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Deciphering the Putative Genes for the Regression of Chicken Right Ovary during Embryonic and Post Hatch Period

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ABSTRACT

Background: The asymmetry in the development of the left and right gonads after E8 (stage 34) causes the right ovary to fully retract in chickens by the adult stage. From embryonic days 6-6.5 (E6-6.5/stage 29-30), sex-specific differentiation of the gonads is evident in chickens.

Methods: To understand why the right ovary recedes over the embryonic and post-hatch periods, the current study compared the differentially expressed genes on the sixth day (E6) with the twelfth day (E12) and on the first day after hatching (D1). This was accomplished using the NCBI datasets (SRR4029458, SRR4029457, SRR4029464, SRR4029463, SRR4029460, SRR4029459). The functional enrichment and differential gene expression analyses were carried out using Galaxy server, ShinyGO 0.76 and g: Profiler over the duration of the experiment.

Result: From E6 to E12, it was found that 373 and 520 were the significantly up- and down-regulated genes that were heterogeneously expressed. Genes such as FGG, APOH, AHSG, HSD17B1, NME7, PROCA1, MLKL and others that were discovered to be highly up-regulated were associated with pathways related to fibrinolysis, endopeptidase inhibitory action, peptidase inhibitory activity, *etc.* when comparing E12 to E6. Likewise, it has been discovered that the genes differentially expressed from E12 to D1 were 708 and 1136, respectively, significantly up- and down-regulated. Comparing D1 to E12 revealed that the genes (TRAF5, CALML3, FGG, APOH, *etc.*) that were markedly down-regulated were involved in the pathway that governed programmed cell death. This implied that the right ovary had total degeneration during the post-hatch phase and that there was increased programmed cell death during E12. The genes HSD17B1, STEAP3, NME7, CALML3, PROCA1 and MLKL were involved in many pathways that resulted in the regression of the right ovary, as per the KEGG pathway analysis.

Key words: Chicken, Differential gene expression, Embryonic, Ovary, Post-hatch, Regression.

INTRODUCTION

Male chickens develop bilateral testes during gonadal differentiation, but females only grow a left ovary (Romanoff *et al*., 1960). According to Van and Van 1960, the majority of bird species, including chickens and ducks, exhibit a notable asymmetry in their gonadal development. Male and female gonad physical appearances are initially comparable during sexual differentiation (HH28 E5.5). Sex-specific differentiation of the gonads is observed in chickens from embryonic day 6-6.5 (E6-6.5/stage 29-30) onwards (Lambeth *et al*., 2013). Furthermore, the right ovary entirely regresses at the adult stage and after E8 (stage 34), there is an imbalance in the growth of the left and right gonads (Smith, 2007). The left and right ovaries of the brown leghorn exhibit notable morphological changes from the ninth day of incubation to adulthood (Brode, 1928). According to Lambeth *et al*. (2013), there are notable differences in the gonads of males and females at stage HH36 (E10.5). According to Guioli *et al*. (2014), at stage HH44 (E18.5), the germ cells of the left ovary are mainly located in the cortex, while those of the right ovary are primarily scattered throughout the core region. According to Andrews *et al*. (1997), Hoshino *et al*. (2005), Guioli *et al*. (2007) and others, the asymmetric growth and degeneration of the right ovary in chickens is caused by many genes. While studies have studied the differential gene expression of the left and right ovaries, the right ovary's distinct gene expression during the embryonic and post-hatch stages has received less attention. Therefore, the current study employs RNA-seq data, providing hitherto unheard-of insights into the transcriptional complexity of a wide range of organisms (Rani and Sharma, 2017), we will be able to look at a possible mechanism controlling the expression of genes related to ovarian development and differentiation in chicken.

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MATERIALS AND METHODS

To generate differentially expressed genes during the embryonic (E6, E12) and post-hatch (D1) stages of the chicken right ovary using RNA-seq data, a simple approach (Fig 1) was utilized. By comparing the differently expressed genes in embryonic 6th day (E6) vs. embryonic 12th day (E12) and post-hatch Day 1 (D1), the current study aimed to identify the regression of the right ovary in the latter embryonic and post-hatch era. In this investigation, the NCBI (https://www.ncbi.nlm.nih.gov/) datasets (SRR4029458, SRR4029457, SRR4029464, SRR4029463, SRR4029460, SRR4029459) were used. The web-based Galaxy server (https://usegalaxy.org/) (Afgan *et al.,* 2018), ShinyGO 0.76 (http://bioinformatics.sdstate.edu/go/) (Ge *et al.,* 2020) and g: Profiler (https://biit.cs.ut.ee/gprofiler/gost) (Raudvere *et al*., 2019) were utilized for differential gene expression and functional enrichment analysis. As part of the present method, transcript levels are measured, low-quality reads are removed, processed reads are aligned with the *Gallus gallus* reference genome (GRCg7b) and statistical tests are run to find genes with differential expression. For enrichment analysis on the genes with differential expression, additional processing is carried out. The right ovary's regression or degeneration in the later embryonic and post-hatch stage was also associated with the differentially expressed genes in E6 vs. E12 and D1.

Quality check and trimming of the data

The raw data was initially processed for its quality using FastQC (Wingett and Andrews 2018, Chang *et al*., 2021) and the poor-quality reads were further processed using Trimmomatic (Bolger *et al*., 2014) with 30 as the average quality of the reads.

Mapping of processed reads

HISAT2 (Kim *et al*., 2015, Wang *et al*., 2023) was used for the alignment of processed reads to the *Gallus gallus* reference genome (GRCg7b), which was uploaded to galaxy server using NCBI FTP URL (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/016/699/485/GCF_016699485.2_bGalGal1.mat.broiler.GRCg7b/GCF_016699485.2_bGalGal1.mat.broiler.GRCg7b_ genomic.fna.gz). Trimmed read files and *Gallus gallus* reference genome is given as input for HISAT2 and obtained output aligned reads as BAM file.

Assignment of mapped reads to genes and quantification of genes

Using feature Counts (Liao *et al.,* 2014), the mapped reads and *Gallus gallus* gene annotation files were used as input to calculate the number of reads per annotated gene (https://ftp.ncbi.nlm.nih.gov/genomes/refseq/vertebrate_other/Gallus_gallus/latest_assembly_versions/GCF_016699485.2_bGalGal.mat.broiler.GRCg7b/GCF_016699485.2_bGalGal1.mat.broiler. GRCg7b_genomic. gtf. gz).

Identification of differentially expressed genes

DESeq2 (Love *et al*., 2014) was used to analyze the differentially expressed genes. Read count files generated by feature Counts were provided as input to DESeq2 and it

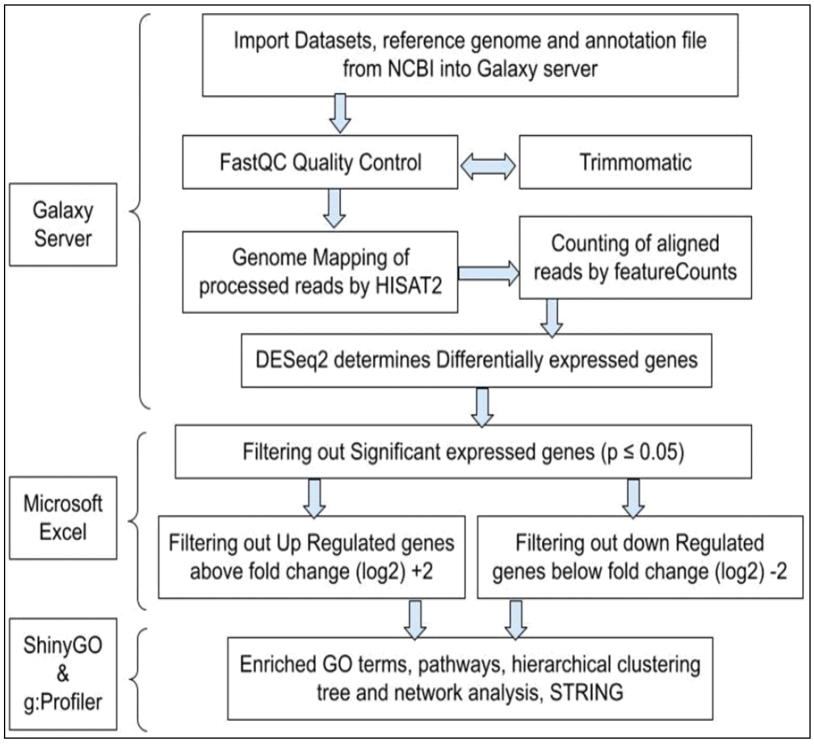


Fig 1: Workflow of the data analysis.

gives differentially expressed genes as an output file. DESeq2 also gives plots on principal component analysis (PCA) and Heatmap of the sample-to-sample distance matrix (with clustering).

Volcano plot

Volcano plot on differentially expressed genes was generated based on the DESeq2 results. A volcano plot is a form of scatter plot that displays differential gene expression, with the fold change on the X-axis and the p-value on the Y-axis.

Filtration of differentially expressed genes

DESeq2 output contains significant ($p \leq 0.05$) and non-significant differentially expressed genes. Up regulated genes are filtered out which are above the +2 fold-change (log2) and down regulated genes are filtered out which are below the -2 fold-change (log2).

Enrichment analysis

ShinyGO 0.76 (Ge *et al*., 2020) was used for in-depth gene list analysis, including a graphical representation of enrichment, pathway, gene features and protein interactions. g:Profiler (g:GOSt) was also used for performing functional enrichment analysis on top differentially expressed genes.

RESULTS AND DISCUSSION

QC check, filtration and alignment

The differential gene expression in the right ovary of chicken on E6, E12 and D1 were studied using data (SRR4029458, SRR4029457, SRR4029464, SRR4029463, SRR4029460, SRR4029459) downloaded from NCBI. The obtained raw data was found to have a total of 121.177678 million reads. The FastQC report was found to have satisfactory quality parameters and do not have any adapter sequences. A total of 108.506697 million processed reads were obtained after filtration for its quality (Q30) using Trimmomatic. When the processed reads were aligned to the *Gallus gallus* reference genome (GRCg7b), 76.836692 million reads were mapped resulting in a mapping percentage of 70.81. The mapped reads with SRA processing summary was given in Table 1.

Quantification of differentially expressed genes

There were 373 and 520 differentially expressed significant up-regulated and down-regulated genes respectively from E6 to E12. Similar to this, 708 and 1136 genes, respectively, were shown to be significantly up-regulated and down-regulated during E12 to D1. The genes that were significantly up-regulated during E12 were FGG, APOH, AHSG, HSD17B1, NME7, PROCA1, MLKL and down-regulated during E12 were NANOG, STEAP3, CALML3. The genes DMRT1, HINTW, FOXL2, CYP19A1 , PIWIL1, TDRD5, DND1, FGG, APOH, AHSG, HSD17B1, NME7, CALML3, PROCA1, MLKL *etc.* were down-regulated during D1 and STEAP3, NANOG, TRAF5 etc were up-regulated during D1. These DEGs genes were discovered to be involved in pathways that inhibit fibrinolysis, thrombosis, lipase activity, regulation of insulin signalling, programmed cell death, ferroptosis, phosphorylation reactions, tumour necrosis factor (TNF)-induced necroptosis *etc*. A volcano plot demonstrating all differentially expressed genes between E6 to E12 and E12 to D1 are shown in Fig 2a and Fig 2b.

Enrichment analysis

HSD17B1 gene was upregulated (0.685248) during early embryonic stage (E6 to E12) and down related (-5.58248) in the late embryonic stages (E12 to D1). The HSD17B1 is involved in estradiol and testosterone synthesis in mouse (Mindnich *et al*., 2004) 11 beta hydroxytestosterone and estradiol 17 beta hormonal production is decreased in the right ovary during later embryonic stages suggesting the regression of the right ovary (KEGG:00140). STEAP3 gene was down regulated (-4.76293) in the early embryonic stage and is then upregulated (1.387534) in the late embryonic stages. STEAP3 involved in fenton reaction triggering ferroptosis suggesting its role in the oxidative damage to the cell membrane which further leads to the cell death in the right ovary in the late embryonic stages (KEGG:04216). Ye *et al*. (2022) reported higher expression of STEAP3 gene increases sensitivity to ferroptosis. The NME7 gene was upregulated (7.082978) in the early embryonic stage and is then down related (-1.75833) in the late embryonic stages. NME7 gene is involved in phosphorylation of dADP to dATP, dGDP to dGTPdIDP to dIT, ADP to ATP, GDP to GTP, IDP to ITP dTDP to dTTP, dUDP to dUTP, dCDP to dCTP and vice versa, suggesting apoptosis due to reduced purine and pyrimidine metabolisim for the DNA synthesis in the later embryonic stages (KEGG:00240, KEGG:00230). CALML3 gene was down regulated (-4.57281) in the early embryonic stage (E6 to E12) and upregulated in late embryonic stages (E12

Table 1: SRA processing summary.

Sample ID	NCBI SRA no	Total RAW sequence reads	Total processed reads	Aligned reads	Mapped %
E6FR_1	SRR4029459	19526456	17749609	12361967	69.64
E6FR_2	SRR4029460	18144127	16523426	11585586	70.11
E12FR_1	SRR4029563	18286342	16694274	12537700	75.10
E12FR_2	SRR4029464	19843600	18078375	13547909	74.93
D1FR_1	SRR4029457	24624235	21362042	13401765	62.74
D1FR_2	SRR4029458	20752918	18098971	13401765	74.05
Total number of reads		121177678	108506697	76836692	70.81

to D1) (-2.41755). Studies reported overexpression of CALML3 significantly prevents the apoptosis (Jia *et al*., 2018). Downregulation of CALML3 is involved in inhibition of glycogen synthesis leading to disruption of glucose homeostasis leading to apoptosis (KEGG: 04910). It also leads to the reduced gonadotropin gene expression and secretion which in turn influences the release of hormones from the ovary (KEGG: 04912). CALML3 is highly down regulated preventing the contraction of vascular smooth muscles, thereby enabling better blood circulation to the ovarian tissue in early embryonic stages than the later embryonic stage (KEGG:04270). PROCA1 gene was upregulated (4.013282) in the early embryonic stage and is then down related (-3.83398) in the late embryonic stages. Downregulation of PROCA1 leading to Ca^{2+} influx into vascular smooth muscle cells enhancing its contraction which may further prevent the blood supply to the ovarian tissue in later embryonic stages (KEGG:04270). The up regulation (4.902271) of MLKL gene during the early embryonic stage resulted in dephosphorylation of Drpl gene leading to tumor necrosis factor (TNF) induced necroptosis through mitochondrial fission (Remijsen *et al*., 2014) (KEGG:04217). The differentially expressed genes and their upregulation and down regulation was given in Fig 3 and Table 2.

The Fibrinogen gamma chain (FGG) gene is involved in the regulation of fibrinolysis and thrombosis (Ma *et al*., 2007). FGG gene was upregulated (5.75) in the early embryonic stage and is then down related (-0.18) in the late embryonic stages. Upregulation of FGG during E12 day in the right ovary suggests increased vasoconstriction that may lead to reduced blood flow in the right ovary causing its regression. Apolipoprotein H (Apo-H) gene was upregulated (5.2) in the early embryonic stage, than during the late embryonic stages (0.47) leading to fibrinolysis (Crook *et al*., 2001). Angiopoietin-like protein 3 (ANGPTL3) regulates the lipid catabolism (Adam *et al*., 2020) and was upregulated (6.32) in the early embryonic stage and is then

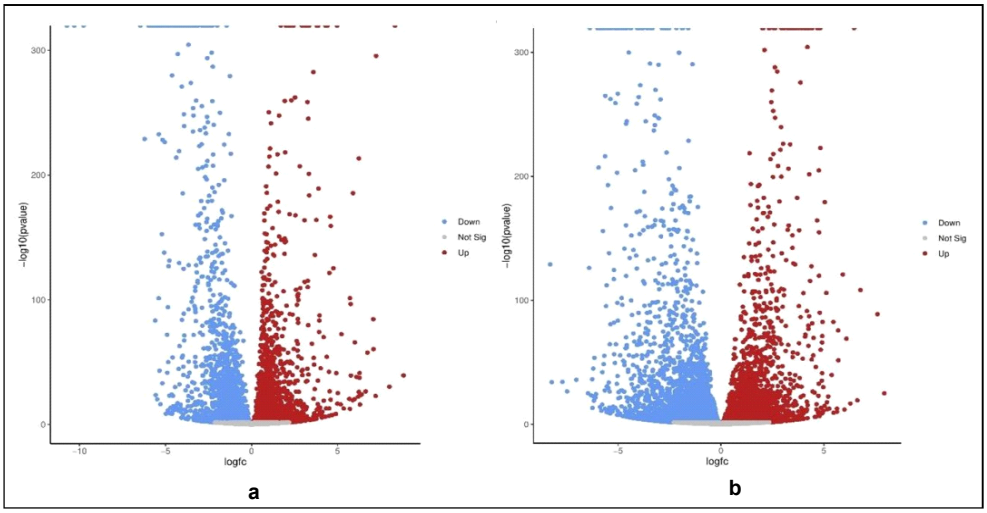


Fig 2: a) (left) and b) (right) representing volcano plot during E6 to E12 and E12 respectively.

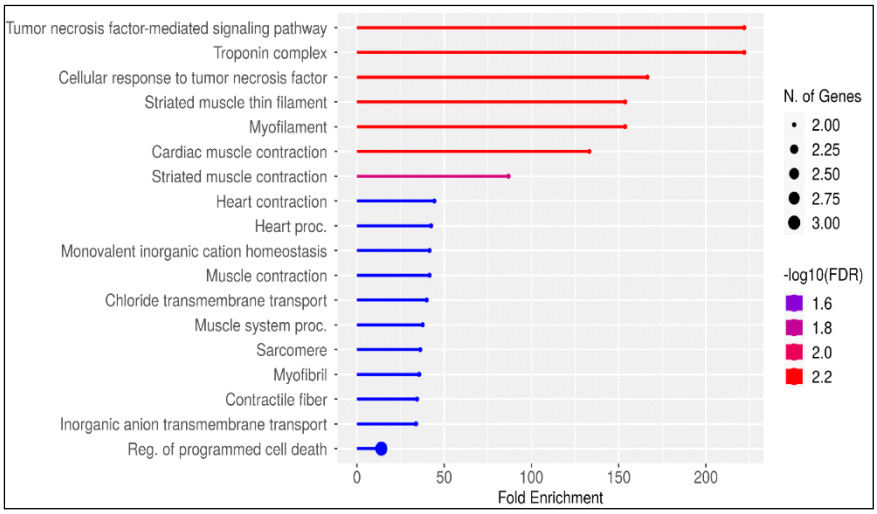


Fig 3: Fold changes of different DEGs.

Table 2: Top up and down regulated genes in right ovary during E6, E12 and D1.

GENE	E12	D1
Genes reported so far in literatures		
DMRT1	Up regulated	Down regulated
HINTW	Up regulated	Down regulated
FOXL2	Up regulated	Down regulated
NANOG	Down regulated	Up regulated
CYP19A1	Up regulated	Down regulated
PIWIL1	Up regulated	Down regulated
TDRD5	Up regulated	Down regulated
DND1	Up regulated	Down regulated
Genes and pathways identified in our study		
FGG	Up regulated	Down regulated
APOH	Up regulated	Down regulated
AHSG	Up regulated	Down regulated
HSD17B1	Up regulated	Down regulated
STEAP3	Down regulated	Up regulated
NME7	Up regulated	Down regulated
CALML3	Down regulated	Down regulated
PROCA1	Up regulated	Down regulated
MLKL	Up regulated	Down regulated

down related (-2.9482) in the late embryonic stages. Overexpression of this gene cause increased activity of lipoprotein lipase that may result in degeneration of the right ovary. Reduced fertility and abnormal follicular development were reported in estrogen receptor-β knockout (BERKO) mice (Cheng *et al*., 2002) and found its correlation with ITIH2 expression (Hamm *et al*., 2008). ITIH2 gene was upregulated (5.99) in the early embryonic stage resulting in reduced oestrogen receptors leading to regression and reduced fertility of right ovary in chicken. Alpha-2-HS-glycoprotein (AHSG) gene was upregulated (7.2) in the early embryonic stage than late embryonic stages (2.43). This gene is involved in glucose metabolism and the regulation of insulin signalling. AHSG may affect glucose uptake and lipid oxidation in adipocytes (Zhuo *et al*., 2015). TRAF5 proteins is involved in programmed cell death (Muzib, 1998) and its overexpression induced the apoptosis of mouse podocytes (Wu *et al*., 2018).

The reduction of the DMRT1 protein expression in-ovo resulted in feminization of the embryonic gonads in genetically male (ZZ) embryos (Smith *et al*., 2009). In early-stage chicken embryos, the HINTW probe ubiquitously and robustly labelled all female cells. According to Nagai *et al*. (2014), this probe can be employed in developmental research to differentiate intraspecific, inter-sex donor/host tissues. FOXL2 (Fork head box L2) is only expressed in female embryos and is essential for controlling ovarian development (Luo *et al*., 2020). A crucial gene for the feminization of the gonad in chicken embryos, Cyp19a1 encodes an aromatase that catalyses the conversion of progesterone to oestrogen (Ellis *et al*., 2012). This gene has been identified as being expressed exclusively in females from the earliest stages of gonadal sex differentiation. PIWIL1 plays a crucial part in gametogenesis by inhibiting transposable elements and blocking their mobilisation. PiRNAs plays an important role in the avian ovarian asymmetry (Shaikat *et al*., 2018, Zhang *et al*., 2021). The major differentially expressed ovarian genes are strongly linked to phagosome function, carbon metabolism, neuroactive ligand-receptor interaction and calcium signalling all of which accelerate the degeneration of the right ovary (Yu *et al*., 2017). The ageing of the cell and the initiation of the inflammatory response results in degeneration of right ovary in ducks and geese (Ran *et al*., 2023).

CONCLUSION

In asymmetric chick gonads, the left and right female gonads go through different developmental processes, producing a functioning ovary exclusively on the left side. Despite substantial advances in recent research, the complete molecular mechanism of right ovary regression has not been identified. Therefore, the current study illustrates the various genes at various stages of embryogenesis and their role in gonad asymmetry during embryonic development. Finding the pathways that contribute to ovarian degeneration is a crucial step in understanding its molecular mechanisms and functions which will be useful for future studies. Furthermore, transcriptome analysis of different time frames of the ovarian development needs to studied for clear understanding of dynamics of genes and to identify hub genes responsible for right ovary regression.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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