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Uterine organoids reveal insights into epithelial specification and plasticity in development and disease

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Understanding how epithelial cells in the female reproductive tract (FRT) differentiate is crucial for reproductive health, yet the underlying mechanisms remain poorly defined. At birth, FRT epithelium is highly malleable, allowing differentiation into various epithelial types, but the regulatory pathways guiding these early cell fate decisions are unclear. Here, we use neonatal mouse endometrial organoids and assembloid coculture models to investigate how innate cellular plasticity and external mesenchymal signals influence epithelial differentiation. Our findings demonstrate that uterine epithelium undergoes marked age-dependent changes, transitioning from a highly plastic state capable of forming both monolayered and multilayered structures to a more restricted fate as development progresses. Interestingly, parallels emerge between the developmental plasticity of neonatal uterine epithelium and pathological conditions such as endometrial cancer, where similar regulatory mechanisms may reactivate, driving abnormal epithelial differentiation and tumorigenesis. These results not only deepen our understanding of early uterine development but also offer a valuable model for studying the progression of reproductive diseases and cancers.

uterus | epithelium | organoid | assembloids | development

Significance

These studies reveal pronounced age-dependent epithelial plasticity in the uterus, driven by intrinsic cellular properties and mesenchymal crosstalk. Using mouse genetic models, neonatal endometrial organoids, and assembloids, we uncover parallels between normal epithelial differentiation and pathological states, highlighting conserved pathways that govern uterine epithelial fate decisions. These findings provide insights into the developmental plasticity of the uterine epithelium and underscore the essential role of epithelial–stromal interactions in regulating uterine development and disease progression.

Epithelial differentiation within the female reproductive tract (FRT) is fundamental to reproductive health and function. The FRT originates from the Müllerian ducts (MD), which give rise to distinct epithelial types in the oviduct, uterus, cervix, and vagina (1, 2). While the specification of the cervical and vaginal epithelium into stratified squamous epithelium is governed by gene regulatory networks activated by p63 (1–4), the mechanisms that suppress p63 and specify uterine epithelial identity remain poorly understood. At birth, FRT epithelial cells exhibit developmental plasticity, marking a critical window allowing for understanding of epithelial lineage determination in both normal development and disease (5, 6). The multipotent epithelial cells can differentiate into a type that is either single-layered (SL) columnar in the oviduct and uterus or multilayered (ML) stratified squamous with basal cells in the cervix and vagina. Despite the significance of these developmental processes, early epithelial fate decisions remain largely uncharacterized, representing a significant gap in our understanding of epithelial biology within the FRT.

Epithelial differentiation is influenced by the local tissue microenvironment, where interactions between epithelial and mesenchymal/stromal cells play a pivotal role in directing tissue morphogenesis and maintaining homeostasis (7, 8). Crosstalk during development is mediated by growth factors, cytokines, and extracellular matrix components and governs the specification of epithelial subtypes (9–12). Disruptions in these interactions, whether due to genetic mutations, hormonal imbalances, or infections, can result in pathological conditions, including neoplastic transformations at epithelial transition zones, such as the squamocolumnar junction between the uterus and cervix (13–15).

Recent advances in 3D organoid cultures provide a powerful platform for modeling epithelial differentiation in vitro (16–18). Organoids, which recapitulate the architecture and functionality of their tissue of origin, enable detailed investigation of the intrinsic and extrinsic factors driving tissue morphogenesis (19, 20). While organoids derived from adult FRT tissues, including the oviduct (21, 22), uterus (12, 23, 24), cervix (13, 25), and vagina (26), have been extensively studied, the developmental potential of neonatal FRT organoids remains largely unexplored. Understanding the intrinsic gene regulatory networks as well as the inductive and instructive actions of the mesenchymal microenvironment is critical for identifying important biological mechanisms governing normal and aberrant development.

These studies integrate advanced multiomic approaches, organoid culture, and assembloid models to address a critical gap in our understanding of epithelial fate specification

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and lineage determination in the FRT. The findings reveal significant age-dependent epithelial plasticity in the uterus that is driven by intrinsic cellular properties and mesenchymal interactions. Notable parallels between normal epithelial differentiation and pathological states suggest that the mechanisms governing neonatal epithelial development may also contribute to the initiation and progression of diseases, such as uterine squamous metaplasia and endometrial cancer.

Results

Development of Neonatal FRT Organoids. Epithelial patterning and differentiation within the FRT are incomplete at birth. To investigate the intrinsic capabilities of FRT epithelial cells to differentiate into the distinct epithelial lineages observed in the adult FRT, organoids were generated from the cervix, uterus, and oviduct of postnatal day 3 (PND3) mice. Epithelial cells, isolated through enzymatic digestion, were expanded in defined WNT-activating culture media within a basement membrane extract (BME; Cultrex) using a previously described method (12, 23). Organoids from the different tissues of the FRT expanded efficiently and displayed distinct morphological differences (*SI Appendix,* Fig. S1*A*). Oviductal epithelial organoids formed spherical structures with discernible lumens, whereas cervical epithelial organoids developed as dense, lobulated structures (*SI Appendix,* Fig. S1*A*). These features align with published phenotypes of organoids derived from the corresponding mouse and human adult tissues (13, 21–26). Notably, endometrial epithelial organoids (EEO) from PND3 mice developed into two distinct populations, forming either spherical structures akin to oviductal epithelial organoids with well-defined lumens or dense lobulated structures reminiscent of cervical epithelial organoids. The developing FRT possesses a squamous stratified type of epithelium in the cervix and a simple or pseudostratified columnar type of epithelium in the oviduct and uterus. Those differences in epithelia type were mirrored in the cervical and oviductal epithelial organoids (Fig. 1*A* and *SI Appendix,* Fig. S1*A*). In contrast, the diversity of epithelial differentiation in neonatal EEOs was not observed during uterine development, supporting the idea the uterine epithelium exhibits developmental plasticity during postnatal development compared to the cervical and oviductal epithelium (Fig. 1 *A* and *B* and *SI Appendix,* Fig. S1*A*).

Organoids were then established from the uterus before (PNDs 3 and 5), during (PND7), and after (PNDs 12 and 15) uterine epithelial fate specification and lineage bifurcation (1, 27–30). The EEOs exhibited pronounced age-dependent morphological variation. Organoids from PNDs 3, 5, and 7 were a mix of both typical spherical and dense, lobulated structures, whereas organoids from PNDs 12 and 15 formed only spherical EEOs with well-defined lumens (Fig. 1*B* and *SI Appendix,* Fig. S1*B*). Spherical EEOs contained a monolayered columnar type of epithelium, typical of EEO established from adult women and mice (12, 23, 24, 31, 32), while the denser, lobulated EEOs exhibited a ML stratified type of epithelium (Fig. 1 *C* and *D*). The number of ML EEOs that developed in culture decreased with age from 12.6% (212/1,683) on PND3 to only 0.1% (11/9,289) by PND15 (Fig. 1 *E* and *F*). The formation of ML organoids was not affected by the removal of WNTs in the media, whereas SL organoids appeared smaller and collapsed (*SI Appendix,* Fig. S1*C*), like EEOs from the human fetal uterus cultured without WNT agonists (33).

Proliferating cells, assessed by Ki67, were present in both monolayered spherical and ML lobulated types of EEOs (Fig. 1*C*). The ML organoids, which were larger in diameter than their SL counterparts, contained a greater number of proliferative cells, suggesting differences in proliferation between EEO types (Fig. 1 *C* and *D*). In the ML EEOs, proliferating cells were predominantly located in the outermost layer. In the neonatal uterus, proliferating Ki67-positive cells were present in both the epithelium and mesenchyme across timepoints (Fig. 1*G*). Notably, at all developmental stages, the LE and GE exhibited a columnar type morphology in vivo (Fig. 1*G*), distinctly different from the ML stratified squamous type of epithelium observed in EEOs, demonstrating the impact of the in vivo microenvironment on epithelial development.

Cellular Heterogeneity of Differentiating EEO. To assess cellular heterogeneity and differentiation dynamics, EEOs were developed from uteri of PND3 and PND15 mice (n = 10 per PND). As expected after 8 d of growth, two distinct types of organoids had formed from PND3 uteri that were SL and ML in nature (*SI Appendix,* Fig. S2*A*). The EEOs were then analyzed via single cell RNA sequencing (scRNA-seq) using the 10X Genomics Chromium System (Fig. 2*A*). Following quality control and filtering, a total of 7,533 cells were used for downstream analysis. Seurat (34) unsupervised clustering analysis identified six distinct clusters (Fig. 2 *B* and *C*). Cell types were defined through a combination of automated clustering and manual annotation (Dataset S1) using established marker genes (27, 28, 35, 36). All clusters were verified as epithelial based on the expression of *Epcam, Cdh1, Klf5, Pax8, Krt7,* and *Krt19* (*SI Appendix,* Fig. S2*B*). As expected, mesenchymal cell populations were absent based on the lack of *Hoxa10, Hoxa11,* and *Hoxd11* expression. Clusters 0, 2, and 3 were designated as luminal epithelium (LE) identified by *Calb1, Cited4* (27), and luminal keratins *Krt8* and *Krt18* expression (Fig. 2*C*). Cluster 2 contained proliferating cells based on *Mki67* and *Top2a* expression.

Clusters 1, 4, and 5 were unique to EEOs derived from PND3 uteri (Fig. 2*B*, highlighted in circled areas of the *Right* panel). Cluster 5, characterized by *Foxj1* and *Sntn* expression, was classified as ciliated epithelia, a cell type that has not been reported in the developing mouse uterus (27, 28, 35). Clusters 1 and 4 were enriched for basal cell markers *Krt5, Krt14, Bcl11b,* and *Sox15* (Fig. 2 *C* and *D*). Abundant *Trp63* expression was an additional defining feature of Clusters 1 and 4. Of note, p63 is a key regulator of cervicovaginal epithelial differentiation in the Müllerian duct (1, 3, 4) and stemness in various other tissues (37–40) (Fig. 2 *C* and *D*). p63 directly regulates the expression of basal keratin genes (41–43) and upstream transcription factor (TF) binding analysis of differentially expressed genes (DEGs) between basal (clusters 1, 4) and luminal (clusters 0, 2, 3, 4) epithelial cells revealed significant enrichment for p63 binding sites (*SI Appendix,* Fig. S2*C*).

Immunofluorescence analyses corroborated the gene expression findings, with ML EEOs from PND3 uteri containing abundant p63, KRT5, and KRT14 protein, whereas SL organoids lacked those markers of basal cells (Fig. 2*E*). Further, EEOs derived from PND15 uteri were consistently SL and devoid of basal cell markers (Fig. 2*E*). Both the PND3 and PND15 uterus contained a single-layer columnar epithelium that lacked basal cell markers (p63, KRT5, KRT14), suggesting that basal cell differentiation occurred only during in vitro EEO development (Fig. 2*E*). scRNA-seq analysis conducted on PNDs 1, 5, 10, and 15 mouse uteri (28) identified 12 distinct clusters containing uterine epithelium (clusters 1, 2, 6), mesenchyme/stroma (clusters 0, 3, 4), myometrium (cluster 5), endothelium (cluster 7, 11), mesothelium (cluster 8), pericytes (cluster 9), and immune cells (cluster 10) (*SI Appendix,* Fig. S2*D*). This analysis found expression of luminal keratins (*Krt7*, *Krt8*, *Krt18*, *Krt19*) and luminal epithelial

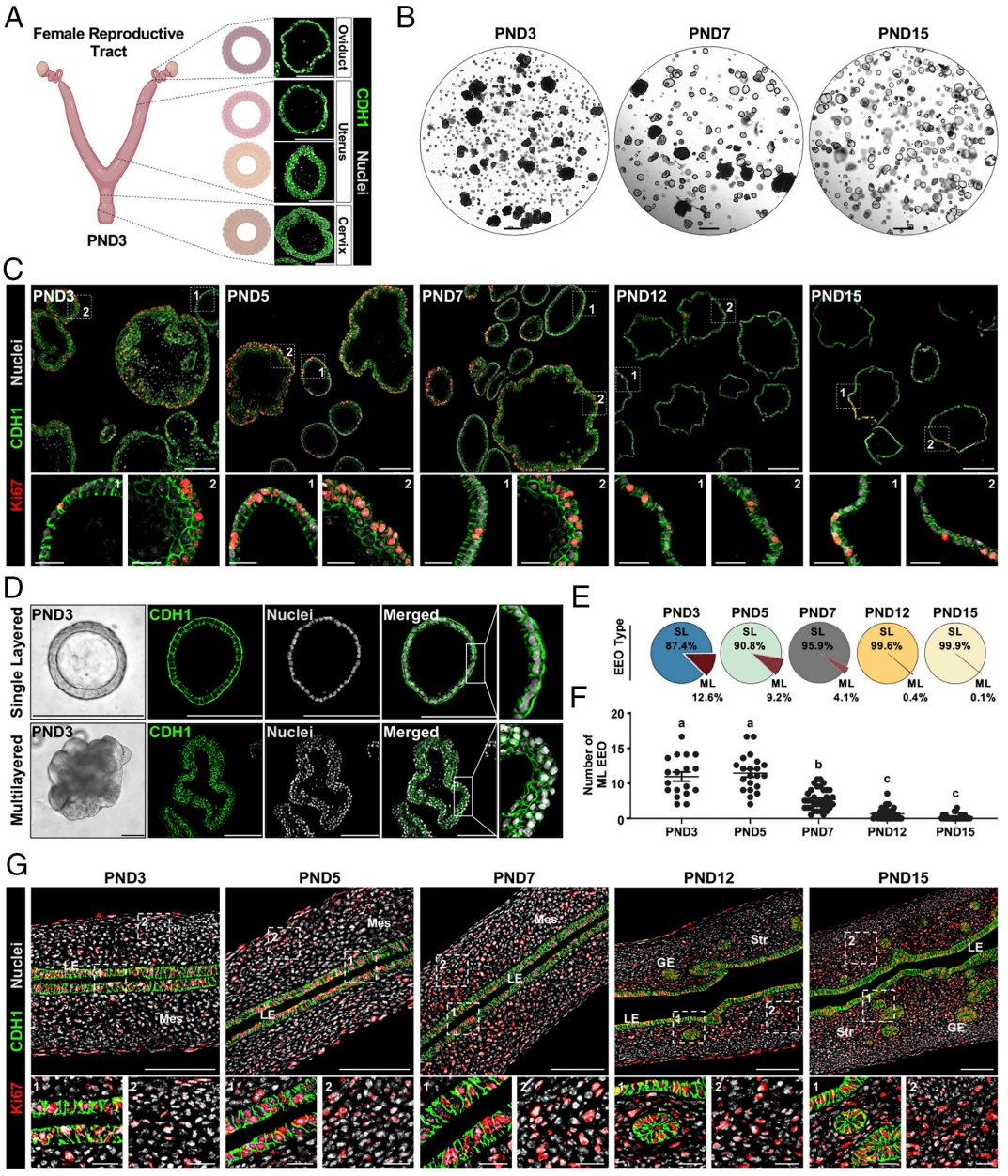

Fig. 1. Establishment and characterization of neonatal uterine organoids. (*A*) Illustration of oviductal, uterine, and cervical organoids established from postnatal day (PND) 3 female reproductive tracts (FRTs); *Right* panels: immunofluorescent localization of CDH1. (Scale bar, 100 μm.) (*B*) Representative low magnification bright-field images of endometrial epithelial organoids (EEO) established from PNDs 3, 7, and 15. (Scale bar, 100 μm.) (*C*) Immunofluorescent localization of Ki67 and CDH1 in EEO across developmental time points (Scale bar, 100 μm); insets represent single-layered (SL) (1) or multilayered (ML) (2) structures. (Scale bar, 20 μm.) (*D*) Representative images for SL and ML EEO. (Scale bar, 100 μm.) (*E* and *F*) Percentage of EEO type (*E*) and number of ML structures (*F*) across time points. All experiments were performed on passage 3 organoids (n ≥ 3 biological replicates per PND). Data are presented as mean ± SEM. Solid dots represent technical replicates (n ≥ 3 biological replicates per PND) and letters indicate statistical differences between groups ($P < 0.001$; ANOVA with the Bonferroni multiple-comparison test). (*G*) Immunofluorescent localization of Ki67 and CDH1 in neonatal uterine tissues (Scale bar, 100 μm); insets represent epithelium (1) or mesenchyme/stroma (2). (Scale bar, 20 μm.) GE, glandular epithelium; LE, luminal epithelium; Mes, mesenchyme; Str, Stroma.

genes (*Epcam*, *Cdh1*, *Wnt7a*, *Calb1*) in both EEO and uterine epithelial cells during postnatal development. In contrast, basal cell marker genes (*Trp63, Sox15, Krt5, Krt14*) were not found in the developing epithelium (*SI Appendix,* Fig. S2*E*). The absence of the basal epithelial gene signature observed in organoid cultures (Fig. 2*B*) from the developing uterine epithelium suggests that, during the first week of life, the postnatal uterus contains a small number of cells that possess developmental plasticity. While most of the undetermined and unspecified epithelia maintain their fidelity to differentiate into a simple SL columnar type of epithelium (Fig. 1 *B–E* and *SI Appendix,* Fig. S1*B*), a subset of the cells can differentiate into a ML stratified type of epithelium containing basal cells (Fig. 2 *C–E*). These observations highlight the usefulness of 3D culture systems to investigate epithelial cell fate dynamics in the uterus and highlight the critical role of the organ and tissue microenvironment in morphogenesis of the epithelium in the developing uterus.

Mesenchymal Induction of Luminal Epithelial Cell Fate in the Uterus. Interactions between the uterine epithelium and mesenchyme/stroma are crucial for epithelial morphogenesis and establishment of normal uterine histoarchitecture (7–11). A 3D coculture assembloid model was developed and used to determine the influence of mesenchymal cells on epithelial fate specification in

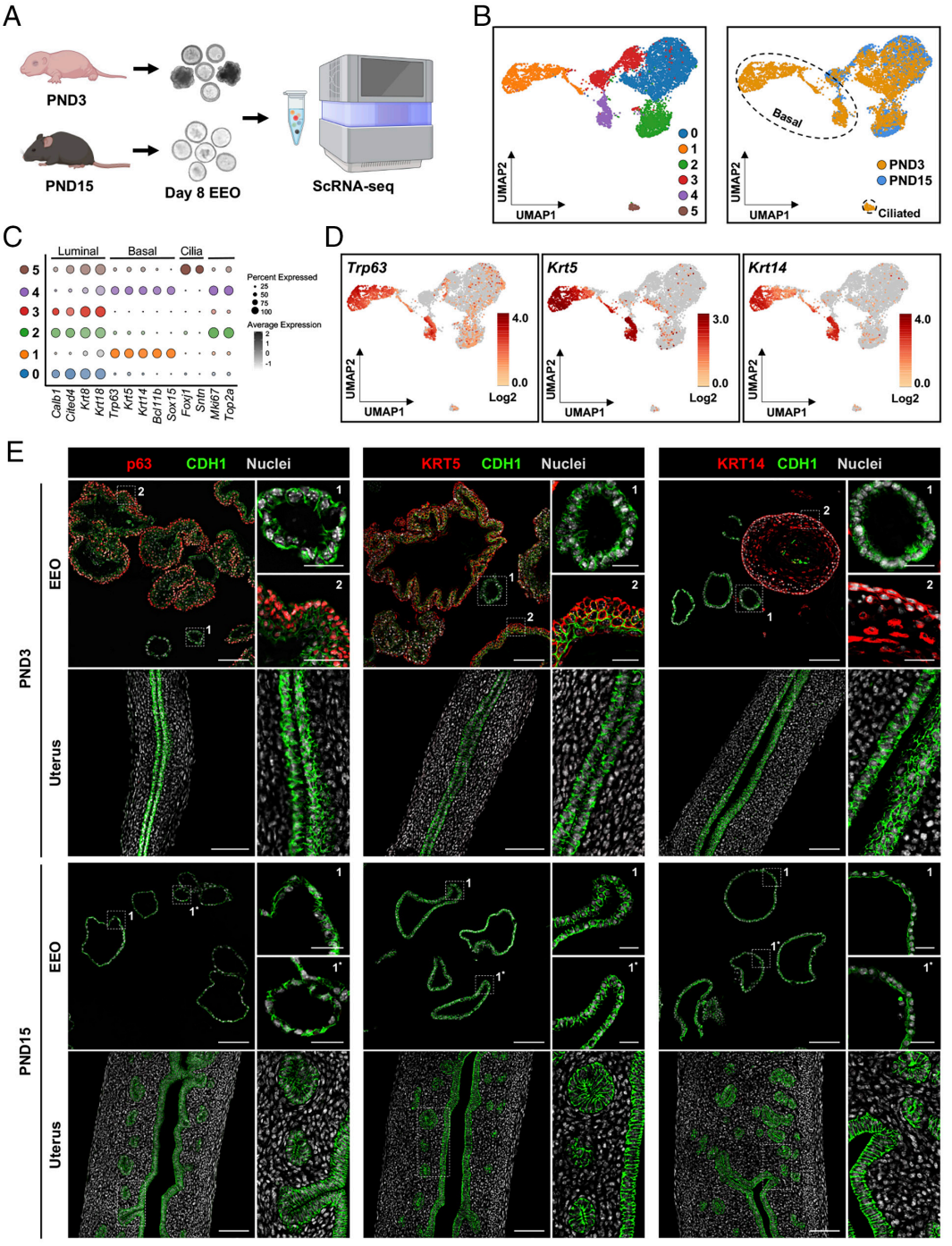

Fig. 2. Cellular heterogeneity of neonatal EEO. (*A*) Schematic illustration of experimental design. EEO established from PND 3 and 15 (n = 10 mice per PND) were collected after 8 d of culture and used for single cell RNA sequencing (scRNA-seq). (*B*) Uniform Manifold Approximation and Projection (UMAP) of cells colored by cell type (*Left*) or PND (*Right*); circled clusters represent basal and ciliated epithelium present only in the PND3 samples. (*C*) Dot plots represent the annotation of cell types based on known marker genes. (*D*) UMAPs of transcripts for the basal cell markers *Trp63*, *Krt5*, and *Krt14* in the clusters unique to PND3 EEO. (*E*) Immunofluorescent localization of p63, KRT5, and KRT14 in EEO at passage 3 or uteri of mice at PND3 (*Top*) and 15 (*Bottom*). (Scale bar, 100 μm.) Basal cell markers were only expressed in ML (2) but not in SL (1, 1*) organoids. (Scale bar, 20 μm.)

the uterus (Fig. 3*A*). Mesenchymal and epithelial cells were isolated from PND3 mouse uteri and then used for assembloid generation (*Methods*). The resulting assembloids contained an outer layer of CDH1-positive epithelium surrounding vimentin (VIM)-positive mesenchymal cells (Fig. 3*B*). Initial characterization and scRNA-seq analysis of PND3 EEOs using standard culture methods indicate the emergence of KRT5-positive cells (*SI Appendix*, Fig. S3*A*) and the formation of ML EEOs after 8 d of culture (*SI Appendix*, Fig. S2*A*). Based on these findings, assembloids were collected and analyzed after 8 d of growth.

Homotypic endometrial assembloids were established using epithelium from PND3 or PND15 uteri and mesenchyme from PND3 uteri (Fig. 3 *C* and *D*). Heterotypic assembloids were also developed using cervicovaginal mesenchyme or skin fibroblasts from PND3 female mice. Homotypic assembloids possessed an epithelium that lacked the p63 and KRT5 basal cell markers

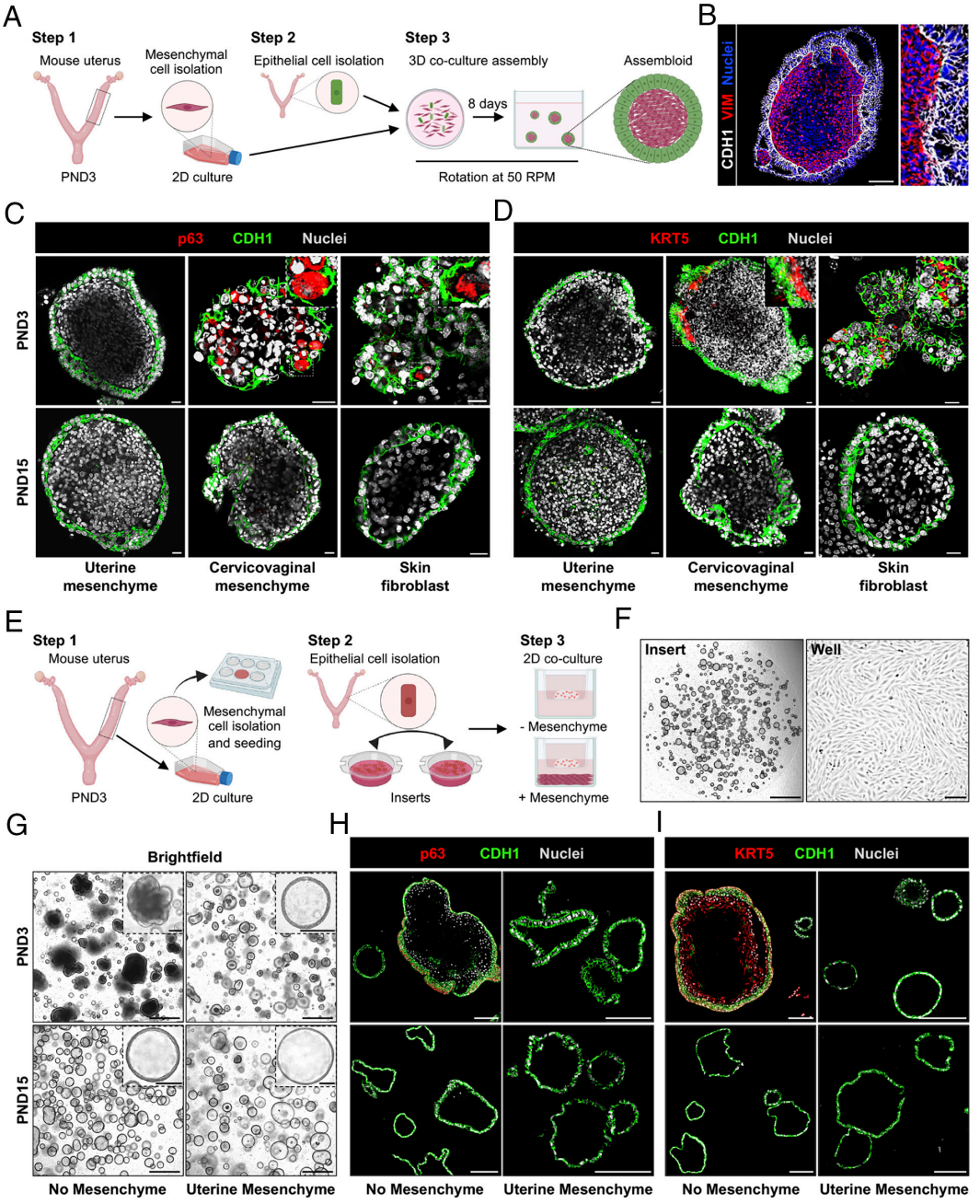

Fig. 3. Mesenchymal induction of uterine luminal epithelial fate. (*A*) Protocol for generating uterine assembloids. (*B*) Representative image of a uterine assembloid stained for epithelial (CDH1) and mesenchymal vimentin (VIM) cell markers. (Scale bar, 100 μm.) (*C* and *D*) Whole mount immunofluorescent localization for the basal cell markers p63 and KRT5 in assembloids. (Scale bar, 50 μm.) Assembloids were generated by combining uterine epithelium isolated from PND 3 (*Top*) or PND15 (*Bottom*) mice with PND3 uterine, cervicovaginal, or skin mesenchyme/fibroblast. (*E*) Experimental design for 2D coculture experiments. Epithelial cells were seeded in Cultrex on transwell inserts over PND3 uterine mesenchyme adhered to the bottom of 6-well plates and cultured for 20 d. (*F*) Representative bright-field images of EEO (*Left*; Scale bar, 500 μm) and mesenchymal cells (*Right*; Scale bar, 100 μm) in 2D coculture. (*G*) Representative bright-field images at day 20 of culture (Scale bar, 500 μm); insets show SL and ML organoids. (Scale bar, 100 μm.) (*H* and *I*) Immunofluorescent localization of p63 and KRT5 in organoids from PND3 (*Top*) and PND15 (*Bottom*) cocultures. (Scale bar, 100 μm.)

(Fig. 3 *C* and *D*). In contrast, heterotypic assembloids with PND3 uterine epithelium and either cervicovaginal mesenchyme or skin fibroblasts developed a ML epithelium with basal cells that expressed p63 and KRT5. The presence of $p63^+$ and $KRT5^+$ basal cells in the epithelium was not observed in heterotypic assembloids from PND15 uterine epithelium (Fig. 3 *C* and *D*). The percentages of luminal and basal structures in epithelial-alone controls were consistent with those observed in standard organoid culture, with approximately 0.3% of PND15 and 21.2% of PND3 EEOs exhibiting basal cells (*SI Appendix,* Fig. S3 *B* and *C*). Likewise, mesenchyme/fibroblast-only aggregates confirmed the absence of epithelial contamination, as no $CDH1^+/p63^+/KRT5^+$ cells were detected (*SI Appendix,* Fig. S3*D*).

Next, a 2D coculture system was utilized to determine whether secreted factors from the mesenchyme influence epithelial cell fate as the system precludes the physical cell–cell interactions that occur in assembloids (Fig. 3 *E* and *F*). As expected, epithelium from PND3 uteri cultured without mesenchyme generated both SL and ML EEOs. In contrast, coculture of PND3 epithelium with uterine mesenchyme yielded only SL organoids that lacked basal cells, whereas coculture with skin fibroblasts resulted in basal cell differentiation (Fig. 3 *G–I* and *SI Appendix,* Fig. S3*E*).

Of note, 2D coculture of PND3 uterine epithelium with cervicovaginal mesenchyme resulted in the development of only SL organoids that lacked basal cells (*SI Appendix,* Fig. S3*F*). However, heterotypic assembloids of PND3 uterine epithelium and cervicovaginal mesenchyme contained basal cells in the developed epithelium that were p63 and KRT5 positive (Fig. 3 *C* and *D*), suggesting that cell–cell interactions are necessary for cervicovaginal mesenchyme induction of basal cell differentiation. Notably and in line with the assembloid studies, the presence of mesenchymal cells did not affect the phenotype of EEOs generated from culture of PND15 uterine epithelium, which was SL and lacked basal cells (Fig. 3 *H* and *I*). These observations underscore the critical role of the mesenchyme in directing epithelial cell differentiation and fate through secreted factors and/or physical interactions. Further, the results support the idea that the epithelium of the neonatal uterus is not specified and exhibits developmental plasticity during the first week of life but becomes progressively restricted to uterine luminal type epithelium by the second week after birth.

Epithelial and Mesenchymal Crosstalk Governs Neonatal Uterine Development. The signaling dynamics and cell–cell interactions that regulate cell fate decisions in the neonatal uterus remain poorly understood. To gain insights into how the mesenchyme regulates epithelial cell differentiation, we performed gene set enrichment analysis (GSEA) using integrated scRNA-seq datasets encompassing PNDs 1, 3, and 5 mesenchyme or epithelium (27, 35) (GSE229790, PRJNA1046685) (Fig. 4*A*). GSEA revealed enrichment of estrogen and androgen response, cholesterol homeostasis, and p53 signaling pathways in epithelial cells, while mesenchymal cells showed enrichment for epithelial–mesenchymal transition, myogenesis, and angiogenesis hallmarks (*SI Appendix,* Fig. S4 *A* and *B*). These findings align with tissue remodeling processes necessary for uterine morphogenesis (30, 44). CellChat (45) analysis was then used to identify candidate pathways regulating cell–cell interactions in the developing uterus (Fig. 4 *A* and *B*). Autocrine and paracrine signaling pathways identified in both epithelial and mesenchymal cells included noncanonical WNT (ncWNT), canonical WNT (cWNT), FGF, PDGF, EGF, BMP, IGF, and TGFB signaling (Fig. 4*B*). Enriched pathways from the epithelium to the stroma included EGF and PDGF signaling, while mesenchymal cells to the epithelium included BMP, IGF, FGF, and TGFB pathways (Fig. 4 *C*–*E* and *SI Appendix,* Fig. S4 *C*–*G*). This mesenchymal-to-epithelial signaling was also confirmed by ligand–receptor analyses (Fig. 4*F* and *SI Appendix,* Fig. S4*C*), which highlighted *Bmp2*, *Igf1*, *Igf2*, *Tgfb2*, and *Tgfbi* ligands in the mesenchyme, and their corresponding receptors in epithelial cells, such as *Bmpr1b*, *Igf1r*, and *Tgfbr2*.

Mass spectrometry was then conducted on conditioned media from PND3 uterine mesenchyme and skin fibroblast cultures to assess the role of in silico identified pathways in our coculture system. This analysis revealed 545 (401 decreased, 144 increased) differentially secreted proteins from the uterine mesenchyme compared to skin fibroblasts, with pathways related to vascular morphogenesis, tube development, and TGFB signaling significantly enriched (Fig. 4*G* and *SI Appendix,* Fig. S4 *H* and *I* and Datasets S2 and S3). Notably, TGFBI, TGFB2, TGFB3, INHBA, and LTBP2 were identified within the TGFB pathway (*SI Appendix,* Fig. S4*I*). Integrated scRNA-seq analysis further confirmed abundant expression of TGFB pathway components in PND3 mesenchymal clusters (subset from GSE229790) (35) and their corresponding receptors in EEO (Fig. 4*H*). Together, these findings reveal a complex interplay between epithelial and mesenchymal cells, with TGFB signaling playing a central role in regulating tissue remodeling and cell fate decisions during neonatal uterine development.

Conserved Epithelial Dynamics in Uterus Development and Disease States. Dysregulation of TGFB signaling in the uterus during neonatal development leads to development of an endometrioid type of uterine cancer (46, 47). Alterations in epithelial ligands such as EGF and PDGF have been linked to cancer progression in multiple organs (48–55), suggesting that signaling pathways active during early development may also play a role in disease states. Therefore, the organoid scRNA-seq dataset (Fig. 2 *A* and *B*) was subset into luminal and basal epithelial cell type clusters (*SI Appendix,* Fig. S5*A*) and compared to the human Uterine Corpus Endometrial Carcinoma (UCEC) data from The Cancer Genome Atlas (TCGA) project (Dataset S4) (56). This analysis revealed shared gene signatures between basal type EEOs and UCEC, including upregulation of *KRT5, PITX1, S100A9, TFAP2A,* and *ALDH3B2*, and downregulation of *TNS1, TRPC1, TIMP2*, *SOCS2,* and *OLFM1* (Fig. 5*A* and *SI Appendix,* Fig. S5 *A* and *B*). Furthermore, analysis of human endometrioid endometrial cancer (EEC) scRNA-seq data (57) (PRJNA786266) confirmed dysregulation of TGFB superfamily signaling, with genes such as *TGFBI, TGFB1, TGFB3, GDF7, SMAD2, SMAD3, ACVR2A, BMP1,* and *BMP2* with decreased expression in EEC stroma compared to normal samples (Fig. 5 *B*–*D*). Similarly, the epithelial gene expression signature in EEC tissues resembled that of the basal cell clusters unique to PND3 organoids (*SI Appendix,* Fig. S5 *A* and *B*).

To explore the relevance of these findings in human disease, we examined endometrium and EEOs derived from normal and endometrial cancer patients. KRT5-positive cells were prevalent in cancerous endometrium and organoids, whereas noncancerous control endometrium and EEOs lacked the KRT5-positive basal cells (Fig. 5 *E* and *F*). These results suggest that, during cancer progression, endometrial epithelial cells differentiate and form abnormal ML epithelium with basal cells (58, 59). Indeed, KRT5-positive basal cells were found in tissues of patients with different grades of endometrioid adenocarcinoma (*SI Appendix,* Fig. S5*C*), and the patient-derived cancer organoids phenocopied the morphology of EEOs derived from PNDs 3, 5, and 7 neonatal mouse uteri.

To further interrogate whether the developmental plasticity observed in neonatal uterine epithelium extends to pathological states, we analyzed uteri and organoids from the $Pgr^{Cre/+}Pten^{f/f}$ mouse model of endometrial cancer (60–63). The uterus of PND30 mice exhibited focal areas of $KRT5^{+}$ cells, indicating the early appearance of basal-like cells in pathological endometrial hyperplasia prior to the onset of neoplasia (60, 63) (Fig. 5*G*). Epithelium from the same PND30 $Pgr^{Cre/+}Pten^{f/f}$ mice developed into a mix of KRT5-positive ML and KRT5-negative SL EEO (Fig. 5*H*). As expected, KRT5 expression was absent in the uterus and EEOs derived from PND30 control wildtype mice (Fig. 5 *G* and *H*).

Collectively, these findings suggest that mechanisms controlling cell fate specification and homeostasis during early uterine development may be co-opted during cancer progression, leading to abnormal differentiation and the emergence of basal-like cells. This transition, observed both in vivo (Fig. 5*G*) and in mouse organoid models (Figs. 1 *B*–*D*, 2*E*, and 5*H*), as well as in human biopsies and in patient-derived cancer EEOs (Fig. 5 *E* and *F*), underscores the persistence of developmental programs in cancer progression (64). These results highlight the potential role of early epithelial dynamics in shaping tumor behavior. Indeed, EEO serve as a valuable model system for investigating the cellular and

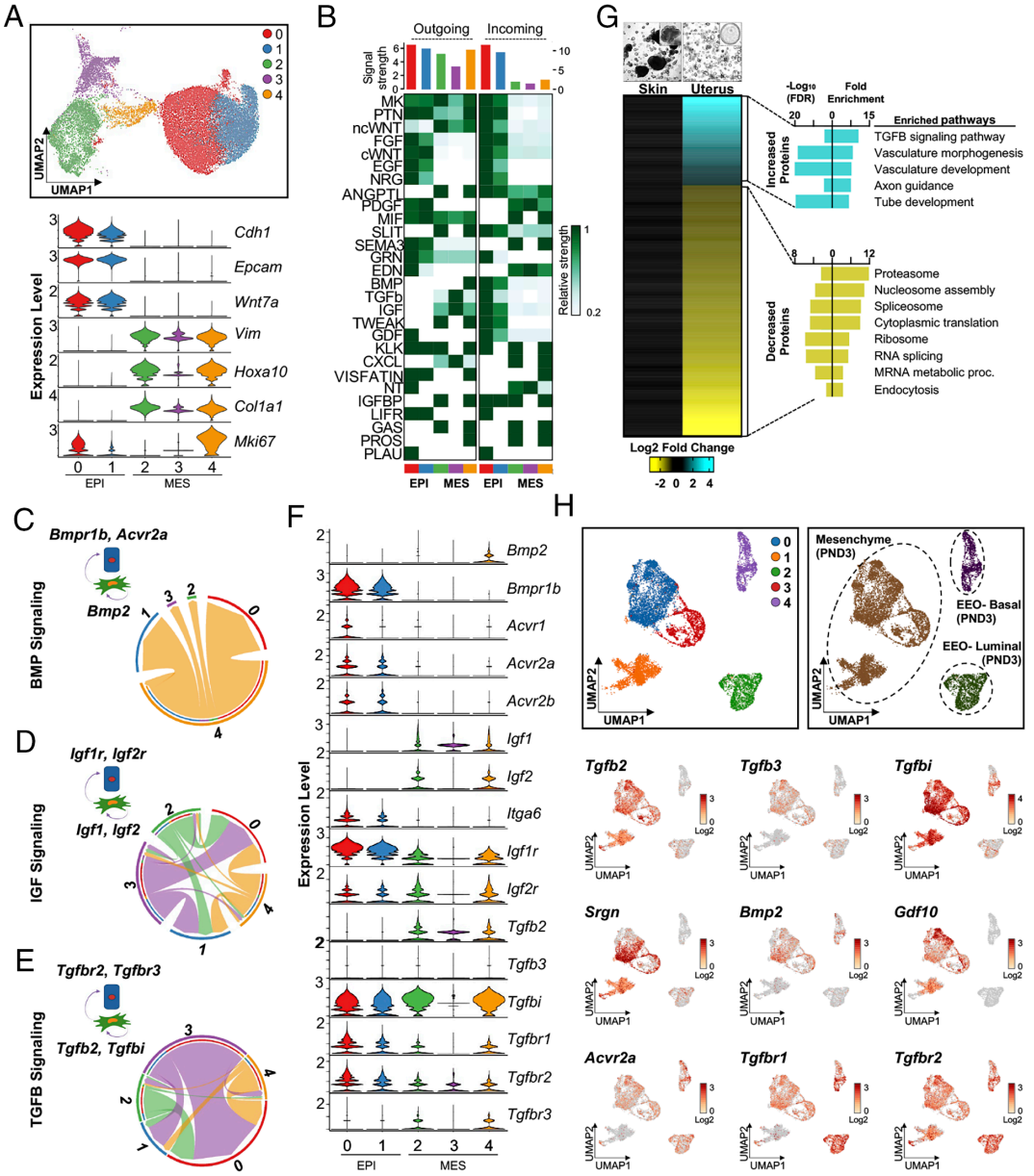

Fig. 4. Epithelial and mesenchymal crosstalk governs neonatal uterine development. (*A*) UMAP plot of integrated scRNA-seq analysis of mesenchyme (35) and epithelium (27) from neonatal uteri (*Top*). Violin plots represent annotation of cell types based on known marker genes (*Bottom*). (*B*) Heatmap comparing the outgoing (*Left*) and incoming (*Right*) signaling patterns associated with both mesenchymal and epithelial cells. Shading denotes the relative signaling strength of a pathway across cell types. Colored bar plots on top depict the signaling strength of a particular cell cluster by summarizing all pathways in the heatmap. (*C–E*) Schematic of outgoing signaling from mesenchymal to epithelial cells for selected pathways. (*F*) Violin plots confirm the expression of ligands and receptors in each cell type. (*G*) Heatmap of differentially abundant proteins determined by mass spectrometry analysis of uterine mesenchyme and skin fibroblast conditioned media. Representative bright-field images illustrating the impact of skin fibroblast and uterine mesenchyme on EEO development (*Top*). (*H*) UMAP plots of integrated scRNAseq analysis of uterine mesenchyme (35) and EEO from PND3 females colored by cluster (*Top Left*) or cell type (*Top Right*). UMAPs in the bottom confirm the expression of ligands in the mesenchyme and receptors in EEOs.

molecular mechanisms underlying this plasticity and its role in the pathogenesis of endometrial cancer (16–18).

Discussion

These studies provide insights into the development of the epithelium in the neonatal FRT, highlight the critical role of the tissue microenvironment in guiding epithelial fate specification, and show a critical period of epithelial plasticity that support foundational theories of FRT development (1, 2, 5, 7, 8). The newborn uterus is marked by a remarkable capacity for cellular differentiation that is evident in the formation of ML p63^{+}KRT5^{+} EEO, which contrasts with the absence of basal epithelial markers in vivo. The progression from development of ML, highly proliferative organoids formed from the epithelium of newborn uterus to only SL structures by PND15 suggests a progressive commitment of the epithelium toward a more defined and stable columnar luminal type fate. These observations indicate that the neonatal uterine epithelium possesses a latent (or intrinsic) plasticity, which is revealed under 3D organoid culture conditions, allowing for an expanded understanding of uterine fate potential. This phenomenon is not exclusive to the FRT as it has been described in other mesoepithelial organs, suggesting the presence of evolutionarily conserved developmental mechanisms (65–67) that have important implications in common endometrial-based diseases such as uterine squamous metaplasia and endometrial cancer.

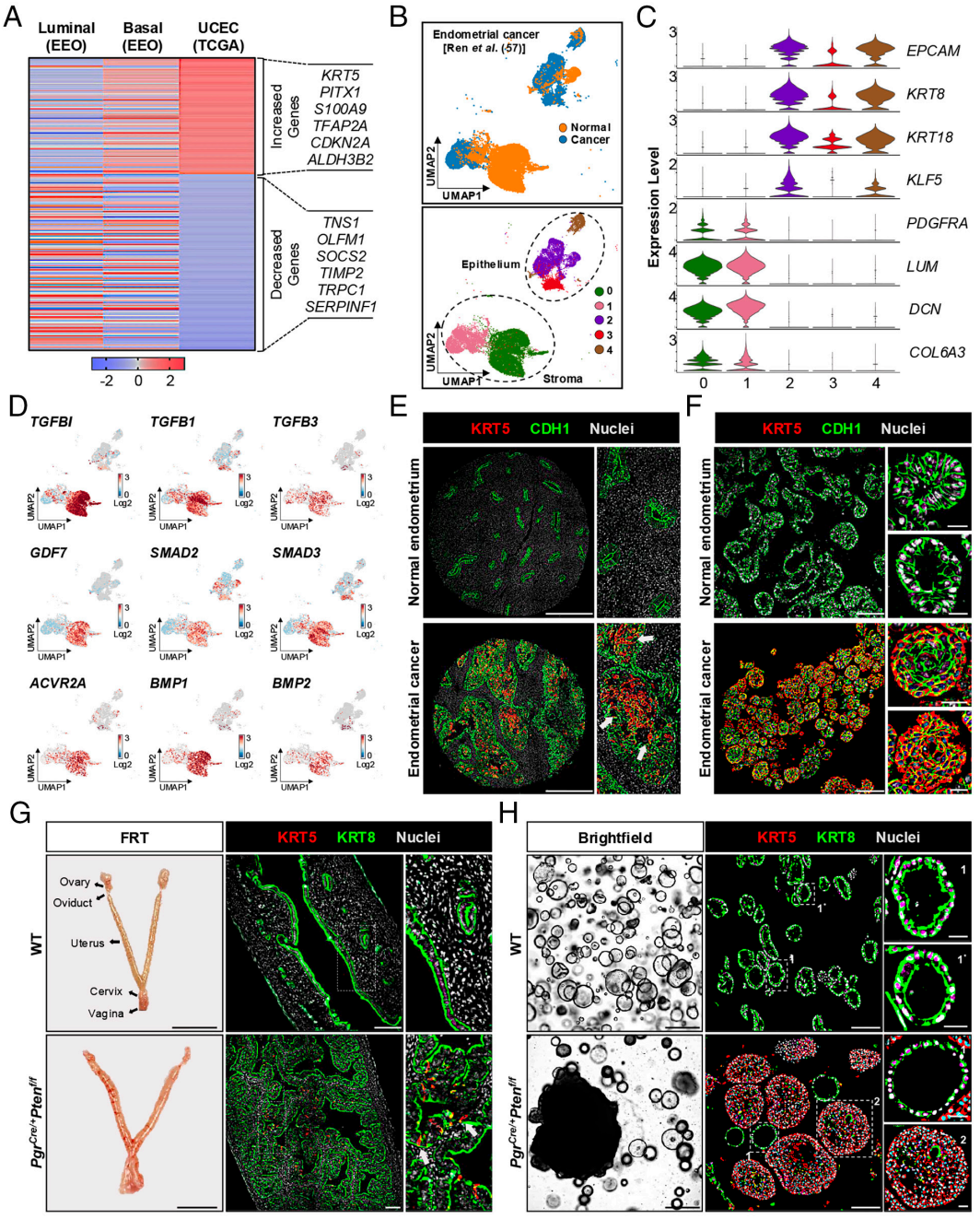

Fig. 5. Conserved epithelial dynamics between uterine development and disease. (*A*) Heatmap comparing the expression of luminal and basal EEO with human Uterine Corpus Endometrial Carcinoma (UCEC) from The Cancer Genome Atlas (TCGA) program. (*B*) UMAP of epithelial and stroma cells from human endometrial cancer (57) colored by disease state (*Top*) and cell type (*Bottom*). (*C*) Violin plots represent annotation of cell types based on known marker genes. (*D*) UMAP plots confirm the downregulation of TGFB superfamily members in the stroma of endometrial cancer samples. (*E* and *F*) Immunofluorescent localization of KRT5 in endometrial biopsies (*E*, Scale bar, 500 μm) and patient-derived endometrial organoids (*F*, Scale bar, 100 μm; insets 20 μm). (*G*) *Right* panels: uteri of wildtype (WT) and $Pgr^{Cre/+}Pten^{f/f}$mice. (Scale bar, 1 cm.) *Left* panels: immunofluorescent localization of KRT5 staining in uterine tissues. (Scale bar, 100 μm.) (*H*) *Left* panels: Representative bright-field images of EEOs established from WT and $Pgr^{Cre/+}Pten^{f/f}$mice. (Scale bar, 500 μm.) *Right* panels: KRT5 staining is present in ML (2) but absent in SL (1) EEOs (Scale bar, 100 μm; insets 20 μm). White arrows indicate KRT5-positive epithelial cells in mouse and human uterine tissues.

Histological and molecular analyses of EEOs underscore a clear age-dependent shift in epithelial differentiation in the uterus after birth, revealing rare basal cell progenitors restricted to early development when environmental cues from the mesenchyme are most influential. The identification of p63 as a marker of these progenitor cells in neonatal EEOs aligns with its established roles in regulating epithelial stemness across various organs (37–40, 68, 69). In the reproductive tract, p63 coordinates squamous epithelial differentiation in the caudal Müllerian duct, resulting in p63-positive stratified squamous cervicovaginal epithelia whereas the uterine epithelium remains a single layer of p63-negative columnar cells (3, 6, 7). Previous studies have linked the focal appearance of basal cells in the uterine epithelium to disruptions in estrogen and retinoic acid signaling during critical windows of uterine development. These disruptions, including vitamin A deficiency, prolonged estrogen exposure, or perinatal exposure to environmental estrogens, have been shown to result in aberrant epithelial differentiation within the FRT (70–72). Indeed, exposure of the newborn mouse to environmental disruptors like diethylstilbestrol, a potent estrogen receptor agonist, induces epithelial differentiation anomalies, consistent with the altered neonatal EEOs observed in the present study (14, 73). Similarly,

daughters of women exposed to diethylstilbestrol have an increased risk of developing uterine squamous metaplasia, a pathology where basal cells are present in the uterus (15). These findings indicate that certain developmental disruptions can activate abnormal basal cell differentiation programs in the uterus parallel to that observed in neonatal EEOs, highlighting the need to understand the cellular and molecular basis of latent epithelial plasticity and its implications for reproductive health and fertility in women.

The mesenchyme–epithelium coculture studies here further demonstrate the critical role of the mesenchyme and its products in directing epithelial fate specification. Uterine mesenchyme, via direct contact and secreted factors, directed and maintained a luminal epithelial fate, while heterotypic interactions fostered basal cell differentiation. Interestingly, cervicovaginal mesenchyme, but not skin fibroblasts, required cell–cell contact to induce basal cell differentiation in neonatal EEOs. These results are consistent with seminal tissue recombination studies showing that the neonatal uterine epithelium (PNDs 1 and 5) adopts a vaginal phenotype when recombined with vaginal mesenchyme, whereas this epithelial differentiation potential is lost by the second week of postnatal life (8). These findings underscore the complex nature of how the mesenchyme regulates the establishment of tissue- and organ-specific epithelial characteristics during organ development (74–76). Thus, transient cell states, possibly within a small progenitor cell population, can contribute significantly to the epithelial heterogeneity of the uterus revealed by EEO, similar to mechanisms reported in the gastrointestinal tract (39, 77), kidneys (78), bladder (38), and trachea (40, 68, 69, 79). Of note, it is possible that the inherent latent plasticity of these putative progenitor cells may be required for epithelium lineage bifurcation that occurs as the glandular epithelial cells differentiate between PNDs 5 and 10 in the developing uterus (27, 80, 81).

Tissue recombination studies determined that the mesenchyme secretes BMP4, INHBA, and FGF7/10 to activate p63 in the cervicovaginal epithelium (7–11), yet the pathways that suppress p63, direct luminal epithelial fate, and specify uterine epithelial identity remain unresolved. Integrated scRNA-seq analyses (27, 28, 35, 57) allowed for the interrogation of mesoepithelial crosstalk and highlighted the roles of pathways such as WNT, FGF, PDGF, EGF, BMP, IGF, and TGFB. Identification of specific ligand–receptor pairs, supported by mouse genetic models (82–93), provides a detailed map of the signaling crosstalk likely guiding uterine luminal epithelial specification, similar to that observed during intestinal and gastroesophageal tract regionalization (74, 94). Indeed, mesenchymal TGFB and its complex signaling regulates tissue morphogenesis in the uterus (95) and other organs (96–98).

Endometrial cancer is the most prevalent gynecologic malignancy in the United States, marked by an increase in both incidence and mortality rates in recent years, highlighting the disease's significant burden. The parallels between the developmental plasticity of neonatal uterine epithelium and pathological states, such as endometrial endometrioid cancer, suggest the conservation of epithelial dynamics across physiological and pathological contexts (65). The dysregulation of TGFB signaling, pivotal during development, becomes aberrant in the mesenchyme of cancerous tissues in both mice and humans (47, 57, 99). This disruption likely influences the tumor microenvironment, contributing to neoplastic progression and affecting epithelial homeostasis in adult tissues (90–93, 100). The alignment of development and disease phenotypes, highlighted in both mouse models and human disease tissues, suggests that the same mechanisms that govern normal epithelial differentiation in the neonatal uterus may also play a role in the emergence of endometrial disease involving abnormal epithelium differentiation. This hypothesis is consistent with reports demonstrating that fetal-like molecular programs are activated during regeneration and disease progression in the gastrointestinal tract (64, 101–103).

Taken together, our findings not only deepen our understanding of uterine epithelial differentiation but also highlight the potential of neonatal EEOs as a model system for elucidating the cellular and molecular underpinnings of tissue development and pathology. Future studies should focus on unraveling the complex biology and nature of mesenchymal regulation of cellular plasticity and epithelial differentiation as well as their contribution to homeostatic states within the uterus. Understanding these developmental pathways offers a promising avenue for developing targeted therapies addressing a spectrum of uterine pathologies (13, 58, 59, 104–107), with interventions informed by their developmental origins.

Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri, Columbia, and were conducted according to the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6 J mice were obtained from The Jackson Laboratory (stock #000664). Floxed *Pten* mice (108) (JAX #004597) were crossed with Pgr^{Cre} mice (109) to generate conditional knockout animals. Pgr^{Cre} mice were provided by Dr. Francesco DeMayo (National Institute of Environmental Health Sciences, Durham, NC) and Dr. John Lydon (Baylor College of Medicine, Houston, TX). Day of birth was considered postnatal day (PND) 0 in all studies. For all other methodological details, please see *SI Appendix*.

Data, Materials, and Software Availability. RNA-seq data have been deposited in NCBI Gene Expression Omnibus (GSE278635) (110). All study data are included in the article and/or supporting information.

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