Published in final edited form as:

Cancer Lett. 2010 March 1; 289(1): 23–31. doi:10.1016/j.canlet.2009.07.012.

Wnt Signaling can Substitute for Estrogen to Induce Division of ER α -positive Cells in a Mouse Mammary Tumor Model

Melissa Mastroianni, Soyoung Kim, Young Chul Kim, Amanda Esch, Caroline Wagner, and Caroline M. Alexander¹

McArdle Lab for Cancer Research, University of Wisconsin, 1400 University Ave, Madison 53706

Abstract

The interaction of estrogen with the estrogen receptor (ER, principally ER α) induces growth of human breast tumor cells. In contrast, ER α -positive cells have been described as non-dividing cells in normal breast (though estrogen stimulation of ER α cells directs the division of neighboring cells). However, there is a small sub-population of cells in normal mammary tissue that are ER α -positive, that can divide, and therefore share this property with human breast tumor cells. In order to investigate their pattern of growth regulation, we measured the fraction of dividing ER α ⁺ cells during normal growth and compared that to glands stimulated by oncogenic Wnt effectors. First, we found there was no difference between the rate of division of ER α ⁺ cells and ER α [−] cells, whether the population was responding to estrogen or Wnt mitogens. The proportion of dividing ER α ⁺ mammary epithelial cells was increased (10 $\times$) in response to pregnancy, and similar increases were observed in response to ectopic Wnt signaling. We propose that Wnt signaling can substitute for estrogen to drive total population growth (that includes ER α ⁺ cells). Although the E-ER α -derived mitogenic response is situated in a minority of the luminal cells, and the Wnt-LRP5/6 –derived mitogenic response is situated in a minority of basal cells, overall, the growth response of the mammary epithelial population is remarkably similar.

Keywords

Breast cancer; Mouse mammary tumor model; Wnt signaling; Estrogen receptor

Introduction

One of the defining features of the majority of human breast tumors is that they express (and over-express) the nuclear hormone receptor estrogen receptor- α (ER α). Non-pregnant levels of estrogen, in combination with ER α , are sufficient to drive cell division in human breast tumor cells, and indeed aromatase inhibitors or tamoxifen (inhibitors of E- ER α trans-activation) arrest or kill ER α ⁺ tumor cells. Interestingly, although growth of normal cells is also estrogen-dependent, the vast majority of dividing cells are ER α -negative. To explain this, one hypothesis suggests that the ER α ⁺ subpopulation (5–10%) induces growth, in paracrine

© 2009 Elsevier Ireland Ltd. All rights reserved.

¹Corresponding author: Alexander@oncology.wisc.edu, ph: 608-265 5182 / 262 1258, FAX: 608-262 2824.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of Interest Statement
None Declared

fashion, in the ER α -negative majority. This is supported by observation of xenografts of human cells in the presence of estrogen, and the growth of ER α -knockout cells in chimeric transplants in mouse [1;2]. Clearly, understanding the local mediators of cell growth are important to understanding the etiology of breast cancer.

A minority of dividing cells (1.0 – 0.05 %; with and without estrogen, respectively) that are ER α^+ / PR $^+$ / Ki67 $^+$ in human [3] and ER α^+ / Ki67 $^+$ in mouse (this study; [4]. Various investigators have proposed that these cells represent a stem/progenitor cell type and suggest that their growth regulation be established separately from the cell majority. For example, using engineered mice, Ewan et al (2005) showed that the division of this sub-population was suppressed by TGF β signaling. Thus, in normal mice, these cells were associated with lower cell-associated TGF β and loss of TGF β signaling (absent nuclear R-SMAD) [4]. In mice with a gain of function in TGF β signaling, the fraction of dividing ER α^+ cells in mammary glands was decreased (6-fold) and, vice versa, increased (16-fold) in mammary glands with loss of function of TGF β -signaling. Thus, the proliferative behavior of these estrogen-dependent cells can be modified by alterations in other signaling pathways.

Here, we have turned to transgenic mouse models with ectopic expression of Wnt signaling to test whether this pathway is important to the control of this ER α^+ cell minority. Wnt signaling is necessary for the growth of mammary ductal trees in virgin mice (at low ambient estrogen), and is key to the maintenance of mammary stem cells [5]. The receptors for canonical Wnt signaling only exist in the basal population (that includes functional regenerative stem cell activity). Ectopic Wnt signaling promotes stem cell accumulation, and is subsequently highly oncogenic [6]. Whereas Ewan et al (2005) showed that TGF β inhibited the division of this group of cells, we show here that ectopic Wnt signaling induces their division to levels usually associated with juvenile ductal development, or pregnancy-associated lobuloalveolar development, the two phases of active growth for mammary glands. We propose that ectopic Wnt signaling can functionally substitute for estrogen-dependent growth, producing estrogen-independent tumors.

Materials and Methods

Mouse mammary samples

Samples were collected at the ages and timepoints indicated from transgenic and control mice. Wnt effector transgenic mice were described in Liu et al (2004); these strains express either Wnt1 ligand or $\Delta N\beta$ catenin (a non-degradable canonical Wnt effector) under the control of the mouse mammary tumor virus LTR (MMTV-LTR). MMTV-driven gene expression is induced at low levels in embryonic mammary placodes, increases during puberty, and rises dramatically during pregnancy and lactation. All the tumors used in this study were analyzed for their H-Ras status, and no mutations were identified. Other later-developing tumors from Wnt1-transgenic mice were mutant for H-Ras (11/18 of those developing after 100 days); unpublished data and [7]). To test the effect of ovariectomy on Wnt-induced hyperplastic glands, 8 week-old mice were ovariectomized and their glands isolated for analysis 4 weeks later. To test the effect of ovariectomy on Wnt-induced tumors, mice with palpable Wnt-induced tumors (0.5–0.8 cm) were anesthetized, their tumors were biopsied to generate pre-ovex samples and simultaneously ovariectomized. When the masses were at least 1 cm diameter, they were excised to compare pre-ovex and post-ovex cell dynamics. All mouse procedures were done under NIH ACUC guidelines.

Immunocytochemistry

Primary antibodies were: anti-ER α from Santa Cruz Biotechnology (rabbit polyclonal antibody made to the C-terminus of ER α , MC20, SC-542; working concentration was 1 μ g/ml in Tris

buffered saline-0.05% Tween; TBS-Tw), Ki67 (mouse monoclonal antibody, proliferation marker; cloneB56, BD Pharmingen; 25 µg/ml), Keratin 5 (Rabbit anti-K5, Covance, Madison, WI) and Keratin 8 (rat anti-K8 (Troma-I), Developmental Studies Hybridoma Bank (University of Iowa). Secondary antibodies were Alexa 546 goat anti-mouse IgG (20 µg/ml) and Alexa Fluor 488 goat anti-rabbit IgG (10 µg/ml) from Molecular Probes (Eugene, OR).

Samples were fixed (overnight) in paraformaldehyde (3.7% in PBS) at 4°C, embedded in paraffin and sectioned (10 µm). Rehydrated sections were treated for antigen retrieval (boiling in sodium citrate buffer (0.01 M, pH 6), as described by Zymed (www.invitrogen.com). Tissues were permeabilized in a solution of 0.2% Triton-X in PBS for 10 mins and washed. Non-specific binding was blocked by incubating slides for at least an hour at room temperature with 10% sheep serum in TBS-Tw. A mixture of both primary antibodies (diluted in TBS-Tween) was incubated with tissues overnight at 4°C, slides were washed three times for 5 minutes in TBS-Tween and incubated with secondary antibodies for 1 hour at room temperature. Slides were washed and counterstained for nuclear DNA with TO-PRO-3 (0.5 µM; Molecular Probes), for 10 minutes at room temperature. Immunofluorescent stains were visualized on a confocal microscope (BioRad MRC1024), using Kr/Ar laser lines at 488, 568 and 647 nm. There was no overlap between fluorescent stains (determined from single stains). Staining was done manually by one operator, and results were scored by 2 blinded operators.

To score ERα⁺, Ki67-positive and ERα⁺ Ki67 double positive nuclei, at least 2000 –5000 (epithelial cell) nuclei (the n (number of nuclei) is shown for all data sets in Supplementary Table 1) were counted separately in up to 30 fields chosen at random from tissue sections spaced approximately 100 µm apart. The statistical analyses that apply to each comparison are shown in Figure Legends.

Results

For this study, we recorded the fractions of ERα⁺ cells, of dividing (Ki67-positive) cells, and the proportion of dividing ERα⁺ cells for each physiological condition. The gross appearance of our sample tissues is shown in Fig. 1 (paraffin-embedded samples stained with H&E).

Patterns of ERα⁺, Ki67⁺ and dividing ERα⁺ cells in normal mammary tissue

During ductal extension and during pregnancy, we confirmed that the majority of ERα⁺ cells were luminal (defined by their position), and that the majority of dividing cells were not ERα⁺ (Fig. 2). Though it is clear that estrogen is absolutely required for cell division (see Fig. 6), and it is likely that cells with nuclear ERα⁺ are the estrogen-responsive population, the dividing luminal cells tend to occur in clusters at a distance from the ERα⁺ cells. These properties are also typical of human breast tissues. Also in common with human breast tissues [3], there were a number of parabasal ERα⁺ cells (Fig. 2) that were also (sometimes) Ki67⁺. Dividing ERα⁺ cells are rare: 10–20% of total cells are ERα⁺, 1 – 10% of total cells are dividing, and only 0.1 – 1.0% of total cells are ERα⁺ Ki67⁺ double positive (DP) (their appearance and distribution is shown in Figs. 2 and 3, and their quantitation in Fig. 4).

The fraction of ERα⁺ cells measured during very different phases of growth and development is remarkably consistent. Thus early in juvenile ductal development, where growth is estrogen-dependent but estrogen concentrations are low, the proportion of ERα⁺ cells is 15– 20% (3 week-old glands). After puberty, when estrogen concentrations increase, this declines to approx. 10% (Fig. 4), as expected from the feedback degradation of ERα⁺ protein after exposure to ligand. The number of dividing cells declines as the outgrowth of the ductal tree slows to a halt in the adult fat pad (from 7 to 1% total). Similarly, during lobuloalveolar development in pregnant mice (when estrogen levels are high) approximately the same proportion of cells- one

out of eight - are $ER\alpha^+$. This is accompanied by a 10 $\times$ increase in cell division (to 4% of the total cell population).

During both ductal extension and pregnancy, the fraction of dividing $ER\alpha^+$ cells was increased 10 fold, from approximately 0.06% of the total $ER\alpha^+$ cell population in quiescent virgin ductal trees to 0.6% in juvenile ductal trees, or late pregnancy ($p=0.0039$; Fig. 4). To address whether the $ER\alpha^+$ cells enter the cell cycle with the same frequency as the total population, the fraction of $ER\alpha^+$ cells was multiplied by the fraction of $Ki67^+$ cells to obtain a “calculated fraction of DP cells” (Fig. 5), for comparison with the observed frequencies of DP cells. Since this frequency was not statistically different from one (DP actual/calculated for ductal populations from juvenile glands= 0.79 ± 0.23 , and for lobuloalveolar populations from pregnant glands= 0.89 ± 0.06), we can conclude that $ER\alpha^-$ cells and $ER\alpha^+$ cells are equally likely to divide. Therefore, the mitotic effect generated by the E- $ER\alpha^+$ -dependent interaction creates an equal chance of cell division in all cells, irrespective of their $ER\alpha^+$ status.

Wnt-induced preneoplastic glands and tumors

In the preneoplastic, virgin glands from transgenic mice expressing Wnt signaling effectors, the growth index was increased compared to non-transgenic controls (Fig. 4), to approximately that of actively growing normal tissues. After 5 months, the mammary epithelial cell populations in both transgenic strains showed the lowest growth indices (data points are color-coded for the age of the mouse at sacrifice; purple spots are 5 month old). Note that for these tumor models, clonal transformation arises in this background of general hyperplasia, resulting in tumor development (median latency of tumor development is 21 weeks (of age) in Wnt1-induced glands and 28 weeks in $\Delta N\beta cat$ -induced glands; [7]).

Both Wnt1- and $\Delta N\beta cat$ -induced preneoplastic glands, and even tumors, contained approximately normal $ER\alpha^+$ cell fractions (ie. approx 1/8, though some showed 1/3 and others 1/20). These tumors have a highly differentiated phenotype that includes cells resembling normal basal and luminal cells. Despite the massive over-production of cells during rapid tumor development characteristic of this model, there is a relatively normal distribution of basal and luminal cells, which even extends to the proportion of $ER\alpha^+$ luminal cells. The distribution of $ER\alpha^+$ cells included (infrequent) patches of cells that were all positive (for example, see bracket in Fig 3A). In contrast, in normal tissues, $ER\alpha^+$ cells tend to be distributed in a salt and pepper pattern. This reflects the disturbance of normal intercellular interaction and polarization that occurs when ectopic Wnt signals are derived from the luminal compartment [8].

Interestingly, the samples from Wnt-induced preneoplastic glands showed higher variance than normal populations from pregnant glands. For example, the $ER\alpha^+$ cell fraction was $14\pm 4.8\%$ for lobuloalveolar populations from pregnant glands, whereas it was $10\pm 102\%$ for Wnt1-induced preneoplasias (f test, 0.001). This variability was also characteristic of the ductal populations in juvenile glands (ER cell population $13.6\pm25.5\%$), though the trend was not statistically significant. The cell division pattern showed a similar trend (pregnant, $4\pm2.4\%$; ductal, $4.36\pm12.6\%$; Wnt-induced, 4.8 ± 6.3 ; f test=0.06). This variance was extended to a bimodal high/low pattern in the tumors (see brackets Fig.4). The significance of this is unknown, but might imply the presence of an unidentified pulsatile growth signal that cooperates with Wnt signals in non-pregnant mice.

A comparison of the calculated DP fractions with those observed (Fig. 5) in Wnt-induced tumors showed that the division rate of $ER\alpha^+$ cells was in striking equilibrium with $ER\alpha^-$ cells (except in $\Delta N\beta cat$ -induced tumors, where $ER\alpha^+$ cells show a 2.2 $\times$ higher mitogenic index than the $ER\alpha^-$ population).

Can Wnt signaling sustain cell division in pre-neoplastic and tumor tissues in the absence of estrogen?

To test whether cell division was estrogen-dependent in Wnt-stimulated transgenic glands, the Ki67+ cell fraction was assessed in Wnt1-transgenic mice after ovariectomy. In contrast to normal tissues, the rate of cell division for these populations did not change (compare Fig. 6C and E with 6B and D), showing that cell division was estrogen-independent.

In order to confirm that the tumors were not estrogen-dependent, mice with palpable lesions were ovariectomized. Unlike human ER α ⁺ tumors, these tumors do not regress; our data was consistent with data published by other groups [9;10]. The variance of these samples was high, and resembled that of the other samples from normal and Wnt-induced tissues (DP fraction either fraction of dividing ER α ⁺ cells in Wnt-induced tumors was either high ($\geq 20\%$) or low ($\leq 5\%$) (Fig. 6A).

Discussion

Wnt signaling can induce the division of ER α ⁺ cells

All mouse mammary growth and expansion has now been shown to be ER α ⁺-dependent [1]. The ER α null mouse strain (ERKO) that was previously studied is now assumed to have hypomorphic ER α function. When that hypomorphic strain was crossed to the Wnt over-expressing strain used here, the partial ductal outgrowth characteristic of this strain was augmented by ectopic Wnt signaling [10;11].

We have shown that mammary epithelial populations can be induced to divide by ectopic Wnt effectors (either secreted Wnt1 ligand, or the intracellular non-degradable effector, Δ N β catenin), to levels associated with normal processes of active growth (either ductal extension in juveniles, or expansion of the lobuloalveolar network during pregnancy). The pattern of division of ER α ⁺ and ER α ⁻ cells resembles that of normal growing epithelial cell populations. Thus, the fraction of dividing ER α ⁺ cells increases from 0.06% in quiescent virgin ductal trees to 0.6% (a 10-fold increase) in juvenile expanding trees, or in glands from pregnant mice. In preneoplastic Wnt and Δ N β cat glands, this fraction can be as high as 1.4%.

Note that Wnt signaling is required for normal ductal stem cell activity [5]. Thus, in the absence of the Wnt signaling receptor LRP5, the ductal tree forms, however the adult ductal regenerative stem cell population is depleted (by $\geq 95\%$). Vice versa, ectopic Wnt signaling over-stimulates this population [6], leading to an accumulation of stem cell activity (10 $\times$) in the total cell population.

Although the overall growth rate and pattern of production of luminal and basal cells is very similar whether growth is induced by estrogen or Wnt ligands, it is unlikely that the cellular target for E-ER α -dependent growth is the same as the Wnt-Lrp5/6-dependent growth. Thus, our work has shown that the canonical (growth/tumor-inducing) receptors for Wnt ligands exist only on basal cells (Badders et al submitted). Vice versa, ER α is clearly only present in the nuclei of luminal cells. The most striking conclusion drawn from this study is that, irrespective of the cell type that receives the signal, the final growth and morphogenesis includes the same proportion of ER α ⁺ and ER α ⁻ cells, and the same overall structure/function (see scheme of Fig. 6F). Wnt signaling can clearly substitute for estrogen to create a mammary epithelial population indistinguishable in most regards from estrogen-dependent normal populations. The growth stimulation is remarkably symmetric whether derived from a basal or luminal origin.

Note that TGF β has directly opposite effects on the division of ER α ⁺ cells compared to Wnt signals. In fact, Wnt and TGF β signaling are often in opposition developmentally [12]. The

growth inhibitory effects of TGF β are known to be very important for maintaining the quiescence of mammary ductal trees [13], and ectopic Wnt signaling may reverse this growth inhibition. Constitutively activated TGF β induced a 6-fold reduction of dividing ER α^+ cells, whereas a 90% ablation of TGF β signaling (in TGF β +/- mice) increased their number 16-fold.

Although it is formally possible that Wnt and estrogen substitute for each other at the molecular level, and indeed there is ample precedent for cross-talk of Wnt and steroid receptor signaling [14] [15] [16], we consider this less likely. Thus, if one or both of these pathways was operating in a non-canonical mode (via the Fzd or other alternate receptors for Wnt signaling, and/or a non-nuclear estrogen function, with or without ER α), they could be using similar/shared transactivation sites to induce growth-associated genes.

Accumulations of ER α^+ cells are typical of post-menopausal women and preneoplastic conditions such as ductal carcinoma in situ, and many are dividing (the fraction of dividing ER α^+ cells increases from 0.01% in premenopausal breast to 11% [17;18]. This is also true for preneoplastic conditions in genetically modified mice [19]. We observed accumulations of ER α^+ cells in [MMTV- Δ N β cat] preneoplastic glands, which deviate from the normal heterogeneous morphogenetic fields that characterize the distribution of ER α^+ cells in mammary gland (ER α^+ cells within each field appear to regulate the proliferation of surrounding ER- cells).

Are dividing ER α^+ cells stem/progenitor cells?

When we compare the rate of division of ER α^+ and ER α^- cells, they are not significantly different. In other words, all mammary epithelial cells (irrespective of their cell-autonomous ER α^+ expression) divide at the same rate after exposure to estrogenic or Wnt-mediated growth stimuli.

Although in theory an ER α^+ estrogen-responsive progenitor cell could orchestrate the endocrine response of mammary tissue (and be subverted during tumorigenesis; [20]), in practice, the ductal stem cell-enriched fractions able to regenerate ductal trees in cleared fat pads do not express ER α^+ . Published data shows that there are at least two stem/progenitor cell types, a ductal stem cell measured using an in vivo isograft assay (mammary repopulating unit; MRU), and another clonogenic cell (colony-forming cell; CFC) that has been scored in vitro using sphere- or colony-forming assay [21;22]. The MRU population sorted by current surface markers is only 5% pure, whereas the CFC fraction is almost pure (ie. most viable cells are clonogenic). None of the MRU fraction expresses measurable ER α protein (by immunohistochemistry) [23;24]. Other sub-populations with a more indirect relationship to stem cells (either human or mouse) have been tested for their expression of ER α , and found to be positive, including SP cells, label-retaining cells (LRC), and parity-induced cells [25;26; 27].

We considered whether there could be two types of ER cells, one terminally differentiated, and a distinct progenitor cell that divides in response to estrogenic stimuli. The frequency of DP cells in this study is 0.06–0.6%. If this is considered to be the growth fraction (5–10% of total cells) of a distinct subpopulation of cells that express ER α^+ , the size of that sub-population might be 0.3–6%, approx 1/50 total mammary epithelial cells. However, given the concordance of the rates of division of ER α^+ and ER α^- cells, it is hard to reconcile the data with this complicated scenario.

Could ectopic Wnt signaling support the growth of tamoxifen-resistant ER α ⁺ breast tumors?

Our results are consistent with those of others in confirming that the majority of biological features of ER α ⁺ cells in mouse mammary glands are similar to those of human breast tissue. Thus, the arrangement and fraction of ER α ⁺ cells is similar, the separation between the majority of ER α -expressing cells and dividing cells is identical, together with the dependence of the minority, ER α ⁺ cells on endocrine support for their division [2;25;28].

When mouse tumors are found to express ER α , they are widely assumed to be estrogen-dependent, without proper functional evaluation. There may be only two cases where mouse models have been rigorously shown to develop ER α ⁺, estrogen-dependent tumors; these are mice that over-express an ER α transgene, and mice with reduced p53 function [29;30]. The fact that tumorigenesis is estrogen-independent in the [MMTV-Wnt1] model has been shown before: Bocchinfuso et al (1999) showed that post-pubertal ovariectomy did not affect Wnt1-induced tumor growth rates, and Zhang et al [9] also showed that the rate of growth of xenotransplanted Wnt1-induced tumors was unaffected by ovariectomy (though the frequency of ER⁺ cells reduced to the normal 8% range if the primary tumor had a high (15%) ER α ⁺ fraction; their data shown without comment, confirmed by this study). We propose that Wnt signaling can substitute for ovarian support to promote mammary growth (though the pattern of outgrowth is abnormal and hyperplastic). Wnt1-induced tumors are known to be “oncogene-addicted”, so that when Wnt is removed, the tumors involute [31].

Unlike other tumor types (like colorectal cancer), the tumor suppressors and oncogenes in the Wnt pathway (Apc, axin and β catenin) appear to be unaffected in breast tumors (except perhaps as a late event). However, genes that regulate extracellular Wnt signaling do show consistent changes in breast tumors (including loss of the inhibitor, SFRP1, [32]. It is therefore possible that ectopic Wnt signaling could be supporting the growth of human breast cancers that are ER α ⁺ but PR-negative, and show tamoxifen-resistance. We propose that the small molecule inhibitors of Wnt signaling that are under development by pharmaceutical companies may show efficacy for this patient group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ER α	estrogen receptor α
MECs	mammary epithelial cells
DP	double positive (ER+ve, Ki67+ve) cells

Acknowledgments

MM was supported by a Hildale scholar grant for undergraduate research, YCK and AE by the McArdle pre-doctoral training grant (T32 CA09681), and the research was supported by grants from the Susan Komen foundation and by NCI-RO1 CA090877.

References

1. Mallepell S, Krust A, Chambon P, Briskin C. Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. *Proc Natl Acad Sci U S A* 2006;103:2196–2201. [PubMed: 16452162]
2. Anderson E, Clarke RB. Steroid receptors and cell cycle in normal mammary epithelium. *J Mammary Gland Biol Neoplasia* 2004;9:3–13. [PubMed: 15082914]

3. Clarke RB, Howell A, Anderson E. Estrogen sensitivity of normal human breast tissue in vivo and implanted into athymic nude mice: Analysis of the relationship between estrogen-induced proliferation and progesterone receptor expression. *Breast Canc. Res. Treat* 1997;45:121–133.
4. Ewan KB, Oketch-Rabah HA, Ravani SA, Shyamala G, Moses HL, Barcellos-Hoff MH. Proliferation of estrogen receptor-alpha-positive mammary epithelial cells is restrained by transforming growth factor-beta1 in adult mice. *Am J Pathol* 2005;167:409–417. [PubMed: 16049327]
5. Lindvall C, Evans NC, Zylstra CR, Li Y, Alexander CM, Williams BO. The Wnt signaling receptor Lrp5 is required for mammary ductal stem cell activity and Wnt1-induced tumorigenesis. *J Biol Chem* 2006;281:35081–35087. [PubMed: 16973609]
6. Liu Y, McDermott SP, Khwaja SS, Alexander CM. The transforming ability of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. *Proc. Natl. Acad. Sci* 2004;101:4158–4163. [PubMed: 15020770]
7. Kim YC, Clark RJ, Ranheim EA, Alexander CM. Wnt1 expression induces short-range and long-range cell recruitments that modify mammary tumor development and are not induced by a cell-autonomous beta-catenin effector. *Cancer Res* 2008;68:10145–10153. [PubMed: 19074881]
8. Rosen JM. Hormone receptor patterning plays a critical role in normal lobuloalveolar development and breast cancer progression. *Breast Dis* 2003;18:3–9. [PubMed: 15687684]
9. Zhang X, Podsypanina K, Huang S, Mohsin SK, Chamness GC, Hatsell S, Cowin P, Schiff R, Li Y. Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations. *Oncogene* 2005;24:4220–4231. [PubMed: 15824740]
10. Bocchinfuso WP, Hively WP, Couse JF, Varmus HE, Korach KS. A mouse mammary tumor virus-Wnt-1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha. *Cancer Res* 1999;59:1869–1876. [PubMed: 10213494]
11. Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic animals. *Annu Rev Physiol* 2005;67:285–308. [PubMed: 15709960]
12. Letamendia A, Labbe E, Attisano L. Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. *J. Bone Joint Surg. (Amer.)* 2001;83-A:S31–S39. [PubMed: 11263663]
13. Gorska AE, Jensen RA, Shyr Y, Aakre ME, Bhowmick NA, Moses HL. Transgenic mice expressing a dominant-negative mutant type II transforming growth factor-beta receptor exhibit impaired mammary development and enhanced mammary tumor formation. *Am J Pathol* 2003;163:1539–1549. [PubMed: 14507660]
14. Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC. Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocr Rev* 2005;26:898–915. [PubMed: 16126938]
15. Kouzmenko AP, Takeyama K, Ito S, Furutani T, Sawatsubashi S, Maki A, Suzuki E, Kawasaki Y, Akiyama T, Tabata T, Kato S. Wnt/beta-catenin and estrogen signaling converge in vivo. *J Biol Chem* 2004;279:40255–40258. [PubMed: 15304487]
16. Hou X, Tan Y, Li M, Dey SK, Das SK. Canonical Wnt signaling is critical to estrogen-mediated uterine growth. *Mol Endocrinol* 2004;18:3035–3049. [PubMed: 15358837]
17. Shoker BS, Jarvis C, Clarke RB, Anderson E, Hewlett J, Davies MP, Sibson DR, Sloane JP. Estrogen receptor-positive proliferating cells in the normal and precancerous breast. *Am J Pathol* 1999;155:1811–1815. [PubMed: 10595909]
18. Shoker BS, Jarvis C, Sibson DR, Walker C, Sloane JP. Oestrogen receptor expression in the normal and pre-cancerous breast. *J Pathol* 1999;188:237–244. [PubMed: 10419589]
19. Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM, Ward JM, Green JE. The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. *Oncogene* 2000;19:968–988. [PubMed: 10713680]
20. Dontu G, El-Ashry D, Wicha MS. Breast cancer, stem/progenitor cells and the estrogen receptor. *Trends Endocrinol Metab* 2004;15:193–197. [PubMed: 15223047]
21. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li H, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. *Nature* 2006;439:993–997. [PubMed: 16395311]

22. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. *Nature* 2006;439:84–88. [PubMed: 16397499]
23. Asselin-Labat ML, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ. Steroid hormone receptor status of mouse mammary stem cells. *J Natl Cancer Inst* 2006;98:1011–1014. [PubMed: 16849684]
24. Sleeman KE, Kendrick H, Robertson D, Isacke CM, Ashworth A, Smalley MJ. Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J Cell Biol* 2007;176:19–26. [PubMed: 17190790]
25. Clarke RB, Spence K, Anderson E, Howell A, Okano H, Potten CS. A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev. Biol* 2005;277:443–456. [PubMed: 15617686]
26. Booth BW, Smith GH. Estrogen receptor-alpha and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. *Breast Cancer Res* 2006;8:R49. [PubMed: 16882347]
27. Booth BW, Boulanger CA, Smith GH. Alveolar progenitor cells develop in mouse mammary glands independent of pregnancy and lactation. *J Cell Physiol* 2007;212:729–736. [PubMed: 17443685]
28. Petersen OW, Hoyer PE, van Deurs B. Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. *Cancer Res* 1987;47:5748–5751. [PubMed: 3664479]
29. Tilli MT, Frech MS, Steed ME, Hruska KS, Johnson MD, Flaws JA, Furth PA. Introduction of estrogen receptor-alpha into the tTA/TA conditional mouse model precipitates the development of estrogen-responsive mammary adenocarcinoma. *Am J Pathol* 2003;163:1713–1719. [PubMed: 14578170]
30. Lin SC, Lee KF, Nikitin AY, Hilsenbeck SG, Cardiff RD, Li A, Kang KW, Frank SA, Lee WH, Lee EY. Somatic mutation of p53 leads to estrogen receptor alpha-positive and -negative mouse mammary tumors with high frequency of metastasis. *Cancer Res* 2004;64:3525–3532. [PubMed: 15150107]
31. Gunther EJ, Moody SE, Belka GK, Hahn KT, Innocent N, Dugan KD, Cardiff RD, Chodosh LA. Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. *Genes Dev* 2003;17:488–501. [PubMed: 12600942]
32. Ugolini F, Charafe-Jauffret E, Bardou VJ, Geneix J, Adelaide J, Labat-Moleur F, Penault-Llorca F, Longy M, Jacquemier J, Birnbaum D, Pebusque MJ. WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type. *Oncogene* 2001;20:5810–5817. [PubMed: 11593386]

Fig. 1. Morphology of samples (illustrated with H&E-stained sections from paraffin-embedded samples)

A. Virgin adult glands, showing the ductal tree, embedded in a predominantly fatty matrix. Total colonization of the fat pad is approximately 5%. B. In pregnant mice (12 days p.c), the ductal tree sprouts lobuloalveolar side-branches. C. After 3 months, transgenic mouse glands expressing the oncogene, $\Delta N\beta$ catenin, develop ductal hyperplasia. D. As early as the mammary rudiment stage, transgenic mice expressing the proto-oncogene, Wnt1, show hyperplastic ductal overgrowth. Shown here is an example from an adult 12 week-old gland. E. After 12 months, 96% of [MMTV- $\Delta N\beta$ cat] mice develop solid, differentiated adenocarcinomas. F. After 8 months, 77% of [MMTV-Wnt1] mice develop differentiated, microacinar adenocarcinomas with highly reactive stromal infiltration.

Fig. 2. Double-staining for ERα- and Ki67-positive cells in normal glands

A. *Virgin ductal trees*. The ductal lumen is indicated, together with the usual bilayered epithelial lining, comprising of luminal cells bounded by myoepithelial cells, which are bounded in turn by the basement membrane. Nuclei are stained blue, and the relative colors of the specific stains are indicated (ERα, green; Ki67, red). In the top panel, * shows an (uncommon) parabasal dividing cell, together with clusters of dividing luminal cells (marked #). Examples of parabasal double positive (DP) cells are shown in the middle and bottom panels. B. *Pregnant glands*. Samples from mice 10 (top) and 15 (bottom panel) days post-coitus are shown. The arrangement of ERα-positive and -negative cells is approximately the same as it is in ducts from virgin mice, together with the distribution of DP cells.

Fig. 3. Double-staining for ERα- and Ki67-positive cells in preneoplastic glands and tumors

A. Glands from pre-neoplastic 6 week-old, and 12 week-old $\Delta N\beta cat$ mice, showing patches of ERα+ve cells (bracket) and DP cells. B. Staining patterns of ERα and Ki67 in $\Delta N\beta cat$ -induced tumors, showing clustering of dividing cells separated from ERα+ve cells. C. Shown here is an example of a reactive site from a hyperplastic gland from a 6 week-old Wnt1-transgenic mouse, where cell-cell contacts are less organized, and dividing, ERα+ and DP cells are less restricted to their appropriate positions, annotated here is an ERα+ basal cell (*) and a giant parabasal cell (DP arrow). D. Two fields from Wnt1-induced tumors, one showing a high DP fraction (top). A large proportion of the cell population expresses some residual ERα (bottom panel).

Fig. 4. Quantitation of ER, Ki67 and DP populations

Each value represents 2000–6000 cells (see Supplemental Table 1) and the samples and methodology is described in Materials and Methods. Normal virgin glands are shown in grey columns, glands from pregnant mice in pink columns. For preneoplastic glands from $\Delta N\beta cat$ - and *Wnt1*-transgenic mice, mice of different ages are shown with different color codes; yellow, 3 week old; green, 6 wk old; blue, 12 wk old; purple, 21 wk old. Tumors are color-coded earliest to latest, white, pale grey, dark grey, black (see Table 1). When populations appeared to fall into two groups, this was indicated with arbitrary brackets.

Fig. 5. Evaluation of the rate of division of ER+ cells compared to the cell majority
If division of cells occurs randomly (irrespective of their ERα⁺ status) then the product of the fraction of cells positive for ERα⁺ × fraction Ki67+ cells (the calculated DP fraction) will be similar to the observed DP fraction. If the actual/calculated DP ratio=1, we conclude that ERα⁺ and ERα⁻ cells divide equally in response to mitogenic signals.

Fig. 6. In the absence of estrogen, ectopic Wnt signaling drives growth

A. Mice with palpable Wnt-induced tumors were biopsied (PRE), ovariectomized, and their growth observed. When the masses were 1 cm diameter, they were excised (POST), and the relative proportions of ERα⁺, Ki67⁺ and DP cells assessed. One tumor was divided into 4 separate samples to assess intra-tumoral variation. B–E. Immunocytochemical evaluation of dividing luminal and basal cell populations in ovariectomized mice. Mammary tissues were stained with lineage-specific markers (keratin 5, basal cells, stained blue; keratin 8, luminal cells, stained red) to provide a cellular context for the Ki67 stain (Ki67, stained green). Two examples are shown for each physiological condition (B–E). Normal ducts from adult virgin mice showed occasional Ki67⁺ cells (B; same frequencies as observed throughout this study),

whereas ovariectomized mice showed no dividing cells (D; the diffuse luminal green stain is non-specific). Wnt1 transgenic glands (13 weeks) showed an increased fraction of Ki67+ cells compared to control (C), that was not reduced after ovariectomy (E). F. Scheme of growth regulation in mammary epithelial cell populations. Basal cells respond to Wnt signals to create the total heterogeneous population, whereas luminal cells respond to estrogenic signals to create a grossly similar population (especially with respect to their ER α expression).