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ORIGINAL RESEARCH

The mouse endometrium contains epithelial, endothelial and leucocyte populations expressing the stem cell marker telomerase reverse transcriptase

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STUDY HYPOTHESIS: The mouse endometrium harbours stem/progenitor cells that express the stem cell marker mouse telomerase reverse transcriptase (mTert).

STUDY FINDING: We used a mouse carrying a transgenic reporter for *mTert* promoter activity to identify rare endometrial populations of epithelial and endothelial cells that express mTert.

WHAT IS KNOWN ALREADY: Stem/progenitor cells are hypothesized to be responsible for the remarkable regenerative capacity of the endometrium, but the lack of convenient endometrial stem/progenitor markers in the mouse has hampered investigations into the identity of these cells.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: A mouse containing a green fluorescent protein (GFP) reporter under the control of the telomerase reverse transcriptase promoter (*mTert*-GFP) was used to identify potential stem/progenitor cells in the endometrium. *mTert* promoter activity was determined using fluorescence microscopy and flow cytometry to identify GFP^+ cells. GFP^+ cells were examined for epithelial, stromal, endothelial, leucocyte and proliferation markers and bromodeoxyuridine retention to determine their identity. The endometrium of ovariectomized mice was compared to that of intact cycling mice to establish the role of ovarian hormones in maintaining mTert-expressing cells.

MAIN RESULTS AND THE ROLE OF CHANCE: We found that *mTert*-GFP is expressed by rare luminal and glandular epithelial cells (0.3% of epithelial cells by flow cytometry), rare $CD45^-$ cells in the stromal compartment (0.028 ± 0.010% of stromal cells by microscopy) and many $CD45^+$ leucocytes. Ovariectomy resulted in significant decrease of *mTert*-GFP^+ epithelial cells ($P = 0.029$ for luminal epithelium; $P = 0.034$ for glandular epithelium) and a decrease in the percentage of *mTert*-GFP^+ $CD45^+$ leucocytes in the stromal compartment ($P = 0.015$). However, $CD45^-$ *mTert*-GFP^+ cells in the stromal compartment were maintained in ovariectomized mice. This population is enriched for cells bearing the endothelial marker CD31 (10.3% of $CD90^-$ $CD45^-$ and 97.8% $CD90^+$ $CD45^-$ by flow cytometry). $CD45^-$ *mTert*-GFP^+ cells also immunostained for the endothelial marker von Willebrand factor. These results suggest that the endometrial epithelium and vasculature are foci of stem/progenitor activity and provide a system to investigate molecular mechanisms involved in endometrial regeneration and repair.

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LIMITATIONS, REASONS FOR CAUTION: The stem/progenitor activity of endometrial *mTert*-GFP^{+} cells needs to be experimentally verified.

WIDER IMPLICATIONS OF THE FINDINGS: The identification and characterization of mTert-expressing progenitor cells in the mouse will facilitate the identification of equivalent populations in the human endometrium that are likely to be involved in endometrial function, fertility and disease.

LARGE-SCALE DATA: Not applicable.

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Key words: endometrium / endothelial / epithelial / leucocyte / mesenchymal stem cell / ovariectomy / progenitor / stromal / telomerase / regeneration

Introduction

The endometrium is the highly regenerative lining of the uterus and undergoes ~400 cycles of shedding and regeneration during a woman's reproductive years. Each month 7–10 mm of endometrial tissue is generated, making the endometrium one of the most cyclically regenerative tissues in the human body. The endometrium is essential for implantation and insufficient regeneration results in infertility (Gargett *et al.*, 2012). Conversely, over exuberant or inappropriate regeneration of the endometrium can cause endometrial hyperplasia, cancer and endometriosis (Gargett, 2007). The regenerative ability of the endometrium has been attributed to endometrial mesenchymal stem/stromal cells (eMSCs) and epithelial progenitors (Gargett *et al.*, 2012).

Evidence for endometrial stem/progenitor cells was demonstrated in cell cloning studies of human endometrial single cell suspensions. Individual colonies generated *in vitro* at very low seeding densities (5–20 cells/cm^2) undergo differentiation, and possess the ability to reconstitute endometrium *in vivo* (Masuda *et al.*, 2012; Gargett *et al.*, 2009). The most studied of these is the eMSC population that expresses a range of MSC markers, including CD90 and CD44 (Gargett *et al.*, 2009). Studies of mouse endometrium have identified CD44^{+} tissue reconstituting epithelial progenitors (Janzen *et al.*, 2013) and label retention studies have identified slow cycling epithelial and stromal cells that participate in endometrial regeneration (Chan and Gargett, 2006; Chan *et al.*, 2012; Cervello *et al.*, 2007). However, the lack of a directly detectable genetic marker for mouse endometrial stem/progenitor cells has hindered progress in understanding the cell biology of endometrial regeneration. To address this shortcoming, we have investigated mouse telomerase reverse transcriptase (mTert) expression as a marker for stem/progenitor cells in the mouse endometrium.

mTert is part of the telomerase complex that maintains telomere length and allows cells to undergo repeated rounds of division without succumbing to senescence triggered by loss of telomeres (Blackburn, 1990). Telomerase reverse transcriptase is the catalytic and rate limiting component of telomerase and is expressed by stem/progenitor cells (Blackburn, 2005). Transgenic reporter constructs driven by the *mTert* promoter mark stem/progenitor cells in the bone marrow, the intestine and testis (Breault *et al.*, 2008; Montgomery *et al.*, 2011). Slow cycling label-retaining cells in the intestine also express *mTert*-green fluorescent protein (GFP) (Breault *et al.*, 2008). Telomerase activity has been reported in the endometrium (Hapangama *et al.*, 2008) and the successful use of mTert expression as a stem cell marker in the examples cited above suggests that mTert expression may be a useful marker of stem/progenitor cells in the endometrium. Here, we examine whether *mTert* promoter activity identifies stem/progenitor cells in the mouse endometrium.

Materials and Methods

mTert reporter mice

mTert-GFP reporter mice on a C57BL/6J background were bred in the Monash Medical Centre Animal Facility. These mice contain a transgene with the *mTert* promoter driving the expression of green fluorescent protein (GFP). *mTert*-GFP expression identifies hematopoietic stem cells and intestinal epithelial stem cells and mice carrying the transgene display normal behaviour and fertility (Breault *et al.*, 2008). C57BL/6J mice were used as a control in flow cytometry experiments. All mouse studies were approved in advance by the Monash Medical Centre Animal Ethics Committee, according to the National Health and Medical Research Council of Australia guidelines.

Fluorescence microscopy

Uteri from *mTert*-GFP mice were immersion fixed in 4% w/v paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 3–5 h or at 4°C overnight, cryoprotected with 30% w/v sucrose in PBS overnight at 4°C, frozen in Optimal Cutting Temperature medium (Sakura Finetek, Netherlands) and cryosectioned with a Leica CM1850 Cryostat (Leica Microsystems GmbH, Wetzlar, Germany). GFP was detected by its endogenous fluorescence unless stated otherwise. Sections were stained with primary antibodies (Table I) for 1 h and visualized either directly or with secondary antibodies (Supplementary data, Table S1). Isotype control antibodies were used to confirm the specificity of CD44, CD45, CD90 and EpCAM immunostaining (Supplementary data, Fig. S1). Nuclei were counterstained with 5 μg/ml Hoechst 33258 (Molecular Probes) and images captured using a Nikon C1 confocal microscope (Nikon, Tokyo, Japan) with a 20× air or 60× oil objective. Captured images were adjusted for brightness and contrast in a linear manner using FIJI (Schindelin *et al.*, 2012).

Bromodeoxyuridine retention

Post-natal bromodeoxyuridine (BrdU) loading was as previously described (Chan and Gargett, 2006). Briefly, female *mTert*-GFP pups were injected twice daily with 100 μg of BrdU (Sigma-Aldrich, St. Louis, MO) in 50 μl PBS on post-natal Days 3–5 via an intraperitoneal route. Mice were killed at 9 weeks of age and uteri prepared for fluorescence microscopy as

Table I Details of antibodies used for immunofluorescence.

Antibody	Clone/product number	Dilution	Supplier	Isotype control	Specificity			
					Epi	Str	Leu	End
GFP	GFP-1020	1:500	Aves Labs	Not used[a]	?	?	±	?
CD45-PE/APC	30-F11	1:100	eBioscience	Rat IgG2b	−	−	+	−
CD44-APC	IM7	1:100	eBioscience	Rat IgG2b	±	±	±	−
von Willebrand factor	A0082	1:800	Dako	Rabbit IgG	−	−	−	+
EpCAM	G8.8	1:200	eBioscience	Rat IgG2a	+	−	−	−
CD90.2-APC	53-2.1	1:50	eBioscience	Rat IgG2a	−	+	±	±
Ki67	MM1	1:500	Leica	Rabbit IgG	±	±	±	±

[a]GFP negative samples used to verify specificity.
Epi, epithelial; Str, stromal; Leu, leucocyte, End, endothelial, PE, phycoerythrin, APC, allophycocyanin; EpCAM, epithelial cell adhesion molecule.

Table II Details of antibodies used for flow cytometry.

Antibody	Clone	Dilution	Isotype control	Supplier	Specificity			
					Epi	Str	Leu	End
EpCAM-eFluor450 or -PE	G8.8	1:50	Rat IgG2a	eBioscience	+	−	−	−
CD90.2-APC	53-2.1	1:100	Rat IgG2a	eBioscience	−	+	±	±
CD44-APC	IM7	1:100	Rat IgG2b	eBioscience	±	±	±	−
CD45-eFluor450 or -PE	30-F11	1:100	Rat IgG2b	eBioscience	−	−	+	−
CD31-eFluor450	390	1:100	Rat IgG2a	eBioscience	−	−	−	+

Epi, epithelial; Str, stromal; Leu, leucocyte; End, endothelial; EpCAM, epithelial cell adhesion molecule; APC, allophycocyanin; PE, phycoerythrin.

described above. To preserve GFP fluorescence, BrdU was detected using treatment with 500 U/ml DNaseI (Worthington Biochemical, Lakewood, NJ, USA) in 40 mM Tris/HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$ for 10 min at room temp, then detected with sheep anti-BrdU (1:100, M20105S, Meridian Life Science, Memphis, TN USA) with chicken anti-sheep AlexaFluor 647.

Oestrous staging and ovariectomy

Vaginal smears were stained with toluidine blue and the oestrous stage determined on the basis of epithelial cell morphology and the absence/presence of leucocytes (Byers *et al.*, 2012). For ovariectomy, mice were anaesthetized with ketamine/xylazine, ovariectomized via a dorsal incision and the endometrium allowed to regress for at least 2 weeks on a soy-free diet to ensure no dietary phytoestrogens contributed to observed responses (Specialty Feeds, Glen Forrest, WA, Australia).

Quantification of *mTert*-GFP, CD44 and CD45 in the endometrium

Longitudinal sections of *mTert*-GFP uterine horns were examined for GFP^+ cells in the epithelial layer or immunostained with CD45-allophycocyanin (APC) (eBioscience) or CD45-phycoerythrin (PE) and CD44-APC as described above. Ten 461 × 461 μm fields sampling the length of the uterus were captured for each mouse. For quantification of GFP and CD45, FIJI (Schindelin *et al.*, 2012) was used to calculate luminal epithelial length and stromal area in each section by manual tracing. The number of nuclei in the defined stromal area was calculated in FIJI using threshold, watershed and analyse particle functions. The area occupied by CD45 immunostaining in the stromal regions was calculated using the threshold and measure area functions. Epithelial *mTert*-GFP^+ and $CD44^+$ cells, *mTert*-GFP^+ $CD45^-$ stromal cells, and *mTert*-GFP^+ $CD45^+$ stromal cells were counted manually. Data were analysed using GraphPad PRISM (GraphPad Software, La Jolla, USA).

Flow cytometry

Fresh uteri were dissected from female *mTert*-GFP mice of mixed oestrous cycle (8–12 weeks old, $n = 12$ as indicated in legends) or age matched GFP negative C57BL6 wild type controls. Uteri for each group were pooled, finely minced and enzymatically digested with 0.5% w/v collagenase Type I (Worthington Biochemical) in PBS with 5 mM glucose and 20 μl/ml deoxyribonuclease type I (Worthington) at 37°C for 1 h on a rotator. Dissociated tissues were then filtered through a 40-μm cell strainer (BD Bioscience), centrifuged at 230*g* for 5 min at 4°C and resuspended in 1% v/v fetal bovine serum (FBS) (Life Technologies) in PBS (FBS/PBS).

Cells were incubated with directly conjugated primary antibodies (Table II). Antibody stained cells were washed and resuspended in 1% FBS/PBS. Fluorochrome-conjugated isotype controls and unlabelled controls were included for each antibody and were used for flow cytometry gating (Table II). Flow cytometry analysis was performed using a FACSCanto II flow cytometer with FACSDiva Software (BD Biosciences, Le Pont-de-Claix, France).

Statistical analysis

Statistical analysis was performed with GraphPad Prism v6 (GraphPad Software) and data were analysed with an unpaired two-tailed *t*-test. Differences

were considered statistically significant when $p < 0.05$. Data are presented as mean ± SEM.

Results

mTert-GFP expression marks stromal and epithelial subpopulations in the endometrium

In adult female cycling mice, *mTert*-GFP marked minor subpopulations in the endometrial stromal compartment and rare epithelial cells in luminal (Fig. 1A and B) and glandular epithelium (not present in Fig. 1, but shown in Fig. 4E). Immunostaining with anti-GFP colocalized with GFP fluorescence and verified the GFP$^+$ status of these stromal (Fig. 1C) and epithelial cells (Fig. 1D). The morphology of stromal *mTert*-GFP$^+$ cells ranged from rounded to flattened with multiple projections and the intensity of GFP detected varied greatly suggesting that the stromal *mTert*-GFP$^+$ population is heterogeneous (Fig. 1C). Flattened *mTert*-GFP$^+$ cells were observed lining voids at the junction of the endometrium with the myometrium (Fig. 1A).

mTert-GFP expression does not predict a label-retaining or proliferative phenotype

A BrdU retention assay was used to identify endometrial label-retaining cells as previously reported (Chan and Gargett, 2006) and determine their relationship to *mTert*-GFP$^+$ cells. Post-natal administration of BrdU and an 8 week chase produced label-retaining cells in the stromal compartment of the endometrium and the myometrium (Fig. 2A and B). BrdU retention and *mTert*-GFP were mutually exclusive in stromal cells as judged by the examination 309 *mTert*-GFP$^+$ cells and 119 BrdU-retaining cells from the endometrium of three mice. Epithelial BrdU-retaining cells were not detected, possibly due to the length of the chase period, and the reduced sensitivity of the DNase-mediated BrdU detection protocol employed to preserve GFP fluorescence, as opposed

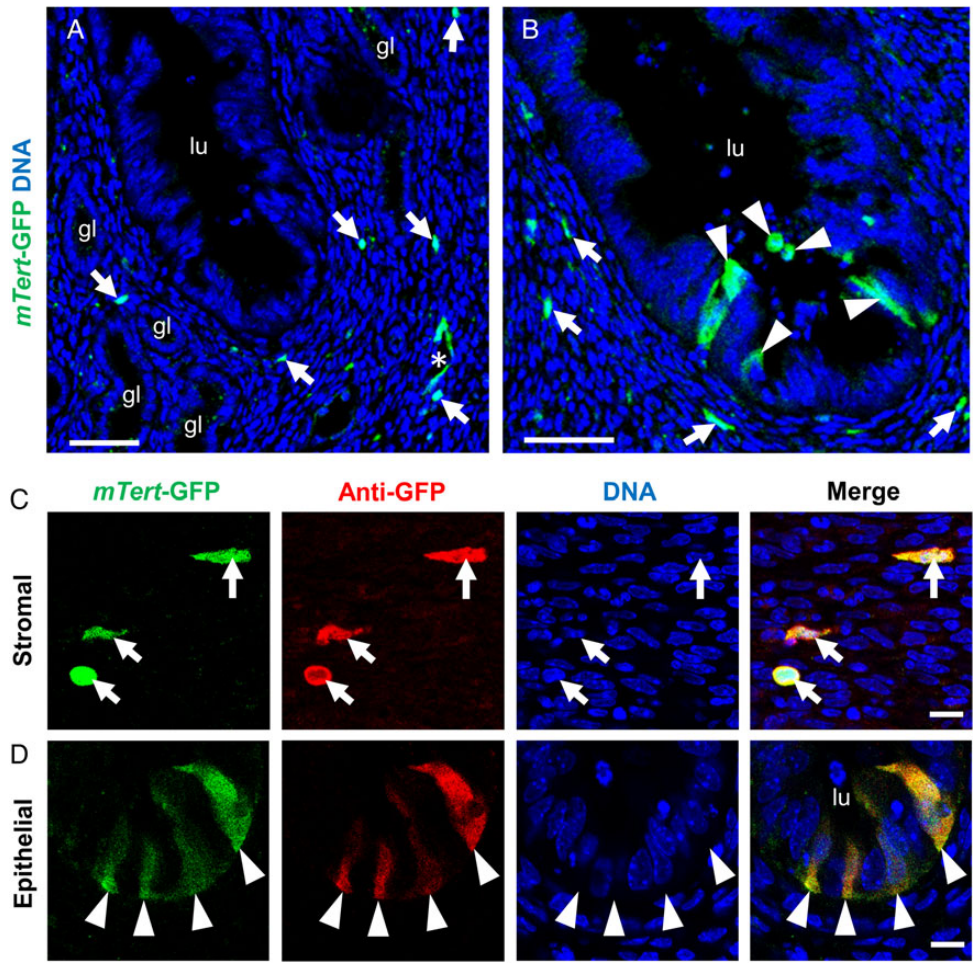


Figure 1 *mTert*-GFP marks cells in the stromal and epithelial compartments of the endometrium. (**A** and **B**) GFP$^+$ cells (green) are scattered throughout the stroma (arrows) including examples surrounding a void that may be a vessel (*, A). Rare epithelial cells are GFP$^+$ (arrowheads, B). (**C** and **D**) GFP expression was confirmed in the stromal cells (arrows, C) and epithelial cells (arrowheads, D) by overlapping patterns of GFP fluorescence (green) and anti-GFP immunostaining (red). DNA is stained with Hoechst 33258 (blue). Scale bars: A and B = 20 μm; C and D = 10 μm. lu, luminal epithelium; gl, gland.

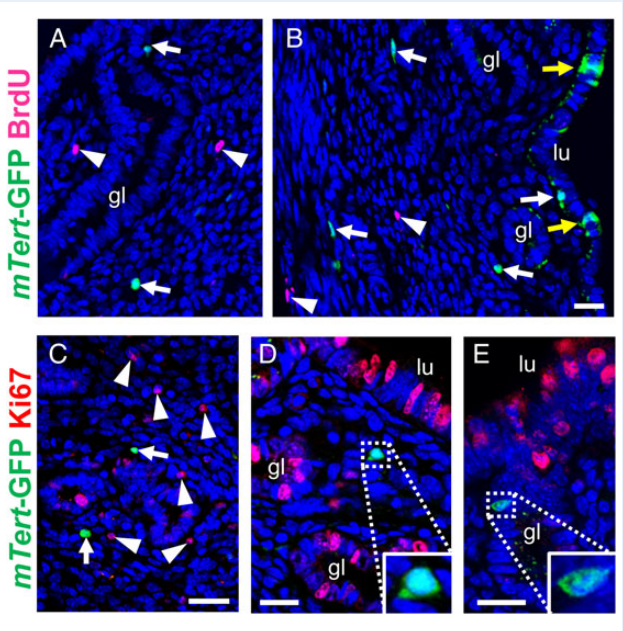


Figure 2 *mTert*-GFP expression does not predict a label-retaining or proliferative phenotype. (**A** and **B**) *mTert*-GFP (green) and BrdU (magenta) in the uterus after an 8 week chase. Stromal *mTert*-GFP$^+$ (white arrows, A and B) and epithelial *mTert*-GFP$^+$ cells (yellow arrows, B) do not overlap with BrdU-retaining cells (A and B, arrowheads). (**C**, **D** and **E**) *mTert*-GFP (green) and the proliferation marker Ki67 (red) in adult cycling endometrium. (C) *mTert*-GFP (arrows) does not overlap with Ki67 immunostaining (arrowheads). (D) Luminal and glandular epithelial cells immunostained for the proliferation marker Ki67 do not express *mTert*-GFP, while a nearby stromal *mTert*-GFP$^+$ cell (inset) does not express Ki67. (E) An *mTert*-GFP$^+$ cell within a gland (inset) that is Ki67$^-$. gl, gland; lu, luminal epithelium. DNA is stained with Hoechst 33258 (blue). Scale bars = 20 μm; A is at the same magnification as B. Insets in (D) and (E) are at an additional 2.5 times zoom.

to HCl used previously (Chan and Gargett, 2006; Cervello *et al.*, 2007; Chan *et al.*, 2012).

We examined whether *mTert*-GFP$^+$ cells were actively proliferating in adult cycling endometrium, a property that might account for their failure to retain BrdU. The nuclear proliferation marker Ki67 immunostained stromal cells (Fig. 2C) and many epithelial cells (Fig. 2D and E). *mTert*-GFP expression did not correlate with Ki67 immunostaining in epithelial or stromal cells (Fig. 2C–E).

Characterization of rare epithelial *mTert*-GFP$^+$ epithelial cells

Epithelial *mTert*-GFP$^+$ cells were detected based on their expression of the epithelial marker EpCAM which is not expressed by leucocytes, vascular or stromal cells. Overlapping immunostaining of EpCAM and GFP was detected infrequently in the luminal epithelium by immunofluorescence microscopy (Fig. 3A). Flow cytometry of dissociated uterus was used to examine EpCAM and GFP co-expression. A population negative for both CD31 and CD90 was examined to exclude endothelial cells, stromal cells and many leucocytes (black box, Fig. 3B). A small population of epithelial *mTert*-GFP$^+$ cells (0.3% of EpCAM$^+$) was detected in *mTert*-GFP uterus (Fig. 3C and D).

The influence of ovarian hormones on endometrial epithelial *mTert*-GFP expression was examined in hormonally intact mice with an oestrous cycle and hormonally deprived ovariectomized mice. No differences in epithelial *mTert*-GFP expression were detected between diestrus, pro-oestrus, oestrus and metestrus phases (data not shown). Mice undergoing an oestrous cycle had significantly higher numbers of *mTert*-GFP$^+$ luminal and glandular epithelial cells than ovariectomized mice (Fig. 3E and F), demonstrating a role for ovarian hormones in promoting epithelial *mTert*-GFP expression.

mTert-GFP$^+$ epithelial cells in the luminal epithelium did not immunostain for the putative epithelial progenitor marker CD44 (Fig. 4A). The absence of CD44 in luminal epithelium was confirmed by carefully examining ten 461 × 461 μm fields from CD45 co-immunostained (to exclude intraepithelial leucocytes) sections of the uterus from six mice in oestrus stage. All CD44$^+$ cells detected in the luminal epithelium were CD45$^+$ intraepithelial leucocytes (Fig. 4B–D). *mTert*-GFP$^+$ and *mTert*-GFP$^-$ glandular epithelial cells both immunostained for CD44 (Fig. 4E). Examination of mice in oestrus stage ($n = 6$) showed that glands were composed entirely of either CD44$^+$ or CD44$^-$ cells, or a combination of CD44$^+$ and CD44$^-$ cells. On average, 1.34 ± 0.75 epithelial cells per gland immunostained for CD44. CD44 was abundant in the endometrial stroma (Fig. 4A, B and E) as examined in more detail below (Fig. 7C and Fig. 8D).

mTert-GFP is expressed by CD45$^+$ leucocytes in the endometrial stroma

mTert-GFP expression marks bone marrow haematopoietic stem cells and is retained in myeloid and lymphoid lineages present in the endometrium (Breault *et al.*, 2008). Immunofluorescence labelling for the pan-leucocyte marker CD45 revealed that the majority of stromal *mTert*-GFP$^+$ cells were CD45$^+$ and a small proportion were CD45$^-$ (Fig. 5A–D).

The endometrium is an immunologically active site and the recruitment of immune cells and accompanying stromal oedema are key components of endometrial regeneration during the oestrous cycle. We examined stromal *mTert*-GFP$^+$ cell abundance by fluorescence microscopy throughout the oestrous cycle but did not detect any differences between the diestrus, pro-oestrus, oestrus and metestrus phases (data not shown).

We then assessed the role of ovarian hormones in the recruitment of *mTert*-GFP$^+$ and CD45$^+$ cells in the endometrium by comparing mice at the oestrus stage of the oestrous cycle and hormonally deprived ovariectomized (non-cycling) mice (Fig. 6). Compared with oestrus phase endometrium, hormonally deprived endometrium had a significantly higher number of nuclei per unit area of stroma (mean 9500 ± 1260 cells/mm^2 versus 15 900 ± 1370 cells/mm^2, $P = 0.009$; Fig 6A–C) and a reduced area of CD45 immunostaining (mean 24 ± 3.4% versus 10 ± 1.1%, $P = 0.005$; Fig. 6A, B and D). These results indicate reduced oedema and reduced leucocyte infiltration, respectively, in hormonally deprived endometrium. Compared with oestrus phase endometrium, hormonally deprived endometrium had a lower percentage of *mTert*-GFP$^+$ CD45$^+$ leucocytes in the total stromal population (mean 0.35 ± 0.071% versus 0.12 ± 0.027%, $P = 0.015$; Fig. 6E). No significant difference was detected in the percentage of *mTert*-GFP$^+$ CD45$^-$ non-leucocytes in oestrus versus ovariectomized (mean 0.03 ± 0.010% versus 0.02 ± 0.009%, $P = 0.639$; Fig. 6F).

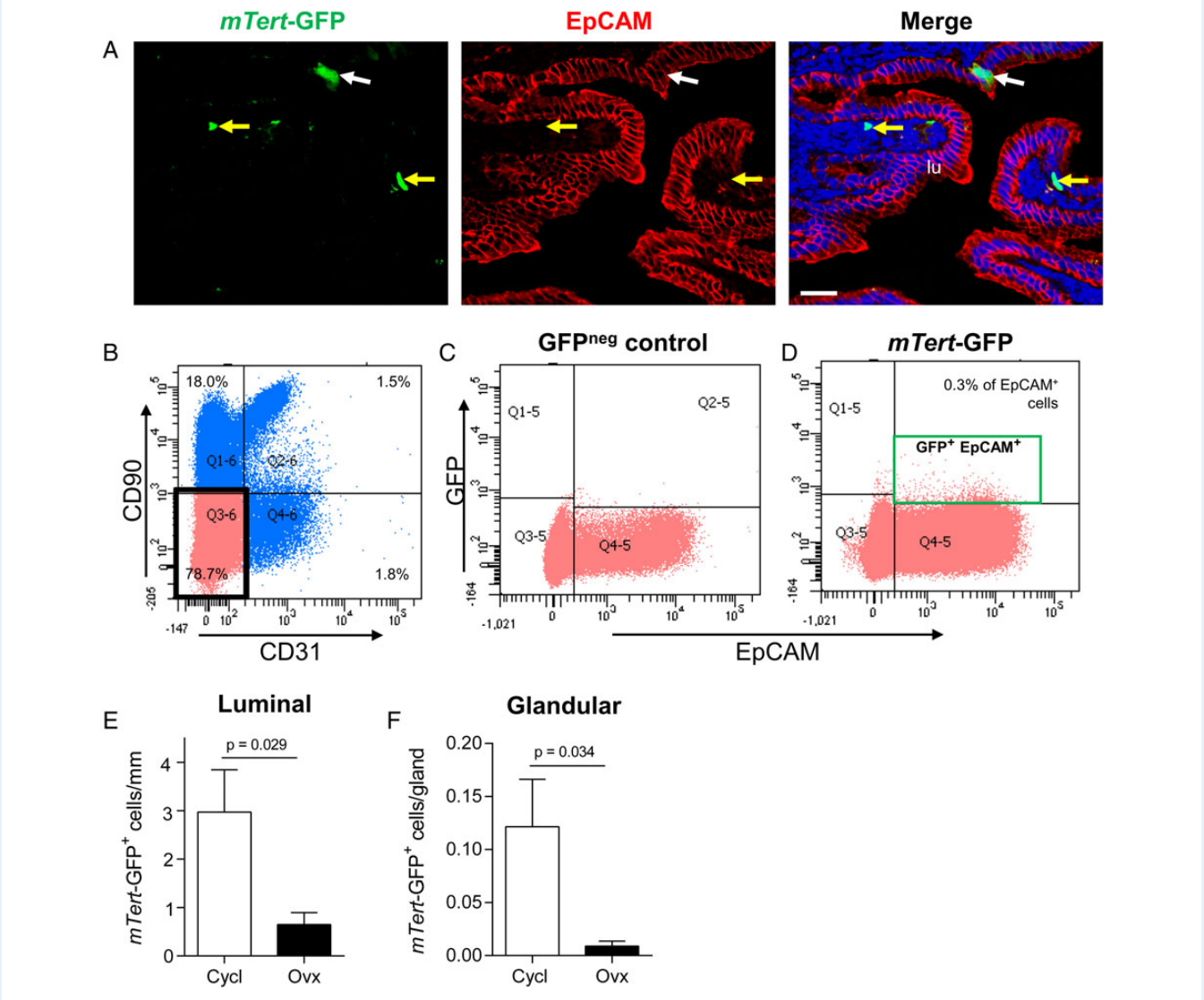


Figure 3 Characterization of epithelial *mTert*-GFP$^+$ cells. (**A**) *mTert*-GFP$^+$ cells immunostained for epithelial cell adhesion molecule (EpCAM) (white arrow) in the luminal epithelium (lu) in contrast to stromal mTert-GFP$^+$ cells (yellow arrows) that are not EpCAM$^+$. DNA is stained with Hoechst 33258 (blue). Scale bar in A = 20 μm. (**B**–**D**) Detection of *mTert*-GFP$^+$ EpCAM$^+$ cells by flow cytometry in a sample derived from 12 uteri. (B) Selection of a double negative population (CD90$^-$ CD31$^-$, black box). (C) CD90$^-$ CD31$^-$ cells from GFP$^-$ wild type mice (without GFP transgene) were used as a control to set the gate for GFP detection. (D) An *mTert*-GFP$^+$ EpCAM$^+$ subpopulation was detected in the *mTert*-GFP uterus (green box). (**E** and **F**) Luminal (E) and glandular (F) *mTert*-GFP expression was quantified using immunofluorescence microscopy in mice with an oestrous cycle (Cycl) and ovariectomized mice (Ovx). Data shown as mean ± SEM, $n = 12$ for Cycl, $n = 11$ for Ovx, *P*-values were calculated using an unpaired two-tailed *t*-test.

CD45$^-$ *mTert*-GFP$^+$ cells are a minor subpopulation of stromal cells in the endometrium

CD45$^-$ *mTert*-GFP$^+$ cells that immunostained for the endometrial stromal marker CD90 were observed (Fig. 7A) as well as CD45$^-$ *mTert*-GFP$^+$ cells that did not immunostain for CD90$^-$ (Fig. 7B). CD90 was also expressed by CD45$^+$ leucocytes (Fig 7A). The MSC marker CD44 immunostained CD45$^+$ leucocytes and some glands as described by Janzen *et al.* (2013), but was not detected on CD45$^-$ cells in the stromal compartment (Fig. 7C–F).

Endometrial *mTert*-GFP$^+$ cells were further examined by flow cytometry for the presence of CD90 and CD44 (Fig. 8). Characterization of the non-epithelial (EpCAM$^-$) *mTert*-GFP$^+$ stromal population (Fig. 8A and B) confirmed that the majority of *mTert*-GFP$^+$ cells in the stromal compartment were CD45$^+$ leucocytes, many of which were also CD90$^+$ or CD44$^+$ (Fig. 8C and D). A subpopulation of *mTert*-GFP$^+$ EpCAM$^-$ CD45$^-$ cells was also detected. These were either CD90$^+$ (5.8% of *mTert*-GFP$^+$ EpCAM$^-$) or CD90$^-$ (2.2% of *mTert*-GFP$^+$ EpCAM$^-$) (Fig. 8C). CD44 was detected on CD45$^+$ *mTert*-GFP$^+$ cells but not on CD45$^-$ *mTert*-GFP$^+$ cells (Fig. 8D).

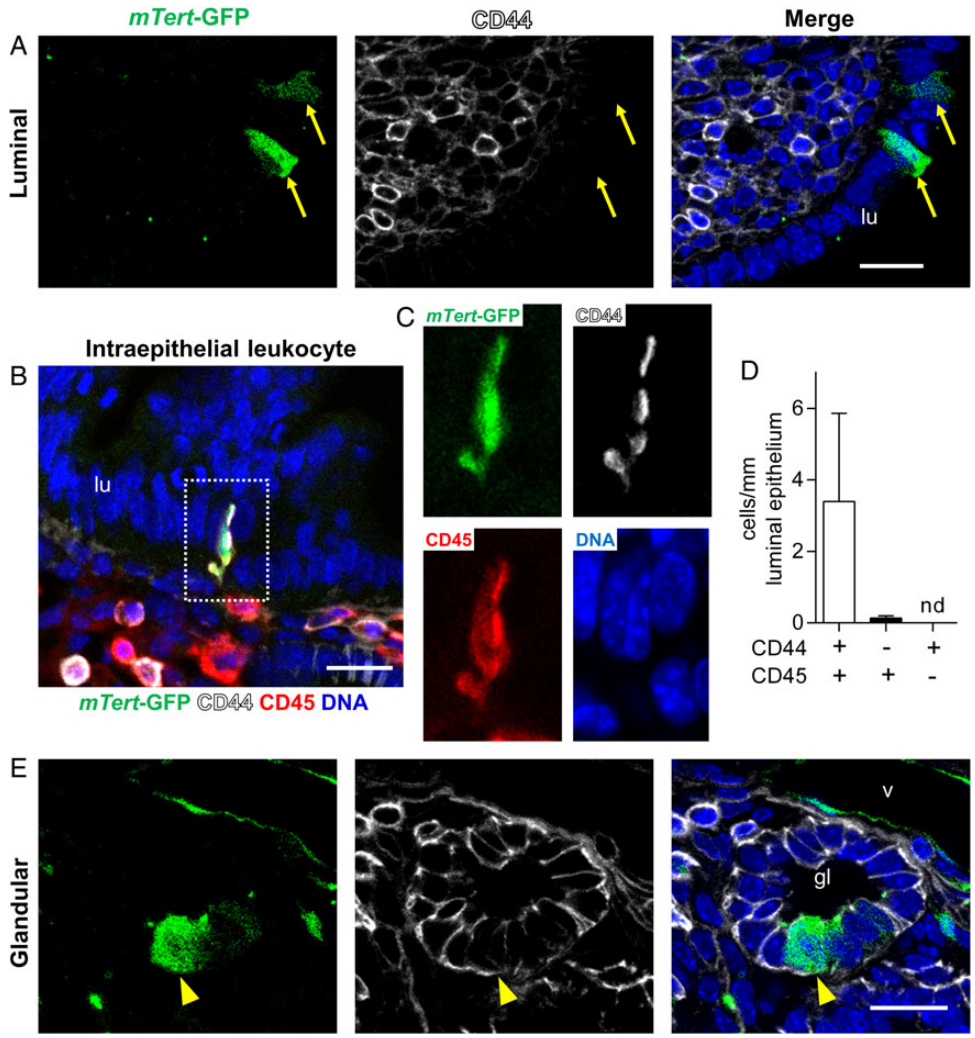


Figure 4 Immunolocalization of the putative epithelial progenitor marker CD44 relative to *mTert*-GFP. (**A**) Luminal epithelial *mTert*-GFP$^+$ (green, arrows) cells do not immunostain for CD44 (white). (**B**) *mTert*-GFP$^+$ CD45$^+$ CD44$^+$ intraepithelial leucocytes are present in the luminal epithelium (box). (**C**) Detail of the *mTert*-GFP$^+$ leucocyte indicated by the box in (B) shows an overlapping distribution of *mTert*-GFP (green), CD44 (white) and CD45 (red). (**D**) Quantification of CD44 and CD45 in the luminal epithelial layer of oestrus phase mice. Data shown as mean ± SEM, $n = 6$, nd, none detected. (**E**) Immunolocalization of CD44 to a glandular *mTert*-GFP$^+$ cell (arrowhead) at a level comparable to *mTert*-GFP$^-$ glandular cells. lu, luminal epithelium; gl, gland; v, vessel. DNA is stained with Hoechst 33258 (blue). Scale bars = 20 μm.

mTert-GFP marks a subpopulation of endometrial endothelial cells

The presence of GFP$^+$ cells lining voids at the endometrial/myometrial junction (Fig. 1A) raised the possibility that *mTert*-GFP marks a subpopulation of endothelial cells in the uterus. CD45$^-$ cells with a flattened morphology immunostained for the endothelial marker von Willebrand factor (Fig. 9A–C). The endothelial cell surface marker CD31 was used to further explore the phenotype of rare CD90$^+$ *mTert*-GFP$^+$ and CD90$^-$ *mTert*-GFP$^+$ cells by flow cytometry (Fig. 9D–F). A small proportion of *mTert*-GFP$^+$ CD90$^-$ CD45$^-$ cells were CD31$^+$ (10.3%) (Fig. 9E), but the majority of *mTert*-GFP$^+$ CD90$^+$ CD45$^-$ cells were CD31$^+$ (97.8%) (Fig. 9F). A substantial *mTert*-GFP$^+$ CD90$^-$ population were neither CD45$^+$ nor CD31$^+$ (Fig. 9E), indicating that these cells are not leucocytes or endothelial cells, and may be epithelial cells described in Fig. 3.

Discussion

For the first time, we have demonstrated that a transgenic *mTert*-GFP reporter allows the detection and analysis of candidate endometrial stem/progenitor cells at the single cell level. This system overcomes the technical shortcomings of immunolocalizing mTert (Wu *et al.*, 2006) and facilitates analysis using flow cytometry. Our study shows that *mTert* promoter activity identifies a heterogeneous endometrial population, including cells from epithelial, haematopoietic and endothelial lineages. Details of the *mTert*-GFP$^+$ populations identified in the mouse endometrium are summarized in Fig. 10.

mTert-GFP expression did not correlate with BrdU retention or the proliferative marker Ki67 in the endometrial epithelium and stroma, indicating that endometrial *mTert*-GFP$^+$ cells are distinct from previously described slow cycling (label-retaining) cells (Chan and

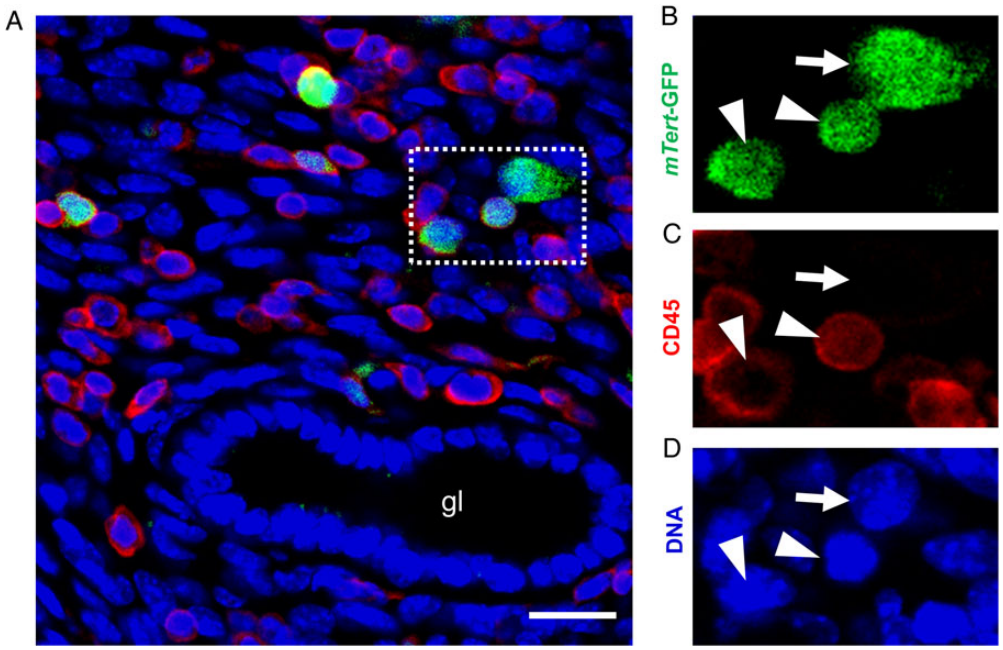


Figure 5 *mTert*-GFP$^+$ CD45$^-$ cells are a rare subpopulation in the endometrial stroma. (**A**) CD45$^+$ leucocytes (red) account for the majority of *mTert*-GFP$^+$ cells in the endometrium. (**B–D**) Detail of the boxed area from in (A) shows an *mTert*-GFP$^+$ CD45$^-$ cell (arrow) and *mTert*-GFP$^+$ CD45$^+$ leucocytes (arrowheads). gl, gland. DNA is stained with Hoechst 33258 (blue). Scale bar = 20 μm.

Gargett, 2006). This suggests that *mTert*-GFP$^+$ endometrial cells do not incorporate BrdU during the post-natal labelling window and indicates a limitation of the label retention method of identifying putative stem/progenitor cells.

Rare *mTert*-GFP$^+$ cells immunostained with EpCAM were observed in the luminal and glandular epithelium of the endometrium. The presence of epithelial *mTert*-GFP$^+$ cells is in line with reports of epithelial progenitor cells with stem-like properties such as clonogenicity and tissue regeneration (Janzen *et al.*, 2013; Gargett *et al.*, 2009). CD44 has been identified as a marker of mouse endometrial epithelial cells with an enhanced ability to reconstitute glands in an *in vivo* assay (Janzen *et al.*, 2013). However, we did not observe a relationship between *mTert*-GFP and CD44 in endometrial epithelial cells. The immunolocalization of CD44 in *mTert*-GFP$^+$ epithelial cells appeared to depend on whether they were luminal or glandular. Using the same CD44 antibody as Janzen *et al.* (2013), we found no evidence for CD44 immunolocalization in the luminal epithelial layer aside from CD45$^+$ intraepithelial leucocytes. In contrast, there were many CD44$^+$ glandular epithelial cells including some that were *mTert*-GFP$^+$. Unlike CD44$^+$ epithelial progenitor cells (Janzen *et al.*, 2013), epithelial *mTert*-GFP$^+$ cells were depleted by hormonal deprivation. Thus, *mTert*-GFP$^+$ epithelial cells do not appear to overlap with the CD44$^+$ putative progenitor population described by Janzen *et al.* (2013) and may represent a different stage of the endometrial stem/progenitor hierarchy.

CD45$^+$ *mTert*-GFP$^+$ cells from the haematopoietic lineage were the most abundant non-epithelial GFP-labelled population in the endometrium. This result is not unexpected as the endometrium is an immunologically active tissue and haematopoietic stem cells and their myeloid and lymphoid progeny are known to express *mTert*-GFP (Breault *et al.*, 2008). Bone marrow-derived cells, including those of the CD45 lineage, have been reported to give rise to endometrial stroma in the mouse and human. The concept that bone marrow stem cells can cross lineage barriers to form non-haematopoietic cells types (transdifferentiation) has been studied in a range of organs and is contentious. If there is a genuine bone marrow-derived contribution to the endometrial stroma and epithelium, it is small and accounts for only a fraction of the profound regenerative potential of the endometrium (reviewed in Gargett *et al.*, 2015). Thus, we suggest that CD45$^+$ *mTert*-GFP$^+$ haematopoietic lineage cells detected in the endometrium are predominantly transient immune cells recruited from the bone marrow rather than an intrinsic part of the endometrium involved directly in regeneration. Expression of the *mTert*-GFP reporter used in this study accurately reflects telomerase activity in CD45$^+$ bone marrow cells (Breault *et al.*, 2008) and human telomerase reverse transcriptase expression and telomerase activity are also elevated upon T cell activation (Hathcock *et al.*, 1998; Chebel *et al.*, 2009). As such, bulk measures of mTert expression and telomerase activity in the endometrium probably reflect levels of leucocyte infiltration and possibly activation rather than intrinsic endometrial stem cell or progenitor activity (Kyo *et al.*, 1997; Kim *et al.*, 2007; Hapangama *et al.*, 2010). Leucocyte abundance in the endometrium, and probably overall endometrial telomerase activity, is modulated by ovarian hormones (Salamonsen and Lathbury, 2000). Thus, we raise the possibility that changes in endometrial telomerase activity observed at the whole tissue level during the human menstrual cycle may reflect leucocyte abundance and type rather than endometrial stem/progenitor cell expansion or activity.

While bone marrow-derived leucocytes are the most likely source of CD45$^+$ *mTert*-GFP$^+$ cells in the endometrium (Givan *et al.*, 1997; Lee *et al.*, 2015), haematopoietic stem cell activity has been reported in the mouse endometrium (Sun *et al.*, 2015). Uterine resident haematopoietic stem cells could also contribute to the population of CD45$^+$ *mTert*-GFP$^+$ cells in the endometrium.

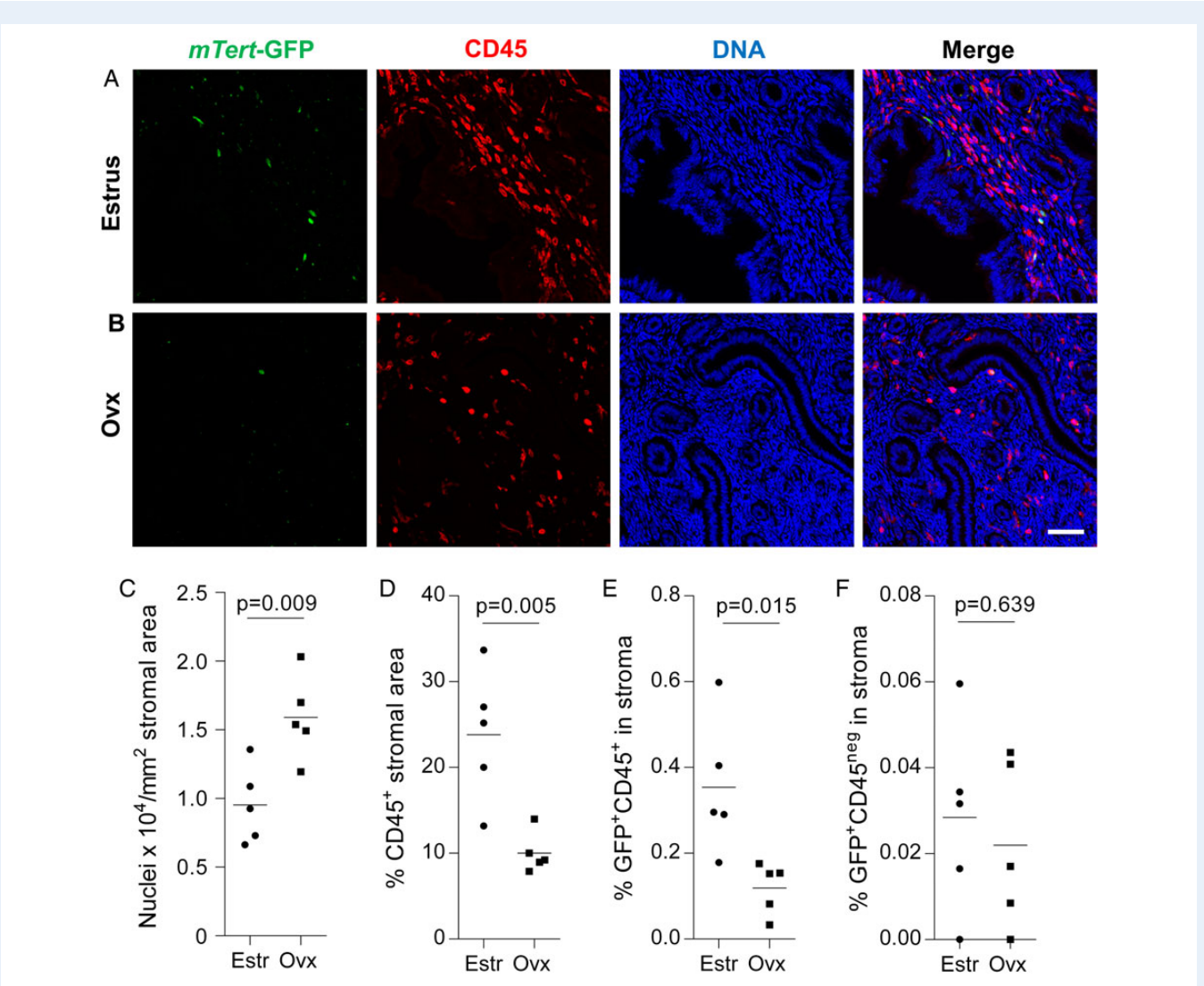


Figure 6 The influence of ovarian hormones on CD45$^+$ *mTert*-GFP$^+$ leucocytes and CD45$^-$ non-leucocyte *mTert*-GFP$^+$ endometrial cells. (**A** and **B**) The uteri of mice at the oestrus stage of the oestrous cycle (A) have wider spaced nuclei in the endometrial stromal region and significantly more CD45$^+$ infiltration compared with ovariectomized (Ovx) hormonally deprived mice (B). DNA is stained with Hoechst 33258 (blue). Scale bar in (B) = 50 μm, (A) is at the same magnification as (B). (**C**–**F**) Quantification of CD45 and *mTert*-GFP in the endometrium of oestrus phase (Estr) and ovariectomized (Ovx) mice: (C) Nuclei per unit stromal area, (D) percentage of stromal area occupied by CD45 immunostaining, (E) percentage *mTert*-GFP$^+$CD45$^+$ cells in the stroma and (F) percentage *mTert*-GFP$^+$CD45$^-$ cells in the stroma. $n = 5$/group in (C–F) with each dot representing a single mouse and mean indicated, *P*-values were calculated using an unpaired two-tailed *t*-test.

mTert-GFP$^+$ cells that are not of the haematopoietic lineage (CD45$^-$) are rare in the stromal compartment and may represent an intrinsic part of the endometrium with roles in tissue maintenance and regeneration. This is the case in the mouse intestine where rare *mTert*-GFP$^+$ cells can give rise to a range of differentiated cell types (Montgomery *et al.*, 2011). Further studies are required to determine whether a similar system operates in the endometrium. Unlike *mTert*-GFP$^+$ leucocytes, the abundance of non-haematopoietic *mTert*-GFP$^+$ cells in the endometrium was resistant to depletion in the absence of ovarian hormones. The atrophied inactive endometrium of ovariectomized mice and post-menopausal women retains its regenerative potential indicating the presence of persistent stem/progenitor populations (Paulson *et al.*, 2002; Gargett and Chan, 2006; Gargett *et al.*, 2012; Ulrich *et al.*, 2014). The observation that the CD45$^-$ *mTert*-GFP$^+$ population is not dependent on ovarian hormones is consistent with the concept that it contains a reserve population of endometrial stem/progenitor cells that survives hormonal deprivation in inactive endometrium (Paulson *et al.*, 2002; Gargett and Chan, 2006; Janzen *et al.*, 2013; Ulrich *et al.*, 2014).

Studies of human endometrium have identified mesenchymal stem/stromal cells (MSC) (Schwab and Gargett, 2007; Schwab *et al.*, 2008; Masuda *et al.*, 2012; Gargett *et al.*, 2015). MSC have been reported to express telomerase reverse transcriptase and have telomerase activity, although levels are low compared to hematopoietic stem cells (Liu *et al.*, 2004; Serakinci *et al.*, 2008). We sought evidence of *mTert*-GFP$^+$ MSC in the mouse endometrium using the endometrial stromal/MSC markers CD90 and CD44. While CD90 marks endometrial

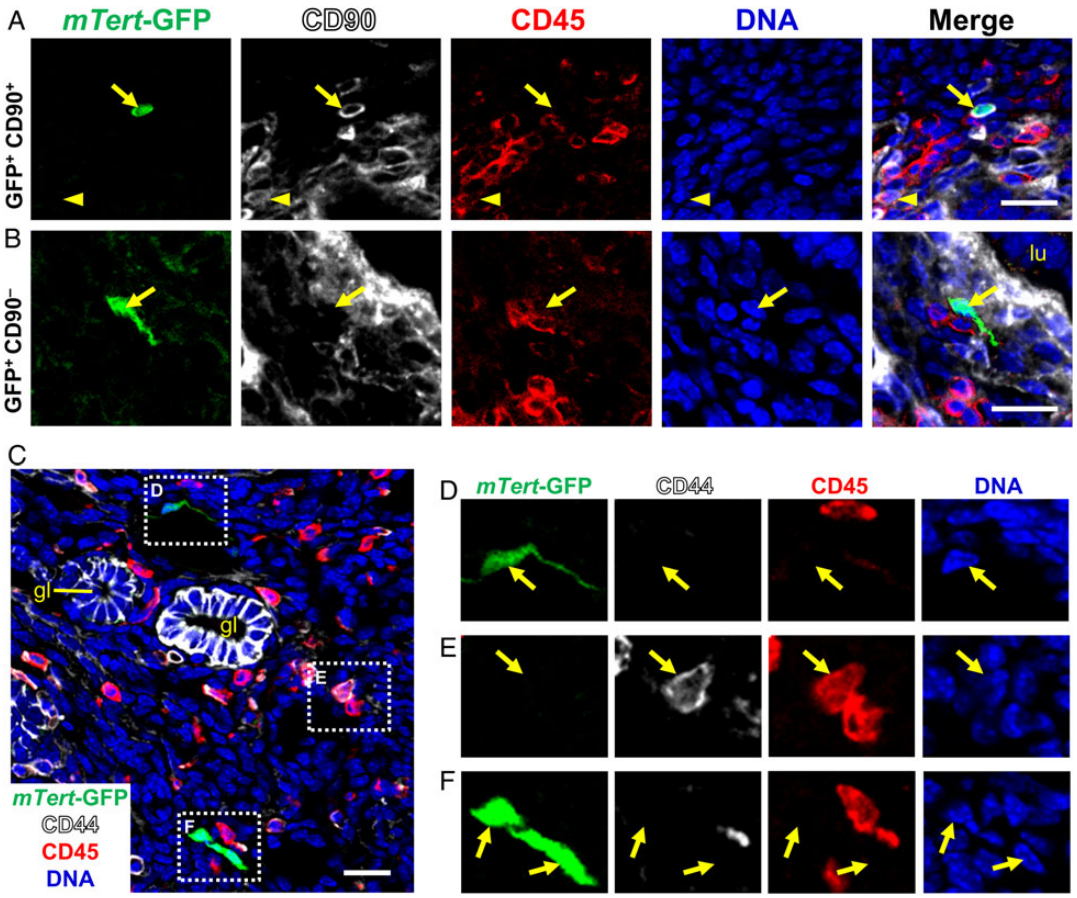


Figure 7 A subpopulation of CD45$^-$ *mTert*-GFP$^+$ cells immunostain for the endometrial stromal marker CD90, but not the mesenchymal stem/stromal cell (MSC) marker CD44. (**A** and **B**) *mTert*-GFP (green), CD90 (white) and CD45 (red) in the endometrium. (A) A CD45$^-$ stromal *mTert*-GFP$^+$ cell (yellow arrow) immunostained for CD90 but not CD45. CD45$^+$ leucocytes also immunostain for CD90 (arrowhead). (B) A stromal *mTert*-GFP$^+$ cell (yellow arrow) that does not immunostain for CD90 or CD45. (**C**–**F**) *mTert*-GFP (green), CD44 (white) and CD45 (red) in the endometrium. (C) CD44 localizes to CD45$^+$ leucocytes and glands, but not CD45$^-$ *mTert*-GFP$^+$ cells. (D and F) Detail of *mTert*-GFP$^+$ CD45$^-$ cells that do not immunostain for CD44. (E) CD44 immunolocalizes to CD45$^+$ leucocytes in the stromal compartment. lu, luminal epithelium; gl, gland. DNA is stained with Hoechst 33258 (blue). Scale bars = 20 μm.

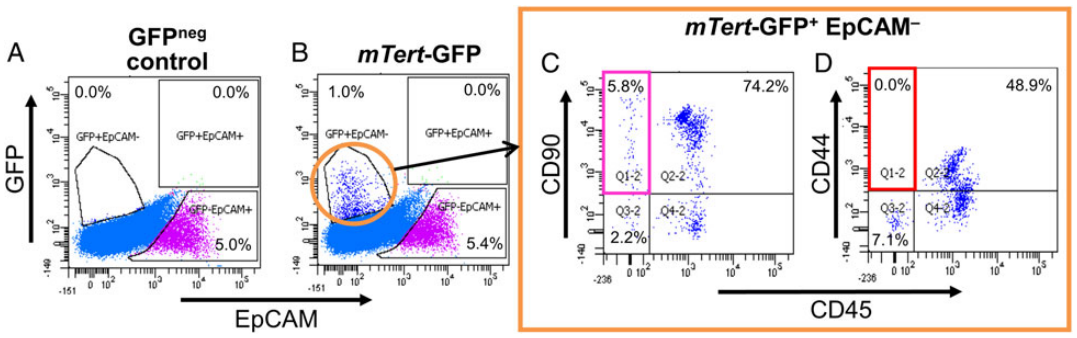


Figure 8 (**A**–**D**) Flow cytometric analysis of *mTert*-GFP$^+$ uterine stromal cells. Results shown are from a sample derived from 12 *mTert*-GFP uteri and are representative of three experiments. (A) Gates for GFP detection were defined using wild type mice lacking GFP (GFP^{neg} control). (B) Flow cytometry identified a population of *mTert*-GFP$^+$ EpCAM$^-$ cells (yellow circle) that were absent in the GFP^{neg} control uterus (A). (**C** and **D**) The *mTert*-GFP$^+$ EpCAM$^-$ population (yellow circle in B) was analysed for the stromal markers CD90 (C) and CD44 (D). (C) 5.8% of *mTert*-GFP$^+$ EpCAM$^-$ cells are CD90$^+$ CD45$^-$ (magenta box). (D) *mTert*-GFP$^+$ EpCAM$^-$ cells were not CD44$^+$ CD45$^-$ (red box).

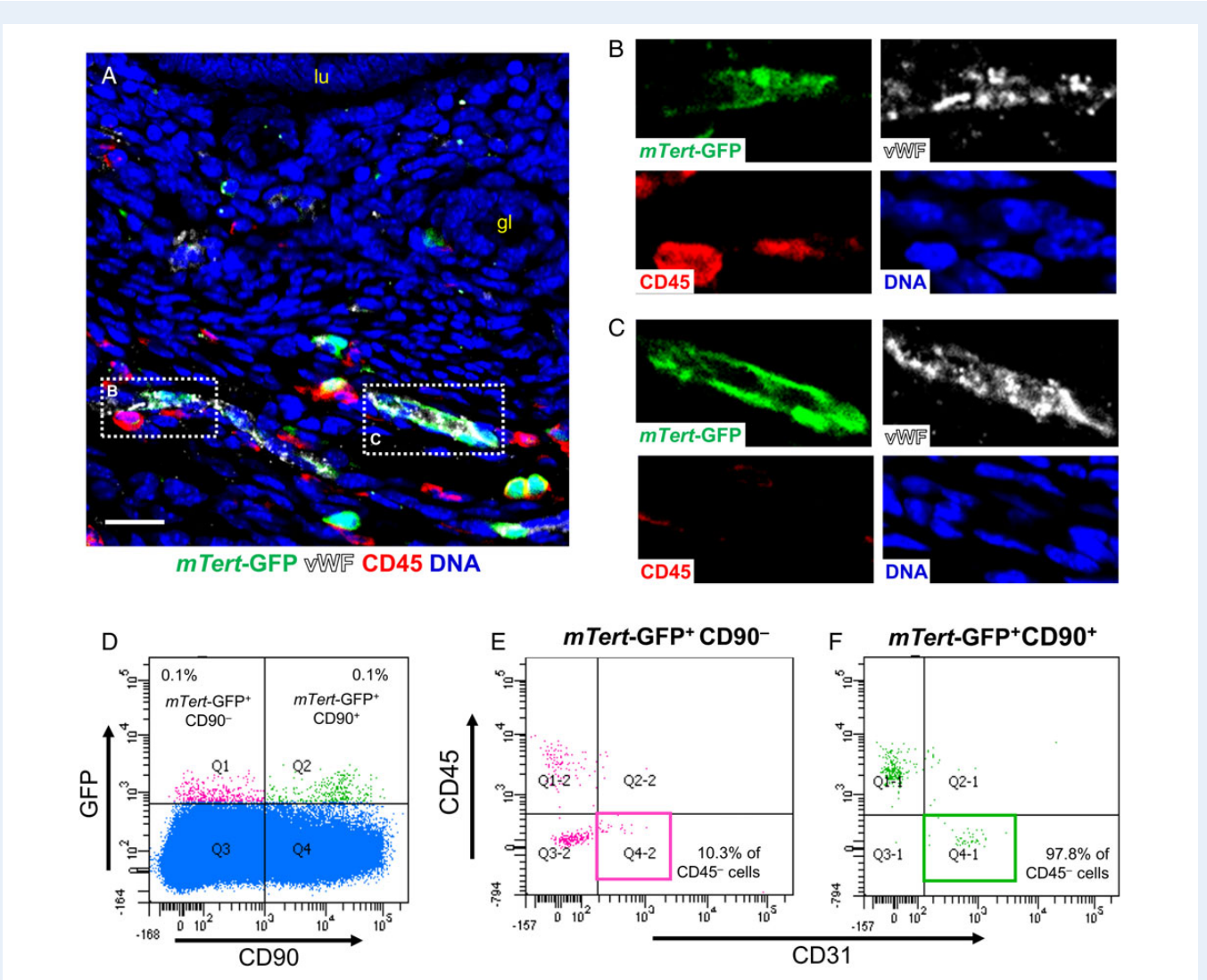
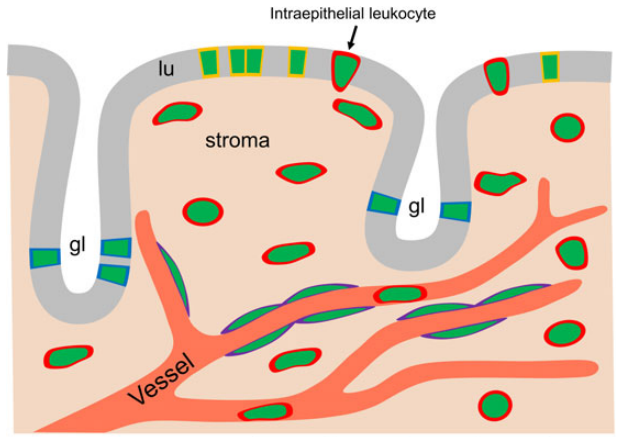


Figure 9 *mTert*-GFP$^+$ cells immunostain for the endothelial markers, von Willebrand factor (vWF) and CD31. (**A**) *mTert*-GFP$^+$ cells near the endometrial/myometrial junction immunostain for vWF (boxed areas). (**B** and **C**) Detail of boxed areas in (A) shows overlapping localization of *mTert*-GFP$^+$ and vWF. DNA is stained with Hoechst 33258 (blue). Scale bar = 20 μm. (**D–F**) Flow cytometry of a dissociated single cell suspension derived from 12 *mTert*-GFP uteri. (D) *mTert*-GFP$^+$ cells gated for CD90 to separate *mTert*-GFP$^+$ CD90$^-$ (magenta) and *mTert*-GFP$^+$ CD90$^+$ (green) populations. (**E** and **F**) CD31$^+$ CD45$^-$ subpopulations are detected in the (E) *mTert*-GFP$^+$ CD90$^-$ (magenta box) and (F) *mTert*-GFP$^+$ CD90$^+$ (green box) populations.

stroma, it does not do so exclusively and CD90 is also expressed by some endothelial cells and leucocytes. Non-leucocyte *mTert*-GFP$^+$ CD90$^+$ cells are also CD31$^+$ and von Willebrand factor positive, suggesting that they are endothelial cells. The only non-epithelial *mTert*-GFP$^+$ CD44$^+$ cells detected were CD45$^+$ leucocytes. A lack of endogenous CD44$^+$ stromal cells may also be due to this MSC marker only being expressed on MSC as a consequence of *in vitro* culture (Qian *et al.*, 2012). Thus, we conclude that mTert expression does not identify an endometrial MSC population in the mouse. This finding does not exclude the possibility that mTert-expressing progenitor cells give rise to an endometrial MSC population.

The existence of CD31$^+$ and von Willebrand factor positive *mTert*-GFP$^+$ endothelial cells in the endometrium is similar to reports of endothelial mTert expression in endothelial cells of the mouse heart (Richardson *et al.*, 2012). Telomerase activity has been linked to the proliferative capacity of endothelial cells and their ability to avoid senescence (Erusalimsky and Skene, 2009). A high prevalence of endothelial cells has also been demonstrated in tissue reconstituting side population cells with telomerase activity from the human endometrium (Masuda *et al.*, 2010; Cervello *et al.*, 2011; Miyazaki *et al.*, 2012). Angiogenesis is an essential component of endometrial repair and endothelial *mTert*-GFP$^+$ cells may represent a progenitor population involved in this process (Fan *et al.*, 2008). Telomerase reverse transcriptase expressing endothelial progenitor cells from the human endometrium may also play a role in the angiogenesis that facilitates the establishment and persistence of endometrial growth outside the endometrium as endometriotic lesions (Laschke *et al.*, 2011). Human telomerase reverse transcriptase expression is up-regulated in cultured endothelial cells by estrogen (Grasselli *et al.*, 2008). However, we did not observe a decline in the predominantly endothelial CD45$^-$ *mTert*-GFP$^+$ population in the stroma of ovariectomized



	CD45	CD90	CD44	CD31/ vWF	EpCAM
Leukocyte ▪ 0.3-0.4% of stromal/vascular	+	±	±	-	-
Luminal epithelium ▪ with glandular, ~ 0.3% of epithelial	-	-	-	-	+
Glandular epithelium ▪ with luminal, ~ 0.3% of epithelial	-	-	±	-	+
Endothelial ▪ ~10% of *mTert*-GFP$^+$CD90$^-$ & ~ 100% of *mTert*-GFP$^+$CD90$^+$ stromal/vascular	-	±	-	+	-

Figure 10 A diagram and table summarizing the localization, abundance and characteristics of *mTert*-GFP$^+$ cells in the mouse endometrium. lu, luminal epithelial layer; gl, gland; vWF, von Willebrand factor; EpCAM, epithelial cell adhesion molecule.

mice, suggesting that these *mTert*-GFP$^+$ endothelial cells are a reserve population that is not reliant on ovarian hormones for survival.

The existence of *mTert*-GFP$^+$ vascular and epithelial putative stem/progenitor populations in the uterus raises questions as to the relationship between these cell types. There is evidence of a stromal/mesenchymal origin for epithelial cells during epithelial repair and regeneration following parturition or induced endometrial shedding (Cousins *et al.*, 2014; Huang *et al.*, 2012; Patterson *et al.*, 2013). However, this process does not occur during the normal oestrous cycle examined in this study (Huang *et al.*, 2012; Patterson *et al.*, 2013). Thus, a direct link between the endometrial *mTert*-GFP$^+$ vascular and epithelial cells we observed is unlikely. Transient reactivation of telomerase activity has been reported in luminal progenitors of mammary epithelium and is thought to represent a telomere salvage mechanism (Kannan *et al.*, 2013). A telomere salvage mechanism may account for the *mTert*-GFP$^+$ epithelial cells we observe in the endometrial epithelium.

In summary, we have shown that *mTert* reporter expression identifies rare epithelial and endothelial populations in the mouse endometrium. This reporter system will allow the role of these previously uninvestigated putative progenitor populations to be examined in endometrial repair, regeneration and experimental models of endometriosis. Understanding the properties of mTert-expressing progenitor cells in the mouse will facilitate the identification of equivalent populations in the human endometrium likely to be involved in endometrial function, fertility and disease. Another important observation is that mTert expression, and most likely telomerase activity, is predominantly due to infiltrating immune cells in the endometrium. This shows that telomerase activity needs to be considered at the cellular level in several discrete endometrial subpopulations with distinct properties, rather than by whole tissue-based measures.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

C.E.G. and D.T.B. conceived the project, C.E.G., D.T.B. and J.A.D. obtained funding; J.A.D., Y.R.O., C.E.G. and D.T.B. designed experiments; J.A.D. and Y.R.O. performed the experiments; J.A.D. and Y.R.O. analysed and assembled data wrote the paper with C.E.G. and D.T.B.; J.E.C., W.S.N.K., D.L.C., D.N.W. provided advice on experimental design and techniques and essential reagents; A.T. advised on experimental design for flow cytometry and contributed to analysis of data. All authors provided feedback on drafts of the manuscript and approved the final version.

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Conflict of interest

The authors have no conflicts of interest to declare.

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