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CTGF Inhibits the Differentiation of Chicken Preadipocytes via the TGFβ/Smad3 Signaling Pathway or by Inducing the Expression of ACTG2

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ABSTRACT: Chicken is the main source of protein for humans in most parts of the world. However, excessive fat deposition in chickens has become a serious problem. This adversely affects the growth of chickens and causes economic losses. Fat formation mainly occurs through preadipocyte differentiation, and excessive fat deposition results from the accumulation of preadipocytes after differentiation. Our previous studies have found that the connective tissue growth factor (CTGF) may be an important candidate gene for fat deposition. However, its function and mechanism in preadipocyte differentiation are still unclear. In this study, the RT-qPCR and Western blot results showed that the expression of CTGF mRNA and protein in the abdominal adipose of lean chickens was significantly higher than that of fat chickens. Therefore, we studied the function and mechanism of the CTGF in the differentiation of chicken preadipocytes. Functionally, the CTGF inhibited the differentiation of chicken preadipocytes. Mechanistically, the CTGF mediated the TGFβ1/Smad3 signaling pathway, thereby inhibiting the differentiation of chicken preadipocytes. In addition, we used the unique molecular identifier (UMI) RNA-Seq technology to detect genes that can be regulated by the CTGF in the whole genome. Through transcriptome data analysis, we selected actin gamma 2 (ACTG2) as a candidate gene. Regarding the function of the *ACTG2* gene, we found that it inhibited the differentiation of chicken preadipocytes. Furthermore, we found that the CTGF can inhibit the differentiation of preadipocytes through the *ACTG2* gene. In summary, this study found the CTGF as a new negative regulator of chicken preadipocyte differentiation. The results of this study help improve the understanding of the molecular genetic mechanism of chicken adipose tissue growth and development and also have reference significance for the study of human obesity.

KEYWORDS: *chicken, adipose, CTGF, preadipocyte, cell differentiation*

1. INTRODUCTION

Broiler chickens are widely cultured and economically important poultry worldwide. Chicken is the main source of protein for humans in most parts of the world. With population growth, rising incomes, and concerns about human health, the global demand for chicken continues to grow.[1] Fast-growing broilers are the main poultry used in broiler chicken production worldwide, but the negative problem of excessive fat deposition is very serious. Excessive fat deposition reduces carcass lean meat rate, which reduces meat production.[2] Simultaneously, the fat content affects the flavor and taste of food.[3] In addition, for consumers, excessive fat intake may lead to chronic diseases such as obesity and aggravate public health problems. High-fat diet is already a public health problem, which has been proven to be related to various digestive system diseases and tumors.[4] Therefore, accurately and effectively controlling the excessive fat deposition in broiler chickens is necessary and critical to improve their carcass quality future.

To solve the problem of excessive fat deposition, we must first understand the influencing factors of adipogenesis. Adipose tissue, mainly composed of fat cells, is an important organ for energy storage in the body and has important endocrine functions. Adipogenesis refers to the differentiation of preadipocytes into mature adipocytes.[5] Many cell regulators are involved in the regulation of this process, such as transcriptional regulators, including peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein (C/EBP), and Fatty acid binding protein A (A-FABP, AP2),[6,7] and many cytokines, including Insulin-like growth factor-1,[8] cyclic adenosine monophosphate[9,10] and glucocorticoid.[11] After acting on specific receptors on the surface of the adipocyte membrane, adipocytokines regulate the activity of transcription factors of preadipocyte differentiation in cells through the transmission of signaling pathways to realize the regulation of preadipocyte differentiation.[12]

Connective tissue growth factor (CTGF), a member of the CCN protein family, is a cysteine-rich secreted protein. Many studies have shown that the *CTGF* gene can regulate various cellular functions, including extracellular matrix formation, cell

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adhesion, migration, apoptosis, cell differentiation and mitosis.[13,14] A study has shown that CTGF can induce hepatic progenitor cells to differentiate into hepatocytes.[15] CTGF can activate BMP2/Smad cascade signal to promote osteoblast differentiation of periodontal ligament stem cells.[16] CTGF induces tendon differentiation of adipose-derived stem cells through FAK and ERK1/2 pathways.[17] Although the effects of CTGF on cell differentiation are very extensive, the effect of CTGF on chicken preadipocyte differentiation has not been reported. CTGF can act in various manners, including binding to cell surface receptors. CTGF can bind to some cell surface receptors and fibronectin and integrin $\alpha_5\beta_1$, thus effectively promoting the adhesion of vascular epithelial cells, microvascular epithelial cells, fibroblasts and human platelets.[18] CTGF can bind to the Wnt receptor low-density lipoprotein (LDL) receptor−associated protein 6, thereby inhibiting the key signaling cascades downstream of LDL receptor−associated protein 6, including c-Jun N-terminal kinase and Wnt/β-catenin.[19] Moreover, CTGF can bind to some growth factors or extracellular matrix proteins to play different roles.[20−22] Furthermore, many previous studies have found that the TGFβ/Smad3 signaling pathway can play a role in regulating the expression of the *CTGF* gene.[23,24]

In our previous study, we used the 60K chicken SNP Beadchip (Illumina) to obtain the genotype of the Northeast Agricultural University broiler lines divergently selected for abdominal fat content (NEAUHLF). We found some important candidate genes, including CTGF, that may affect abdominal fat deposition using genome-wide association analysis.[25] Subsequently, we found that the mRNA expression of the *CTGF* gene in the abdominal fat of lean chickens was significantly higher than that of fat chickens. Therefore, in this study, the *CTGF* gene was selected as an important candidate gene affecting chicken fat deposition. We found that CTGF can inhibit the differentiation of preadipocytes through the TGFβ/Smad3 signaling pathway and by inducing the expression of actin gamma 2 (ACTG2).

2. MATERIALS AND METHODS

2.1. Experimental Chickens and Ethical Statement. The male chickens used in this study were from the 23th (lean line, $n = 4$, and fat line, $n = 4$) and 26th (lean line, $n = 6$, and fat line, $n = 6$) generations of the NEAUHLF. NEAUHLF have been established since 1996, using abdominal fat percentage (AFP) = abdominal fat weight (AFW)/body weight at 7 weeks of age (BW7) and plasma very low-density lipoprotein (VLDL) levels as selection criteria.[26] All chickens were maintained under similar environmental conditions and had free access to feed and water. Commercial corn−soybean−based diets that met all NRC (1994) requirements were provided in the study. All animal work was conducted according to the guidelines for the Care and Use of Experimental Animals established by the Ministry of Science and Technology of the People's Republic of China (approval number 2006−398) and was approved by the Laboratory Animal Management Committee of Northeast Agricultural University.

2.2. Collection of Adipose Tissue. The male chickens were slaughtered after fasting for 12 h at 7 weeks of age. The live weight (BW7) of chickens was measured before slaughtering. Abdominal fat (AF) was collected and weighed after slaughter. The collected adipose tissue was washed with 0.75% NaCl, immediately frozen in liquid nitrogen, and stored at −80 °C. The phenotype data of the BW7 and AF weight (AFW) was showed in Supporting Information Table 1.

2.3. Cell Culture and Differentiation Induction. Chicken stromal-vascular cells (SV) and fat cells (FC) were obtained through type I collagenase digestion. Abdominal adipose tissue (3−5 g) was isolated from 12-day-old chickens, chopped, added with collagenase I (2 mg/mL), and incubated at 37 °C for 1 h. The suspension was then passed through 100-mesh and 600-mesh nylon cell filters (BD Falcon, New York) to remove undigested tissue. The filtrate was centrifuged at 200 × *g* for 10 min. The top layer (fat cell fraction) and precipitate (stromal-vascular cell part) were collected as chicken mature adipocytes and preadipocytes, respectively. Chicken preadipocytes ICP2 were constructed in our laboratory.[27] All cells were cultured in a basic medium (DMEM/F12, Gibco) containing 10% fetal bovine serum (FBS, BI, Israel) and 1% penicillin−streptomycin (Gibco). The cells were inoculated into a 25 cm^2 culture flask and cultured at 37 °C, and 5% CO_2 in an incubator.

Cells were inoculated on cell plates. When the confluence reached 60%, the medium was removed, and the oleic acid (Sigma) induction medium with a concentration of 200 μM was added to induce cell differentiation. The oleic acid medium was replaced every 24 h.

2.4. Transfection. All siRNA sequences used in this study are listed in Table 1. The expression plasmids of CTGF and ACTG2 were constructed by inserting expanded CTGF and ACTG2 cDNA fragments into the pCMV-HA vector, respectively. Primer sequences are listed in Table 2. The ICP2 cells were inoculated in 12-well plates until the cell confluence reached 60%. Then, plasmids or siRNAs were introduced into cells using Lipofectamine 3000 (Invitrogen).

Table 1. SiRNA Sequences Used in This Study

name	sequences (5′→3′)
si-CTGF	sense: CCAGGGUCACCAACGAUAATT
	antisense: UUAUCGUUGGUGACCCUGGTT
si-TGFβ1	sense: GCAUCUUCUUCGUGUUCAAdTdT
	antisense: UUGAACACGAAGAAGAUGCdTdT
si-ACTG2	sense: GCACCAUCUACAGGGAGACTT
	antisense: GUCUCCCUGUAGAUGGUGCTT

2.5. Oil red O Staining and Extraction. The medium in the cell plate was removed and washed three times with phosphate-buffered saline (PBS) for 3 times. Each well was fixed for 30 min at 4 °C after adding 4% paraformaldehyde. After discarding the fixative, the cell wells were washed three times with PBS. Subsequently, cells were stained with an Oil red O (Sigma) working solution (Oil red O stock solution/distilled water = 3:2) at room temperature for 15 min. After removing the staining solution and washing three times with PBS, 60% isopropanol was added to each well for 10−20 s. After removing isopropanol, cells were washed three times with PBS. Finally, the staining was observed using an inverted microscope. After photographing, Oil red O was dissolved in 100% isopropanol, and the optical density (OD) was recorded at 510 nm using an enzyme reader. The total protein concentration was determined using a BCA protein quantitative analysis kit (Beyotime, China). The lipid droplet accumulation was presented as a ratio of OD_{510} per mg protein.

2.6. RNA Extraction and Reverse Transcription−Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted from tissues (100 mg each) and cells using the RNAiso reagent kit (TaRaKa, China). First strand cDNA synthesis was performed with 1 μg of total RNA (TaKaRa, China). A SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology) was used for RT-qPCR. The reaction conditions were as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Data analysis was performed using the average of the threshold cycle (ct) of each sample. The relative expression level of the gene was calculated using the $2^{-\Delta\Delta ct}$ method. The internal reference gene was a TATA-box binding protein (TBP). The primers used for RT-qPCR are shown in Supporting Information Table 2.

2.7. UMI RNA-Seq. UMI (Unique Molecular Identifier) RNA-Seq was performed by SeqHealth company (Wuhan, China). The quantity and purity of total RNA were controlled using a Nanophotometer (N60, Germany), and the integrity of RNA was detected using a Qsep-100. After the total RNA samples were qualified, the library was constructed. Briefly, the enriched mRNA was disrupted into short fragments, which were used as templates to synthesize one-strand

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Table 2. Primer Sequences of Plasmid Construction[a]

name	sequences (5′→3′)
CTGF	sense: tggccatggaggcccgaattcCAGCGCACAGCAACCCCA
	antisense: ccgcggccgcggtacctcgagGTTAGCGTCTCCTTCTGGCTTTA
ACTG2	sense: ggccatggaggcccgaattcggATGTGCGAGGAGGAGACCACCGCCCTG
	antisense: ccgcggccgcggtacctcgagTTAGAAGCACTTTCGGTGGACGAT

[a]The lowercase bases include homologous arms and restriction sites.

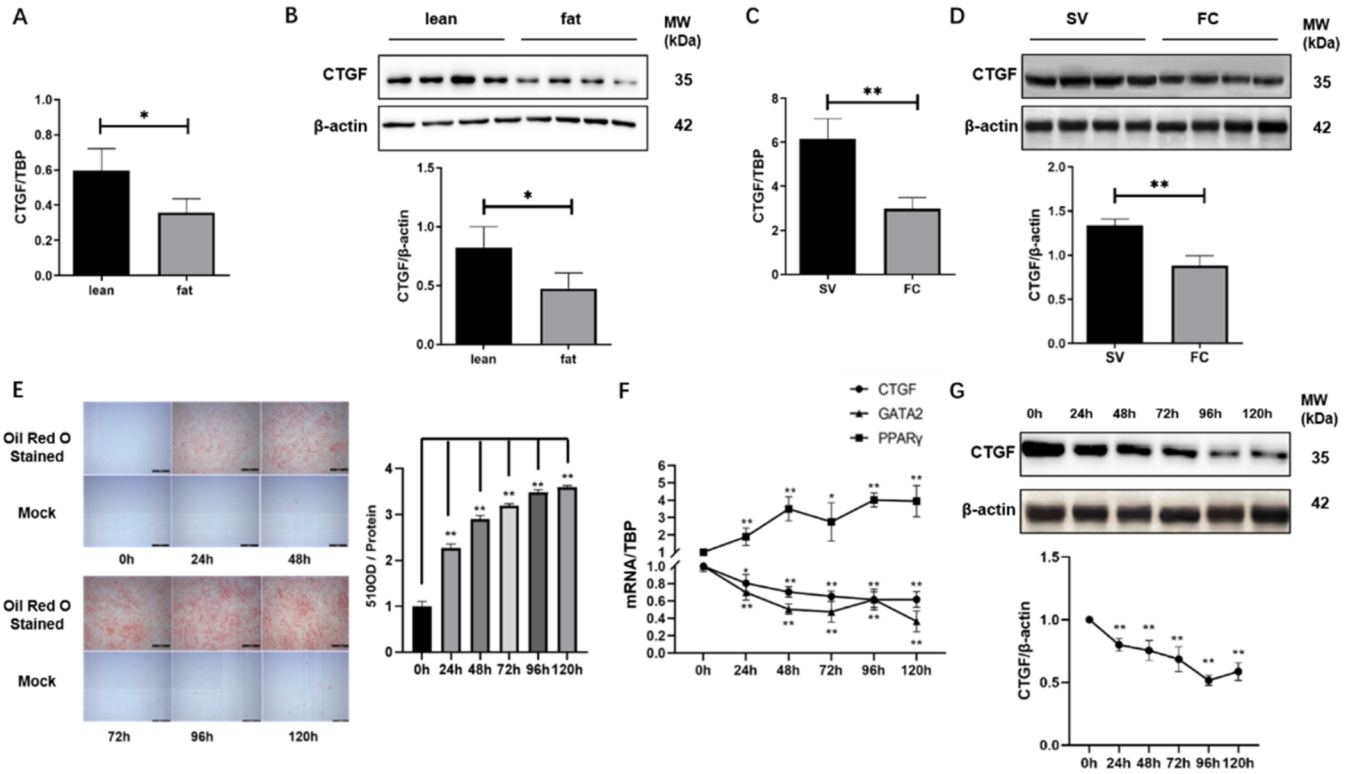

Figure 1. Expression characteristics of the *CTGF* gene in the chicken adipose tissue and adipocytes. (A) The expression of CTGF mRNA in the abdominal adipose tissue of fat and lean chickens. (B) The expression of CTGF protein in abdominal adipose tissue of fat and lean chickens. (C) The expression of CTGF mRNA in primary chicken preadipocytes (SV) and mature adipocytes (FC). (D) The expression of CTGF protein in primary chicken SV and FC. (E) Oil red O staining (left) to detect lipid droplet deposition and Oil red O extraction colorimetric extraction (right). (F) The mRNA expression of the *CTGF* gene and adipocyte differentiation marker gene during chicken preadipocyte differentiation. (G) The expression of CTGF protein during the differentiation of chicken preadipocytes.

cDNA with hexanucleotide random primers, and then buffer, dNTPs and DNA polymerase I were added to synthesize two-strand cDNA. Before amplification, the end of each double-stranded cDNA was repaired, base A was added, and the UMI of 8 random bases was added at the 5′ end. The fragment size was screened and purified using magnetic beads before PCR amplification. The quality of the constructed library was tested and the library products of 200−500 bps were enriched and quantified. Finally, the NovaSeq6000 sequencer (Illumina) was used for sequencing according to the standard operation. The raw data were filtered using fastp (version 0.23.0) to obtain high-quality sequencing data (clean data). KcUID software was used to deduplicate clean data. Then similar reads were merged under the same UID to read error correction and removal of duplicate reads. STAR (version = 2.7.10a) software was used to align the clean data with the chicken reference genome (GRCg7a), and the expression profile of all transcripts was calculated using FeatureCounts software. The mRNA expression was detected by calculating TPM (transcripts per million), and the differentially expressed genes were screened using DESeq2 ($P < 0.05$, |FoldChange| > 1.2).

2.8. Western Blot. Total protein was extracted from cells using RIPA buffer (Beyotime, China) supplemented with a protease inhibitor (Beyotime). The protein sample was added to 5× denaturing loading buffer (Beyotime), boiled for 5 min, separated using 10% SDS-PAGE, and transferred onto a 0.22-μM NC membrane. The antibody of CTGF was purchased from Novus (1:1200, NB100-724). The antibody of PPARγ was purchased from Santa Cruz Biotechnology (1:1000, sc-7273). The antibody of C/EBPβ was purchased from MyBioSource (1:1000, MBS9204875). The antibody of β-actin was purchased from Beyotime (1:800, AA128, China). Horseradish peroxidase−labeled goat antibody of rabbit IgG (1:1000, Beyotime, A0208, China) and horseradish peroxidase−labeled goat antibody of Mouse IgG (1:1000, Beyotime, A0216, China) were used as secondary antibodies. Specific protein bands were visualized using the ECL detection kit (HaiGene, China) in a chemiluminescence system (Sagecreation).

2.9. Statistical Analysis. All data are presented as mean ± SD. Student's *t*-test analysis was used to assess the significant differences between the two groups. Differences between multiple groups were assessed using one-way analysis of variance. The Pearson correlation coefficient was used to analyze the correlation between the mRNA expression level of the *CTGF* gene and the mRNA expression level of its candidate downstream genes. The differences between the groups were considered significant at a P value of <0.05 (*) and highly significant at a P value of <0.01 (**).

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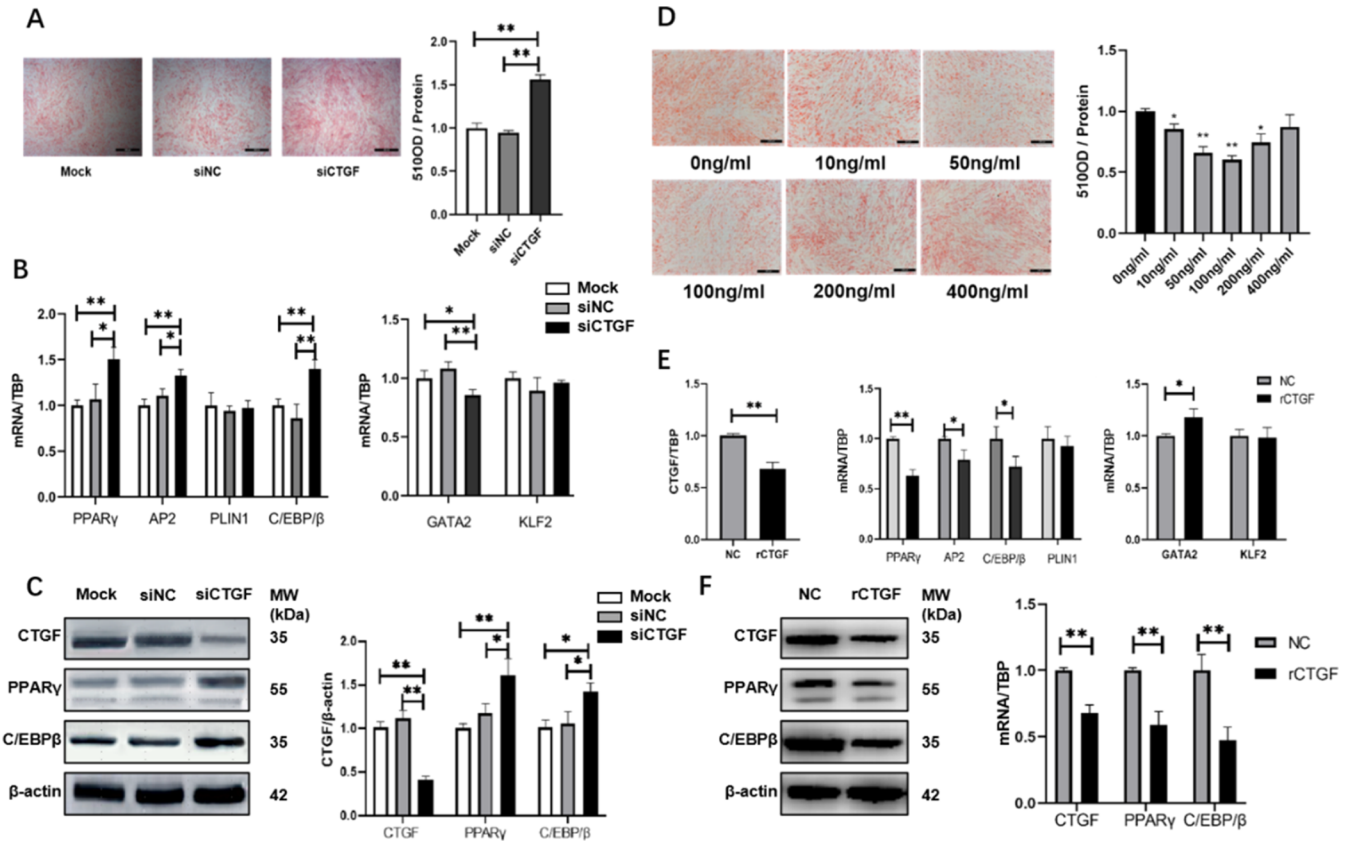

Figure 2. *CTGF* gene inhibits the differentiation of chicken preadipocytes. (A) Oil red O staining was used to detect the effect of CTGF knockdown on lipid droplet deposition. (B) Effect of knocking down chicken CTGF on mRNA expression of differentiation marker genes in ICP2. (C) Effect of knocking down chicken CTGF on protein expression of differentiation marker genes in ICP2. (D) The effect of adding rCTGF on lipid droplet deposition in preadipocytes was detected by Oil red O staining. (E) The effect of adding rCTGF on the mRNA expression of CTGF and differentiation marker gene in ICP2. (F) The effect of adding rCTGF on the protein expression of CTGF and differentiation marker gene in ICP2.

3. RESULTS

3.1. Expression Characteristics of the *CTGF* Gene in the Chicken Adipose Tissue and Adipocytes. To explore whether the *CTGF* gene affects abdominal fat deposition in chickens, we used the adipose tissue of 7-week-old male chickens of the 23th generation (G23) of NEAUHLF as the experimental material to determine the tissue expression of the *CTGF* gene. RT-qPCR and Western blot results showed that the mRNA and protein expression levels of the *CTGF* gene in the adipose tissue were different between lean and fat lines (Figure 1A,B). These findings suggested that the disparity in *CTGF* gene expression may be associated with the variation in abdominal fat content between lean and fat lines. To determine whether *CTGF* gene is involved in the differentiation of chicken adipocytes, we detected the expression levels of CTGF in primary chicken preadipocytes (SV) and mature adipocytes (FC). RT-qPCR and Western blot results showed that the expression level of *CTGF* gene in preadipocytes was significantly higher than that in mature adipocytes (Figure 1C,D). To explore whether CTGF is involved in preadipocyte differentiation, we used oleic acid to induce ICP2 cell differentiation to obtain differentiated chicken preadipocytes. It was found that as differentiation progressed, there was an increase in the deposition of lipid droplets (Figure 1E), along with an increase in the expression of PPARγ and a decrease in the expression of GATA2 (Figure 1F). RT-qPCR and Western blot results showed that the mRNA and protein expression levels of the *CTGF* gene decreased gradually during chicken preadipocyte differentiation (Figure 1F,G). These results indicate that CTGF is involved in the differentiation of chicken preadipocytes.

3.2. *CTGF* Gene Inhibits the Differentiation of Chicken Preadipocytes. To clarify the effect of the *CTGF* gene on chicken preadipocyte differentiation, we designed and synthesized three siRNAs targeting the *CTGF* gene to knockdown the expression level of the *CTGF* gene in ICP2 (Supporting Information Figure 1). In contrast, the interference effect of siCTGF-812 was relatively stable at all time the points. Therefore, siCTGF-812 was used to knockdown the expression level of CTGF. After knocking down the *CTGF* gene, the deposition of lipid droplets during chicken preadipocyte differentiation was significantly increased (Figure 2A). Moreover, RT-qPCR results showed that the mRNA expression levels of preadipocyte differentiation marker genes PPARγ, AP2 and C/EBPβ were significantly increased and GATA2 was decreased (Figure 2B). Western blot results showed that the expression levels of PPARγ and C/EBPβ were significantly increased (Figure 2C). The above results indicate that the knockdown of *CTGF* gene can promote the differentiation of chicken preadipocytes.

We overexpressed the *CTGF* gene in chicken preadipocytes by adding CTGF recombinant protein (rCTGF) and by transfecting the pCMV-CTGF plasmid, respectively. The results of Oil red O staining showed that compared with 0 ng/mL, rCTGF at concentrations of 10, 50, 100, 200 ng/mL could inhibit lipid droplet deposition during chicken preadipocyte differentiation, and the inhibition effect of 100 ng/mL rCTGF

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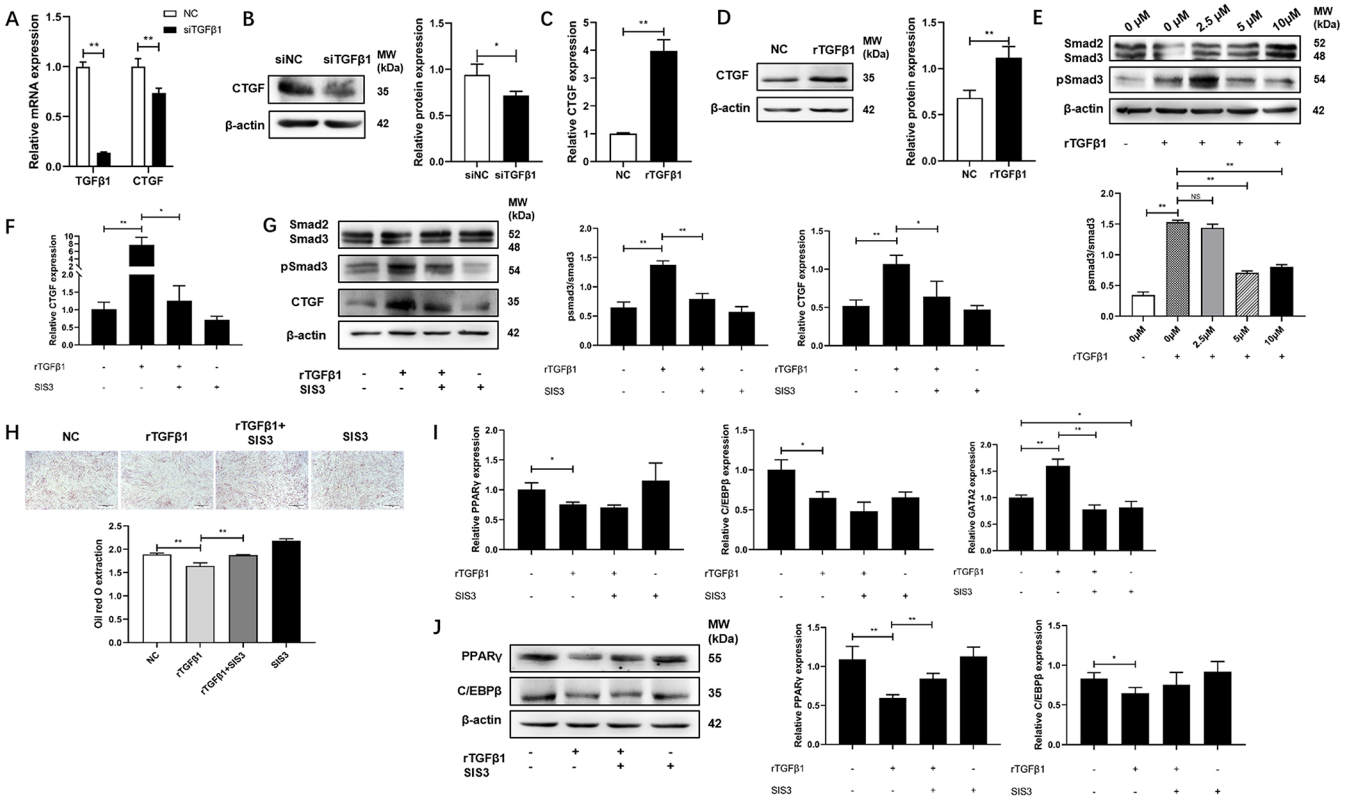

Figure 3. TGFβ1/Smad3 signaling pathway inhibited the differentiation of chicken preadipocytes. (A) The mRNA expression of CTGF after knocking down TGFβ1. (B) The protein expression of CTGF after knocking down TGFβ1. (C) The mRNA expression of CTGF was detected after adding rTGFβ1. (D) The protein expression of CTGF was detected after adding rTGFβ1. (E) Optimum concentration of the inhibitor SIS3. (F) The mRNA expression of the *CTGF* gene was inhibited after inhibition of Smad3 phosphorylation. (G) The protein expression of the *CTGF* gene was inhibited after inhibition of Smad3 phosphorylation. (H) Lipid droplet deposition after inhibition of Smad3 phosphorylation was detected using Oil red O assay. (I) The mRNA expression of differentiation marker genes after inhibition of Smad3 phosphorylation. (J) The protein expression of differentiation marker genes after inhibition of Smad3 phosphorylation.

was the most significant (Figure 2D). After adding 100 ng/mL rCTGF, the mRNA and protein expression levels of CTGF in the cells decreased significantly (Figure 2E,F). RT-qPCR results showed the mRNA expression levels of PPARγ, AP2 and C/EBPβ were significantly decreased, and the mRNA expression level of GATA2 was significantly increased (Figure 2E). The Western blot results showed that the protein expression levels of PPARγ and C/EBPβ were significantly decreased (Figure 2F). Based on the above results, it was concluded that CTGF was a negative regulator of chicken preadipocyte differentiation.

3.3. *CTGF* Gene Affects the Differentiation of Chicken Preadipocytes via the TGFβ/Smad3 Signaling Pathway. In mammals, CTGF is considered a downstream factor of TGFβ, which can be directly regulated by the TGFβ/Smad3 signaling pathway and participates in various biological processes regulated by TGFβ.[23,24] RT-qPCR and Western blot results showed that the mRNA and protein expression levels of *CTGF* gene were significantly decreased after knocking down TGFβ1 in ICP2 (Figure 3A,B). The expression of *CTGF* gene was significantly increased after adding rTGFβ1 (Figure 3C,D). Subsequently, to explore whether the TGFβ/Smad3 signaling pathway affects the expression of CTGF during chicken preadipocyte differentiation, we treated cells with different concentrations (0, 2.5, 5, and 10 μM) of Smad3 phosphorylation inhibitor (SIS3). Western blot results showed that 5 μM SIS3 could significantly inhibit the phosphorylation of Smad3 (Figure 3E). Inhibition of Smad3 phosphorylation in the TGFβ/Smad3 signaling pathway could inhibit TGFβ1-induced CTGF expression (Figure 3F,G). In addition, we demonstrated that the TGFβ1/Smad3 signaling pathway inhibited the differentiation of chicken preadipocytes (Figure 3H–J). The above results indicate that TGFβ1/Smad3 and CTGF could form the TGFβ1/Smad3/CTGF signal axis.

Subsequently, we evaluated whether interference with CTGF could attenuate the inhibitory effect of TGFβ1 on chicken preadipocyte differentiation. First, rTGFβ1 with biological activity was added to ICP2 cells for 6 h, and then CTGF was knocked down using siRNA. After induction of differentiation, it was found that knockdown of CTGF significantly attenuated the inhibitory effect of TGFβ1 on preadipocyte differentiation (Figure 4A–C). The above results indicated that the TGFβ1/Smad3/CTGF signaling axis inhibited the differentiation of chicken preadipocytes.

3.4. UMI RNA-Seq Was Used to Screen CTGF-Regulated Genes in the Whole Genome. To explore the genes that CTGF can regulate during the differentiation of chicken preadipocytes, we performed UMI RNA-seq on ICP2 cells after knocking down CTGF. The total RNA of the control and the CTGF knockdown groups was extracted, and the concentration, purity, and integrity of the total RNA were detected. The results are shown in Supporting Information Figure 2.

The results of the principal component analysis showed that 6 samples were clustered into 2 groups, which proved that the

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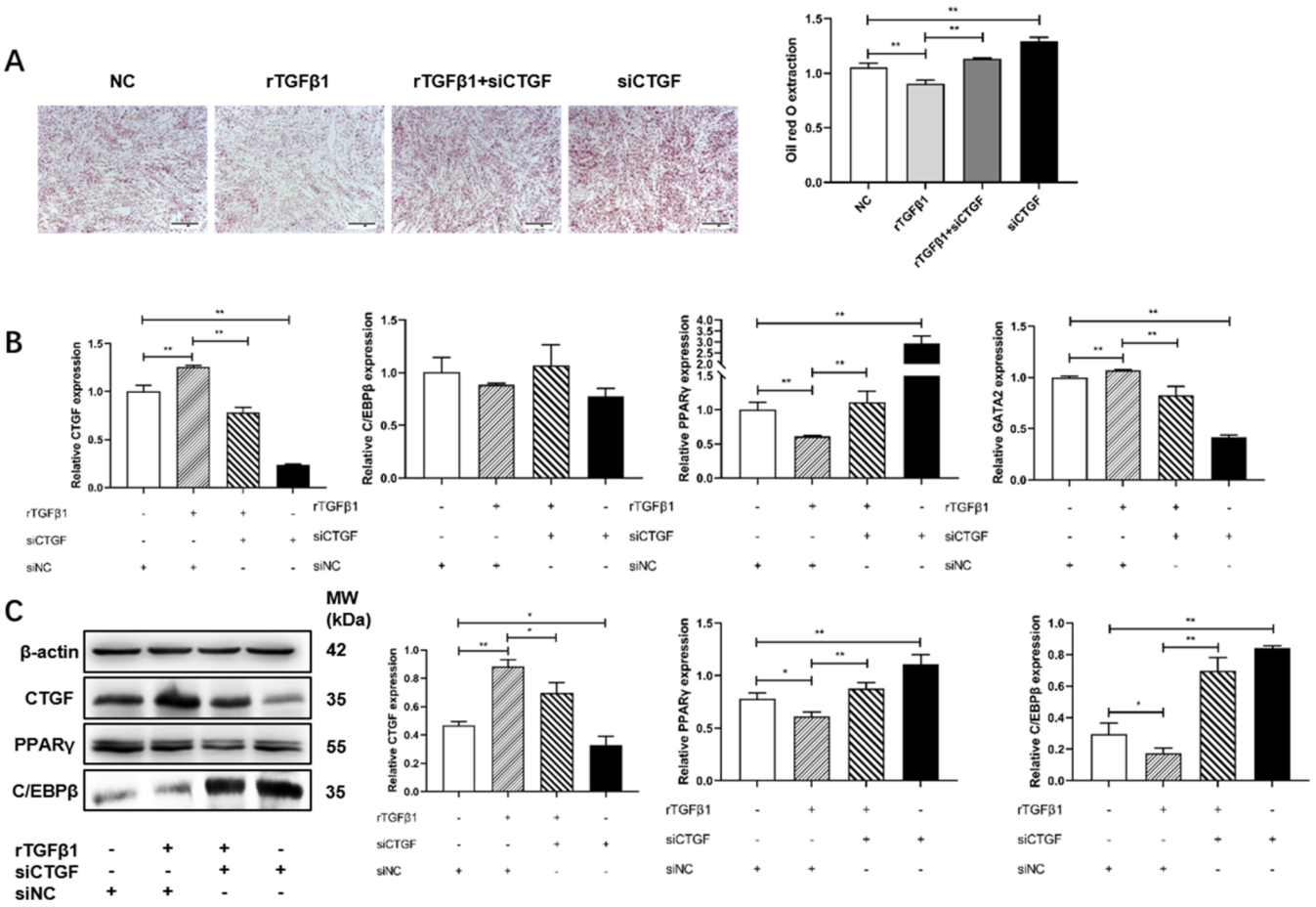

Figure 4. Effect of CTGF knockdown on the differentiation of chicken preadipocytes regulated by TGFβ1. (A) Lipid droplet deposition after the knockdown of TGFβ1-induced CTGF expression. (B) Effect of the knockdown of CTGF induced by TGFβ1 on mRNA expression of differentiation marker genes in chicken preadipocytes. (C) Effect of the knockdown of CTGF induced by TGFβ1 on protein expression of differentiation marker genes in chicken preadipocytes.

sequencing results were reliable and available (Figure 5A). DESeq2 software was used to screen differentially expressed genes (DEGs) between the experimental and the control groups with |FoldChange| of >1.2 and P value <0.05 as the standard. A total of 95 DEGs were screened (Figure 5B, Supporting Information Document 2), including 53 upregulated genes and 42 downregulated genes. To better understand which biological functions are significantly related to DEGs, we performed Gene Ontology and Kyoto Encyclopedia of Genes and Genomes functional enrichment analyses on the identified DEGs. Gene Ontology analysis showed that these genes were significantly enriched in biological processes, stress response, biological tissue development, multicellular homeostasis and osteoclast differentiation (Figure 5C). Kyoto Encyclopedia of Genes and Genomes analysis showed that DEGs were significantly enriched in four pathways: focal adhesion signaling pathway, Wnt signaling pathway, actin cytoskeleton regulation pathway, and adhesion junction (Figure 5D). The functional and pathway analysis results of DEGs showed great differences between the experimental and the control groups in biological tissue development and cell differentiation. Based on the above results and literature reports, 39 candidate genes with significant differences (|FoldChange| of >1.2 and P < 0.05) related to classical pathways such as fat and insulin were found. Among them, there were 21 upregulated genes and 18 downregulated genes. Then cluster analysis was performed on these 39 candidate genes. As shown in Figure 5E, the genes with the same or similar expression patterns were clustered into the same class, and the genes clustered into the same class may have similar functions.

Among the 39 candidate genes, 9 genes have relatively special coding sequences (such as a high GC content) for which specific primers could not be designed for amplification. Therefore, we finally verified the remaining 30 candidate genes (16 upregulated genes, 14 downregulated genes) using RT-qPCR. The verification results of 21 genes were consistent with the sequencing results (Figure 5F,G). As shown in Figure 5G, the expression of the ACTG2 gene decreased most significantly after CTGF knockdown. In order to further screen out the key genes regulated by CTGF, we detected the mRNA expression of CTGF and the above 21 differential genes in the abdominal adipose tissue of the 26th generation of 7-week-old fat and lean chickens and performed the Pearson correlation analysis. The results of the correlation analysis showed that the expression levels of 6 genes of the upregulated genes were significantly correlated with the expression level of CTGF (Supporting Information Figure 3). Among the downregulated genes, the expression levels of 8 genes were significantly correlated with the expression level of CTGF, and the expression level of the *ACTG2* gene was significantly positively correlated with that of CTGF (Figure 5H, Supporting Information Figure 4). Moreover, the expression levels of 9 genes, including ACTG2 in the

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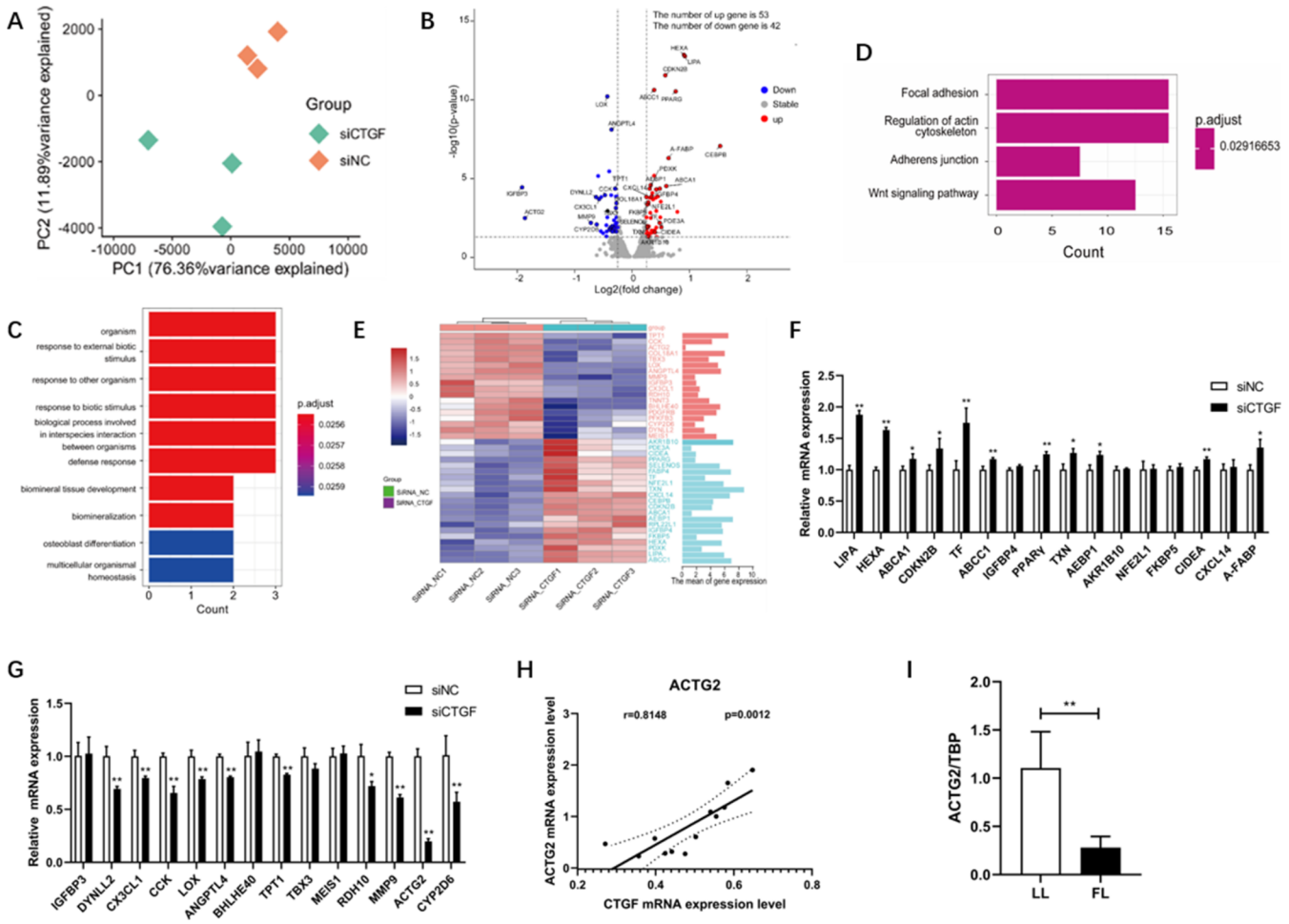

Figure 5. UMI RNA-seq technology was used to screen CTGF-regulated genes in the whole genome. (A) Principal component analysis. (B) Volcano plot of differential gene expression. The red dots represent the upregulated genes in the group, the blue dots represent the downregulated genes in the group, and the gray dots represent nonsignificant differences. (C) The significantly enriched GO terms of DEGs. The horizontal axis indicates the significance of enrichment (expressed by p.adjust; the smaller the value, the more the significance), and the vertical axis represents the enriched GO terms. (D) Pathway analysis of the DEGs. The horizontal axis indicates the significance of enrichment (expressed by p.adjust; the smaller the value, the more the significance), and the vertical axis represents the enriched KEGG pathway. (E) Differential gene cluster map. Red indicates highly expressed genes, and blue indicates low expressed genes. The *x*-axis represents different samples and the *y*-axis represents the gene name. (F and G) Verification results of mRNA-seq. (H) Pearson correlation analysis of CTGF and ACTG2 mRNA expression levels. (I) The expression difference of the *ACTG2* gene in the abdominal adipose tissue of fat and lean chickens.

adipose tissue of lean-line chickens were significantly higher than those of fat-line chickens (Figure 5I, Supporting Information Figure 5). According to the above results, the mRNA expression of the *ACTG2* gene in abdominal adipose tissue of lean-line chickens was significantly higher than that of fat-line. Simultaneously, the expression of the *ACTG2* gene was positively correlated with that of the *CTGF* gene in adipose tissue. Moreover, we found that the *ACTG2* gene was significantly enriched in the actin cytoskeleton regulation pathway. Previous studies have shown that the actin cytoskeleton is a marker of adipocyte differentiation.[28] In addition, after interfering with the *CTGF* gene, the mRNA expression level of ACTG2 changed most significantly. These results suggest that ACTG2 may play an important role in adipocyte differentiation. Therefore, we conducted a follow-up study on the *ACTG2* gene.

3.5. CTGF Affects Chicken Preadipocyte Differentiation by Inducing the Expression of the *ACTG2* Gene. To further confirm that ACTG2 can be regulated by CTGF, we examined the effect of CTGF overexpression on the expression of the ACTG2 *gene.* The results showed that the overexpression of CTGF induced *ACTG2* gene expression (Figure 6A). Then we used RT-qPCR to detect the mRNA expression pattern of the *ACTG2* gene during chicken preadipocyte differentiation. The results showed that with the progression of cell differentiation, the *ACTG2* gene showed a trend of decreasing first and then increasing (Figure 6B). This result suggested that ACTG2 was likely to be involved in the regulation of preadipocyte differentiation. Therefore, we further studied the effect of ACTG2 on chicken preadipocyte differentiation. First, we used si-gga-ACTG2-1 to knockdown the ACTG2 gene in ICP2 cells and then detected the changes in cell differentiation (Figure 6C). Oil red O results showed that the cell lipid droplet deposition increased after the knockdown of ACTG2 (Figure 6D). The results of RT-qPCR showed that the expression levels of *PPARγ* and *AP2* genes were significantly increased (Figure 6E). The above results showed that the knockdown of the *ACTG2* gene could promote the differentiation of chicken preadipocytes. Therefore, we speculate that CTGF can affect the differentiation of chicken preadipocytes by affecting the

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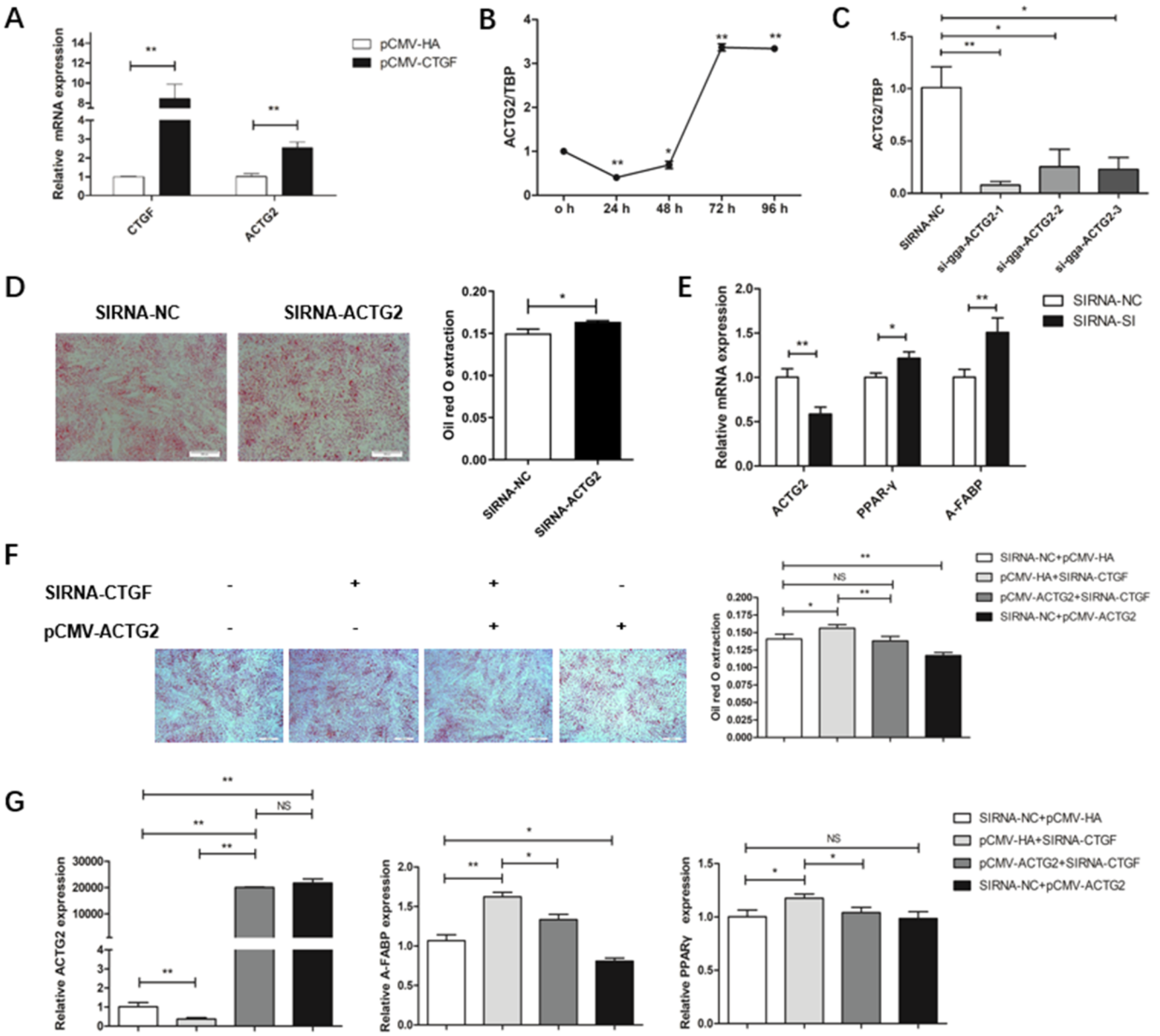

Figure 6. CTGF affects chicken preadipocyte differentiation by inducing the expression of the *ACTG2* gene. (A) The mRNA expression of the *ACTG2* gene after overexpression of CTGF. (B) The expression pattern of the *ACTG2* gene during the differentiation of chicken preadipocytes. (C) Screening of *ACTG2* gene siRNA. (D) Lipid droplet deposition after knocking down the *ACTG2* gene. (E) The effect of knocking down ACTG2 on differentiation marker genes. (F) The effect of overexpression of ACTG2 on lipid droplet deposition after knockdown of the *CTGF* gene. (G) The effect of overexpression of ACTG2 on differentiation marker genes after knockdown of the *CTGF* gene.

expression of ACTG2. We first knocked down the expression of CTGF and then overexpressed the ACTG2 gene in ICP2. Oil red O results showed that overexpression of ACTG2 attenuated the promotion of lipid droplet deposition after the knockdown of CTGF (Figure 6F). In addition, RT-qPCR results showed that the overexpression of the ACTG2 attenuated the mRNA expression of *PPARγ* and *AP2* genes after knockdown of CTGF (Figure 6G). The above results showed that overexpression of *ACTG2* gene could weaken the promoting effect of CTGF knockdown on the differentiation of chicken preadipocytes.

4. DISCUSSION

Adipose tissue is the center of fat accumulation, energy expenditure, glucose and insulin metabolism, and hormone regulation.[29] However, excessive fat deposition in chickens reduces meat quality and yield. Adipocytes are differentiated from preadipocytes and are the main components of adipose tissue.[30] Excessive fat deposition results from excessive accumulation of FC. Therefore, preadipocyte differentiation plays an important role in adipogenesis.

As a growth factor with complex functions, the role of CTGF in chicken adipogenesis has not been reported. Our study found that the mRNA and protein expression of the *CTGF* gene in abdominal adipose tissue was significantly different between fat and lean chickens, suggesting that the *CTGF* gene may be related to abdominal fat deposition in chickens. The results of previous studies showed that the expression of the *CTGF* gene in human subcutaneous adipose precursor cells is significantly higher than that in mature adipocytes.[31] Similarly, the expression of CTGF in chicken primary preadipocytes was

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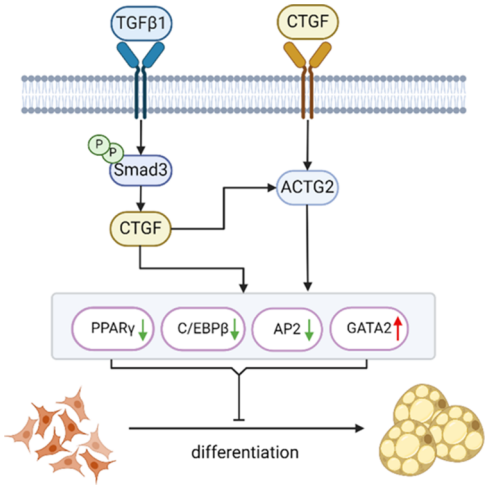

Figure 7. Model of CTGF affecting the differentiation of chicken preadipocytes.

significantly higher than that in mature adipocytes (Figure 1C,D). These results suggest that CTGF may play an important role in chicken adipogenesis. Previous studies have shown that CTGF can inhibit the differentiation of 3T3-L1 preadipocytes.[32] Similarly, the results of this study showed that CTGF could inhibit the differentiation of chicken preadipocytes.

Subsequently, we explored how CTGF inhibits preadipocyte differentiation. A large number of studies have shown that CTGF can be regulated by TGFβ/Smad3 signaling and plays a role in pulmonary and liver fibrosis.[33−35] The expression of CTGF in Smad3 knockout mice was lower than that in normal mice because TGFβ could not induce the expression of CTGF in Smad3 knockout mice.[36] In primary dermal fibroblasts, TGFβ1-induced CTGF expression was significantly inhibited by siSmad3.[24] TGFβ1 can induce human trophoblast cells to secrete CTGF protein, but this induction is significantly reduced after Smad3 knockdown.[23] Therefore, we treated ICP2 cells with the TGFβ/Smad3 signaling pathway inhibitor SIS3 and found that the induction of CTGF by TGFβ1 was significantly attenuated (Figure 3G). Through rescue experiments, it was found that the knockdown of CTGF could rescue the inhibitory effect of TGFβ1 on preadipocyte differentiation (Figure 4). These results suggested that CTGF was the downstream gene of TGFβ1, and TGFβ/Smad3/CTGF formed a signal axis to regulate preadipocyte differentiation.

In addition, the candidate gene *ACTG2* was screened by UMI RNA-seq. ACTG2 is a highly conserved smooth muscle actin, which belongs to the γ class of the actin family. Whether the structure and function of actin are normal is closely related to the health status of the growth and development of biological tissues and organs.[37] There are a few reports on the role of the *ACTG2* gene in fat. Only some actin families have been reported in adipogenesis.[38−40] Previous studies in our laboratory have found that ACTG2 was a target gene of TCF21, a negative regulator of preadipocyte differentiation.[41] Therefore, we studied the role of ACTG2 in chicken preadipocyte differentiation. This study confirmed that ACTG2 was a new regulator of preadipocyte differentiation. At present, there are a few reports on the relationship between CTGF and the *ACTG2* gene. Only a few studies have found that there is a specific binding between actin and CTGF/Hcs24 in chondrocytes isolated from chondrosarcoma.[42] In this study, we demonstrated that CTGF can induce the expression of ACTG2 in preadipocytes and then affect the differentiation of preadipocytes.

In summary, this study found CTGF as a new negative regulator of preadipocyte differentiation. Mechanistically, on the one hand, the *CTGF* gene can inhibit the differentiation of chicken preadipocytes via the TGFβ/Smad3 signaling pathway. On the other hand, it can also inhibit the differentiation of chicken preadipocytes by inducing the expression of ACTG2 (Figure 7).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c04233.

Table 1. The phenotype data for the body weight of 7-weeks-old(BW7) and adipose fat weight (AFW). Table 2. Primer sequences for RT-qPCR. Table 3. Determination of RNA concentration and purity. Figure 1. Screening of effective siRNA for CTGF. Figure 2. Quality detection of RNA. (A) RNA agarose gel electrophoresis (B) Quality control of RNA sample for sequencing. Figure 3. The correlation between CTGF mRNA expression level and Upregulated gene mRNA expression level. Figure 4. The correlation between CTGF mRNA expression level and Downregulated gene mRNA expression level. Figure 5. Differential expression of genes in adipose tissue of fat and lean chickens (PDF)

The fold change and *P* value of 95 differentially expressed genes (XLSX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Kim, M.; Voy, B. H. Fighting Fat With Fat: n-3 Polyunsaturated Fatty Acids and Adipose Deposition in Broiler Chickens. *Front. Physiol.* **2021**, *12*, No. 755317, DOI: 10.3389/fphys.2021.755317.

(2) Li, L. L.; Zhang, H. H.; Yao, Y.; Yang, Z. M.; Ma, H. T. (−)-Hydroxycitric Acid Suppresses Lipid Droplet Accumulation and Accelerates Energy Metabolism via Activation of the Adiponectin-AMPK Signaling Pathway in Broiler Chickens. *J. Agric. Food Chem.* **2019**, *67* (11), 3188−3197.

(3) Chevance, F. F. V.; Farmer, L. J.; Desmond, E. M.; Novelli, E.; Troy, D. J.; Chizzolini, R. Effect of some fat replacers on the release of volatile aroma compounds from low-fat meat products. *J. Agric. Food Chem.* **2000**, *48* (8), 3476−3484.

(4) Tong, Y.; Gao, H.; Qi, Q.; Liu, X.; Li, J.; Gao, J.; Li, P.; Wang, Y.; Du, L.; Wang, C. High fat diet, gut microbiome and gastrointestinal cancer. *Theranostics* **2021**, *11* (12), 5889−5910.

(5) Lefterova, M. I.; Lazar, M. A. New developments in adipogenesis. *Trends Endocrinol. Metab.* **2009**, *20* (3), 107−114.

(6) Darlington, G. J.; Ross, S. E.; MacDougald, O. A. The role of C/EBP genes in adipocyte differentiation. *J. Biol. Chem.* **1998**, *273* (46), 30057−30060.

(7) Lehrke, M.; Lazar, M. A. The many faces of PPARgamma. *Cell* **2005**, *123* (6), 993−999.

(8) Yu, W. H.; Chen, Z. G.; Zhang, J. L.; Zhang, L. R.; Ke, H.; Huang, L. H.; Peng, Y. W.; Zhang, X. M.; Li, S. N.; Lahn, B. T.; Xiang, A. P. Critical role of phosphoinositide 3-kinase cascade in adipogenesis of human mesenchymal stem cells. *Mol. Cell. Biochem.* **2008**, *310* (1−2), 11−18.

(9) Zhang, J. W.; Klemm, D. J.; Vinson, C.; Lane, M. D. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein β gene during adipogenesis. *J. Biol. Chem.* **2004**, *279* (6), 4471−4478.

(10) Tseng, Y. H.; Butte, A. J.; Kokkotou, E.; Yechoor, V. K.; Taniguchi, C. M.; Kriauciunas, K. M.; Cypess, A. M.; Niinobe, M.; Yoshikawa, K.; Patti, M. E.; Kahn, C. R. Prediction of preadipocyte differentiation by gene expression reveals role of insulin receptor substrates and necdin. *Nat. Cell Biol.* **2005**, *7* (6), 601−611.

(11) Fève, B. Adipogenesis:: cellular and molecular aspects. *Best Pract. Res., Clin. Endocrinol. Metab.* **2005**, *19* (4), 483−499.

(12) Karaskova, E.; Velganova-Veghova, M.; Geryk, M.; Foltenova, H.; Kucerova, V.; Karasek, D. Role of Adipose Tissue in Inflammatory Bowel Disease. *Int. J. Mol. Sci.* **2021**, *22* (8), No. 4226, DOI: 10.3390/ijms22084226.

(13) Chen, C. C.; Lau, L. F. Functions and mechanisms of action of CCN matricellular proteins. *Int. J. Biochem. Cell Biol.* **2009**, *41* (4), 771−783.

(14) Fu, M.; Peng, D.; Lan, T.; Wei, Y.; Wei, X. Multifunctional regulatory protein connective tissue growth factor (CTGF): A potential therapeutic target for diverse diseases. *Acta Pharm. Sin B* **2022**, *12* (4), 1740−1760.

(15) Yang, A. T.; Wang, P.; Tong, X. F.; Cong, M.; Liu, T. H.; Cong, R.; Wu, P.; Jia, J. D.; Wang, B. E.; You, H. Connective tissue growth factor induces hepatic progenitor cells to differentiate into hepatocytes. *Int. J. Mol. Med.* **2013**, *32* (1), 35−42.

(16) Yan, S. Y.; Zhang, M.; Yang, G. M.; Sun, Y. M.; Ai, D. M. CTGF Promotes the Osteoblast Differentiation of Human Periodontal Ligament Stem Cells by Positively Regulating BMP2/Smad Signal Transduction. *Biomed Res. Int.* **2022**, *2022*, No. 2938015, DOI: 10.1155/2022/2938015.

(17) Li, X. N.; Pongkitwitoon, S.; Lu, H. B.; Lee, C.; Gelberman, R.; Thomopoulos, S. CTGF induces tenogenic differentiation and proliferation of adipose-derived stromal cells. *J. Orthop. Res.* **2019**, *37* (3), 574−582.

(18) Arnott, J. A.; Lambi, A. G.; Mundy, C.; Hendesi, H.; Pixley, R. A.; Owen, T. A.; Safadi, F. F.; Popoff, S. N. The role of connective tissue growth factor (CTGF/CCN2) in skeletogenesis. *Crit. Rev. Eukaryotic Gene Expression* **2011**, *21* (1), 43−69.

(19) Johnson, B. G.; Ren, S.; Karaca, G.; Gomez, I. G.; Fligny, C.; Smith, B.; Ergun, A.; Locke, G.; Gao, B.; Hayes, S.; MacDonnell, S.; Duffield, J. S. Connective Tissue Growth Factor Domain 4 Amplifies

Fibrotic Kidney Disease through Activation of LDL Receptor-Related Protein 6. *J. Am. Soc. Nephrol.* **2017**, *28* (6), 1769−1782.
(20) Nishida, T.; Kubota, S.; Aoyama, E.; Janune, D.; Maeda, A.; Takigawa, M. Effect of CCN2 on FGF2-induced proliferation and MMP9 and MMP13 productions by chondrocytes. *Endocrinology* **2011**, *152* (11), 4232−4241.
(21) Abreu, J. G.; Ketpura, N. I.; Reversade, B.; De Robertis, E. M. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. *Nat. Cell Biol.* **2002**, *4* (8), 599−604.
(22) Kubota, S.; Takigawa, M. The CCN family acting throughout the body: recent research developments. *Biomol. Concepts* **2013**, *4* (5), 477−494.
(23) Cheng, J. C.; Chang, H. M.; Leung, P. C. K. TGF-β1 Inhibits Human Trophoblast Cell Invasion by Upregulating Connective Tissue Growth Factor Expression. *Endocrinology* **2017**, *158* (10), 3620−3628.
(24) Purohit, T.; Qin, Z.; Quan, C.; Lin, Z.; Quan, T. Smad3-dependent CCN2 mediates fibronectin expression in human skin dermal fibroblasts. *PLoS One* **2017**, *12* (5), No. e0173191, DOI: 10.1371/journal.pone.0173191.
(25) Zhang, H.; Shen, L. Y.; Xu, Z. C.; Kramer, L. M.; Yu, J. Q.; Zhang, X. Y.; Na, W.; Yang, L. L.; Cao, Z. P.; Luan, P.; Reecy, J. M.; Li, H. Haplotype-based genome-wide association studies for carcass and growth traits in chicken. *Poult. Sci.* **2020**, *99* (5), 2349−2361.
(26) Guo, L.; Sun, B.; Shang, Z.; Leng, L.; Wang, Y.; Wang, N.; Li, H. Comparison of adipose tissue cellularity in chicken lines divergently selected for fatness. *Poult. Sci.* **2011**, *90* (9), 2024−2034.
(27) Wang, W.; Zhang, T. M.; Wu, C. Y.; Wang, S. S.; Wang, Y. X.; Li, H.; Wang, N. Immortalization of chicken preadipocytes by retroviral transduction of chicken TERT and TR. *PLoS One* **2017**, *12* (5), No. e0177348, DOI: 10.1371/journal.pone.0177348.
(28) Cristancho, A. G.; Lazar, M. A. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat. Rev. Mol. Cell Biol.* **2011**, *12* (11), 722−734.
(29) Ferhat, M.; Funai, K.; Boudina, S. Autophagy in Adipose Tissue Physiology and Pathophysiology. *Antioxid. Redox Signaling* **2019**, *31* (6), 487−501.
(30) Tang, Q. Q.; Lane, M. D. Adipogenesis: from stem cell to adipocyte. *Annu. Rev. Biochem.* **2012**, *81*, 715−736.
(31) Yoshino, J.; Patterson, B. W.; Klein, S. Adipose Tissue CTGF Expression is Associated with Adiposity and Insulin Resistance in Humans. *Obesity* **2019**, *27* (6), 957−962.
(32) Tan, J. T. M.; McLennan, S. V.; Song, W. W.; Lo, L. W. Y.; Bonner, J. G.; Williams, P. F.; Twigg, S. M. Connective tissue growth factor inhibits adipocyte differentiation. *Am. J. Physiol. Cell Physiol.* **2008**, *295* (3), C740−C751.
(33) Ou, S. C.; Bai, K. J.; Cheng, W. H.; Chen, J. Y.; Lin, C. H.; Wen, H. C.; Chen, B. C. TGF-β Induced CTGF Expression in Human Lung Epithelial Cells through ERK, ADAM17, RSK1, and C/EBPβ Pathways. *Int. J. Mol. Sci.* **2020**, *21* (23), No. 9084, DOI: 10.3390/ijms21239084.
(34) Wei, P.; Xie, Y.; Abel, P. W.; Huang, Y. P.; Ma, Q.; Li, L. H.; Hao, J. F.; Wolff, D. W.; Wei, T. T.; Tu, Y. P. Transforming growth factor (TGF)-β1-induced miR-133a inhibits myofibroblast differentiation and pulmonary fibrosis. *Cell Death Dis.* **2019**, *10* (9), No. 670, DOI: 10.1038/s41419-019-1873-x.
(35) Li, X. J.; Liu, H.; Sun, L.; Zhou, X.; Yuan, X. K.; Chen, Y. S.; Liu, F. Y.; Liu, Y.; Xiao, L. MicroRNA-302c modulates peritoneal dialysis-associated fibrosis by targeting connective tissue growth factor. *J. Cell. Mol. Med.* **2019**, *23* (4), 2372−2383.
(36) Xu, F. Y.; Liu, C. W.; Zhou, D. D.; Zhang, L. TGF-/SMAD Pathway and Its Regulation in Hepatic Fibrosis. *J. Histochem. Cytochem.* **2016**, *64* (3), 157−167.
(37) Perrin, B. J.; Ervasti, J. M. The Actin Gene Family: Function Follows Isoform. *Cytoskeleton* **2010**, *67* (10), 630−634.
(38) Nobusue, H.; Onishi, N.; Shimizu, T.; Sugihara, E.; Oki, Y.; Sumikawa, Y.; Chiyoda, T.; Akashi, K.; Saya, H.; Kano, K. Regulation of MKL1 via actin cytoskeleton dynamics drives adipocyte differentiation. *Nat. Commun.* **2014**, *5*, No. 3368, DOI: 10.1038/ncomms4368.
(39) Yang, W. L.; Thein, S.; Wang, X. R.; Bi, X. Z.; Ericksen, R. E.; Xu, F.; Han, W. P. BSCL2/seipin regulates adipogenesis through actin cytoskeleton remodelling. *Hum. Mol. Genet.* **2014**, *23* (2), 502−513.
(40) Uetaki, M.; Onishi, N.; Oki, Y.; Shimizu, T.; Sugihara, E.; Sampetrean, O.; Watanabe, T.; Yanagi, H.; Suda, K.; Fujii, H.; Kano, K.; Saya, H.; Nobusue, H. Regulatory roles of fibronectin and integrin α5 in reorganization of the actin cytoskeleton and completion of adipogenesis. *Mol. Biol. Cell* **2022**, *33* (9), No. ar78, DOI: 10.1091/mbc.E21-12-0609.
(41) Zhang, X. Y.; Cheng, B. H.; Ma, Y. Y.; Liu, Y. M.; Wang, N.; Zhang, H.; Li, Y. M.; Wang, Y. X.; Luan, P.; Cao, Z. P.; Li, H. Genome-wide survey and functional analysis reveal TCF21 promotes chicken preadipocyte differentiation by directly upregulating HTR2A. *Biochem. Biophys. Res. Commun.* **2022**, *587*, 131−138.
(42) Yosimichi, G.; Kubota, S.; Hattori, T.; Nishida, T.; Nawachi, K.; Nakanishi, T.; Kamada, M.; Takano-Yamamoto, T.; Takigawa, M. CTGF/Hcs24 interacts with the cytoskeletal protein actin in chondrocytes. *Biochem. Biophys. Res. Commun.* **2002**, *299* (5), 755−761.