We have studied the role of autocrine transforming growth factor-β (TGF-β) signaling on antiestrogen-mediated growth inhibition of hormone-dependent T47D and MCF-7 human breast carcinoma cells. Tamoxifen treatment increased the secretion of TGF-β activity into serum-free cell medium and the cellular content of affinity cross-linked type I and III TGF-β receptors in both cell lines. Anti-parietal TGF-β antibodies did not block antiestrogen-induced recruitment in G1 and inhibition of anchorage-dependent and -independent growth of both cell lines. Early passage MCF-7 cells, which exhibit detectable type II TGF-β receptors at the cell surface and exquisite sensitivity to exogenous TGF-β1, were treated with a tetracycline-controllable dominant-negative TGF-βRII (ΔRII) construct. Although the TGF-β1 response was blocked by removal of tetracycline in MCF-7/ΔRII cells, tamoxifen-mediated suppression of Rb phosphorylation, recruitment in G1, and inhibition of cell proliferation were identical in the presence and absence of tetracycline. TGF-β1 treatment up-regulated the Cdk inhibitor p21 and induced its association with Cdk2 in MCF-7 cells; these responses were blocked by the ΔRII transgene product. In MCF-7 cells with a functional TGF-β signaling pathway, tamoxifen did not up-regulate p21 nor did it induce association of p21 with Cdk2, suggesting alternative mechanisms for antiestrogen-mediated cytostasis. Finally, transfection of late passage, TGF-β1 unresponsive MCF-7 cells with high levels of TGF-βRII restored TGF-β1-induced growth inhibition but did not enhance tamoxifen response in culture. Taken together these data strongly argue against any role for TGF-β signaling on tamoxifen-mediated growth inhibition of hormone-dependent breast cancer cells.

Transforming growth factor-βs (TGF-βs) are potent regulators of cellular proliferation, differentiation, morphogenesis as well as extracellular matrix formation, extracellular proteolysis, and inflammation (1–3). In epithelial cells a major effect of TGF-β is its ability to inhibit cell proliferation (4). Three different mammalian TGF-β isoforms (TGF-β1, -β2, and -β3) encoded by different genes have been identified, and they exhibit similar effects in a variety of biological assays (5). Three membrane ligand-binding proteins with sizes of 53, 73, and 250 kDa have been reported as TGF-β receptors type I, II, and III, respectively. Type I and II receptors are transmembrane serine/threonine kinases directly involved in signal transduction, while the type III receptor functions mainly by presenting the ligand to the signaling type I and II receptors and as a storage protein (6, 7). Both TGF-β and its receptor molecules are expressed ubiquitously by normal and transformed cells.

Both signaling receptors seem to be needed for TGF-β responsiveness (8), and type II receptor expression correlates with the anti-proliferative activity of TGF-β (9, 10). TGF-β arrests cell growth in the G1 phase of the cell cycle and most probably affects multiple signaling pathways (11). TGF-β has been shown to retain the retinoblastoma susceptibility gene (pRb) in a hypophosphorylated form, which prevents cells from entering the S phase (12). By regulating the formation of Cdk-cyclin complexes and their inhibitor levels, TGF-β contributes to the accumulation of hypophosphorylated pRb (13). In several cell lines, TGF-β1 also induces rapid down-regulation of c-myc expression (14, 15), suggesting this is an additional mechanism by which these peptides suppress cell growth.

All three TGF-β isoforms are expressed in mouse mammary gland, and there are data supporting their role in the development of the mouse mammary gland (16, 17). Exogenous TGF-β administered by slow release pellets or tissue-specific expression of active TGF-β1 in the mammary gland of transgenic mice leads to ductal hypoplasia and suppression of ductal branching (16, 18, 19).

Normal and tumorigenic human breast epithelial cells in culture express TGF-β1 mRNA and secrete TGF-β receptor binding activity into their medium (20, 21). Published data support the notion that endogenous TGF-βs function as autocrine growth regulators of breast cancer cell proliferation (22, 23). Antibodies that neutralize mature TGF-βs stimulate the proliferation of estrogen-independent breast cancer cell lines (23). Growth stimulation of estrogen-dependent breast cancer cells with estradiol or the testosterone derivative norethindrone is associated with down-regulation of TGF-β2 and -β3 receptors in the tumor (24).
mRNAs (24–26). Growth inhibition of these cell lines by the antiestrogens tamoxifen or toremifene and the progesterin analogue gestodene is associated with enhanced TGF-\(\beta\) mRNA expression or increased secretion of TGF-\(\beta\) bioactivity or protein synthesis without associated mRNA changes (22, 27, 28), thus leading to the hypothesis that autocrine TGF-\(\beta\) signaling contributed to antiestrogen's actions. Some reports, however, argue against TGF-\(\beta\)'s role in the growth inhibitory response to antiestrogens. MCF-7 and T47D breast cancer cells can exhibit resistance to TGF-\(\beta\)-mediated growth inhibition despite retaining sensitivity to tamoxifen (29, 30). In addition, T47D and CAMA-1 breast cancer lines, which lack mRNA for TGF-\(\beta\)RII and hence response to exogenous TGF-\(\beta\), remain sensitive to the cytostatic effect of tamoxifen (31, 32). By using anti-TGF-\(\beta\) neutralizing antibodies as well as dominant negative TGF-\(\beta\)RII constructs in estrogen-dependent, tamoxifen-sensitive human breast cancer cells, we have formally tested in this study the role of endogenous TGF-\(\beta\) signaling on the cellular response to antiestrogens in breast carcinoma.

MATERIALS AND METHODS

Cell Lines and Antibodies—MCF-7 cells were provided by C. K. Osborne (University of Texas Health Science Center, San Antonio, TX) and have been described previously (33). T47D cells were derived from ATCC (Rockville, MD). They were both cultured in IMEM (Life Technologies, Inc.) supplemented with 5% (MCF-7) or 10% (T47D) fetal calf serum (JRH Biosciences, Lenexa, KS) and 10 mM insulin. The late passage MCF-7 cells transfected with a TGF-\(\beta\)RII expression vector (MCF-7/RII) were described previously (9). These cells as well as early passage MCF-7 cells transfected with a dominant negative tetracycline-repressible type II TGF-\(\beta\) receptor (MCF-7/\(\alpha\)RII) were cultured in IMEM containing 10% FCS and 500 \(\mu\)g/ml G418 (Life Technologies, Inc.). The \(\alpha\)-human pRb monoclonal antibody was from Pharmingen (San Diego, CA). The 2G7, 121H5, and 4A11 antibodies (provided by B. M. Fendly, Genentech, South San Francisco, CA) were raised against human recombinant TGF-\(\beta\) and have been characterized previously (34). The 121H5 IgG\(_a\) is devoid of TGF-\(\beta\) neutralizing activity, while the 4A11 IgG\(_a\) and the 2G7 IgG\(_\alpha\) neutralize the growth inhibitory activity of TGF-\(\beta\)1 and TGF-\(\beta\), \(\beta_1\), and \(\beta_2\) on Mv1Lu mink epithelial cells, respectively (34). The Cdk2 polyclonal IgG (M2), raised against carboxy-terminal residues 283–298, and the p27 polyclonal IgG (C-10) were from Santa Cruz Biotechnology (Santa Cruz, CA). The p21\(^{\text{SAF/CC}}\) monoclonal antibody was purchased from Abcam (Cambridge, MA). The polyclonal antibody C-16 (Santa Cruz Biotechnology) was used for immunoprecipitation of TGF-\(\beta\)RII.

Collection of Cell-conditioned Medium (CM) and TGF-\(\beta\) Radioreceptor Assay—Secreted TGF-\(\beta\) bioactivity was measured in serum-free IMEM conditioned for 24 h by adherent breast cancer cells as described previously (23). When indicated, the CM was acidified with 1 N NaOH before testing. Bound 125I-TGF-\(\beta\)1 for 4 h at 4 °C was neutralized with 1 N NaOH before testing.

RESULTS

Tamoxifen Increases Secreted TGF-\(\beta\) Bioactivity and TGF-\(\beta\) Binding in MCF-7 and T47D Cells—We first examined the modulation of TGF-\(\beta\)-secretion by tamoxifen in the ER-positive MCF-7 and T47D human breast carcinoma cell lines. Exponentially growing cells were treated with 0.1% ethanol (controls) or 1 \(\mu\)M tamoxifen for 24 h in serum-free medium. TGF-\(\beta\) activity was measured in a 125I-TGF-\(\beta\)1 radioreceptor assay. In the absence of acid activation, TGF-\(\beta\)-activity was below the detection limit of the binding assay, indicating that the majority of the secreted TGF-\(\beta\) was in a latent form. Tamoxifen induced increases of approximately 30- and 4-fold in the secretion of acid-activatable TGF-\(\beta\)-activity in MCF-7 and T47D cultures, respectively (Table I).

To test whether endogenous TGF-\(\beta\)-ligands in response to the antiestrogens were masking endogenous TGF-\(\beta\) receptors, we examined the effect of tamoxifen on 125I-TGF-\(\beta\)1 binding and the cellular content of affinity cross-linked TGF-\(\beta\) receptors. Both MCF-7 and T47D cells exhibit type I and type III TGF-\(\beta\) receptors at the cell surface, whereas TGF-\(\beta\)RII was

2 Y. Ko, K. Koli, W. Li, J. K. V. Willson, M. G. Brattain, and C. L. Arteaga, submitted for publication.

3 C. L. Arteaga and T. C. Dugger, unpublished results.
TABLE I
TGF-β and Tamoxifen Action in Breast Cancer

Subconfluent exponentially growing breast cancer cells in 100-mm tissue culture dishes were washed with serum-free IMEM and incubated overnight in serum-free IMEM containing 0.1% ethanol or 1 μM tamoxifen. The cell CM was collected after 24 h, acidified with 1 N HCl to pH 1.5 for 1 h on ice, and reneutralized to pH 7.6 with 1 N NaOH. TGF-β activity in CM was measured in a 125I-TGF-β1 radioreceptor assay with AKR-2B mouse fibroblasts as indicator cells. TGF-β1 equivalents of receptor binding activity in CM were extrapolated from a competition standard curve with recombinant unlabeled TGF-β1 and standardized to cell number.

| Treatment          | TGF-β1a |
|--------------------|---------|
| Control (0.1% EtOH) | 0.06 0.22 |
| Tamoxifen (1 μM)   | 1.85 0.87 |

*The results are expressed as nanograms of TGF-β1 equivalents/10⁶ cells/24 h.*

FIG. 1. Tamoxifen-induced up-regulation of TGF-β binding in MCF-7 and T47D cells. Subconfluent cell monolayers were treated for 48 h with 0.1% ethanol (−) or 1 μM tamoxifen (Tam, +) and then labeled with 1 ng/ml 125I-TGF-β1 in the presence or absence of 100-fold excess unlabeled TGF-β1 for 4 h at 4 °C. Labeled receptors were cross-linked with 50 mM disuccinimidyl suberate as described under “Materials and Methods.” Colonies measuring ≥50 μm were counted 10 days later. Each data point represents the mean ± S.D. of three dishes.

Fig. 2. Anti-TGF-β neutralizing antibodies do not abrogate tamoxifen-mediated cytostasis. A, cells were treated in regular growth medium with 0.1% ethanol or 1 μM tamoxifen plus the indicated IgGs (100 μg/ml) for 48 h. After this incubation, the monolayers were trypsinized, cell nuclei labeled with PI, and DNA histograms analyzed by flow cytometry. B, a suspension of 3 × 10⁵ MCF-7 or T47D cells was plated in a soft agarose colony forming assay in the presence of 0.1% ethanol or 1 μM tamoxifen (Tam) plus the indicated IgGs (100 μg/ml) as described under “Materials and Methods.” Colonies measuring ≥50 μm were counted 10 days later. Each data point represents the mean ± S.D. of three dishes.

antibodies have been shown to stimulate the proliferation of breast tumor cells with an operative autocrine TGF-β pathway by neutralizing endogenous mature TGF-βs (23). Cells were treated with 1 μM tamoxifen or 0.1% ethanol (control) in the presence of IgGs (100 μg/ml) for 3 days and cell cycle distribution analyzed by flow cytometry of PI-labeled DNA. In both lines, tamoxifen induced a marked decrease of the percentage of cells in S phase and an accumulation in G1. Neither the anti-pan-TGF-β 2G7 nor the anti-TGF-β 4A11 antibodies nor their respective controls, the 12H5 IgG2 and an irrelevant IgG1, altered tamoxifen-mediated cytostasis (Fig. 2A). Identical results were obtained in a colony-forming soft agarose assay. In this assay, both MCF-7 and T47D cells were markedly inhibited by the antiestrogen; this inhibition was not altered by any of the TGF-β antibodies (Fig. 2B). By themselves, the IgGs utilized had no growth effects on either cell line (data not shown).

High Expression of Functional TGF-βRII Does Not Enhance Tamoxifen Action in MCF-7 Cells—Transfection with high levels of a tetracycline-repressible TGF-βRII expression vector into MCF-7 cells (MCF-7/RII cells, Ref. 9) restores sensitivity to growth inhibition by exogenous TGF-β. Therefore, in these cells, we tested whether up-regulation of an operative TGF-β signaling pathway would enhance antiestrogen-induced growth inhibition. MCF-7/RII cells were plated with or without 0.1 μM tetracycline. The following day, 1 μM tamoxifen or 0.1%
Tamoxifen-mediated up-regulation of TGF-β sensitivity by TGF-β-colony-forming assays to test the modulation of tamoxifen senescence ability for anchorage-independent growth. Soft agarose assay of conditioned medium. Since these cells have an impaired ability for anchorage-independent growth, soft agarose colony-forming assays to test the modulation of tamoxifen sensitivity by TGF-βRII were not useful.

Expression of a Dominant Negative TGF-βRII Does Not Block TGF-βRII protein at the cell surface and are markedly growth inhibited by exogenous TGF-βRIs. Expression of a tetracycline-repressible dominant negative type II TGF-β receptor in these cells (MCF-7/ΔRII) blocks cellular responses to exogenous TGF-β1. We first studied the proliferation effects of prolonged tamoxifen treatment in cells preincubated (for 24 h) or not with 0.1 μM tetracycline, concentration known to maximally induce the ΔRII mutant protein. Both anchorage-dependent and -independent proliferation were inhibited by 1 μM tamoxifen in the presence or absence of tetracycline (Fig. 4). Similar results were obtained with 5–10 μM amounts of the antiestrogen toremifene (27) in monolayer culture (data not shown).

Both TGF-β1 (4, 12) and antiestrogens (38) can recruit sensitive cells in the G1 phase of the cell cycle while suppressing Rb phosphorylation (12, 39). Therefore, we studied the impact of the ΔRII mutant on tamoxifen-mediated cell cycle arrest and Rb phosphorylation in MCF-7/ΔRII cells. A 72-h incubation with 1 μM tamoxifen markedly increased the proportion of cells in G1, while reducing those in G2M and S phases of the cycle. These changes in response to antiestrogen were almost identical in the presence or absence of tetracycline (Fig. 5). Consistent with this result, a 24-h incubation with 1 μM tamoxifen reduced Rb hyperphosphorylation in MCF-7/ΔRII cells under conditions in which the ΔRII mutant type II receptor was expressed or not (Fig. 6) further arguing against any role for endogenous TGF-β signaling on antiestrogen-mediated G1 arrest.

TGF-β1 but Not Tamoxifen Induces p21WAF1/CIP1 in MCF-7 Cells—TGF-β1 has been shown to inhibit the kinase activity of cyclin E-Cdk2 complexes (7) and hence suppress Rb phosphorylation. One reported mechanism for such inhibition is induction of the Cdk inhibitor p21, which then associates with the
FIG. 5. Tamoxifen-induced G1 arrest is not blocked by the ΔRII mutant receptor. MCF-7/ΔRII cells were seeded in regular growth medium with or without 0.1 μM tetracycline. The following day 0.1% ethanol (−) or 1 μM tamoxifen (+) were added for 72 h. After this incubation, the monolayers were trypsinized, cell nuclei labeled with PI, and DNA histograms generated in a FACScan flow cytometer. A, DNA histograms of PI-labeled MCF-7/ΔRII nuclei. B, table of cell cycle distribution of MCF-7/ΔRII cells under different treatment conditions based on analysis of 50,000 PI-labeled nuclei as indicated under “Materials and Methods.”

table of cell cycle distribution of MCF-7/ΔRII cells under different treatment conditions.

| Treatment | Tetracycline | Tamoxifen | G1 | G2-M | S |
|-----------|--------------|-----------|----|------|---|
| 1         | −            | −         | 57.8 | 14.2 | 28.0 |
| 2         | −            | +         | 84.4 | 6.1  | 9.5  |
| 3         | +            | −         | 56.0 | 14.4 | 29.6 |
| 4         | +            | +         | 82.4 | 5.7  | 11.8 |

FIG. 6. Suppression of Rb phosphorylation by antiestrogens is not blocked by the ΔRII dominant negative receptor. MCF-7/ΔRII were plated in regular growth medium with or without 0.1 μM tetracycline. The following day 0.1% ethanol or 1 μM tamoxifen were added for 24 h. Cells were washed twice with ice-cold PBS and solubilized with EBC buffer as described under “Materials and Methods.” 150 μg of protein (BCA method) were then subjected to 7% SDS-PAGE followed by an Rb immunoblot procedure. Molecular size markers in kDa are indicated on the left.

cyclin E-Cdk2 complex and inhibits its kinase (40, 41). A similar induction of p21 has been reported recently by the antiestrogen ICI182,780 in MCF-7 breast cancer cells (39). Therefore, we examined whether TGF-β1 and tamoxifen suppressed Rb phosphorylation by inducing p21 in MCF-7/ΔRII cells. An overnight incubation with 1 ng/ml TGF-β1 in the presence of tetracycline markedly up-regulated p21 protein levels and induced its association with Cdk2, as supported by coprecipitation of p21 with Cdk2 antibodies. This induction and association with Cdk2 were abrogated by removal of tetracycline, which eliminates endogenous TGF-βRII signaling (Fig. 7). Interestingly, 1 μM tamoxifen did not induce p21 nor did it induce its association with Cdk2, suggesting its suppressive action on Rb phosphorylation is mediated by a mechanism(s) other than up-regulation of autocrine growth inhibitory TGF-βs. Prolongation of tamoxifen treatment to 72 h still did not induce p21 protein. A 24-h treatment of MCF-7/ΔRII cells with 1 μM tamoxifen or 1 ng/ml TGF-β1 in the presence or absence of tetracycline, did not induce the Cdk inhibitor p27 as measured by immunoblot analysis (data not shown).

DISCUSSION

It is proposed that antiestrogens induce growth inhibition of human breast tumor cells by up-regulating expression and/or secretion of TGF-βs. We have directly tested this hypothesis in MCF-7 and T47D breast carcinoma cells, which exhibit enhanced secretion of TGF-β bioactivity upon treatment with the antiestrogen tamoxifen. All this secreted TGF-β activity was in a latent form requiring acid activation in vitro for it to be detected. This may reflect the inability of the radioreceptor assay to detect TGF-βs already utilized by the cells as well as to estimate in situ activation of TGF-βs at 37 °C over a more prolonged time and in the presence of a potential target cell. Supporting the latter possibility, medium conditioned by MCF-7 cells in the presence of tamoxifen inhibits the growth of co cultured tamoxifen-insensitive MDA-231 breast cancer cells. This inhibition by MCF-7 cell medium was reversed by anti-TGF-β antibodies (22).

MCF-7 cells but not the T47D line express TGF-βRII mRNA (32). Although the growth inhibitory response of both cell lines to high concentrations of exogenous TGF-β1 and TGF-β2 is minor (36, 37), this does not rule out a potential response to lower concentrations of endogenous TGF-βs. The absence of TGF-βRII mRNA and protein, presumably indispensable for TGF-β cellular responses, in tamoxifen-sensitive T47D cells argue s per se against TGF-β’s involvement in antiestrogen response. However, these cells bind TGF-β1 (37, Fig. 1) and, in response to the progestin analog gestodene, secrete 90-fold higher levels of TGF-β1 and -β2 proteins and become growth arrested (28). This inhibitory effect of gestodene in T47D cells is partially reversed by a polyclonal TGF-β antisera, suggesting these cells are responsive to autocrine TGF-βs
MCF-7 cells to exogenous TGF-β. These cells are more sensitive than late passage cells, however, failed to show an increase in steady-state TGF-β levels despite a simultaneous increase in expression/secretion of ICI182780 and TGF-β. On the other hand, other steroid molecules can up-regulate TGF-β receptors at the protein and/or mRNA level despite a simultaneous increase in expression/secretion of TGF-β ligands (44). Further work is needed to study the mechanism(s) by which tamoxifen up-regulates TGF-β receptors in MCF-7 and T47D cells. Preliminary experiments with MCF-7 cells, however, failed to show an increase in steady-state TGF-βRII mRNA levels after a 24-h incubation with 1 μM tamoxifen, arguing against a transcriptional effect to explain the result with MCF-7/RII cells (Fig. 3B).

TGF-β activation can occur locally within the cell surface of target cells (45, 46). Neutralizing anti-TGF-β antibodies may not be able to block TGF-β activity to a threshold required for the reversal of growth inhibitory signals or alter a ligand-independent direct effect of tamoxifen on TGF-βRII signaling. Therefore, we examined the effect of a kinase negative truncated TGF-βRII (ΔRII) on tamoxifen response in early passage MCF-7 cells. These cells are more sensitive than late passage MCF-7 cells to exogenous TGF-β1 and exhibit detectable levels of TGF-βRII at the cell surface thus providing an appropriate model to test directly both TGF-β1 and antiestrogen response. TGF-β1 responses were abrogated by the dominant negative ΔRII mutant. However, tamoxifen-mediated cell cycle arrest, suppression of Rb phosphorylation, and antiproliferative effects in these MCF-7 cells were identical with or without endogenous TGF-βRII signaling, disproving any major role for autocrine TGF-β in the response to antiestrogens.

Once a dissociation between TGF-β- and tamoxifen-mediated growth inhibition was established, we studied whether they independently suppressed Rb phosphorylation by similar mechanisms in the MCF-7/ΔRII cells. The pure antiestrogenICI182780 and TGF-β1 induce p21 and p27, which, by complexing with the cyclin E-Cdk2 complex, prevent Rb phosphorylation and hence progression beyond the G1 phase of the cell cycle (7, 39–41, 47). Exogenous TGF-β1 but not tamoxifen induced p21 as well as association of p21 with Cdk2. These responses were abrogated by the ΔRII mutant receptor, supporting the need of intact TGF-βRII signaling to elicit ligand-mediated effects on the Cdk2 inhibitor p21. Neither tamoxifen nor TGF-β1 induced p27 in MCF-ΔRII cells. In addition to the dissociation between both growth inhibitory pathways at a cellular level, these data with p21 further suggest that TGF-βs and tamoxifen suppress Rb phosphorylation by independent molecular mechanisms.

These data, generated with cell-autonomous experimental systems, do not rule out a possible role for antiestrogen-induced TGF-β in the anti-tumor response to tamoxifen in clinical breast carcinoma by a paracrine/endoctrine mechanism. Conflicting data have been published on this topic. Butta et al. reported that 3 months of tamoxifen therapy resulted in ER-independent enhanced TGF-β1 staining around stromal fibroblasts in breast tumor biopsies (48). The correlation between antiestrogen-induced enhancement of peritumoral TGF-β1 protein and a clinical response was not reported in this study. In two other studies, a rise in the circulating level of TGF-β2 (49) or in the tumor levels of TGF-β2 mRNA (50) correlated with a clinical response to antiestrogens, suggesting up-regulation of TGF-βs is a surrogate marker or epiphenomenon of an anti-tumor effect. On the other hand, a more recent immunohistochemical study in 19 patients failed to show alterations in TGF-β1 staining with intervening tamoxifen therapy, despite a >50% clinical response rate (51). Transfection of MCF-7 cells with a TGF-β1 expression vector does not alter tamoxifen sensitivity (52). Finally, breast tumors unresponsive to tamoxifen, when rebiopsied, expressed significantly higher levels of TGF-β1 mRNA than clinically responsive tumors (53). A causal association between ligand overexpression and the antiestrogen-resistant phenotype, if any, would require additional mechanistic studies.

In any event, the data presented strongly argue against a significant involvement of TGF-β ligands and receptor signaling on the growth inhibition of human breast carcinoma cells by antiestrogens. Prospective epidemiologic studies will likely address whether treatment-induced up-regulation of TGF-βs expression in tumors in situ can be used as a marker of response (or lack of response) to antiestrogens. Although it is still possible that autocrine/paracrine TGF-βs can be involved in antiestrogen response in some mammary carcinomas, the effect of TGF-βs on the host’s immune system and on tumor’s stroma, cell adhesion, and angiogenesis (reviewed in Ref. 54) can easily mask the net contribution of this putative autocrine pathway to breast tumor cell viability and progression by indirectly favoring breast tumor maintenance.

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