

# Distinct stem cells contribute to mammary gland development and maintenance

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The mammary epithelium is composed of several cell lineages including luminal, alveolar and myoepithelial cells. Transplantation studies have suggested that the mammary epithelium is maintained by the presence of multipotent mammary stem cells. To define the cellular hierarchy of the mammary gland during physiological conditions, we performed genetic lineage-tracing experiments and clonal analysis of the mouse mammary gland during development, adulthood and pregnancy. We found that in postnatal unperturbed mammary gland, both luminal and myoepithelial lineages contain long-lived unipotent stem cells that display extensive renewing capacities, as demonstrated by their ability to clonally expand during morphogenesis and adult life as well as undergo massive expansion during several cycles of pregnancy. The demonstration that the mammary gland contains different types of long-lived stem cells has profound implications for our understanding of mammary gland physiology and will be instrumental in unravelling the cells at the origin of breast cancers.

The mammary gland is composed of epithelial cells and mesenchymal cells, including adipocytes, fibroblasts, blood vessels and immune cells<sup>1</sup>. Initially visible as placode-like structures, mammary glands are specified along the ventral epidermis during embryonic development and progressively invade the underlying mesenchyme, called the mammary fat pad. At puberty, the mammary gland expands considerably to form a highly branched tubular structure that progressively fills the fat pad. During pregnancy, the mammary gland expands further and the terminal end tubular structures differentiate into milk-producing cells. Two main cellular subtypes comprise the mammary gland epithelium: the basal myoepithelial cells and luminal cells, which can differentiate either into ductal cells or milk-producing cells (Supplementary Fig. 1). Whereas alveoli and luminal cells secrete the water and nutrients, the myoepithelial cells, through their contraction, guide the circulation of the milk throughout the ductal tree<sup>1–3</sup>.

Different assays have been developed to define the differentiation potential of mammary epithelial cells (MECs)<sup>2,4,5</sup>. *In vitro* assays indicated that both luminal cells and myoepithelial cells can be maintained with their lineage-restricted differentiation potential in a specific medium but only luminal cells can be forced to differentiate into myoepithelial cells upon medium switch<sup>6</sup>. Culture of fluorescence-activated cell sorting (FACS)-isolated human MECs gives rise to either luminal or myoepithelial colonies as well as some bipotent colonies<sup>6–8</sup>. Culturing MECs as non-adherent cells, called mammospheres, allowed renewal and differentiation of cells with unipotent and bipotent differentiation potential<sup>9</sup>. Transplantation of primary MECs at limiting dilutions suggested the presence of multipotent mammary stem cells and more committed progenitors<sup>10,11</sup>, and a single MEC is able to reconstitute an entire functional mammary gland in serial transplantation<sup>10</sup>. Transplantation of a single FACS-isolated MEC can reconstitute, although at low frequency, a normal mammary gland<sup>12,13</sup>, indicating that rare multipotent mammary stem cells reside at the top of the cellular hierarchy within the mammary gland. Although

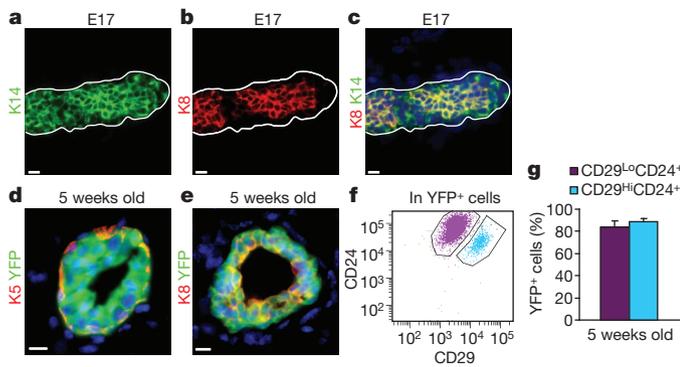
transplantation studies are important to define the differentiation potential of stem cells, these assays mimic a regenerative state that in certain circumstances forces stem cells to differentiate into lineages for which they usually do not contribute to under physiological conditions. For example, hair follicle bulge stem cells give rise to all epidermal lineages upon transplantation and wound healing, but only to hair follicle regeneration under physiological conditions<sup>14</sup>. The definitive demonstration that, under physiological conditions, multipotent stem cells are responsible for the development and adult maintenance of the mammary epithelium awaits genetic lineage-tracing experiments<sup>3</sup>. Here we developed novel lineage-tracing approaches in mice to decipher the cellular hierarchy of the mammary epithelium during physiological conditions.

## Multipotent embryonic K14 progenitors

We first assessed the contribution of K14-derived cells to mammary gland development and adult life using K14-Cre/Rosa-YFP mice. The mammary placode arises from the embryonic epidermis at embryonic day 14 (E14). At E17, all MECs expressed K14 and were YFP<sup>+</sup> in K14-Cre/Rosa-YFP mice and remained YFP<sup>+</sup> thereafter (Supplementary Figs 2 and 3). During the early stage of mammary gland development, K14 expression encompassed both myoepithelial cells and a fraction of luminal cells (Fig. 1a–c). At birth, the mammary gland consisted of a tubular epithelial structure composed of basal myoepithelial cells expressing K5, K14 and SMA and luminal cells expressing K8 and K19. At the beginning of puberty and thereafter, K14 expression was restricted to the myoepithelial lineage (Supplementary Figs 1 and 2). FACS analysis of mammary gland from K14-Cre/Rosa-YFP mice revealed that all YFP-labelled cells expressed CD24, as previously suggested<sup>15</sup>, and could be divided into two populations: CD29<sup>Hi</sup>CD24<sup>+</sup> enriched for myoepithelial cells and CD29<sup>Lo</sup>CD24<sup>+</sup> enriched for luminal cells<sup>12,13</sup> (Supplementary Figs 4 and 5). Because K14-Cre is expressed in myoepithelial cells after birth, we investigated whether all MECs derive from embryonic K14<sup>+</sup> progenitors by administering

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**Figure 1 | All mammary epithelial lineages derive from embryonic K14-expressing progenitors.** **a–c**, Immunostaining of K8 and K14 in mammary glands at E17. **d, e**, Immunostaining of K5 (**d**) or K8 (**e**) and YFP in K14-rtTA/TetO-Cre/Rosa-YFP MECs 5 weeks after doxycycline administration. **f**, FACS analysis of CD24 and CD29 expression in Lin<sup>-</sup>YFP<sup>+</sup> cells 5 weeks after doxycycline administration. **g**, YFP expression in Lin<sup>-</sup>CD29<sup>Lo</sup>CD24<sup>+</sup> and Lin<sup>-</sup>CD29<sup>Hi</sup>CD24<sup>+</sup> populations 5 weeks after doxycycline administration, showing that embryonic K14 tracing marked the vast majority of MECs ( $n = 3$  mice). Scale bars, 10  $\mu$ m; error bars indicate s.e.m.

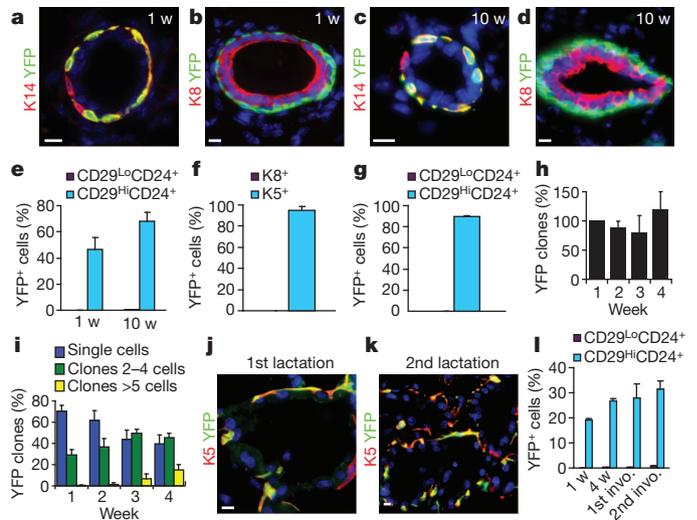
a single dose of doxycycline to pregnant K14-rtTA/TetO-Cre/Rosa-YFP mice at E17 to label MECs during development (Supplementary Fig. 6). Analysis of the mammary gland at puberty showed that embryonic labelling marked the majority of MECs, including both myoepithelial cells and luminal cells, demonstrating that embryonic K14<sup>+</sup> progenitors give rise to all MEC lineages (Fig. 1d–g).

### K14 myoepithelial stem cells

The transcriptional profiling of CD29<sup>Hi</sup>CD24<sup>+</sup> cells revealed that the putative multipotent mammary stem cells<sup>12,13</sup> are enriched for K5 and K14 (refs 12, 13, 16, 17). To determine whether postnatal K14<sup>+</sup> cells contain multipotent mammary stem cells, we performed inducible genetic lineage-tracing experiments of K14-expressing cells during puberty and in adult virgin mice. Doxycycline administration over 5 days in K14-rtTA/TetO-Cre/Rosa-YFP mice induced YFP expression in about 40% of myoepithelial cells but did not label luminal cells (Fig. 2a, b, e and Supplementary Figs 7 and 8). Surprisingly, 10 weeks after doxycycline administration, YFP was still exclusively expressed by myoepithelial cells (Fig. 2c–e and Supplementary Figs 7 and 8). To rule out that the remaining CD29<sup>Hi</sup>CD24<sup>+</sup> myoepithelial cell population that was not labelled in the previous experiments contains multipotent mammary stem cells, we administered doxycycline continuously to K14-rtTA/TetO-Cre/Rosa-YFP mice during the whole process of pubertal development and found that almost all myoepithelial cells (>97%) but no luminal cells were labelled (Fig. 2f, g and Supplementary Fig. 7m). These data demonstrate that the K14-expressing cells do not contribute to the luminal lineage during mammary gland expansion that occurred during pubertal development.

Administration of a low dose of doxycycline to K14-rtTA/TetO-Cre/Rosa-YFP 4-week-old mice resulted in the labelling of isolated myoepithelial cells 1 week after doxycycline administration. These cells were maintained for several weeks and about 10% of them expanded over time (Fig. 2h, i and Supplementary Fig. 9). YFP<sup>+</sup> myoepithelial cells expanded further during pregnancy and lactation. Some of them escaped mammary gland involution and reinitiated another cycle of expansion during the following pregnancy and were still present after the second mammary gland involution (Fig. 2j–l and Supplementary Fig. 10). The proportion of YFP-labelled cells was stable over time (Fig. 2l), showing that these unipotent stem cells undergo long-term self-renewal and are not replaced by multipotent stem cells over time.

Doxycycline administration to K14-rtTA/TetO-Cre/Rosa-YFP mice at postnatal day 1 (P1) marked mostly myoepithelial cells and



**Figure 2 | K14-expressing stem cells ensure mammary myoepithelial lineage expansion and maintenance during puberty, adult life and pregnancy.** **a–d**, Immunostaining of K14 (**a, c**) or K8 (**b, d**) and YFP 1 week (**a, b**) and 10 weeks (**c, d**) after doxycycline administration. **e**, Percentage of YFP<sup>+</sup> cells in Lin<sup>-</sup>CD29<sup>Hi</sup>CD24<sup>+</sup> and Lin<sup>-</sup>CD29<sup>Lo</sup>CD24<sup>+</sup> populations 1 week and 10 weeks after doxycycline administration at puberty ( $n = 3$  mice per time point). **f, g**, YFP expression in myoepithelial cells and luminal cells as determined by immunofluorescence (**f**) and FACS analysis (**g**) after 1 month of continuous doxycycline administration during puberty ( $n = 2$  mice per time point). **h**, Percentage of YFP myoepithelial clones observed at different time points. **i**, Temporal distribution of clone size observed as single cell, 2–4 cells and 5 cells or more ( $n =$  at least 470 clones from 3 different mice were counted at each time point). **j, k**, Immunostaining of K5 and YFP in MECs during first and second lactation after 1 mg doxycycline administration at puberty. **l**, Percentage of YFP<sup>+</sup> cells in MECs at different time points after doxycycline administration at puberty ( $n =$  at least 2 mice per time point). Scale bars, 10  $\mu$ m unless stated; error bars indicate s.e.m.

only a small proportion of luminal cells (Supplementary Fig. 11), indicating that the lineage restriction of K14-expressing cells occurred rapidly after birth.

To determine whether K14-rtTA/TetO-Cre targeted a subpopulation of myoepithelial cells distinct from the putative mammary stem cells, we repeated these experiments using K5-CreER mice, another marker preferentially expressed by putative multipotent mammary stem cells<sup>12,13,16,17</sup>. Tamoxifen administration to P1 and 4-week-old K5-CreER/Rosa-YFP mice initially labelled exclusively myoepithelial cells, giving rise several weeks later to myoepithelial cells only and not luminal cells. Similarly to K14-rtTA/TetO-Cre targeted cells, a fraction of K5-CreER targeted cells was able to clonally expand during puberty and pregnancy (Supplementary Figs 12–15), confirming the presence of myoepithelial stem cells and the apparent absence of multipotent mammary stem cells among postnatal MECs expressing K5 and K14.

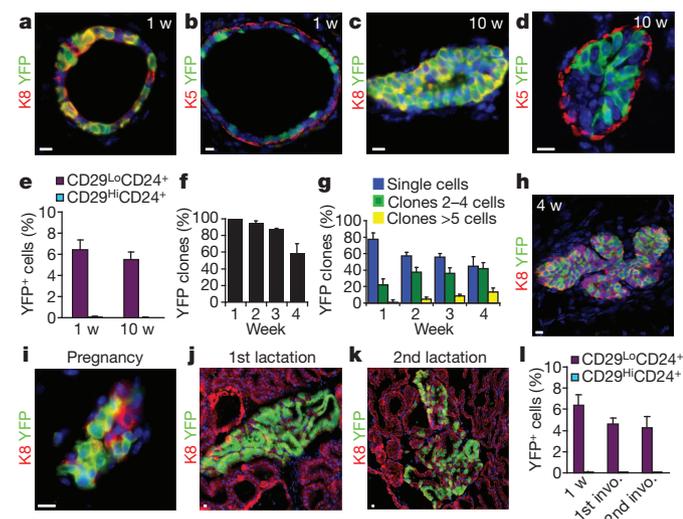
Lgr5, a marker of epithelial stem cells in different tissues<sup>18</sup>, has been reported to be enriched in the CD29<sup>Hi</sup>CD24<sup>+</sup> population<sup>12</sup>. Lgr5 is expressed in only 2–3% of MECs, localized to the nipple region. At puberty, the vast majority of Lgr5<sup>+</sup> cells within the mammary gland were myoepithelial cells, although a small fraction of Lgr5<sup>+</sup> cells was also luminal (Supplementary Fig. 16a–d). Tamoxifen administration to 4-week-old Lgr5-GFP-CreER/Rosa-Tomato mice preferentially labelled myoepithelial cells, giving rise to myoepithelial cells several weeks later (Supplementary Fig. 16e–m). Functionally, Lgr5<sup>+</sup> myoepithelial cells were indistinguishable from K14<sup>+</sup> cells except for their clustering close to the nipple region. The only difference between Lgr5 and K14 is the Lgr5 expression and tracing of rare luminal cells.

### K8 luminal stem cells

Lineage-tracing experiments using WAP-Cre mice, which is expressed and active in luminal cells during pregnancy, identified

long-lived cells that display the ability to clonally expand during pregnancy and give rise to luminal and alveolar cells, and are therefore called parity-induced cells<sup>19</sup>. *Ex vivo* culture of WAP-Cre mammary explants, in the presence of several growth factors, suggested that cells with similar renewal and differentiation potential as parity-induced mammary progenitors may already exist in nulliparous mice<sup>20</sup>. To determine whether, under physiological conditions without *ex vivo* manipulations, luminal cells contain stem cells before pregnancy and whether these cells represent unipotent or multipotent stem cells, as has been previously suggested for human MECs<sup>6</sup>, we generated transgenic mice expressing CreER in the luminal lineage using the K8 promoter (Supplementary Fig. 17a). Administration of tamoxifen to K8-CreER/Rosa-YFP mice at P1 only traced luminal cells (Supplementary Fig. 17). Similarly, tamoxifen administration to 4-week-old and adult virgin mice induced YFP expression only in luminal cells (Fig. 3a, b and Supplementary Figs 18 and 19), and after 10 weeks, YFP<sup>+</sup> cells had expanded but were still luminal cells (Fig. 3c, d and Supplementary Figs 18 and 19), indicating that K8<sup>+</sup> cells contain luminal stem cells.

Clonal analysis of K8<sup>+</sup> cells by administration of a low dose of tamoxifen revealed that not all YFP<sup>+</sup> cells persist long-term and at 4 weeks after tamoxifen administration 40% of YFP luminal clones were lost, indicating that K8-CreER also targets more committed luminal cells (Fig. 3e–h and Supplementary Figs 20–22). Temporal analysis of clone size revealed that about 10% of the YFP clones contained more than 5 YFP<sup>+</sup> cells, some of which could be even much larger (Fig. 3g and Supplementary Fig. 21), consistent with the targeting of luminal stem cells that clonally expand and participate in luminal cell expansion during puberty and maintenance during adult life.



**Figure 3 | K8-expressing stem cells ensure mammary luminal lineage expansion and maintenance during puberty, adult life and pregnancy.** a–d, Immunostaining of K8 (a, c) or K5 (b, d) and YFP in MECs 1 week (a, b) and 10 weeks (c, d) after 15 mg tamoxifen administration at puberty. e, Percentage of YFP<sup>+</sup> cells in Lin<sup>-</sup>CD29<sup>Lo</sup>CD24<sup>+</sup> and Lin<sup>-</sup>CD29<sup>Hi</sup>CD24<sup>+</sup> populations 1 week and 10 weeks after 1 mg tamoxifen administration to K8-CreER/Rosa-YFP mice at puberty ( $n = 7$  and 4 mice at 1 week and 10 weeks). f, g, Number of YFP clones (f) and distribution of clone sizes (g) observed as single cell, 2–4 cells, and 5 or more cells at different time points after 1 mg tamoxifen administration at puberty ( $n =$  at least 526 clones analysed from three different mice at each time point). h–k, Immunostaining of K8 (red) and YFP (green) in the mammary gland 4 weeks after induction (h), during pregnancy (i), lactation (j) and second lactation (k) in K8-CreER/Rosa-YFP mice treated with 1 mg tamoxifen at puberty. l, Percentage of YFP<sup>+</sup> cells in MECs at different time points after tamoxifen administration in K8-CreER/Rosa-YFP mice treated with 1 mg tamoxifen at puberty ( $n =$  at least 2 mice per time point). Scale bars, 10  $\mu$ m unless stated; error bars indicate s.e.m.

To establish further the renewal capacities of K8<sup>+</sup> luminal stem cells and their contribution to milk-producing cells, we induced clonal YFP expression in luminal cells during puberty and followed their fate during pregnancy and lactation (Fig. 3i–l and Supplementary Fig. 23). During pregnancy, only clones of YFP<sup>+</sup> luminal cells were found. During lactation, very large YFP clones were observed with some lobules that were almost entirely YFP<sup>+</sup> whereas others were either negative or mosaic for YFP expression (Fig. 3j and Supplementary Fig. 23). K8-CreER-targeted cells differentiated into both luminal and milk-producing cells, as revealed by the co-expression of YFP with K8 and NaPiIIb in the fat-milk-producing cells; no myoepithelial cells were YFP<sup>+</sup> (Fig. 3j and Supplementary Fig. 23d). After involution, some YFP<sup>+</sup> cells persisted and were able to reinitiate another round of expansion during the following pregnancy and lactation and to escape cell death during the second involution (Fig. 3k and Supplementary Fig. 23). Even after three consecutive cycles of pregnancy and lactation, K8-derived cells were found in luminal cells and milk-producing cells (Supplementary Fig. 23j–m). The percentage of YFP<sup>+</sup> cells was stable over time (Fig. 3l), indicating that these cells are self-renewing long-term and are not progressively replaced by multipotent stem cells.

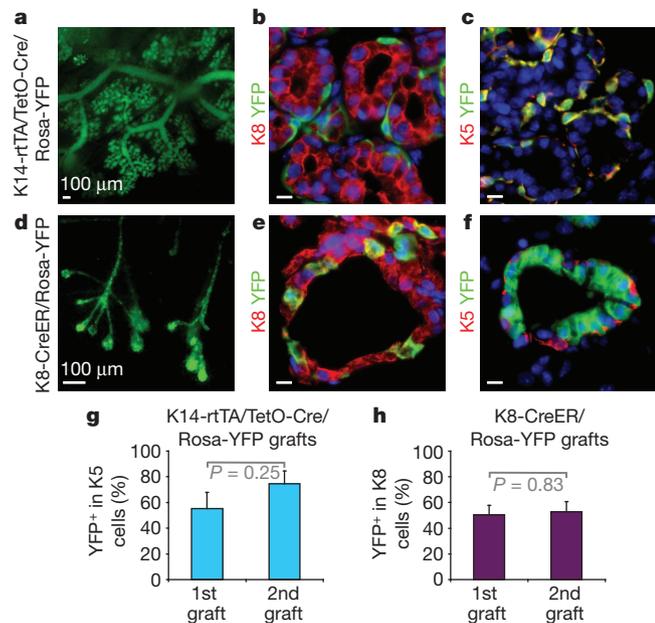
### K18 committed luminal cells

Different studies suggested that luminal cells are composed of morphologically distinct cell types that are thought to display different proliferation and differentiation capacities<sup>21–23</sup>. We used another luminal inducible CreER (K18-CreER)<sup>24</sup> to determine whether all luminal cells presented similar renewal and differentiation potential as compared to K8-CreER-targeted cells (Supplementary Figs 24–28). Administration of tamoxifen to 4- and 8-week-old K18-CreER/Rosa-YFP mice resulted in a patchy expression of YFP in the mammary gland. Only luminal cells were initially labelled and 10 weeks after tamoxifen administration, YFP-marked luminal cells were still present whereas no myoepithelial cells were YFP<sup>+</sup> (Supplementary Figs 24 and 25). Clonal analysis during puberty and in adult virgin mice revealed no sign of important clonal expansion, even during pregnancy and lactation (Supplementary Figs 26–28). Together, these data suggest that the luminal cells targeted by K18-CreER display a low cellular turnover, and should be considered as more committed luminal cells, possibly representing the cells that are lost and/or failed to expand in K8 lineage-tracing experiments.

### Transplantation and stem cell differentiation

To clarify the discrepancy between the results obtained in transplantation assays<sup>12,13</sup> and our lineage-tracing experiments, we performed mammary reconstitution assays with cells labelled by our different myoepithelial- and luminal-specific Cre. Transplantation of  $5 \times 10^4$  dissociated mammary gland cells—which represent about  $5 \times 10^4$  living MECs from 4-week-old K14-Cre/Rosa-YFP mice—into the fat pad of NOD/SCID mice allowed the reconstitution of a morphologically normal mammary gland with YFP<sup>+</sup> myoepithelial cells and luminal cells (Supplementary Fig. 29a–d).

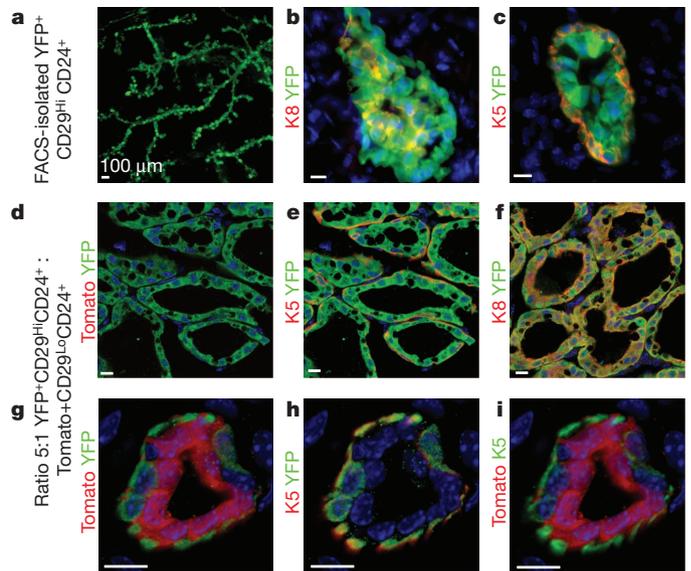
To determine whether the transplantation procedure itself can promote the differentiation potential of YFP-labelled stem cells into the other lineage, we induced YFP expression in myoepithelial cells by administering doxycycline to 4-week-old K14-rtTA/TetO-Cre/Rosa-YFP mice. One week later the mammary gland was dissociated into single cells and a mixture of YFP<sup>+</sup> myoepithelial cells together with unlabelled luminal cells was transplanted into the mammary fat pad of NOD/SCID mice (Supplementary Fig. 29a). Seven weeks after transplantation, the grafted cells regenerated a new mammary gland expressing YFP in the ducts and growing alveoli (Fig. 4a). Microscopic examination revealed that the vast majority of YFP<sup>+</sup> cells were myoepithelial cells (Fig. 4b, c and Supplementary Fig. 29e–h) and only very rare clones expressing YFP in both myoepithelial cells and luminal cells were identified (Supplementary Fig. 29i, j and



**Figure 4 | Myoepithelial and luminal stem cells maintain their unipotent fate in mammary reconstitution assay when co-transplanted in non-limiting conditions.** **a**, Whole-mount of the reconstituted mammary gland after transplantation of mammary cells in which myoepithelial cells have been induced to express YFP. **b, c**, Immunostaining of K8 (**b**) or K5 (**c**) and YFP in the K14-rtTA/TetO-Cre/Rosa-YFP graft. **d**, Whole-mount of the reconstituted mammary gland after the transplantation of breast cell suspensions in which a fraction of luminal cells has been induced to express YFP. **e, f**, Immunostaining of K8 (**e**) or K5 (**f**) and YFP in the K8-CreER/Rosa-YFP graft. **g, h**, Percentage of YFP<sup>+</sup> cells in K5<sup>+</sup> (**g**) and K8<sup>+</sup> (**h**) cells in the first and second transplants quantified by immunofluorescence in serial transplantation of unsorted cells from K14-rtTA/TetO-Cre/Rosa-YFP (**g**) and K8-CreER/Rosa-YFP (**h**) mice. Scale bars, 10  $\mu$ m unless stated; error bars indicate s.e.m.

Supplementary Table 1). Similar results were obtained after the transplantation of MECs labelled by K5-CreER (Supplementary Fig. 29k–m). Transplantation of YFP-marked luminal cells together with unlabelled myoepithelial cells after tamoxifen administration to K8-CreER/Rosa-YFP mice resulted in the regeneration of a new mammary gland expressing YFP in the duct and growing alveoli, but in this case only luminal cells and not myoepithelial cells were YFP<sup>+</sup> (Fig. 4d–f). Both luminal and myoepithelial YFP-marked cells could be serially transplanted without a significant decrease in the overall YFP chimaerism and with similar differentiation potential as obtained in the first transplantation. This suggests that both types of stem cells possess a sustained renewal potential and are not progressively replaced by multipotent stem cells (Fig. 4g, h and Supplementary Fig. 30). These experiments clearly demonstrate that luminal and myoepithelial stem cells actively participate in epithelial regeneration in mammary reconstitution assays while still exhibiting the similar lineage-restricted differentiation as observed in unperturbed mammary gland when the two types of unipotent stem cells are present together at non-limiting dilutions.

To determine whether the presence of only one type of mammary stem cell during the transplantation procedure can expand their differentiation potential, FACS-isolated YFP<sup>+</sup> myoepithelial or luminal cells were transplanted into the mammary fat pad of NOD/SCID mice (Supplementary Fig. 31a). As previously shown<sup>12,13</sup>, transplantation of FACS-isolated CD29<sup>Lo</sup>CD24<sup>+</sup> luminal cells alone failed to reconstitute a new mammary gland upon transplantation whereas CD29<sup>Hi</sup>CD24<sup>+</sup> myoepithelial cells alone were able to regenerate a new mammary gland independently of the presence of luminal cells (Fig. 5a–c and Supplementary Table 1). Finally, we assessed whether there is a critical threshold of luminal/myoepithelial cell ratio that



**Figure 5 | Myoepithelial but not luminal stem cells can be forced to adopt a multipotent fate in mammary reconstitution assays.** **a**, Whole-mount of the graft obtained after the transplantation of FACS-isolated YFP<sup>+</sup>CD29<sup>Hi</sup>CD24<sup>+</sup> cells. **b, c**, Immunostaining of K8 (**b**) and K5 (**c**) and YFP. **d–i**, Transplantation of 10<sup>4</sup> FACS-isolated YFP<sup>+</sup>CD29<sup>Hi</sup>CD24<sup>+</sup> cells together with 2,000 Tomato<sup>+</sup>CD29<sup>Lo</sup>CD24<sup>+</sup> cells into the mammary fat pads of NOD/SCID mice. Immunostaining of Tomato and YFP (**d, g**), K5 and YFP (**e, h**), K8 and YFP (**f**) and K5 and Tomato (**i**). Scale bars, 10  $\mu$ m unless stated.

uncovers the bipotentiality of stem cells. Transplantation of FACS-isolated YFP<sup>+</sup> myoepithelial cells and Tomato<sup>+</sup> luminal cells showed that decreasing the luminal/myoepithelial cell ratio to 1/5, which is about ten times lower than physiological conditions, markedly increased the ability of myoepithelial cells to differentiate into luminal cells, as about half of the grafts were exclusively derived from YFP<sup>+</sup> myoepithelial cells (Fig. 5d–f and Supplementary Table 1), whereas the other half was composed of a mixture of YFP<sup>+</sup> myoepithelial cells and Tomato<sup>+</sup> luminal cells (Fig. 5g–i and Supplementary Fig. 31).

## Discussion

Our study shows that the mammary gland initially develops from multipotent embryonic K14<sup>+</sup> progenitors, which give rise to both myoepithelial cells and luminal cells, as has been suggested previously<sup>25</sup>. During puberty and homeostasis, the expansion and maintenance of each lineage is ensured by the presence of two types of lineage-restricted stem cell, able to differentiate into either myoepithelial or luminal lineages, rather than being maintained by rare multipotent stem cells (Supplementary Fig. 32). Our data cannot rule out that some rare multipotent stem cells that were not targeted by the multiple different Cre lines used in this study exist in the mammary epithelium. However, the long-term maintenance and stable frequency of YFP<sup>+</sup> cells, their ability to be serially transplanted as well as their massive expansion during pregnancy and lactation clearly show that unipotent stem cells are not replaced by multipotent stem cells over time and that the contribution of multipotent stem cells to mammary gland morphogenesis and adult maintenance, if it exists, is very limited under physiological conditions.

Consistent with our data, previous experiments in which labelled and non-labelled MECs have been transplanted into the fat pad showed that the new mammary glands were composed of mixture of labelled and unlabelled cells<sup>11,19,26–29</sup>, suggesting that multiple progenitors contribute to mammary gland development and adult maintenance rather than being performed by rare stem cells. In humans, analysis of X-chromosome inactivation indicates that breast epithelium is organized into multiple discrete regions sharing the same inactive X chromosome

and suggested the existence of multiple stem cells scattered throughout the gland<sup>30</sup>.

Our study also demonstrates that transplantation assays, although extremely informative about the differentiation potential of tissue-specific stem cells, can be misleading in extrapolating the differentiation potential of stem cells under physiological conditions. These results may explain why genetically altered strains of mice that lack basal mammary stem cells in transplantation assays show no particular defects in development or pregnancy<sup>31,32</sup>. The very low frequency of luminal differentiation of transplanted unipotent myoepithelial stem cells when luminal cells are present within the graft at the physiological ratio, and the increase in the multipotent differentiation of myoepithelial cells when luminal cells were depleted, suggested that either luminal cells restrict the differentiation potential of myoepithelial stem cells or that the differentiation of myoepithelial stem cells into luminal cells is a relatively rare event compared to the natural differentiation of luminal stem cells, and consequently these rare multipotent clones were out-competed by clones originating from luminal stem cells in these non-limited dilution experiments. The reason why myoepithelial cells can adopt a multipotent fate and are able to regenerate a complete mammary gland upon transplantation remains elusive. One possibility would be that mammary reconstitution assays recapitulate the process of mammary gland development and adult K14/K5<sup>+</sup> stem cells are more prone to dedifferentiate into K14/K5 embryonic multipotent mammary progenitors under these conditions.

## METHODS SUMMARY

YFP expression was induced in K8-CreER/Rosa-YFP, K18-CreER/Rosa-YFP and K5-CreER/Rosa-YFP female mice by intraperitoneal tamoxifen injection and in K14-rtTA/TetO-Cre/Rosa-YFP mice by oral administration of doxycycline food or by intraperitoneal injection. Immunostaining was performed as described<sup>24</sup>. Mammary glands were dissected and lymph nodes removed. Tissues were cut in pieces of 1 mm<sup>3</sup> and digested in HBSS plus 300 U ml<sup>-1</sup> collagenase plus 300 µg ml<sup>-1</sup> hyaluronidase for 2 h at 37 °C under shaking. EDTA was added for 10 min, followed by trypsin-EGTA for 2 min. Cell labelling, flow cytometry and sorting were performed as described<sup>12,13</sup>. Dead cells were excluded with DAPI; CD45<sup>+</sup>, CD31<sup>+</sup> and CD140a<sup>+</sup> cells were excluded (Lin<sup>-</sup>) before analysis. Unsorted mammary cells or FACS-isolated cells were re-suspended in 10 µl PBS and injected into the cleared mammary fat pad of NOD/SCID female mice. Recipient mice were mated 4 weeks after the transplantation and analysed 3 weeks later.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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## METHODS

**Mice.** Rosa-YFP<sup>33</sup>, Rosa-Tomato<sup>34</sup>, and Lgr5-GFP-CreER mice<sup>18</sup> were obtained from the Jackson Laboratory. K14-Cre transgenic mice<sup>35</sup> and K14-rtTA<sup>36</sup> mice were provided by E. Fuchs. TetO-Cre mice<sup>37</sup> were provided by A. Nagy. The generation of K18-CreER mice was as previously described<sup>24</sup>. Mice colonies were maintained in a certified animal facility in accordance with European guidelines. These experiments were approved by the local ethical committee (CEBEA).

**Generation of K8-CreER mice.** The CreERT2 fragment (supplied by P. Chambon) preceded by the  $\beta$ -globin intron and followed by a SV40 polyA signal was subcloned into pBluescript II SK<sup>+</sup>. The 3.5-kb sequence upstream the ATG codon of the murine K8 gene, obtained from the BAC clone RP23-254K21 (BACPAC Resources Center, Children's Hospital Oakland Research Institute) using the forward primer 5'-GGTGGATCACTTGCCCCCTCCGTTTG-3' and the reverse primer 5'-GGGACAGCGCCAGCGAAGGCC-3', was cloned upstream of the  $\beta$ -globin intron. The resulting K8-CreER fragment of 6.3 kb was released from the backbone by NotI digestion and was microinjected into fertilized oocytes to generate transgenic mice (Jacquemin laboratory). Seven transgenic founders were first identified by PCR, out of 27 mice born. Expression profiles of the K8-CreER founders were screened with reporter Rosa-YFP mice. Four founders expressed the YFP in cells expressing the endogenous K8, and one founder K8 was used throughout this study.

**Generation of K5-CreER mice.** The CreERT2 fragment, preceded by IRES, was inserted into the 3' UTR of *Krt5* in 129-derived ES cells. Correctly targeted cells were injected into B6 blastocysts. The *neo* selection cassette was removed by crossing a chimaeric K5-CreER male to a  $\beta$ -actin-Flp female (B6;SJL-Tg(ACTFLPe)9205Dym/J). First generation of animals backcrossed to B6 were crossed with Rosa-YFP mice.

**Targeting YFP or Tomato expression.** K14-Cre/Rosa-YFP female mice express YFP in all cells derived from K14-expressing cells, whereas K14-Cre/Rosa-Tomato mice express Tomato in all cells derived from K14-expressing cells. For lineage tracing induced at 4 weeks or at 8 weeks, K8-CreER/Rosa-YFP, K18-CreER/Rosa-YFP, K5-CreER/Rosa-YFP and Lgr5-GFP-CreER/Rosa-Tomato female mice were induced with 15 mg of tamoxifen (Sigma, diluted in sunflower seed oil, Sigma) by intraperitoneal injection (three injections of 5 mg every other day) and K14-rtTA/TetO-Cre/Rosa-YFP female mice were induced by oral administration of doxycycline food diet (1 g kg<sup>-1</sup>, BIO-SERV) during 5 days, or 1 month. For lineage tracing induced at E17, pregnant females of K14-rtTA/TetO-Cre/Rosa-YFP mice were fed with a doxycycline food diet at E17 for 3 days. For lineage tracing induced at birth, K8-CreER/Rosa-YFP and K5-CreER/Rosa-YFP newborn mice were induced with 125  $\mu$ g tamoxifen (5  $\mu$ l of 25 mg ml<sup>-1</sup> solution) and K14-rtTA/TetO-Cre/Rosa-YFP female mice were induced with 25  $\mu$ g doxycycline (Sigma) (5  $\mu$ l of 5 mg ml<sup>-1</sup> solution diluted in sterile PBS) by intraperitoneal injection. For two-dimensional clonal analysis, K8-CreER/Rosa-YFP and K18-CreER/Rosa-YFP female mice were induced respectively with 1 mg and 10 mg tamoxifen and K14-rtTA/TetO-Cre/Rosa-YFP female mice were induced with 1 mg of doxycycline by intraperitoneal injection. For clonal analysis by confocal microscopy, K8-CreER/Rosa-YFP and K5-CreER/Rosa-YFP female mice were induced respectively with 0.2 and 7.5 mg tamoxifen by intraperitoneal injection.

**Histology and immunostaining.** Dissected mammary glands were pre-fixed for 2 h in 4% paraformaldehyde at room temperature. Tissues were washed three times with PBS for 5 min and incubated overnight in 30% sucrose in PBS at 4 °C. Tissues were then embedded in OCT and kept at -80 °C. Sections of 5  $\mu$ m (for epifluorescence analysis) or 50  $\mu$ m (for confocal analysis) were cut using a HM560 Microm cryostat (Mikron Instruments).

Five-micrometre sections were incubated in blocking buffer (5% NDS/1% BSA/0.2% Triton in PBS) for 1 h at room temperature. The different primary antibody combinations were incubated overnight at 4 °C. Sections were then rinsed three times for 5 min in PBS and incubated with proper secondary antibodies diluted at 1:400 in blocking buffer for 1 h at room temperature. Fifty-micrometre sections were incubated in blocking buffer (5% NDS/1% BSA/0.5% Triton/0.1% Tween 20 in PBS) for 2 h at room temperature. The different primary antibody combinations were incubated overnight at 4 °C. Sections were then rinsed three times in PBS for 45 min and incubated with proper secondary antibodies diluted at 1:400 in blocking buffer overnight at 4 °C. The following primary antibodies were used: anti-GFP (rabbit, 1:1,000, Molecular Probes), anti-GFP (chicken, 1:4,000, abcam), anti-K8 (rat, 1:500, Developmental Studies Hybridoma Bank), anti-K14 (rabbit, 1:2,000, Covance), anti-K14 (chicken, 1:1,000, Covance), anti-K5 (rabbit, 1:1,000, Covance), anti-K19 (rat, 1:500, Developmental Studies Hybridoma Bank), Cy3-coupled-anti-SMA (mouse, 1/1,000, Sigma), NaPiIIb (rabbit, 1/300, provided by J. Biber). The following secondary antibodies were used: anti-rabbit, anti-rat, anti-chicken conjugated to AlexaFluor 488 (Molecular Probes), to rhodamine Red-X or to Cy5 (JacksonImmunoResearch). Nuclei were stained in Hoechst solution and

slides were mounted in DAKO mounting medium supplemented with 2.5% Dabco (Sigma).

**Microscope image acquisition.** For whole-mount, freshly dissected mammary glands were squeezed between two coverslips and were imaged using a Zeiss EC Plan-NEOFLUAR  $\times 2.5$  objective. Pictures of immunostaining were acquired using  $\times 10$  and  $\times 40$  Zeiss EC Plan-NEOFLUAR objectives. Pictures were acquired on an Axio Observer Z1 microscope, with the AxioCamMR3 camera and using the Axiovision software (Carl Zeiss). Confocal pictures were acquired at room temperature using a Zeiss LSM780 multiphoton confocal microscope fitted on an Axiovert M200 inverted microscope equipped with C-Apochromat ( $\times 40 = 1.2$  numerical aperture) water immersion objectives (Carl Zeiss). Optical sections, 1,024  $\times$  1,024 pixels, were collected sequentially for each fluorochrome. The data sets generated were merged and displayed with the ZEN software.

**Mammary cell preparation.** Mammary glands were dissected and the lymph nodes removed before processing. Tissues were washed in HBSS, and cut in pieces of 1 mm<sup>3</sup> with scissors. The tissues were placed in HBSS plus 300 U ml<sup>-1</sup> collagenase (Sigma) plus 300  $\mu$ g ml<sup>-1</sup> hyaluronidase (Sigma) and digested for 2 h at 37 °C under shaking. EDTA at a final concentration of 5 mM was added for 10 min to the resultant organoid suspension, followed by 0.25% Trypsin-EGTA for 2 min before filtration through a 40- $\mu$ m mesh, two successive washes in 2% FBS/PBS and labelling.

**Cell labelling, flow cytometry and sorting.** Two–five million cells per condition were incubated in 250  $\mu$ l of 2% FBS/PBS with primary antibodies for 30 min, with shaking every 10 min. Primary antibodies were washed with 2% FBS/PBS, and cells incubated for 30 min with the appropriate secondary antibodies, with shaking every 10 min. Secondary antibodies were washed with 2% FBS/PBS and cells were resuspended in 2.5  $\mu$ g ml<sup>-1</sup> DAPI (Invitrogen) before analysis. Primary antibodies used were: biotinylated anti-CD24 (1/50, clone M1/69, BD Biosciences), APC-conjugated anti-CD29 (1/50, clone eBioHMB1-1, eBiosciences), PE-conjugated anti-CD45 (1/50, clone 30-F11, eBiosciences), PE-conjugated anti-CD31 (1/50, clone MEC 13.33, BD Biosciences), PE-conjugated anti-CD140a (1/50, clone APA5, eBiosciences). PE-Cy7 coupled Streptavidin (1/400, BD Biosciences) was used as secondary antibody. Data analysis and cell sorting was performed on a FACSaria sorter using the FACS DiVa software (BD Biosciences). Dead cells were excluded with DAPI; CD45<sup>+</sup>, CD31<sup>+</sup> and CD140a<sup>+</sup> cells were excluded (Lin<sup>-</sup>) before analysis of the YFP<sup>+</sup> cells. For Lgr5-GFP-CreER/Rosa-Tomato and K14-Cre/Rosa-Tomato mice, the same procedure was followed, except that no Lin<sup>-</sup> exclusion was performed. A minimum of 1,000 YFP<sup>+</sup> cells was analysed per mouse.

**Mammary fat-pad transplantation and analysis.** One-million unsorted mammary cells (around 5  $\times$  10<sup>4</sup> MECs) (for primary grafts), 10<sup>5</sup> unsorted cells from the primary graft (for secondary grafts) or 10,000 sorted cells (either only YFP<sup>+</sup>, or a combination of luminal Tomato<sup>+</sup> and myoepithelial YFP<sup>+</sup>) as indicated were resuspended in 10  $\mu$ l PBS and injected into the number 4 glands of 4-week-old NOD/SCID female mice that had been cleared of endogenous epithelium. Recipient mice were mated 4 weeks after the transplantation, and were killed 3 weeks later, when usually fully pregnant. Recipient glands were dissected, analysed by whole-mount, fixed and placed in OCT for further staining. An outgrowth was defined as an epithelial structure comprising ducts and lobules and/or terminal end buds. The percentage of YFP<sup>+</sup> luminal or myoepithelial cells was analysed on a minimum of 1,000 YFP<sup>+</sup> cells on an average of 12 sections per graft. For the grafts of unsorted cells from K8-CreER/Rosa-YFP mice previously treated with 15 mg tamoxifen, a total of 7,885 and 6,591 YFP<sup>+</sup> cells were analysed for the primary and secondary transplants, respectively. For the grafts of unsorted cells from K14-rtTA/TetO-Cre/Rosa-YFP mice previously treated with doxycycline food for 1 week, a total of 9,352 and of 5,505 YFP<sup>+</sup> cells were analysed for the primary and secondary transplants, respectively. For the graft of mixed YFP<sup>+</sup> and Tomato<sup>+</sup> sorted cells, a total of 4,714 luminal cells was analysed for expression of YFP or Tomato.

**Quantification of keratin expression in YFP<sup>+</sup> cells.** A minimum of 20 sections and about 10,000 YFP<sup>+</sup> cells per mouse were analysed for K5 and K8 expression. For lineage tracing of K14-rtTA/TetO-Cre/Rosa-YFP, a total of 32,853, 31,429, 31,633 and 34,002 cells from three different mice per condition were analysed respectively at 1 week and 10 weeks after tamoxifen administration at 4 weeks, and during first lactation and second lactation. No K8-expressing cells were observed among the YFP<sup>+</sup> cells in these conditions. For lineage tracing of K8-CreER/Rosa-YFP, a minimum of 10,000 YFP<sup>+</sup> cells per mouse from three different mice per condition were analysed respectively at 1 week and 10 weeks after tamoxifen administration at puberty, during first lactation and during second lactation. No K5-expressing cells were observed among the YFP<sup>+</sup> cells in these conditions.

**Quantification of the number and size of clones induced in the mammary gland.** A minimum of 500 YFP<sup>+</sup> clones were analysed per time point for each CreER/Rosa-YFP lineage in three independent mice. For each mammary tube section, the number, size (number of cells) and keratin expression (myoepithelial (K5) or luminal (K8)) of YFP clones was scored. The clones were grouped in three

classes according to their size, and their frequency was calculated as the percentage of the total number of clones. The mean number of clones per 1,000 cells was calculated at the different time points studied and normalized as the percentage of the number of clones counted 1 week after tamoxifen administration. For the confocal analysis of size of clones in K8-CreER/Rosa-YFP mice, 69 and 197 clones were analysed respectively 1 week and at 4 weeks after tamoxifen induction.

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