PAX8 expressing epithelial cells are a cancer-prone source of clonal cyclical regeneration of endometrial epithelium

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Abstract

Humans and mice have cyclical regeneration of the endometrial epithelium. It is expected that such regeneration is ensured by tissue stem cells. However, their location remains debatable. A number of recent studies have suggested the presence of stem cells in the mouse endometrial epithelium. At the same time, it has been reported this tissue can be regenerated by stem cells of stromal/mesenchymal or bone marrow cell origin. Here we show that cells expressing transcription factor PAX8 are the main contributor to the homeostatic regeneration of endometrial epithelium. In the uterus, based on a single-cell transcriptome and immunostaining analyses, PAX8 expression is limited to the endometrial epithelium. According to lineage tracing, PAX8 positive (PAX8+) epithelial cells are responsible for long-term maintenance of the epithelium. Furthermore, multicolor tracing shows that individual glands are formed by clonal expansion of cells labeling both glandular and luminal epithelial components. Inactivation of tumor suppressor genes *Trp53* and *Rb1* in PAX8+ cells but not FOXJ1+ cells leads to formation of neoplasms with features of serous endometrial carcinoma, the most aggressive type of human endometrial malignancy. Taken together, our results show that a progeny of single PAX8+ cells represent the main source of cyclical regeneration of the endometrial epithelium. They also provide direct experimental genetic evidence for the key roles of the P53 and RB pathways in the pathogenesis of serous endometrial carcinoma, and suggest that PAX8+ cells represent the cell-of-origin of this neoplasm.

Introduction

During reproductive age, in both humans and mice, the endometrial epithelia and stroma undergo cell loss and regrowth in a cyclical manner in response to steroid hormones. During ovulation, there is an increase in progesterone levels resulting in differentiation of the endometrium with subsequent turnover in the absence of embryo implantation. In humans, this turnover involves endometrial shedding manifested by menstruation. In mice, the turnover is accomplished by cell apoptosis and reabsorption. In both species cyclical homeostatic regeneration of the endometrial epithelium is expected to be maintained by stem cells. However, the presence and location of such cells has been actively debated for the last several decades (reviewed in Gargett et al., 2016; Wang et al., 2019).

A number of recent studies have suggested location of stem cells in either the glandular (Kaitu'u-Lino et al., 2010) or luminal (Chan and Gargett, 2006; Huang et al., 2012; Jin, 2019) compartments of the mouse endometrial epithelium. In humans, such cells are commonly thought to be located in the basalis segment of the endometrial glands (Nguyen et al., 2012; Patterson et al., 2013; Valentijn et al., 2013). However, according to a recent single-cell transcriptome study of secretory phase human endometrium, cells showing characteristics of stem/progenitor cells are located in the upper region of the functionalis (Wu et al., 2018). At the same time, several studies suggested that endometrial epithelium can be regenerated by stem cells of stromal/mesenchymal (Huang et al., 2012; Patterson et al., 2013) or are of bone marrow cell origin (Bratincsak et al., 2007; Du and Taylor, 2007; Ikoma et al., 2009; Mints et al., 2008; Taylor, 2004).

By analogy with stem cells in other organs and tissues, aberrations in mechanisms governing endometrial epithelial stem cells may lead to a number of pathological conditions, including cancer. Uterine cancer is the 4th most frequent malignancy and the 6th cause of cancer-related deaths in women in the US (Siegel et al., 2019). While the incidence and mortality rates of some cancers, such as lung and colorectal cancers, are declining, they are both increasing for endometrial carcinoma (Felix et al., 2017; Siegel et al., 2019). Endometrioid and serous endometrial carcinomas are the most common subtypes, characterized by distinct genetic alterations, pathological phenotypes and clinical behavior. Serous endometrial carcinoma (SEC) is the second most common type of endometrial carcinoma accounting for approximately 10-15% of all cases. SEC often presents at high stage, recurs even after aggressive, adjuvant therapy, and is responsible for the majority of deaths associated with endometrial carcinoma.

Recent extensive integrated genomic analyses of endometrial carcinomas have provided important insights into the repertoire of molecular aberrations characteristic of this malignancy (Berger et al., 2018; Kandoth et al., 2013). According to TCGA data 77% of endometrioid carcinomas contain mutations in *PTEN*, but few alterations in *TP53* (1.1%) and RB pathway, such as upregulation of *CCNE1* (none), *CDKN2A* (3%), *E2F1* (2.2%), *CDK2* (none), *CDK4* (1.1%), *CDK6* (1.1%), and deletion of *RB1* (2.2%). SEC have *TP53* mutations in 95% of cases (Berger et al., 2018; Kandoth et al., 2013). Additionally, as the second most common alteration, over 70% of SEC have aberrations, in the RB pathway, involving upregulation of *CCNE1* (35%), *CDKN2A* (18%), *E2F1* (18%), *CDK2* (17%), *CDK4* (7%), and *CDK6* (12%), and deletion of *RB1* (7%). Unfortunately, utilization

of this information is compromised because the originating cell types have not been determined.

Endometrial epithelium-specific knockout of *Trp53* in Ksp1.3-Cre expressing cells results in the development of endometrial intraepithelial carcinoma (the precursor of serous carcinoma) and serous carcinoma at 14-16 months of age (Wild et al., 2012). The use of this model for studies of adult endometrial carcinoma pathogenesis is complicated by constitutive expression of Ksp1.3-Cre in the embryonic precursor structures that give rise to most of the epithelia of the adult genitourinary system.

In the current report we identify PAX8+ cells as the main contributor to homeostatic regeneration of the endometrial epithelium. We also show that neoplasms associated with conditional inactivation of *Trp53* and *Rb1* in PAX8+ endometrial epithelial cells of adult mice recapitulate human serous endometrial carcinoma.

Results and Discussion

An attempt to establish cell lineage hierarchy of the mouse uterus during development has been reported (Wu et al., 2017). Unfortunately, the study was focused only on CD326+ epithelial cells isolated by FACS and therefore did not allow identification of other epithelium-specific markers. To identify cells with expression limited to endometrial epithelium, we have analyzed single-cell sequencing data published in the Mouse Cell Atlas (Han et al., 2018). This atlas contains more than 400,000 single-cell transcriptomic profiles from 51 mouse tissues, organs, and cell cultures, including 3,761 cells from the uterus. For the uterus, in depth analysis of the data was not performed. However, two epithelial clusters were identified and annotated. One cluster was characterized by high *Ltf* expression, while the other had high expression of *Spr2f*. To improve the resolution of this data we have re-analyzed the Mouse Cell Atlas uterus dataset using our bioinformatic pipeline and a new data normalization method that better accounts for technical noise while preserving biological heterogeneity (Hafemeister and Satija, 2019). We then prepared a UMAP atlas for the main cell types of the mouse uterus and epithelial subset (Figure 1 and Figure S1). Furthermore, we performed unsupervised SNN clustering and revealed 3 subpopulations (Figure 1B). Two groups (0 and 1) had expression of *Tacstd2*, *Klf5*, *Ly6a*, *Met*, *Slc1a3*, *Wnt7a*, *Wnt7b*, *Lpar3*, *Krt13*, and *Spr2f* genes. Expression of *Itga6* was mainly localized to group 0. According to luminal-specific expression of *Tacstd2*, *Lpar3* and *Krt13*, this group was annotated as luminal epithelium group. Group 2 was characterized by high expression of *Aldh1a1*, *Foxa2*, *Lgr 4*, *Lgr5*, *Sox17*, *Axin2*, *Wif1*, *Prss29*, and *Ltf*. According to glandular-specific expression of *Foxa2* and *Prss29* it was annotated as glandular epithelium group. In sum, some markers

associated with cell stemness and fate (*Tacstd2*, *Klf5*, *Ly6a*, *Met*, *Slc1a3*, *Wnt7a*, *Wnt7b*) were detected in the luminal subpopulations while others (*Aldh1a1*, *Foxa2*, *Lgr 4*, *Lgr5*, *Sox17*, *Axin2*) were present in the glandular group. This suggests a possibility that endometrium may contain not one but 2 stem cell pools.

According to single-cell transcriptome analysis, expression of transcription factor PAX8 was largely present in the endometrial epithelium. Immunostaining has shown presence of PAX8 in luminal and glandular epithelial but not stromal cells of the endometrium (Figure 2). To further support this observation we used Tg(Pax8-rtTA2S*M2)1Koes/J (Pax8-rtTA) Tg(tetO-Cre)1Jaw/J (Tre-Cre) mice (Perets et al., 2013) crossed to *Gt(ROSA)26Sor^{tm9(CAG-tdTomato)}Hze/J* (Ai9) reporter mice (Jax Stock No 007909). In these mice *Pax8* promoter drives expression of reverse transactivating protein, rtTA, which in the presence of doxycycline binds to the tetracycline response element (TRE), thereby leading to Cre-*loxP* mediated activation of the reporter gene in Ai9 mice. In Ai9 mice expression of red fluorescent protein variant (tdTomato) under the control of CAG promoter at the *Rosa26* locus is possible only after Cre-mediated deletion of the stop codon flanked by *loxP* sites (Madisen et al., 2010). Lineage tracing, using the *Pax8-rtTA Tre-Cre* (Ai9) mice revealed that the majority of the luminal and glandular epithelium continue to express tdTomato at least for 300 days after the doxycycline pulse. These results support the notion that epithelial cells are responsible for long-term maintenance of the epithelium throughout the estrous cycle.

Using the same *Pax8-rtTA Tre-Cre* model but with Confetti mice (*Gt(ROSA)26Sor^{tm1(CAG-Brainbow2.1)}Cle/J*; Jax Stock No 013731) instead of Ai9 reporter mice, we have observed clonal expansion of cells labeling both glands and luminal epithelium

by 90 days after Cre induction (Figure 3). Recent study has reported clonal expansion of normal endometrial epithelial cells with cancer-associated mutations (Suda et al., 2018). Thus, our findings provide direct support to the previous report and previously proposed clonal expansion of endometrial epithelium. Further studies will be required to test if such expansion is a result of a neutral competition between endometrial epithelial stem cells, as has been shown for Lgr5+ cells in the small intestine (Snippert et al., 2010).

Since SEC have *TP53* mutations in 95% of cases and alterations in Rb pathway in over 70% of cases, we have conditionally inactivated *Trp53* and *Rb1* in the endometrial epithelium in composite Pax8-rtTA TRE-Cre *p53*^{loxP/loxP} *Rb*^{loxP/loxP} Ai9 mice (Figure 4). Use of these mice allows direct visualization of cells exposed to Cre expression (Figure S2). We administered a single I.P. injection of doxycycline to adult mice (6-8 weeks old) and observed formation of neoplasms starting 120 days after doxycycline administration. Histological characterization of uteri revealed invasion of malignant glands into the myometrium and serosa, marked cytologic atypia and morphological similarity to human SEC (Figure 4). Consistent with this observation, mouse neoplasms expressed markers characteristic of human SEC, such as cytoplasmic p16, reduced expression of ER, and PR, and increased number of Ki67 positive cells. No advanced lesions were observed in other tissues expressing PAX8, such as kidneys and tubal epithelium, by 300 days after doxycycline administration. Taken together, this mouse model mimics many features of the human disease and is well suited for studying serous endometrial carcinoma. To test if PAX8+ cells are preferentially susceptible to *Trp53* and *Rb1* mutations, we have inactivated these genes in FOXJ1+ ciliated endometrial epithelial cells using *Foxj1*^{tm1.1(cre/ERT2/GFP)Htg/J} (FoxJ1CreERT2::GFP) mice. According to both mRNA levels

and tdTomato expression in FoxJ1CreERT2::GFP Ai9 mice frequency of FOXJ1+ cells was about one third of that of PAX8+ glandular epithelial cells (Figure S2B and C, and Bidarimath et al., in preparation). However, none of FoxJ1CreERT2::GFP *p53^{loxP/loxP}* *Rb^{loxP/loxP}* Ai9 mice (n=14) developed any pathological lesions by 400 days after a single pulse of tamoxifen. Thus, our findings suggest that transformation of FOXJ1 ciliated cells may require other initiating genetic alterations. Indeed, it has been proposed that clear cell endometrial carcinomas may arise from ciliated endometrial cells (Cochrane et al., 2017). As compared to SEC, clear cell endometrial carcinomas have lower frequency of TP53 mutations (30-40% vs 95%) but contain other alterations, such as microsatellite instability (15% vs 5%) and PTEN mutations (30% vs 10%)(An et al., 2004; DeLair et al., 2017; Lax et al., 1998). We also cannot exclude that luminal not glandular cells represent the cell of origin of SEC.

Our current analysis of single-cell transcriptome data was based on metestrus epithelium. Further studies will evaluate cell lineage dynamics during the full estrous cycle. Our findings support existence of endometrial epithelial stem cells. However they cannot completely exclude non-epithelial contributions under non-homeostatic conditions, such as postpartum endometrial regeneration and artificial decidualization (Huang et al., 2012; Patterson et al., 2013). None of the studies reporting contributions of bone-marrow derived cells (Bratincsak et al., 2007; Du and Taylor, 2007; Ikoma et al., 2009; Mints et al., 2008; Taylor, 2004) included experiments testing for cell fusion, which is a common reason for detection of bone-marrow-specific markers in non-hematopoietic tissues. Thus, these results await further confirmation.

The main strengths of autochthonous genetically modified mouse models of cancer include precise control of initiating genetic alterations and ability to study cancer initiation and progression in an immunocompetent environment (Day et al., 2015; HogenEsch and Nikitin, 2012). Our new model of SEC confirms critical role of TP53 mutations and RB1 pathway alterations in the pathogenesis of this disease. It also offers a valuable tool for future fundamental and translational studies.

Methods

Experimental animals

The Tg(Pax8-rtTA2S*M2)1Koes/J (Pax8-rtTA) Tg(tetO-Cre)1Jaw/J (Tre-Cre) mice (Perets et al., 2013), *Foxj1*^{tm1.1(cre/ERT2/GFP)Htg/J} (FoxJ1CreERT2::GFP; Stock number 027012), *Gt(ROSA)26Sor*^{tm9(CAG-tdTomato)Hze} (*Rosa-loxp-stop-loxp*-tdTomato/Ai9) mice (Stock number 007909) and Confetti mice (*Gt(ROSA)26Sor*^{tm1(CAG-Brainbow2.1)Cle/J}; Jax Stock No 013731) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The *Trp53*^{loxP/loxP} and *Rb1*^{loxP/loxP} mice, which have *Trp53* and *Rb1* genes flanked by *loxP* alleles, respectively, were a gift from Dr. Anton Berns. All the experiments and maintenance of the mice were following with the recommendations of the Guide for the Institutional Laboratory Animal Use and Care Committee.

Doxycycline and tamoxifen induction

For lineage tracing experiments Pax8-rtTA Tre-Cre Ai9 and Pax8-rtTA Tre-Cre, *Gt(ROSA)26Sor*^{tm1(CAG-Brainbow2.1)Cle/J} 6 week-old mice received a single dose (12 μ l g⁻¹ body weight) of doxycycline (6.7 mg ml⁻¹ in sterile PBS) by intraperitoneal injection. For tumor induction experiments, doxycycline was administered to 6 to 10 week-old Pax8-rtTA, Tre-Cre, Ai9*Lgr5*^{eGFP-Ires-CreERT2}*Trp53*^{loxP/loxP} *Rb1*^{loxP/loxP} mice and control mice. For tamoxifen induction of Cre expression in FoxJ1CreERT2::GFP mice, 6 week-old mice received a single dose (8 μ l g⁻¹ body weight) of tamoxifen (25 mg ml⁻¹ in corn oil, Sigma-Aldrich, St. Louis, MO, USA, T5648) by intraperitoneal injection. All mice were euthanized by CO₂ and further analyses were carried out.

Histology, immunohistochemistry and image analysis

All tissues were fixed in buffered 4% paraformaldehyde overnight at 4°C followed by standard tissue processing and paraffin embedding. Histology and immunohistochemistry stainings were carried out on 4 µm-thick tissue sections. For immunohistochemistry, antigen retrieval was performed by incubation of deparaffinized and rehydrated tissue sections in boiling 10 mM sodium citrate buffer (pH 6.0) for 10 minutes. The primary antibodies against ER, FOXA2, Ki67, P16, PAX8, PR, tdTomato/RFP, TP53 and TROP2 were incubated at 4°C for overnight, followed by incubation with secondary biotinylated antibodies (30 minutes, at room temperature, RT). Modified Elite avidin-biotin peroxidase (ABC) technique (Vector Laboratories, Burlingame, CA, USA; pk-6100) was performed at RT for 30 minutes. Hematoxylin was used as the counterstain. All primary antibodies used for immunostaining are listed in Supplementary Table 1.

Single cell transcriptome analysis

The Mouse Cell Atlas (Han et al., 2018) digital expression matrices for uterus (Uterus1 GEO: GSM2906478 and Uterus2 GEO: GSM2906479) and related cell type metadata were downloaded (GEO: GSE108097) and reanalyzed using a custom version of Seurat version 3.1.0 R package (Stuart et al., 2019). Seurat was used for data integration and normalization, principal component reduction, shared nearest neighbor (SNN) clustering, UMAP visualization, and differential gene expression analysis. We combined the Uterus1 and Uterus2 datasets and normalized the data using a regularized negative binomial regression model (sctransform R package) in order to remove technical effects while preserving biological variability (Hafemeister and Satija, 2019). On the normalized data,

we performed SSN clustering with a resolution of 0.4 and we visualized the data using uniform manifold approximation and projection (UMAP) (Becht et al., 2018). Differential expression analysis was performed using Seurat's "FindAllMarkers" function using a wilcoxon signed-rank test and only considering genes with $>\log_2(0.25)$ fold-change and expressed in at least 25% of cells in the cluster.

Patient materials

All human specimens were de-identified. They were not collected specifically for the purpose of this research.

Statistical analyses

Statistical comparisons were performed using a two-tailed unpaired t test and a Chi square test with InStat 3 and Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Survival curves were computed using the Kaplan-Meier method and the survival comparisons were analyzed by log-rank tests. Significance was determined as $P < 0.05$.

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Author contributions

AFN and AYN designed experiments, DJF, AFN, and MB performed experiments, AJDM and BDC carried out bioinformatics analyses, LHE and AYN performed pathological evaluations, LHE and BDC participated in conceptualization and editing of the manuscript, AFN and AYN wrote the paper.

Conflict of interest

All authors declare no competing interests or conflicts of interest.

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Figure Legends

Fig. 1. Single-cell transcriptome analysis of the mouse uterus. (A) UMAP projection of single-cell transcriptomic data generated from the mouse uterus and highlighting the main cell types as annotated by the Mouse Cell Atlas. (B) UMAP projection of Luminal (LE) and Glandular (GE) epithelial populations labeled by group as identified by unsupervised SNN clustering. (C) Heatmap representing the top 55 differentially expressed genes across the 3 subpopulations identified in (B) and independent from prior labels. (D-G) Expression of genes in luminal and glandular clusters. (F and G. right panels) immunostaining for TROP2 encoded by *Tacstd2* (F) and FOXA2 (G). ABC Elite. Hematoxylin counterstaining. Scale bar 200 μ m.

Fig. 2. Characterization of PAX8⁺ cells. (A) Average normalized PAX8 mRNA expression across uterine cells following the Mouse Cell Atlas annotation (Fig. 1A). (B) Normalized PAX8 mRNA expression in luminal (LE) and glandular (GE) epithelial cells. (C) Transgenic mouse design for lineage tracing of PAX8⁺ cells. Dox, doxycycline. (D)

Immunohistochemical detection of PAX8 expression in both luminal (LE) and glandular (GL) epithelial cells. Hematoxylin counterstaining. ABC Elite. (E) Lineage tracing of PAX8+ cells in mouse endometrium collected 300 days after a single doxycycline pulse. tdTomato is expressed in both LE and GE cells. DAPI counterstaining, blue. (D and E). Scale bar 200 μ m.

Fig. 3. Clonal expansion of PAX8 cells in Confetti mice. (A) Experimental design (B-D) Detection of nGFP (green), RFP (red) and YFP (yellow) 3 (B) and 90 (C and D) days after administration of doxycycline (Dox). Note that a subset of PAX8+ cells has the ability to proliferate resulting in large monochromatic clones. Counterstaining with DAPI, blue (B, C) and grey (D). Confocal microscopy. Scale bar 100 μ m (B and C) and 800 μ m (D).

Figure 4. Mouse model of endometrial serous carcinoma. (A) Mouse model design.

(B) Survival of Pax8-rtTA TRE-Cre $p53^{loxP/loxP}$ $Rb^{loxP/loxP}$ Ai9 mice that were treated with

doxycycline (n=14) or vehicle (n=5). (log-rank $P=0.0193$). (C-F) Mouse tissues. (C-E) Glandular pattern with marked cytologic atypia (D, arrow) and invasion into myometrium and perimetrium (E, arrow). Arrow and arrowhead in (C) indicate location of (D) and (E), respectively. (F) Detection of proliferation marker Ki67, p16, ER and PR in tumor (top row) and normal tissue (bottom row). Arrowheads, Ki67 positive cell and cytoplasmic staining of p16. (G) Human serous endometrial carcinoma. Glandular pattern and marked cytologic atypia (inset, arrow; HE), intense, diffuse staining of p53, Ki67 positive cells, lack of expression of ER, strong, diffuse staining of p16 and lack of PR expression. Arrowheads, normal cells with expression of ER and PR near tumor. (HE) Hematoxylin and eosin. Other images, ABC Elite method, hematoxylin counterstaining. Scale bar (C-D): 600 μm (B), 100 μm (D and E), (F, G) 60 μm (F) and 200 μm (G).