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Spatiotemporal Transcriptome Analysis Reveals Activation of the AP1 Pathway in the Ovarian Microenvironment during the Transition from Premenopause to Postmenopause

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MATERIALS AND METHODS

Sample preparation and ethical statement

The sample collection was approved by the Research Ethics Committee of the Peking University Third Hospital (IRB00006761-M2022616) and was conducted following approved institutional guidelines. The donated human ovaries were obtained from women who had undergone oophorectomy due to benign lesions. The samples were cut into 6 x 6 x 5 mm pieces and dried with lab blotting paper to prevent ice crystal formation. Subsequently, we embedded the tissue in OCT in the embedding box in dry ice and stored it at -80°C.

Spatial RNA-seq on the 10× Genomics platform

Spatial transcriptomics analysis was carried out using the 10x Genomics Visium platform [1]. Ten micrometre tissue sections from OCT-embedded fresh frozen human ovaries were mounted onto Visium Spatial slides and permeabilized for 30 min to release mRNAs, which bound to the spatially barcoded oligos present in the underlying spots and were reverse transcribed according to the manufacturer's protocol. Libraries prepared from the cDNAs were sequenced on the Illumina NovaSeq 6000 platform at >50,000 reads per spot, generating >400 M reads per section.

Single-cell RNA-seq analysis

Single-cell RNA-seq data of young (n = 4) and aged (n = 4) nonhuman primates, which were generated using the STRT-seq protocol, were downloaded from GSE130664 [2]. In total, 2,601 cells from primate ovaries were processed by Seurat, with the following criteria: (1) log2 (the mean expression of the cluster (transcripts per kilobase million [TPM]) greater than 0.5, (2) power value greater than 0.25, and (3) percentage of cluster cells (PCT.1) greater than 0.3.

Spatial RNA-seq analysis

Space Ranger software (version 1.3.0, 10x Genomics) was used to align and obtain raw counts from each of the spots on the Visium spatial transcriptomics slides against the GRCh38 human genome reference data. The Space Ranger output files were then imported into the R environment (V4.0.5) and analysed using the R package Seurat v4.0.1 (https://github.com/satijalab/seurat/). We obtained information about the number of spots, UMIs under each tissue, median/mean genes and reads per spot, etc. SCTransform was used to normalize the data, followed by PCA. The spatial expression of selected genes was visualized by using the normalized data.

Cell-type annotation of the spatial transcriptomic data

Cell2location [3] performs deconvolution in two steps, i.e., training the regression model and building the cell2location model. In training the regression model, the function "filter_genes" was used to filter the genes based on default parameters. Then, the function "cell2location.models. RegressionModel.setup_anndata" with default parameters was used to build an annData object. After applying the function "RegressionModel" to create a regression model, the function "mod.train" with the parameters "max_ epochs = 1000, use_gpu=False" was used to train the regression model. Based on the trained model, functions "adata_scrna_raw. varm" and "adata_scrna_raw.var" were implemented to export the estimated expression in each cell subpopulation. The function "np.intersect1d" was used to find shared genes and subset both the spatial Visium data and reference signatures. For the model construction, the function "cell2location.models.Cell2location.setup_anndata" was used to prepare the anndata to construct the cell2location model. Functions "cell2location.models. Cell2location" and "mod.train" were used to create and train this model. Finally, the function "plot_spatial" was used to visualize cell abundance in spatial coordinates.

Analysis of DEGs and GO and pathway enrichment analysis

We used the FindMarkers function to perform differential gene expression analysis between regions and set p_val_adj = 0.05 as the cut-off value. DEGs were visualized as a heatmap with the R package ComplexHeatmap. GO analysis of these DEGs was performed by Metascape (version 3.5, http://metascape.org/), and selected pathway terms are displayed in the heatmap.

Spatial trajectory analysis

The pseudotime trajectories of all spatial spots were generated with the Monocle2 package in R4.0.3. The newCellDataSet(), estimateSizeFactors(), and estimateDispersions() were used to perform these analyses. DetectGenes() was used to filter low-quality cells with "min_expr = 0.1".

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PROGENy analysis

We used a footprint-based method called PROGENy (Pathway RespOnsive GENes for activity inference) [4] to estimate signalling pathway activities based on consensus gene signatures obtained from perturbation experiments. PROGENy contains signatures for 14 signalling pathways (Androgen, EGFR, Estrogen, Hypoxia, JAK-STAT, MAPK, NFkB, p53, PI3K, TGFb, TNFa, Trail, VEGF, and WNT). In data preprocessing, Seurat function "SCTransform" was used to normalize spatial data. Then, the SCT assay data was used as input to the function "progeny" of PROGENy. The pathway activity score was calculated with the function progeny from the PROGENy R package v1.16.0 using the 500 most responsive genes per pathway. Average pathway activity scores for different cell types or samples were used to assess the differences. Heatmaps were generated using the R package PHEATMAP v1.0.12.

Cell communication analysis

Raw sequence reads in FASTQ format from ovaries were processed and aligned to the human reference transcriptome (https://www.10xgenomics.com/) using the Cellranger v4.0.0 pipeline with the default settings. The cell types were annotated by the Cell2location according to the single-cell transcriptomic data of GSE130664. The interactions between cell types identified in ovary samples were analysed by CellPhoneDB v.3.0 (https://github.com/ventolab/CellphoneDB) [5,6]. We retrieved the interacting pairs of ligands and receptors satisfying the following criteria: all members were expressed in at least 10% of the cells in the cluster under consideration. We used iTALK (https://github.com/Coolgenome/iTALK) and igraph software for cellphoneDB results to demonstrate the cell type interaction networks. The shell diagram is a split presentation of the network diagram.

Histological evaluation and immunohistochemistry

For histological examination, the indicated tissues were harvested, fixed with 4% paraformaldehyde in PBS, embedded into paraffin blocks, sectioned and then stained with H&E following standard protocols. For immunohistochemistry (IHC) analysis, deparaffinized sections were incubated with primary antibodies at 4°C overnight and then incubated with species-appropriate secondary antibodies (1:200, Servicebio). The antibodies used are listed in the Supplementary Table 5. The images were taken with an inverted microscope.

Immunofluorescence

After deparaffinization and rehydration, sections were subjected to antigen retrieval using citrate antigen repair buffer (pH 6.0) or EDTA antigen repair buffer (pH 9.0) at 100°C for 20 min, blocked with 3% BSA for 30 min at room temperature, and incubated in primary antibodies at 4°C overnight. Then, the sections were incubated with the corresponding secondary antibodies (1:200 dilution) for 1 h at room temperature. Polychromatic immunofluorescence staining for CD3, CD68, ZP1, and ZP3 was performed with the tyramide signal amplification (TSA) technique. In brief, tissue sections were incubated with primary antibodies as described in the above IHC protocol in two sequential cycles before the application of corresponding secondary antibodies and TSA solution for iF488 (G1231, Servicebio) and CY3 (G1223, Servicebio). After the last TSA cycle, DAPI was counterstained for 10 min. Fluorescent images were obtained with an automatic digital slide scanner (Pannoramic MIDI). The mean fluorescence intensities were measured using ImageJ software. Antibody information is listed in the Supplementary Table 5.

Masson's staining

The ovary sections were brought to room temperature and incubated in potassium dichromate overnight. After a wash in distilled water, the sections were stained in iron haematoxylin working solution for 1 min and rinsed with running tap water. Then, the slides were stained in Ponceau-acid fuchsin solution for 6 min. After a wash with running tap water, the sections were differentiated in the phosphomolybdic acid solution for 1 min and directly transferred to an aniline blue solution for 30 s. Next, the sections were briefly rinsed in distilled water, followed by differentiation in 1% acetic acid solution for 2-5 min. Finally, the slides were dehydrated in absolute ethyl alcohol, mounted with resinous mounting medium, and examined by microscopy.

Statistical analyses

Data are presented as the mean ± SD. All experimental data were analysed using an unpaired two-sided Student's t test or one-way analysis of variance (ANOVA) to assess differences between groups (GraphPad 7.0 Software). *p value < 0.05 was considered to indicate statistical significance.

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Ethics approval

The human sample collection was approved by the Research Ethics Committee of the Peking University Third Hospital (IRB00006761-M2022616) and was conducted following approved institutional guidelines. The animal experiments were approved by the Animal Experiment Ethics Committee of Peking University Third Hospital (A2022001) and performed according to the AVMA guidelines.

Data availability statements

Spatial transcriptomic sequencing of human pre/postmenopausal ovaries is available at the Genome Sequence Archive (HRA004049). Single-cell RNA-seq data were downloaded from GSE130664. Cell clusters of single-cell transcriptomic data could be found at the website, https://doi.org/10.7910/DVN/D8RZKK. Any additional information is available from the lead contact upon request.

References

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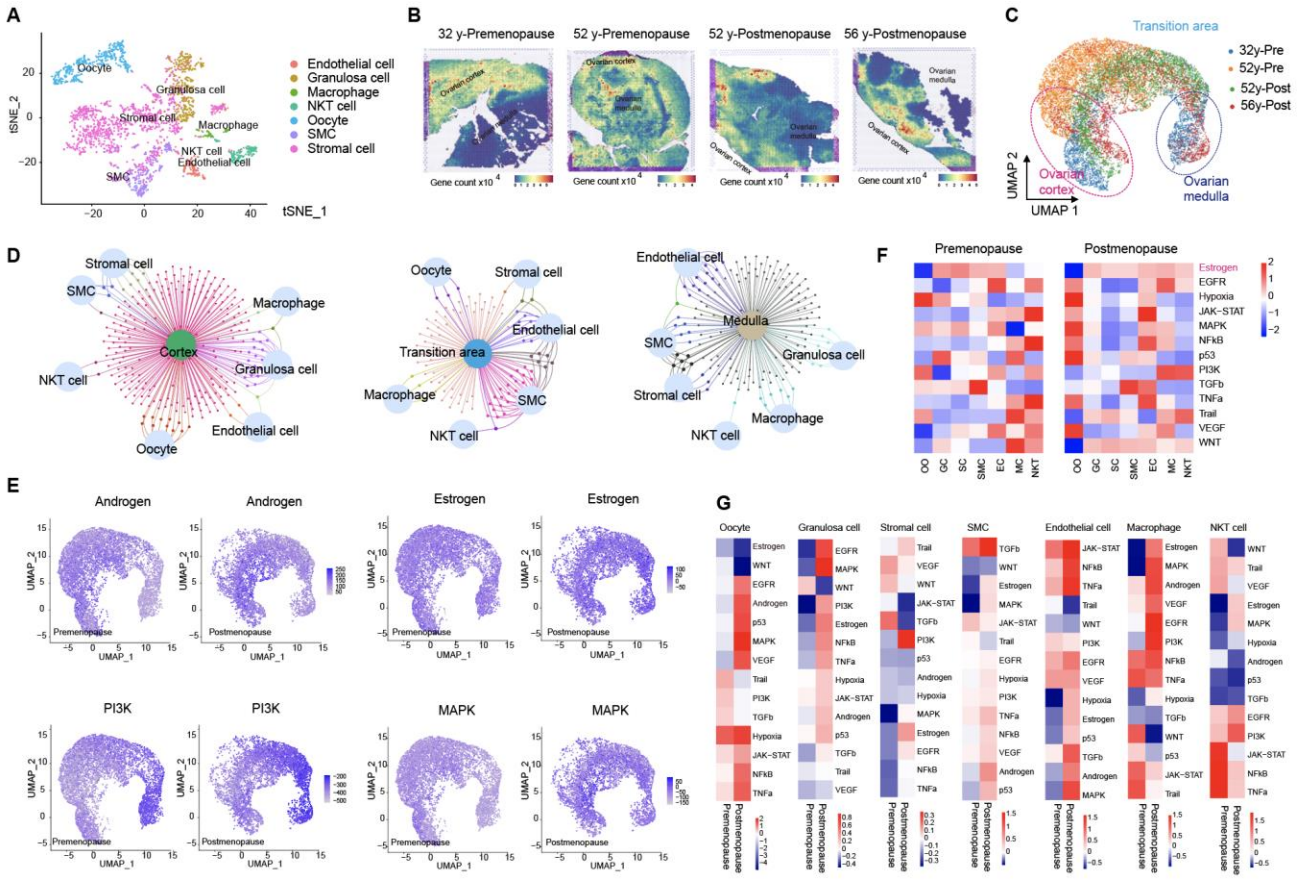

Supplementary Figure 1. Spatial transcriptomic characteristics of samples and PROGENy analysis of cell types. (**A**) Single-cell transcriptomic analysis of NHP ovaries from GSE130664. Seven types of cell clusters are indicated, and each colour represents a cell cluster. (**B**) Gene expression levels of spatial spots in different samples. (**C**) UMAP plot of spatial spots of samples. Each colour represents a sample. (**D**) Venn network analysis of differentially expressed genes (DEGs) of the ovarian cortex, transition area, and medulla and marker genes of each cell type. (**E**) PROGENy analysis of the androgen, estrogen, PI3K, and MAPK pathways between premenopause and postmenopause. (**F**) PROGENy analysis of different pathways in premenopause and postmenopause. (**G**) PROGENy analysis of different pathways in different cell clusters between premenopause and postmenopause.

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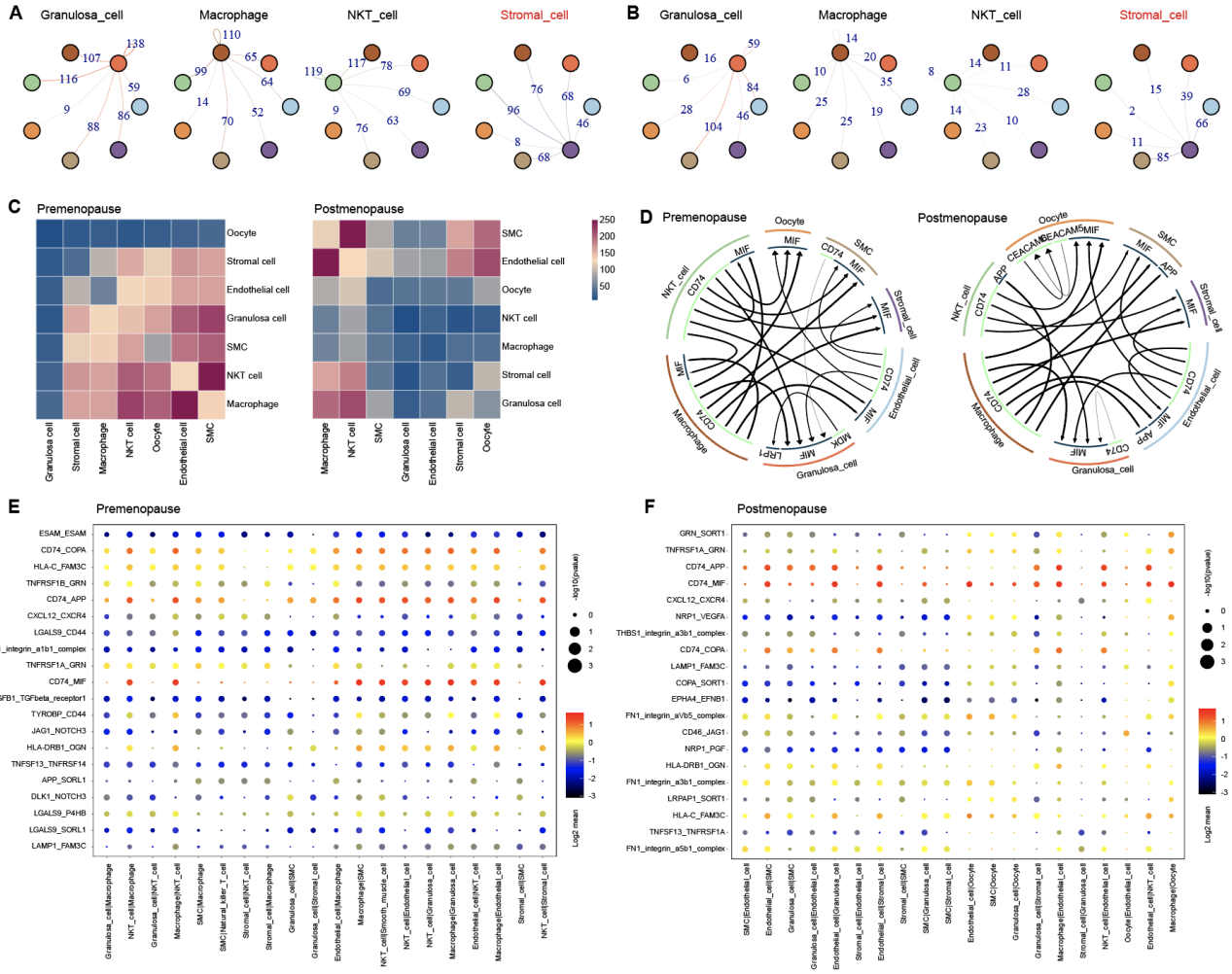

Supplementary Figure 2. Intercellular communication analysis of the cell clusters. Communications among cell types in premenopausal samples (**A**) and postmenopausal samples (**B**) at the spatial transcriptomic level. We used a shell diagram to show the communication networks of each cell type. Each line represents the relationship from cell type A to cell type B. The number represents ligand–receptor pairs. The higher the number, the thicker the line. (**C**) Heatmaps of communication among cell types in pre- and postmenopausal samples. (**D**) The top ligand–receptor networks of cell types in pre- and postmenopausal samples. The arrow represents the pointing relationship, the thickness of the line represents the expression of the departure gene, and the size of the arrow represents the expression of the gene in the accepting direction. The light green represents the departure direction, and the dark green represents the acceptance direction. If a protein/complex is both a ligand and an acceptor, it is light green. Dot plot of the top ligand–receptor pairs among cell types in premenopausal samples (**E**) and postmenopausal samples (**F**).

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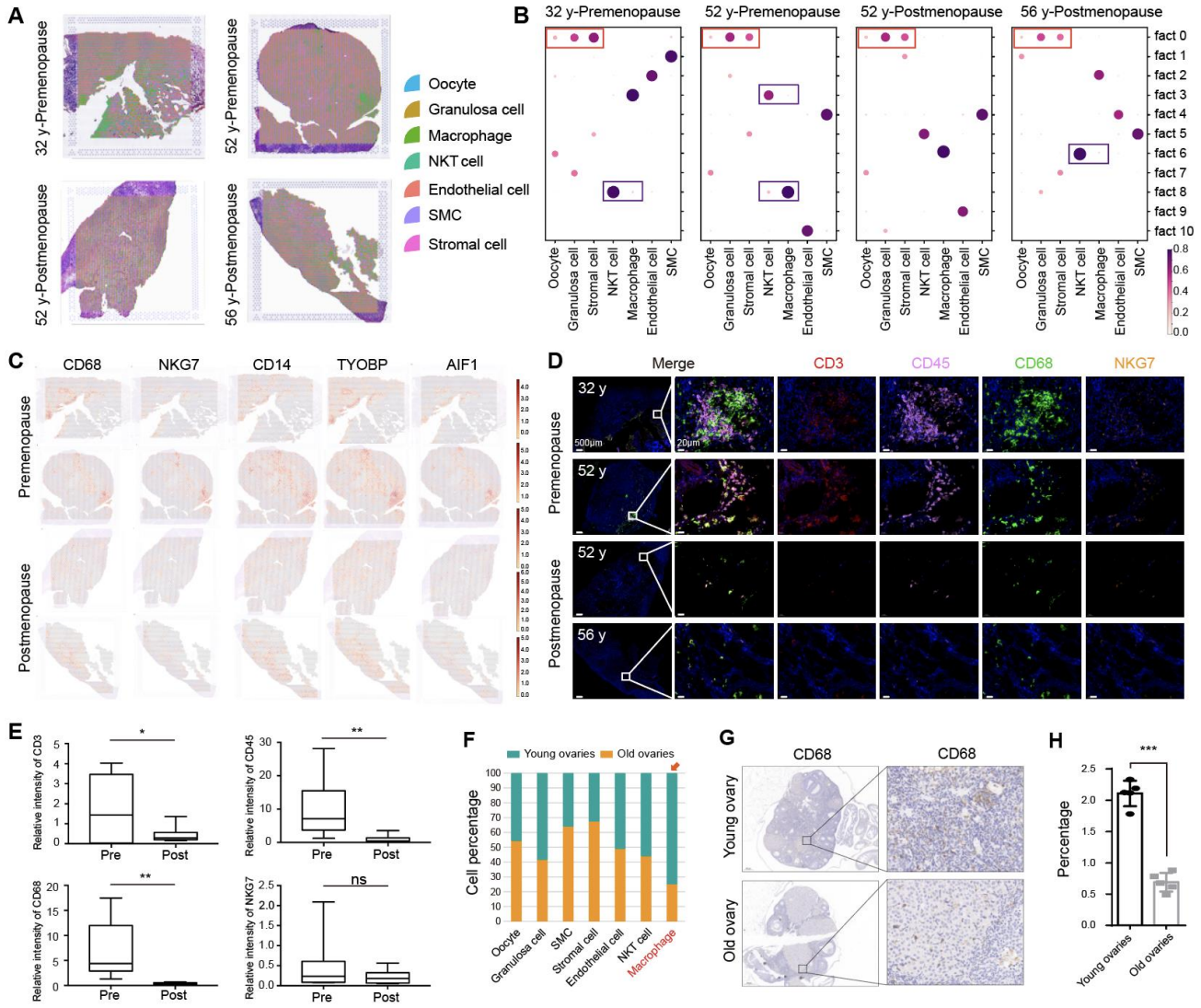

Supplementary Figure 3. The expression features of immune cells in human, NHP and mouse ovaries. (**A**) Using the single-cell RNA-seq data, we performed factor analysis to determine the likely single-cell composition of each spot via Cell2location. (**B**) Nonnegative matrix factorization (NMF) weight analysis among the 7 cell types in the spatial transcriptomic spots. Dot colour shade and size indicate the weight obtained for each cell type evaluated in each NMF component. (**C**) The expression levels of immune cell markers at the spatial transcriptomic level. (**D**) fm-IHC staining of immune cells among samples. (**E**) Statistical analysis of immune cell markers between pre/postmenopausal samples. (**F**) Cell proportion analysis of cell types between young and old ovaries from the single-cell dataset GSE130664. IHC staining (**G**) and statistical analysis (**H**) of Cd68 between young and old mouse ovaries.

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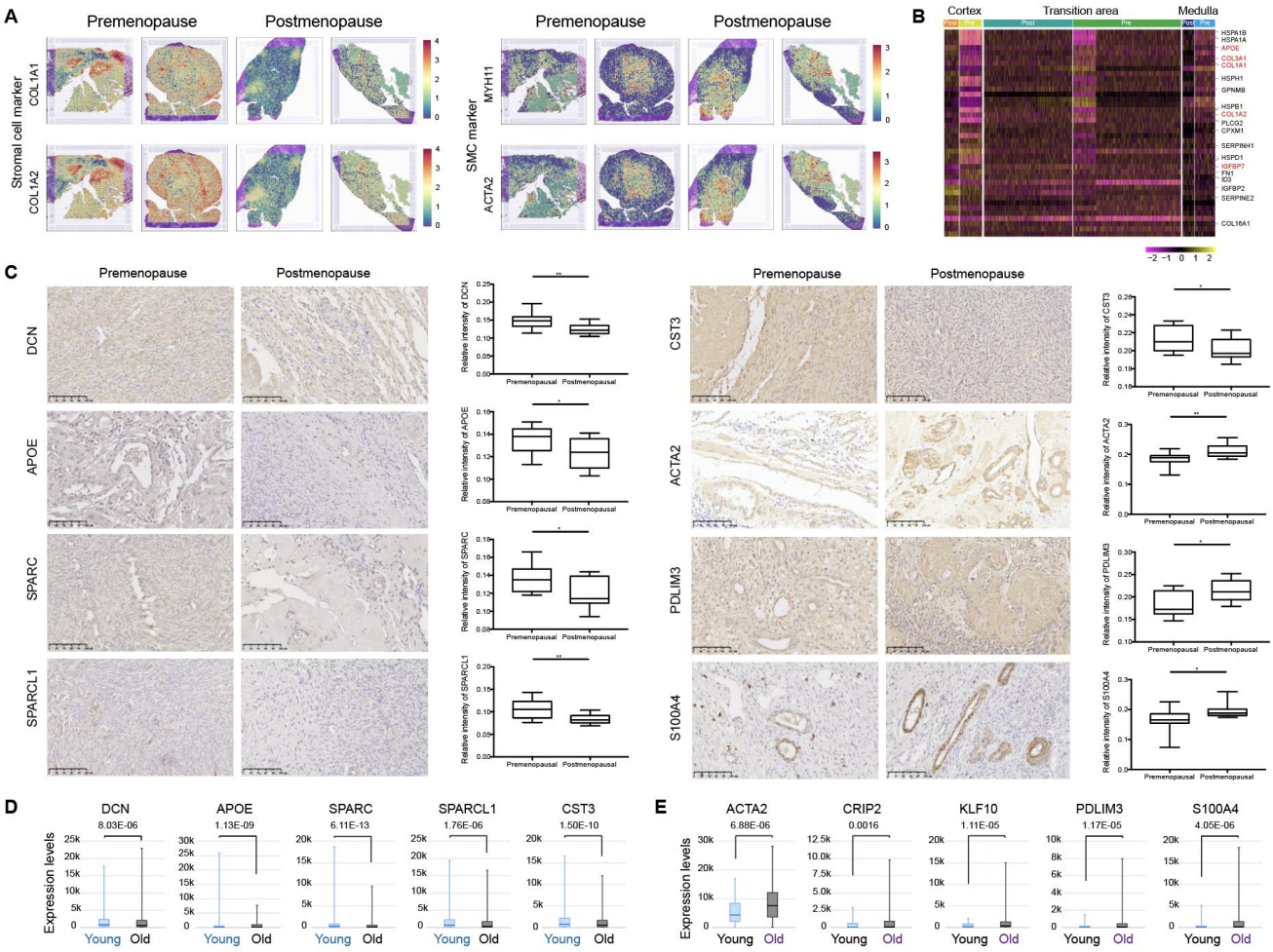

Supplementary Figure 4. Expression patterns of stromal cells and SMCs in the human and NHP ovaries. (**A**) The expression levels of immune cell markers at the spatial transcriptomic level. (**B**) Heatmap of the cortex, transition, and medulla area in human ovaries between premenopause and postmenopause. (**C**) IHC staining and statistical analysis of makers of stromal cells and SMCs between premenopause and postmenopause. The genes upregulated in stromal cells in young NHP ovaries (**D**) and the genes upregulated in SMCs in old NHP ovaries (**E**) at the single-cell transcriptomic level.

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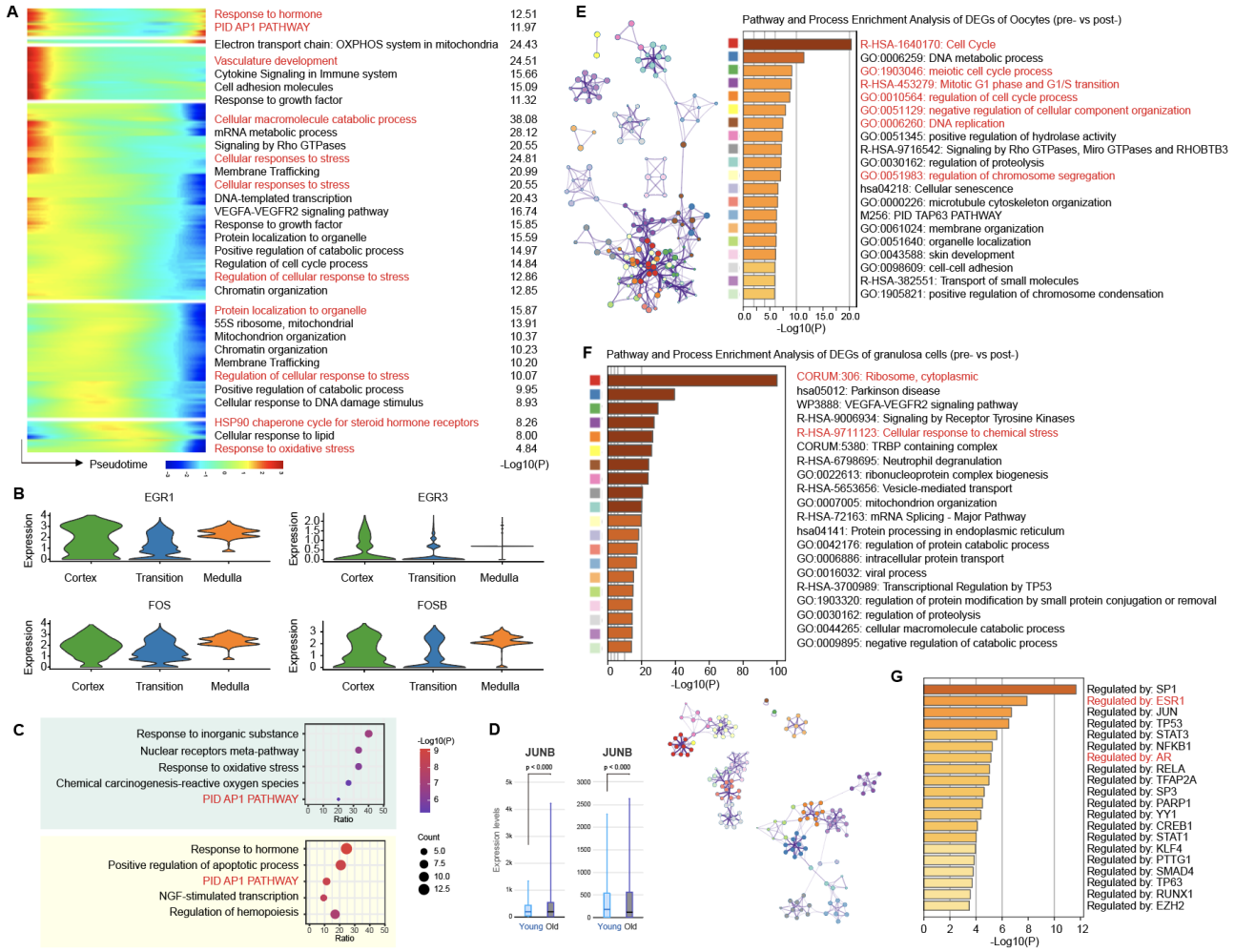

Supplementary Figure 5, Pseudotime trajectory analysis, AP1 pathway analysis, and enrichment analysis of DEGs of oocytes and granulosa cells. (**A**) Heatmap showing the dynamic DEGs along the pseudotime trajectory. According to the cluster analysis, the distribution of DEGs was divided into 6 clusters. The processes enriched in each subtype are listed on the right. (**B**) The different expression patterns of AP1 pathway-associated genes along the pseudotime trajectory. (**C**) Representative pathway and process enrichment terms for upregulated genes of old NHP ovaries in stromal cell (top) and NKT cell (bottom). (**D**) The AP1 pathway-associated genes upregulated in SMCs (left) and stromal cells (right) in old NHP ovaries at the single-cell transcriptomic level. Pathway and process enrichment analysis of the DEGs of oocytes (**E**) and granulosa cells (**F**) between premenopause and postmenopause. (**G**) Transcription factors analysis in TRRUST (v2.0) of DEGs in granulosa cells between premenopause and postmenopause.

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Supplementary Table 1. Quality control analysis of spatial transcriptomic data.

Summary	32y-Pre	52y-Pre	52y-Post	56y-Post
Number of Reads	245,284,460	205,352,644	243,576,742	212,291,518
Number of Spots Under Tissue	3,206	4,242	3,212	2,431
Fraction Reads in Spots Under Tissue	91.30%	92.00%	92.70%	90.80%
Mean Reads per Spot	76,508	48,409	75,833	87,327
Mean Reads Under Tissue per Spot	67,823	43,596	68,477	76,653
Median UMI Counts per Spot	8,571	10,503	6,666	7,323
Median Genes per Spot	2,868	3,202	2,320	2,467
Total Genes Detected	23,470	24,113	22,728	22,459
Reads Mapped to Genome	93.10%	95.90%	93.80%	92.20%
Reads Mapped Confidently to Genome	90.10%	92.10%	89.20%	87.20%
Reads Mapped Confidently to Intergenic Regions	1.80%	1.60%	2.10%	1.80%
Reads Mapped Confidently to Intronic Regions	3.20%	3.50%	3.90%	4.10%
Reads Mapped Confidently to Exonic Regions	85.10%	87.10%	83.30%	81.30%
Reads Mapped Confidently to Transcriptome	83.30%	85.20%	81.50%	79.60%
Reads Mapped Antisense to Gene	0.50%	0.60%	0.60%	0.50%

Supplementary Table 2. DEGs of macrophages between the young and old NHP ovaries. Cell clusters of single-cell transcriptomic data. Found at: https:// doi.org/10.7910/DVN/ D8RZKK.

Tissue	Cell Type	Class	Gene	Avg_logFC	P_val
Ovary	M	OvsY.up	ADAMTS1	0.778194	0.00066693
Ovary	M	OvsY.up	ADH5	1.67125	0.02514208
Ovary	M	OvsY.up	AICDA	1.03086	0.04173586
Ovary	M	OvsY.up	APOD	2.21561	0.00150051
Ovary	M	OvsY.up	AQP11	0.949935	0.00436886
Ovary	M	OvsY.up	ARID1B	3.69342	0.01534768
Ovary	M	OvsY.up	ARID5B	1.90255	0.00630426
Ovary	M	OvsY.up	ARPC4	0.959754	0.00751762
Ovary	M	OvsY.up	ATG101	4.49833	0.01395118
Ovary	M	OvsY.up	ATP5C1	0.897773	0.0363182
Ovary	M	OvsY.up	ATP6V0C	0.558482	0.0305432
Ovary	M	OvsY.up	BAZ2B	2.38025	0.00081652
Ovary	M	OvsY.up	BCL2L11	0.590356	0.01915052
Ovary	M	OvsY.up	BHLHE40	1.9011	0.00766345
Ovary	M	OvsY.up	BNIP3	2.88783	0.02349596
Ovary	M	OvsY.up	BRI3	2.07367	0.04021393
Ovary	M	OvsY.up	C15orf48	2.58014	0.00459349
Ovary	M	OvsY.up	C7	1.62252	0.01412396
Ovary	M	OvsY.up	CALD1	0.961784	2.21E-06
Ovary	M	OvsY.up	CALR	1.04225	0.02970558
Ovary	M	OvsY.down	CAPG	-0.76021	0.04498583
Ovary	M	OvsY.up	CCL11	0.641224	0.03025845
Ovary	M	OvsY.up	CD14	0.56101	0.00908249
Ovary	M	OvsY.up	CD44	1.23342	0.04575892
Ovary	M	OvsY.up	CDC5L	2.86859	0.03385815
Ovary	M	OvsY.up	CFAP36	2.05834	0.02637569
Ovary	M	OvsY.up	CHD2	1.12725	0.0151845
Ovary	M	OvsY.up	CLU	0.541448	0.00565296
Ovary	M	OvsY.up	CNN1	2.02825	0.00517442
Ovary	M	OvsY.up	COX17	0.98789	0.04326221
Ovary	M	OvsY.up	COX20	1.67633	0.04064835
Ovary	M	OvsY.up	COX6B1	1.34332	0.00270915
Ovary	M	OvsY.up	COX8A	0.819429	0.00593168
Ovary	M	OvsY.up	CRHBP	1.1357	0.02605035
Ovary	M	OvsY.up	CSRNP1	0.588363	0.04510988

Ovary	M	OvsY.up	CST3	1.01993	0.00050318
Ovary	M	OvsY.down	CTSB	-0.86908	0.01671242
Ovary	M	OvsY.down	CTSD	-1.13379	0.00773248
Ovary	M	OvsY.up	CUTA	2.75957	0.00183615
Ovary	M	OvsY.down	CYTB	-1.67742	0.00967385
Ovary	M	OvsY.up	DCN	1.18153	0.00948083
Ovary	M	OvsY.up	DDAH2	1.70125	0.00399521
Ovary	M	OvsY.up	DDRGK1	1.23076	0.00929375
Ovary	M	OvsY.up	DPYD	0.847349	0.00387975
Ovary	M	OvsY.up	DST	0.517928	0.01582068
Ovary	M	OvsY.up	EBNA1BP2	0.958167	0.04138999
Ovary	M	OvsY.up	EEF1D	0.717503	9.26E-05
Ovary	M	OvsY.up	EEF1G	0.615812	0.02405955
Ovary	M	OvsY.up	EFNA1	1.39246	0.00774823
Ovary	M	OvsY.up	EGR1	0.843017	2.93E-05
Ovary	M	OvsY.up	EGR2	2.41593	0.00425322
Ovary	M	OvsY.up	EIF4A3	1.12467	0.0059723
Ovary	M	OvsY.up	ENSMFAG00000000366	0.545139	0.00284407
Ovary	M	OvsY.up	ENSMFAG00000000594	1.02195	0.00019986
Ovary	M	OvsY.up	ENSMFAG00000001185	3.02371	0.01321284
Ovary	M	OvsY.up	ENSMFAG00000002766	0.982351	0.00166303
Ovary	M	OvsY.up	ENSMFAG00000002778	1.28735	0.00213808
Ovary	M	OvsY.up	ENSMFAG00000007994	1.0867	0.00028717
Ovary	M	OvsY.up	ENSMFAG00000011609	3.67107	0.00963559
Ovary	M	OvsY.up	ENSMFAG00000018170	0.684274	0.02238595
Ovary	M	OvsY.up	ENSMFAG00000024955	0.738411	0.04173895
Ovary	M	OvsY.up	ENSMFAG00000026096	1.18186	0.01595272
Ovary	M	OvsY.up	ENSMFAG00000027717	0.523236	0.00529406
Ovary	M	OvsY.up	ENSMFAG00000030096	0.991923	0.00039395
Ovary	M	OvsY.up	ENSMFAG00000030228	1.695	0.00315781
Ovary	M	OvsY.up	ENSMFAG00000030582	0.906578	0.00701329
Ovary	M	OvsY.up	ENSMFAG00000033495	0.901842	0.00223778
Ovary	M	OvsY.down	ENSMFAG00000034345	-1.01984	0.02761902
Ovary	M	OvsY.up	ENSMFAG00000035459	0.754122	0.02677971
Ovary	M	OvsY.up	ENSMFAG00000035836	1.10475	0.00046307
Ovary	M	OvsY.up	ENSMFAG00000036749	1.10593	0.01359847
Ovary	M	OvsY.up	ENSMFAG00000036985	1.28784	0.02695623
Ovary	M	OvsY.up	ENSMFAG00000037878	1.10098	8.96E-05
Ovary	M	OvsY.up	ENSMFAG00000039100	0.843533	0.00279888
Ovary	M	OvsY.up	ENSMFAG00000039291	3.22417	0.02036549
Ovary	M	OvsY.up	ENSMFAG00000040276	1.45959	0.01458812
Ovary	M	OvsY.up	ENSMFAG00000040595	1.16887	0.00074705
Ovary	M	OvsY.up	ENSMFAG00000041383	0.736064	0.00346151
Ovary	M	OvsY.up	ENSMFAG00000042314	1.55437	1.71E-05
Ovary	M	OvsY.up	ENSMFAG00000042652	0.593242	0.01446712
Ovary	M	OvsY.down	ENSMFAG00000043121	-0.6985	0.01489661
Ovary	M	OvsY.up	ENSMFAG00000043412	0.520291	0.01130395
Ovary	M	OvsY.up	ENSMFAG00000044548	1.6866	0.03253923
Ovary	M	OvsY.down	ENSMFAG00000044694	-1.21426	0.01703951
Ovary	M	OvsY.up	ENSMFAG00000045551	1.49954	0.005272
Ovary	M	OvsY.up	ENSMFAG00000045648	1.16619	0.00597113
Ovary	M	OvsY.up	ENSMFAG00000045819	0.838923	0.01237762
Ovary	M	OvsY.down	FCER1G	-1.33846	0.00212333
Ovary	M	OvsY.up	FERMT3	1.74686	0.0222265
Ovary	M	OvsY.up	FN1	0.644118	0.02001111
Ovary	M	OvsY.up	FOS	1.74519	0.0288182
Ovary	M	OvsY.up	FOSB	1.5524	0.00032709
Ovary	M	OvsY.up	FRMD8	2.92936	0.01755202
Ovary	M	OvsY.up	FRZB	1.56709	0.00149809

Ovary	M	OvsY.up	G3BP2	1.92084	0.04981849
Ovary	M	OvsY.up	GADD45B	1.50788	9.22E-05
Ovary	M	OvsY.up	GAPDH	0.519429	0.01567379
Ovary	M	OvsY.up	GLUL	1.21836	0.00821632
Ovary	M	OvsY.up	GNAS	0.840639	0.03226885
Ovary	M	OvsY.up	GOLPH3	0.920164	0.02966904
Ovary	M	OvsY.up	GPR183	2.25194	0.00487212
Ovary	M	OvsY.up	GRIPAP1	1.73366	0.01099072
Ovary	M	OvsY.up	GSTA5	2.11243	0.01149713
Ovary	M	OvsY.up	GSTM3	2.5182	0.0114052
Ovary	M	OvsY.up	GSTM5	0.892979	0.01331677
Ovary	M	OvsY.up	GTF2B	1.31703	0.00804124
Ovary	M	OvsY.up	HES1	1.23001	0.04728321
Ovary	M	OvsY.up	HIGD2A	2.25502	0.03141508
Ovary	M	OvsY.up	HNRNPC	0.749923	0.01394309
Ovary	M	OvsY.up	HNRNPH1	1.038	0.00216813
Ovary	M	OvsY.up	HSPA9	0.608933	0.02021266
Ovary	M	OvsY.up	HSPB1	1.04498	2.26E-05
Ovary	M	OvsY.up	ID2	0.962655	0.00130023
Ovary	M	OvsY.up	IER3	1.60405	0.03010508
Ovary	M	OvsY.up	IFNGR2	0.744807	0.0169405
Ovary	M	OvsY.up	IFRD1	0.757958	0.00545007
Ovary	M	OvsY.up	IL13RA1	0.624494	0.00728684
Ovary	M	OvsY.up	IRF2BP2	1.933	0.00900651
Ovary	M	OvsY.up	ITGAM	1.39838	0.01406867
Ovary	M	OvsY.up	KDM6B	1.09782	0.00808602
Ovary	M	OvsY.up	LIPN	0.754295	0.03095927
Ovary	M	OvsY.up	LITAF	0.524994	0.0031625
Ovary	M	OvsY.up	LMAN1	1.63686	0.00273481
Ovary	M	OvsY.up	LMCD1	1.97803	0.00053432
Ovary	M	OvsY.up	LYZ	1.09563	0.02945215
Ovary	M	OvsY.up	MALAT1	1.64654	0.00243826
Ovary	M	OvsY.up	MAP2K3	1.05551	0.03613875
Ovary	M	OvsY.up	MDK	0.613626	0.01639406
Ovary	M	OvsY.up	METAP2	2.42442	0.00822955
Ovary	M	OvsY.down	MS4A7	-1.02304	0.04431127
Ovary	M	OvsY.up	MTHFD2	2.115	0.00074357
Ovary	M	OvsY.up	MYL9	2.44107	6.90E-05
Ovary	M	OvsY.up	MYLK	1.30981	0.01743301
Ovary	M	OvsY.up	NASP	2.2446	0.04006268
Ovary	M	OvsY.up	NDUFS5	1.03626	0.02064821
Ovary	M	OvsY.up	NEIL3	0.941497	0.00395334
Ovary	M	OvsY.up	NEK2	3.01423	0.01461861
Ovary	M	OvsY.up	NFE2L2	1.98423	0.01930207
Ovary	M	OvsY.up	NFKBIA	0.578516	0.00010408
Ovary	M	OvsY.up	NIPBL	1.62478	0.0144073
Ovary	M	OvsY.up	NKTR	0.888633	0.02533087
Ovary	M	OvsY.up	PCBP2	1.52718	0.00970698
Ovary	M	OvsY.up	PEG3	0.878794	0.03980873
Ovary	M	OvsY.up	PHPT1	0.979232	0.02513146
Ovary	M	OvsY.up	PIM3	1.86459	0.00189737
Ovary	M	OvsY.up	PKN2	2.07836	0.02193272
Ovary	M	OvsY.up	PLD3	2.60373	0.00545393
Ovary	M	OvsY.up	PNPLA8	1.23179	0.00497023
Ovary	M	OvsY.up	POLR2E	0.686783	0.02593443
Ovary	M	OvsY.up	PPAP2C	1.23938	0.00828414
Ovary	M	OvsY.up	PPDPF	0.95692	0.04784275
Ovary	M	OvsY.up	PPIA	0.907847	0.00558788
Ovary	M	OvsY.up	PPP1R14A	1.75656	0.00068789

Ovary	M	OvsY.up	PRDX1	1.2054	0.04351836
Ovary	M	OvsY.up	PRDX4	0.598569	0.00816558
Ovary	M	OvsY.up	PSMD4	2.00156	0.00775036
Ovary	M	OvsY.up	PTP4A1	0.762515	0.01945902
Ovary	M	OvsY.up	PYURF	1.14464	0.02057917
Ovary	M	OvsY.up	RAB33A	1.09008	0.00517286
Ovary	M	OvsY.up	RAC1	0.934717	0.01924061
Ovary	M	OvsY.up	RACK1	0.996933	0.01714369
Ovary	M	OvsY.up	RAD21	0.618381	0.00308785
Ovary	M	OvsY.down	RAMP2	-1.87502	0.04027059
Ovary	M	OvsY.up	REXO2	2.84786	0.00615662
Ovary	M	OvsY.up	RPL24	0.742032	0.00014464
Ovary	M	OvsY.up	RPL41	0.939276	6.71E-05
Ovary	M	OvsY.up	RPL7A	0.74315	0.00078786
Ovary	M	OvsY.up	RPLP2	1.01998	0.00118842
Ovary	M	OvsY.up	RPS2	1.04209	0.0141093
Ovary	M	OvsY.up	RPS23	0.6177	0.04755735
Ovary	M	OvsY.up	RPS24	0.617578	0.00031341
Ovary	M	OvsY.up	RPS29	0.905888	0.00018685
Ovary	M	OvsY.up	RPS8	0.629402	0.00830688
Ovary	M	OvsY.up	SAMD4B	1.83227	0.00457234
Ovary	M	OvsY.up	SDC2	0.700234	0.04016952
Ovary	M	OvsY.up	SERP1	0.830625	0.03054197
Ovary	M	OvsY.up	SERPINB1	1.5428	0.00342572
Ovary	M	OvsY.up	SERTAD1	0.882494	0.00464793
Ovary	M	OvsY.up	SH3GLB1	0.854983	0.03812893
Ovary	M	OvsY.up	SIVA1	1.40094	0.02229872
Ovary	M	OvsY.up	SLC25A3	0.865038	0.00169989
Ovary	M	OvsY.up	SLC38A2	1.38711	0.00258813
Ovary	M	OvsY.up	SNU13	1.33939	0.00349024
Ovary	M	OvsY.up	SPAG9	3.14354	0.00136283
Ovary	M	OvsY.up	SPARCL1	0.968941	4.84E-05
Ovary	M	OvsY.up	SPART	0.626656	0.04618237
Ovary	M	OvsY.up	SPINT2	1.22148	0.01181223
Ovary	M	OvsY.up	SRPRA	2.44218	0.00919714
Ovary	M	OvsY.up	SRSF1	0.999032	0.00912985
Ovary	M	OvsY.up	SWAP70	1.46647	0.02053285
Ovary	M	OvsY.up	TBCA	1.33826	0.01121352
Ovary	M	OvsY.up	TBCB	1.43584	0.03489264
Ovary	M	OvsY.up	TDG	1.23922	0.02608692
Ovary	M	OvsY.up	THBS2	0.94607	0.00137354
Ovary	M	OvsY.up	TIMP1	0.888242	0.00173954
Ovary	M	OvsY.up	TMEM120A	1.80338	0.0389777
Ovary	M	OvsY.up	TMEM14A	1.17649	0.02388428
Ovary	M	OvsY.up	TMEM14B	0.712379	0.00196401
Ovary	M	OvsY.up	TMEM169	0.806019	0.03110226
Ovary	M	OvsY.up	TMEM176B	3.1122	0.00690821
Ovary	M	OvsY.up	TSC22D1	0.999942	0.0070494
Ovary	M	OvsY.up	TSPAN6	1.74028	0.00751025
Ovary	M	OvsY.up	TUBB3	1.05296	0.01619611
Ovary	M	OvsY.up	UBA1	0.774928	0.00470263
Ovary	M	OvsY.up	UBE2D2	1.20989	0.02012158
Ovary	M	OvsY.up	UBE2K	1.76081	0.0205189
Ovary	M	OvsY.down	UBE2L3	-2.04239	0.02370725
Ovary	M	OvsY.up	UGDH	0.631174	0.02254947
Ovary	M	OvsY.up	VCAN	1.57162	0.04306227
Ovary	M	OvsY.up	VEGFA	1.34029	0.00449882
Ovary	M	OvsY.up	VMP1	0.850501	0.02855451
Ovary	M	OvsY.up	VPS13C	1.34713	0.0492799

Ovary	M	OvsY.down	VPS29	-3.10794	0.01232905

Supplementary Table 3. DEGs of smooth muscle cells between the old and young NHP ovaries. Gene expression matrix along the pseudotime heatmap. Found at: https:// doi.org/10.7910/DVN/ D8RZKK.

Tissue	Cell Type	Class	Gene	Avg_logFC	P_val
Ovary	SMC	OvsY.up	ACTA2	0.678349	6.88E-06
Ovary	SMC	OvsY.down	AICDA	-1.57754	0.0009049
Ovary	SMC	OvsY.down	ANKRD35	-1.49226	0.02946943
Ovary	SMC	OvsY.up	APOD	1.21845	0.00024339
Ovary	SMC	OvsY.down	ARID5B	-0.50326	0.01555397
Ovary	SMC	OvsY.down	ATF4	-0.724	4.14E-06
Ovary	SMC	OvsY.up	ATP5A1	0.693943	0.04657936
Ovary	SMC	OvsY.down	ATP5G2	-0.586	0.00815078
Ovary	SMC	OvsY.up	ATP5I	0.860874	0.00211421
Ovary	SMC	OvsY.up	ATP6	1.15101	3.20E-09
Ovary	SMC	OvsY.up	B2M	0.967341	0.0145217
Ovary	SMC	OvsY.up	BTG2	0.703694	0.00018061
Ovary	SMC	OvsY.down	C7	-0.79072	2.00E-14
Ovary	SMC	OvsY.down	CCDC80	-1.10138	0.0346835
Ovary	SMC	OvsY.down	CD74	-1.1459	0.04999628
Ovary	SMC	OvsY.up	CHD2	0.741299	0.01357294
Ovary	SMC	OvsY.up	CNBP	0.664387	0.00258875
Ovary	SMC	OvsY.up	COX2	1.11173	7.07E-08
Ovary	SMC	OvsY.up	COX3	0.947244	1.08E-06
Ovary	SMC	OvsY.up	COX7B	0.803316	0.02672011
Ovary	SMC	OvsY.up	CRIP2	0.588661	0.00158476
Ovary	SMC	OvsY.up	CSRP1	0.584884	0.01780457
Ovary	SMC	OvsY.down	CST3	-1.36085	8.99E-05
Ovary	SMC	OvsY.down	DCN	-3.05192	6.55E-08
Ovary	SMC	OvsY.down	DDIT4	-1.98378	4.55E-07
Ovary	SMC	OvsY.up	EGFR	3.27833	1.67E-31
Ovary	SMC	OvsY.down	EIF3D	-1.16643	0.00139145
Ovary	SMC	OvsY.up	EIF4A1	0.923249	0.00109042
Ovary	SMC	OvsY.up	EIF4A3	0.781518	0.04878032
Ovary	SMC	OvsY.up	EIF4H	0.569801	0.0076308
Ovary	SMC	OvsY.down	ELF4	-1.81208	0.00086687
Ovary	SMC	OvsY.up	ELOB	0.794235	0.0231853
Ovary	SMC	OvsY.down	ENSMFAG00000001097	-0.5219	0.00637826
Ovary	SMC	OvsY.down	ENSMFAG00000001318	-0.75808	0.00156721
Ovary	SMC	OvsY.down	ENSMFAG00000001552	-0.80831	0.0414128
Ovary	SMC	OvsY.down	ENSMFAG00000002899	-0.55171	0.00058527
Ovary	SMC	OvsY.up	ENSMFAG00000015501	0.932351	0.00027197
Ovary	SMC	OvsY.down	ENSMFAG00000016983	-2.49498	4.72E-05
Ovary	SMC	OvsY.down	ENSMFAG00000021078	-1.89554	0.0032968
Ovary	SMC	OvsY.down	ENSMFAG00000021547	-0.69324	0.0076811
Ovary	SMC	OvsY.up	ENSMFAG00000026126	0.659182	0.00363251
Ovary	SMC	OvsY.up	ENSMFAG00000029971	0.939994	1.22E-05
Ovary	SMC	OvsY.down	ENSMFAG00000030186	-1.782	6.32E-15
Ovary	SMC	OvsY.up	ENSMFAG00000030267	0.504473	0.00367622
Ovary	SMC	OvsY.up	ENSMFAG00000030534	0.631055	0.00071722
Ovary	SMC	OvsY.up	ENSMFAG00000035941	1.03229	0.00149153
Ovary	SMC	OvsY.up	ENSMFAG00000036093_RHOA	0.745485	0.00092482
Ovary	SMC	OvsY.up	ENSMFAG00000036985	0.58104	0.00307063
Ovary	SMC	OvsY.down	ENSMFAG00000037443	-2.9195	7.30E-07
Ovary	SMC	OvsY.up	ENSMFAG00000038131	1.39231	2.33E-06
Ovary	SMC	OvsY.up	ENSMFAG00000039219	0.506021	0.01955355
Ovary	SMC	OvsY.up	ENSMFAG00000039395	1.38542	3.46E-07

Ovary	SMC	OvsY.up	ENSMFAG00000040760	0.850986	0.02883261
Ovary	SMC	OvsY.up	ENSMFAG00000043193_NDUFA4	0.766489	0.01741999
Ovary	SMC	OvsY.up	ENSMFAG00000045720	0.884084	0.01510664
Ovary	SMC	OvsY.down	FCGRT	-0.72903	0.01334557
Ovary	SMC	OvsY.down	GLUL	-0.70758	0.00010259
Ovary	SMC	OvsY.down	GRK6	-1.49687	0.01405835
Ovary	SMC	OvsY.up	GSTP1	0.677417	0.00341286
Ovary	SMC	OvsY.down	GTF2H1	-1.55438	0.00033076
Ovary	SMC	OvsY.down	GZMB	-1.41049	0.03265704
Ovary	SMC	OvsY.up	HMGN3	0.635188	0.01725802
Ovary	SMC	OvsY.down	HSP90AA1	-0.61118	0.01509599
Ovary	SMC	OvsY.down	HSPD1	-1.00065	0.00672231
Ovary	SMC	OvsY.down	IGFBP6	-2.45977	0.03447367
Ovary	SMC	OvsY.down	IGFBP7	-0.65104	0.00234881
Ovary	SMC	OvsY.up	IRF1	0.996931	0.00132716
Ovary	SMC	OvsY.up	ISYNA1	0.903091	0.00036036
Ovary	SMC	OvsY.up	JUNB	0.653412	0.01979956
Ovary	SMC	OvsY.up	KLF10	1.05606	1.11E-05
Ovary	SMC	OvsY.up	KRTCAP2	0.663798	0.01755475
Ovary	SMC	OvsY.down	LAPTM4A	-0.79696	0.00119439
Ovary	SMC	OvsY.down	Mafa-DRA	-1.25722	0.00264916
Ovary	SMC	OvsY.down	MDK	-0.88881	2.46E-06
Ovary	SMC	OvsY.up	MFGE8	0.796755	0.01184893
Ovary	SMC	OvsY.up	MGST3	0.609316	0.03364205
Ovary	SMC	OvsY.up	MRPL51	1.11047	0.00331256
Ovary	SMC	OvsY.up	MYC	0.680635	0.00118994
Ovary	SMC	OvsY.up	ND2	0.564061	0.00947173
Ovary	SMC	OvsY.up	ND3	3.04726	4.66E-19
Ovary	SMC	OvsY.up	ND4	1.01825	2.86E-05
Ovary	SMC	OvsY.up	NDUFA1	0.610248	0.0008077
Ovary	SMC	OvsY.up	NDUFA11	0.631398	0.02586826
Ovary	SMC	OvsY.up	NR4A1	0.827791	0.00797687
Ovary	SMC	OvsY.up	NR4A2	1.23009	0.00413498
Ovary	SMC	OvsY.down	OGN	-1.27057	2.26E-07
Ovary	SMC	OvsY.down	PALLD	-0.76882	0.00453869
Ovary	SMC	OvsY.up	PDLIM3	1.08656	1.17E-05
Ovary	SMC	OvsY.up	PFN1	0.841991	1.02E-05
Ovary	SMC	OvsY.down	PLA2G1B	-2.06076	0.00069938
Ovary	SMC	OvsY.down	PNRC1	-0.53501	0.02977418
Ovary	SMC	OvsY.down	PPP1R10	-0.60171	7.01E-05
Ovary	SMC	OvsY.up	PPP1R15A	0.547289	0.00632066
Ovary	SMC	OvsY.down	PSAP	-0.66287	0.04680705
Ovary	SMC	OvsY.down	PSMD3	-1.54224	0.00077869
Ovary	SMC	OvsY.down	RACK1	-0.67458	0.00157815
Ovary	SMC	OvsY.up	RAP1B	0.542375	0.02298961
Ovary	SMC	OvsY.up	RBM8A	1.02795	0.00470735
Ovary	SMC	OvsY.up	RBX1	1.06432	0.04527277
Ovary	SMC	OvsY.up	RGS16	1.87878	0.00214154
Ovary	SMC	OvsY.up	RHEB	0.774314	0.0047312
Ovary	SMC	OvsY.down	RPL28	-0.62883	0.00428551
Ovary	SMC	OvsY.down	RPL9	-0.55593	1.11E-05
Ovary	SMC	OvsY.down	RPS11	-0.56403	4.20E-05
Ovary	SMC	OvsY.down	RPS7	-0.80326	0.02571838
Ovary	SMC	OvsY.down	RPSA	-0.6185	0.01977225
Ovary	SMC	OvsY.down	RSRP1	-1.27897	3.43E-06
Ovary	SMC	OvsY.up	S100A4	1.13435	4.05E-06
Ovary	SMC	OvsY.up	SARAF	0.509079	0.04348822
Ovary	SMC	OvsY.down	SERPINE2	-2.15637	5.38E-05
Ovary	SMC	OvsY.down	SPARC	-1.02419	0.00944246

Ovary	SMC	OvsY.up	SQSTM1	0.825957	0.00185928
Ovary	SMC	OvsY.up	SRP14	0.6339	0.01038047
Ovary	SMC	OvsY.up	SUB1	0.964319	0.00193604
Ovary	SMC	OvsY.up	TAGLN2	1.1536	6.10E-05
Ovary	SMC	OvsY.down	TPT1	-0.61994	0.00262251
Ovary	SMC	OvsY.down	TXNIP	-1.34853	8.23E-15
Ovary	SMC	OvsY.up	VDAC2	0.710707	0.00733689
Ovary	SMC	OvsY.up	YWHAQ	0.56093	0.00175672

Supplementary Table 4. DEGs of stromal cells between the young and old NHP ovaries.

Tissue	Cell Type	Class	Gene	Avg_logFC	P_val
Ovary	SC	OvsY.down	AICDA	-0.80063	1.45E-05
Ovary	SC	OvsY.up	APOD	1.7504	1.17E-14
Ovary	SC	OvsY.up	APOE	0.874626	1.13E-09
Ovary	SC	OvsY.down	ATP5A1	-0.52271	0.04934536
Ovary	SC	OvsY.up	ATP5D	0.798107	1.43E-08
Ovary	SC	OvsY.up	BTG2	0.568507	7.30E-09
Ovary	SC	OvsY.down	CD74	-0.7567	1.15E-11
Ovary	SC	OvsY.up	CLU	1.15425	2.41E-07
Ovary	SC	OvsY.down	COX6A1	-0.52117	1.49E-06
Ovary	SC	OvsY.down	CST3	-1.03092	1.50E-10
Ovary	SC	OvsY.down	CTSK	-0.68885	1.20E-10
Ovary	SC	OvsY.down	DCN	-0.86659	8.03E-06
Ovary	SC	OvsY.up	EGFR	1.09867	7.69E-39
Ovary	SC	OvsY.up	EIF4A3	0.598141	0.0036785
Ovary	SC	OvsY.down	EIF5	-0.56433	0.00380578
Ovary	SC	OvsY.down	ENSMFAG00000001022	-0.58749	0.02502341
Ovary	SC	OvsY.down	ENSMFAG00000001143	-0.5842	0.00883344
Ovary	SC	OvsY.down	ENSMFAG00000001318	-0.56749	0.00044983
Ovary	SC	OvsY.up	ENSMFAG00000003168	0.572757	5.71E-08
Ovary	SC	OvsY.up	ENSMFAG00000007994	0.630161	1.72E-15
Ovary	SC	OvsY.up	ENSMFAG00000011451_RPL32	0.521913	1.89E-08
Ovary	SC	OvsY.up	ENSMFAG00000011609	3.32299	1.12E-30
Ovary	SC	OvsY.up	ENSMFAG00000012114	0.701585	1.11E-11
Ovary	SC	OvsY.up	ENSMFAG00000015501	0.722035	8.51E-12
Ovary	SC	OvsY.down	ENSMFAG00000021078	-1.37899	1.88E-05
Ovary	SC	OvsY.up	ENSMFAG00000027351	0.566119	6.44E-08
Ovary	SC	OvsY.up	ENSMFAG00000027717	0.60156	9.12E-10
Ovary	SC	OvsY.up	ENSMFAG00000030096	0.634781	9.07E-13
Ovary	SC	OvsY.up	ENSMFAG00000030598	0.750616	4.05E-21
Ovary	SC	OvsY.down	ENSMFAG00000030943	-0.64663	0.00022713
Ovary	SC	OvsY.down	ENSMFAG00000033843	-0.52627	0.00905864
Ovary	SC	OvsY.down	ENSMFAG00000035478	-0.581	0.00992812
Ovary	SC	OvsY.up	ENSMFAG00000043631	0.538052	1.24E-12
Ovary	SC	OvsY.down	ENSMFAG00000044097	-0.57563	1.38E-06
Ovary	SC	OvsY.up	ENSMFAG00000045720	0.747852	2.78E-13
Ovary	SC	OvsY.down	FBXO21	-0.53941	0.01053495
Ovary	SC	OvsY.down	GLUL	-1.42555	1.03E-11
Ovary	SC	OvsY.down	GNAS	-0.5744	8.13E-06
Ovary	SC	OvsY.down	GRK6	-1.55085	0.00634979
Ovary	SC	OvsY.up	GSTM5	1.05457	9.94E-09
Ovary	SC	OvsY.down	GZMB	-1.05983	0.00031619
Ovary	SC	OvsY.down	IDO2	-0.57109	0.00402219
Ovary	SC	OvsY.up	JUNB	0.547117	9.06E-09
Ovary	SC	OvsY.down	MDK	-0.66343	2.89E-06
Ovary	SC	OvsY.up	MT2	1.20194	6.57E-06
Ovary	SC	OvsY.up	MYC	0.728057	3.03E-11

Ovary	SC	OvsY.up	ND3	1.35182	2.05E-15
Ovary	SC	OvsY.down	NR2F2	-0.95278	6.05E-05
Ovary	SC	OvsY.down	PDK4	-1.03301	1.61E-13
Ovary	SC	OvsY.up	PRDX1	0.617236	0.00023654
Ovary	SC	OvsY.down	PSMD3	-1.13931	0.0024302
Ovary	SC	OvsY.down	RBM8A	-0.56304	0.00521863
Ovary	SC	OvsY.up	RBP1	0.50939	2.33E-07
Ovary	SC	OvsY.up	RPS15A	0.574898	4.23E-10
Ovary	SC	OvsY.down	SON	-0.61538	0.00597948
Ovary	SC	OvsY.down	SPARC	-1.20052	6.11E-13
Ovary	SC	OvsY.down	SPARCL1	-0.55893	1.76E-06
Ovary	SC	OvsY.down	SSR4	-0.59639	0.00647388
Ovary	SC	OvsY.down	TAGLN	-0.55453	0.00074739
Ovary	SC	OvsY.down	TSC22D3	-0.64507	1.00E-09
Ovary	SC	OvsY.down	TXNIP	-0.66529	1.50E-07
Ovary	SC	OvsY.down	WDR83OS	-0.61485	0.00153142
Ovary	SC	OvsY.down	YBX3	-0.5986	0.00098666

Supplementary Table 5. Antibodies used in this study.

Antibodies	Source	Identifier
Rabbit anti-Decorin (DCN)	Abcam	Cat# ab277636
Rabbit anti-Apolipoprotein E (APOE)	Abcam	Cat# ab183597
Rabbit anti-SPARC	Immunoway	Cat# YT5521
Rabbit anti-SPARCL1	Abcam	Cat# ab255597
Mouse anti-Cystatin C (CST3)	Immunoway	Cat# YM3626
Rabbit anti-α-SMA (ACTA2)	CST	Cat# 19245T
Rabbit anti-PDLI3	Immunoway	Cat# YT6467
Rabbit anti-S100A4	Abcam	Cat# ab197896
Mouse anti-DNA/RNA damage (8-OHdG)	Abcam	Cat# ab62623
Rabbit anti-ZP1	Immunoway	Cat# YT4994
Rabbit anti-Histone H2A.X, phospho (Ser139) (γH2AX)	CST	Cat# 9718
Rabbit anti-ZP3	Proteintech	Cat# 21279-1-AP
Rabbit anti-CD3	Abcam	Cat# ab16669
Rabbit anti-CD68	Servicebio	Cat# GB113150
Mouse anti-CD8	Servicebio	Cat# GB12068
Mouse anti-Collagen I (COL1A1)	Immunoway	Cat# YM3764
Rabbit anti-MIS (AMH MIF)	Abcam	Cat# ab229212
Rabbit anti-CD45	Servicebio	Cat# GB113885