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# Comprehensive Transcriptomic Analysis for Thymic Epithelial Cells of Aged Mice and Humans

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**Conflict of Interest**
The authors declare no potential conflicts of interest.

## ABSTRACT

Thymic epithelial cells (TECs) play a critical role in thymic development and thymopoiesis. As individuals age, TECs undergo various changes that impact their functions, leading to a reduction in cell numbers and impaired thymic selection. These age-related alterations have been observed in both mice and humans. However, the precise mechanisms underlying age-related TEC dysfunction remain unclear. Furthermore, there is a lack of a comprehensive study that connects mouse and human biological processes in this area. To address this gap, we conducted an extensive transcriptome analysis of young and old TECs in mice, complemented by further analysis of publicly available human TEC single-cell RNA sequencing data. Our analysis revealed alterations in both known and unknown pathways that potentially contribute to age-related TEC dysfunction. Specifically, we observed downregulation of pathways related to cell proliferation, T cell development, metabolism, and cytokine signaling in old age TECs. Conversely, TGF-β, BMP, and Wnt signaling pathways were upregulated, which have been known to be associated with age-related TEC dysfunctions or newly discovered in this study. Importantly, we found that these age-related changes in mouse TECs were consistently present in human TECs as well. This cross-species validation further strengthens the significance of our findings. In conclusion, our comprehensive analysis provides valuable insight into the biological and immunological characteristics of aged TECs in both mice and humans. These findings contribute to a better understanding of thymic involution and age-induced immune dysfunction.

**Keywords:** Thymic epithelial cell (TEC); Transcriptome analysis; Aging

## INTRODUCTION

Thymic epithelial cells (TECs) play a crucial role in thymic development and thymopoiesis (1). They can be classified into cortical TECs (cTECs) and medullary TECs (mTECs) based on anatomical position and functional characteristics. These distinct subsets of TECs are responsible for important functions in the thymus, such as regulating negative and positive selection of thymocytes (2). During the aging process, TECs undergo a decrease in cell number and become dysfunctional in thymocyte selection. These changes contribute

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**Abbreviations**

cTEC, cortical thymic epithelial cell; DEG, differentially expressed gene; GSEA, gene set enrichment analysis; mTEC, medullary thymic epithelial cell; KEGG, Kyoto Encyclopedia of Genes and Genomes; OXPHOS, oxidative phosphorylation; PRC2, polycomb repressive complex 2; RNA-seq, RNA sequencing; RPMI, Roswell Park Memorial Institute; scRNA-seq, single-cell RNA sequencing; SNUH, Seoul National University Hospital; STRING, search tool for recurring instances of neighboring genes; TEC, thymic epithelial cell.

**Author Contributions**

Conceptualization: Chung DH; Data curation: Lee S, Song SG; Formal analysis: Lee S, Song SG; Funding acquisition: Chung DH; Investigation: Lee S, Song SG; Visualization: Lee S, Song SG; Writing - original draft: Lee S, Song SG; Writing - review & editing: Lee S, Song SG, Chung DH.

to thymic involution, characterized by thymus shrinking, loss of tissue structure, and architectural distortion, such as adipose tissue accumulation (3-5). Thymic involution disrupts thymic development, leading to impaired clearance of self-reactive T cells, reduced production of naïve T cells, and limited diversity of TCRs (4). In humans, age-related thymic involution is a major risk factor for cancer, infection, and rheumatoid arthritis (6-8). Several factors have been proposed as potential causes of TEC dysregulation in old age, including sex hormones, Wnt/β-catenin signaling, TGF-β signaling, and the decrease in the transcription factor FOXN1, a key regulator of TECs (9-12). However, the specific mechanisms underlying the reduction and dysfunction of TECs induced by aging are not yet fully understood.

Age-related dysfunctions of TECs are widely observed and evolutionarily conserved in vertebrates. While the thymus undergoes involution in mice after 4–6 wk of age, this process occurs after 1 year of age in humans (4,13,14). Most investigations of age-related TEC dysfunctions have been conducted using mouse models. However, the suitability of the mouse system for understanding age-related thymic alterations in humans remains uncertain due to species differences, including disparities in TCR excision circles, thymic output, and peripheral division of naïve T cells (11,13,15-17). Moreover, certain critical pathways implicated in age-related TEC dysfunctions in mice have not been adequately validated in TECs from aged humans, such as TGF-β signaling (11). Therefore, there is an urgent need for a comprehensive analysis that compares the mouse and human systems to identify common pathways across species (6,13,18).

To address this issue, our study conducted a comprehensive transcriptome analysis using TECs obtained from young and old age mice and compared these results with publicly available single-cell RNA sequencing (scRNA-seq) data for human TECs. Our analysis revealed distinct transcriptome profiles in TECs from old mice, including alterations in cell cycle regulation, epigenetic modifications, signaling pathways, metabolism, cytokine signaling, and protein modification. These age-related distinctions in mouse TECs were then compared with human scRNA-seq data. Overall, our study provides a comprehensive analysis of aging-related changes in the TEC transcriptome.

## MATERIAL AND METHODS

### Mice

C57BL/6 (B6) mice (4 wk old and 9 months) were purchased from Koatech (Pyeongtaek, Korea). All mice were bred and maintained under specific pathogen-free conditions at the Clinical Research Institute, Seoul National University Hospital (SNUH). All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute, SNUH and conducted in accordance with relevant guidelines and regulations.

### Cell preparation

$CD45^{-}EpCAM^{+}$ TECs were isolated from mouse thymus using the FACS Aria III Cell Sorter (BD, San Diego, CA, USA). Fresh thymic tissues were obtained from both young and old mice and finely chopped in Roswell Park Memorial Institute (RPMI) 1640 medium using scissors. Subsequently, the tissue was incubated at 37°C in a shaking incubator with RPMI 1640 medium supplemented with 1 mg/ml Collagenase-IV (Sigma-Aldrich, St Louis, MO, USA) and 50 μg/ml DNase I (Sigma-Aldrich) for 30 min. After the incubation, the samples were sequentially passed through needles of decreasing size (18 gauge, 21 gauge, 23 gauge, and 26

gauge) using a 1 cc syringe, followed by an additional 20-min incubation at 37°C in a shaking incubator. To remove $CD45^+$ cells, MojoSort mouse CD45 microbeads (BioLegend, San Diego, CA, USA) were used, and the surface proteins were stained for 30 min using the following antibodies: APC-conjugated anti-mouse CD45 (BioLegend), PerCP-conjugated anti-mouse EpCAM (BioLegend), and Brilliant Violet 421™-conjugated anti-MHC-II (BioLegend).

### RNA sequencing (RNA-seq)

The quality and quantity of the purified RNA were assessed using the Agilent BioAnalyzer 2100. For RNA library construction, the Illumina SMARTer Stranded Total RNA-Seq Kit v2—Pico Input Mammalian kit was utilized. A minimum of 1 ng of RNA from each sample was mixed with a Removal mix to eliminate rRNA, followed by cDNA synthesis. Library preparation involved adenylating the 3' ends, adding adaptors during the ligation step, and conducting PCR amplification for 15 cycles. The resulting libraries were purified using AMPured beads. Subsequently, the fragment size and concentration of the libraries were assessed using the Agilent TapeStation 4200 with the D1000 screen tape. The desired fragment size distribution ranged from 250–350 bp, with a final concentration above 5 ng/μl. Each sample was quantified using the KAPA Library Quantification Kit. High-quality libraries were pooled and sequenced on the Illumina NovaSeq6000 platform, following the manufacturer's protocols for 150 bp paired-end reads. Image analysis was performed using the NovaSeq6000 control Software version 1.3.1, and the base calling data output was demultiplexed using bcl2fastq version v2.20.0.422, resulting in the generation of fastQC files.

### RNA-seq data analysis

For the analysis of RNA-seq data, adapter sequences were trimmed, and reads shorter than 50 bp or those with ends having a Phred quality score lower than 20 were removed using the cutadapt v.2.8 tool. The filtered reads were aligned to the reference genome of the respective species using the STAR v.2.7.1a aligner (19). The alignment process followed the ENCODE standard options described in the "Alignment" section of the HTML report. The alignment was performed with the "-quantMode TranscriptomeSAM" option to estimate the expression levels. Gene expression was estimated using RSEM v.1.3.1, which considered the direction of the reads based on the library protocol using the "--strandedness" option (20). The accuracy of the measurement was improved by applying the "--estimate-rspd" option while keeping the remaining options at their default values. To normalize the sequencing depth across samples, FPKM and TPM values were calculated. Differentially expressed genes (DEGs) were identified using the R package TCC v.1.26.0 (21). The TCC package utilizes robust normalization techniques to compare tag count data. Normalization factors were computed using the iterative DESeq2/edgeR method (22,23). DEGs were determined based on a p-value threshold of less than 0.05 to account for multiple-testing errors.

### Gene set enrichment analysis (GSEA)

GSEA was conducted using GSEA software version 4.1.0 (24). Gene sets from Hallmark, Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, and WikiPathway were obtained from the Molecular Signatures Database. Permutations of gene sets were performed 10,000 times, and significant gene sets were identified based on a p-value threshold of less than 0.05.

### scRNA-seq analysis

Processed scRNA-seq data of TECs from human thymus samples were retrieved from the Gene Expression Omnibus database using the accession code GSE147520 (18). A subset containing only postnatal and adult samples was generated based on the provided annotation

data from the authors. The data underwent the standard normalization workflow using Seurat R-package version 4.0.2. DEGs and pathways were compared between TECs from the young group (samples "Postnatal 6 days" and "postnatal 10 months") and the adult group (sample "adult 25 years"). The average gene expression level within each group was calculated using the "AverageExpression" function. DEGs were identified using the "FindMarkers" function with an adjusted p-value threshold of less than 0.05.

For TEC subset analysis, we classified TECs into nine subsets by following the classification method described by Bautista et al. (18). Briefly, according to their expression levels of HLA class II molecules, cortical and medullary TECs were further divided into high-expressing groups (cTEC$^{hi}$, Aire$^{+}$ mTEC$^{hi}$) and low-expressing groups (cTEC$^{lo}$, mTEC$^{lo}$), respectively. Additionally, TECs that expressed the typical TEC genes but did not exhibit distinct functional gene expression patterns were classified as immature TECs. Subsets with corneocyte-like, neuroendocrine, myoid, or myelin$^{+}$ characteristics were also categorized accordingly.

### Search tool for recurring instances of neighboring genes (STRING) database analysis

For the analysis of protein-protein interactions, we utilized the STRING database version 11.5 (https://string-db.org/) (25). In order to focus on genes with potentially significant functions, we performed cluster analysis using genes that demonstrated at least one interaction with other genes when the interaction score cutoff was set to high confidence (i.e., an interaction score higher than 0.700).

## RESULTS

### The transcriptomic profiles of TECs in aged mice

To investigate the distinct transcriptomic profiles of TECs in young and old age mice, TECs were collected from mice at two different stages: 4 wk (referred to as young) and 9 months (referred to as old) using FACS. It has been reported that thymic involution starts after 4–6 wk of age in mice and thymic cellularity is robustly reduced at 9 months (4,13). Based on these reports, we selected 4 wk as the young control group and 9 months as the old age control group. Bulk RNA-seq was performed on the isolated TECs, followed by data preprocessing, alignment to the mouse genomes, and generation of gene expression profiles. DEGs were identified, and pathway analysis was conducted using GSEA to understand the associated biological processes and pathways (**Fig. 1A**).

The analysis revealed a reduction in the numbers of TECs and thymocytes in old age mice (**Fig. 1B and C**). Additionally, a decrease in the expression of MHC-II was observed in TECs of old age mice (**Fig. 1D**). Analyzing the DEGs, it was found that a higher number of genes were downregulated in old age TECs, with 2,904 genes downregulated and 1,547 genes upregulated (**Fig. 1E and F**). Notably, the expression of *Aire* was significantly decreased, and *Foxn1* expression showed a decreasing trend, which is consistent with previous reports on old age TECs (**Fig. 1G**) (12,18).

Furthermore, several important genes were categorized and summarized in **Table 1**, and their expression patterns were annotated on the volcano plot (**Fig. 1H**, **Table 1**). In old age TECs, a decrease in the expression of various genes related to negative selection for TECs (*Aire, Fezf2*), protein modification and histone modification (*Dusp4*, *Hdac9*), co-stimulatory and MHC

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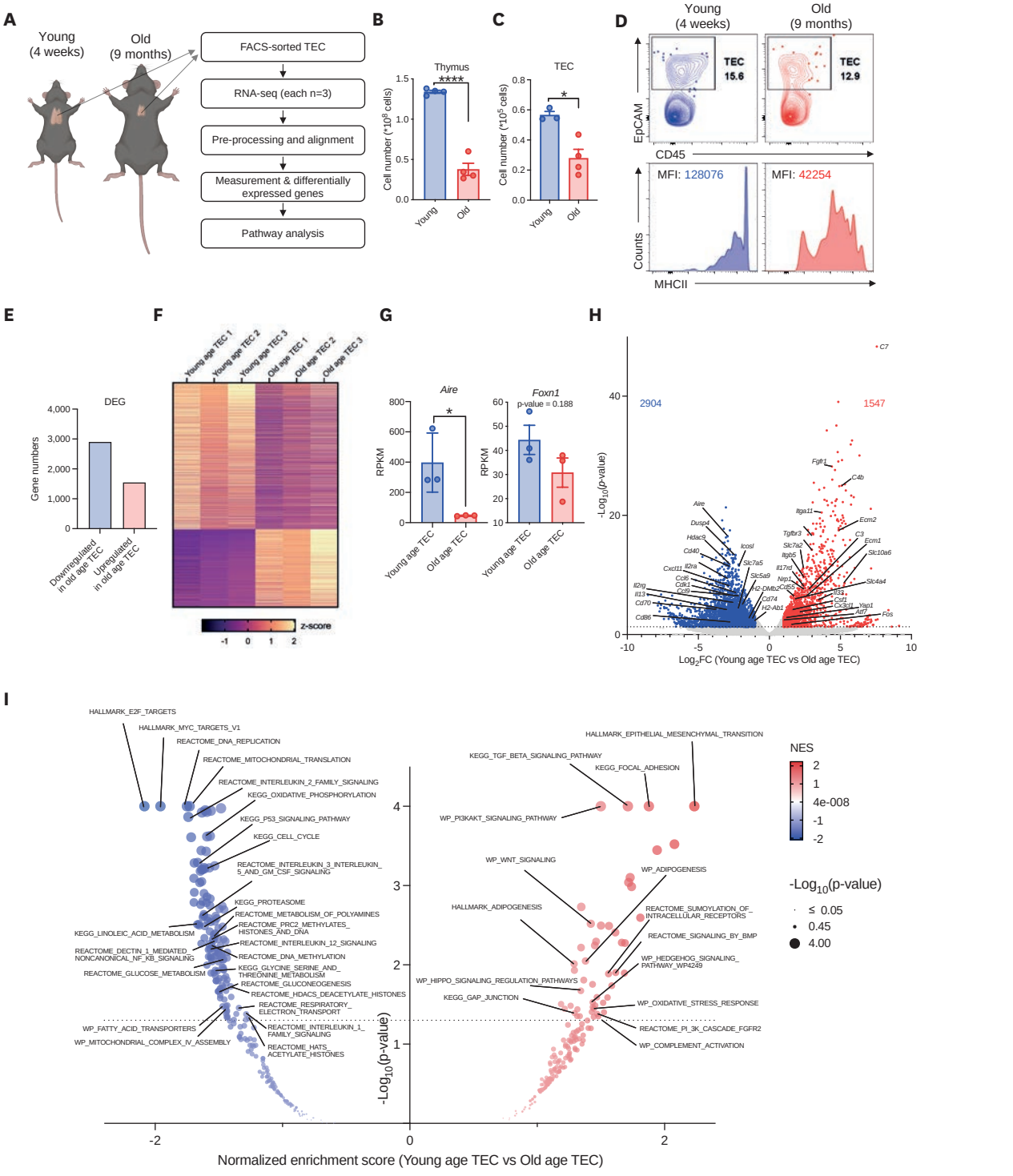


**Figure 1.** Overall distinct transcriptomic profiles in TECs of old mice. (A) Experimental scheme. (B) Total cell numbers in the thymus. (C) Numbers of TECs. (D) Population and MHC-II expression of TECs. (E) Number of DEGs identified in RNA-seq analysis, and (F) heatmap displaying the expression pattern of DEGs. (G) Expression levels of *Aire* and *Foxn1* in TECs of young and old mice. (H) Volcano plot representing the DEGs in TECs. (I) Bubble plot showing the top altered pathways identified through GSEA of RNA-seq data from mouse TECs. Each dot represents an individual mouse (B, C, and G). Flow cytometry plots shown are representative of at least two independent experiments. The data are presented as mean±SEM. Statistical significance was determined using an unpaired two-tailed *t*-test.
RPKM, reads per kilobase of transcript per million reads mapped; NES, normalized enrichment score.
*p<0.05; ****p<0.0001.


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**Table 1.** Genes downregulated (≤0.5-fold) or upregulated (≥2-fold) in TECs from old mice

| Gene category | Gene |
|---|---|
| Downregulated in old age TECs (≤0.5-fold) | |
| TEC negative selection | *Aire*, *Fezf2* |
| Protein ubiquitination & histone modification | *Dusp4*, *Hdac9* |
| Co-stimulatory molecule & MHC | *Cd40*, *Icosl*, *Cd70*, *Cd86*, *H2-Dmb2*, *H2-Ab1*, *Cd74* |
| Cytokine & cytokine receptor | *Il13*, *Il2ra*, *Il2rg* |
| Chemokine | *Cxcl11*, *Ccl6*, *Ccl9* |
| Cell cycle | *Cdk1* |
| Metabolite transporter | *Slc7a5*, *Slc5a9* |
| Upregulated in old age TECs (≥2-fold) | |
| Cytokine & cytokine receptor | *Il33*, *Csf1*, *Il17rd* |
| Chemokine | *Cx3cl1* |
| Metabolite transporter | *Slc7a2*, *Slc10a6*, *Slc4a4* |
| Complement pathway | *C7*, *C4b*, *C3*, *Cd55* |
| Integrin & extracellular protein | *Itga11*, *Itgb5*, *Ecm1*, *Ecm2* |
| TGF-β signaling & hippo signaling | *Tgfbr3*, *Yap1* |
| Cell surface receptor | *Fgfr1*, *Nrp1* |
| Transcription factor | *Atf7*, *Fos* |

molecules (*Cd40*, *Icosl*, *Cd70*, *Cd86*, *H2-Dmb2*, *H2-Ab1*, *Cd74*), cytokines and cytokine receptors (*Il13*, *Il2ra*, *Il2rg*), chemokines (*Cxcl11*, *Ccl6*, *Ccl9*), cell cycle regulation (*Cdk1*), and metabolite transporters (*Slc7a5*, *Slc5a9*) was observed (26,27). Conversely, increased expression was found in genes associated with certain cytokines and cytokine receptors (*Il33*, *Csf1*, *Il17rd*), chemokines (*Cx3cl1*), metabolite transporters (*Slc7a2*, *Slc10a6*, *Slc4a4*), the complement pathway (*C7*, *C4b*, *C3*, *Cd55*), integrins and extracellular proteins (*Itga11*, *Itgb5*, *Ecm1*, *Ecm2*), TGF-β signaling and hippo signaling (*Tgfbr3*, *Yap1*), cell surface receptors (*Fgfr1*, *Nrp1*), and transcription factors (*Atf7*, *Fos*) (**Fig. 1H**, **Table 1**). These findings indicate significant transcriptomic changes in various cellular phenotypes, including protein and histone modification, cytokine and cytokine receptors, chemokines, metabolism, and signaling pathways in TECs from aged mice.

Next, we performed GSEA using four gene set databases: Hallmark, KEGG, Reactome, and WikiPathway. This analysis revealed that various gene sets were either downregulated or upregulated in old age TEC. In old age TEC, gene sets related to cell cycle, epigenetic modification, signaling pathways, fatty acid metabolism, amino acid metabolism, oxidative phosphorylation (OXPHOS) & mitochondria, glucose metabolism, cytokine signaling, and protein modification were downregulated (**Fig. 1I**, **Table 2**). Conversely, gene sets associated with thymic involution, signaling pathways, complement, ROS, cell surface interaction, and protein modification were upregulated in old age TECs (**Fig. 1I**, **Table 2**).

Our transcriptomic analysis confirmed previous reports of increased TGF-β and Wnt signaling pathways in old age TECs, which have been suggested to be involved in age-related TEC dysfunction (**Fig. 1I**, **Table 2**, and **Supplementary Fig. 1A**) (10,11). Additionally, we observed a decrease in cell cycle and proliferation, which may contribute to the reduction in the number of TECs in aged mice (**Fig. 1I**, **Table 2**, and **Supplementary Fig. 1B**). Epigenetic changes, including DNA methylation and histone modification, were also detected in old age TECs (**Fig. 1I**, **Table 2**). Various signaling pathways, such as Myc, NF-κB, p53, hippo, PI3K-AKT, and hedgehog signaling, exhibited alterations in old age TECs, suggesting their potential roles in age-related TEC dysfunction (**Fig. 1I**, **Table 2**). Notably, metabolic pathways related to fatty acids, amino acids, OXPHOS & mitochondria, and glucose metabolism were significantly downregulated in old age TECs, indicating a decrease in metabolic processes in

**Table 2.** Pathways downregulated or upregulated in TECs from old mice (p-value<0.05)

| Gene set category | Gene set |
|---|---|
| Downregulated pathways | |
| Cell cycle | HALLMARK_E2F_TARGETS, KEGG_CELL_CYCLE, REACTOME_DNA_REPLICATION |
| Epigenetics | REACTOME_DNA_METHYLATION, REACTOME_PRC2_METHYLATES_HISTONES_AND_DNA, REACTOME_HDACS_DEACETYLATE_HISTONES, REACTOME_HATS_ACETYLATE_HISTONES |
| Signaling pathway | HALLMARK_MYC_TARGETS_V1, REACTOME_DECTIN_1_MEDIATED_NONCANONICAL_NF_KB_SIGNALING, KEGG_P53_SIGNALING_PATHWAY |
| Metabolism | |
| Fatty acid metabolism | KEGG_LINOLEIC_ACID_METABOLISM, WP_FATTY_ACID_TRANSPORTERS |
| Amino acid metabolism | REACTOME_METABOLISM_OF_POLYAMINES, KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM |
| OXPHOS & Mitochondria | KEGG_OXIDATIVE_PHOSPHORYLATION, REACTOME_MITOCHONDRIAL_TRANSLATION, REACTOME_RESPIRATORY_ELECTRON_TRANSPORT, WP_MITOCHONDRIAL_COMPLEX_IV_ASSEMBLY |
| Glucose metabolism | REACTOME_GLUCONEOGENESIS, REACTOME_GLUCOSE_METABOLISM |
| Cytokine signaling | REACTOME_INTERLEUKIN_1_FAMILY_SIGNALING, REACTOME_INTERLEUKIN_2_FAMILY_SIGNALING, REACTOME_INTERLEUKIN_3_INTERLEUKIN_5_AND_GM_CSF_SIGNALING, REACTOME_INTERLEUKIN_12_SIGNALING |
| Protein modification | KEGG_PROTEASOME |
| Upregulated pathways | |
| Thymic involution | HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION, HALLMARK_ADIPOGENESIS, WP_ADIPOGENESIS |
| Signaling pathway | KEGG_TGF_BETA_SIGNALING_PATHWAY, REACTOME_SIGNALING_BY_BMP, WP_WNT_SIGNALING, WP_HIPPO_SIGNALING_REGULATION_PATHWAYS, REACTOME_PI_3K_CASCADE_FGFR2, WP_HEDGEHOG_SIGNALING_PATHWAY_WP4249 |
| Complement | WP_COMPLEMENT_ACTIVATION |
| ROS | WP_OXIDATIVE_STRESS_RESPONSE |
| Cell surface interaction | KEGG_FOCAL_ADHESION, KEGG_GAP_JUNCTION |
| Protein modification | REACTOME_SUMOYLATION_OF_INTRACELLULAR_RECEPTORS |

old aged TECs (**Fig. 1I**, **Table 2**, and **Supplementary Fig. 1C and D**). Finally, cytokine signaling pathways including IL-1, IL-2, IL-3, IL-5, GM-CSF, and IL-12 were also decreased in old age TECs (**Fig. 1I**, **Table 2**, and **Supplementary Fig. 1E**). Previous studies have demonstrated that IL-7 and IL-15 produced by TECs regulate the development of thymocytes and innate lymphoid cells (28,29). Based on these results, it is reasonable to hypothesize that the decreased cytokine signaling in old age TECs may contribute to the development of these subsets. In summary, our GSEA analysis identified various alterations in biological pathways, including epigenetic changes, signaling pathways, metabolic pathways, and cytokine signaling, in old age TECs.

## Analysis for publicly available scRNA-seq data of TECs from young and adult humans

To investigate the differences in aged human TECs and determine if the transcriptomic changes observed in aged mouse TECs are also present in humans, we reanalyzed a human thymic scRNA-seq dataset that included fetal, postnatal (6 days and 10 months), and adult (25 years) TECs (**Fig. 2A**) (18). Fetal cells were excluded from the analysis for comparability with the mouse data. Moreover, considering that thymic involution in humans typically begins around the age of 1 year, the postnatal 6-day and 10-month samples were categorized as the young group (3). Then, the differences in transcriptomic profiles between the young and adult age groups of TECs were analyzed.

Analysis of gene expression levels associated with TEC function revealed a reduction in the expression of MHC molecules (*HLA-A* and *HLA-DRA*), key regulators for thymic development (*FOXN1*), and tissue-specific Ag expression regulators (*AIRE* and *FEZF2*) in adult TECs, similar to the findings observed in mice (**Fig. 2B and C**). Subset analysis of TECs showed a significant decrease in cTECs within the adult thymus. Instead, the majority of adult TECs were found

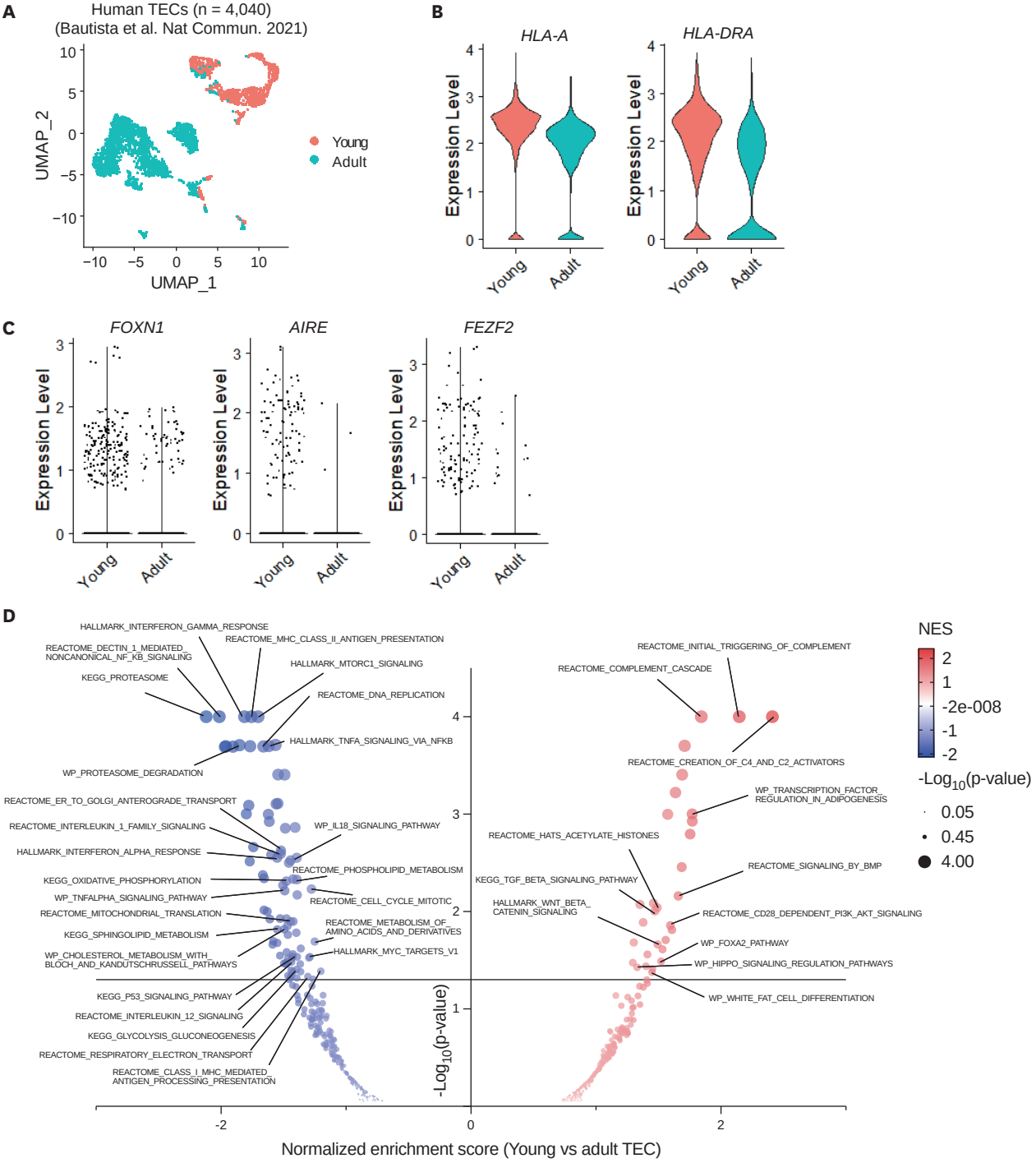


**Figure 2.** Analysis of TEC scRNA-seq data from young and adult humans. (A) UMAP visualization of young (n=1,409) and adult (n=2,631) human TECs. (B) Violin plots depicting the expression of MHC genes. (C) Violin plots showing the expression levels of *FOXN1*, *AIRE*, *FEZF2* in young and adult human TECs. (D) Bubble plot illustrating the GSEA results for human TEC scRNA-seq data.
UMAP, uniform manifold approximation and projection; NES, normalized enrichment score.

to be immature TECs that lacked typical gene characteristics of both cTECs and mTECs, which may contribute to the impaired T cell developmental function in the adult thymus (**Supplementary Fig. 2A and B**) (18).


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**Table 3.** Pathways downregulated or upregulated in TECs of adult humans (p-value<0.05)

| Gene set category | Gene set |
|---|---|
| Downregulated pathways | |
| Cell cycle | REACTOME_DNA_REPLICATION, REACTOME_CELL_CYCLE_MITOTIC |
| Ag presentation & processing | REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION, REACTOME_MHC_CLASS_II_ANTIGEN_PRESENTATION, WP_IL18_SIGNALING_PATHWAY, REACTOME_ER_TO_GOLGI_ANTEROGRADE_TRANSPORT |
| Signaling pathway | HALLMARK_MTORC1_SIGNALING, KEGG_P53_SIGNALING_PATHWAY, HALLMARK_MYC_TARGETS_V1, REACTOME_DECTIN_1_MEDIATED_NONCANONICAL_NF_KB_SIGNALING, HALLMARK_TNFA_SIGNALING_VIA_NFKB |
| Metabolism | |
| Fatty acid metabolism | KEGG_SPHINGOLIPID_METABOLISM, WP_CHOLESTEROL_METABOLISM_WITH_BLOCH_AND_KANDUTSCHRUSSELL_PATHWAYS, REACTOME_PHOSPHOLIPID_METABOLISM |
| Amino acid metabolism | REACTOME_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES |
| OXPHOS & Mitochondria | KEGG_OXIDATIVE_PHOSPHORYLATION, REACTOME_RESPIRATORY_ELECTRON_TRANSPORT, REACTOME_MITOCHONDRIAL_TRANSLATION |
| Glucose metabolism | KEGG_GLYCOLYSIS_GLUCONEOGENESIS |
| Cytokine signaling | HALLMARK_INTERFERON_GAMMA_RESPONSE, REACTOME_INTERLEUKIN_1_FAMILY_SIGNALING, HALLMARK_INTERFERON_ALPHA_RESPONSE, WP_TNFALPHA_SIGNALING_PATHWAY, REACTOME_INTERLEUKIN_12_SIGNALING |
| Protein modification | KEGG_PROTEASOME, WP_PROTEASOME_DEGRADATION |
| Upregulated pathways | |
| Thymic involution | WP_TRANSCRIPTION_FACTOR_REGULATION_IN_ADIPOGENESIS, WP_WHITE_FAT_CELL_DIFFERENTIATION |
| Epigenetic | REACTOME_HATS_ACETYLATE_HISTONES |
| Signaling pathway | KEGG_TGF_BETA_SIGNALING_PATHWAY, REACTOME_SIGNALING_BY_BMP, WP_HIPPO_SIGNALING_REGULATION_PATHWAYS, WP_FOXA2_PATHWAY, HALLMARK_WNT_BETA_CATENIN_SIGNALING |
| Complement | REACTOME_COMPLEMENT_CASCADE, REACTOME_INITIAL_TRIGGERING_OF_COMPLEMENT, REACTOME_CREATION_OF_C4_AND_C2_ACTIVATORS |

Next, GSEA was performed to identify gene sets that were either upregulated or downregulated in human adult TECs. Consistent with the GSEA results in mice, a downregulation was observed in gene sets related to cell cycle, signaling pathways, various metabolic processes, cytokine signaling, protein modification, and Ag presentation and processing in adult TECs (**Fig. 2D**, **Table 3**). Conversely, biological processes related to thymic involution, epigenetic modifications, complement reaction, and several other signaling pathways were upregulated (**Fig. 2D**, **Table 3**). These findings indicate that the transcriptomic alterations in human TECs during aging were similar to the changes observed in murine TECs, highlighting a conserved pattern across species.

## Integrated analysis of mouse and human sequencing data of TECs

To investigate aging-related transcriptomic changes in TECs that are shared between mice and humans, an integrated analysis of RNA-seq data from mice and scRNA-seq data from humans was conducted. Comparative analysis of GSEA results revealed significant age-related declines in key signaling pathways such as Myc, p53 signaling, and NF-κB, which are associated with thymic development, proliferation, and thymopoiesis (30-32). Aged TECs exhibited upregulation of TGF-β and Wnt signaling, known to be involved in age-related TEC dysfunction. Additionally, we identified transcriptomic alterations of BMP and hippo signaling pathways, which have not been previously reported in TECs (**Tables 2** and **3**, **Fig. 3A**, and **Supplementary Fig. 3A and B**).

A set of 196 upregulated genes and 454 downregulated genes in aged TECs was identified (**Fig. 3B**), and functional enrichment analysis was performed using the STRING database (**Fig. 3C**, **Supplementary Fig. 4A and B**) (25). In young TECs, gene clusters related to cell proliferation (*CDK1*, *MKI67, BIRC5, CCNB2*), chemokine/chemokine receptors (*CCL5, CCL19, CXCL11, XCR1, CCR10*), NF-kB signaling (*NFKB2, RELB, NFKBID, NFKBIE, TRAF1, TNFRSF11A*), myoid differentiation (*MYOD1, MYOG, ACTA1, ACTC1, MYH7, TNNT2*), and terminal epidermal differentiation (*IVL, SPRR1A, SPRR1B, SPRR2D, SPRR3, RPTN, CASP14)* were observed


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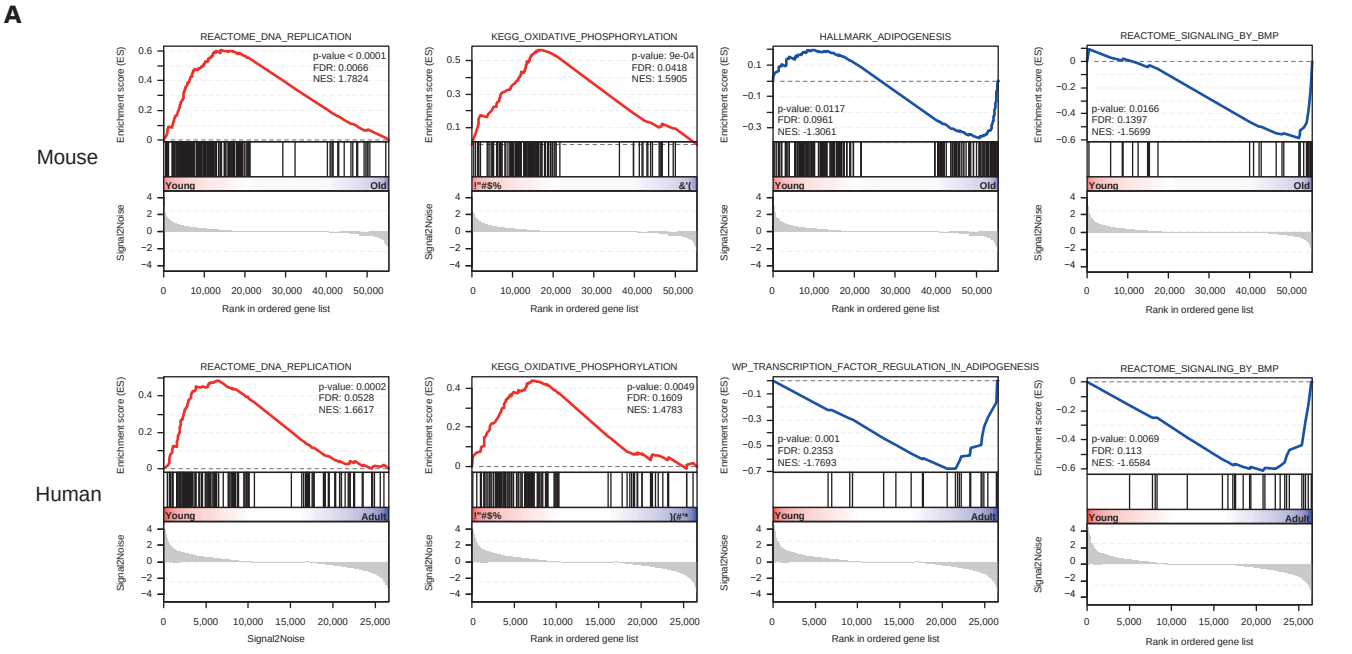


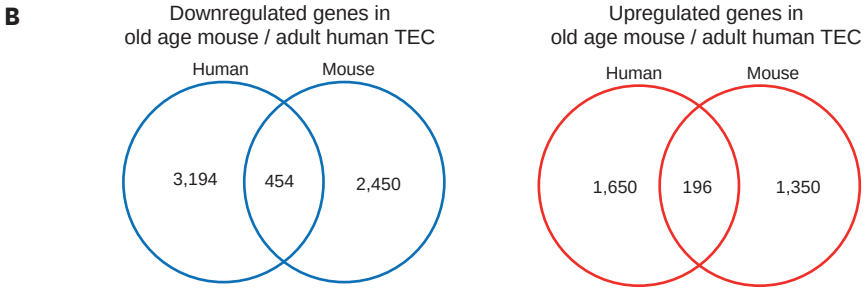


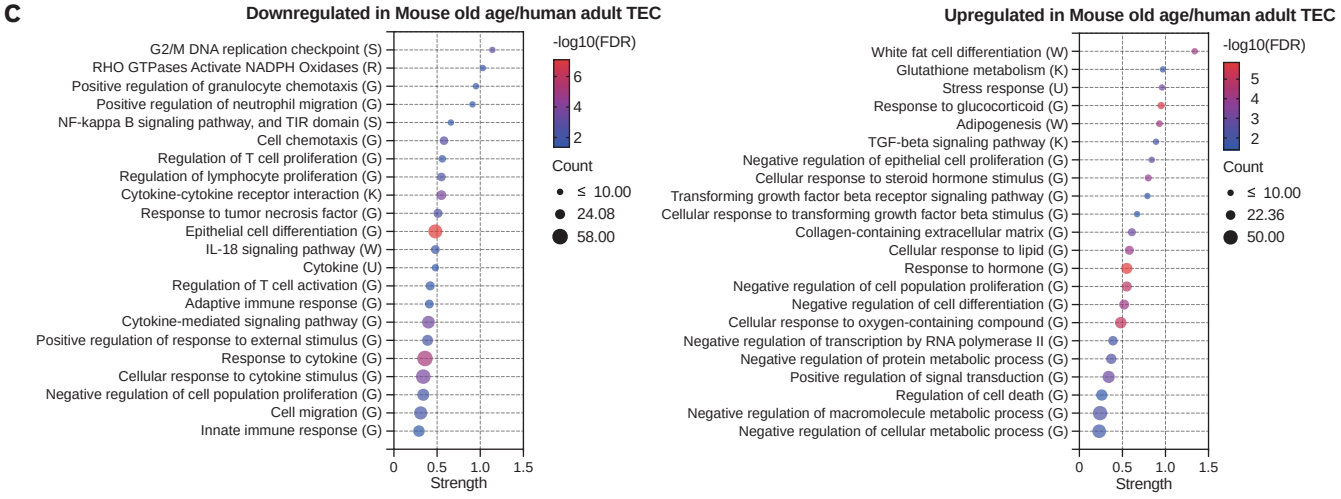


**Figure 3.** Integrated analysis of mouse and human sequencing data. (A) GSEA plots of mice and humans. (B) Venn diagram illustrating the common DEGs in TECs from old mice and adult humans. (C) Bubble plot displaying the STRING-based analysis of functionally enriched pathways in TECs of old mice and adult humans. Strength indicates the logarithm of the ratio of the number of DEGs in the network to the number of genes expected to be annotated in a random network. S, STRING clusters; R, reactome; G, gene ontology process; K, KEGG; W, WikiPathways; U, UniProt keywords; FDR, false discovery rate.


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(**Supplementary Fig. 4A**) (33). In contrast, cluster analysis of upregulated genes in aged TECs revealed pathways associated with epithelial-to-mesenchymal transition (*ZEB1, TWIST1, FOS*), postnatal maintenance of thymic medulla (*EGFR*), TGF-β signaling (*TGFBR2, TGFBR3*), and BMP signaling (*BMP4, FST*) (**Supplementary Fig. 4B**) (34-37).

Further functional enrichment analysis of the DEGs revealed that cell cycle and proliferation, cell migration, regulation of thymocyte differentiation (including NF-κB signaling pathway), and cytokine response were upregulated in young TECs (**Fig. 3C**, left), while pathways related to thymic involution (white fat cell differentiation, adipogenesis), TGF-β signaling, and negative regulation of metabolic processes were upregulated in aged TECs (**Fig. 3C**, right).

Overall, the comprehensive analysis of mouse and human transcriptomic data recapitulated aging-induced transcriptomic changes in TECs, highlighting shared pathways and processes across species.

## DISCUSSION

Our study revealed distinct transcriptomic profiles in old age mouse TECs compared to their young counterparts, indicating the involvement of several potential pathways in age-related TEC dysfunction. Notably, we observed a decrease in polycomb repressive complex 2 (PRC2) activity in old age TECs. Barthlott et al. (38) have previously suggested that PRC2 regulates the development and function of TECs by catalyzing epigenetic marks known as H3K27me3. Loss of PRC2 activity in TECs led to a phenotype similar to age-related TEC dysfunction, including thymic atrophy, decreased thymic cellularity, and reduced TCR diversity (38). Furthermore, our investigation revealed significant epigenetic changes in both mouse and human TECs during aging, which can impact various aspects of TEC function and senescence. Previous studies have demonstrated the influence of epigenetic changes on the expression of Foxn1, the epithelial-to-mesenchymal transition of TECs to adipocytes, and the expression of tissue-restricted self-Ags (39,40). Moreover, our analysis also highlighted potential involvement of other unstudied epigenetic factors, such as histone methylation, histone acetylation, and DNA methylation, which may contribute to age-related TEC dysfunction. Therefore, our analysis of mouse RNA-seq data suggests a potential role of epigenetic changes in age-related TEC dysfunction. Age-related dysfunction of TECs contributes to various contradictory characteristics observed in the immune system of aged individuals, including a reduced ability to combat infections and tumors, diminished efficacy of vaccines, and increased susceptibility to autoimmune diseases (41,42). These contrasting features arise from inadequate naive T cells and an upsurge in self-reactive T cells, which result from age-related TEC dysfunction (42-44). In our study, the comparative analysis of transcriptome data for human and mouse TECs supported the overlapping nature of age-related pathways between mice and humans, providing a clearer understanding of the key pathways involved in age-related TEC dysfunction.

Our study revealed significant transcriptomic alterations in metabolic processes among various biological processes, including oxidative stress response, fatty acid metabolism, amino acid metabolism, OXPHOS and mitochondria function. These findings suggest a comprehensive metabolic reprogramming in old age TECs. Consistent with our findings, previous studies have implicated mTOR signaling, ROS, and autophagy in the regulation of thymic stromal environment (45). Increased ROS levels in TECs, resulting from a deficiency in catalase, have been associated with thymic atrophy in aged mice (46).

Furthermore, our comprehensive GSEA analysis conducted on both mouse and human TECs revealed shared alterations in signaling pathways associated with aging across species. We confirmed the upregulation of TGF-β and Wnt signaling pathways, which are known to contribute to age-related TEC dysfunction. Moreover, our analysis also uncovered the upregulation of BMP and hippo signaling pathways in aged TECs, which have not been previously reported in TECs (3). These findings provide valuable insight into the potential roles of BMP and hippo signaling in thymic aging. Previous studies have highlighted the importance of fine-regulation of the Wnt pathway for proper thymus morphogenesis and function, controlling the expression of Foxn1. Disruption of the delicate balance through elevated Wnt signaling has been shown to contributed to TEC dysfunction (10,47). Similarly, BMP signaling has been recognized as a requirement for normal thymic development, but there are also reports suggesting its involvement in the SNAIL1-mediated epithelial-to-mesenchymal process (48,49). In mice, the initial activation of BMP4 signaling plays a crucial role in thymopoiesis by stimulating the expression of Foxn1. Additionally, increased BMP4 signaling within TECs facilitates thymic regeneration after exposure to radiation-induced thymic damage (50-52). These findings suggest that BMP signaling might be essential for biological processes of TECs. Consistently, our analysis suggests that appropriate levels of BMP and Wnt signaling are critical for old age TEC functions, although the role of excessive BMP signaling in TECs has not been previously reported. Moreover, the hippo signaling pathway has been reported to regulate organ size and tissue homeostasis (53). Recent research has indicated that hippo signaling is higher in the thymosphere of thymus from the adult compared to that of the fetal thymus (54). Based on these findings, it is hypothesized that dysregulated hippo signaling may contribute to a reduction in thymus size during the aging process. However, further investigation is necessary to gain a deeper understanding and confirm this hypothesis.

In conclusion, this study represents a pioneering investigation that sheds light on the transcriptomic alterations and shared pathways associated with age-related TEC dysfunction in both mice and humans. It provides a comprehensive overview of the distinctive changes observed in age-related TEC dysfunction, validating previous findings in mice and uncovering novel pathways, including epigenetic changes, metabolic pathways, and BMP signaling, that may contribute to TEC dysfunction during aging. These findings significantly contribute to our understanding of the underlying mechanisms driving age-related TEC dysfunction in both mice and humans. Furthermore, the transcriptomic data obtained from mouse TECs in this study serve as a valuable resource for future investigations into TEC biology and the dysfunction of TECs associated with aging.

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## SUPPLEMENTARY MATERIALS

### Supplementary Fig. 1

KEGG pathway analysis of mouse TEC RNA-seq. (A) KEGG_TGF_BETA_SIGNALING_PATHWAY. (B) KEGG_DNA_REPLICATION. (C) KEGG_OXIDATIVE_PHOSPHORYLATION. (D) KEGG_FAT_DIGESTION_AND_ABSORPTION. (E) KEGG_CYTOKINE-CYTOKINE RECEPTOR_INTERACTION. (A-E) Green indicates enriched in young mouse TECs, and red indicates enriched in old mouse TECs.

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### Supplementary Fig. 2

Analysis of human TEC single cell RNA-seq. (A) UMAP visualization of human TEC subsets. Based on their expression levels of HLA class II molecules, the cTECs and mTECs were divided into high-expressing groups (cTEC$^{hi}$, Aire$^{+}$ mTEC$^{hi}$) and low-expressing groups (cTEC$^{lo}$, mTEC$^{lo}$), respectively. (B) Composition of TEC subsets in young and adult human TECs.

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### Supplementary Fig. 3

GSEA plots of commonly altered pathways from mouse and human sequencing data. (A) Pathways downregulated in both old mouse TECs and adult human TECs. (B) Pathways upregulated in both old mouse and adult human TECs.

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### Supplementary Fig. 4

PPI network analysis. (A) Downregulated genes in old mouse and adult human TECs. (B) Upregulated genes in both old mouse TECs and adult human TECs.

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