, and 36.6 µl of H₂O. The cycling conditions for the PCR were 94°C for 5 min, followed by 33 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, followed by 72°C for 10 min. The product was stored at −20°C.

\textit{Construction of chicken IGFBP2-expressing plasmid}

\textit{Eco}RI and \textit{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™ DNA Polymerase, 5 µl of 10 × Pyrobest Buffer, 4 µl of 2 mmol/l dNTPs, and 36.6 µl of H2O. The cycling conditions for the PCR were 94°C for 5 min, followed by 33 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min.

The IGFBP2 CDS and pcDNA3.1+ vector (Invitrogen, Shanghai, China) were both digested with EcoRI and XhoI. 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°C. The hepatocytes were released from the digested livers and suspended in 80 ml of precooled (4°C) serum-free Williams’ E medium (Invitrogen) and filtered through nylon mesh (150 µm pores). The hepatocytes were then incubated for 30 min at 37°C 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/ml
insulin, 5 mg/l transferrin, 3 g/l glutamate amine, $10^{-7}$ mol/l dexamethasone, 100 U/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (FBS) at 37°C 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™ 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™ 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/ml insulin, 5 mg/l transferrin, 3 g/l glutamate amine, $10^{-7}$ mol/l dexamethasone, and 10% FBS without antibiotics, and incubated at 37°C 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 (ABI, Beijing, China).

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/ml 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/ml 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 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 pcDNA3.1–*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*, *IGF1*, and *IGF1R* 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*, *IGF1*, and *IGF1R* 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* 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).

**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.

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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: 936bp full length of IGFBP2 coding sequence, lane9: 536bp coding sequence of IGFBP2. (B) Map of chicken IGFBP2 expression vector. (C) Recombinant plasmid pcDNA3.1–IGFBP2 was digested with EcoRI and Xhol.

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.

Fig. 4. **Expression of the PPARG, IGF1, and IGFIR genes in chicken hepatocytes.** (A) Expression of the PPARG gene in chicken hepatocytes. (B) Expression of the IGF1 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.

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.
Fig. 1

(A)  
(B)  

100 µm

50 µm
Fig. 3

mRNA expression of IGFBP-2 (fold)

| Culture time of hepatocyte | IGFBP2 | Control | Blank |
|---------------------------|--------|---------|-------|
| 24h                       | *      |         |       |
| 48h                       | *      |         |       |
| 72h                       | *      |         |       |

Fig. 3
Fig. 5

(A) mRNA expression of IGFBP2 (fold)

(B) mRNA expression of Aplp2 (fold)

Culture time of hepatocyte

24h  48h  72h
Fig. 6
| No | Name | Sequence (5’ to 3’) |
|----|------|---------------------|
| 1  | BP2-OF1 | ATGGCGCTCGGCGGGGTCGGTCGCGGCGGCGGCGGCCGGGCGCTTGGCC |
| 2  | BP2-OF2 | GCGGCGCGCGGCCGCTTGGCCGCGTGCTGCTGGCGGCTGGCGGCGGCGGGCCGTGCGG |
| 3  | BP2-OF3 | GTGGCGCCCGGCGCTGCGGGCGGGGCCGGCGCGGGCCGTGCGG |
| 4  | BP2-OF4 | TGCCCGAGTGTCGCTGTCGCCTGCGCGGCGCGGTACGGCGGCGGGTCGGGGT |
| 5  | BP2-OF5 | GACCGGTCATCACCGTTGTCGTCGGGGGCCGGGGTACGGCGGCGGGTCGGGGT |
| 6  | BP2-OF6 | GGCGGCGCGCGGCGGCCGCCGCGCGCGGCCGCCGCCGCGGCCGCCGCCGCCGCCG |
| 7  | BP2-OR1 | GACCGGTCATCACCGTTGTCGTCGGGGGCCGGGGTACGGCGGCGGGTCGGGGT |
| 8  | BP2-OR2 | TGCCCGAGTGTCGCTGTCGCCTGCGCGGCGCGGTACGGCGGCGGGTCGGGGT |
| 9  | BP2-OR3 | GGCGGCGCGCGGCGGCCGCCGCGCGCGGCCGCCGCCGCGGCCGCCGCCGCCG |
| 10 | BP2-OR4 | GACCGGTCATCACCGTTGTCGTCGGGGGCCGGGGTACGGCGGCGGGTCGGGGT |
| 11 | BP2-OR5 | GGCGGCGCGCGGCGGCCGCCGCGCGCGGCCGCCGCCGCGGCCGCCGCCGCCG |
| 12 | BP2-OR6 | GGCGGCGCGCGGCGGCCGCCGCGCGCGGCCGCCGCCGCGGCCGCCGCCGCCG |
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    | AGCAAGCAGGACCACTCC          |               |
| 2  | IGFBP2 | Clone | IGFBP2EcoRI-F | GAGAATTCATGGCGCTGCGGCGGTCG | 936           |
|    |         |       | IGFBP2XhoI-R | CGCTCGCGGGACCACATCCATCTACTGC |               |
| 3  | PPARγ  | Expression | PPARγ-F | GTGCAATCAAATGGAGCC         | 170           |
|    |         |       | PPARγ-R     | CTCAACCTTCACATGCAT          |               |
| 4  | IGF1    | Expression | IGF1-F | TTCTTCTACCTGGCTGTG         | 147           |
|    |         |       | IGF1-R      | CATAACCCTGTAGGCTTACTG        |               |
| 5  | IGF1R   | Expression | IGF1R-F | TGGGCTAATGGCTACTTTG         | 177           |
|    |         |       | IGF1R-R     | GTGGGCTATATTATCTTTGC         |               |
| 6  | LFABP   | Expression | LFABP-F | TCACTGGAAAGTACGAGC         | 390           |
|    |         |       | LFABP-R     | GCAATGCGGTTCTAGATT          |               |
| 7  | ApoAI   | Expression | ApoAI-F | CATCTGGGATGGATG             | 133           |
|    |         |       | ApoAI-R     | CTCAGCTGCTCCAGGTTT          |               |
| 8  | IGFBP2 | Expression | IGFBP2-F | TGC CGG ATG AGC GAG GTC     | 117           |
|    |         |       | IGFBP2-R    | CCA TTC ACC GAC ATCTTGC     |               |
| 9  | 18S     | Expression | 18S-F | TAGATAACCTCGAGCCATCGCA      | 321           |
|    |         |       | 18S-R       | GACTTGCCCTCCATGGATCTC       |               |