BMC Genomics

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Chromatin accessibility and transcriptomic profiles of sheep pituitary function associated with fecundity

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Abstract

Background The pituitary gland, a central regulator of the hypothalamic-pituitary-gonadal (HPG) axis, plays a pivotal role in reproductive efficiency by precisely controlling the secretion of gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Chromatin accessibility enables physical interactions between promoters and chromatin-binding factors to drive the gene expression. Despite this mechanistic insight, the chromatin accessibility landscape of the sheep pituitary and its functional implications for reproductive traits remain largely unexplored. To address this knowledge gap, we performed an integrated multi-omics analysis of ATAC-seq and RNA-seq profiling of pituitary from sheep with divergent fecundity phenotypes.

Results We identified 1,567 differential accessibility regions (DARs) and 768 differentially expressed genes (DEGs). Functional enrichment analysis revealed that the DEGs were significantly associated with key signaling pathways, including neuroactive ligand-receptor interactions, the cAMP signaling pathway, and the calcium signaling pathway, suggesting their critical roles in pituitary-regulated reproductive functions. Based on integrative analysis of ATAC-seq and RNA-seq, we revealed several potentially key genes involved in gonadotropin secretion, such as *CMKLR1*, *TAFA1*, and *PPP1R17*. Furthermore, we identified novel transcription factors (TFs), including NR4A2 and MEF2, which may influence pituitary hormone secretion by modulating chromatin accessibility and gene expression.

Conclusions This study systematically delineated the gene expression and chromatin accessibility of the pituitary and identified some key regulatory genes associated with gonadotropin secretion in sheep. Our integrated multi-omics analysis identifies critical molecular markers that may contribute to the genetic improvement of reproductive efficiency in ovine species.

Keywords Pituitary, Chromatin accessibility, Gene expression, Fertility

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Introduction

The pituitary gland serves as the central regulator of the hypothalamus-pituitary-gonadal (HPG) axis in mammals and regulates various physiological functions including growth, fertility, puberty, and lactation [1]. Gonadotropes are stimulated by the pulsing gonadotropin-releasing hormone (GnRH) released by neurons in the hypothalamus, subsequently producing and releasing the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [2]. These pituitary hormones then mediate gametogenesis and steroidogenesis in the gonads, thereby directly controlling animal reproductive performance. Recent studies have identified several molecular regulators of gonadotropin secretion. EGF could govern FSH synthesis and secretion via the EGFR/miR-27b-3p/FOXO1 pathway [3]. BDNF [4] and EZH2 [5] have been shown to influence ovine pituitary cell proliferation, apoptosis, and gonadotropin secretion via the AKT/ERK signaling pathway.

More and more studies have found that transcription factors can directly or indirectly affect endocrine regulation. By binding to a specific regulatory sequence, transcription factors are the key drivers of cell function and phenotype. Various elements can be involved in the regulation of gene expression, thereby influencing the phenotype [6]. It has been proved that many transcription factors are involved in producing FSH and LH. FOXL2 specifically binds to a cis-regulatory element within the proximal *FSHB* promoter region, which is essential for activin A mediated induction of murine *FSHB* subunit transcription [7]. In *FOXL2* conditional knockout (cKO) mice, both testicular and ovarian weight exhibited significant reduction compared to wild-type controls, indicative of impaired gonad development [8, 9]. Differences in chromatin accessibility landscapes determine *FOXP2* regulators specific to the three cell types in the pituitary gland and regulate distinct biological processes in each cell type [10]. NR5A1 can affect the synthesis of FSH and LH by affecting the expression of *LHB*, *FSHB*, and *CGA* [11]. Studies on *NR5A1* knockout mice reveal that they are sterile, fail to reach sexual maturity, and exhibit significantly reduced levels of LH and FSH [12]. Subsequently, LH and FSH regulate the production of gametes and sex hormones in the gonads. Several sequencing techniques have been developed to evaluate chromatin accessibility. Among these, ATAC-seq is widely used for mapping open chromatin regions and predicting transcription factor (TF) binding sites. This technique has been employed to analyze chromatin accessibility across various tissues, such as ovine skeletal muscle [13] and human pituitary adenoma [14]. However, the chromatin accessibility landscape of the pituitary of ewes has not been systematically investigated. Therefore, understanding the epigenetic mechanisms that influence the fate of pituitary cells is essential for gaining insights into the reproductive processes governed by the hypothalamic-anterior pituitary-gonadal axis.

Hu sheep, a renowned indigenous breed in China, are distinguished by their exceptional prolificacy traits [15]. This characteristic has established them as a valuable model organism for deciphering the molecular mechanisms governing high fecundity in ruminants. To systematically investigate the epigenetic and transcriptional regulation underlying reproductive performance, we conducted integrated ATAC-seq and RNA-seq analyses to characterize genome-wide chromatin accessibility landscapes and transcriptomic profiles in pituitary glands of Hu sheep exhibiting divergent fertility phenotypes. These findings are not only essential for enhancing our understanding of the molecular mechanisms underlying sheep reproduction but also provide valuable insights for improving domestic animal breeding strategies.

Materials and methods

Sample collection and Preparation

Collection of pituitary tissue

Six healthy Hu sheep were were purchased from Jiangsu Qianbao Sheep Industry Limited Company (Yancheng, China) and divided into two groups ($n = 3$). The sheep with lambing number ≥ 3 in three consecutive lambing records were assigned to the high fecundity group, and lambing number = 1 as the low fecundity group. The sheep was administered 3% pentobarbital sodium intravenously, inducing deep anesthesia before being euthanized by exsanguination. After euthanasia, the pituitary glands were carefully dissected and immediately snap-frozen in liquid nitrogen for subsequent RNA extraction and analysis.

RNA sequencing and data analysis

The RNA sequencing and data analysis followed previously established protocols [16]. In summary, RNA was extracted from pituitary and sequenced on the Illumina NovaSeq 6000 platform to produce raw data. Following quality control and alignment, gene expression levels were quantified and prepared for further analysis.

Assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)

Library Preparation and sequencing

Pituitary was washed with 0.09% NaCl solution and then grinded into powders in liquid nitrogen. Lysis buffer were added to the powders and incubated for 10 min at 4 ℃ on the rotation mixer. cell suspension was filtered with 40 μm cell strainer and then washed with cold PBS buffer three times. After inspecting nuclei purity and intactness under microscopy, approximate 50,000 nuclei were allidote to perform tagmentation according to standard

protocols [17]. Tn5-transposed DNA was purified using AMPure magnetic beads. A qPCR reaction was performed on a subset of the DNA to determine the optimum number (average 11) of PCR cycles, and amplified libraries were run on an Agilent Tapestation 2200 (Agilent Technologies) using a D5000 DNA ScreenTape to assess quality by visualizing nucleosomal laddering. Trimmed reads were aligned to the reference genome using Bowtie2 [18] (version 2.2.6), excluding reads mapping to the mitochondrial genome. Duplicate reads were removed, and mapped reads were downsampled to approximately 20 M using Picard (version 1.126).

Quality control and data assessment

Insert size distribution was evaluated, revealing a clear periodicity of ~200 bp, indicative of nucleosome-protected fragments. Transcription Start Site (TSS) Enrichment Scores were calculated as a signal-to-noise ratio. Peaks in TSS signals were used as a metric for data quality.

Peak detection and analysis

Peaks were called using MACS2 [19] with parameters (--nomodel --extsize 200 --shift −100). High-confidence peaks were identified by assessing overlaps between replicates and pseudoreplicates, with an IDR threshold of 0.05. Differentially accessible regions (DARs) were identified using the R package DESeq2 [20], with criteria of log2 fold enrichment > 1 and FDR < 0.05. Motif analyses of DARs were performed using the MEME suite [21], retaining motifs with $P < 0.01$. Gene ontology enrichment analyses were conducted with ClusterProfiler [22], and sequencing tracks were visualized using the Integrated Genomic Viewer [23] (IGV, Version 2.18.2).

Integration analysis of ATAC-seq and RNA-seq

ATAC-seq peaks were annotated to the nearest genes using the ChIPseeker [24] R package (v1.40.0) with default parameters. To investigate the potential interplay between transcriptional regulation and chromatin accessibility, genes associated with differentially accessible regions (DARs) were intersected with differentially expressed genes (DEGs) identified from RNA-seq analysis. This integrative approach facilitated the identification of candidate genes exhibiting both differential chromatin accessibility and altered transcriptional activity. To further elucidate the regulatory patterns of these genes, representative genomic loci were visualized using the Integrative Genomics Viewer [23] (IGV, v2.18.2). Core transcription factors regulating the representative genes were predicted using the HOMER [21] software(v5.1), and the regulatory network was constructed and visualized using Cytoscape [25] (Version 3.10.2).

Results

The profile of mRNA expression in the pituitary

To investigate pituitary gene expression dynamics associated with fecundity variation, we performed RNA sequencing on pituitary from two distinct Hu sheep populations: high-fecundity and low-fecundity groups. Transcript abundance was quantified using transcripts per million (TPM) normalization, showing consistent expression patterns within biological replicates (Fig. 1A, B). The Pearson correlation heatmap demonstrated strong repeatability within groups, while significant differences were observed between the groups, indicating the dataset's reliability for subsequent bioinformatics analyses (Fig. 1C). Differential expression analysis identified 668 differentially expressed genes (DEGs) between the two groups, with 295 upregulated and 373 downregulated genes (Fig. 1D-F). A protein-protein interaction network was constructed based on these DEGs to identify key proteins potentially influencing pituitary function in Hu sheep with differing fecundity (Figure S1). Key reproduction-related genes, including *BDNF*, *FOS*, and *CALB1*, were identified as central nodes in the network. KEGG pathway enrichment demonstrated significant involvement of cAMP signaling, calcium signaling, and circadian entrainment pathways (Fig. 2A). Concurrently, GO enrichment analysis highlighted significant terms related to Toll-like receptor binding, regulation of sterol transport, and rhythmic process (Fig. 2B). Gene Set Enrichment Analysis (GSEA) was performed on all DEGs ranked by log fold change (logFC) and indicated that pathways associated with reproduction, including the neuroactive ligand-receptor interaction, cAMP signaling pathway, and calcium signaling pathway, were downregulated in the pituitary of high-fecundity Hu sheep (Fig. 2C).

The chromatin accessibility profile of the pituitary gland

To elucidate the transcriptional mechanisms underlying fertility levels of Hu sheep, we conducted ATAC-seq profiling of pituitary glands from high- and low-fecundity groups. Genome-wide mapping of all peaks revealed that most peaks were predominantly distributed in promoter-TSS regions(18.72%), introns(38.21%), and intergenic regions(34.69%) (Fig. 3A). Additionally, a significant enrichment of peaks was observed within ±3 kb of the TSS (Fig. 3B), suggesting preferential positioning of regulatory elements near transcriptional initiation sites. Comparative analysis identified 1,567 differentially accessible regions (DARs), including 767 diminished DAR (loss DAR) and 800 Enhanced DAR (gain DAR) (Fig. 3C, D). Further analysis demonstrated that the majority of DARs were located within intronic and distal intergenic areas (Fig. 3E), indicating that these DARs could serve as distal regulatory elements, potentially functioning

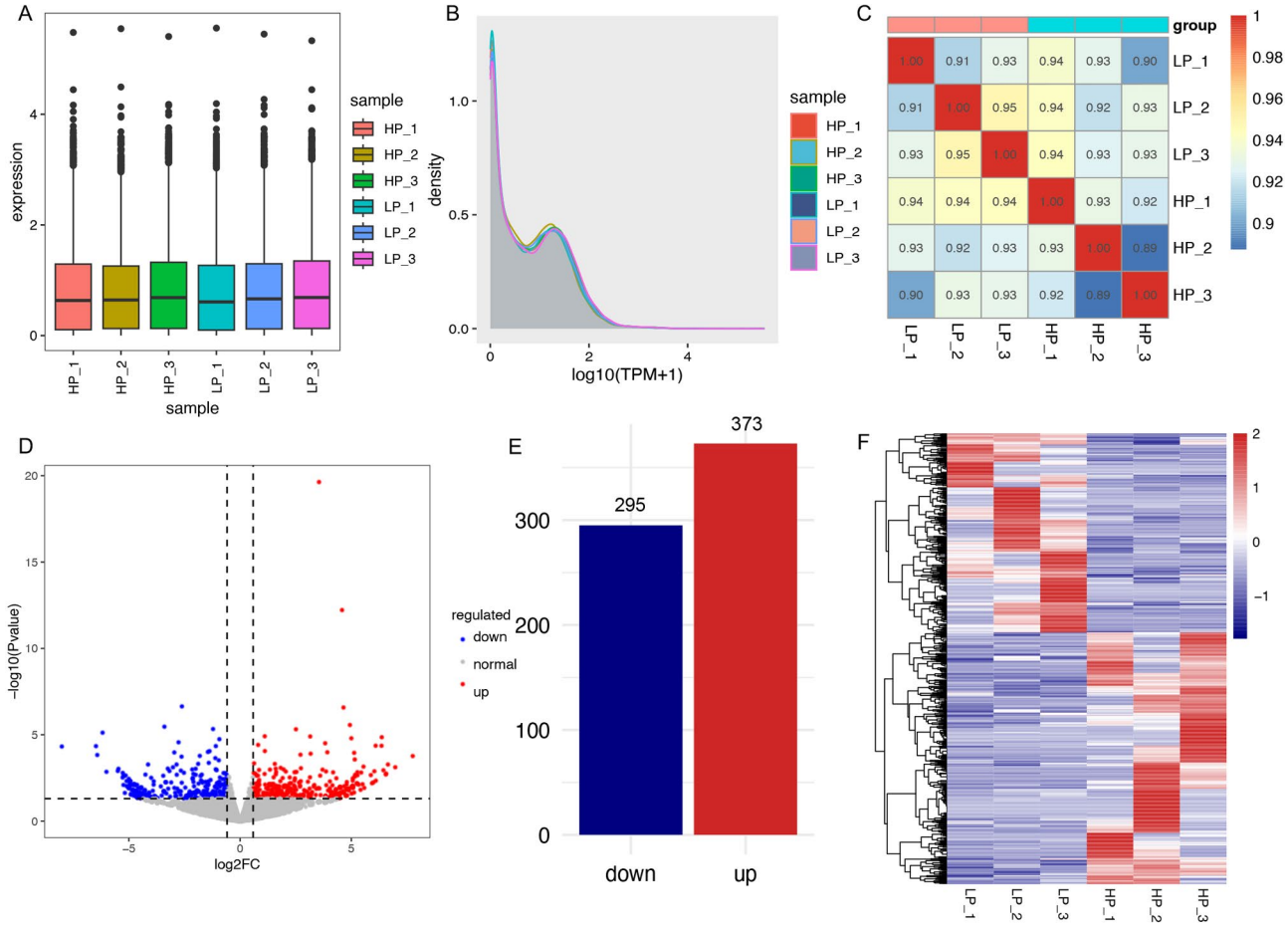

Fig. 1 Characteristics of mRNA expression in the sheep pituitary. (**A**) Gene expression levels in each sample (HP: high fecundity, LP: low fecundity). (**B**) Distribution of gene expression density across samples. (**C**) Pearson correlation heatmap between different samples. (**D**) Volcano plot showing differentially expressed genes (DEGs) between the pituitaries of high and low fecundity Hu sheep. (**E**) Number of DEGs. (**F**) Hierarchical clustering heatmap of all DEGs

as enhancers. To gain insight into the functional roles of DARs and their associated signaling pathways, GO enrichment analysis was performed. The genes associated with gain DAR were mainly enriched in cell development, cell adhesion, and BMP receptor binding (Fig. 3F). The genes associated with loss DAR were mainly enriched in Wnt signaling pathways and nerve development (Fig. 3G).

Transcription factor analysis of differentially accessible regions between different pituitary

The binding of regulatory proteins such as transcription factors to DNA requires recognition of specific motifs to perform regulatory functions. Homer software was used to analyze the sequences corresponding to DARs, and the enriched Motif and corresponding transcription factors could be obtained. A total of 44 transcription factors were identified in gain DARs, while 47 transcription factors were identified in loss DARs. As shown in Fig. 4, proximal peaks in gain DARs were highly enriched for transcription factors such as *BMYB*, *GRHL2*, and *IK2F1*, whereas proximal peaks in loss DARs were enriched for *CDX4*, *DMRT6*, and *ELF1*. In addition, we also performed transcription factor analysis on the distal peak of the gene—the concentration of the ZNF family transcription factors mainly enriched at the distal peaks (Fig S2). Together, these findings provide a comprehensive overview of the transcription factors associated with differential chromatin accessibility in the pituitary of Hu sheep with varying fecundity, offering valuable insights into the regulatory mechanisms governing reproductive traits.

Integrative analysis of gene expression and chromatin accessibility

To assess the relationship between changes in gene mRNA expression and chromatin accessibility, we performed an integrated analysis of RNA-seq and ATAC-seq data. By mapping differentially accessible regions to the nearest genes, we identified 15 differentially expressed genes (DEGs) associated with these specific chromatin accessibility regions. There were differentially accessible sites in the distal and proximal parts of 16 genes (Fig. 5A),

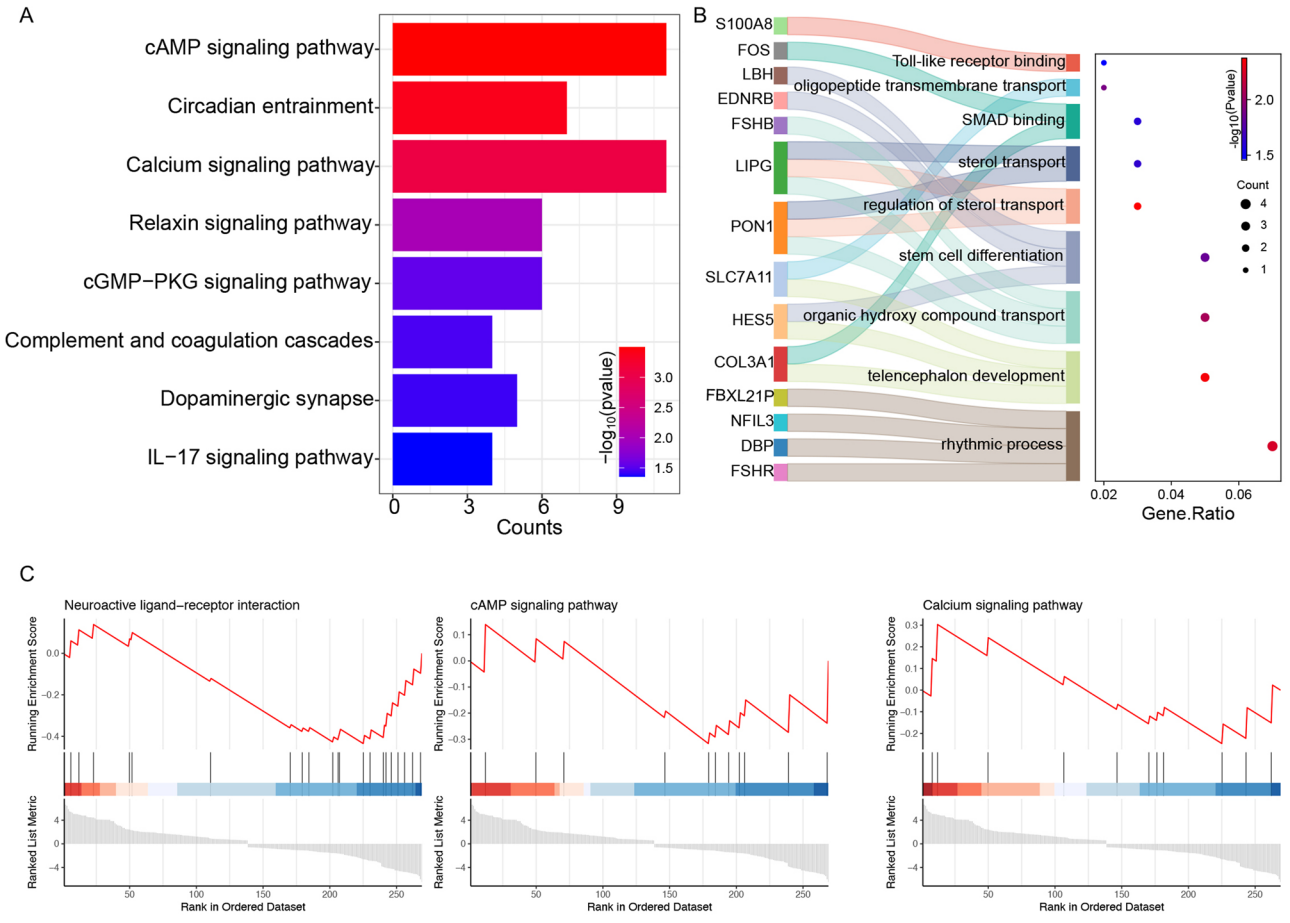

Fig. 2 Enrichment analysis of genes identified by RNA-seq. (**A**) Histogram of GO terms of up-regulated genes. (**B**) Dot plot of GO terms of down-regulated genes. (**C**) The top three pathway identified by GSEA

Among these, the mRNA expression of *LOC114118859* was up-regulated, suggesting that increased chromatin accessibility plays a critical role in the upregulation of *LOC114118859*. Additionally, 19 key DEGs were associated with alterations in chromatin accessibility(Fig. 5B). Based on previous studies, three genes (*CMKLR1*, *TAFA1*, and *PPP1R17*) were screened as potential regulators of hormone secretion. Notably, the differentially accessible regions were primarily located within these genes' exons, introns, and promoter-TSS regions (Fig. 5C). Based on this, we predicted some transcription factors and constructed a network of the interactions between these genes and transcription factors(TFs) (Figure S3). The genes were found to interact with TFs closely and are likely to play significant roles in ewe's fecundity. These findings provide insight into the relationship between chromatin accessibility and gene expression, highlighting key genes potentially involved in hormone regulation in Hu sheep.

Discussion

As a neuroendocrine tissue, the anterior pituitary is unique and contains a variety of endocrine cells that produce hormones affecting ewe fertility. ATAC-seq is an effective technique for detecting chromatin accessibility, which has been widely used to map chromatin accessibility of tissues and cells in different physiological states [26]. Previous ATAC-seq analyses of ovine skeletal muscle across developmental stages have revealed conserved regulatory elements associated with muscle hypertrophy and fiber-type transformation [27]. Previous studies have described pituitary gene expression at different stages of development and during different estrus cycles [16, 28], but the variation of chromatin state in the pituitary of different fertility ewes has never been investigated. To this end, we used ATAC-seq analysis to study chromatin accessibility and elucidate the differential regulation of the pituitary in ewes with different fecundity. In addition, we integrated RNA-seq to pinpoint key factors that may potentially affect pituitary function.

We identified 668 DEGs between high- and low-fecundity sheep groups. Functional enrichment analysis

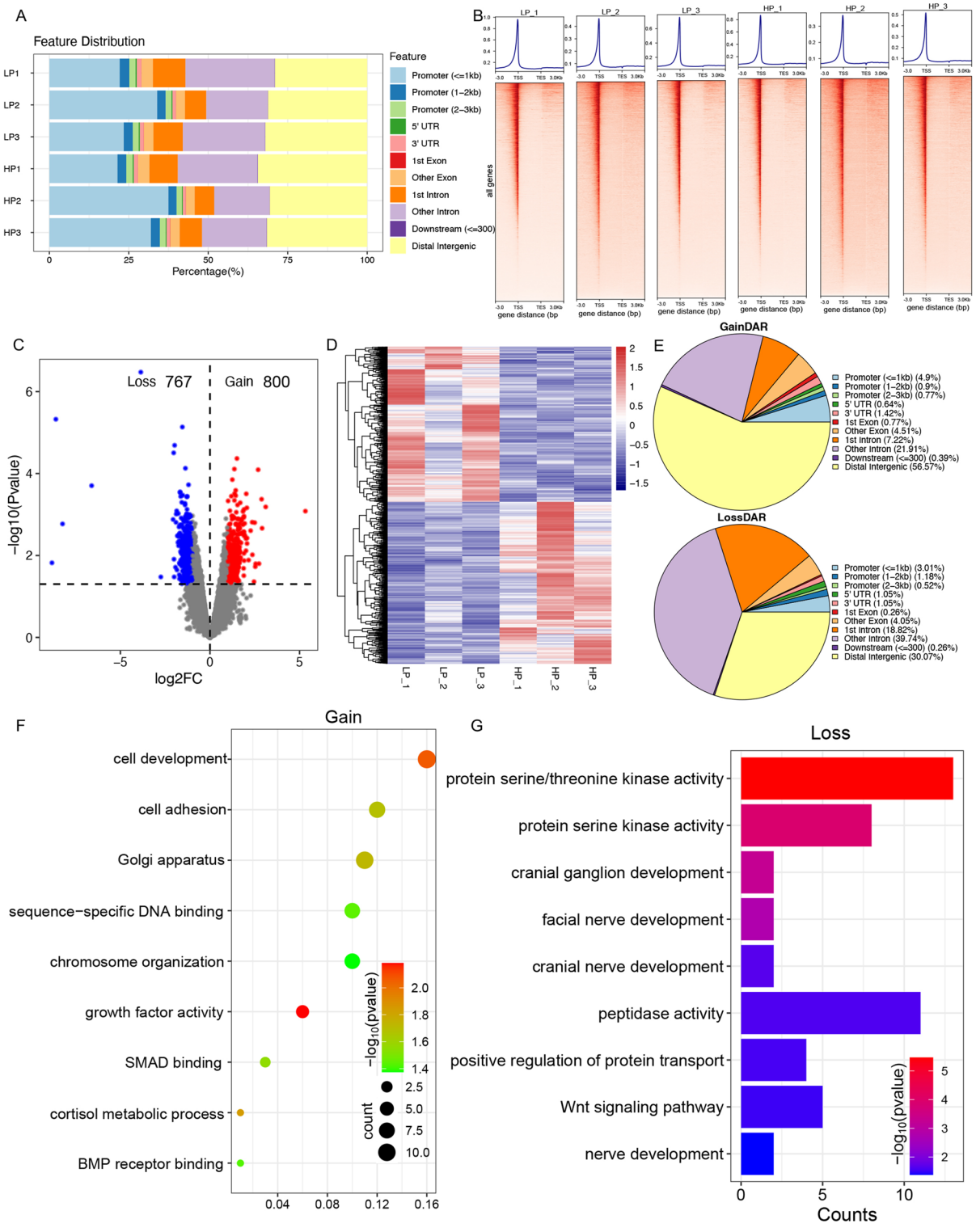

Fig. 3 Accessible chromatin landscape of pituitary. (**A**) Annotation of peaks to genomic features. (**B**) Heatmaps illustrating ATAC-seq signal across a three Kb genomic window, extending three Kb upstream of the TSS and three Kb downstream of the TES. (**C**) Volcano plot displayed the differentially accessible regions (DARs) between the pituitaries of high and low fecundity Hu sheep. (**D**) Hierarchical clustering heatmap of all DARs. (**E**) Pie chart showing the gain/loss DARs proportion within the indicated genomic regions. (**F**) Dot plot of GO terms of gain DARs-associated genes. (**G**) Histogram of GO terms of loss DARs-associated genes

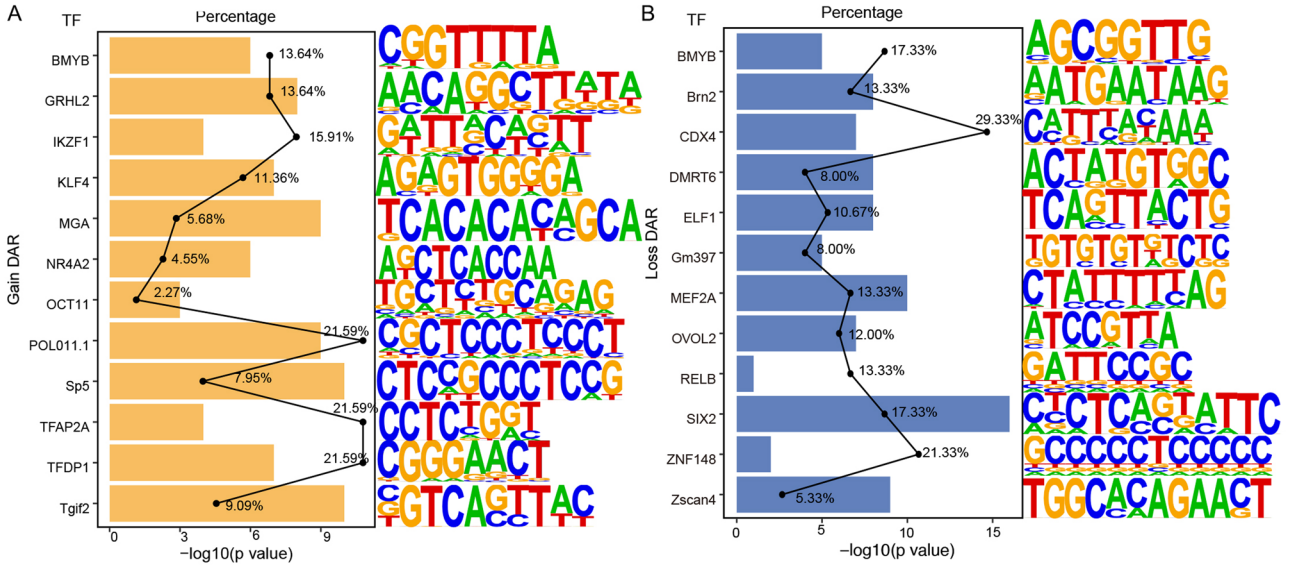

Fig. 4 Prediction of transcription factors involved in chromatin accessibility changes in the pituitary. (**A**) The top 12 enriched known TF motifs of gain proximal peak sites. (**B**) The top 12 enriched known TF motifs of gain distal peak sites

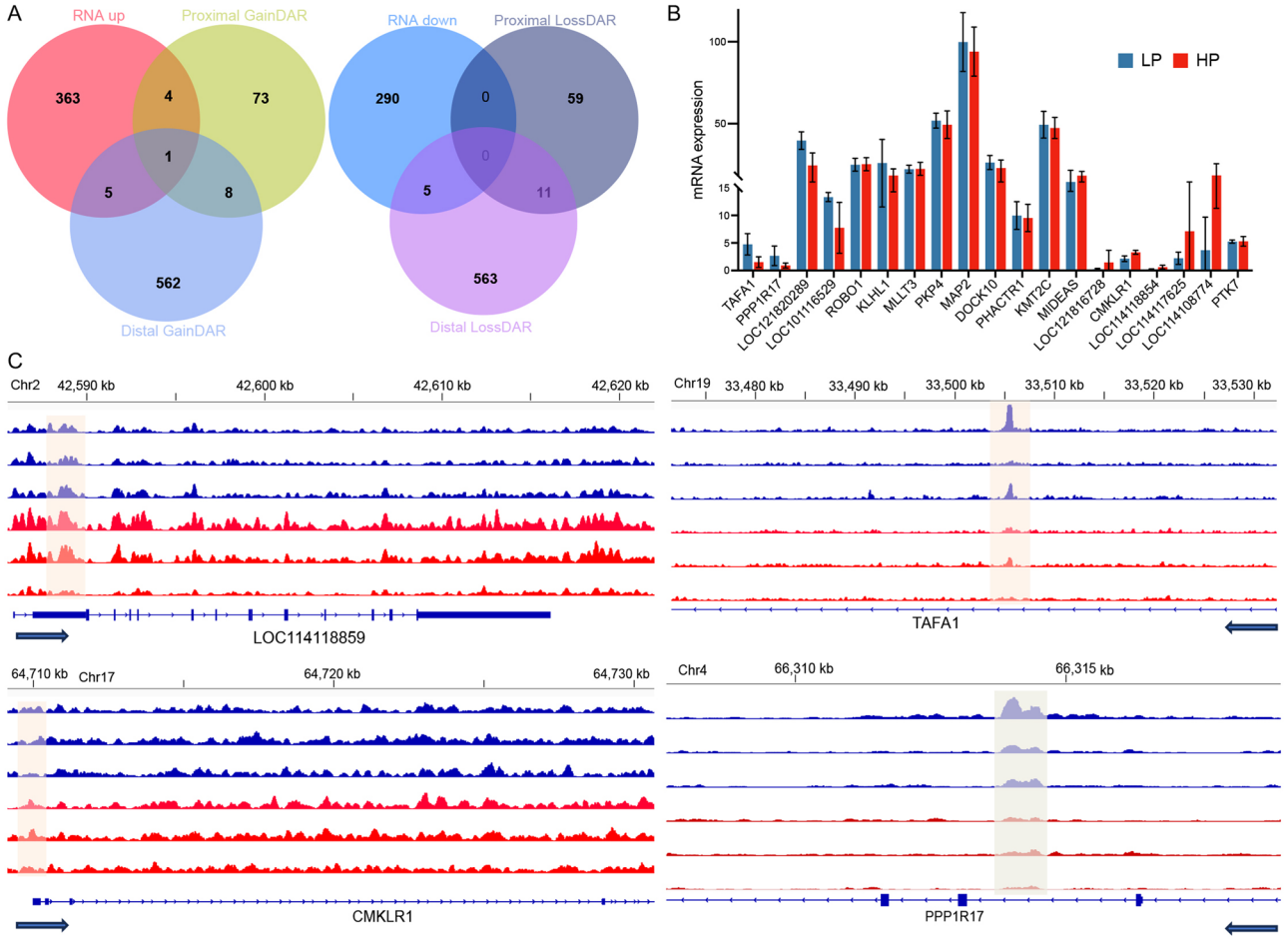

Fig. 5 Combined ATAC-seq and RNA-seq analysis. (**A**) Overlapping analyses of DEGs and the genes closest to the DARs. (**B**) Bar plot showing the expression of key DEGs related to DARs. (**C**) Visualization of chromatin accessibility region of selected genes

was performed for these genes, the results showed that some genes were concentrated in the circadian entrainment and rhythmic process pathways. Previous researchers performed RNA-seq on the hypothalamus, pituitary, pineal, and ovary of Tan sheep during estrus and anestrus. A total of 750 differentially expressed genes were identified in the pituitary, among which several hub genes potentially involved in the regulation of seasonal reproduction in Tan sheep were identified [29]. These findings indicate that the pituitary gland plays a crucial role in regulating seasonal estrus in ewes. Besides, B*DNF*, *FOS*, *CALB1*, and *NPPA* were significantly differentially expressed in the pituitary with different fecundity. The study demonstrated that exogenous BDNF influences the expression of *GnRH* and *KNDy* genes, as well as modulates the secretory activities of LH and FSH in pituitary cells [30]. *CALB1* is mainly expressed in dopamine neurons and has a unique set of responses to external stimuli [31], it may play a role in the secretion of pituitary hormones. *NPPA* encodes ANP (atrial natriuretic peptide), these hormones are secreted by the heart and regulate extracellular fluid volume and electrolyte homeostasis [32]. The difference in NPPA expression suggests that the homeostasis of pituitary cells with different fecundity may be inconsistent. GSEA analysis found that DEG expression was positively correlated with neuroactive ligand-receptor interaction, cAMP signaling pathway, and calcium signaling pathways. The neuroactive ligand-receptor interaction and calcium signaling pathways have been implicated in signaling molecule interactions and signal transduction processes [33]. It has been reported that intracellular Ca2 + concentration plays a crucial role as a signaling molecule in regulating exocytosis, which in turn controls the release of neurotransmitters and endocrine hormones. Therefore, the effect of these signaling pathways may cause the difference in pituitary hormone secretion and then affect the reproductive performance of ewes.

Chromatin accessibility is directly tied to gene transcriptional regulation. Therefore, we comprehensively analyzed the dynamic changes of chromatin accessibility in pituitary of Hu-sheep with different fecundity. ATAC-seq analysis revealed 767 down-regulated differential peaks and 800 up-regulated differential peaks in various groups. Notably, several reproduction-related transcription factors were identified in predicted transcription factor binding motifs that showed an increase or decrease in peaks, such as *NR4A2* and *MEF2*. The study found that NR4A2 binds to the *PRL* promoter at a site near *POU1F1* in a cell culture model and works synergistically with *POU1F1* to drive *PRL* transcription in the pituitary [34]. *MEF2* factors are key regulators of various developmental programs, including muscle and neural differentiation and the responsiveness to growth factors [35]. We have also identified many transcription factors that have been poorly studied in reproduction and whose functions can be explored in subsequent experiments.

To explore the potential relationship between chromatin accessibility and gene expression, we performed an integrative analysis of ATAC-seq and RNA-seq data. This analysis identified 15 differentially expressed genes (DEGs) that were also associated with differentially accessible chromatin regions, suggesting a coordinated regulation at both the epigenetic and transcriptional levels. We hypothesize that these genes may contribute to the differences in fecundity between the two groups. Next, we focus on three genes: *CMKLR1*, *TAFA1*, *PPP1R17*. CMKLR1 is a receptor for Chemerin, a member of the adipokines family, has pleiotropic effects on different cell types [36]. Recent studies have demonstrated that chemerin plays a role in regulating female reproductive processes by acting as a mediator that links metabolic function, steroidogenesis, and reproductive activity in the ovaries [37]. *CMKLR1* is mainly expressed in gonadotrophs and thyrotrophs. The expression of CMKLR1 was different in different estrus cycles and gestation periods [38]. Furthermore, our results revealed a region of differential accessibility in the promoter of the C gene, suggesting that variations in transcription factor binding may influence the expression of this gene. TAFA1, also known as FAM19A1, is a secreted protein primarily expressed in various regions of the central nervous system (CNS). Recent research indicates that FAM19A1 actively participates in several neurophysiological processes and interacts with its binding partners, GPR1 and NRXNs [39, 40]. Studies suggest that FAM19A1 may play a critical role in preserving the physiological integrity of dendritic spines and promoting neurite development during neurogenesis. Additionally, TAFA1 could be pivotal in the development of nerve cells within the pituitary gland [41]. The hypothalamus mainly regulates the process of regulating the production of various hormones by the pituitary. PPP1R17 is predominantly expressed in a neuronal subpopulation of the dorsomedial hypothalamus, which has been implicated in regulating aging and longevity in mice [42]. However, its role in the reproductive process remains unclear. In this study, we identified several key genes, and future research should focus on validating the potential regulatory mechanisms of transcription factors (TFs) and their influence on key genes involved in pituitary hormone secretion.

Conclusions

In conclusion, this study identified 1,567 differential chromatin accessibility regions and 768 differentially expressed genes in the pituitary of Hu sheep with varying fertility through integrated ATAC-seq and RNA-seq analyses. This multi-omics approach enhances our

understanding of the epigenetic and transcriptional mechanisms regulating ovine reproductive physiology and highlights potential molecular targets for improving sheep breeding efficiency. Notably, the joint analysis revealed key candidate genes involved in gonadotropin secretion, including *CMKLR1*, *TAFA1*, and *PPP1R17*, as well as transcription factors *NR4A2* and *MEF2*, which are implicated in pituitary hormone production. These findings provide valuable biomarkers for marker-assisted selection programs aimed at enhancing reproductive performance in sheep. Furthermore, the identified genes and regulatory elements may serve as targets for genetic testing to identify high-fertility breeding stock or guide precision breeding strategies through genomic editing.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11621-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements
The authors sincerely thank all members of the Jiangsu Livestock Embryo Engineering Laboratory for their support in sample collection and technical assistance throughout the preparation of this research. The consumables used in this study were all provided by Guangzhou Jet Bio-Filtration Co., Ltd.

Author contributions
ZYL and WF designed the study. LSL and ZBR performed bioinformatics analyses and drafted manuscript. CPY, CY, and XH collected the samples. YCB, WF and ZYL supervised the research and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by the National key research and development program (No. 2021YFF1000702), Project of Seed Industry Revitalization in Jiangsu Province (JBGS [2021]113), and the high-performance computing platform of Bioinformatics Center, Nanjing Agricultural University.

Data availability
All data in this study are available from the Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) under accession number: PRJNA1236023.

Declarations

Ethics approval and consent to participate
The study adheres to the guidelines set by the Animal Care and Use Committee of Nanjing Agricultural University, ensuring that all procedures involving the ewes were conducted in strict compliance with the Animal Experiments guidelines (SYXK2022-0031). As the animals were acquired from a commercial supplier, informed consent was implicitly obtained through the purchase agreement, which allows for the use of the animals in research. The supplier was aware that the animals would be used for experimental purposes, and all ethical considerations were followed throughout the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 26 December 2024 / Accepted: 21 April 2025
Published online: 20 May 2025

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