Spatially Resolved Transcriptomic Profiling of Developing Primary Follicles in the Chicken Ovary Reveal a Potential Developmental Checkpoint

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The female reproductive lifespan relies on the controlled activation of primordial follicles and regulation of primary follicle development. In avian species, a sufficient number of primary follicles need to be recruited into advancing growth phases for the maintenance of the follicular hierarchy. Despite its importance, the process regulating the coordinated activation and development of avian follicles has received little attention. Elucidating the complex interactions in the ovary responsible for initiating and maintaining primary follicle development would benefit from retaining the original tissue morphology. The objective of this study was to characterize the transcriptional landscape of developing primary follicles (<2 mm diameter) in the chicken ovary. Whole ovaries were harvested from two 42-week-old White Leghorn hens with a normal laying cycle. Large follicles (>3 mm diameter) were removed before processing and the remaining samples were embedded in optimal cutting temperature (OCT) compound and snap frozen. Four 12 μm frozen sections, one from layer 1 and three from layer 2, containing primary follicles at various stages of development were placed onto a single Visium Spatial Gene Expression Slide (10X Genomics). Sections were fixed in methanol, stained with hematoxylin and eosin, and imaged followed by tissue permeabilization, reverse transcription, and second strand synthesis for cDNA amplification and library construction. The sequenced reads were aligned to the *Gallus gallus* reference genome using cellRanger v7.1.0. Gene expression was

normalized across the four tissue sections with individual chickens as covariate followed by principal component analysis for dimensionality reduction and unsupervised spot clustering using Seurat. Differentially expressed genes (DEG; p adj<0.05 and |log2FC|>0.25) of relevant clusters were annotated using Gene Ontology (GO) and visualized using Kyoto Encyclopedia of Genes and Genomes (KEGG) to gain insight into their functional significance during early follicle development. Distinct transcriptional signatures associated with small (<600 μm diameter) and larger (>600 μm diameter) primary follicles were identified. Notably, smaller primary follicles exhibited enrichment in pathways related to controlled degeneration, or atresia. In contrast, larger primary follicles showed enrichment in pathways involved in mRNA surveillance, transport, and processing. Furthermore, a subset of DEGs were selected and plotted individually across the tissue sections to explore their similarity to the histological features. Genes involved with cell cycle progression (e.g., CCNO, CCNA1, CKS1B), vesicular trafficking (e.g., MARCKSL1, EHD3, DAB2), and maintenance of follicle identity (e.g., FOXL2) were spatially enriched within the oocytes of primary follicles while genes related to oxidative phosphorylation (e.g., COX1, COX2, COX3) were highly enriched in the area directly surrounding large primary follicles. Genes encoding ribosomal proteins (e.g., RPS17, RPS28, RPL35A) showed spatial expression in the surrounding stroma. These findings suggest a potentially important developmental checkpoint during the development of primary follicles that may lead to either their atresia or proceed in preparation for later stages of development. A deeper understanding of these processes not only has implications for improving reproductive success in poultry but also advancing our broader knowledge of avian reproductive biology.