104 (2025) 105027

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj

Exploring the functional variations of key candidate genes affecting egg production by hypothalamic-pituitary-ovarian axis in chickens

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ARTICLE INFO

Keywords:
Chicken
Egg production
Functional variants
Genetic regulation

ABSTRACT

Potential genetic variants associated with chicken egg production traits have been extensively identified, most of which are located in the non-coding regions of the genome. However, which functional variants really drive the egg-laying phenotype change remain elusive. In the present study, by integrating the previously screened egg-laying related candidate genetic variants, transcriptome data derived from 16 high- and low-yield Gushi chickens, and epigenomic analyses, 22 potential functional variants (PFVs) were systematically identified. These PFVs potentially drive the differences in egg production phenotypes by affecting the expression of 10 egg-laying related key candidate genes in the hypothalamus (*ZNF804B, DPP10, NEO1* and *GABRG1*), pituitary (*DPP10* and *GNG7*), and ovary (*PHIP, OSTN, GADD45B, NFXL1* and *ADAMTS17*). Subsequently, the regulatory activity and function of one PFV, chr3:79510218A>T, located in the third intron of the pleckstrin homology domain interacting protein gene (*PHIP*), were investigated in chicken ovarian granulosa cells. Association analysis confirmed the significant association of chr3:79510218A>T with egg number from 21 to 43 week of age in Gushi chicken ($P = 0.0022$) and Guangxi Yao chicken ($P = 0.0388$), as well as with ovarian *PHIP* expression levels ($P = 0.0010$). Functional analysis indicated that the T allele of chr3:79510218A>T enhanced the transcriptional activity and upregulated *PHIP* expression in vitro by binding transcription factor forkhead box I1 (FOXI1). Furthermore, the knockdown of *PHIP* led to the inhibition of ovarian granulosa cell proliferation and a reduction in the synthesis and secretion of progesterone (PROG) hormones. Collectively, this study unveil the egg-laying related functional variants and illustrate a potential genetic regulation mechanism, and will help accelerate the molecular design breeding process of chicken egg production.

Introduction

Egg production is economically most important trait in both layers and broilers. The egg production level not only serves as an indicator of the reproductive capacity of breeder hens but also has a direct impact on the economic benefits of breeding enterprises. Consequently, enhancing chicken egg production has long been a primary objective in poultry breeding. Nevertheless, due to the relatively low heritability of this trait, genetic progress via traditional breeding strategies relying on phenotypic selection has been slow (Du et al., 2020). Currently, understanding the genetic basis of chicken egg production traits and mining effective molecular markers for genetic breeding selection are the focal points of research for poultry breeders.

Over the past few decades, numerous genetic variants widely located on multiple chromosomes were excavated based on multiple egg-laying related traits including age at first egg, egg number, clutch size and egg-laying rate (Wolc et al., 2019; Du et al., 2020; Ding et al., 2022; Wang et al., 2024). These findings suggest that the genetic control of egg production traits is minor and polygenic. However, among the multitude of potential variants, it remains unclear which ones are the actual drivers of the egg-laying phenotype change. Studies have shown that almost 90% of all phenotype-associated variants identified by genome-wide association studies (GWAS) are located in non-coding regions of the genome (Pan et al., 2023). This implies that the regulatory activity of genetic variants in non-coding regions, namely functional variation, is the main driver of phenotypic variation. Therefore, the identification of functional variants and the elucidation of their genetic regulatory mechanisms will help expedite the molecular design breeding

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https://doi.org/10.1016/j.psj.2025.105027
Received 24 December 2024; Accepted 11 March 2025
Available online 13 March 2025
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process for chicken egg production.

Chicken egg-laying process is a highly coordinated reproductive activity strictly controlled by the hypothalamic-pituitary-ovary (HPO) axis. This process involves the regulation of various secreted hormones to facilitate a series of activities, including ovarian development and ovulation (Kanda et al., 2019; Shi et al., 2024).

In HPO axis, the hypothalamus functions as the central regulator that activates the HPO axis. It integrates internal and external signals to synthesize and secrete neuroendocrine hormones, such as Gonadotropin-releasing hormone (GnRH). Meanwhile, the pituitary receives neuroendocrine signals from the hypothalamus to activate the function of hormone-secreting cell populations to regulate the levels of reproductive hormones, such as Follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in the bloodstream (Tsutsui et al., 2022). The development and maturation of ovarian follicles directly determine the age at first egg and the overall egg production (Johnson et al., 2009). Previous studies, based on HPO axis tissues from multiple chicken breeds, have explored numerous potential candidate genes involved in the regulation of egg-laying difference, follicle selection, and reproductive hormone synthesis (Ma et al., 2021; Wang et al., 2022; Li et al., 2024; Li et al., 2025). Recently, by integrating GWAS of egg production traits in Gushi chicken, comparative genomic analysis of layers and native chickens, and transcriptome data of HPO axis tissues in high- and low-yield Gushi hens, we have identified the potential candidate genes that may be regulated by genetic variation in driving egg production differences (Wang et al., 2024). However, it has not been clarified which functional variants influence egg production traits by affecting the expression of candidate genes in the hypothalamus, pituitary or ovary.

In the present study, a systematic screening of key candidate genes in the HPO axis and their adjacent genetic variants influencing egg production was conducted. Subsequently, potential functional variants (PFVs) affecting the expression of key candidate genes were explored through epigenetic annotation and association analysis between genotypes and gene expression. Functional experiments were carried out on a PFV, chr3:79510218A>T, located in the intron of the pleckstrin homology domain interacting protein gene (*PHIP*), for further verification. The results demonstrated that the intronic SNP chr3:79510218A>T functions as a functional variant to regulate the expression of PHIP in the ovary, thereby influencing egg production in chickens.

Materials and methods

Ethics statement

All animal experiments and sample collection were performed in accordance with the guidelines of Institutional Animal Care and Ethics Committee, Permit No. LLSC2024042, Henan Institute of Science and Technology.

Principal component analysis of egg-laying related significant SNPS mapping to potential candidate genes in multiple chicken breeds

Egg-laying related significant SNPs ($P < 0.01$) mapping to the 2 kb promoter region and gene body of potential candidate genes were screened based on the genetic association with egg production traits, including egg number (EN) at different laying periods (EN21-25w, EN26-30w, EN31-35w, EN36-43w, EN31-43w and ENT), average clutch size (ACS) at different laying periods (ACS21-25w, ACS26-30w, ACS31-35w, ACS36-43w, ACS31-43w and ACST) and maximum clutch size (MCS) (Wang et al., 2024). Genotypes of these SNPs were extracted from the whole-genome resequencing data of three different type breeds including layer breeds, native breeds and a wild breed (n = 65) and high- (n = 120) and low-yield (n = 119) Gushi chicken population, respectively (Wang et al., 2024). WL, White Leghorn layer; RIR, Rhode Island Red layer; GS, Gushi chicken; LS, Lushi chicken; XCBB, Xichuan Black Bone chicken; ZYSH, Zhengyang San Huang chicken; HNG, Henan Gamecock; RJF, Red jungle fowl. Principal component analysis (PCA) were used to identify of population characteristics of these SNPs in the above multiple chicken breeds or population.

Screening of key candidate genes affecting egg production via Hypothalamus-pituitary-ovarian axis

RNA-seq data of hypothalamus, pituitary and ovary from 43-week-old high- (n =8) and low-yield (n =8) Gushi chicken (NCBI-SRA: PRJNA953784) were used to screen key candidate genes from potential candidate genes. The information on population screening for 43-week-old high- and low-yield Gushi chicken were described previously (Wang et al., 2024). Key candidate genes were identified according to the criteria of Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) ≥ 1, |fold change between high- and low-yield group| ≥ 1.2 and Q-value (FDR) < 0.05.

Functional Epigenetic annotation of egg-laying related SNPs mapping to key candidate genes

We annotated functional regulatory features for egg-laying related SNPs using the epigenetic data of hypothalamus and shellgland from ChickenGTEx (https://ngdc.cncb.ac.cn/chickengtex/), including enhancer markers (H3K27ac and H3k4me1) or promoter markers (H3K27me3 or H3K4me3).

Experimental birds and sample preparation

In total, 874 female birds from the 12th generation of Gushi chickens and 283 female birds from the 3th generation of Guangxi Yao chickens were used separately for association analyses of SNPs with between egg number from 21 to 43 weeks of age (EN21-43w). All birds used in this study were kept in the similar environmental conditions with free water and a commercial diet. All birds were recorded for individual egg number until 43 weeks of age. Blood samples of all individuals were collected at 43 weeks of age by wing vein. Blood genomic DNA was extracted by using the phenol-chloroform method.

Thirty-five out of 283 female individuals of Guangxi Yao chickens from the 3th generation were used to validate the association of SNPs with *PHIP* expression level. They were humanely slaughtered to collect ovarian tissues at 43 weeks of age. All tissue samples were snap-frozen in liquid nitrogen, and stored at -80 °C until use.

Association study of the potential functional variation in PHIP with egg production traits or PHIP expression

An egg-laying related potential functional SNP chr3:79510218A>T mapping to the intron 3 of *PHIP* gene were used as an example to verify its functionality. The genotypes of chr3:79510218A>T in the 874 individuals of Gushi chicken population and 283 individuals of Guangxi Yao chicken population were detected by using Kompetitive Allele Specific PCR (KASP) technique (He et al., 2014). The association analysis between SNP genotypes and EN21-43w or *PHIP* expression were carried out by mixed linear model.

Chicken ovarian Granulosa cell isolation, culture and identification

The 6-12 mm prehierarchical follicles of 35-week-old hens was used to isolate granulosa cells according to our previous protocol (Wang et al., 2024). In brief, the intact ovarian tissue isolated from chicken abdominal cavity was rapidly placed into PBS supplemented with 3% penicillin/streptomycin (Solarbio, Beijing, China). The follicle granular layer was isolated and washed in PBS supplemented with 1% penicillin/streptomycin, then placed in a 1.5 ml centrifuge tube and cut into pieces.

The cell suspension digested with 1 mg/ml collagenase type II

(Solarbio, Beijing, China) was filtered through a 200-mesh cell sieve, then resuspended with M199 medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and 1% penicillin/streptomycin to obtain dispersed granulosa cells. The cells were incubated at 37 °C with 5% CO2.

The granulosa cells were identified by two methods. 1. Monitoring the specificity of a granulosa cell marker gene, follicle-stimulating hormone receptor gene (*FSHR*) expression by comparing mRNA levels of *FSHR*, in granulosa cells with other cells such as LMH cell lines, and chicken abdominal preadipocytes. 2. FSHR is a granulosa membrane receptor protein, and its immunofluorescence localization on cells can be used as a visualization method for chicken ovarian granulosa cell identification.

Plasmid construction and siRNA synthesis

The adjacent 100 nucleotides of chr3:79510218A>T was amplified from genomic DNA and cloned into the pGL3-promoter vector (Promega, WI, USA) upstream of the SV40 promoter to construct luciferase reporter plasmids. The primers are listed in Table S1.

The small interfering RNA (siRNA) of *FOXI1* (si-FOXI1) and a negative control (si-NC) were synthesized from Beijing Tsingke Biotech Co., Ltd. (Tianjin, China) to investigate the effects of *FOXI1* on *PHIP* expression and the transcriptional activity of reporter gene carrying chr3:79510218A>T. The *PHIP* siRNA (si-PHIP) and a negative control (si-NC) were synthesized to investigate the biological function of *PHIP* in chicken ovarian granulosa cells.

Dual-Luciferase reporter assay

For independent transfection, 0.9 μg of the constructed luciferase reporter plasmids and 0.1 μg of pRL-TK Renilla luciferase vector (Promega, WI, USA) as an internal control were transfected into chicken ovarian granulosa cells by using the transfection reagent LipofectamineLTX (Invitrogen, Carlsbad, CA). For the cotransfection, 0.2 μg of the constructed luciferase reporter plasmids, 0.02 μg of pRL-TK Renilla luciferase vector, and 0.78 μg of si-FOXI1 were transfected into granulosa cells. After 36 h of transfection, luciferase activity was detected by using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). Fireflyluciferase activity was normalized to Renilla luciferase.

Functional validation of PHIP in vitro

Granulosa cells cultured in 12-well plates were transfected with si-PHIP and si-NC, respectively. After 36 h of transfection, The cells were collected to detect the expression of cell proliferation marker genes, apoptosis related genes, follicle growth and differentiation related genes, and steroid hormone synthesis pathway genes. The cell supernatants were collected to measure progesterone (PROG) and estradiol (E2) hormone levels by using PROG ELISA kit (MM-114302, Jiangsu, China) and E2 ELISA kit (MM-078702, Jiangsu, China), respectively.

Granulosa cells cultured in 96-well plates were transfected with si-PHIP and si-NC, respectively. Cell proliferation was evaluated at 12, 24, 36, and 48 h after transfection using cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan).

Granulosa cells cultured in 24-well plates were transfected with si-PHIP and si-NC, respectively. The numbers of living granulosa cells were counted using 5-ethynyl-2'-deoxyuridine (EDU) assay at 36 h post-transfection. The cells were first incubated with 10 μM EdU medium (Ribobio, Guangzhou, China) at 37°C, and then fixed with 4% paraformaldehyde at room temperature. The fixed cells were stained, and then imaged by a fluorescence microscope (Olympus, Tokyo, Japan).

cDNA synthesis and gene expression analysis

Total RNA of ovarian tissue and granulosa cells were extracted with TRIzol reagent (Vazyme, Nanjing, China), and 1 μg of total RNA per reaction was used to synthesize the first-strand cDNA (Vazyme, Nanjing, China). To detect the gene expression levels, quantitative real-time PCR (qRT-PCR) was performed with ChamQ Universal SYBR qPCR Master Mix kit (Vazyme, Nanjing, China) by LightCycler® 96 Instrument (Roche Applied Science, IN, USA). All samples were tested in triplicate. The relative expression level of each gene to the internal control gene (Glyceraldehyde-3-phosphate dehydrogenase, GAPDH) was determined using the $2^{-\Delta\Delta ct}$ method. The specific primers used for qRT-PCR were designed across the intron (Table S1).

Statistical analysis

SPSS 23.0 (IBM, Chicago, IL, USA) were used for all statistical analyses. The generalized linear mixed model was used to analyze the association between the genotype of chr3:79510218A>T and EN21-43w or *PHIP* expression.

The model used was as follows:

$$Y_{ijl} = \mu + G_j + f_l + e_{ijl}$$

In the model, Y_{ijl} was the individual phenotypic value of EN21-43w or *PHIP* expression, μ was the observation mean, G_j was the fixed effect of genotype (j = genotypes), f_l was the fixed effect of hatching batch (j =1, 2) and e_{ijl} is the random error (Wang et al., 2022). And Least significant difference (LSD) test was used to calculate the *P* value of the least squares means of the different genotypes.

An independent-samples T-test was used to calculate the *P* value of the differences between the two groups. The results were presented as the mean ± SEM. * $P < 0.05$, and ** $P < 0.01$.

Results

Key candidate genes affecting egg production via Hypothalamus-

Pituitary-ovarian axis

Based on three GWAS analysis methods, we had screened the potential small-effect and pleiotropic variants related to chicken egg production traits in our previous study, and obtained 1479 annotated GWAS overlapping genes (GWAS-OGs) (Fig. S1, Table S2) (Wang et al., 2024). These GWAS-OGs were further intersected with 1165 potential selected genes (PSGs) during domestication or breeding of layer breed, we obtained 94 overlapping genes as potential candidate genes, which were strongly selected and significantly associated with egg production traits (Fig. 1A, Table S3).

921 significant association SNPs ($P < 1 \times 10^{-4}$) distributing in 2 kb promoters and gene body of 67 of 94 potential candidate genes were extracted from genomic sequencing data of multiple chicken breeds (Table S4). Principal component analysis (PCA) based on the 921 SNPs in multiple breeds showed that two layer populations (WL and RIR) were separated from wild and native populations (Fig. 1B), implying that these egg-laying related genetic variations in the specialized layer breeds were indeed subject to intensive artificial selection during breeding. In Gushi (GS) chicken population, there was also some degree of genetic differentiation between high- and low-yield GS chickens, but the genetic diversity was relatively high (Fig. 1C), hinting at the potential for the laying-oriented genetic selection in the further.

In the 67 potential candidate genes, only 10 genes were significantly differentially expressed in hypothalamus (*ZNF804B, DPP10, NEO1* and *GABRG1*), pituitary (*DPP10* and *GNG7*) or ovary (*PHIP, OSTN, GADD45B, NFXL1* and *ADAMTS17*) of the 43-week-old high- and low-yield GS chickens (Fig. 2, Table S5-7), were therefore considered as key candidate genes for screening functional variants.

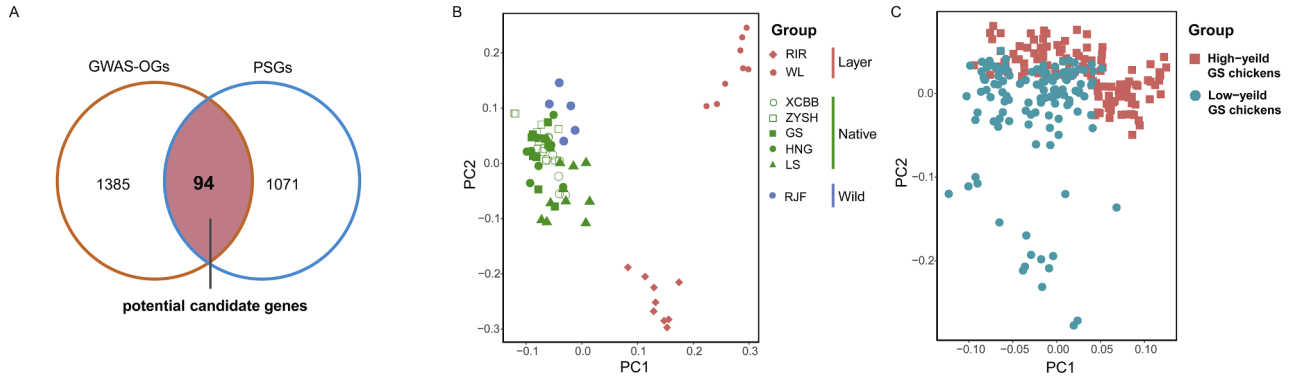

Fig. 1. Screening of potential candidate genes related to chicken egg production traits and population characteristics of their nearby variants. (A) Screening potential candidate genes associated with egg production traits by intersecting GWAS overlapping genes (GWAS-OGs) and potential selected genes (PSGs). GWAS-OGs represent genes based on annotation of potential variants associated with chicken egg production traits via three GWAS analysis methods. PSGs represent genes based on annotation of selected region during domestication or breeding of layer breed. (B) and (C) Principal component analysis (PCA) plots based on SNPs mapping to the 2 kb promoter region and gene body of 94 potential candidate genes in multiple breeds and high- and low-yield Gushi chicken population. WL, White Leghorn layer; RIR, Rhode Island Red layer; GS, Gushi chicken; LS, Lushi chicken; XCBB, Xichuan Black Bone chicken; ZYSH, Zhengyang San Huang chicken; HNG, Henan Gamecock; RJF, Red jungle fowl.

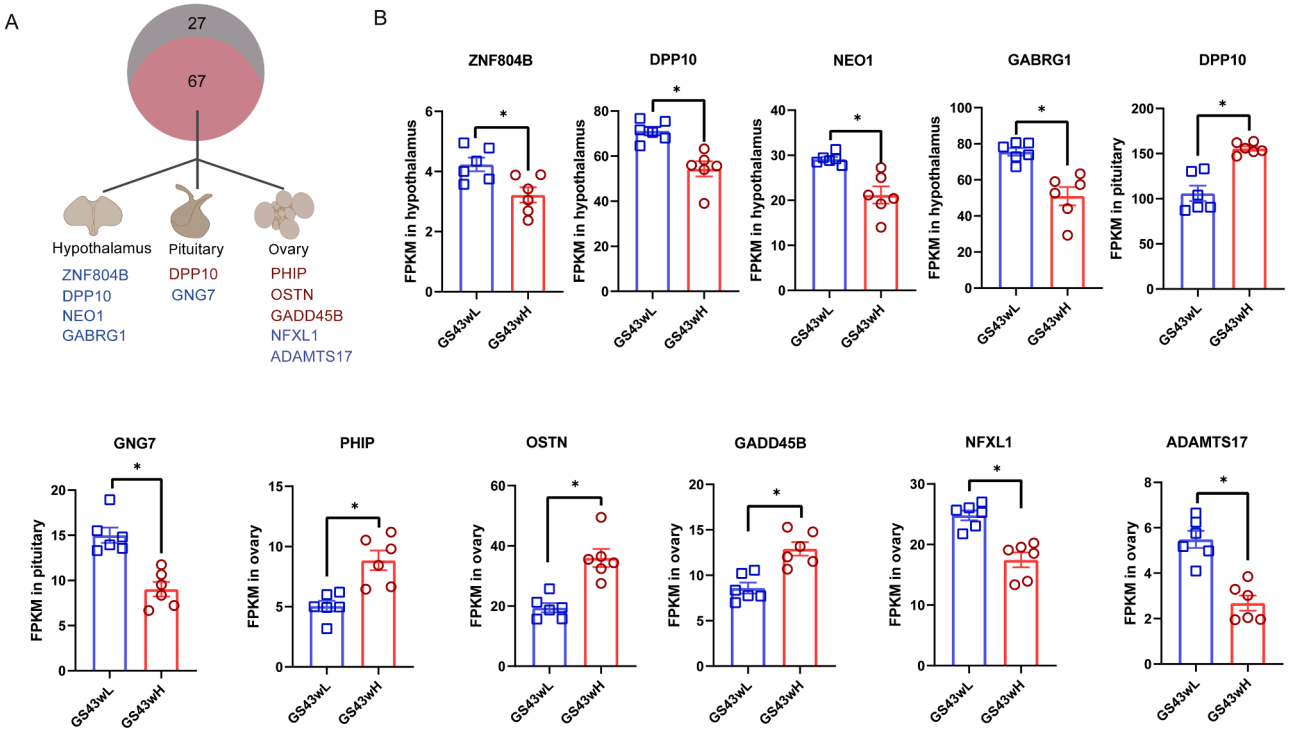

Fig. 2. Key candidate genes affecting egg production via hypothalamus-pituitary-ovarian axis in chicken. (A) Screening of key candidate genes by overlapping the potential candidate genes and the RNA-seq data of hypothalamus, pituitary and ovary in 43-week-old high- and low-yield Gushi chicken. Red and fonts indicate the differentially expressed genes (DEGs) that were significantly up-regulated and down-regulated expression in the high-yield group, respectively. (B) Expression of 10 key candidate genes in hypothalamus, pituitary or ovary of 43-week-old high- (GS43wH) and low-yield Gushi chicken (GS43wL). The data are presented as mean ± SEM (n =6 for each group). * Q-value < 0.05.

Potential functional variations affecting the expression of key candidate genes

There were 599 egg-laying related SNPs ($P < 1 \times 10^{-2}$) in Gushi chicken mapping to the 10 key candidate genes (Fig. 3, Table S8). To evaluate the functionality of SNPs, we used the epigenetic data of hypothalamus and shellgland in ChickenGTEx (https://ngdc.cncb.ac.cn/chickengtex/) to annotate these variant sites. 22 SNPs were located within the genomic regions of the strong epigenetic enhancer markers (H3K27ac and H3k4me1) or epigenetic promoter markers (H3K27me3 or H3K4me3) (Fig. 4A, Fig. S2), and therefore were prioritized as potential functional variations (PFVs).

As an example, one PFV (chr3:79510218A>T) locating in the third intron of *PHIP* gene was selected for further validation (Fig. 3A). Association analysis showed that chr3:79510218A>T were significantly associated with egg number from 21 to 43 week of age (EN21-43w) in Gushi chicken ($P = 0.0022$) and Guangxi Yao chicken ($P = 0.0388$) (Fig. 4B and C), and a consistent significant correlation between the variant site and *PHIP* expression levels in chicken ovary tissues was also established (Fig. 4D). The genotype TT of chr3:79510218A>T was more

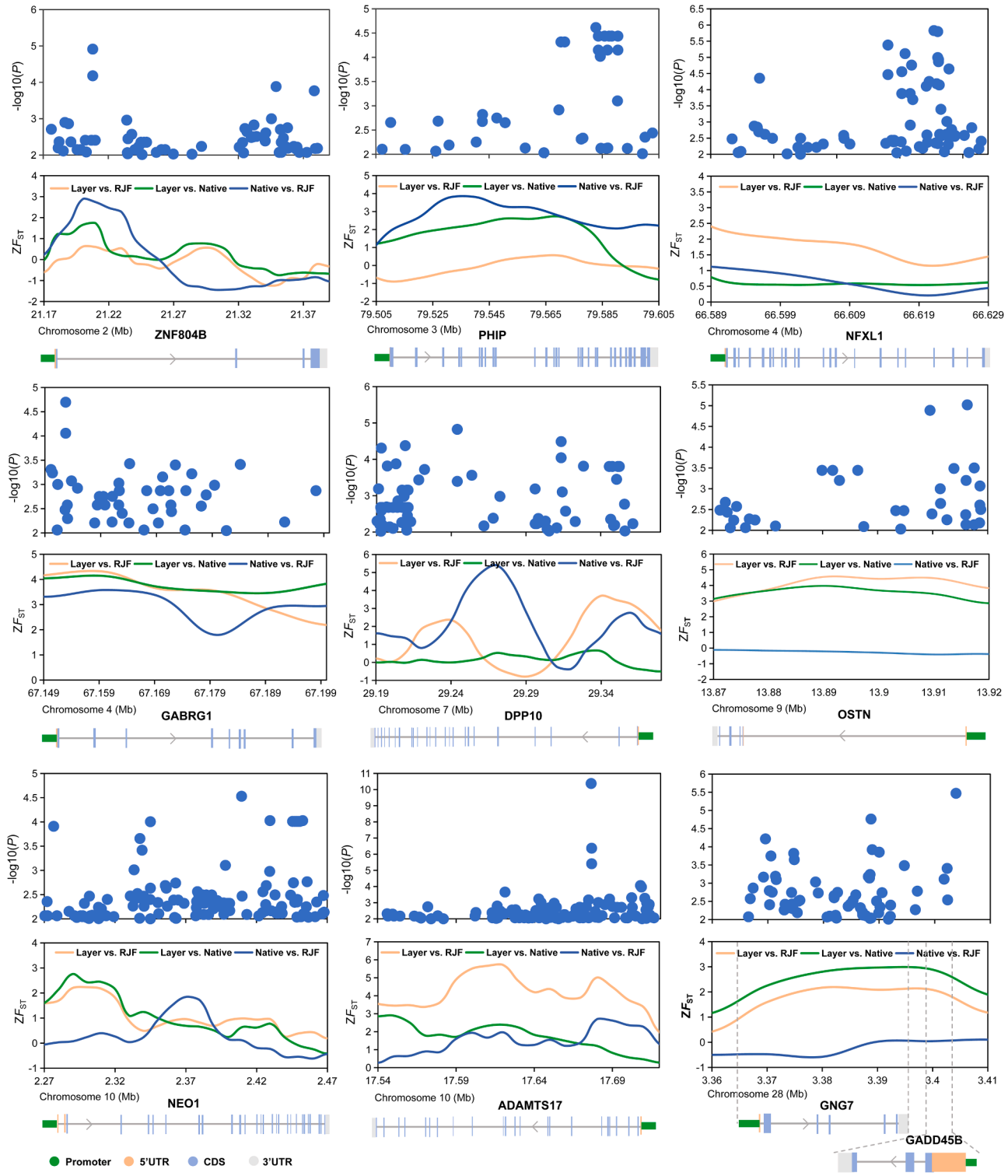

Fig. 3. Egg-laying related GWAS significant signals of SNPs mapping to 10 key candidate genes in Gushi chickens and their genetic differentiation characteristics in different breeds.

conducive to the increase of egg production and the expression of *PHIP*, compared to the other genotypes (Fig. 4D). These results preliminarily suggest that chr3:79510218A>T may affect egg production by altering *PHIP* expression in chickens.

chr3:79510218A>T regulate PHIP expression by changing the binding affinity to transcription factor FOXI1

To examine the allelic regulatory effect between chr3:79510218A>T and *PHIP* expression, we compared the transcriptional activity of genomic fragments including two different genotype of the site by using dual-luciferase reporter assays in chicken ovarian granulosa cells. Result showed that the luciferase activity of both alleles was enhanced ($P <$

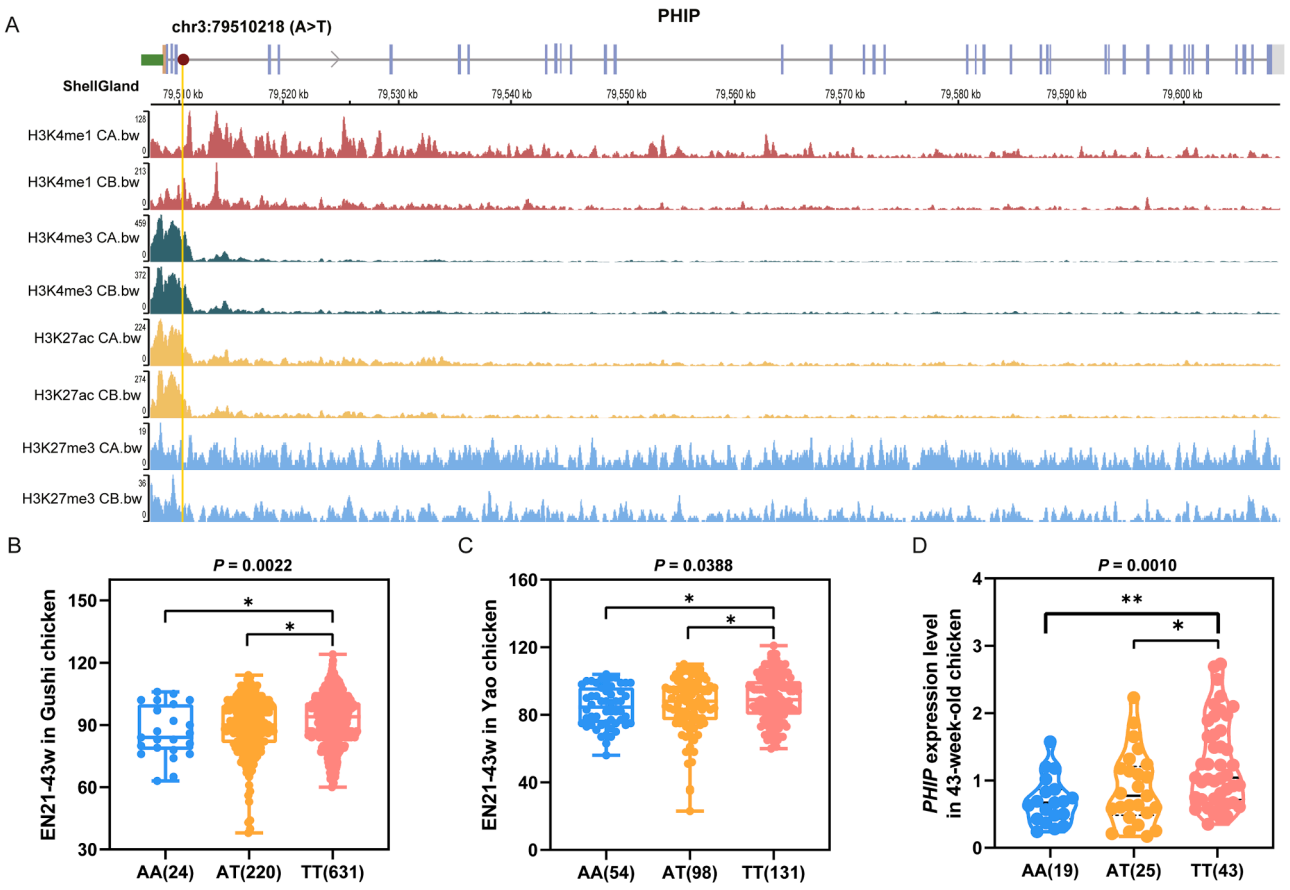

Fig. 4. Screening of potential functional variations affecting the expression of key candidate genes. (A) Epigenetic annotation in chicken shellgland for egg-laying related SNPs mapping to *PHIP*, visualized using ChickenGTEx. (B) and (C) Association analysis between the genotypes of chr3:79510218A>T and egg number from 21 to 43 weeks of age (EN21-43w) in Gushi chicken and Guangxi Yao chicken, respectively. (D) Comparison of *PHIP* expression in ovary of 43-week-old hens (Guangxi Yao and Gushi chickens) among the individuals with different genotypes for chr3:79510218A>T. Each dot represents an individual. * $P < 0.05$; ** $P < 0.01$.

0.01), with the chr3:79510218T allele having significantly enhanced effect compared with the chr3:79510218A allele ($P < 0.01$; Fig. 5A).

We next explored the underlying molecular mechanism that the regulation of PHIP transcriptional efficiency by chr3:79510218A>T. Transcription factor binding motifs in intron 3 of PHIP containing the site were predicted by JASPAR (http://jaspardev.genereg.net). The motif analysis showed that FOXI1 could specifically bind to the T allele of chr3:79510218A>T (Fig. 5B). Subsequently, *FOXI1* knockdown experiments were performed in ovarian granulosa cells, and we detected the significant decline of *PHIP* expression in *FOXI1* knockdown group (si-*FOXI1*) compared with the control group (si-NC) ($P < 0.05$; Fig. 5C). Cotransfection experiments with *FOXI1* interference and the pGL3 plasmid of both alleles (chr3:79510218A or chr3:79510218T) were further performed in ovarian granulosa cells. We detected that when *FOXI1* expression was suppressed, the luciferase activity of the T allele was significantly reduced, while that of the A allele did not significantly change ($P < 0.05$; Fig. 5D). These results supported chr3:79510218A>T acts as a potential functional variant site mediated by transcription factor *FOXI1* to regulate *PHIP* expression.

Knock down of PHIP inhibited the synthesis and secretion of PROG hormones and proliferation of chicken ovarian Granulosa cells

To further investigate the biological role of *PHIP*, chicken ovarian granulosa cells were isolated, cultured and characterized for its loss-of-function assay (Fig. S3). The mRNA level of *PHIP* was significantly decreased approximately by 65% after transfecting with si-PHIP (Fig. 6A). *PHIP* knockdown reduced the mRNA levels of cell proliferation marker genes such as cyclin dependent kinase 1 (*CDK1*), cyclin dependent kinase 2 (*CDK2*) and tumor protein P53 (*TP53*) ($P < 0.01$; Fig. 6B). CCK-8 and EdU assays showed that *PHIP* knockdown markedly decreased the numbers of living granulosa cells and EdU-positive granulosa cells ($P < 0.05$; Fig. 6C and D). These results suggested that *PHIP* functions to promote ovarian granulosa cell proliferation.

Meanwhile, we also observed that *PHIP* knockdown could down-regulate the mRNA expression of the follicle selection and granulosa cell differentiation genes including follicle stimulating hormone receptor (*FSHR*), growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 gene (*BMP15*) ($P < 0.05$; Fig. 7A), indicating that *PHIP* has a potential function of promoting granulosa cell differentiation. Further, *PHIP* knockdown significantly reduced the mRNA levels of steroid hormone synthesis pathway genes (steroidogenic acute regulatory protein gene (*STAR*), cytochrome P450 subfamily a member gene (*CYP11A1*) and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 gene (*3β-HSD*)) ($P < 0.01$; Fig. 7B) and the protein levels of *STAR* ($P < 0.05$; Fig. 7C) in granulosa cells, and down-regulated progesterone (PROG) hormone level in cell supernatant ($P < 0.01$; Fig. 7D and E).

Discussion

Egg production trait is a complex characteristic that is driven by multiple small-effect variation sites and is coordinately regulated by multiple tissues. Gaining a profound understanding of the genetic regulatory mechanisms underlying such complex traits remains a

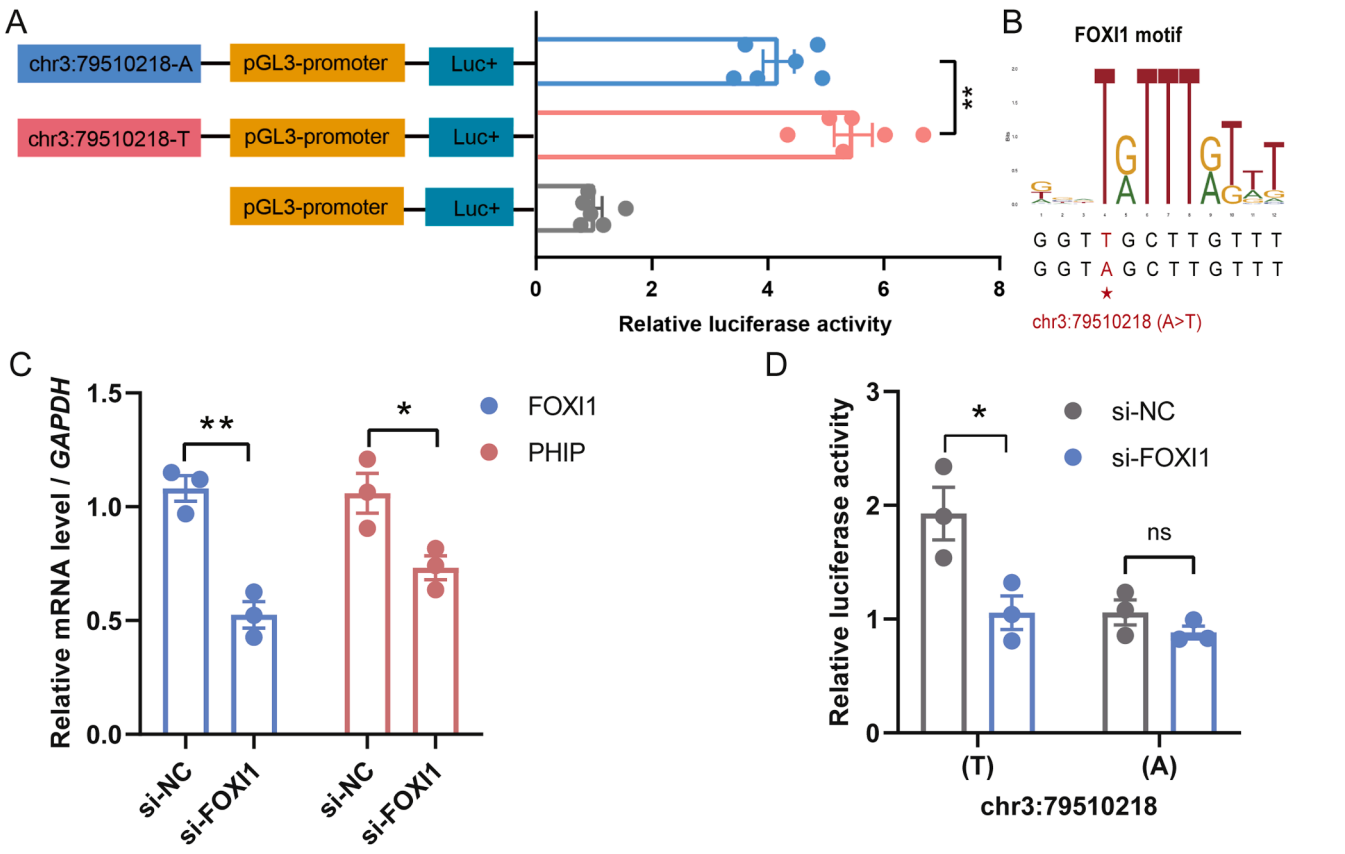

Fig. 5. Potential molecular mechanism by which alleles of chr3:79510218A>T regulate *PHIP* expression. (A) The dual-luciferase assay for the pGL3 promoter containing the genomic region surrounding chr3:79510218A or chr3:79510218T. (B) FOXI1 motif binding sequence containing chr3:79510218A>T. (C) Effect of *FOXI1* knockdown on *PHIP* expression in chicken ovarian granulosa cells. (D) Effect of *FOXI1* knockdown on relative luciferase activity of vectors containing chr3:79510218A or chr3:79510218T allele in chicken ovarian granulosa cells. The data are presented as mean ± SEM (n =6 for each group in A; n =3 for each group in C and D). * $P < 0.05$; ** $P < 0.01$.

significant challenge. To uncover the black box between genetic variants and complex traits, researchers have increasingly focused on intermediate phenotypes such as gene expression levels, to identify these variation sites (functional variants) and elucidate their potential regulatory mechanisms (Zhong et al., 2024). In this study, through bioinformatics analyses of previously screened candidate genetic variants/genes associated with egg production traits and a series of functional experiments, 22 potential functional variants (PFVs) that affect the expression of 10 genes in the hypothalamic, pituitary, and ovarian tissues were identified. Additionally, the regulatory function of one of these PFVs in chicken ovarian granulosa cells was confirmed. The identification of these PFVs is critical to translate GWAS findings into laying-oriented breeding design information.

During the process of integrated bioinformatics analysis, 10 key candidate genes that were differentially expressed in high - and low - yield Gushi chickens were screened for functional variation detection. Among them, five genes were concentrated in chicken hypothalamus and pituitary, which played important roles in the regulation of alzheimer's disease (ZNF804B, NEO1, GNG7) (Chung et al., 2018; Magalingam et al., 2023; Willett et al., 2024), anorexia nervosa (ZNF804B) (Wang et al., 2011), neuronal excitability (DPP10) (He et al., 2024), reproduction (ZNF804B, GABRG1) (Smitchger et al., 2024; Wang et al., 2024), and blood-brain barrier (NEO1) (Wei et al., 2024) in human or mammalian. While the other five genes concentrated in the ovary functioned in cell growth and apoptosis (GADD45B, PHIP) (Liao et al., 2020), granulosa cell proliferation and synthesis of sex steroid hormones (OSTN) (Wang et al., 2024), and oocyte maturation (ADAMTS17) (Zhan et al., 2024). Based on these key candidate genes in chicken HPO axis and their nearby egg-laying related SNPs, we expected to find functional variants that regulate the expression of these genes. The recently released Chicken Genotype-Tissue Expression (ChickenGTEx) project analyzed 377 epigenome, transcriptome and chromatin conformation data sets, and constructed a map of regulatory variants across 28 distinct chicken tissues, thus completing the systematic annotation of functional variation in the chicken genome (Pan et al., 2023). The release of ChickenGTEx has facilitated our detectives for functional variants associated with egg production. Here, we identified 22 potential functional variants, located in the strong epigenetic enhancer markers or epigenetic promoter markers, and we demonstrated that the intronic SNP chr3:79510218A>T acts as a functional variant to regulate the expression of *PHIP* in ovary and thus affect egg production in chickens.

Up to now, the study on *PHIP* genes has mainly focused on mammals, especially humans, and have rarely been reported in poultry. The variation or function of *PHIP* are related to the occurrence and development of diseases such as neurodevelopmental disorder, developmental delay, early-onset obesity, dysmorphic features and cancers (Webster et al., 2016; Ren et al., 2023; Pascolini et al., 2024; Loid et al., 2024). In this study, although the positive correlation between ovarian PHIP expression and egg production was determined, the specific function of *PHIP* on the ovary still needs to be explored. In avian, the ovary is the reproductive determining tissue of poultry and consists of follicles and ovarian stroma. The follicle is the basic unit of ovum maturation to ovulation and sex steroid hormone synthesis, which consists of the oocyte and the surrounding somatic layer (granulosa cells and theca cells) (Zou et al., 2025). And the status of ovarian granulosa cells plays an important role in follicular development (Zou et al., 2025). Therefore, we used chicken primary ovarian granulosa cells to explore the regulatory function of *PHIP*.

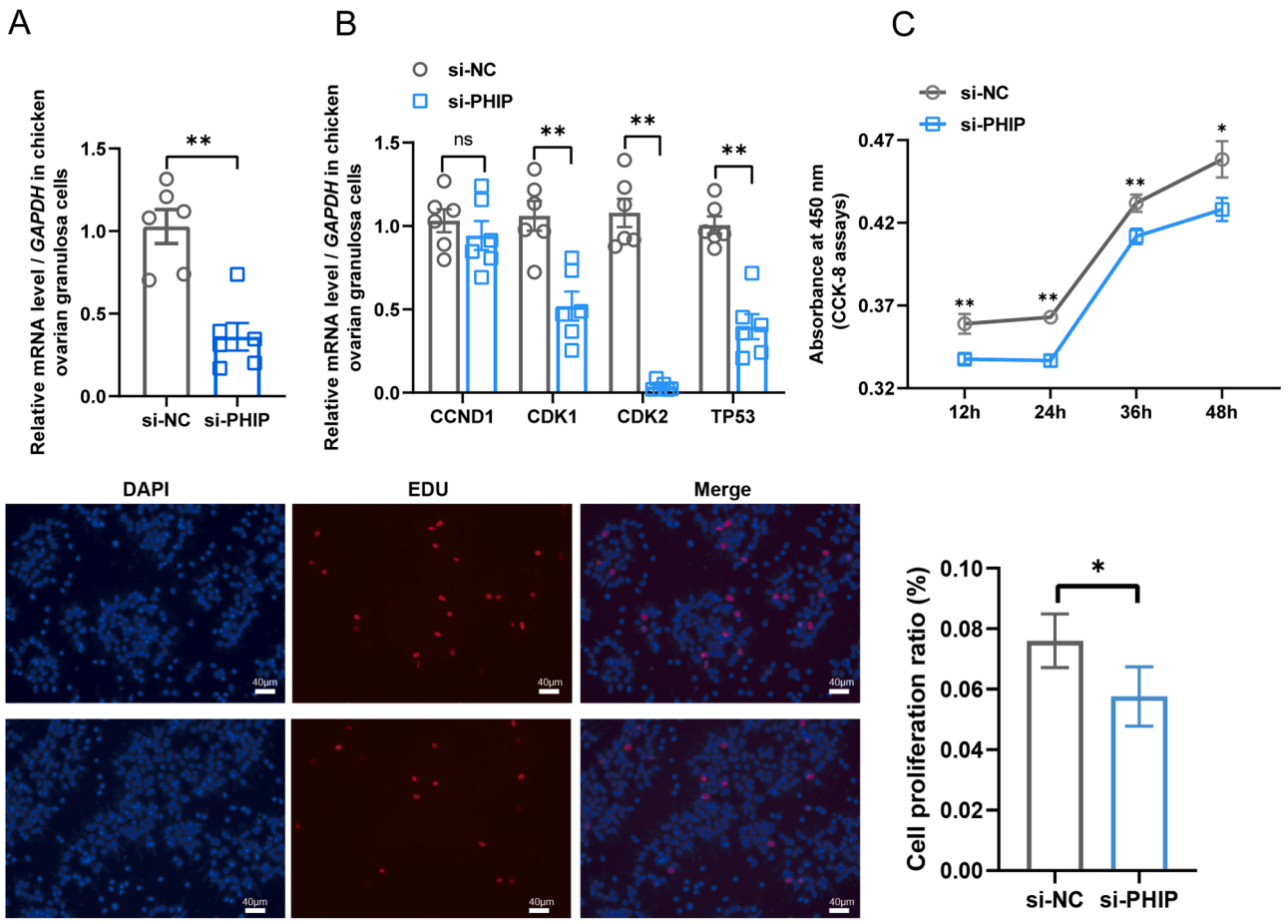

Fig. 6. Effect of *PHIP* knockdown on proliferation of chicken ovarian granulosa cells. (A) Detecting the interference efficiency of *PHIP* gene. (B) The expression of cell proliferation marker genes after *PHIP* knockdown. Cell proliferation was detected using (C) cell counting kit-8 (CCK-8) and (D) 5-ethynyl-2'-deoxyuridine (EDU) assay after *PHIP* knockdown. The data are presented as mean ± SEM (n =6 for each group). * $P < 0.05$; ** $P < 0.01$.

Proliferation and differentiation of granulosa cells promote follicle growth and selection, while apoptosis accelerates follicle atresia (Zhang et al., 2024). In our study, *PHIP* knockdown inhibited the expression levels of cell proliferation marker genes and reduced the number of the living granulosa cells, which is consistent with the role of *PHIP* in the proliferation in mammals. For instance, *PHIP* could affect the transcription level of cyclin D2 (CCND2) by binding to insulin receptor substrate 2, thereby promoting the growth and proliferation of pancreatic beta cells (Podcheko et al., 2007). Knock down of PHIP expression could inhibit the proliferation and invasion of three types of cancer cells (breast cancer, lung cancer and melanoma) (de Semir et al., 2018). In addition, our results also showed that the expression levels of *FSHR, GDF9* and *BMP15* were also inhibited when *PHIP* was interfered. FSHR plays a key role in the maintenance of prehierarchical follicle activity and hierarchical follicle selection in chickens (Lee et al., 2024). BMP15 and GDF9 regulate follicle development and maturation by promoting granulosa cell differentiation and steroid hormone secretion (Stephens et al., 2016; Hlokoe et al., 2022). Therefore, these results indicated that *PHIP* has a potential function of promoting the normal proliferation and differentiation of ovarian granulosa cells.

Follicles are selected to enter the hierarchical follicle, granulosa cells have the ability to synthesize sex steroid hormones, including PROG and E2, to regulate follicle maturation and ovulation (Johnson et al., 2009). The synthesis of sex steroid hormones is mainly dependent on the catalysis of steroid synthase on the mitochondrial membrane. In poultry granulosa cells, cholesterol is transported by STAR to the inner mitochondrial membrane and catalyzed to pregnenolone by CYP11A1, which is then catalyzed to PROG by 3β-HSD via the Δ4 pathway. The synthesis of E2 is catalyzed twice by 17β-HSD and CYP19A1 on the basis of PROG (Lee et al., 1998). In this study, *PHIP* knockdown significantly reduced the mRNA levels of steroid hormone synthesis pathway genes (*STAR, CYP11A1* and *3β-HSD*) and the protein levels of *STAR*, and slightly reduced CYP11A1 protein levels, resulting in a decrease in PROG hormone levels in cell supernatant. Our results suggest that *PHIP* may promote the expression of catalytic enzyme in PROG synthesis pathway to varying degrees, but does not affect the expression activity of the catalytic enzyme in E2 synthesis pathway.

We have preliminarily clarified the regulatory function of *PHIP* in granulosa cells, and we also hope to explain how the functional variation chr3:79510218T regulates the PHIP expression. Our study indicated that transcription factor FOXI1 could preferentially bind to the chr3:79510218T allele to increase the expression of *PHIP* gene. FOXI1 belongs to the FOX family protein family, which involved in many biological processes, such as embryonic development, immune regulation, carbohydrate and lipid metabolism, and cell cycle regulation (Blomqvist et al., 2006; Jackson et al., 2010). The relationship between FOXI1 and reproduction regulation in chicken is still unclear, but FOXI1 protein deficiency have been proved to cause immature sperm and male infertility in mice (Blomqvist et al., 2006). Our results showed that knockdown of FOXI1 in chicken ovarian granulosa cells resulted in a significant decline of PHIP expression, supporting its involvement in the regulation of egg production. In addition, the TT genotype of chr3:79510218A>T had a relatively higher egg number, PHIP expression levels, and transcriptional activity, which supported that it was the dominant genotype in the direction of chicken egg production selection.

In conclusion, we report 22 potential functional variants that may

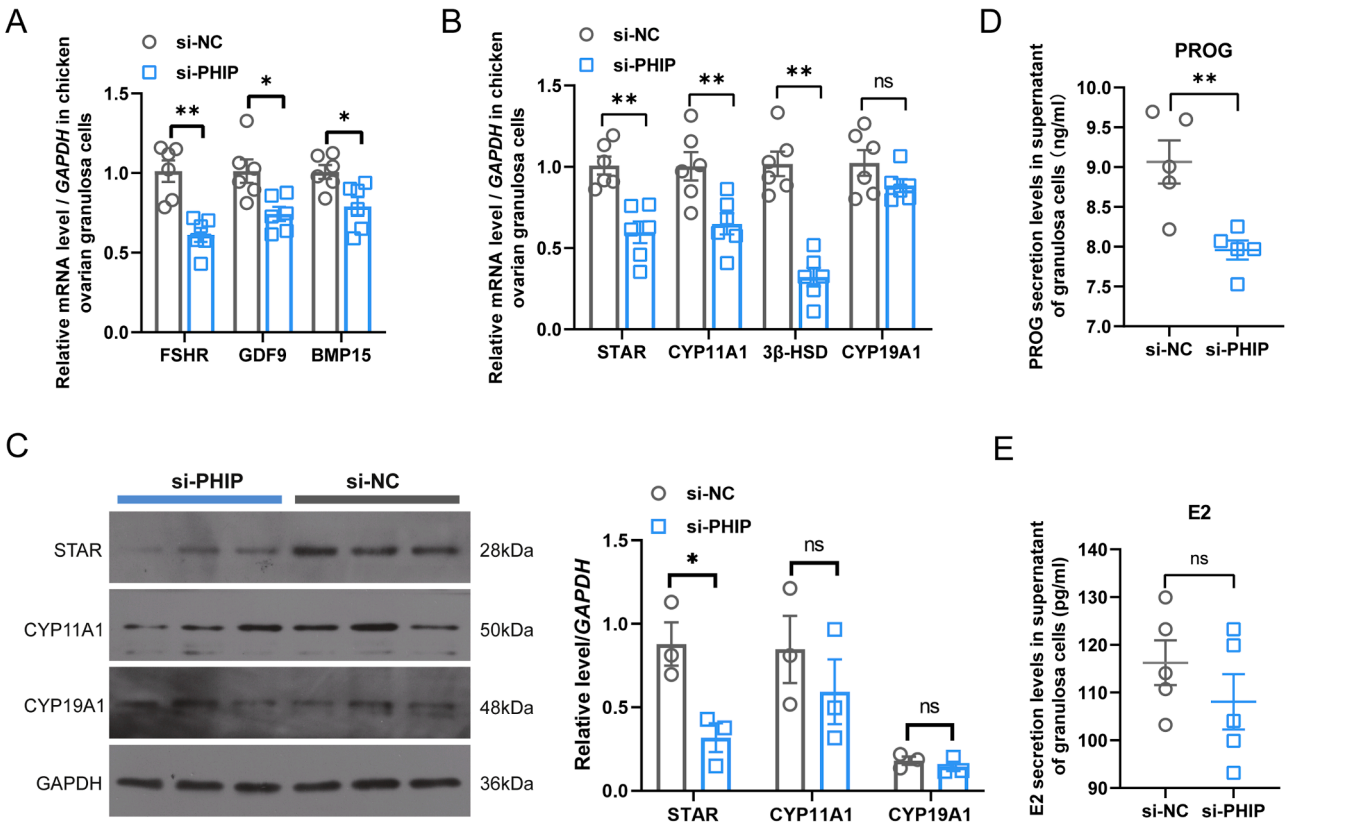

Fig. 7. Effect of *PHIP* knockdown on steroid hormone synthesis of chicken ovarian granulosa cells. (A) The mRNA expression of follicle growth or differentiation related genes after *PHIP* knockdown. (B) and (C) The mRNA and protein expression of steroid hormone synthesis pathway genes after *PHIP* knockdown. (D) and (E) PROG and E2 hormone levels in chicken ovarian granulosa cell supernatant after *PHIP* knockdown. The data are presented as mean ± SEM (n =6 for each group in A and B; n =3 for each group in C; n =5 for each group in D and E). * $P < 0.05$; ** $P < 0.01$.

drive the differences in egg production phenotypes by affecting the expression of 10 egg-laying related key candidate genes in the hypothalamus, pituitary, and ovary. We also provide a mechanistic insight that the intronic SNP chr3:79510218A>T acts as a functional variant to regulate *PHIP* expression mediated by FOXI1. These potential functional molecular markers will contribute to the improvement of chicken egg production traits in molecular breeding selection.

Disclosures

The authors declare no conflict of interest.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

The work was supported by the Henan Provincial Science and Technology Research Project (105020224082), the National Natural Science Foundation of China (32272867) and the National Key Research and Development Program (2022YFF1000202).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105027.

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