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

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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 restraints 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 *Tgfβ1* 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 *Tgfβ1* 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 hormones \(^{6,10}\). Studies in estrogen receptor α (ERα) knockout mouse mammary outgrowths indicate that both stromal and epithelial ERα, in cooperation with epithelial progesterone receptor (PR), govern mammary gland development in adult mice \(^{11}\). 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 cells \(^{12-17}\). Interestingly, increased proliferation in the hormone receptor positive population is associated with breast cancer risk \(^{18}\), while a transgenic mammary model of dysregulated ERα expression results in hyperplasia and ductal carcinoma in situ \(^{19}\).

Our TGFβ immunolocalization revealed striking epithelial heterogeneity during mammary stages characterized by proliferation \(^{7}\). For example, during estrus many cells downregulate TGFβ, consistent with it's action as a growth inhibitor, but some mammary epithelial cells maintain prominent TGFβ1 activation. This observation suggested to us the hypothesis that TGFβ 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 TGFβ1-positive and steroid hormone receptor epithelial subpopulations using dual immunolocalization, and second, to determine whether TGFβ depletion specifically affects proliferation of hormone receptor positive cells.

METHODS

Mice

All experiments were conducted with LBNL institutional review and approval.
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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-Tgfβ223-225 transgenic mice were previously described. 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 described. 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 GS. 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
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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 TGFβ1, 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
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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.

\textbf{RESULTS}

\textit{Autocrine TGF\textbeta}1 \textit{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 TGF\textbeta}1 immunoreactivity is reduced in most cells \textsuperscript{7}, but approximately 35\% of luminal epithelial cells show prominent immunoreactivity (Figure 1). All cells secrete latent TGF\beta and the extracellular matrix is a reservoir of latent protein, thus it is difficult to ascertain its original cellular source. Further, TGF\textbeta}1 activity is controlled by extracellular activation and depending on the mode of latent TGF\textbeta}1 activation, TGF\textbeta}1 can signal in both autocrine \textsuperscript{22} or paracrine \textsuperscript{23} fashion. Thus, it is important to determine when TGF\textbeta}1 is actively signaling and to which cells. Upon ligand binding, TGF\beta type I receptor phosphorylates Smad 2 and 3 (R-Smad), which leads to nuclear translocation and initiation of transcription (reviewed in \textsuperscript{24}). We asked whether TGF\beta was acting in an autocrine or paracrine fashion upon activation by using dual immunofluorescence to localize both TGF\beta and nuclear R-Smad. The frequencies of cells positive for active TGF\textbeta}1 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 TGF\textbeta}1 and nuclear Smad. Co-localization of active TGF\textbeta}1/nuclear R-Smad cells showed nearly perfect correspondence (Figure 1B). Most cells positive for active TGF\beta were Ki-67 negative (91\%, n=2 mice). These data indicated that cell-restricted TGF\textbeta}1 activation during estrus results in triggering of the TGF\beta signaling pathway in the same cells.
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Ovarian Hormone Receptor Positive Cells are Positive for Active TGFβ1 and Exhibit Nuclear R-Smad

It is well established that estrogen action is mediated through its cognate receptors, ERα and ERβ, but that ERα is essential for mammary epithelial cell proliferation. ERα localization by immunohistochemistry is heterogeneous within the mammary epithelium. To determine the relationship between TGFβ activation and the ERα positive cell populations, we used dual immunofluorescence to determine co-localization of ERα and R-Smad. Almost all ERα positive cells were positive for nuclear R-Smad (Figure 2A). The same was true when ERα was co-localized with active TGFβ1 (data not shown). Dual immunofluorescence of progesterone receptor (PR) and active TGFβ1(Figure 2B) also demonstrated that most PR positive cells were positive for active TGFβ1. ERα co-localized with PR(Figure 2C), as is found in human and rat mammary gland. These data indicated that most cells that are ERα and PR positive maintain TGFβ activation during estrus, suggesting that TGFβ may inhibit their ability to respond to ovarian hormone-induced proliferation.

Proliferation of ERα Positive Mammary Epithelial Cells is Increased in Tgfβ1 Heterozygote Mice

The infrequent co-localization of proliferation markers with ERα positive cells, despite the presence of estradiol, raises two possibilities: either the inability to proliferate is intrinsic to these cells independent of TGFβ, or that TGFβ1 selectively restrains ERα positive cells from proliferating. If it is the latter, then it is reasonable to expect that changes in the endogenous levels of TGFβ would lead to changes in the proliferation of ERα positive cells. TGFβ1 protein is reduced by more
than 90% in mice lacking one Tgfβ1 allele, whether measured by immunoreactivity or by quantifying TGF-β biological activity in tissue extracts. Therefore, to determine the relationship between TGFβ and proliferation of ERα positive cells, we used dual immunofluorescence localization of nuclear ERα and the cell cycle marker Ki-67 and scored dual-labeled epithelial cells in Tgfβ1 heterozygote and wildtype mammary glands (Figure 3A, B). The overall frequency of Ki-67 marked cells at estrus was increased 24-fold in Tgfβ1 heterozygote mammary epithelium compared to wildtype mice, consistent with our previous results using PCNA as a marker of cell cycle. The frequency of cells exhibiting dual localization of Ki67 and ERα increased 16-fold in Tgfβ1 heterozygote (Figure 3C). Although the degree of response was modified by genetic background, the consequence of TGFβ1 depletion was similar in two other mouse strains (i.e. Balb/c and FvB, data not shown).

To confirm that these ERα 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 ERα positive subpopulation was also significantly increased in Tgfβ1 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 ERα positive cells are capable of entering the cell cycle and undertaking DNA synthesis during estrus, but that concomitant TGFβ activation functionally restrains them.

ERα is found in both stromal and epithelial cells of the mammary gland. The role of stromal versus epithelial ERα 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 mice. Their results indicated that stromal, but not epithelial, ERα is
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 maturation. To examine whether regulation of proliferation of ERα positive cells was intrinsic to the Tgfβ1 heterozygote epithelium or is mediated by depletion of TGFβ1 in the stroma, we conducted similar transplant studies of Tgfβ1 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 Tgfβ1 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 Tgfβ1 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 Tgfβ1 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.

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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- *Tgfβ*223-225 transgenic mice that expresses constitutively-active TGFβ1 in the mammary epithelium. 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-*Tgfβ*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 women. Similarly, parous rats and rats exposed to a hormone regime that mimics pregnancy and subsequent involution 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 *Tgfβ1* wildtype mice was similar to that in estrus-stage, nulliparous *Tgfβ1* wildtype mice, which is already low (i.e. 0.1%). However, the frequency of
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ERα co-localization with Ki-67 in parous mammary epithelial cells was four-fold higher Tgfβ1 heterozygote mice than in Tgfβ1 wildtype mice. As we reported for mammary proliferation overall, these data indicate that ERα positive cells are restrained from proliferating in the parous gland through mechanism(s) mediated by TGFβ1. Consistent with this conclusion, conditional deletion of Smad4 in the mammary gland as a function of pregnancy leads to hyperplastic foci with increased cell proliferation.

**Regulation of Proliferation by TGFβ1 in ERα Positive Cells is Absent at Puberty**

In contrast to the foregoing data showing that TGFβ1 depletion increased proliferation overall and of ERα positive mammary epithelial cells at estrus, in hormone-treated ovariectomized mice, and following pregnancy, TGFβ1 depletion did not increase the proliferation of ERα positive cells that are present in the pubertal mammary gland. The frequency of Ki-67 positive cells was significantly higher in Tgfβ1 heterozygote endbuds and ducts than in Tgfβ1 wildtype, as expected from our previous analysis, but the frequency of ERα positive co-localization with Ki-67 in either structure was not significantly affected by TGFβ depletion (Figure 6). These data suggest that proliferation of ERα 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. 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
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 Tgfb1 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 (reviewed 26 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 cells 34.

Reproductive history with an early first pregnancy is a strong factor in assessing breast cancer risk (reviewed in 35). 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 restraints
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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. 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.

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. 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. Japanese women living in Hawaii have more ERα cells than those in Japan, which parallels cancer risk. ERα positive cells are increased in normal tissue of tumor-bearing breasts, in postmenopausal women, and in post-menopausal women using hormone replacement therapy. Consistent with the human data, a mouse mammary model of dysregulated ERα predisposes to hyperplasia and ductal carcinoma in situ. 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.
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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 TGFβ1\(^{42-44}\). 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 senescence\(^{45}\). 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 cells\(^{48}\). 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 signaling\(^{49}\). 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 progeny\(^{50-55}\). Dysregulation of stem cell populations in transgenic mouse models of breast cancer can predispose the mammary gland to carcinogenesis\(^ {56}\), 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
cells (green nuclei) are also positive for cytoplasmic active TGFβ1 (red). Column graph shows mean co-localization frequency ± 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 ± S.E.M. (n=3). Bar = 20 µm 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
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*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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TGFβ1 Restrains ERα Cell Proliferation

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TGFβ1 Restrains ERα Cell Proliferation
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FIGURE 1

A

[Images of DAPI, Act. TGFβ1, Smad, and Merge]

B

<table>
<thead>
<tr>
<th></th>
<th>% Positive</th>
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<tbody>
<tr>
<td>Active TGFβ1</td>
<td>30</td>
</tr>
<tr>
<td>Smad</td>
<td>25</td>
</tr>
<tr>
<td>Active TGFβ1/Smad</td>
<td>32</td>
</tr>
</tbody>
</table>

FIGURE 1
FIGURE 2
FIGURE 3

C

D

% Positive

0 2 4 6 8 10

+/-  +/-       +/+   +/-

Ki67  ER

α

% Positive

0 2 4 6 8 10

+/-    +/-       +/+    +/-

BrdU                   ERα/Ki67

% Positive

0 2 4 6 8 10

+/-  +/-       +/+  +/-

BrdU                   ERα/BrdU

FIGURE 3