Distinct stem cells contribute to mammary gland development and maintenance

Alexandra Van Keymeulen1,*, Ana Sofia Rocha1,*, Marielle Ousset1, Benjamin Beck1, Gaëlle Bouvencourt1, Jason Rock2, Neha Sharma1, Sophie Dekoninck1 & Cédric Blanpain1,3

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 cells1. 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 tree1–3.

Different assays have been developed to define the differentiation potential of mammary epithelial cells (MECs)4–6. 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 switch6. Culture of fluorescence-activated cell sorting (FACS)-isolated human MECs gives rise to either luminal or myoepithelial colonies as well as some bipotent colonies6–8. Culturing MECs as non-adherent cells, called mammospheres, allowed renewal and differentiation of cells with unipotent and bipotent differentiation potential6. Transplantation of primary MECs at limiting dilutions suggested the presence of multipotent mammary stem cells and more committed progenitors10,11, and a single MEC is able to reconstitute an entire functional mammary gland in serial transplantation12. Transplantation of a single FACS-isolated MEC can constitute, although at low frequency, a normal mammary gland12,13, 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 conditions14. 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 experiments15. 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+ in K14-Cre/Rosa-YFP mice and remained YFP+ 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 2 and 3). FACS analysis of mammary gland from K14-Cre/Rosa-YFP mice revealed that all YFP-labelled cells expressed CD24, as previously suggested15, and could be divided into two populations: CD24+B2CD24+ enriched for myoepithelial cells and CD24+B2CD24− enriched for luminal cells15 (Supplementary Figs 4 and 5). Because K14-Cre is expressed in myoepithelial cells after birth, we investigated whether all MECs derive from embryonic K14 progenitors by administrating

---

1Université Libre de Bruxelles, IRIBHM, Brussels B-1070, Belgium. 2Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, USA. 3Welbio, Université Libre de Bruxelles, IRIBHM, Brussels B-1070, Belgium.

*These authors contributed equally to this work.
K14 myoepithelial stem cells

The transcriptional profiling of CD29\(^+\)CD24\(^-\) cells revealed that the putative multipotent mammary stem cells\(^{2,3}\) are enriched for K5 and K14 (refs 12, 13, 16, 17). To determine whether postnatal K14\(^-\) cells contain multipotent mammary stem cells, we performed inductive 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 and was not labelled in the previous experiments contains multipotent mammary stem cells, we administrated 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\(^-\) 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. 2i), 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 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 K14 tracing marked the vast majority of MECs (>97%) but did not label luminal cells (Supplementary Fig. 16a–d). Tamoxifen administration to K5-CreER mice, another marker preferentially expressed by putative multipotent mammary stem cells\(^{2,13,16,17}\), to 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\(^{15}\), has been reported to be enriched in the CD29\(^+\)CD24\(^-\) population\(^{12}\). Lgr5 is expressed in only 2–3% of MECs, localized to the nipple region. At puberty, the vast majority of Lgr5\(^+\) cells within the mammary gland were myoepithelial cells, although a small fraction of Lgr5\(^+\) 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\(^+\) myoepithelial cells were indistinguishable from K14\(^+\) 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. 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. 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, 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 cells had expanded but were still luminal cells (Fig. 3c, d and Supplementary Figs 18 and 19), indicating that K8+ cells contain luminal stem cells.

Clonal analysis of K8+ cells by administration of a low dose of tamoxifen revealed that not all YFP+ 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+ 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.

To establish further the renewal capacities of K8+ 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+ luminal cells were found. During lactation, very large YFP clones were observed with some lobules that were almost entirely YFP+ 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+ (Fig. 3j and Supplementary Fig. 23d). After involution, some YFP+ 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+ 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.

K818 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. We used another luminal inducible CreER (K18-CreER) 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+ (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 and our lineage-tracing experiments, we performed mammary reconstitution assays with cells labelled by our different myoepithelial- and luminal-specific Cre. Transplantation of dissociated mammary gland cells—which represent about 5 × 10⁶ 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+ 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 administrating doxycycline to 4-week-old K14-rTA/Tet-O-Cre/Rosa-YFP mice. One week later the mammary gland was dissociated into single cells and a mixture of YFP+ 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+ 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...
Fraction of luminal cells has been induced to express YFP in the mammary gland after the transplantation of breast cell suspensions in which a K14-rtTA/TetO-Cre/Rosa-YFP graft was introduced to express YFP. 

CD29^HiCD24^-positive cells contribute to the new mammary gland upon transplantation whereas CD29^LoCD24^-negative cells, as about half of the grafts were exclusively derived from YFP^+ cells, their ability to be serially transplanted as well as their massive expansion during pregnancy and lactation clearly uncovers the bipotentiality of stem cells. Transplantation of FACS-isolated YFP^+ myoepithelial cells and Tomato^+ 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^+ myoepithelial cells (Fig. 5d–f and Supplementary Table 1), whereas the other half was composed of a mixture of YFP^+ myoepithelial cells and Tomato^+ luminal cells (Fig. 5g–i and Supplementary Fig. 31).

Discussion

Our study shows that the mammary gland initially develops from multipotent embryonic K14^+ progenitors, which give rise to both myoepithelial cells and luminal cells, as has been suggested previously.25 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 Cre recombinase are present in the 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, 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.

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^+ (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^+ myoepithelial or luminal cells were transplanted into the mammary fat pad of NOD/SCID mice (Supplementary Fig. 31a). As previously shown,11,13,17,18,19,26,29, transplantation of FACS-isolated CD29^+CD24^- myoepithelial cells alone failed to reconstitute a new mammary gland upon transplantation whereas CD29^+CD24^- 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
and suggested the existence of multiple stem cells scattered throughout the gland.

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. 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 outcompeted 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 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-rta/TetO-Cre-Rosa-YFP mice by oral administration of doxycycline food or by intraperitoneal injection. Immunostaining was performed as described. Mammary glands were dissected and lymph nodes removed. Tissues were cut in pieces of 1 mm and digested in HBSS plus 300 U ml⁻¹ collagenase plus 300 μg ml⁻¹ 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. Dead cells were excluded with DAPI, CD45⁻, CD3¹ and CD140a⁻ cells were excluded (Lin⁻) before analysis. Untsorted mammary cells or FACS-isolated cells were re-suspended in 10 ml 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.

Received 4 March; accepted 21 September 2011.


Acknowledgements We thank our colleagues who provided us with reagents, which are cited in the text, and B. Hogan for sharing unpublished mice. We thank our colleagues who provided us with reagents, which are cited in the text, and B. Hogan for sharing unpublished mice. We thank our colleagues who provided us with reagents, which are cited in the text, and B. Hogan for sharing unpublished mice. We thank our colleagues who provided us with reagents, which are cited in the text, and B. Hogan for sharing unpublished mice.

Author Contributions C.B., A.V.K, A.S.R, D.C. and N.S. performed the experiments. K.S.W. and M.O. performed most of the experiments. J.R. generated the K5-CreER knockin mice. B.B., S.D. and A.V.K. performed the FACS analysis and cell sorting. G.B. and N.S. provided technical support. C.B. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to C.B. (cdeb@pasteur.fr).
METHODS

Mice. Rosa-YFP®, Rosa-Tomato®, and Lgr5-GFP-CreER mice® were obtained from the Jackson Laboratory. K14-Cre transgenic mice® and K14-rTA™ mice were provided by E. Fuchs. TetO-Cre mice® were provided by A. Nagy. The generation of K18-CreER mice was as previously described®, 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. Chambron) preceded by the β-globin intron and followed by a 5′-SA40A signal was subcloned into pBlueScript II SK+®. 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′-GGTTGACATGGCTCCCTCCGTTTG-3′ and the reverse primer 5′-GGGACAGGCGCCAGGAGGCC-3′, was cloned upstream of the β-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 K5 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 β-actin-Flp female (B6SJL-Tg(ACTFLP)9205Sdyjm). 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 injected with 15 mg of tamoxifen (Sigma, diluted in 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 with PBS for 5 min and incubated overnight in 30% sucrose in PBS at 4 °C. Five micrometre sections were incubated in blocking buffer (5% NDS/1% BSA/0.5% Triton/0.1% Tween 20) for 1 h at room temperature. The forward primer 5′-GGTTGACATGGCTCCCTCCGTTTG-3′ and the reverse primer 5′-GGGACAGGCGCCAGGAGGCC-3′, was cloned upstream of the β-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.

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 K5 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 β-actin-Flp female (B6SJL-Tg(ACTFLP)9205Sdyjm). 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 injected with 15 mg of tamoxifen (Sigma, diluted in 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 with PBS for 5 min and incubated overnight in 30% sucrose in PBS at 4 °C. Five micrometre sections were incubated in blocking buffer (5% NDS/1% BSA/0.5% Triton/0.1% Tween 20) for 1 h at room temperature. The forward primer 5′-GGTTGACATGGCTCCCTCCGTTTG-3′ and the reverse primer 5′-GGGACAGGCGCCAGGAGGCC-3′, was cloned upstream of the β-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 K5 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 β-actin-Flp female (B6SJL-Tg(ACTFLP)9205Sdyjm). 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 injected with 15 mg of tamoxifen (Sigma, diluted in 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 with PBS for 5 min and incubated overnight in 30% sucrose in PBS at 4 °C. Five micrometre sections were incubated in blocking buffer (5% NDS/1% BSA/0.5% Triton/0.1% Tween 20) for 1 h at room temperature. The forward primer 5′-GGTTGACATGGCTCCCTCCGTTTG-3′ and the reverse primer 5′-GGGACAGGCGCCAGGAGGCC-3′, was cloned upstream of the β-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 K5 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 β-actin-Flp female (B6SJL-Tg(ACTFLP)9205Sdyjm). First generation of animals backcrossed to B6 were crossed with Rosa-YFP mice.
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.


Supplementary Figure 1. Summary of keratin expression in the MG during morphogenesis and adult life.

a-d. Scheme summarizing the expression of different keratins expressed in the MG during morphogenesis, adult life and pregnancy. During embryogenesis, K14 is uniformly expressed in the mammary bud (green), while K8 is expressed preferentially in the inner cell of the bud (yellow) (a). At birth, MCs express K14 and K5, while LCs express K8 and K19. At this stage, K14 is expressed more broadly than K5 and is expressed in a subpopulation of K8 expressing cells (b). In adult virgin (c) and during pregnancy (d), LCs express K8 and K19, while MCs express K14, K5 and SMA. Embryonic mammary progenitors can be labelled for lineage tracing by the constitutive K14CRE and the inducible K14rtTA/TetOCRE. After birth, the myoepithelial cells can be labelled using the K14rtTA/TetOCRE or the K5CREER, while the luminal cells can be labelled using the K8CREER transgenic mice.
Supplementary Figure 2. Analysis of keratin expression during MG morphogenesis.
a-e. Immunostaining of K8 (red) and K14 (green) (a), K8 (red) and K5 (green) (b), K14 (red) and K5 (green) (c), SMA (red) and K14 (green) (d), SMA (red) and K5 (green) (e) in WT MG at E17, birth (P1), 10 days old (P10) and puberty (5w old). Scale bars, 10 μm.
Supplementary Figure 3. All mammary epithelial lineages derive from K14 expressing cells.

a. Scheme summarizing the genetic strategy used to target YFP expression in all K14 expressing cells and their progeny. b. Scheme summarizing the protocol used to study mammary epithelial lineages derived from K14 expressing cells at different stages of development. c. Wholemount of the MG of 4w old K14CRE/RosaYFP mice showing YFP expression throughout the MG. d-h. Immunostaining of K14 (d), K5 (e), SMA (f), K8 (g), or K19 (h) (red) and YFP (green) in K14CRE/RosaYFP MECs during embryogenesis (E17), at birth (P1), puberty (4w old), adult virgin (8w old) and mid-pregnancy. Rectangles highlight the areas shown at higher magnification. Altogether, these data show that during embryonic development K14CRE targets all future MECs of the MG, including MCs and LCs. Scale bars, 10 μm, unless stated.
Supplementary Figure 4. FACS analysis of CD29 and CD24 expression in MECs from K14CRE/RosaYFP mice. 

a-e. Unicellular suspension of mammary cells from K14CRE/RosaYFP mice stained for CD24, CD29 and Lin (CD31, CD45 and CD140a) were gated in P1 to eliminate debris (a), doublets were discarded in P2 (b), the living cells were gated in P3 by DAPI dye exclusion (c), the non-epithelial Lin positive cells were discarded in P4 (d), and the YFP positive cells were gated in P5 (e). f, g. CD24 and CD29 expression was studied in P5 to analyze only YFP+ cells (f) and in P4 to analyze the lin- cells (g). h. Gating tree showing the gating strategy used for FACS analysis and showing the proportion of parent and total cells for each gates. These data demonstrate that living YFP+ MECs (Lin-CD29HiCD24+ and Lin-CD29LoCD24+ population) represent overall 7% of the total cells of the MG. i. Distribution of YFP positive cells in Lin-CD29HiCD24+ and Lin-CD29LoCD24+ populations at puberty (4w) and adult virgin females (8w), showing a ratio of 2-3 LCs for 1MC. Histograms and error bars represent the mean and sem.
Supplementary Figure 5. Strategy used for the determination of Lin negative gate for flow cytometry analysis using fluorescently labelled MECs from K14CRE/RosaYFP mice. 

a, e, i. 3 different choices of Lin- gates are shown in non debris, singlet, and living cells. Dot plots showing the relative abundance of YFP+ MECs (green) and YFP- non-MECs (red) at different levels of Lin- gate. 

b, f, j. Dot plot showing YFP+ MECs (green) and YFP- non-MECs (red) at different levels of the Lin- gate. 

c, g, k. Histograms showing YFP expression at different levels of the Lin- gate. 

d, h, l. Dot plot showing CD29LoCD24+ (purple) and CD29HiCD24+ (blue) populations at different levels of the Lin- gate. These data show that most of the MECs express very low level of the Lin- markers (<1,000 of fluorescence), while there are a lot of non-MECs expressing low level of the Lin- markers (fluorescence between 1,000 to 10,000). Using the gate at the natural break between the populations would include all the red non-MECs and the MEC purity of the isolated population would drop to 28% while still excluding 5% of the YFP+ cells. By setting the gate at 1,000, where most of the YFP epithelial cells reside, the MEC purity is much higher (44%) and only 12% of YFP expressing cells are excluded from the analysis. Setting the gate further lower at 100 only resulted in a marginal increase in cell purity while massively increasing the number of cells excluded from the analysis (50%). In conclusion, gate 2 represents the best compromise between cell purity and cell exclusion to preferentially purify MECs by FACS. Modification of the Lin- gate at the natural break between Lin low and Lin High did not modify the proportion of YFP cells expressing CD29HiCD24+ and CD29LoCD24+ and thus enlarging the Lin- gate does not add or remove any meaningful data from the analysis.
Supplementary Figure 6. Scheme summarizing the protocol used to induce CRE activity in K14 expressing cells at E17.
Supplementary Figure 7. K14 expressing unipotent stem cells ensure mammary myoepithelial lineage maintenance and expansion during puberty.

a. Scheme summarizing the genetic strategy used to target YFP expression in K14 expressing cells. b. Scheme summarizing the protocol used to induce the CRE activity in K14 expressing cells during puberty and to analyze the differentiation potential of these cells at different time points. c. Wholemount of the MG 5 days after DOX administration in K14rtTA/TetOCRE/RosaYFP mice at puberty. d-i. Immunostaining of K5 (d, g), SMA (e, h), or K19 (f, i) (red) and YFP (green) 1w (d-f) or 10w (g-i) after DOX administration for 5 days in 4w old K14rtTA/TetOCRE/RosaYFP mice. Rectangles highlight areas shown at higher magnification, showing that YFP is expressed only in MCs. j-m. FACS analysis of CD24 and CD29 expression in Lin- cells (j, l) or in Lin-YFP positive cells (k, m) of K14rtTA/TetOCRE/RosaYFP mammary cells, 1w (j, k) or 10w (l, m) after DOX induction at puberty, showing that all YFP+ cells are present in the Lin-CD29HiCD24+ population. n. Scheme summarizing the protocol used to induce CRE activity in almost all K14 expressing cells during puberty. Scale bars, 10 μm.
Supplementary Figure 8. K14 expressing unipotent stem cells ensure mammary myoepithelial lineage maintenance and expansion in adult virgin mice.

a. Scheme summarizing the protocol used to induce the CRE activity in K14 expressing cells in adult virgin mice and to analyze the differentiation potential of these cells at different time points. b. Histogram representing the percentage of YFP positive cells within CD29Hi CD24+ and CD29LoCD24+ populations in adult virgin K14rtTA/TetO CRE/RosaYFP mice 1w and 10w after DOX administration (n= 2 and 3 mice analyzed respectively at 1w and 10w). c-l. Immunostaining of K5 (c, h), K14 (d, i), SMA (e, j), K8 (f, k) or K19 (g, l) (red) and YFP (green) 1w after DOX administration for 5 days in 8w old K14rtTA/TetO CRE/RosaYFP mice (c-g) and 10w after induction (h-l). Rectangles highlight areas shown at higher magnification. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 9. Clonal expansion of K14 marked cells at puberty visualized by confocal microscopy.

3D confocal analysis of immunostaining of K5 (red) and YFP (green) performed on thick sections of K14rtTA/TetOCRE/RosaYFP MG 4w (in 6 Z-stacks) after YFP initiation at puberty by injection of 1mg DOX, showing firstly that only MCs express YFP and secondly, that large YFP+ coherent clones containing more than 30 cells can be found along MCs surrounding a mammary duct, consistent with the targeting of a MC with high self renewal potential. Scale bars, 10 μm.
Supplementary Figure 10. K14 expressing unipotent stem cells ensure long-term maintenance and expansion of the mammary myoepithelial lineage during several cycles of pregnancy.

a. Scheme summarizing the protocol used to induce the CRE activity in K14 expressing cells at puberty and to analyze the differentiation potential of these cells during pregnancy, lactation, after involution, during a 2nd lactation and after a 2nd involution. 

b-i. Immunostaining of K8 (c, d, f, g, i) or K5 (b, e, h) (red) and YFP (green) in K14rtTA/TetOCRE/RosaYFP MG during pregnancy (b, c), lactation (d), after involution (e, f), during 2nd lactation (g) and after 2nd involution (h, i), showing that myoepithelial stem cells ensure long term maintenance of the myoepithelial lineage but do not contribute to LCs during cycles of expansion/regression. Scale bars, 10 μm.
Supplementary Figure 11. K14 lineage tracing at birth preferentially marks prospective MCs.

a. Scheme summarizing the protocol used to induce the CRE activity in K14 expressing cells at birth and to analyze the differentiation potential of these cells 5 weeks later. b-e. Immunostaining of K5 (b, c) or K8 (d, e) (red) and YFP (green) in the MG 5w after DOX injection in K14rtTA/TetOCRE/RosaYFP P1 mice. f, g. Histogram representing the ratio of K5 and K8 expression in YFP+ cells (f) and histogram representing immunostaining analysis of YFP expression in K5+ and K8+ cells (n=4 mice). h. Histogram representing FACS analysis of YFP positive expression in CD29HiCD24+ and CD29LoCD24+ populations 5w after neonatal DOX injection of 25 μg (n= 5 mice). The data illustrate that lineage restriction in K14 expressing progenitors arise early during the neonatal period and at P1, the vast majority of K14 expressing cells give rise 5 weeks later to MCs, although rare YFP+ LCs can also be found. Scale bars, 10μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 12. K5 lineage tracing at birth marks prospective MCs.

a. Scheme summarizing the genetic strategy used to target YFP expression in K5 expressing cells.
b. Scheme summarizing the protocol used to induce the CRE activity in K5 expressing cells at birth and to analyze the differentiation potential of these cells 5w later.
c, d. Immunostaining of K5 (c) or K8 (d) (red) and YFP (green) in K5CREER/RosaYFP MG 5w after 125 μg TAM administration at birth.
e. Histogram representing the percentage of YFP positive cells in CD29LoCD24+ and in CD29HiCD24+ populations from K5CREER/RosaYFP mice 5w after TAM administration at birth (n= 3). Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 13. K5CREER targeted MCs during puberty give rise to MCs only.

a. Scheme summarizing the protocol used to induce the CRE activity in K5 expressing cells at puberty and to analyze the differentiation potential of these cells at different time points.

b. Histogram representing the percentage of YFP positive cells in CD29HiCD24+ and CD29LoCD24+ populations from K5CREER/RosaYFP mice 1w and 8w after 15 mg TAM administration at puberty (n= 3 mice per time point).

c-l. Immunostaining of K5 (c, h), K14 (d, i), SMA (e, j), K8 (f, k) or K19 (g, l) (red) and YFP (green) in K5CREER/RosaYFP MG 1w (c-g) and 8w after 15 mg TAM administration at puberty (h-l), showing that 8w after CRE-mediated YFP expression, only MCs are YFP+. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 14. K5CREER targets MCs with high clonogenic potential during puberty.

a, b. 3D confocal analysis of immunostaining of K5 (red) and YFP (green) of a thick section of the MG of K5CREER/RosaYFP MG 1w (a) and 4w (in 7 z-stacks) (b) after 7.5 mg TAM administration at puberty, showing the important clonal expansion of YFP-marked MCs 4w later. The white line delineates the limits around a coherent large clone. Scale bars, 10 μm.
Supplementary Figure 15. K5CREER marked cells during puberty give rise to only MCs during pregnancy.

a. Scheme summarizing the protocol used to induce the CRE activity in K5 expressing cells at puberty and to analyze the differentiation potential of these cells during the 1st lactation and 2nd lactation. b-e. Immunostaining of K5 (b, d) or K8 (c, e) (red) and YFP (green) in K5CREER/RosaYFP MG during first lactation (b, c) and during 2nd lactation (d, e), showing that some K5CREER marked cells survive long term, and contribute to the MC lineage during two consecutive cycles of pregnancy followed by a MG involution. Scale bars, 10 μm.
Supplementary Figure 16. Lgr5CREER targeted cells during puberty are mostly MCs giving rise to MCs.

a. Scheme summarizing the genetic strategy used to target Tomato expression in Lgr5-GFP-CREER expressing cells. b, c. Immunostaining of K5 (b) or K8 (c) (red) and Lgr5 (revealed with GFP, green) in 1-month-old Lgr5GFP-CREER mice. d. Histogram representing the percentage of GFP+ cells in CD29HiCD24+ and in CD29LoCD24+ populations from 4w, 8w or 12w old Lgr5GFP-CREER mice (n= 5, 2 and 2 mice analyzed respectively at 4w, 8w and 12w). e. Scheme summarizing the protocol used to induce the CRE activity in Lgr5 expressing cells at puberty and to analyze the fate of these cells at different time points. f. Wholemount of a 5w Lgr5GFP-CREER/RosaTomato MG 1w after 15 mg TAM administration, showing the Tomato expression in the MECs in the ducts close to the nipple. White line delineates the ducts. g. Histogram representing the percentage of Tomato positive cells in CD29HiCD24+ and in CD29LoCD24+ populations from Lgr5GFP-CREER/RosaTomato mice 1w and 4w after 15 mg TAM administration at puberty (n=4 and 2 mice analyzed respectively at 1w and 4w). h-k. Immunostaining of K5 (h, j) or K8 (i, k) (green) and Tomato (red) in Lgr5GFP-CREER/RosaTomato MG 1w (h, i) and 4w after induction (j, k) after 15 mg TAM administration at puberty. Rectangles highlight areas shown at higher magnification. l, m. Immunostaining of K5 (l) or K8 (m) (green) and Tomato (red) in Lgr5GFP-CREER/RosaTomato MG during lactation. Scale bars, 10 μm, unless stated. Histograms and error bars represent the mean and sem.
Supplementary Figure 17. K8CREER targeted cells at birth give rise essentially to LCs.

a. Scheme summarizing the genetic strategy used to target YFP expression in K8 expressing LCs. b. Scheme summarizing the protocol used to induce the CRE activity in K8 expressing cells at birth and to analyze the differentiation potential of these cells 5w later. c, d. Immunostaining of K8 (c) or K5 (d) (red) and YFP (green) in K8CREER/RosaYFP MG 5w after 125 μg TAM administration at birth. e. Histogram representing the percentage of YFP positive cells in CD29LoCD24+ and in CD29HiCD24+ populations from K8CREER/RosaYFP mice 5w after TAM administration at birth (n= 3), showing that the vast majority of YFP+ cells are LCs. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 18. K8CREER targets LCs during puberty that give rise only to LCs.

a. Scheme summarizing the protocol used to induce the CRE activity in K8 expressing cells at puberty and to analyze the differentiation potential of these cells at different time points. 
b-e. FACS analysis of CD24 and CD29 expression in Lin-cells (b, d) or in Lin-YFP+ cells (c, e) of K8CREER/RosaYFP MG, 1w (b, c) and 10w (d, e) after TAM administration at puberty. 
f-k. Immunostaining of K19 (f, i) K14 (g, j) or SMA (h, k) (red) and YFP (green) in K8CREER/RosaYFP MG 1w (f-h) and 10w (i-k) after 15 mg TAM administration at puberty. Rectangles highlight areas shown at higher magnification. 
l, m. Immunostaining of K8 (l) or K5 (m) (red) and YFP (green) in K8CREER/RosaYFP MG 7 months after 15 mg TAM administration at puberty. 
n. Histogram representing the percentage of YFP positive cells in CD29Hi CD24+ and in CD29Lo CD24+ populations from K8CREER/RosaYFP mice 1w and 7 months after 15 mg TAM administration at puberty (n= at least 3 mice per time point), showing the long-term maintenance of these cells. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 19. K8CREER targets LCs in adult virgin mice that give rise only to LCs.

a. Scheme summarizing the protocol used to induce the CRE activity in K8 expressing cells in adult virgin mice and to analyze the differentiation potential of these cells at different time points. b-k. Immunostaining of K8 (b, g), K19 (c, h), K5 (d, i), K14 (e, j) or SMA (f, k) (red) and YFP (green) in K8CREER/RosaYFP MG 1w (b-f), and 10w (g-k) after 15 mg TAM administration in 8w old mice. Rectangles highlight areas shown at higher magnification. l, m. Immunostaining of K8 (l) or K5 (m) (red) and YFP (green) in K8CREER/RosaYFP MG 6 months after 15 mg TAM administration in adult virgin mice. n. Histogram representing the percentage of YFP positive cells in CD29HiCD24+ and CD29LoCD24+ populations from K8CREER/RosaYFP mice 1w and 6 months after 15 mg TAM administration in adult virgin mice (n= at least 3 mice per time point). These data show that LCs give rise only to LCs in adult virgin mice and are not replaced by other cells overtime. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 20. Clonal analysis of K8CREER labelled cells during puberty.

**a.** Scheme summarizing the protocol used to induce the CRE activity in isolated K8 expressing cells at puberty and to analyze the fate of these cells at different time points. **b, c.** Immunostaining of K8 (b) or K5 (c)(red) and YFP (green) in K8CREER/RosaYFP mice treated with 1 mg TAM at puberty and analyzed 1, 2, 3, 4 and 12w later, showing the clonal expansion of some K8 targeted cells. Scale bars, 10 μm.
Supplementary Figure 21. Clonal expansion of K8CREER labelled cells during puberty analyzed by confocal microscopy.

a-d. 3D confocal analysis of immunostaining of K8 (red) and YFP (green) in thick section of MG of K8CREER/RosaYFP mice treated with 0.2 mg TAM at puberty and analyzed 1w (a) and 4w later (b-d). These data show that only isolated YFP marked cells were present 1w following TAM administration. Three weeks later some YFP marked cells had considerably expanded and formed coherent clones containing many YFP+ cells. b. represents a large YFP coherent clone found in the main duct, c. represents a very large clone found in the terminally end structure and d. represents a large luminal clone found in a secondary duct. e. Frequency of large YFP+ clones (more than 5 cells) as quantified by confocal microscopy in K8CREER/RosaYFP 1w and 4w following 0.2 mg TAM administration at puberty (n=197 counted clones). Scale bars, 10 μm.
Supplementary Figure 22. K8CREER targets unipotent stem cells that ensure mammary luminal lineage maintenance and expansion in adult virgin mice.

a. Scheme summarizing the protocol used to induce the CRE activity in K8 expressing cells in adult virgin mice (8w) and to analyze the fate of these cells at different time points. b. Histogram representing the percentage of YFP positive cells in CD29HiCD24+ and CD29LoCD24+ populations 1w and 10w after 1 mg TAM administration to 8w old K8CREER/RosaYFP mice (n=7 and 4 mice analyzed respectively at 1w and 10w). c, d. Immunostaining of K8 (c) or K5 (d) (red) and YFP (green) in MG of K8CREER/RosaYFP mice treated with 1 mg TAM during homeostasis and analyzed 1, 2, 3, 4, 6 and 8 w later. e. Frequency of YFP clones at different time points in K8CREER/RosaYFP induced with 1 mg TAM at 8w. f. Distribution of clone sizes observed as single cells, 2-4 cells, or 5 or more cells in K8CREER/RosaYFP at different time points following 1 mg TAM administration at 8w (n=482, 516, 670, 670, 312 and 195 clones analyzed from 2 different mice at 1w, 2w, 3w, 4w, 6w and 8w respectively). Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 23. K8CREER targets unipotent stem cells that ensure mammary luminal lineage expansion during pregnancy.

a. Scheme summarizing the protocol used to induce the CRE activity in K8 expressing cells at puberty and to analyze the fate of these cells during pregnancy, lactation or after involution. b-i. Immunostaining of K5 (b, c, e, g, i), K8 (f, h) or NAPI IIB (d) (red) and YFP (green) in K8CREER/RosaYFP MG during pregnancy (b), lactation (c, d), after involution (e, f), during 2nd lactation (g) or after the 2nd involution (h, i), showing the long term renewing potential and unipotent fate of K8CREER marked LCs during the successive rounds of pregnancy. j-m. Immunostaining of K8 (j, k) or K5 (l, m) (red) and YFP (green) in K8CREER/RosaYFP MG of lactating mice that were induced with 1 mg TAM at puberty, mated at 8w, and which then underwent 3 successive pregnancies. Scale bars, 10 μm.
**Supplementary Figure 24. K18CREER targets committed LCs during puberty.**

**a.** Scheme summarizing the genetic strategy used to target YFP expression in K18 expressing LCs.  

**b.** Scheme summarizing the protocol used to induce the CRE activity in K18 expressing cells at puberty and to analyze the fate of these cells at different time points.

**c-i.** Immunostaining of K8 (c, h), K19 (d, i), K5 (e, j), K14 (f, k) or SMA (g, l) (red) and YFP (green) in K18CREER/RosaYFP MG 1w (c-g) and 10w (h-l) after 15 mg TAM administration during puberty. Rectangles highlight areas shown at higher magnification. Scale bars, 10 μm.
Supplementary Figure 25. K18CREER targets committed LCs in adult virgin mice.

a. Scheme summarizing the protocol used to induce the CRE activity in K18 expressing cells at 8w and to analyze the differentiation potential of these cells at different time points. b-k. Immunostaining of K8 (b, g), K19 (c, h), K5 (d, i), K14 (e, j) or SMA (f, k) (red) and YFP (green) in K18CREER/RosaYFP MG 1w (b-f) and 10w (g-k) after 15 mg TAM administration at 8w. Rectangles highlight areas shown at higher magnification. Scale bars, 10 μm.
**Supplementary Figure 26. Clonal analysis of K18CREER labelled cells during puberty.**

**a.** Scheme summarizing the protocol used to induce the CRE activity in K18 expressing cells at puberty and to analyze the fate of these cells at different time points. **b.** Histogram representing the percentage of YFP positive cells in CD29HiCD24+ and in CD29LoCD24+ populations from K18CREER/RosaYFP mice 1w and 4w after 10 mg TAM administration at puberty (number of mice analyzed is respectively 4 and 3 for 1w and 4w). **c, d.** Immunostaining of K8 (c) or K5 (d) (red) and YFP (green) in K18CREER/RosaYFP mice treated with 10 mg TAM during puberty and analyzed 1, 2, 3, 4, 6 and 8w later. **e.** Normalized frequency of YFP clones observed at different time points in K18CREER/RosaYFP mice induced with 10 mg TAM at puberty. **f.** Distribution of clone sizes observed as single cell, 2-4 cells, and 5 or more cells in K18CREER/RosaYFP at different time points following 10 mg TAM at puberty (number of clones analyzed is respectively 79, 269, 257, 181, 135 and 93 from 2 different mice in 1w, 2w, 3w, 4w, 6w and 8w). These data indicated that K18CREER targeted cells present a low cellular turnover during pubertal development. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 27. Clonal analysis of K18CREER labelled cells in adult virgin mice.

**a.** Scheme summarizing the protocol used to induce the CRE activity in K18 expressing cells at 8w and to analyze the fate of these cells at different time points. **b.** Histogram representing the percentage of YFP positive cells in CD29HiCD24+ and CD29LoCD24+ populations from K18CREER/RosaYFP mice 1w and 10w after 10 mg TAM administration at 8w (n= 6 and 2 mice analyzed respectively at 1w and 10w). **c, d.** Immunostaining of K8 (c) or K5 (d) (red) and YFP (green) in K18CREER/RosaYFP mice treated with 10 mg TAM at homeostasis and analyzed 1, 2, 3 and 4w later. **e.** Frequency of YFP clones observed at different time points in K18CREER/RosaYFP mice induced with 10 mg TAM. **f.** Distribution of clone sizes observed as single cells, 2-4 cells, or 5 or more cells in K18CREER/RosaYFP at different time points following 10 mg TAM administration at homeostasis (n= 210, 189, 129 and 151 clones analyzed from 3 different mice at 1w, 2w, 3w and 4w respectively). These data indicate that K18CREER targeted cells present a low cellular turnover in adult virgin mice. Scale bars, 10 μm. Histograms and error bars represent the mean and sem.
Supplementary Figure 28. K18CREER targets committed LCs that present little expansion during pregnancy.

a. Scheme summarizing the protocol used to induce the CRE activity in K18 expressing cells at puberty and to analyze the fate of these cells during pregnancy, lactation or after involution. b-h. Immunostaining of K8 (b, c, e), NAPI IIB (d) or K5 (f-h) (red) and YFP (green) in K18CREER/RosaYFP mice treated with 10 mg TAM at puberty, during pregnancy (b, f), lactation (c, d, g) and after involution (e, h). i. Histogram representing the percentage of YFP positive cells in CD29HiCD24+ and CD29LoCD24+ populations 1w and 4w after induction, and after involution, in K18CREER/RosaYFP mice treated with 1 mg TAM at puberty (number of mice analyzed is respectively 4, 3 and 3 for 1w, 4w and invo). Scale bars, 10 μm. Histograms and error bars represent mean and sem.
Supplementary Figure 29. Myoepithelial and luminal SCs maintain their unipotent fate in mammary reconstitution assay when transplanted together at the physiological ratio under non-limiting conditions.

a. Scheme summarizing the protocol used to graft unicellular suspensions of 5w old MGs into cleared mammary fat pads of 4w old NOD SCID mice. b. Wholemount analysis of the reconstituted MG formed from the transplantation of K14CRE/RosaYFP MG shows YFP expression in the newly formed MG. c, d. Immunostaining of K8 (c) or K5 (d) (red) and YFP (green) of K14CRE/RosaYFP graft showing that LCs and MCs express YFP. e, f. Low magnification confocal analysis of immunostaining of K8 (e) or K5 (f) (red) and YFP (green) in K14rtTA/TetOCRE/RosaYFP graft showing that only MCs express YFP. g, h. Low magnification of immunostaining of K8 (g) or K5 (h) (red) and YFP (green) in K14rtTA/TetOCRE/RosaYFP graft showing that most MCs express YFP. i, j. Immunostaining of K8 (i) or K5 (j) (red) and YFP (green) in K14rtTA/TetOCRE/RosaYFP graft showing some LCs expressing YFP. k. Wholemount analysis of the reconstituted MG after the transplantation of MG from K5CREER/RosaYFP mice that have been previously treated with TAM as to induce YFP expression only in MCs, shows YFP expression in the reconstituted MG. l, m. Low magnification immunofluorescence analysis of K8 (l) or K5 (m) (red) and YFP (green) in the K5CREER/RosaYFP graft showing that only MCs express YFP. Scale bars, 10 μm unless stated.
Supplementary Figure 30. Myoepithelial and luminal SCs maintain their unipotent fate in serial transplantation assay when transplanted together at the physiological ratio under non-limiting conditions.

a. Scheme summarizing the protocol used to serially transplant unicellular suspensions of primary transplanted glands. b-g Wholemount (b, e) and immunostaining analysis of K8 (c, f) or K5 (d, g) and YFP in the reconstituted MG formed from the 2nd transplantation from K14rtTA/TetOCRE/RosaYFP (b-d) and K8CREER/RosaYFP (e-g) mice. Scale bars, 10 μm unless stated.
Supplementary Figure 31. Myoepithelial but not luminal stem cells can be forced to adopt a multipotent fate in mammary reconstitution assay.

**a.** Scheme summarizing the protocol used to transplant 104 FACS isolated YFP+ MECs obtained from MGs of K14rtTA/TetOCRE/RosaYFP mice previously treated with DOX.

**b-d.** Transplantation of 104 FACS isolated YFP+CD29HiCD24+ cells together with 2,000 Tomato+CD29LoCD24+ cells into the mammary fat pads of NOD/SCID mice. Immunostaining of Tomato and YFP (b), K8 and tomato (c) and K8 and YFP (d). Scale bars, 10 µm.
Supplementary Figure 32. Model of the breast cellular hierarchy.
The K14+ multipotent mammary progenitors exist only during a very restricted period during embryonic development and are rapidly replaced after birth by two distinct types of unipotent SCs that ensure MG expansion during puberty and pregnancy.
### a. 1st transplant

$10^6$ cells ($5 \times 10^4$ MECs) isolated from glands

<table>
<thead>
<tr>
<th></th>
<th>Grafts with outgrowth</th>
<th>YFP+ grafts</th>
<th>Grafts showing YFP+ luminal cells</th>
<th>% of YFP+ luminal cells (individual score for each graft presenting YFP+ luminal cells)</th>
<th>Mean +/- sem % YFP+ luminal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>K14rtTA/TetOCre/RosaYFP</td>
<td>15</td>
<td>15</td>
<td>4/15</td>
<td>1.7</td>
<td>0.1 +/- 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>K5CREER/RosaYFP</td>
<td>6</td>
<td>6</td>
<td>1/6</td>
<td>2.3</td>
<td>0.4 +/- 0.4</td>
</tr>
<tr>
<td>K8CREER/RosaYFP</td>
<td>14</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K14CREER/RosaYFP</td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### b. 2nd transplant

$10^5$ cells ($5 \times 10^3$ MECs) isolated from primary transplants

<table>
<thead>
<tr>
<th></th>
<th>Grafts with outgrowth</th>
<th>YFP+ grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>K14rtTA/TetOCre/RosaYFP</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>K8CREER/RosaYFP</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

### C. Sorted cells

<table>
<thead>
<tr>
<th></th>
<th>Grafts with outgrowth</th>
<th>YFP+ grafts</th>
<th>Grafts showing YFP+ luminal cells</th>
<th>% YFP+ luminal cells (individual score for each graft presenting YFP+ luminal cells)</th>
<th>Mean +/- sem % YFP+ luminal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^4$ YFP+ CD29HighCD24+</td>
<td>7</td>
<td>7</td>
<td>7/7</td>
<td>100</td>
<td>100 +/- 0</td>
</tr>
<tr>
<td>$10^4$ YFP+ CD29LowCD24+</td>
<td>0/10</td>
<td>0</td>
<td>0/10</td>
<td>0</td>
<td>0 +/- 0</td>
</tr>
<tr>
<td>Mix of $10^4$ YFP+ CD29HighCD24+ and $2 \times 10^3$ TOM+ CD29LowCD24+</td>
<td>7</td>
<td>7</td>
<td>7/7</td>
<td>100</td>
<td>60.6 +/- 15.7</td>
</tr>
</tbody>
</table>

Supplementary Table S1. Summary of results of transplant experiments.