LETTER

GNAS deficiency is associated with premature ovarian insufficiency phenotype via regulating ovarian steroidogenesis

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Dear Editor,

Premature ovarian insufficiency (POI) is a condition in which the ovaries stop functioning normally before the age of 40, leading to a reduced quantity and quality of oocytes, and a decrease in the production of estrogen and other sex hormones.[1] While there are several causes of POI, including autoimmune disorders, chemotherapy, and radiation therapy, genetic mutations are believed to be major contributors to the development of POI.[1] Since pathogenic mutations in POI have been identified via the largest-scale whole-exome sequencing analysis in POI cohorts (1,030 cases and 5,000 normal controls), the genetic basis of POI has become increasingly clear.[1] However, the mechanisms and expression patterns of these pathogenic mutation genes remain to be determined, especially at the single-cell and spatial transcriptomic resolution.

Herein, we performed single-cell transcriptomic analyses of ovaries from non-human primates (NHP)[2] and the adult mouse,[3,4] combined with spatial transcriptomic data of human ovaries,[5] to demonstrate the expression patterns of pathogenic mutant genes for POI. Multicolor immunohistochemistry (M-IHC) was used to validate the oocyte-specific expression patterns of mutant genes. Furthermore, pathway analysis and a conditional knockout mouse model (*Gnas*$^{flox/flox}$, *Cyp17a1*^{Cre}) were used to identify potential mechanisms underlying POI pathogenesis (Figure 1A).

To identify the expression patterns of POI-pathogenic mutant genes, we carried out an expression analysis of 79 POI-pathogenic mutant genes using single-cell transcriptomic data from NHP ovaries. After processing using a reproducible workflow,[4] NHP ovarian cells were clustered into eight cell types (Figure 1B). Notably, we found that most of the POI-pathogenic mutant genes were enriched in the oocyte population. *GNAS* and *LMNA* were found in all cell populations, and *STAR* was mainly found in stromal cells (Figure 1B). Next, we used single-cell transcriptomic data of the adult mouse ovary that did not include oocytes in the sequencing library[5] to explore the expression patterns of POI-pathogenic mutant genes. By integrating the expression matrix, we found that the oocyte-specific mutation genes were not enriched in any of the 14 cell clusters (Figure 1C).

To further validate the oocyte-specific expression patterns of POI-pathogenic mutant genes, we validated the expression levels of these genes in spatial transcriptomic spots from human ovaries.[5] By clustering 13,091 spatial transcriptomic spots into 20 clusters, of which cluster 11 showed high expression of oocyte markers (Figure 1D), we found that POI-pathogenic mutant genes were relatively bright in cluster 11 and associated with *ZP3* expression, such as *LMNA*, *GNAS*, *STAR*, *FIGLA*, *ZAR1*, *ZP3*, and *STAG3* (Figures 1D-E). Moreover, visualization of spatial transcriptomic spots in the human follicle validated the oocyte-specific expression patterns of POI-pathogenic mutant genes (Figure 1F). Single-cell and spatial transcriptomic results suggest that POI-pathogenic mutant genes show oocyte-specific expression patterns in ovaries.

As oocytes show dynamic changes in gene expression during folliculogenesis,[2] we subsequently explored the dynamic expression characteristics of POI-pathogenic mutant genes in oocytes. The four subtypes (C1 to C4) of oocytes were obtained from the previous study[2] and their developmental trajectories correspond to primordial (C1), primary (C2), secondary (C3), and antral (C4) follicles, respectively. We rewired the expression matrix at the four stages and found that the overall gene expression pattern progressively increased during stepwise developmental stages, especially at the antral follicular stage (Figure 1G), such as *ZP3*, *GDF9*, *GNAS*, and *ZAR1* (Figure 1H). Moreover, the developmental trajectory of the four subtypes could be reorganized using the POI-pathogenic mutant genes in unsupervised dimension reduction analysis (Figures 1I-J). These results suggest that POI-pathogenic mutant genes in oocytes show dynamic gene expression during folliculogenesis and developmental trajectories.

To identify potential mechanisms of pathogenic mutations in POI, we explored pathway enrichment analysis and found that POI-pathogenic mutant genes were enriched in the ovarian steroidogenesis (P = 6.10E-06), and cortisol synthesis and secretion (P = 4.50E-02) pathways (Figure 1K). By visualizing the pathway information and mutation frequency of the related genes in the POI cohort, we found that *GNAS* and *STAR* were involved in the two pathways and 17β-estradiol was the main product related to the POI-pathogenic mutant genes (Figure 1K). Importantly, the gene expression levels of *GNAS* were progressively increased during stepwise developmental stages both in young and old ovaries (Figure 1J). M-IHC staining in different follicular stages validated the increased oocyte-specific expression patterns of Gnas (Figure 1L). We then generated a conditional knock-out mouse model (*Gnas*$^{flox/flox}$, *Cyp17a1*^{Cre}) in exon 1 of *Gnas* gene (Figure 1M). Results showed that *Gnas* disruptions show POI phenotype in mouse ovaries (Figure 1N). IHC staining showed that *Gnas* deficiency decreased the produce of 17β-estradiol during folliculogenesis, while not influenced the expression of upstream receptors, such as Fshr (Figure 1N). These results suggest that Gnas deficiency is associated with POI phenotype via regulating ovarian steroidogenesis in oocytes.

In most cases, the cause of POI is unknown, but it can be caused by genetic factors, autoimmune disorders, chemotherapy or radiation therapy, or other environmental factors.[1] Recently, the largest POI cohort indicated that at least 18-23% of patients have genetic variations.[1] Previous studies have demonstrated the essential role of some pathogenic genes, such as *BRCA2*,[6] *CHEK1/2*,[7] and *EGFR*,[4] in ovarian development and function. However, high-resolution expression patterns and changes during folliculogenesis remain to be determined. Here, we identified the oocyte-specific expression patterns of POI-pathogenic mutant genes and showed a dynamic increase during folliculogenesis.

Among the POI-pathogenic mutant genes, although some were not enriched in oocyte populations, they have been implicated in the follicle development process via the regulation of steroidogenesis in granulosa cells, such as *HSD17B1*, *BMP15*, *FHSR*, and *BMP6*, which is consistent with previous studies.[1,8] Some genes that are involved in oocyte maturation or ovulation via homologous recombination (HR) repair processes are significantly enriched in oocyte populations in our results, such as Fanconi anemia complementation group (FANC) and *BRCA2*.[6] Given that early menopause and POI have similar patterns of inheritance and genetic backgrounds,[7,9]

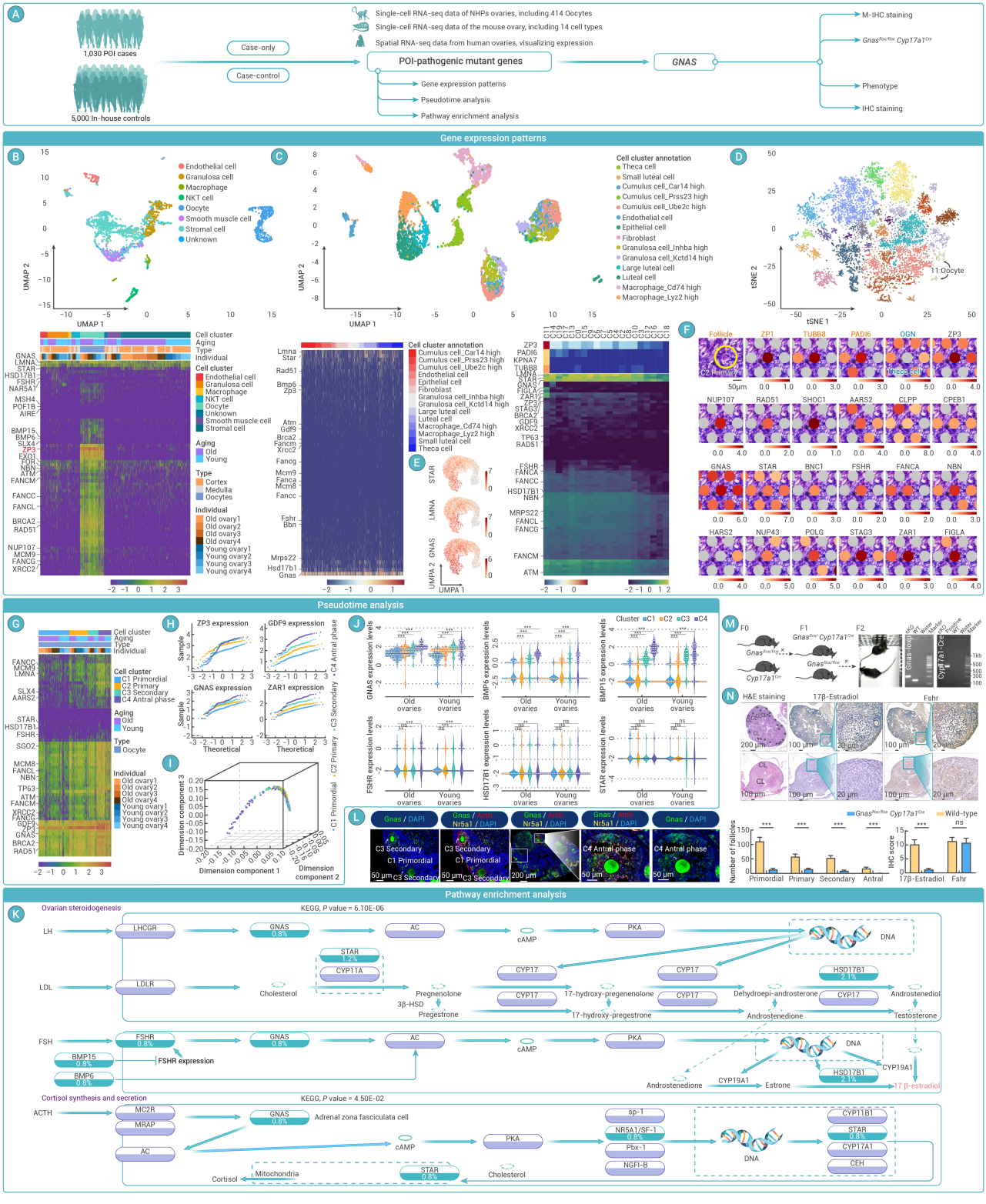

Figure 1. Single-cell and spatial transcriptomics reveal *GNAS* deficiency associated with POI via ovarian steroidogenesis (A) Summary of the analysis approach and findings. (B) The UMAP map of single-cell RNA-seq data of NHP ovaries. Heatmap shows POI-pathogenic mutant genes in the single-cell RNA-seq of ovaries from young (4-5 years old, 1,122 cells) and old (18-20 years old, 1,479 cells) cynomolgus monkeys. (C) The UMAP map of single-cell RNA-seq data of the mouse ovary (4,363 cells). Heatmap shows POI-pathogenic mutant genes in the single-cell RNA-seq from adult mouse ovary. Each column represents a cell, and each line represents a gene. The cell clusters are indicated. (D) The tSNE map of spatial transcriptomic data of human ovaries. The oocyte cluster is indicated. Heatmap shows POI-pathogenic mutant genes in the spatial transcriptomic data from four human ovaries. Spatial transcriptomic spots are clustered into 20 clusters, and the expression levels of oocyte markers and POI-pathogenic mutant genes are shown. (E) The gene expression levels in UMAP plot. (F) The expression levels of POI-pathogenic mutant genes in the spatial transcriptomic spots near the follicle. (G) Heatmap of POI-pathogenic mutant genes in the single-cell RNA-seq of four type oocytes from the young and old cynomolgus monkeys. Each column represents a cell, and each line represents a gene. The cell cluster and the annotations are indicated. (H) Dot plots of *ZP3*, *GDF9*, *GNAS*, and *ZAR1* in the oocytes (from C1 to C4). (I) Developmental trajectory analysis of oocytes by using the expression matrix of POI-pathogenic mutant genes. (J) The group violin plot of POI-pathogenic mutant genes in oocytes. The *p*-value is calculated by the Wilcoxon test. (K) Pathway information and mutation frequency of related genes in the POI cohort. (L) M-IHC staining Gnas, Amh, and Nr5a1 in the different stages of follicles. (M) Schematic diagram and polymerase chain reaction results of the conditional knockout mouse model. (N) Hematoxylin-Eosin (H & E), IHC staining, and statistical analysis of follicles in wild-type and conditional knockout mice. Data are represented as mean ± SEM. CL: corpus luteum, * indicates follicles. The *p*-value is calculated by Wilcoxon text. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

oocyte-specific expression patterns for POI-pathogenic mutant genes were also consistent with the results of the risk genes for early menopause identified in our previous report.[10] This suggests that common genetic factors may be involved in the development of both POI and early menopause, which could have important implications for future research.

It is important to note the limitations of this study, including the fact that not all POI-pathogenic mutant genes were analyzed, and the mouse single-cell transcriptomic data did not include mouse oocytes due to the limitations of the sequencing technology. The dynamic gene expression and roles of *GNAS* in human oocytes at different stages should be determined in future studies. In addition, the mechanisms of *GNAS* in the POI phenotype should be validated in the human ovaries.

In summary, by comprehensively exploring the expression patterns of POI-pathogenic mutant genes in multiple species using multi-dimensional data, we identified oocyte-specific pathogenic mutations in POI, and *Gnas* deficiency is associated with POI phenotype via regulating ovarian steroidogenesis production, providing potential biomarkers for the diagnosis of POI and targets for the development of therapeutic interventions to treat POI-associated infertility.

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AUTHOR CONTRIBUTIONS

Y. Y. and C. C. developed the concepts and supervised the overall experiments. C. C., P. W., and P. Z. performed bioinformatics analysis and ovary histological analysis. Y. Y. and C. C. performed manuscript writing, review, and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

The animal experiments were approved by the Animal Experiment Ethics Committee of Peking University Third Hospital (A2022001) and performed according to the AVMA quidelines.

DATA AND CODE AVAILABILITY

Spatial transcriptomic sequencing of human pre/postmenopausal ovariesis available at the Genome Sequence Archive (HRA004049). Single-cell RNA-seq data were downloaded from GSE130664 and GSE134355. Any additionalinformation is available from the lead contact upon request.

LEAD CONTACT WEBSITE

https://www.puh3.net.cn/lcgxbyjzx/info/1251/1981.htm