Overexpression of Aromatase Leads to Hyperplasia and Changes in the Expression of Genes Involved in Apoptosis, Cell Cycle, Growth, and Tumor Suppressor Functions in the Mammary Glands of Transgenic Mice

Nameer Kirma, Kiran Gill, Usha Mandava, and Rajeshvar Rao Tekmal

Department of Gynecology and Obstetrics [N. K., K. G., U. M., R. R. T.] and Winship Cancer Institute [R. R. T.], Emory University, Atlanta, Georgia 30322

ABSTRACT

Our previous studies have shown that overexpression of aromatase results in increased tissue estrogenic activity and induction of hyperplastic and dysplastic lesions in aromatase transgenic mammary glands. In this study, we have examined the effects of aromatase overexpression on biochemical changes in the aromatase transgenic mice. Our results show an increase in the expression of both estrogen and progesterone receptors, and their expression is maintained in the transgenic mammary tissue even without circulating ovarian estrogens. Our results also show an increase in the expression of several growth factors and cell cycle genes in the aromatase transgenic mammary glands, which is consistent with the observed increase in proliferating cell nuclear antigen levels and cellular proliferation. Interestingly, we have also observed a decrease in the expression of epidermal growth factor receptor and its ligands, epidermal growth factor and transforming growth factor α, as well as several tumor suppressor genes such as p53 and retinoblastoma. This study presents novel and interesting findings that are consistent with the current models of aromatase influence and the complex interactions of biochemical pathways leading to mammary tumorigenesis.

INTRODUCTION

Breast cancer is one of the most prevalent types of cancer observed in women. The mitogenic and proliferative effects of estrogen have long been recognized to correlate with ER and PR in breast tumor tissue. Interestingly, the proportion of patients with hormone-sensitive tumors is higher among postmenopausal patients than premenopausal patients (1, 2). Breast tumors from postmenopausal women maintain a high estrogen content, although the plasma levels of estradiol fall to low levels after menopause. Previous studies have demonstrated that the presence of aromatase, the rate-limiting enzyme responsible for estrogen biosynthesis, is increased in breast tumors. Assessing the functional significance of aromatase in breast tumors has attracted considerable attention and controversy. A number of recent studies have provided evidence to support a biological role for tumor aromatase (3).

Estrogen exerts its mitogenic effect on mammary tissue through its interaction with the ER. Several studies have shown that the proliferative effect of estrogen on epithelial cells is indirect and may involve a paracrine mechanism of regulation (4–7). Russo and Russo (8) hypothesized that the estrogen-activated ER in the ER-positive epithelial cells induces the expression of growth factors, which in turn stimulate the proliferation of the ER-negative epithelial cells. The observation that estrogen-dependent tumors eventually lose this dependence suggests the presence of other factors involved in cellular proliferation and tumor maintenance. Among these factors are growth factors (e.g., EGF, TGF-α, and VEGF), cell cycle factors (e.g., PCNA and cyclin D1), and apoptosis factors (e.g., bcl-2 and bax), whose expression may be initially dependent on estrogen induction. The role of some of these factors and their regulation by estrogen in normal mammary development and tumorigenesis have been the focus of several studies (9–12).

Although the use of breast cancer cell lines has provided valuable information regarding the relationship between estrogen and breast cancer, very little is known about how breast tissue estrogen affects the initiation and progression of breast cancer and its involvement in normal development of mammary glands. To address this question, we have developed a mouse transgenic model overexpressing aromatase (13). Our previous studies with these animals have shown that the mammary glands of the females exhibited hyperplasia and other preneoplastic characteristics (13). The aim of this study was to determine which estrogen-dependent factors are responsible for the changes observed in the aromatase transgenic mammary glands. Our results show an increase in both ER and PR expression. In addition, we have identified several genes that are differentially expressed in the mammary glands of aromatase transgenic female mice. These genes are involved in various pathways, such as cell cycle control, cellular proliferation, apoptosis, and tumor suppression.

MATERIALS AND METHODS

Materials. The antibodies used for Western analysis and immunoprecipitations are listed below. Mouse anti-Bcl-XL (Ab-1), Bcl-2 (Ab-2), and Bax (Ab-1) antibodies were purchased from Oncogene Research Products (Boston, MA). Mouse-specific antibodies for anti-ERα (Ab-10; clone TE111.5D11), ERβ (PA1–310), PR (Ab-7; clone hPRa7), p27 (M-197), PCNA (Ab-1; clone PC10), and cyclin D1 (Ab-1; DCS-6) were purchased from Neomarkers (Fremont, CA). Mouse anti-TGF-β (AB-100-NA)-specific antibodies were purchased from R&D Systems (Minneapolis, MN). Mouse-specific antibodies, anti-actin (1–19), anti-cyclin E (M-20), aggarose-conjugated anti-CDK2 (M2), agarose-conjugated anti-CDK4 (C-22), agarose-conjugated anti-p27 (F-8), and agarose-conjugated normal IgG were purchased from Santa Cruz Biotechnol-
were washed again and coverslipped, and the staining pattern was observed. Incubation of mammalian tissue was performed by embedding in paraffin, sectioning at 5 μm, and staining with H&E. To immunolocalize p27, 5-μm-thick sections of aromatase and nontransgenic mammary gland tissue were used. After deparaffinization and rehydration in xylene and ethanol, nonspecific sites were blocked by incubating with 1% normal goat serum in 0.05 M Tris-HCl buffer. After decanting the reagents, the sections were covered with anti-p27 in 1:100 dilution. The samples were incubated overnight at 4°C in a humidified chamber. After three washings with 0.05 M Tris-HCl buffer, the slides were incubated with alkaline phosphate-conjugated secondary antibody for 30 min at room temperature, washings were repeated, and sections were incubated with 1% fast red/naphthol AS phosphate solution for 5 min. Sections were washed again and coverslipped, and the staining pattern was observed using a light microscope.

Serum and Tissue Estradiol Levels. Serum concentrations of estradiol were measured by double-antibody radioimmunoassay using commercially available reagents (Diagnostic Products, Corp., Los Angeles, CA). Using an equivalent of 200 μl of serum in duplicate, the assay had a sensitivity of 2.5 pg/ml and an upper limit of 500 pg/ml. Assaying increasing volumes of serum from 50–200 μL produced a displacement line parallel to the standard curve. Intra- and interassay coefficients of variations averaged <10% and 6.7%, respectively. Tissue estradiol concentrations were determined as described previously (14).

RNA Analysis. Total RNA from mice mammary glands was isolated using Tri Reagent (Sigma-Aldrich) according to the manufacturer’s protocol. mRNA was purified using the Oligotex purification kit (Qiagen Inc., Valencia, CA). To identify growth factors whose expression is affected by aromatase overexpression, we used two identical cDNA array membranes (DNA Technologies, Inc., Gaithersburg, MD) for hybridization with two specific radiolabeled cDNA probes, which were synthesized from purified mRNA isolated from nontransgenic and aromatase transgenic mammary tissue. The expression of specific candidate growth factors was then verified by RT-PCR, using the primer set sequences.

RESULTS

Overexpression of Aromatase in Transgenic Mammary Glands Contributes to Persistent Hyperplasia Even after Several Months of Postlactational Involution. To genotype the aromatase transgenic mice, Southern blot hybridization was routinely used to determine the presence of the transgene (data not shown). RT-PCR was used routinely to examine the overexpression of the aromatase in the mammary gland tissue of all transgenic mice along with control nontransgenic mice. All aromatase transgenic mice were identified by genotyping, consistently exhibited aromatase overexpression in the mammary glands at levels three to six times higher than those in the nontransgenic animals (data not shown), which is consistent with our previous observations (13). Our previous studies (13) have shown that mammary glands from aromatase transgenic virgin females of 8 weeks exhibited increased and enlarged ductal growth compared to nontransgenic litters. Several of these ducts had hyperplastic and dysplastic lesions and fibroadenomas. Mammary glands from post-lactational nontransgenic females consisted of extensive ducts with the presence of minimal lobulo-alveolar growth. In contrast, mammary glands of transgenic females showed significant ductal and alveolar hyperplasia throughout all of the mammary glands. In general, the involuted mammary glands of transgenic mice contain a pattern of postpregnancy involution different from that seen in the control animals. There is a significant increase in periductal, perilobular, and intralobular fibrosis. Interestingly, these changes are similar to what is observed in the breast tissue of women with a history of breast cancer. Persistence of ductal hyperplasia and dysplasia (Fig. 1, A and B) was evident even after several months of involution in mammary glands from transgenic females. In many postlactational females, the progression of hyperplastic and dysplastic changes to more prominent neoplastic/neoplastic changes was very significant with age (Fig. 1, C and D). Our data also show that neoplastic and neoplastic changes persist even in the absence of ovarian estrogen in ovariectomized aromatase transgenic females (Fig. 1, E–H). These results clearly demonstrate that overexpression of aromatase, which contributes to increased mammary estrogen, is sufficient to maintain various neoplastic/neoplastic changes without the influence of circulating ovarian estrogen.

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Estradiol Serum and Tissue Levels in the Aromatase Transgenic versus Nontransgenic Mice. To determine whether aromatase overexpression in the mammary tissue changes the hormonal milieu in the transgenic animals, serum and mammary tissue estradiol levels were estimated from aromatase transgenic and nontransgenic littermate females of same age ($n = 6$). Although these animals overexpress aromatase in mammary tissue, we have seen an insignificant increase in the serum estradiol levels in the transgenic animals (12.7 ± 1.61 pg/ml) as compared to the nontransgenic animals (11.4 ± 1.23 pg/ml). However, about a 2-fold increase was observed in the mammary tissue estradiol levels of the transgenic female mice (286 pg/gram) as compared with the nontransgenic animals (146 pg/gram). These results suggest that the increase in mammary tissue estrogen does not contribute to the endocrine pool, and based on histological and morphological observations among other indications, this moderate increase is sufficient for inducing various biological changes.

Aromatase Overexpression Results in the Increased Expression of ER and PR. Because the mitogenic effects of estrogen are mediated via the ER, it was of importance to determine how the overexpression of aromatase that results in tissue estrogen production in the transgenic mammary glands affects the expression of both $\text{ER}_\alpha$ and $\text{ER}_\beta$. Fig. 2A shows that mRNA levels of both $\text{ER}_\alpha$ and $\text{ER}_\beta$ are higher (2-fold and 3-fold, respectively) in the transgenic mammary gland than in the nontransgenic gland. Protein levels of these receptors were analyzed using Western blot analysis with antibodies specific to $\text{ER}_\alpha$ and $\text{ER}_\beta$ separately, which also demonstrated that their expression is higher (2-fold and 3-fold, respectively) in the transgenic mammary gland compared than in the nontransgenic gland (Fig. 2B).

The up-regulation of ER is known to influence the expression of genes involved in cellular proliferation, mammary gland development, and other physiological changes. The induction of PR expression by ER and the involvement of PR in normal mammary development as well as tumorigenesis have been well documented (24, 25). To determine whether the overexpression of aromatase resulting in the increase of ER in the mammary glands of the transgenic animals also affects PR expression, we examined PR expression at both the transcriptional and translational levels. Our results show a 1.5–2-fold increase in PR mRNA and protein levels in the transgenic mammary tissue as compared with those in nontransgenic tissue (Fig. 2, A and B).

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Fig. 1. Histological sections of the mammary glands of aromatase transgenic female mice. A and B, ductal hyperplasia and dysplasia are evident in the mammary glands of aromatase transgenic females after several months of involution. Note the evident extensive hyperplasia (C) and ductal carcinoma-like changes (D) in the mammary glands of aged females. E and F, mammary glands of ovariectomized nontransgenic mice exhibit a few rudimentary ductal glandular epithelial growths. G and H, continuous presence of hyperplastic and dysplastic lesions in aromatase transgenic glands 8 weeks after ovariectomy. Magnification, ×25.
indicating that elevated estrogen production due to aromatase overexpression in these mice induced PR expression in their mammary glands.

To further examine the effects of aromatase overexpression in mammary glands without the influence of circulating ovarian estrogens, the expression of ER and PR was examined in both ovariectomized nontransgenic and transgenic mice. The results show that both ER and PR are expressed in the aromatase-overexpressing mammary gland, and these levels are higher than the levels present in nontransgenic mammary gland (Fig. 3, A and B), confirming the direct influence of mammary estrogen on these receptors.

Aromatase Overexpression Leads to Changes in the Expression of Genes Involved in Apoptosis. Our previous studies suggest that the continued proliferation of mammary epithelium even during the postlactational phase may be due to continuous estrogen stimulation as a result of aromatase overexpression in breast tissue (13). Estrogens are also known to be involved in the regulation of genes involved in apoptosis (26). To understand how the continuous estrogenic stimulation affects the genes involved in apoptosis, we have examined the steady-state protein levels of Bcl-XL, Bcl-2, and Bax in mammary tissue of aromatase transgenic animals after 6 weeks of involution along with control tissues. Data shown in Fig. 4 suggest that the levels of Bcl-XL and Bcl-2 are higher in transgenic mammary tissue as compared to nontransgenic tissue, whereas the levels of Bax are higher in nontransgenic mammary tissue, suggesting that the ratio of Bcl:Bax favors the bypassing of apoptosis in aromatase transgenic mammary tissue, whereas it favors apoptosis and tissue remodeling in nontransgenic mice, as expected.

Aromatase Overexpression in Mammary Glands Leads to Changes in the Expression of Various Growth Factors. The mitogenic effects of estrogen on mammary glands in normal development as well as carcinogenesis are likely to be mediated by downstream factors, such as growth factors and their receptors, under the control of ER induction. We screened for growth factors that are differentially expressed in the mammary glands of aromatase transgenic female mice as compared to nontransgenic females by array hybridization as described in “Materials and Methods.” After normal-
izing for the signal obtained for the housekeeping genes actin and GAPDH, the extent of probe hybridization to the target genes on the transgenic membrane was compared to that of the nontransgenic membrane. Our results show the overexpression of a number of growth factors in the aromatase transgenic mammary gland as compared to nontransgenic mammary glands (data not shown). We used both RT-PCR and Western blot analysis to further confirm the differential expression of various growth factors between nontransgenic and aromatase-transgenic mice mammary glands. TGF-β1 mRNA and protein levels are higher (about 3–5-fold) in the transgenic mammary gland as compared to the nontransgenic gland (Fig. 5A). A 1.5-fold and 4-fold increase in mRNA expression is also observed for TNFα and VEGF (Fig. 5B), respectively, indicating that the transcripts of these two growth factors are also elevated in the transgenic mammary tissue. On the other hand, the RT-PCR results for EGF show a 2-fold decrease in mRNA levels of the growth factor in the transgenic mammary glands as compared to the nontransgenic glands (Fig. 5C). We further examined whether the decrease in EGF expression corresponds to a decrease in the expression of its receptor, EGFR. The results (Fig. 5C) show that EGFR mRNA levels are 2-fold lower in the transgenic mammary tissue as compared with nontransgenic tissue. Similarly, the mRNA levels of TGF-α, an EGFR ligand, are lower in the majority of the transgenic tissue (Fig. 5C); however, in mammary tissue from very few animals, we observed an increase in TGF-α expression (data not shown), suggesting a possible variable regulation of this gene in the transgenic mammary gland.

**Aromatase Overexpression Affects the Expression Levels of Genes Involved in the Cell Cycle.** To determine the effect of increased in situ estrogen production in the mammary glands of aromatase transgenic mice on the extent of cellular proliferation, we examined the expression of genes involved in cell cycle (cyclin D1, cyclin E, PCNA, and p27) using Western blot analysis. Fig. 7 shows a significant increase in the levels of cyclin D1 (6-fold), cyclin E (6-fold), and PCNA (10-fold) along with a slight increase in the levels of p27 (1.5-fold) in the transgenic as compared to the nontransgenic mammary glands. We have also seen a 2-fold increase in the levels of c-jun (Fig. 6, bottom panel), which is a downstream early estrogen-responsive gene, in aromatase transgenic mammary tissue compared to nontransgenic mammary gland tissue. This indicates that increased

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**Fig. 5.** Expression analysis of growth factors in the mammary glands of transgenic mice. A, 50 and 70 ng of total RNA from the mammary glands of nontransgenic and transgenic mice were used for GAPDH and TGF-β1, respectively, in RT-PCR as described in “Materials and Methods.” Western analysis was carried out as described in “Materials and Methods.” B, 400 and 500 ng of total RNA were used for RT-PCR using TNFα- and VEGF-specific primers, respectively, as described in “Materials and Methods.” The corresponding Southern blot of the VEGF PCR is shown below the corresponding RT-PCR panel. C, the expression of EGFR and its ligands in the mammary glands of transgenic mice was analyzed by RT-PCR. Fifty ng of total RNA were used for cDNA synthesis and PCR amplification for GAPDH and EGF. A total of 200 ng of total RNA was used for TGF-α and EGFR. Southern hybridization was carried out for the TGF-α and EGFR PCR products to determine the specificity of the products as described in “Materials and Methods.”

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**Fig. 6.** Expression of cell cycle genes in the mammary glands of transgenic mice. Western analysis for actin, p27, cyclin D1, cyclin E, PCNA, and c-jun in mouse mammary glands was carried out as described in “Materials and Methods.”
that of the nontransgenic gland (magnification, ×100). It is evident that the overexpression of p27 in the aromatase glands by immunohistochemistry (Fig. 7). Whereas moderate staining of p27 in the transgenic mammary glands as compared to nontransgenic glands, whereas the levels of p27 bound to cyclin D1 bound to cyclin E in the nontransgenic mice were examined the levels of p27 bound to cyclin D1 and cyclin E in both animal strains by coimmunoprecipitation experiments. The results in Fig. 8A show that the levels of p27 bound to cyclin D1 in the aromatase transgenic mammary glands are 1.6-fold higher than those in the nontransgenic glands, whereas the levels of p27 bound to cyclin E were 1.25-fold lower in the aromatase transgenic glands than in the nontransgenic glands. In addition, we have also examined the association of p27 to CDKs by immunoprecipitating p27 with CDK2, CDK4, and CDK6 (Fig. 8B). The data show that the levels of p27 complexed with CDK4 are 1.7-fold higher in the transgenic mammary glands as compared to the nontransgenic glands. The levels of p27 complexed to CDK2 and CDK6, on the other hand, are 1.2- and 1.4-fold lower, respectively, in the aromatase transgenic gland as compared to the nontransgenic gland. Along with these experiments, immunoprecipitation with normal IgG was used as a control for the specificity of the reactions and has shown negative results (data not shown). Combined, the data suggest that the release of p27 from cyclin E-CDK2 complexes and increased association of p27 to the cyclin D1-CDK4 complex due to the increase in cyclin D1 levels favor cell cycle progression rather than inhibition.

Expression of Tumor Suppressor Genes in the Mammary Glands of Aromatase Transgenic Mice. Previous reports (28, 29) have shown that estrogen up-regulates BRCA1 levels. To determine whether BRCA1 expression is affected by the overexpression of aromatase in the mammary glands, we compared the levels of BRCA1 mRNA in aromatase transgenic mice with those in nontransgenic mice by RT-PCR. The results show that the expression of the BRCA1 mRNA 2-fold higher in aromatase transgenic gland than in nontransgenic mammary gland (Fig. 9A).

p53 is another tumor suppressor gene that is mutated in many types of cancer, including familial breast cancer. We examined the expression levels of p53 in the transgenic mice by RT-PCR. The results show that the p53 message is decreased in the mammary glands of transgenic animals as compared to that of nontransgenic animals (Fig. 9B).

The activity of the Rb gene product plays an important role in cell cycle progression from G1 to S phase and, consequently, cellular proliferation. To determine the effect of aromatase overexpression on Rb expression, we examined the mRNA and protein levels of this gene. The results (Fig. 9C) show a drastic decrease in Rb mRNA expression in the transgenic mammary tissue as compared to nontransgenic mammary tissue. We also tested for the expression of transcription factor E2F1, a binding target for Rb. The RT-PCR results show a corresponding decrease in the expression of E2F1 in the transgenic animals as compared to the nontransgenic animals (Fig. 9C).

DISCUSSION

Our previous studies (13) have shown that aromatase overexpression, which contributes to increased estrogenic activity in mammary tissue, leads to hyperplastic, dysplastic, and other premalignant changes in the mammary glands of aromatase transgenic mice. The studies presented here not only confirm our earlier observations but also show (Fig. 1) that these changes are present even several months after postlactational involution. Furthermore, the development of hyperplastic lesions is localized to the hyperplastic lesions, as shown by the highly stained epithelial cells (Fig. 7B). To determine the association of p27 to cyclin D and cyclin E in the aromatase transgenic mice as compared to the nontransgenic mice, we examined the levels of p27 bound to cyclin D1 and cyclin E in both animal strains by coimmunoprecipitation experiments. The results in Fig. 8A show that the levels of p27 bound to cyclin D1 in the aromatase transgenic mammary glands are 1.6-fold higher than those in the nontransgenic glands, whereas the levels of p27 bound to cyclin D1 were 1.25-fold lower in the aromatase transgenic glands than in the nontransgenic glands. In addition, we have also examined the association of p27 to CDKs by immunoprecipitating p27 with CDK2, CDK4, and CDK6 (Fig. 8B). The data show that the levels of p27 complexed with CDK4 are 1.7-fold higher in the transgenic mammary glands as compared to the nontransgenic glands. The levels of p27 complexed to CDK2 and CDK6, on the other hand, are 1.2- and 1.4-fold lower, respectively, in the aromatase transgenic gland as compared to the nontransgenic gland. Along with these experiments, immunoprecipitation with normal IgG was used as a control for the specificity of the reactions and
Although the mammary glands of the aromatase transgenic mice exhibited hyperplastic and dysplastic changes at the histological level, we have not observed any palpable (frank) mammary tumor development even in older mice (≥2 years old), which suggests that other cooperating factor(s) or carcinogenic events are necessary for further cancer development. To this end, our recent studies (34) have shown that a single dose of DMBA leads to the induction of frank mammary tumors in about 25% of the aromatase transgenic mice, and all of the aromatase transgenic animals that received DMBA had microscopic evidence of tumor formation, whereas no tumor formation was observed in DMBA-treated nontransgenic mice. This suggests that the preneoplastic changes induced by breast tissue estrogen increase susceptibility to environmental carcinogens. It may also be likely that DMBA develops preneoplastic lesions in which the elevated estrogen levels in the aromatase transgenic mice promote mammary carcinogenesis. However, the observed hyperplastic lesions in aromatase transgenic mice, along with increased levels of PCNA protein (an indicator of cell proliferation) and cell cycle proteins even before DMBA treatment, suggest that higher levels of estrogen could result in an increased number of carcinogen-susceptible proliferating cells.

In the mitogenic pathway initiated by estrogen, the initial step is the induction of ER expression and its activation by ligand binding. In turn, the activated ER, a transcription factor, depending on the milieu of coregulators, activates or represses the expression of growth factor genes and genes involved in cell cycle control. These estrogen-regulated genes play a direct role in downstream pathways leading to the regulation of cell cycle and, consequently, proliferation (8).

We have identified several growth factor genes with altered expression in the aromatase transgenic mammary gland. Our results also support the notion that estrogenic action results in the overexpression of cellular factors responsible for the suppression of apoptosis and promotion of cellular proliferation during tumorigenesis, including cyclin D1, cyclin E1, and PCNA. In addition, consistent with our results showing an increase in the expression of the angiogenic factor VEGF, other studies have shown that increased estrogenic activity can up-regulate the expression of VEGF in mammary glands (11). Interestingly, the levels of EGFR expression and that of its ligand, EGF, were lower in the transgenic mammary tissue. The study by Yarden et al. (10) has shown that estrogenic action is bimodal with respect to regulation of EGFR expression. The first phase is a transient induction that is not dependent on protein synthesis, whereas the other phase is a repression that is dependent on protein synthesis. The latter obser-

perplasia and other changes are persistent even without circulating ovarian estrogens in ovariectomized transgenic mice, suggesting that breast tissue estrogen has direct effects on the induction and maintenance of these changes in mammary glands. These observations, along with our recent studies (30–32) that show induction of gynecomasia and testicular cancer in male aromatase transgenic mice, suggest that tissue estrogens play a direct role in mammary tumorigenesis. Recent studies by Fisher et al. (33), which have shown that the absence of aromatase in the aromatase knockout mice led to underdeveloped genitalia and immature mammary glands, are consistent with our observation that aromatase plays a direct role in mammary development and other preneoplastic and neoplastic changes.

The increase of ERα and ERβ mRNA and protein levels in transgenic mammary tissue suggests that both ER isotypes have an important role in the mediation of the estrogenic response resulting in preneoplastic development of the mammary gland in our transgenic animal model. In addition, our results also showed an increase in PR levels in the transgenic mammary glands. The expression of both ER (ERα and ERβ) and PR in the absence of ovarian estrogen and their up-regulation in aromatase transgenic mice underscore the relevance of aromatase overexpression in increased estrogenic activity and the regulation of these receptors in mammary tissue. Consistent with these observations, we have also seen up-regulation of ERα in testicular tissue of aromatase transgenic mice. These observations further suggest that tissue estrogen has a role in the up-regulation of the steroid receptors.

![Fig. 9. Expression of tumor suppressor genes in the mammary glands of transgenic mice. A, 400 ng of total RNA were used for RT-PCR using BRCA1-specific primers as described in “Materials and Methods.” Southern hybridization of the PCR product was carried out as described in “Materials and Methods.” B, 1 μg of total RNA was used in RT-PCR using RB- and E2F1-specific primers as described in “Materials and Methods.” Southern hybridization of the PCR products was carried out as described in “Materials and Methods.” C, 300 ng of total RNA were used in RT-PCR using p53-specific primers as described in “Materials and Methods.”](image)

![Fig. 10. Model for the regulation of the cell cycle by cyclin D1, cyclin E, and p27 in the mammary glands of aromatase transgenic mice. In the mammary glands of aromatase transgenic animals, both cyclin D1 and cyclin E are overexpressed. The increase in cyclin D1 levels would sequester the p27 away from cyclin E1-CDK2 complexes. Due to this and the increased levels of cyclin E, p27-free cyclin E-CDK2 complexes can then hyperphosphorylate Rb and inhibit the latter’s negative control on cell cycle progression.](image)
vation suggests that the synthesis of an additional factor is required for estrogen repression of EGFR (10). The expression of the EGFR ligand TGF-α is also decreased in the transgenic mammary tissue, except in the very few cases in which we have seen either no decrease or a slight increase in the transgenic mammary gland as compared to the nontransgenic gland. This variability could reflect the multilevel gene regulation observed between ER and EGFR.

Surprisingly, TGF-β1 showed a 3–5-fold overexpression in the transgenic mammary gland as compared to the nontransgenic control. TGF-β1 is known to play a negative role in cellular proliferation by promoting cell cycle arrest at the G₁ checkpoint (35, 36). However, recent studies (37) suggest that TGF-β1 is a pleiotropic protein that also has positive effects on cellular growth and may, under normal conditions, be involved in maintaining homeostasis. Recent evidence has shown that the CDKI (p27) that is induced by TGF-β1 prevents the progression of cell cycle past the G₁ phase by interfering with the activity of cyclin-CDK complexes (27). Consistent with the previously observed induction of p27 by TGF-β1, our results also show an increase in p27 levels in the mammary glands of aromatase transgenic animals. We have not observed a difference in the expression of p21 in the transgenic mammary gland as compared to the nontransgenic gland (data not shown). Despite the increase in expression of the CDKI p27, the observed net effect exerted by aromatase overexpression in our model results in abnormal cellular proliferation in the transgenic mammary tissue. Previous evidence (38) has shown that estrogenic stimulation of breast cancer cell lines results in increased cellular proliferation and an increase in the levels of cyclin D1 as well as the CDKI p21 and that the mitogenic response in these cells is dependent on the relative induction of both of these genes. Current studies (27) have also suggested that the increase in cyclin D1 levels results in the sequestration of p27 (and/or p21, depending on the cellular context) away from cyclin E-CDK2 complexes, resulting in the release of the inhibitory effects on cyclin E-CDK2, which can then phosphorylate Rb and inactivate it (summarized in Fig. 10). The inactivation of Rb by hyperphosphorylation results in the progression of cell cycle past the G₁ checkpoint. We have observed increased levels (6-fold) of both cyclin D1 and cyclin E in the aromatase transgenic mammary glands as compared to the nontransgenic glands, whereas only about a 1.5-fold increase was observed in the levels of p27. Our studies also show that the increase in the levels of p27 in the aromatase transgenic mammary gland (compared to that in nontransgenic mice) corresponds to a similar increase in p27 levels associated with cyclinD1-CDK4 complexes. These results are consistent with the study by Prall et al. (39) and other studies reviewed by Pestell et al. (40), which show that in breast cancer cells, estrogen stimulation results in increased cyclin D1 expression and an increase in the association of CDKI to cyclin D-CDK4 complexes. In addition to the possible regulation of Rb phosphorylation, the expression of Rb mRNA is down-regulated in the mammary glands of aromatase transgenic mice, which can contribute to the reduction of Rb activity in the inhibition of cell cycle progression. Also decreased in the aromatase transgenic mammary gland is the expression of tumor suppressor p53, which is involved in cell cycle control and apoptosis. However, a slight increase is seen in BRCA1 expression (2-fold) in our animal model, which is consistent with recent studies. Romagnolo et al. (29) have shown, using an in vitro system, that estrogen up-regulates the expression of BRCA1. Bennet et al. (41) have also shown that the administration of synthetic estrogen leads to proliferating mammary glands in the presence of the wild-type BRCA1+/+ and BRCA2+/+ alleles but not in the presence of heterozygous BRCA1+/− or BRCA2+/−. Furthermore, studies by Aritzi et al. (42) have shown that p53 induces the transcriptional repression of BRCA1, linking the expression of both genes in a common pathway of cellular growth. Our observed changes in the expression of BRCA1 and increased mammary proliferation are consistent with these observations.

In summary, we have shown that overexpression of aromatase, which results in increased estrogen levels, in the mammary tissues of aromatase transgenic mice acts a direct mitogen and leads to the induction of genes involved in cell cycle and down-regulation of tumor suppressor genes, shifting the balance toward increased cellular proliferation.

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