Genome-wide survey and functional analysis reveal TCF21 promotes chicken preadipocyte differentiation by directly upregulating HTR2A

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ABSTRACT

Background/aim: Previously, we showed that transcription factor 21 (TCF21) promotes chicken pre-adipocyte differentiation. However, the genome-wide TCF21 binding sites and its downstream target genes in chicken adipogenesis were unknown.
Methods: ChIP-Seq and RNA-Seq were used to screen candidate targets of TCF21. qPCR and luciferase reporter assay were applied to verify the sequencing results. Western blotting, oil red-O staining and pharmacological treatments were performed to investigate the function of 5-hydroxytryptamine receptor 2A (HTR2A), one of the bonafide direct downstream binding targets of TCF21.
Results: A total of 94 candidate target genes of TCF21 were identified. ChIP-qPCR, RT-qPCR, and luciferase reporter assay demonstrated that HTR2A is one of the bonafide direct downstream binding targets of TCF21. HTR2A expression in adipose tissue was upregulated in fat line broilers. Also, the abundance of HTR2A gradually increased during the adipogenesis process. Interestingly, pharmacological enhancement or inhibition of HTR2A promoted or attenuated the differentiation of preadipocytes, respectively. Furthermore, HTR2A inhibition impaired the TCF21 promoted adipogenesis.
Conclusions: We profiled the genome-wide TCF21 binding sites in chicken differentiated preadipocytes revealing HTR2A as the direct downstream target of TCF21 in adipogenesis.

1. Introduction

Obesity, a great burden to mankind's health, is closely related to many metabolic diseases, such as hypertension, hyperlipidemia, and coronary heart disease [1]. In many studies, chicken has been used as an animal model to investigate the biology of adipose tissue, metabolism, and obesity-related issues since its metabolic peculiarities and approximately 70% genetic makeup is homologous to humans [2,3]. Besides, continued selection for the broiler's productive performance has resulted in excessive fat deposition causing economic and environmental problems, such as reduced feed utilization, increased incidence of physiological disorders, difficult processing, and environmental pollution [4–6]. Therefore, investigating the biomolecular mechanisms underlying the chicken adipose tissue deposition will not only help in reducing fat deposition in the broiler industry but also bring new insights into human obesity and related diseases.

The adipose tissue fat deposition is closely correlated with adipocyte differentiation involving a cascade of transcription factors regulated gene expressions [7]. Previously, we showed that

transcription factor 21 (TCF21) was involved in promoting chicken preadipocyte differentiation [8]. Notably, TCF21 is known to regulate the transcription of various target genes in distinct cell types and biological processes [9,10]. However, to the best of our knowledge, the genome-wide TCF21 binding sites and the downstream target genes of TCF21 in adipogenesis are uncertain in both chicken and mammals.

In this study, we used ChIP-Seq and RNA-Seq tools to identify the genome-wide TCF21 binding sites and its potential targets in differentiated chicken preadipocytes. A total of 94 candidate target genes were found. Furthermore, we performed ChIP-qPCR, RT-qPCR, and luciferase reporter assay to find that 5-hydroxytryptamine receptor 2A (HTR2A) was one of the direct downstream targets of TCF21. Moreover, HTR2A involvement in TCF21 promoted adipogenesis was validated using the functional analysis, and rescue studies.

2. Materials and methods

2.1. Ethics statement

The care and use of broilers were conducted following the guidelines published by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). Also, all the experiments involving broilers were approved by the Laboratory Animal Management Committee of the Northeast Agricultural University (Harbin, China).

2.2. Animals and tissue collection

Broiler chickens, 23rd generation (G_{23}), were obtained from the Northeast Agricultural University broiler lines which were divergently selected for abdominal fat content (NEAUHLF). The detailed selection procedure was as described by Guo et al. [11]. At 7 weeks of age, 8 male broilers (4 per line) were sacrificed to isolate abdominal fat (AF), crop fat (CF), gizzard fat (GF), and subcutaneous fat (SF). These were washed and snap-frozen with liquid nitrogen before storage at $-80\ °C$ for further use.

2.3. Cell fraction preparation and cell culture

Chicken stromal-vascular fractions (SVF) and fat cells (FC) were obtained from AF of 10-days-old Ross 308 broilers according to Zhang et al. [12]. Immortalized chicken preadipocytes (ICP) and the preadipocyte cell line stably overexpressing TCF21 (LV-TCF21) were previously developed in our laboratory [5,8]. The culture and induced differentiation methods of chicken preadipocytes refer to our previous study [8].

2.4. Lipid staining and measurement of lipid droplet accumulation

Lipid droplets were stained with oil red O refer to our previous study [8]. Lipid droplet accumulation was measured by Oil red O extraction assay [8]. The lipid droplet accumulation was presented as a ratio of OD_{510}/mg protein.

2.5. qPCR

Cells were washed thrice with PBS. Then, to extract the cellular RNA by TRIzol (Invitrogen, CA, USA). The method of reverse transcription and qPCR refer to our previous study [8]. Samples were analyzed in triplicates and TATA-box binding protein (*TBP*) was used for normalization. The relative gene expression was quantified by the $2^{-\triangle CT}$ method [13]. Primer sequences are listed in Additional file 1: Table S1.

2.6. ChIP-Seq and RNA-Seq

ChIP-Seq and RNA-Seq services were provided by SeqHealth (Wuhan, China). ChIP assay, in triplicate, was performed as detailed in Zhang et al. [8]. The obtained ChIP DNA libraries were sequenced on a Hiseq X Ten system (Illumina, CA, USA). After quality control and data filtering, reads were mapped to the chicken genome (Gallus_gallus 5.0). MACS2 software was used for peak calling with control inputs. Already known motifs and *de novo* motifs were identified by HOMER software. The ChIP-Seq results were validated by ChIP-qPCR and the results are presented as % of input. The primers used in ChIP-qPCR are listed in Additional file 1: Table S2.

For RNA-Seq, total RNA of the LV-TCF21 (N = 3) and LV-control (N = 3) preadipocytes cultured in differentiation media for 24 h was extracted using TRIzol (Invitrogen, CA, USA). This was used to prepare the mRNA sequencing library which was sequenced on a Hiseq X Ten system (Illumina, CA, USA). After quality control and data filtering, reads were mapped to the chicken genome (Gallus_gallus 5.0). Significant differences in gene expressions were filtered with criterion P-value < 0.05 and Fold change >2.

2.7. Luciferase reporter assay

The effect of over-expressed TCF21 on the promoter activities of candidate target genes identified by ChIP and RNA-Seq were investigated by luciferase reporter assay. For that, promoter-reporter gene vectors with wild or mutated TCF21 binding sites of the candidate target genes were constructed by GENEWIZ (Suzhou, China). These vectors were co-transfected with pRL-TK Renilla luciferase vector into LV-control and LV-TCF21 preadipocytes which were plated in 24 well plates. After 48 h of transfection, the luciferase activities were detected by the Dual-Luciferase Assay System (Promega, WI, USA).

2.8. Western blotting

Whole-cell extracts were prepared from SVF, FC, and ICP preadipocytes by homogenization in RIPA buffer (Santa Cruz, sc-364162) with PMSF (Beyotime, ST506). The sample protein concentration was determined by the BCA protein assay (Thermo Fisher, 23227). An equal amount of respective protein samples were resolved with SDS-PAGE and then transferred onto the nitrocellulose membranes. Then, the membranes, blocked with 5% (wt/vol) BSA in TBST, were overnight incubated at 4 °C with corresponding specific primary antibodies, anti-HTR2A, 1:100, Santa Cruz, sc-166775; anti-β-actin, 1:1000, Beyotime, AA128. This was followed by the incubation with goat anti-mouse IgG, 1:5000, ZSGB-BIO, ZB-2305. Lastly, the protein bands were visualized using the BeyoECL Plus kit (Beyotime, P0018S) and ImageQuant LAS 500 system (GE, NJ, USA). The greyscale quantitative analysis of the band intensities was performed using the Image J 1.46r software (NIH, Bethesda, MD, USA).

2.9. Preadipocyte treatment with HTR2A agonist and antagonist

At 50% confluency, ICP cells were treated with fresh differentiation media containing distinct concentrations (0, 5, 50, or 100 μM) of HTR2A agonist (DOI) or antagonist (Ketanserin) [14] for 24 h. Likewise, for the rescue experiment, LV-control, and LV-TCF21 preadipocytes were treated with fresh differentiation media containing 100 μM DOI or Ketanserin for 24 h at 50% confluency.

Availability of data and materials

ChIP-Seq data has been deposited in GEO database with the accession ID GSE179259. RNA-Seq data has been deposited in NCBI

database with the accession ID PRJNA740053. All data generated or analyzed during this study are included in this manuscript and its supplementary information files.

2.10. Statistical analysis

All data were shown as mean ± SEM. Comparisons between the two groups were calculated using Student's t-tests. Comparison among multiple groups were analyzed by Turkey's HSD test. All analyses were performed with JMP v11.0 (SAS Institute Inc., NC, USA). P < 0.05 or P < 0.01 denote the statistical significance of the data.

3. Results

3.1. Genome-wide binding sites of TCF21 in differentiated chicken preadipocytes

To identify the direct target genes of TCF21, ChIP-Seq was performed with LV-TCF21 preadipocytes after inducing differentiation for 24 h. A total of common 37420 peaks (Fig. S1A) were obtained, of which 48.09% were located in the intergenic regions (Fig. S1B). Notably, CAGCTG, a known TCF21 binding site, was the most significant enriched motif (Fig. S1C). For the subsequent analysis, we selected a total of 1879 genes with peak located in the promoter region (3000 bp upstream of transcriptional start site).

3.2. Identification of genes regulated by TCF21

To examine the TCF21 mediated gene regulation in adipogenesis, we employed RNA-Seq for LV-control and LV-TCF21

preadipocytes in which differentiation was induced for 24 h. We identified a total of 1312 differentially expressed genes (DEGs), including 848 upregulated and 464 downregulated genes (Fig. S2). Finally, 720 DEGs were used for subsequent analysis, while the remaining 592 DEGs were not considered because of their extremely low expression levels (RPKM <1).

3.3. Integrative analysis and validation of ChIP-Seq and RNA-Seq

To further examine the candidate target genes of TCF21, we intersected these 1879 genes with peaks located in the promoter region with the 720 DEGs. We found a total of 94 candidate TCF21 target genes at the intersection. After eliminating the previously studied A-FABP and LPL [8], and 15 other unannotated transcripts, 77 candidate TCF21 target genes were validated by ChIP-qPCR, RT-qPCR, and luciferase reporter assays.

The workflow of candidate target gene selection and verification is depicted in Fig. S3. Firstly, based on criterion fold enrichment >5 [15], a total of 52 candidate target genes were verified (Fig. S4). Next, we performed RT-qPCR to validate the RNA-Seq results. We found that the RT-qPCR results of 32 genes were completely consistent with the sequencing results (Fig. S5). Among these, the expression levels of chemerin chemokine-like receptor 1 (CMKLR1), fasciculation and elongation protein zeta 1 (FEZ1) and runt-related transcription factor 1 (RUNX1) were too low as revealed by RT-qPCR (Fig. S5). Therefore, these genes were not selected for luciferase reporter assays.

To further verify the TCF21 mediated transcriptional regulation of the candidate target genes, we cloned the promoter sequences of 29 candidate target genes harboring the wild-type or mutated TCF21 binding motifs up-stream of a luciferase reporter gene into
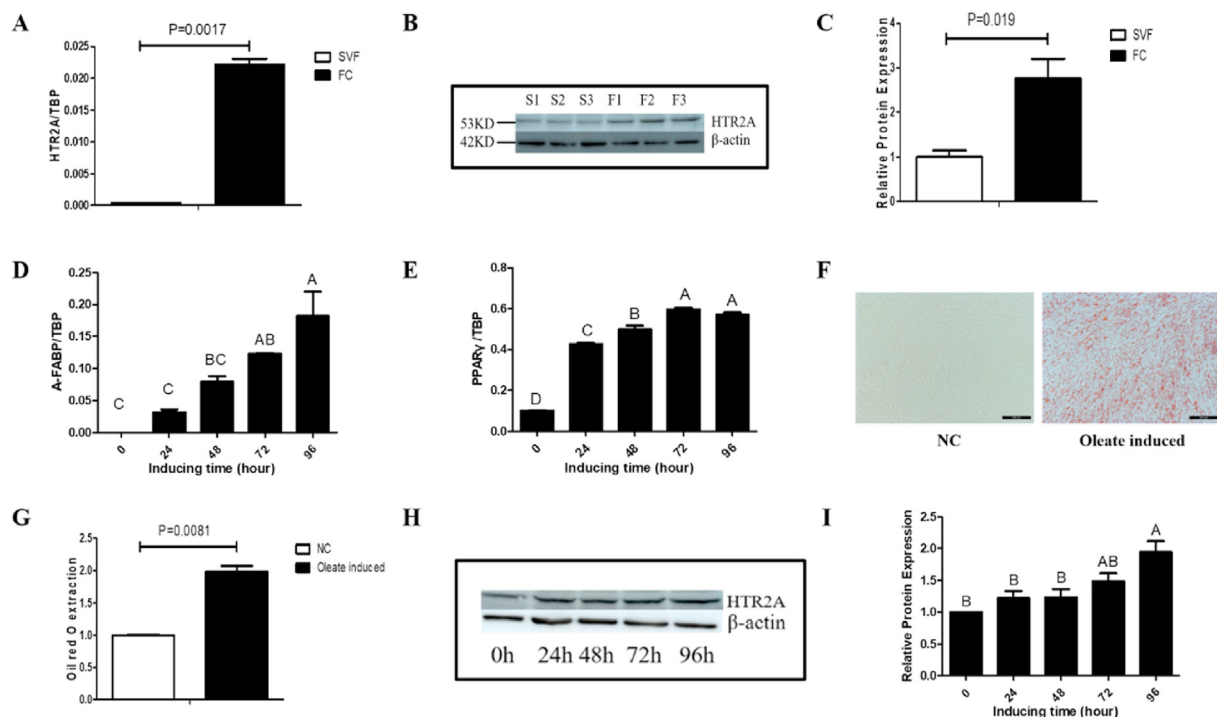

Fig. 1. HTR2A expression increases during chicken preadipocyte differentiation. (A) The mRNA levels of HTR2A in chicken preadipocytes (SVF) and mature adipocytes (FC) were assessed by RT-qPCR; (B) HTR2A protein levels in SVF and FC were measured by western blotting; (C) The band intensities were quantified by Image J software; (D-E) The mRNA levles of A-FABP and PPARγ were assessed by RT-qPCR; (F-G) Lipid droplet accumulation was investigated by oil red-O staining and extraction. Scale bar: 200 μm; (H) HTR2A protein expression patterns during preadipocyte differentiation were determined by western blotting; (I) The band intensities were quantified by Image J software. Graphs are plotted as mean ± SEM from three independent experiments. The uppercase letters above respective columns denote the significance of the differences (P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the pGL3-basic vector. Luciferase reporter assays showed that TCF21 over-expression significantly increased the wild-type promoter activities of 28 candidate target genes (Fig. S6). However, when the TCF21 binding site was mutated, the promoter activities of the 13 genes decreased significantly. These genes included mitochondrial ribosomal protein L33 (MRPL33), regulator of calcineurin (RCAN1), ADAM metallopeptidase with thrombospondin type 1 motif 2 (ADAMTS2), integrin subunit alpha 4 (ITGA4), transient receptor potential cation channel subfamily C member 6 (TRPC6), FYVE, RhoGEF and PH domain containing 3 (FGD3), HMG-box containing (BBX), melanogenesis associated transcription factor (MITF), rhomboid 5 homolog 1 (RHBDF1), HTR2A, adenosine deaminase (ADA), sequestosome 1 (SQSTM1) and interferon regulatory factor 1 (IRF1) (Fig. S7). Notably, RHBDF1, ADA, SQSTM1, and IRF1 showed conflicting results between RT-qPCR (Fig. S5) and luciferase reporter assay (Fig. S6) and therefore were not considered for further study.

Next, 9 genes, including MRPL33, HTR2A, RCAN1, ADAMTS2, TRPC6, ITGA4, FGD3, BBX, and MITF, were identified as *bonafide* targets of TCF21 in differentiated preadipocytes. Interestingly, very recently in 2020, Shong et al. [16] reported that adipose tissue-specific HTR2A KO mice fed a high-fat diet showed reduced lipid accumulation in white adipose tissue and resistance to obestiy. However, the role of HTR2A in chicken has not been investigated so far. Thus, HTR2A was selected for the subsequent studies.

3.4. HTR2A is a positive regulator of chicken preadipocyte differentiation

Firstly, we determined the mRNA levels of *HTR2A* in the adipose tissues (AF, CF, GF, and SF) of NEAUHLF individuals, in which AFP of fat birds is 8.8 times more than the lean ones (Fig. S8A). We found that HTR2A was expressed in multiple chicken adipose tissues with the highest and lowest levels in SF and AF, respectively. Also, among
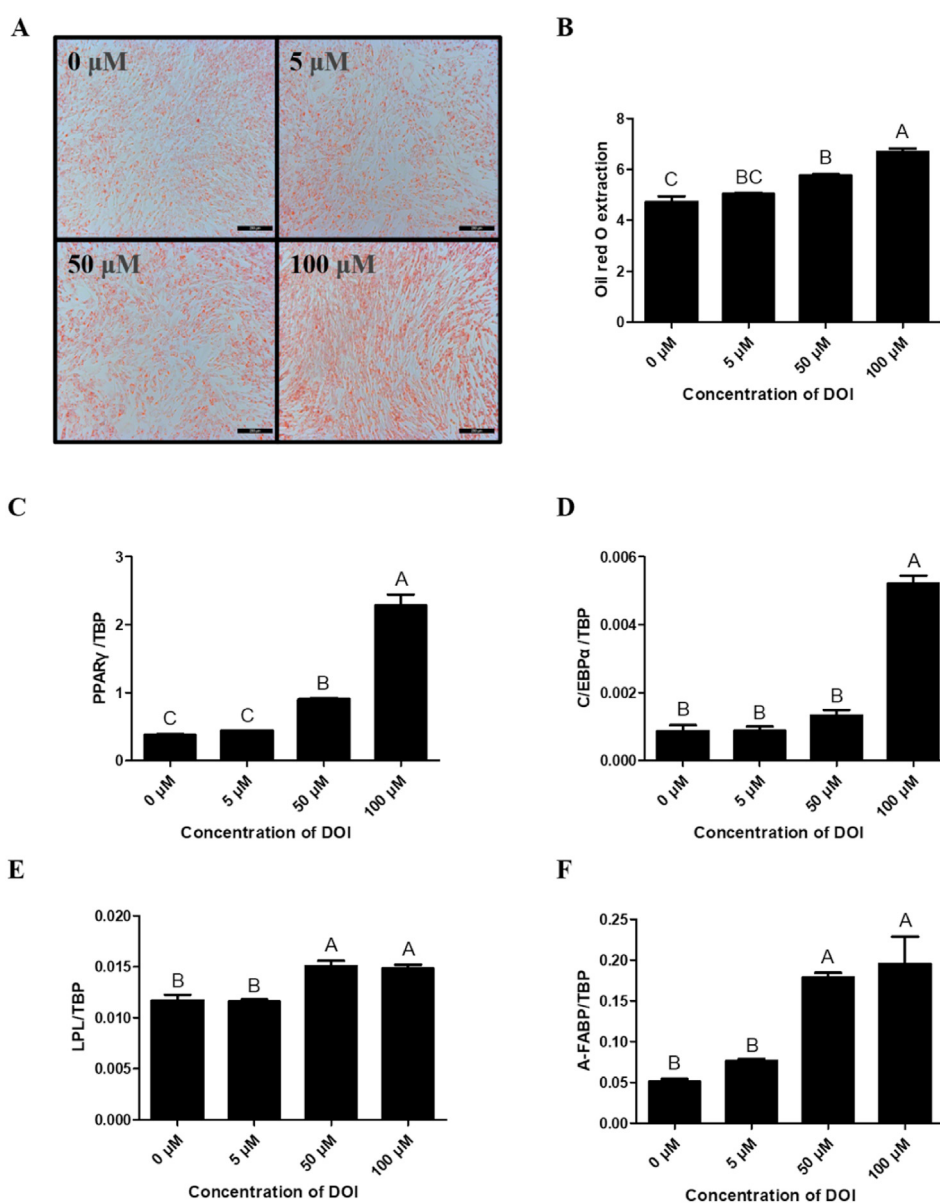

Fig. 2. HTR2A agonist DOI promotes preadipocyte differentiation. ICP preadipocytes were treated with different concentrations of DOI. (A-B) The accumulation of lipid droplets was assessed by oil red-O staining and extraction. Scale bar: 200 μm; (C−F) The expression of PPARγ, C/EBPα, LPL and A-FABP were determined by RT-qPCR. Graphs are plotted as mean ± SEM from three independent experiments. The uppercase letters above columns denote the statistical significance (P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
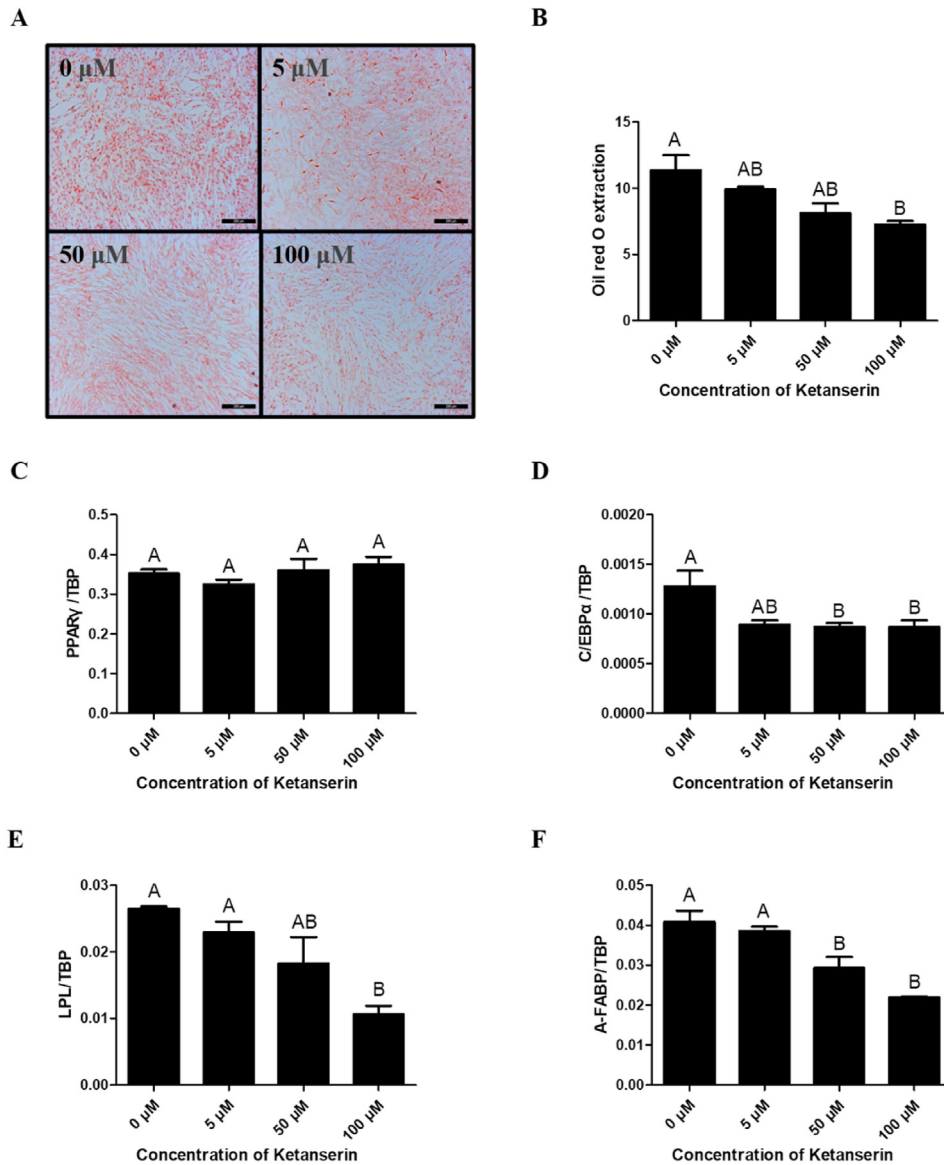
Fig. 3. HTR2A antagonist Ketanserin inhibits preadipocyte differentiation. ICP preadipocytes were treated with different concentrations of Ketanserin. (A-B) The accumulation of lipid droplets was assessed using the oil red-O staining and extraction. Scale bar: 200 μm; (C—F) The expression of PPARγ, C/EBPα, LPL and A-FABP were determined by RT-qPCR. Graphs are plotted as mean ± SEM from three independent experiments. The uppercase letters above columns denote the significance of differences (P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

all these detected adipose tissues, fat birds showed higher levels of HTR2A than the lean ones, especially with significant differences in AF and GF (Fig. S8B). Furthermore, HTR2A protein levels in AF of fat birds were higher than lean birds (Figs. S8C—D). Overall, these results suggest that HTR2A may be involved in the growth and development of fat tissues.

Next, we examined the HTR2A expression during preadipocyte differentiation. Importantly, the abundance of HTR2A was remarkably elevated in FC than in SVF *in vivo* (Fig. 1A—C). *In vitro* analysis for adipocyte fatty acid-binding protein (*A-FABP*) mRNA expression (Fig. 1D), peroxisome proliferator-activated receptor-gamma (*PPARγ*) mRNA expression (Fig. 1E), and oil red-O staining and extraction (Fig. 1F and G) revealed that ICP preadipocytes were well-differentiated. Fascinatingly, HTR2A protein levels gradually increased with the progress of differentiation (Fig. 1H and I), suggesting its regulatory role in adipogenesis.

To validate the above said results, ICP preadipocytes, treated with an HTR2A agonist DOI or HTR2A antagonist Ketanserin, were

assessed for adipogenic genes expression and lipid accumulation. We found that both DOI and Ketanserin treatment affected the lipid droplets deposition in a dose-dependent manner. DOI or Ketanserin significantly increased or decreased the accumulation of lipid droplets in the final concentration of 50 and 100 μM, respectively (Fig. 2A and B and Fig. 3A and B). Besides, administration of DOI and Ketanserin influenced the expressions of pro-adipogenic genes, including *PPARγ*, CCAAT/enhancer-binding protein alpha (*C/EBPα*), lipoprotein lipase (*LPL*), and A-FABP. (Fig. 2C—F and Fig. 3C—F). These findings indicate that HTR2A is a positive regulator of chicken adipogenesis.

3.5. TCF21 promotes adipogenesis by targeting HTR2A

Finally, we performed a rescue experiment to explore whether TCF21 regulated preadipocyte differentiation through HTR2A. The inhibition of HTR2A activity attenuated the promoting effects of overexpression of TCF21 on chicken preadipocytes differentiation.
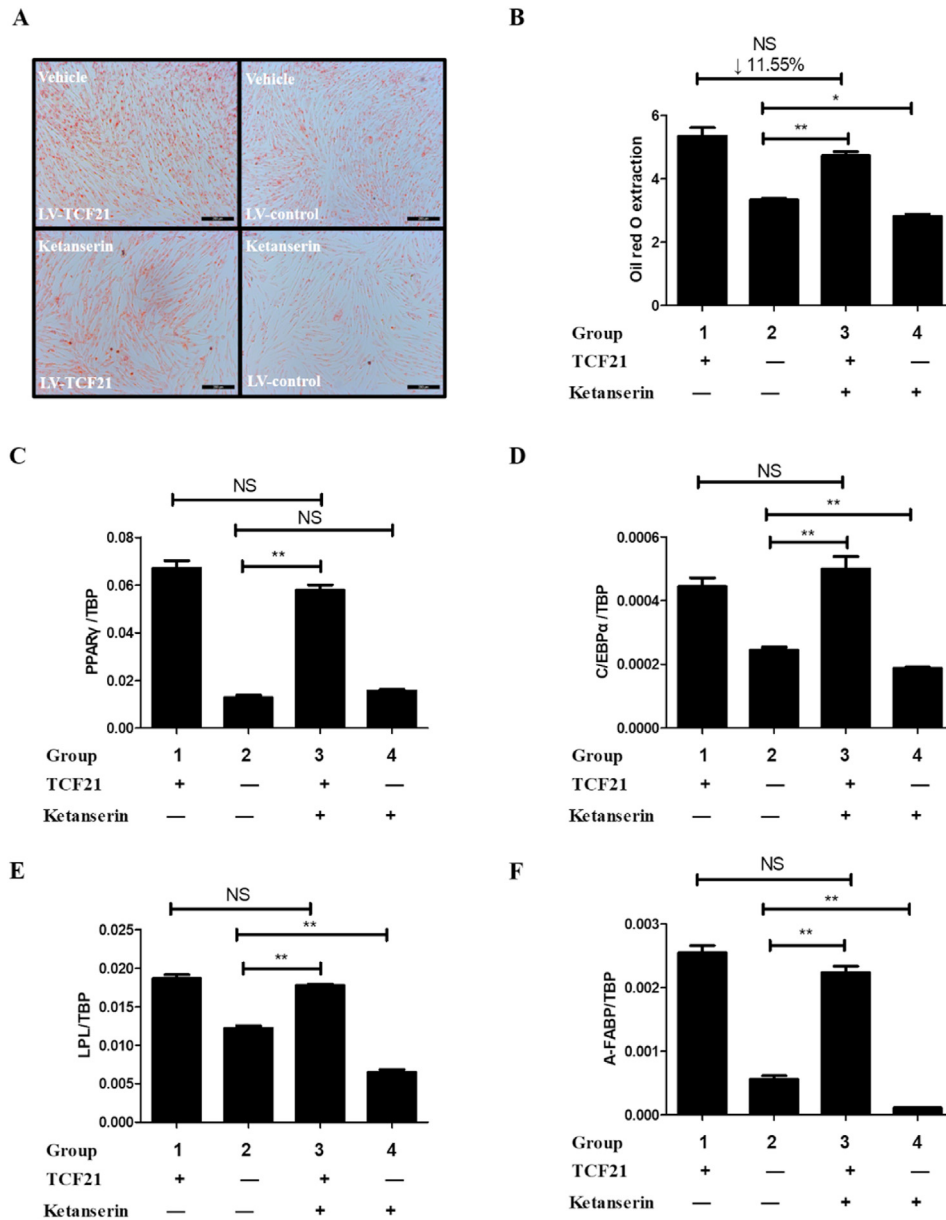
Fig. 4. TCF21 promotes adipogenesis by targeting HTR2A. LV-control and LV-TCF21 preadipocytes were treated with 0 or 100 μM Ketanserin. (A-B) The lipid droplet accumulation was assessed by oil red-O staining and extraction. Scale bar: 200 μm; (C−F) The expression of PPARγ, C/EBPα, LPL, and A-FABP was estimated by RT-qPCR. Graphs are plotted as mean ± SEM from three independent experiments. NS, no significance, *P < 0.05, **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

After the TCF21 overexpression preadipocytes were treated with Ketanserin, lipid accumualtion and the expression levels of *PPARγ*, *LPL* and *A-FABP* decreased, but the inhibition of HTR2A could not completely neutralize the differentiation promoting effect of over-expressed TCF21 (Fig. 4). These results indicated that TCF21 pro-motes adipogenesis, at least in part, by targeting HTR2A.

4. Discussion

Recently, our group showed that over-expression of TCF21 ele-vates the accumulation of lipid droplets and the expression of several pro-adipogenetic genes. Additionally, using the ChIP-PCR and luciferase reporter assays, we found that LPL was a target of TCF21 in chicken adipogenesis [8]. To further unravel the

transcriptional regulatory mechanisms of TCF21, here we inte-grated ChIP-Seq with RNA-seq to identify the genome-wide downstream targets of TCF21 in chicken adipogenesis.

Firstly, we identified the TCF21 interacting genes using ChIP-Seq. Notably, the successful ChIP-Seq largely depends on the correct se-lection of antibodies [17−19]. In lack of commercial ChIP-grade anti-bodies against the chicken TCF21, we first tried IP-grade antibodies against human TCF21. However, western blotting revealed that the antibodies failed to recognize the chicken TCF21 (data not shown). Therefore, in this study, we used the ChIP-grade anti-HA tag anti-bodies to recognize the HA-TCF21 fusion protein in chicken pre-adipocytes. Additionally, considering that different transfection efficiencies in ICP preadipocytes may lead to poor reproducibility in ChIP, we used a preadipocyte cell line stably expressing HA-TCF21 [8].

Using ChIP-Seq, we found a total of common 37420 peaks in three independent samples. Notably, most of the peaks were located in the intergenic region (Fig. S1B). Recently, multiple studies reported that lincRNAs located in the intergenic region could be involved in transcription-mediated regulation of various biological processes [20—22]. This suggests that apart from protein-coding genes, lincRNAs may also be the direct transcriptional targets of TCF21 in chicken adipocytes, and must be explored in the future. Furthermore, we found that the canonical TCF21 binding site (CAGCTG) [10] was the most enriched motif in our study (Fig. S1C). Additionally, the other identified binding motifs (Fig. S1C) suggest that TCF21 may interact with distinct transcription factors while the potential regulation mediated by the novel non-canonical binding motifs needs further study. Here, we focused on the 94 candidate target genes with peaks located in the promoter region (3000 bp upstream of 5′UTR) and showed significant differential expressions in RNA-Seq between the LV-control and LV-TCF21 preadipocytes.

Next, the sequencing results were validated by ChIP-qPCR, normal RT-qPCR, and luciferase reporter assays. From the ChIP-qPCR experiment, we got 52 candidate genes. Notably, while designing the primers for ChIP-qPCR, we found that there were no canonical TCF21 binding motifs in the peaks located in the promoters of 18 candidate target genes (Fig. S3), which may also be caused by the interaction of TCF21 with other transcription factors or novel non-canonical TCF21 binding sites discussed above. Furthermore, from normal RT-qPCR analysis, we got 32 candidate genes that showed consistent expression patterns with the RNA-Seq data. Lastly, luciferase reporter assays confirmed the binding and the transcriptional regulation between TCF21 and candidate genes. Interestingly, the luciferase reporter assay results of 8 genes (Fig. S6) did not correspond to their mRNA levels (Fig. S5). Since only a part of promoter sequences was amplified for constructing the promoter-reporter gene vectors, we believe that the conflict between the two experiments could have resulted from the exclusion of some other important regulatory elements in the amplified products. Taken together, MRPL33, MITF, RCAN1, ADAMTS2, TRPC6, ITGA4, FGD3, BBX, and HTR2A were identified as the *bonafide* target genes of TCF21 in chicken preadipocytes.

Based on previous literature, we selected HTR2A for further study. HTR2A is one of the serotonin receptors that has multiple biological functions regulating temperature, mood, sleep, pain, or energy balance [23—25].Notably, in humans, HTR2A polymorphisms has been correlated with obesity [26]. Also, HTR2A is reported to promote adipogenesis in mice [14] and bovine [27]. Very recently in 2020, it was reported that adipose tissue-specific HTR2A KO mice fed an HFD showed a reduction in adipose tissue fat deposition [16]. However, the role of HTR2A in fat deposition was not explored yet in chicken.

Accordingly, we first estimated the HTR2A expression levels in various adipose tissues of lean and fat line chickens. We found that compared to lean birds, HTR2A expressions were higher in all detected adipose tissues of the fat birds (Fig. S8B). This suggests that HTR2A is closely related to fat deposition in chickens. Since adipose tissues are mainly composed of adipocytes, we next detected the cellular levels of HTR2A expression and found a markedly higher abundance of HTR2A in FC, compared to SVF (Fig. 1A—C). Moreover, the HTR2A expression levels were positively correlated with the progress of differentiation (Fig. 1H and I), indicating its involvement in chicken adipogenesis.

To test our hypothesis, we used an agonist (DOI) and antagonist (Ketanserin) of HTR2A in the subsequent studies. We observed that cells treated with DOI showed increased lipid accumulation (Fig. 2A and B) while the cells treated with Ketanserin showed the opposite (Fig. 3A and B). These findings are in agreement with the similar findings of a mice study [14]. Additionally, the pro-adipogenic genes, such as *C/EBPα*, *LPL*, and *A-FABP* were either upregulated or downregulated after DOI (Fig. 2D—F) and Ketanserin administration, respectively (Fig. 3D—F). All these results demonstrate that HTR2A promotes chicken adipogenesis.

To investigate whether TCF21 regulates chicken adipogenesis through HTR2A, we performed a rescue experiment. We found that Ketanserin mediated inhibition of HTR2A impaired but not completely offset the adipogenesis promoting effect of over-expressed TCF21 (Fig. 4), suggesting that TCF21 promotes chicken adipogenesis at least in part via HTR2A.

In conclusion, for the first time, we identified MRPL33, MITF, RCAN1, ADAMTS2, TRPC6, ITGA4, FGD3, BBX, and HTR2A as the *bonafide* targets of TCF21 in differentiated chicken preadipocytes. Furthermore, we verified that HTR2A could partially mediated promotive effect of TCF21 in chicken adipogenesis. These findings improve our knowledge of TCF21 related transcriptional regulatory network in adipogenesis and may provide novel insights into fat deposition in both chicken and human.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.11.103.

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