

## Role of Label Retaining Cells in Estrogen Induced Endometrial Regeneration

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7 **Role of Label Retaining Cells in Estrogen Induced Endometrial**  
8 **Regeneration**

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28  
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31 epithelium and stroma in prepubertal and adult cycling mice.  
32

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**ABSTRACT**

Candidate stem/progenitor cells have been identified in mouse endometrium as label-retaining cells (LRC). The role of endometrial stem/progenitor cells in initiating estrogen-stimulated endometrial growth in pre-pubertal and cycling mice was investigated following a single  $17\beta$ -estradiol (E2) injection in BrdU-labelled and chased (LRC), ovariectomised mice. Proliferating ( $\text{BrdU}^+/\text{Ki-67}^+$ ) and mitotic ( $\text{BrdU}^+/\text{PH3}^+$ ) epithelial LRC were first detected in pre-pubertal mice 8 hours following E2 treatment, initiating the proliferative response. In contrast, all epithelial LRC and 16% of epithelial cells in cycling mice proliferated within 2 hours. In cycling mice, 12% of stromal LRC initiated a proliferative response 8 hours after E2. Proliferating epithelial LRC and most stromal LRC (85%) lacked estrogen receptor- $\alpha$  (ESR1). These findings suggest that endometrial epithelial LRC function as stem/progenitor cells by receiving proliferative signals from neighboring  $\text{ESR1}^+$  niche cells to initiate growth of the epithelium during development, while mature epithelial cells may undergo self replication in cycling endometrium.

## INTRODUCTION

The endometrium is one of the few tissues in the adult human that undergoes cyclical shedding and rapid regeneration. Each month, the human endometrial mucosa grows 4 to 10 mm over a period of 4-7 days following menses for over 400 cycles during the reproductive years.<sup>1-3</sup> This regenerative capacity of human endometrium is similar to that of epidermis, intestine and bone marrow. Recent evidence suggests that adult stem/progenitor cells present in human and mouse endometrium may be responsible for this remarkable regenerative capacity (reviewed in Gargett 2007, Gargett et al 2007).<sup>3,4</sup> Cell cloning analysis identified a rare population of human endometrial epithelial and stromal cells which exhibited high proliferative potential in both normal cycling<sup>5</sup> and inactive endometrium.<sup>6</sup> The large colony forming epithelial cells (CFU) exhibit self renewal and differentiate into large gland-like structures in 3D culture, while stromal CFU underwent substantial self renewal and multilineage differentiation into 4 mesodermal lineages<sup>7</sup> suggesting that human endometrium harbors epithelial progenitor and a mesenchymal stem cell-like cell populations.<sup>8</sup> Likewise both epithelial and stromal label-retaining cells (LRC) were identified in mouse endometrium and myometrium.<sup>9-11</sup> Epithelial and most stromal LRC did not express estrogen receptor- $\alpha$  (ER- $\alpha$ , ESR1) although 16% of stromal LRC were ESR1<sup>+</sup>.<sup>9</sup> Epithelial and stromal LRC underwent proliferation within 8 hr of 17- $\beta$  estradiol administration, suggesting that they have capacity to act as stem/progenitor cells and may be responsible for endometrial regeneration.<sup>9</sup> E2 is obligatory for uterine function, with defined roles in endometrial cell viability, survival and proliferation.<sup>12</sup> E2 elicits its effects via two estrogen receptors (ESR), ESR1 (ER- $\alpha$ ) and ESR2 (ER- $\beta$ ), both members of the steroid hormone superfamily of transcription factors. Although both isoforms are expressed in the uterus, ESR1 predominates and mediates the majority of E2 signals in mouse endometrium, while ESR2 may modulate the

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3 proliferative effects of E2<sup>13,14</sup>. ESR are first detected in fetal Müllerian duct,<sup>15</sup> however this  
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6 80 expression diminishes in the neonatal period and reappears in both endometrial epithelial and  
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8 stromal cells of juvenile and adult mice.<sup>16</sup>  
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10 Mice undergo cycles of growth and regression during each 4-5 day estrus cycle rather than a  
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12 menstrual cycle as seen in humans and other primates. The luminal epithelium proliferates in  
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14 response to increasing plasma E2 levels and undergoes apoptosis when E2 levels decline.<sup>17</sup>  
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17 85 These cellular dynamics observed during the estrus cycle can be reproduced in ovariectomized  
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19 (OVX) mice by exogenous sex steroid hormone administration, providing a tractable system for  
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21 examining cellular responses and their interplay with fluctuating hormone levels.  
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24 It is well accepted that stem/progenitor cells are found within the LRC population,<sup>18</sup> since LRC  
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26 are clonogenic,<sup>19</sup> demonstrate plasticity<sup>20</sup> and are involved in the regeneration of tissues.<sup>21</sup>  
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29 90 However, not all LRC are stem cells and it is important to demonstrate their functionality. In  
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31 several tissues where LRC have been identified, substantial damage or tissue ablation has been  
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33 used to provoke quiescent LRC into cell cycle to initiate tissue replacement.<sup>22</sup> LRC have also  
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35 been shown play a key role during natural growth and regression phases of the hair cycle without  
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37 resorting to ablation strategies.<sup>23,24</sup> The endometrium serves as another model for investigating  
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40 95 the role of LRC/stem/progenitor cells under dynamic physiological conditions without the need  
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42 to inflict tissue damage by radio- or chemotherapy. The fate of LRC can then be examined under  
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44 steady state conditions in one of the few actively remodeling mammalian tissues. In this study,  
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46 we hypothesized that endometrial LRC respond to E2 via ESR1<sup>+</sup> niche cells to enter the cell  
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48 cycle and initiate the process of cellular replacement associated with endometrial growth and  
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50 regeneration. Since there are differences in the sensitivity of endometrial epithelial cells to E2 in  
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52 100 neonatal and adult uteri,<sup>25</sup> the overall objective of this study was to compare the effect of E2 on  
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3 the proliferation kinetics of mouse endometrial epithelial and stromal LRC in prepubertal non-  
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5 cycling mice with those in mice undergoing regular estrus cycles. More specifically we wished to  
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7 examine whether epithelial and stromal LRC function as adult stem cells and proliferate in  
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9 response to E2 to initiate endometrial regeneration in an OVX mouse model after a single  
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11 105 physiological dose of E2. In this study, we showed a difference in the proliferative response to  
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13 estrogen in endometrium from animals that have previously cycled versus those that had not. Our  
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15 data suggest that whereas epithelial LRC play a major role in initiating E2-stimulated epithelial  
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17 cell growth during development in juvenile non-cycling endometrium they appear to have a  
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19 minor role in cycling endometrium. E2-induced stromal regeneration is considerably less than for  
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21 110 epithelium involving a minority of the stromal LRC.  
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## 27 **MATERIALS AND METHODS**

### 28 29 30 *Animals and Housing Conditions*

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33 All mouse husbandry and experimental procedures were conducted in compliance with the  
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35 115 protocols approved by the Monash Medical Centre Animal Ethics Committee A. Mice had access  
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37 to food and water *ad libitum* and were placed on a soy-free diet at ovariectomy. Mice were  
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39 housed under controlled environmental conditions at 20°C with a 12-hour dark/light cycle.  
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### 43 44 *Estrogen Treatment of BrdU Pulse Labeled and Chased Mice*

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46 All BrdU labeled mice (C57BL/6J) were generated by administering 6 subcutaneous injections of  
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48 120 BrdU in 0.9% saline from postnatal day (P) 3 to 5 (twice daily, at 9am and 4 pm for 3  
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50 consecutive days, 50µg/g of body weight) using our established protocol<sup>9</sup> and randomly  
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52 distributed into 2 groups. Mice were then allowed to grow without further labeling. One group  
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54 was chased for 4 weeks and the other 8 weeks after which mice were OVX and the endometrium  
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3 allowed to regress for 2 weeks, producing prepubertal never cycled LRC mice and cycling LRC  
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6 125 mice, respectively (Fig 1). These 2 groups of mice were randomly allocated into subgroups of 5-6  
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8 mice/group (104 mice used in total; CP4 wk, n=50; CP8wk, n=54) and given a single  
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10 subcutaneous E2 injection (17 $\beta$ -estradiol, 100ng/100 $\mu$ l, Sigma-Aldrich, St Louis, MO, USA) or  
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12 vehicle treatment (100 $\mu$ l peanut oil) and chased a second time for 0, 2, 8, 16, 24, 48, 72, 96 and  
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14 120 hours (n=5-6/timepoint/treatment, Fig 1A). Mice were euthanized by cervical dislocation and  
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17 130 the uterine horns harvested, the right horn fixed in 4% paraformaldehyde, the left in 10%  
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19 formalin for 2 hrs, and processed into paraffin blocks by standard techniques.  
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### 23 *BrdU/Ki-67 Immunohistochemistry and Immunofluorescence Protocols*

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26 To visualize proliferating LRC and their progeny, double immunohistochemistry (IHC) and  
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28 immunofluorescence (IF) protocols for BrdU/Ki67 were undertaken as previously described<sup>9</sup>  
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30 135 with several modifications. Following antigen retrieval (0.1 M citrate buffer, pH 6.0, microwave  
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32 for 20 min) and mild acid hydrolysis (0.1M HCl for 45 min), sheep anti-BrdU antibody (8.4  
33  
34  $\mu$ g/ml (Biodesign International, Saco, ME) was applied for 1 hr, then LSAB alkaline  
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36 phosphatase-conjugated streptavidin (Dako Cytomation, Glostrup, Demark) was incubated for 15  
37  
38 min followed by the Vector alkaline phosphatase substrate Kit III (Vector Laboratories Inc.,  
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40 Burlingame, CA, USA) for 10 min to visualize the BrdU<sup>+</sup> cells. Endogenous peroxidase was then  
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42 140 quenched (0.3% H<sub>2</sub>O<sub>2</sub>, Orion Laboratories Pty. Ltd., Welshpool, WA, Australia) for 10 min and  
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44 blocked with Protein Blocking Agent (Immunon Thermo Shandon, Pittsburgh, PA) for 10 min  
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46 before incubation with rabbit polyclonal Ki-67 antibody 0.15  $\mu$ g/ml, Novocastra Laboratories  
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48 Ltd, Newcastle upon Tyne, England) diluted in 0.1% BSA/PBS for 1 hr, followed by biotinylated  
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50 145 swine anti-rabbit IgG (Dako Cytomation) for 30 min, then peroxidase-conjugated streptavidin  
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52 (Dako Cytomation) for 15 min, and visualized with diaminobenzidine chromogen (Sigma-  
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3 Aldrich) for 5 min. Washing steps using PBS were conducted between each step and all  
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5 incubations were performed at room temperature unless otherwise specified. Isotype matched  
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7 negative controls, sheep IgG for BrdU (Sigma-Aldrich), and rabbit IgG for Ki-67 (Sigma-  
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9 Aldrich), at the same concentration as the primary antibodies were included in every staining run.  
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12 To examine for true co-localization of the proliferation marker Ki-67 and BrdU in LRC a double  
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14 IF protocol for BrdU/Ki67 was conducted using Alexa Fluor 488- and Alexa Fluor 568-  
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16 conjugated secondary antibodies respectively (Molecular Probes, Invitrogen Corp, Carlsbad CA)  
17  
18 as described.<sup>9</sup>  
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### 23 155 *Detection of ESR1 in Proliferating LRC using Immunofluorescence*

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26 To determine whether proliferating endometrial LRC co-expressed ESR1, a 3 µm serial section  
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28 was double IF stained with the proliferation marker Ki-67 and BrdU (as above) and a second 3  
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30 µm serial section was double IF stained with ESR1 (mouse anti-human estrogen receptor clone  
31  
32 6F11, 3.5 µg/ml) and BrdU as described.<sup>9</sup> The M.O.M basic kit (Vector Laboratories Inc.) was  
33  
34 used to prevent nonspecific binding of the ESR1 monoclonal antibody to mouse tissue Negative  
35  
36 160 control was an isotype matched mouse IgG. After staining with BrdU, sections were incubated  
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38 for 1 hr with M.O.M. mouse Ig blocking reagent, incubated with ESR1 antibody in M.O.M.  
39  
40 diluent for 30 min and incubated with M.O.M. biotinylated anti-mouse IgG for 10 min followed  
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42 by streptavidin Alexa Fluor 488 conjugate (Molecular Probes Inc.) for 30 minutes to detect  
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47 165 ESR1.  
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### 51 *BrdU/phosphorylated histone H3 (PH3) Immunohistochemistry and Immunofluorescence*

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53 To visualize mitotic LRC, double IHC and IF protocols for BrdU/PH3 were established. Paraffin  
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55 sections (5µm) were dewaxed, antigen retrieval and pre-treatment were as described for the BrdU  
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3 protocol.<sup>9</sup> Sections were then blocked with 10% donkey serum in 10% fetal calf  
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5 170 serum/0.1%BSA/PBS for 30 min and then incubated with rabbit polyclonal Phospho Histone-3  
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7 antibody (PH3, 0.02 µg/ml Cell Signaling Inc., Danvers, MA) in 0.1% BSA/PBS overnight at  
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9 4°C, followed by biotinylated swine anti-rabbit secondary antibody (Dako Cytomation) for 30  
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11 mins. Slides were washed with Tris buffered saline (TBS), incubated with ABC reagent (Vector  
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13 Laboratories Inc.) for 30 min and then with DAB chromogen (Sigma-Aldrich) for 5 min. The  
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15 175 subsequent BrdU staining was carried out as described<sup>9</sup> using Vector alkaline phosphatase  
16  
17 substrate Kit III as chromogen. To determine true co-localization of PH3 and BrdU in LRC, the  
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19 above protocol was adapted by substitution of secondary antibodies conjugated with  
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21 fluorochromes ie Alexa Fluor 568-conjugated donkey anti-sheep IgG (Molecular Probes) for  
22  
23 BrdU and Alexa Fluor 488-conjugated donkey anti-rabbit (Molecular Probes) to detect PH3.  
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25 180 Nuclei were counterstained with Hoechst 33258 (4 µg/ml, Molecular Probes) for 1 min, washed  
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27 with dH<sub>2</sub>O and coversliped using fluorescent mounting medium (Dako Cytomation). Isotype  
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29 matched negative controls: sheep IgG for BrdU (Sigma-Aldrich), and rabbit IgG for PH3 (Sigma-  
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31 Aldrich), at the same concentration as the primary antibodies were included in every staining run.  
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33 All IHC slides were examined under a Zeiss microscope (Axioskop, West Germany) and images  
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35 185 captured using a digital video camera (Fujix, West Germany). Dual immunofluorescence was  
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37 detected using a Leica confocal microscope (Leica Microsystem, Heerbrugg, Switzerland).  
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39 Images were captured with Leica confocal software version 2.5 and subsequently imported into  
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41 Adobe Photoshop (version 8.0, Adobe Systems Inc., San Jose, CA, USA) where they were  
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43 pseudocoloured, images processed and merged. Optical sectioning on the entire cell or nucleus  
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45 through its z plane was done to provide 3 dimensional images (x/y/z plane) to detect co-  
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localization of nuclear markers.

### *Assessment of Labeling Indices*

The number of BrdU<sup>+</sup>, Ki-67<sup>+</sup>, BrdU<sup>+</sup>Ki-67<sup>+</sup>, PH3<sup>+</sup>, BrdU<sup>+</sup>PH3<sup>+</sup> endometrial epithelial and stromal cells were counted separately in the entire area of single transverse and longitudinal sections from one uterine horn of 5-6 mice for each post-E2 time point for both 4 and 8 week chased groups in a blinded manner. The total number of epithelial and stromal nuclei were counted in each section of the double labeled chromogen-stained sections by RWS Chan using the AIS software (version 3.0; AIS, Ontario, Canada); at least 200-1600 nuclei per uterine horn per mouse at each time point. All epithelial and stromal LRC (BrdU<sup>+</sup>, BrdU<sup>+</sup>Ki-67<sup>+</sup>, BrdU<sup>+</sup>PH3<sup>+</sup>) nuclei were counted in each section examined. The labeling index (LI) for BrdU<sup>+</sup>, Ki-67<sup>+</sup>, BrdU<sup>+</sup>Ki-67<sup>+</sup>, PH3<sup>+</sup>, BrdU<sup>+</sup>PH3<sup>+</sup> cells was calculated as a percentage of total epithelial or stromal cells for each section and reported as means  $\pm$  SEM for each experimental group per time point. Only heavily immunostained BrdU, Ki-67 and PH3 nuclei were counted as labeled cells and those that had a speckled or faded appearance were considered to have undergone several cell divisions with subsequent dilution of the BrdU label and were not counted. Double immunofluorescence slides were not assessed for LI in this study.

### *Statistical Analysis*

Statistical analysis was performed using GraphPad Prism (version 4.00, GraphPad Software, San Diego, CA). Data were tested for homogeneity of variance using Bartlett test and was found significant, therefore nonparametric tests were used. The Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparison test were used for comparison between post-E2 time points within the treatment groups. Data are presented as mean  $\pm$  SEM. Results were considered statistically significant when  $P < 0.05$ .

## **RESULTS**

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3 215 *Role of LRC in Initiating Epithelial Proliferation in Prepubertal Endometrium*  
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6 To establish the role of LRC in mediating E2 stimulated endometrial epithelial growth during the  
7 final stages of endometrial development, prepubertal LRC mice were OVX to regress the  
8 endometrium and treated with a single 17 $\beta$ -estradiol injection (Fig 1A). Within 14 days of OVX,  
9 the luminal epithelium is substantially diminished, glands have virtually disappeared and there is  
10 dense packing of the remaining stromal cells (Fig 1B-C). On administration of E2 the  
11 endometrium rapidly regenerated with growth of glands, expansion of luminal epithelial cells to  
12 pre-OVX numbers, and production of an enlarged edematous stroma (Fig 1D). The proliferation  
13 kinetics of endometrial epithelial cells and LRC were then examined during the first 120 hr of  
14 E2-induced endometrial regeneration in OVX mice. The percentage of epithelial LRC (BrdU<sup>+</sup>)  
15 220 was constant over this period with an average LI of  $1.29 \pm 0.18\%$  (n=9 time points, 4-6  
16 animals/group) (Fig 2A-F). The irregular shape of many BrdU<sup>+</sup> nuclei (eg Fig 2) is due to the  
17 incorporation of BrdU into the non-uniformly distributed chromatin. As BrdU<sup>+</sup> cells undergo cell  
18 division and the BrdU label dilutes, the heterogeneity of staining becomes more obvious.<sup>26,27</sup>  
19 Proliferating epithelial cells (Ki-67<sup>+</sup>) were absent during the first 2 hours (Fig 2A, E) and were  
20 first detected in the luminal epithelium 8 hours after E2 (Fig 2B, D, E). Proliferating epithelial  
21 230 cells increased significantly over time (P<0.05, 0 and 2 hr vs 72, 96, 120 hr), reaching a  
22 maximum of  $18.7 \pm 1.8\%$  (n=5) at 120 hours (Fig 2E). By 16 hr Ki-67<sup>+</sup> epithelial cells were  
23 observed directly adjacent to epithelial LRC (results not shown) and at 24 hr nascent glands  
24 began invaginating into the stroma (results not shown). Ki-67<sup>+</sup> epithelial cells were not detected  
25 in vehicle control prepubertal LRC mice 24 hr following injection with oil (n=3) (data not  
26 shown). These results confirm that in prepubertal mice, luminal and glandular epithelium  
27 proliferate in response to E2.<sup>28-30</sup> Proliferating epithelial LRC (BrdU<sup>+</sup>Ki-67<sup>+</sup>) were first detected  
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3 at 8 hr (Fig 2B, D, E), peaked at 8 hr and remained relatively constant thereafter (Fig 2E).

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5 Nuclear co-localisation of BrdU and Ki-67 is indicated by optical sectioning in confocal images

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8 240 and shown as strong yellow signals in the XZ and YZ planes (Fig 2D). BrdU<sup>+</sup>Ki-67<sup>+</sup> cells were

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10 located in the luminal epithelium rather than the glands (Fig 2D) as previously reported.<sup>9</sup> Since

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12 all epithelial LRC (BrdU<sup>+</sup>) at 8 hr were found to co-localise with Ki-67<sup>+</sup>, this indicates that the

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14 first cells to proliferate in prepubertal mice after E2 treatment were the epithelial LRC.

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16 Since Ki-67 is expressed throughout the cell cycle (late G1, S, G2 and M phase) we used the

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19 245 mitosis marker, PH3 to examine a more precise stage of the cell cycle. As expected fewer PH3<sup>+</sup>

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21 were observed compared to Ki-67<sup>+</sup> cells (Fig 2E, F, note scale differences in the Y axes). There

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24 were no PH3<sup>+</sup> epithelial cells at 0 and 2 hr after E2 (Fig 2F) and they first appeared in the luminal

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27 epithelium at 8 hr (Supplementary Fig 1A) reaching a peak of  $6.73 \pm 0.40\%$  (n=4) at 24 hr (Fig

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30 2C, F) ( $P < 0.05$ , 0, 2 and 8 hr vs 24 hr), gradually declining to 1.8% by 120 hr (Fig 2F). There

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32 250 was one round of mitosis (Fig 2F). Mitotic epithelial LRC (PH3<sup>+</sup>BrdU<sup>+</sup>) first appeared at 8 hr

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34 (Fig 2F, Supplementary Fig 1A) reached a maximum at 24 hr ( $P < 0.01$ , 0 and 2 hr vs 24 hr) and

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36 declined thereafter (Fig 2F). Thus in non-cycling juvenile endometrium, luminal epithelial LRC

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39 were the first epithelial cells to enter cell cycle and undergo mitosis in response to E2.

#### 40 41 42 *Role of LRC in Initiating Epithelial Proliferation in Adult Cycling Endometrium*

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45 255 The role of epithelial LRC in mediating E2-stimulated endometrial epithelial regeneration in

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47 adult LRC mice, which had experienced 5-6 estrus cycles, was examined in 8 week BrdU pulse-

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49 labeled and chased, OVX mice given a single E2 injection using a similar protocol as for

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51 prepubertal mice (Fig 1A). As expected epithelial LRC (BrdU<sup>+</sup>) were present at a similar

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54 frequency ( $1.81 \pm 0.28\%$ , n=9 time points, 4-6 animals/group,  $P > 0.05$ ) as in the prepubertal mice

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57 260 (Fig 2G-L), despite significant luminal epithelial proliferation that occurs with each estrus cycle.

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3 At 0 hr,  $5.4 \pm 1.6\%$  (n=6) of epithelial cells were Ki-67<sup>+</sup> (Fig 2G, K) despite 14 days without  
4 ovarian E2 and a phytoestrogen-free diet. Ki-67<sup>+</sup> epithelial cells were only found in the luminal  
5 epithelium as most glands had regressed following OVX (Fig 2G). In adult control mice injected  
6 with vehicle, a similar small percentage of KI-67<sup>+</sup> epithelial cells were detected (data not shown).  
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13 265 Two hr after E2,  $16.3 \pm 6.6\%$  of epithelial cells were Ki-67<sup>+</sup> and at 8 hr many were found in the  
14 lumen (Fig 2H, Supplementary Fig 1B) and newly forming glands (Fig 2H). The majority of  
15 epithelial cells were in cell cycle by 48 hr (LI =  $88.9 \pm 4.7\%$ , Fig 2K). Proliferating epithelial  
16 LRC (BrdU<sup>+</sup>Ki-67<sup>+</sup>) were not detected at 0 time, but by 2 hr most LRC were Ki-67<sup>+</sup> and the LI  
17 for BrdU<sup>+</sup>Ki-67<sup>+</sup> epithelial cells was similar ( $0.57 \pm 0.28\%$ ) to that for epithelial LRC ( $1.75 \pm$   
18  $0.57\%$ , n=5, P=0.50) (Fig 2K inset) indicating that all epithelial LRC participated in the  
19 proliferative response to E2.  
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30 There was also a basal level of mitotic epithelial cells in 14 day regressed endometrium (0 hr  
31 after E2, LI  $1.16 \pm 0.37\%$  n=6) (Fig 2L) increasing to  $6.43 \pm 0.97\%$ , n=6) at 8 hr (Fig 2L),  
32 reaching a maximum of  $7.65 \pm 1.81\%$ , (n=6) at 16 hr (Fig 2I, L), decreasing to baseline levels at  
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37 275 96 hours (Fig 2L) and showing one round of mitosis after a single E2 injection. BrdU<sup>+</sup>PH3<sup>+</sup>  
38 epithelial cells were not observed until 8 hr after E2 (Fig 2H, L), and all LRC were in mitosis at  
39 this time point. This E2-induced increase in mitotic LRC was significant at 8, 16, 24 and 48 hr  
40 compared to 0 and 2 hr (P < 0.05) and all epithelial LRC underwent mitosis between 16 ( $0.91 \pm$   
41  $0.16\%$ , n=6) (Fig 2I, J, L) and 96 hr ( $0.59 \pm 0.16\%$ , n =6) (Fig 2L). It appears that in cycling  
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49 280 endometrium, luminal and glandular epithelial cells are primed to rapidly respond to E2 by  
50 dramatically increasing their numbers.  
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### *Role of LRC in Initiating Stromal Proliferation in Adult Cycling Endometrium*

The proliferative response of stromal LRC to E2 stimulation in regressed endometrium was only examined in the 8 wk chased BrdU-labeled mice, since stromal cell turnover was inadequate in pre-pubertal mice to sufficiently dilute the BrdU label. This is reflected in the high LI of stromal LRC ( $23.7 \pm 5.7\%$ , n=9) for prepubertal mice after a 4 wk chase. In cycling endometrium, stromal LRC (BrdU<sup>+</sup>) were relatively constant in number over the 0-120 hr period after E2 (Fig 3A, C, D, F, G) with an average LI of  $9.14 \pm 0.87\%$  (n=9 time points, 6 animals/group) confirming previously published data.<sup>9</sup> Proliferating (Ki-67<sup>+</sup>) stromal cells were first detected 8 hr after E2 treatment with a LI of  $2.55 \pm 0.89\%$ , n=6 (Fig 3A, D, F). Proliferating stromal cells gradually increased reaching significance at 16 hr ( $16.6 \pm 3.4\%$ , n= 6, P <0.05) and a maximum at 48 hr ( $23.6 \pm 7.46\%$ , n= 6, P < 0.05) after E2 and returned to baseline by 120 hours (Fig 3C, F). Ki-67<sup>+</sup> stromal cells were not detected in control adult LRC mice 24 hr after injection with oil (data not shown). Proliferating stromal LRC (BrdU<sup>+</sup>/Ki-67<sup>+</sup>) were first detected 8 hr after E2 treatment (Fig 3A, D, F) and were situated near the endometrial-myometrial junction (Fig 3A, D) or adjacent to the luminal epithelium. In contrast to proliferating epithelial LRC, only approximately one in 8 (12%) of stromal LRC commenced proliferation at 8 hr (LI of  $1.22 \pm 0.43\%$ , n=6) after E2 treatment (Fig 3F). The BrdU<sup>+</sup>/Ki-67<sup>+</sup> LI for stromal cells then gradually increased over time peaking at 24 hours ( $6.52 \pm 1.44\%$ , n = 6, P < 0.001) (Fig 3B) after E2, remaining significantly elevated at 48 and 72 hr compared to 0 and 2 hr (P < 0.05) and declining to baseline by 120 hours (Fig 3F). BrdU<sup>+</sup>/Ki-67<sup>+</sup> stromal cells were not detected in adult control LRC mice injected with oil (data not shown). These data suggest that a small proportion of stromal LRC are gradually recruited into the cell cycle after E2 treatment of regressed adult mouse endometrium.

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3 305 Very few stromal cells underwent mitosis and these were first detected 8 hours (LI  $0.57 \pm 0.07\%$ ,  
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16 310 cells were not observed in control adult LRC mice injected with vehicle (data not shown). In  
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Very few stromal cells underwent mitosis and these were first detected 8 hours (LI  $0.57 \pm 0.07\%$ ,  $n=6$ ) (Fig 3E, G), gradually increasing to  $3.46 \pm 1.29\%$   $n=6$ ,  $P < 0.001$ ) at 96 hr after E2 and diminishing to baseline at 120 hr (Fig 3G). Mitotic stromal LRC were not detected until 24 hr after E2 (Fig 3G) ( $0.32 \pm 0.04\%$   $n=6$ ) and these gradually increased until 96 hr ( $1.17 \pm 0.40\%$ ,  $n=6$ ,  $P < 0.01$ ) and then declined (Fig 3G) in parallel with mitotic stromal cells. BrdU<sup>+</sup>KI-67<sup>+</sup> cells were not observed in control adult LRC mice injected with vehicle (data not shown). In general all mitotic stromal cells were LRC, suggesting that this subpopulation of LRC may be the mesenchymal stem-like cells previously observed in mouse and human endometrium<sup>9,31</sup> likely responsible for initiating stromal replacement during E2-induced regeneration in mouse endometrium.

### 315 *Association of ESR1 Expression with Proliferating Endometrial LRC*

Our previously published data showed that epithelial LRC did not express ESR1 and that 16% of stromal LRC were ESR1<sup>+</sup>.<sup>9</sup> We therefore examined ESR-1 expression in proliferating epithelial and stromal LRC from juvenile and cycling mouse endometrium in 3  $\mu\text{m}$  serial sections, double IF stained with BrdU/Ki-67 and BrdU/ESR-1 respectively. In prepubertal endometrium, proliferating luminal epithelial LRC (Fig 4A) did not immunostain with ESR-1 despite neighboring non-proliferating, non-LRC epithelial cells showing cytoplasmic immunostaining as previously reported<sup>14</sup> (Fig 4B). In contrast, some stromal LRC located near the endometrial-myometrial junction (data not shown) and near the luminal epithelium in cycled adult endometrium co-localised both Ki-67 (Fig 4C) and ESR1 (Fig 4D), suggesting that E2 can directly induce a subpopulation of ESR1<sup>+</sup> stromal LRC to proliferate. In contrast, it appears that epithelial LRC respond indirectly to E2 via paracrine signals from surrounding ESR-1<sup>+</sup> stromal niche cells to initiate endometrial regeneration.



## DISCUSSION

Epithelial and stromal LRC have recently been identified as candidate stem/progenitor cells in mouse endometrium, the cyclically regenerating mucosal lining of the uterus.<sup>9-11</sup> In the present study, we have shown that in a prepubertal model of endometrial regeneration ESR1<sup>-</sup> epithelial LRC function as progenitor cells by initiating E2-stimulated growth of glands and luminal epithelium, suggesting an important role for epithelial progenitor cells in regulating endometrial gland development. In contrast, in adult cycling mice, both epithelial LRC and non LRC rapidly entered the cell cycle and underwent mitosis in response to estrogen to replenish glandular and luminal epithelium in estrogen-depleted mice. These findings suggest that in cycling mice, LRC play a minor role in the cyclical production of endometrial epithelium and that mature epithelial cells may undergo self-duplication to replenish estrogen-sensitive epithelial cells depleted in the ovariectomy model. Alternatively there may be many more epithelial progenitors in adult mouse endometrium than are detectable as LRC. While stromal growth during the cyclical turnover of endometrial epithelium is modest in comparison, our data shows that a small proportion (12%) of stromal LRC function as stromal stem/progenitor cells (or mesenchymal stem/stromal cells) enabling a modest expansion of the mouse endometrial stromal compartment diminished by estrogen deprivation. Thus a proportion of the stromal LRC proliferated in response to E2 to provide adequate stromal support tissue for the rapidly expanding nascent glands and luminal epithelium. These differential responses of endometrial epithelial and stromal LRC at different physiological developmental stages suggests unique regulation of down-stream effectors of estrogen signaling in stimulating and maintaining cellular homeostasis in this dynamically remodeling tissue.



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3 350 While there is evidence that adult stem cells are relatively quiescent compared to their progeny  
4 and can be detected as LRC in situ, it is essential to demonstrate their functionality as  
5 stem/progenitor cells.<sup>19</sup> A cell undergoing its final cell division at the time of BrdU labeling will  
6 become a LRC, but has no capacity for cell division or tissue regeneration, no matter the length  
7 of the chase period. A key function of adult stem cells is to maintain tissue homeostasis and  
8 regenerate tissue following injury or tissue loss.<sup>3</sup> In this study we capitalized on the estrogen  
9 sensitivity of endometrial cells and used ovariectomy to regress the endometrium and then  
10 administered a single 17 $\beta$ -estradiol injection to study the cellular kinetics of estrogen-stimulated  
11 endometrial growth. We showed, through co-localisation of LRC and the proliferation marker Ki-  
12 67 or mitosis marker PH3, that almost all epithelial LRC proliferated in response to 17 $\beta$ -estradiol  
13 in both the prepubertal and cycling mouse models at the commencement of the epithelial  
14 proliferative response (8 hr and 2 hr respectively), indicating their capacity to function as adult  
15 stem/progenitor cells and restore endometrial epithelial homeostasis. In contrast, only 12% of  
16 stromal LRC initiated stromal cell proliferation upon estrogen replacement. Many of these  
17 stromal LRC were at the endometrial-myometrial junction or directly beneath the luminal  
18 epithelium suggesting that these cells have stromal/mesenchymal stem cell activity and are  
19 responsible for stromal tissue homeostasis. These data also indicate the relative cellular turnover  
20 rates in mouse endometrium, with significantly greater rates observed for the epithelium than  
21 stroma as previously reported.<sup>29,30</sup> We predict that these same stromal LRC would also proliferate  
22 in response to progesterone to initiate stromal expansion associated with the decidual response,  
23 particularly the periluminal stromal LRC. Our recent unpublished observations on stromal LRC  
24 distribution using a mouse model of menstrual breakdown and repair (MMBR)<sup>26</sup> suggest that  
25 stromal cells proliferate more readily in a protocol where progesterone is administered to induce  
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3 a decidual response. Therefore we consider that the 12% of endometrial stromal LRC that  
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5 initiated estrogen-induced endometrial stromal regeneration are likely candidate  
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8 375 stromal/mesenchymal stem cells because of their ability to proliferate and generate endometrial  
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10 stroma, an adult stem cell property. In contrast, we consider the 88% of stromal LRC that failed  
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12 to proliferate in response to estrogen are likely transit amplifying cells and that an 8 week chase  
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14 was insufficient to fully dilute the label due to their relatively low cell turnover rate. We also do  
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16 not consider this population to be candidate endometrial mesenchymal stem/stromal cells.  
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20 380 Together these studies indicate that all epithelial LRC have proliferative capacity and are likely  
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22 stem/progenitor cells initiating regenerative responses after estrogen stimulation in both the  
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24 prepubertal and adult cycling models, while only a small proportion of stromal LRC function as  
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26 stem/progenitor cells.  
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29 Many different protocols have been used to assess uterine proliferative responses to sex steroid  
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32 385 hormones. Some use multiple priming doses of estrogens, short or long acting estrogens in both  
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34 immature or cycling rodents, and yet others use single dose estrogen exposures.<sup>32,33</sup> In order to  
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36 assess the whether LRC functioned as stem/progenitor cells, we chose a single dose (5 µg/kg) of  
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38 17β-estradiol, sufficient to initiate a robust proliferative response as shown in the present study.  
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40 In using both immature and adult cycling mice in our models of endometrial regeneration, we  
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44 390 demonstrated a striking difference in the magnitude of the proliferative response as measured by  
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46 Ki-67 immunohistochemistry, and mitoses by PH3 immunoreactivity. A slower and protracted  
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48 proliferative response was observed in prepubertal LRC mice with one round of mitosis (Fig 2F),  
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50 in which all epithelial LRC proliferated and drove the response. Non-labeled epithelial cells also  
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52 participated in the regenerative response subsequent to LRC proliferation suggesting that  
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56 395 daughter cells generated from LRC also proliferated. In adult mice which had undergone at least  
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3 4 estrus cycles prior to ovariectomy, there was a basal level of Ki-67<sup>+</sup> and mitotic epithelial cells  
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5 in estrogen-depleted mice, and the proliferative response to 17 $\beta$ -estradiol was extremely rapid  
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7 with 16% of epithelial cells Ki-67<sup>+</sup> within 2 hours and 60% within 16 hr of treatment, similar to  
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9 previously reported values.<sup>34,35</sup> This rapid proliferation of epithelial cells may result from the  
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11 shortening of the cell cycle; mainly at the expense of G1, S and possibly G0 phase.<sup>36</sup> While all  
12 400  
13 epithelial LRC proliferated, the majority of glandular and luminal epithelial cells had proliferated  
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15 by 48 hours (Fig 2L), and undergone one or perhaps 2 rounds of mitosis. This differential  
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17 response suggests that even in previously cycled OVX mice, the remaining epithelial cells are  
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19 very sensitive to rising estrogen levels and are primed for proliferation. Our data also suggests  
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21 that while all epithelial LRC proliferate in response to E2 stimulation, they do not appear to  
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23 initiate epithelial proliferation in regressed adult cycling mouse endometrium. It is possible that  
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25 BrdU-label retention may not be as sensitive or as specific as a marker of stem/progenitor cells in  
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27 adult (compared to juvenile) endometrium as is the case for haemopoietic stem cells.<sup>37</sup> Further, it  
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29 is possible that maintenance of cycling mouse endometrial epithelium does not rely primarily on  
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31 stem/progenitor cells, but rather depends on expansion of differentiated mature epithelial cells via  
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33 self-replication as previously shown for the pancreas.<sup>38,39</sup> These possibilities can only be  
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35 distinguished using genetic lineage tracking systems such as *Cre-lox* where the *Cre* gene is  
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37 expressed under an endometrial epithelial specific promoter to excise floxed stop codons flanking  
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39 an *EGFP* or *LacZ* reporter gene.<sup>40</sup>  
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48 415 An alternate hypothesis emanating from our data is that there are 2 populations of epithelial  
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50 stem/progenitor cells with 2 different niches in endometrial epithelium, with different roles in  
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52 maintaining epithelial cell homeostasis. One population may engage in the long term growth  
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54 associated with development of the glands, while the second population rapidly contributes to the  
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3 immediate production of epithelial cells as has been demonstrated in other adult stem cell niches,  
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6 420 including the bone marrow, intestine, hair follicle and brain.<sup>40-43</sup> We suggest that the longterm  
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8 endometrial epithelial stem/progenitor cells are detectable as LRC in the luminal epithelium of  
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10 mouse endometrium labeled with BrdU during postnatal life (this study).<sup>9</sup> The short term, rapidly  
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12 responsive endometrial stem/progenitor cell population has not yet been definitively identified to  
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14 date, although previous studies using a mouse model of menstrual breakdown and repair suggest  
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17 425 this population may be found within a glandular niche.<sup>26</sup> A feature of the rapidly responsive  
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19 stem/progenitor population is their responsiveness to tissue damage, which is often a key  
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21 component of the models used to study this population in bone marrow and intestine. The  
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23 induction of a menstruation-like event through progesterone administration and subsequent  
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25 withdrawal causing decidual tissue breakdown and shedding in mice that do not normally  
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27 menstruate is akin to invoking tissue damage. In this model where BrdU labeling is done in adult  
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29 430 mice during an artificially induced “proliferative” stage, it appears that the cells ultimately  
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31 responsible for repairing luminal epithelium might come from a second population of slowly  
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33 cycling cells within the glands in a repair process that does not require estrogen<sup>44</sup>. It would  
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35 appear that the luminal epithelium contains both a rapidly dividing transit amplifying population  
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41 435 that respond rapidly to cell shedding in regions where the lumen is undergoing repair, and a long  
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43 term progenitor cell population that selectively responds to estrogen-induced growth signals. In  
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45 adult mice, this population may be located in segments of the lumen distant to the shedding  
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47 regions and not undergoing repair. As mentioned above, genetic lineage tracing analysis is  
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49 required to determine the relative roles of the potentially 2 epithelial stem/progenitor cell  
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53 440 populations in mouse endometrium and their precise niche locations.  
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3 In this study we have demonstrated that the LRC populations rapidly proliferate in response to E2  
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5 in estrogen-depleted mice, despite their lack of ESR-1 expression. It would appear that epithelial  
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7 LRC do not respond directly to E2 but rather may receive E2 signals indirectly from surrounding  
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9 ESR-1<sup>+</sup> stromal niche cells via paracrine signals to initiate endometrial regeneration and regulate  
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11 cell fate decisions.<sup>3</sup> Thus epithelial LRC responsiveness to E2 mirrors earlier studies  
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13 445 demonstrating that the mitogenic effect of E2 on endometrial epithelial cells is indirect and  
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15 mediated via stromal cell ESR-1.<sup>45,46</sup> Furthermore, ESR-2, present in endometrium at very low  
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17 levels<sup>47</sup> may modulate estrogen activity by inhibiting ESR-1 to maintain proliferation and  
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19 differentiation of the endometrium.<sup>14,48</sup> Further studies are required to conclusively demonstrate a  
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21 role for ESR<sup>+</sup> niche cells in regulating epithelial LRC function in regenerating endometrial  
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23 450 epithelium.  
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29 Characterisation of endometrial stromal LRC for pluripotency or adult stem cell markers has  
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31 demonstrated that a small proportion co-express OCT-4 (6%) and c-KIT (20%).<sup>10</sup> Telomerase  
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33 which functions to maintain telomeres in proliferating stem cells has been detected in human  
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35 455 endometrium, particularly in the basalis layer<sup>49</sup>. Telomerase activity has also been identified in  
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37 human endometrial Side Population (SP) cells, a small population considered to be adult stem  
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39 cells.<sup>50</sup> It would be of interest to determine whether telomerase is expressed in murine  
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41 endometrial epithelial or stromal LRC. Similarly, Musashi-1, an RNA binding protein which  
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43 functions in epithelial stem/progenitor cell self renewal has been identified in single epithelial  
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45 460 cells and small clusters of stromal cells in the basalis of human endometrium,<sup>51</sup> suggesting that  
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47 Musashi-1 may be a marker of endometrial stem/progenitor cells. Given the known role of  
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49 Musashi-1 in stem/progenitor cell function, it will be important that future studies examine  
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51 whether mouse endometrial epithelial and stromal LRC co-express Musashi-1. Further, markers  
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3 that have been used to purify human endometrial mesenchymal stem-like cells should also be  
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6 465 examined for colocalisation with BrdU<sup>+</sup> stromal LRC in mouse endometrium.<sup>31</sup> Such studies will  
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8 determine if these markers are conserved between human and mouse endometrium.  
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11 In conclusion, we have demonstrated distinct proliferative responses of candidate endometrial  
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13 epithelial stem/progenitor cells (LRC) to E2 in prepubertal non-cycled and adult cycling  
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15 endometrium. Our data has shown that ESR-1<sup>-</sup> epithelial LRC drive E2-induced epithelial growth  
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18 470 in the developing endometrium of juvenile mice, but play a minor role in regenerating the  
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20 endometrium in cycling mice. Alternatively, there may be many more epithelial stem/progenitor  
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22 cells in cycling mouse endometrium than LRC and fewer stromal stem/progenitors than stromal  
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24 LRC. Our data also demonstrate that epithelial and stromal LRC survive periods of E2  
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26 withdrawal as expected of candidate stem/progenitor cells. Despite the lack of ESR-1 on  
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29 475 epithelial LRC, both epithelial and stromal LRC have the capacity to rapidly respond to E2  
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31 replacement, proliferate and participate in endometrial expansion, an important feature if LRC  
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33 harbor the endometrial stem/progenitor cell population. Furthermore, the lack of ESR-1 suggests  
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35 an important role of stromal niche cells in mediating epithelial LRC proliferative responses to  
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38 estrogenic stimuli. Future studies will seek to identify the niche cells of endometrial  
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41 480 stem/progenitor cells and to investigate whether mature epithelial cells contribute to endometrial  
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43 regeneration in the estrus cycles of adult female mice.  
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## FIGURE LEGENDS

### **Figure 1 – Experimental Strategy for examining the role of LRC in mediating Estrogen Induced Endometrial Regeneration.**

(A) C57BL6 mice were given 6 bromodeoxyuridine (BrdU) injections for 3 days, commencing postnatal day 3 (P3). The BrdU label was chased for 4 (prepubertal non-cycled) or 8 (adult cycling) wk, then mice were ovariectomized (OVX) to allow the endometrium to regress for 14 days and then given a single injection of 17- $\beta$  estradiol (E2). Tissue was harvested 0 – 120 hr later as indicated ( $\blacktriangledown$ ). (B-D) Hematoxylin and eosin staining of transverse mouse uterine sections. (B) Normal 35 day old (P35) prepubertal endometrium, comprising an extensive luminal epithelium, glands, stroma and myometrium. (C) Regressed endometrium from mouse OVX (14 days later) at P49, showing a decreased diameter, a contracted lumen, few glands and densely packed stroma. (D) E2 induced endometrial regeneration (8 hour after E2 injection) showing extensive growth of luminal epithelium, nascent glands and edematous stroma. Scale bar = 50 $\mu$ m. Abbreviations: ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium.

### **Figure 2 – E2-induced Epithelial Proliferation in Non-Cycled Prepubertal and Adult Cycling Endometrium.**

(A-D) **Non-cycled prepubertal endometrium:** 4-week chased OVX mice double immunostained for BrdU (blue) and Ki67 (brown) to visualize epithelial LRC (BrdU, blue arrows), proliferating epithelial cells (Ki67, brown arrows), and proliferating epithelial LRC (BrdU/Ki67, black arrows) at (A) 0 and (B) 8 hr after E2. Double immunostaining for BrdU (blue) and PH3 (brown) to visualize epithelial mitotic epithelial cells (PH3, brown arrows) and

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3 mitotic epithelial LRC (BrdU/PH3, black arrows) at (C) 24 hr after E2 Double  
4 immunofluorescence staining of BrdU (red) and Ki67 (green) to visualize (D) proliferating  
5 epithelial LRC (white arrow) 8 hrs post-E2 treatment. The x/z and y/z planes obtained from  
6 optical sectioning are shown on the far right and beneath the merged images and demonstrate true  
7 co-localisation of both markers. Negative control (D) for red (inset) and green (inset)  
8 fluorochrome. Labeling Indices (LI) of endometrial epithelial cells for (E) Ki67 and (F).PH3  
9 shown as mean  $\pm$  SEM (n=4-6 animals/time point). (G-J) **Adult cycling endometrium:** 8-week  
10 chased OVX mouse uteri double immunostained for BrdU (blue) and Ki67 (brown) to visualize  
11 epithelial LRC (BrdU, blue arrows), proliferating epithelial cells (Ki67, brown arrows),  
12 proliferating epithelial LRC (BrdU/Ki67, black arrows) at (G) 0 and (H) 8 after E2. Section  
13 double immunostained for BrdU (blue) and PH3 (brown) to visualize epithelial LRC (BrdU, blue  
14 arrows) and mitotic epithelial cells (PH3, brown arrows) and mitotic epithelial LRC (BrdU/PH3,  
15 black arrows) at (I) 16 hrs post-E2 treatment. Double immunofluorescence staining of BrdU (red)  
16 and PH3 (green) to visualize (J) mitotic epithelial LRC (white arrow) at 16 hrs post-E2 treatment.  
17 LI of endometrial epithelial cells for (K) Ki67, 0-120 hr (inset shows the first 8-16 hr); (L) PH3  
18 shown as mean  $\pm$  SEM (n=6 animals/time point). White dotted lines outline the epithelium in  
19 (D,J). Scale bars; 50 $\mu$ m (A-C, G-I), 20 $\mu$ m (D) and 40 $\mu$ m (J) ge, glandular epithelium; le,  
20 luminal epithelium; s, stroma.  
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### 48 **Figure 3- E2-induced Stromal Proliferation in Adult Cycling Endometrium.**

49 (A-E) **Adult cycling endometrium:** 8-week chased OVX mouse uteri double immunostained for  
50 BrdU (blue) and Ki67 (brown) to visualize stromal LRC (BrdU, blue arrows), proliferating  
51 stromal cells (Ki67, brown arrows), proliferating stromal LRC (BrdU/Ki67, black arrows), at (A)  
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3 8 and (C) 120hr after E2. Double immunostained for BrdU (blue) and PH3 (brown) to visualize  
4 stromal LRC (BrdU, blue arrows), mitotic stromal cells (PH3, brown arrows), mitotic stromal  
5 LRC (BrdU/PH3, black arrows), at (B) 24hr post-E2 treatment. Double immunofluorescence  
6 staining of BrdU (red) and Ki67 (green) to visualize (D) proliferating stromal LRC (white arrow)  
7 and (E) mitotic stromal LRC (white arrow) at 8 hrs post-E2 treatment. LI for (F) Ki67; (G) PH3  
8 shown as mean  $\pm$  SEM (n=6 animals/time point). Scale bar; 50 $\mu$ m (A-C) and 40 $\mu$ m (D, E).  
9 Dotted line indicates endometrial-myometrial junction. ge, glandular epithelium; le, luminal  
10 epithelium; s, stroma; myo, myometrium.  
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25 **Figure 4 - ESR1 Expression in Proliferating Mouse Endometrial Epithelial and Stromal**  
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29 Endometrial serial sections (3 $\mu$ m) at 8 hrs post-E2 treatment from prepubertal non-cycled (CP4  
30 wk) (A,B) and adult cycling (CP8 wk) (C,D) mice double immunofluorescence stained with (A)  
31 BrdU (red), Ki67 (green) and (B) BrdU (red), ESR1 (green). The x/z and y/z planes shown on the  
32 far right and underneath the merged pictures, demonstrate the co-expression of BrdU/Ki67 (A),  
33 and lack of BrdU/ESR1 (B) co-expression of the same epithelial LRC nucleus located in the  
34 luminal epithelium (white arrow), and co-expression of BrdU/Ki-67 (C) with BrdU/ESR1 (D) in  
35 the same stromal LRC nucleus located near the luminal epithelium (white arrow). Both (A, B)  
36 and (C, D) are images of the same field of view of serial sections of mouse endometrium.  
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55 **Supplementary Figure 1**



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3 (A) Double immunofluorescence staining of BrdU (red) and PH3 (green) to visualize mitotic  
4 epithelial LRC (white arrow) 8 hrs post-E2 in non-cycled prepubertal endometrium. (B) Double  
5 immunofluorescence staining of BrdU (red) and Ki-67 (green) to visualize proliferating epithelial  
6 LRC (white arrow) and proliferating epithelial cells (yellow arrows) 8 hrs post-E2 treatment in  
7 adult cycling endometrium.  
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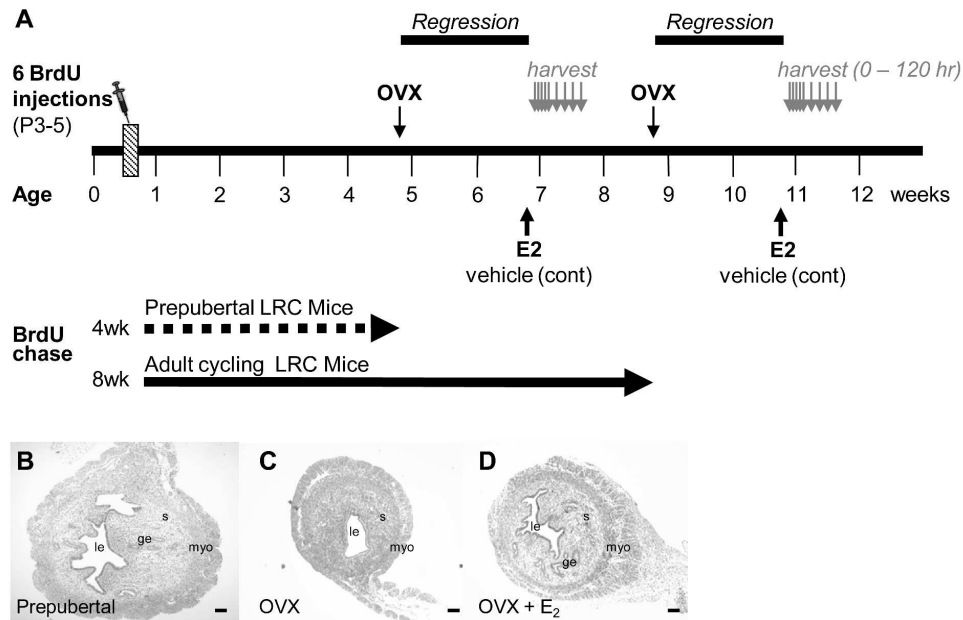


Figure 1 – Experimental Strategy for examining the role of LRC in mediating Estrogen Induced Endometrial Regeneration.

(A) C57BL6 mice were given 6 bromodeoxyuridine (BrdU) injections for 3 days, commencing postnatal day 3 (P3). The BrdU label was chased for 4 (prepubertal non-cycled) or 8 (adult cycling) wk, then mice were ovariectomized (OVX) to allow the endometrium to regress for 14 days and then given a single injection of 17- $\beta$  estradiol (E<sub>2</sub>). Tissue was harvested 0 – 120 hr later as indicated (□). (B-D) Hematoxylin and eosin staining of transverse mouse uterine sections. (B) Normal 35 day old (P35) prepubertal endometrium, comprising an extensive luminal epithelium, glands, stroma and myometrium. (C) Regressed endometrium from mouse OVX (14 days later) at P49, showing a decreased diameter, a contracted lumen, few glands and densely packed stroma. (D) E<sub>2</sub> induced endometrial regeneration (8 hour after E<sub>2</sub> injection) showing extensive growth of luminal epithelium, nascent glands and edematous stroma. Scale bar = 50 $\mu$ m. Abbreviations: ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium.

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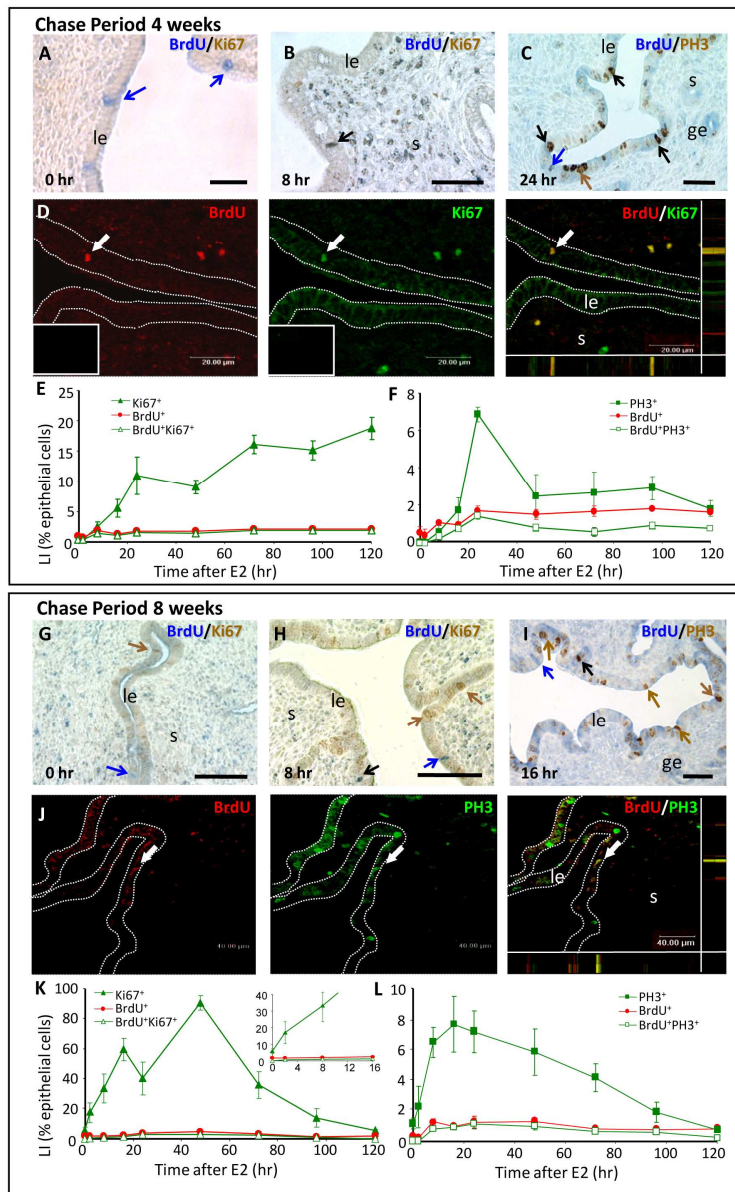


Figure 2 – E2-induced Epithelial Proliferation in Non-Cycled Prepubertal and Adult Cycling Endometrium.

(A-D) Non-cycled prepubertal endometrium: 4-week chased OVX mice double immunostained for BrdU (blue) and Ki67 (brown) to visualize epithelial LRC (BrdU, blue arrows), proliferating epithelial cells (Ki67, brown arrows), and proliferating epithelial LRC (BrdU/Ki67, black arrows) at (A) 0 and (B) 8 hr after E2. Double immunostaining for BrdU (blue) and PH3 (brown) to visualize epithelial mitotic epithelial cells (PH3, brown arrows) and mitotic epithelial LRC (BrdU/PH3, black arrows) at (C) 24 hr after E2. Double immunofluorescence staining of BrdU (red) and Ki67 (green) to visualize (D) proliferating epithelial LRC (white arrow) 8 hrs post-E2 treatment. The x/z and y/z planes obtained from optical sectioning are shown on the far right and beneath the merged images and demonstrate true co-localisation of both markers. Negative control (D) for red (inset) and green (inset) fluorochrome. Labeling Indices (LI) of endometrial epithelial cells for (E) Ki67 and (F).PH3 shown as mean  $\pm$  SEM (n=4-6 animals/time point). (G-J) Adult cycling endometrium: 8-week

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3 chased OVX mouse uteri double immunostained for BrdU (blue) and Ki67 (brown) to visualize  
4 epithelial LRC (BrdU, blue arrows), proliferating epithelial cells (Ki67, brown arrows), proliferating  
5 epithelial LRC (BrdU/Ki67, black arrows) at (G) 0 and (H) 8 after E2. Section double immunostained  
6 for BrdU (blue) and PH3 (brown) to visualize epithelial LRC (BrdU, blue arrows) and mitotic  
7 epithelial cells (PH3, brown arrows) and mitotic epithelial LRC (BrdU/PH3, black arrows) at (I) 16  
8 hrs post-E2 treatment. Double immunofluorescence staining of BrdU (red) and PH3 (green) to  
9 visualize (J) mitotic epithelial LRC (white arrow) at 16 hrs post-E2 treatment. LI of endometrial  
10 epithelial cells for (K) Ki67, 0-120 hr (inset shows the first 8-16 hr); (L) PH3 shown as mean  $\pm$  SEM  
11 (n=6 animals/time point). White dotted lines outline the epithelium in (D,J). Scale bars; 50 $\mu$ m (A-C,  
12 G-I), 20 $\mu$ m (D) and 40 $\mu$ m (J) ge, glandular epithelium; le, luminal epithelium; s, stroma.  
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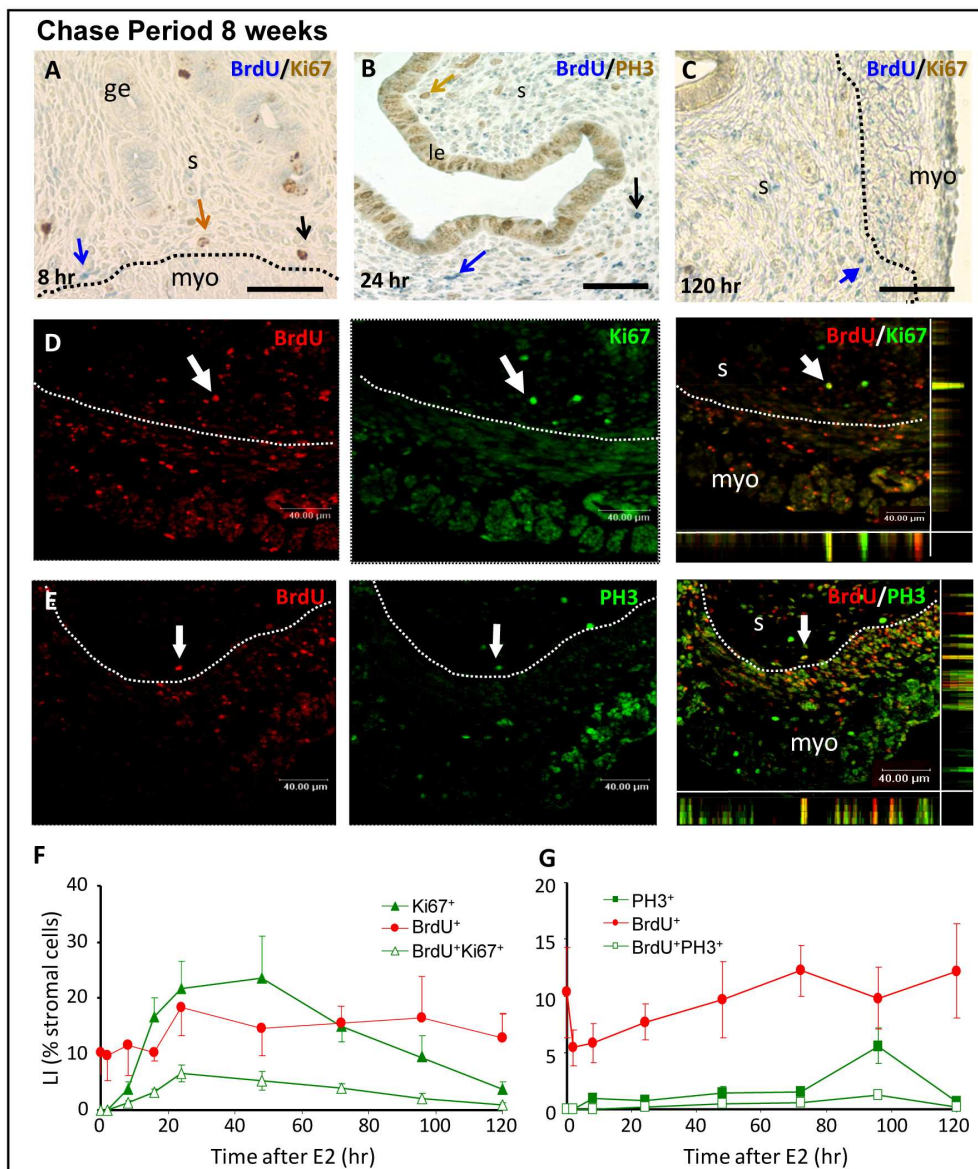


Figure 3- E2-induced Stromal Proliferation in Adult Cycling Endometrium. (A-E) Adult cycling endometrium: 8-week chased OVX mouse uteri double immunostained for BrdU (blue) and Ki67 (brown) to visualize stromal LRC (BrdU, blue arrows), proliferating stromal cells (Ki67, brown arrows), proliferating stromal LRC (BrdU/Ki67, black arrows), at (A) 8 and (C) 120hr after E2. Double immunostained for BrdU (blue) and PH3 (brown) to visualize stromal LRC (BrdU, blue arrows), mitotic stromal cells (PH3, brown arrows), mitotic stromal LRC (BrdU/PH3, black arrows), at (B) 24hr post-E2 treatment. Double immunofluorescence staining of BrdU (red) and Ki67 (green) to visualize (D) proliferating stromal LRC (white arrow) and (E) mitotic stromal LRC (white arrow) at 8 hrs post-E2 treatment. LI for (F) Ki67; (G) PH3 shown as mean  $\pm$  SEM (n=6 animals/time point). Scale bar; 50 $\mu$ m (A-C) and 40 $\mu$ m (D, E). Dotted line indicates endometrial-myometrial junction. ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium. 152x180mm (300 x 300 DPI)

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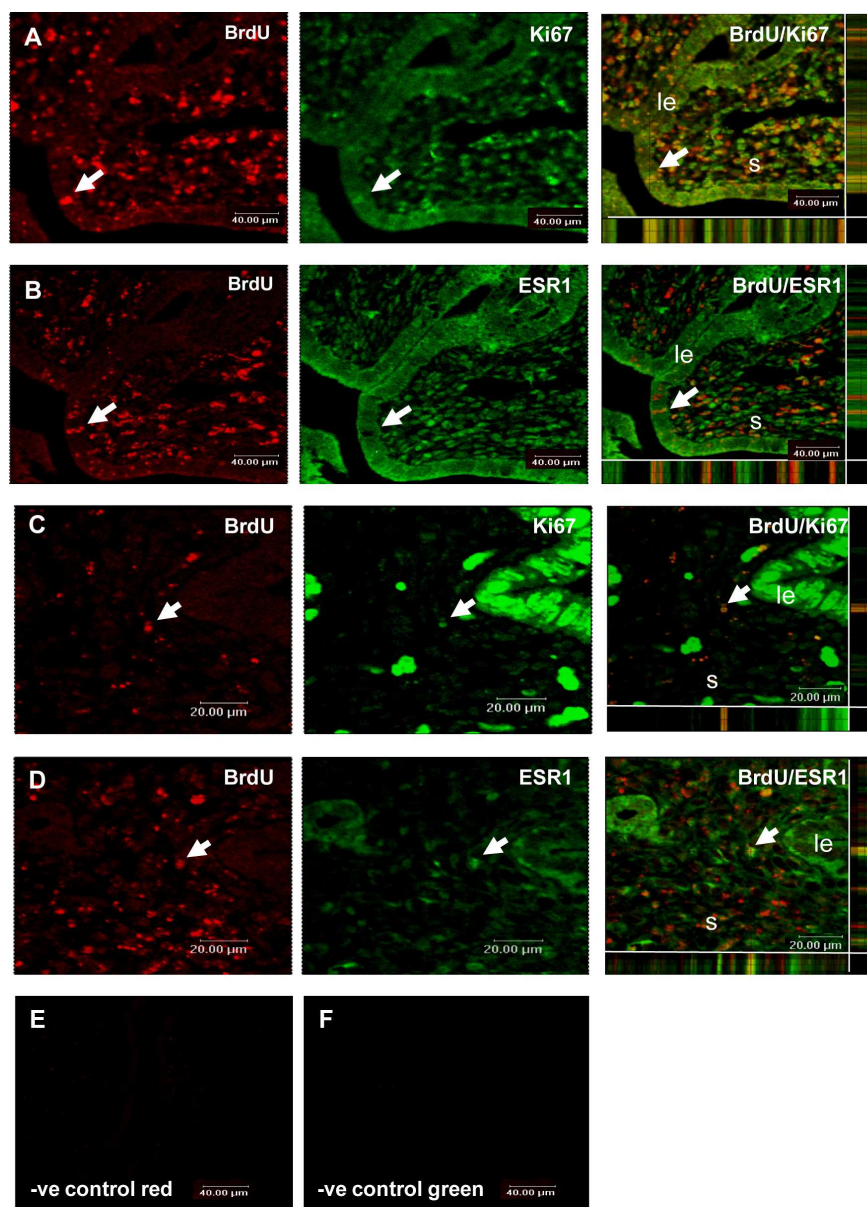


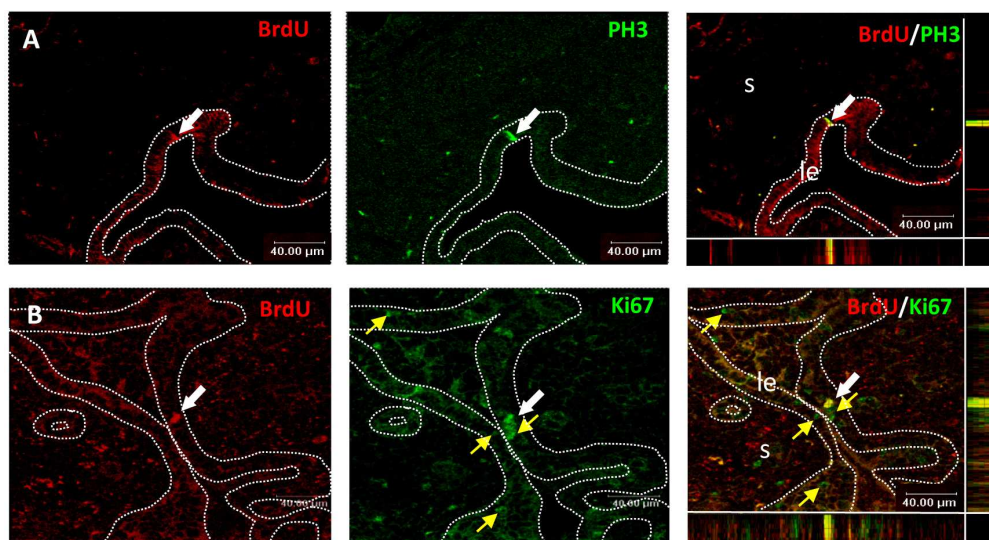
Figure 4 - ESR1 Expression in Proliferating Mouse Endometrial Epithelial and Stromal LRC. Endometrial serial sections (3 $\mu$ m) at 8 hrs post-E2 treatment from prepubertal non-cycled (CP4 wk) (A,B) and adult cycling (CP8 wk) (C,D) mice double immunofluorescence stained with (A) BrdU (red), Ki67 (green) and (B) BrdU (red), ESR1 (green). The x/z and y/z planes shown on the far right and underneath the merged pictures, demonstrate the co-expression of BrdU/Ki67 (A), and lack of BrdU/ESR1 (B) co-expression of the same epithelial LRC nucleus located in the luminal epithelium (white arrow), and co-expression of BrdU/Ki-67 (C) with BrdU/ESR1 (D) in the same stromal LRC nucleus located near the luminal epithelium (white arrow). Both (A, B) and (C, D) are images of the same field of view of serial sections of mouse endometrium. Negative (isotype) control for (E) BrdU (red) and (F) ESR1 (green). Scale bars: 40 $\mu$ m (A, B, E, F) and 20 $\mu$ m (C, D). le, luminal epithelium; s, stroma.

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Supplementary Figure 1: (A) Double immunofluorescence staining of BrdU (red) and PH3 (green) to visualize mitotic epithelial LRC (white arrow) 8 hrs post-E2 in non-cycled prepubertal endometrium. (B) Double immunofluorescence staining of BrdU (red) and Ki-67 (green) to visualize proliferating epithelial LRC (white arrow) and proliferating epithelial cells (yellow arrows) 8 hrs post-E2 treatment in adult cycling endometrium.  
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