BMP4/SMAD8 signaling pathway regulated granular cell proliferation to promote follicle development in Wanxi white goose

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ABSTRACT Granular cells proliferation in goose regulated by bone morphogenetic proteins (**BMPs**) signaling pathway is still unknown. In this experiment, BMPs and their receptor, and receptor activated mothers against decapentaplegic homologs (**SMADs**) were quantitatively expressed in granular cell layer of prehierarchycal and hierarchycal follicles in Wanxi White goose. The screened BMP was then used for construction of overexpressed and knockdown vectors and transfected into granular cells of goose to assess the cell proliferation and apoptosis. Granular cells with BMP-overexpressed were then used for ChIP-Seq analysis to elucidate the molecular mechanism of BMP affecting granular cell proliferation. The results showed that the mRNA expression of BMP4 was significantly expressed in prehierarchical follicles, and also highly expressed in hierarchical follicles than other BMPs, while the Ⅰ and Ⅱ type of BMP receptors were expressed in basic level. The mRNA expression of SMAD8 was significantly elevated in pre-hierarchical follicles. Overexpression of BMP4 could promote the proliferation of granular cells and inhibited the expression of BMP4 caused a higher cell apoptosis. ChIP-Seq identified multiple regulatory targets of SMAD4, which were mostly related to cell cycle and lipid metabolism according to the GO and KEGG pathway enrichment. From the five most significant binding motif and quantitative expression verification, the activin membrane binding inhibitor (**BAMBI**) was down regulated in BMP4 overexpressed granular cells. In conclusion, the BMP4 was highly expressed in granular cells and phosphorylates SMAD8, the activated SMAD8 combined with SMAD4 transfers into nucleus to regulate the expression of BAMBI to promote lipid synthesis.

Key words: Goose, BMP/SMAD signal pathway, granular cell, ChIP-Seq, activin membrane binding inhibitor

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INTRODUCTION

Goose, especially Chinese indigenous mid-type breeds like Wanxi white goose (Chen et al., 2015), Zhedong goose (Bao et al., 2022), has low egg production of 15 to 40 eggs per year, which caused a high cost of goslings. Egg production is mainly dependent on follicle development and maturation, during which the follicular granular cells secret growth factors so as to ensure the normal development of follicles (Schütz et al., 2022). Therefore, the proliferation of follicle granular cells is crucial for follicle development. It is demonstrated that about ten percent of apoptosis occurred in granular cells could cause atresia of follicle (Yeung et al., 2017). Granular cells proliferate in rapid speed during follicle development and then slow down in follicles tend to maturation (Dong et al., 2014).

It was found that bone morphogenetic proteins (**BMPs**) could promote the proliferation of granular cells and secretion of steroids, and then regulate the growth of follicles through BMPs/SMAD pathway (Cui et al., 2017; Liu et al., 2019). Through transfecting recombinant human BMP4 into immortalized human granular cell lines, granular tumor cell lines and primary granular lutein cells, it was found that BMP4 phosphorylated mothers against decapentaplegic homolog (**SMAD**) of 1/5/9 through anaplastic lymphoma kinase3 (**ALK3**) and ALK6, and inhibited the expression of pentraxin 3 (**PTX3**) (Chang et al., 2015). The BMP4 and BMP7 bound to different Ⅰ-type receptor, phosphorylated SMAD1/5/9 to down regulate the expression of PTX3 so as to promote the proliferation of granular cells (Chang et al., 2015). Thus BMPs that in high expression in

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follicle granular layer might be the most important gene responsible for follicle development.

So far, there were 8 SMADs have been found, of which SMAD1, 2,3, 5, and 9 are receptor activated (Pangas et al., 2012). The BMPs could phosphorylate the receptor activated SMAD1, 5 and 9 through binding and phosphorylating its receptors (Nakano et al., 2020). The activated SMADs then binding to SMAD4 to form heterodimer and transfers into nucleus as transcriptional factor to activate the expression of related genes (Derynck et al., 2003). Transcriptome analysis of porcine follicle granular cells with SMAD4 knockdown found that differentially expressed genes were mainly enriched in cell cycle related pathways (Zhang et al., 2016). The SMAD4 could initiate the transcription of frizzled homolog 4 (**FZD4**), and then activate the FZD4-dependent Wnt signal to prohibit the apoptosis of granular cells (Du et al., 2020), indicating that SMAD4 promotes follicle development through the regulation of granular cells proliferation.

It is still unclear about the BMPs signal regulatory pathway related to the proliferation of follicle granular cells in poultry. In this research, BMP and its receptor that could participate in proliferating granular cells and follicle development of goose were first screened by qRT-PCR, and then demonstrated by overexpression and knockdown method. The ChIP-Seq was used to analyze the downstream genes and binding motif of SMAD4. The results could provide the regulatory mechanism of BMPs in follicle development of goose.

MATERIALS AND METHODS

Ethics Standards

All animal experimental procedures in this study were approved by the Animal welfare committee of Anhui Agricultural University with the assurance number of SYDW-P20210823021.

Tissue Sample Preparation

Follicles at pre-hierarchical and hierarchical were obtained from ovaries of 10 Wanxi White geese. Granular layer was separated and immediately snap-frozen in liquid nitrogen, and then stored at −80°C freezer for RNA and protein extraction.

RNA Extraction, cDNA Synthesis, and qRT-PCR

Total RNA were extracted from granular cell layers using Trizol Reagent (Shanghai Yisheng Biotechnology Co., LTD, China). The quality and quantity of RNA samples were determined using Nanodrop2000 spectrophotometer (Thermo, former Savant, MA), and the value of OD260/280 within 1.8 to 2.0 was considered as qualified. The cDNA synthesis was performed using the PrimeScript RT Reagent Kit with the gDNA Eraser Kit (Hifair, Shanghai Yisheng Biotechnology Co., LTD).

Quantitative RT-PCR was performed by using SYBR Green Master Mix (Low Rox Plus) from Hifair, Shanghai Yisheng Biotechnology Co., LTD, on a cycler (ABI 7500, Massachusetts, MA). The reaction system was 10 μL, including 5.0 μL Hieff qRT-PCR SYBR Green Mix, 0.2 μL upstream primer and 0.2 μL downstream primer, 1 μL total RNA template, and 3.6 μL RNase free ddH2O. The reaction conditions were set as follows, 95°C for 5 min, followed by 40 cycles (95°C for 10 s, 60°C for 20 s, and 72°C for 20 s), and then 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The gene β-actin was set as internal control. Primers used for relative gene expression were designed by Primer Premier 5 software according to the gene sequence in NCBI and listed in Table 1.

Recombinant Plasmid Construction

For overexpression plasmids construction, the coding sequence of goose BMP4 was synthesized by Sangon Biotech (Shanghai, China). The synthesized cDNA of BMP4 was cloned into HindIII and XhoIpolyclonal site of PCDNA3.1-EGFP plasmid to generate the pEGFP-BMP4 overexpressed vector. The PCDNA3.1-EGFP plasmid with no cDNA insertion was set as negative control (**NC**).

Two sequences of short hairpin RNAs (**shRNA**) were designed to knock down the expression of BMP4. Two shRNAs were cloned by seamless cloning of enzyme digestion sites of pLKO.1-EGFP-puro plasmid and the other site of EcoRI to form BMP4 knockdown vectors of shRNA232 and shRNA742. The shRNAs were synthesized by Sangon Biotech.

Granular Cell Culture and Transfection

Geese at their peak laying period were used for follicle collection and granular cell isolation, culture and identification was performed according to Du et al. (2022). The isolated granular cells were cultured in 6-well culture plate for plasmid transfection according to Du et al. (2022). The transfection efficiency was quantified using Image J software and presented as % transfected cells/NC cells. The expression level of BMP5, PCNA, and Caspase3 was determined by qRT-PCR.

Cell Proliferation Assay

Cell counting kit-8 (**CCK-8**) assay was used to assess cell proliferation activity. The granular cells transfected with overexpressed plasmids were plated into 96-well plates and incubated for 36 h. Cells with 10 μL CCK-8 reagent (Coolaber, Beijing, China) in each well was cultured for 6, 12, 24, 48 h. The absorbance at 450 nm was measured using a Microplate Reader (SUNRISE, Tecan Austria GmbH, Austria). Each treatment group had 6 independent replicates and cells set as blank control was added with only 100-μL cell culture medium and 10-μL CCK-8 reagent.

Table 1. Primers for quantitative RT-PCR of each gene.

Genes	Gene ID	Sequence (5'-3')	Length (bp)
BMP2	106016176	AGTGACGTTTGGGCATGATG CCCACGTCATTGAAGTCCAC	135
BMP4	101803077	AAAGCCATGAACTCTTGCGG CCGGTAGAGATCCAGCATGT	119
BMP5	101798837	AAAGATCGAAGCAACAGCCG GCCCACATCAGAAGCTTGAG	135
BMP6	101804730	CCACTAGGTCAGCAACAAGC AGTCGACACCCTGGAAACAT	83
BMP7	101789810	CAACATCGCAGAGAACAGCA AATACGCAGCATAGCCCTCT	125
BMP15	101794104	CTCCAGAGACAGGGACAGAC CAGCTCTGGGAATTTTGGGG	174
BMPR1B	101794439	AATTTGCTGCCCTCTTTGGG TCCTTTGGGACTGGGTTTGT	157
TGFBR1	101805328	CCTCGCTAGATCGTCCCTTT TTCCCTCGCCATACTTCTCC	178
BMPR2	101793707	GCACCTGCTATGGACTTTGG ATCACAGAAGGTGTGGTCGT	136
SMAD1	101795825	GACCCAGGAAGTCCTTTCCA TGTCAGGATGGATTGCAGGT	133
SMAD2	101805334	TTGCCTCCTGTACTAGTGCC GTTCAACTGCTGGTCACTCG	192
SMAD3	101801230	ATCTGGATCTGCAGCCTGTT GACAGCAAACCAAGGCAGAA	172
SMAD4	101805274	CCAGAATGGCCATCTTCAGC GGAATGCGAGTTCGTTGTGA	77
SMAD5	101795209	CGCAGTCTATGGACACAAGC CGAACACCAGTGTTTGGGTT	107
SMAD8	101800239	CAGGGCTTTCCAAACAGCTT TGTACAGGCACCAAGTTTGC	155
β-actin	101800437	ACACTGTGCCCATCTACGAA TCGAAATCCAGGGCGACATA	s152
PCNA	119713445	AGAAATGAATGAGCCAGTCCAGC TTCAATCTTTGGAGCCAGGTAGT	178
Caspase-3	101805296	CTGGTATTGAGGCAGACAGTGG CAGCACCCTACACAGAGACTGAA	158
Bax	106045425	GAAGCATTTACAGTTGCCATTACAG CCACAAGCAAGCAAAGAGCC	162
CPEB2	101801772	ACAAGCCAGTTCAGATCCGT CCTGCGTAACAAACTCCACC	175
BAMBI	101792754	TACATCTTCGTCTGGCTGCA CAAGCAGCCGTGAGTAAGTG	192
CDC73	101804180	AAGGAGATCGTGGTGAAGGG GCACGACGGACATAAACAGG	188

Granular cells were seeded in 12-well plate for adhesion, and the proliferating cells were detected using Abbkine TUNEL Apoptosis Detection Kit (Orange Fluorescence, CA). The cells were incubated with 500 μL 4% polyformaldehyde for 30 min at room temperature and permeabilized with 0.2% Triton X-100 for 30 min. The cells were added 500 μL reaction buffer with 10 μL TdT enzyme and incubated for 2 h at 37°C and then DAPI was added for cell staining and standing for 10 min. The stained cells were observed by fluorescence microscope (IX73, Olympus, Japan)

Western Blotting

Granular cells growth at their logarithmic period were collected and lyzed by cell lysis buffer. The lysis was centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected and the protein concentration was determined using BCA method. After denaturation, 20 μg sample together with loading buffer was loaded on 10% SDS-PAGE gel for electrophoresis. The gel was then transferred on a polyvinylidene difluoride (**PVDF**) membrane (MIllipore, Birrika, Massachusetts, USA) which was blocked for 2 h using 5% skimmed milk and incubated with anti-SMAD4 (10231-1-AP, Wuhan Sanying Biotechnology Co., LTD., 1:600 dilution) at 4°C overnight. The PVDF was washed by tris-buffered saline (**TBST**) 3 times and then was incubated with the second antibody (2729S, Cell Signaling Technology, Boston, USA, 1:10,000 diluton) for 2 h. Proteins were visualized under chemiluminescence imaging system (MINI HD4, UVITEC, Cambridge, UK) after exposure in developing solution.

ChIP-seq With Anti-SMAD4 Antibody

ChIP assay was performed on granular cells with BMP4 overexpressed by SeqHealth (Wuhan, China). Granular cells with 1×10^6 incubated in cell plate were washed twice in cold PBS buffer and crosslinked with 1% (w/v) formaldehyde for 10 min at 37°C and then terminated by 0.125 M glycine. The cells were treated with cell lysis buffer and nucleus was collected by centrifuging

at 2,000 × g for 5 min. Then, nucleus was treated with nucleus lysis buffer and sonicated to produce fragment chromatin DNA. The 10% lysis sonicated chromatin was stored and named "input", and 80% was used for immunoprecipitation reactions with anti-SMAD4 antibody (#10231-1-AP, Proteintech, Wuhan, China) and named "IP", and 10% was incubated with rabit IgG (Cell Signaling Technology, Boston, USA) as a negative control and named "IgG", respectively. The DNA of input and IP was extracted by phenol-chloroform method. The high-throughput DNA sequencing libraries were prepared by using VAHTS Universal DNA Library Prep Kit for Illumina V3 (Catalog No. ND607, Vazyme, Nanjing, China). The library products corresponding to 200 to 500 bps were enriched, quantified and finally sequenced on MGISEQ-T7 sequencer (MGI) with PE150 model.

Data Analysis

Raw sequencing data was first filtered by Trimmonatic (version 0.36), low quality reads were discarded and the reads contaminated with adaptor sequences were trimmed.

The clean reads were used for protein binding site analysis. They were mapped to the reference genome of GooseV1.0 (GCA_002166845.1) from http://pub/release-104/fasta/anser_cygnoides/dna/ (ensembl.org) using STAR software (version 2.5.3a0) with default parameters. The RSeQC (version 2.6) was used for reads distribution analysis. The MACS2 software (Version 2.1.1) was used for peak calling. The bedtools (Version 2.25.0) was used for peaks annotation and peak distribution analysis. The differentially binding peaks were identified by a python script, using Fisher test. The Homer (version 4.10) was used for motifs analysis. Gene ontology (**GO**) analysis and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis for annotated genes were both implemented by KOBAS software (version: 2.1.1) with a corrected P-value cutoff of 0.05 to judge statistically significant enrichment.

Gene expression level was calculated by using the $2^{-\triangle\triangle ct}$ method. Gene expression and transfect efficiency was analyzed by one way ANOVA (SAS 9.1 edition) followed by Duncan's multiple comparison test. Values were expressed at means ± standard error. Significance was set at $P < 0.05$.

RESULTS

The mRNA Expression Level of BMPs and Its Receptors in Each Follicle

The mRNA Expression of BMPs in Each Follicle At peak laying, a total of eight hierarchical follicles could be observed (Figure 1). The BMP2 expression showed the

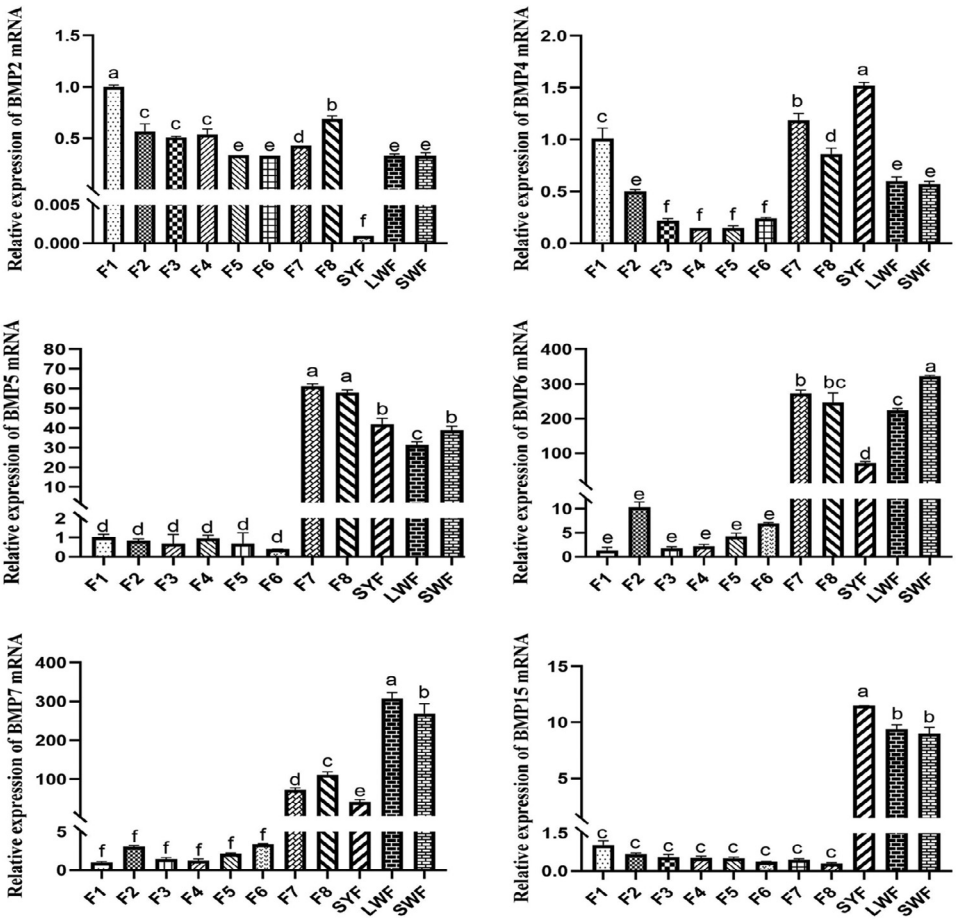

Figure 1. Analysis of the relative expression level of BMPs in pre-hierachycal and hierachycal follicles. F1 to F8 represent hierachycal follicles, SYF means small yellow follicle, LWF means large white follicle, SWF means small white follicle.

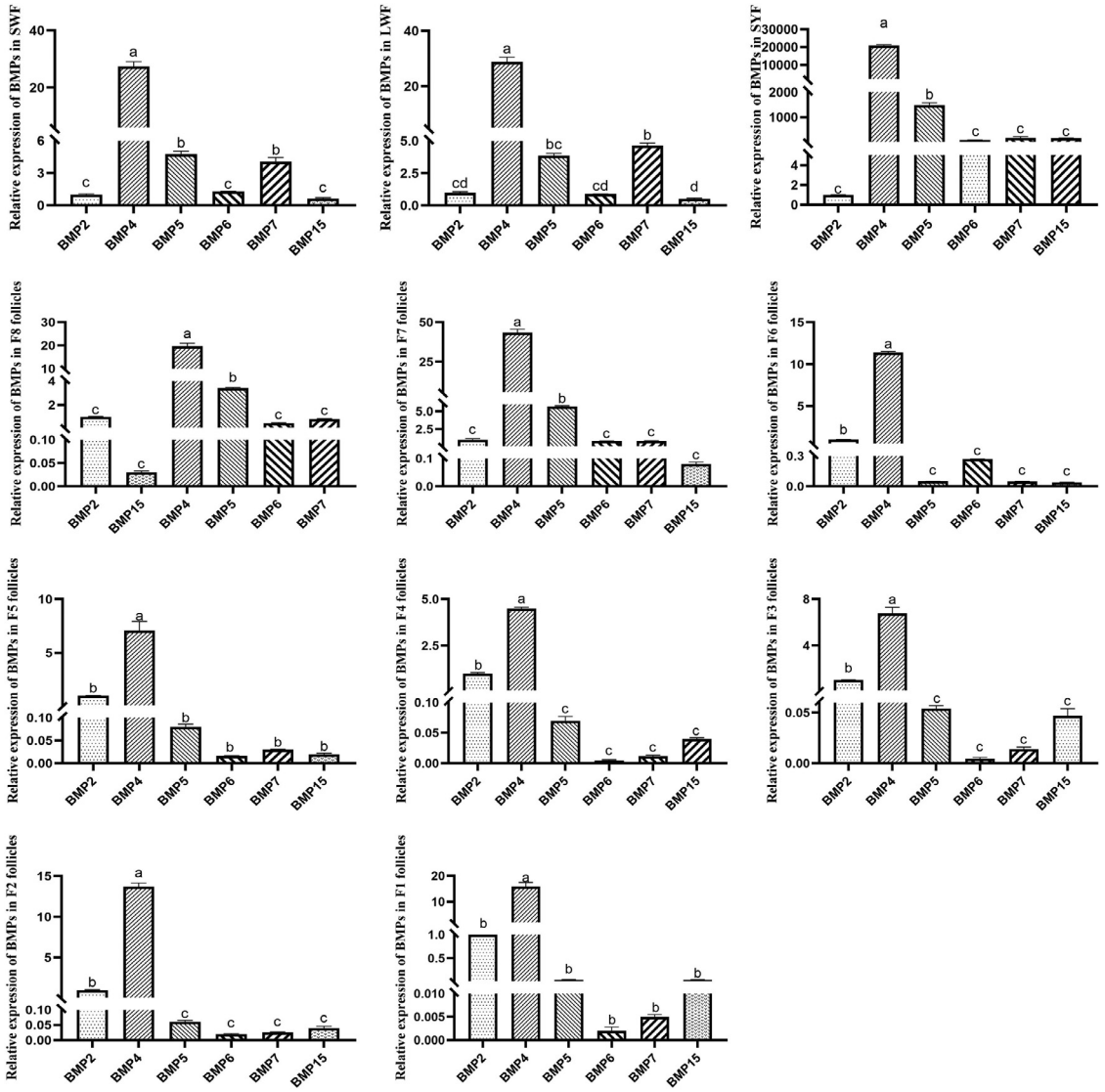

Figure 2. Comparison of the relative expression level of BMPs among each other in each follicle. F1 to F8 represent hierachycal follicles, SYF means small yellow follicle, LWF means large white follicle, SWF means small white follicle.

highest at F1 follicle which was near maturation and ovulation, and showed the lowest expression in pre-hierarchical follicles (Figure 1). The BMP4 expression showed the highest in small yellow follicle (**SYF**), and then the F7 follicle. The pre-hierarchical follicles of large white follicle (**LWF**) and small white follicle (**SWF**) showed lower expression of BMP4 as compared with F1 follicle, and higher expression as compared with F3 to F6 follicles. The BMP5 expression showed higher in F7, F8, and pre-hierarchical follicles as compared with hierarchical follicles of F1 to F6. The BMP7 expression showed highest in LWF, and then SWF, which was significantly higher than that of SYF, F7 and F8 follicles. The BMP15 expression showed only higher expression at pre-hierarchical follicles of SYF, LWF, and SWF as compared with that of hierarchical follicles of F1 to F8. The expression of BMP6 was highest in SWF, and also showed the lowest expression in F1 to F6 hierarchical follicles.

The expression level of BMPs in each follicle was also compared (Figure 2). The expression of BMP4 was significantly higher in all types of follicles than that of other BMPs ($P < 0.05$). While the expression of BMP5 was significantly higher in SYF, F7, and F8 follicles than that of other BMPs ($P < 0.05$).

The mRNA Expression Level of BMP Receptors in Each Follicle The BMP receptor Ⅱ, BMPR2, and BMP receptor Ⅰ, BMPR1B, were both exhibited basal expression in pre-hierarchical and hierarchical follicles although higher expression were exhibited in follicles F1 to F6 than that of F7, F8, LWF, and SWF. The BMP receptor Ⅰ, ALK2 and TGFBR1, were both exhibited basal expression in all of the follicles, and F1 follicle showed the highest expression level (Figure 3).

The mRNA Expression of SMAD8 Was Significantly Elevated in Pre-hierarchical Follicles

The expression of SMAD1 was highest in F1 follicle, and then the F2 to F5 follicles (Figure 4). Its expression

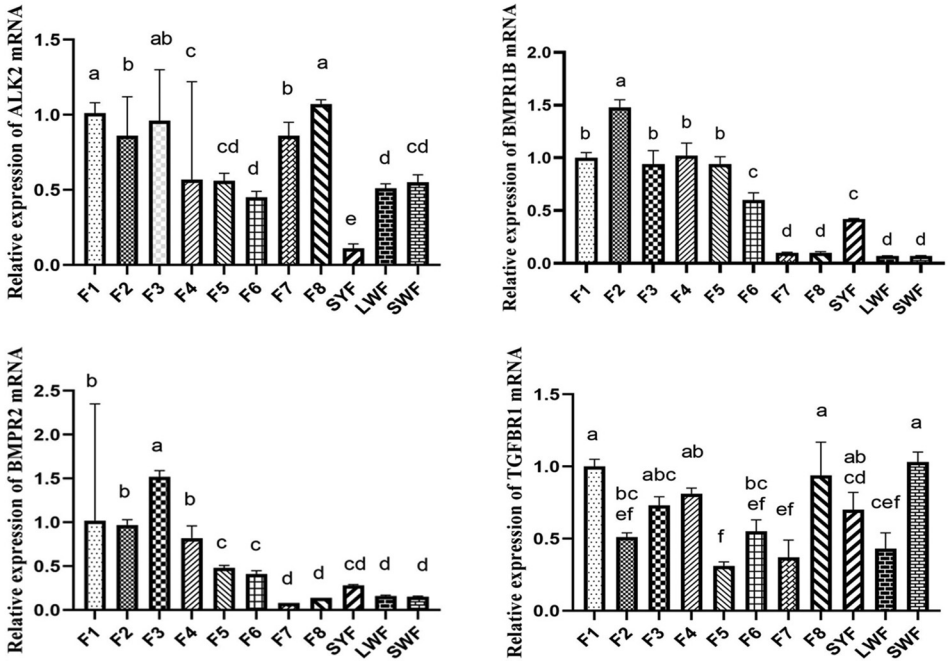

Figure 3. Analysis of the relative expression level of BMPs receptors in pre-hierachycal and hierachycal follicles. F1 to F8 represent hierachycal follicles, SYF means small yellow follicle, LWF means large white follicle, SWF means small white follicle.

level in F6 follicle was also higher than that of F7, F8, and SYF (Figure 4). The expression of SMAD2 was highest in F2 and F3 follicles. The expression of SMAD2 was higher in F4 and F5 follicles than that of F7, F8, LWF, and SWF. The expression of SMAD3 was highest in F3 follicle, and its expression in F1 to F4 follicles was higher than that of F7 to SWF. The expression of SMAD4 was higher in F2 follicle and SYF than that of

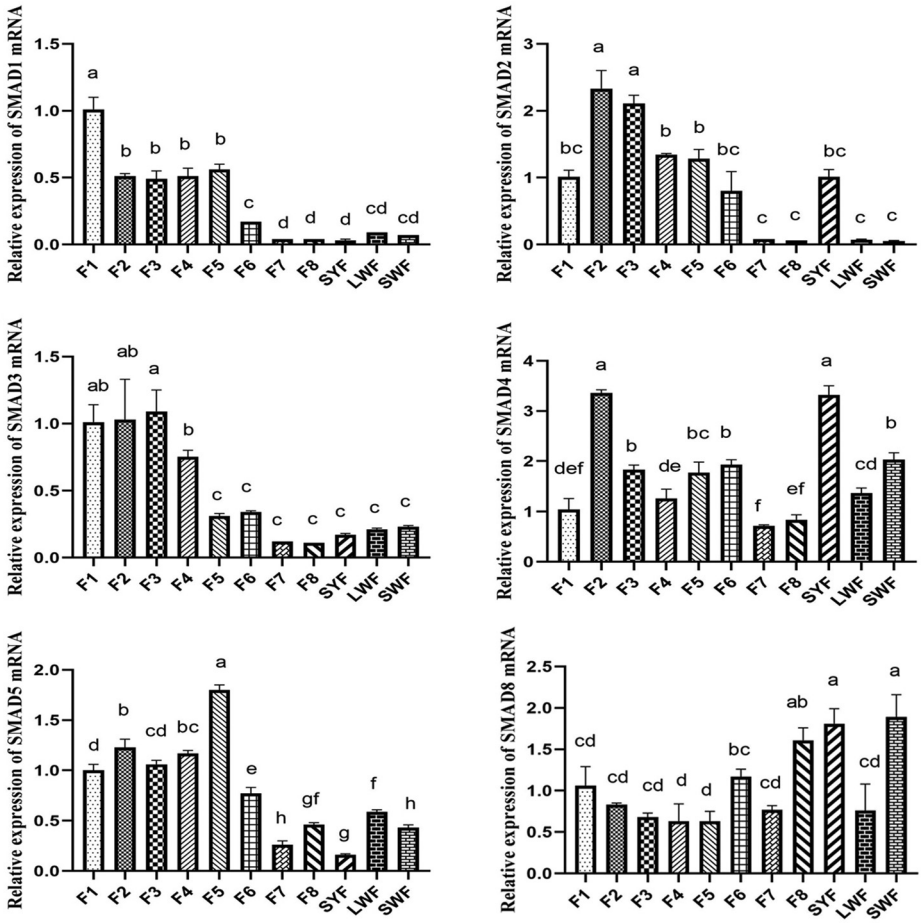

Figure 4. Analysis of the relative expression level of SMADs in pre-hierachycal and hierachycal follicles. F1 to F8 represent hierachycal follicles, SYF means small yellow follicle, LWF means large white follicle, SWF means small white follicle.

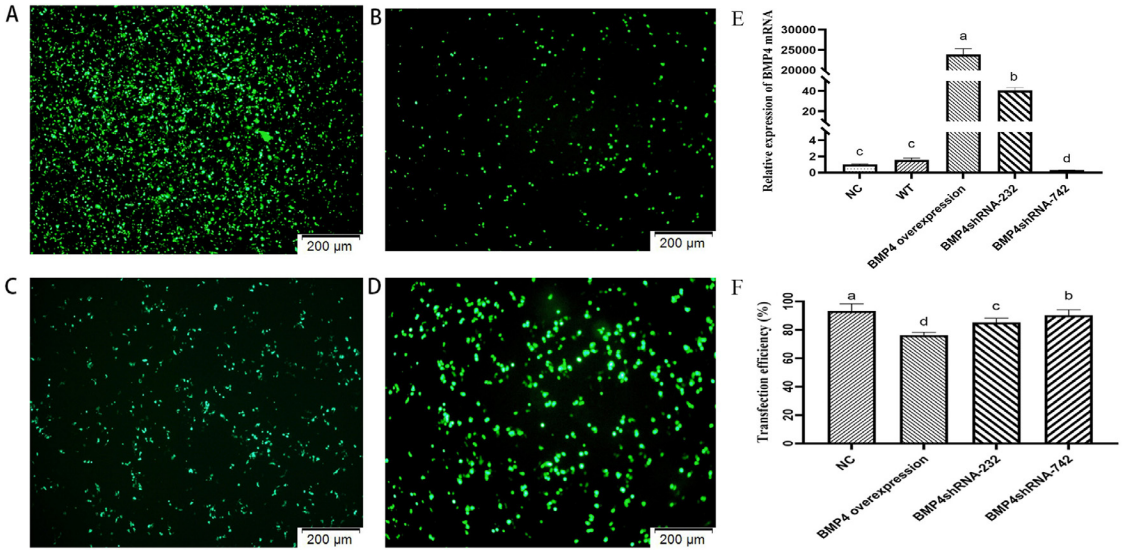

Figure 5. The efficiency of BMP4 overexpression and knockdown plasmid transfection into granular cells. (A) Granular cells transfected with PCDNA3.1-EGFP as NC. (B) Granular cells transfected with shRNA232. (C) Granular cells transfected with shRNA742. (D) Granular cells transfected with pEGFP-BMP4. (E) Transfection efficiency of granular cells with BMP4 overexpression or knockdown plasmids. (F) Relative expression of BMP4 in granular cells transfected with each plasmid, WT means granular cells without any transfection treatment.

other follicles. The expression of SMAD5 was higher in F5 follicle than that of other follicles. The expression of SMAD5 was higher in hierarchical follicles of F1 to F6 than that of pre-hierarchical follicles. The expression of SMAD8 was higher in SYF and SWF than that of hierarchical follicles of F1 to F7 (Figure 4).

Confirmation of Overexpression and Interference Efficiency of BMP4

To investigate the role of BMP4 on the proliferation of follicle granular cells in goose, the granular cells were transfected with pEGFP-BMP4, over-NC, shRNA232, and shRNA742 (Figures 5A−5E). The expression of BMP4 was significantly upregulated in cells transfected with pEGFP-BMP4 as compared with over-NC (Figure 5F). The shRNA742 had the higher interference effect on the expression of BMP4 and was selected for the following experiment.

BMP4 Overexpression Promoted the Proliferation of Follicle Granular Cells in Goose

The proliferation and apoptosis of goose granular cells after transfection with pEGFP-BMP4, PCDNA3.1-EGFP as NC and shRNA742 were assessed by CCK-8 assay and TUNEL assay (Figure 6). The results obtained from CCK-8 assay showed that the proliferation of granular cells was significantly higher in pEGFP-BMP4 as compared with the NC and shRNA742 (Figure 6A). The expression level of cell proliferation related gene of PCNA also showed highest in pEGFP-BMP4 transfected cells as compared with the NC and shRNA742 transfected groups (Figure 6B). The TUNEL assay showed that granular cells transfected with shRNA742 had a higher number of cell apoptosis as compared with the NC and pEGFP-BMP4 transfected groups (Figures 6C−6F). However, the expression level of Caspase-3 and Bax showed no difference between pEGFP-BMP4 and shRNA742 transfected cells (Figures 6G−6H), which suggested that the apoptosis of granular cells with BMP4 knockdown was not regulated by Caspase-3 signaling pathway.

SMAD4 Positively Regulate the Expression of Genes in Cell Cycle

Western blotting was first used to detect whether the anti-SMAD could effectively bind to SMAD4. As shown in Figure 7A, the anti-SMAD4 bound to the prepared samples in high affinity.

The genome-wide target sites of SMAD4 in BMP4 overexpressed granular cells was analyzed using the ChIP-seq approach. There were 4,653 peaks corresponding to 13,737 RefSeq genes (Figures 7B and 7C). The peaks over chromosomes suggested different peak values with high values of peaks on chromosome 1 (Figure 7B). The BMP4 binding sites located in the transcription start site (**TSS**) accounted for 2.5% of the total readings (Figure 7D). Gene ontology (GO) analysis of the peak-related genes showed that the BMP4 peak-related genes were involved in various biological processes, such as biological regulation, regulation of biological process, regulation of cellular process, signal transduction, signaling (Figure 7E). Kyoto Encyclopedia of Genes and Genomes (**KEGG**) analysis showed that the BMP4 peak-related genes were significantly enriched in Phosphatidylinositol signaling system, Glutamatergic synapse, Adherens junction, cGMP-PKG signaling pathway, Ras signaling pathway, etc (Figure 7F). The motifs shared between the peaks were scanned by

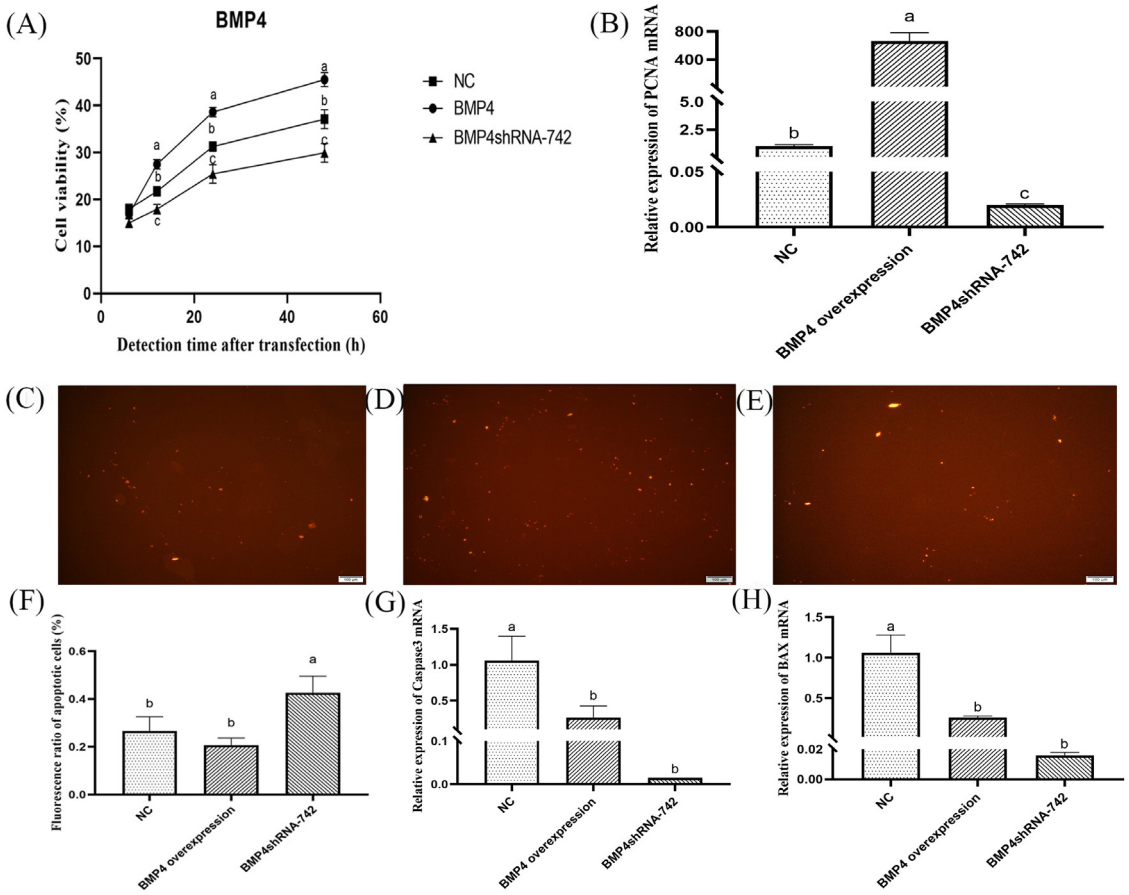

Figure 6. The proliferation or apoptosis of granular cells transfected with BMP4 overexpression or knockdown plasmids. (A) Cell viability after transfected with plasmids of BMP4 overexpressed or knockdown. (B) Effects of BMP4 overexpression or knockdown on *PCNA* expression. (C) Apoptosis of granulosa cells after transfected with BMP4 overexpressed plasmid. (D) Apoptosis of granulosa cells after transfected with BMP4 knockdown plasmid shRNA742. (E) Apoptosis of granulosa cells after transfected with PCDNA3.1-EGFP. (F) Granular cell apoptotic ratio after transfected with BMP4 overexpressed or knockdown plasmid. (G) The expression level of Caspase3 on granular cells transfected with BMP4 overexpressed or knockdown plasmid. (H) The expression level of Bax on granular cells transfected with BMP4 overexpressed or knockdown plasmid.

HOMER motif scanning from the ChIP-seq data, a variety of known and de novo motifs were found. Known motifs enriched in the top ten were all belonged to TFs, which could all be classified into basic leucine zipper factors (**bZIP**) and zinc finger (**ZF**) families. Five motifs with the most significant differences were selected for display (Figure 7G). The motifs could bind to many TFs, and an interaction network diagram was shown according to the corresponding relationship between TFs and genes (Figure 7H). The five motifs all located at the TSS of BAMBI and CPEB2 genes. The qRT-PCR of BAMBI and CPEB2 genes in over-expressed granular cells found that BAMBI was in low expression in BMP4 overexpressed granular cells and in high expression in BMP4 knockdown granular cells, while CPEB2 was in high expression in both BMP4 over-expressed and knockdown granular cells (Figure 7I).

DISCUSSION

Follicular development, maturation and ovulation directly determine the number of egg production in birds. In goose, especially the indigenous breeds, egg production is much lower as compared with that of hen and duck (Xia et al., 2019; Zhai et al., 2022). Therefore, study of the regulatory mechanism of follicular development could provide signals for promoting follicular development and improving egg production in indigenous goose breed.

It is demonstrated that BMP/SMAD signaling pathway related factors play a regulatory role in promoting the entry of pre-hierarchical follicles into hierarchical selection (Xu et al., 2015). The lack of SENP1 in mouse ovarian stromal cells could lead to the downregulation of BMP4, which delayed oocyte growth and follicle maturation, and reduced follicle number and size in the early stage of oocyte development (Tan et al., 2017), indicating that BMP4 plays a key role in the early stage of oocyte selection. It was also found that exogenous addition of BMP4 could significantly increase the transition from primordial follicles to primary follicles in mouse ovary (Ding et al., 2013). These suggested that BMP4 need to be maintained in high expression level especially in the early pre-hierarchical follicles in responsible for follicle selection in goose. However, BMP4 was expressed in low level in all follicle granular layers and only showed a higher expression in SYF, which suggested that BMP4

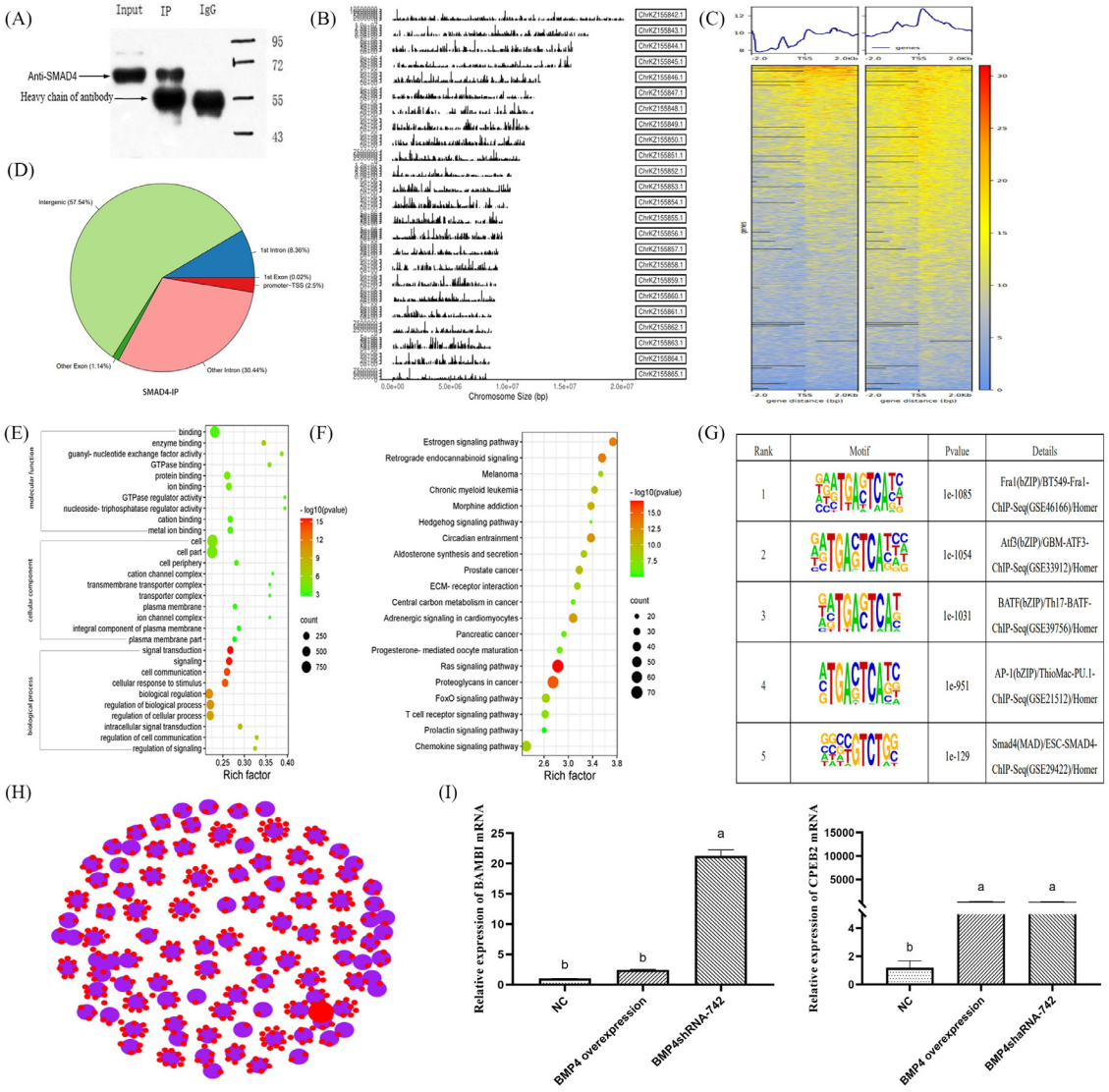

Figure 7. Identification of genome-wide DNA binding sites and transcription targets for BMP4 by ChIP-seq. (A) Detection of the immunoprecipitation efficiency of BMP4 overexpressed granular cells with SMAD4 antibody binding. (B) ChIP peaks over chromosomes by ChIP-seq using the primary antibody against BMP4 (the right represents the chromosome number, the left ordinate represents the peak value of each chromosome). (C) The distribution of reads on both sides of the transcription start site (TSS). (D) Pie diagram showed the ratios of BMP4 binding sites located relative to a transcription unit including intergenic, 1st intron, 1st exon, promotor, other intron and other exon. (E) GO enrichment of Peak related genes (top ten terms of each category). (F) KEGG enrichment map of metabolic pathways of Peak related genes (top 20 pathways). (G) Five common motifs with the most significant differences among peaks. (H) Interaction network diagram between TFs and genes based on sequencing results, purple represented the transcription factor or transcription factor family, red represented the target gene that TF may act through motifs. (I) Validation of the expression level of annotated genes BAMBI and CPEB2 in granular cells with BMP4 overexpression or knockdown plasmid.

might not be the only regulator for follicle selection in Wanxi white goose.

BMP6 is selectively expressed in bovine granular cells and oocytes through autocrine or paracrine, thus to promote granular cells proliferation and steroidogenesis, to activate the follicle development (Glister et al., 2004). In poultry, the growth of follicle diameter from 0.5 to 1 mm is a crucial transition which determined the development or atresia of follicles (Anthong et al., 2009). As compared with follicles at 0.5 mm diameter, the expression level of BMP6 decreased 50 to 90% of follicles at 1 to 4 mm diameter (Anthong et al., 2009), which suggested that BMP6 is mainly expressed in pre-hierarchical follicles. In this study, BMP6 was only highly expressed in SWF which indicated that BMP6 might responsible for promoting folliculogenesis in goose. Similar to the expression pattern of BMP6, the expression of BMP7 and BMP15 were also in high level in SWF and LWF. BMP7 was exclusively expressed in follicle cells while BMPRs were expressed in both oocytes and follicle cells. BMP7 might play a role in the suppression of DHP-induced oocyte maturation by affecting autocrine/paracrine pathways in S. paramamosain (Yang et al., 2018). In buffalo granular cells, the expression of BMP7 increased with the follicle diameter and stimulated the secretion of estradial in cultured granular cells (Rajesh et al., 2018). In this study, the high level of BMP7 in SWF and LWF might indicate its function for initiating follicle activation in goose. BMP15 was expressed in all levels of follicles in buffalo with a higher expression level

in early stage of follicle and decreased expression during follicle growth, which indicated that BMP15 play regulatory role in the formation and maintenance of follicle development in buffalo (Liu et al., 2019). In this research, BMP15 only expressed in pre-hierarchical follicles also indicating its function in folliculogenesis in goose. BMP5 was expressed in high level of pre-hierarchical follicles and F7 and F8 hierarchical follicles in this research, suggesting its particular function in regulating folliculogenesis, follicular selection and maintaining hierarchical follicle development in the early stage of goose.

Through binding to BMPR1B, type Ⅱ receptor, BMP could phorsphorylate type Ⅰ receptor and promote the phosphorylation of downstream Smads. Silencing the expression of BMPR1B significantly inhibited the proliferation and estrodial secretion of granular cells, and induced the apoptosis of granular cells in porcine (Zhao et al., 2014). In mice, lack of BMPR1B exhibited infertility also suggesting the important role of BMPR1B in regulating follicle development (Renault et al., 2020). In porcine, the expression of TGFBR1 was highly upregulated during follicle selection and the expression could be detected in oocyte, granular cells and membrane cells, while BMPR2 showed no expression change in granular cells during follicle development (Paradis et al., 2009). Different from mammalian follicle, the expression of TGFBR1 and ALK2 were in background levels at all stage of goose follicles. In the bovine estrous cycle, the expression of BMPR2 decreased gradually with the increase of follicle diameter from third to sixth day (Spicer et al., 2013), while the expression of BMPR2 was elevated with follicle diameter of goose in this study, which might indicate that BMPR2 phosphorylates TGFBR1 or ALK2, and then promotes follicle selection and development.

SMAD family members are the key factors mediating the function of BMPs. SMAD8 was involved in goose follicle initiation. The polymorphism of SMAD8 could directly influence the egg production performance in goose (Xu et al., 2015). In this study, SMAD8 was highly expressed in the early stage of follicle further proved its involvement in goose follicle initiation. It was reported that SMAD1 was in quite different expression level in small tailed Han sheep with different fertility. The polymorphism of SMAD1 g.12487190G>T was related to the reproductive performance of sheep(Tian et al., 2019). It was also found that the expression of SMAD5 could affect the follicle development and ovulation, and expression of SMAD5 was regulated by miR-23a and miR-27a thus to regulate the apoptosis of granulosa cells in human (Nie et al., 2015). SMAD2 showed specific expression in granular cells of anterior lobe and early sinus follicles of mouse. Knockdown the SMAD2 could reduce P450 aromatase expression, indicating its regulatory role of hormone production (Nomura et al., 2013). Study has found that SMAD3 deficient mice could not ovulate and the expression of FSH receptor and aromatase was in low level, suggested its function in regulating the response of ovarian follicles to FSH (Gong et al., 2009). In this study, SMAD1, SMAD2, SMAD3, and SMAD5 were all highly expressed in the hierarchical follicles, indicating their function in follicular maturation in goose.

It is demonstrated that activated SMADs bind to SMAD4, and the heterodimer transports to nucleus and binds to the transcriptional regulatory region to activate the expression of downstream genes. SMAD4 is widely expressed in porcine ovary and is mainly located in oocytes, granular cells and membrane cells at different follicle stages (Xing et al., 2014). Silencing SMAD4 in porcine granular cells could inhibit its proliferation and cell cycle process (Wang et al., 2011), indicating that SMAD4 is a key regulator for follicle growth and involved in follicle regulation at all stages. Knockdown of SMAD4 could not induce the expression of stearoyl CoA desaturase (**SCD**) in granular cells, while overexpression of SMAD4 could restore the expression of SCD (Samuel et al., 2002), indicating that SMAD4 participates in the lipid metabolism. In this study, SMAD4 showed a basal expression in all follicle stages and a little higher in SYF, F7 and F1 follicles, this might responsible for lipid synthesis and follicle maturation and ovulation.

In BMP4 overexpressed granular cells, we found that SMAD4 could effectively combine to the TSS region of BAMBI and CPEB2 genes. Cytoplasmic polyadenylation element binding protein 2(**CPEB2**) regulates the translation of maternal mRNAs controlling meiotic cell cycle progression, and also expressed in various combinations in regulation of mitosis-related gene expression, especially in sustaining polyadenylation dynamics during the M-phase of cell cycle (Yu et al., 2019). Activin membrane binding inhibitor (BAMBI) is a transmembrane and acts as an antagonize signal of TGF superfamily member, including TGF-β and BMPs, to negatively regulates adipogenesis, and modulates as an anti proadipogenic effect of BMPs (Luo et al., 2012). It is therefore speculated that SMAD4 could bind to the TSS region of CPEB2 so as to ensure the granular cell proliferation, and the down regulation of BAMBI in granular cells with BMP4 overexrpession could transcriptionally maintain the normal lipid synthesis of granular cells.

CONCLUSIONS

High expression of BMP4 in granular cells of SYF of goose could bind to BMPR2 and then phosphorylate Ⅰ type receptor, this phosphorylated heterodimer further phosphorylates SMAD8, the phosphorylated SMAD8 bind to SMAD4 and transfers to the nucleus, and bind to the TSS region of BAMBI gene, regulating lipid synthesis of granular cells.

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DISCLOSURES

The authors have no conflicts of interests.

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