Deciphering the endometrial niche of human thin endometrium at single-cell resolution

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Thin endometrium has been widely recognized as a critical cause of infertility, recurrent pregnancy loss, and placental abnormalities; however, access to effective treatment is a formidable challenge due to the rudimentary understanding of the pathogenesis of thin endometrium. Here, we profiled the transcriptomes of human endometrial cells at single-cell resolution to characterize cell types, their communications, and the underlying mechanism of endometrial growth in normal and thin endometrium during the proliferative phase. Stromal cells were the most abundant cell type in the endometrium, with a subpopulation of proliferating stromal cells whose cell cycle signaling pathways were compromised in thin endometrium. Both single-cell RNA sequencing and experimental verification revealed cellular senescence in the stroma and epithelium accompanied by collagen overdeposition around blood vessels. Moreover, decreased numbers of macrophages and natural killer cells further exacerbated endometrial thinness. In addition, our results uncovered aberrant SEMA3, EGF, PTN, and TWEAK signaling pathways as causes for the insufficient proliferation of the endometrium. Together, these data provide insight into therapeutic strategies for endometrial regeneration and growth to treat thin endometrium.

thin endometrium | cell proliferation | cellular senescence | single-cell sequencing

Successful pregnancy requires a receptive endometrium of adequate thickness and an appropriately developed embryo (1). Physiologically, the human endometrium is a highly regenerative tissue during each cycle under estrogen stimulation during the proliferative phase of the menstrual cycle. The responsiveness of endometrial tissue to sex steroid hormones is dependent on the state of the local microenvironment, which is created by stromal, epithelial, endothelial, and immune cells in endometria (2, 3). The proliferative phase, characterized by endometrial cellular proliferation and rapid tissue growth, is the critical phase for determining endometrial thickness (4). Insufficient endometrial thickness, diagnosed as thin endometrium with a maximum thickness ≤ 7 mm on an ultrasound scan accompanied by a normal uterine cavity, is closely associated with pregnancy failure (5, 6).

The most common causes of thin endometrium arise from inappropriate endometrium repair after curettage and surgical separation of intrauterine adhesion accompanied by disrupted blood vessel distribution and sparse glands (7, 8). At present, the cellular and molecular mechanisms of thin endometrium remain ambiguous, and therapeutic options for thin endometrium are limited and controversial due to its complex pathogenesis. Hence, unraveling the function of different cell types, the regulation of cell proliferation and the features of thin endometrium are urgently required to develop specific and effective therapies.

To depict the sophisticated alterations of endometrial cells and the local microenvironment in thin endometrium, 10X Genomics single-cell RNA sequencing (scRNA-seq) was applied to analyze the discrepancy in gene transcription between normal and thin endometrium during the late proliferative phase. We identified 15 distinct cell types with their own unique characteristics of gene expression profiles in the endometrium. The number of stromal, proliferating stromal (pStrs), epithelial, natural killer (NK), and T cells was reduced in thin endometrium accompanied by increased cellular senescence in perivascular cells. An analysis of cell interactions revealed that signaling pathways related to cell growth were markedly interrupted in thin endometrium, especially in stromal niches (stromal cells, pStrs, and perivascular cells). These findings indicated a potential mechanism of thin endometrium pathogenesis and provide insight into improving fertility.

Results

Cellular Heterogeneity in the Proliferating Endometrium Characterized by scRNA-seq. To elucidate the cellular dynamics of the endometrium during the proliferative phase at single-cell resolution, scRNA-seq was applied to three normal endometrial samples (Fig. 1*A* and *SI Appendix*, Table S1). After stringent cell

Significance

Thin endometrium is the most common reason for uterine infertility and refractory gynecological diseases due to its complexity in pathogenesis and adverse pregnancy outcomes. Here, we profile cells from normal and thin endometrium at single-cell resolution to investigate the sophisticated alterations in the local microenvironment that occur in thin endometrium. Increased cellular senescence, collagen overdeposition, and significant down-regulation of gene expression related to cell proliferation are observed and confirmed. Moreover, we demonstrate aberrant activation of the SEMA3 pathway accompanied by dampened EGF, PTN, and TWEAK signaling pathways in thin endometrium. These findings aid in understanding the mechanisms of thin endometrium and provide new tools to rejuvenate the atrophic endometrium for female fertility preservation and successful pregnancy.

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filtration and batch correction, graph-based clustering was performed, which cataloged a total of 29,393 cells into 15 distinct clusters in normal proliferative endometrium (Fig. 1 *B* and *C* and *SI Appendix*, Fig. S1*A* and Table S2). Stromal cells were the most abundant cell type and made up the mass of the regenerative endometrial functionalis (9) as evidenced by HAND2 and WT1. The immunostaining of HAND2 and WT1 with CD45 revealed many stromal cells dispersed with some CD45-positive immune cells in between (Fig. 1*D*). pStrs possessed high proliferative potential with potentiated expression of *MKI67* in normal endometrium (Fig. 1*C* and *SI Appendix*, Fig. S1*C*). Moreover, perivascular cells in the endometrium specifically expressing PDGFRB, COL4A1, and SUSD2 were confirmed in normal endometrium, consistent with previous findings (*SI Appendix*, Fig. S1 *B* and *C*). We also captured lymphatic endothelial cells in our scRNA-seq data, characterized by the expression of FLT4 and PROX1, which were validated by PROX1 staining in normal endometrium (Fig. 1*C* and *SI Appendix*, Fig. S1 *B* and *C*). Epithelia, including luminal cells (LE), glandular cells (GE), and ciliated epithelial cells (Cili_Epi), were identified by E-cadherin (E-CAD) staining with appropriate proliferation marked by phosphorylated histone 3 (pH3) in normal endometrium (Fig. 1*E*). In addition to these cell types, the distributions of T cells marked by CD8, NK cells marked by CD56 and GNLY, and macrophages marked by CD14 and CD163 were verified by immunofluorescence staining in normal endometrium (Fig. 1*F*).

Gene Expression Signatures of Stromal Cells and Epithelial Cells. After menstruation, endometrial cells undergo extensive proliferation to support the restoration and regeneration of the functional layer. Stromal cells, pStrs, and perivascular cells, marked by the high expression of COL1A1 (Fig. 1*G*), might be key contributors to this proliferative process to achieve adequate endometrial thickness. To thoroughly depict the signature of these three cells globally, genes of stromal cells, pStrs, and perivascular cells were clustered based on their expression patterns (Fig. 1*H* and *SI Appendix*, Fig. S2*A*). Using Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of highly expressed genes specific to stromal cells (*SI Appendix*, Fig. S2 *A* and *B*), we found that the terms "protein processing in endoplasmic reticulum (ER)," "endocytosis," "apoptosis," and "mitophagy" as well as the "FOXO signaling pathway" were highly enriched, suggesting active metabolism in mature stromal cells. KEGG analysis of highly expressed genes specific to pStrs showed that it possessed genes responding to cell proliferation, including PCNA and CCNB1 (Fig. 1 *H–K* and *SI Appendix*, Fig. S2*C*). To corroborate the proliferation of stromal cells, pH3, a marker of mitosis, was costained with CD10, a well-recognized human endometrial stromal cell marker (10), demonstrating that proliferating cells were widely distributed in the stroma (Fig. 1*K*). In addition, we verified that RGS5 and NOTCH3, identified as perivascular markers in murine endometrium, were also specifically expressed in the perivascular cells of human endometrium (11, 12) (*SI Appendix*, Fig. S2*D*).

GE was marked by Leukemia inhibitory factor (LIF) expression (Fig. 1*L*), and KEGG analysis showed that highly expressed genes specifically in GE were primarily associated with protein processing in the ER, lysosome, sphingolipid metabolism, *N*-glycan biosynthesis, and glycosaminoglycan degradation (Fig. 1 *M* and *N*) in accordance with the higher capacity for GE protein secretion. Moreover, KEGG terms including "IL-17 signaling pathway" and "TNF signaling pathway" were also enriched in GE, suggesting a secretion property of inflammatory-related proteins in GE at the proliferative phase (Fig. 1*N* and *SI Appendix*, Fig. S2*E*). AXIN2-positive cells were observed in the epithelia of the human endometrium (*SI Appendix*, Fig. S2*F*), consistent with findings in the murine uterus (13). FOXJ1 marking Cili_Epi was in line with a previous study (14). With respect to immune cells, to explore whether these cells expanded during the proliferative phase, pH3 staining was performed, revealing proliferation only rarely detected in CD163+ macrophages, CD8+ T cells, and CD56+ NK cells in normal endometrium (*SI Appendix*, Fig. S2*F*).

Increased Cellular Senescence and Collagen Deposition in Thin Endometrial Cells. We next dissected the changes that occurred in endometrial cells in three thin endometria during the same phase as the normal samples (*SI Appendix*, Table S1). We found that the thin endometria exhibited comparable cell types, but the number of cells was changed in certain types (Fig. 2*A* and *SI Appendix*, Fig. S1*A* and Table S2). pStrs were decreased in thin endometrium, suggesting that the growth capacity of the endometrium was impaired in this disease (Fig. 2 *A* and *B*). To gain insight into the dynamic cellular activities between normal and thin endometrium, we performed KEGG analysis of differentially expressed genes (DEGs) between the two groups. The expression of genes related to RNA polymerase was high in pStrs of normal endometrium and was significantly down-regulated in thin endometrium (Fig. 2*C*). Among these genes, the expression of POLR2I and POLR3K responding to messenger RNA (mRNA) synthesis in eukaryotes was significantly decreased in thin endometrium (Fig. 2 *D* and *F* and *SI Appendix*, Fig. S3*A*), and expression of NME4, associated with nucleotide metabolism, was significantly reduced in thin endometrium (*SI Appendix*, Fig. S3*B*). The down-regulated expression of these proliferation-related genes (*POLR2I*, *NME4*, and *MKI67*) was validated by RT-qPCR (Fig. 2*H*). Cell cycle assays revealed that most pStrs were in the S and G2/M phase, with most stromal and perivascular cells in the G1 phase (Fig. 2*I*). Expression of the proliferation marker PCNA was observed in G1, S, and G2/M phases of pStrs in normal endometrium but was markedly decreased in thin endometrium (Fig. 2 *I* and *J*). These results suggested that disruption of pStrs was one of the potential reasons for thin endometrium.

Interestingly, functional enrichment analysis also showed that cellular senescence was highly enriched in the stroma of thin endometrium along with increased thyroid hormone signaling (Fig. 2*C*). *DIO2*, identified as a major branch gene in the senescence pathway in decidual cells (15), was aberrantly increased in the stroma of thin endometrium during the proliferative phase (Fig. 2 *E* and *G*). Cellular senescence was also observed in thin endometrium as evidenced by increased expression of p21 or p16 at the mRNA and protein levels (*SI Appendix*, Fig. S3 *C–E*). Perivascular cells exhibited the highest expression of cellular senescence-related genes (Fig. 2*C*), and the colony formation ability of these perivascular cells sorted by SUSD2+CD31− from thin endometrium was significantly impaired compared to that of normal endometrium (Fig. 2*K*).

Type IV collagen is the primary component of basement membranes. Expression of COL4A1, a gene encoding the type IV collagen alpha 1 chain, was higher in perivascular cells of thin endometrium than in those from normal endometrium, indicating a thicker basement membrane (*SI Appendix*, Fig. S4 *A* and *B*). This may contribute to the impairment of angiogenesis and new blood vessel sprouting and ultimately result in insufficient vascularization, which has been reported as a key cause for thin endometrium (16, 17). Expression of VCAN and MGP was significantly up-regulated in the stroma of thin endometrium (*SI Appendix*, Fig. S4*C*), implying overdeposition of ECM in the endometrium, which was further confirmed by Masson staining and collagen1 expression (*SI Appendix*, Figs. S3*E* and S4*D*).

In epithelial cells, we observed decreased LE with relatively increased GE (Fig. 3*A*). Functional enrichment analysis of DEGs between normal and thin endometrium suggested that glycolysis/gluconeogenesis and the cell cycle were inhibited in epithelial cells, characterized by decreased expression of

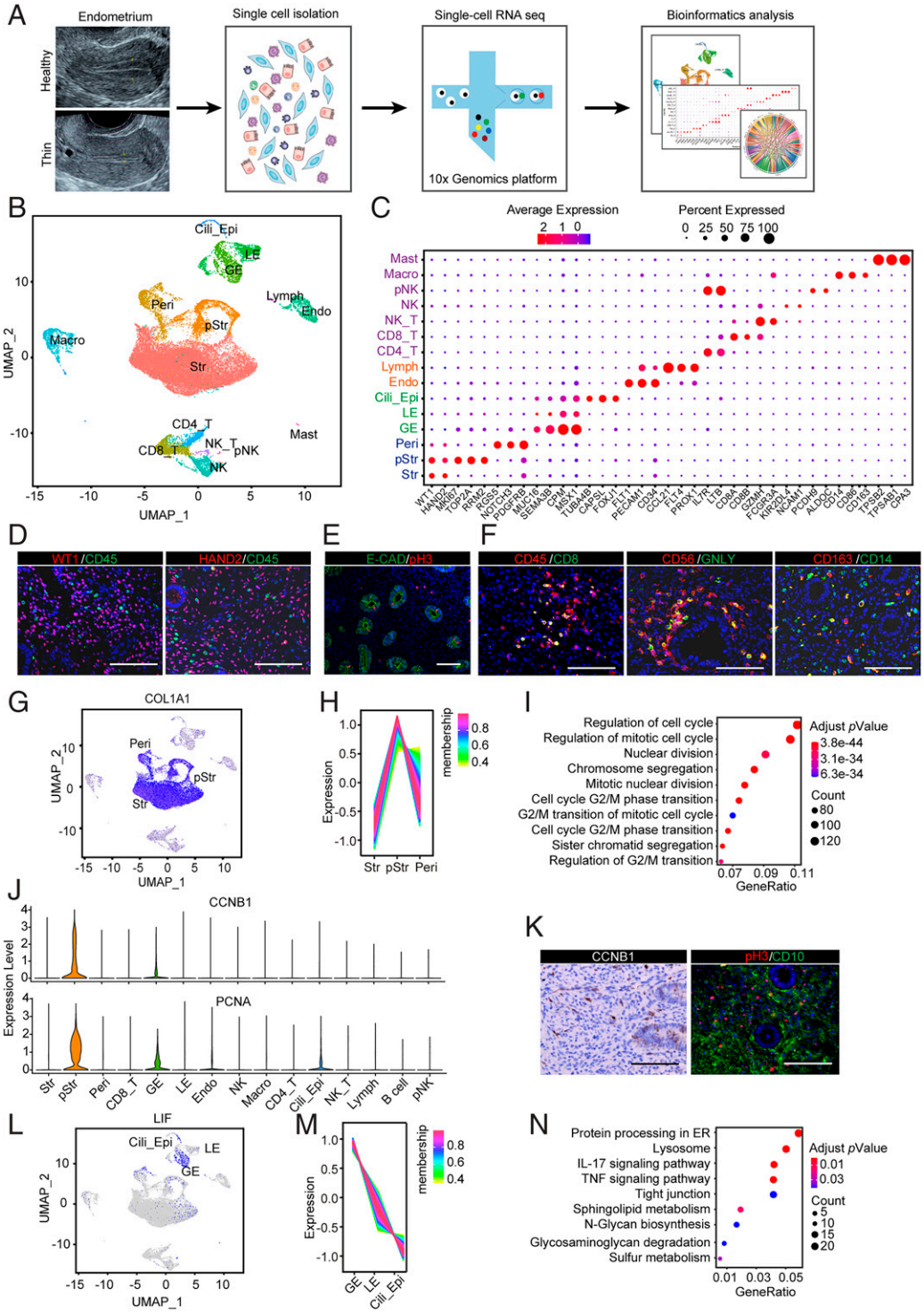

Fig. 1. Characterization of the different types of cells in normal endometrial samples. (*A*) Summary of the sample origins and analysis workflow. (*B*) UMAP of cells with the associated cell types in samples of normal endometrium (n = 3). Macro, macrophage; Lymph, lymphatic endothelial cell; Endo, endothelial cell; Peri, perivascular cell; Str, stromal cell. (*C*) Expression of classical marker genes of each cell type in the endometrial samples (n = 3). (*D–F*) Representative immunofluorescence images of markers for stromal cells (WT1, HAND2) and immune cells (CD45) (*D*), epithelial cells (E-CAD) and cell mitosis (pH3) (*E*), T cells (CD8), NK cells (CD56 and GNLY), and macrophages (CD163 and CD14) (*F*) in normal endometrium (n = 5). (*G*) The distribution of COL1A1 in normal endometrium by UMAP. (*H*) The expression pattern of highly expressed genes in pStr analyzed using the TCSeq package in R. The expression of these genes was normalized to the Z-score, and the color indicated the membership values representing the degree of genes belonging to this cluster. (*I*) Functional enrichment of highly expressed genes in pStr compared to Str and Peri based on *H*. (*J*) Expression of CCNB1 and PCNA in distinct types of cells shown by violinplot. (*K*) Immunohistochemistry of CCNB1 and immunofluorescence markers of stromal cells (CD10) and pH3 in normal endometrium (n = 5). (*L*) The distribution of LIF in normal endometrium by UMAP. (*M*) The expression pattern of highly expressed genes in GE compared to LE and Cili_Epi analyzed using the TCSeq package in R. Expression of these genes was normalized to Z-score, and the color indicated the membership values representing the degree of genes belonging to this cluster. (*N*) The functional enrichment of specific highly expressed genes in GE compared to LE and Cili_Epi based on *M*. (Scale bars, 100 μm.)

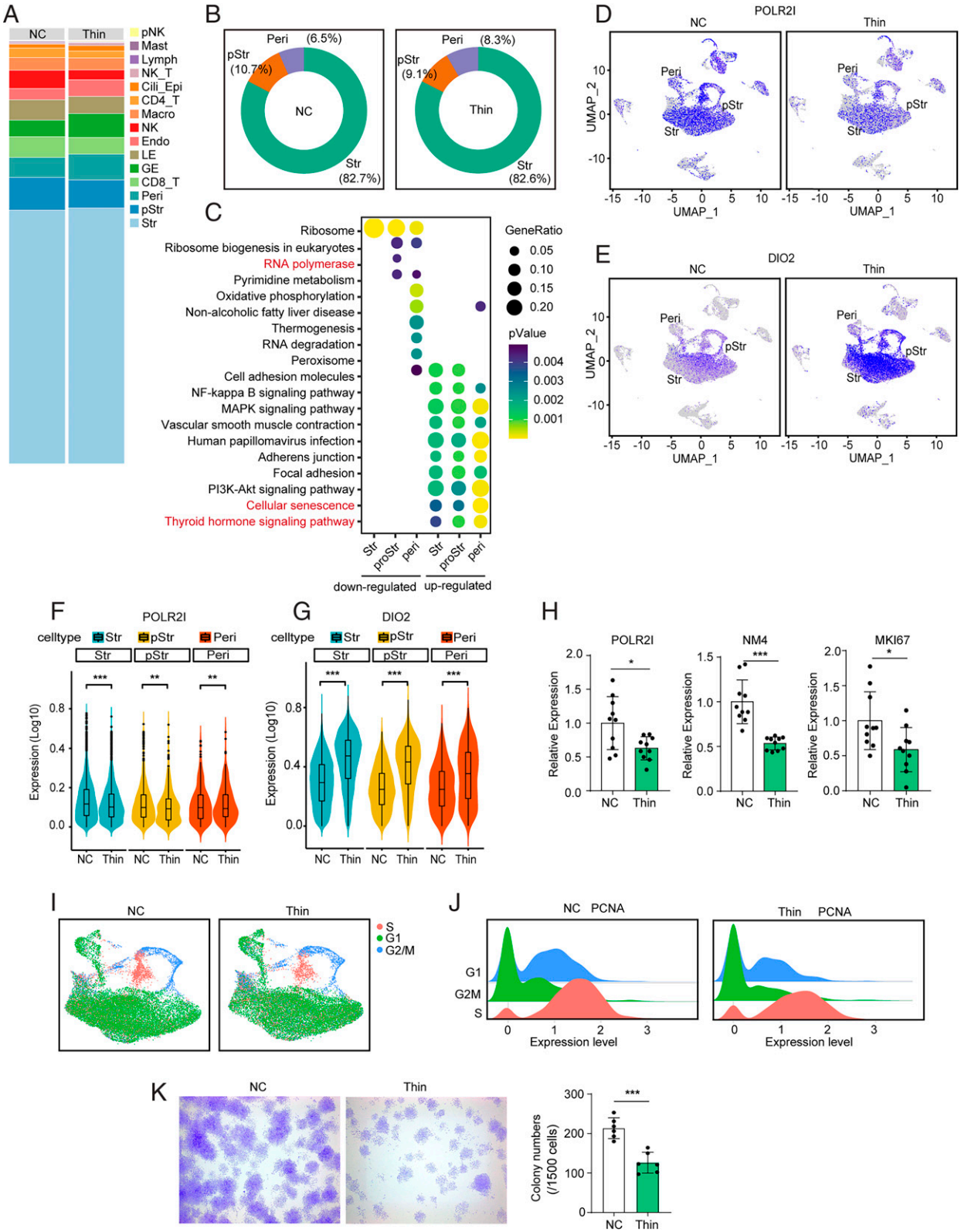

Fig. 2. Aberrant changes in cell proportions and gene expression patterns of stroma in thin endometrium. (*A*) The proportion of each cell type in normal and thin endometrium. (*B*) The fractions of each type of cell in stromal niches (Str, pStr, and Peri) in normal and thin endometrium. (*C*) Functional enrichment analysis of DEGs among Str, pStr, and Peri. (*D* and *E*) The distribution of POLR2I (*D*) and DIO2 (*E*) in normal and thin endometrium by UMAP. (*F* and *G*) Expression of POLR2I (*F*) and DIO2 (*G*) in Str, pStr, and Peri between normal and thin endometrium shown by violinplot. Data are presented as the mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$. (*H*) mRNA expression levels of *POLR2I*, *NM4*, and *MKI67* between normal and thin endometrium examined by RT-qPCR ($n = 10$ per group). Data are presented as the mean ± SEM, $^{*}P < 0.05$, $^{***}P < 0.001$. (*I*) Cell cycle analysis of Str, pStr, and Peri in normal and thin endometrium. (*J*) Expression of PCNA in G1, S, G2/M phase in pStr between normal and thin endometrium. (*K*) Colony numbers counted by clone formation assay ($n = 6$ per group). Data are presented as the mean ± SEM, $^{***}P < 0.001$.

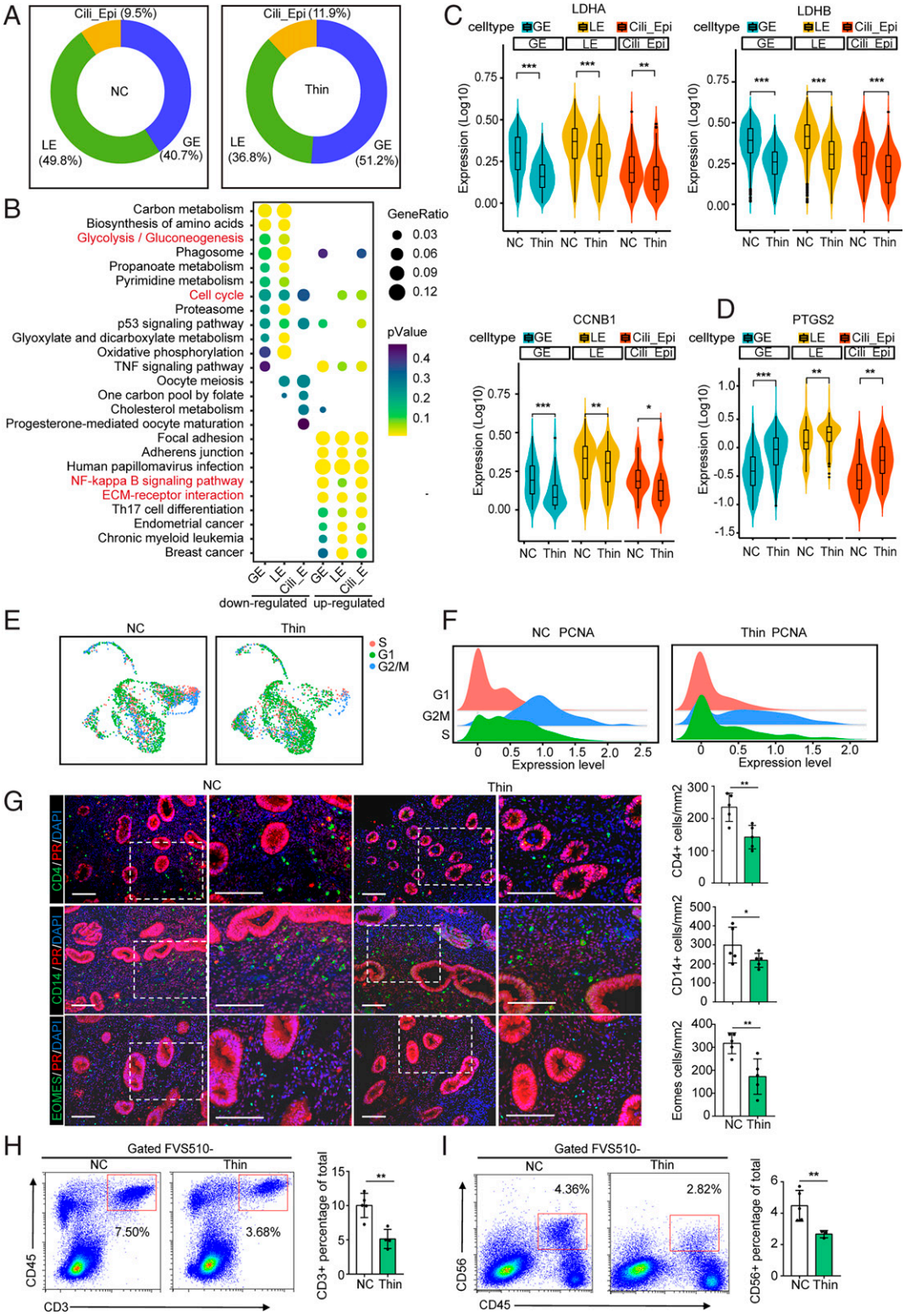

Fig. 3. Abnormal changes in cell proportions and gene expression patterns of epithelia and immune cells in thin endometrium. (*A*) The fractions of each cell type in epithelial niches (GE, LE, and Cili_Epi) in normal and thin endometrium. (*B*) KEGG enrichment of up-regulated and down-regulated DEGs in GE, LE, and Cili_Epi. (*C*) Expression of LDHA, LDHB, and CCNB1 in GE, LE, and Cili_Epi of normal and thin endometrium shown by violinplot. Data are presented as the mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*D*) Expression of PTGS2 in GE, LE, and Cili_Epi of normal and thin endometrium shown by violinplot. (*E*) Cell cycle analysis of GE, LE, and Cili_Epi in normal and thin endometrium. (*F*) Expression of PCNA in G1, S, G2/M phase in GE, LE, and Cili_Epi between normal and thin endometrium. (*G*) Costaining of progesterone receptor with CD4, CD14, or EOMES in normal and thin endometrium by immunofluorescence ($n = 5$ per group). (Scale bars, 100 μm.) Data are presented as the mean ± SEM, $*P < 0.05$, $**P < 0.01$. (*H* and *I*) Flow cytometry analysis of the proportion of T cells (CD3+ cells) and NK cells (CD56+ cells) between normal and thin endometrium ($n = 5$ per group). Data are presented as the mean ± SEM, $**P < 0.01$.

LDHA, LDHB, and CCNB1, indicating impaired cell proliferation (Fig. 3 *B* and *C*). Up-regulated genes in thin endometrium were enriched in the terms "NF-kappa B signaling pathway" and "ECM receptor interaction," indicating collagen overdeposition in epithelial cells of the thin endometrium (Fig. 3*B*). Immunostaining for p16 and p21 was also positive in the luminal epithelium of thin endometrium (*SI Appendix*, Fig. S3*D*), suggesting that increased cellular senescence was also present in epithelial cells. Senescence denotes a cellular stress response triggered by replicative exhaustion, which can be induced by inhibition of the cell cycle (18), and the ECM plays an important role in signaling and affecting senescent cells (19). Thus, impaired cell proliferation and ECM overdeposition in epithelial cells of thin endometrium may contribute to the senescence of epithelial cells, which ultimately results in the loss of LE. Expression of *PTGS2*, a profibrotic gene reported in many studies (20–22), was highly up-regulated in the epithelium of thin endometrium, indicating the fibrotic microenvironment in this disease (Fig. 3*D* and *SI Appendix*, Fig. S3*F*). Cell cycle analysis showed that although there was cell proliferation in GE, LE, and Cili_Epi, expression of PCNA in the S and G2/M phase was significantly decreased in thin endometrium (Fig. 3 *E* and *F*). In addition, it was also notable that T cells, NK cells, and macrophages were reduced in thin endometrium as determined by scRNA-seq (Fig. 2*A*), which was further confirmed by immunofluorescence staining and flow cytometry (Fig. 3 *G*–*I*).

Altogether, these findings revealed diminished cell proliferation in both stromal and epithelial cells with increased cellular senescence and collagen overdeposition and decreased numbers of T cells, NK cells, and macrophages in thin endometrium compared to control samples.

Aberrant Cell Connections in Thin Endometrium. Cell–cell communication plays a critical role in maintaining homeostasis. How aberrant cell connections in endometrial cells lead to inadequate growth of the endometrium remains largely unknown. To dissect out the complex interactions among different cell types, we inferred all potential intercellular communications by analyzing the expression of ligand–receptor pairs using CellPhoneDB (Fig. 4 and *SI Appendix*, Fig. S5 *A* and *B*). As ranked by the number of connections between different cell types, GE and endothelial cells were the most active cell types in both normal and thin endometrium (Fig. 4 *A* and *B*), and there were marked changes in outgoing and incoming signaling in perivascular cells, lymphatic cells, peripheral blood-derived NK (pNK), and LE (*SI Appendix*, Fig. S5 *A* and *B*). In addition, stromal cells and perivascular cells expressed a number of cytokines related to cell growth in normal endometrium, such as WNT5A, WNT4, PTN, FGF2, and HGF (Fig. 4*C*).

As a "noncanonical" WNT ligand, WNT5A is suggested to exert different effects depending on its receptors, whose deficiency largely compromises embryo implantation by regulating epithelial planar cell polarity through FZDs/ROR1/2 (23). The presence of disrupted WTN5A-FZD5 signaling between stromal and epithelial cells in thin endometrium indicated impaired epithelial polarity establishment (Fig. 4*D*). The significantly reduced activity of VEGFA-FLT1, NOTCH1-WNT4, PDGFB-PDGFRB, and PDGFB-PDGFRA, which participated in angiogenesis and cell proliferation in endothelial cells, perivascular cells, and pStrs, contributed to the weakened capacity of endometrial regeneration in thin endometrium. There was also some previously unappreciated cross-talk observed between endothelial cells and epithelial cells, such as *SEMA3A-NRP1*. The major function of *SEMA3A* was reported to inhibit cell motility and angiogenesis (24); thus, the up-regulated activity of SEMAs would contribute to insufficient angiogenesis in thin endometrium (Fig. 4*D*).

Aberrant connections between endometrial immune cells (NK cells, macrophages, and T cells) and stromal cells were also observed in thin endometrium (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S5 *A* and *B*). To explore the influence of immune cells on stromal cells, supernatants of macrophages induced from THP1 cells, Jurkat cells (T cell line), and human primary uterine NK cells (uNKs) were administered to the culture medium of human primary endometrial stromal cells (hESCs). The results showed that the supernatants from macrophages and uNK cells significantly promoted the proliferation of hESCs, indicating that the loss of immune cells impaired the growth of endometrium in thin endometrium (*SI Appendix*, Fig. S5 *C*–*H*).

EGF and IHH Signaling Pathways in Endometrium Regeneration. To globally interrogate the incoming and outgoing signaling in endometrial cells, CellChat was applied to investigate cellular cross-talk among different cell types in normal endometrium (25). Cell types that had a similar enriched signaling pattern were clustered together on the left, while the detailed molecules enriched in each pattern were shown on the right (Fig. 5*A*). For incoming signaling, stromal cells and pStrs primarily accepted signals of EGFs and BMPs (Fig. 5*A*), and the primary EGF producers were GE, LE, and NK targeting stromal cells and pStrs (Fig. 5*B*). Gene expression analysis further revealed that AREG and HBEGF, which are the dominant family members of EGF, were predominantly secreted from GE, LE, pNK, and NK cells (Fig. 5*C*). Furthermore, decreased EGF downstream signaling pathways, such as ERK, AKT, mTORC1, and STAT3, were observed in thin endometrium by Western blot and immunostaining (Fig. 5 *D* and *E* and *SI Appendix*, Fig. S6*A*). To directly investigate the role of ERK, AKT, mTORC1, and STAT3 signaling on hESC growth, we treated hESCs with their corresponding inhibitors (3CAI and LY294002 for AKT, PD98059 and U0126 for ERK, PF-4708671 and rapamycin for mTOR, and cryptotanshinone and S3I for STAT3) for 48 h. The abolishment of stromal cell proliferation and the cell cycle was revealed by CCK-8 and cell cycle analysis (Fig. 5*F* and *SI Appendix*, Fig. S6 *B*–*J*) when the activation of these pathways was inhibited. Furthermore, decidualization of stromal cells was also impaired in the presence of the corresponding inhibitors (*SI Appendix*, Fig. S7 *A*–*H*), indicating the essential role of EGF downstream signaling pathways in the proliferation and decidualization of stromal cells.

Indian hedgehog (IHH) is expressed in the murine uterus and is induced by steroids to stimulate endometrial cell proliferation and differentiation via its downstream targets SMO, PTCH1, GLI1, and GLI2 (26). In our multilineage interactome analysis, epithelial cells sent strong HH signaling to pStrs in normal endometrium, indicating the important role of this signaling pathway in cell proliferation during the proliferative phase (Fig. 6 *A*–*C*). In contrast, cell proliferation and decidualization were compromised in the presence of the IHH inhibitor cyclopamine and were rescued by the administration of recombinant IHH (Fig. 6 D–F and *SI Appendix*, Figs. S7 *I* and *J* and S8 *A* and *B*). These results suggested that the EGF and IHH signaling pathways were critical for endometrial stromal cell proliferation and subsequent decidualization.

Abnormal Cellular Communication in Thin Endometrium. Although we demonstrated that IHH signals were essential for stromal proliferation, this pathway was comparable between normal and thin endometrium. To identify novel therapeutic targets, we investigated other potential interactions important for endometrial growth. In outgoing signaling, SEMA3B was transmitted from epithelial cells to its receptors NRP1 and NRP2 in stromal cells and endothelial cells (Fig. 7 *A* and *B*) but was increased in both LE and GE of thin endometrium, which was

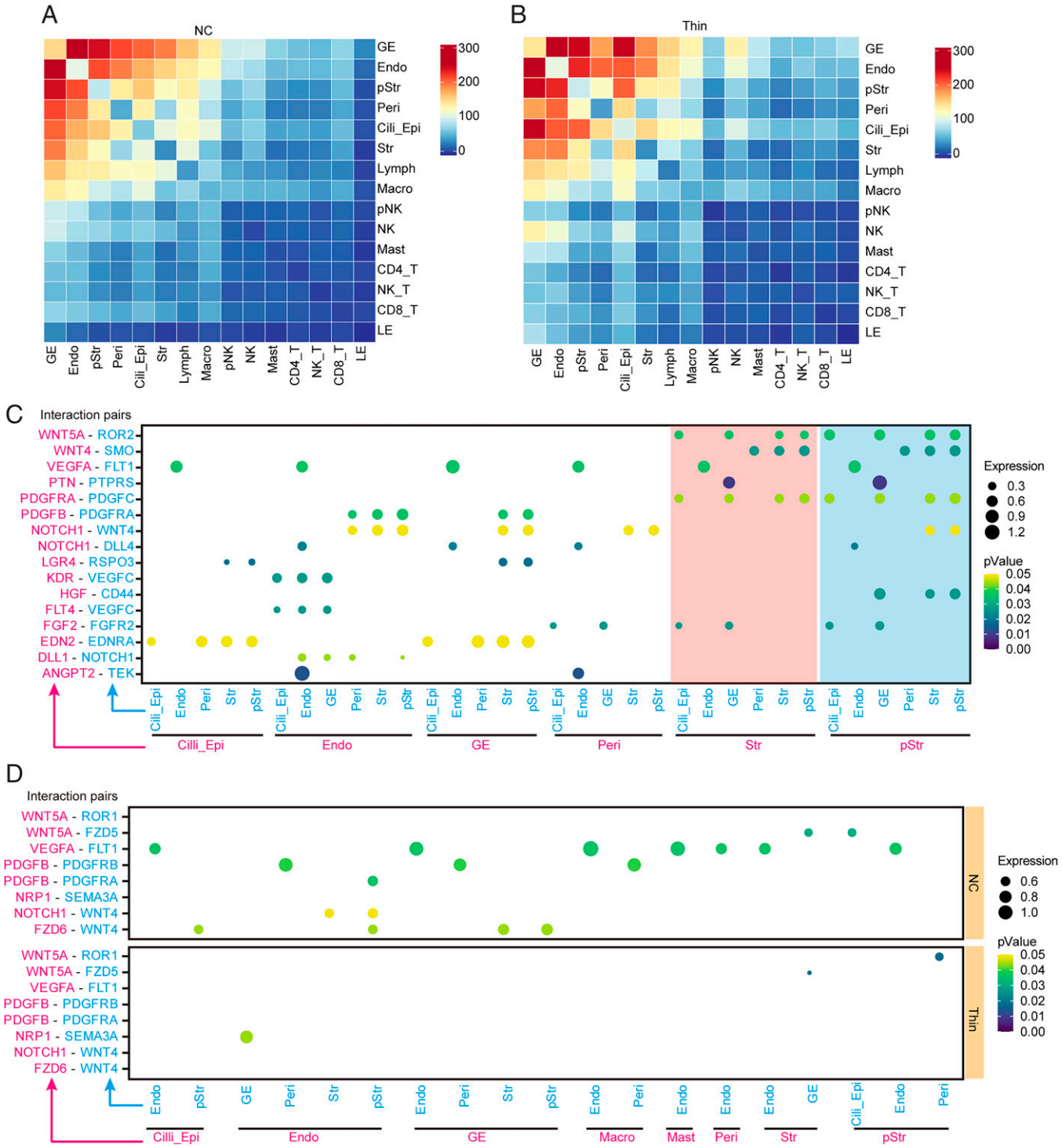

Fig. 4. Cell-to-cell connections in normal and thin endometrium. (*A* and *B*) Abundance of connections between different cell types in normal (*A*) and thin (*B*) samples shown by CellphoneDB. (*C*) Detailed connections between the indicated cells in normal endometrium. (*D*) Comparisons of dot plots for the indicated ligand–receptor interactions between normal and thin endometrium.

confirmed by immunostaining (Fig. 7 *C* and *F* and *SI Appendix*, Fig. S8*C*). As SEMA3B is an inhibitor of cell proliferation (27), our in vitro results showed that the proliferation of hESCs was indeed significantly reduced in the presence of SEMA3B (Fig. 7*G* and *SI Appendix*, Fig. S8*D*).

In addition, the pleiotrophin (PTN) signaling pathway network was only activated in normal endometrium, with its outgoing signals and receptors both primarily expressed in stromal cells (Fig. 7*E*). Consistent with the Uniform Manifold Approximation and Projection (UMAP) distribution (*SI Appendix*, Fig. S8*E*), expression of PTN was significantly reduced in thin endometrium as verified by immunohistochemical staining, Western blot, and RT-qPCR (Fig. 7*F* and *SI Appendix*, Figs. S3*E* and S8*F*). Moreover, the proliferation of hESCs was increased in the presence of PTN recombinant protein (*SI Appendix*, Fig. S8*G*). In response to PTN knockdown, hESC proliferation was significantly blunted

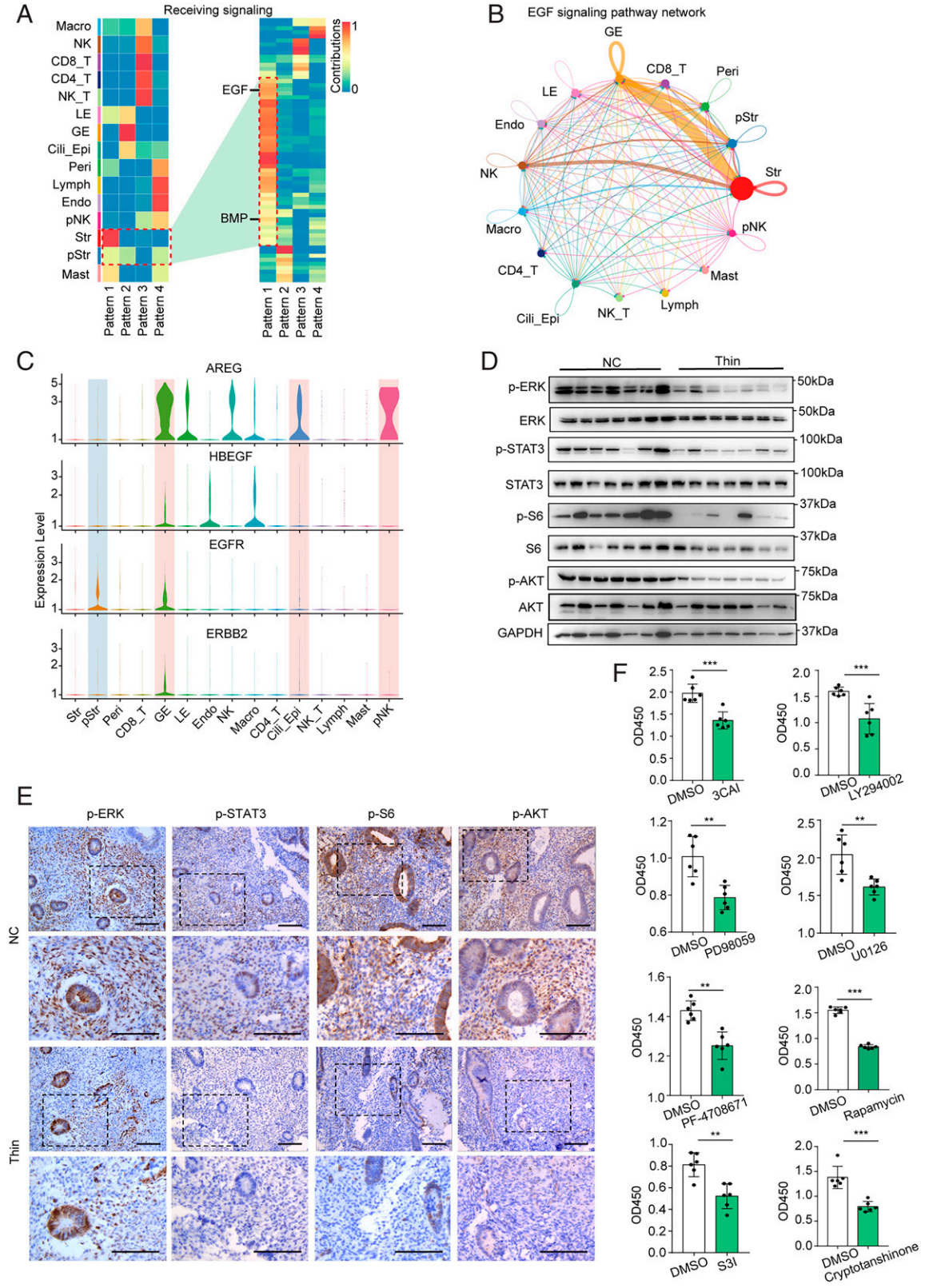

Fig. 5. Abnormal incoming signaling leads to insufficient thickness in thin endometrium. (*A*) Heatmap of incoming signal patterns of all cell types in normal endometrium shown by CellChat. (*B*) Circle plot showing the inferred EGF signaling networks among different cell types in normal endometrium. (*C*) Expression of ligand–receptor pairs of EGF signaling among each cell type in normal endometrium. (*D*) Western blot analysis of the phosphorylation of ERK, STAT3, mTOR, and AKT (p-ERK, p-STAT3, p-S6, and p-AKT) in normal and thin endometrium ($n = 7$ per group). (*E*) Immunohistochemical staining for p-ERK, p-STAT3, p-S6, and pAKT in both normal and thin endometrium ($n = 5$ per group). (Scale bars, 100 μm.) (*F*) CCK-8 analysis of hESCs after treatment with signaling inhibitors of AKT (3CAI or LY294002), ERK (PD98059 or U0126), mTOR (PF-4708671 or rapamycin), and STAT3 (cryptotanshinone or S3I) for 48 h. Data are presented as the mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$.

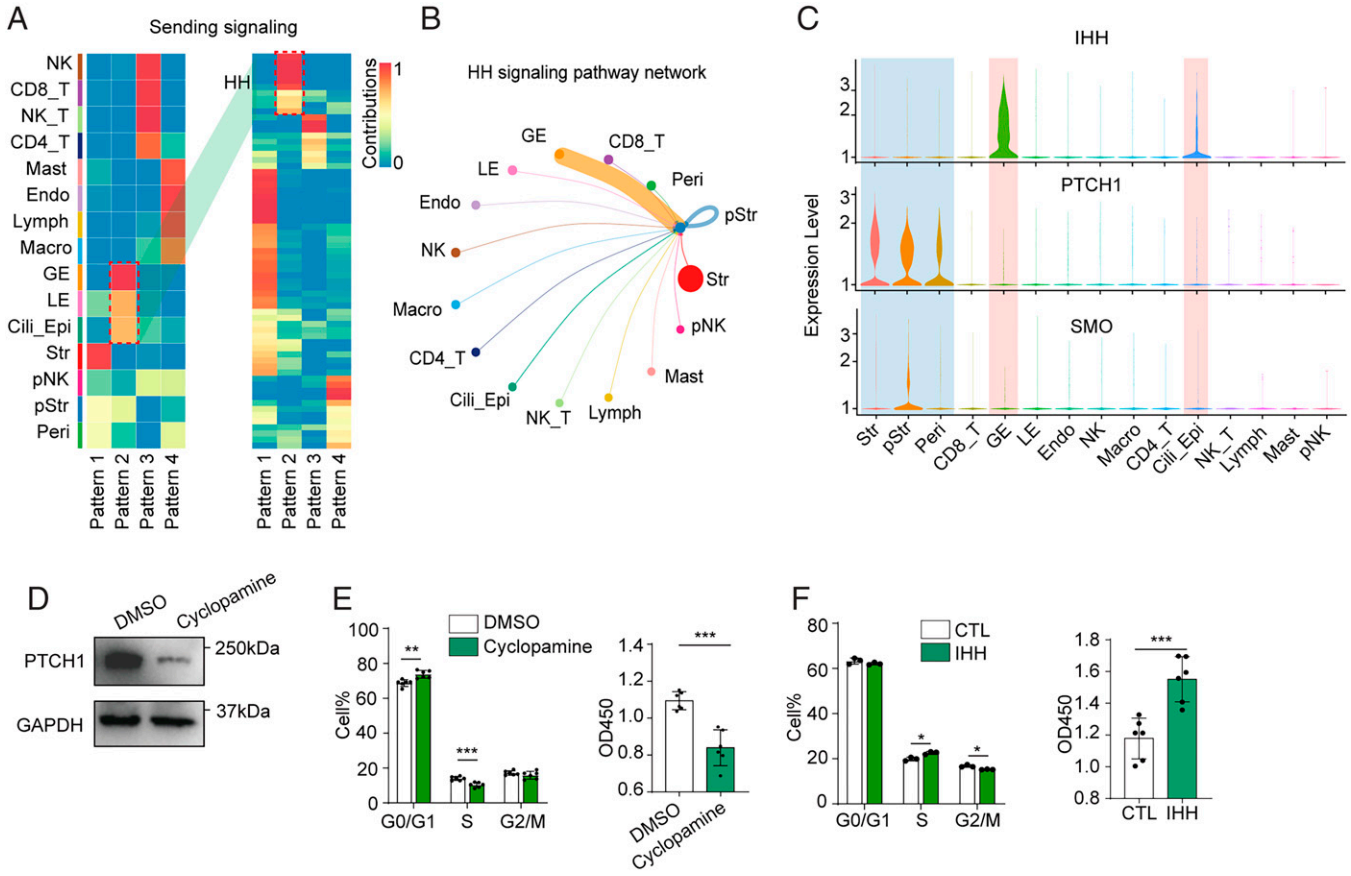

Fig. 6. The role of IHH in the growth of the endometrium. (*A*) Heatmap of the outgoing signaling patterns of all cell types in normal endometrium as shown using CellChat. (*B*) Circle plot showing the inferred HH signaling networks among different cell types in normal endometrium. (*C*) Expression of the ligand–receptor pair of HH signaling among different cell types in normal endometrium. (*D*) The effects of the signaling inhibitor cyctopamine on HH signaling were confirmed by Western blot. (*E* and *F*) Cell cycle and CCK-8 analysis of hESCs after treatment with cyclopamine (*E*) or recombinant IHH (*F*) for 48 h. Data are presented as the mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

(*SI Appendix*, Fig. S8*H*). TWEAK was another signaling pathway that was only enriched in normal endometrium (*SI Appendix*, Fig. S8*I*). Expression of TNFSF12, the primary ligand in the TWEAK signaling pathway, was reduced in thin endometrium as verified by immunohistochemical staining (*SI Appendix*, Fig. S8*J*). The proliferative ability of hESCs was significantly up-regulated after treatment with TNFSF12 recombinant protein (*SI Appendix*, Fig. S8*K*). The effect of SEMA3B, PTN, and TNFSF12 on the decidualization of stromal cells was shown in *SI Appendix*, Fig. S9. In summary, our unbiased investigation of the signaling pathways between the stroma and other types of cells highlighted that SEMA3B, PTN, and TWEAK signaling pathways were important regulators of hESC proliferation and that their aberrant expression contributed to insufficient endometrial growth during the proliferative phase.

Discussion

Thin endometrium is characterized by insufficient cell proliferation and dysfunctional cells (7). Although bulk tissue-based genomic studies have revealed some information on the molecular mechanism related to this disease (28), convincing evidence and verification of molecular biology remain largely elusive. In this study, we demonstrated and confirmed impaired EGF, PTN, and TWEAK signaling pathways and overactivation of the SEMA3B pathway, which inhibited the proliferation of stromal cells in the endometrium. Moreover, increased cellular senescence and collagen overdeposition with decreased gene expression related to cell proliferation led to defective growth of the endometrium during the proliferative phase in thin endometrium (Fig. 7*H*).

Endometrial physiology relies on a dynamic cell-to-cell dialogue between the stroma and epithelium compartments with a mixture of vascular and immune cells (11). Many studies have focused on re-epithelization and vascularization during the regeneration of the endometrium after menstruation (29), confirming that the stroma, which has the highest proportion of endometrial cells, is a feeder that orchestrates endometrial repair and growth during the proliferative phase (30). Although a recent study investigated endometrial transformation with respect to cell types, especially epithelial cells, across the menstrual cycle (14), our research highlighted fundamental understanding of the cross-talk between stromal cells and other cell types for endometrial regeneration in both normal and thin endometrium and unraveled the defective signaling pathways related to cell proliferation in stromal cells of thin endometrium. In addition, our results revealed that the stroma closely interacts with epithelia through WNT5A-FZD5, which has been reported to be critical for epithelial polarity and differentiation (31, 32).

The blood vessel wall harboring perivascular cells is considered a place that gives rise to multipotent cells (33, 34). A recent study using scRNA-seq of the mouse uterus confirmed that there were putative mesenchymal progenitor cells located in the perivascular niche of the endometrium that exhibited high proliferative potential and the capacity for multilineage differentiation (11, 35). Therefore, cellular senescence in the

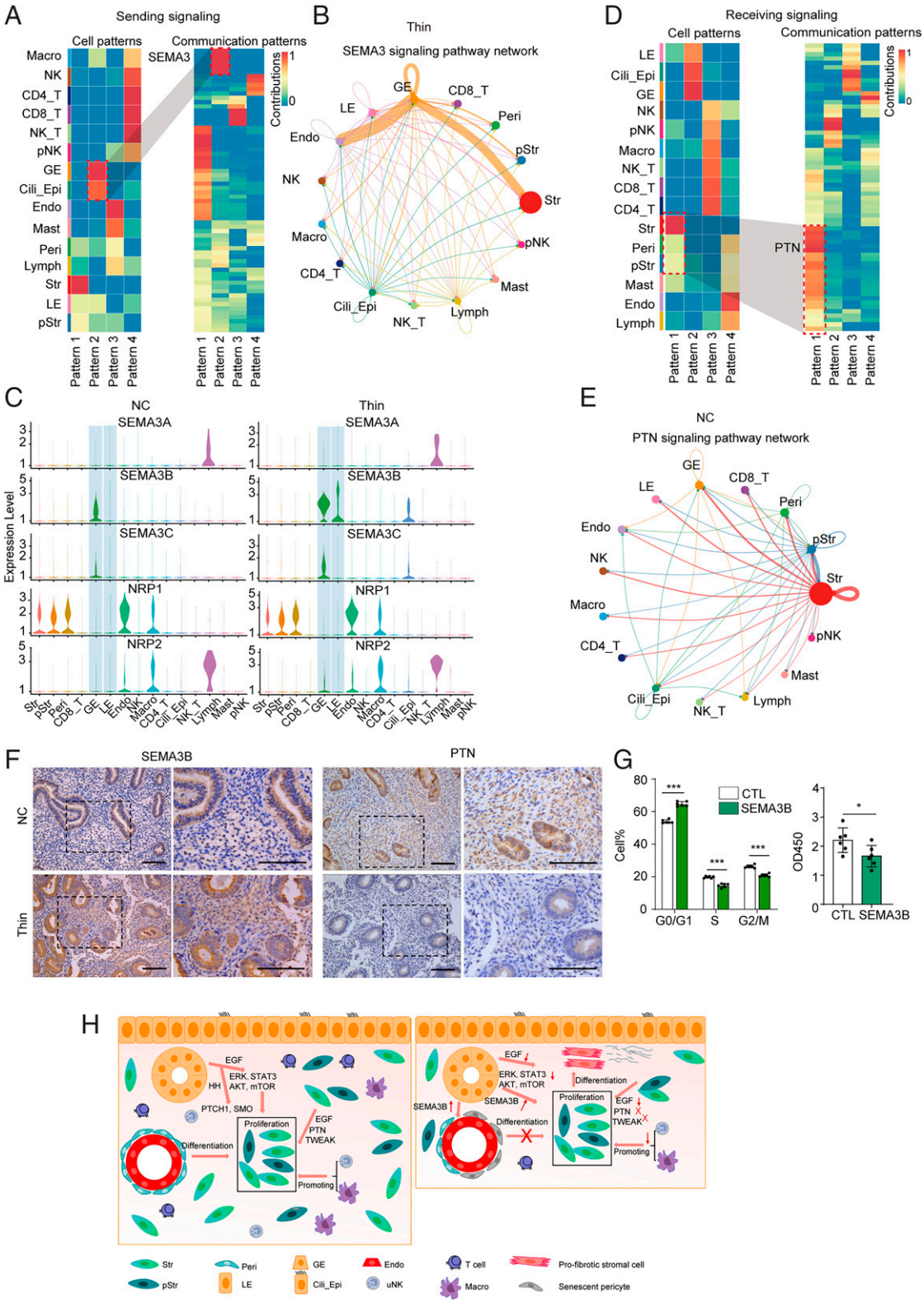

Fig. 7. Disrupted outgoing signaling contributes to insufficient thickness in thin endometrium. (*A*) Heatmap of the outgoing signaling patterns of all cell types in thin endometrium as shown using CellChat. (*B*) Abnormal SEMA3 signaling networks among each cell type in thin endometrium. (*C*) Violinplot showing expression of the ligand–receptor pair of SEMA3 signaling among different cell types in normal and thin endometrium. (*D*) Heatmap of incoming signal patterns of all cell types in normal endometrium as shown using CellChat. (*E*) Circle plot showing the inferred PTN signaling pathway network in normal endometrium. (*F*) Immunohistochemical staining for SEMA3B and PTN in normal and thin endometrium during the proliferative phase ($n = 5$ per group). (Scale bars, 100 μm.) (*G*) Cell cycle and CCK-8 analysis of hESCs after treatment with SEMA3B protein for 48 h. Data are presented as the mean ± SEM, $^{*}P < 0.05$, $^{***}P < 0.001$. (*H*) A schematic illustration showing cell type–specific genes involved in endometrial homeostasis in normal and thin endometrium.

perivascular niches of thin endometrium supported that deficient sources of cells led to inadequate growth of the endometrium. Moreover, the thickening of the basement membrane, a hallmark of microvascular aging characterized by overdeposition of ECM around the vasculature (36, 37), rendered thin endometrium more vulnerable to proliferation. However, the causal relationship between defective cell proliferation, overdeposition of ECM, and cellular senescence of the stroma in human thin endometrium remains unconfirmed. In addition, in this study, we identified a decreased number of LEs in thin endometrium. It has been reported that after menstruation, some epithelial cells may derive from the differentiation of stromal cells through epithelial–mesenchymal transition (38, 39), but whether the senescence of stroma contributes to the loss of LE in thin endometrium is unknown and deserves further exploration.

Collectively, we probed the human endometrial cell composition during the proliferative phase at single-cell resolution in both normal and thin endometrium and described detailed cell type–specific gene signatures and communications between different cell types. The mechanistic insights arising from this study could establish new avenues for intervention development targeting "promoting proliferation of stromal cells," "anti-senescence," and "anti-collagen overdeposition" to protect against insufficient growth of the endometrium and related diseases and for developing new tools to rejuvenate the atrophic endometrium for female fertility preservation and successful pregnancy.

Materials and Methods

Detailed descriptions of materials and methods used in this study, including the endometrium samples collections, isolation and culture of endometrial cells, scRNA-seq data processing and analysis, RT-qPCR, Western blot, Masson staining, immunohistochemistry, flow cytometry, and statistical analysis are presented in *SI Appendix, Materials and Methods*. This study was approved by the Scientific Research Ethics Committee of the Drum Tower Hospital (No. 2016–129-01), and informed consent was obtained from each participant.

Data Availability. All original sequence datasets have been submitted to the database of the National Center for Biotechnology Information Sequence Read Archive under accession number PRJNA730360.

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