LETTERS

Progesterone induces adult mammary stem cell expansion

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Reproductive history is the strongest risk factor for breast cancer after age, genetics and breast density[1,2]. Increased breast cancer risk is entwined with a greater number of ovarian hormone-dependent reproductive cycles, yet the basis for this predisposition is unknown[3–5]. Mammary stem cells (MaSCs) are located within a specialized niche in the basal epithelial compartment that is under local and systemic regulation[6]. The emerging role of MaSCs in cancer initiation warrants the study of ovarian hormones in MaSC homeostasis. Here we show that the MaSC pool increases 14-fold during maximal progesterone levels at the luteal dioestrus phase of the mouse. Stem-cell-enriched CD49f^{hi} cells amplify at dioestrus, or with exogenous progesterone, demonstrating a key role for progesterone in propelling this expansion. In aged mice, CD49f^{hi} cells display stasis upon cessation of the reproductive cycle. Progesterone drives a series of events where luminal cells probably provide Wnt4 and RANKL signals to basal cells which in turn respond by upregulating their cognate receptors, transcriptional targets and cell cycle markers. Our findings uncover a dynamic role for progesterone in activating adult MaSCs within the mammary stem cell niche during the reproductive cycle, where MaSCs are putative targets for cell transformation events leading to breast cancer.

Mammary stem cells (MaSCs) are widely considered to be constant and quiescent unless activated at puberty and pregnancy, which are brief and isolated developmental periods[7–9]. In contrast, the reproductive cycle is continuously present from puberty to menopause. Women are repeatedly exposed to ovarian hormones during the reproductive lifespan, and a higher number of menstrual cycles correlates with increased risk of developing breast cancer[3–5]. Given the highly responsive nature of the mammary gland to ovarian hormones, which also alter breast cancer risk, it is important to elucidate the impact of the reproductive cycle on MaSC homeostasis.

We elucidated changes in MaSCs using the mouse oestrous cycle as a model, given its parallels with the human menstrual cycle: the human follicular phase has a surge of circulating oestrogen that is reflected in murine pro-oestrus/oestrus, whereas the progesterone peak during the human luteal phase is initiated at late murine metoestrus with maximal levels at dioestrus[10]. Oestrous cycle phases were followed precisely by vaginal cytology of individual, adult cycling FVB mice (Supplementary Fig. 1) and confirmed by serum ovarian hormone measurements. Progesterone levels rose fourfold at dioestrus (Fig. 1a). A number of distinct cell lineages constitute a mammary gland including epithelial, stromal, haematopoietic and endothelial cells. Dissociated phase-specific glands showed a significant increase in total cell numbers at dioestrus compared with oestrus (1.9-fold) and metoestrus (Fig. 1b), and dioestrus displayed a unique morphology with pronounced tertiary branching and lobuloalveolar structures (Fig. 1c). The progesterone peak and mammary architecture[10] at dioestrus are consistent with the known role of progesterone in mediating lobuloalveolar growth during pregnancy in mice and humans[11].

We investigated whether MaSC numbers alter as a function of the oestrous cycle by testing the *in vivo* functional capacity of phase-specific cells to repopulate the mammary gland. Dioestrus and oestrus represented the luteal and follicular phases of the menstrual cycle, respectively. Limiting dilution assays were performed by transplanting total mammary cells from dioestrus or oestrus glands into contralateral cleared mammary fat pads of 21-day-old prepubescent recipient mice. Figure 1d depicts the robustness of outgrowths, which were comparable for both cell populations when they generated outgrowths. Dioestrus cells showed a mammary repopulating unit frequency of 1 in 579 compared to 1 in 4,409 for oestrus cells based on positive outgrowths scored for each cell dilution, demonstrating a 7.6-fold increase at dioestrus (Fig. 1e and Supplementary Table 1). Because the total cell number was 1.9-fold higher at dioestrus compared to oestrus, this translates into a remarkable 14-fold increase in the absolute number of mammary repopulating units at dioestrus.

MaSCs have been localized to the basal compartment of the mouse mammary gland and have the cell surface signature CD45$^-$Ter119$^-$CD31$^-$ (collectively called Lin$^-$) CD24$^+$CD49f^{hi}/CD29^{hi} (refs 8, 12). Single mammary cells from individual, oestrous-staged mice were analysed by flow cytometry for Lin$^-$ luminal (CD24$^+$CD49f^{lo}) and basal (CD24$^+$CD49f^{hi}) epithelial cells, and stromal cells (CD24$^-$CD49$^-$). The stromal fraction fluctuates during the oestrous cycle but not in absolute numbers (Supplementary Fig. 2). With respect to epithelial cell populations, we observed an increase in the number of luminal (threefold) and stem-cell-enriched basal (sixfold) cells at dioestrus compared with oestrus (Fig. 2a, b). This epithelial expansion at dioestrus was coincident with the extensive lobuloalveolar morphology observed in the matched thoracic mammary gland whole mounts as in Fig. 1c. The basal cell population is enriched in MaSCs, which have the capacity to generate a functional mammary gland *in vivo*[8,12]; the sixfold increase in MaSC-enriched basal cells is consistent with the elevated mammary repopulating units at dioestrus.

We next examined the effect of progesterone on stem-cell-enriched basal cells. Bilaterally ovariectomized mice were subjected to classic hormone regimens: 17β-oestradiol, progesterone, 17β-oestradiol plus progesterone, and controls. 17β-oestradiol, a potent mitogen in the pubertal gland[13], is merely permissive in the adult gland[14,15] as it induces progesterone receptor (PR) expression. Therefore, 17β-oestradiol plus progesterone represents progesterone-directed effects. At the morphological level, vehicle controls showed the ductal arrest typical of

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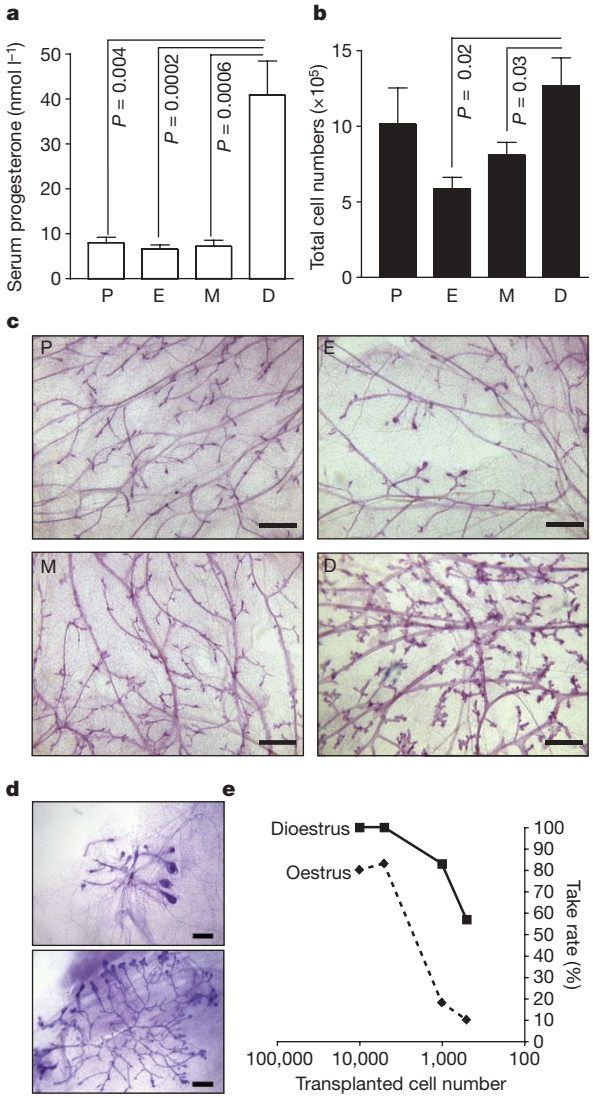

Figure 1 | MaSCs fluctuate during the reproductive cycle. **a**, Serum progesterone levels during the oestrous cycle (mean ± s.e.m.). The four phases of the cycle are denoted as: P, pro-oestrus; E, oestrus; M, metoestrus; D, dioestrus; $n = 5$ (P), $n = 8$ (E), $n = 7$ (M), $n = 6$ (D) mice. **b**, Total cell numbers generated from oestrous-staged inguinal glands (mean ± s.e.m.); $n = 5$ (P), $n = 4$ (E), $n = 7$ (M), $n = 5$ (D) mice. **c**, Mammary whole mounts from mice at the indicated oestrous phases show pronounced lobuloalveolar morphology at dioestrus. Scale bars, 500 μm. **d**, Two examples of positive ductal outgrowths from limiting dilution analyses into cleared fat pads, reconstituted with 500 (top) or 5,000 (bottom) dioestrus cells. Scale bars, 500 μm. **e**, Comparison of take rates of dioestrus and oestrus mammary cells at limiting dilutions show a 7.6-fold increase in the mammary repopulating unit frequency of dioestrus cells. Take rate indicates positive outgrowths as detailed in Supplementary Table 1. Data are consistent with a single-hit process ($P = 0.9998$, dioestrus cells; $P = 0.7534$, oestrus cells).

ovariectomized females, 17β-oestradiol led to bud formation, progesterone induced lateral branching, whereas 17β-oestradiol plus progesterone triggered extensive lobuloalveologenesis (Supplementary Fig. 3a) as found at dioestrus. Flow analysis of MaSC markers showed that 17β-oestradiol plus progesterone led to a large expansion of MaSC-enriched basal ($CD24^+CD49^{hi}$) and luminal ($CD24^+CD49^{lo}$) cells, whereas 17β-oestradiol or progesterone alone had no effect (Fig. 2c, e and Supplementary Fig. 3b). Further segregation of the $CD24^+$ subset using CD61 (β3 integrin)[16] showed a significant increase in progesterone-driven stem ($CD61^+CD49f^{hi}$) and differentiated ($CD61^-CD49f^{lo}$) cells (Fig. 2d, f and Supplementary Fig. 3c), but little effect on progenitor ($CD61^+CD49f^{lo}$) numbers. Stromal cells ($CD24^-CD49f^-$) showed changes in relative percentages in response to hormonal supplementation, but not in absolute numbers (not shown). Taken together, progesterone is responsible for dynamic shifts in specific populations within the mammary epithelial cell hierarchy which are consistent with MaSC alterations in the natural reproductive cycle. Stem cells are known for their ability to undergo symmetrical and asymmetrical cell division to achieve their fundamental properties of self renewal and multipotency[17]. The increase in the MaSC-enriched basal population implicates symmetric cell division, whereas the increase in mature cells along with differentiated morphology reflects asymmetric cell division, all caused by progesterone during the reproductive cycle.

The colony forming cell (CFC) assay provides an *in vitro* readout for progenitor cells that can form discrete colonies[12]. It has been shown that solid colonies arise from cells of basal/myoepithelial origin, whereas acinar structures are characteristic of luminal cells[18]. Recent work reported that sorted cells from the brightest tip of the $CD24^+CD49f^{hi}$ basal population formed solid colonies whereas those from $CD24^+CD49f^{lo}$ luminal cells were responsible for >90% CFC capacity and mostly gave rise to acinar colonies in Matrigel[12]. To preserve *in vivo* luminal–basal cross talk, we used total mammary cells to compare CFCs from dioestrus and oestrus. We consistently obtained more solid colonies with dioestrus than oestrus, indicating a preponderance of cells of the basal/myoepithelial origin in dioestrus (Fig. 2g, h). To determine whether progesterone was responsible, at least in part, for generating solid colonies at dioestrus, we assayed CFCs from glands of ovariectomized mice treated with 17β-oestradiol versus 17β-oestradiol plus progesterone. The latter induced a significant increase in solid colonies while reducing acinar colonies (Fig. 2i, j). Thus, specific progenitors of a basal/myoepithelial lineage are probably enhanced in a progesterone-enriched microenvironment as encountered at dioestrus or with exogenous progesterone.

In histology (Supplementary Fig. 4a), we noted increased cellularity of dioestrus and 17β-oestradiol plus progesterone glands, with expected distribution of K18-expressing luminal and K14-expressing basal cells. Progesterone receptor-A (PR-A) immunostaining indicated abundant PR^+ cells in these glands; however, quantification showed that the percentage of PR positive cells was comparable to control groups (Fig. 3a, b). The percentage of $Ki67^+$ proliferating cells was increased at dioestrus and upon 17β-oestradiol plus progesterone treatment (Figs 3c, d), whereas apoptosis (cleaved caspase 3) was only detectable at dioestrus but not at oestrus (Supplementary Fig. 4b). This heightened proliferative state accompanied by cell death provides evidence for cell turnover with progesterone exposure in the adult gland. This led us to rationalize that each reproductive cycle may culminate with the restoration of stem cell homeostasis, to begin the next cycle. If this were not the case, a gradual accumulation of MaSC-enriched basal cells would be expected with ageing. We tested this hypothesis using 6- and 20-month-old mice. Vaginal cytology and serum progesterone confirmed that 6-month-old females continued to cycle and flow cytometry analysis showed an expansion of $CD24^+CD49f^{hi}$ cells at dioestrus comparable to 10-week-old mice (not shown). In contrast, 20-month-old mice had unchanging vaginal smears, low circulating progesterone levels and dormant mammary morphology, indicating cessation of their reproductive cycle (Fig. 3e, f). Total mammary cells derived from these mice had reduced CFC activity *in vitro* compared with even oestrus 10-week-old mice (Fig. 3g). More importantly, MaSC-enriched $CD24^+CD49f^{hi}$ cells did not show an accumulation with age (Fig. 3h, i).

MaSCs are oestrogen-receptor-negative and PR-negative (ER^-PR^-) and reside in a specialized niche surrounded by luminal and myoepithelial cells, some of which are hormone-receptor positive[6,19]. We sought to identify the mechanism for progesterone-mediated MaSC expansion by quantitative gene expression analyses of sorted luminal ($CD24^+CD49f^{lo}$) and basal ($CD24^+CD49f^{hi}$) cell fractions from oestrous-staged or hormone-treated mice. Basal (*K14* (also called *Krt14*), *Sma* (also called *Acta2*)) and luminal (*K18* (also called *Krt18*)) markers were highly expressed in their respective compartments,

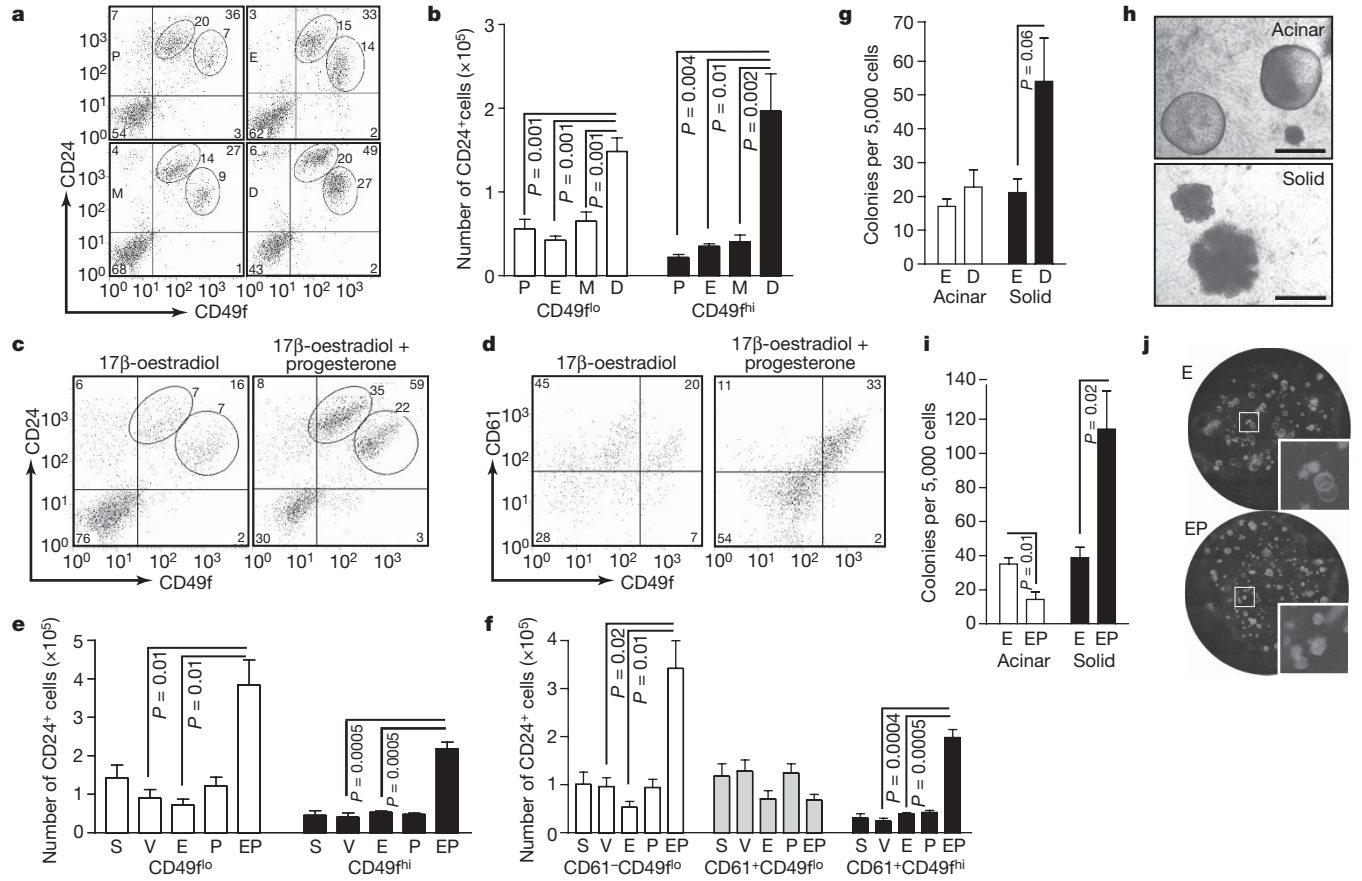

Figure 2 | Progesterone drives expansion of the MaSC-enriched subpopulation *in vivo*. **a**, Representative FACS plots of $CD24^+CD49f^{lo}$ (luminal) and $CD24^+CD49f^{hi}$ (basal) populations generated from oestrous-staged glands. **b**, Histogram showing total numbers of $CD24^+CD49f^{lo}$ and $CD24^+CD49f^{hi}$ cells at stages of the oestrous cycle (mean ± s.e.m.); $n = 5$ (P), $n = 4$ (E), $n = 7$ (M), $n = 5$ (D) cycling mice. (See Fig. 1 legend for definitions.) **c**, $CD24^+CD49f^{lo}$ and $CD24^+CD49f^{hi}$ populations after 17β-oestradiol treatment alone or with progesterone of ovariectomized mice. **d**, Further segregation of stem ($CD24^+CD61^+CD49f^{hi}$), progenitor ($CD24^+CD61^+CD49f^{lo}$) and differentiated ($CD24^+CD61^-CD49f^{lo}$) cells using CD49f and CD61 markers within the $CD24^+$ subset. **e**, **f**, Histograms showing quantification of the indicated cell populations after hormone treatments. E, 17β-oestradiol; EP, 17β-oestradiol plus progesterone; P, progesterone; S, sham; V, vehicle (sesame oil). Data represent mean ± s.e.m. of $n = 4$ (S), $n = 3$ (V), $n = 3$ (E), $n = 3$ (P), $n = 4$ (EP). **g**, The colony-forming capacity of 5,000 total mammary cells in Matrigel from dioestrus or oestrus glands (mean ± s.e.m., $n = 3$ per group). **h**, Representative gross morphology of acinar and solid colonies arising in Matrigel cultures. Scale bars, 500 μm. **i**, **j**, Histogram showing colonies formed from 17β-oestradiol versus 17β-oestradiol plus progesterone cells (**i**) and representative tiled image composites spanning a Matrigel droplet of 1.2-cm diameter, with insets showing a 3.5× magnification (**j**). Data represent mean ± s.e.m., $n = 3$ mice per group.

indicating purity of sorted populations (Fig. 4a, b). *Gata3*, a luminal cell fate determinant[16], increased in the dioestrus or 17β-oestradiol plus progesterone luminal cell fraction. The PR gene expresses two distinct isoforms: *PR-A* (the amino-terminally truncated variant of full-length *PR-B*) is reported in luminal cells[20,21] and *PR-B* in luminal and myoepithelial/basal cells[21]. Gene deletion studies show a requirement for *PR-B*, but not *PR-A*, in mammary morphogenesis[22]. We observed a pronounced induction of both isoforms in 17β-oestradiol plus progesterone luminal cells, and of *PR-B* in 17β-oestradiol plus progesterone basal cells (Fig. 4c). This analysis provides new evidence for progesterone-induced PR expression which occurs without altering the percentage of PR^+ cells.

Progesterone exerts mitogenic effects predominantly by paracrine signalling in the mammary gland[14,23]. Wnt4[24] and RANKL[14,22,25] are implicated in mediating the paracrine effect, and both were elevated several thousand-fold exclusively in sorted luminal cells from 17β-oestradiol plus progesterone, but not 17β-oestradiol, glands (Fig. 4d, e). Also notable was the surge in *Rank* (also called *Tnfrsf11a*) expression in MaSC-enriched basal cells with 17β-oestradiol plus progesterone treatment (Fig. 4d). Thus, RANKL from luminal cells, binding to its receptor RANK on basal cells, is a likely paracrine effector of progesterone for MaSC-enriched basal cells. Because *PR-B* is also induced in basal cells, it is tempting to speculate that systemic progesterone may directly turn on *Rank* in basal cells. LRP5 is one of two essential co-receptors for canonical Wnt signalling required for maintenance of the mammary basal lineage[26]. Relevant to luminal-derived Wnt4, *Lrp5* was significantly upregulated in basal cells with 17β-oestradiol plus progesterone (Fig. 4e). Furthermore, Wnt target genes were activated in sorted 17β-oestradiol plus progesterone luminal (*Axin2*, *Mmp7*) and basal (*Axin2*, *Tcf1* (also called *Tcf7*)) cells. It was recently shown that progesterone requires cyclin D1 to induce proliferation in a cell-autonomous manner[14], and indeed 17β-oestradiol plus progesterone increased cyclin D1 expression in luminal cells (Fig. 4f). Notable was the 17β-oestradiol plus progesterone-induced rise in cyclin D2 that was restricted to basal cells. Cyclin D2 is a cell cycle activator and provides further evidence for the non-quiescent state of the MaSC-enriched population upon progesterone exposure.

This study provides the first evidence, to our knowledge, for progesterone-driven dynamic shifts in the MaSC pool of the adult female. A natural progesterone peak during the reproductive cycle triggers alveologenesis with a remarkable expansion in $CD24^+CD49f^{hi}$ basal cells and mammary repopulating units. Complementing this, exogenous progesterone in ovariectomized mice induces a marked oscillation in the stem cell–progenitor-differentiated cell balance. Although previous studies have suggested a cycling property for mouse MaSCs[12] and progesterone induction of human bipotent progenitors *in vitro*[27], we demonstrate an inherent instructive role of progesterone in propelling MaSC expansion *in vivo* during the reproductive cycle,

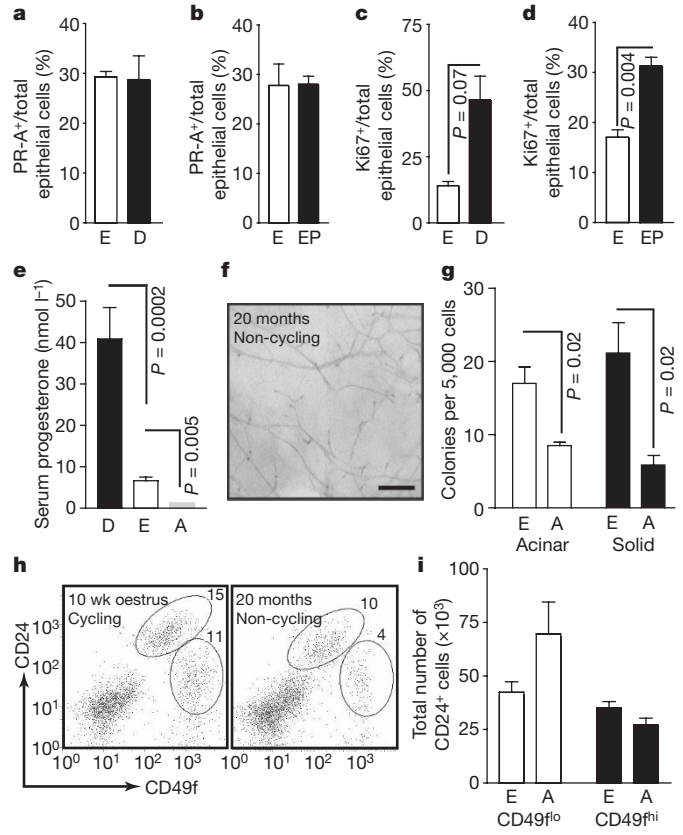

Figure 3 | Dynamic mammary cell turnover in cycling females whereas stem-cell-enriched basal cells show stasis in aged mice. **a**, **b**, The percentage of PR^+ cells in oestrous-staged and hormone-treated mice as quantified after immunostaining. **c**, **d**, Histograms showing the percentage of $Ki67^+$ cells at dioestrus and after 17β-oestradiol plus progesterone treatment. In **a**–**d**, data represent mean ± s.e.m.; $n = 2$ (**a**, **c**), $n = 3$ (**b**, **d**); E, oestrus and D, dioestrus in **a**, **c**; E, 17β-oestradiol and EP, 17β-oestradiol plus progesterone in **b**, **d**. **e**, Diminished serum progesterone levels in non-cycling, 20-month-old aged (A) mice compared with those in 10-week-old oestrus (E) and dioestrus mice (D). Data are mean ± s.e.m.; $n = 6$ (D), $n = 8$ (E), $n = 3$ (A). **f**, Mammary whole mount from a 20-month-old non-cycling female mouse. **g**, Histogram showing the clonogenic capacity of cells derived from 10-week-old oestrus (E) versus 20-month-old (A) glands. Results are mean ± s.e.m. of $n = 3$ mice each. **h**, FACS profiles of luminal $CD24^+CD49f^{lo}$ and basal $CD24^+CD49f^{hi}$ cell populations in 20-month-old mice are comparable to 10-week-old oestrus mice. **i**, Histogram showing the number of $CD24^+CD49f^{lo}$ and $CD24^+CD49f^{hi}$ cells in 20-month-old (A) mice compared to those at oestrus (E). Data are mean ± s.e.m.; $n = 4$ (E) and $n = 3$ (A).

and further elucidate probable downstream mechanisms. Progesterone probably acts via paracrine effectors in individual epithelial compartments to elicit specific mitogenic responses, as modelled in Supplementary Fig. 5a.

Both early menarche and late menopause translate into a greater number of reproductive cycles, and are recognized risk factors in breast cancer[2–5]. Furthermore, the inclusion of progestin in hormone replacement therapy is implicated in increased breast cancer risk[28]. The importance of progesterone in breast cancer is also illustrated in carcinogen- or *Brca1*-mediated mammary tumour models where proliferative signals from the PR are required for robust tumorigenesis[29,30].

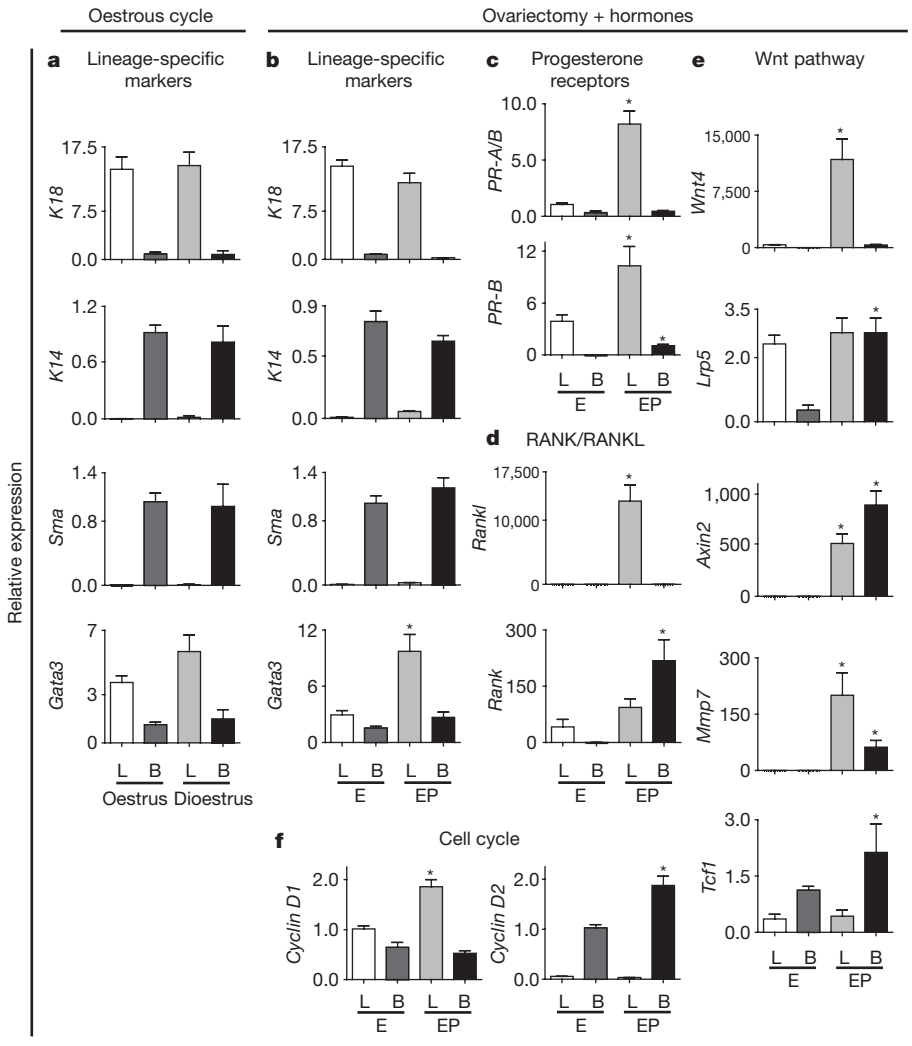

Figure 4 | RANKL and Wnt4 as paracrine effectors of progesterone-induced MaSC expansion. **a**–**f**, Luminal (L, $CD24^+CD49f^{lo}$) and MaSC-enriched basal (B, $CD24^+CD49f^{hi}$) populations were sorted by flow cytometry from dioestrus, oestrus, 17β-oestradiol (E) and 17β-oestradiol plus progesterone (EP) mammary glands and analysed by qRT–PCR for gene expression relative to β-actin. Expression is relative to that of $CD24^+CD49f^{hi}$ basal cells (B) from oestrus mice or ovariectomized mice with 17β-estradiol treatment, set at 1, except where it was undetectable (*PR-B*, *Wnt4*). In this case, expression is relative to $CD24^+CD49f^{hi}$ (B) cells from 17β-oestradiol plus progesterone glands. Data represent mean ± s.e.m.; $n = 3$ each for oestrus and dioestrus; $n = 4$ each for E and EP. Statistically significant differences ($P < 0.05$) are denoted with an asterisk.

Our study highlights the role of progesterone in MaSC regulation. Given the long-lived nature of the stem cell, MaSCs provide seeds for accumulating additional mutagenic hits in sporadic and/or familial breast cancer. We propose a concept that an expanded and cycling MaSC population, driven by progesterone, presents putative targets for cell transformation events at specific windows of the adult reproductive cycle (Supplementary Fig. 5b). Our findings provide new insights into the aetiology of breast cancer risk where progesterone is a complex and crucial mediator of mammary gland stem cell fate.

METHODS SUMMARY

All methods used in this study are illustrated in Supplementary Fig. 1 and are described in Supplementary Information. These include vaginal cytology, mammary cell dissociation, *in vivo* limiting dilution analyses, Matrigel colony-forming assays, FACS analysis and sorting, ovarian hormone treatments, quantitative RT–PCR, and immunohistochemistry.

Received 19 March; accepted 19 April 2010.
Published online 5 May; corrected 10 June 2010 (see full-text HTML version for details).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by grants from the Canadian Breast Cancer Research Alliance. P.A.J. holds a Terry Fox Foundation studentship through an award from the National Cancer Institute of Canada; H.W.J. holds a studentship and A.G.B. a fellowship from the Canadian Breast Cancer Foundation, Ontario. The authors thank F. Tong and R. Nayyar of the OCI FACS facility for cell sorting, and M. Monroy and S. Yousef of the UHN Animal Resources Center for performing ovariectomies.

Author Contributions P.A.J. designed and performed majority of the experiments and data analysis; H.W.J. conducted CFC assays and contributed to transplantation experiments; A.G.B. extracted RNA and performed quantitative RT–PCR; M.A.D.G. administered hormones and designed graphics; P.M. and C.C. provided PR antibody and advice; J.S. advised on multiple aspects of stem cell analyses; P.D.W. conceptualized the importance of the reproductive cycle; and R.K. directed the study. P.A.J. and R.K. wrote the paper.

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