Preliminary insights into the molecular mechanism of energy metabolism on sex differentiation in chickens based on transcriptome sequencing

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Abstract

Mammalians sex determination is well-known, and sophisticated mechanisms to sex control have been developed. However, effective sex control in poultry has not yet been developed. Through RNA-sequencing of the male gonads and ovarian tissues of chicken embryos up to 18.5 days, we identified the primary metabolic factors affecting male and female sex differentiation and gonadal development. Pkm2, an essential glycolysis-related protein, influences sex differentiation in chickens via Gapdh, a crucial hormone gene. Glycolysis-related sex regulation also involves epigenetic alterations such as transcription factor (Srf, Myb, Tead1), acetylation (Hdac3), and phosphorylation (Nfkbia, Ikbkb) modifications. Our findings support the concept that glycolysis and oxidative phosphorylation contribute upstream to sexual phenotypic development and maintenance.

Introduction

Genders have a strong correlation with animal production efficiency. For example, female calves are in more demand than male calves, male pigs are in greater demand than female pigs in pig breeding farms. Similar conditions occur with chicken. Egg production needs the preservation of egg-laying females while males are removed to produce chicken eggs. The production of broilers relies mostly on males due to their rapid growth and meat output. The development of effective sex control technology is crucial to the economic growth of the livestock and poultry industries in an era of rapid industrialization. In mammals, sex control technologies, such as flow cytometric analysis, are relatively mature, ensuring nearly 100% birth rate for female calves[1]. However, there are currently no effective sex control technologies in poultry[2], which rely solely on the Vent sexing[3] of chicks and identification of the matching line. The discrepancy in the study advancement of sex determination and differentiation processes in mammals and poultry is the main cause of this scenario.

Recently, our understanding of the mechanisms underlying sex determination in many species has advanced greatly. In mice, the SRY gene was found on the Y chromosome (Berta et al., 1990). The sex-determining region Y protein, encoded by SRY gene, which was found on the Y chromosome, acts as a transcription factor and promotes male gonad (testes) development while preventing female reproductive structure (uterus and fallopian tubes) development by inducing Sox9 and Fgf9. In amphibians, the sex of larvae reared at ambient temperature is generally determined by GSD (Nakamura, 2009). Even though many sex determination-related genes have still been reported to involve in the regulation of sex determination in chickens, the mechanism of sex determination in chickens is still inconclusive DMRT1 (Smith et al., 2003, 2009a; Lambeth et al., 2014; Ioannidis et al., 2021), SOX9 (Kent et al., 1996, 9; Yamashita et al., 2019), HEMGN(Nakata et al., 2013) and AMH (Nishikimi et al., 2000; Takada et al., 2006) were known to regulate the male testicular development, and HINTW (Ceplitis and Ellegren, 2004; Backström et al., 2005; Smith et al., 2009b), FOXL2 (Govoroun et al., 2004, 2; Hudson et al., 2005; Major et al., 2019), WNT4 (Bernard and Harley, 2007; Ottolenghi et al., 2007) mediate the female ovarian development. However, these genes don't directly control sex orientation. There is still a need to further study the mechanism of sex determination in chickens.

Our team has been researching the mechanism of sex determination in chickens for a long time and has discovered that Jun and Ube21[4, 5] can participate in the female sex determination process during the development of the chicken embryo whereas Hmgcs, Spin1z, and Tle4z1[6–8] can take part in the male sex determination process. However, the efficacy of sex reversal by interfering with these genes is more than 40 percent[9], showing that there is still a considerable amount of work to be done to isolate and identify the critical genes for chicken sex determination. This study aims to examine the differentially expressed genes in chicken male gonads and female gonads tissues by high-throughput sequencing, and to initially construct a regulatory network for sex determination and differentiation in chickens by GO analysis and Protein-Protein Interactions Network, to lay the theoretical groundwork for further screening key genes for sex determination and establishing a sex control system in chickens.

Results

1. Differentially expressed genes in the male gonads and female gonads

To elucidate the molecular processes underlying the process of male and female sex determination in chickens, we sequenced the transcriptomes of chicken male gonads and female gonads including differentially expressed genes. At 18.5 days of development, male gonads and left female gonads tissues were obtained, the male gonads are full and rice-like in both gonads, while the female gonads are atrophied in the right gonad. (Fig. 1A) and HE stains revealed that the distribution of varicocele was evident in the male gonads but not in the female gonads (Fig. 1B). Comprehensive transcriptome analysis of male gonads and female gonads using Illumina sequencing (Fig. 1C). Further gene expression studies revealed that male marker genes such as Amh and Sox9 were strongly expressed in male gonads, whereas female marker genes such as Foxl2 and Cyp19a1 were substantially expressed in female gonads (Fig. 1D). RNA was taken from male gonads and female gonads for transcriptome sequencing, and preliminary analysis yielded a total of 3684 differentially expressed genes, of which 2337 were highly expressed in females and 1347 were highly expressed in male (Fig. 2A). The male gonads and the female gonads converged in two separate locations on the two-dimensional graphs created by our UMAP analysis (Fig. 2B). This suggests that sample differences are evident, which is consistent with our assumption. The Pearson coefficients of the two groups were near to 1 (Fig. 2C), indicating that the test is repeatable. Amh, Dmrt1, and Gata4 were strongly expressed in male, whereas Foxl2, Wnt4, and Cyp19a1 were substantially expressed in females (Fig. 2D), agreeing with the qRT-PCR results. All these results indicate that our sequencing of the transcriptome is authentic and precise enough to proceed to the next stage of analysis.

2. Patterns of differentially expressed genes in the male gonads and female gonads

To further study the differences and reasons throughout male gonads and female gonads development, GO functional annotation and enrichment were performed on 3684 differentially expressed genes. The

data indicated that a total of 7294 GO items, including 4998 for biological processes (Supplementary material Table 1). We observed that the entries connected to hormone synthesis were considerably enriched among the GO terms engaged in biological processes, such as "hormone activity," "gonadotropin hormone-releasing hormone activity," "reaction to hormone," etc. (Supplementary material Table 2). This is since the male gonads and female gonads are already developed and may be regulated by hormone production via self-feedback. Tshb, C1qtnf9, Ren, and Gnrh1 are highly expressed in females among these genes. (Fig. 3A), and their high expression is implicated in the manufacture of estrogen and the maintenance of female sex. Inha, Adra1b, and others are strongly expressed in men, and their expression levels are tightly associated to the function of androgens, indicating that the expression of these genes facilitates the production and functional maintenance of androgens (Fig. 3B). We focused on the gonadal morphogenesis-associated gene Amh, whose expression leads to the degeneration of Müllerian ducts in male embryos that would otherwise develop into uterus and oviducts, and is hence highly expressed in males. (Fig. 3C, Supplementary material Table 3), and the KEGG analysis results validated these findings: In the KEGG enrichment analysis bubble diagram, hormone-related pathways such as TGF-β pathway, PPAR signaling route, Steroid hormone biosynthesis, and Fatty acid biosynthesis are considerably enriched (Fig. 3D).

Importantly, the proportion of "Metabolism" items in the GO analysis is reached 26.55 percent (Fig. 4A), showing that metabolic processes play a crucial role in male gonads and female gonads development. From the data of the GO analysis, we determined that "glycolytic process", "positive regulation of glycolytic process", "tricarboxylic acid cycle", and "energy metabolism" were associated with energy metabolism. Expression of "One-carbon metabolic process" and "tricarboxylic acid cycle" genes differed considerably between genders (Supplementary material Table 4), suggesting that energy metabolic processes such as glycolysis (Fig. 4B), tricarboxylic acid cycle (Fig. 4C) differ in the development of male gonads and female gonads.

To further determine the differences in cellular metabolism between males and females during sex determination in chickens, analyzed transcriptomic data from 0d and 4.5d male and female cells. The data can be accessed from National Center for Biotechnology Information (NCBI) under study accession number PRJNA608148. Both 0d and 4.5d male and female cells had differential expression of genes involved in glycolytic and oxidative phosphorylation pathways, with the glycolytic pathway being enhanced in males. It is notable that glycolytic rate-limiting enzymes PKM, HK1 and ENO1 were more prevalent in males than females in 0d (Fig. 4D) and in 4.5d (Fig. 4E). While the TCA-related genes do not show the similar features (Fig. 4F 4G). Above all, Throughout the embryonic phase, there are unique features of glycolysis expression that are shared by both sexes.

3. Energy metabolism is involved in the process of sex determination and differentiation in chickens.

To further clarify the dynamic regularity of energy metabolism during the differentiation of chicken males and females, the key genes of glycolysis and oxidative phosphorylation were marked in the metabolic pathway map, and it was discovered that the differentially related genes occupied important nodes in the

metabolism gene expression levels demonstrated that the glycolytic pathway was dominant during male gonads development, whereas the oxidative phosphorylation process was dominant during female gonads development (Fig. 5A). These findings clearly imply that the processes of sex determination and differentiation in chickens rely on distinct metabolic pathways. The differential expression of Cox family genes (Fig. 5B) and folate metabolism genes (Fig. 5C) in the two sexes, which are essential glycolysis-related enzymes, confirmed that energy metabolism is certainly engaged in the development of gonads in both sexes during data analysis. Downstream folic acid metabolism may generate SAM and CoA, with the latter playing a crucial role in methylation and acetylation modification. Xinxin Wang et al.[10] demonstrated that DNA methylation on zebrafish sex can affect zebrafish sex; Houng-wei Tsai et al.[11] demonstrated that acetylation modification differed in chicken sex and location, suggesting that folate metabolism may exert epigenetic regulation of sex differentiation and gonadal development via downstream products. To further determine the accuracy of the analysis, we collected different types of cells from male and female, respectively, and measured the expression of key enzymes of glycolysis and oxidative phosphorylation in male gonads and female gonads using quantitative polymerase chain reaction (qPCR) (Fig. 5D). PKM and LDH associated with glycolysis demonstrated significant differential expression. This shows that male gonads and female gonads produce glycolysis in separate ways. In addition, LD was assessed in the male gonads and female gonads using an LD testing kit and was shown to be considerably overexpressed in the male gonads, agreeing with the gene expression findings. To further confirm the effects of glycolysis and oxidative phosphorylation on gonadal development, we injected the glycolysis inhibitor 2DG and the oxidative phosphorylation inhibitor rotenone through the air chambers of 0d chicken embryos, respectively, and observed the effects on gonadal development at 18.5d using PAS staining. EdU cell proliferation studies were used to determine the optimal concentration of 2DG and rotenone (Figure S2); the final injection concentration of 2DG and rotenone was 1 mM and 0.5 mM, respectively. We discovered that the testicular spermatophore structure was more pronounced in the 2DG-treated group compared to the control group, and had cleaner borders compared to the rotenone-treated group veins; however, there were no significant alterations in the female gonads (Fig. 5E). This suggests that inhibiting oxidative phosphorylation might facilitate testicular growth. Therefore, we concluded that the male sex determination and differentiation process in chickens is dependent on the glycolytic process, while the female sex determination and differentiation process is dependent on the oxidative phosphorylation process.

To further establish the mechanism by which energy metabolism affects sex determination and differentiation in chickens, we collected the genes relevant to sex determination and differentiation in chicken males and females by GO analysis and did PPI analysis with genes linked to glycolysis and oxidative phosphorylation processes, respectively, and the findings indicated that the key gene Pkm2 as an essential hub was coupled with Dmrt1, an important gene in sex diff, through a positive correlation (Fig. 5F). Thus, the glycolytic key gene Pkm2 maintained the physiological features of male development by associating with genes involved in male sex maintenance, such as Dmrt1, Gata4, Sox9, and Hemgn (Fig. 5G); Similarly, the glycolytic key gene Pkm2 maintained the physiological characteristics of female

development by connecting with Gata4 with female sex-specific genes, whereas oxidative phosphorylation-related genes were less connected to sex-specific genes.

4. Energy metabolism regulates chicken sex determination and differentiation processes through key transcription factors.

Previous Studies have shown that transcription factors are necessary for embryonic development and sexual differentiation. Through the transcription factor database, we evaluated 273 transcription factors in the male and female transcriptome data, of which 68 were significantly expressed in the male gonads and 68 in the female gonads (Supplementary material Table 4). Protein interaction studies indicated that the glycolytic process regulates the transcription factors *Myb* and *Pax2* (Fig. 6A). *Srf, Myb*, and *Pax2* transcription factors can interact with male sex-determining Wnt4 and Sox9 genes, as demonstrated by the interaction of these transcription factors with sex-determining genes (Fig. 6B). Therefore, we propose that glycolysis activates the transcription factors *Srf, Myb*, and *Pax2*, which in turn controls *Wnt4* and is involved in female development and regulates *Sox9* to impact male development. To further demonstrate the accuracy of the analysis, we examined the expression levels of transcription factors and associated signaling pathways in male gonads and female gonads. The results revealed that *Myb* transcription factor and the glycolytic signaling pathway were highly expressed/activated in male cells (Fig. 6C), whereas *Pax* transcription factor was activated in female cells (Fig. 6D). Therefore, we infer that the glycolytic process may influence female sex through the transcription factor Pax and determine male sex via the *Myb* transcription factors.

5. Energy metabolism maintains gonadal development by affecting acetylation and phosphorylation.

Recent research has shown that the metabolome may influence gene expression through epigenetic regulation. By influencing epigenetics, metabolic processes may impact sexual differentiation. *Hdac3*, a crucial enzyme associated to acetylation, was considerably differently expressed in male and female samples, as were *Nfkbia* and *Ikbkb*, which are connected to phosphorylation, suggesting that acetylation and phosphorylation potentially play a role in sex differentiation and gonadal development. To further clarify the relationship between epigenetic regulation such as acetylation and phosphorylation and sex differentiation, we performed a String interaction analysis of the key acetylation enzyme *Hdac3* and the key phosphorylation enzymes *Nfkbia* and *Ikbkb* with genes involved in energy metabolism and genes involved in sex determination and gonadal development (Fig. 6E). The results demonstrate that *Nfkbia* and *Ikbkb* may interact through the glycolysis essential gene *Gapdh* to influence sex determination and gonadal development.

6. Energy metabolism maintains the gonadal development process through hormone synthesis.

To investigate the connection between sexual differentiation and gonad development. According to extant hypotheses or research, in several animals, early sex-determining genes control whether individuals grow into males or females, but beyond a certain point, hormones are required to preserve secondary sexual traits. This was also verified by our sequencing data, which indicated more substantial

variations in hormone-related signaling pathways at the transcriptome level and significant changes in the expression of hormone-related genes in the 18d male gonads and female gonads (Figure S3). We also analyzed by GO the entries "hormone activity", "steroid hormone receptor activity", and "hormone-mediated signaling pathway" (Supplementary material Table 5), which were significantly differentially expressed in male and female samples, with Inha, Nr5a2, and Adra1b highly expressed in males and Tshb, Ren, and Gnrh1 highly expressed in females (Fig. 3B). We grouped hormone-related genes, gonadal development genes, and sex differentiation genes for String interaction analysis. PPI results revealed that the hormone-related gene *Pparg* can interact with sex differentiation-related genes *Sox9* and *Cyp19a1*, thereby regulating chicken sex differentiation and gonadal development (Fig. 7A), and *Cyp19a1* has been reported to have a similar effect on human sex differentiation and gonadal development. *Cyp19a1* has been found to play a significant role in chicken sex differentiation[12], which indirectly confirms the veracity of our findings. This further demonstrates that sex-determining genes may impact gonadal development by modulating hormone expression.

To investigate how sex differentiation and gonadal development are connected through glycolysis-related genes, sex differentiation-related genes, gonadal development-related genes, and sex hormone-regulated genes, a string interaction analysis was conducted (Fig. 7B). It was shown that glycolysis-related genes may influence *Gapdh* through Pkm2 and interact with hormone-related genes *Pparg* to influence sex-regulated *Sox9*, ultimately controlling the whole system for sex maintenance. To verify the accuracy of the data, we injected 0d chicken embryos with 2DG, a glycolysis inhibitor, and rotenone, an oxidative phosphorylation inhibitor, through the air chamber. We then measured the blood hormone levels in male and female individuals at 18.5d, and found that testosterone levels (Fig. 7C) in males and estradiol levels (Fig. 7D) in females were lower in the 2DG group than in the rotenone group. We also discovered the expression levels of hormone production and gonadal development genes. The qPCR results revealed that in males, the hormone-regulated gene PPARG and sex-regulated genes, such as SOX9 and GATA4, were downregulated after addition of 2DG(Fig. 7E), indicating the inhibition of hormone synthesis after blocking the glycolysis process; in females, after the addition of Rotenone, PPARG was upregulated, and SOX9 and GATA4 were upregulated(Fig. 7F), which indicates promoting glycolysis increases the expression of genes involved in male and female gonad development. We inferred, based on the morphological differences between the two groups, that the addition of glycolysis inhibitors resulted in a compensatory enhancement of spermatogonia structures to maintain the original testosterone levels, which partially supports our conclusion that glycolysis and oxidative phosphorylation ultimately influence sex differentiation and gonadal development by influencing changes in hormone levels. Again, no significant changes in female estradiol levels were seen. Additional examination is required for validation.

Discussion

The process of sexual differentiation is a multifactorial regulation mechanism. According to our findings, the sex determination process is controlled by hormones, metabolic levels, and environmental cues. In previous studies, it was generally believed that hormones are involved in the regulation of chicken sex.

However, Kozelka and Gallagher et al.[13] added estrogen during sex differentiation, which resulted in the formation of left female gonads in male embryos; Müllerian ducts were found to persist in male embryos with added estrogen; and high expression of *Amh* (the gene that causes degeneration of Müllerian ducts) was also observed in males in our metabolic data Similarly, we discovered that the expression of glycolytic metabolism-related genes varied across male and female organs. This suggests that glycolytic processes are involved in sex differentiation and maintenance, adding to the theories regarding sex differentiation and maintenance and providing a starting point for future research into the regulatory mechanisms of sex differentiation in chickens, beginning with cellular energy metabolism.

In our study, we found that Pkm2, a key gene for glycolysis, acts as a critical factor to influence male sex development. The mechanism of sex determination for birds is still obscure. Although new sex-determining genes, such as Sry gene, have been reported in mammalians, it is believed that these genes are not involved in the sex-determination process in chickens as research progresses. The reason for this is the belief that finding a key gene can solve the sex differentiation problem while ignoring the influence of intracellular metabolic processes on sex differentiation. The high-glucose environment promotes the activation of Sox9 in mice during the sexual differentiation. So, based on that, our study completed the theory that energy metabolism acts as an upstream factor regulating sex differentiation involved in the formation and maintenance of the sex phenotype in chickens.

By analyzing the sequencing results, we were able to identify the control of several epigenetic alterations. The transcription factors play a significant role in the differentiation of males and females. According to our findings, *Myb* serves as a link between glycolysis-related genes and sex differentiation-related genes. Numerous studies have demonstrated the association of transcription factors with both metabolic regulation and sex determination; Shawlot et al.[14] demonstrated that the transcription factor *Lim1* has a link between hormones and sex determination and plays an important role in *Lim1* knockout mice in which gonads fail to develop; Keith L. Parker et al.[15] identified transcription factor Sf-1 as a key determinant of steroid hormone synthesis regulating gonadotropin and ghrelin And according to our research, the glycolytic process interacts with transcription factors *Srf, Myb*, and *Tead1* through the important gene *Pkm2*, consequently altering the main gene for sex determination, *Sox9*. Consequently, our data provides more evidence that transcription factors play a crucial part in the sex determination process.

Hormones have a significant impact on the process of gonad development. Although there are two main hypotheses for sex determination in chickens, one indicates that genes on one or both sex chromosomes direct differentiation of the embryonic gonads into either an female gonads (ZW) or a male gonads (ZZ), and steroid hormones secreted from the gonads then feminize or masculinize other parts of the body. Alternatively, sex determination in birds may be cell-autonomous, as shown by the production of male-female chimeric chickens. Current research suggests that the metabolic environment has a greater impact on sex control in chickens than gene expression, and that genes govern gonad genesis through hormone levels. Numerous studies have emphasized the significance of hormones in sex determination and gonad genesis. Scheib. D et al.[16] reported that estrogen plays a decisive role in female gonad

development; Nathalie Jasso et al.[17] reported that anti-Müllerian duct hormones secreted by supporting cells in males play an important role in the downstream effector Samd1, and Samd1 has also been linked to sex differentiation. This is reflected in our data as well: the sex-determining gene *Sox9* regulates the gonadal development genes *Sox9* and *Cyp9a1* via the key hormone gene *Gapdh*, while our study enriches the theory of gene-determined sex: hormones regulate sex differentiation and gonadal development by affecting sex- and gonad-related genes. Thus, metabolic control may play a significant role in sex differentiation as a pre-transcriptional component.

There may be a role for epigenetic alterations such as acetylation and phosphorylation in sex-regulatory systems. Zhexu Chi et al.[18] found that histone acetylation is involved in mitochondrial regulation of fatty acids, indicating that cellular metabolism may regulate cellular processes via acetylation. Yunqi Jiang et al.[19] discovered that histone H3K27ac is involved in the regulation of asymmetric development of the left and right gonads in chickens, indicating that cellular metabolism may regulate gonadal development via acet *Hdac3*, a key enzyme associated with acetylation, was significantly differentially expressed in male and female samples, and our results corroborate the current study; Mo-Han Zhang et al.[18] discovered that *Ampk* can regulate mitochondrial metabolism and glycolytic pathways under high glucose conditions, and we discovered that phosphorylation-related *Nfkbia, Ikbkb* were significantly differentially expressed. These results imply that the glycolytic process may be further controlled by sex through epigenetic alterations, indicating the direction of future research on sex differentiation and maintenance.

Materials And Methods

Chicken embryo incubation and gonad collection

Fertilized eggs of White Leghorn chickens were incubated at 37°C and 60% humidity until HH stage 44 (embryonic day 18.5, E18.5). Embryos were exposed by knocking and opening the blunt end of eggshell. The left and right gonads were detached from the mesonephros (primitive kidneys) under a stereo microscope and washed with cold PBS [20]. The isolated gonads were either snapped frozen in liquid nitrogen and stored at -80°C for RNA-Seq and RT-qPCR. At the same time, a small piece of embryonic heads or other tissues were collected and stored at -20°C separately for PCR sexing. All animal procedures were performed according to the protocols of the Yangzhou University and the Institutional Animal Care and Use Committee.

Genetic sexing

For the genetic sexing of embryos, a small piece of tissue was digested to extract DNA with a genomic DNA extraction kit (Tiangen). Genetic sexing was carried out by a standard genotyping PCR protocol focused on the chicken CHD1 (Chromo-helicase-DNA-Binding) gene. The primers are listed in Supplementary material Table 6. These primers are targeting the introns of the CHD1 gene, which is located on the Z (CHD1Z, 482 bp) and W-chromosome (CHD1W, 326 bp)[21]. PCR reactions were 95 °C

for 5 min followed by 30 cycles of 95 °C for 30 s, 51°C for 30 s, 72 °C for 30 s, and a final extension step of 72 °C for 10 min. The amplicons were separated from one band (Z) in the case of male or two bands (Z + W) in the case of female on agarose gel.

Periodic acid–Schiff (PAS) staining

Embryos were fixed prior to performing gradient dehydration with different ethanol concentrations. The embryos were further transparentized with xylene, immersed in paraffin and embedded with paraffin. Paraffin sections were dewaxed with xylene and rehydrated with different ethanol concentrations, then stained with PAS staining kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

Quantitative real-time PCR

Dissected gonads from embryonic stage HH24 were pooled according to sex and side. Each sample included more than three biological replicates and one replicate contained at least three gonads of the same type. Total RNA was isolated by using the TRIzol reagent. The cDNA was prepared using PrimeScript RT reagent Kit with gDNA Eraser (Takara, RR047A). Analysis of mRNA expression was performed with SYBR green, fluorescent dye (ABclonal, RM21203). Quantitative PCR analysis was performed with ABclonal qPCR reagent and Bio-Rad CFX96/384 fluorescent quantitative PCR instrument. The sequences of the primers that worked for each gene, with the control DNA, were provided in Supplementary material Table 6.

RNA Isolation and Library Preparation

Total RNA was extracted using the TRIzol reagent according to the manufacturer’s protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then the libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China)

RNA Sequencing and Differentially Expressed Genes Analysis

The libraries were sequenced on an Illumina HiSeq X Ten platform and 150 bp paired-end reads were generated. About 291.72M raw reads for each sample were generated. Raw data (raw reads) of fastq format were firstly processed using Trimmomatic and the low-quality reads were removed to obtain the clean reads. Then about 284.54 clean reads for each sample were retained for subsequent analyses.

The clean reads were mapped to the human genome (GRCh38) using HISAT2. FPKM of each gene was calculated using Cufflinks, and the read counts of each gene were obtained by HTSeq-count. Differential

expression analysis was performed using the DESeq (2012) R package. P value < 0.05 and foldchange > 2 or foldchange < 0.5 was set as the threshold for significantly differential expression. Hierarchical cluster analysis of differentially expressed genes (DEGs) was performed to demonstrate the expression pattern of genes in different groups and samples. GO enrichment and KEGG pathway enrichment analysis of DEGs were performed respectively using R based on the hypergeometric distribution.

After reads ressembled by StringTie, gene structure extension and novel transcripts identification were performed by comparing the reference genome and the known annotated genes using Cuffcompare software.

Hormone Level Detection

Use a serum separator tube (SST) and allow samples to clot for two hours overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately with Zcbio® Chicken T ELISA Kit (ZC-51745) and Cusabio® Chicken Estradiol ELISA Kit (CSB-E12013C). Absorbance was detected by SPARK® Multimode Microplate Reader.

Determination of the Concentration of Injectable Drug

Chicken DF-1 cells were seeded in a 24-well plate of 50% confluency and treated with 2DG and rotenone at gradient doses, 0.5mM, 1mM, 2mM, 4mM, and 8mM for 2DG, and 100μM, 300μM, 500μM, 700μM, and 900μM for rotenone. Beyond EdU-488 kit was utilized to determine the cell viability by accessing Azide-488 positive cells.

Embryo Injection Experiment

Collecting freshly fertilized eggs, the blunt end was sterilized with 70% ethanol and embryos were opened for microinjection into vessels, 30 eggs per treatment in 3 separate repeats of the experiment. Each treatment of embryos was injected with 2DG(1mM), Rotenone(500μM) and ddH2O of 2μl in total. After injection, Paraffin wax is used to seal and use 1% penicillin and streptomycin wipe the surface. The embryonic genital ridges were collected at 4.5 days. Male gonads and female gonads separated at 18.5 days after incubation for qRT-PCR, PAS staining (Figure S1).

Conclusions

Sex determination in chickens is a complex process. Through transcriptome sequencing analysis of chicken male gonads and female gonads, we discovered that energy metabolism-related genes are differently expressed in both sexes, with glycolysis-related genes being strongly expressed in males. The actual mechanism is that the crucial gene for glycolysis, Pkm2, impacts the critical gene for hormone control, GAPDH. In turn, GAPDH regulates the genes that regulate sex, such as SOX9 and Cyp19a1.

Declarations

Ethical Approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee (Permit Number: SYXK (Su) IACUC 2016–0019).

Consent to Publication

Not applicable.

Data Availability statement

The dataset generated and/or analysed during the current study is available in the NCBI repository. The data can be accessed by BioProject submission: PRJNA871063.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Tables 1 to 6 are available in the Supplementary Files section

Figures

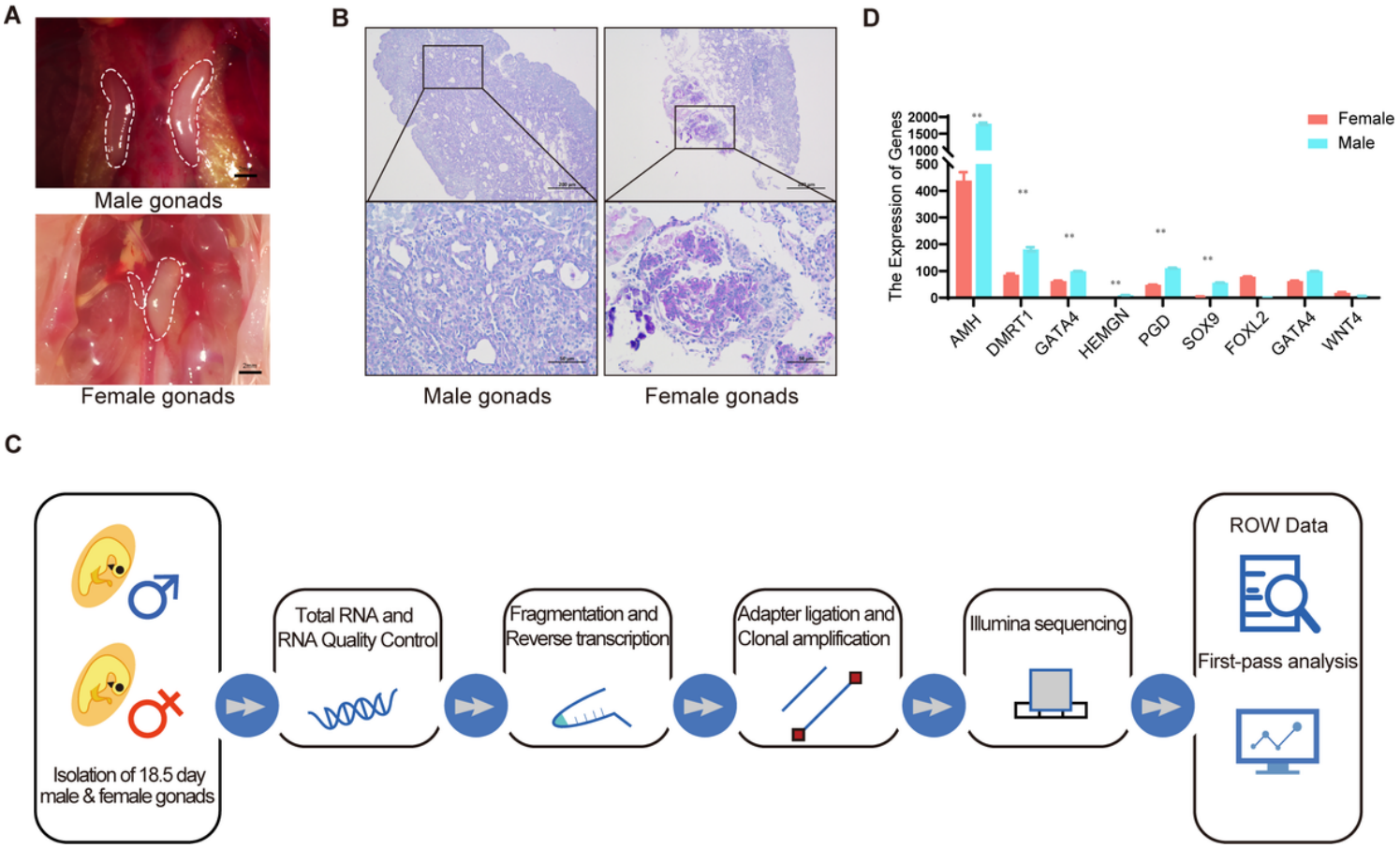

Figure 1

Male and female gonads sampling, transcriptome sequencing, and validation. A. The white dashed lines indicate the position of the male and female gonads. (Up: male, down: female). B.PAS staining of chicken male gonads and female gonads. Scale bar: 50 μm. C. Flow chart for sampling and sequencing, and data analysis of RNA-seq. D. qPCR of key genes involved in sex differentiation in male gonads and female gonads tissues at 18.5 days (n=3). *p < 0.05 and **p < 0.01 (Student's t-test).

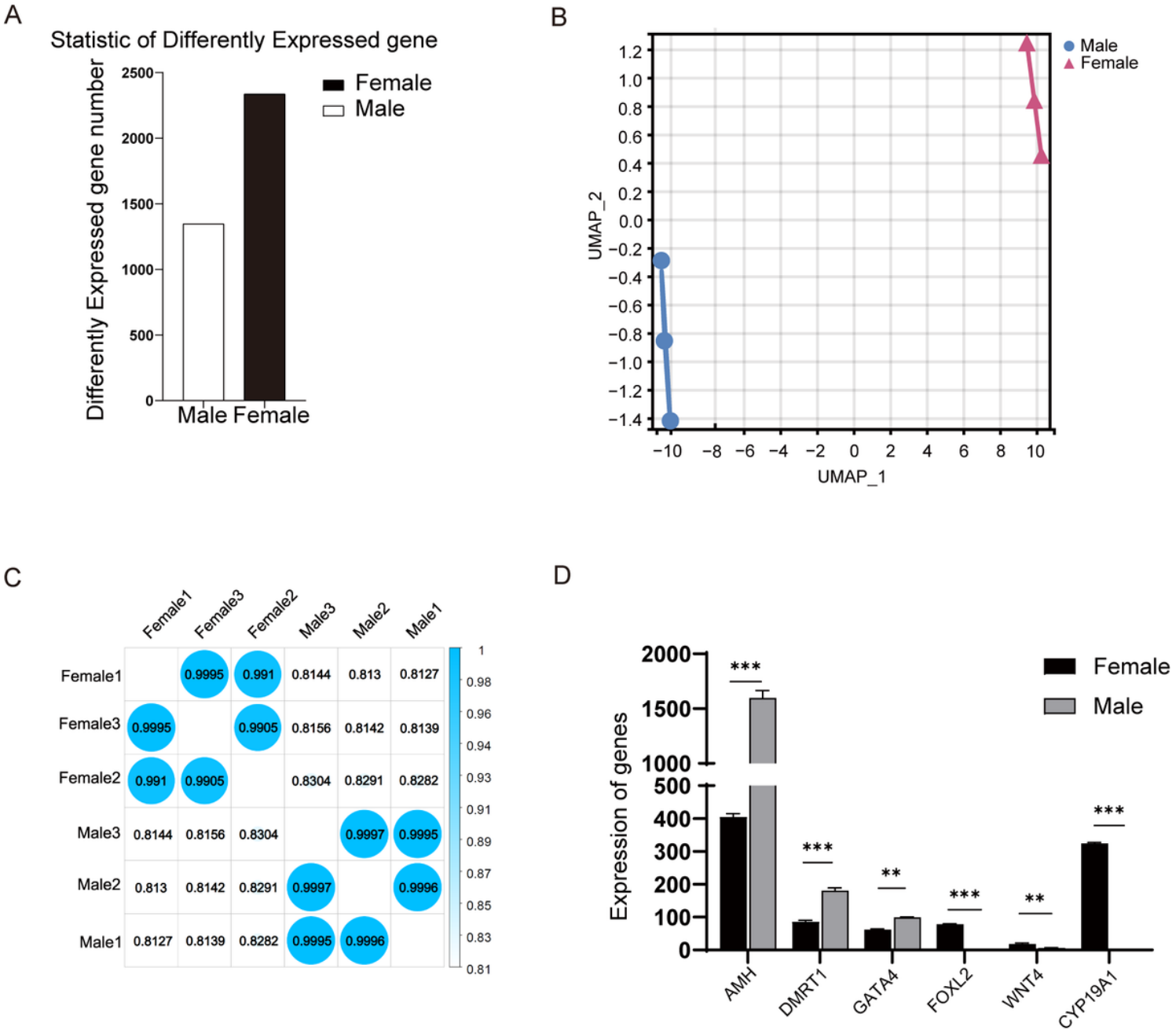

Figure 2

Differentially expressed genes (DEGs) in male and female gonads. A. The number of DEGs in male and female gonads. B. PCA analysis of male and female gonads transcriptomes C. Correlation analysis of male and female gonads transcriptomes. D. qPCR of Amh, Dmrt1, Gata4, Foxl2, Wnt4, and Cyp19a1 in male and female gonads (n=3).*p < 0.05, and **p < 0.01 (Student's t-test).

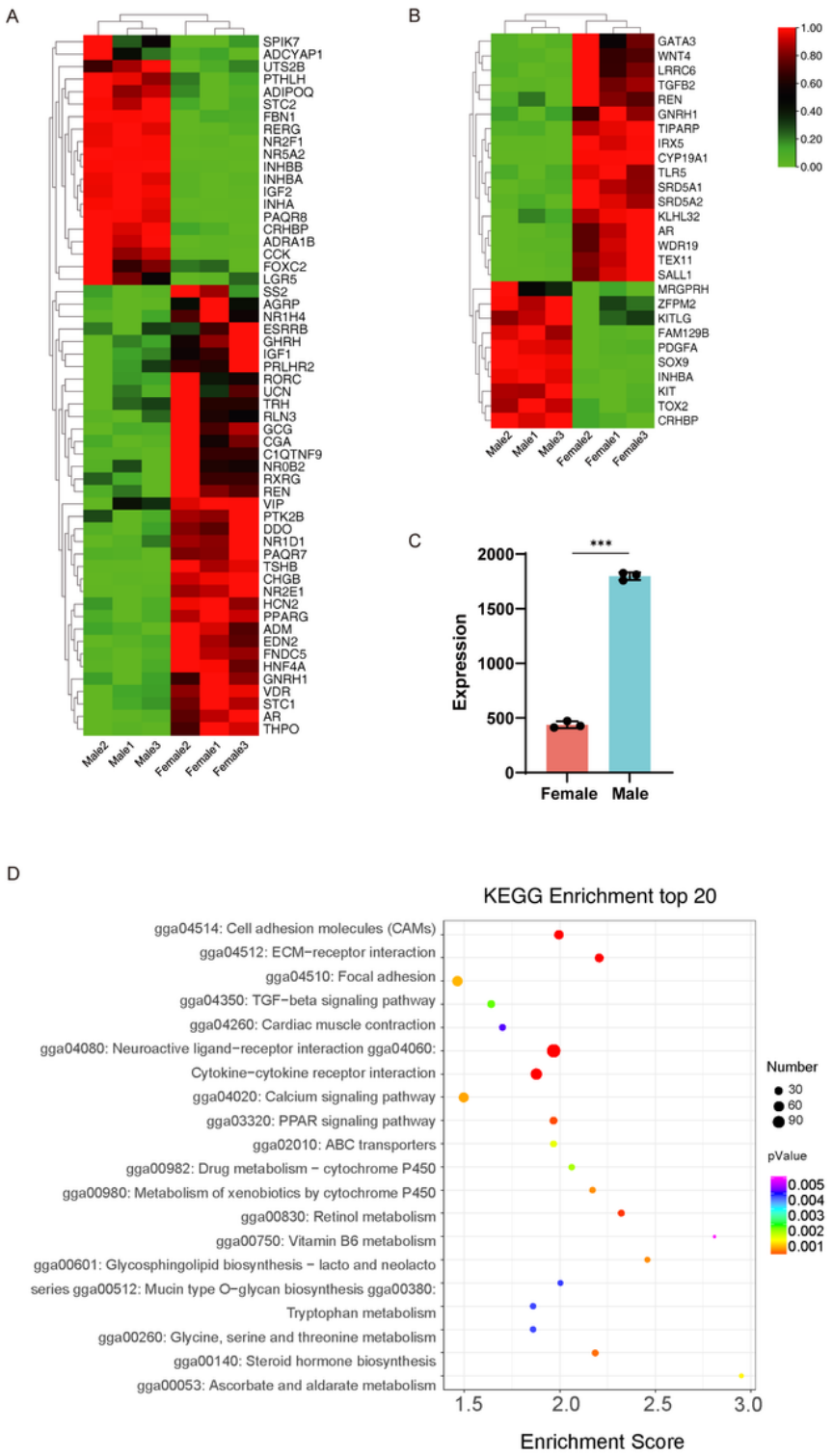

Figure 3

Characteristics of differentially expressed genes in male gonads and female gonads A. Hormone-related genes are differentially expressed in male gonads and female gonads. B. Male and female gonadal development genes are expressed in male gonads and female gonads. C. qRT-PCR results of AMH expression in male gonads and female gonads (n=3). **p<0.001 (Student's t-test). D. Bubble chat of testicular and ovarian differential genes.

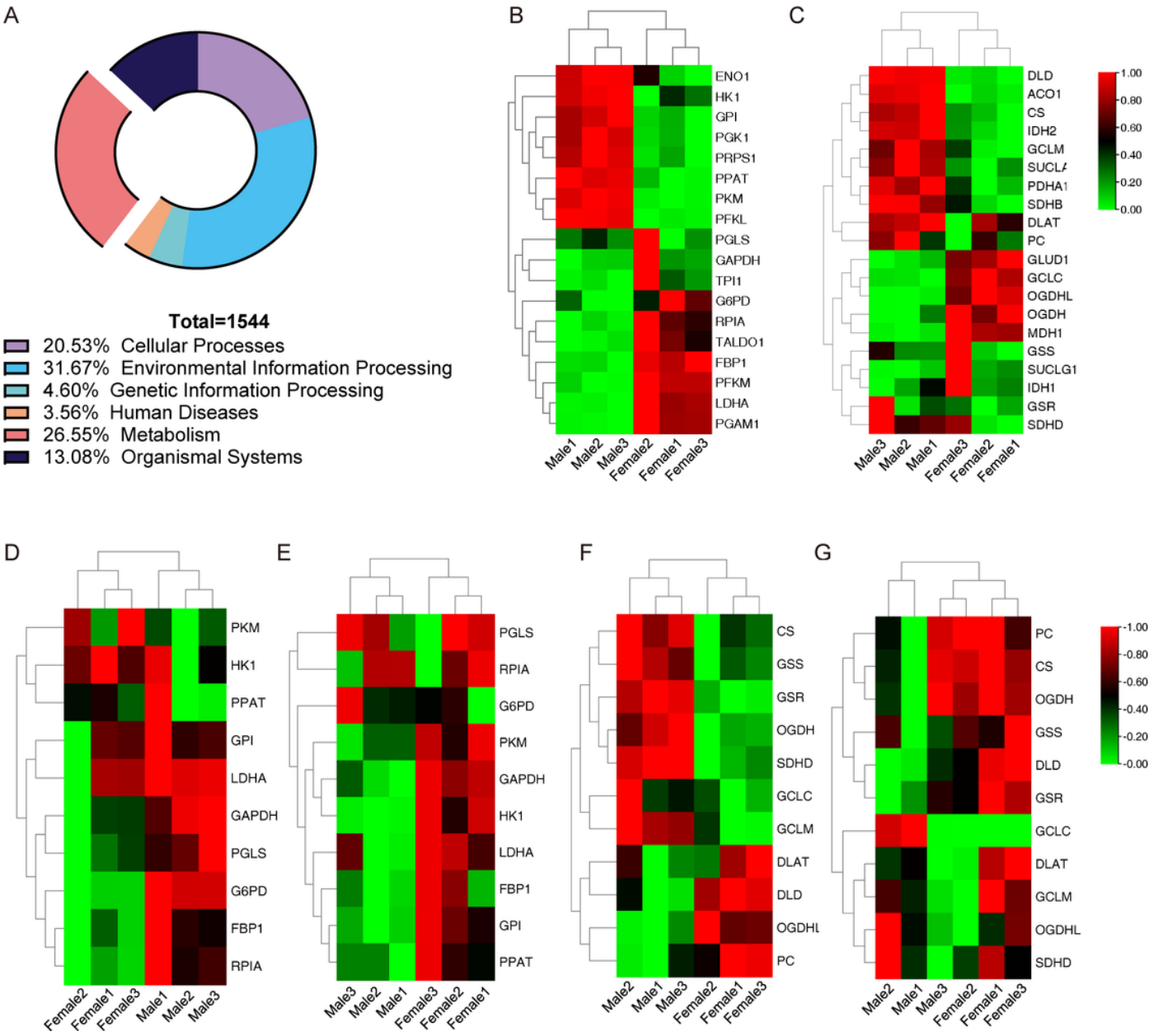

Figure 4

Characteristic of metabolism-related genes A. Pie chart showing 26.55% of differential genes are metabolism-related genes. B. Heat map of glycolysis-related genes expression in 18.5d. C. Heat map of tricarboxylic acid cycle-related genes expression in 18.5d. D. Heat map of glycolysis-related gene expression in 0d. E. Heat map of glycolysis-related gene expression in 4.5d. F. Heat map of tricarboxylic acid cycle-related gene expression in 0d. G. Heat map of tricarboxylic acid cycle-related gene expression in 4.5d.

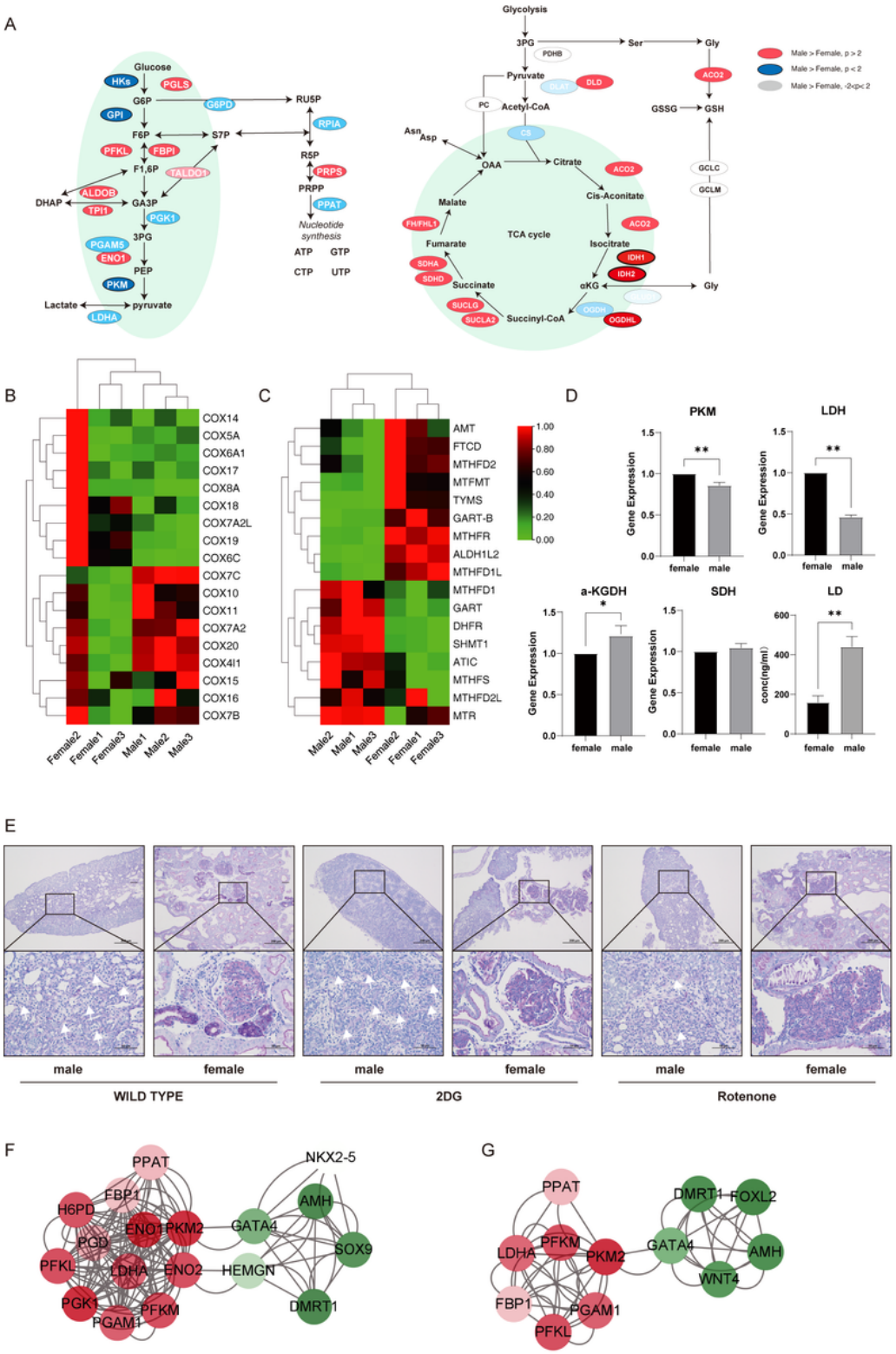

Figure 5

Energy metabolism and sex determination in chickens A. Expression of essential glycolysis and oxidative phosphorylation genes in the female gonads and male gonads. B. Heat map of Cox family gene expression. C. Heat map of one carbon metabolism gene expression. D. RT-qPCR analysis for expression of indicated genes (PKM, LDH, a-KGDH and SDH). Relative expression to ACTB. Concentration of LD. *p < 0.05, **p < 0.01 and ***p<0.001 (Student's t-test). E. PAS staining demonstrated that 2DG enhanced

testicular spermatophore structure relative to the control group. The male gonads spermatic cord structure was more apparent in the 2DG-added group compared to the control group, whereas the female gonads revealed no significant alterations. F. PPI analysis linked glycolysis- and sex-related genes via PKM2 and DMRT1. G. PKM2 is related with male gonad genes Dmrt1, Gata4, Sox9, and Hemgn, according to PPI analysis.

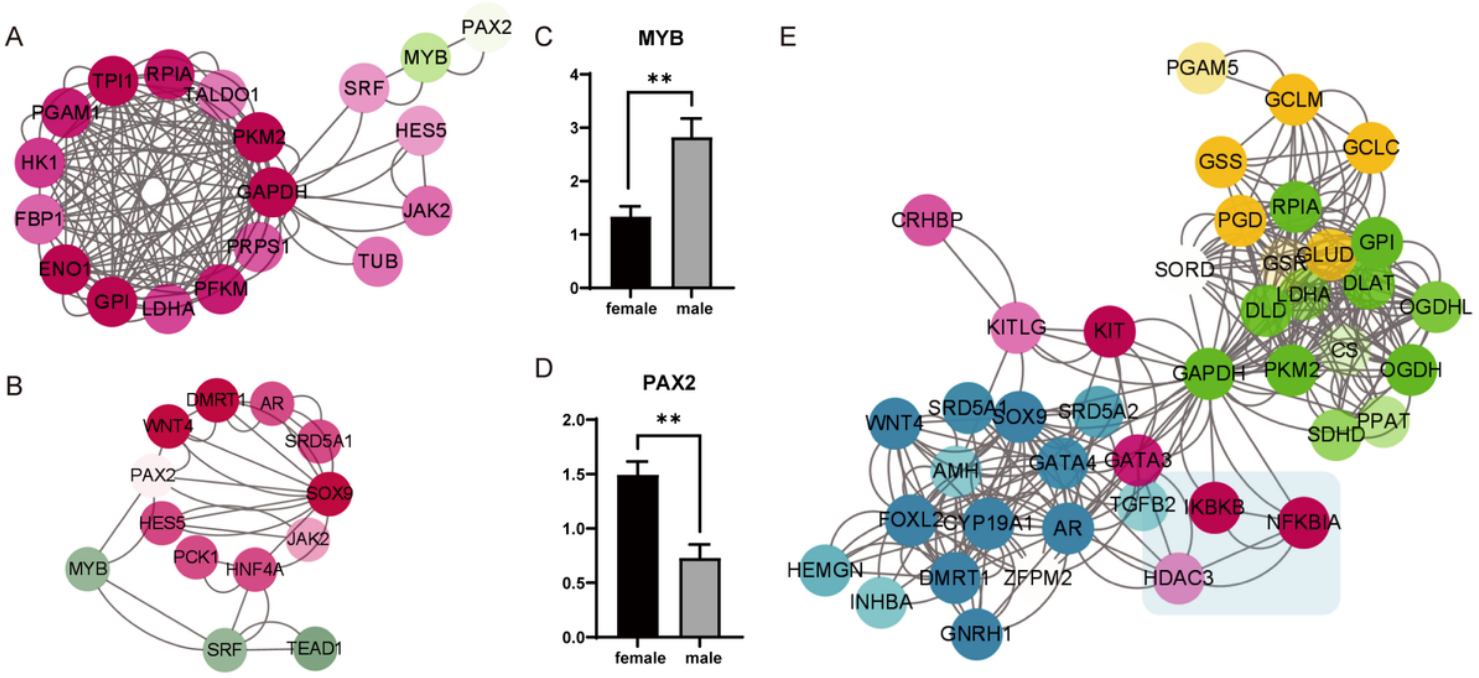

Figure 6

Energy metabolism regulates chicken sex determination and differentiation. A. PPI results show association of Myb, Pax2 and glycolysis-related genes. B. PPI results showed the association of transcription factors such as Srf, Myb, Pax2 and male sex determination related genes Wnt4, Sox9. C. qRT-PCR results showed that MYB was differentially expressed in the male gonads and female gonads. D. qRT-PCR results showed that Pax was differentially expressed in the male gonads and female gonads. E. PPI results show the involvement of IKBKB, NFKBIA, and HDAC in the process of glycolysis and sex determination (n=3). *p < 0.05, **p < 0.01 and ***p<0.001 (Student's t-test).

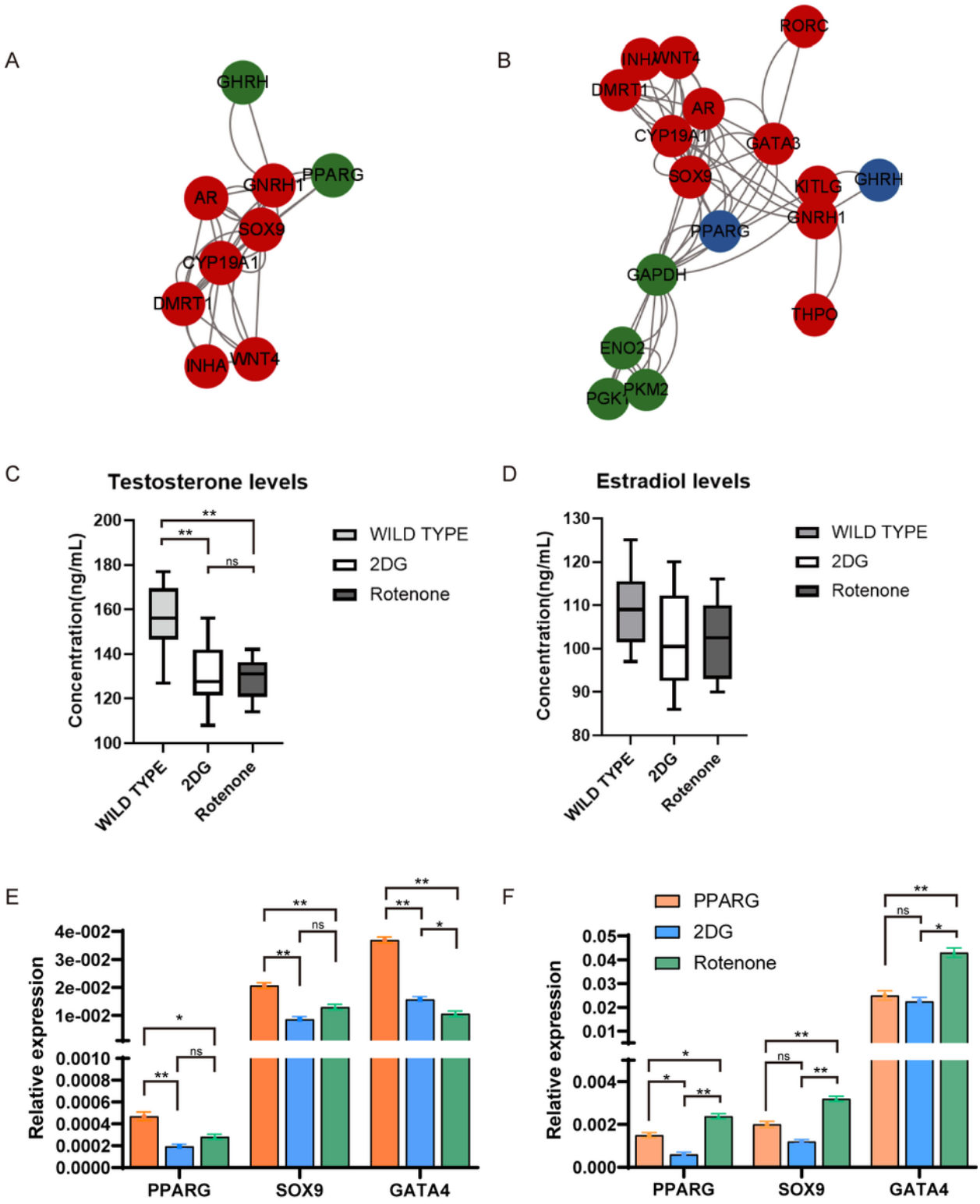

Figure 7

Energy metabolism maintains gonadal development through hormone synthesis. A. PPI data demonstrate that the hormone-related gene PPARG interacts with SOX9 and CYP19a1. B. PPI data reveal that glycolysis-related genes may alter GAPDH through Pkm2, which interacts with PPARG to affect sex-regulated SOX9. C. Box blot shows testosterone levels were lower in the 2DG group than in the rotenone group. D. Box blot shows estradiol levels were lower in the 2DG group than in the rotenone group. E.

Expression of hormone-related gene PPARG and sex regulation-related genes SOX9 and GATA4 in males after adding 2DG and Rotenone. F. Expression of hormone-related gene PPARG and sex regulation-related genes SOX9 and GATA4 in females after adding 2DG and Rotenone. (Data are shown as mean ± SEM, n = 3 independent experiments, **P < 0.01, ****P < 0.0001, one-way ANOVA.).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table1.xlsx
- table2.xlsx
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- table4.xlsx
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