

Proliferation of Estrogen Receptor α Positive
Mammary Epithelial Cells is Restrained by TGFβ1 in Adult Mice

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ABSTRACT

Transforming growth factor β1 (TGFβ1) is a potent inhibitor of mammary epithelial proliferation. In human breast, estrogen receptor α (ERα) cells rarely co-localize with markers of proliferation, but their increased frequency correlates with breast cancer risk. To determine whether TGFβ1 is necessary for the quiescence of ERα-positive population, we examined mouse mammary epithelial gland at estrus. Approximately 35% of cells showed TGFβ1 activation, which co-localized with nuclear receptor-phosphorylated Smad 2/3, indicating that TGFβ signaling is autocrine. Furthermore, nuclear Smad co-localized with nuclear ERα. To test whether TGFβ was functional, we examined genetically engineered mice with different levels of TGFβ1. ERα co-localization with markers of proliferation (i.e. Ki-67 or BrdU) at estrus was significantly increased in the mammary glands of *Tgfβ1* C57/bl/129SV heterozygote mice. This relationship was maintained following pregnancy, but was absent at puberty. Conversely, mammary epithelial expression of constitutively active TGFβ1 via the MMTV promoter suppressed proliferation of ERα positive cells. Thus, TGFβ1 activation functionally restrains ERα positive cells from proliferating in adult mammary gland. Accordingly, we propose that TGFβ1 dysregulation may promote proliferation of ERα positive cells associated with breast cancer risk in humans.

INTRODUCTION

The pluripotent cytokine, transforming growth factor β1 (TGFβ1) has been widely implicated in mammary epithelial growth (reviewed in ^{1,2}), in cancer (reviewed in ³), and in response of breast cancer cells to estrogen and progesterone (reviewed in ⁴). TGFβ family members can modify cell behaviors via autocrine, paracrine, and endocrine mechanisms of action (reviewed in ⁵). A primary control of TGFβ1 activity is its secretion as a latent complex, which consists of the 24 kD cytokine and a 80 kD dimer of its pre-pro region containing the signal sequence for secretion ⁶. Extracellular events release TGFβ from the latent complex so that it can bind to expressed cell surface receptors that then initiate signaling cascades that modulate gene expression. To our knowledge, all cells secrete latent TGFβ and all cells express TGFβ receptors, underscoring the necessity of identifying the cell and tissue conditions leading to release of TGFβ as an essential control of its bioactivity.

To evaluate cause-and-effect relationships between TGFβ1 activation and epithelial cell proliferation in the mammary gland, we characterized spatial and temporal patterns of TGFβ1 activation using immunostaining in conjunction with functional assessment using *Tgfb1* knockout mice ⁷. Immunolocalization revealed that TGFβ1 production and activity are differentially regulated during mammary gland development, such that periods of proliferation were accompanied by decreased TGFβ1 activation in most cells. TGFβ1 depletion results in significantly accelerated morphogenesis during puberty and pregnancy and increased mammary epithelial proliferation during proliferative stages. However, TGFβ depletion is insufficient to initiate proliferation since ovariectomized *Tgfb1* heterozygote mice did not show a phenotype, although proliferation increased more than 10-fold compared to wildtypes following administration of estrogen and progesterone. These data indicate that TGFβ specifically inhibits the proliferative potential of mammary epithelial cells in response to ovarian steroids.

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While estrogen and progesterone are critical for mammary epithelial proliferation, it is clear that cells differ in their ability to respond to these signals. During both ductal and lobular-alveolar mammary growth, the distribution of proliferating cells is heterogeneous, suggesting the involvement of local factors in dictating the specific response to systemic hormones⁸⁻¹⁰. Studies in estrogen receptor α (ER α) knockout mouse mammary outgrowths indicate that both stromal and epithelial ER α , in cooperation with epithelial progesterone receptor (PR), govern mammary gland development in adult mice¹¹. Ovarian steroid hormone receptors are also heterogeneously expressed by a subpopulation of the mammary epithelium, but these receptors rarely co-localize with markers of proliferation in human cells¹²⁻¹⁷. Interestingly, increased proliferation in the hormone receptor positive population is associated with breast cancer risk¹⁸, while a transgenic mammary model of dysregulated ER α expression results in hyperplasia and ductal carcinoma in situ¹⁹.

Our TGF β immunolocalization revealed striking epithelial heterogeneity during mammary stages characterized by proliferation⁷. For example, during estrus many cells downregulate TGF β , consistent with its action as a growth inhibitor, but some mammary epithelial cells maintain prominent TGF β 1 activation. This observation suggested to us the hypothesis that TGF β acts to restrain such cells from proliferating particularly in the presence of ovarian steroids. Therefore, in this study we first sought to determine the relationship between the TGF β 1-positive and steroid hormone receptor epithelial subpopulations using dual immunolocalization, and second, to determine whether TGF β depletion specifically affects proliferation of hormone receptor positive cells.

METHODS

Mice

All experiments were conducted with LBNL institutional review and approval.

TGFβ1 Restrains ERα Cell Proliferation

Animals were killed by CO₂ inhalation and cervical dislocation at the indicated times in accordance with AAALAC guidelines. Mammary glands were collected from *Tgfβ1* heterozygote and wildtype mice bred in house in the C57BL/6-129SV mixed background unless otherwise noted. In some experiments, *Tgfβ1* knockouts crossed back to FvB or Balb/c background mice were used, which were obtained respectively from Drs. Lalage Wakefield and Adam Glick at NCI. FvB *MMTV-Tgfβ*²²³⁻²²⁵ transgenic mice were previously described²⁰. All specimens were collected from animals reared and housed at LBNL. Estrus was determined by cytological characteristics of vaginal smears and confirmed postmortem by uterine wet weight. Nulliparous animals approximately 10 weeks of age were killed in estrus. Ovariectomy and estradiol and progesterone treatment of ovariectomized mice was carried out as previously described⁷. At least three animals were used for each treatment group. The inguinal mammary glands were dissected free of the skin and embedded in OCT compound (Miles Inc., Elkhart, IN). Frozen OCT-embedded tissue blocks were stored at -70°C until the time of sectioning.

Antibodies

TGFβ1 was detected using polyclonal, affinity-purified chicken anti-TGFβ1 (AF-101-NA, lot FS08; R&D Systems, Minneapolis, MN), which preferentially reacts with the active form of TGFβ1. We used monoclonal antibody NCL-ER-6F11 to ERα and rabbit polyclonal antibody to Ki-67 both from Novacastra (Newcastle, UK). Antibody FL-425 (#SC-8332 Santa Cruz Biotechnology, Santa Cruz, CA) recognizes receptor-phosphorylated Smad proteins. The rabbit polyclonal antibody to progesterone receptor was developed in house by GS²¹. For secondary antibodies, Alexa 488 labeled goat anti mouse IgG (Molecular Probes, Eugene, OR) FITC-labeled anti-mouse (Pierce, Rockford, IL), Texas red labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and Texas red labeled rabbit anti-chicken IgY (Sigma, St. Louis, MO) were

used.

Immunohistochemistry

Frozen OCT-embedded mammary glands were sectioned onto gelatin-coated coverslips, then fixed using 2% buffered paraformaldehyde for 20 minutes at room temperature, followed by a 0.1M glycine in phosphate-buffered saline (PBS) washes. For estrogen receptor α co-localization with Ki-67, Smad or progesterone receptor, sections were treated with prewarmed 0.1% Triton X100 in PBS at 37°C for 20 minutes followed by PBS washes. Nonspecific sites were blocked using the supernatant from a 0.5% casein/PBS solution (pH 7.4) for 60 minutes. Endogenous mouse IgG was blocked using “Mouse-on-Mouse” blocking agent (Vector Laboratories, Burlingame, CA) diluted 50% in 0.5% casein/PBS solution for 4 hours at room temperature. Sections were incubated in primary antibodies diluted in 0.5% casein/PBS solution and incubated overnight at 4°C. After washes, each primary was detected by sequential incubations with species-specific secondary antibodies. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and stored at -20°C until evaluated. For progesterone receptor colocalization with active TGF β 1, sections were blocked with 0.5% casein/PBS solution, reacted with primary antibodies overnight at 4°C. After washes, each primary was detected by sequential incubations with species-specific secondary antibodies.

Microscopy and Image Analysis

Immunofluorescence images were obtained and processed as previously described ⁷. Color images of ducts were split into their constituent channels and scored for total and positive cells using the text annotation feature in Corel Photopaint 7 (Corel, Dallas TX) without knowledge of tissue source of images. Three animals per genotype/treatment and at least 300 cells per animal were

scored for presence of the marker. Prism 3 (GraphPad Inc, San Diego CA) was used to conduct two-tailed Student t-tests to evaluate whether mean frequency differed significantly between treatment groups.

RESULTS

Autocrine TGFβ1 Action in the Mammary Epithelium

Mammary epithelial proliferation is dictated by the endogenous ovarian steroid milieu such that it is maximal at estrus. At estrus activation state-specific TGFβ1 immunoreactivity is reduced in most cells⁷, but approximately 35% of luminal epithelial cells show prominent immunoreactivity (Figure 1). All cells secrete latent TGFβ and the extracellular matrix is a reservoir of latent protein, thus it is difficult to ascertain its original cellular source. Further, TGFβ1 activity is controlled by extracellular activation and depending on the mode of latent TGFβ1 activation, TGFβ1 can signal in both autocrine²² or paracrine²³ fashion. Thus, it is important to determine when TGFβ1 is actively signaling and to which cells. Upon ligand binding, TGFβ type I receptor phosphorylates Smad 2 and 3 (R-Smad), which leads to nuclear translocation and initiation of transcription (reviewed in²⁴. We asked whether TGFβ was acting in an autocrine or paracrine fashion upon activation by using dual immunofluorescence to localize both TGFβ and nuclear R-Smad. The frequencies of cells positive for active TGFβ1 and nuclear R-Smad were similar. Figure 1A shows single fluorochrome images and a merged image of a mammary duct containing cells positive for active TGFβ1 and nuclear Smad. Co-localization of active TGFβ1/nuclear R-Smad cells showed nearly perfect correspondence (Figure 1B). Most cells positive for active TGFβ were Ki-67 negative (91%, n=2 mice). These data indicated that cell-restricted TGFβ1 activation during estrus results in triggering of the TGFβ signaling pathway in the same cells.

Ovarian Hormone Receptor Positive Cells are Positive for Active TGF β 1 and Exhibit Nuclear R-Smad

It is well established that estrogen action is mediated through its cognate receptors, ER α and ER β , but that ER α is essential for mammary epithelial cell proliferation²⁵. ER α localization by immunohistochemistry is heterogeneous within the mammary epithelium^{26,27}. To determine the relationship between TGF β activation and the ER α positive cell populations, we used dual immunofluorescence to determine co-localization of ER α and R-Smad. Almost all ER α positive cells were positive for nuclear R-Smad (Figure 2A). The same was true when ER α was co-localized with active TGF β 1 (data not shown). Dual immunofluorescence of progesterone receptor (PR) and active TGF β 1 (Figure 2B) also demonstrated that most PR positive cells were positive for active TGF β 1. ER α co-localized with PR (Figure 2C), as is found in human and rat mammary gland²⁷. These data indicated that most cells that are ER α and PR positive maintain TGF β activation during estrus, suggesting that TGF β may inhibit their ability to respond to ovarian hormone-induced proliferation.

Proliferation of ER α Positive Mammary Epithelial Cells is Increased in Tgf β 1 Heterozygote Mice

The infrequent co-localization of proliferation markers with ER α positive cells, despite the presence of estradiol, raises two possibilities: either the inability to proliferate is intrinsic to these cells independent of TGF β , or that TGF β 1 selectively restrains ER α positive cells from proliferating. If it is the latter, then it is reasonable to expect that changes in the endogenous levels of TGF β would lead to changes in the proliferation of ER α positive cells. TGF β 1 protein is reduced by more

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than 90% in mice lacking one *Tgfb1* allele, whether measured by immunoreactivity or by quantifying TGF- β biological activity in tissue extracts⁷. Therefore, to determine the relationship between TGF β and proliferation of ER α positive cells, we used dual immunofluorescence localization of nuclear ER α and the cell cycle marker Ki-67 and scored dual-labeled epithelial cells in *Tgfb1* heterozygote and wildtype mammary glands (Figure 3 A, B). The overall frequency of Ki-67 marked cells at estrus was increased 24-fold in *Tgfb1* heterozygote mammary epithelium compared to wildtype mice, consistent with our previous results using PCNA as a marker of cell cycle⁷. The frequency of cells exhibiting dual localization of Ki67 and ER α increased 16-fold in *Tgfb1* heterozygote (Figure 3C). Although the degree of response was modified by genetic background, the consequence of TGF β 1 depletion was similar in two other mouse strains (i.e. Balb/c and FvB, data not shown).

To confirm that these ER α positive cells were actively undergoing DNA synthesis, animals were injected with bromodeoxyuridine (BrdU) 1 hr before death. The frequency of BrdU-labeled cells in the ER α positive subpopulation was also significantly increased in *Tgfb1* heterozygote mice (Figure 3D), in a fashion similar to what was observed with the Ki-67 labeling index. The relative frequency of labeled cells is less than that for Ki-67 because BrdU marks a narrower window of the cell cycle (i.e. S phase). Thus, these studies indicated that more ER α positive cells are capable of entering the cell cycle and undertaking DNA synthesis during estrus, but that concomitant TGF β activation functionally restrains them.

ER α is found in both stromal and epithelial cells of the mammary gland. The role of stromal versus epithelial ER α was first addressed by Cunha and colleagues who used recombined mammary stromal and epithelial tissue from neonatal Balb/c and ERKO mice transplanted under the renal capsule of intact athymic nude mice²⁸. Their results indicated that stromal, but not epithelial, ER α is

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required for ductal growth of immature mammary epithelium. Subsequent studies by Korach and colleagues in a mature mouse demonstrated the lack of outgrowth of ERα-deficient epithelial cells injected into an ERα-positive stroma, which suggests that compartment-specific requirement for ERα is dependent on maturation¹¹. To examine whether regulation of proliferation of ERα positive cells was intrinsic to the *Tgfb1* heterozygote epithelium or is mediated by depletion of TGFβ1 in the stroma, we conducted similar transplant studies of *Tgfb1* Balb/c heterozygote or wildtype fragments to wildtype stroma. We found that the frequency of cells dual labeled for ERα and Ki67 obtained at 8 weeks post-transplantation was 5-fold greater in *Tgfb1* heterozygote outgrowths (2.5 ± 1.06 , N=5; $p < 0.05$, Mann-Whitney U test) compared to wildtype (0.47 ± 0.19 , N=4). Thus depletion of TGFβ1 in the epithelium was sufficient to significantly increase proliferation of ERα positive cells at estrus.

Next we examined if proliferation of ERα positive cells in the *Tgfb1* heterozygotes was regulated by ovarian steroids. Accordingly, we depleted endogenous levels of estrogen and progesterone by ovariectomy and examined the proliferative status of ERα positive cells in these mammary glands. ERα/Ki67 dual-labeled cells were not evident in the ovariectomized mammary gland. Daily treatment of ovariectomized mice with estrogen and progesterone for three days resulted in 17-fold higher frequency of double-labeled ERα/Ki-67 mammary epithelial cells in *Tgfb1* heterozygote mammary epithelium versus wildtype epithelium (2.2 ± 0.35 S.E.M. vs 0.13 ± 0.07 S.E.M., $p = 0.02$; unpaired t-test with Welch's correction). Thus, as with ERα negative cells, TGFβ depletion promotes proliferation in ERα positive cells only in response to estrogen and progesterone.

Decreased Proliferation of ERα Positive Mammary Epithelial Cells in MMTV- TGFβ²²³⁻²²⁵

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Gain of Function Mice

If TGFβ1 depletion results in greater proliferation of ERα positive mammary epithelial cells, then one would predict that increased TGFβ1 activity might suppress the ability of ERα positive cells to enter the cell cycle. To test this, we examined FvB MMTV- *Tgfb*²²³⁻²²⁵ transgenic mice that expresses constitutively-active TGFβ1 in the mammary epithelium²⁰. Mice were studied at 12 weeks of age to ensure fully developed nulliparous tissue, which was confirmed by wholemount. The frequency of dual-localization of Ki67 and ERα was decreased six-fold in MMTV-*Tgfb*²²³⁻²²⁵ transgenic mice compared to estrus-stage wildtype mice (Figure 4), which indicates that expression of activated TGFβ1 can override hormone-induced proliferation of these cells.

Parous TGFβ1 Heterozygote Mice Exhibit Increased Frequency of ERα Positive Cells in Cycle

Parous women have fewer proliferating ERα positive mammary epithelial cells compared with nulliparous women²⁹. Similarly, parous rats³⁰ and rats exposed to a hormone regime that mimics pregnancy and subsequent involution³¹ exhibit fewer proliferating ERα positive mammary epithelial cells than age-matched nulliparous rats. Thus, ERα positive mammary epithelial cells in the parous gland appear to be either less susceptible to hormonal signals to proliferate or are more stringently restrained from proliferation than in the nulliparous condition. To test the latter possibility, we determined the frequency of proliferating ERα positive mammary epithelial cells in TGFβ1 depleted C57/bl parous animals (Figure 5). The frequency of dual ERα/Ki67 positive mammary epithelial cells in estrus-stage parous *Tgfb*1 wildtype mice was similar to that in estrus-stage, nulliparous *Tgfb*1 wildtype mice, which is already low (i.e. 0.1%). However, the frequency of

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ER α co-localization with Ki-67 in parous mammary epithelial cells was four-fold higher *Tgf β 1* heterozygote mice than in *Tgf β 1* wildtype mice. As we reported for mammary proliferation overall, these data indicate that ER α positive cells are restrained from proliferating in the parous gland through mechanism(s) mediated by TGF β 1. Consistent with this conclusion, conditional deletion of Smad4 in the mammary gland as a function of pregnancy leads to hyperplastic foci with increased cell proliferation³².

Regulation of Proliferation by TGF β 1 in ER α Positive Cells is Absent at Puberty

In contrast to the foregoing data showing that TGF β 1 depletion increased proliferation overall and of ER α positive mammary epithelial cells at estrus, in hormone-treated ovariectomized mice, and following pregnancy, TGF β 1 depletion did not increase the proliferation of ER α positive cells that are present in the pubertal mammary gland. The frequency of Ki-67 positive cells was significantly higher in *Tgf β 1* heterozygote endbuds and ducts than in *Tgf β 1* wildtype, as expected from our previous analysis⁷, but the frequency of ER α positive co-localization with Ki-67 in either structure was not significantly affected by TGF β depletion (Figure 6). These data suggest that proliferation of ER α positive cells is differentially regulated during puberty compared to estrus.

DISCUSSION

The developmentally discrete patterns of TGFβ1 activation during mammary gland maturation underscores the exquisite context and cell dependent regulation of TGFβ1 activation and activity. It also raises the question of which of its many roles TGFβ1 plays at different stages of mammary morphogenesis and differentiation. The feature that is most striking about the immunostaining for active TGFβ1 in the mammary gland at estrus is that certain cells activate more TGFβ1 at estrus than at any other time in the estrus cycle ⁷. As we show here, those cells are almost exclusively ERα positive. Based on this we infer that the TGFβ1 activity is greater in the ERα positive cells than in the ERα negative cells, and thus its ability to block proliferation is likely greater. As shown in our previous paper, TGFβ1 is severely depleted in the heterozygote so that the observed effect is the result of the relative reduction of TGFβ1 on each population. Thus, we have determined that TGFβ1 at estrus is stringently restraining ERα positive cells from proliferating in response to hormonal stimulation.

TGFβ appears to be acting in an autocrine fashion since mammary epithelial cells positive for TGFβ1 by immunostaining also exhibit nuclear R-Smad protein indicative of TGFβ signaling. The idea of autocrine action within the epithelium is further supported by the observation that 5-fold more ERα cells proliferated in *Tgfb1* heterozygote epithelium transplanted to wildtype stroma than similarly treated wildtype epithelium. The question of whether TGFβ activation in these cells arose stochastically or is associated with known heterogeneity of epithelial cell types was resolved by the demonstration that nearly all ERα positive mammary epithelial cells exhibited TGFβ1 activation and nuclear R-Smad immunoreactivity. The functional significance of the correlation between ERα and TGFβ was evident upon examination of the *Tgfb1* transgenic mouse mammary glands. We found that, with the important exception of pubertal mammary gland, TGFβ1 depletion resulted in

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increased frequency of co-localization of ERα positive epithelial cells with markers of cell cycle traversal in adult tissue. Even though the original cellular source of latent TGFβ is obscure since it is a secreted molecule produced by essentially all cells, it is clear from our transplantation study that epithelial depletion of TGFβ1 is sufficient for increased proliferation of the ERα positive cells. Analysis of ovariectomized *Tgfβ1* heterozygote mice demonstrated that an increase in proliferation of the ERα positive subpopulation requires estradiol and progesterone under the protocol of daily injections. Conversely, transgenic overexpression of active TGFβ1 resulted in reduced co-localization of ERα and markers of proliferation. Observations in human breast that epithelial cells expressing either ERα or PR rarely co-localize with makers of proliferation led to the proposal by Anderson and Clarke that these cells are actively restrained from proliferation by a growth inhibitor^{12,18}. Based on our present studies we propose that TGFβ1 is the growth inhibitor that restrains ERα positive cells in adult mammary gland from responding to signals to proliferate.

The validity of extending our observations in the rodent to the human breast is based on the following salient features shared between mouse mammary gland and human breast. 1) ERα positive cells are almost all PR positive (reviewed²⁶ for human); 2) ERα positive cells are heterogeneously distributed^{26,27}; 3) ERα positive cells rarely proliferate^{12,18}; 4) estrogen upregulates PR expression^{26,33}. This accord supports the use of mouse models to understand the regulation and responses of ERα positive mammary epithelial cells. Anderson and Clarke proposed that ERα positive cells are sensors that indirectly, via growth factors, regulate proliferation in ERα negative effector cells³⁴. Reproductive history with an early first pregnancy is a strong factor in assessing breast cancer risk (reviewed in³⁵). Parity in women or rodents results in less proliferation of ERα positive mammary epithelial cells^{29,30}. We found that the frequency of dual ERα/Ki67 positive cells was increased in TGFβ-depleted mice, which suggests that TGFβ1 also restrains

proliferation of ERα positive mammary epithelial cells in parous mice. Likewise, conditional deletion of Smad 4 by a pregnancy-induced promoter leads to increased proliferation, alveolar hyperplasia and, after repeated pregnancies, squamous cell carcinoma ³². Gene expression microarray analysis suggests that other members of the TGFβ family may also have a role in restricting proliferation after parity since TGFβ3 and its transcriptional targets are up-regulated in parous glands ³⁶.

Our novel finding that adult mammary epithelial ERα positive cells are restrained by TGFβ1 from proliferating in the presence of estrogen also has implications for understanding the biology of ERα positive cells in human breast cancers. The frequency of ERα positive cells increases with age in human breast, which parallels increased breast cancer risk ¹⁸. Lawson has shown that increased frequency of ERα positive cells is associated with increased breast cancer risk. Women at higher risk of breast cancer have more ERα positive cells compared with those women in a low risk population ³⁷. Japanese women living in Hawaii have more ERα cells than those in Japan, which parallels cancer risk ³⁸. ERα positive cells are increased in normal tissue of tumor-bearing breasts ³⁹, in postmenopausal women ¹⁸, and in post-menopausal women using hormone replacement therapy ⁴⁰. Consistent with the human data, a mouse mammary model of dysregulated ERα predisposes to hyperplasia and ductal carcinoma in situ ¹⁹. Together these data indicate that the size of the ERα positive subpopulation is modulated by tissue or host factors associated with age and environment, but they have yet to be identified specifically. Based on our present observations it is reasonable to suggest that decreased responsiveness to, or activation of, TGFβ1 may be one of the earliest events in dysregulating ERα cells. Consistent with this is the finding that ERα positive breast cancer lines are less sensitive to TGFβ1 mediated growth inhibition than are ERα negative breast cancer cell lines ⁴¹.

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We suggest that these data support a model in which the ERα positive population represents a distinct mammary lineage. Stem cell behaviors, such as cell cycle entry, regeneration and formation of niches, have been postulated to involve regulation by TGFβ1⁴²⁻⁴⁴. Boulanger and Smith have shown that ectopic expression of constitutively active TGFβ1 in mammary gland leads to decreased serial transplantation capacity which they hypothesize is due to premature stem cell senescence⁴⁵. TGFβ1 has been implicated in telomerase expression^{46,47}, which is thought to be characteristic of stem cells, and, interestingly, telomerase has been implicated in TGFβ1 insensitivity of human mammary epithelial cells⁴⁸. Recently, Tumbar et al. used a novel label-retaining approach in a transgenic mouse model to mark epidermal stem cells in the follicle bulge; these cells highly express TGFβ regulated proteins and are more likely than progeny to exhibit phosphorylated Smad proteins indicative of TGFβ signaling⁴⁹. These examples of early progenitors regulated by TGFβ1 suggest the possibility that the ERα positive population may represent a similar mammary gland progenitor population; indeed, their low proliferation frequency is consistent with that of progenitor cells in other tissues. Tissue-specific stem cells or early progenitors are thought to be the critical cellular target in carcinogenesis based on their ability to produce unlimited progeny⁵⁰⁻⁵⁵. Dysregulation of stem cell populations in transgenic mouse models of breast cancer can predispose the mammary gland to carcinogenesis⁵⁶, thus dysregulation of ERα progenitors may be one route to ERα positive breast tumors.

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FIGURE LEGENDS

Figure 1: Nuclear R-Smad protein co-localizes in mammary epithelial cells that are positive for active TGFβ1. A. Individual channel images of DAPI-stained nuclei (DAPI), R-Smad and TGFβ1 immunostaining in a transverse section of a duct. Merged image shows R-Smad immunoreactivity as green and active TGFβ1 as red. Bar = 20 μm. B. Active TGFβ1 and nuclear R-Smad are markers of the same cell population in the mammary epithelium at estrus. Shown are means ± S.E.M. from 3 C57Bl/6-129SV mixed background animals. At least 250 cells were scored per animal.

Figure 2: Subpopulations of the mammary epithelium of the C57Bl/6-129SV mixed background mouse at estrus. A. Example of dual immunofluorescence localization of ERα and R-smad immunoreactivity shows the gray-scale individual images of DAPI-stained nuclei, ERα, R-smad and a merged color image showing ERα immunoreactivity as green and nuclear Smad as red which makes nuclei positive for both appear yellow/orange. Column graph shows mean co-localization frequency ± S.E.M. (n=3). B. Gray scale images of DAPI-stained nuclei, PR and active TGFβ1 immunostaining in ductal epithelium. The merged color image shows that most PR positive

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cells (green nuclei) are also positive for cytoplasmic active TGFβ1 (red). Column graph shows mean co-localization frequency $\pm$ S.E.M. (n=3). C. Individual channels and merged image of ERα and PR immunoreactivity in a transverse section of a duct. ERα positive cells all exhibit PR and the nuclei appear orange in the merged image. Column graph shows mean co-localization frequency $\pm$ S.E.M. (n=3). Bar = 20 μ m for all images.

Figure 3: TGFβ1 depletion results in increased frequency of ERα positive mammary epithelial cells in cycle. A. Dual immunofluorescence localization of ERα (green; non-nuclear staining is non-specific) and Ki67 (red) in mammary epithelium. Nuclei are counterstained with DAPI (blue). B. Dual immunofluorescence localization of ERα and Ki67, as in A, of higher magnification of a mammary duct with an example of double-labeled nucleus (yellow). C. Ki-67 and ERα/Ki-67 co-localization frequency in mammary epithelium of nulliparous *Tgfβ1* heterozygote (+/- in figure) and *Tgfβ1* wildtype (+/+) C57Bl/6-129SV mice at estrus. Three animals per genotype and at least 300 cells per animal were scored for presence of ERα and Ki-67 immunoreactivity. Asterisks indicate significance difference from *Tgfβ1* wildtype mean frequency ($P < 0.01$; t-test). D. BrdU and ERα/BrdU co-localization frequency in mammary epithelium of the same nulliparous *Tgfβ1* heterozygote (+/-) and *Tgfβ1* wildtype (+/+) C57Bl/6-129SV mice at estrus. Asterisks indicate significance difference from *Tgfβ1* wildtype mean frequency ($P < 0.01$; t-test).

Figure 4. Expression of constitutively active TGFβ1 results in decreased mammary epithelial co-localization of ERα and proliferation. Ki-67 and ERα/Ki-67 co-localization frequency in mammary epithelium of 12 week old nulliparous MMTV- TGFβ²²³⁻²²⁵ FvB transgenic mice (TG +) and age-matched wildtype mice (TG -) mice at estrus. Asterisks indicate significant difference from

Tgfβ1 wildtype mean frequency ($P<0.05$; t-test).

Figure 5. TGFβ1 depletion results in increased frequency of ERα positive mammary epithelial cells in cycle in parous mice. Ki-67 and ERα/Ki-67 co-localization frequency in mammary epithelium of parous *Tgfβ1* heterozygote (+/- in figure) and *Tgfβ1* wildtype (+/+) C57Bl/6-129SV mice at estrus. These animals were 3 weeks post weaning. Four animals per genotype and at least 250 cells per animal were scored for presence of ERα and Ki-67 immunoreactivity. Asterisk indicates significant difference from *Tgfβ1* wildtype mean frequency ($P<0.05$; t-test).

Figure 6. TGFβ1 depletion in pubertal glands results in increased frequency of cells in cycle but not in proliferating ERα positive mammary epithelial cells. A. Ki-67 and ERα /Ki-67 co-localization frequency in endbud mammary epithelium of TGFβ1 heterozygote (+/- in figure) and TGFβ1 wildtype (designated +/+) C57Bl/6-129SV mice at 6 weeks of age. Three animals per genotype and at least 300 cells per animal were scored for presence of ERα and Ki-67 immunoreactivity. B. Ki-67 and ERα /Ki-67 co-localization frequency in the mammary epithelium of subtending ducts in the same tissues. Asterisks indicate significant difference from *Tgfβ1* wildtype mean frequency ($P<0.01$; t-test).

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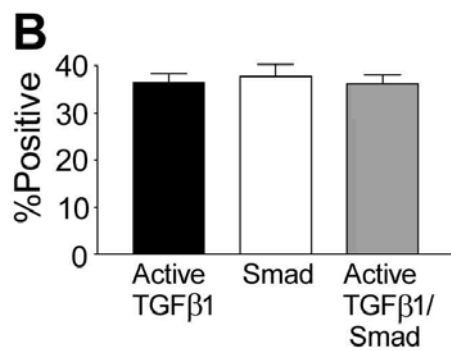
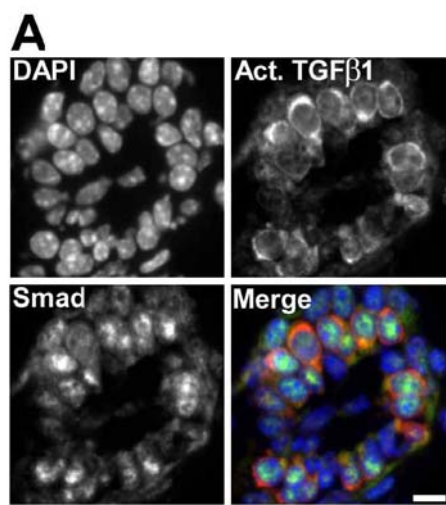


FIGURE 1

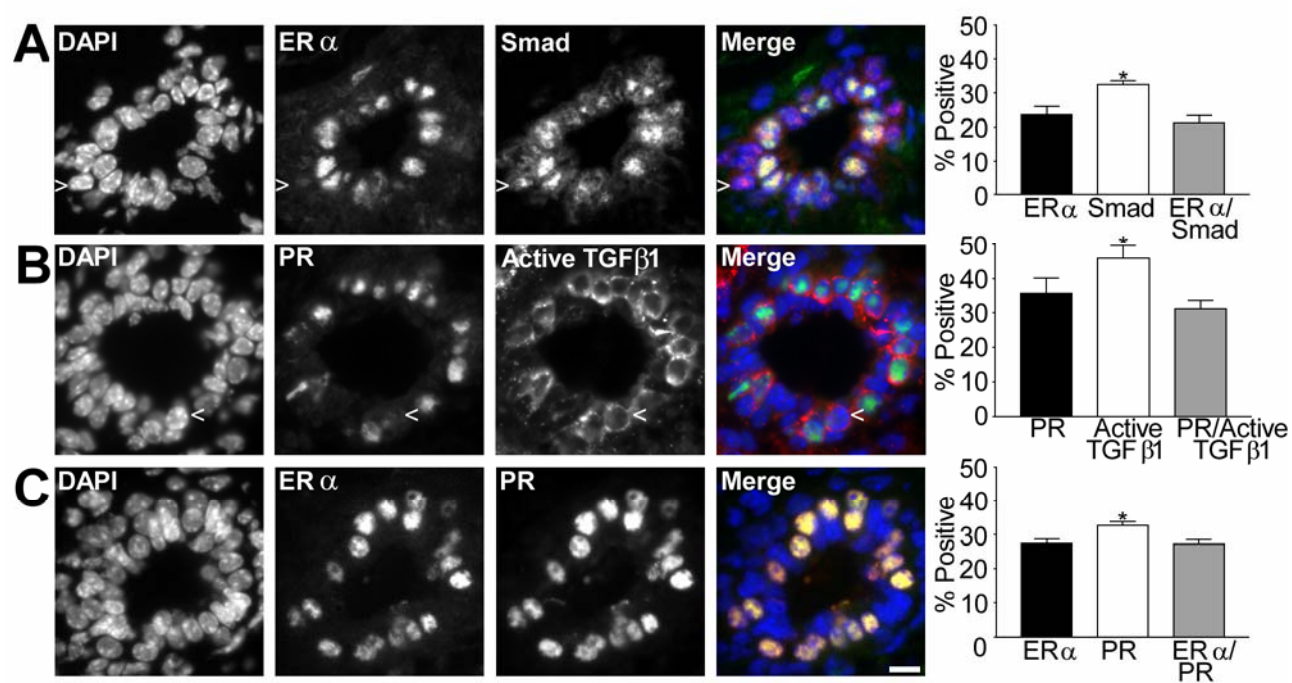


FIGURE 2

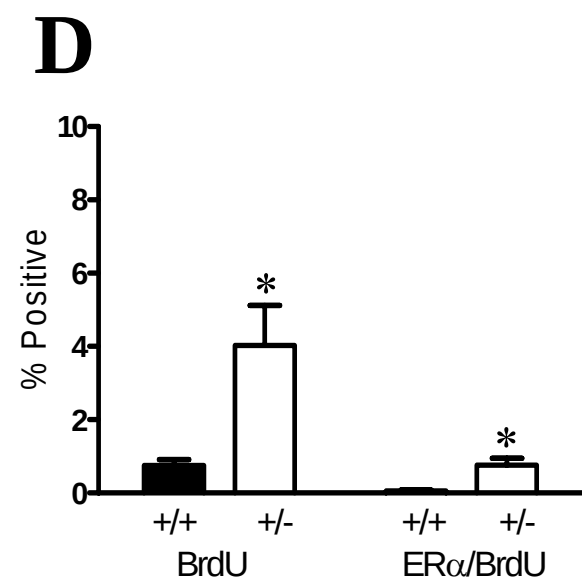
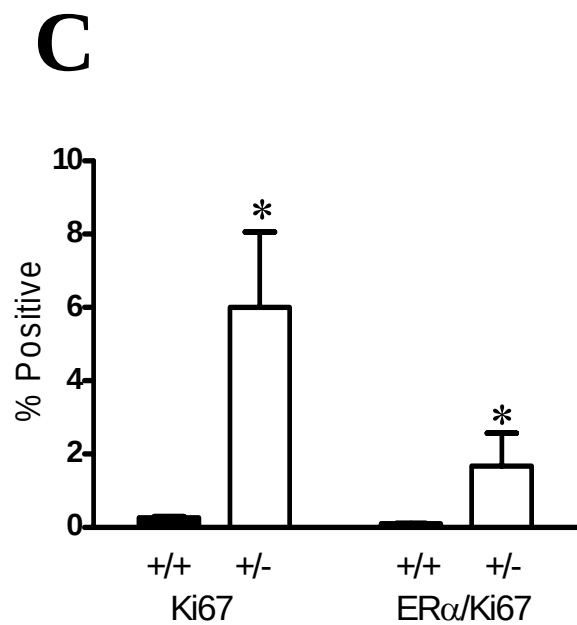
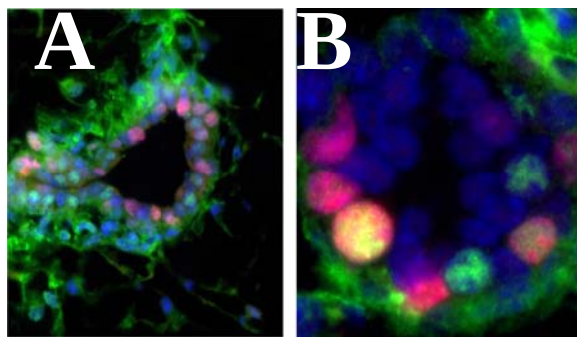


FIGURE 3

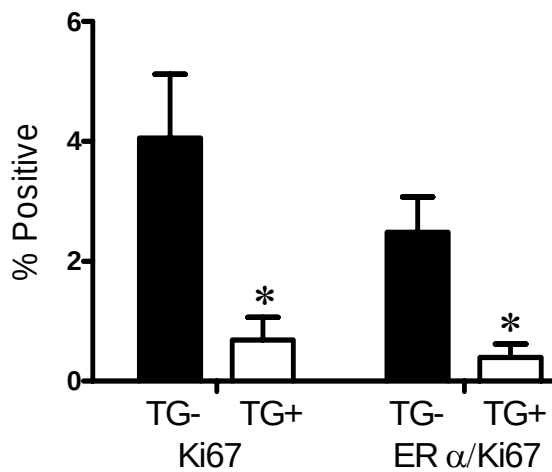


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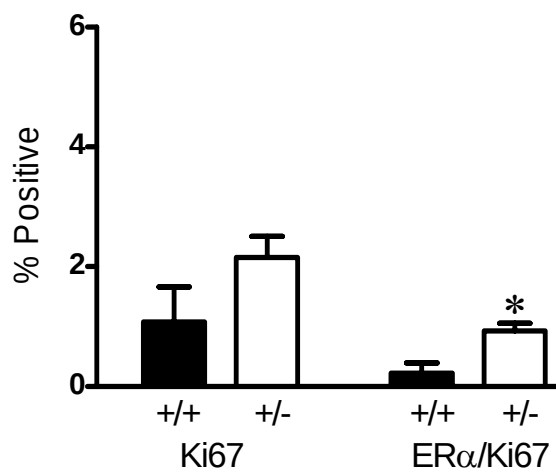
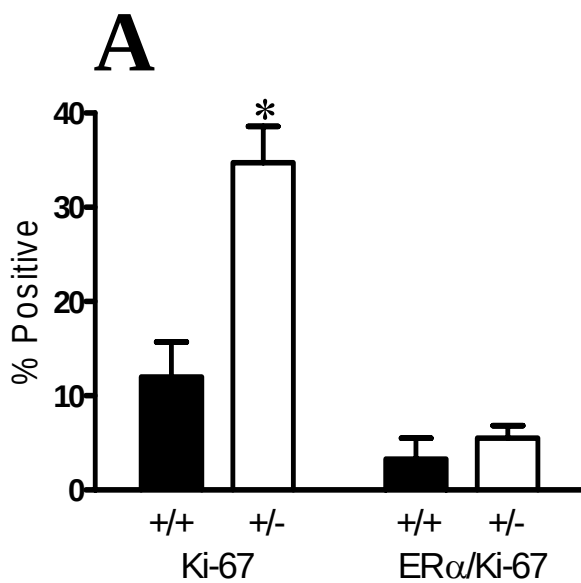


FIGURE 5

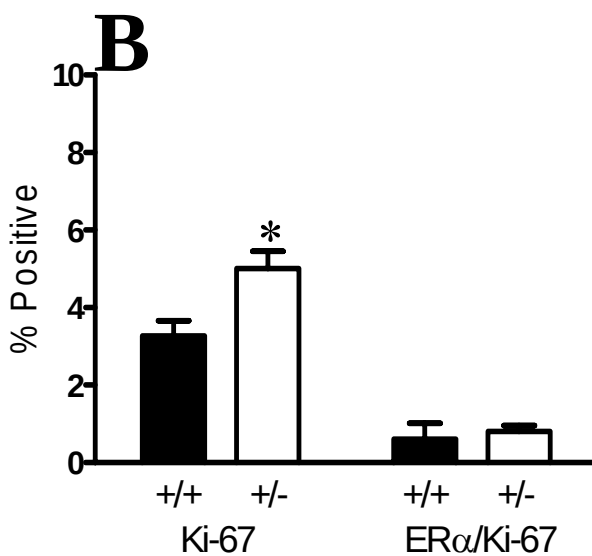


FIGURE 6