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Estrogen and Progesterone Together Expand Murine Endometrial Epithelial Progenitor Cells

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ABSTRACT

Synchronous with massive shifts in reproductive hormones, the uterus and its lining the endometrium expand to accommodate a growing fetus during pregnancy. In the absence of an embryo the endometrium, composed of epithelium and stroma, undergoes numerous hormonally regulated cycles of breakdown and regeneration. The hormonally mediated regenerative capacity of the endometrium suggests that signals that govern the growth of endometrial progenitors must be regulated by estrogen and progesterone. Here we report an antigenic profile for isolation of mouse endometrial epithelial progenitors. These cells are $EpCAM^{+}CD44^{+}ITGA6^{hi}Thy1^{-}PECAM1^{-}PTPRC^{-}Ter119^{-}$, comprise a minor subpopulation of total endometrial epithelia and possess a gene expression profile that is unique and different from other cells of the endometrium. The epithelial progenitors of the endometrium could regenerate in vivo, undergo multi-lineage differentiation and proliferate. We show that the number of endometrial epithelial progenitors is regulated by reproductive hormones. Co-administration of estrogen and progesterone dramatically expanded the endometrial epithelial progenitor cell pool. This effect was not observed when estrogen or progesterone was administered alone. Despite the remarkable sensitivity to hormonal signals, endometrial epithelial progenitors do not express estrogen or progesterone receptors. Therefore their hormonal regulation must be mediated through paracrine signals resulting from binding of steroid hormones to the progenitor cell niche. Discovery of signaling defects in endometrial epithelial progenitors or their niche can lead to development of better therapies in diseases of the endometrium.

INTRODUCTION

Hormonally regulated diseases of the endometrium are common. Endometriosis, a

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benign chronic condition characterized by endometrial growth outside of the uterus, affects up to 10% of reproductive age women causing pain, infertility and tumors [1]. Endometrial carcinoma, the leading gynecologic cancer in developed countries [2], results from overgrowth of endometrial epithelium. Studies dating back to the 1970s hypothesized the existence of uterine epithelia with stem activity that could regenerate the endometrial epithelial lining in response to estrogen and progesterone [3]. These cells were hypothesized to be a precursor for endometrial cancer when exposed to imbalances of the steroid hormones [3].

The evidence for existence of adult endometrial epithelial stem cells has been indirect and debated [4-6]. Subsets of short-lived label retaining cells (LRC) were identified in the mouse endometrium suggesting that endometrial cells have varying proliferative capacity [7, 8]. Only 0.2% of human endometrial epithelia formed colonies in a 2-dimensional assay suggesting that not all endometrial epithelia have equal regenerative potential [9]. Subpopulations of Hoechst dye-excluding human endometrial cells, composed of mixtures of stroma and epithelium, formed endometrial glands in vivo [10] and colonies in vitro [11]. These data support the presence of an adult progenitor cell pool in the endometrium but do not provide direct evidence for their existence. A possible extra-uterine endometrial precursor has been proposed based on identification of bone marrow derived cells in the endometrium of transplantation recipients [12, 13]. While cells from the bone marrow may contribute to the endometrial cell pool, direct evidence demonstrating stem-like activity in the epithelium derived from these cells is lacking.

There are similarities and differences between the mouse and human endometrium. In both species during reproductive years the stroma and epithelia undergo cell loss and re-growth in a cyclic manner in response to steroid hormones [7, 14]. Ovulation induces a rise in progesterone, and in the absence of implantation progesterone levels decline resulting in endometrial turn over. Humans undergo endometrial shedding while in mice apoptosis and re-absorption of the endometrium occurs [15, 16]. To support cyclic endometrial regeneration, a residual pool of progenitors must be maintained in both species.

Using an *in vivo* transplantation and regeneration assay established by our group, here we provide evidence for the existence of an adult mouse endometrial epithelial progenitor population. The total number of these epithelial progenitors fluctuated in response to two reproductive hormones: estrogen and progesterone. While sensitive to hormonal signals, endometrial epithelial progenitors did not express estrogen receptor α (ERα) or the progesterone receptor isoforms (PRA and PRB) suggesting that estrogen and progesterone regulate the total number of these cells via paracrine signals.

MATERIALS AND METHODS

Animals. WT C57BL/6, β-actin green fluorescent protein (C57BL/6-Tg[ACTbEGFP]1Osb), β-actin DsRed [C57BL/6-Tg(ACTB-DsRed.MST)1Nagy/J], and CB17Scid/Scid mice were from Jackson Laboratory. Mice were maintained in accordance with University of California Los Angeles (UCLA), Division of Laboratory Animal Medicine guidelines. All animal experiments were approved by the UCLA Animal Research Committee.

Preparation of dissociated uteri. Mouse uteri were dissected, cut into fragments, washed in 5 mM EDTA, and incubated in 1% trypsin for 45min at 4°C. Trypsinization was stopped with DMEM/10% FBS. Luminal epithelia was separated from underlying stroma with a fine forceps and saved. Remaining uterine tissue was minced. Both uterine fractions (luminal epithelium and minced uterine fragments) were digested with 0.8 mg/ml collagenase in DMEM/10% FBS with 5 μg/ml insulin and 0.5 mg/ml DNase for 1.5h at 37C. Digested fractions

were passed through 22-gauge syringes, filtered through 40 μm cell strainers and recombined.

Human endometrial specimens from hysterectomies performed for benign indications were obtained through UCLA IRB approved protocols. The endometrial lining was isolated from specimens by scraping the endometrium and some of the underlying myometrium. Isolated tissue was minced and digested with 0.8 mg/ml collagenase in DMEM/10% FBS with 5 μg/ml insulin and 0.5 mg/ml DNase for 1.5h at 37C. Digested tissue was washed with 1X PBS, then incubated 5 minutes in pre-warmed 0.05% trypsin-EDTA and passed 5 times through a 20 gauge needle. After trypsin was neutralized with DMEM/10% FBS, cells were passed through a 40 μm cell strainer yielding single cells.

Endometrial Regenerations. Endometrial regeneration was performed as described [17]. FACS isolated epithelia and cultured neonatal stroma were mixed and re-suspended in collagen (BDBiosciences; 354236) and dispensed into grafts. Endometrial grafts were implanted under the kidney capsule of oophorectomized CB17Scid/Scid mice and regenerated for 8 weeks with an estrogen pellet (0.72-mg β-estradiol/pellet). A progesterone pulse (2.5 mg/d) was administered for the final 8 days of regeneration.

For serial transplantation, regenerated primary endometrial grafts were minced and digested into single cells. Dissociated total and DsRed single cells from the primary grafts were counted, combined with WT neonatal stroma and re-implanted sub-renally as a secondary graft. Similarly cells harvested from secondary grafts were used to generate tertiary grafts.

For limiting dilution experiments, enumerated sorted DsRed epithelia were placed in 10-fold serial dilutions in the regeneration assay. Regeneration, defined as the formation of pankeratin or DsRed positive glandular structures, was scored for each graft and analyzed using extreme limiting dilution (http://bioinf.wehi.edu.au/software/elda/ [18]).

Immunohistochemistry. Frozen or formalin-fixed and paraffin-embedded tissue was stained with antibodies (Supporting Information Table 1). To quantify expression of antigens, 5-10 high-power fields of view were scored per sample and averaged.

FACS Fractionation. Endometrial cells were suspended in DMEM/5% FBS and stained with antibody (Supporting Information Table 2). For cell cycle analysis, cells were incubated with 20 uM Hoecsht 33342 at 37C for 30min, then washed and stained with cell surface antibodies. Intracellular FACS Ki67 staining was performed using the FITC-Ki67 kit from BDBiosciences (BD 556026).

Hormone ablation and supplementation in experimental mice. Hormone deprivation was achieved by surgical removal of ovaries followed by a 3-4 week rest period. For hormone supplementation, hormone pellets (90-d time-release, 0.72-mg β-estradiol/pellet; 60-d time-release, 100-mg progesterone/pellet; Innovative Research of America) were implanted subcutaneously for 2-3 weeks. Serum plasma hormone levels were measured using estradiol and progesterone EIA kits (Cayman Chemical).

Quantitative PCR. Quantitative PCR was performed as described previously [17]. Primers are outlined in Supporting Information Table 3.

Western Blot. Isolated cell fractions were lysed and equal amounts of protein were fractionated on SDS-PAGE gels and transferred to nitrocellulose membrane. Blocked blots were incubated overnight at 4C in primary antibody and 1h at RT in secondary antibody (Supporting Information Table 4).

RNA Seq Analysi. Uterine tissue from three separate groups of mice was FACS sorted to yield biologic triplicates for EEPC, non-EEPC and stromal cell fractions. RNA was isolated

using the Qiagen RNAmicro kit, and RNA integrity was confirmed on an Agilent 2100 Bioanalyzer. Index-tagged cDNA libraries were generated from 250ng RNA samples using the TruSeq RNA Sample Preparation kit (Illumina). Index-tagged libraries were pooled and sequenced at three per lane on an Illumina HiSeq 2000 to generate single-end 50-bp reads. RNA-seq reads were mapped against the mouse genome using TopHat allowing only unique reads with up to 2 mismatches [19], using the Ensembl annotation as a guide. Gene expression values were calculated with HTSeq, and adjusted p-values for differential expression between groups were calculated with DESeq. Gene expression was compared using RPKM values [19].

For hierarchal clustering, the gene expression set was first filtered. Genes with RPKM values <1 were eliminated from the analysis set, and gene expression differences of greater than 2-fold between EEPC and non-EEPC with adjusted p-values <0.05 were selected. Clustering was performed using Cluster3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Genes were centered and normalized, and average linkage clustering was performed. Resulting heat maps were visualized using JavaTreeview (http://jtreeview.sourceforge.net/). Gene clusters were analyzed using the DAVID Bioinformatics resource [20, 21]. Plots of RNA seq reads were examined using the UCSC genome browser (http://genome.ucsc.edu [22]).

In vitro Cell Growth Assays. To culture mouse and human endometrial cells in 3-dimensional assays, FACS isolated single cell epithelial preparations were suspended in equal volumes of PrEGM media (Lonza) and Matrigel (BD Biosciences). Suspensions were plated around the rim of 12-well tissue culture plate, allowed to solidify, and then overlaid with warm PrEGM. Hollow spheres were counted after 11 days. To test for self-renewal, spheres were released from matrigel by incubation in 1 mg/ml dispase in PrEGM at 37C. Liberated spheres were pelleted by centrifugation, washed in PBS, incubated for 5 min in 0.05% Trypsin-EDTA, and passed through a 22 gauge syringe 5 times to yield single cells. Cells were counted by hemocytometer and re-plated. For CD44 blocking experiments, cells were incubated with anti-CD44 or IgG2b isotype at 10 μg/ml throughout the duration of in vitro culture. For adherence assays, sterilized glass coverslips were coated with fibronectin or collagen. Enumerated cells were plated on coated cover slips in PrEGM. After 24h, unbound cells were counted and used to calculate percentage of bound cells by subtraction from original number of input cells.

Statistical analysis. Results are expressed as mean±SD. To determine significance, comparisons were performed using a two-tailed T-test for groups of two or one-way ANOVA with Tukey HSD test for three or more groups.

RESULTS

Subpopulations of mouse endometrial epithelia have long-term self renewing *in vivo* regenerative activity

The uterus is composed of three predominant cellular compartments: endometrial epithelium, endometrial stroma (together called the endometrium) and the myometrium. To identify endometrial epithelial progenitors, we established *in vivo* assays for growth of total endometrial epithelia. Traditionally the endometrial epithelium is broken down into two compartments, glandular and luminal, based on their geographic localization in the endometrium. This geographic sub-division may have functional implications as the formation of endometrial glands by luminal branching is required for establishment of an endometrium receptive to implantation [reviewed in[23&24]]. Based on immunohistochemistry all endometrial epithelia, glandular and luminal, expressed epithelial cell adhesion marker (EpCAM) (Fig. 1A a,b&e and Supporting Information Fig. S1A a&b). Endometrial epithelia expressed integrin α6 (ITGA6) and integrin β1 (ITGB1) at their

basal surface and were bound to a laminin marked basement membrane (Supporting Information Fig. S1A). Endometrial stroma was marked with Thy1 (Fig. 1Ac&f). EpCAM was used to isolate all epithelia from whole mouse uteri dissociated in to single cells (Supporting Information Fig. S1B). Hematopoietic and endothelial cells were excluded using the lineage depletion markers protein tyrosine phosphatase receptor C (PTPRC, lymphoid), platelet/endothelial cell adhesion marker 1 (PECAM1, endothelial) and Ter119 (red blood cells [17]). Thy1 was used to exclude endometrial stroma. Since a small fraction of $EpCAM^{+}Thy1^{-}$ cells were Lin positive, (Supporting Information Fig. S1C), the $EpCAM^{+}Thy1^{-}Lin^{-}$ fraction was used to stringently isolate endometrial epithelia (Fig. 1A). The $EpCAM^{+}Thy1^{-}Lin^{-}$ cells were confirmed to be epithelial based on expression of E-cadherin and a lack of expression of stromal (desmin) or myometrial (smooth muscle actin-SMA Fig. 1Ad) markers in a Q-PCR assay (Fig. 1A). The $EpCAM^{-}Thy1^{+}Lin^{-}$ and $EpCAM^{-}Thy1^{-}Lin^{-}$ fractions were identified as endometrial stromal and myometrial cells based on expression of desmin and SMA respectively (Fig. 1A).

To assay for cells capable of forming functional endometrial epithelial structures, an *in vivo* regeneration model from dissociated uterine epithelia and stroma was utilized (Fig. 1B). This assay is adapted from a model previously reported by our group where luminal epithelia from an adult DsRed mouse in combination with neonatal wild type (WT) stroma regenerated into endometrial tissue in the sub-renal space of an immunodeficient mouse [17]. Here, to assay for growth of all endometrial epithelia (glandular and luminal), FACS isolated endometrial epithelia from dissociated uterine cellular preparations were regenerated *in vivo* in a similar assay (Fig. 1B). This approach ensured that all endometrial epithelia (glandular and luminal) would be included in this *in vivo* regeneration assay. As reported previously [17], endometrial epithelia regenerated in this assay were marked with red fluorescent protein (RFP) demonstrating that regenerated epithelia derive from the DsRed adult donor tissue (Fig. 1B).

The antigenic profile $EpCAM^{+}Thy1^{-}Lin^{-}$ was used for isolation of endometrial epithelia from group housed mice. The mouse reproductive estrous cycle lasts 4-5 days [16] and depending on the stage of the estrous cycle there may be variations in the growth capacity of the endometrial epithelia. Based on daily examination of vaginal cytology [25], mice housed in groups of five were in various stages of the estrous cycle (Supporting Information Fig. S1D). Therefore, endometrial epithelia harvested from 5-10 un-staged mice were pooled for most experiments. This approach ensured that a representative sample of endometrial epithelia from various stages of the estrous cycle would be used in our analyses.

One x 10^5 $EpCAM^{+}Thy1^{-}Lin^{-}$ or $1x10^5$ $EpCAM^{-}Thy1^{+/-}Lin^{-}$ cells from DsRed uteri were combined with $2.5x10^5$ WT neonatal stroma and placed in the regeneration assay. $EpCAM^{+}Thy1^{-}Lin^{-}$ cells contained almost all the *in vivo* regenerative activity demonstrated by formation of multiple endometrial epithelial structures (35±2 $EpCAM^{+}Thy1^{-}Lin^{-}$ vs. 2±2 $EpCAM^{-}Thy1^{+/-}Lin^{-}$, P=0.015) (Fig. 1C a vs. b). These findings confirm that the $EpCAM^{+}Thy1^{-}Lin^{-}$ antigenic profile marks epithelial cells capable of regenerating endometrial epithelial structures. We hypothesized that a sub-population of endometrial epithelia would have stem-like activity with a capacity to self-renew and proliferate *in vivo*. To test this hypothesis regenerated endometrial epithelial cells were serially passaged *in vivo* as secondary and tertiary grafts (Fig. 1D). Isolated color marked endometrial epithelia from DsRed transgenic mice were used to regenerate primary grafts. One in 3570 endometrial epithelia were capable of forming a gland in vivo and this number is comparable to previously reported mammary repopulating units of 1/1400-2000 [26] to 1/4900 [27]. Regenerated tissue was dissociated into single cells and re-transplanted with fresh WT

stroma as secondary and then tertiary grafts. Importantly, new epithelia were not supplemented in these grafts to assess the serial re-transplantability of existing regenerated epithelia. Based on an approximate estimation, 1 in 3500 endometrial epithelia was capable of serial re-transplantation *in vivo* (Fig. 1D). The fact that endometrial epithelia were capable of *in vivo* serial transplantation, establishes the existence of a long-term self-renewing endometrial epithelial population.

Hormonal withdrawal enriches for a population of endometrial epithelia with increased regenerative activity

To isolate and identify endometrial epithelial progenitors, we set out to define a population of endometrial epithelia enriched for regenerative activity. With the cessation of ovarian hormones, endometrial atrophy ensues in human and mouse; yet hormone supplementation causes remarkable regeneration of the remaining endometrial epithelia [28, 29]. These observations suggest that subsets of adult endometrial epithelia have stem-like activity and can survive without ovarian hormones. We hypothesized that hormonal deprivation would lead to loss of differentiated endometrial epithelia causing a relative increase in the percentage of endometrial epithelial progenitors. Reproductive age mice were hormonally deprived by surgical removal of their ovaries. The growth of endometrial epithelia from hormonally deprived and reproductive hormonally intact mice was compared. Hormonal deprivation dramatically decreased uterine size, accompanied by a 10-fold reduction in total number of endometrial epithelia (Fig. 2A). The surviving endometrial epithelia were enriched for a population of cells capable of *in vivo* regenerative activity demonstrated by increased growth potential of hormonally deprived compared to equal numbers of hormonally intact epithelia (1 in 69 hormonally deprived vs. 1 in 862 reproductive endometrial epithelia, $p < 0.0001$) (Fig. 2B and Supporting Information Fig. S2). These findings suggest that subsets of endometrial epithelial progenitors are resilient to hormonal deprivation. Results also demonstrate that hormonally deprived endometrial epithelia were enriched for progenitors capable of *in vivo* regeneration.

Signaling through the Wnt pathway is critical for the uterine development [30, 31]. For example, deletion of Wnt7a resulted in developmental abnormalities in the endometrial epithelium and infertility [30]. The expression of β-catenin, a downstream target of Wnt [*reviewed in [32]*] was examined in intact and hormonally deprived uteri. Concentrated expression of membranous β-catenin was detected in the endometrial epithelial glands and luminal crypts of hormonally intact mice (Fig. 3A a-d). A similar distribution of increased but also nuclear β-catenin was detected in hormonally deprived mice (Fig. 3A e-h). The mRNA expression level of Wnt/β-catenin target genes [33-40] was compared in endometrial epithelia of hormonally deprived vs. hormonally intact uteri (Fig. 3B). Higher expression of several Wnt/β-catenin target genes was observed in hormonally deprived endometrial epithelia (Fig. 3B) previously shown to be enriched for increased *in vivo* regenerative activity (Fig. 2B). These findings suggest the Wnt pathway may be activated in endometrial epithelial progenitors.

Basally located CD44 marked cells are the endometrial epithelial progenitors

The cell surface marker CD44 was one of the differentially expressed Wnt regulated genes found through Q-PCR analysis, with a 12-fold increase in CD44 transcript levels detected in hormonally deprived epithelia (Fig. 3B). Over-expression of CD44 has been reported in human endometrial carcinoma [41] and more recently, this cell surface marker has been used to isolate a cancer initiating population in endometrial cancer cell lines [42]. To test if CD44 was a candidate cell surface marker of normal mouse endometrial epithelial progenitors, its expression was examined in hormonally deprived and intact mouse uterine sections (Fig 4A). In both hormonal conditions CD44 positive cells were distributed as single cells in the luminal

epithelium, in some luminal epithelial crypts and in some but not all glands (Fig. 4A). These cells were in close contact with the basement membrane marked with ITGA6 (Fig. 4A b&e). Similar to the human endometrium [43], CD44 was detected not only in the endometrial epithelia but also in endometrial stroma ($EpCAM^{-}CD44^{+}$ cells, Fig 4A); however, these stromal cells were not included in our regenerations as they were EpCAM negative. We quantified the number of CD44 positive cells in hormonally intact and deprived endometrial epithelia by immunohistochemistry and FACS analysis (Fig 4B&C). By both methods, hormonal deprivation led to an approximate two-fold increase in CD44 positive endometrial epithelia (Fig 4B&C).

We hypothesized that CD44 positive cells in contact with the basement membrane contained the endometrial epithelial progenitors. We examined the *in vivo* growth of $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ endometrial epithelia compared to $EpCAM^{+}Lin^{-}CD44^{-}ITGA6^{hi}$/$EpCAM^{+}Lin^{-}CD44^{+/-}ITGA6^{lo}$ epithelia (Fig. 5A and Supporting Information Fig. S3A). To exclude endometrial stroma, Thy1 was added to the lineage depletion markers (Supporting Information Fig. S3A). Enrichment for CD44 was verified in the $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ cellular pool (Supporting Information Fig. S3B). To estimate the number of endometrial epithelia capable of forming endometrial epithelial structures *in vivo*, 10-fold serial dilutions of epithelia were placed in the regeneration assay (Fig. 5A). The $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ population was clearly enriched for cells capable of *in vivo* growth compared to the depleted fraction (Fig. 5A). One in 109 $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ cells formed an endometrial epithelial structure *in vivo* compared to one in 3124 $EpCAM^{+}Lin^{-}CD44^{-}ITGA6^{hi}$/$EpCAM^{+}Lin^{-}CD44^{+/-}ITGA6^{lo}$ epithelia (Fig. 5B). Previously we found that one in 862 hormonally intact endometrial epithelia regenerated endometrial epithelial structures *in vivo* (Fig. 2B). Collectively, these results suggest that selection with $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ antigenic profile resulted in an approximate 8-fold enrichment in endometrial epithelia with *in vivo* regenerative activity.

Analysis of grafts regenerated from $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ cells revealed that majority of regenerated epithelia were EpCAM and ITGA6 positive but CD44 negative (Fig. 5C). Only a small fraction of regenerated cells maintained the EpCAM/CD44 double positive progenitor signature (0.8 ± 0.1%) (Fig. 5C a-c). Similarly, these grafts contained a small subpopulation of ITGA6/CD44 dual positive cells (Fig. 5C d-f). Pankeratin marked all regenerated endometrial epithelia similar to the native endometrium (Supplementary Figure S3C). These results demonstrate that $EpCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ cells are capable of self-renewal, proliferation and multi-lineage differentiation *in vivo*. The 8-fold increase in the *in vivo* regenerative activity of the $EPCAM^{+}Lin^{-}CD44^{+}ITGA6^{hi}$ cells coupled with their ability to undergo multi-lineage differentiation suggests that this population is enriched for murine endometrial epithelial progenitor cells (EEPC). The remaining fraction of endometrial epithelia ($EpCAM^{+}Lin^{-}CD44^{-}ITGA6^{hi}$/$EpCAM^{+}Lin^{-}CD44^{+/-}ITGA6^{lo}$) had an approximately 4-fold decrease in their *in vivo* regenerative potential compared to all endometrial epithelia and this cellular fraction will be called non-EEPC.

To assess if the Wnt signaling pathway was activated in endometrial progenitors the expression of β-catenin protein, a well known target of Wnt signaling [32] was measured by western blot (Figure 5D). A relatively greater expression of total β-catenin was detected in the EECP cells compared to the non-EEPC and endometrial stroma (Figure 5D). A β-catenin degradation product was detected only in the non-EEPC fraction [44]. These findings suggest that the Wnt/β-catenin pathway is primarily activated in the endometrial epithelial progenitors.

The transcriptome of uterine epithelial progenitors is unique in the endometrium

Thus far, we have demonstrated that the EEPC are the cellular population in the endometrium capable of regenerating endometrial epithelial structures. To test if this biologic behavior is accompanied with a unique transcriptional profile, RNA-sequencing was performed to compare the transcriptomes of EEPC, non-EEPC and endometrial stroma. Among 36,872 genes examined, 11,140 transcripts were expressed in the epithelial cell fractions (RPKM>1.0). Of these transcripts, 332 were differentially expressed in EEPC vs. non-EEPC. Differentially regulated genes were defined as transcripts that varied by greater than 2-fold and were significantly different among sample replicates (adjusted p-value<0.05).

Hierarchal clustering of the differentially regulated gene set revealed four distinct groups of genes (Fig. 6Aa and Supplementary Table S5). Cluster A contained 135 genes more highly expressed in non-EEPC compared to EEPC and stroma. This cluster was enriched for genes encoding secreted proteins, and genes involved in immune response and host defense (Fig. 6Ab). The detection of secreted protein genes may reflect the differentiated nature of non-EEPC as a secretory epithelium. Expression of immune defense related genes in non-EEPC may be a protective mechanism to prevent uterine infections given that the uterus is regularly exposed to foreign fluids [45, 46].

The 60 genes in cluster B had greater expression in EEPC relative to non-EEPC and stroma. This cluster was enriched for transcripts involved in the cell cycle and cell division (Fig. 6Ab). Traditionally regenerative cells were considered to be quiescent, but recent studies have shown that in tissues with rapid cellular turnover regenerative cells are cycling [47, 48]. To determine if EEPC were quiescent or cycling two assays were performed: (a) cell cycle analysis and (b) measurement of proliferation using Ki67 expression. A higher percentage of cells were found in G2M/S in the EEPC compared to non-EEPC (Supporting Information Fig. S4A). Similarly, a greater percentage of cells were Ki67 positive in the EEPC pool compared to other endometrial epithelia (Supporting Information Fig. S4B). These findings suggest that EEPC are not quiescent but actively cycling. The high rate of proliferation in EEPC may support the rapid turn-over of the endometrial lining during reproductive years. Higher mRNA levels of telomerase reverse transcriptase enzyme (TERT), the catalytic component of telomerase, have been reported in rapidly dividing stem cells such as those in the intestinal epithelium [49]. Given that many EEPC were found to be cycling, the expression of TERT mRNA was measured by Q-PCR and compared in EEPC vs. non-EEPC cells. TERT transcript levels were significantly elevated in EEPC compared to non-EEPC (Supplementary Figure S4C). Telomerase activity may help protect dividing EEPC from DNA attrition [50].

Cluster C contained 119 genes whose transcripts were highest in stroma and higher in EEPC compared to non-EEPC. Analysis of this cluster showed enrichment for transcripts involved in signal transduction, pattern binding, cell adhesion and components of the extracellular matrix (Fig. 6Ab). These genes may be involved in localizing and maintaining the EEPC in its niche. Cluster D contained only 14 genes that were enriched in EEPC and stroma compared to non-EEPC. Similar to genes in cluster C, these transcripts were associated with cell surface proteins (Fig. 6Ab).

CD44 was expressed in all EEPC and some endometrial stromal cells (Fig 4A). Multiple isoforms of CD44 exist based on splicing of its ten variant exons [reviewed in [51]]. The differential expression of CD44 splice variants in specific cells can regulate their adhesion and interaction with the microenvironment [52, 53]. We compared the expression of CD44 variants in EEPC, non-EEPC and endometrial stroma by examining plots of the CD44 RNA sequencing reads. Clear differences between the abundance of CD44 variant exons were observed between

the three cellular populations and increased levels of CD44 variant transcripts were detected in EEPC (Fig. 6Bb). Functionally, EEPC had greater in vitro adherence capacity to fibronectin [54] and collagen [55, 56] compared to non-EEPC (Supplementary Figure S4D). This differential adherence capacity may be due to increased expression of CD44 and CD44 variants in the EEPC.

Collectively we demonstrate that four cell surface markers (EpCAM, Thy1, CD44 and ITGA6) can divide the endometrium into three distinct cellular compartments: EEPC (epithelial progenitors), non-EEPC (differentiated epithelia) and stroma. These three compartments have different biologic properties *in vivo* coupled with distinct transcriptional profiles.

The numbers of endometrial epithelial progenitors are regulated by estrogen and progesterone through paracrine signals

The endometrium undergoes numerous cycles of breakdown and regeneration during reproductive years. This activity is tightly regulated with two steroid hormones: estrogen (E) and progesterone (P). Both hormones are necessary for embryonic implantation [57]. To examine effects of E and P on the progenitors, the percentage of EEPC was measured in reproductive mice supplemented with placebo, E, P or a combination of both hormones. Serum levels of E and P were measured in each experimental condition (Supporting Information Fig. S5A). The percentage of EEPC remained unchanged in placebo, E or P treated uteri (Fig. 7A and Supporting Information Fig S5B). In contrast, when E and P were co-administered the percentage of EEPC doubled (Fig. 7A and Supporting Information Fig S5B). Previously we observed a similar increase in the percentage of EEPC in hormonally deprived mice (Fig 4B&C, Fig. 7A). To determine the biologic explanation for this unexpected result, a rise in the percentage of EEPC seen with both hormone deprivation and supplementation, we examined the total numbers of EEPC per uterus. To estimate the total number of EEPC per uterus, the percentage of EEPC was multiplied with total number of endometrial epithelia per uterus. Dual hormone supplementation resulted in a 2-fold rise in the number of EEPC while hormonal deprivation caused a 2-fold drop in EEPC compared to hormonally intact control (Fig 7A). With hormonal deprivation the increased percentage of EEPC is explained by massive loss of the differentiated endometrial epithelia coupled with relative survival of many EEPC (Fig. 7A and Fig. 2A). In contrast, with dual hormone supplementation, the total number of endometrial epithelial remained unchanged while the number of progenitors doubled resulting in a net increase in the percentage of EEPC (Fig. 7A and Supporting Information Fig S5B&C).

Given these results, we hypothesized that exposure to E and P would enhance the growth of endometrial epithelia. To test this hypothesis the *in vivo* regenerative activity of E and P vs. placebo treated endometrial epithelia was compared (Fig. 7B & Supporting Information Fig S5D). Estrogen and progesterone treatment enhanced the *in vivo* growth potential of endometrial epithelia 6-fold (Fig. 7B and Supporting Information Fig S5D). This hormone-induced growth augmentation could be due to (a) an increase in progenitors and/or (b) a heightened growth of existing progenitors. To test this hypothesis, the growth of EEPC was compared to total endometrial epithelia using an *in vitro* growth assay (Supporting Information Fig S6 and Fig. 7C) adapted from previously established protocols [58, 59]. In this assay isolated epithelia give rise to spheroid structures that express EpCAM similar to the native endometrial epithelium (Supplementary Figure S6A&B). The EEPC were the cells with sphere forming capacity in vitro (Supplementary Figure S6C). Blocking CD44 with a neutralizing antibody [60] decreased the sphere forming capacity of endometrial epithelia in this assay, suggesting a possible role for CD44 signaling in the proliferation of sphere forming cells (Supplementary Figure S6D). Cell growth in this assay was clonal (Supplementary Figure

S6E&F). Cells within spheres were capable of serial passaging in vitro (Supplementary Figure S6G) suggesting that subsets of endometrial epithelia maintain clonal growth with time.

Co-administration of E and P doubled the growth of total endometrial epithelia but did not change the growth of EEPC (Fig. 7C). These results suggest that the enhanced growth potential induced by E and P co-treatment primarily results from increased numbers of EEPC but not qualitative changes in these progenitors. Pregnancy is a physiologic state accompanied with elevated levels of both E and P resulting in expansion and biochemical changes in the endometrial stroma called decidualization [57]. The E and P induced expansion of EEPC could be a physiologic adaption for a concomitant expansion of endometrial epithelia in a rapidly growing uterus during pregnancy.

To determine if the mechanisms controlling the expansion of EEPC are cell autonomous or paracrine, the expression of ERα and progesterone receptor (PR) was examined. Basally located CD44 positive cells did not express ERα or PR based on immunohistochemistry (Fig. 7D). These findings were confirmed by immunocytochemistry on isolated EEPC (Supporting Information Fig. S7). ERα and the progesterone receptor isoforms PRA and PRB were detected in non-EEPC and endometrial stroma but were absent in EEPC in western blots (Fig. 7E). We did not detect a difference between mRNA levels of these hormone receptors in EEPC vs. non-EEPC cells suggesting modulation of hormone receptors at a post-transcriptional level. These results suggest that hormonal signals promoting the proliferation of EEPC are paracrine and arise either from neighboring epithelia or uterine stroma.

Similar to the mouse endometrium, using the markers EpCAM and membrane metallo-endopeptidase (MME), we have fractionated human endometrium into an epithelial ($EpCAM^+MME^-$), stromal ($EpCAM^-MME^+$) and myometrial ($EpCAM^-MME^-$) compartments (Supplementary Figure S8A). Expression of CD44 has been reported in the secretory phase of the normal endometrium and human endometrial carcinoma [41, 61]. We detected basally located CD44 positive cells in both the proliferative and the secretory phase of the human endometrium (Supplementary Figure S8B). To assess if, basally located CD44 positive human endometrial epithelia were candidate progenitors, these cells were isolated from three normal reproductive age women undergoing hysterectomy for benign indication. Lineage depletion markers PTPRC (lymphoid), PECAM1 (endothelial), glycophorin A (GYPA, red blood cell) and MME (stroma) were utilized to exclude non-epithelial cells from human dissociated endometrial cellular preparations. The in vitro growth capacity of $EpCAM^+CD44^+ITGA6^{hi}Lin^-$ human endometrial epithelia was compared to $EpCAM^+CD44^-ITGA6^{hi}Lin^-$ /$EpCAM^+CD44^+ITGA6^{lo}Lin^-$ and unfractionated endometrial epithelia (Supplementary Figure S8C). In all three clinical specimens, predominance of the *in vitro* sphere forming activity was found in the $EpCAM^+$ $CD44^+ITGA6^{hi}Lin^-$ fraction, suggesting that this cell surface signature enriches for cells progenitor activity (Supplementary Figure S8C). Similar to mouse endometrial epithelial progenitors, basally located human endometrial epithelia marked with CD44 were predominantly ER α and PR negative (Supplementary Figure S8D).

DISCUSSION

Over the last decade investigators have attempted to identify adult endometrial progenitors with marginal success [reviewed in [6]]. This is surprising given the remarkable strides made in defining the cellular hierarchy in other hormonally regulated tissues such as breast [62, 63] or the prostate epithelium [64-66]. Recent work has identified candidate cell surface markers for isolation of stromal stem-like populations in the human endometrium. Human stromal cells marked with W5C5 (marking an unknown antigen) or the CD146 and PDGFRβ

were found to have stem-like activity based on *in vitro* assays, differentiation potential and limited *in vivo* growth [67, 68]. These cells did not differentiate into endometrial epithelia. Putative markers for isolation of human endometrial epithelial stem cells remain unknown.

Minimal progress has been made in identification of murine endometrial epithelial or stromal progenitors [reviewed in [6]]. Epithelial or stromal LRCs in the endometrium or the fallopian tube have been considered as potential endometrial progenitors [7, 8, 69, 70] but functional *in vivo* stem activity of these cells has not been demonstrated. The challenges for analysis of LRCs as progenitors are two-fold: (a) lack of *in vivo* growth assays that can examine regenerative activity of candidate progenitors [6, 69], and (b) absence of cell surface markers for live isolation of BrdU marked LRCs. Therefore, a cell surface antigenic profile for isolation of murine endometrial epithelia or stromal cells has been unavailable.

Similar to the human endometrium [9, 71], we find that mouse endometrial epithelia express EpCAM. Subpopulations of EpCAM positive cells were capable of regenerative activity in an *in vivo* functional assay designed to test for clonal growth of endometrial epithelia. Some EpCAM positive cells could undergo serial transplantation, establishing proof for the existence of a long-term self-renewing endometrial epithelial progenitor population. To isolate the progenitor cells we fractionated EpCAM positive endometrial cells based on expression of two other makers: CD44 and ITGA6. A minor subpopulation of the mouse endometrium, the EEPC marked with $EpCAM^{+}CD44^{+}ITGA6^{hi}Thy1^{-}PECAM1^{-}PTPRC^{-}Ter119^{-}$ contained predominance of epithelia with *in vivo* growth capacity. This cellular pool must contain the endometrial epithelial stem cells demonstrated by the capacity of these cells to self-renew, differentiate and proliferate *in vivo*. One in 109 EEPC were capable of regenerating an endometrial gland *in vivo*. In the mammary system, where epithelial progenitors are also regulated with reproductive hormones [72, 73], one in 91 [26] and one in 64 [27] epithelial stem cells could regenerate a mammary gland *in vivo* as determined by limiting dilution analyses. Thus our purification of EEPC is comparable to earlier studies in the mammary system. We plan to refine isolation of our epithelial progenitor population by mining the transcriptome of EEPC for identification of cell surface markers that could be used to further purify the stem population.

Here we show that a greater proportion of the EEPC are cycling and proliferating compared to the differentiated endometrial epithelia. The high rate of cellular division in these progenitors must be a biologic adaptation required to keep up with the demands of rapid turn-over in the reproductive endometrium. In this respect the endometrial progenitors may behave similar to intestinal epithelial and hematopoietic stem populations where a pulsed label such as BrdU is not retained [48, 74]. Many studies had attempted to utilize the label retention for identification of endometrial epithelial progenitors [7, 69, 70, 75]. These studies showed that LRCs could be detected up to 8 weeks in the endometrial epithelium [7] but failed to identify a long-term LRC in this compartment [7, 69, 70, 76]. Because long-term LRCs were not observed in the endometrial epithelium it has been proposed that the progenitor/stem cell pool for these cells must reside elsewhere, either in the stroma/perivascular niche [6] or in the distal end of the fallopian tube [69] where LRCs can be detected. A major assumption in these studies is that endometrial epithelial progenitors are quiescent.

An alternative method for identification of stem cells is lineage tracing, but it is only feasible when relevant transgenic mice are available. There were two limitations with our combination of makers which made lineage tracing not possible: (a) there are no transgenic mice with promoters relevant to our markers, and (b) because CD44 is also expressed in the endometrial stroma, lineage tracing or depletion

of cells using the CD44 promoter would lead to difficulties interpreting the data. In previous studies, lineage tracing has been compared to *in vivo* regeneration as a method for identification of somatic stem cells. In a murine prostate model, the same epithelial stem cell population was capable of self-renewal and multi-lineage differentiation regardless of the method tested [77]. While *in vivo* regeneration and lineage tracing did not yield identical results in terms of differentiation capacity of mouse mammary cells, the stem cell subset identified using limiting dilution transplantation was confirmed to be a stem cell population by lineage tracing [78]. Taken together, *in vivo* regeneration coupled with serial transplantation is consistent with standards in the field for the identification of self-renewing epithelial progenitor and stem-like cells. We hope that analysis of the EEPC transcriptome will reveal promoters that can be used for lineage tracing in future studies.

Similar to the endometrial epithelial progenitors, mammary stem cells are ER/PR negative and expanded when E and P were co-administered [72, 73]. However, unlike mammary tissue where E and P increase the risk of breast cancer [79], P concomitant with E has protective effects on the endometrium [80]. These differences may result from selective use of PR isoforms, PRA in the uterus and PRB in the mammary tissue, resulting in different transcriptional signals [81]. The exquisite sensitivity of EEPCs to E and P suggests that the balance of these hormones may be critical for maintaining endometrial homeostasis. In fact, resistance of endometrial epithelium to progesterone has been implicated as a central mechanism in the pathophysiology of endometriosis, infertility and endometrial cancer [82]. Mechanisms leading to progesterone resistance remain unknown. Estrogen induced proliferation of the endometrial epithelium occurs even with epithelial specific deletion of ERα, suggesting that the effects of estrogen on endometrial epithelium are paracrine and mediated through the stroma [83]. During pregnancy, the anti-proliferative effects of progesterone on the endometrial epithelium are proven to be paracrine [84]. We find that murine EEPC and basally located CD44 marked human endometrial epithelia are ERα and PR negative, suggesting that signals regulating their growth may also be paracrine. Two plausible mechanisms for progesterone resistance may include (a) cell autonomous defects in EEPC that make them unable to respond to paracrine signals, and/or (b) defects in stromal or surrounding non-EEPC rendering them incapable of PR mediated signaling and thus paracrine regulation of EEPC. Understanding hormone-mediated mechanisms that regulate growth of EEPC in their niche will likely have major implications in designing therapies for common yet poorly understood diseases of the endometrium.

CONCLUSIONS

We report the first description for dissociating the mouse uterus into three major cellular compartments: endometrial epithelium, endometrial stroma and myometrium. We subdivide the endometrial epithelium into two functionally distinct cellular fractions: (a) the epithelial progenitor pool capable of self-renewal, multi-lineage differentiation and propagation *in vivo* and, (b) the differentiated endometrial epithelia. The progenitors comprise a small fraction of total endometrial epithelia and their numbers are tightly regulated with two female reproductive hormones estrogen and progesterone through paracrine signals. We suspect that defects in the normal cross-talk between these progenitors and their niche may play a critical role in endometriosis, endometrial cancer and infertility. Findings in this manuscript are currently being used as a stepping stone for discovery of these defects and therapeutic approaches that can correct them.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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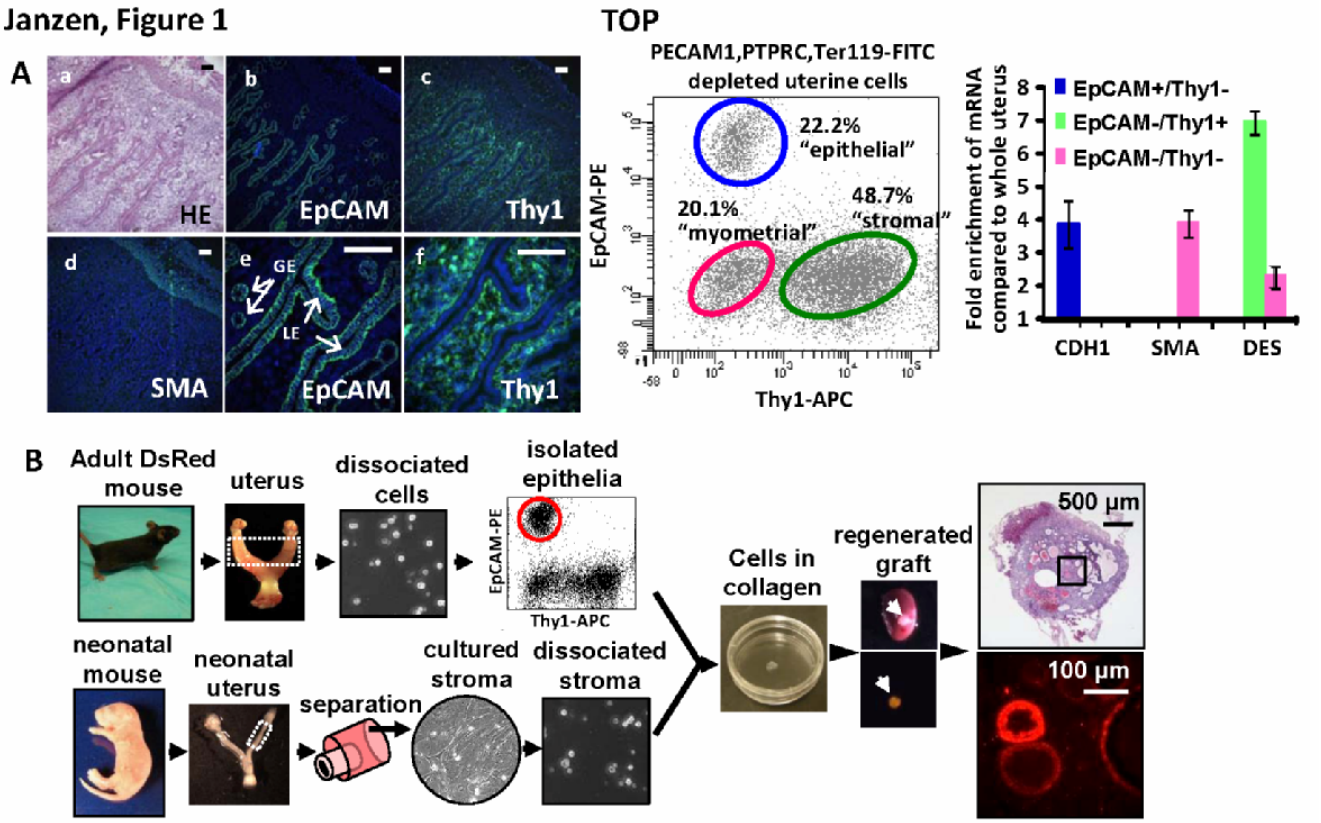
Figure 1. Adult mouse endometrial epithelia contain subpopulations of self-renewing cells. (A) Immunohistochemical staining showed that EpCAM marked all uterine epithelia: glandular epithelium (GE) and luminal epithelium (LE) (a,b&e). Thy1 marked the endometrial stroma (c&f) and smooth muscle actin (SMA) marked the myometrium (d). Epithelial ($EpCAM^{+}Thy1^{-}$), stromal ($EpCAM^{-}Thy1^{+}$) and myometrial ($EpCAM^{-}Thy1^{-}$) cells were isolated from dissociated uterine cellular preparations after exclusion of endothelial (PECAM1), lymphoid (PTPRC) and red blood cells (Ter 119). Quantitative PCR analysis confirmed identity of each cellular fraction based on enrichment for transcripts of E-cadherin (CDH1 epithelial), smooth muscle actin (SMA myometrial) and Desmin (DES stromal). **(B)** Combinations of FACS isolated total endometrial epithelia from adult DsRed mice and cultured neonatal stroma were implanted under the kidney capsule of an immunodeficient mouse. These cells regenerated into endometrial tissue. Epithelia in this regenerated tissue were marked with DsRed indicating their origin from isolated adult endometrial epithelia. **(C)** Only the $EpCAM^{+}Thy1^{-}$ cells could regenerate *in vivo* into endometrial epithelial structures (a-c vs. d-f) measured by the formation of hollow, RFP positive glands (b&c vs. e&f). $EpCAM^{-}Thy1^{-/+}$ cells predominantly gave rise to stromal cells (e&f). **(D)** A sub-population of endometrial epithelia could serially re-transplant *in vivo* demonstrating the existence of a long-term self-renewing population of endometrial epithelia. Primary grafts (a&d), when dissociated and re-implanted with equal numbers of fresh stroma, gave rise to secondary grafts (b&e). Secondary grafts likewise gave rise to tertiary grafts (c&f). All scale bars are 100 μm and results are mean ± SD.

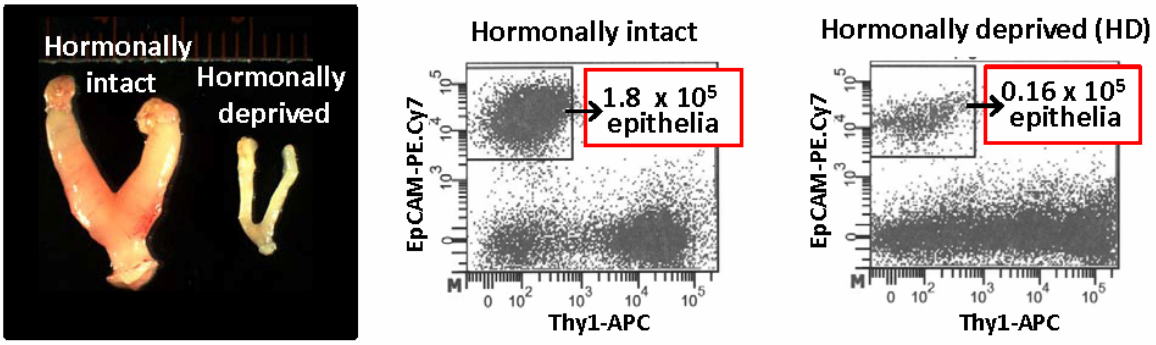


Epithelial cells implanted	250,000	50,000	50,000
Glands detected	70	15	13
Approximate number of gland forming cells	1 in 3570	1 in 3330	1 in 3850

Figure 2. Hormonal deprivation resulted in a massive loss of endometrial epithelia but enrichment for endometrial epithelial progenitors. (A) Hormonal deprivation with surgical removal of the ovaries resulted in the shrinkage of the uterine horns and an approximate 10-fold drop in the number of total endometrial epithelia. **(B)** Endometrial epithelia that survived hormonal deprivation were enriched for epithelial progenitors. Equal numbers of endometrial epithelia from intact or hormone depleted uteri were placed in serial dilution in the *in vivo* regeneration assay. Regeneration was scored by the presence of pankeratin positive epithelial structures (glands). Representative regenerated grafts and the average number of glands detected per graft from the serial dilution series are shown. The *in vivo* growth capacity of hormonally deprived endometrial epithelia was superior to age matched hormonally intact cells at all serial dilutions tested. Scale bars equal 100 μm.

Janzen, Figure 2 **TOP**

A



B

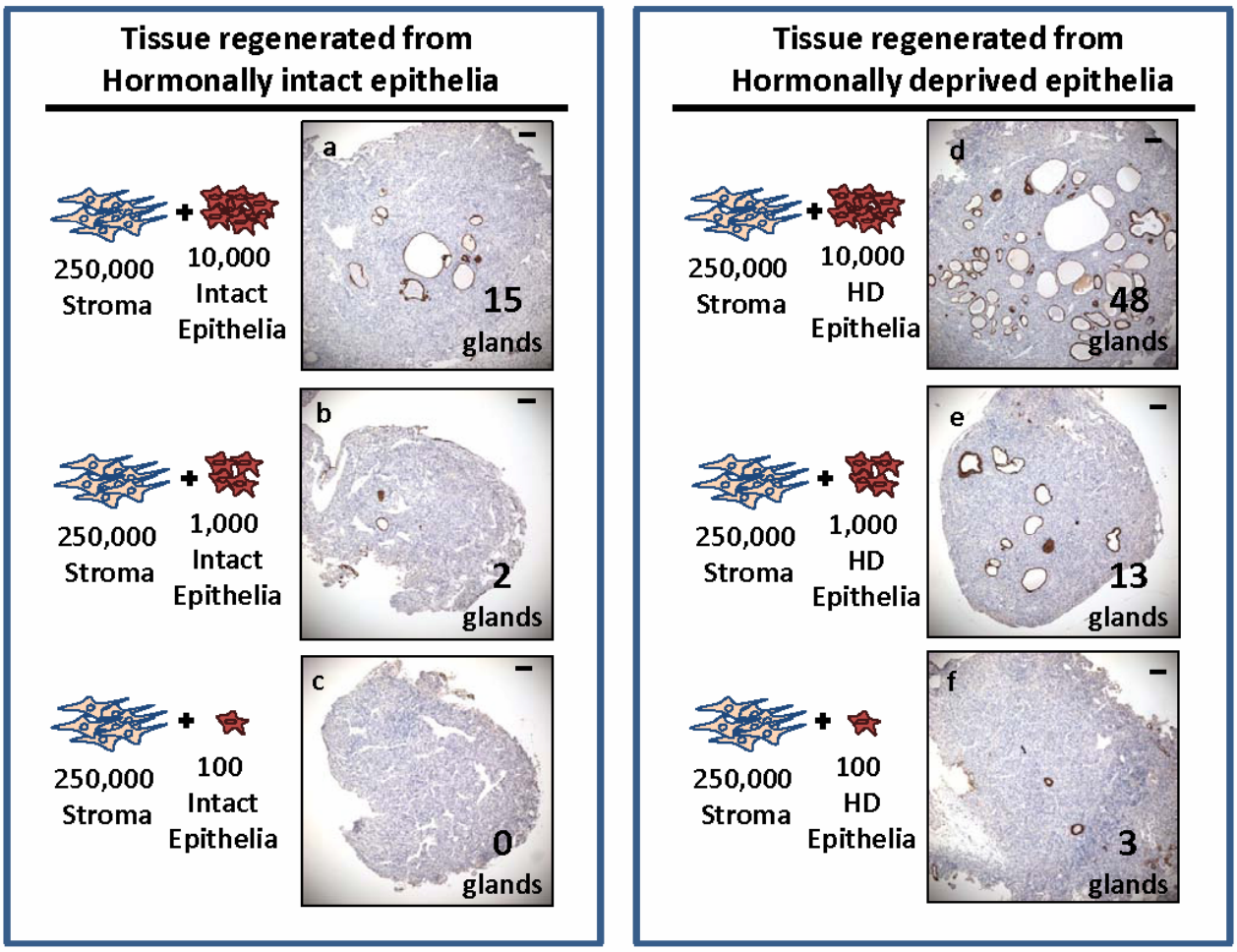
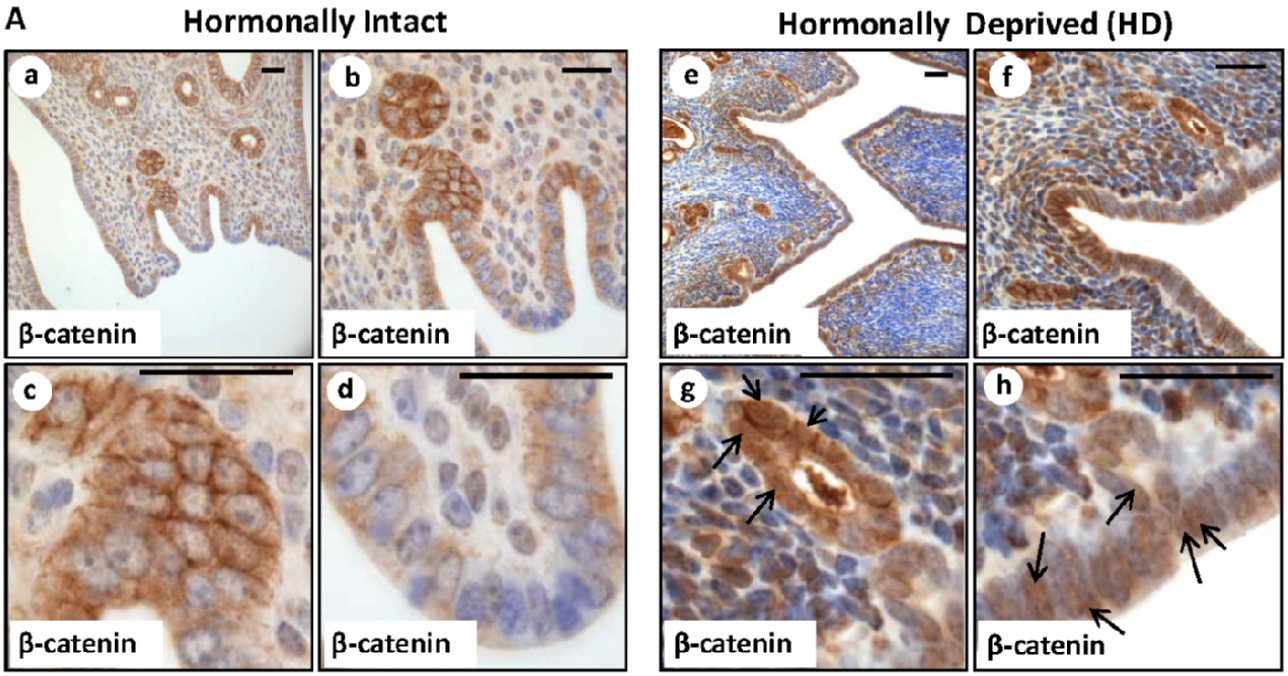


Figure 3. The Wnt/β-catenin pathway is activated in populations of hormonally deprived endometrial epithelia. (A) β-catenin was detected in the endometrial epithelial luminal crypts and glands of the endometrium of hormonally intact (a-d) and deprived (e-h) mice. Nuclear localization of β-catenin was detected in the endometrial epithelia of hormonally deprived (g&h) but not hormonally intact endometrium (c&d). **(B)** An increase in the transcript levels of multiple Wnt/β-catenin regulated genes was detected by QPCR in hormonally deprived compared to hormonally intact endometrial epithelia. All scale bars are 25 μm. Results are mean ± SD.

Janzen, Figure 3 TOP



B QPCR Analysis of selected Wnt/β-catenin regulated genes in reproductive vs. hormonally deprived isolated uterine epithelia

Class	Gene	Relative level in HD vs Reproductive endometrial epithelia (fold increased in HD)
Wnt Target Genes	c-Myc	2.0 ± 0.4
	Jun	7.6 ± 3.0
	Cyclin D1	8.6 ± 1.8
	Axin2	14.7 ± 3.3
	CD44	11.6 ± 4.5
	ID2	17.1 ± 3.9

Figure 4. Subsets of endometrial epithelia express CD44 and these cells are enriched in the hormonally deprived endometrium. **(A)** Isolated CD44 positive cells were seen in the epithelial lumen and in some but not all glandular epithelia (a-f). CD44 positive epithelial cells were in close contact with the basement membrane marked with ITGA6 (b&e). Arrows denote CD44 positive luminal epithelial cells (a,b,d&e). Filled arrow heads indicate CD44 positive epithelial glands (a&d). Empty arrow heads mark CD44 negative epithelial glands (b,c&f). Stars indicate positive luminal epithelial crypts (c&f). Boxed areas in b & e are shown as magnified insets. **(B)** An increase in the proportion of CD44 positive cells was detected upon hormonal deprivation in the endometrial epithelium. Based on immunohistochemistry, a higher percentage of CD44 positive epithelia were detected in hormonally deprived uteri. **(C)** FACS analysis also demonstrated an approximate doubling in the percentage of CD44 positive epithelia in the hormonally deprived endometrium compared to the hormonally intact conditions. All scale bars equal 50 μm and results are mean ± SD.

Janzen, Figure 4 TOP

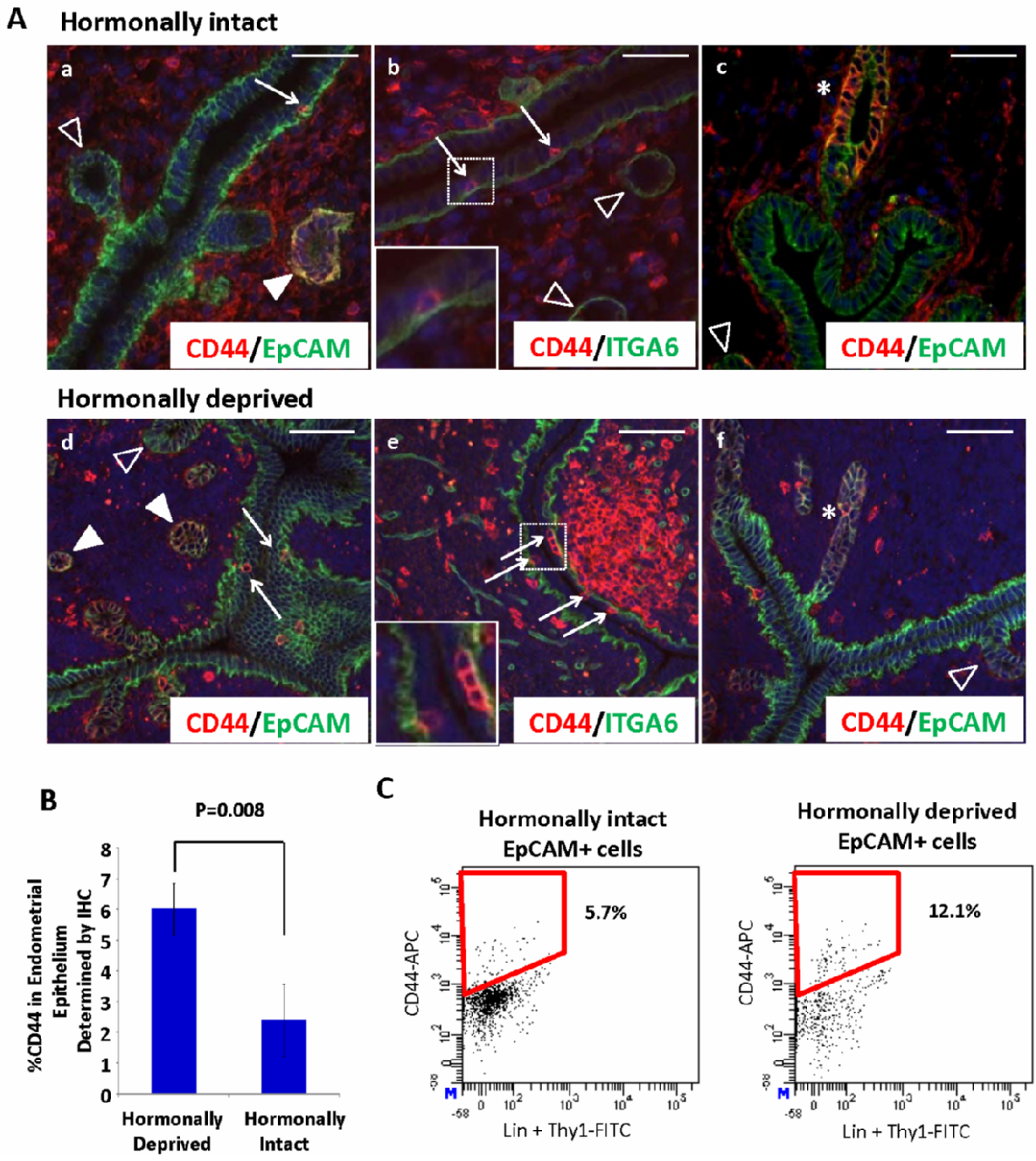
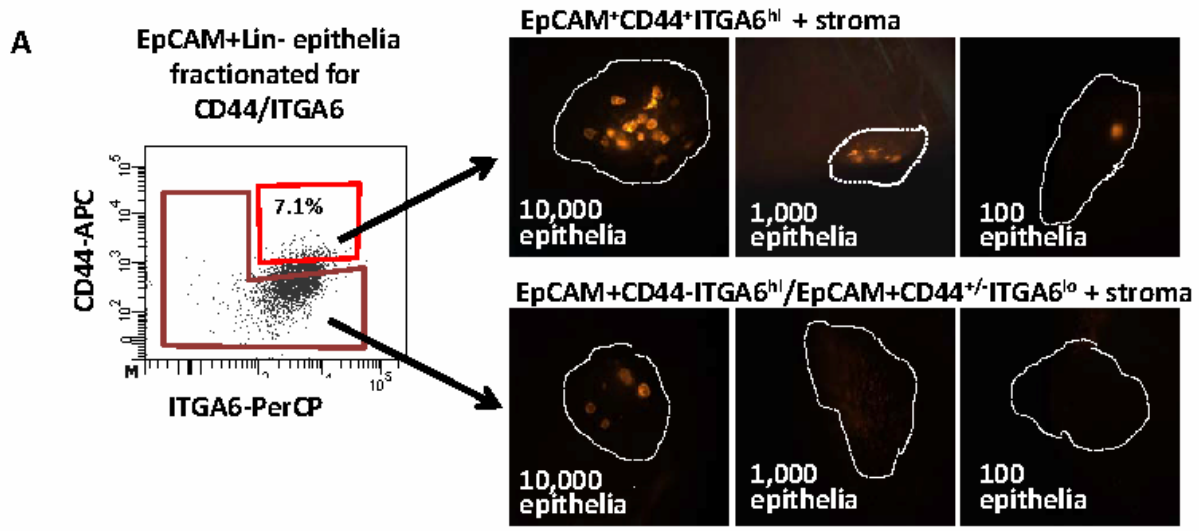


Figure 5. Minor subpopulations of endometrial epithelia are EpCAM^{+}CD44^{+}ITGA6^{hi}Thy1^{-} PECAM1^{-}PTPRC^{-}Ter119^{-} and these cells are the endometrial epithelial progenitors (EEPC). (A) After depletion of Thy1^{+}PECAM1^{+}PTPRC^{+}Ter119^{+} cells, endometrial epithelial were divided into two populations: (1) EpCAM^{+}CD44^{+}ITGA6^{hi}, and (2) remaining endometrial epithelia comprised of EpCAM^{+}CD44^{-}ITGA6^{hi} and EpCAM^{+}CD44$^{+/-}$ITGA6^{lo} cells. Logarithmic dilutions of equal numbers of these cells were grown *in vivo*. Regeneration was scored as the presence of RFP positive glandular structures. Predominance of the regenerative activity was detected in the EpCAM^{+}CD44^{+}ITGA6^{hi} endometrial cells suggesting that these cells are the endometrial epithelial progenitors (EEPC). **(B)** A significant difference in the regenerative activity of these two subpopulations was detected *in vivo* based on a limiting dilution analysis. **(C)** EpCAM^{+}CD44^{+}ITGA6^{hi} cells undergo multi-lineage differentiation *in vivo*. Majority of cells regenerated from this cellular pool were EpCAM/ITGA6 positive but CD44 negative (solid arrow heads) while a minor subpopulation of EpCAM/CD44 dual positive (a-c) and CD44/ITGA6 dual positive (d-f) cells were detected (arrow). **(D)** Higher levels of total β-catenin (90 kDa) were detected in EEPC vs. non-EEPC and stroma. Degraded β-catenin (66 kDa) was only detected in the non-EEPC. Scale bars equal 50 μm.

Janzen, Figure 5

TOP



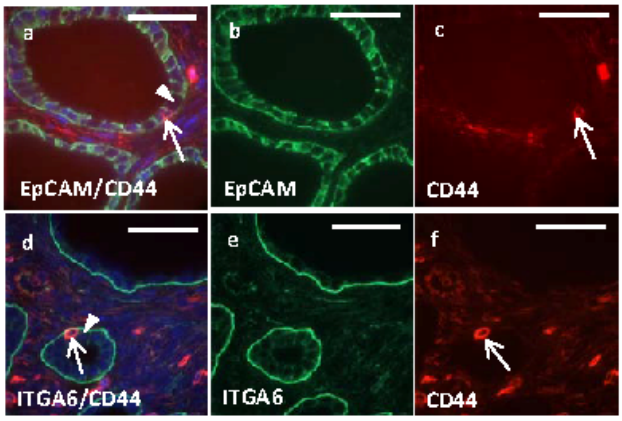
B Estimation of progenitors based on in vivo serial dilution assay

Cell Number	Grafts with Regeneration	
	EpCAM+CD44+ITGA6fhi	EpCAM+CD44-ITGA6hi & EpCAM+CD44+/-ITGA6lo
10000	2/2	2/2
1000	4/4	1/4
100	3/5	0/5

EpCAM+CD44+ITGA6fhi	1 in 109
EpCAM+CD44-ITGA6hi & EpCAM+CD44+/-ITGA6lo	1 in 3124

P = 0.002

C Graft regenerated from EpCAM+CD44+ITGA6hi cells



D

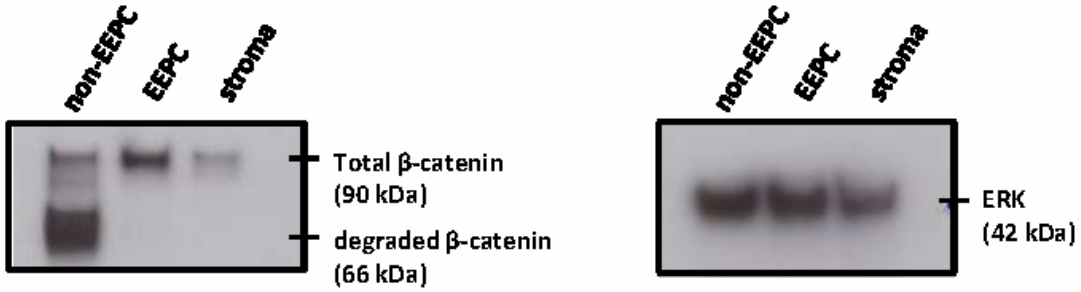
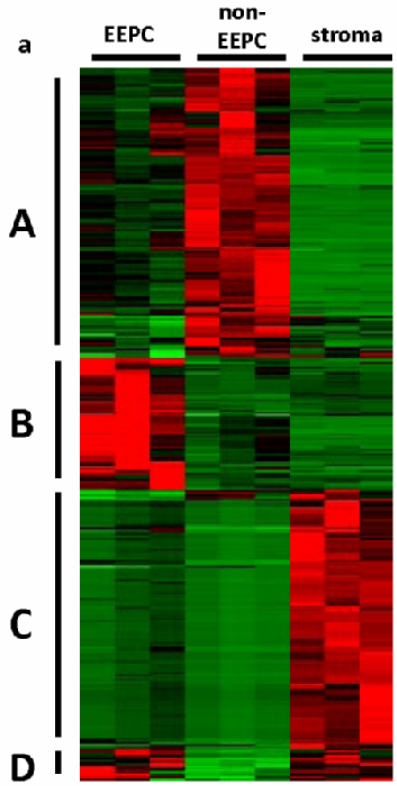


Figure 6. The transcriptome of EEPC is different and distinct compared to other endometrial cells. (A) Differentially expressed genes between EEPC and non-EEPC were identified. Based on this gene set a hierarchal clustering was performed comparing EEPC, non-EEPC and stroma. Four transcript clusters were detected (a) Higher expression (red) and lower expression (green) is indicated by color and intensity. The gene sets in each of these four clusters were associated with distinct and different pathways (b). **(B)** The frequency of each CD44 variant and standard exon was cross compared in these three populations. A higher frequency of variant exons was detected in the EEPC compared to non-EEPC and stromal cells.

Janzen, Figure 6 **TOP**

A Heat map of genes differentially expressed by 2-fold or more in EEPC compared to non-EEPC



b

Cluster	Pathway (adjusted p-value)
Cluster A	Secreted Proteins (4.3×10^{-11}) Extracellular Region (5.2×10^{-10}) Signal peptides (6.4×10^{-8}) Immune response (4.7×10^{-5}) Defense response (2.7×10^{-3})
Cluster B	Cell cycle (1.1×10^{-7}) Cell division (1.6×10^{-7}) Signal (5.0×10^{-4})
Cluster C	Signal (1.6×10^{-15}) Extracellular Matrix (1.9×10^{-17}) Pattern binding (2.0×10^{-12}) Cell adhesion (1.2×10^{-7}) ECM-receptor (2.3×10^{-5})
Cluster D	Cell surface (9.2×10^{-2})

B Relative transcript levels of CD44 isoforms in EEPC, non-EEPC and stroma

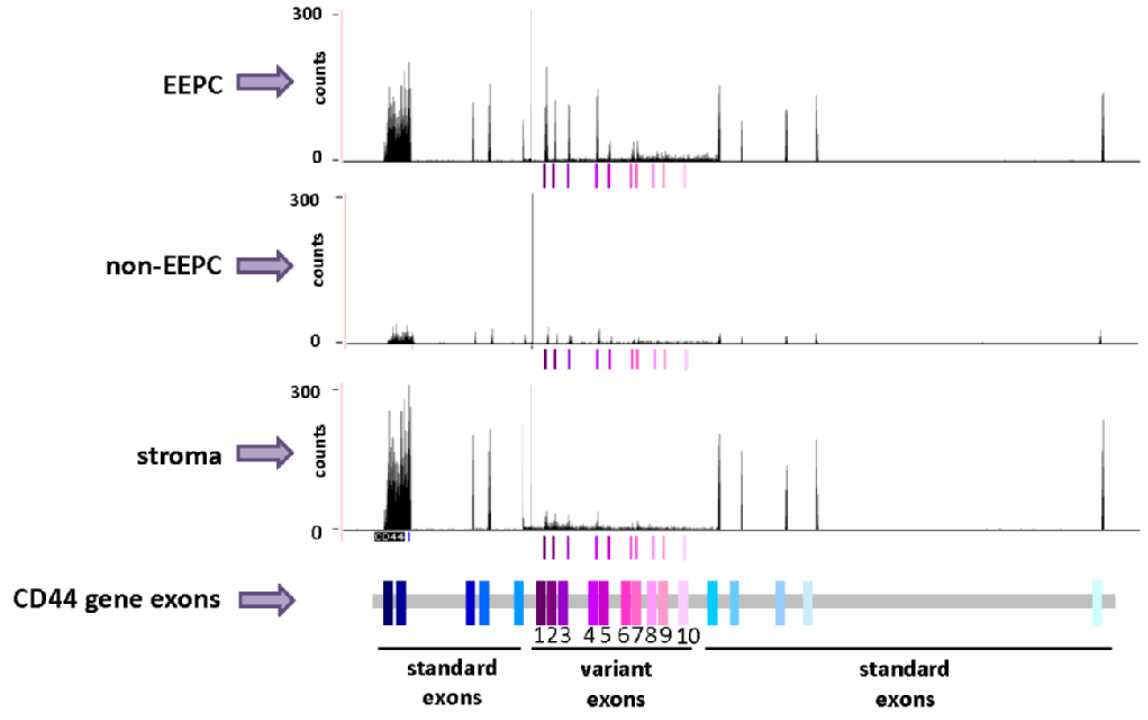


Figure 7. Estrogen and progesterone expand EEPC through paracrine signals. (A) To measure number of EEPC in each hormonal condition, the percentage of EEPC was multiplied by total number of endometrial epithelia per uterus. Hormonal supplementation with estrogen or progesterone alone did not significantly alter the number of EEPC. In contrast, when estrogen and progesterone were co-administered, the number of EEPC increased 2-fold compared to placebo treated controls. Hormonal deprivation yielded a 2-fold drop in total number of EEPC but a relative increase in the percentage of EEPC due to massive loss of many differentiated endometrial epithelia. **(B)** Endometrial epithelia isolated from estrogen and progesterone co-treated mice had an enhanced regenerative potential compared to placebo treated controls based on a serial dilution growth assay *in vivo*. Regeneration in this assay was scored as the presence of RPF positive glandular structures. **(C)** A 2-fold increase in the *in vitro* growth potential of total endometrial epithelium was noted upon co-treatment with estrogen and progesterone. The growth of EEPC was equivalent in both hormonal conditions. The non-EEPC rarely grew in this assay. Findings suggest that the increased growth capacity of estrogen and progesterone treated endometrial epithelia is due to an increase in the number of EEPC and not due to qualitative differences in the epithelial progenitors. **(D)** Majority of basal CD44 positive (brown) cells detected in the endometrium were ERα (a) and PR (b) negative. (E) Western blot analysis demonstrated that EEPC do not express ERα or PRA or PRB at the protein level. Scale bars equal 50 μm and results are mean ± SD.

Janzen, Figure 7 TOP

A

Hormonal milieu in reproductive mice	Total epithelia per uterus	Percent EEPC	Total EEPC per uterus (epithelia X %EEPC)
placebo hormonally intact	98,000 ± 10,000	5.8 ± 0.5	5500 ± 480
estrogen hormonally intact	118,000 ± 12,000	5.2 ± 0.4	6,100 ±470
progesterone hormonally intact	89,000 ± 2,700	5.5 ± 0.7	4900 ±120
estrogen +progesterone hormonally intact	98,000 ± 12,000	10.6 ± 1.1	10,400 ±1020
placebo hormonally deprived	20,000 ± 3,500	11.9 ± 0.8	2400 ± 340