Membrane expression of the estrogen receptor ERα is required for intercellular communications in the mammary epithelium

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**Abstract:**

17β-estradiol (E2) induces the post-natal development of mammary gland and influences breast carcinogenesis by binding to the estrogen receptor ERα. ERα acts as a ligand activable transcription factor but also elicits rapid signaling through a fraction of ERα expressed at the membrane. Here, we used the C451A-ERα mouse model mutated for the palmitoylation site to understand how ERα membrane signaling affects mammary gland development. While the overall structure of physiological mammary gland development is slightly affected, both epithelial fragments and basal cells isolated from C451A-ERα mammary glands failed to grow when engrafted into cleared WT fat pads, even in pregnant hosts. Similarly, basal cells purified from hormone-stimulated ovariectomized C451A-ERα mice did not produce normal outgrowths. Ex vivo, C451A-ERα basal cells displayed reduced matrix degradation capacities, suggesting altered migration properties. More importantly, C451A-ERα basal cells recovered in vivo repopulating ability when co-transplanted with WT luminal cells and specifically with ERα-positive luminal cells. Transcriptional profiling identified crucial paracrine luminal-to-basal signals. Altogether, our findings uncover an important role for membrane ERα expression in promoting intercellular communications essential for mammary gland development;
Key words: Estrogen Receptor alpha/ Membrane initiated signaling/ Mammary gland/ Stem cells/ Paracrine signals

Summary Statement:
ERα palmitoylation is important for mammary epithelial cell regenerative functions, especially for the ERα expressing luminal cells to expand and promote the paracrine signaling for epithelial cell-cell communications and cell-ECM communication.

Introduction:
Estrogens, particularly 17β-estradiol (E2), are sex hormones that are widely implicated in mammary gland development, which occurs mostly postnatally under endocrine control (Brisken and O'Malley, 2010). E2 binds to two main receptors, the estrogen receptors ERα and ERβ. ERα is required for normal ductal development during puberty (Dupont et al., 2000) while the deletion of ERβ has no effect on postnatal development (Antal et al., 2008). In addition to the crucial role of ERα in mammary gland development, ERα is a key factor in breast cancer diagnosis and treatment. Based on its expression in 70% of breast cancers, hormonotherapy using anti-estrogens, such as Tamoxifen and Fulvestrant, or aromatase inhibitors are efficacious in reducing recurrence and cancer-related deaths. However, 40% of ERpositive tumors develop resistance and recur. Therefore, studies aiming to identify the mechanisms of ERα action in mammary gland development are important to obtain a better understanding of the genesis of breast cancers.
The mammary gland is composed of an inner luminal layer (LCs) surrounded by an outer layer of myoepithelial/basal cells enriched by mammary stem cells (MaSCs). Basal cells appear ERα-negative by immunohistochemistry, and have regenerative properties into basal and luminal cells in transplantation assays (Visvader and Stingl, 2014, Van Keymeulen et al., 2011) In contrast, approximately 30-50% of luminal cells are ERα positive by immunohistochemistry, most of them co-express the progesterone receptor (PR) (Petersen et al., 1987, Clarke et al., 1997). A second population of luminal cells shows ER expression at mRNA level but the protein is not detected by IHC (Cagnet et al., 2018). Transplantation of the epithelium from ERα knockout (KO) mice into cleared fat pads revealed a requirement for ERα expression in the epithelium for ductal outgrowth (Mallepell et al., 2006). Moreover, transplantation of a mixture of WT and ERα-KO cells induced the proliferation of ERα-deficient cells, showing that E2 exerts its mitogenic effects on the mammary gland through paracrine signaling to promote proliferation and morphogenesis (Mallepell et al., 2006, Brisken and O'Malley, 2010). The expression of amphiregulin, an epidermal growth factor receptor ligand, is highly induced by E2, and this ligand is an important paracrine mediator of estrogen function (Ciarloni et al., 2007). Progesterone receptor (PR) is also an ER target and promotes the expression of strong inducers of mammary development such as Wnt4 and RANKL in adulthood (Beleut et al., 2010, Fata et al., 2000, Rajaram et al., 2015). Thus, ERα-positive cells have been dubbed “sensor cells” as they sense the systemic signals and translate them into paracrine cells for neighboring basal and luminal cells (Pond et al., 2013, Gjorevski and Nelson, 2011).

In response to E2, ERα modulates the transcriptional activity of target genes via its nuclear actions. Over the last two decades, ERα has been shown to associate with plasma membrane caveolae/lipid rafts and to activate non-nuclear signaling, the so-called rapid/non-genomic/membrane initiated steroid signaling (MISS), in a variety of cell types ( Arnal et al., 2017, Levin, 2011, Madak-Erdogan et al., 2008). Post-translational modifications, such as palmitoylation which occurs on cysteine 447 (451 in mice) as part of a nine-aminoacid motif
in the ligand binding domain of all steroid receptors, were shown to be crucial for anchoring ERα to the membrane (Acconcia et al., 2005, Pedram et al., 2007). Following association with the Heat shock protein Hsp27, presumably opening up the structure of the receptor, two palmitoyl acyltransferases DHHC-7 and DHHC-21 (Pedram et al., 2012) attach the palmitoyl acid to the N-terminal Cys of the motif, promoting the physical interaction of ER with the caveolin-1 protein and the transport to the plasma membrane (Acconcia et al., 2005). The membrane rapid initiated estrogen signaling indirectly regulates transcription (Madak-Erdogan et al., 2008, Arnal et al., 2017). These MISS effects also act in concert with growth factors, modulating their signaling in certain tissues and cells (Hawsawi et al., 2013, Tian et al., 2012). They appear to play a major role in breast cancer (Levin and Pietras, 2008), and interactions of ERα with Src and PI3K have been observed in aggressive tumors (Poulard et al., 2012). To gain mechanistic insights into the physiological roles of MISS in vivo, our laboratory (Adlanmerini et al., 2014) and Levin et al. (Pedram et al., 2014) generated mouse models expressing ERα carrying a mutation of cysteine 451 to alanine, thus abrogating this palmitoylation site and membrane ERα expression (named C451A-ERα and NOER mice, respectively). The C451A-ERα mouse model has revealed a major role for MISS in the vasculature, where it mediates the estrogen effects on endothelial cells (Adlanmerini et al., 2014). Levin and his collaborators reported that the mammary glands of homozygous NOER female mice completely filled the fat pad but showed diminished ductal side branching and the formation of blunted duct termini (Pedram et al., 2014).

In the present study, we analyze mammary gland development in C451A-ERα mice. There is a transient delay in development during puberty. Intriguingly, C451A-ERα mammary stem/CD24+/CD29hi cell MaSC) cells fail to outgrow in in vivo transplantation experiments. This default is rescued by co-injection with wild type luminal cells, and specifically ERα-positive luminal cells. Altogether, these data indicate that stem cell properties are not cell intrinsic but rely on intercellular communications which in turn are controlled by the membrane ERα in epithelial mammary cells.
Results:

C451A-ERα delays pubertal mammary gland development

To assess the effects of the C451A-ERα germline mutation on mammary gland development, we analyzed mammary glands from C451A-ERα female mice and their WT littermates at critical developmental stages. At puberty (5 weeks), fat pad filling was delayed in C451A-ERα females compared to their WT littermates (Fig. 1A-C). At the adult stage (3 months), no difference in fat pad filling was observed between the two genotypes (Fig. 1B, C), but C451A-ERα glands showed fewer side branches (Fig. 1E) and thinner ducts observed on transverse sections (Fig. 1D, F), as reported for NOER mice (Pedram et al., 2014).

Histological analysis revealed a normal architecture of the ductal tree, attested by the presence of double layer structure by immunohistochemistry with anti-K5 and K8 cytokeratins (Fig. S1A,B). At 5 weeks of age, when puberty had occurred, C451A-ERα mice presented a significant small decrease of PR-positive cells, with 57.5 %± 1.1 in the WT and 51.7± 2.1 in the C451A-ERα mice. However, the expression of ERα, the proliferation and the apoptotic rates (KI-67 and active caspase-3 stainings, respectively) were not altered neither at puberty nor in adult animals. Western blot analysis of ERα expression confirmed these data (Fig. S1C). Steroid hormone levels were also measured in 5-week-old and 3-6-month-old mice. E2 and progesterone levels were comparable in 5-week-old C451A-ERα and WT mice, but in the adult in C451A-ERα mice serum E2 levels were increased, and the progesterone levels were substantially decreased compared to their WT littermates (Fig. 1G).

Altogether, these results indicate a delay in mammary gland outgrowth during puberty, when serum estrogen and progesterone levels are still similar, and a defect in ductal side branching that may be attributable to decreased serum progesterone levels.
Transplanted C451A-ERα ducts fail to grow in WT mice

The virgin adult C451A-ERα mice showed altered serum hormone profiles, particularly a substantial decrease in the circulating progesterone level, which might impact the observed alterations in mammary gland morphology (Need et al., 2014). To reveal the epithelial-intrinsic role of the C451A-ERα mutation in mammary gland development, we performed transplantation experiments. These engraftments also allowed us to study the normal development of C451A-ERα mammary glands during regular estrous cycles and pregnancy (alveogenesis), circumventing the infertility of C451A-ERα females (Adlanmerini et al., 2014).

A piece of mammary epithelium from C451A-ERα mice was engrafted into a cleared inguinal fat pad from 3-week-old WT (Rag1−/− or C57BL/6N) mice, whereas the contralateral fat pad was engrafted with WT epithelium, as previously described (Mallepell et al., 2006). We used donors that ubiquitously expressed the GFP transgene and visualized the epithelium under a fluorescence stereomicroscope to ensure that comparable amounts of mammary epithelia were engrafted. Eight weeks after surgery, fluorescence stereomicroscopy of grafted glands showed the growth and extension of the WT epithelium and the presence of terminal end buds (TEBs), whereas the C451A-ERα epithelium completely failed to grow (Fig. 2A). A nearly total absence of mammary fat pad filling was observed in more than 22 mice engrafted with the C451A-ERα epithelium (Fig. 2B). C451A-ERα epithelium development remained rudimentary, with on average less than 10% of the fat pad filled.

On day 16.5 of pregnancy, when intense hormonal stimulation occurs, alveoli formed all over the ductal tree in the WT grafts but not in C451A-ERα epithelia (Fig. 2A) yet the graft expanded (Fig. 2B). Histologically, in WT mice, we observed the formation of alveoli lined by a single layer of low columnar epithelial cells, containing lipid droplets (Fig. 2C). Immunofluorescence following the cytokeratins 5 and 8 labeling attested a double layer structure (Fig. S2A). Expression of ERα and PR by immunohistochemistry was limited to rare luminal cells in WT mice, as expected with the known-decrease of ER positive cells during pregnancy (Fig. 2C, D) (Van Keymeulen et al., 2017). Noteworthy, ERα was significantly
expressed in a percentage of luminal cells while PR was absent in pregnant C451A-ERα mice (a positive control of the PR labeling was presented in Fig. S2B). These observations revealed the importance of palmitoylation of ERα in the mammary epithelium to repopulate the fat pad, and a role in alveologenesis during pregnancy.

C451A-ERα mutation alters the balance of luminal/basal mammary epithelial cells and the regenerative potential of MaSCs

To test the hypothesis that a lack of stem cells may underly the transplantation defect, we monitored different populations of mammary epithelial cells using flow cytometry. The luminal (CD29loCD24+) cell population was increased in C451A-ERα mice, whereas a decrease in the MaSC-enriched (CD29hiCD24+) subpopulation occurred when these populations were isolated by cell sorting (Fig. 3A). We further investigated whether cell-sorted MaSC from intact C451A-ERα mice were able to repopulate the mammary gland in vivo. Transplantation of limited numbers of CD29hiCD24+ GFP cells from C451A-ERα mice into cleared mammary fat pads revealed an absence of outgrowth compared to similar gate-sorted WT cells even when 5000 cells were injected (Fig. 3B,C). Control MaSCs gave rise to extensive outgrowth when ≥300 cells were injected, whereas 98% of outgrowths from MaSCs isolated from C451A-ERα virgin mice filled less than 2% of the fat pad. Only one C451A-ERα mouse presented 5% outgrowth when 2000 CD29hiCD24+ cells from mutant C451A-ERα mice were transplanted. The mammary repopulating unit frequency was 1/701 for WT cells compared to 1 in 28189 for C451A-ERα cells, representing a 40.2-fold decrease in the absolute number of mammary repopulating units. Immunohistochemical staining of this small outgrowth using specific anti-cytokeratin K5 and K8 antibodies revealed the presence of both luminal and basal cells (Fig. S3A). Interestingly, ERα immunostaining on this small outgrowth demonstrated that C451A basal cells were able to differentiate into both ERα positive and ERα negative luminal cells although the ductal elongation was absent (Fig. S3B). Thus, membrane ER is required for the outgrowth of CD29hiCD24+ cells.
Engrafted C451A-ERα MaSC cells do not recover their extensive outgrowth ability following hormonal supplementation.

Progesterone is responsible for dynamic shifts in specific populations within the mammary epithelial cell hierarchy (Joshi et al., 2010, Asselin-Labat et al., 2010). To investigate whether the shift in cell populations observed in the C451A-ERα mammary glands was secondary to lower progesterone levels, when the hormone has stem cell promoting effects; mice of both genotypes were ovariectomized at 26 days of age and treated with both E2 and progesterone for 3 weeks. The exposure of C451A-ERα mice to E2 and progesterone did not modify the ability of their mammary ducts to invade the fat pad compared to their control WT littermates (Fig. 4A, B). However, carmine staining revealed an important difference in the architecture of virgin mammary glands. A significant decrease in the thickness of ducts was observed, although this combined treatment efficiently released similar doses of E2 and progesterone in both WT and mutant mice (Fig. 4C). According to the immunohistochemistry, obvious changes in the numbers of ERα and PR-positive cells were not observed (an average of 30-40% positive cells in both and C451A-ERα WT mice, Fig. 4D). The proliferation index was not affected. Importantly, hormone treatments restored the balance between luminal (CD29loCD24+ ) and MaSC-enriched basal (CD29hiCD24+) subpopulations to WT ratios (Fig. 4E). Again, we assessed the repopulating MaSC capacities and transplanted limited numbers of CD29hiCD24+GFP positive C451A-ERα cells into cleared mammary fat pads and CD29hiCD24+GFP positive WT cells into the contralateral fat pads. Still, the CD24+CD29hi cells from C451A-ERα mice were unable to generate a functional mammary gland, in contrast to WT (Fig. 4F, G). 99% of outgrowths from MaSC isolated from C451A-ERα virgin mice filled less than 2% of the fat pad, whereas control MaSC yielded extensive outgrowths in 56% of cases. The mammary repopulating unit frequency was 1 in 987 in ovariectomized WT mice supplemented with E2 and progesterone (Fig. 4H), a value that was very similar to intact mice. However, in C451A-ERα mice, this frequency was 1 in 51750, representing a remarkable 52.4-fold decrease in the absolute number of mammary...
repopulating units. Thus, inability of C451A-ERα CD24⁺CD29⁺⁺ cells to reconstitute cleared fat pads is independent of previous intrinsic hormone exposures of stem cells.

C451A-ERα epithelial cell populations undergo clonal expansion in vitro but exhibit defects in matrix degradation

Next, we analyzed the potential of mammary epithelial cells to form colonies (CFC) and mammospheres in vitro as a readout for the number of progenitor cells in each population (Stingl et al., 2006). First, FACS-sorted luminal cells of both genotypes were cultured on irradiated fibroblasts in growth factor-enriched medium. After 8 days, no differences in the number and size of CFCs were observed between C451A-ERα cells and their controls (Fig. S4A). When cells were grown in medium enriched with growth factors containing 4% matrigel, mammospheres were obtained from both sorted luminal and basal cells. We cultured the two subpopulations for more than 3 generations and did not observe any difference between C451A-ERα and WT cells (Fig. S4B). CD24⁺CD29⁺⁺ cells yielded an average of 300, 200 and 150-200 spheres from 5000 cells seeded at the 1st, 2nd and 3rd generations, respectively. Clonal expansion of the luminal and basal cells was not impacted by the different passages (generations 1 to 3). Having ascertained that clonogenicity is unaffected, we went on to ask whether an inability to invade the mammary stroma may underlie the in vivo/in vitro discrepancy. We plated equivalent numbers of CD24⁺CD29⁺⁺ cells onto a fluorescent gelatin matrix in vitro. Five days later, the area of degraded gelatin appeared black. The area of degraded gelatin observed with C451A-ERα cells was 2-fold lower than that of WT (Fig. S5A, B) and was completely abrogated using a nonselective metalloproteinase (MMP) inhibitor (marimastat, Fig. S5C). Degradation was not obtained in experiments using luminal cells (data not shown). In summary, in vitro studies do not reveal a clonogenic difference between populations of WT and C451A-ERα luminal and basal epithelial cells. Basal cells harbor outgrowth-matrix interaction defects, suggesting that the inability of C451A-ERα epithelial cells to repopulate fat pads is linked not to the clonogenicity...
of stem cells but rather to perturbed capacities in establishing interactions with the surrounding tissue \textit{in vivo}.

WT luminal cells mediate the expansion of C451A-ER\(\alpha\) MaSCs in mixed cell transplantation assays. Abundant literature has demonstrated that some basal cells are multipotent and able to give rise to both luminal and basal lineages upon transplantation (Stingl et al., 2006, Shackleton et al., 2006), while they remain lineage-restricted in physiological conditions (Van Keymeulen et al., 2011, Prater et al., 2014, Wuidart et al., 2016). Moreover, paracrine signaling between luminal and basal cells is critically important for mammary epithelial development (Brisken and O’Malley, 2010, Van Keymeulen et al., 2011). To clarify the discrepancy between the normal mammary gland development in hormonally adjusted C451A-ER\(\alpha\) females and the absence of outgrowth in reconstitution assays with C451A-ER\(\alpha\) basal cells, we transplanted a mixture of FACS-sorted (CD29\(^{hi}\)CD24\(^{+}\)) basal cells from C451A-ER\(\alpha\).GFP\(^{+}\) mice and GFP-negative CD29\(^{hi}\)CD24\(^{+}\) luminal WT or C451A-ER\(\alpha\) cells into cleared mammary fat pads of WT mice. We used different ratios of C451A basal cells with luminal cells (1/1 or 5/1) because decreasing the number of luminal cells has a tendency to preserve the pluripotency of basal cells (Van Keymeulen et al., 2011). Transplantation of GFP-negative WT luminal cells with GFP-positive C451A-ER\(\alpha\) basal cells restored their regenerative potential, since extensive outgrowth was observed in 33\% of cases (Figs. 5B and S6A). In contrast, transplantation of GFP-positive C451A-ER\(\alpha\) mutant basal cells mixed with GFP-negative C451A-ER\(\alpha\) luminal cells failed to regenerate mammary glands. The absolute number of repopulating cells in the pool from C451A basal/WT luminal mice was increased compared to MaSCs alone with 1 in 3154 cells, (Fig. 5C), but remained lower than WT MaSCs with 1 in 701 (Fig. 3D). Analysis of the reconstituted mammary gland under a fluorescence stereomicroscope indicated that the green fluorescent signal was discontinuous (higher magnification image in the left panel of Fig. 5A). Analysis by confocal microscopy of
reconstituted mammary glands 8 weeks after transplantation with basal and luminal markers revealed that the vast majority of the basal, cytokeratin 5-positive cells, were GFP positive in 5 out of 9 mice; these cells originated from the engrafted GFP positive MaSCs (Figs. 5D (left panel) and S6B). Very few luminal cells were GFP positive. In the other four mice (Fig. 5D, right panel), GFP positive (K5-positive) basal and (K8-positive) luminal cells were observed, indicating that the C451A-ERα MaSCs differentiated into luminal cells in response to paracrine signaling from WT luminal cells. To assess whether the GFP positive-C451A basal cells can give rise to ERα positive cells when mixed with WT luminal cells; we performed confocal microscopy analysis using anti-GFP and anti-ERα antibodies (Figs. 5E and S6B-D). We found that the percentage of ERα positive cells was similar in all the outgrowths (Fig. S6C). However, in the majority of outgrowths, GFP positive C451A-ERα basal cells gave rise to ERα negative luminal cells (Fig. 5E and Movie S1) while GFP and ERα double-positive cells were rarely observed (Fig. S6D).

Thus, the C451A-ERα mutation alters the properties of mammary stem cells as assessed by in vivo cell reconstitution assays, possibly due to absence of ERα positive luminal cells.

The absence of palmitoylated ERα affects the paracrine signaling of luminal cells

To identify transcriptional changes in C451A-ERα luminal cells that may underlie the observed phenotype (Fig. 5A), we performed a global gene expression analysis on FACS-sorted luminal cells from ovariectomized C451A-ERα and WT mice treated with E2 alone or in combination with progesterone for 3 weeks. Two hundred thirty one genes were differentially expressed between WT and C451A-ERα mice in response to E2 (>1.5-fold, Adj P value <0.05; Fig. 6A,B). The addition of progesterone along with E2 differentially regulated the expression of 100 genes between the 2 genotypes. More precisely, in response to the progesterone/E2 treatment, only a limited number of genes (6 genes, with 1 gene shared with cells treated with E2 alone) were upregulated in C451A-ERα cells compared to WT cells, whereas most genes (94, with 7 common genes). Among these down-regulated
common genes, *Grebl* is one strongly down-regulated (fold change of 11 in response to E2, and fold change of 17 in response to E2+ progesterone) and is well-known as an estrogen-responsive gene that is an early response gene in the estrogen receptor-regulated pathway (Fig. 6B). According to the gene ontology analysis, most of the differentially expressed genes encode proteins that are integral components of membrane, part of extracellular matrix or display kinase activity (Figs. 6C,D,E and S7A).

The results of the microarray analysis were validated by qRT-PCR using Fluidigm Biomark Real-Time PCR. Among the panel of analyzed genes, we also included several genes known to be regulated by ERα and PR in the mammary gland, specifically *Areg* and *Wnt4* (Fig. 6F). *Esr1*, *Pgr*, and *Areg* were not differentially expressed in mutant mice, consistent with the immunostaining analyses of ERα and PR protein expression (Fig. 4D). The RankL gene, known to be induced by PR signaling, was indeed upregulated following progesterone treatment, indicating that the progesterone pathway is partially conserved. Interestingly, the *Snail*, which encodes a zinc finger transcription factor also known as Snal1 involved in different processes controlling cell differentiation and apoptosis (Come et al., 2004), was significantly decreased in C451A mice by E2 treatment (Fig.S7A-C). *Fn1*, *Jak2* and *Stat5a* genes, were also downregulated by the E2 treatment in C451A-ERα cells. Importantly, a strong gene-gene interaction network was found among these E2-downregulated genes in C451A-ERα cells. These interactions point genes belonging to the positive regulation of cell migration (with *Snail*, *Fn1*, and *LambB* being the most down-regulated genes on this pathway) and involved in the JAK-STAT signaling cascade (a pathway induced by growth-hormone receptors (GH-R) and Fibroblast-growth-factor receptors (FGF-R, (Furth et al., 2011)). Among the differentially expressed genes observed following addition of progesterone treatment, genes such as *Mmp7*, *Bmp1*, *Bmp3*, *Tgfβ* and *Clca1* (involved in mammary gland development and extracellular matrix modifications) were downregulated in C451A-ERα mice (Fig. S8A,B). A predicted gene-gene interaction network was found between the morphogens *Bmp1*, *Bmp3* and the *Wnt* signaling pathway (*Dkk3*), the *MMP-7*...
metalloprotease that is important for duct morphogenesis. Interestingly, RT-PCR also confirmed down-regulation of the growth factor Tgfβ3 or the growth factor receptor Fgfr3 (involved in paracrine signaling of ER-positive luminal cells). In contrast, the gene whose expression was upregulated to the greatest extent by the progesterone treatment in C451A-ERα mice was Fgb encoding the extracellular matrix protein Fibrinogen, which is exclusively expressed in luminal ER positive cells (Kendrick et al., 2008). Altogether, this gene profile analysis indicated alteration of signaling pathways involving growth factors, extracellular matrix and paracrine signals in the luminal compartment of the C451A-ERα mammary gland. Finally, in order to analyze which subpopulation of WT luminal cells was important for the signaling to basal cells, we separated by flow cytometry the Sca1+/CD133+ from the Sca1−/CD133− luminal cells, corresponding respectively to the ERα-positive and ERα-negative luminal cells that was confirmed by qRT-PCR (Sleeman et al., 2007, Kendrick et al., 2008, Van Keymeulen et al., 2017) (Figs.7A, B) This WT luminal subpopulation of cells was co-transplanted with C451A-ERα MACS at a luminal/basal ratio of 1/5 (See supplementary Table 1). While transplantation of C451A-ERα basal cells mixed with the Sca1−/CD133− WT luminal cells cannot reconstitute a normal mammary gland, outgrowths were obtained when C451A-ERα basal cells were mixed with Sca1+CD133+ luminal cells (Fig.7C). After labeling with anti-GFP and anti-ERα antibodies, GFP C451A-ERα basal cells did not give detectable double positive GFP-ERα luminal cells (Fig.7D). These additional transplantation assays strongly indicate that there is a failure of mutant ERα-positive luminal cells at the origin of the phenotype.
Discussion:

Our study of mammary gland development in C451A-ERα mice provides evidence that ERα palmitoylation is important for mammary epithelial cell functions, both for the ability of mammary epithelial cells to establish themselves in the fat pad to promote the outgrowth of ducts, but also for paracrine signaling emanating from ERα positive luminal cells.

Mammary gland development is slightly delayed at puberty, while in adult C451A-ERα mice the mammary gland completely filled the fat pad with decreased side branching. Strikingly, this almost normal in situ mammary gland development was substantially different from the total absence of ductal outgrowth observed when C451A-ERα basal cells were transplanted on wild type stroma, even under the hormonal stimulation of pregnancy. In fact, this dichotomy in the properties of MaSCs between their regenerative potential in transplants and their natural fate under physiological conditions has already been largely described for WT MaSCs (Van Keymeulen et al., 2011, van Amerongen et al., 2012, de Visser et al., 2012, Blanpain and Fuchs, 2014). Moumen and its collaborators (Moumen et al., 2012) have also reported that deletion of the proto-oncogene Myc from the mammary stem cell layer impaired stem cell self-renewal, while it did not prevent physiological mammary gland fat pad filling. Here, compensatory mechanisms in the germline knockouts from the embryonic C451A-ERα mammary gland likely reconcile these apparently contradictory results.

Since C451A-ERα mice exhibited decreased circulating progesterone levels which was shown to activate adult MaSCs expansion within the mammary cell niche during the reproductive cycle (Joshi et al., 2010, Asselin-Labat et al., 2010), ovariectomized C451A-ERa mice were supplemented with E2 and progesterone. MaSCs from these mice were still unable to repopulate the mammary gland in an endocrine intact host in vivo. By contrast, no clonogenic difference between both populations of luminal and basal C451A-ERα epithelial cells was observed in vitro in CFC assays or mammosphere cultures. The presence of abundant growth factors in the culture medium used for the in vitro clonogenic studies might bypass the missing paracrine signaling required in vivo, and might explain this discrepancy.
This hypothesis was confirmed by our *in vivo* findings showing that the addition of WT luminal cells to C451A-ERα MaSCs restores their regenerative function by potentially secreting the missing factors. Although it was recently established that the ERα positive and ERα negative luminal populations are maintained by lineage restricted stem cells (Scheele et al., 2017, Dekoninck and Blanpain, 2019, Van Keymeulen et al., 2017), it was shown that ERα-positive cells (sensor cells) are the ones responding to hormone stimulation by sending paracrine signals to the ERα-negative cells to ensure ductal elongation (described as responder cells) (Mallepell et al., 2006, Sternlicht et al., 2006). Our analysis of the rare tiny outgrowths obtained after C451A-ERα MaSCs transplantation alone strongly indicated that C45A-ERa MaSCs can give rise to luminal cells including ERα-positive luminal cells. This initial differentiation is also maintained when these MaSCs cells are mixed with WT luminal cells, but double positive ERα-GFP cells were very rarely founded. Altogether, these data strongly indicated that the observed phenotype is not due to an inherent failure of MaSCs to perform the initial differentiation, but rather to the alteration of the paracrine signaling from mutated ERα positive sensing luminal cells which prevents their expansion and impairs the function of basal cells required for the final ductal outgrowth. This conclusion is reinforced by the engraftement of isolated ERα positive luminal cells from WT mice.

To try to understand the missing paracrine signaling, we performed large scale gene analysis in luminal cells. The transcriptional profiles of the C451A-ERα luminal cells indicate that the primary responses to hormones were conserved. Indeed, the main factors induced by ERα were not disturbed in C451A-ERα mice; *PgR*, *Areg* and the response to progesterone appear to be preserved because *RankL* expression was increased in C451A-ERα cells treated with E2+ progesterone. However, expression of *Greb-1*, well-known as an early response gene in the estrogen receptor-regulated pathway is highly affected (Mohammed et al., 2013). Moreover, expression of several major effectors, such as the morphogens BMP-1 and BMP-3, were substantially decreased by the progesterone treatment in C451A-ERα cells. Importantly, the expression of fibroblast-growth factor receptor FgfR3 was down-regulated as
These key paracrine signaling pathways were already described to be required for normal mammary morphogenesis and stem cell function (Sternlicht et al., 2006, Gjorevski and Nelson, 2011, Pond et al., 2013). More precisely, non genomic signaling of ERα was shown to play a pivotal role at puberty in concert with IGF-1 to activate the PI3K/Akt pathway (Tian et al., 2012). Expression of some proteases, such as the metalloprotease MMP-9 and the serine protease Tmprss6 (matriptase-2), was substantially decreased by the progesterone treatment in C451A-ERα cells pointing membrane ERα in luminal cells being a critical regulator of this paracrine signaling to MaSCs. Indeed, MMPs are ECM-degrading enzymes involved in branching morphogenesis that require epithelial invasion of adipose tissue (Fata et al., 2004). Moreover, a gene network was found in the GO category of genes belonging to the positive regulation of cell migration, involving in particular Fn1, Snai1, Jak2 and LamB (Fig. S7B). Among these genes similarly down-regulated by E2 in the C451A-ERα mice, a picture emerges that link membrane ERα signaling in luminal cells with the Jak2 and Stat5a genes (Fig. S7A-C). Interestingly, the Stat5a signaling pathway is known to be at the crossroad of hormonal and growth factor signalings which uncovers an important role of membrane ERα in the paracrine signaling of luminal cells and points membrane ERα as a key regulator of this growth factor sensitivity. Moreover, this Stat5a dysregulation might explain the decrease side branching observed (Furth et al., 2011, Santos et al., 2010). The Fn1 and LamB genes encoding respectively fibronectin and laminin B1, two proteins of the extracellular matrix, were also part of this gene network downregulated when membrane ERα was lost (Fig. 6B-F and S7). It turns out that fibronectin was recently described to be involved in the recycling pathway of membrane ERα in MCF7 cells, rescuing ERα from lysosomal degradation, and enhancing its transcriptional activity in response to E2 (Sampayo et al., 2018). The Snai1 gene on this network is also largely involved in different processes controlling cell differentiation and apoptosis, and acts as a major effector of epithelial cells migration (Come et al., 2004). Downregulation of this set of genes probably contributes to the observed alterations in the capabilities of basal cells to migrate into gelatin and might also
explain the delayed ductal invasion of the fat pad observed at puberty. Overall, these gene pathways and the transplantation experiment of gated ERα-positive WT luminal cells demonstrate that the absence of phenotype on the transplantation experiment is rather a failure of mutant ERα expressing luminal cells to expand and promote the paracrine signaling for epithelial cell-cell communications and cell-ECM communication. Altogether, these data strongly indicate that mutation C451A of ERα affects intrinsic properties and paracrine functions of mammary epithelial cells.

An important unsolved question is how MaSCs sense the initial hormone signals in transplantation experiments. Within MaSCs, Sca1⁺ expression separates ERα positive cells from ERα negative cells. Sca1⁺ ER⁻ cells exhibit a higher proliferation rate than Sca1⁺ ER⁺ cells (Dall et al., 2017). Whether these ERα negative MaSCs cells express membrane ERα at very low levels is difficult to solve, because membrane ERα expression was detectable by immunohistochemistry only in overexpressing conditions in CHO or HEK293 cells (Pedram et al., 2007). Assays in these MaSCs cell lines, even in WT, were unsuccessful (data not shown). In parallel, a single cell analysis recently performed has revealed the presence of a rare basal subset that displays features of mixed-lineage cells that can respond to ovarian hormones and generate luminal progenitors (Pal et al., 2017). However, this subset was not observed in another analysis with settings that are more stringent, arguing that mammary epithelial cells display a differentiation continuum (Bach et al., 2017). While our microarray analysis was performed using bulk luminal cells analysis, performing a single cell RNA profiling on the mammary gland from C451A-ERα mutant mice at different stages of mammary gland development would better refine how absence of membrane ERα affects the differentiation of progenitors cells and the signaling pathways between ERα⁺/ ERα⁻ luminal and basal cells.

In conclusion, our study reveals a key role for membrane ERα in the outgrowth abilities of CD24⁺CD29⁺ cells in transplantation assays, indicating that membrane ERα is required in both luminal and basal cells, particularly for the signaling of ERα-expressing
luminal cells in order to expand and then to activate MaSC in a paracrine manner. Our results provide some mechanistic insights into the nature of the interaction between ERα-negative and ERα-positive epithelial cells that should improve our understanding of the intercellular communication involved in breast development and carcinogenesis.

**Materials and Methods:**

**Mice**

The procedures involving experimental animals were performed in accordance with the principles established by the Institut National de la Santé et de la Recherche Médicale (INSERM) and were approved by the local Ethical Committee of Animal Care (CEA-122-DAP-2015-05). The C451A-ERα knock-in mouse line was generated on a C57BL6/N background at the Mouse Clinical Institute as previously described (Adlanmerini et al., 2014). These mice were bred with the C57BL/6 TgN(act-EGFP), GFP-positive mice (Okabe et al., 1997) that were kindly provided by Masaru Okabe (University of Osaka, Japan). Estrous cycle phases were determined in individual adult cycling WT and C451A-ERα mice using vaginal cytology (Hennighausen and Robinson, 2005). C451A-ERα mice and corresponding wild-type littermates (WT-ERα) were ovariectomized at 26 days old. For chronic E2 treatment, ovariectomized mice were implanted with subcutaneous pellets releasing either vehicle or E2 combined with progesterone (P) (0.01 mg/60 days for E2, 1.5 mg/60 days for progesterone; Innovative Research of America, USA).

**Determination of serum hormone levels**

Gas chromatography coupled with mass spectrometry (GC-MS) was used to determine serum E2 and progesterone levels using previously described methods (Giton et al., 2015). After clotting, sera were stored at −80 °C until hormone assays. E2 levels were determined in two steps.
Mammary gland whole mounts

Mammary glands whole mounts were generated as previously described (Brisken et al., 1998). Mammary glands of GFP positive mice were fixed with 4% PFA. Digital images were captured using a Leica Macrofluo microscope equipped with Planapo 1.0x objective. For fluorescent images, an L5 cube (Ex 480/40x, Em 527/30m) was used and images were analyzed using the ImageJ software.

Immunohistochemistry

Paraffin-embedded transverse sections (5 µm) from formalin-fixed mammary gland specimens were stained with anti-Ki-67 (RM-9106; ThermoScientific), anti-PR (sc-7208; Santa Cruz Biotechnology) antibodies or anti active caspase-3 (AF835, R&D Systems), as previously described (Abot et al., 2013). For ERα detection (ER-6F11, NCL-ER-6F11, Leica), immunohistochemistry was performed with a Dako Autostainer Link 48 on 3-µm sections. Antigen retrieval was performed using a Dako PT Link pressure cooker in pH 6.0 citrate buffer and an EnVision™ system for antibody detection. Images were acquired using a NanoZoomer Digital Pathology Scanner and NDPView software (Hamamatsu Photonics) for quantification.

Western Blot

Total proteins were separated on 10% SDS/PAGE gels and transferred to nitrocellulose membranes. The following primary antibodies were used: anti-ERα (60C, 04-820, Millipore) and anti-GAPDH (sc-32233; Santa Cruz Biotechnology). Bands were revealed using HRP-conjugated secondary antibodies and visualized through ECL detection, according to the manufacturer’s instructions (Amersham Biosciences/GE Healthcare), using a ChemiDoc Imaging System (Bio-Rad). Bands were quantified using densitometry in the ImageJ software.
Mammary cell preparation

Mammary glands #2, 3, 4, and 5 were dissected from 8- to 12-week-old female mice, and the lymph nodes were removed before processing. After mechanical dissociation into pieces, the tissue was digested in CO\textsubscript{2}-independent culture medium (Gibco) containing 3mg/mL collagenase A (Roche) and 100U/mL hyaluronidase (Sigma), supplemented with 5% bovine calf serum for 90 min at 37°C, followed by 0.25% trypsin-EDTA for 1–2 min, 5mg/mL dispase (Roche) and 0.1mg/mL DNase (Roche) for 5 min, and 0.64% NH\textsubscript{4}Cl for 3 min. Samples were then filtered through a 40µm mesh and labeled.

Cell labelling, flow cytometry and sorting

All labeling steps were performed in PBS supplemented with 2.5% bovine serum albumin (Sigma-Aldrich) and 50µM EDTA. Cells were first incubated with blocking anti-CD16/CD32 antibodies (14-0161-82, eBioscience) for 10 min at RT before incubation with primary antibodies for 40 min on ice. Primary antibodies included CD24-PerCP-Cy5.5(M1/69, 45-0242, eBioscience), CD29-PE (HMb1, 12-0291, eBioscience), CD31-APC (390, 17-0311, eBioscience), CD45-APC (30-F11, 17-0451, eBioscience), CD133-SuperBright436 (13A4, 62-1331-82, ThermoFisher Scientific) and Sca1-BV711 (D7, 108131, BioLegend). Cells were washed, resuspended in PBS supplemented with 2.5% BSA and 50µM EDTA before analysis.

Cells were sorted on an INFLUX flow cytometer (BD Bioscience, pressure 20psi, nozzle 100µm) using FACS DiVa software. The purity of sorted populations was routinely greater than 95%. Data from live cells, which were initially gated using FACS DiVA software, were analyzed.
Mammary epithelium transplants

For transplants, the fat pads of 3-week-old Rag1\textsuperscript{-/-} or C57BL/6N females were cleared. Pieces of mammary tissue of 1mm in diameter were prepared from the mammary epithelium of 3-month-old WT-ER\textalpha/GFP and C451A-ER\textalpha/GFP females under a fluorescence stereomicroscope (Nikon SMZ1500) and inserted into the inguinal prepared fat pads, as previously described (Brisken et al., 1998).

Mammary epithelial cell transplants

Sorted GFP-positive cells (either GFP-positive basal cells or a mix of GFP-positive basal cells with GFP-negative luminal cells, as indicated) were resuspended in 10 µL of PBS containing 0.04% trypan blue (Sigma) and 50% heat-inactivated fetal calf serum (BWCC) and injected into the inguinal glands of 3-week-old C57BL/6N female mice that had been cleared of endogenous epithelium. Recipient mice were sacrificed 8 weeks after transplantation, unless indicated otherwise. Recipient glands were dissected and analyzed using a Leica Macrofluo microscope with a Planapo 1.0x objective. Outgrowth was defined as an epithelial structure comprising ducts arising from a central point with lobules and/or terminal end buds. For further analysis, some glands were fixed and embedded in paraffin for immunostaining. Limiting dilution transplantation assays of basal cells sorted by flow cytometry were performed to determine MaSC functionality and the mammary repopulating unit number \textit{in vivo}. MaSC frequency was calculated at website:http://bioinf.wehi.edu.au/software/elda/.

Confocal microscopy analysis

Paraffin-embedded transverse sections (10 µm) from formalin-fixed mammary gland specimens were dewaxed, washed with PBS, and subjected to antigen retrieval by boiling in 0.1M sodium citrate buffer, pH6, for 20 min and blocking with 2.5%BSA.
Cells were fixed with PFA 4%, permeabilized with PBS containing 0.2% Triton X-100 for 3 min, rinsed three times with PBS and blocked with PBS containing 3% BSA, 0.05% Tween20 and 0.08% sodium azide for 20 min before being incubated with primary antibodies diluted in blocking solution for 1 hour.

Staining was performed overnight at 4°C with the following primary antibodies: Anti-GFP (goat polyclonal, 1/500, ab6673, Abcam), Anti-K5 (rabbit Poly19055, 1/500, 905501-Biolegend), Anti-K8 (Rat, TROMA-1, 1/7, DSHB), Anti-K14 (rabbit, EPR17350, 1/250, ab181595, Abcam) and Anti-K18 (rabbit polyclonal, 1/250, ab24561, Abcam). The following secondary antibodies were incubated with the sections for 1 hour at room temperature: AlexaFluor 488-conjugated donkey anti-goat (705-545-147, 1/500, Jackson ImmunoResearch), AlexaFluor 594-conjugated donkey anti-rabbit (711-585-152, 1/500, Jackson IR), AlexaFluor 647-conjugated donkey anti-rat (712-605-153, 1/500, Jackson IR). DAPI was included in the Fluoromount medium. The double staining with anti-GFP (Goat Poly, 1/500, ab6556, Abcam) and anti-ERα (rabbit polyclonal MC-20, 1/200, Santa Cruz) was performed using the Opal™ Multiplex IHC kit with the OPAL 520 and OPAL570 respectively following the manufacturer’s recommendations (Perkin Elmer). Sections were imaged using a Zeiss LSM780 confocal microscope. The Z-series were reconstructed into a 3D movie using the Imaris 9.1.2 software.

In vitro assays

Freshly sorted luminal cells (1000 cells) were resuspended in culture medium (DMEM/F12 lacking phenol red supplemented with 5µg/mL insulin (Sigma), 10ng/mL EGF (Sigma), 100ng/mL cholera toxin (Sigma) and 5% FCS) and seeded onto 24-well plates in the presence of 5000 irradiated NIH-3T3 cells, as previously described (Sleeman et al., 2007). Five days later, colonies were fixed with 4% PFA, stained with hematoxylin and eosin (H&E), and counted.
For three-dimensional mammosphere assays, FACS-sorted luminal or basal cells (10000 cells) were resuspended in culture medium (DMEM-F12 lacking phenol red supplemented with B27 (1x, Gibco), 20ng/mL EGF (Sigma), 20 ng/mL bFGF (Gibco), 4µg/mL heparin (Sigma), 10 µg/mL insulin (Sigma) containing 4% Matrigel) as previously described (Dontu et al., 2003, Spike et al., 2012). After 15 days in culture, mammospheres were imaged using a stereomicroscope (Nikon SZM800). Three independent experiments were analyzed. For each traced organoid, the size and number of clones were measured using ImageJ software (NIH). For serial passaging, mammospheres were collected by centrifugation and incubated with 0.05%trypsin/EDTA (Gibco) to obtain a single cell suspension. Cells were replated in 4% Matrigel (BD Pharmingen) at a density of 5000 cells/mL, as stated above. All cultures were maintained in a 5% CO2 atmosphere at 37°C.

Gelatin degradation assay
Coverslips were cleaned overnight in 1 M HCl, washed four times with ddH2O and then successively coated with 50 µg/mL poly-L-lysine, 0.5% glutaraldehyde, fluorescent gelatin (1:10 mixture of Oregon green 488-conjugated gelatin from pig skin (G13186, Molecular Probes) and 0.2% gelatin from bovine skin (Sigma G1393), and 5 mg/ml sodium borohydride (Sirmans et al., 2014). Between each coating, coverslips were washed three times with PBS. Coverslips were then sterilized with 70% ethanol and 30,000 basal cells were seeded and incubated for 5 days. When mentioned, medium was supplemented with marimastat (a non selective MMP inhibitor, BB25.16, Euromedex, 5 µM). Culture medium and drugs were replaced every two days, cells were fixed with 4% PFA and stained.
Micro-array analysis

Luminal cells (4 samples for WT luminal cells and 5 for the other conditions) were sorted into 0.04 M RLT-DTT medium (Qiagen Gmbh) and stored at -20°C. A Qiagen RNeasy micro-kit (Qiagen Gmbh) was used to extract mRNAs. Gene expression profiles were analyzed at the GeT-TRiX facility (GénoToul, Génopole Toulouse Midi-Pyrénées) using Agilent Sureprint G3 Mouse GE V2 microarrays (8x60K, design 074809) according to the manufacturer’s instructions. For each sample, Cyanine-3 (Cy3)-labeled cRNAs were prepared from 25 ng of total RNA using the One-Color Quick Amp Labeling kit (Agilent) according to the manufacturer’s instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were examined using a Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). Six hundred nanograms of Cy3-labelled cRNAs were hybridized on the microarray slides according to the manufacturer’s instructions. Immediately after washing, slides were scanned on an Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software, and fluorescence signals were extracted using Agilent Feature Extraction software v10.10.1.1 with the default parameters.

Microarray data were analyzed using R (R Development Core Team, 2008, http://www.R-project.org) and Bioconductor packages (www.bioconductor.org, v 3.0, (Gentleman et al., 2004) as described in GEO accession GSE142297). Raw data (median signal intensity) were filtered, log2 transformed, corrected for batch effects (microarray washing bath and serial labeling) and normalized using the quantile method (Bolstad et al., 2003). The list of selected genes was established from microarray analyses with a fold change <1.5 or >1.5 and an adjusted $P$ value <0.05. Functional analyses were performed using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov), and comparisons were achieved with the Venn Diagrams plug-in based upon the VENNY tool developed by J. C. Oliveros.
Gene expression analysis using qRT-PCR

For luminal gene expression profiling, we performed a quantitative PCR (Fluidigm Dynamic Array, Fluidigm platform, GeT facility, GenoToul) on a set of 37 genes selected from the microarray data and a literature search. Primers were validated by testing PCR efficiency using standard curves (95% < efficiency < 105%). Gene expression was quantified using the comparative Ct (threshold cycle) method. HPRT1, β2M and GUSb were used as reference genes.

Statistics

Statistical analyses were performed using Prism 5 software (GraphPad). Data are presented as the means ± SEM. Comparisons between two specific groups were performed using Student’s t test. To test the effect of treatments or genotypes, data were compared between multiple groups with one variable a using one-way ANOVA followed by a Mann–Whitney test post-hoc multiple comparison test. To test the interaction between treatments and genotypes, a two-way ANOVA was used, followed by the Bonferroni post hoc test when an interaction was observed. A P value < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001, ns= not significant).

Authors’ contributions:

F.L designed the study with helpful discussions with C. Brisken. L.G., M.R., M.A., F.B., and A.W. performed the experiments. S.C performed the grafts of duct fragments. P.J. and A.B. assisted with the gelatin degradation assay. I.R-L. and F.G. performed the immunohistochemistry and performed the hormone dosages, respectively. N. Gaide helped us with immunostaining, L.G., S.C., M.R., and F.L. analyzed data. F.L. wrote the paper with input from C.B., A.B. and J.F.A. All authors discussed the results and commented on the manuscript.
Acknowledgments

The authors thank Masaru Okabe (University of Osaka, Japan) for providing the C57BL/6 TgN(act-EGFP) mice, Jason Iacovoni (INSERM U1048) and Marie-Ange Deugnier (Institut Curie, France) for participating in helpful discussions, Juliette Paunet and Guy Carcassès for providing animal care at the INSERM US006 platform ANEXPLO Genotoul (Toulouse, France), Alexia Zakaroff and Elodie Riant for their assistance and advices on flow cytometry and cell sorting (the cytometry platform, TRI-Genotoul, Toulouse), Dr. Romina D’Angelo, E. Vega and R. Flores-Flores (Cellular Imaging Facility-I2MC/TRI Platform) and E. Bellard (Imagery platform-IPBS-TRI-Genotoul, Toulouse) for their assistance and advices on imaging; R. Flores-Flores (Cellular Imaging Facility-I2MC/TRI Platform) for creating the movie using Imaris 9.1.2 sofware, J.J. Maoret and F. Martins from GeT Platform Genotoul (Toulouse), P. Rochaix, Armelle Gaston and Audrey Benest who assisted with the ERα immunohistochemistry. We are also grateful to Y. Lippi and Claire Naylies for their excellent contributions to the microarray analysis performed at the GeT-TQ Genopole Toulouse Facility and to Isabelle Bleuart and Isabelle Pardo for providing excellent technical support and advice regarding the histology (ENVT).

Funding information:

The work at I2MC-INSERM U1048 is supported by Institut National de la Santé et de la Recherche Médicale, Université et CHU de Toulouse, Faculté de Médecine Toulouse-Rangueil, Fondation pour la Recherche Médicale, Fondation de France, Association pour la Recherche Contre le Cancer and La ligue Contre le Cancer. L.G and M.A. were supported by grants from the Ministère de la Recherche. S.C. was supported by the Swiss Cancer Ligue KFS-3701-08-2015.

Declaration of Interests:

None
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Figure 1: The invasion of mammary fat pad is delayed at puberty in C451A-ERα mice.

A-B) Representative images of whole-mount mammary glands from (A) 5-week-old (WT- n=8; C451A-ERα, n=8) and adult, 3- to 6-month-old virgin C451A-ERα and WT mice (B) (WT- n=9; C451A-ERα, n=8) (scale bar=10 mm). Higher magnification images of the ductal tree are also shown (scale bar=1 mm). C) Quantification of fat pad filling in 5-weeks (two-way ANOVA, $P < 0.01$, interaction $P=0.0082$) and 3-months old ($P$ value not significant) C451A-ERα and WT mice D) Representative images of hematoxylin-eosin stained transverse sections of mammary glands from 3- to 6-month-old virgin WT and C451A-ERα mice (scale bars=250µm and 50µm (higher magnification)). Quantification of number of branching points
(E) and of ductal diameters (F) on the ductal tree in three-month-old WT and C451A- ERα mice (WT- n=7; C451A-ERα- n=7, t-test, ns= not significant, **P <0.01). G) Circulating levels of E2 and progesterone in 5-week and 3-month-old mice. Expression levels above the dotted line were considered detectable.
Figure 2: Absence of development of the C451A-ERα/GFP mammary epithelium after transplantation of ducts in WT mice

A) Fluorescence stereomicrographs of contralateral inguinal WT mammary fat pads engrafted with mammary epithelium from C451A-ERα or WT littermates. Images of virgin or day 16-18 pregnant recipients obtained 8 weeks after transplantation (scale bar=1mm). B) Dot plots showing the extent of fat pad filling by the engrafted epithelia in virgin mice (n= 22, non-parametric Mann-Whitney T-test, *** P value<0.001) or pregnant mice (n=15, non-parametric Mann-Whitney T-test, ** P value<0.01). Experiments were repeated with 4 independent donors. C) Representative immunostaining using hematoxylin coloration (upper panel), or an anti-ERα (middle panel) or PR (lower panel) antibody in mammary glands from 16.5 day pregnant mice engrafted with epithelium from C451A-ERα or WT mice (scale bar = 100µm). D) The proportion of epithelial cells expressing ERα and PR following immunohistochemistry with anti-ERα and PR antibodies was quantified as percentage of total epithelial cells.
Figure 3: The frequency and regenerative potential of CD29hiCD24+ basal cells are impacted by the C451A-ERα mutation

A) Representative flow cytometry dot plots of CD24+CD29lo (luminal) and CD24+CD29hi (basal) populations gated on CD45- and CD31- from mouse mammary glands obtained from 3-month-old WT and C451A-ERα mice. The percentages of luminal or basal cells were calculated within the CD31 and CD45-negative populations (WT- n=25; C451A-ERα- n=25, T-test, ** P<0.01)

B) Representative images of GFP-positive outgrowths arising from transplantation of 2000 double-sorted CD29hi CD24+ cells from glands of virgin adult WT or C451A-ERα mice (scale bar = 2 mm). The virgin recipient tissue was collected 8 weeks after transplantation into WT and C451A-ERα mice (right panel).

C) Dot plots show the percentages of fat pad filling by outgrowths 8 weeks after transplantation of different numbers of double-sorted CD29hiCD24+ cells. Cells were injected into the cleared mammary fat pads of 3-week-old syngeneic recipients and collected 8 weeks after transplantation. Data were pooled from 2 independent experiments (two-way ANOVA, *** P<0.001).

D) Repopulating frequency of the transplantation of limited numbers of double-sorted CD29hiCD24+ cells from the mammary glands of 12-week-old WT or C451A-ERα female mice (ELDA statistical test).
Figure 4: Hormone supplementation in C451A-ERα mice restores the frequency, but not the regenerative potential of CD29^{hi}CD24^{+}basal cells

A) Representative images of whole-mount mammary glands from ovariectomized WT and C451A-ERα female mice captured after 3 weeks treatment with a combination of 17β-estradiol and progesterone; higher magnification images of the ductal tree are also shown (scale bar=2 mm).

B) Bar plots show the percentages of fat pad filling and thickness of ducts.
(μm) in ductal trees of ovariectomized mice treated with E2+progesterone for 3 weeks (WT- n=8; C451A-ERα- n=11, t-test, the P was not significant or *** P <0.001). C) Circulating levels of E2 and progesterone in ovariectomized mice treated with E2 and progesterone for 3 weeks. (Levels above the dotted red line were considered detectable) (E2- WT-n=7; C451A-ERα- n=5, E2+Pg WT-n=6; C451A-ERα-n=7, t-test, the P was not significant). D) Representative images of Ki67, ERα, and PR immunostaining in mammary glands from ovariectomized WT and C451A-ERα mice treated with E2 and progesterone for 3 weeks. The percentages of epithelial cells positive for Ki-67, ERα or PR are expressed relative to the number of total epithelial cells (WT- n=6; C451A-ERα- n=6, t-test, the P was not significant - scale bar=200 μm). E) Flow cytometry dot plots of CD24+CD29lo (luminal) and CD24+CD29hi (basal) populations gated on CD45- and CD31- cells from mouse mammary glands removed from ovariectomized WT and C451A-ERα mice following 3 weeks treatment with the combination of 17β-estradiol and progesterone (WT- n=19; C451A-ERα- n=21, t-test, the P was not significant). F) Representative images of GFP-positive outgrowths arising from transplantation of 2000 double-sorted CD29hiCD24+ cells from the mammary glands of ovariectomized WT (left panel) and C451A-ERα (right panel) mice treated with E2+ progesterone (scale bar=2mm). The virgin recipient tissue was collected 8 weeks after transplantation. G) Percentages of fat pad filling by outgrowths 8 weeks after the transplantation of different numbers of double-sorted CD29hiCD24+ cells. Cells were injected into the cleared mammary fat pads of 3-week-old syngeneic recipients and collected 8 weeks after transplantation. Data were pooled from 2 independent experiments (two-way ANOVA, ***P <0.001). H) Repopulation frequency of the transplantation of a limited number of double-sorted CD29hiCD24+ cells from these mammary glands (ELDA statistical test).
Figure 5: Co-injection of WT CD24⁺CD29⁻ luminal cells with CD29⁺CD24⁺ basal cells from C451A-ERα mice restores their regenerative ability in transplantation assays

A) Representative images of GFP-positive outgrowths arising from the transplantation of 2500 double-sorted CD24⁺CD29⁻ luminal cells from WT (left panel) or C451A-ERα (right panel) mice co-injected with 2500 GFP-positive CD29⁺CD24⁺ basal cells from C451A-ERα mice. Cells from ovariectomized WT or C451A-ERα mice treated with E2+ progesterone (Pg) for 3 weeks were sorted by flow cytometry (scale bar=2mm). The WT virgin recipient tissue was collected 8 weeks after transplantation.

B) Percentage of fat pad filling by outgrowths at...
8 weeks after the transplantation of different numbers of double-sorted CD24⁺CD29⁻ luminal cells from WT or C451A-ERα mice mixed with GFP positive CD29⁺CD24⁺ cells basal cells from C45A-ERα mice. Cells were injected into the cleared mammary fat pads of 3-week-old syngeneic recipients and collected 8 weeks after transplantation. Data were pooled from 2 independent experiments (two-way ANOVA, ***P <0.001). C) Repopulating frequency of the transplantation of limited numbers of double-sorted CD29⁺CD24⁺ cells from these mammary glands (ELDA statistical test). D) Confocal images of mammary gland epithelium after immunostaining with anti-GFP (Green), K5 (red) and K8 (magenta) primary antibodies (scale bar=20μm). E, F) Confocal images of mammary gland epithelium after immunostaining using anti-GFP (Green), -ERα (MC20- red) and Dapi (Cyan) in epithelium 8 weeks after co-injection of GFP-positive C451A-ERα MaSCs mixed with GFP negative luminal cells from WT mice. Representative sections of ducts when only MaSCs C451A gave rise to basal cells (E), or when MaSCs C451A gave rise to both basal and luminal GFP positive, but no ERα positive luminal cells were observed (F) (scale bar= 10µm or 25 µm).
Figure 6: Large-scale analysis of the effects of C451A-ERα on gene expression in the CD24⁺CD29lo luminal cells

A) Heatmap of the global gene expression analysis in CD24⁺CD29lo luminal cells from WT and C451A-ERα ovariectomized mice treated with E2 or E2+ progesterone (Pg) for 3 weeks (WT- n=5; C451A-ERα-n=5 in each condition). B) Venn diagram of differentially expressed (up- and downregulated) genes. C-E) Gene ontology analysis of the dysregulated genes...
using the GO database. F) Gene expression analysis using qRT-PCR. Relative mRNA levels were normalized and presented as relative levels compared to expression in WT mice treated with E2. Samples used in the large-scale analysis were included and complemented with 2 additional samples prepared using the same conditions (WT- n=7; C451A-ERα-n=7; two-way ANOVA, * P<0.05, ** P<0.01, *** P<0.001).
Figure 7:

A) Representative gating strategy illustrating luminal cells (CD24+ CD29-) being subgated for Sca1+ CD133+ and Sca1- CD133- subpopulations. 

B) Relative quantity of *Esr1*, *Pgr* and *Areg* RNA normalized to β2m, Hprt, GusB and Tbp in WT Sca1+CD133+ luminal cells as compared to WT Sca1-CD133- luminal cells. 

C) Representative images of GFP positive outgrowths arising from the transplantation of C451A-ERα MACS with sorted Sca1+ CD133+ or Sca1- CD133- WT luminal cells. (scale bar=2 mm).

D) Confocal images of mammary gland epithelium after immunostaining with anti-GFP (green), anti-K5(Green) and K8 (magenta) primary antibodies (scale bar=20 μm).

E) Confocal images of mammary gland epithelium after immunostaining with anti-GFP (green) and anti ERα (red) antibodies.
**Figure S1: A-B** Representative images of Ki67, ERα, PR and active-caspase 3 immunostaining of mammary glands from 5-week-old (A) and 3 to 6-month-old (B) virgin WT and C451A-ERα mice (t-test, *P* <0.05; scale bar=100 µm). The proportion of epithelial cells expressing Ki67 (both on distal ducts and terminal end buds (TEB), ERα, PR and active caspase 3 was quantified as percentage of total epithelial cells. On B) Confocal images of mammary gland epithelium after immunostaining with anti-K5(Green) and K8 (magenta) primary antibodies (scale bar=20µm or 5µm). **C** Levels of the ERα protein normalized to GAPDH in mammary glands from 5-week-old or 3-6-month-old C451A-ERα and WT mice were assessed using Western blotting. Uteri were used as positive controls (C+) (two-way ANOVA, ns: not significant).
Figure S2: Immunostaining of pregnant 3-week-old WT mice mammary glands engrafted with a piece of mammary epithelium from C4514-ER\(\alpha\) or WT mice

A) Confocal imaging of immunostainings using anti-GFP (green), -K5 (red) and -K8 (magenta) primary antibodies on mammary glands from 16 days pregnant mice grafted with ducts from WT or C451A-ER\(\alpha\) mice (scale bar=26 \(\mu\)m).

B) Positive control of the PR staining from experiment in Figure 2C (scale bar=200 \(\mu\)m and 50 \(\mu\)m)
Figure S3: Immunostaining of the mammary epithelium 8 weeks after transplantation of basal cells from virgin C451A-ERα or WT mice

A) Confocal images after immunostaining using anti-GFP (green), -K5 (red), -K8 (magenta) and Dapi (blue). (Scale bar=20µm and in the high magnification image=5µm).

B) Immunohistochemistry using anti-ERα (6F11) antibody. (scale bar=200 µm).
Figure S4: The C451A-ERα mutation does not modify the clonogenic capacity of luminal and basal cells in vitro, but impacts matrix degradation

A) Hematoxylin-eosin staining was performed to evaluate the clonogenic capacity of luminal CD24+CD29lo cells seeded on irradiated fibroblast feeder cells after 7 days in culture. Box plots show the number of luminal colonies generated when 1000 cells were plated, and area of the different obtained clones (n=3 independent experiments from luminal WT or ERα-C451A cells pooled from 2 to 4 mice (t-test, the P value was not significant)).

B) Representative images of mammosphere colony forming assays from luminal and basal cells grown on matrigel. After one generation, cells from the colonies were collected and plated again for the colony forming assay at generations II and III. The data indicate the number of mammospheres obtained from 5000 cells (n=4 independent experiments from luminal WT or C451A-ERα cells pooled from 2 to 4 mice each (t-test, the P value was not significant)).
Figure S5: Images of immunofluorescence staining of green-fluorescent gelatin degradation by double-sorted CD29\textsuperscript{hi}CD24\textsuperscript{+} basal cells in vitro

A) Representative images of degraded A488-labeled gelatin appearing in black. The areas of degraded gelatin were measured in at least 18 fields per genotype in each experiment and an average of 281 cells was counted from each genotype in each experiment (scale bar=50µm).

B) The area of gelatin degraded by gate-sorted C451A-ER\textalpha basal cells was compared to the area of gelatin degraded by cells purified from their WT littermates within the same experiment. The graph shows the means of four independent experiments using WT or C451A-ER\textalpha cells pooled from 2 to 4 mice each (t-test, **P value<0.01).

C) Inhibition of gelatin degradation was observed when the non selective MMP inhibitor (marimastat) was added on the culture medium.
**Figure S6:**

A) Recapitulative table of the co-transplantation experiments performed with different ratios of C451A GFP positive MaSCs with ERα-WT luminal cells and number of outgrowth success obtained for each conditions

| Experiment | Ratio BCs/LCs | Number cells | Nb of outgrowths/ Total grafts | % outgrowth success |
|------------|--------------|--------------|--------------------------------|---------------------|
| 1          | 4/1          | 2000 BCs/500 LCs | 1/10                            | 10%                 |
|            | 1/1          | 2000 BCs/2000 LCs | 2/10                            | 20%                 |
| 2          | 1/1          | 2000 BCs/2000 LCs | 4/8                             | 50%                 |
| 3          | 5/1          | 5000 BCs/1000 LCs | 3/6                             | 50%                 |
|            | 5/1          | 2500 BCs/500 LCs  | 2/6                             | 33%                 |
|            | 1/1          | 2500/2500        | 3/5                             | 60%                 |

B) Number of grafts containing only GFP positive basal cells or giving bipotent GFP positive cells (i.e. both basal and luminal cells) obtained after co-transplantations of GFP-positive C451A-ERα basal cells with GFP-negative ERα-WT luminal cells, in the regenerated mammary gland 8 weeks after transplantation. Four of 9 mammary glands contained GFP-positive luminal cells, whereas when only GFP-negative luminal cells were injected while GFP-positive luminal cells were not detected in 5 out of 9. In grey, are underlined conditions given the higher proportion of success obtained.

| Ratio BCs/LCs | Number cells | Number analyzed | GFP+ BCs only (Unipotency) | GFP+ BCs and LCs (Pluripotency) |
|---------------|--------------|-----------------|---------------------------|---------------------------------|
| 1/1           | 2000 BCs/2000 LCs | 2               | 1                         | 1 high LCs                      |
| 5/1           | 2500 BCs/500 LCs  | 2 *              | 1                         | 1 (few LCs)                    |
| 1/1           | 2500 BCs/2500 LCs | 3               | 2                         | 1 (few + 1 high LCS)           |
| 5/1           | 5000 BCs/1000 LCs | 3               | 1                         | 2 (1 few + 1 high LCS)         |
| **Total**     |               | **5/9**         | **4/9**                   |                                 |

* among 2 analyzed, ducts were only visible in 1 sample

C) Confocal image of mammary gland epithelium after immunostaining using anti-GFP (Green), -ERα (MC20, red) and Dapi (Cyan) in epithelium 8 weeks after co-injection of GFP-positive C451A-ERα MaSC mixed with GFP negative luminal cells from WT mice when MaSCs C451A gave rise to basal and few luminal GFP positive cells (right), with very rare GFP luminal cells were found ERα positive (white arrows).

D) The proportion of ERα positive cells following immunohistochemistry using anti-ERα antibody was quantified in the regenerated mammary gland obtained 8 weeks after co-transplantations of GFP-positive C451A-ERα or WT basal cells with GFP-negative ERα-WT luminal cells.
Figure S7: **A)** Predicted gene-gene interaction network (Search tool for the Retrieval of interacting Genes/StringV10) among genes significantly down-regulated in C451A versus WT mice by E2 (FC>1.5, P>0.05). Genes coloured in red belong to the GO category of «extracellular region», in blue to the «Plasma membrane» and in green to the «basal membrane protein». **B)** Gene network among the 9 genes found in the GO category of genes belonging to the positive regulation of cell migration, including Stat5a, a related gene with **C)** the corresponding Log fold change indicated in the right. **D)** Model of impact of membrane ERα on stem cells interfering with pathways induced by Growth-Hormone Receptors (GH-R) and FGF-R receptors impacting the Jak2/Stat5 signalling pathways, modified from Furth et al., 2011.
Figure S8: 

A) Predicted gene-gene interaction network (Search tool for the Retrieval of interacting Genes/StringV10) among genes significantly down-regulated in C451A versus WT mice by E2+Progestosterone (Pg) (FC>1.5, P>0.05). Genes coloured in red belong to the GO category of «extracellular matrix protein», in blue to the «membrane protein». In B) the Log fold change of genes found in the network are indicated.
Table S1: Summary of co-transplantation experiments performed mixing C451A-ERα MaSCs with either Sca1\(^+\) CD133\(^+\) or Sca1\(^-\) CD133\(^-\) WT luminal cells at a ratio of 5 basal/1 luminal cells.

| Experiment | Ratio BCs/LCs | Number cells | Mice grafted | Right | Left | Outgrowth right | Outgrowth left |
|------------|--------------|--------------|--------------|-------|------|----------------|---------------|
| 1          | 5/1          | 5000/1000    | 5            | MaSC C451A-ERα LCs WT Sca1\(^+\) | MaSC C451A-ERα LCs WT Sca1\(^+\) | 1/5 (70% outgrowth-BCs GFP only) | 0%            |
| 2          | 5/1          | 3000/600     | 10           | MaSC C451A-ERα LCs WT Sca1\(^+\) | MaSC C451A-ERα LCs WT Sca1\(^+\) | 1/10 (80% outgrowth- rare GFP LCs) | 0%            |
Movie 1: Movie showing different angles of the confocal images of the 3D-reconstructed outgrowths obtained 8 weeks after transplantation of a mixture of GFP positive C451A MaSCs with WT luminal cells, after labeling with anti-ERα (red) and anti-GFP (green) antibodies.