GPNMB promotes abdominal fat deposition in chickens: genetic variation, expressional profile, biological function, and transcriptional regulation

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ABSTRACT Glycoprotein nonmetastatic melanoma protein B (**GPNMB**) is a vital secreted factor that promotes the occurrence of obesity in mammals. However, the effects of GPNMB on abdominal fat deposition is still unknown in chickens. In this study, we looked into the genetic and expression association of *GPNMB* gene with abdominal fat traits in chicken, and found that a genetic variation rs31126482 in *GPNMB* promoter was significantly associated with abdominal fat weight (**AFW**, $P <$ 0.05) and abdominal fat percentage (**AFP**, $P < 0.01$). Express profile analysis of the *GPNMB* indicated that the gene was mainly expressed in abdominal fat tissue, and its expression level was strongly positively correlated with AFW ($R^2 = 0.6356$, $P = 4.10E{-}05$) and AFP ($R^2 = 0.6450$, $P = 2.90E{-}05$). We then investigated biological function of GPNMB on adipogenesis in chicken, and found that GPNMB could inhibit abdominal preadipocyte proliferation, but promote abdominal preadipocyte differentiation and lipid deposition. Furthermore, we explored regulatory mechanism of *GPNMB* gene in chicken, and detected one nonclassical estrogen regulatory element (**AP1**) and one peroxisome proliferator-activated receptor α (**PPARα**) responsive element in the 2 kb promoter region of *GPNMB* gene, and demonstrated that estrogen could up-regulate *GPNMB* mRNA expression in adipose tissue and primary abdominal preadipocytes, while PPARα could down-regulate *GPNMB* expression in primary preadipocytes. Taken together, this study brings new insights into understanding the function and transcriptional control of *GPNMB* gene, and provides genetic markers for breeding selection to improve abdominal fat traits in chicken.

Key words: chicken, GPNMB, abdominal fat deposition, expression regulation

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INTRODUCTION

Most of the Chinese indigenous chicken breeds show excellent meat and egg quality and are favored by local consumers. However, the native chickens often have excessive abdominal fat deposition properties, especially in late stage of growth. Excessive abdominal fat deposition generally accompanies with low immune response (Guo et al., 2021), poor feed conversion rate (Moreira et al., 2018), reduced carcass quality and reproductive performance (Zhang et al., 2018), undesirable production efficiency, and leading to restricted development of native chicken industry. Therefore, controlling the excessive abdominal fat deposition is an important long-term goal of native chicken breeding. Over the past several decades, though a number of candidate genes and genetic variations that could potentially affect fat deposition have been identified by different genetic analysis approaches in various chicken populations (Jin et al., 2017; Chen et al., 2019; Mu et al., 2019; Na et al., 2019; Xing et al., 2020; Zhou et al., 2020; Wang et al., 2021a; Wang et al., 2021b; Xing et al., 2021; Yuan and Lu, 2021), the mechanisms underlying the abdominal fat deposition still remain poorly understood in chickens.

Glycoprotein nonmetastatic melanoma protein B (**GPNMB**) is a type I transmembrane glycoprotein released into blood circulation to exert its function under the hydrolysis of the integrin metalloproteinase 10 (**ADAM10**) (Rose et al., 2010). It has been revealed that GPNMB plays important roles in many biological processes, such as melanin deposition (Zhang et al., 2013), bone mineral deposition (Frara et al., 2016), regulation of inflammation (Neal et al., 2018), induction of

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tumor growth and invasion (Maric et al., 2019). Meanwhile, accumulating evidences have demonstrated that GPNMB also involved in lipid metabolism (Gabriel et al., 2014; Choi et al., 2015; Gong et al., 2019). It was reported that the expression level of *GPNMB* gene in white adipose tissue and concentration of GPNMB protein in serum were higher in diet-induced obesity mice than those in the normal control group (Choi et al., 2015), and serum GPNMB level was positively correlated with body mass index (Gabriel et al., 2014), suggesting a potential role of GPNMB in obesity. Recent study showed that the ectodomain of GPNMB protein could promote lipid synthesis of primary adipocytes through the CD44 molecule (**CD44**)−AKT serine/threonine kinase 1 (**AKT**)−sterol regulatory element binding transcription factor 1 (**SREBP1c**) pathway, and the liver-specific overexpression of GPNMB in mouse significantly increased the weight of white adipose and enlarged the area of adipocytes (Gong et al., 2019), indicating that GPNMB might promote obesity by increasing lipid accumulation in white adipose tissue.

However, in poultry, the functions of GPNMB on lipid metabolism have been rarely reported. Our previous work found that the expression level of *GPNMB* gene was highly significantly up-regulated in the mature adipocytes in comparison to that in preadipocytes of Gushi chickens (Zhang et al., 2019). Therefore, we hypothesized that *GPNMB* might also exert important functions during abdominal fat deposition in Chinese indigenous chickens. In this study, we systematically investigated genetic and expressional association of *GPNMB* with chicken abdominal fat traits, explored its biological role in abdominal preadipocyte proliferation and adipocyte differentiation via the gain or loss of function strategy, analyzed the regulatory mechanism of *GPNMB* gene expression in vivo and vitro. Our findings bring new insight into understanding the function and transcriptional control of *GPNMB* in adipogenesis, and facilitate the marker-assisted selection for improvement of abdominal fat traits in chicken.

MATERIALS AND METHODS

Ethics Statement

All animal experiments and sample collection were performed in accordance with the guidelines of Institutional Animal Care and Use Committee, Permit No.11-0085, Henan Agricultural University.

Animals and Sample Preparation

A total of 439 birds from the Gushi-Anka F2 chicken resource population of Henan Innovative Engineering Research Center of Poultry Germplasm Resource were used for association analysis between genotype and abdominal fat traits. The information of Gushi-Anka F2 chicken resource population were described previously (Han et al., 2010). All birds were humanely slaughtered at 12 wk, and the phenotypes used in this study including abdominal fat weight (**AFW**) and abdominal fat percentage (**AFP**, abdominal fat weight/body weight).

A total of 89 female Gushi chickens at 43 wk old from Poultry Germplasm Resource farm of Henan Agricultural University were used to validate the association of single nucleotide polymorphism (**SNP**s) with abdominal fat traits and analyze *GPNMB* expression characteristics. All birds were raised in the similar environmental conditions with free water and a commercial diet, which contained 2,650 kcal kg^{-1} metabolic energy and 16% crude protein after 35 wk of age. They were humanely slaughtered, blood samples were taken, abdominal fat traits including AFW and AFP were measured, and 10 tissues including abdominal fat, pancreas, subcutaneous fat, duodenum, ovary, jejunum, liver, shell gland, pectoralis, and kidney were immediately collected, snap-frozen in liquid nitrogen, and stored at −80°C until use.

To investigate the effect of estrogen on *GPNMB* expression, a total of 24 pullets at the age of 10 wk old were randomly divided into 3 groups with 8 birds every group. According to our previously optimized experimental protocol (Li et al., 2020; Ren et al., 2021), the birds in the first 2 groups were intramuscularly injected with 0.5 and 1.0 mg/kg of body weight of 17 β-estradiol (Sigma, St. Louis, MO) which was dissolved in olive oil, respectively. The third group was served as a control, only injected with the same amount of olive oil. The abdominal fat tissues were collected and stored as mentioned above after injection for 12 h.

Association Study Between SNPs in the Promoter Region of GPNMB and Abdominal Fat Traits

To explore the effect of genetic variations in the promoter region of *GPNMB* gene on abdominal fat traits, the SNPs in promoter region of *GPNMB* gene were detected based on double-digest genotyping-by-sequencing (**ddGBS**) in Gushi-Anka F2 chicken population (Zhang et al., 2021) and genetic variant table in Ensembl database (https://asia.ensembl.org/). All genetic variant information was obtained based on chicken reference genome Ensembl Release 105. A total of 6 SNPs were screened and used for linkage disequilibrium (**LD**) analysis. The genotypes of the 6 SNPs in the 439 individuals of Gushi-Anka F2 resource population were done by using Kompetitive Allele Specific PCR (**KASP**) technique (He et al., 2014). The SNPs that were significantly associated with AFW and AFP in the Gushi-Anka F2 resource population were further used to perform the association analysis with the 89 Gushi chickens as mentioned above.

Plasmid Construction and siRNA Synthesis

To construct an overexpression plasmid for *GPNMB* gene, coding sequence (**CDS**) of *GPNMB* gene was amplified from Gushi chicken abdominal fat cDNA by PCR using gene specific homologous recombinant clone

primers (Table S1). The CDS sequence was then cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) by homologous recombination method, named pcDNA3.1-GPNMB. The correctness of the construction of pcDNA3.1-GPNMB was confirmed by agarose gel electrophoresis and sequencing. The siRNA oligonucleotides specifically against chicken *GPNMB* (**si-GPNMB**) and a nonspecific duplex (si-NC, negative control) were synthesized from GenePharma Co., Ltd. (Shanghai, China).

Chicken Primary Preadipocyte Isolation, Culture and Transfection

The primary preadipocytes were isolated from abdominal fat of 2-wk-old Gushi chickens according to our previous protocol (Zhang et al., 2019). In brief, abdominal fat tissue was separated under sterile condition. The tissue was washed repeatedly with phosphate-buffered saline (**PBS**) containing penicillin (100 units/mL) and streptomycin (100 μg/mL), and cut into pieces, then digested with 1 mg/mL collagenase type II (Solarbio, Beijing, China) at 37°C for 1 h with a gentle shaking every 5 min. The digested cell suspension was filtered using 100-, 200- and 500-mesh screens, respectively. The filtrated suspension was then centrifuged at 2,000 rpm for 10 min, and the precipitate was suspended with red blood cell lysate and incubated at room temperature for 10 min. The cell suspension was centrifuged, and the cell pellet was re-suspended to obtain preadipocytes. The preadipocytes were plated onto a 12-well culture plate at a density of 1×10^5 cells/mL and cultured in high-glucose Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 supplemented with 10% Fetal Bovine Serum (**FBS**) (Gibco, Gaithersburg, MD) and 0.2% penicillin/streptomycin at 37°C with 5% CO_2. When the cells reached 80% confluence, they were divided into 2 groups. The first group was used to induce preadipocyte differentiation by adding sodium oleate medium to base medium to a final concentration of 160 μM. Cells were collected after induction for 48 h. The second group was used for overexpression and knockdown experiments of the *GPNMB* gene. Transfection of plasmid DNA including pcDNA3.1 vector (control), pcDNA3.1-GPNMB overexpression vector, si-NC and si-GPNMB were carried out using the transfection reagent Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following the manufacturer's instruction, respectively.

Cell Proliferation Assays

Preadipocytes cultured in 96-well plates were transfected with the above constructed plasmids and siRNA oligonucleotides, respectively. Cell proliferation was detected at 12, 24, 36, and 48 h post-transfection using cell counting kit-8 (**CCK-8**) reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction.

Preadipocytes cultured in 24-well plates were transfected with the above constructed plasmids and siRNA oligonucleotides, respectively. At 36 h post-transfection, the cells were cultured with 10 μM 5-Ethynyl-2'-deoxyuridine (**EdU**) medium (Ribobio, Guangzhou, China) at 37°C for 6 h. The incubated cells were fixed with 4% paraformaldehyde at room temperature for 40 min. The fixed cells were then stained using the cell-Light EdU Apollo 567 in vitro kit (Ribobio, Guangzhou, China), imaged using a fluorescence microscope (Olympus, Tokyo, Japan).

Cell Cycle Analysis

The preadipocytes cultured in 6-well plates were transfected with the above constructed plasmids and siRNA oligonucleotides, respectively. At 36 h post-transfection, the cells were collected and fixed with 75% ethanol at −20 °C for 24 h. The fixed cells were further stained with propidium iodide (Solarbio, Beijing, China), and then incubated for 30 min under dark conditions. The cell cycle was subsequently analyzed by using BD Accuri C6 flow cytometer (BD Biosciences, San Diego, CA), and the data was analyzed in FlowJo 7.6 software (TreeStar Inc., Ashland, OR).

Oil Red O Staining and Triglyceride Content Determination

After transfection with the above constructed plasmids and siRNA oligonucleotides for 24 h, the preadipocytes were treated with sodium oleate with final concentration of 160 μM (Sigma Life Science, St. Louis, MO) to induce adipogenic differentiation (Chen et al., 2019). At 24 h postdifferentiation, the cells were washed with PBS and fixed with 4% paraformaldehyde solution for 30 min, then the cells were stained with oil Red O working solution (Solarbio, Beijing, China) and incubated for 20 min at room temperature. After washed with PBS, the cells were photographed under fluorescence inverted microscope (Sony, Tokyo, Japan). Then, the oil Red O dyes were extracted with isopropanol solution and measured at 500 nm absorbance by multi-mode microplate reader (BioTek, Vermont). The triglyceride (**TG**) content in the adipocytes were detected using tissue and cell triglyceride enzyme kit (Applygen Technologies Inc., Beijing, China) following the manufacturer's instruction.

Transcription Factor Binding Elements Prediction in Promoter Region of GPNMB Gene

The promoter sequence (2 kb) upstream from the transcription start site of *GPNMB* gene were retrieved from Ensembl database. The transcription factor binding elements were predicted using online bioinformatics tool JASPAR (http://jaspar.genereg.net/). The element matrixes and promoter sequence were then submitted to MEME FIMO (http://meme-suite.org/tools/fimo) to further scan for individual matches to each binding elements.

Estrogen and PPARα Agonist Treatment of Chicken Primary Preadipocytes

Chicken preadipocytes were cultured in 12-well plates were divided into four groups (3 replicates/group). The first 3 groups were treated with 17β-estradiol dissolved in ethanol at final concentrations of 100 nM, 150 nM, and 200 nM, respectively, and the last group was treated with same amount of ethanol only as a control. The cells were harvested after the treatment for 24 h.

In order to establish whether peroxisome proliferator-activated receptor alpha (PPARα) regulate the expression of *GPNMB*, chicken preadipocytes cultured in 12-well plates were divided into 4 groups (3 replicates/group). The first three groups were treated with PPARα agonist WY14,643 (Sigma) dissolved in dimethylsulfoxide (**DMSO**) at final concentration of 10 nM, 50 nM, and 100 nM respectively, and the fourth group was treated with the same amount of DMSO only as a control. The cells were harvested after the treatment for 24 h. All experiments were repeated in triplicate independently.

RNA Preparation, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted from the collected tissues or cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using HiScript III RT SuperMix with gDNA wiper (Vazyme Biotech Co., Ltd) according to the manufacturer's protocol. The gene expression levels were detected using quantitative real-time PCR (**qPCR**) in LightCycler 96 Instrument (Roche Applied Science, IN) with 2 × SYBR Premix Ex TaqTM II (TaKaRa). The specific primers were designed by NCBI Primer-BLAST tool (Table S1). All reactions were test in triplicate. The $2^{-\Delta\Delta ct}$ method was used to measure the relative level of each gene with *GAPDH* (For abdominal fats and chicken preadipocytes) as the internal control gene.

Statistical Analysis

The generalized linear mixed model (**GLM**) included with SPSS 23.0 (IBM, Chicago, IL) was used to analyze the association of SNPs with abdominal fat traits. The model used was as follows:

$$Y_{iklm} = \mu + G_i + H_k + f_l + b\,(W_{iklm} - \overline{W}) + e_{iklm}$$

Carcass weight as a covariate was used for SNP associations with abdominal fat traits. In these models, Y_{iklm} was the dependent variable (phenotypic value), μ was the observation mean and e_{iklm} is the random error, G_i was the fixed effect of genotype (i = genotypes), H_k was the fixed effect of hatching (k =1, 2), f_l was the fixed effect of family (l = 1, 7), b was the regression coefficient for the carcass weight, W_{iklm} was the individual slaughter weight, and Wwas the average slaughter weight (Zhang et al., 2014). Significant differences between least squares means of the different genotypes were calculated using the least significant difference (**LSD**).

Statistical significance of the comparison between the two groups was examined by an independent-samples *t*-test, and statistical analysis among more than two groups was performed by one-way ANOVA. The results were presented as the mean ± SEM. The correlation between the gene expression and phenotypes was analyzed using Pearson correlation. $^*P < 0.05$ indicated the significant difference, and $^{**}P < 0.01$ indicated the extremely significant difference.

RESULTS

Association of SNPs in the Promoter Region of GPNMB with Abdominal Fat Traits

A total of 6 SNPs in the promoter region of *GPNMB* gene were detected by integrative analysis of genomic sequencing data of Gushi-Anka F2 population and genetic variant table in Ensembl database. The LD analysis showed that 3 SNPs including rs31126482, rs31126558, and rs31126843 were located in a 361bp LD block with strong LD (D' > 0.98 and $R^2 > 0.77$) (Figure S1). Therefore, rs31126482 was considered as a tag SNP. The rs31126482, together with the other three SNPs were genotyped in Gushi-Anka F2 population. Association analysis showed that only rs31126482 was significantly associated with AFW (P = 0.018) and AFP (P = 0.003) (Table 1). Meanwhile, a consistent significant correlation between rs31126482 and abdominal fat traits was also established in Gushi chicken (Figure 1A and B). And the genotype AA of rs31126482 was more conducive to abdominal fat deposition, compared to the other genotypes.

Expression Characteristics of GPNMB Gene

The expression profiles of *GPNMB* were analyzed in 10 tissues including abdominal fat, pancreas, subcutaneous fat, duodenum, ovary, jejunum, liver, shell gland, pectorals, and kidney from 43-wk-old chickens. The results showed that *GPNMB* was expressed with the highest level in abdominal fat (Figure 2A). Further, the *GPNMB* expression level was significantly higher in abdominal fat tissue in the Gushi chicken with high abdominal fat percentage (GSH) than those with low abdominal fat percentage (GSL) ($P < 0.05$, Figure 2B). And there was highly positive Pearson's correlation between *GPNMB* expression level in abdominal fat and AFW ($R^2 = 0.6356$, P = 4.10E−05, Figure 2C) or AFP ($R^2 = 0.6450$, P = 2.90E−05, Figure 2D). The expression levels of *GPNMB* gene in chicken abdominal fat tissues were significantly higher in the individuals with AA genotype compared with those whose genotypes were AG and GG in rs31126482 ($P < 0.05$; Figure 2F).

In addition, the expression level of *GPNMB* was significantly higher in mature abdominal adipocytes than

Table 1. Association of SNPs in the promoter region of GPNMB with abdominal fat traits.

SNPs	Genotypes (Number)	AFW (g)	*P*-value (AFW)	AFP (%)	*P*-value (AFP)
rs31126482	GG (146)	8.53 ± 2.37^{b}	0.018	1.17 ± 0.21^{b}	0.003
	AG (272)	8.95 ± 1.13^{b}		1.18 ± 0.09^{b}	
	AA (52)	14.82 ± 1.31^{a}		2.25 ± 0.11^{a}	
rs31126789	CC (367)	8.27 ± 0.86	0.706	0.69 ± 0.07	0.355
	CT (92)	8.45 ± 1.32		0.83 ± 0.14	
	TT (11)	6.68 ± 1.86		1.01 ± 0.22	
rs31127050	TT (454)	7.64 ± 0.72	0.592	0.84 ± 0.08	0.101
	CC (16)	7.98 ± 1.42		0.90 ± 0.17	
rs31127108	TT (296)	7.84 ± 0.79	0.464	0.87 ± 0.09	0.396
	TC (167)	6.74 ± 0.92		0.81 ± 0.13	
	CC (7)	9.82 ± 1.54		1.12 ± 0.17	

[a-b]Values within a column with different superscripts differ significantly at $P < 0.05$.
AFP, abdominal fat percentage; AFW, abdominal fat weight.

that in abdominal preadipocytes ($P < 0.01$, Figure 2E). These results suggest that the high expression of *GPNMB* may promote abdominal fat deposition in chickens.

GPNMB Inhibits the Proliferation but Promotes Differentiation and Lipid Droplet Accumulation in Chicken Abdominal Preadipocytes

To explore the biological role of *GPNMB* in adipogenesis in chicken, the abdominal preadipocytes were transfected with *GPNMB* overexpression vector and siRNA oligonucleotides, respectively. The mRNA expression level of *GPNMB* gene in preadipocytes was increased approximately by 400 folds after transfecting with pcDNA3.1-GPNMB (Figure 3A), while decreased approximately by 50% after transfecting with si-GPNMB (Figure 3B).

GPNMB overexpression inhibited the mRNA expression of marker genes reflecting cell proliferation such as cyclin dependent kinase 1 (***CDK1***) and cyclin D1 (***CCND1***), and promoted the mRNA expression of cell cycle arresting factor (*P21*) ($P < 0.\ 01$; Figure 3C). In contrast, *GPNMB* knockdown upregulated the mRNA expression of marker genes of cell proliferation and downregulated the expression of cell cycle arresting factor ($P < 0.\ 01$; Figure 3D). Additionally, *GPNMB* overexpression increased the cell populations in the G1 phase but decreased the cell populations in the S phase ($P < 0.\ 01$; Figure 3E), and *GPNMB* knockdown showed an opposite effect ($P < 0.\ 01$; Figure 3F), suggesting that *GPNMB* inhibits cell proliferation characterized by blocking cell cycle transition from G1 to S phase. In addition, CCK-8 and EdU assays also showed that *GPNMB* overexpression markedly decreased numbers of living preadipocytes and EdU-positive preadipocytes, and vice versa ($P < 0.01$, Figure 3G, H, I, and J).

Meanwhile, compared with the control group, the mRNA expression levels of the marker genes for adipocyte differentiation such as peroxisome proliferator activated receptor γ (***PPARγ***), CCAAT/enhancer-binding protein α (***C/EBPα***), fatty acid binding protein 4 (***FABP4***) and lipoprotein lipase (***LPL***) were significantly upregulated in the *GPNMB* overexpression group ($P < 0.\ 05$, Figure 4A), while opposite results were found in the *GPNMB* knockdown group (Figure 4B).

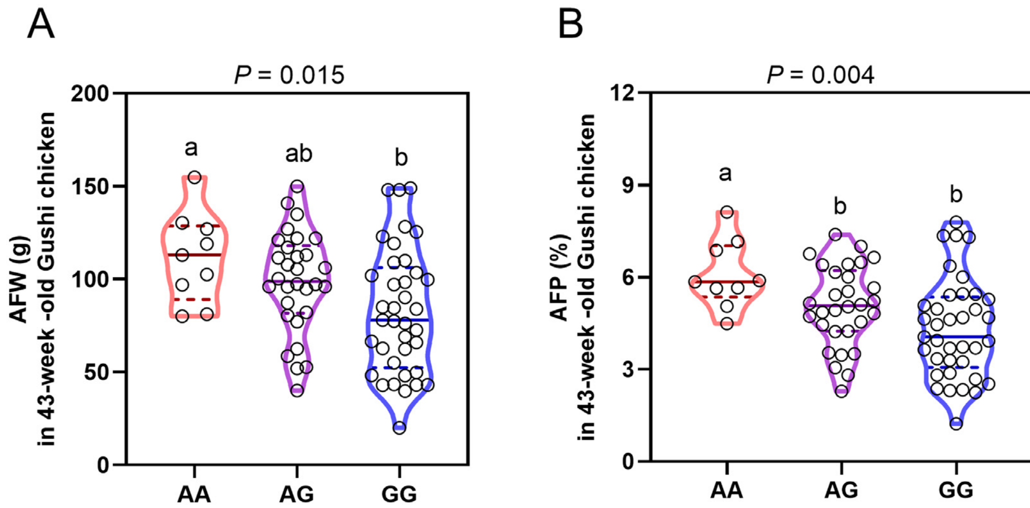

Figure 1. Association of SNP rs31126482 in the promoter region of glycoprotein non-metastatic melanoma protein B gene (*GPNMB*) with abdominal fat traits in Gushi chickens. (A) and (B) Association analysis between the genotypes of rs31126482 and abdominal fat weight (AFW) or abdominal fat percentage (AFP) in 89 Gushi chicken population at 43 wk old. Different letters mean significant difference, same letters mean no significant difference.

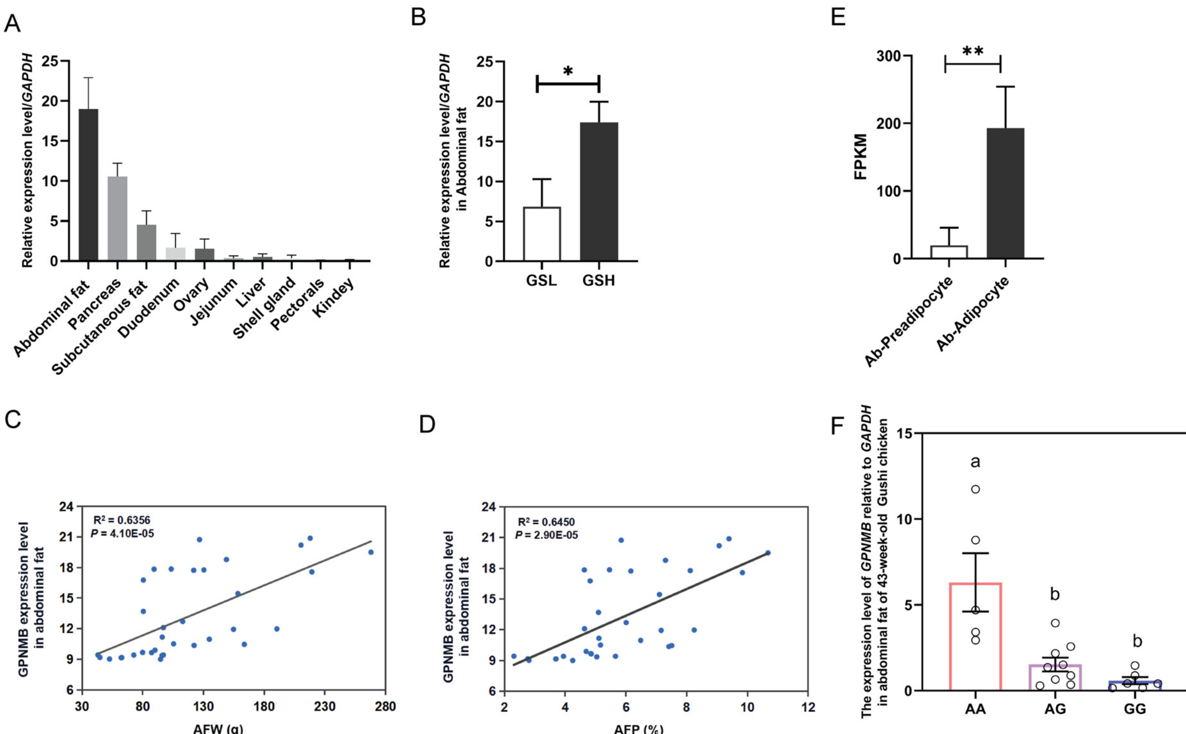

Figure 2. Expression characteristics of glycoprotein non-metastatic melanoma protein B gene (*GPNMB*). (A) Tissue expression profile of *GPNMB* in 43-wk-old Gushi hens. (B) Different expression of *GPNMB* in Gushi chicken with high abdominal fat percentage (GSH) and low abdominal fat percentage (GSL). (C) and (D) The correlation analysis between GPNMB expression levels in abdominal fat and abdominal fat weight (AFW) or abdominal fat percentage (AFP) in 43-wk-old Gushi chickens. (E) The expression changes of *GPNMB* in Gushi chicken abdominal preadipocytes (Ab-Preadipocyte) and adipocytes (Ab-Adipocyte). (F) The expression levels of *GPNMB* in Gushi chicken abdominal fat among individuals with different genotypesof rs31126482. *GAPDH* was used as the internal control gene to estimate the mRNA relative level. Data are presented as mean ± SEM (n = 6 for each group). Each blue dot represents an individual. $^*P < 0.05$, $^{**}P < 0.01$.

Moreover, *GPNMB* overexpression significantly increased expression levels of genes involving triglyceride synthesis, including fatty acid synthase (***FASN***), stearoyl-CoA desaturase (***SCD***), acetyl-CoA carboxylase alpha (***ACACA***) and acyl-CoA synthetase long-chain family member 1 (***ACSL1***), and genes involving lipid droplet formation, including SREBF chaperone (***SCAP***), sterol regulatory element binding transcription factor 1 (***SREBP1***) and perilipin 2 (***PLIN2***) in differentiated adipocytes (Figure 4C). The results were reversed after *GPNMB* gene was knocked down (Figure 4D). Further, *GPNMB* overexpression significantly increased the lipid droplets content, whereas *GPNMB* knockdown reduced the lipid droplets content, as determined by Oil red O staining (Figure 4E, F). Similarly, the TG and cholesterol (TC) contents of differentiated adipocytes were remarkably enhanced upon GPNMB stimulation ($P < 0.05$, Figure 4G), whereas TG and TC were remarkably reduced in the *GPNMB* knockdown group ($P < 0.01$, Figure 4H). These data indicate that *GPNMB* promotes chicken abdominal adipocyte differentiation.

Regulatory Characteristics of Chicken GPNMB Gene

The potential transcription factor binding elements were predicted within 2 kb of upstream sequences from the transcription start sites of the *GPNMB* gene (Figure 5A, Table S2). Four types of transcription factor binding elements including the nonclassical estrogen response element (AP1), RXR-related receptor element (**RXRA**), PPARα response element, and thyroid hormone receptor-related factor element (**RORA**) were identified.

The mRNA levels of *GPNMB* were significantly up-regulated in the abdominal fat in the chickens treated with 1 mg/kg 17β-estradiol compared with those in the control group ($P < 0.05$, Figure 5C), as did hepatic apovitellenin very-low-density lipoprotein Ⅱ (*ApoVLDL II*), a marker gene of estrogen response ($P < 0.05$, Figure 5B). This result was further confirmed in chicken preadipocytes treated with 17β-estradiol ($P < 0.05$, Figure 5D). The results suggested that 17β-estradiol might up-regulate the expression of *GPNMB* via AP1 in chicken abdominal fat tissues and abdominal preadipocytes.

To investigate whether the expression of *GPNMB* is affected by other 3 transcription factors as well, the expression levels of *PPARα, RXRA* and *RORA* were detected in chicken abdominal preadipocytes and the differentiated adipocytes. The results showed that *PPARα* expression was significant decreased in the abdominal adipocytes compared to abdominal preadipocytes ($P < 0.01$, Figure 6A), while the expression levels of *RXRA* and *RORA* did not change significantly (Figure 6B and C). To verify the effect of PPARα on

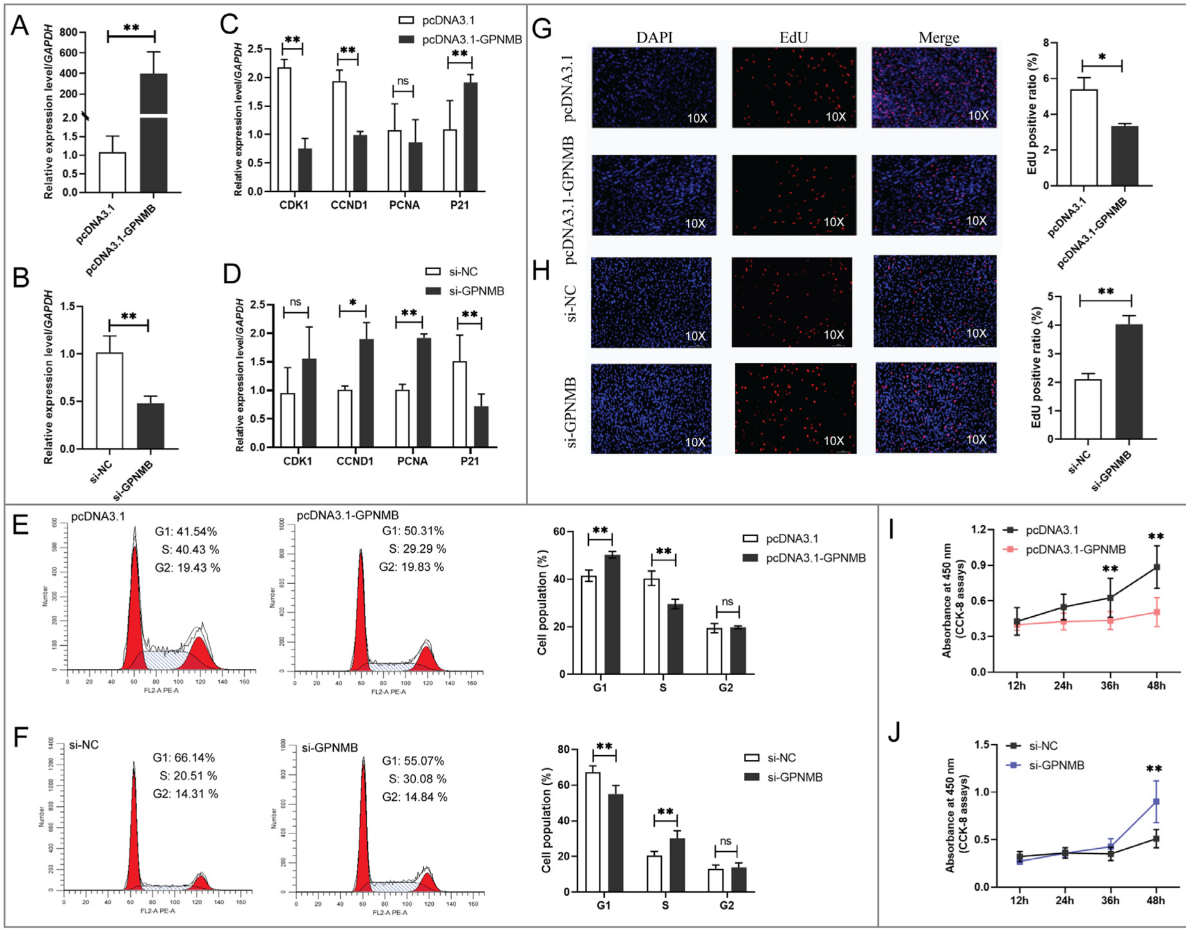

Figure 3. Glycoprotein non-metastatic melanoma protein B gene (*GPNMB*) inhibited the proliferation of chicken primary abdominal preadipocytes. (A) and (B) The mRNA expression of *GPNMB* after 36 h transfection of *GPNMB* overexpression vector (pcDNA3.1-GPNMB) and si-GPNMB in preadipocytes. (C) and (D) The expression of cell cycle related genes after transfection of pcDNA3.1-GPNMB and si-GPNMB in preadipocytes. (E) and (F) Cell cycle changes of abdominal preadipocytes after transfection with pcDNA3.1-GPNMB and si-GPNMB as assayed by flow cytometry. (G) and (H) Proliferation of abdominal preadipocytes was assayed using 5-Ethynyl-2'-deoxyuridine (EdU) after transfection with pcDNA3.1-GPNMB and si-GPNMB. (I) and (J) Cell proliferation was detected using cell counting kit-8 (CCK-8) after transfection with pcDNA3.1-GPNMB and si-GPNMB in abdominal preadipocytes. The data are presented as Mean ± SEM (n ≥ 3 for each treatment group). $^{*}P < 0.05$; $^{**}P < 0.01$.

regulating *GPNMB* expression, the abdominal preadipocytes were treated with different doses of the PPARα agonist WY14,643. The mRNA level of acyl-coenzyme A oxidase 1 (*ACOX1*), a marker gene of PPARα response, was significantly up-regulated, but mRNA level of *GPNMB* was significantly down-regulated. It indicated that PPARα negatively regulates *GPNMB* expression in abdominal preadipocytes ($P < 0.05$, Figure 6D, E) in chicken.

DISCUSSION

Excessive abdominal fat deposition presents a great obstacle to chicken health, productivity and reproductive efficiency (Moreira et al., 2018; Zhang et al., 2018; Guo et al., 2021). In the present study, we firstly analyzed the association between SNPs in *GPNMB* promoter and abdominal fat traits, and found that the SNP rs31126482 was significantly associated with AFW and AFP in Gushi-Anka F2 population and Gushi chicken population. The result indicate that the genetic variation in *GPNMB* potentially influence chicken abdominal fat deposition. We then analyzed the expression profiles of *GPNMB* gene, and detected the *GPNMB* was highly expressed in abdominal fat, which was consistent with the expression pattern in mammals (Colwill, 2011). Meanwhile, we looked into the expression differences of the gene in abdominal fat between the birds with high and low AFPs, and demonstrated a strong positive correlation between the *GPNMB* mRNA expression levels and AFW or AFP. Additionally, our previous study revealed *GPNMB* expression level was significantly higher in the differentiated adipocytes in comparison to that in preadipocytes of abdominal fat in Gushi chickens (Zhang et al., 2019). In addition, we also validated the genotypes of rs31126482 in *GPNMB* promoter affected its gene expression. These findings suggested that *GPNMB* gene could play a crucial role in abdominal fat deposition in chicken.

To verify the role of GPNMB in adipogenesis in chicken, we adopted gain- and loss-function strategy to investigate the effects of *GPNMB* gene on proliferation, differentiation and lipid deposition in abdominal

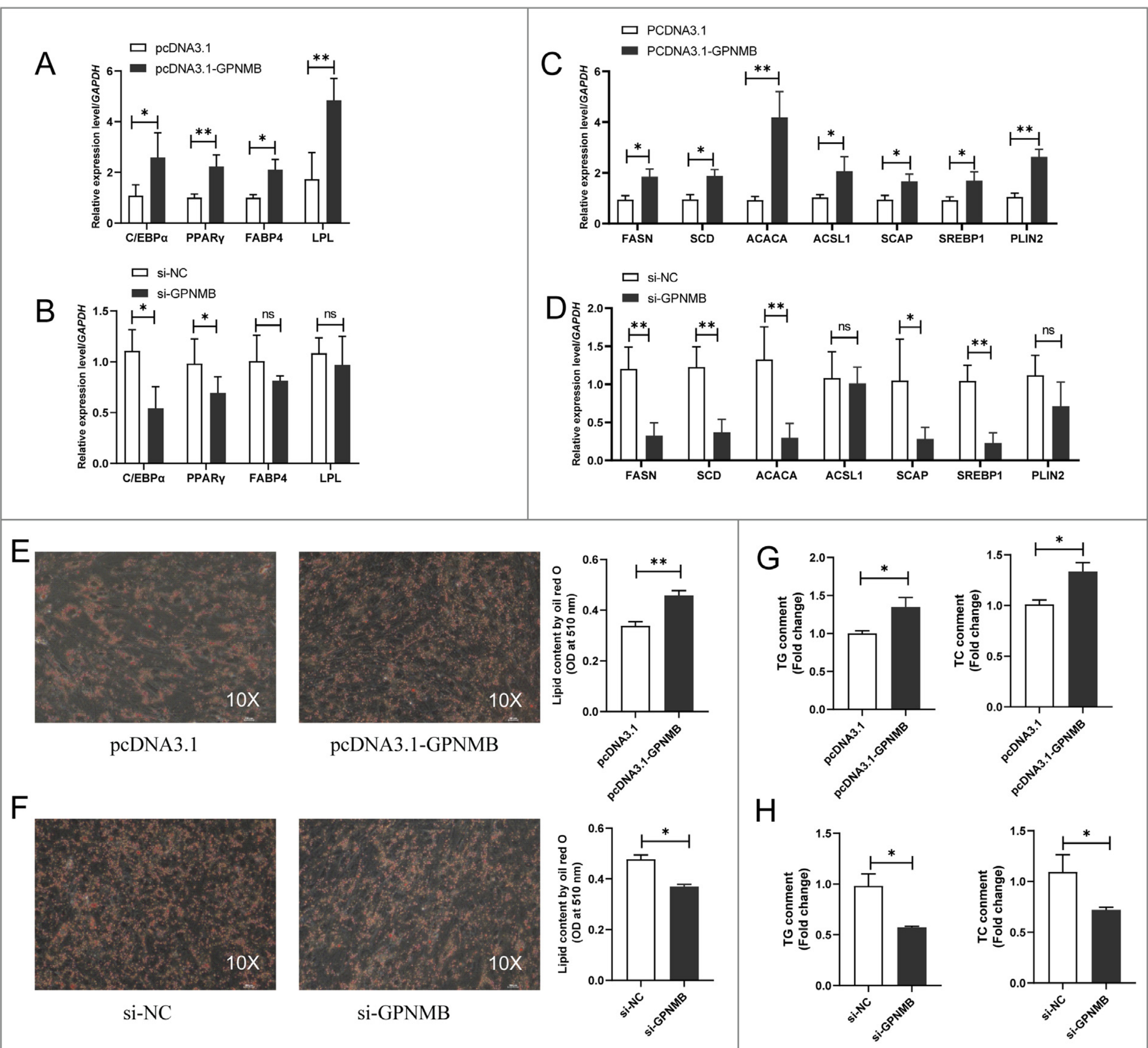

Figure 4. Glycoprotein nonmetastatic melanoma protein B gene (*GPNMB*) promoted chicken primary abdominal adipocyte differentiation and increased lipid droplet accumulation. (A) and (B) The expression of genes related to adipocyte differentiation after *GPNMB* overexpression and inhibition in chicken adipocytes. (C) and (D) The expression of genes related to triglyceride synthesis and lipid droplet formation after *GPNMB* overexpression and inhibition in chicken adipocytes. (E) and (F) Representative images of oil red O staining (red) and Lipid droplet content after overexpression and inhibition of *GPNMB* in chicken adipocytes. scale bar: 100 μm (10-magnification). (G) and (H) TG and TC contents of adipocytes transfected with with pcDNA3.1-GPNMB and si-GPNMB. The data are presented as Mean ± SEM (n ≥ 6 for each treatment group). $^{*}P < 0.05$; $^{**}P < 0.01$.

preadipocytes. Overexpressed *GPNMB* promoted the cell cycle arrest, decreased cell number in abdominal preadipocytes and blocked cell proliferation, meanwhile, the overexpression of *GPNMB* promoted the differentiation of abdominal preadipocytes to adipocytes, and increased lipid droplet accumulation. The knockdown of *GPNMB* showed the opposite results to the above overexpression. These results validated that *GPNMB* could inhibit the proliferation, but promote differentiation and accumulation of lipid drops in abdominal preadipocytes in chicken.

Previous studies had demonstrated that GPNMB was a vital secretory factor promoting the occurrence of obesity in humans and mice (Gabriel et al., 2014; Choi et al., 2015; Gong et al., 2019). The GPNMB, as type I membrane glycoprotein, was secreted by liver, and could be proteolytically cleaved to release a large ectodomain to serum, and stimulated lipogenesis through the CD44-AKT-SREBP1c axis in white adipose tissue (Gong et al., 2019). However, *GPNMB* was mainly directly expressed in adipose tissue in chicken. The pathways or mechanisms by which *GPNMB* promotes abdominal fat accumulation still need to be further investigated.

To explore the potential regulatory mechanism of *GPNMB* expression, we detected the transcription factor binding elements in the 2 kb promoter region of *GPNMB* and found 4 potential binding elements for the factors of AP1, RXRA, PPARα and RORA in the region. Of the factors, AP1 is a nonclassical pathway binding factor of estrogen, which can be tethered with estrogen receptor (**ER**) to co-regulate transcription of target genes (Jakacka et al., 2001), which are mainly involved in lipid metabolism in chicken (Ren et al.,

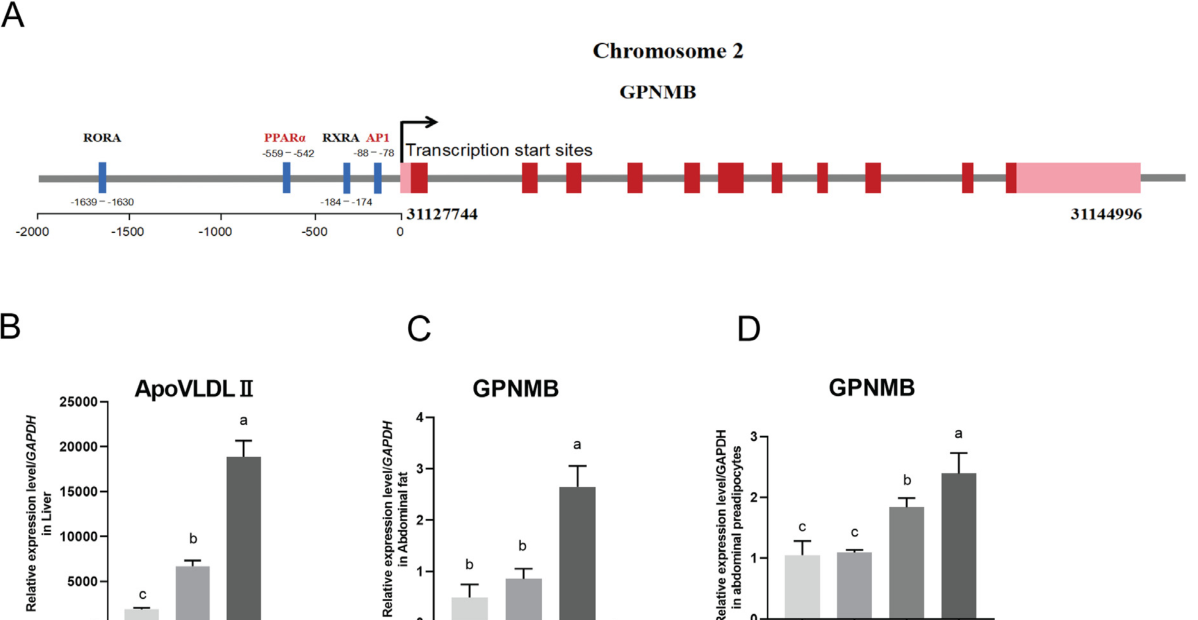

Figure 5. Transcription factor binding element prediction and estrogen response of chicken glycoprotein non-metastatic melanoma protein B gene (*GPNMB*). (A) Predicted the transcription factor binding elements in 2 kb promoter region of *GPNMB* gene. (B) 17β-estradiol treatment significantly up-regulated the expression of hepatic apovitellenin very-low-density lipoprotein Ⅱ (*ApoVLDL II*) in 10-wk-old Gushi hens, a marker gene of estrogen response. (C) Effect of 17β-estradiol treatment on *GPNMB* expression in 10-wk-old Gushi hens. (D) Effect of 17β-estradiol treatment on *GPNMB* expression in chicken primary abdominal preadipocytes. The data are presented as Mean ± SEM (n ≥ 6 for each treatment group). $^{*}P < 0.05$; $^{**}P < 0.01$.

2021). In fact, our study demonstrated that the expression level of *GPNMB* was significantly up-regulated by estrogen (17β-estradiol) in chicken abdominal fat tissue and abdominal preadipocytes. 17β-estradiol, the most active estrogen, regulates gene transcription by the classical nuclear estrogen receptors (estrogen receptor 1 [**ERα**] and estrogen receptor 2 [**ERβ**]) and cell surface receptor (G-protein coupled estrogen receptor [GPER, known as GPR30]) (Vasudevan and Pfaff, 2008; Sharma et al., 2018). Estrogen-binding nuclear receptors can exert their effects through the classical ERE element binding pathway and the nonclassical AP1 binding pathway (Jakacka et al., 2001). However, the classical estrogen response ERE element is not predicted in the *GPNMB* gene promoter region, so if estrogen plays its regulatory function through the nuclear receptors, it may only be through the nonclassical AP1 pathway. Meanwhile, estrogen can signal rapidly through GPR30 receptor which is expressed in multiple tissues including liver and abdominal fat, and other tissues (Sharma et al., 2018). And GPR30-mediated signaling can also activate the downstream AP1 transduction signaling (Santolla et al., 2012). Therefore, *GPNMB* may be regulated by estrogen via a nonclassical AP1 pathway or crosstalk with cell surface receptor GPR30 in abdominal fat.

The *PPARα* is a ligand-activated nuclear receptor, and its target genes are involved in peroxisomal and mitochondrial β-oxidation in tissues with high oxidative rates such as muscle, brown adipose tissue and liver (Xu et al., 2002). It has been reported that PPARα and RXRA could form heterodimers, and further bind with peroxisome proliferator-activated responsive element (**PPRE**) and retinoid X responsive element (**RXRE**) in target genes to exert their regulated functions (Boergesen et al., 2012). Activating PPARα signaling enhances fatty acid oxidation and reduces lipogenesis, and activating PPARα/RXRA signaling optimizes the homeostasis of lipid metabolism (Xue et al., 2020). The retinoic-acid-related orphan receptor α (**RORA**) also act as a nuclear transcription factor that modulates various target genes related to lipid metabolism (Goldbraikh et al., 2020). It has demonstrated that RORA reduced hepatic lipid accumulation by disturbing liver X receptor-mediated lipogenesis and enhancing AMPK-mediated fatty acid oxidation (Jetten, 2009). However, in this study, the expression levels of PPARα was significantly reduced in abdominal adipocytes compared with that in abdominal preadipocytes, and PPARα agonist WY14,643 down-regulated *GPNMB* transcription in preadipocytes. Therefore, *GPNMB* was probably regulated by PPARα via PPARα response element (**PPRE**). The opposite regulatory effects on *GPNMB* expression by estrogen and PPARα implied a dynamic balance mechanism on regulating abdominal fat deposition in chicken.

It was because that the genotypes of SNP rs31126482 had significantly effects on *GPNMB* expression, we looked into the positional relationship of the 3 SNPs in the rs31126482 LD block and AP1 and PPARα binding elements. We found that the position of SNPs in the LD block was concentrated in the interval from −1262 bp to −901 bp upstream of the transcription start sites, which was far away from the predicted positions of the AP1

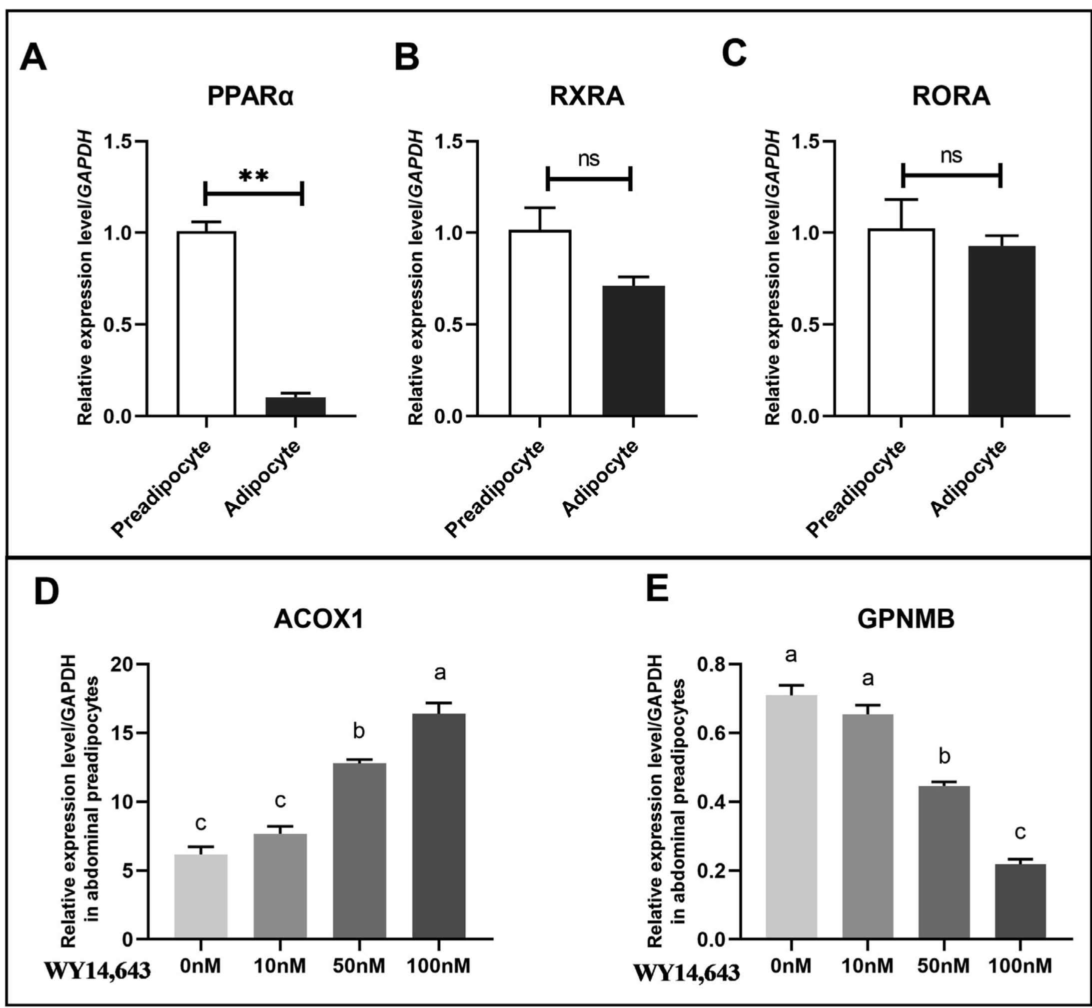

Figure 6. Effect of transcription factors on glycoprotein nonmetastatic melanoma protein B gene (*GPNMB*) expression in chicken primary abdominal preadipocytes. (A), (B), and (C) The expression levels of peroxisome proliferator activated receptor alpha gene (*PPARα*), retinoid X receptor alpha gene (*RXRA*) and RAR related orphan receptor A gene (*RORA*) in chicken abdominal preadipocytes and differentiated adipocytes. (D) and (E) Effect of PPARα agonist (WY14,643) treatment on *GPNMB* expression in chicken primary abdominal preadipocytes. Acyl-coenzyme A oxidase 1 (ACOX1) is a marker gene of PPARα response. The data are presented as Mean ± SEM (n ≥ 6 for each treatment group). $^{*}P < 0.05$; $^{**}P < 0.01$.

and PPARα binding elements. Therefore, the SNP rs31126482 might linked with other factors to affect the GPNMB expression, it needs to be investigated further.

In conclusion, we demonstrated for the first time that *GPNMB* regulates abdominal fat deposition by inhibiting the proliferation and promoting differentiation and accumulation of lipid drops in abdominal preadipocytes in chicken. A dynamic balance mechanism, in which the expression of *GPNMB* was up-regulated by estrogen through AP1 element, and down-regulated by PPARα via PPRE, could be adopted to control abdominal fat deposition in chicken. Our findings not only bring new insight into the regulatory mechanism of adipogenesis in chicken, but also facilitate the marker-assisted selection of abdominal fat traits in chicken.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

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